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Extraction of Algal Pigments and Their Encapsulation

in Lyotropic Liquid Crystalline Nanoparticles for Anticancer Applications

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Abstract

Drug resistance, various side effects, long treatment time increasing the risk of metastasis represent just a few examples of the limitations imposed by traditional anticancer therapies. Despite the rapid development of science and technology in the field of pharmacy and medicine, many malignancies remain a huge problem for humanity, and at the same time, a challenge prompting the development of modern approaches, such as photodynamic therapy (PDT) combined with nanomedicine. Moreover, in the current climate crisis, there is an increasing search for sustainable solutions based on the principles of *Green Chemistry*. As an alternative to standard chemical syntheses, a potential source of drugs is a widely available biomass of marine algae, rich in a whole range of valuable bioactive compounds. Modern green extraction techniques, such as microwave-assisted extraction (MAE), allow for highly efficient isolation of natural products with low energy and solvent input, supporting their environmental friendliness. The management of invasive species biomass constitutes an additional advantage, since it helps to stabilize and protect the marine environment. Nevertheless, many of the natural compounds, such as pigments being the subject of this thesis, have certain limitations due to their hydrophobicity, thermo- or photosensitivity. Hence, the instability in aqueous cellular environments may lead to degradation or aggregation before reaching the target, which hinders their therapeutic application. Furthermore, addressing tumors' highly complex and heterogeneous microenvironment remains a major obstacle for effective drug delivery.

Intending to find a solution for these problems, the thesis presents an approach based on non-lamellar lyotropic liquid crystalline nanoparticles (LLCNPs), specifically cubosomes, as nanocarriers suitable for encapsulation of the extracted algal pigments to improve their solubility, stability, and delivery to pancreatic cancer cells, thus enhancing their anticancer efficiency. It highlights the benefits of novel biocompatible Pluronic-free cubosomes (TS-CUB) compared to the standard Pluronic-based ones (CUB), focusing on their physicochemical characteristics through scattering and microscopic techniques, along with the biocompatibility and anticancer activity evaluation in a series of *in vitro* assays upon human pancreatic cancer BxPC-3 cells. Combined with the TS-CUB, the cytotoxicity, photocytotoxicity, cellular uptake, or morphological analysis via the chosen cell line bioimaging have proved promising anticancer effects of the potential drugs derived from marine algae. Namely, the natural photosensitizers – chlorophylls, and a bisindole alkaloid, caulerpin, characteristic of *Caulerpa* species. The provided investigation indicated that the sustainable nanoplatfroms represent a promising step toward advanced nanomedicine applications combined with the valorization of marine biomass.

List of papers included in the thesis

Paper I

Cubosomes and Hexosomes Stabilized by Sorbitan Monooleate as Biocompatible Nanoplatfoms against Skin Metastatic Human Melanoma

Marco Fornasier, **Karolina Krautforst**, Julita Kulbacka, Peter Jönsson, Sergio Murgia, Urszula Bazylińska; *Journal of Colloid and Interface Science* 677 (2025) 842-852 (DOI: 10.1016/j.jcis.2024.08.126)

Paper II

Delivery of Ulva rigida Extract by Bicontinuous Cubic Lipid Nanoplatfoms for Potential Photodynamic Therapy against Pancreatic Cancer

Karolina Krautforst, Julita Kulbacka, Marco Fornasier, Rita Mocci, Debora Dessì, Andrea Porcheddu, Davide Moccia, Antonio Pusceddu, Giorgia Sarais, Sergio Murgia, Urszula Bazylińska; *Colloids and Surfaces B: Biointerfaces* 253 (2025) 114754 (DOI: 10.1016/j.colsurfb.2025.114754)

Paper III

Caulerpin Delivery via Pluronic-Free Cubosomes: Unlocking the Therapeutic Potential of a Pigment from an Invasive Marine Algae

Karolina Krautforst, Julita Kulbacka, Marco Fornasier, Rita Mocci, Andrea Porcheddu, Antonio Pusceddu, Davide Moccia, Sergio Murgia, Urszula Bazylińska; *Molecular Pharmaceutics* 22 (2025) 4747–4761 (DOI: 10.1021/acs.molpharmaceut.5c00340)

Additional papers not included in the thesis

Brown Seaweed – Fucus Vesiculosus as a Feedstock for Agriculture and Environment Protection

Karolina Krautforst, Anna Szymczycha-Madeja, Maja Wełna, Izabela Michalak; *Scientific Reports* 13 (2023) 10065 (DOI: 10.1038/s41598-023-36881-z)

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Chapter 1 – Introduction

1.1. Algae as a biomass for bioactive compounds extraction

Thanks to the growing understanding of the natural biomass economy, aligned with the principles of *Sustainable Development* and *Green Chemistry*, biomass is increasingly utilized for the extraction of biologically active compounds rather than traditional chemical synthesis [1–3]. The biomass of marine macroalgae (seaweeds) constitutes a widely available and cheap source of a wide variety of bioactive compounds useful for applications in several industries, including the food, chemical and pharmaceutical ones [4]. In general, seaweeds are abundant in polysaccharides, vitamins, and minerals, high in protein content and low in fat [5]. Despite being exposed to harsh environmental factors like intense light and high oxygen levels that can generate free radicals, seaweeds do not show significant photodynamic damage [6]. These marine organisms, often living in variable, extreme environmental conditions of the deep oceans characterized by tides and waves, have adapted their metabolism to produce compounds that are intended to protect them from UV radiation, environmental stress, and herbivores [7]. The concentration of these bioactive metabolites in algae biomass can vary depending on factors such as water temperature, salinity, nutrient availability, and light conditions [6]. In this respect, invasive species seem to be extremely interesting because they are characterized by a high ability to survive in difficult conditions and, therefore, should present a high content of such compounds with a strong protective bioactivity [8]. Among others, these secondary metabolites include the group of alkaloids, terpenoids, flavonoids, steroids and tannins, presenting biological effects, and therefore constituting a potential source of medicines [9,10].

1.1.1. Green marine algae

1.1.1.1. Definition and division

Seaweeds are photoautotrophic plant-like marine organisms inhabiting seas, oceans and coastal areas, usually attached to rocks or other hard substrate [11]. These marine macroalgae are divided into three main groups based on their pigment composition: green algae (*Chlorophyta*), brown algae (*Phaeophyta*), and red algae (*Rhodophyta*) [12] (**Figure 1.1**). The green color of the *Chlorophyta* is related to the high content of pigments belonging to the chlorophyll group, mainly chlorophyll a and chlorophyll b. They are not masked by pigments such as fucoxanthin characteristic for *Phaeophyta*, which stains algae cells brown, or phycoerythrin protein-pigment complex responsible for the red color of *Rhodophyta* [13]. Thanks to the specific chemical structure responsible for the ability to produce reactive oxygen species (ROS), chlorophylls

constitute a valuable group of bioactive compounds referred to as natural photosensitizers [14] (explained in more detail in the paragraph **1.1.3.2. Natural photosensitizers**). A great example of a potential source of these photoactive pigments is the biomass of a widely available green alga named *Ulva rigida* [2]. In addition, green marine algae may contain compounds characteristic of a given species, such as caulerpin from *Caulerpa cylindracea*, with anti-inflammatory, antimicrobial, antiviral or even anticancer properties [9,15]. These two species of green algae are described in more detail in the following paragraphs.

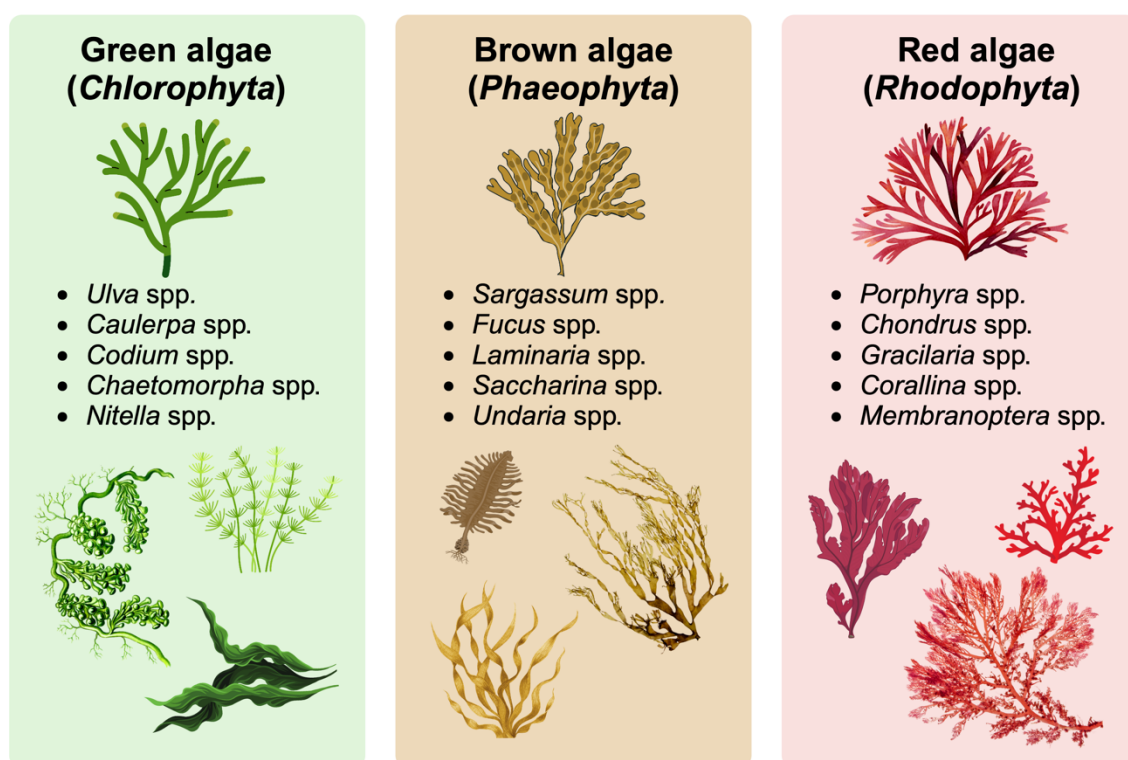


Figure 1.1. Division of marine macroalgae into three main groups: *Chlorophyta*, *Phaeophyta*, *Rhodophyta*.

1.1.1.2. *Ulva rigida*

Ulva rigida, (*U. rigida*; C. Agardh, 1823, **Figure 1.2A**), common and widely distributed on rocky shores along many seas and oceans, including the Mediterranean coast, belongs to the group of green algae, *Ulva* spp. commonly known as “sea lettuce” due to its expanded leaf-like structures, resembling a garden lettuce [12,16]. Persisting throughout the whole year by growing on rocks, stones and as an epiphyte, *U. rigida* has an emerald-green, sheet-like thallus that is firm at the base and more delicate toward the growing edges, occasionally featuring irregular elliptical holes [17].

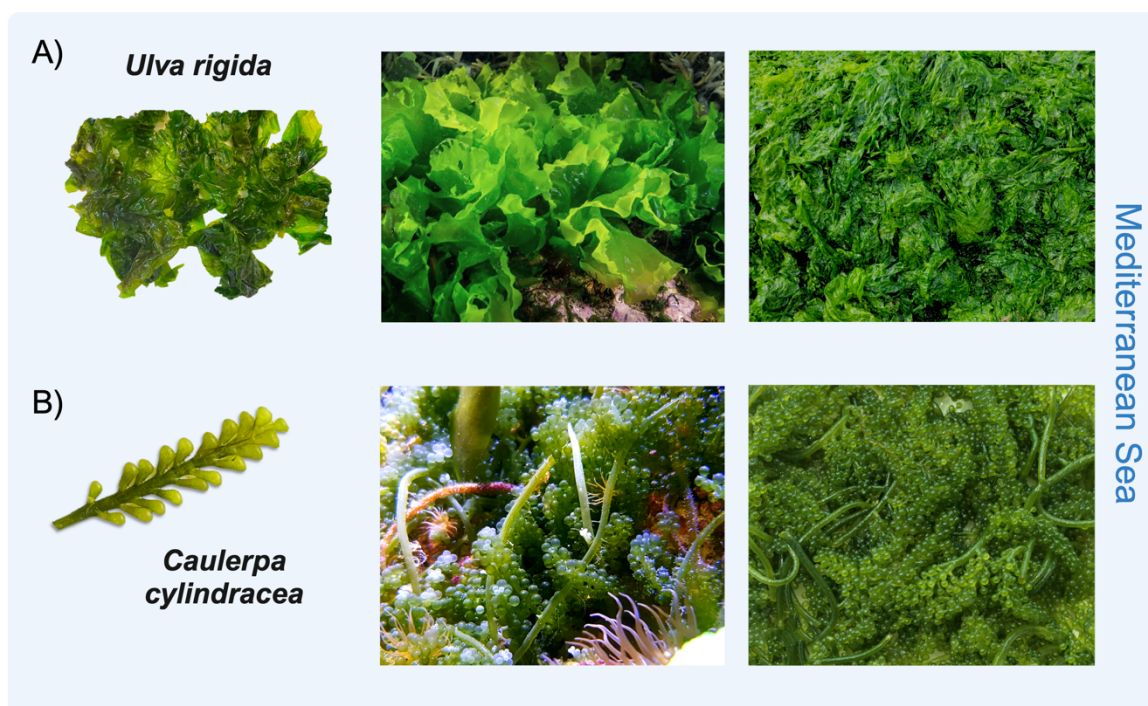


Figure 1.2. Representation images of *U. rigida* (A) and *C. cylindracea* (B), green marine algae of the Mediterranean Sea. Own elaboration based on own photos, from [18] and [19].

Ulva spp. is known for its nutraceutical properties, due to the presence of valuable compounds such as vitamins, proteins, minerals, carbohydrates and fibre [5,20]. These species are among the most traded edible seaweeds, due to their widespread distribution across the globe [21]. On the other hand, as mentioned in the previous paragraph, it is important to notice that *U. rigida* presents a high content of natural photosensitizers, chlorophylls [2]. These photoactive pigments present potential pharmacological effects, for instance, in combination with photodynamic therapy (PDT) for anticancer treatment [22] or as bioimaging agents [23].

1.1.1.3. *Caulerpa cylindracea*

Another example of an interesting group of green macroalgae is the genus *Caulerpa*, which consists of more than 100 species, occurring in various forms, from oval grapes and flat blades to feather-like structures [24]. Some of them are known for their nutritional value, are thus eaten around the world, commonly called “sea grapes” or “green caviar” [25]. In addition to the nutritional components, due to intertidal zone habitation, this species has adapted its metabolism to produce secondary metabolites in response to stress, such as high salinity, UV radiation, and variable temperature [26]. Such compounds include alkaloids and terpenoids, which, due to their specific chemical structure, exhibit bioactive effects such as anti-

inflammatory or antimicrobial ones [27,28]. Moreover, it turns out that *Caulerpa* spp. contains a whole range of molecules with potential anticancer properties due to their cytotoxic, antiproliferative, and antimetastatic activities [9]. Therefore, as *Caulerpa* seaweeds are edible and contain pharmacologically active compounds, they may offer both preventive and therapeutic benefits.

Caulerpa cylindracea (*C. cylindracea*; Sonder, 1845, **Figure 1.2B**) is a green alga, that occurs abundantly in tropical and warm-temperate areas, native to Southwestern Australia, where it tends to grow in mixed groups with other algae, rather than forming pure meadows, and can be found from the intertidal zone to depths of 6 meters on reef flats and in intertidal pools [29]. Since the early '90s, *C. cylindracea* has been spreading in the Mediterranean Sea [30]. In contrast to its native habitat, it forms extensive and dense monocultures, thriving in various environmental conditions on a range of substrates, including pebbles, rocks, decaying mats of the seagrass *Posidonia oceanica*, sand, mud, as well as detrital and coralligenous environments at depths from 0 to even 70 meters [30,31]. *C. cylindracea* has a slender thallus, fixed to the substratum by thin rootlike rhizoids that are closely arranged along the stolon, from which the branches extend vertically, forming round bubbles (ramuli), resembling a bunch of grapes, thus commonly being called “sea grapes” [24]. This seaweed forms a dense “green carpet” on different substrates, with a substantial rhizoid development, and survives winter temperatures down to 10.5 °C [32].

As mentioned earlier, *Caulerpa* spp. similarly to *U. rigida* contains many compounds with potential pharmacological applications (see **Table 1.1**), such as bisindole alkaloids: caulerpin and caulersin, or a sesquiterpene: caulerpenyne, presenting many biological activities, thus being good candidates for anticancer drugs [9] (explained in more detail in the chapter **1.1.3. Bioactive algae pigments with pharmaceutical applications**). On the other hand, it belongs to the list of the world’s worst invasive algal species, due to their harmfulness to the aquatic environment [33]. The expansion of *C. cylindracea* in the Mediterranean Sea is regarded as one of the most significant invasive occurrences globally, characterized by its rapid spread, extensive area covered, and detrimental effects on [34].

Table 1.1. Various bioactive compounds extracted from *Ulva* and *Caulerpa* marine algae.

Biomass	Extracted compounds	Bioactivity	References
<i>Ulva rigida</i>	polyssacharides	antioxidant activity	[35]
<i>Ulva pertusa</i>	ulvan, other polysaccharides	antioxidant activity	[36]
<i>Ulva rigida</i>	chlorophylls, xanthophylls	-	[2]
<i>Ulva rigida</i>	proteins	-	[37]
<i>Ulva lactuca</i>	ulvan	antioxidant activity	[38]
<i>Ulva lactuca</i>	quercetin, other antioxidants	antioxidant activity	[39]
<i>Ulva lactuca</i>	carotenoids, phenolics	antioxidant activity	[40]
<i>Ulva lactuca</i>	chlorophylls, carotenoids	inhibition of breast cancer cells proliferation (MDA-231 cell line)	[23]
<i>Ulva prolifera</i>	polysaccharides	antioxidant; pancreatic lipase inhibition activity	[41]
<i>Ulva</i> (not specified)	proteins, glycoproteins	no cytotoxicity against Vero cells	[37]
<i>Caulerpa racemosa</i>	caulerpin	antinociceptive and anti-inflammatory activity	[27]
<i>Caulerpa cylindracea</i>	lipidic extract	antibacterial activity, antioxidant activity	[42]
<i>Caulerpa cylindracea</i>	caulerpin	inhibition of mitochondrial complex II (potential anti-tumor activity)	[8]
<i>Caulerpa cylindracea</i>	caulerpin, monomethyl caulerpinate	anti-SARS-CoV-2 (inhibition of the spike-ACE2 complex formation); strong cytotoxic effects against breast cancer cell lines (MDA-MB-231 and MCF-7)	[43]
<i>Caulerpa</i> (not specified)	caulerpin	hypoxia-inducible transcription factor (HIF-1) inhibitory activity (potential anti-tumor activity)	[44]

<i>Caulerpa taxifolia</i>	caulerpenyne	antiproliferative activity on tumor cell line SK-N-SH (neuroblastoma cell line); the microtubule network modification	[45]
<i>Caulerpa racemosa</i>	racemosin A, racemosin B	neuro-protective activity against A β ₂₅₋₃₅ -induced SH-SY5Y (neuroblastoma cell line from a metastatic bone tumor) cell damage	[46]
<i>Caulerpa racemosa</i>	racemosin C	protein tyrosine phosphatase-1B inhibitory activity	[47]
<i>Caulerpa cylindracea</i>	methanolic extract, caulerpenyne	antiproliferative and apoptotic activity on SH-SY5Y and Kelly cells (neuroblastoma cell lines)	[48]
<i>Caulerpa cylindracea</i> , <i>Caulerpa prolifera</i>	proteins	-	[49]

1.1.2. Sustainable and green management of algae biomass

1.1.2.1. Sustainability and *Green Chemistry* principles

In 2015, the United Nations (UN) established 17 Sustainable Development Goals (SDGs) aimed at securing a sustainable future for our planet [50]. These goals address a wide range of interconnected global challenges, such as population growth, urbanization, industrialization, climate change, food security and healthcare, all in pursuit of sustainable development. Chemistry plays a dual role as both a non-normative science and a key economic sector, making it essential for achieving the SDGs [51]. Chemical and related industries, such as the pharmacy or the food industry, contribute to improved living standards and longer life expectancies. However, the growing extraction of non-renewable resources, along with the waste and environmental pollution generated throughout synthesis, manufacturing, usage, and disposal, still stands in stark contrast to sustainability.

In the era of rapidly growing technological and industrial development, sustainability is a key aspect when designing chemical processes. Terms such as *Sustainable Development* or *Green Chemistry* are becoming inseparable elements considered when designing an environmentally friendly process consistent with the protection of our planet, and therefore future generations

[52]. According to Anastas et al., *Green Chemistry is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances* [53]. To gather knowledge on this subject, in 1998 the *12 principles of Green Chemistry* (**Figure 1.3**) were formulated to provide guidelines for a conscious and ecological approach when designing and performing chemical experiments, waste management, as well as many others [54]. Biomass management is consistent with principle no. 7: *Use of renewable feedstocks*. Additionally, if the biomass is used to produce potential drugs based on bioproducts isolated from it, it is crucial to comply with the remaining principles of *Green Chemistry*, including principle no. 5 regarding the reduction of the use of harmful solvents. Wherever possible, the use of auxiliary substances (such as solvents and separation agents) should be avoided, and when necessary, innocuous when used [54]. Therefore, modern extraction techniques are increasingly being developed, which, while maintaining high efficiency, reduce the duration of the process associated with the lower consumption of costs and energy [6] (explained in more detail in the paragraph *1.1.2.3. Novel green extraction techniques*).

THE CHEMISTRY OF NATURE

Definition of Green Chemistry:
The design, development and implementation of chemical products and processes that reduce or eliminate the use and generation of hazardous substances

Green Chemistry is doing chemistry the way nature does chemistry - using renewable, biodegradable materials which do not persist in the environment.

Green Chemistry is a proven systems approach.

Green Chemistry reduces negative human health and environmental impacts.

Green Chemistry offers a strategic pathway to build a sustainable future.

The 12 Principles of Green Chemistry provides a framework for learning about green chemistry and designing or improving materials, products processes and systems.

The 12 Principles of Green Chemistry

1. Prevent Waste
2. Atom Economy
3. Less Hazardous Synthesis
4. Design Benign Chemicals
5. Benign Solvents & Auxiliaries
6. Design for Energy Efficiency
7. Use of Renewable Feedstocks
8. Reduce Derivatives
9. Catalysis (vs. Stoichiometric)
10. Design for Degradation
11. Real-Time Analysis for Pollution Prevention
12. Inherently Benign Chemistry for Accident Prevention

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Figure 1.3. The definition of *Green Chemistry* and the *12 principles of Green Chemistry*. Own elaboration reproduced with permission from (ACS Chemistry of Life website).

The management of marine algae biomass washed up by the sea helps to get rid of waste, and at the same time, by utilizing it to produce potential drugs, it fits into the principles of circular economy, which holds a remarkable promise for achieving many of the SDGs [56,57]. In addition, invasive species are increasingly encountered, and their development has a negative impact on the marine ecosystem. Therefore, the management of the invasive species biomass helps to protect the environment, with the additional advantage of being a huge source of products for various branches of industry, including pharmacy and medicine.

1.1.2.2. Invasive algae species

Many papers have been published concerning the understanding of the invasive strategies of *Caulerpa* spp., being a great example of the invasive algae species [29,30,34,58–61]. Nowadays, besides the well-known as “killer alga” *Caulerpa taxifolia* [62], one of the most infamous and threatening invasive species in the Mediterranean Sea is described here *C. cylindracea* (paragraph 1.1.1.3. *Caulerpa cylindracea*), rapidly spreading and causing many ecological changes by affecting marine ecosystems at the global scale [60]. This species of algae can alter the native species' behavior, with alleged adverse repercussions on fish growth patterns and dynamics of population [59]. Invasive species usually spread very quickly, so the availability of biomass for the extraction of valuable raw materials is very high. As mentioned earlier, *C. cylindracea* has been spreading in the Mediterranean Sea since the early '90s, and it has been noticed that its overgrowth in Italy has reduced diversity and abundance of the native macroalgae [29,30]. Highly competitive ability of the *C. cylindracea* invasive algae, concerning native species, caused a conviction that the spread of this green macroalga is considered one of the most threatening invasions in the Mediterranean Sea [34]. Nowadays, the occurrence of this species has also been recorded even beyond the Mediterranean area, reaching by the year 1997-1998 even the North-East Atlantic Ocean regions, the Canary Islands [63]. Pereira et al. have characterized 5 species of invasive algae typical for the waters of northwestern Spain: *Asparagopsis armata*, *Codium fragile*, *Gracilaria vermiculophylla*, *Sargassum muticum*, and *Gratelopia turuturu*, as a source of bioactive compounds for industrial applications, which may be an appropriate strategy to limit their populations, obtaining both economic and environmental benefits [64]. Therefore, if it is possible to limit the development of invasive algae biomass through its management for various useful applications, it should be used on a large scale.

1.1.2.3. Novel green extraction techniques

Algae extracts may be isolated from the biomass in an ecological way using modern extraction methods, e.g. enzyme-, ultrasound-, microwave-assisted extraction or supercritical fluid extraction [1,6,65]. These techniques have many advantages, including the possibility of replacing harmful organic solvents (used in traditional extraction) with environmentally friendly ones, hence, among others, making them green techniques [66]. So-called green solvents are selected for biomass extractions according to one of the recently published *12 green extraction principles* [67], such as water, ethanol, ethyl acetate, ethyl lactate, supercritical fluids or deep eutectic solvents [68–70]. In addition, the novel extraction techniques are characterized by higher efficiency, reduced processing time and lower cost compared to traditional ones [6]. For instance, Cantarino et al. proved that microwave-assisted extraction is a suitable method for caulerpin extraction from *Caulerpa racemosa* with improved yield efficiency [3]. Many other papers have been published concerning innovative extraction techniques being applied to obtain different valuable compounds from marine algae biomass such as polysaccharides, phenolic compounds, omega-3 and omega-6 fatty acids, peptides, proteins and bioactive pigments such as carotenoids and chlorophylls [1,2,6,65,71–73] (**Table 1.2**).

Table 1.2. Various green extraction techniques applied for the bioactive compounds extraction/quantification. MAE = microwave-assisted extraction, UAE = ultrasound-assisted Extraction, SFE = supercritical fluid extraction, EAE = enzyme-assisted extraction.

Biomass	Extracted bioactive compounds	Green extraction technique	References
<i>Fucus vesiculosus</i>	fucoidan	MAE	[74]
<i>Caulerpa racemosa</i>	caulerpin	MAE	[3]
<i>Porphyra</i> , <i>Palmaria</i> , <i>Undaria pinnatifida</i> , <i>Himanthalia</i> <i>elongata</i> , <i>Laminaria</i> <i>ochroleuca</i> , <i>Ulva</i> <i>rigida</i>	iodine	MAE	[75]
<i>Ulva prolifera</i>	polysaccharides	MAE	[41]
<i>Ulva pertusa</i>	ulvan, other polysaccharides	MAE	[36]
<i>Ulva lactuca</i>	quercetin, other antioxidants	UAE	[39]

<i>Ascophyllum nodosum, Laminaria hyperborea</i>	laminarin extract	UAE	[76]
<i>Ulva rigida</i>	polysaccharides	UAE	[35]
<i>Sargassum echinocarpum</i>	fucoïdan	UAE	[77]
<i>Palmaria palmata, Porphyra</i>	antioxidants	UAE	[78]
<i>Sargassum muticum</i>	polyphenols	SFE	[79]
<i>Undaria pinnatifida</i>	fucoxanthin	SFE	[80]
<i>Saccharina japonica</i>	fucoxanthin, other carotenoids, phlorotannin	SFE	[81]
<i>Undaria pinnatifida</i>	fucoxanthin, lipids (including omega -3 and omega-6 fatty acids)	EAE	[82]
<i>Ulva armoricana</i>	antiviral and antioxidant extracts (ulvans-rich)	EAE	[83]

1.1.3. Bioactive algae pigments with pharmaceutical applications

1.1.3.1. General characteristics

The substances that can impart their color to a given substrate, such as paper or cotton, called colorants, are used to add or alter the color of different materials. Such abilities are demonstrated by the so-called dyes and pigments, which have certain significant differences. Dyes must be soluble in the medium, typically water, at certain stages of the coloring process, and they generally show a degree of affinity for the material being dyed (substantivity), allowing them to be absorbed from the aqueous solution [84]. In contrast, pigments are colorants made up of particles that are insoluble in the medium used for application, have no substantivity for the material, and since the particles are too large to penetrate the substrate, they stay on the surface and can be easily removed unless bonded with an adhesive [85]. Apart from the production of paints, inks and other products for the textile, art or photographic industry, these compounds are also widely used in the food, cosmetics and pharmaceutical fields [84].

Bioactive pigments from marine algae

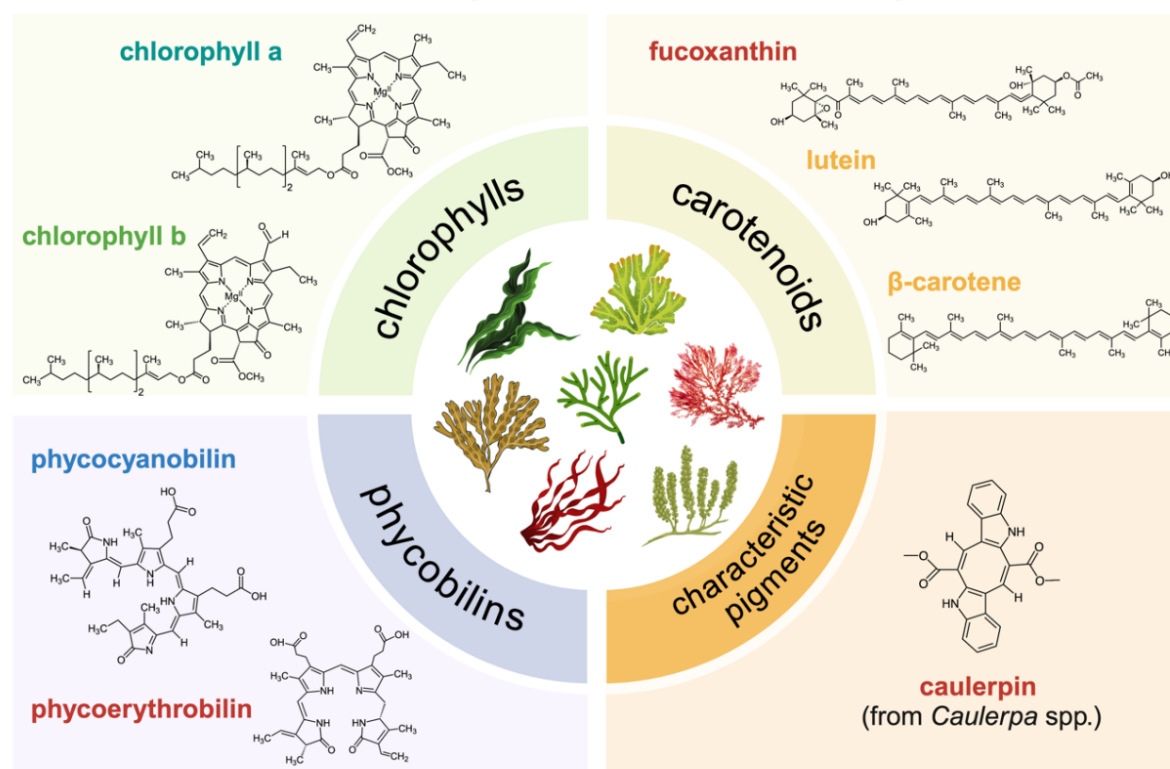


Figure 1.4. Chemical structures of some bioactive pigments from marine algae.

Natural pigments are an attractive group of compounds present in algae due to their significant photoactive potential [2]. Patel et al. claim that algal pigments offer 2–10 folds higher bioactivity than synthetic ones with no side effects [86]. Algae use pigments like chlorophylls, phycobilins, and carotenoids (see **Figure 1.4**) for photosynthesis, allowing them to capture sunlight for energy [87].

For instance, due to the presence of double bonds in their chemical structures, carotenoids such as astaxanthin, lutein, fucoxanthin, and zeaxanthin offer a wide range of health benefits, including antioxidant and anti-inflammatory properties, by protecting other molecules from oxidative damage caused by free radicals through various mechanisms [86]. On the other hand, chlorophylls, with the most well-known chlorophyll a, due to their porphyrin-based structure, after irradiation with light at a proper wavelength, can produce ROS capable of destroying cancer cells and therefore being a natural photosensitizer potentially useful for anticancer PDT [14,22]. Some algae pigments, such as characteristic of *Caulerpa* spp., an orange-red pigment named caulerpin, present anticancer activity by targeting and modulating multiple cancer cell-specific signalling pathways and cascades [9,10]. Caulerpin was found in *C. cupressoides*, *C. paspaloides*, *C. prolifera*, *C. sertularioides* and *C. cylindracea* [27,88,89], and its chemical

structure was first elucidated by Aguilar-Santos in 1970 and later revised by Maiti and Thomson in 1977 [15,90]. Caulerpin molecule (**Figure 1.4**) consists of two indole groups separated by a central cyclooctatetraene ring with two methyl ester groups [9,28]. This specific structure classifies it into the natural bisindole alkaloids, characterized by two indole units, more potent concerning the biological activity than their corresponding monomeric units [91]. Various naturally derived medical products are based on indolic alkaloids, like the most studied vincamine, vincristine or vindasine, mainly due to their activities as cerebral vasodilators, antiarrhythmics, antihypertensives, and anticancer agents [92,93].

1.1.3.2. Photosensitizers

A **photosensitizer** (PS) is defined as a chemical compound that, when it absorbs light, causes a chemical or physical change in another substance [94]. This ability is related to its specific chemical structure (a chromophore), very often based on the porphyrin ring [95]. The PSs are categorized into three generations (**Figure 1.5**): first, second, and third-generation PSs [96].

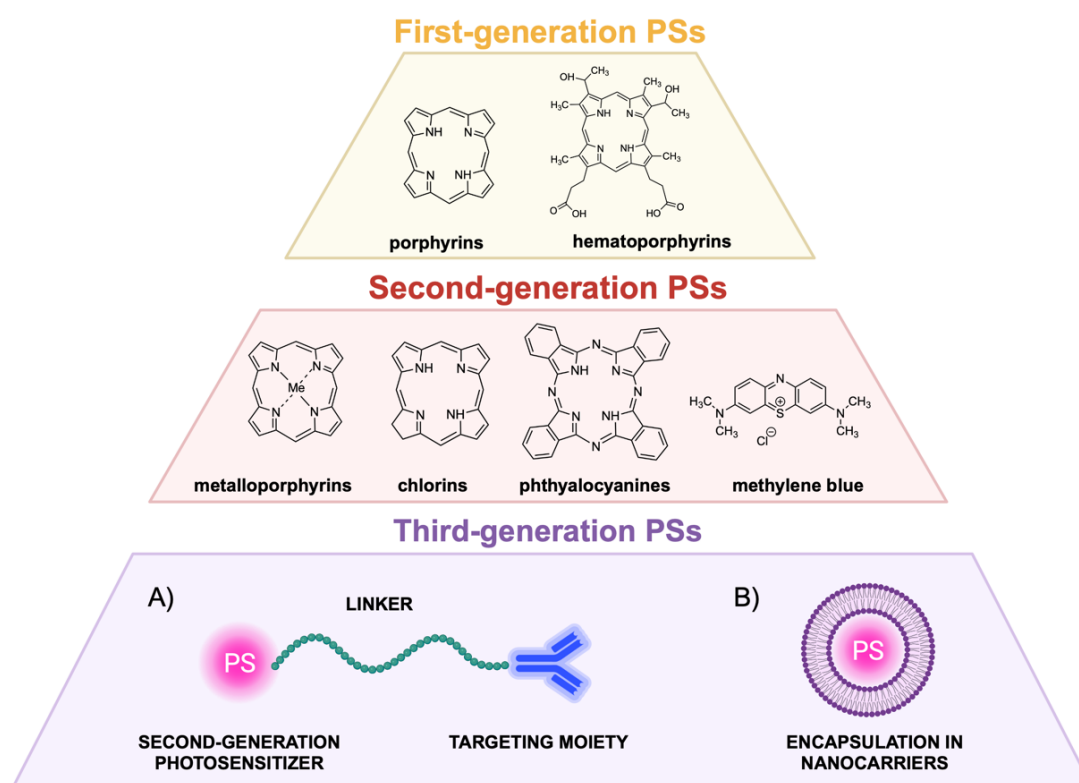


Figure 1.5. Three generations of photosensitizers with some example molecules.

The first-generation PSs are water-soluble porphyrins and their derivatives, such as hematoporphyrins, while second-generation PSs include compounds with improved properties and overcoming the limitations indicated by the firstly mentioned group of PSs, with examples such as metalloporphyrins (Lutrin® and Lutex®), methylene blue, toluidine blue, sulphonated metallo-phthalocyanines (Photosense®), Foscan® belonging to chlorins, and Rose Bengal belonging to xanthenes [97–99].

The second-generation PSs are characterized by improved light absorption, usually with the maximum in range 650-800 nm (allowing to penetrate deeply located tissues), as well as higher singlet oxygen quantum yield, greater chemical purity, better cancer tissue selectivity with reduced side effects, and improved elimination of the PS from the body [100,101]. Shakhova et al. claim that the use of chlorin-based PSs in PDT offers added benefits, as apart from their light absorption at 660 nm, allowing to reach deeper tissues, the absorption peak around 405 nm enables effective superficial impact beneficial for antimicrobial and antiaging PDT [102].

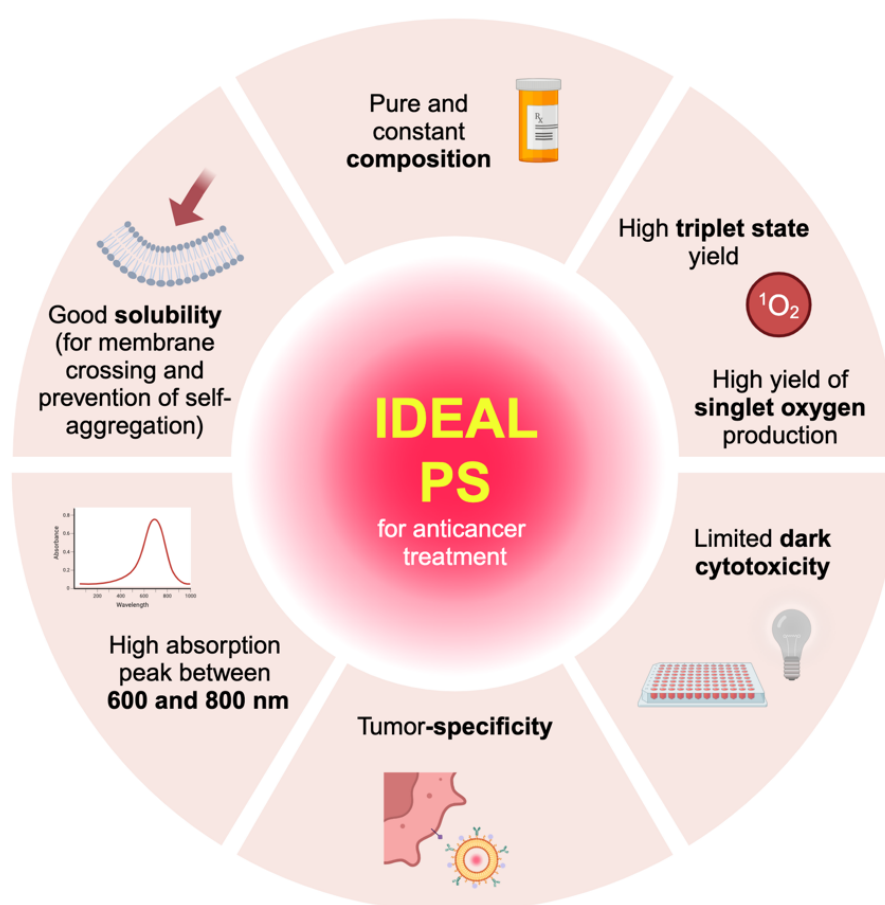


Figure 1.6. Characteristics of an ideal photosensitizer for anticancer treatment.

Chlorin e6 is a second-generation PS approved by the FDA (Food and Drug Administration) and widely used in PDT [103]. Since the PSs described above still have some limitations, like a well-known problem of their low solubility in water being a limiting factor in the intravenous administration [100]. Furthermore, the new methods of their delivery into targeted tissue in proper bioactive form are still being sought. Recently, third-generation PSs have been developed to enhance the treatment by reducing damage to healthy cells and improving PSs bioavailability [104]. These often involve drug delivery systems, gene engineering technologies, or monoclonal antibody receptor targeting [95,105]. Semeraro et al. claim that a good PS should have pure and constant composition, high triplet state yield, high quantum yield of singlet oxygen production, and efficient energy transfer for $^1\text{O}_2$ formation [22,106]. Moreover, for the anticancer treatment, an ideal PS should present limited dark toxicity, selective accumulation in tumor sites (tumor-specificity), high absorption peak between 600 and 800 nm, as well as good solubility to ensure lipophilic properties for membrane crossing and to prevent self-aggregation [100,105–107] (**Figure 1.6**).

It turns out that to produce a potential photosensitizing agent, it is not necessary to perform special chemical syntheses, because nature is full of them. In recent years, a shift towards using natural substances in medicine and pharmacy over traditional synthetic chemotherapeutic drugs is becoming increasingly noticeable, as they are widely available, more environmentally sustainable, therefore considered as “green”, and mostly have no side effects [108]. These natural PSs are produced by algae and plants as a defense strategy against excessive sunlight, which is why they are commonly included in sunscreens and cosmetics for protection [109]. It turns out, however, that these properties can be used in medicine and pharmacy, in particular for PDT in the treatment of cancer (described in more detail in the paragraph **1.2.4.3. Photodynamic therapy (PDT)**). Natural PSs with potential anticancer activity, belonging to the groups of curcumins, furanocoumarins, thiophenes, polyacetylenes, alkaloids and the largest group of anthraquinones, have been characterized by Muniyandi et al. as potential candidates for PDT [110]. Kubrak et al. claim that between 1981 and 2019, around 25% of all newly approved anticancer drugs were obtained from natural products [109]. A great example of photoactive pigments ubiquitous in nature are chlorophylls, present in large quantities in algae from the *Chlorophyta* group, as mentioned earlier. Chlorophylls, due to their features of a good PS, such as a strong photosensitizing effect by high ROS production after irradiation and low toxicity in dark conditions, are classified as natural PSs, and with their several derivatives are nowadays in advanced clinical trials or investigations for the use in PDT [22].

These pigments with a porphyrin structure, occur widely in most plants and algae, and can be obtained quickly, cheaply and environmentally friendly from their biomass [2,71]. Moreover, Abrahamse and Hamblin presented some other examples of naturally occurring PSs such as well-known curcumin, hypericin from the St John's wort, or hypocrellins from the parasitic fungi *Hypocrella bambuase sacc* and *Shiraia bambusicola* [99].

Therefore, the combination of naturally derived compounds with a strong photosensitizing effect, with biocompatible drug delivery system, may constitute an effective therapeutic approach in the form of third-generation natural PS for fighting cancer by PDT, additionally characterized by a sustainable and environmentally friendly approach.

1.1.3.3. Applications in medicine and pharmacy

PSs are used in various therapies, including antimicrobial and anticancer ones, by irradiating the appropriate tissue treated with the PS with light of the appropriate wavelength triggering the production of ROS, which destroy disease cells, the so-called photodynamic therapy (PDT) (described in more detail in the paragraph 1.2.4.3. *Photodynamic therapy (PDT)*) [95]. In addition, these compounds can fulfill diagnostic functions due to their fluorescence ability, e.g., in cell bioimaging [111]. As already mentioned, due to the presence of a specific porphyrin chain in chlorophylls structure, they demonstrate the ability to generate ROS, which is a potential application in research on the treatment of cancer by PDT [14,22]. Due to certain limitations of the PSs related to their chemical nature, which will be discussed in more detail in the next chapter, various types of drug delivery systems are used to protect them and enhance their action (**Table 1.3**).

Table 1.3. Various types of drug delivery systems for photosensitizers encapsulation, along with their potential applications.

Photosensitizer	Drug delivery system	Application	References
curcumin-supported Methylene Blue	bilosomes	antimicrobial phyto-photodynamic activity	[112]
verteporfin or meso-tetraphenylporphyrin	multilayer nanocapsules	PDT	[113]
Rose Bengal	multiple nanoemulsions	NIR-induced imaging and PDT, theranostics	[114]

chlorin e6 or meso-tetraphenylporphine-Mn (III) chloride	cubosomes	PDT and bioimaging of skin malignant melanoma	[115]
daunorubicin	cubosomes	NIR-Induced imaging and PDT against human skin melanoma (MeWo) and ovarian (SKOV-3) cancer cells, theranostics	[116]
chlorophyll a	cyclodextrin supramolecular complex	PDT of colorectal adenocarcinoma (HT-29 cell line)	[22]
chlorophylls, carotenoids (extract)	chitosan-based hydrogel	potential targeted therapy and bio-imaging for breast carcinoma (MDA-231 cell line)	[23]
chlorophyll a (extract)	polyamidoamine-based hydrogel	PDT	[14]
chlorin e6	liposomes	PDT, diagnostics, theranostics	[117]
indocyanine green	liposomes	dual-mode imaging and photodynamic/photothermal therapy of ovarian cancer	[118]
curcumin	nanoemulsion	PDT against cervical carcinoma	[119]
acitretin	niosomes (nanovesicular gel)	antipsoriatic activity (<i>ex vivo-in vivo</i>) in HaCaT cells (a keratinocyte cell line)	[120]
ferrous chlorophyllin	transethosomes	PDT against resistant animal melanoma (B16-F10 cells)	[121]
ruthenium (II) polypyridyl complexes with π -expansive ligands	cubosomes	PDT against non-melanoma skin cancer	[122]
diketopyrrolopyrrole-porphyrin	cubosomes	chemo- and photodynamic synergistic cancer therapy	[123]
indocyanine green + doxorubicin	cubosomes	chemo-photothermal therapy of melanoma	[124]

For instance, Rizzi et al. enclosed chlorophyll a extract obtained from *Spirulina* algae in polyamidoamine-based hydrogel scaffold for potential application in PDT [14], as well as Semeraro et al., who have studied the same pigments for PDT, but encapsulated in cyclodextrin supramolecular complexes [22]. In addition, these algal pigments loaded into chitosan were also investigated for diagnostic purposes, due to their fluorescence ability, in bioimaging for breast carcinoma [23].

As for caulerpin, the pigment characteristic for *Caulerpa* spp., many works have been published regarding the verification of its bioactivity. It has been reported that caulerpin presents antinociceptive and anti-inflammatory activities [27], antimicrobial [28], antiviral [43,125], and anticancer [88,126] as well. Some papers have been published to understand the origin of these caulerpin bioactive properties, for instance by presenting its ability to affect mitochondrial health, various metabolic and stress pathways through interaction with signaling proteins like AMPK, PTP1B or HIF-1, as well as cell cycle progression [9,44]. According to Liu et al., caulerpin can disrupt mitochondrial ROS-regulated HIF-1 activation and HIF-1 downstream target gene expression by inhibiting the transport or delivery of electrons to complex III [44]. As proved by Cheng et al., this anticancer strategy based on overcoming *Hypoxia-Induced Resistance* mechanism may be responsible for sensitizing cancer cells to the action of a potential drug [127]. Another anticancer strategy may be related to caulerpin's PTP1B inhibitory activity [128,129], since Xu et al. proved in 2019 that inhibition of PTP1B protein blocks pancreatic cancer progression by targeting the PKM2/AMPK/mTOC1 pathway [130].

1.1.3.4. Limitations

Unfortunately, the effectiveness of extracted pigments is sometimes limited due to their potential instability, especially in the case of photosensitive compounds (see **Figure 1.7**). This is most often due to their high sensitivity to temperature, light and oxygen [2,131]. Moreover, due to their hydrophobic nature, when exposed to an aqueous environment, they can quickly aggregate or degrade, losing their biological properties [14]. Thus, to properly deliver these compounds to pathological tissues in their active monomeric form, there is a need to find suitable carriers for them capable of ensuring the solubilization of encapsulated active substances, as well as protection against their aggregation or degradation in the cellular environment. Referring to the features of the ideal PS described above (paragraph **1.1.3.2. Natural photosensitizers**), it is worth mentioning that the increasingly researched third-generation PSs based on nanotechnology, thanks to the unique structure of the nanocarriers

or their modifications, present features such as better tissue selectivity (targeting ability) or improved membrane crossing [116,132], allowing to bypass the above-mentioned limitations, which often occur in the case of compounds in free form, and at the same time getting closer to design an ideal PS.

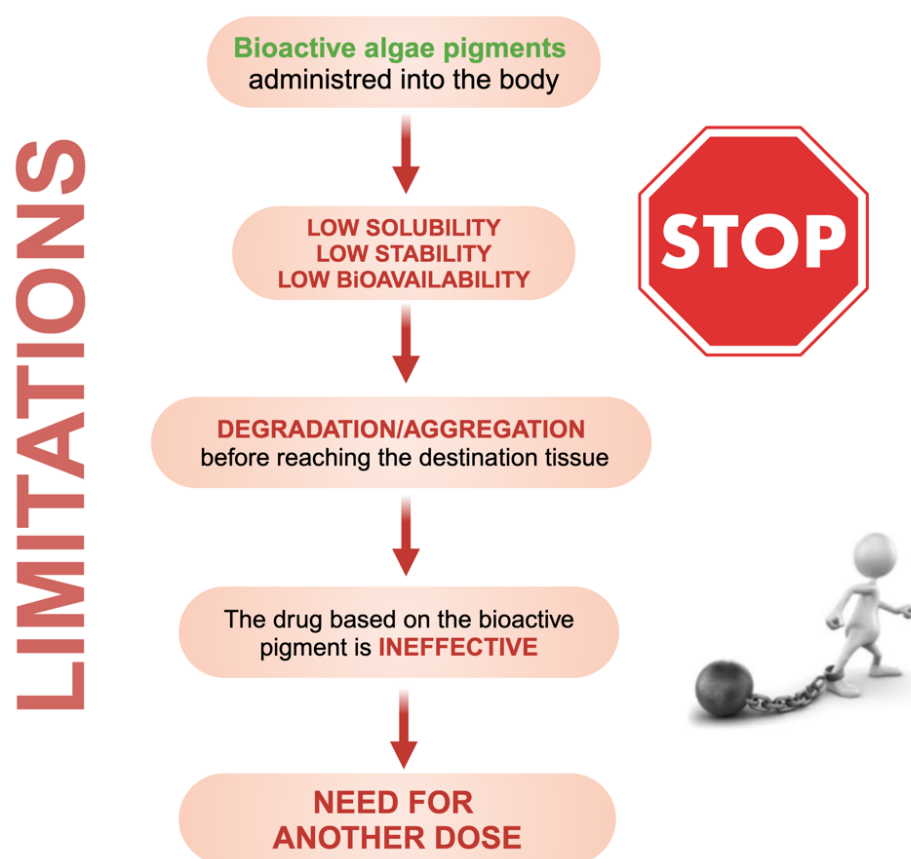


Figure 1.7. Limitations of the bioactive algae pigments.

1.2. Surfactants and self-assembly

The concept of **self-assembly** is gaining traction across various disciplines, each with its own focus and perspective. In chemistry, self-assembly is typically associated with energy minimization and thermodynamic equilibrium [133]. Israelachvili defines it as the spontaneous, thermodynamically driven organization of atoms and molecules into multimolecular structures [134]. The term itself reflects this nature: *self*, meaning an independent process occurring without external intervention, and *assembly*, referring to the formation of structures from simpler building blocks [135]. Historically, the first observation of self-assembled monolayers was recorded by Benjamin Franklin in 1774 when he noted the spreading of oil on water. This was followed by the emergence of nanochemistry in 1818, when the color change of

colloidal gold was recognized as dependent on particle size [136]. In nanochemistry, self-assembly allows atoms, molecules, or nanoscale components to spontaneously organize into ordered structures without human intervention, representing one of the most promising and cost-effective approaches for nanofabrication [135]. Nature offers many examples of self-assembled structures, where stability over time is ensured by building blocks such as phospholipids, nucleic acids, and peptides. These structures form through thermodynamically driven processes that harness energy released during nutrient breakdown in catabolic reactions [137]. The self-assembly of biological components has inspired scientists to design artificial systems based on this principle [138]. In the realm of soft matter and colloidal nanotechnology, self-assembly leads to the formation of diverse nanostructures, primarily driven by the hydrophobic effect of amphiphilic compounds, known as surfactants [139].

Surfactants, or surface-active agents, accumulate at interfaces to minimize unfavourable interactions, thereby altering properties such as surface tension [140]. They can be active at solid-liquid, liquid-liquid, and liquid-gas interfaces [141,142]. Most surfactants dissolve in at least one liquid phase, typically water [143]. Their behaviour is dictated by their amphiphilic nature, characterized by a hydrophilic (polar) head and a hydrophobic (hydrocarbon) tail [144] (see **Figure 1.8A**). The term *amphiphilic* originates from the Greek words *amphi* (“on both sides”) and *philos* (“loving” or “fond of”), highlighting the dual affinity of these molecules - one part interacts with the solvent while the other does not (**Figure 1.8B**).

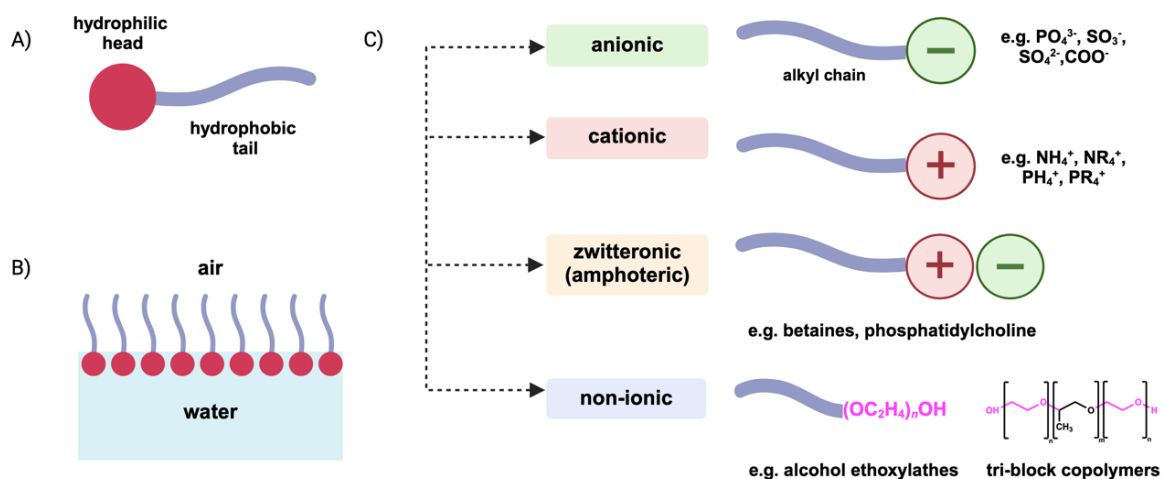


Figure 1.8. Schematic representation of the surfactant structure (**A**) arrangement of the surfactants molecules at the air-water interface (**B**) and the division of surfactants with some examples (**C**).

In aqueous environments, surfactants self-assemble due to the hydrophobic effect, forming aggregates at the critical micellar concentration (CMC), reducing unfavorable interactions with water [145,146]. Based on the charge of their hydrophilic head, surfactants are categorized as anionic, cationic, non-ionic, or zwitterionic [143] (**Figure 1.8C**). The simplest self-assembled structures are micelles (**Figure 1.9**), but various aggregates can form, depending on the molecular geometry and the balance between hydrophilic and hydrophobic moieties [147–149]. The supramolecular organization of surfactant aggregates is primarily dictated by the critical packing parameter (P), a geometric factor defined as:

$$P = \frac{v}{a_0 l_c} \quad (\text{Eq.1})$$

where v is the volume of the hydrophobic chain, a_0 is the area of the headgroup, and l_c is the critical length of the hydrocarbon chain, approximately its fully extended length [150,151].

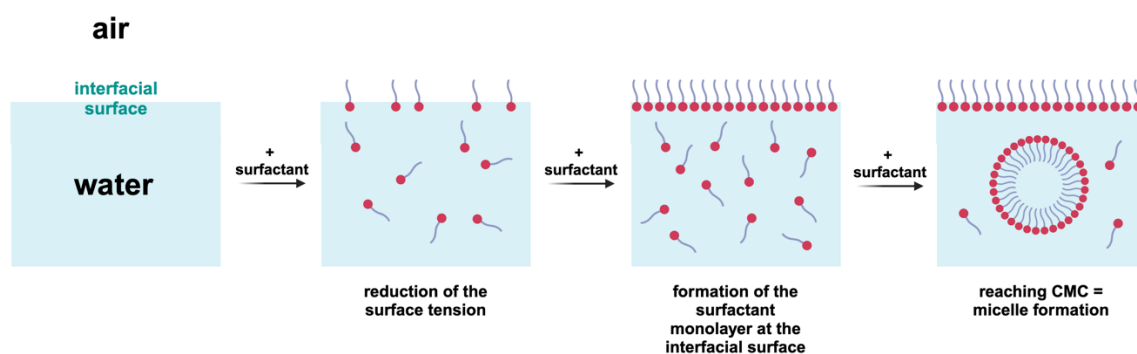


Figure 1.9. Schematic representation of the micelle formation by self-assembly of surfactant molecules upon reaching CMC.

Surfactants are abundant in nature and play essential roles in biological systems. Examples include phospholipids, which constitute cell membranes, fatty acids in sebum and intestines, and bile salts in the gallbladder [143]. Their widespread application extends to the cosmetic and chemical industries, where they are key ingredients in detergents, emulsifiers, and cleaning products, owing to their surface-active properties [152].

1.2.1. Lyotropic liquid crystalline (LLC) phases

The term liquid-crystalline is related to the nanostructure's features, typical for solid crystals – long-range organizational order, and at the same time, some fluidity characteristics related to liquids [146]. In this thesis, we will focus on **lyotropic liquid crystals** (LLC) rather than thermotropic liquid crystals. The former form in the presence of a solvent, typically water, and

their structure and phase behaviour depend on the concentration of amphiphilic molecules (such as surfactants or lipids) and the solvent. Differently, thermotropic liquid crystals form purely as a function of temperature without requiring a solvent, and they transition between different liquid crystalline phases (e.g., nematic, smectic, or cholesteric) (see **Figure 1.10**) when heated or cooled. Usually, they consist of rod-like (calamitic) or disc-like (discotic) organic molecules, which exhibit thermotropic liquid crystalline behaviour due to molecular anisotropy.

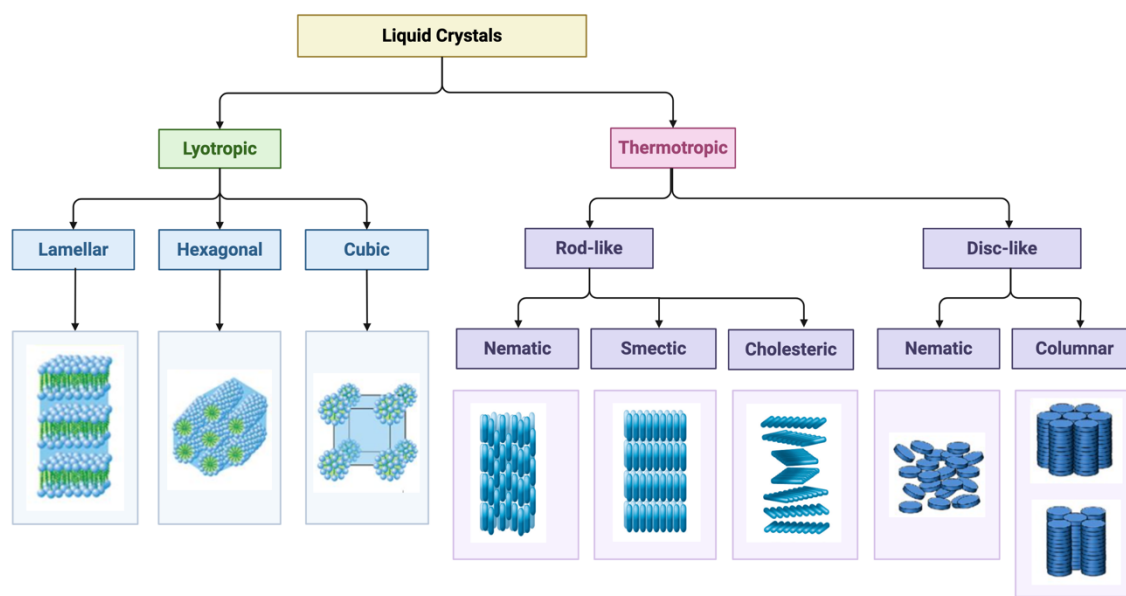


Figure 1.10. Classification of liquid crystals. Own elaboration reproduced with permission from [153,154].

The final LLC phase architecture depends on the mutual interactions between the building components and the surfactant's shape and concentration. The most characteristic and well-studied LLC phases are lamellar, hexagonal and bicontinuous cubic [146,151,155]. The lamellar phase consists of one-dimensional stacked bilayers, while in the hexagonal one, water cylinders encased by a lipid monolayer are arranged in a two-dimensional hexagonal pattern [151]. Bicontinuous cubic phases are constituted by a curved bicontinuous, nonintersecting lipid bilayer organized in three dimensions, forming two continuous, interpenetrating, but non-interconnected water channels [146]. Particularly, the lipid bilayer is draped on mathematical surfaces known as *infinite periodic minimal surfaces* (IPMSs), with the hydrophilic heads facing outward, while water molecules occupy the maze-like system on both sides [146,151]. Three types of IPMSs are important for lipid self-assembly in water: the primitive (body-centered lattice, $Im3m$), the double diamond (primitive lattice, $Pn3m$), and the double gyroid (body-centered lattice, $Ia3d$) (see **Figure 1.13B** in the paragraph 1.2.2.1. *Cubosomes*) [155].

LLC phase can be formulated as colloidal dispersions, and in the next paragraphs will be presented two non-lamellar LLC nanoparticles, cubosomes and hexosomes, characterized by an inner nanostructure of the inverse bicontinuous cubic or inverse hexagonal type, as well as an example of the dispersion of a less organized LLC phase, so-called “melted cubic phase” – the sponge nanoparticles.

1.2.2. Non-lamellar LLC nanoparticles

Lipid-based LLC nanoparticles (LLCNPs) are nanostructures formed through the self-assembly of amphiphilic lipid molecules in water, driven by the hydrophobic effect [156]. Initially, they organize into LLC bulk phases, which are then fragmented through energy input, resulting in LLCNPs – non-viscous, diluted colloidal dispersions of LLC phases in water [151]. An example of the lipid able to form different LLC phases by self-assembly in water depending on its concentration (**Figure 1.11**) [157], and widely used (also in this thesis) as the main building block of the LLCNPs, is glycerol monooleate (commonly known as monoolein, MO) [158].

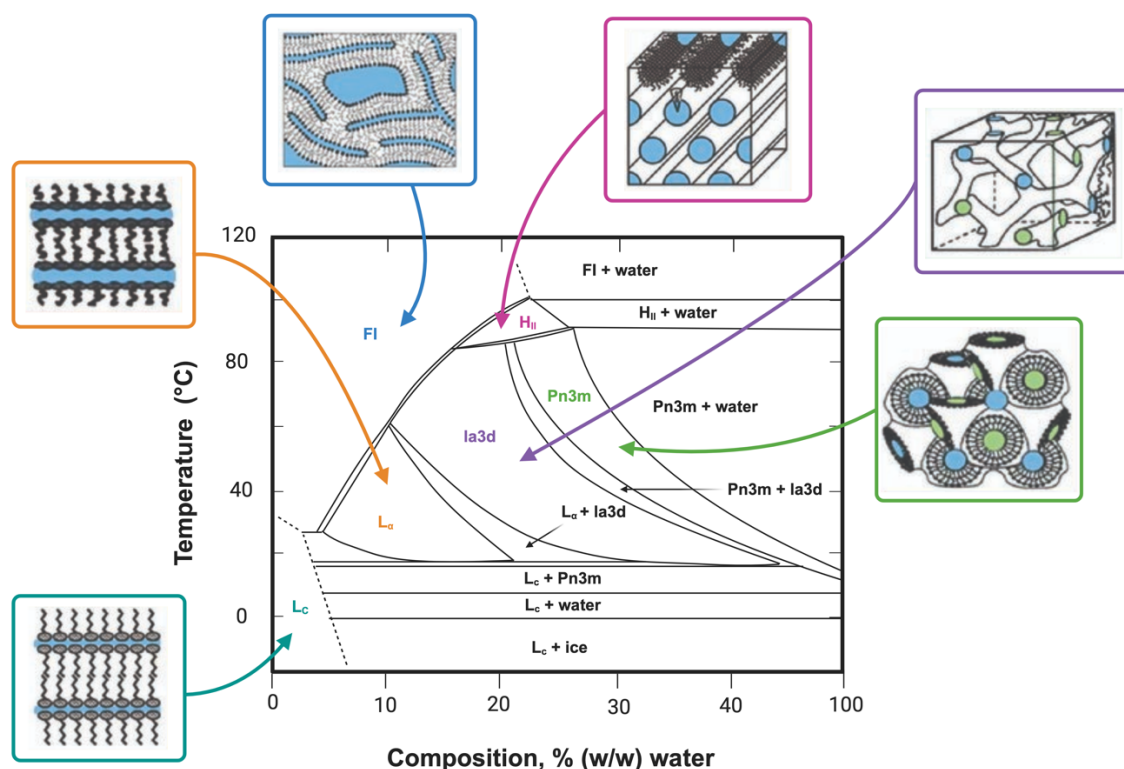


Figure 1.11. MO/water system temperature-composition phase diagram with the schematic representation of various phases formed, namely the lamellar crystal (L_c), fluid isotropic phase (FI), and several LLC phases: lamellar liquid crystal (L_α), inverse hexagonal (H_{II}), double gyroid (Ia3d) and double diamond (Pn3m) bicontinuous cubic phases. Own elaboration reproduced with permission from [157,159].

In contrast to the LLC phases, the LLCNPs are only kinetically stable, not thermodynamically. Therefore, to maintain the thermodynamic stability of such dispersions and avoid their flocculation or coalescence, LLCNPs are stabilized by various molecules, such as well-studied non-ionic tri-block copolymers known as Pluronics, with the most common Pluronic F127 [160,161]. Nevertheless, other approaches have been reported to stabilize those nanosystems, namely polysorbate 80 (Tween 80) [162], brush copolymers [163], PEGylated-phytanyl copolymers [164], citrem-phospholipids [165] or lipidated polymers [166]. Some stabilizers, such as Pluronics, tend to interact with MO, and therefore, apart from Pn3m formed by MO, they originate a second cubic phase, namely Im3m [161], resulting in two LLC phases coexisting in the nanosystem. Since Pluronics does not biodegrade *in vivo* and in case of sensitive patients, may induce a cardiopulmonary distress [165], other compounds have been introduced to lower the cytotoxicity of the developed dispersions [167–169], with the example in polyphosphoesters tested by Fornasier et al. in 2020, as well as increase their biodegradability [170] and biocompatibility [115,171], such as recently developed cubosomes and hexosomes stabilized by sorbitan monooleate (Span 80, Sp80), and tuned with the bile salt, sodium taurocholate (TC) for topical administration [172], which will be described in more detail in Chapter 3 referred to **Paper I** [171]. Given the above information, **Figure 1.12** represents the molecules used for the preparation of the non-lamellar LLCNPs described in the thesis.

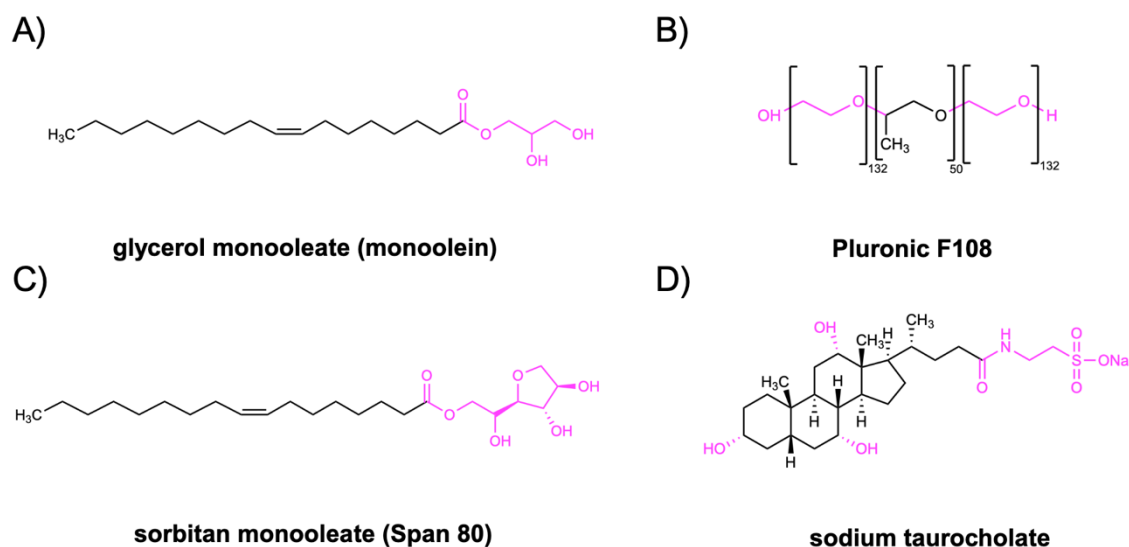


Figure 1.12. Chemical structures of the non-lamellar LLCNPs' components used in this thesis: glycerol monooleate (**A**) tri-block copolymer Pluronic F108 (**B**), sorbitan monooleate (**C**) and sodium taurocholate (**D**).

Overall, the final structure of LLCNPs is influenced by temperature and additives, such as salts, affecting ionic strength and leading to changes in the final P value of the nanosystem. It is important since the final topology may influence their properties, and thus differentiates their applications.

1.2.2.1. Cubosomes

The colloidal dispersion of the inverse bicontinuous cubic LLC phase (Q_{II}) in water with the use of suitable surfactants and stabilizers results in nanostructured systems, referred to as cubosomes, having sizes ranging from 100 to 400 nm [146]. Inverse bicontinuous cubic phases have been observed to form spontaneously in mitochondrial membranes, as well as in cells that are stressed or infected by viruses, playing a crucial role in understanding vital processes such as cell fusion and food digestion [156,173]. These honeycomb-like structures comprise a curved three-dimensional bicontinuous lipid bilayer, forming two continuous and not-interconnected water channels, that can be exploited to encapsulate various bioactive ingredients, such as chemical drugs, peptides and proteins (see **Figure 1.13A**).

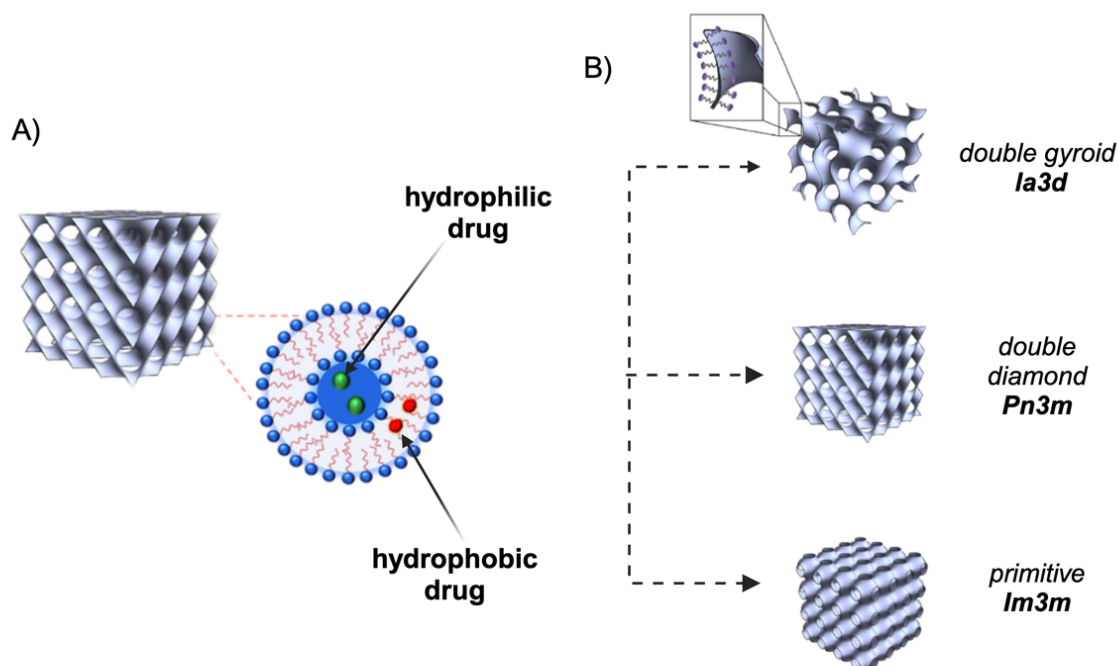


Figure 1.13. Schematic representation of the cubosome structure with hydrophobic and hydrophilic drug encapsulated within (A); and the three types of typical cubic IPMSs (B). Own elaboration reproduced with permission from [146].

1.2.2.2. Hexosomes

Inverse hexagonal phase (H_{II}) characterizes another example of LLCNPs, called hexosomes. For drug delivery applications in nanomedicine, the hydrophilic regions can encapsulate various water-soluble molecules, while the large hydrophobic portion can accommodate hydrophobic drugs [174]. Beyond conventional drugs, hexosomes can also, in principle, encapsulate larger biomolecules, including peptides, proteins, and nucleic acids such as DNA or siRNA [175,176]. Similarly to cubosomes, their hydrodynamic diameter is typically in between 100 nm and 400 nm, thus suitable for intravenous administration [146].

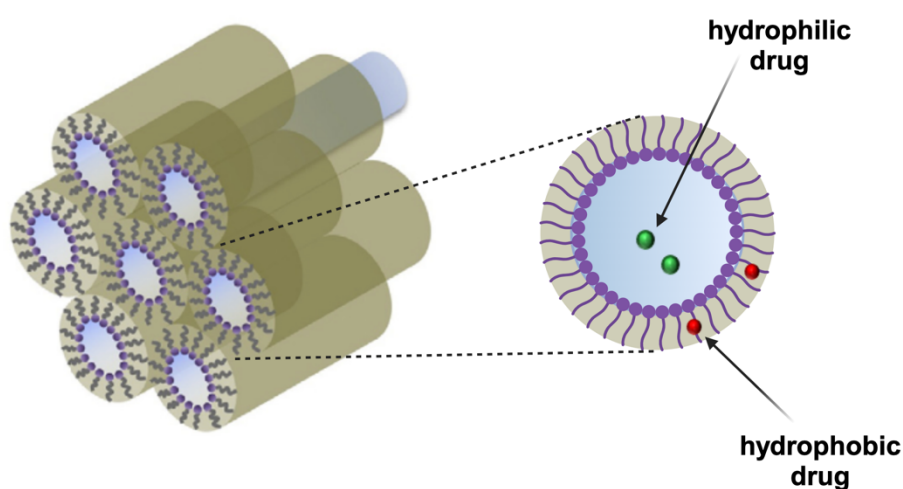


Figure 1.14. Schematic representation of the hexosome structure with hydrophobic and hydrophilic drugs encapsulated within. Own elaboration reproduced with permission from [146].

1.2.2.3. Sponge nanoparticles

A less ordered cubic phase, also referred to as a “melted bicontinuous phase”, is known as the sponge (L_3) phase [151]. When dispersed in water, the L_3 phase forms sponge nanoparticles [177,178]

Similar to the cubic phase, the L_3 phase consists of a non-intersecting bicontinuous bilayer and two unconnected water channels. However, it is less organized, lacking long-range order (see **Figure 1.15**). This structure allows it to accommodate large amounts of water due to its large aqueous pores (with diameters up to 13 nm) and provides high fluidity, making it suitable for encapsulating large hydrophilic molecules, such as proteins [151,178].

The L_3 interface is highly flexible and thermosensitive. Upon heating, its long-range order is disrupted, causing the interface to lose its lattice arrangement [179]. Barauskas et al. studied L_3 dispersions and concluded that sponge nanoparticles have a complex, disordered, and mostly dense inner core, surrounded by a spongy surface composed of intersecting lamellae, with an average size ranging from 100 to 150 nm [177]. Larger L_3 nanoparticles, measuring between 100 and 250 nm, can be obtained [178].

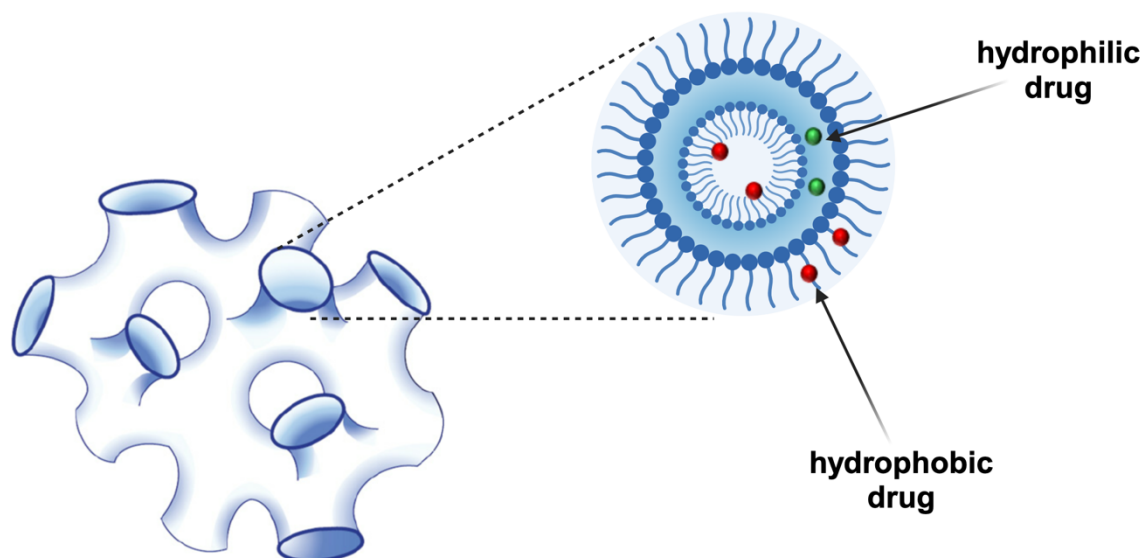


Figure 1.15. Schematic representation of the sponge nanoparticle structure with hydrophobic and hydrophilic drugs encapsulated within. Own elaboration reproduced with permission from [180].

1.2.3. Applications

LLC phases are highly attractive for pharmaceutical and nanomedicine applications, particularly as advanced drug delivery systems (see **Figure 1.16**) [146,151]. Particularly, non-lamellar LLCNPs provide larger hydrophobic surface area per unit of volume compared to traditional liposomes [181], and therefore offer possible higher encapsulation efficiency [182]. These nanocarriers may deliver bioactive cargo to the target disease tissue, protecting it from the degrading cellular environment, and therefore increasing its stability and bioavailability, as well as, in the case of water-insoluble compounds, preventing their aggregation [146,183]. An increased absorption of a potential drug into cellular structures is associated with the biocompatible construction of the nanoparticles [184], which are capable of penetrating cell membranes and controlling the release of the drug inside the cell [185,186].

As already mentioned, LLCNPs are often prepared with monoolein, a monoglyceride that is easily biodegraded by lipases in the cellular environment [187]. LLCNPs enable the simultaneous encapsulation of two bioactive cargos within hydrophobic and hydrophilic compartments. This dual loading can enhance drug efficacy by leveraging synergistic effects, ultimately improving the therapeutic potential of formulations that combine both compounds within a single nanocarrier [186].

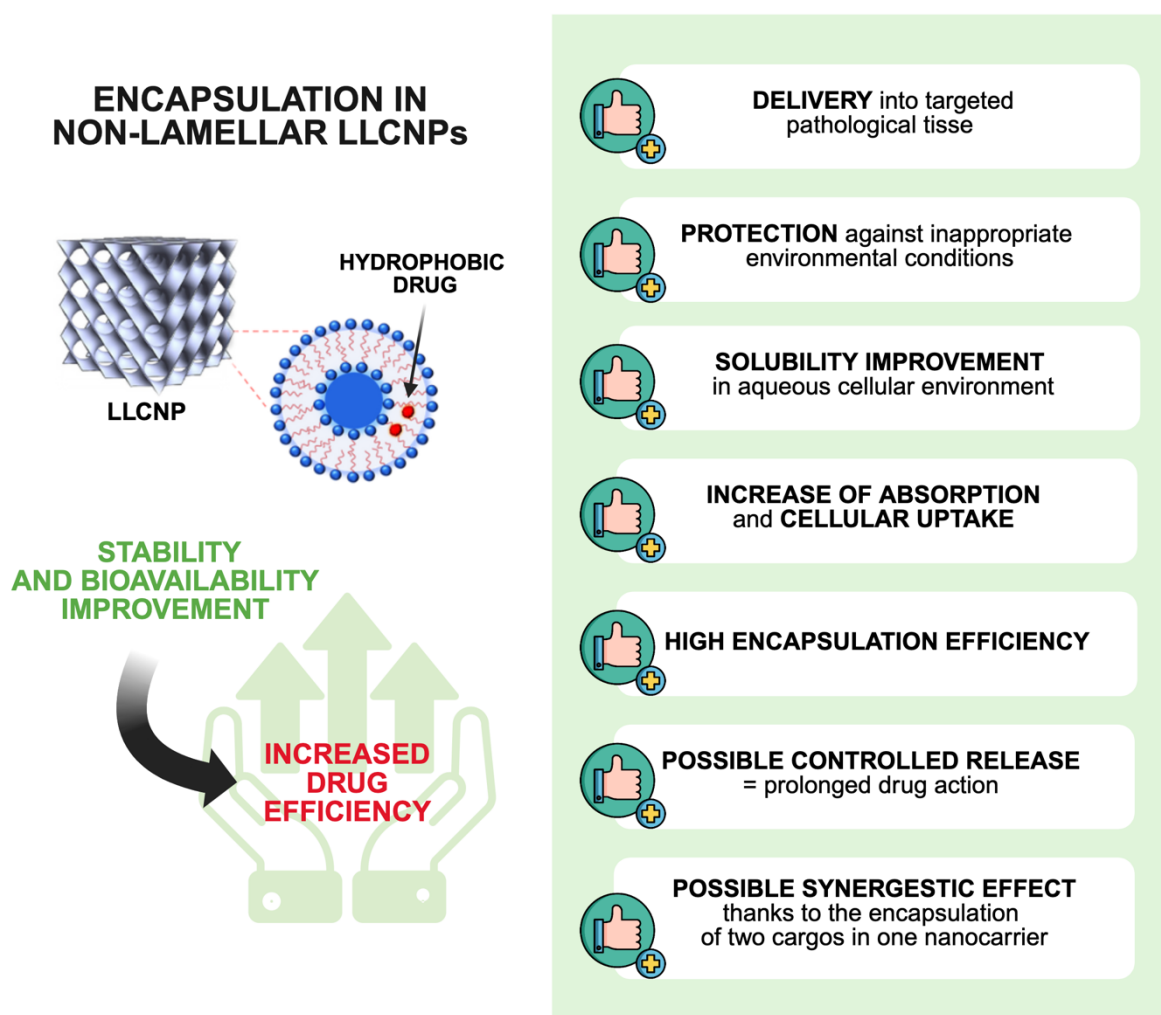


Figure 1.16. Advantages of potential drug encapsulation in non-lamellar LLCNPs-based nanocarriers.

Due to the unique characteristics of tumor blood vessels and the appropriate size of the nanocarriers (20–200 nm), LLCNPs can passively accumulate in tumor tissues, so-called *Enhanced Permeability and Retention* (EPR) effect (see **Figure 1.19** in paragraph 1.2.4.4. *Theranostics*) [188,189]. This effect results from the rapid and irregular growth of tumor blood vessels, which feature unusually large pores between endothelial cells, enabling the particles to pass through due to enhanced permeability of tumor vessels, in contrast to normal blood

vessels [190]. Moreover, the dysfunction of lymphatic drainage (poor convective flow of interstitial fluids) in tumors hinders the effective removal of particles, leading to their extended retention. Therefore, the EPR effect is used in targeted drug delivery and cancer therapy to increase the concentration of therapeutic agents at tumor sites [189,191].

Non-lamellar LLCNPs have been also proposed as anticancer drug delivery nanosystems, [116,186,192] with possible theranostic applications [193–195] (described in more detail in paragraph **1.2.4.4. Theranostics**).

1.2.3.1. Bioimaging

It is common knowledge that early cancer detection significantly increases the chances of the treatment. Nowadays, in medical diagnosis, the main techniques used are based on optical imaging (fluorescence or luminescence-based) for biological tissue visualization both *in vitro* and *in vivo*, as well as magnetic resonance imaging appropriate especially for soft tissues [196]. Nevertheless, fluorescence microscopy is currently one of the most powerful non-invasive diagnostic techniques used for various purposes. The continuous improvement of this method, such as combination with increasingly developing nanotechnology or different therapies, results in advanced approaches, with the example of an image-guided cancer surgery [196–198]. The action of fluorophores (fluorescent probes) used in optical imaging is based on the emission of light of a higher wavelength after absorption of the radiation with higher energy (lower wavelength) [199]. In addition to the targeted killing of cancer cells, a very important application of non-lamellar LLCNPs is also their diagnostic approach in the form of cancer bioimaging, due to the ability of these nanoparticles to encapsulate imaging agents such as fluorescent probes [146,151]. As an example, Murgia et al. in 2013 developed fluorescent cubosomes for single living cell bioimaging co-loaded with a potential anticancer drug [182], thus ultimately combining diagnostic and therapeutic effects (theranostics approach), proved as an efficient potential application of the non-lamellar LLCNPs through *in vivo* tests by Muir et al. in 2012 [200] (described in more detail in paragraph **1.2.4.4. Theranostics**). This approach is very often studied for application in anticancer nanomedicine with the use of LLCNPs, due to the diverse possibilities offered by these nanostructures [182,186,195,201]. An encapsulation in proper nanocarrier allows to overcome the limitations such as low photostability or low solubility in aqueous cellular environment of the diagnostic imaging agent in its free form [196], additionally enabling the targeted action with improved cell selectivity, thanks to the specific modifications in the structure of nanocarrier [116,194].

1.2.3.2. Examples of anticancer therapies

There are currently three main traditional approaches to cancer treatment: chemotherapy, radiotherapy and surgery. Chemotherapy is based on the ability of cytostatic drugs to kill or inhibit the growth of cancer cells, but non-selectively, which can also lead to destruction in healthy tissues, leading to various side effects [202]. Radiotherapy uses high-energy radiation (X-rays) to damage or destroy cancer cells [203]. It can be targeted at specific areas of the body where cancer cells are located, helping to shrink or eliminate tumors, unfortunately, just like in the case of chemotherapy, very often destroying neighboring healthy tissue. Surgery, an approach with high invasiveness, involves the physical removal of cancerous tissue, used for localized, mostly early-stage tumors and aims to eliminate as much of the cancer tissue as possible, therefore not guaranteeing complete removal [202,203]. As mentioned above, many of these therapies are characterized by limitations related to, among others, lack of tumor-selectivity, drug resistance, risk of cancer recurrence and metastasis, long time action, high costs, or inadequacy of the treatment method for the patient or type of cancer [202,204]. In addition, very often, short-term and long-term side effects are an inherent element of these therapies, such as weight loss, hair loss, vomiting, heart problems, or e.g. post-radiation dermatitis associated with radiotherapy [202,203,205]. Therefore, new types of anticancer treatments are being developed, such as PDT (described in more detail in the paragraph 1.2.4.3. *Photodynamic therapy (PDT)*), which bypass many of the limitations of traditional therapies, namely it is characterized by low invasiveness and low associated risk of side effects, short time, as well as possibility of combining with other techniques, giving e.g. selective targeting (protection of healthy tissues) after coupling with nanocarriers [206] (see **Figure 1.17**).

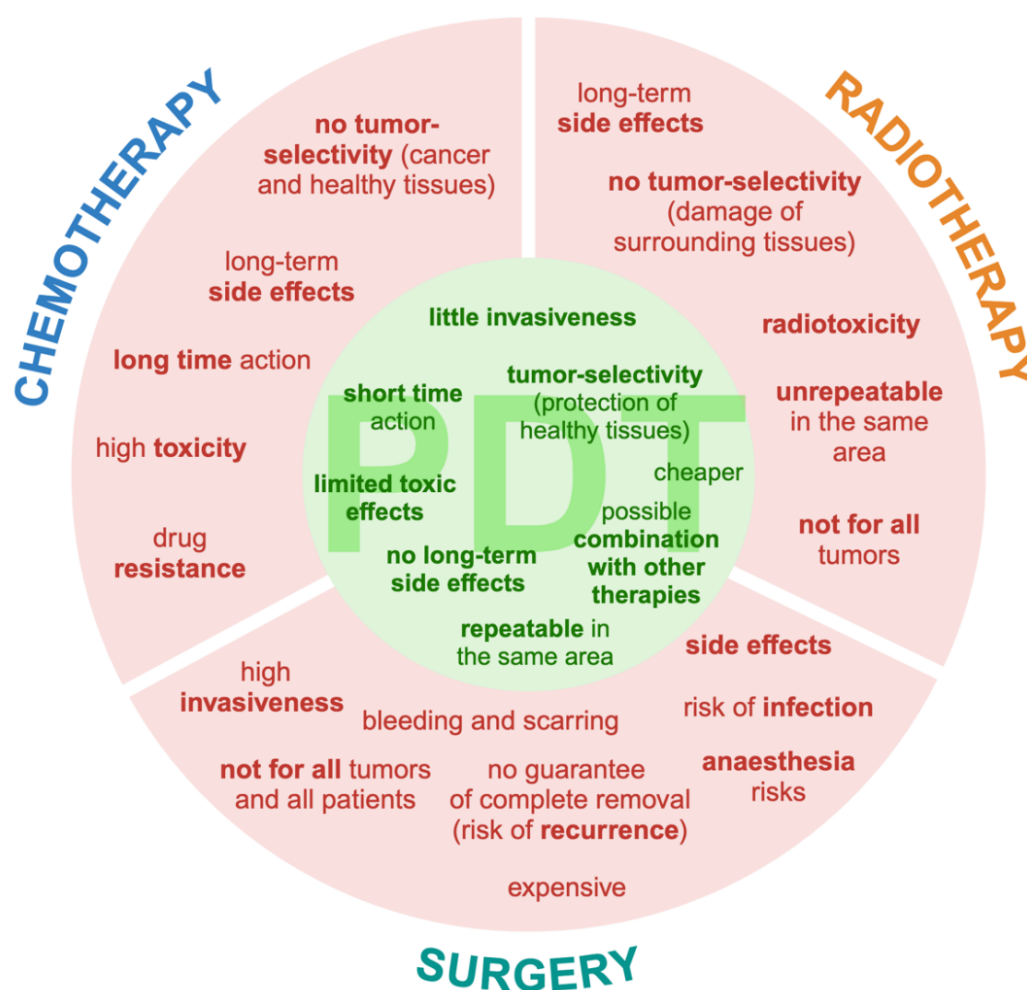


Figure 1.17. Disadvantages of traditional anticancer therapies in comparison with some advantages of PDT.

Traditional anticancer chemotherapies are often ineffective and additionally characterized by several side effects caused by their harmfulness not only against cancer cells but also against healthy ones. Therefore, in the era of enormous scientific development, innovative solutions are sought to effectively fight cancer with the elimination of side effects, among which stand-out techniques such as PDT, very often combined with nanotechnology, which together provide a potential anticancer approach with increased biocompatibility and bioavailability, for diagnostic and therapeutic applications. Non-lamellar LLCNPs are increasingly used in research on potential anticancer drugs (see **Table 1.4**).

Table 1.4. Examples of various non-lamellar LLCNPs assessed in anticancer activity studies.

Type of non-lamellar LLCNPs	Bioactive cargo	Anticancer activity evaluation	References
cubosomes	doxorubicin + indocyanine green	improved cellular uptake, cytotoxicity <i>in vitro</i> , and improved <i>in vivo</i> anti-tumor efficacy through the synergistic effects of chemo-photothermal therapy on melanoma tumor (B16F10) – bearing C57BL/6 mice	[124]
cubosomes	ruthenium (II) polypyridyl complexes with π -expansive ligands	improved cytotoxicity and photoactivity against non-melanoma skin cancer – <i>in vitro</i> studies on A431 cells	[122]
cubosomes	daunorubicin + Er^{3+} , Yb^{3+} codoped NaYF_4	improved cellular uptake and NIR-induced photodynamic activity – <i>in vitro</i> cytotoxicity studies, F-actin bioimaging of human skin melanoma (MeWo) and ovarian (SKOV-3) cancer cells	[116]
cubosomes	cisplatin + paclitaxel	impairment of HeLa cells (cervical cancer) related to the reduction in impedance in cells proved by impedance measurement and fluorescent bioimaging – <i>in vitro</i> studies	[186]
cubosomes	ruthenium (II) polypyridyl complex	significant phototoxic activity against Calu-3 (lung cancer cell line) – cytotoxicity studies	[207]
cubosomes	chlorin e6 or meso-tetraphenylporphine-Mn (III) chloride	significant photodynamic activity against Me45 and MeWo melanoma cell lines – bioimaging, cytotoxicity	[115]
cubosomes	genistein + frankincense oil	selective anticancer activity on pancreatic cancer cells (PANC-1), cellular uptake and anti-migratory effect – <i>in vitro</i> cytotoxicity + <i>in vivo</i> studies (tumor growth suppression)	[208]
cubosomes	panobinostat	improved cellular uptake and cytotoxicity against HER2-	[209]

		positive breast cancer cells (SKBR3) – <i>in vitro</i> studies	
cubosomes	quercetin + modified fluorescein/dansyl (fluorescent probe)	-	[182]
cubosomes	docataxel	significant <i>in vitro</i> cytotoxicity and cellular uptake studies of HeLa cells (cervical cancer)	[195]
hexosomes	fluoxetine hydrochloride	improved cellular internalization and cytotoxicity against HepG2 cells (hepatocellular carcinoma) – <i>in vitro</i> studies	[210]
hexosomes	gemcitabine + thymoquinone	improved <i>in vitro</i> cellular uptake and cytotoxicity against breast cancer cells (MCF7)	[211]
cubosomes, hexosomes	<i>Brucea javanica</i> oil + doxorubicin hydrochloride	enhanced antitumor efficacy against MCF-7 cells; potential reversal of the doxorubicin resistance in MCF-7/DOX cells (MCF-7 cells with doxorubicin resistance) – <i>in vitro</i> cytotoxicity and apoptosis studies	[212]
spongosomes	<i>Brucea javanica</i> oil + baicalin	improved efficacy of the multi-drug delivery in comparison to the single-drug delivery, against A549 cells (lung cancer) – <i>in vitro</i> cytotoxicity and apoptosis studies	[213]
spongosomes, cubosomes	curcumin + Fish oil	neuroprotective potential against H ₂ O ₂ -induced oxidative stress in differentiated SH-SY5Y cells (neuroblastoma cell line from a metastatic bone tumor) – <i>in vitro</i> cytotoxicity and apoptosis studies	[214]
spongosomes	<i>Brucea javanica</i> oil	enhanced anti-tumor efficiency against A549 cells (lung cancer) – <i>in vitro</i> cytotoxicity and apoptosis studies	[215]

Unfortunately, the source of treatment failure is not always the therapy itself. A very common limitation is an extremely high resistance of a given type of malignancy associated with a specific tumor microenvironment, like in some types of pancreatic cancer, which is on the list of most lethal cancers with one of the lowest survival rates [216,217]. Many papers concerning this unique resistance have been published to explore the multitude of mechanisms behind it [201,216–219]. Pancreatic cancer is characterized by extraordinary resistance to most conventional treatment methods [220], due to its resilience related to its remarkable capacity to orchestrate a special tumor protective microenvironment full of different components with a huge impact on cancer cell biology, and therefore on treatment resistance [201,218]. One of the pancreatic cancer cells' mechanisms described by Chand et al. in 2016 called *Hypoxia-Induced Resistance*, is related to the fact that the nutrient and oxygen-poor tumor microenvironment imparts a selective pressure, favoring the growth of the most aggressive and fit pancreatic cancer cells, tends to be the most elusive to cytotoxic chemotherapeutic agents [216]. To overcome the harsh stress imposed by chronic hypoxia, pancreatic cells orchestrate a multifaceted response by activating hypoxia-inducible factors (HIFs), special protective proteins able to regulate tumor cell biology [221]. It turns out that this transcriptional response is involved in drug resistance, demonstrated in 2014 by Cheng et al., who proved that under hypoxic conditions, siRNA inhibition of either HIF-1 α or NF κ B, have sensitized pancreatic cells to gemcitabine, a commonly used cytostatic drug [127]. Taking into account the fact that the mechanism described here is only one of many reasons for the exceptional resistance of pancreatic cancer cells, as well as their ability to acquire or exert these mechanisms [216], poor patients' prognosis due to resistance to many types of therapies can be understood. Scientists are studying different types of potential anticancer treatments, some of them are based on targeting specific pathways involved in the tumor development. Namely, as already mentioned in the previous part of the Introduction (paragraph *1.1.3.3. Applications in medicine and pharmacy*) it has been reported that pancreatic cancer progression can be blocked through the inhibition of PTP1B protein by targeting the PKM2/AMPK/mTOC1 pathway [130]. Throughout encapsulation in nanocarriers, bypassing the complex tumor microenvironment and delivering the drug in an active form to the target cell can significantly affect the effectiveness of therapy. Although, many works are describing the potential use of nanocarriers in the treatment of pancreatic cancer [201], including many works about liposomes [222,223], there is still a gap in research considering the application of the non-lamellar LLCNPs in this field. To the best of my knowledge, there is only one recently published work regarding the potential use of cubosomes in pancreatic cancer treatment [208].

Apart from the genetic factors, frequent exposure to sunlight without proper skin protection is considered as a major risk factor for the highly dangerous skin cancer called melanoma, which can spread very quickly to other tissues causing metastases [224]. According to data from 2018, melanoma causes 55,500 deaths per year, considering differences resulting from the level of health care in each place in the world, and therefore, the possibility of early detection [225–227]. Only in the last 20 years the scientific advancement allowed for the development of new treatment methods that offer hope for stopping metastases, long-term tumor suppression, extending relapse-free survival, improving prognosis, and thus possibly curing patients with melanoma [225]. One of the innovative types of studied melanoma treatments involves the use of nanocarriers [226], including the LLCNPs loaded with suitable drugs, very often in combination with other types of therapy such as PDT [115,116,124,171]. Generally, the aforementioned works showing cubosomes as suitable drug carriers for melanoma treatment, indicating biocompatibility, increasing the efficiency of drug absorption and cellular uptake.

1.2.3.3. Photodynamic therapy (PDT)

It turns out that non-lamellar LLCNPs work very well as drug carriers for PDT, by protecting unstable PSs from the unfavorable conditions of the cellular environment and delivering them in a photoactive monomeric form, as well as presenting an increase in their effectiveness in the target tissue after irradiation [115,116]. The idea of using tumor-targeting drugs that can be activated by visible light to selectively cause tumor cell death has intrigued scientists since the beginning of the 20th century. The term *photodynamic therapy* was introduced by von Tappeiner in 1907, 3 years after von Tappeiner and Jodlbauer in 1904 discovered that oxygen is essential for the photosensitization process [228]. Therefore, PDT is an approach to anticancer therapy which involves the administration of a photosensitizing agent that targets tumors, followed by oxygen-dependent photosensitization of the agent, activated by light of a specific wavelength [96]. This process triggers a series of photochemical and photobiological reactions that lead to irreversible damage to tumor tissues, mainly by the production of ROS that induce oxidative stress in cancer cells [229]. Upon absorbing light, from the ground singlet state, the PS enters an excited singlet state, then converts to a triplet excited state via intersystem crossing, and can react further in two ways: with biomolecules (e.g. proteins, lipids, nucleic acids) by transferring an electron to form free radicals, which through a reaction with oxygen result in various types of ROS (e.g. $\cdot\text{OH}$, $\cdot\text{O}_2^-$, H_2O_2) (Type I), or by energy transfer directly to molecular oxygen (Type II) to produce another type of ROS, a singlet oxygen $^1\text{O}_2$ (see **Figure 1.18**) [108,109,206]. The production of all these types of ROS results in cellular damage of cancer tissue.

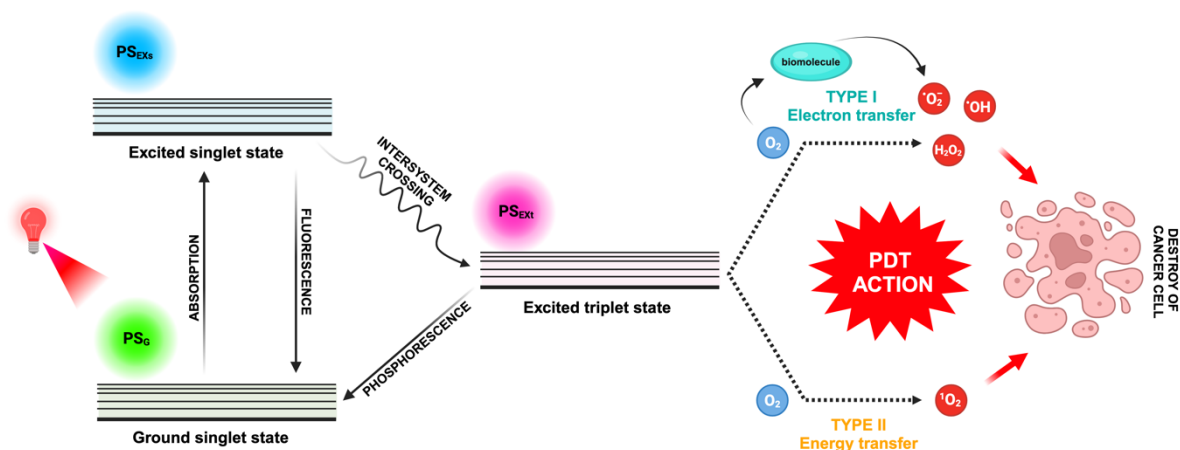


Figure 1.18. Schematic representation of the PDT Action: Type I and Type II with different ROS produced, based on the Jablonski diagram. Own elaboration reproduced with permission from [206].

The significant destructions in cells are related to the acceleration of accumulative ROS, thereby disrupting the cellular reduction-oxidation (redox) balance [230]. When the level of ROS surpasses the redox capacity of cancer cell, it triggers an intense oxidative stress, leading to various cell death processes, such as apoptosis, autophagic cell death, or necroptosis [229]. While PDT is primarily used to treat cancer, it is also being explored for the treatment of premalignant and benign conditions [228]. Due to the frequent instability of photoactive compounds in their free form, the approach based on the combination of PDT with non-lamellar LLCNPs-based nanocarriers has been studied by many scientists [115,116,123,171,207,231]. The promising conclusions coming from these works give hope for improving the anticancer treatment with PDT and increasing the effectiveness of potential drugs through encapsulation in proper nanocarriers.

1.2.3.4. Theranostics

As already mentioned, non-lamellar LLCNPs can be applied in cellular bioimaging for theranostic purposes, including potential anticancer treatment [116,182,186,193–195,200,232]. PDT is an increasingly utilized example of theranostics, as a PS can serve both diagnostic and therapeutic functions, such as the fluorescence properties of chlorophylls. Furthermore, when encapsulated in nanocarriers, PSs with multifaceted actions can be employed in a targeted approach that combines nanomedicine and theranostics (nanotheranostic

carriers). This approach enables simultaneous cancer imaging and therapy through PDT, while also reducing, among other benefits, the systemic side effects commonly associated with drug accumulation throughout the body in traditional chemotherapy (see **Figure 1.19**).

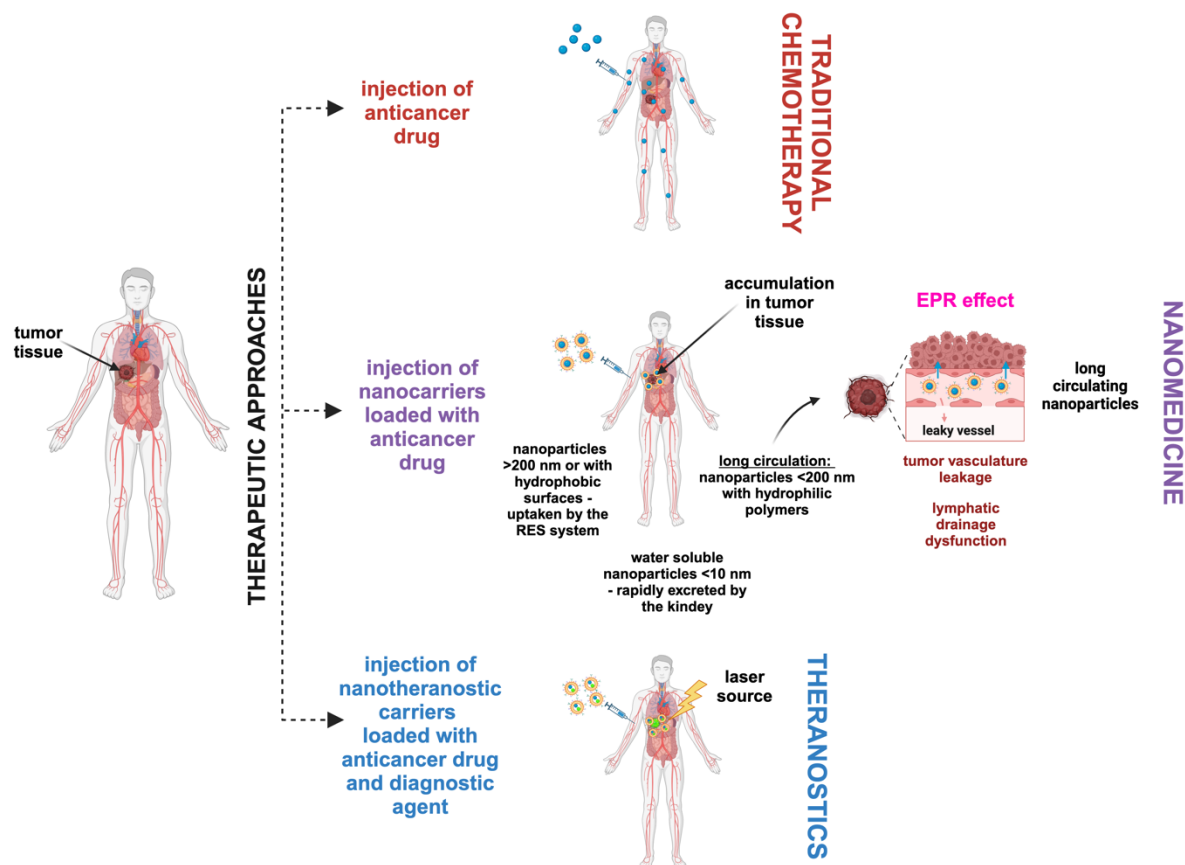


Figure 1.19. Schematic comparative representation of three types of therapeutic approaches for anticancer treatment.

In 2013, the quercetin-loaded fluorescent cubosomes were investigated for a theranostic approach [182]. For this purpose, in addition to encapsulation of the potential anticancer drug, the cubosomes were doped with the modified fluorescent probes to facilitate the accommodation of the fluorophores within the monoolein palisade, from which the modified fluorescein turned out to be a successful approach for single living cell imaging [182]. Moreover, Zhang et al. in 2020 have developed cubosome-based nanosystems, that act like theranostic probes against HeLa cells (cervical cancer), loaded with anticancer drugs with synergistic action: cisplatin and paclitaxel, and coated with a layer of poly- ϵ -lysine to achieve slow and sustained drug release over increased time [186]. An organic/inorganic hybrid theranostic cubosomes were also evaluated by Bazylińska et al. for NIR-induced PDT against MeWo (melanoma) and SKOV-3 cells (ovarian cancer) [116]. The innovative cubosomes were

loaded with a therapeutic agent – daunorubicin, a cytostatic drug co-encapsulated with inorganic up-converting nanoparticles Er^{3+} and Yb^{3+} codoped NaYF_4 , acting as an energy harvester and diagnostic agent. In addition, to increase the cellular uptake and promote specific interactions with pathological tissue, including targeted delivery or cell adhesion, the surface of nanocarriers was functionalized with a shell of alternate layers of chitosan, single-strand DNA, and a folic acid–chitosan conjugate [116]. These works show that due to the specific complex structure of the non-lamellar LLCNPs, these nanostructures are capable of being loaded with many compounds of different chemical characteristics at once, as well as endowed with additional fluorescent probes or the nanoparticle's surface functionalization, thus significantly broadening their field of application in nanomedicine.

1.3. Objectives of the PhD thesis

The aim of the PhD thesis is to investigate the potential of non-lamellar LLCNPs (cubosomes and hexosomes), endowed with high colloidal stability and high encapsulation efficiency, for the encapsulation, protection, and release of bioactive agents extracted from marine algae: *Ulva rigida* and *Caulerpa cylindracea*, for potential application in the pharmaceutical industry and nanomedicine in anticancer therapies, including PDT. The final formulations are expected to reduce the susceptibility to degradation, oxidation or aggregation of the bioactive cargo, by improving its stability in the cellular environment, with the final increase of the potential drug effectiveness. The main consequence of such encapsulation in nanocarriers is more favorable delivery of the bioactive cargo to the target cells due to its better bioavailability and biocompatibility. The application of modern, efficient, and green extraction (e.g. microwave-assisted) techniques make the process of obtaining bioactive compounds from the algae biomass fast, relatively cheap, and environmentally friendly, following the rules of *Green Chemistry*. Moreover, the management of invasive algae biomass helps to protect the marine environment, which is an additional advantage in line with the principles of *Sustainable Development*. The physicochemical characterization of the obtained nanoformulations is presented along with their biocompatible aspects. Furthermore, several biological tests on cancer cell lines are performed to confirm their bioactivity, to produce potential anticancer drugs, including the evaluation of PDT action preceded by an assessment of ROS production.

In order to achieve the above objectives, the following three main research stages were carried out (with the detailed steps presented in **Figure 1.20**):

- I.** Extraction of bioactive pigments from algae biomass
- II.** Synthesis, optimization of nanocarriers and encapsulation of bioactive compounds
- III.** Biocompatibility and anticancer activity evaluation of the formulated nanosystems

In the first part of the thesis (Chapter 3, referred to **Paper I**), the comparative and optimization studies of the nanocarriers synthesized in different ways and loaded with PSs, are discussed upon tests against melanoma cells (MeWo) for potential anticancer PDT. A traditional way of non-lamellar LLCNPs preparation, using a standard polymer, is compared with the innovative method using biodegradable surfactants, to obtain nanocarriers with improved biocompatibility. Further, in Chapter 4 (referred to **Paper II**), the two types of cubosome formulations are tested after loading with a photoactive extract from *U. rigida*, rich in chlorophylls, for potential PDT against human pancreatic cancer cells (BxPC-3). The next chapter (Chapter 5, referred to **Paper III**) describes the studies concerning an isolation of caulerpin, the pigment with potential anticancer activity from the invasive alga, *C. cylindracea*, followed by its encapsulation in freshly developed cubosomes (optimized in Chapter 3), with increased efficiency and biocompatibility, selected based on previous studies (Chapters 3 and 4). The efficiency of the developed nanoformulation is presented in a series of biological tests on BxPC-3 cells, including the modern technique, a holotomographic microscopy (HTM), suitable for the assessment of the apoptotic features of cancer cells induced by the developed nanosystem.

Thus, this PhD thesis exemplifies the first use of non-lamellar LLCNPs for encapsulation of diverse natural bioactive compounds extracted from marine algae biomass in a green way for applications in anticancer therapies, demonstrating the advantages of modern drug delivery nanocarriers such as innovative biocompatible cubosomes.

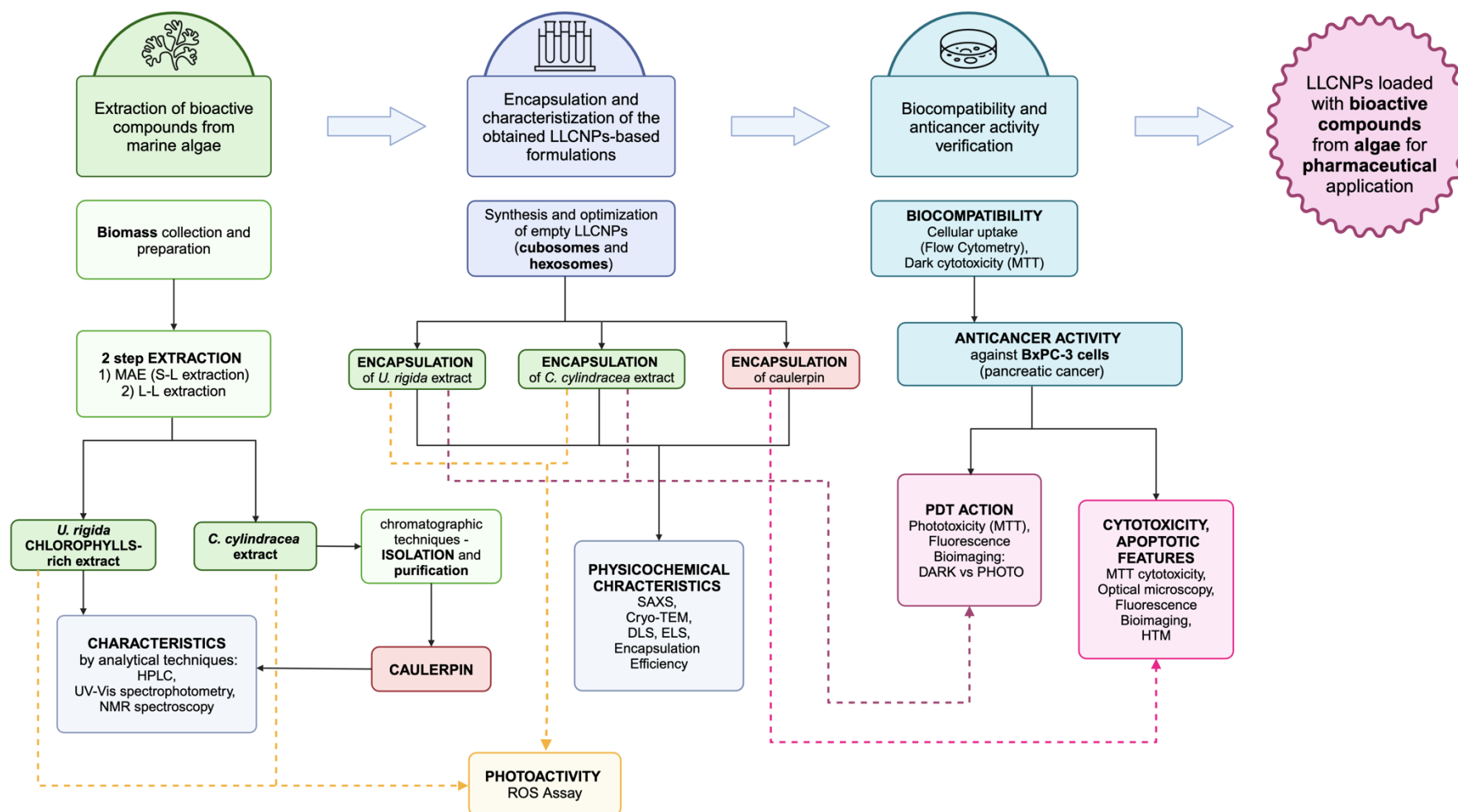


Figure 1.20. General schematic representation of the research steps related to the PhD thesis.

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Chapter 2 – Experimental

2.1. Extraction

Various extraction techniques are used to obtain specific natural products derived from plants or algae. To prevent structural changes of naturally occurring compounds, gentle and specific sample pre-treatment procedures are required [1]. Wet biomass is unstable, since it increases the risk of enzymatic degradations or microbial fermentations within [2], and it is usually not desirable in further extraction with organic solvents. Therefore, an appropriate preparation of the biomass usually consists of its cleaning, drying, and then grinding. Drying of the biomass can traditionally take place in the air, on sun or by heating in the oven, although due to the high water content in algae biomass, these processes are time-consuming, and the risk of sensitive compounds degradation is increased, which further limits the final quality of the product [3]. Therefore, to overcome these limitations, a fast, gentle and effective technique commonly used for algae biomass pre-treatment is **freeze-drying**. This process of removing water by sublimation is usually preceded by rapid deep freezing at -80 °C to crystallize the intracellular fluid and preserve the cell constituents [4] for further longer storage without loss of quality and to increase the reproducibility of the milling and extraction processes applied on the same biomass. Working with dry, stable, and equally grinded (homogenous) material ensures that each extraction starts from a consistent "baseline". Moreover, freeze-drying helps to preserve the photo- or thermosensitive molecules, such as phenolic compounds or pigments, without their degradation, related to oxidation, exposure to light or too high temperature during traditional drying processes [5,6]. Further, to increase the extraction surface and facilitate the solid-liquid extraction process from the dried algal biomass, various grinding techniques are applied, including traditional mechanical millers, as well as innovative methods such as **ball-milling**, characterized by ultrafine grinding of the biomass with even submicrometric particle size, thus enabling an increase in the solubilization level of the ultrafine powder [7]. This powerful tool, through the material grinding, enables breaking down plant or algae tissues along with the integrity of cells, which further has an impact on the increase of solvent penetration and the extraction efficiency, thus shortening its duration [2,8].

After biomass pre-treatment, to isolate the compounds of interest, a two-stage extraction process is usually applied: from solid, dried and grinded matrix to liquid (**solid-liquid (S-L) extraction**), and then from liquid to liquid (**liquid-liquid (L-L) extraction**), to fractionate the specific components of the extract [2]. From the mostly used classical S-L extraction methods, the Soxhlet extraction, maceration or traditional mixing are distinguished, among others [9,10].

Nevertheless, as mentioned in the Introduction (paragraph *1.1.2.3. Novel green extraction techniques*), these techniques are increasingly replaced by modern, green approaches, such as ultrasound- or microwave-assisted extraction (MAE). For the extraction of various bioactive compounds from seaweeds, these techniques are often preferred due to their high extraction efficiency combined with environmental sustainability. They typically involve lower power and energy consumption, minimal or no use of harmful solvents, reduced operational costs, and shorter processing times [1,9,11]. Importantly, the reduced thermal load also minimizes the risk of degradation of thermolabile compounds – a key limitation of traditional, time-consuming methods such as Soxhlet extraction [12].

2.1.1. S-L extraction: MAE

Usually, bioactive compounds of natural origin are located inside the cells of the biomaterial, therefore, the key step is to release them into the solution by breaking the cell wall. As already mentioned, an additional procedure that can support this process, and thus increase S-L extraction efficiency, is appropriate pre-treatment by freeze-drying and ball-milling. After that, the obtained ultrafine biomass powder is subjected to the S-L extraction process, which can be assisted by microwaves (MAE). This step aims to inactivate degrading enzymes, destroy the cell wall and release valuable compounds of interest into the solution [13–16].

The operation of **microwave-assisted extraction (MAE)** is based on the action of electromagnetic radiation with a frequency of 0.3-300 GHz (microwaves) on the sample, by penetrating it and causing the rapid oscillation of the polar molecules inside the sample-solvent mixture, leading therefore to the collisions with surrounding molecules and the release of thermal energy into the medium [10]. Usually, 2.45 GHz (ISM: Industrial, Scientific, and Medical frequency) is applied to avoid interference with other devices, such as radio [17]. Heating occurs through two simultaneous processes: a **dipolar rotation**, caused by the alignment of molecules characterized by the dipole moment, in the electric field, as well as through the movement of ions in a solution, an **ionic conduction** [10,18,19]. The alignment of the molecules followed by their return to disorder takes place approximately 5×10^9 times (cycles) per second for the aforementioned frequency of 2.45 GHz [20], hence the heating occurs very quickly, and the entire sample is heated simultaneously, in contrast to classical conductive heating methods (see **Figure 2.1**). The oscillating molecules generate heat within the material through the intermolecular friction, which results in the thermal energy generation by conversion of kinetic energy into heat (**dielectric heating**) [18].

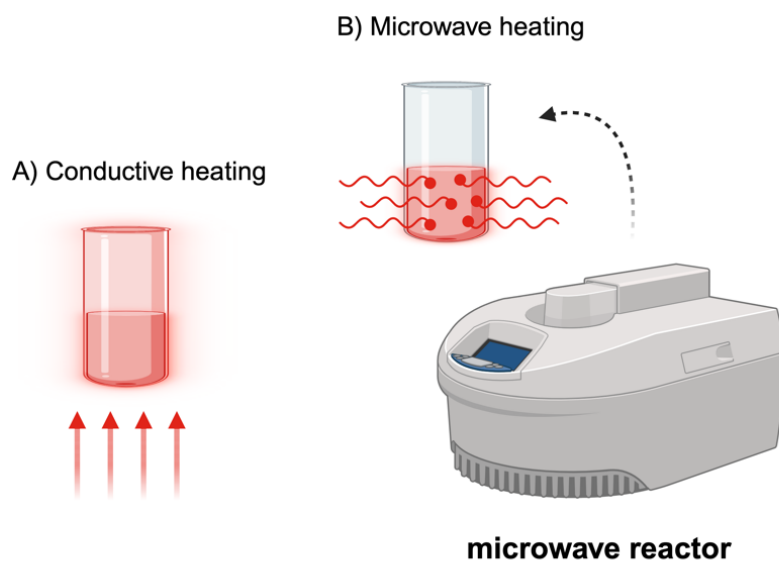


Figure 2.1. Schematic representation of conventional conductive heating (A) and the heating by microwaves (B). Own elaboration reproduced with permission from [12].

This localized heating within the material leads to a rapid temperature increase inside the sample and speeds up the extraction process by enhancing the solubility and diffusion of the target compounds [9,12]. Moreover, the temperature increase created inside the biomaterial helps to break down the cell walls or other barriers, releasing molecules of interest inside the solution more efficiently than with conventional extraction methods [21]. This reduces the extraction time, as the heat is generated directly within the sample, compared to external heating methods where heat must be transferred to the solution from outside and through the vessel, wasting, therefore, time and energy [22]. Thus, this process can be done in minutes, whereas conventional methods might take hours. Moreover, an advantage of the MAE is breaking down the weak hydrogen bonds promoted by the dipole rotation of the sample molecules [9,23]. Additionally, the movement of dissolved ions enhances the penetration of the solvent into the solid biomass matrix, thereby aiding in the solvation of the sample [9,21].

The properly prepared raw material (e.g. plant or algae biomass) is placed in a closed or semi-closed extraction vessel along with the relevant solvent (water or organic solvents like ethanol, methanol, or hexane), which helps to dissolve the compounds of interest. Then, the sample-solvent mixture is exposed to microwave radiation, and thus heated to the applied temperature. In some systems, pressure may also be applied to further increase the extraction efficiency. After the extraction is completed, the sample is cooled down, and the solvent containing the

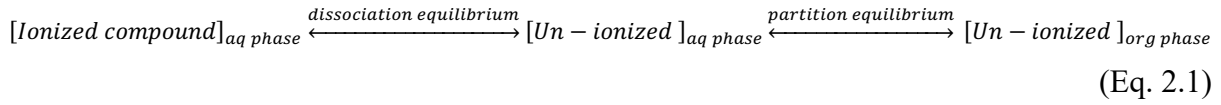
extracted compounds is separated from the remaining solid biomaterial, usually by centrifugation or filtration.

MAE is widely used to extract natural products from various groups, such as flavonoids and other phenolic compounds, alkaloids, polysaccharides, essential oils, pigments, lipids or proteins [11,21,24–28]. By controlling the microwave power and extraction conditions (temperature, pressure), MAE minimizes the thermal degradation of sensitive compounds, such as antioxidants, vitamins or pigments, that might degrade at higher temperatures applied in traditional extraction methods [12,16]. This, and the previously mentioned advantages related to the reduced process time and ecological values, make MAE an approach currently used for processing various types of plant or algae biomasses, very often for the extraction of natural-origin potential drugs (as described in more detail in the Introduction: **paragraph 1.1.2.3. Novel green extraction techniques**).

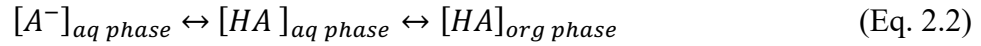
2.1.2. L-L extraction

Due to the diverse chemical nature of the compounds present in the extracted biomass, the next key stage is their separation in the L-L extraction process, namely usually the crude extract obtained earlier by S-L extraction, is dissolved in an appropriate solvent and then subjected to further fractionation.

L-L extraction, also known as solvent extraction, is one of the oldest and most commonly used methods for preparing samples for qualitative and quantitative analysis by separation of the homogeneous liquid mixtures (solutions) constituents [29]. This separation technique relies on the varying distribution of the components between two liquid phases, which create definite contact areas and, due to their specific properties, can either not mix with each other at all or only mix to a limited degree [30]. To give a degree of such separation, the ratio of such component concentrations in two immiscible solvents at equilibrium, reflecting its solubility in each, is designated, and so-called **partition coefficient (P)** or **distribution coefficient (D)** [29,31]. The partition coefficient refers to the ratio of un-ionized species (partition equilibrium), while the distribution coefficient also includes the ionized species related to dissociation equilibrium between un-ionized and ionized compound within the aqueous phase (see **Eq. 2.1**), which makes it pH-dependent [31]. Therefore, to achieve a successful separation, various components of a homogeneous mixture must have different distribution coefficients between the two liquid phases.



For example, an organic acid (HA) partitioning between the two phases is expressed as follows (**Eq. 2.2**), with its partition (**Eq. 2.3**) and distribution coefficients (**Eq. 2.4**) [31]:



$$P = \frac{[HA]_{org\ phase}}{[HA]_{aq\ phase}} \quad (\text{Eq. 2.3})$$

$$D = \frac{[HA]_{org\ phase}}{[HA]_{aq\ phase} + [A^-]_{aq\ phase}} \quad (\text{Eq. 2.4})$$

Typically, in chemical and pharmaceutical sciences, the two phases are solvents, one solvent is water or water-soluble, and the other (organic phase) is very often hydrophobic, like 1-octanol [32]. As a result, the partition coefficient indicates whether a substance is more hydrophilic ("water-loving") or hydrophobic ("water-repellent"). Moreover, the partition coefficients help estimate how drugs are distributed in the body. Hydrophobic drugs with high octanol-water partition coefficients tend to accumulate in hydrophobic areas like cell lipid bilayers, while hydrophilic drugs (with low partition coefficients) are mostly found in aqueous regions, such as blood serum [33].

L-L extraction typically involves more than three components and works as presented in **Figure 2.2** [30]. The feed is the solution to be separated, with component A, being the main liquid phase and C, being the solute (the transferred substance of interest). The extraction solvent added to extract C from the feed is very often a pure substance B, but can constitute a recycled liquid phase from the recovery system containing small amounts of A and C. The extract, a phase which leaves the extractor, contains mostly B and C, sometimes with a small amount of A, while the raffinate (the liquid phase left from the feed after extraction) consists mainly of A, with small amounts of C and B.

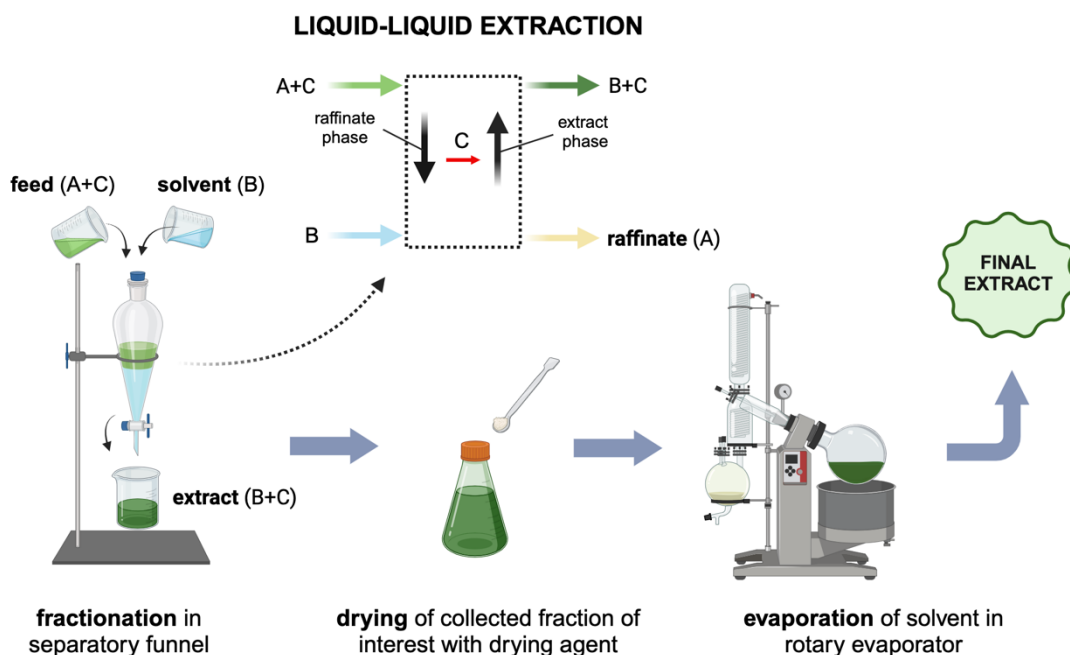


Figure 2.2. Extract development procedure with the schematic representation of the L-L extraction principle of operation. Own elaboration reproduced with permission from [30].

In the traditional form, the process is carried out in a special glass separatory funnel, using appropriate solvents in the relevant proportion. The mixture is there agitated with the excess CO_2 release, often repeated a couple of times, to disperse droplets of one liquid into the other, and then allowed to rest, so the droplets can coalesce. Next, the two liquid phases are separated, and the successively parted fraction of interest is slowly poured through a funnel [34]. Such retained one is usually subjected to solvent evaporation to give the final extract, or further separated and purified, e.g. using chromatographic techniques (described in more detail in paragraph 2.1.3.1. *Chromatographic techniques*), to isolate the pure compounds. In addition, in the case of obtaining hydrophobic extracts, the fraction of interest can be dried before evaporation using compounds with hygroscopic properties such as anhydrous sodium sulfate, which, by converting to the hydrated form, absorbs water molecules to avoid them in the final product [35].

This type of extraction is currently often used as the next step after S-L extraction in obtaining extracts from plant or algae biomass, for various applications in the food or pharmaceutical industry [36–39].

2.1.3. Separation and identification of bioactive compounds

Since plant or algae biomass extracts usually constitute a mixture of compounds with various characteristics, such as different polarity or hydrophobicity, to separate and isolate their components or characterize them in terms of quantitative composition, after prior fractionation by L-L extraction, various chromatographic methods are subsequently applied, such as **liquid chromatography (LC)**, **thin layer liquid chromatography (TLC)** or **high-performance liquid chromatography (HPLC)**. An identification of the obtained products can be carried out by analytical techniques such as ultraviolet/visible (**UV-Vis**) **spectrophotometry**, **mass spectrometry (MS)** or **nuclear magnetic resonance (NMR) spectroscopy**. Combinations of various techniques, such as HPLC-UV-Vis, are also frequently used.

2.1.3.1. Chromatographic techniques

For even more detailed separation and identification of the natural products extracted from the biomass, various chromatographic techniques are applied. By comparing the separated components of the mixture with appropriate standards, a detailed characterization of the tested extracts' composition can be obtained through qualitative and quantitative analysis [40].

Chromatographic techniques operate by separating components of a mixture based on their differential interactions with a **stationary phase** (solid or liquid), and a **mobile phase** (liquid or gas), which moves in a definite direction [41]. A stationary phase constitutes the material that stays fixed in place, often in the form of a packed column or a thin layer, solid or liquid-coated solid particles, while a mobile phase is a solvent or a mixture of solvents that moves through or over the stationary phase, carrying the sample components with it [41,42].

Differences in the affinity of the individual compound for the stationary and the mobile phase lead to their distribution between the two phases. These interactions may include adsorption, partition, size exclusion, ion exchange, or specific affinities (e.g., by antibodies) [41], as presented in **Figure 2.3**.

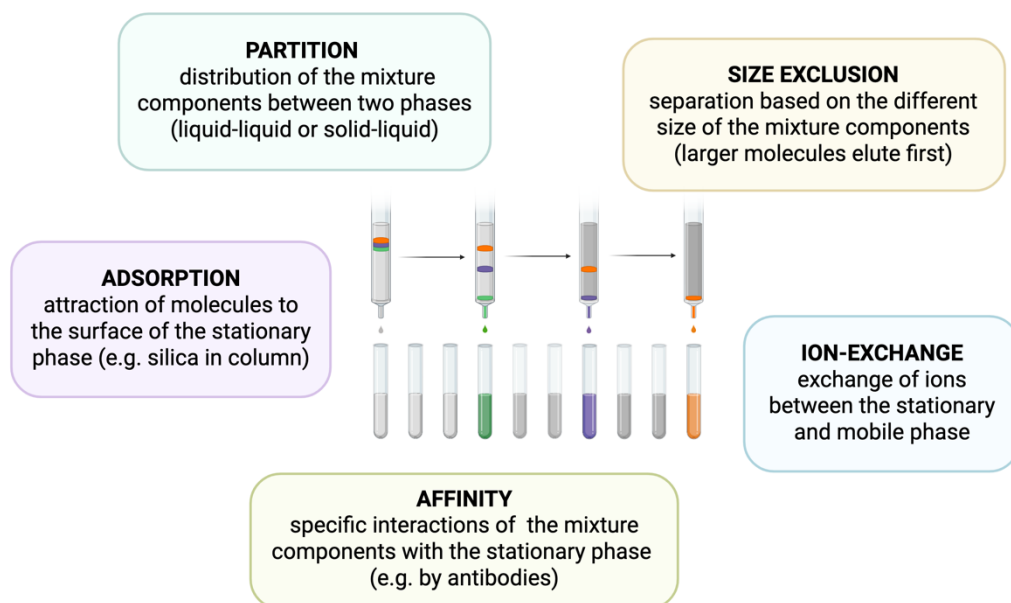


Figure 2.3. Different types of interactions based on which the mixture compartments are separated through various chromatographic techniques [41].

When the studied sample mixture is introduced into the chromatographic system, it begins moving through the stationary phase along with the mobile phase, in the process of so-called **elution** [42]. **Retention time** describes the time it takes for a particular compound to travel through the column (elute) and be detected [41,43]. Each compound has a characteristic retention time to reach the detector under the same conditions, which is crucial in its identification. Different components of the sample interact with the stationary phase in varying degrees – those that interact strongly with the stationary phase move more slowly, in contrast to those with weaker interactions, which move faster [44].

In some types of chromatographic techniques, a detector is used to detect the components as they exit the column or system and create the chromatogram based on different retention times of individual components, presenting the intensity of the signal (y-axis) versus time (x-axis). Each peak represents a different compound, and the area under the peak correlates with the concentration of that compound in the studied sample. Sometimes the chromatographic techniques can be combined with UV-Vis, fluorescence, or MS detectors, depending on the application [45]. For instance, with the UV-Vis detector, when the sample components emerge from the column, the detector generates signals related to their absorbance at specific wavelengths, and the graph based on the detector's response is plotted in the form of the

absorbance dependence against time. The resulting peaks correspond to different components in the mixture, and the results can be used to qualify and quantify such molecules in the sample.

Chromatographic techniques are classified based on the type of stationary and mobile phases used, as well as the method of separation. Therefore, the most common types include [46]:

- ♦ **Liquid chromatography (LC):** a broad category where the mobile phase is a liquid, and the stationary phase is typically a solid; separation is based on the differences in polarity (partition) and the adsorption properties
- ♦ **Thin-layer liquid chromatography (TLC):** a type of LC, a simple method using a thin layer of stationary phase on a flat surface, often used for quick, qualitative analysis
- ♦ **Ion exchange chromatography (IEC):** another type of LC based on the ions and polar compounds separation due to their differences in charge by the charged stationary phase, used to attract or repel such components of the sample
- ♦ **Size exclusion chromatography (SEC):** a type of LC technique also known as “gel filtration”, that separates molecules based on their differences in size by the porous stationary phase, retaining particles smaller than its pores
- ♦ **Gas chromatography (GC):** in this type of chromatography, the stationary phase is a solid or liquid coating on a solid support, while the mobile phase is a gas; therefore, GC is widely used for volatile compounds

The LC is one of the simplest and oldest used chromatographic techniques, which can operate on the normal-phase or reversed-phase principle. In **normal-phase chromatography**, the stationary phase is polar (such as silica), while the mobile phase is relatively non-polar (such as hexane modified with slightly polar solvents) [42,47]. In this case, polar compounds interact strongly with the polar stationary phase and move more slowly. In contrast, the IUPAC defines **reversed-phase chromatography** as an elution method where the mobile phase is considerably more polar than the stationary phase [41]. Therefore, reversed-phase chromatography separates molecules according to their hydrophobicity; thus, the most hydrophilic molecules elute first, while those with stronger hydrophobic characteristics elute later [48].

As already mentioned, the key to separation lies in the fact that each component has a unique rate of travel through the column based on the differential interactions, and this results in the components being separated over time, with each one emerging at a different time from the column. In traditional column LC, as the components elute, they are collected in separate

subsequent fractions, each one containing different components of the mixture. These fractions may be subjected to solvent evaporation and quantified, or further analyzed and purified if needed, by repeating the chromatographic method, or e.g. by recrystallization to isolate pure compounds [49].

The presence of expected compounds of interest in individual fractions can be verified by quick and simple TLC analysis, using a thin layer of the stationary phase on a flat surface. After visualization of the spots, the value of the **retention factor (R_f)** for each spot is measured as the division of the distance travelled by each compound by the distance travelled by the solvent front (see **Figure 2.4**), which determines how strongly the molecule is retained on the adsorbent stationary phase in a given solvent system [50]. Therefore, the compounds, which migrate higher up the TLC plate, are characterized by larger R_f values. Thus, for quick identification of a compound by TLC, the comparison of the calculated for each compound separation parameter R_f with the literature data, or with the value of a standard molecule, is applied. Very often, the end of the path (a spot) is visualized by the UV light, or after a characteristic reaction with specific salts and heating if the given spot is not possible to observe in ordinary light [50,51].

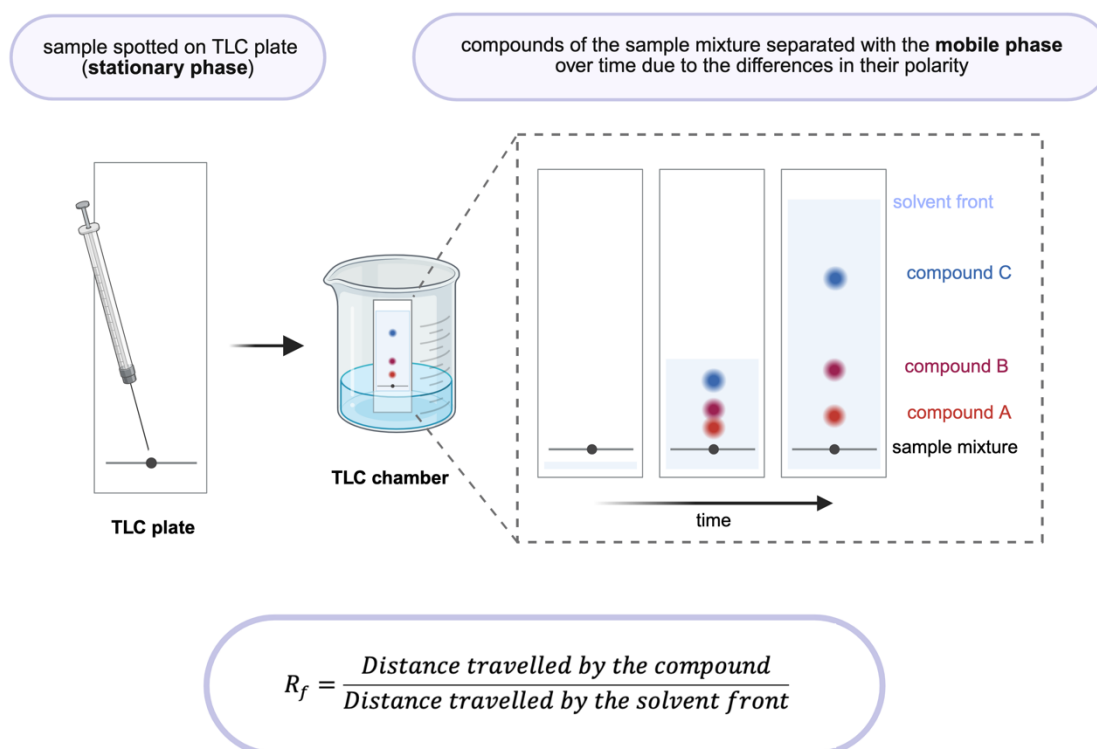


Figure 2.4. Schematic representation of the TLC process with the calculation of R_f parameter.

An advanced form of the LC is **high-performance liquid chromatography (HPLC)**, where the separation is performed under high pressure (up to 400 bar) and with the use of smaller particles (2–10 μm) of the stationary phase material, which increases surface area for interactions and improves the separation efficiency [46,47,52]. The smaller particle size requires high pressure to push the mobile phase through the column, allowing for faster and more efficient separations with greater precision [53]. In HPLC, buffers are used primarily to maintain a stable pH in the mobile phase and minimize variations during the analysis, which is crucial for ensuring consistent separation and reproducible results [47]. The pH of the mobile phase can affect the ionization of analytes, influencing their retention and behavior on the column, especially in techniques like reversed-phase chromatography, where the interaction between the analytes and the stationary phase is pH-dependent. In addition to pH control, buffers can also help to enhance the resolution of certain compounds, improve peak shape, and prevent unwanted interactions between the analytes and the column, particularly when dealing with sensitive or polar compounds [52].

HPLC is a reliable and versatile, highly automated method, with detectors and systems that control the pressure, flow rate, solvent composition, which makes it more convenient for routine, repetitive and high-volume broad application range. HPLC is particularly useful for high-precision and high-throughput complex separations, namely for pharmaceutical analysis, environmental testing, and bioanalytical studies [54]. Moreover, this chromatographic technique is widely used in natural product isolation by their separation from mixtures extracted from biomass, commonly employed in phytochemical and analytical chemistry to purify, identify, and quantify individual components [2,55]. In HPLC, the reversed-phase method is mostly used, accounting for 70-80% of all separations, preferred due to its simplicity, versatility, and ability to handle compounds of varying polarity and molecular mass [47], making it an ideal tool for identifying secondary plant metabolites, such as phenolic compounds, flavonoids, alkaloids, and steroids [56]. As already mentioned, to streamline the process for the simultaneous identification of separated compounds, HPLC can be combined with identification techniques such as UV-Vis spectroscopy or MS, using various types of appropriately selected detectors, giving respectively an HPLC coupled with UV-Vis (HPLC-UV-Vis), or with MS (HPLC-MS) [54]. The UV-Vis detectors are commonly used due to their high sensitivity, and therefore the advantage of identifying the compounds in trace amounts, in the case of the molecules that naturally absorb UV-Vis light [57], such as algae pigments. Some other detectors, such as Diode Array Detectors (DAD) are also increasingly used to analyze phytochemicals [58] or Photodiode Array (PDA), which can be employed within

the 190-380 nm wavelength range, thus being a good option for the analysis of phenolic compounds mostly present in various biomasses [59]. MS detectors enable rapid and accurate identification of different molecules, even when pure standards are unavailable [56].

Generally, HPLC is a powerful method used in various fields, including pharmaceuticals, environmental analysis, food testing, and more, due to its ability to provide high-resolution separations with precision and reliability. For instance, this technique was widely applied for the characterization and identification of algal bioactive compounds [60–63].

2.1.3.2. UV-Vis spectroscopy

UV-Vis spectroscopy is a technique that measures the interaction between a chemical compound and light within the ultraviolet (200-400 nm) and visible (400-780 nm) ranges of the wavelength spectrum, by the instrument called a spectrophotometer [64,65]. This technique involves passing light through a sample and measuring the amount of light absorbed at different wavelengths. The principle of UV spectroscopy operation is based on the **electronic excitation** within the studied sample [65]. When the light interacts with a molecule, the energy from the photons can excite its electrons to higher energy levels, promoting an electronic transition between different orbitals. The absorption of light is present when the energy of the photons matches the energy difference between two electronic energy levels within the molecule, related to its specific chemical structure, such as the presence of chromophores [66]. A **chromophore** is a region of a molecule responsible for its color, where the energy required to transfer an electron from the ground state to its excited state is in the range of visible light [67]. These regions often involve conjugated double bonds or lone pairs of electrons. The extent of conjugation (alternating single and double bonds) can affect the wavelength of light absorbed, shifting it to longer wavelengths (red shift) or shorter wavelengths (blue shift) [68]. The more conjugated a molecule is, the less energy is required to excite electrons, which means it absorbs light at longer wavelengths (for example, in the visible range).

The absorption occurs in discrete jumps (quantized), where an electron in the molecule absorbs energy and moves from a lower energy state (ground state) to a higher energy state (excited state) [66]. This transition is specific to the molecule and depends on the types of bonds, atomic orbitals, and functional groups present. In UV-Vis spectroscopy, transitions typically involve π -electrons (common in conjugated organic molecules (e.g., aromatic compounds; transition $\pi \rightarrow \pi^*$) or non-bonding n-electrons (e.g. lone pairs of atoms like oxygen or nitrogen; transition $n \rightarrow \pi^*$), while the transitions $\sigma \rightarrow \sigma^*$ typically occur at higher energies (shorter wavelengths) which are often outside the UV-Vis range (**Figure 2.5**) [64,65].

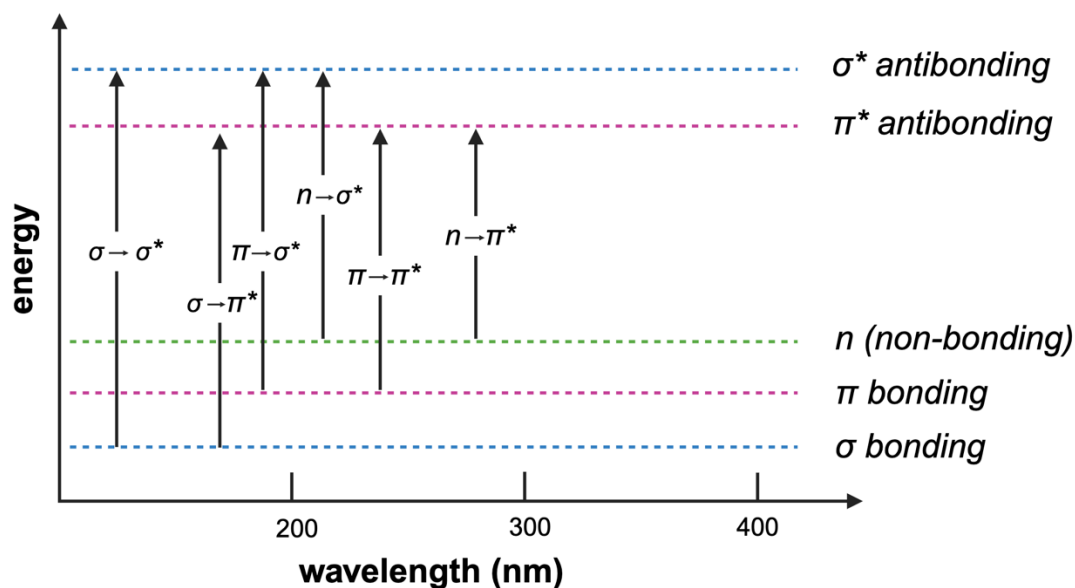


Figure 2.5. Schematic representation of different electronic transitions [65].

A specific light source (usually a deuterium lamp for UV and a tungsten lamp for visible light) emits light, which is passed through a monochromator, to isolate specific wavelengths from the broad light spectrum [64,65]. The selected wavelength(s) of light is directed at the sample, and through their interaction, certain wavelengths of light are absorbed by the molecules within. The amount of absorption depends on the chemical composition of the sample and its electronic structure. After passing through the sample, the remaining light that wasn't absorbed is measured by a detector, which records how much light has been transmitted through the sample at each wavelength. The collected data is used to create an absorption spectrum, which presents the dependence of absorbance on wavelength. Therefore, the peaks in the spectrum correspond to specific electronic transitions in the studied molecules of the sample, related to their specific electronic structure, providing a "fingerprint" for identifying various substances. It is important to notice that the absorption spectrum can be influenced by the solvent or the surrounding environment, as they may interact with the molecule, leading to shifts in the absorption maxima (known as **solvatochromism**) [68]. This is particularly important for compounds that are sensitive to solvent polarity, or polar molecules where solvation alters the electronic states.

By analyzing the absorption spectrum after the UV-Vis spectroscopy experiment, various properties of the tested sample can be evaluated, like concentration, the identity and nature of compounds, or the presence of specific functional groups based on characteristic absorption peaks. The **Beer-Lambert law** proves that absorbance is directly proportional to sample

concentration and optical path length, making UV-Vis spectroscopy useful for quantitative analysis (see **Fig. 2.5**) [69]. Therefore, the relationship between the concentration of a substance in solution and the amount of light absorbed is described by the following equation:

$$A = \varepsilon \cdot c \cdot l \quad (\text{Eq. 2.5})$$

where A is the absorbance, ε is the molar absorption coefficient (a constant specific to the substance at a given wavelength) [$\text{dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$], c is the molar concentration of a sample [mol/dm^3], and l is the optical path length [cm].

Since this technique enables qualitative and quantitative analysis of the compounds with specific absorption properties, and algae biomass is full of different molecules with given abilities, such as pigments, UV-Vis spectroscopy constitutes a suitable method for their characteristics. Therefore, this method is frequently used by scientists for the identification and quantification of different molecules extracted from such biomasses [2].

2.1.3.3. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical technique that provides detailed information about the structure, dynamics, and composition of molecules, particularly organic compounds [70–73]. NMR spectroscopy works by applying a strong magnetic field (usually between 7 and 21 Tesla) [73] to a sample, causing certain nuclei to resonate at specific frequencies depending on their chemical environment and the magnetic field strength. The most commonly analyzed atomic nuclei are **hydrogen (^1H NMR)** and **carbon-13 (^{13}C NMR)** because of their abundance in organic compounds and strong magnetic properties. These nuclei have a property called **spin**, which is a vector sum of the spins of their elementary particles, such as electrons, and makes them behave like tiny magnets, creating a **magnetic moment** [74]. When placed in a magnetic field, the magnetic nuclei align with the field, either in a lower energy state (parallel alignment) or a higher energy state (anti-parallel alignment). The nuclei can be flipped from the lower to higher energy state by applying a **radiofrequency (RF) pulse** at a specific frequency, which leads to the energy absorption and transition between energy levels [70–73]. Therefore, the principle of the NMR spectrometer operation is based on the application of an RF pulse that excites the sample, and after the pulse is turned off, the excited nuclei relax back to their lower energy state, releasing energy in the form of radiofrequency signals. These signals are detected by the instrument and analyzed to produce the NMR spectrum.

The frequency at which a nucleus resonates depends on its chemical environment. The magnetic field around a nucleus is influenced by nearby electrons, which shield the nucleus. This results in a **chemical shift**, usually expressed in parts per million (ppm) and is measured relative to a reference compound, usually tetramethylsilane (TMS), which is assigned a chemical shift of 0 ppm [70–73,75]. In the NMR spectrum, the position of peaks along the x-axis (in ppm) indicates the chemical environment of the nuclei. Each type of hydrogen or carbon in a molecule will have a unique chemical shift. Another feature of the NMR spectrum is the **multiplicity (splitting pattern)**, due to spin-spin coupling between adjacent nuclei, resulting in splits of peaks into multiple smaller ones and provides information about the number of adjacent equivalent nuclei. The distance between the split peaks in such multiplets provides information about the strength of the interaction between coupled nuclei, measured in Hz, and known as **J-coupling**. This parameter helps to identify how atoms are connected by the bonds within the molecule. Moreover, the area under each peak is proportional to the number of nuclei responsible for that signal (**integration**). By integrating the peaks, the relative number of hydrogens (or carbons) in different environments in the molecule can be determined; thus, the integration of peaks can be used to evaluate the relative amounts of different components in a mixture, making NMR a tool useful also for quantification [70–73]. Besides this, NMR spectroscopy can be applied to determine the preferred 3D structure of a molecule (conformational analysis), especially in the case of nucleic acids, proteins, and other large biomolecules [76–78].

Given the aforementioned features, the NMR spectrum provides crucial information about the chemical environment, the number and types of nuclei, and how atoms are connected in the molecule. This makes NMR spectroscopy an invaluable tool in the detailed identification of single compounds, as well as the evaluation of their purity. Thus, it is an ideal technique for studying the structure of the unknown, or rarely encountered and not yet detailed researched compounds, e.g. those characteristics of a given species of plant or algae, such as bisindole alkaloids: caulerpin or racemosins A and B [39,49,61,79,80]. Various natural products may be identified by this technique, even from a mixture [2].

^1H NMR, the most common type of NMR spectroscopy, provides detailed information about each hydrogen atom in a molecule (e.g. if it is a part of a specific chemical group, such as OH, CH_3 or an aromatic ring). While the ^{13}C NMR is useful to evaluate the characteristics of carbon atoms, giving thus information about the “carbon skeleton” of an organic molecule, and providing an identification of various functional groups. ^1H spectra are usually more complex

than ^{13}C spectra, because hydrogen atoms generally couple with more nuclei. Apart from that, NMR spectroscopy provides also advanced approaches, namely two-dimensional (2D) NMR, which compared to the described earlier one-dimensional (1D) NMR, provides more comprehensive analysis of the complex molecular structures, due to signals distribution across two frequency axes (instead of one) and therefore improved resolution, as well as separation of the overlapping peaks, among others [71]. 2D NMR involves techniques like correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY) or heteronuclear single quantum coherence (HSQC), particularly useful for the biomolecules' characteristics, such as peptides, proteins and nucleic acids [72]. Such approaches are used, for instance, for the determination how different nuclei are connected in the complex molecule (e.g., proton-carbon correlations or even between ^{15}N and proton).

2.2. LLCNPs formulations and characterization

2.2.1. LLCNPs formulations

As mentioned in the Introduction (chapter 1.2.3. *Non-lammellar LLC nanoparticles*), formulations consisting of LLCNPs are obtained by dispersion of liquid crystalline bulk phases in water. There are two main approaches for the preparation of these dispersions: **top-down** and **bottom-up** methods (Figure 2.6) [81–83].

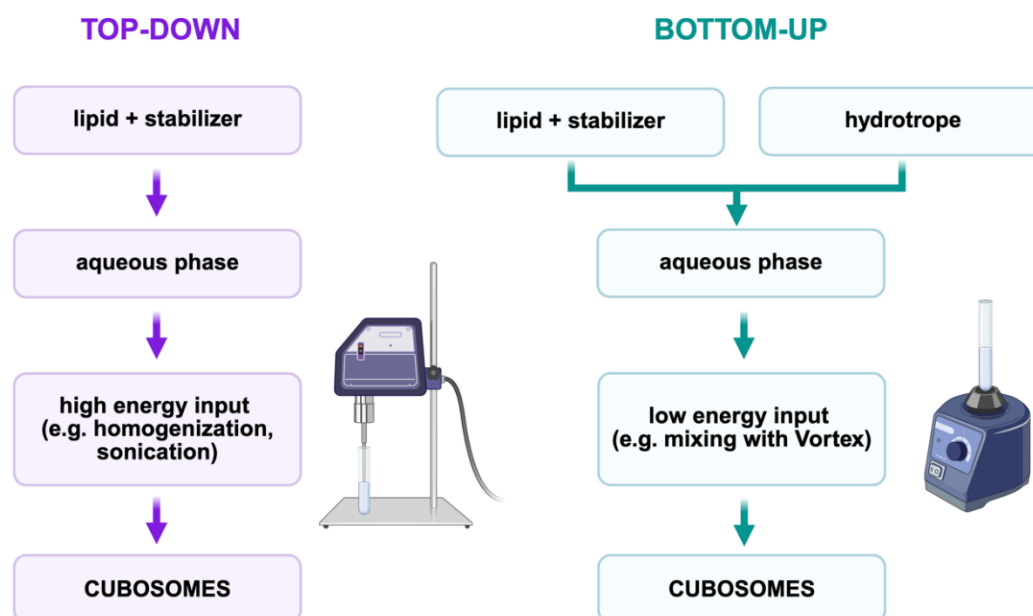


Figure 2.6. Schematic representation of the top-down and bottom-up approach for cubosomes preparation. Own elaboration reproduced with permission from [81].

Both methods are widely used to produce those systems, but they differ significantly in their fundamental principles and processes. The top-down approach is the most common technique and involves breaking down (fragmentation) of the bulk phase material in water into smaller nanoscale aggregates (LLCNPs) with the presence of an emulsifier (stabilizer) (e.g. tri-block copolymer) to circumvent the aggregation problem and stabilize the formulation kinetically [82–84]. Essentially, this method starts with a larger entity and reduces in size through emulsification using high energy input, e.g., by ultrasonic processors or other homogenizing devices (e.g. with high-pressure) [85–87]. The energy input leads to the dispersion of LLCNPs in excess of water, leading to the formation of the desired nanocarriers in a short time with relative size uniformity, below 200 nm, thus suitable for the sterilization by filtration and intravenous administration [83,87]. On the other hand, the bottom-up approach involves constructing the LLCNPs from individual molecules or atoms, which self-assemble into more complex structures, by mixing of two water-like solutions with minimal energy input (dilution method) developed by Spicer et al., in 2001, thanks to applying the hydrotrope not displaying surfactant phase behavior (e.g. ethanol) to dissolve the lipid in aqueous solution [82,83,87,88]. In this way, specific molecules such as surfactants, amphiphilic lipids, or block copolymers used as building blocks, spontaneously organize themselves into well-defined, ordered nanostructures (like the cubosomes or hexosomes) under the right, controllable thermodynamic conditions [89] (as described in more detail in the Introduction: chapter *1.2.2. Surfactants and self-assembly*). Spicer et al. proved that the dilution approach may offer stable, smaller cubosomes with lower polydispersity and fewer vesicles formed, through lower energy input in comparison to the previously described method [88,90], therefore easier to scale up, thus eliminating the problems related to the labile compounds incorporation (e.g. proteins or peptides) due to high thermal/mechanical energy in the top-down approach [83]. Nevertheless, nowadays, after many years of various experiments, it is reported that the top-down method usually provides smaller cubic nanoparticles with a lower polydispersity index than the ones obtained by bottom-up approaches [82,83]. Moreover, it is important to notice that the preparation methods are very often modified in some way to increase the control of the particle size and size distribution, such as the microfluidics-based approach [91]. This indicates a large diversity, and the influence of various factors during the preparation method, on the average size of the studied nanoparticles, and overall polydispersity of the formulation. The particles obtained by the top-down approach are mostly endowed with reproducibility and stability, although the heat produced by high energy input may have an impact on thermosensitive compounds encapsulated within, thus it must be well-controlled,

for instance by applying cooling procedures or an appropriate low-volume ultrasonication tip with pulse mode [90]. Moreover, the bottom-up method provides the risk associated with residual hydrotropes, such as the impact on phase behavior or the increase of cellular toxicity, which constitutes a key disadvantage in biological applications [90]. Therefore, both methods have their pros and cons, and often, a hybrid approach is used depending on the application and the desired properties of the obtained LLCNPs.

The encapsulation of molecules (such as drugs, proteins, or other bioactive cargos) into LLCNPs can be quite different depending on whether a top-down or bottom-up method is used, as well as the type of cargo to load within (hydrophobic or hydrophilic). As already described in the Introduction (chapter *1.2.3. Non-lamellar LLC nanoparticles*), thanks to a specific structure of these nanoparticles, for instance, in the case of cubosomes: there are three loading mechanisms distinguished: within the water channels, within the lipid membrane or tethered to the lipid membrane [90]. This process can be done by adding the cargo to molten lipid, pre-dispersion with co-lyophilizing the drug molecules with the lipid film, or by post-dispersion by loading the nanocarriers after their formation through incubation [90,92]. In the top-down method, encapsulation generally occurs during the mechanical disruption of the bulk material into smaller nanoparticles; thus, the cargo molecules are entrapped within the newly formed structures. In the bottom-up method, encapsulation happens through self-assembly, where molecules are assembled into nanostructures from smaller building blocks. Particularly, the top-down approach allows for easier entrapment of hydrophilic drugs (such as water-soluble peptides, oligonucleotides, and proteins), which are hardly introduced by preparing the nanocarriers directly through the bottom-up approach [84].

In conclusion, while both methods can successfully encapsulate molecules in liquid crystalline nanocarriers, the bottom-up approach generally offers better control over the encapsulation of labile compounds due to lower energy input. Moreover, it can be useful when the particle-size criteria are not crucial. On the other hand, the top-down approach usually offers smaller nanoparticles suitable for pharmaceutical application, making it a preferred method when the formulation uniformity and low polydispersity are important, or the hydrophilic drugs are incorporated within.

2.2.2. Dialysis and encapsulation efficiency

Dialysis is a technique commonly used in the preparation and purification of colloidal nanoformulations, to remove unexpected small molecules, such as residual surfactants, stabilizers, solvents, salts, or other impurities that might be present after the synthesis process, while preserving the larger nanocarriers without compromising their integrity [90,93]. Such excess reagents might otherwise interfere with the NPs structure, formulation's stability, toxicity profile, or efficiency. Moreover, if the formulation involves encapsulation of potential drugs (loaded nanocarriers), dialysis helps to purify it from unencapsulated cargo molecules. This is particularly useful in the development of drug delivery systems, since the goal is to load the drug inside the nanocarrier, not in the solution (free form) [94–96].

The basic principle of dialysis relies on the selective permeation of molecules through a semi-permeable membrane with a specific molecular weight cut-off (e.g. cellulose membrane) smaller than the size of the nanocarriers, but large enough to allow the diffusion of smaller molecules [93]. This membrane allows small molecules to diffuse out of the dialysis chamber while retaining larger particles, such as the nanocarriers (e.g. cubosomes, hexosomes) with the molecules encapsulated within (see **Figure 2.7**). The choice of dialysis membrane is crucial, since if it is not appropriate, it might not be effective in removing the unwanted molecules without also losing part of the nanocarriers.

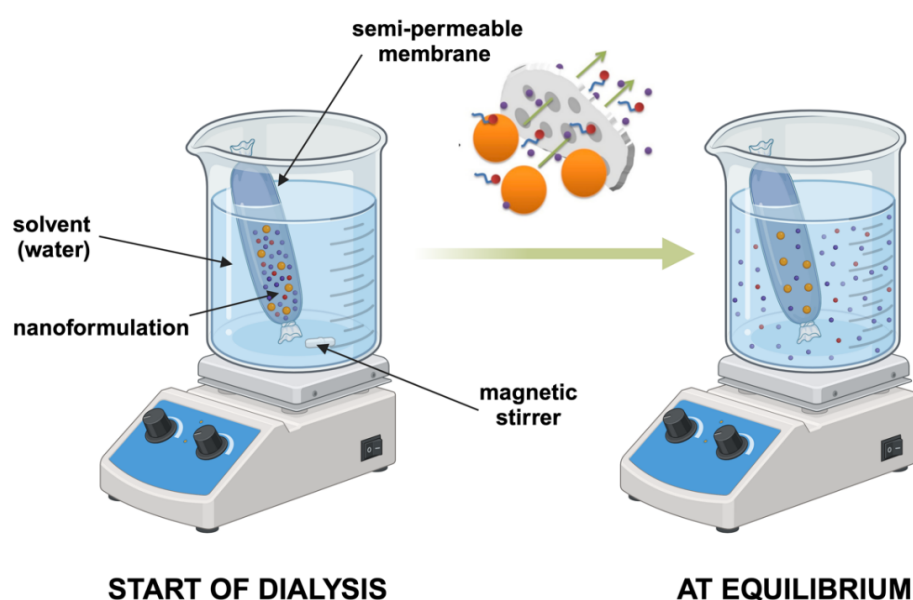


Figure 2.7. Schematic representation of the nanoformulation purification by dialysis. Own elaboration reproduced with permission from [93].

The formulation containing the nanocarriers is placed inside a dialysis bag, which is submerged in a larger volume of buffer or water (the dialysis medium). Smaller molecules (e.g., residual building molecules, unencapsulated drug) diffuse through the membrane into the surrounding dialysis medium, forced by the concentration difference between the two environments separated by the membrane (**diffusion (concentration) gradient**), while the nanocarriers with encapsulated molecules remain inside the bag [97,98]. Moreover, water osmosis is involved in the dialysis process, since when the small solutes initially move from the compartment with higher concentration (diffusion phenomenon), the solvent molecules travel in the opposite direction to “dilute” the compartment with higher concentration, driven by the goal of balancing solute concentrations on both sides of the membrane (osmosis phenomenon) [93]. The surrounding medium is continuously exchanged or stirred to maintain the concentration gradient, and thus facilitate the removal of smaller molecules from the formulation during the specified dialysis time. At the end, the purified formulation is collected from the dialysis bag.

Overall, dialysis is a gentle, easy and low-cost technique that does not require harsh conditions, preserving the integrity and stability of the nanocarriers [93]. It allows for the efficient removal of small impurities, leading to high-purity formulations. The process does not introduce harmful or harsh chemicals, making it suitable for preparing formulations for biological or pharmaceutical applications. Dialysis can be easily scaled up for larger volumes, making it suitable for both laboratory research and large-scale manufacturing. This method ensures that the final product is free from unwanted molecules and is suitable for use in a variety of applications, including drug delivery and biomedical research.

To quantify the amount of encapsulated cargo, the **encapsulation efficiency (EE)** is determined, usually by dialysis followed by UV-Vis spectrophotometry, LC-MS, or by pressure ultrafiltration followed by reverse-phase HPLC [90]. Namely, in the case of the firstly mentioned method, EE of the encapsulated compounds presenting absorption in the UV-Vis regions of light wavelength may be evaluated by UV-Vis spectroscopy after dissolving in proper solvent, e.g. ethanol [99]. Firstly, the standard calibration curve of the encapsulated cargo is prepared to calculate further the weight of the cargo in the formulation before and after dialysis based on its Absorbance in the same solvent. Briefly, the EE is calculated through the following equation [99]:

$$EE [\%] = \frac{\text{weight of the cargo after dialysis}}{\text{weight of the cargo before dialysis}} * 100 \quad (\text{Eq. 2.6})$$

2.2.3. Physicochemical characterization

The prepared nanoformulations are subjected to a series of physicochemical tests aimed at assessing the basic features occurring in the formulation of LLCNPs in terms of their structure, morphology, size, polydispersity, etc. There are three frequently used scattering techniques: **small-angle X-ray scattering (SAXS)** for surface structure determination, **electrophoretic light scattering (ELS)** and **dynamic light scattering (DLS)** for determination of stability, polydispersity, size and electrophoretic mobility [85,90,100–102]. In addition, through the **cryogenic transmission electron microscopy (cryo-TEM)** experiments, it is possible to assess the morphology and confirm the internal structure and phase of the studied nanoparticles [87,100,102–104]. Nanocarriers are typically designed for drug delivery; therefore, understanding their characteristics is crucial for optimizing their performance.

2.2.3.1. SAXS

One of the key experimental tools useful for investigating LLCNPs is **small-angle X-ray scattering (SAXS)**, a powerful method for characterizing their structural properties, typically in the range of 1–100 nm (or higher with the synchrotron radiation). Particularly, type of the bulk phase, including local order, defined by lattice parameter, water channel radius and the overall nanoparticle dimension, are evaluated [100,101,105,106]. Based on the scattering pattern analysis, various phases can be identified by their characteristic peak arrangement, such as Im3m, Ia3d, Pn3m inverse bicontinuous cubic or inverse hexagonal (H_{II}) phase.

SAXS is sensitive to the polydispersity of the studied nanosystems. The width of the SAXS peak can indicate the degree of uniformity in size, with broader peaks indicating greater size distribution. This is important for formulations where uniformity is critical to performance. This technique can also provide insights into the surface properties of nanocarriers, including the presence of any surface modifications, coatings, or functional groups. These features are key for improving the bioavailability and targeting capabilities of nanocarriers.

The principle of SAXS operation is based on the measurements of the X-ray scattering emitted from a special X-ray lamp, as it passes through the sample and interacts with its electrons [107]. The X-rays are scattered at small angles (typically less than 5°) in various directions and are detected by the detector [101]. The amount of scattering depends on the electron density of the sample; thus, its different regions (e.g., related to various structures and phases in one sample) cause the scattering of the X-rays to different extents. The scattering angle, along with the

intensity of the scattered X-rays, is measured. Therefore, the scattering data is plotted as intensity dependent on the scattering vector (q), related to the scattering angle (2θ), and the wavelength of the X-ray radiation (λ), expressed by the following equation [101,107]:

$$q = \frac{4\pi \sin \theta}{\lambda} \quad (\text{Eq. 2.7})$$

Well-ordered objects with periodic arrangement present sharp peaks (Bragg peaks), detected by SAXS, in the pattern described by the Bragg equation [101,107]:

$$n\lambda = 2d \sin \theta \quad (\text{Eq. 2.8})$$

where n is the order of diffraction, and d is the inter-crystalline spacing, the distance between the lattice planes identified by Miller indices (hkl).

The ratios between the Bragg peaks are related to specific LLC phase, characterized by the **lattice parameter** (a) of the elementary cell with specific Miller's indices (hkl), while the **water channel radius** (r_w) can be calculated through the following equations [105]:

A) in case of the inverse hexagonal phase (H_{II}):

$$a = \frac{2}{\sqrt{3}} * d * \sqrt{h^2 + k^2 + hk} \quad (\text{Eq. 2.9})$$

$$r_w = \sqrt{(1 - \phi)} * \left(\frac{\sqrt{3}}{2}\right)^{\frac{1}{2}} \quad (\text{Eq. 2.10})$$

hkl (H_{II}): 100, 110, 200, 210, 220, 300 lattice dimension: 2D ($l=0$)

where ϕ is the volume fraction (lipid fraction), described by following equation:

$$\phi = \left(\frac{\frac{w_{lip}}{\rho_{lip}}}{\frac{w_{lip}}{\rho_{lip}} + \frac{w_{solv}}{\rho_{solv}}} \right) \quad (\text{Eq. 2.11})$$

where w_{lip}, ρ_{lip} correspond to the weight and density of the building lipid, and respectively w_{solv}, ρ_{solv} correspond to the weight and density of the solvent, e.g. water).

B) in case of the inverse cubic phases ($Im3m$, $Ia3d$, $Pn3m$):

$$a = d * \sqrt{h^2 + k^2 + l^2} \quad (\text{Eq. 2.12})$$

$$r_w = a * \left(\frac{A_o}{-2\pi\chi} \right)^{\frac{1}{2}} - l \quad (\text{Eq. 2.13})$$

- ♦ hkl (Im3m): 110, 200, 211, 220, 310, 222 lattice dimension: 3D
- ♦ hkl (Ia3d): 211, 220, 321, 400, 420, 332 lattice dimension: 3D
- ♦ hkl (Pn3m): 110, 111, 200, 211, 220, 221 lattice dimension: 3D

where l is the lipid hydrophobic chain length (thickness of the monolayer) at 25 °C, χ is the Euler-Poincaré characteristic [105,108], and A_o is the ratio of the minimal surfaces in a unit cell, useful for the calculation of the cubic nanoparticle r_w (see **Eq. 2.13**), and are presented for various cubic phases in **Table 2.1**.

Table 2.1. Values of Euler-Poincaré characteristics χ , along with the ratio of the various minimal surfaces in a unit cell A_o , for different cubic phases [105,108].

Cubic phase	Minimal surface	χ	A_o
Im3m	Primitive	-4	2.345
Ia3d	Gyroid	-8	3.091
Pn3m	Diamond	-2	1.919

Moreover, the r_{w-L_3} represents the water channel radius of the sponge phase (L_3), and is calculated by following equation [109]:

$$r_{w-L_3} = \frac{d_{L_3}}{d_{cubic}} r_{w-cubic} \quad (\text{Eq. 2.14})$$

where d_{L_3} and d_{cubic} are d-spacings of the sponge phase and the previous cubic phase, respectively, and $r_{w-cubic}$ is the water channel radius of the cubic phase.

Therefore, the scattering pattern contains information about the size, shape, internal structure organization, type of the bulk phase and dimension of the nanoparticles [100,101,105]. Moreover, SAXS enables the assessment of possible changes in the nanostructure under different conditions. For instance, this technique can be applied to monitor the stability of the drug nanocarriers over time, as well as at different temperatures, which is an important investigation for potential *in vivo* applications [95]. Changes in the scattering pattern can indicate alterations in the nanocarrier structure, such as aggregation, disintegration or phase

transition. Thus, SAXS is a versatile and valuable technique for quality control and optimization of the newly developed nanoformulations. It's especially useful in the pharmaceutical and biomedical fields for investigating the structure and stability of drug-loaded nanosystems. In contrast to being forced to solid-state preparations, studying the samples in solution without damage is an advantage crucial in the case of biological and sensitive materials, and enables the evaluation of the sample's behavior in conditions closer to the aqueous cellular environment of *in vivo* applications. Moreover, the data collection and analysis can be quite rapid, making SAXS an excellent tool for high-throughput and complex structural analysis.

2.2.3.2. Cryo-TEM

In addition to standard optical microscopy, some materials such as nanoparticles require devices capable of imaging these nano-scale structures in higher resolution, such as electron-based techniques, namely **scanning electron microscopy (SEM)** or **transmission electron microscopy (TEM)**. SEM enables imaging of the sample surface by scanning it with a focused electron beam, leading to the emission of secondary electrons and backscattered electrons by the excited atoms, which are received by the detector and imaged to assess the sample surface topography [110–112]. TEM, on the other hand, is more useful for the visualization of the internal morphology of the studied material at atomic resolution (0.1 nm), thanks to the transmission of the electrons [111]. TEM is a technique which images the ultra-thin sample (around 100 nm) by passing through a focused beam of electrons (transmission), which interact with the sample and the scattered electrons are collected to form an image, which is magnified and focused on the imaging device, such as fluorescent screens or digital cameras [111,112]. The contrast in the final image is acquired from the differences in electron scattering in different parts of the sample: regions that backscatter more electrons (e.g., denser or heavier elements with higher atomic numbers) appear brighter, while areas that scatter fewer electrons appear darker (e.g. water) [111,113].

These techniques are based on focusing the emitted radiation using special electromagnetic lenses, and then the focused beam of electrons hits the sample. A high vacuum is necessary to maintain the electron beam focus; therefore, the limitation of these techniques is the dryness of the tested materials [111,112]. Hence, TEM is useful for solid-state samples, which can be thinly sliced, chemically stained or dehydrated, nevertheless, this may change or destroy their internal structure. Nevertheless, in case of the liquid wet samples such as soft colloidal nanosystems composed of LLCNPs, the technique called **cryogenic transmission electron microscopy (cryo-TEM)** is applied, due to the possibility [100,114,115].

Cryo-TEM is an imaging technique used to observe the morphological and structural details of formulations at high resolution, even at the atomic or sub-nanometer scale [103]. In contrast to traditional TEM, it involves observation of the samples at cryogenic temperatures (around liquid nitrogen temperature, -196 °C), which helps to preserve the sample's natural, hydrated state without the need for staining or drying. Since Cryo-TEM operates at very low temperatures, there is minimal damage from electron beam exposure [116], which is a common problem with traditional TEM. This allows for high-resolution imaging of sensitive samples. The contrast in cryo-TEM images and typical TEM, comes from the various electrons scattering by different regions of the sample, depending on their density and structures presented [117].

All of this is possible thanks to the application of the **vitrification method** developed by James Dubochet for organic materials observation, around the 1980s [116]. The water-based sample is placed on a metal grid, which is typically covered with a thin layer of carbon or other materials to support the sample, and rapidly frozen in liquid ethane (-190 °C) and then stored in liquid nitrogen between the measurements [100,117]. The rapid freezing occurs at very high speeds (milliseconds), necessary to prevent the formation of ice crystals. The vitrification method turns the water in the sample into vitreous ice, a disordered glass state (non-crystalline ice), rather than forming ice crystals that could destroy the sample's structure, and diffract the microscope's electron beam, thus disturbing the results [103,117]. The grid is transferred into the vacuum chamber of the cryo-TEM microscope, preventing water evaporation from the sample and thus preserving its stability [115]. During and between the measurements, the sample is cooled by liquid nitrogen. In this way, the native, hydrated state of the studied liquid nanoformulations is preserved, as well as the bulk state; therefore, various structural and morphological aspects of the studied nanoparticles can be evaluated by cryo-TEM, such as size and shape [103,115,118]. Further investigations, such as fast Fourier transforms of the cryo-TEM images, enable a more detailed assessment of the crystallographic structure and arrangement of the studied nanoparticles [119]; therefore, it may be very useful for the confirmation of the results obtained by SAXS. In addition, the advantage of studying water solutions close to the physiological environment of cells provides insights into the behavior of future nanocarriers in the conditions of biological applications, and thus, the observations are crucial for nanomedicine development. In addition to the evaluation of nanomaterials, this technique is also applied to image various types of biological macromolecules that require an atomic scale, such as proteins, viruses, lipid bilayers or other cellular organelles [103,117,120].

2.2.3.3. ELS, DLS

Electrophoretic light scattering (ELS) and **dynamic light scattering (DLS)** provide two techniques based on light scattering, measured in one instrument, useful for the research on colloidal nanoparticles in nano- and submicrometer ranges, focused on different aspects of the particle behavior and characteristics [101,121]. Namely, ELS is used for the electrophoretic mobility evaluation, providing information about the stability of the colloidal nanosystem by the zeta potential parameter indicating particle surface charge, while DLS, by analysis of the particle's diffusion in a solution, measure its size, as well as the overall polydispersity of the formulation [101,122,123].

DLS, also known as **photon correlation spectroscopy (PCS)**, is a technique used to evaluate the size distribution of small particles in the formulation by the analysis of fluctuations in the scattered light intensity caused by the random Brownian motions of particles in a liquid medium [101,121–125]. Namely, a monochromatic laser light (e.g. $\lambda = 632.8$ nm, depending on the instrument used) is directed at the studied sample, and the light is scattered by various particles of the formulation in different directions. Due to the Brownian motions of the particles in solution [126], the intensity of the scattered light fluctuates. While smaller particles move faster, leading to rapid fluctuations, the larger ones move more slowly, with slower fluctuations. The scattered light is detected at a specific angle by a detector, which measures the changes in its intensity over time and calculates an autocorrelation function, describing how the intensity of the scattered light varies (fluctuates) over time [101,121–125]. Then, from this function, the **diffusion coefficient (D)** of a particle can be determined, and thanks to the Stokes-Einstein equation (**Eq. 2.15**), it is possible to determine the effective size of the particles, namely the **hydrodynamic diameter (d)**, typically in the range up to 1-3 μm [121]:

$$D = \frac{k_B T}{3\pi\eta d} \quad (\text{Eq. 2.15})$$

where D is the diffusion coefficient, k_B is the Boltzmann constant, T is the temperature, η is the viscosity of medium.

The hydrodynamic diameter is an **apparent hydrodynamic diameter** of the particle because it does not exactly describe the real diameter of a particle, but the diameter of a theoretical sphere that diffuses with the same diffusion coefficient as the particle in solution, due to the approximation of the hard-sphere model (apparent diffusion coefficient) [124,127]. The apparent hydrodynamic radius is dependent on the hydrodynamic forces, as well as inter-

particle interactions [101]. Therefore, since this parameter also considers ions and water molecules adhering to the nanoparticle surface, which influence the fluctuations in the scattered light, they are considered as a part of the particle.

ELS is a technique used to measure the electrophoretic mobility of particles in a liquid medium after applying an electric field (electrophoresis), where the light scattered by the moving particles is detected by the photodetector system, as well as in DLS [101,123,128,129]. The frequency of the scattered light changes due to the particle's movement, and this frequency shift (the Doppler shift) is measured in relation to the velocity of the particle [130]. The method relies on the fact that the charged particles move under the influence of an applied electric field towards the oppositely charged electrode, opposed by the viscous forces [101,123,128,129]. By reaching the equilibrium between these two, the constant velocity of that movement is achieved, namely the **electrophoretic mobility** (μ) described by the Henry equation (**Eq. 2.16**), depending on particle's charge (the zeta potential, described further in more detail), size, and the viscosity of the medium [101]:

$$\mu = \frac{2\zeta\varepsilon}{3\eta} f(\kappa R) \quad (\text{Eq. 2.16})$$

where ζ is the zeta potential, ε is dielectric constant of the medium, η is the viscosity of medium, and $f(\kappa R)$ is the Henry's function. κR describes the shape of the electrical double layer of curved surfaces by the ratio between the particle radius R , and the double layer thickness, estimated by the Debye screening length κ^{-1} . Therefore, for this equation, there are some limiting values, due to specific particle characteristics [101]:

- ♦ In case of particles small enough to not modify the applied field, thus treated as a point charge in an unperturbed electric field, but large enough to apply Stoke's law describing the frictional force of colloidal spherical particle moving in a medium ($f = 6\pi\eta R$), the $\kappa R \ll 1$, κ^{-1} is very big, therefore $\kappa R \rightarrow 0$, and $f(\kappa R) = 1$ (Hückel approximation).
- ♦ If the radius R of the particle is large enough, it is approximated as a planar (flat) charged surface exposed to flowing liquid, and the double layer thickness κ^{-1} is small, therefore $\kappa R \gg 1$ and $f(\kappa R) = 1.5$ (Smoluchowski approximation; applied in the thesis).

Zeta potential (ζ) is the parameter which represents the actual potential at the shear (slipping) plane of the **electrical double layer** (see **Figure 2.8**) surrounding the particle in a liquid medium, which represents the theoretical boundary between the movement of ions with

the solution (mobile diffuse layer) and the outer, diffuse and less strongly bounded ions region with the immobile counter-ions bounded to the particle surface (Stern layer) [128,131]. This parameter is a key indicator of colloidal stability, namely the higher the absolute values, the more stable the formulation, since the particles will repel each other (due to higher repulsive forces) instead of flocculating, while low values suggest the possible aggregation of the studied particles due to attractive forces [132].

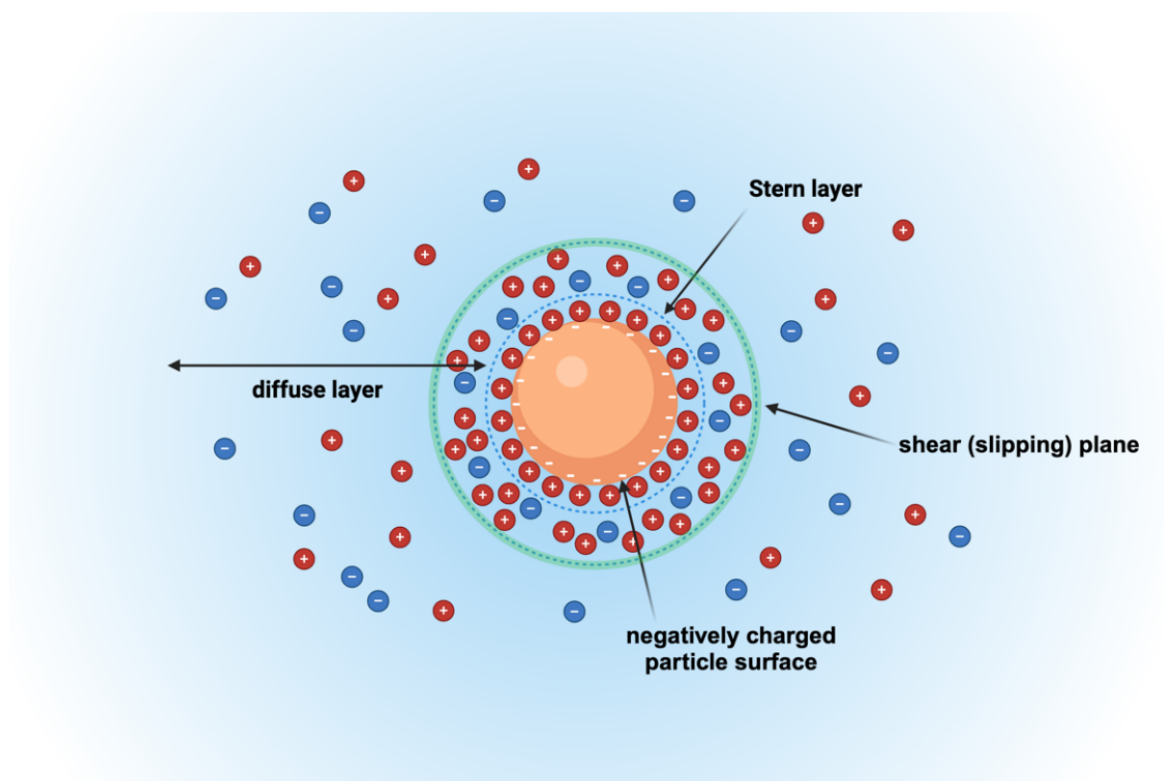


Figure 2.8. Schematic representation of the electrical double layer surrounding the particle in a liquid medium. Own elaboration reproduced with permission from [131].

Besides the characteristics of soft colloidal formulations, DLS and ELS are frequently used for other types of nanoparticles, as well as molecules such as proteins and polymers, mainly in terms of their size or stability [122,123]. In the case of the future pharmaceutical applications of the studied nanosystems, the assessment of their stability is crucial to know how long the developed formulation will be useful, without losing its initial properties responsible for its action, like in the case of drug delivery nanocarriers. Therefore, DLS and ELS measurements of the same sample done over time help to track potential changes in the formulation, such as aggregation and precipitation of the studied particles. ELS identifies the surface charge of potential drug nanocarriers, or biological structures, such as proteins. It is useful for predicting their behavior in the cellular environment and some interactions with other molecules.

2.3. Bioactivity evaluation

2.3.1. ROS assay

As already mentioned in the Introduction (chapter 1.2.4.3. *Photodynamic therapy (PDT)*), reactive oxygen species (ROS) are highly reactive molecules that can cause cellular damage associated with various diseases such as neurodegenerative disorders, cardiovascular diseases, or cancers, through increased oxidative stress caused by the imbalance between ROS production and antioxidant defenses. Therefore, their levels are tightly regulated under normal conditions in cells. Nevertheless, ROS play a dual role in cancer, because they are one of the promoting factors of tumorigenesis, but at the same time, ROS production by the molecules with this specific ability is used as a mechanism for anticancer therapy-induced cell death [133,134]. Thus, various ROS assays are implemented in the research, depending on its goal.

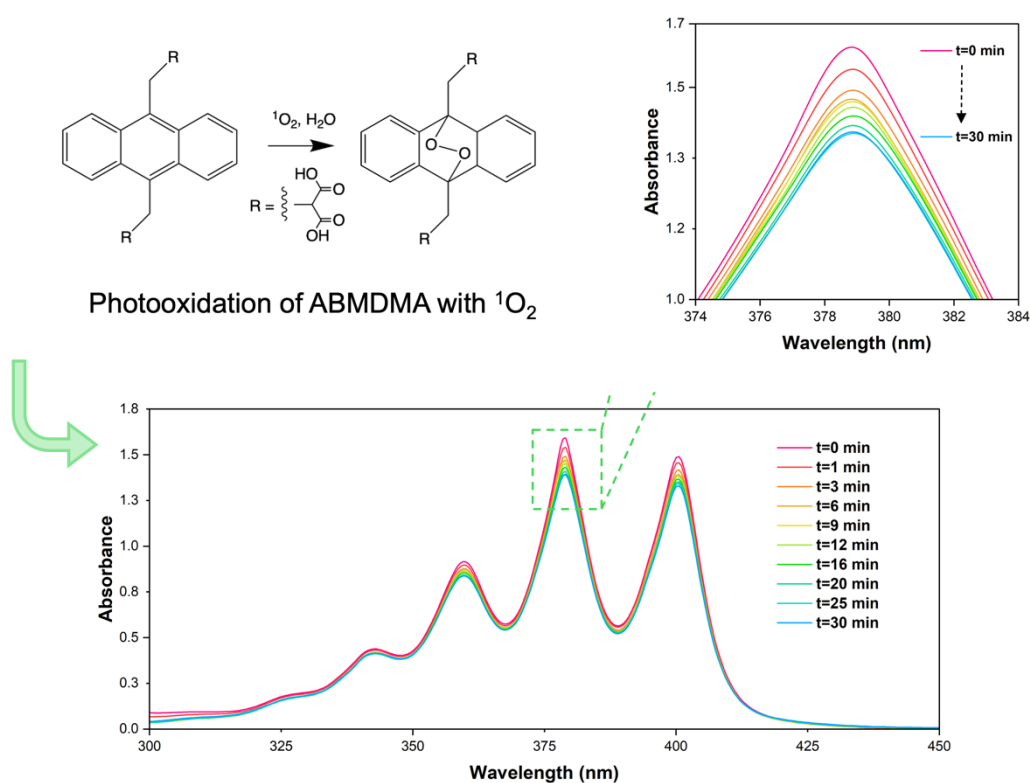


Figure 2.9. Schematic representation of the ROS assay for the evaluation of $^1\text{O}_2$ production in the studied sample, based on the photooxidation of the scavenger molecule 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA), leading to the decrease of its absorbance, measured in time.

Some of them are used to evaluate the efficacy of antioxidant compounds or supplements in reducing ROS levels and oxidative damage in normal cells [135,136], while other ones provide the preliminary assessment of the photosensitizers' ability to generate ROS, which would ultimately induce cancer cell death through oxidative stress in future anticancer treatments, such as PDT [137–140]. Therefore, for future application in PDT of the developed nanosystems, the necessary primary information about the potential photoactive action of the studied photosensitizers, as well as the influence of the encapsulation on this activity, is studied by the **ROS assay** presented in **Figure 2.9**. This technique provides information about the levels of ROS generation by the studied samples and enables the evaluation of an efficient concentration of the photoactive cargo for the next biological experiments on cells. Experiments are based on the reaction of highly reactive singlet oxygen ($^1\text{O}_2$) with the scavenger molecule 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA). Singlet oxygen causes photooxidation of ABMDMA, resulting in a decrease of its absorbance measured in time at specific wavelength by UV-Vis spectroscopy. The remaining ABMDMA is calculated in % for each sample. The greater the decrease in the scavenger absorbance, the greater the ability of the studied sample to generate ROS, corresponding to its potential photodynamic anticancer activity, which is the case studied in the next experiments on cells for the development of therapies targeting oxidative damage, such as PDT.

2.3.2. Cell cultures

The possibility of growing and manipulating cells outside their natural environment in the form of **cell cultures** constitutes an essential tool in biological research, which enables *in vitro* studies, an integral part of the research on various potential drugs. Cell culture, originating from tissue culture in a drop of medium developed by Ross Harrison, the so-called "hanging drop technique," was further advanced by Alexis Carel and Charles Lindbergh to grow tissue on glass plates and find methods for continuous growth, thus evolved into the process of culturing cells from various sources in a monolayer in an appropriate medium [141].

Several different types of cell cultures are presented and categorized based on their origin and growth (see **Figure 2.10**). The **primary cell cultures** are the firstly cultured cells directly taken from living tissues or organs of an organism and characterized by the same features as cells in the original tissue [142]. The primary cell cultures are also categorized based on their growth characteristics, namely **suspension cells** and **adherent cells** [142–144]. The suspension cultures grow in a liquid medium, free-floating, since they do not require attachment to grow,

therefore their growth is limited to the concentration of cells in the medium and subculturing does not require enzymatic dissociation [143]. The adherent cultures require a solid surface to attach and properly grow in the form of monolayer in culture flasks (plastic bottles) or Petri dishes [145]. The growth of adherent cells is limited to the surface area of the culture vessel. Thus, to prevent their overgrowth or expand cell population needed for various experiments, adherent cells should be subcultured (passaged) to a new culture vessel to provide space and fresh nutrients for further growth. In short, subculture refers to the process where cells detach from one another, as well as from the surface, creating a single-cell suspension that can be measured, and then reseeded at the lower concentration into a new vessel, forming a **secondary culture** [144]. The process can be repeated to produce tertiary cultures and beyond, providing a **finite cell line** with a limited number of passages (lifespan), in contrast to **continuous cell lines** (e.g. cancerous cells), capable of an unlimited number of population doublings (indefinite rapid divisions), thus being known as immortal cell culture [142–144]. Passaging of adherent cells involves trypsinization, namely an enzyme-assisted detachment of the cells from the substrate (using trypsin) [146]. Then, after subsequent neutralization with a medium, centrifugation and resuspension in a new medium, cells are transferred to a new vessel [142] or to a specific plate e.g. a multi-well plate in the case of using the cells for an assay. Cells are cultured in the appropriate media (culture growth media) selected based on a given type of cell line, to preserve proper cell growth and maintain stable pH and osmolality [147].

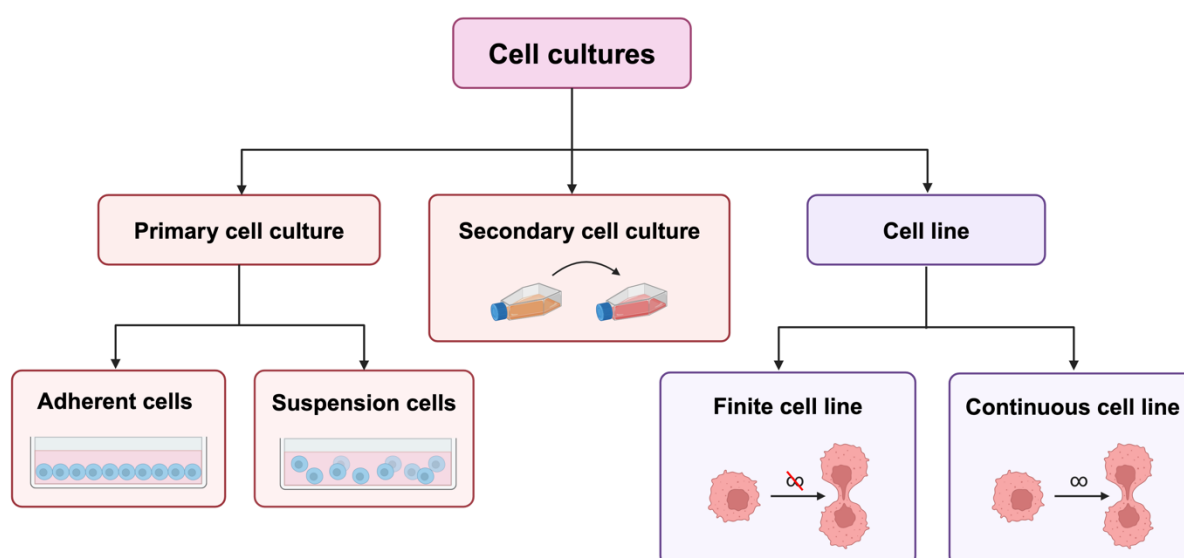


Figure 2.10. The classification of cell cultures.

Some commonly used media with a wide range of applications, including cancer cell culture, are DMEM or RPMI-1640, characterized by specific composition, including vitamins, carbohydrates, amino acids, glucose, salts, and a buffering system [143,147]. To ensure a continuous supply of essential nutrients for the growth and cell line development, the culture medium should be systematically replaced. The decision is often based on its color due to pH indicators (e.g. phenol red) causing the medium color change along with pH shifts, indicating, for example, high content of metabolites, dead cells, contaminants or waste products, leading to acidification (yellow color) [142,143,148]. Moreover, cell culture requires specific conditions for proper growth, such as a stable temperature 37 °C, pH at 7.2–7.4, controlled CO₂ level at 5% and relative humidity at 95%; thus, the cultured cell lines are incubated in appropriate (CO₂)-humidified incubators that ensure the conditions control [142].

Each type of cell culture has its specific applications depending on the research focus, whether it is studying basic cellular mechanisms, drug testing, or disease modelling. *In vitro* studies enable testing biochemical mechanisms in various conditions, through the possibility of manipulating various controlled factors affecting the culture. In this way, *in vitro* tests constitute an invaluable tool for assessing detailed cellular processes induced, for example, by the action of a potential anticancer drug, leading to various types of cell death [95,149–151]. Those can be examined by cytotoxicity tests such as MTT assay, as well as by the assessment of morphological changes through techniques such as optical and fluorescence microscopy, but also recently discovered, modern method for imaging living cells in real-time without the need for staining, a holotomographic microscopy (HTM), which, in addition to standard visualization techniques, gives the possibility of developing results in the form of 3D structures.

2.3.3. MTT cytotoxicity

One of the most common, rapid, versatile, highly reproducible, and relatively simple quantitative methods for cytotoxicity evaluation, including testing of substances to be used as drugs, is the **MTT cytotoxicity** test. It provides information about cell viability after treatment with various samples, in comparison to untreated control cells [152,153]. According to McCauley et al., the MTT *in vitro* cell proliferation assay is a reliable, colorimetric method to assess the preliminary anticancer potential of both synthetic and naturally derived compounds, applied to a variety of cell lines, indicating the overall cytotoxicity [154]. In addition, this test can also be used to assess the biocompatibility of a potential photosensitizer

using an experiment in dark conditions, where low or no cytotoxicity is expected, while it supposed to be induced only by irradiation (phototoxicity) [96,155,156].

The idea of the MTT test is based on the ability of living cells to convert the yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) into a purple formazan product (**Figure 2.11**), which amount is directly proportional to the number of viable cells [153,157,158].

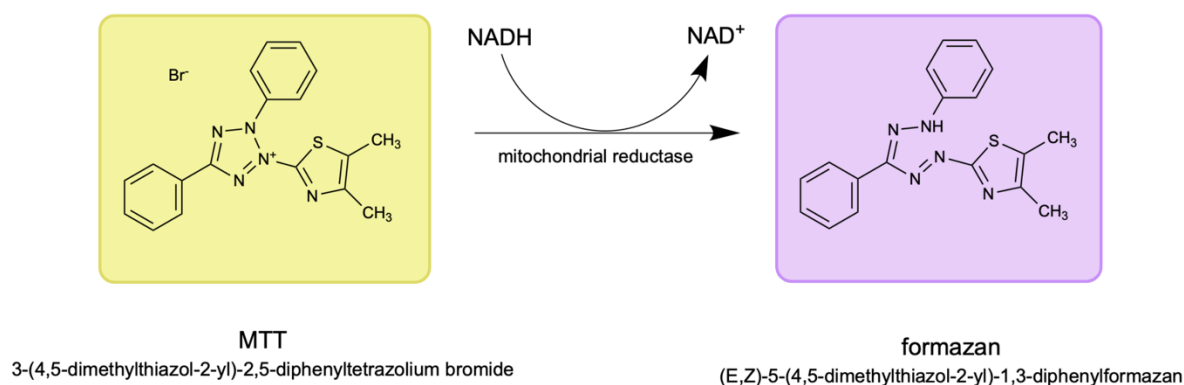


Figure 2.11 Reduction of yellow MTT salt into purple crystals of formazan by mitochondrial reductase (succinate dehydrogenase). Own elaboration reproduced with permission from [157].

The reduction of MTT salt to formazan crystals is done by **mitochondrial enzymes**, such as succinate dehydrogenase, which are present only in viable, metabolically active cells [142,152]. The product of this reaction is insoluble in water, so it must be dissolved in a suitable solvent, usually isopropanol or DMSO, to measure its absorbance (the intensity of purple color) proportional to its amount produced by cells, and therefore correlating with the number of viable cells [153,158]. This measurement is performed at 570 nm in a specific UV-Vis spectrophotometer with a 96-well plate reader (the microplate reader), and compared to the control, to calculate % of cell viability from the following equation:

$$[\%] \text{ cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} * 100\% \quad (\text{Eq. 2.17})$$

Firstly, the cells are cultured in a 96-well plate. Once they stick to the substrate, the culture medium is replaced with different concentrations of the tested substance diluted with the medium. Cells treated with the tested samples are incubated for a given time. Sometimes, several texts are performed at different incubation times to show the dependence of cytotoxicity

on the time of action of a potential drug [159]. After that, MTT reagent is added to each well, which during incubation, usually lasting 1.5-2 hours, leads to the formation of formazan crystals by viable cells. The purple formazan is dissolved by the appropriate solvent, and the absorbance is measured at 570 nm in each well. The low intensity of the purple color in the transparent solution, related to lower mitochondrial activity, indicates that such treatment has induced cell death, indicating cytotoxicity. Then, the cytotoxic effect is higher when the absorbance is lower.

MTT assay provides quantitative data on cell viability, allowing for comparison between different treatments with various substances at different concentrations, such as potential drugs. Sometimes the effectiveness of a given substance is assessed for killing, e.g., cancer cells, then the higher the cytotoxicity, the more effective the drug. However, as already mentioned, in cases such as the assessment of formulations useful for PDT, after the MTT test is performed in the dark, low or no cytotoxicity is expected, in contrast to the phototoxicity evaluated by the MTT test after irradiation of a photosensitizer with the light of an appropriate wavelength. Besides the potential drug testing, MTT cytotoxicity also provides a useful tool for the empty nanocarriers biocompatibility determination [95,160–162], as well as the toxicity assessment of environmental pollutants such as industrial-derived chemicals [163,164].

2.3.4. Flow cytometry

Flow cytometry is used to analyze cells suspended in a fluid stream for their physical and chemical characteristics, such as the number of specific cells, their size, complexity, granularity, or presence of specific biomarkers, making it a crucial tool in immunology, cell biology, and diagnostics [165]. This technique relies on passing a stream of cells through a laser beam, one at a time, and analysis of the individual cell's characteristics in a population thanks to the light scattering and the presence of specific fluorescence markers [166], as described thoroughly in the next paragraph.

For this purpose, a cell suspension is injected into the flow cytometer, and with the use of a fluidics system, it is hydrodynamically focused into a single stream alignment, which ensures that cells are analyzed individually [166]. The lasers pass through the flow of cells, and when the beam hits the cell, it scatters light and emits a fluorescence signal, due to the excitation of specific fluorochromes bound, and therefore, the cellular markers or other fluorescent molecules (e.g., taken up by the cell) are detected. The scattered light and emitted signals of fluorescence are collected by specific detectors (photomultiplier tubes and photodiodes) [167]. Then, the measured light signals are converted into digital data, processed and analyzed

by specialized software, providing the results in the form of graphs, scatter plots or histograms. Each type of detector provides different information about the cell characteristics, due to various types of light measured, such as forward scatter (FSC), side scatter (SSC) and fluorescence (**Figure 2.12**) [165,166].

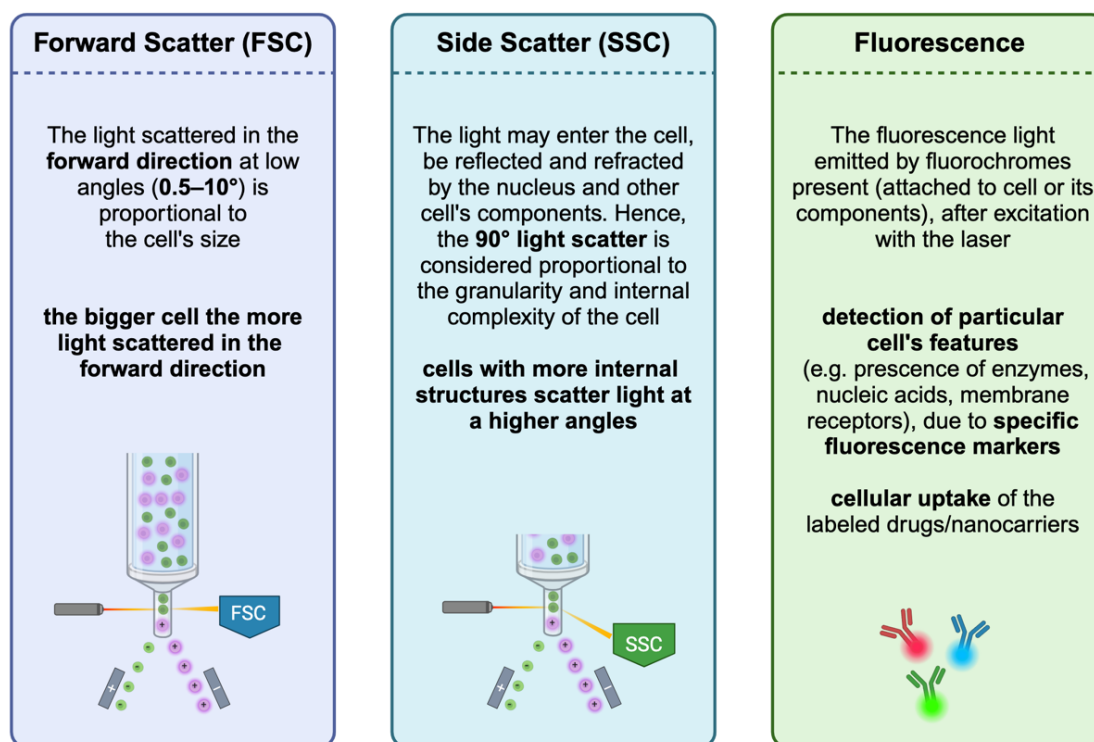


Figure 2.12. Three types of light measured by the detectors of a flow cytometer [165,166].

Usually, to characterize cells, they are labelled with specific fluorochrome-linked antibodies that bind to the surface of the cell or inside through specific molecules of interest or are stained by membrane-, cytoplasmic-, or nuclear-fluorescent dyes [166]. The markers allow for the detection of specific cell populations (e.g. immune cells), the content of DNA for cell cycle analysis, detection of apoptosis, evaluation of specific proteins and gene expression, as well as many others [165]. Moreover, it is a valuable technique for the evaluation of the cellular uptake of the fluorescent compounds, or for instance, in the case of nanotheranostics, the uptake of nanocarriers loaded with them [161,168,169]. Such an approach makes it possible to evaluate the benefits of encapsulating a potential drug. One of the tools applied for this purpose, and measured by flow cytometer, is FACS, a **Fluorescence-Activated Cell Sorting**, useful for the separation of cells based on their characteristics, for their further analysis (e.g. quantification) or experiments [167].

Overall, flow cytometry is an essential technique for the detailed analysis of individual cells in a population, offering insights into their size, complexity, granularity, or specific features. Its high sensitivity, speed, and ability to simultaneously analyze multiple parameters make it a powerful tool for applications in various pharmaceutical, medical and molecular biology fields.

2.3.5. Optical microscopy

One of the simplest microscopic techniques used to visualize and study the morphology, structure and behavior of various cell lines, tissues, and microorganisms is the **optical microscopy** [170,171]. This technique is the most commonly used imaging method in biological research, allowing for a quick assessment of the appearance of a cell culture. It provides a lot of information about the condition and the culture development. Consequently, it is possible to decide about the need to replace the growing medium, cell passaging, or the possibility of an experiment conducted using a given culture.

Optical microscopy consists of a specific lamp or LED source, responsible for the visible light emission, and a system of lenses for the magnification to capture detailed images directly by eye (through the ocular) or by camera for further analysis [172]. For this purpose, the sample is illuminated by a beam of visible light. The image is magnified by the objective lenses positioned close to the sample, and the ocular lenses located in the ocular [170,173]. This combination allows the image of the sample to be magnified, with the resolution depending on the light wavelength and the lens's quality. The maximum magnification of most optical microscopes is around 1000x [111], but higher values can be achieved thanks to techniques, such as oil immersion [172].

Since samples can be observed in real-time without the need for fixation, and thus, are vital and undamaged, this technique offers a multitude of applications in biological research. For example, it can be used to preliminarily visualize the structure and behavior of cells or their organelles, in various conditions (e.g. after different incubation times with the potential drug). Time-lapse microscopy allows the observation of living cells over time (real-time observation), providing insights into dynamic processes like cell migration, growth, and differentiation [174–176]. In clinical approaches, optical microscopy helps pathologists examine tissue samples to diagnose diseases like cancer by examining tissue morphology, cellular structure, and the presence of abnormal cells [177]. Therefore, optical microscopy is a fundamental tool in biological studies, providing insights into cellular structures and dynamics, as well as an overall assessment of the cell culture condition. Nevertheless, this technique is limited in terms

of the size of visualized objects, due to the diffraction limit of light and therefore limits in resolution of about 200 nm [111]. Hence, for the detailed observation of smaller structures, an advanced imaging technique is required.

2.3.6. Fluorescence microscopy

Fluorescence microscopy constitutes a tool very often applied in biological research to image individual cellular structures with increased resolution, such as nuclei or the cytoskeleton, after specific staining. This advanced form of optical microscopy is based on the fluorescence ability of the dyes (fluorophores) used to label specific molecules and cellular structures of interest and, therefore, visualize and study them with high sensitivity and specificity [178]. This makes it possible to track the presence and localization of various cellular components within cells, such as proteins and nucleic acids, or to study the morphology of cells treated with a potential drug [179–181].

The fluorophores used in fluorescence microscopy are either naturally origin molecules with fluorescence ability (e.g., green fluorescent protein (GFP), particularly useful for specific protein localization and cell dynamics investigation by protein-protein interactions) [182] or synthetic fluorescent dyes, such as DNA-specific 4',6-diamidino-2-phenylindole (DAPI) for the nuclei visualization [183]. Moreover, for cytoskeleton imaging, a mushroom-origin molecule, phalloidin is used for F-actin binding [184], conjugated with the fluorescent dye (e.g. iFluor® 488, Alexa Fluor® 488 (green) or Alexa Fluor® 546 (red)) for staining and the actin filaments visualization by fluorescent microscopy with high specificity [185–187]. It is possible to use different fluorophores to obtain a multicolor image, allowing the observation of different structures at once. The light with high intensity is emitted from the light source (such as a mercury lamp or LED) to excite specific fluorophores [188]. After the absorption of light by the fluorophores at specific wavelengths (excitation light with shorter wavelengths), the emitted fluorescence (emission light with longer wavelengths) is then passed through a set of optical filters and dichroic mirrors. They block out the excitation light by its reflection towards the sample and allow only the emitted light of the desired wavelength to pass through [178,188]. Then, the light is captured by the detector to generate a detailed image at a high resolution.

Standard fluorescence microscopy, where the entire field of view is excited by light and fluorescence is captured across the whole sample area, is called wide-field fluorescence microscopy [189]. Unfortunately, its limitations include non-specific background fluorescence, e.g., from the cell environment or out-of-focus light, disturbing the image of interest [189].

An advanced form of fluorescence microscopy that allows for higher resolution images is **confocal laser scanning microscopy (CLSM)**, which uses laser light to scan the sample point by point and eliminates the problem of out-of-focus light by a pinhole [190,191]. There are also other forms of refinement of this technique, selected due to the application, or type of cellular feature/structure studied. These include total internal reflection fluorescence (TIRF) microscopy, for selective imaging of events such as structure interactions and dynamics within the cell membrane [189,192,193], or multi-photon microscopy, which allows for deeper penetration of thick samples [189,194].

In addition to the wide range of various applications of fluorescence microscopy, it constitutes a valuable tool used to observe the behavior of cancer cells or changes in their morphology, such as F-actin organization [161,195,196], and therefore investigate the action of potential anticancer drugs or drugs-loaded nanocarriers, in the cancer research [197–200]. Since the emitted high-intensity light can lead to the induction of the destruction process of the tested cells (phototoxicity) with the possible emission of fluorescence, this technique can also be used to assess the action and distribution of a given third-generation photosensitizer in targeted disease cells [201,202].

Overall, this technique is characterized by high selectivity, sensitivity and resolution, thus, it is possible to visualize specific structures difficult to observe with a traditional optical microscope, to get detailed insights into cellular organization, molecular processes and interactions, gene expressions, or overall condition of cells. In addition, this technique enables live-cell imaging of cells over time, giving an overview, for instance, of dynamic and signaling processes [203,204].

2.3.7. Holotomographic microscopy

One of the recently developed modern techniques for imaging biological samples is **holotomographic microscopy (HTM)**, a non-invasive, label-free method that combines digital holography and optical tomography to produce high-resolution images, without the need for staining or using contrast agents, providing a whole range of advantages, including simpler and faster sample preparation or real-time non-invasive imaging of living cells [205,206]. The resolution of HTM can reach below the diffraction limit of traditional optical microscopy, allowing for the imaging of very small cellular structures, namely organelles like mitochondria or endoplasmic reticulum at submicrometric resolution [207–209]. The huge advantage of this

technique is the ability to view the specimen intravitaly and generate 3D images that provide quantitative information about the structures and composition of the studied sample.

As well as in the other microscopic techniques, in holotomographic microscopy, the light scattering phenomena are used to create information about the sample's structures. For this purpose, as it is illuminated by coherent light (such as a laser), a series of interference patterns (holograms) is captured. The holography technique measures the optical phase delay of the light scattered after passing through each part of the specimen, related to the distribution of its refractive index (RI) values, and the sample thickness, then the 3D distribution of those RI values is reconstructed through optical diffraction tomography technique by creation of cross-sectional images from the gathered data [210]. Therefore, those parameters provide information about the studied internal structure at high resolution [205]. For instance, while studying cells, this quantitative analysis discloses details about the volume, shape, and density of intracellular components [211]. For the reconstruction of the RI 3D distribution within the sample, advanced computational algorithms are used to create 3D image, presenting detailed morphological features of the studied cells, such as organization of various cellular structures, namely the organelles, cytoplasm, membranes, or extracellular matrix components [205,209,210].

The ability of real-time and non-invasive imaging of live cells or tissues is one of the key advantages of HTM, making it a particularly valuable tool for assessing dynamic biological processes, like molecular interactions, intracellular transport, cell divisions or migrations [205,206,212,213]. Moreover, this technique may provide information about the effects of drug treatments, such as studying various external and internal morphological changes (e.g. cytoskeleton organization), growth and cell survival behavior over time, related to the drug-induced cell death or stress responses to anticancer treatments [211]. For instance, it provides a quick distinction between healthy cells and the apoptotic ones [210,211]. According to the studies of Salucci et al., HTM seems to be a reliable method for analyzing well-established apoptotic features, including chromatin condensation, micronuclei, cell blebbing, and apoptotic bodies [210]. Therefore, HTM constitutes a valuable tool in the science of drug discovery and development, to monitor the response of cells to different drug treatments, such as novel drug-loaded nanocarriers, in a non-invasive, relatively fast manner, with high resolution and possibility of real-time imaging.

2.4. Techniques employed in the papers

Paper I

SAXS, DLS, ELS, cryo-TEM, dialysis, UV-Vis spectroscopy, fluorescence microscopy, MTT cytotoxicity, HTM, ROS assay

Paper II

S-L extraction (MAE), L-L extraction, HPLC-MS, HPLC-UV-Vis, dialysis, UV-Vis spectroscopy, SAXS, DLS, ELS, cryo-TEM, ROS assay, MTT cytotoxicity, flow cytometry, fluorescence microscopy

Paper III

S-L extraction (MAE), L-L extraction, MAE, LC, dialysis, UV-Vis spectroscopy, NMR, SAXS, DLS, ELS, cryo-TEM, MTT cytotoxicity, fluorescence microscopy, optical microscopy, HTM

2.5. References

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Chapter 3 – Novel cubosomes with increased biocompatibility loaded with photosensitizers for melanoma PDT

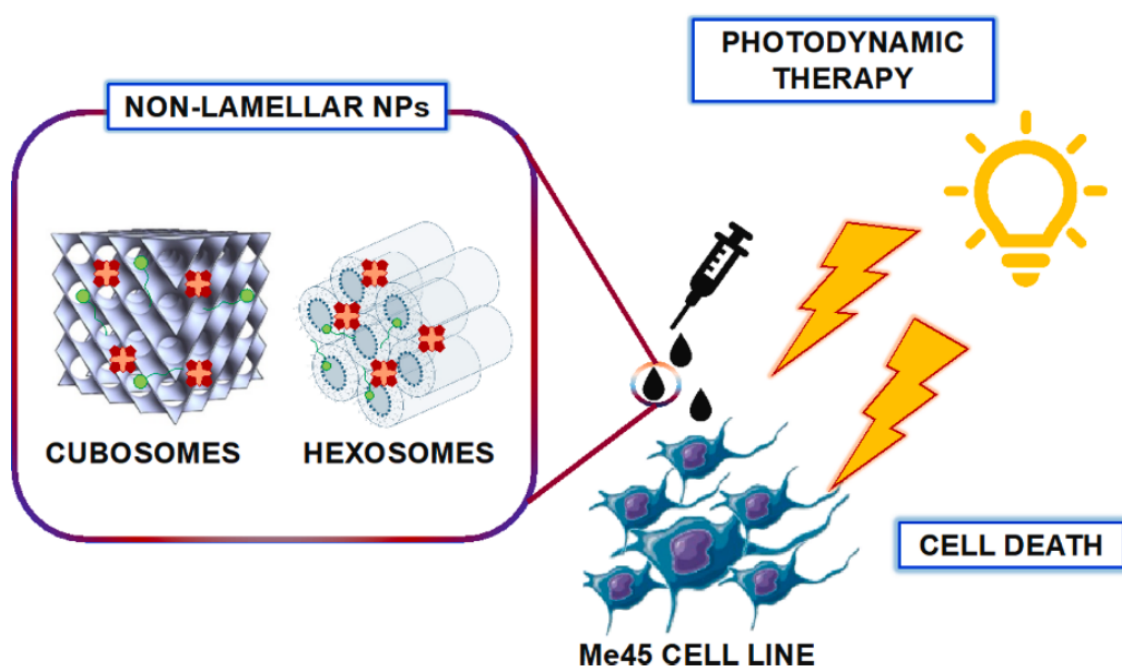
3.1. Cubosomes and hexosomes stabilized by sorbitan monooleate tuned with sodium taurocholate

3.2. Co-encapsulation of Ce6 and CoQH

3.3. Biocompatibility and photodynamic action against skin metastatic human melanoma

3.4. Conclusions and future perspectives

3.5. References



This chapter (refer to **Paper I**) investigates the development of novel monoolein-based, Pluronic-free lyotropic liquid crystalline nanoparticles (LLCNPs) – specifically, cubosomes (TS-CUB) and hexosomes (TS-HEX) – stabilized by sorbitan monooleate (Span 80, Sp80) and incorporating the bile salt sodium taurocholate (TC). These nanocarriers were designed as potential nanomedicines for photodynamic therapy (PDT) targeting skin metastatic melanoma. The systems were co-loaded with chlorin e6 (Ce6), a second-generation commercial photosensitizer, and the antioxidant coenzyme QH (CoQH), known for its potential anticancer properties. The active agents were embedded within the nanocarrier matrix to improve biocompatibility (via Sp80) and enhance skin penetration (via TC), supporting their potential for topical PDT application in the treatment of skin metastatic melanoma (see **Figure 3.1**).

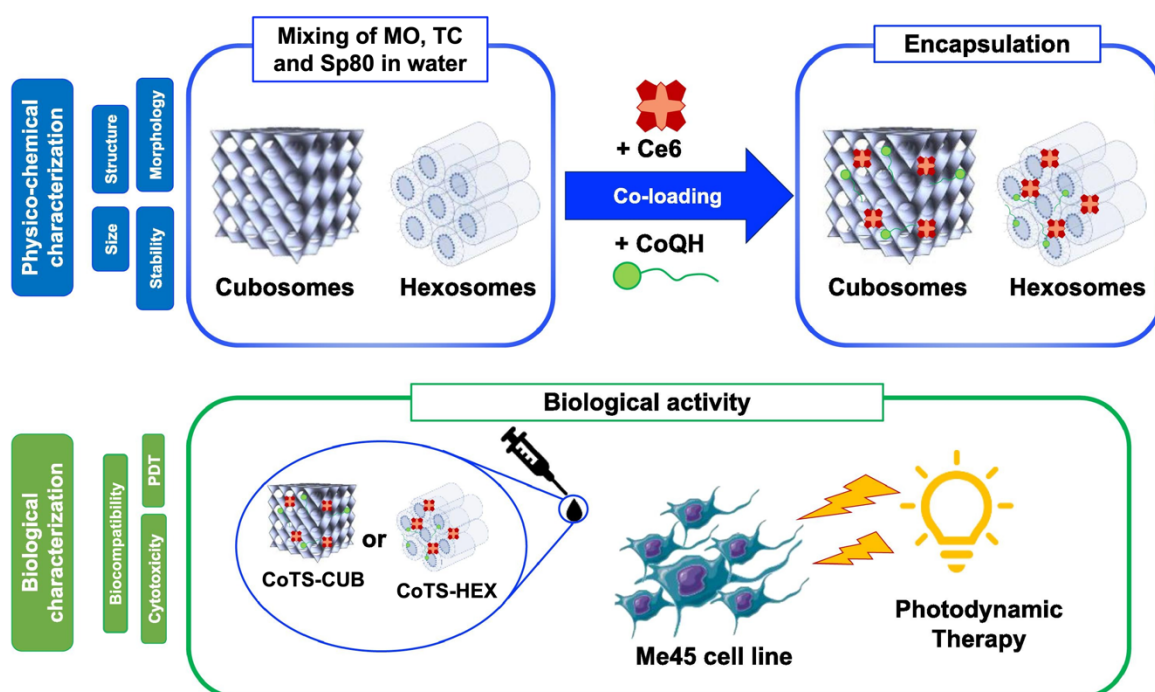


Figure 3.1. Schematic representation of the study. (**Paper I**)

3.1. Cubosomes and hexosomes stabilized by sorbitan monooleate tuned with sodium taurocholate

The stratum corneum (SC), the skin's outermost layer, acts as a crucial barrier that prevents water loss and shields the body from external threats like UV radiation chemicals, and pathogens [1,2]. Structurally, it resembles a “brick and mortar” system, with dead corneocytes as the “bricks” and rich in collagen, lamellar phase as the “mortar” [3]. To overcome this barrier for the topical administration of the developed formulations, permeation enhancers [4,5], like lipids, able to fluidify the SC, making it easier for the encapsulated drug to penetrate the skin layers. Furthermore, acting as softeners of the lipid membranes, edge activators are incorporated in the nanosystem [6,7]. Namely, compounds like bile salts, cholesterol, and polyethoxylated lipids [7–10] may increase the flexibility and elasticity of lipid carriers.

Since both Sp80 and TC can influence the MO phase behavior in water [11], an initial investigation was carried out to optimize the new formulations. As presented in previous studies, adding TC reduces the MO effective packing parameter, followed by an increase in interfacial positive curvature (toward water), thus leading to the transition from cubosomes to vesicles [8,9]. Here, Sp80 was added as stabilizer to counteract the lamellar-forming effect of TC, thus restoring the bicontinuous cubic nanostructure, or promoting the inverse hexagonal nanostructure, depending on the Sp80 concentration.

Table 3.1 Investigated samples' compositions: MO, Sp80, TC and water (W) presented in wt%, LLC phase, calculated lattice parameter (a), and water channel radius (r_w). (**Paper I**)

Sample	MO	Sp80	TC	W	Sp80:TC ratio	Phase	a (Å)	r_w (Å)
1	3.50	0.20	0.05	96.25	4:1	H _{II}	59.5 ± 0.3	30.7 ± 0.2
2	3.50	0.20	0.10	96.20	2:1	Pn3m	n.d.	n.d.
3	3.50	0.20	0.20	96.10	1:1	Vesicles	-	-
4	3.50	0.20	0.40	95.90	0.5:1	Vesicles	-	-
5	3.50	0.20	0.50	95.80	0.4:1	Vesicles	-	-
6	3.50	0.10	0.10	96.20	1:1	Vesicles	-	-
7	3.50	0.40	0.10	95.90	4:1	H _{II}	59.3 ± 0.3	30.6 ± 0.2
8	3.50	0.50	0.10	95.80	5:1	H _{II}	61.0 ± 0.2	31.5 ± 0.1

n.d. (not defined) – due to the low resolution of the SAXS pattern

Hence, for the optimization purposes, the mass ratio (wt%) of MO/TC or MO/Sp80 was kept constant, while varying the amounts of other components, as presented in **Table 3.1**. To investigate the combined effect of TC and Sp80 on the final nanostructure of the obtained LLCNPs, a preliminary assessment by SAXS measurements was conducted. The scattering diffractograms (see **Figures 3.2AB**) reveal that the native inverse cubic Pn3m nanostructure is lost, even at very low concentrations of 0.2 wt% Sp80 and 0.05 wt% TC (sample 1), since the SAXS pattern exhibits Bragg peaks characteristic of an inverse hexagonal (H_{II}) phase.

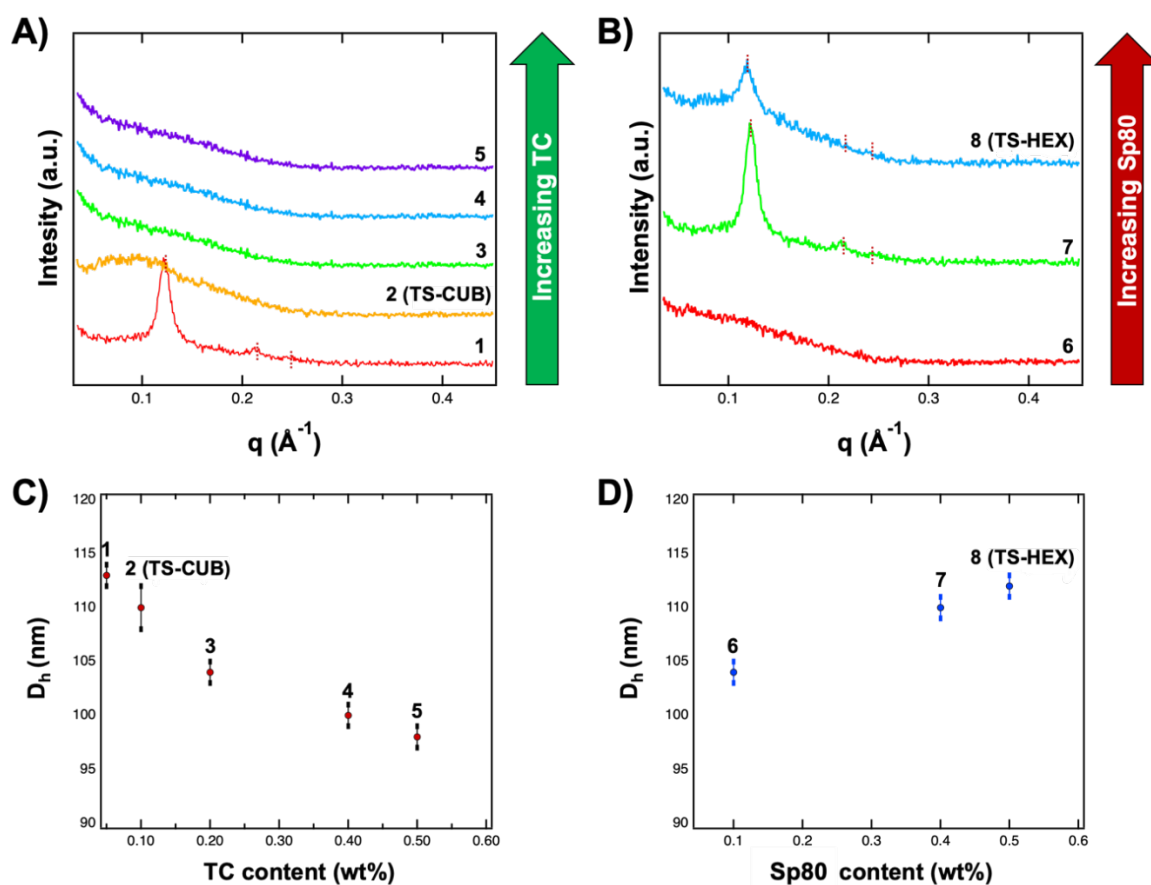


Figure 3.2. SAXS diffractograms recorded at 25 °C of the investigated samples with varying TC content (A) or Sp80 content (B). The presence of H_{II} phase is indicated by the dotted red lines of the assigned peaks. The dependence of an apparent hydrodynamic diameter (D_h) from various TC and Sp80 content, is presented on (C) and (D), respectively. (**Paper I**)

As observed in the SAXS pattern of sample 2, a slight increase in TC content leads to changes in the NPs internal structure. Despite the low resolution of the patterns, no Bragg peaks were observed, although the recorded signal closely resembles what is typically seen in the presence of cubosomes [12–14]. Additional TC amounts likely triggered the transition to unilamellar

vesicles, indicated by the broad, diffuse scattering pattern, as observed in formulations with similar lipid compositions [8,15,16]. Samples tested with higher content of Sp80 significantly highlight the role of TC in promoting negative interfacial curvature, as shown by the formation of an inverse hexagonal phase over the concentration 0.40 wt% of Sp80.

These initial findings prove that the combined effect of TC and Sp80 can be applied to modify the curvature of MO bilayers, even with small amounts of such surface active agents. To confirm the predicted internal structure, along with the presence of cubosome and hexosome LLCNPs, the formulations were analyzed by cryo-TEM (**Figure 3.3**). Due to the expected presence of cubosomes, sample 2 (TS-CUB) was chosen for further experiments, while sample 8 (TS-HEX) was selected because of its highest colloidal stability over time, compared to the other samples with H_{II} phase. Visual inspection confirmed the stability of both samples for about a month.

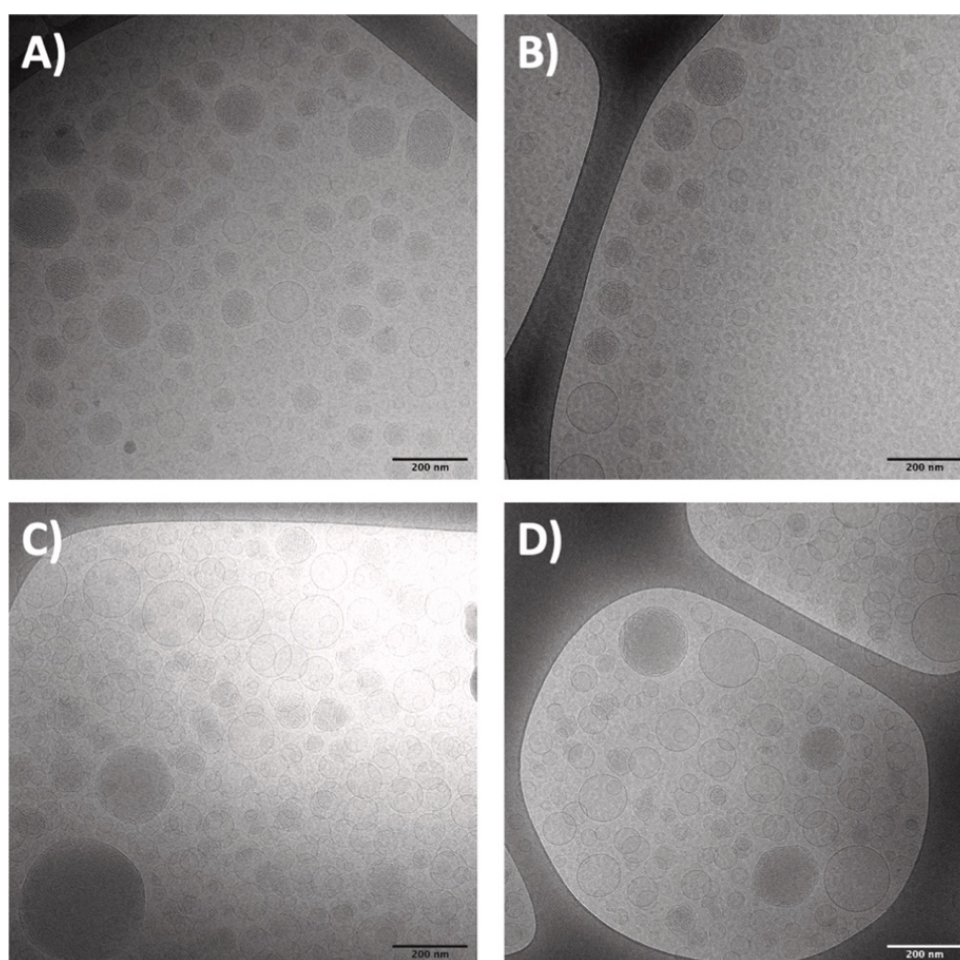


Figure 3.3. Cryo-TEM images of the selected sample 2 (TS-CUB, MO/Sp80/TC/W = 3.5/0.2/0.1/96.2) (A,B) and 8 (TS-HEX, MO/Sp80/TC/W = 3.5/0.5/0.1/95.8) (C,D). (**Paper I**)

As shown in **Figures 3.3A,B**, TS-CUB consists of many polydisperse vesicles and LLCNPs characterized by the honeycomb-like inner structure, typical for cubosomes [17]. The sample's heterogeneity is expected, as TC influences the self-assembly of MO, leading to the formation of various vesicles, as reported by Fornasier et al. in 2021 [8]. Nevertheless, this lamellar-forming tendency is counterbalanced by Sp80, enabling the formation of cubosomes, as anticipated. Consistent with the previous SAXS studies, **Figures 3.3C,D** confirm that the TS-HEX formulation is composed of hexosomes and vesicles, without the cubosomes presence. Similarly to TS-CUB, a large population of vesicles can be observed, indicating the formulation's heterogeneity. Overall, the SAXS and cryo-TEM screenings validated the optimal Sp80:TC ratios needed to form cubosomes and hexosomes with the MO as their building lipid. DLS measurements further revealed that increasing the Sp80:TC ratio led to a rise in the apparent hydrodynamic diameter (**Figures 3.2C,D**). Notably, the polydispersity index of these formulations was slightly higher (around 0.22–0.25) compared to those prepared with MO and other stabilizers (PDI around 0.05–0.15) [8,12,18–20], which can be attributed to the broad distribution of vesicles observed by cryo-TEM investigation, affecting the overall NPs size distribution in the studied samples.

3.2. Co-encapsulation of Ce6 and CoQH

With the aim of developing effective nanocarriers for drug delivery in PDT of skin metastatic human melanoma, previously optimized formulations were evaluated in this context. Thus, TS-CUB and TS-HEX formulations were chosen for the simultaneous encapsulation of Ce6, second-generation PS, and the antioxidant, CoQH, resulting in the two final nanosystems tested: cubosome-based CoTS-CUB with EE_{Ce6} of $(95 \pm 2)\%$ and EE_{CoQH} of $(93 \pm 2)\%$ and hexosome-based CoTS-HEX with EE_{Ce6} of $(90 \pm 1)\%$ and EE_{CoQH} of $(90 \pm 1)\%$. The compositions of TS-CUB and TS-HEX were MO/Sp80/TC/W = 3.5/0.2/0.1/96.2, and MO/Sp80/TC/W = 3.5/0.5/0.1/95.8, respectively. The high encapsulation efficiencies, measured by UV-Vis spectroscopy, indicate significant capacity of such novel cubosomes and hexosomes, to encapsulate the hydrophobic cargos (Ce6 and CoQH). This conclusion agrees with other studies indicating great ability of such LLCNPs to incorporate hydrophobic molecules into the lipid bilayer [21–25]. TS-CUB and TS-HEX loaded with the two hydrophobic cargos, were characterized by SAXS, DLS, ELS and cryo-TEM, followed by the biocompatibility, and anticancer photoactivity assessment through cyto-, phototoxicity and bioimaging studies.

SAXS results performed with higher resolution and intensity (**Figure 3.4**), revealed five low but distinct Bragg peaks, assigned to the Pn3m phase in the case of TS-CUB formulation, loaded and empty as well, as presented in **Figure 3.4A**. Hence, it could be stated that co-encapsulation of Ce6 and CoQH had minimal impact on the internal structure of both nanoformulations, with only a small shift of the peaks towards higher q values. This led to a slight reduction in the calculated lattice parameters, as well as the water channel radii, as presented in **Table 3.2**, which is consistent with other LLCNPs-based nanosystems loaded with hydrophobic drugs [17,26].

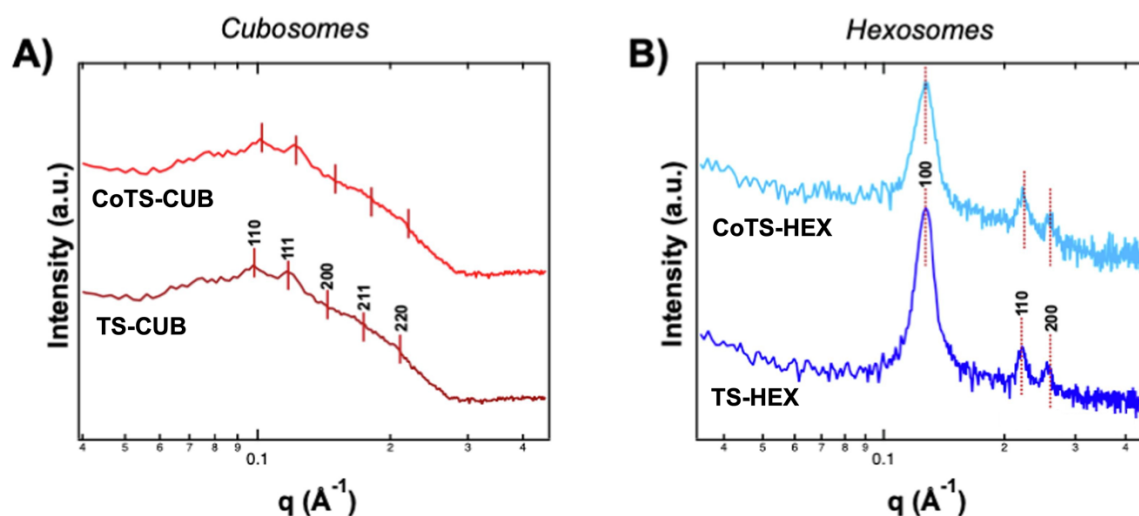


Figure 3.4. Log-log SAXS diffractograms of the loaded and empty TS-CUB (A) and TS-HEX (B). The peaks assigned to Pn3m and H_{II} phase, are marked with red line and dotted red line, respectively. Miller's indices are also reported. (**Paper I**)

Table 3.2. Structural parameters calculated from the SAXS patterns: lattice parameter (a) and water channel radius (r_w), of the empty and loaded TS-CUB and TS-HEX at 25 °C. (**Paper I**)

Sample	a ($\text{\AA}$)	r_w ($\text{\AA}$)
TS-CUB	92.1 ± 0.1	22.0 ± 0.3
CoTS-CUB	90.2 ± 0.1	21.2 ± 0.2
TS-HEX	59.5 ± 0.3	30.6 ± 0.2
CoTS-HEX	57.2 ± 0.4	28.2 ± 0.3

Regarding the assessment of apparent hydrodynamic diameter, PDI, and ζ -potential values for both loaded formulations (**Table 3.3**), these parameters remained unchanged after co-encapsulation. Moreover, the observed values are consistent with those reported in the

literature for cubic and hexagonal LLCNPs prepared using different lipid compositions [27–30]. Additionally, to simulate topical administration, the stability of the loaded and empty TS-CUB and TS-HEX nanosystems was examined by DLS and ELS measurements at pH 5, imitating the skin's pH, as presented in **Table 3.3** and **Figure 3.5** (evaluated from Table 3.3). At this specific pH, no significant changes in both formulations' characteristics were observed after comparison with standard water dilutions (pH 7), supporting their potential topical application.

Table 3.3. Apparent hydrodynamic diameter (D_h), polydispersity index (Pdl), and ζ -potential of the empty and loaded TS-CUB and TS-HEX at Milli-Q water dilution of pH 7 (control) and at skin's pH 5 adjusted with 0.01M HCl, controlled by a pH meter) for comparison. (**Paper I**)

Sample	D_h (nm)		Pdl		ζ -potential (mV)	
	water	pH 5	water	pH 5	water	pH 5
TS-CUB	114 $\pm$ 2	113 $\pm$ 2	0.21 $\pm$ 0.01	0.22 $\pm$ 0.01	-39 $\pm$ 1	-39 $\pm$ 1
CoTS-CUB	115 $\pm$ 1	113 $\pm$ 2	0.21 $\pm$ 0.00	0.21 $\pm$ 0.01	-40 $\pm$ 1	-38 $\pm$ 1
TS-HEX	122 $\pm$ 1	123 $\pm$ 1	0.27 $\pm$ 0.01	0.24 $\pm$ 0.01	-42 $\pm$ 0	-43 $\pm$ 1
CoTS-HEX	135 $\pm$ 1	132 $\pm$ 1	0.28 $\pm$ 0.00	0.27 $\pm$ 0.01	-40 $\pm$ 1	-38 $\pm$ 0

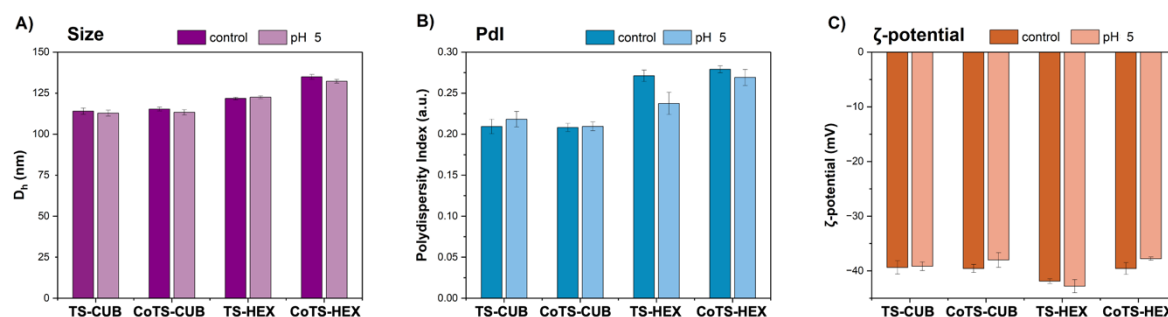


Figure 3.5. Assessment of the TS-CUB and TS-HEX (empty and loaded) formulations' stability by DLS and ELS measurements of an apparent hydrodynamic diameter (D_h) (A), polydispersity index (Pdl) (B) and ζ -potential (C) at skin pH 5 in comparison to standard water pH 7 (control).

3.3. Biocompatibility and photodynamic action against skin metastatic human melanoma.

The next research step involved the bioimaging studies of empty and loaded TS-CUB and TS-HEX formulations, performed on human skin melanoma cells. Namely, the nanosystems were tested against the malignant Me45 cell line, originating from a lymph node metastasis of skin

melanoma, highly metastatic and resistant to standard cancer treatments [31]. It was previously proved that Me45 cells are more sensitive to PDT in comparison to less aggressive primary melanoma cells (MeWo) [32], particularly when Ce6 is encapsulated within cubosome nanocarriers [33]. Therefore, in this research, the anticancer potential combined with PDT, of novel co-loaded TS-CUB and TS-HEX formulations against the resistant melanoma was examined by *in vitro* MTT cytotoxicity tests, followed by HTM and fluorescence bioimaging, for the biocompatibility evaluation purposes.

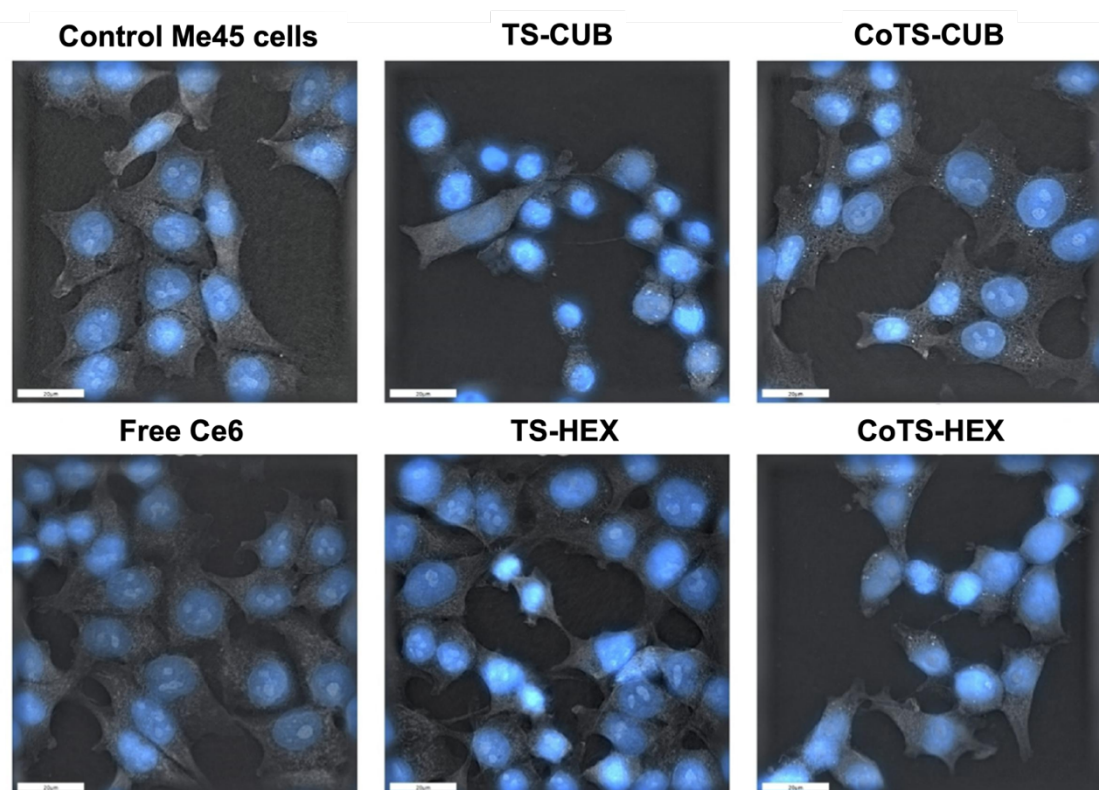


Figure 3.6. HTM bioimaging of Me45 cells morphology, after treatment with empty and loaded TS-CUB and TS-HEX, as well as free Ce6, with the final PS concentration of 2 μM , corresponding to 117 mg/mL of MO. The scale bar represents 20 μm . (**Paper I**)

Initially, the morphological assessment of melanoma cells treated with the empty and loaded TS-CUB and TS-HEX, as well as free PS (Ce6) at the concentration of 2 μM , followed by 24 hours of incubation, was performed by HTM studies with the nuclei staining, as presented in **Figure 3.6**. Notably, exposure to the empty and loaded TS-CUB did not lead to any visible changes in cell morphology, while the TS-HEX formulations caused a slight elongation in their shape, reducing the cells' area. Moreover, CoTS-HEX treatment induced lysosomes accumulation, indicating a lysosomal stress response (LSR), an early marker of autophagy, and

depending on the severity of this stress, the cell either undergoes death or returns to homeostasis [34]. Previous works have also shown that self-assembled nanosystems composed of Ce6 and an autophagy promoter (celastrol) extracted from plant roots, induced autophagic cell death, activated by the PSs photodynamic action [35].

Furthermore, to evaluate the influence on the cytoskeleton's structure after treatment with the studied samples, F-actin staining was carried out to analyze the cells by fluorescence microscopy. As presented in **Figure 3.7**, no significant changes in the actin fibers' organization were observed in the case of exposure to both empty and loaded TS-CUB formulations, indicating no significant impact on the dynamics of cells' cytoskeleton.

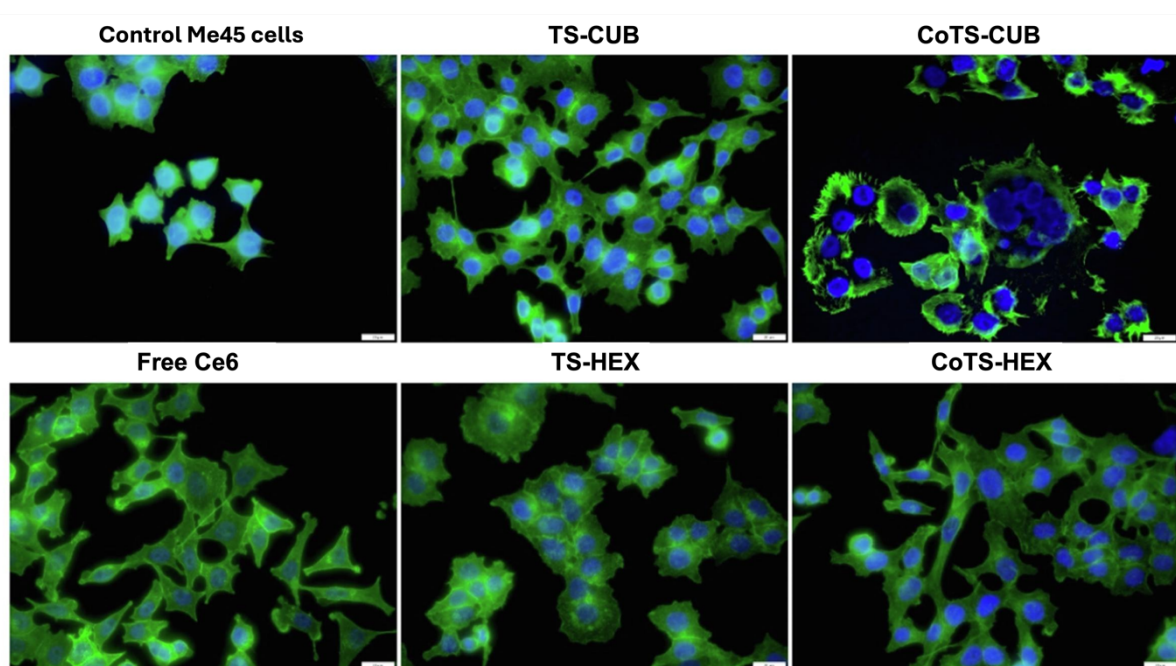


Figure 3.7. F-actin fluorescence bioimaging of Me45 cells, after treatment with empty and loaded TS-CUB and TS-HEX, as well as free Ce6, with the PS concentration of 2 μM , corresponding to 117 mg/mL of MO. The scale bar represents 20 μm . (**Paper I**)

It is important to notice that similarly to the results obtained by HTM, free Ce6 and CoTS-HEX provoked cells elongation. After exposure to free PS, a notable stress tension of the fibers was observed. The cellular features and alterations induced by the hexosomes might be attributed to the higher concentration of Sp80 in such nanosystem, which could influence cell viability. Overall, these results suggest that the formulations developed here (TS-CUB and TS-HEX) formulated with Sp80 and TC did not significantly affect cellular morphology or the dynamics of the actin cytoskeleton, supporting their safety profiles and following the previously indicated biocompatibility requirements for biomedical applications [36].

The CoTS-CUB and CoTS-HEX nanosystems were developed for potential application in PDT. Accordingly, their cytotoxicity and photodynamic activity were evaluated against Me45 melanoma cells. In parallel, the cytocompatibility of the corresponding empty formulations (TS-CUB and TS-HEX) was assessed using primary human cutaneous keratinocytes (HaCaT) as a representative normal control cell line. **Figure 3.8** represents the cytotoxicity assay of the two empty novel TS-CUB and TS-HEX formulations examined at three different dilutions (1:300, 1:160, and 1:80; starting concentration of 640 μ M) after incubation with HaCaT cells for 24 hours in the dark, focused on the concentration-dependent effects. It can be observed that cubosomes exhibited no significant cytotoxicity across all concentrations, with cell survival of about 100% of the control, indicating their safety and biocompatibility. Notably, the concentrations found to be non-toxic in this study were higher than those reported for MO-based cubosomes tested against other cell lines [37,38], suggesting that the biological behavior of cubosomes might depend on the specific cell line tested, and not only on their composition.

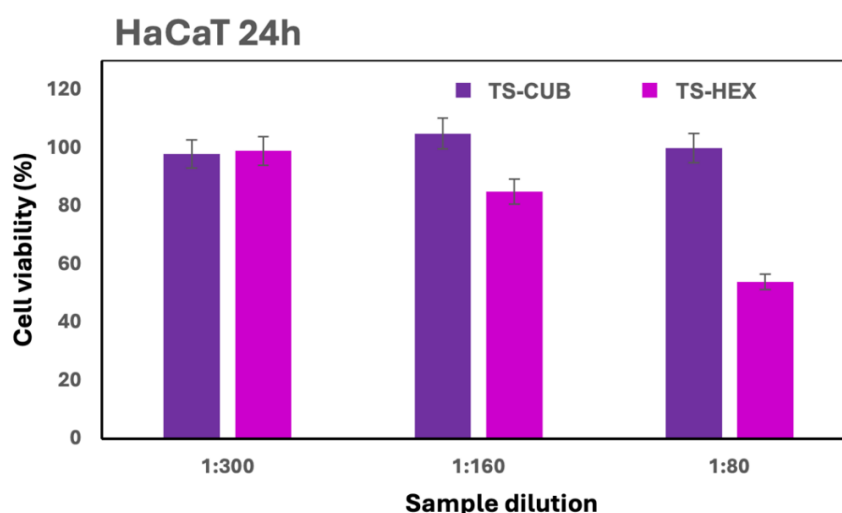


Figure 3.8. Cytocompatibility evaluation by MTT cytotoxicity test on human HaCaT cells after incubation for 24 hours with empty TS-CUB and TS-HEX at different dilutions. (**Paper I**)

In contrast, TS-HEX promoted a more significant reduction in cell viability, particularly at the highest concentration (with the cytotoxicity dropping below 50% of the control cells). Such a result could be attributed to the induced LSR effect and the specific elongation of cells, previously observed by bioimaging studies of the cells treated with hexosomes.

For the preliminary photoactivity assessment, the singlet oxygen $^1\text{O}_2$ generated by the studied samples (CoTS-CUB, CoTS-HEX and free Ce6) with the PS concentration of 2 μ M, was evaluated by ROS assay (**Figure 3.9**), based on the photooxidation of the 9,10-

anthracenediyl-bis(methylene)dimalonic acid (ABMDMA), applied as a scavenger molecule. Such experiment indicated that non-loaded PS presents the lowest efficiency in terms of ROS production, which might be related to its possible aggregation or degradation, highlighting the benefits of encapsulation in nanocarriers, such as the protection of photosensitive bioactive cargo. Moreover, CoTS-CUB demonstrated higher ROS production, and therefore provided the most efficient photoactivity of the loaded PS.

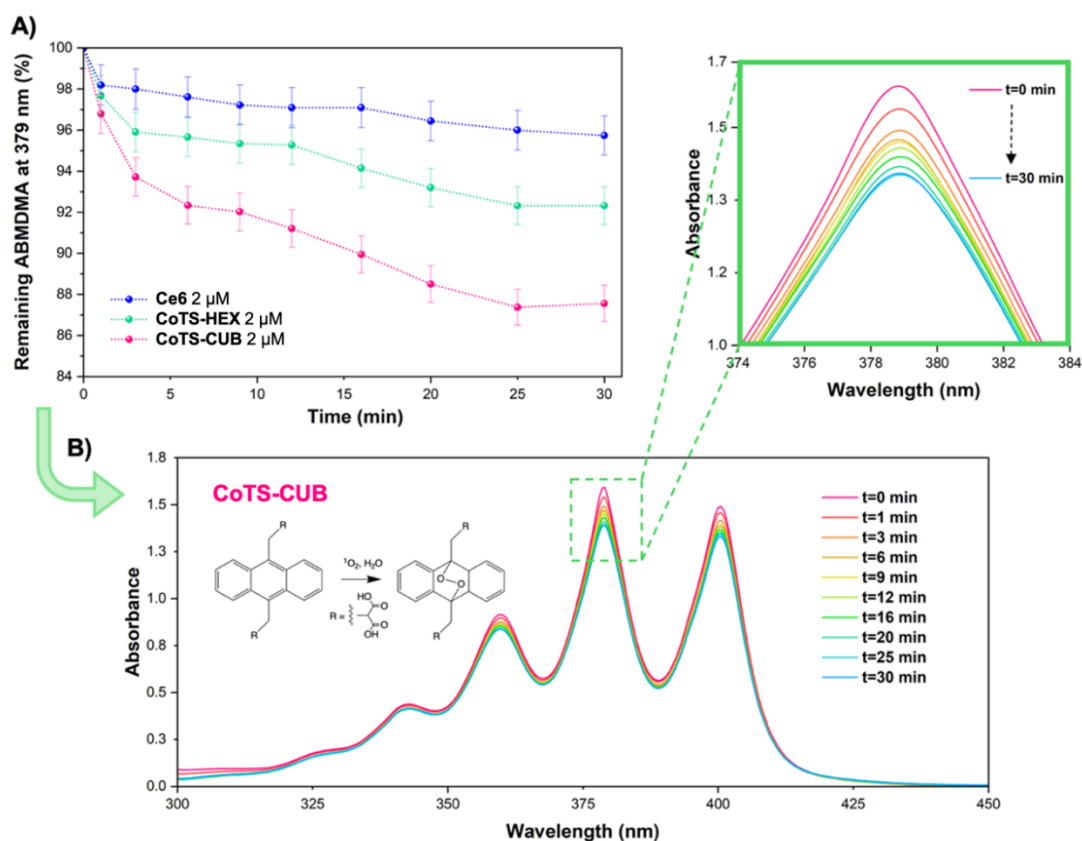


Figure 3.9. ROS assay for the evaluation of 1O_2 production by the studied samples: CoTS-HEX and CoTS-CUB and free Ce6, with the PS concentration of 2 μ M (A) based on the photooxidation of scavenger (ABMDMA) leading to decrease in its absorbance (B). (**Paper I**)

Finally, the primary goal of the subsequent time-dependent photodynamic activity experiment on Me45 cells was to evaluate the efficiency of PDT action of the TS-CUB and TS-HEX co-loaded with Ce6 and CoQH, with the PS concentration of 2 μ M. As shown in **Figure 3.10**, exposure of melanoma cells to red light (10 minutes with a total light dose of 10 J/cm², λ_{max} 600–630 nm), followed by 4 and 24 hours of incubation with the co-loaded formulations, led to a significant reduction in mitochondrial activity, and the decrease in the number of viable cells, for both CoTS-CUB and CoTS-HEX, compared to free PS.

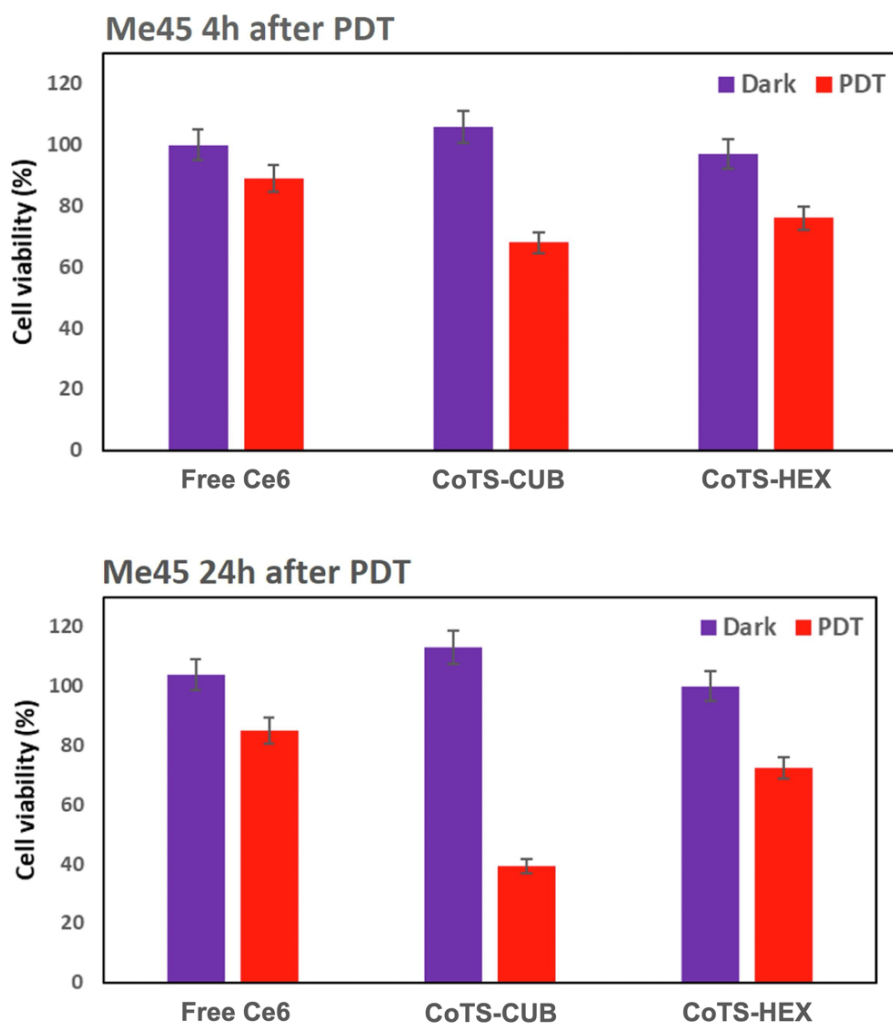


Figure 3.10. Dark cytotoxicity (without irradiation) and photocytotoxicity (PDT effect) of loaded cubosomes (CoTS-CUB) and hexosomes (CoTS-HEX) evaluated in comparison to free Ce6 molecules (final PS concentration: 2 μ M) on the human melanoma Me45 cell line. Incubation was carried out for 4 hours (top panel) and 24 hours (bottom panel) following red light irradiation. Cell viability in each group was expressed as a percentage relative to the control group (Me45 cancer cells untreated with the tested samples). (**Paper I**)

The photodynamic effect was particularly notable after exposure to CoTS-CUB, aligning with the increased photoactivity of this formulation evaluated by ROS assay, thus resulting in a significant reduction of cell viability after irradiation (less than 65%), even more pronounced than the effect observed with CoTS-HEX with the viability remained about 80% of the control. Furthermore, in the case of cells treated with CoTS-CUB, after 24 hours of incubation, an enhancement of the photocytotoxicity was observed, with the cell viability dropping below 40%. In contrast, CoTS-HEX or free Ce6 did not lead to a significant reduction in cell survival over the additional incubation time.

Overall, it can be stated that the photodynamic effect was improved after encapsulation of Ce6 within the studied formulations, which is consistent with findings from other studies reported on various nanosystems [39,40]. *In vivo* experiments on micelles also indicated the promising photodynamic efficacy of Ce6 against melanoma [41]. Furthermore, Tan et al. highlighted the potential of cubosomes and hexosomes, particularly for applications involving bioactive compounds for skin and melanoma treatments [30].

3.4. Conclusions and future perspectives

The study presented in this chapter introduced a novel approach for the formulation of non-lamellar LLCNPs, specifically cubosomes and hexosomes, using MO as molecular building block and stabilized by Sp80, tuned with TC acting as an edge activator. After carefully adjusting the ratio of these two additives, two promising formulations, cubosome-based (TS-CUB) and hexosome-based (TS-HEX) co-loaded with bioactive agents, were tested as potential nanocarriers for PDT of the malignant skin melanoma. The studied formulations exhibited a mix of lamellar and non-lamellar NPs, influenced by the presence of TC and Sp80. TC may facilitate these nanosystems for topical use [8,9], while Sp80 stabilizes the NPs interface. After co-encapsulation of the PS (Ce6) with the adjuvant (CoQH), the formulations maintained their core physicochemical properties. The high biocompatibility of the cubosomes stabilized by Sp80 and TC was reflected in the cell viability data. Furthermore, it was demonstrated that CoTS-CUB was more effective in photodynamic treatment against Me45 cells than CoTS-HEX. Since metastatic cells are highly invasive and mobile, such findings could be crucial for the potential application of these novel non-lamellar nanosystems in skin melanoma treatment. The high ability of the studied cubosome and hexosome formulations to induce photodamage in cancer cells is comparable to other nanocarriers reported in the literature [42]. Since the samples in this study consisted of both non-lamellar and lamellar NPs, distinguishing the specific contribution of each form is challenging. Nevertheless, it can be hypothesized that cubosomes and hexosomes, carrying a higher dosage of the PS, may be responsible for stronger PDT effects over time compared to vesicles. The role of lamellar versus non-lamellar NPs in PDT and cancer treatment remains an open question in the field [43–46]. Further studies focused on understanding the role NPs composition could help to clarify this issue. The liquid dispersions of CoTS-CUB and CoTS-HEX proposed here are suitable for direct topical application to the target area, especially when a short contact time is required. To further assess the formulations' potential to penetrate superficial or deeper skin layers, *in vitro* dermal or transdermal release tests on skin would be necessary to evaluate this ability, particularly

concerning the TC content. On the other hand, since the new cubosomes showed greater biocompatibility and effectiveness in the evaluation of PDT action after loading PS within, TS-CUB constitute a potential model nanoplatfrom for encapsulation of various types of bioactive compounds. An unconventional approach involves the encapsulation of natural photosensitizers obtained from algae biomass with potential anticancer properties, which therefore turned out to be the subject of the research described in the next chapter, being also a comparative study between the standard Pluronic-based cubosomes with the Pluronic-free TS-CUB.

3.5. References

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Chapter 4 – Cubosomes loaded with *Ulva rigida* extract rich in natural photosensitizers for pancreatic cancer PDT

4.1. Chlorophylls-rich *U. rigida* photoactive extract isolation by green MAE

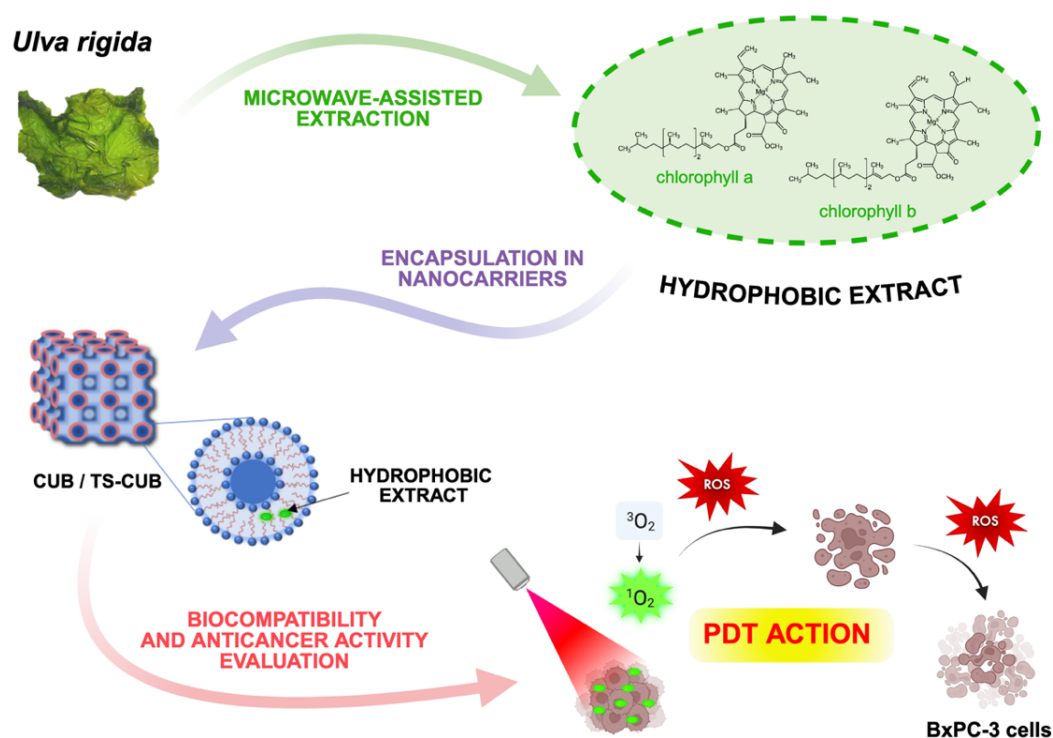
4.2. Extract encapsulation in two types of cubosomes for PDT – a comparative study

4.2.1. Physicochemical characterization

4.2.2. Biocompatibility and photodynamic activity against pancreatic cancer cells

4.3. Conclusions and future perspectives

4.4. References



As described in the Introduction (paragraph **1.1.1.2. *Ulva rigida***), the biomass of green marine algae named *Ulva rigida* serves as a sustainable source of natural photosensitizers, specifically chlorophylls with potential anticancer activity when combined with PDT. Therefore, Chapter 4 (refer to **Paper II**) describes a green MAE-based approach of the photoactive pigments-rich extract development from algae biomass, followed by encapsulation in two types of MO-based cubosome nanocarriers. These nanoparticles were applied for the protection and efficient delivery of the photosensitive cargo, with the final aim to test their anticancer potential through photodynamic action on highly resistant pancreatic cancer cells (see **Figure 4.1**). For this purpose, two distinct cubosome formulations differing in the composition and loaded with *U. rigida* extract were compared: standard CUB, stabilized with Pluronic F108, and the new formulation discussed in the previous chapter, TS-CUB, stabilized with Sp80 and TC. Both formulations demonstrated high encapsulation efficiency. Extensive physicochemical characterization was performed using SAXS, cryo-TEM, DLS, and ELS techniques, demonstrating structural stability of the nanocarriers, also at body temperature (37 °C), and supporting their potential *in vivo* application. Interestingly, apart from the typical Pn3m cubic phase, encapsulation of the extract led to the emergence of a sponge phase (L₃) in the TS-CUB-based formulation. Furthermore, ROS generation provides the information about preliminary photoactivity of the tested formulations, followed by the biocompatibility studies by MTT cytotoxicity in the dark, and cellular uptake determination by flow cytometry. Finally, the photodynamic activity was evaluated by *in vitro* F-actin fluorescence bioimaging. All biological experiments were performed on the human pancreatic cancer cell line, BxPC-3.

Overall, in relation to standard CUB and free extract molecules, novel TS-CUB loaded with the natural algae pigments presented greater photoactivity, improved biocompatibility, and stronger photodynamic anticancer action, highlighting its promising application as an effective delivery nanoplatform for PDT-based treatment of pancreatic cancer. Based on these results, TS-CUB was also tested to encapsulate another algal pigment, caulerpin, a bisindole alkaloid with potential anticancer properties, isolated from an invasive *C. cylindracea*, as presented in Chapter 5.

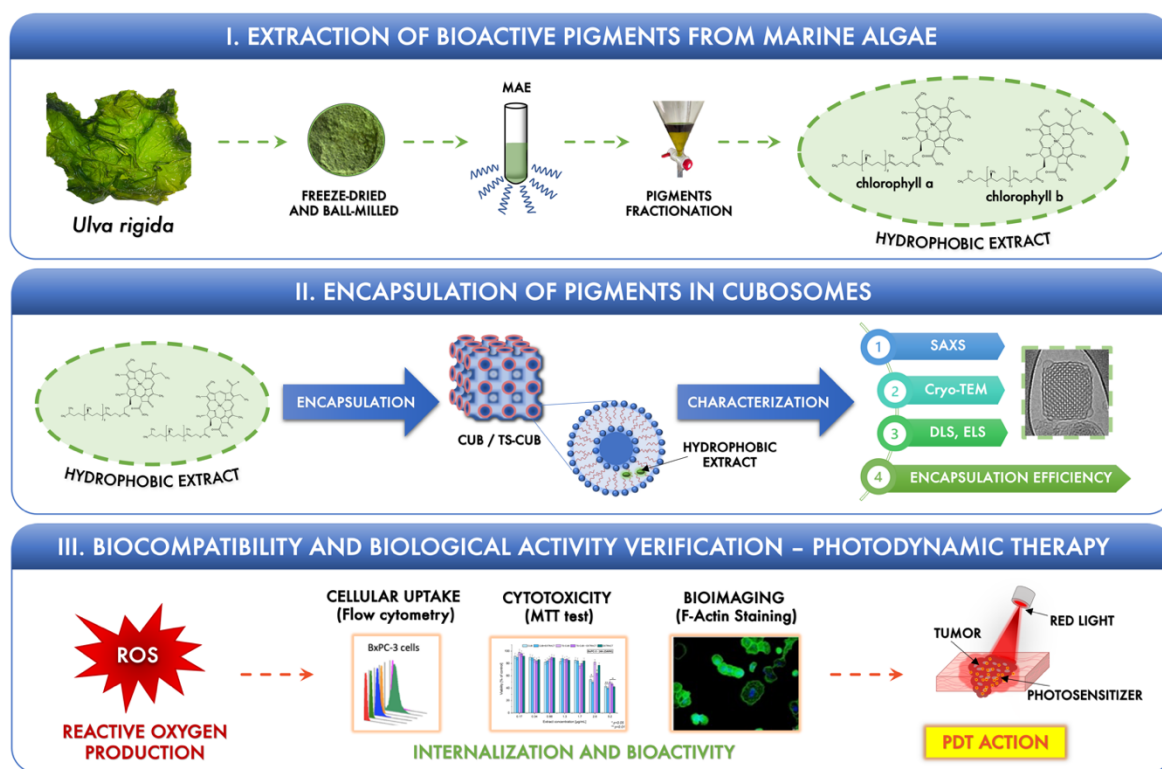


Figure 4.1. Schematic representation of the study.

4.1. Chlorophylls-rich *U. rigida* photoactive extract isolation by green MAE

Firstly, *U. rigida* biomass was collected and properly prepared (washing, freeze-drying, ball-milling). Then the powdered material was subjected to the two-step extraction procedure: S-L extraction by MAE to disrupt the algae cell walls and release the pigments into the extraction solvent (ethanol), followed by L-L extraction in a separatory funnel for the pigments fractionation. The efficient system was composed of 50% ethanolic extract, 30% hexane and 20% water, suitable for pigments recovery from *U. rigida* biomass as proved by Martins et al. [1] to obtain the fraction of interest, rich in natural photosensitizers: chlorophylls. A schematic representation of the extraction process is presented in **Figure 4.2**. As already mentioned in the Introduction (paragraph *1.1.2.3. Novel green extraction techniques*), natural bioactive products isolation from the biomass should prioritize both efficiency and sustainability, therefore, novel green extraction techniques are applied for this purpose.

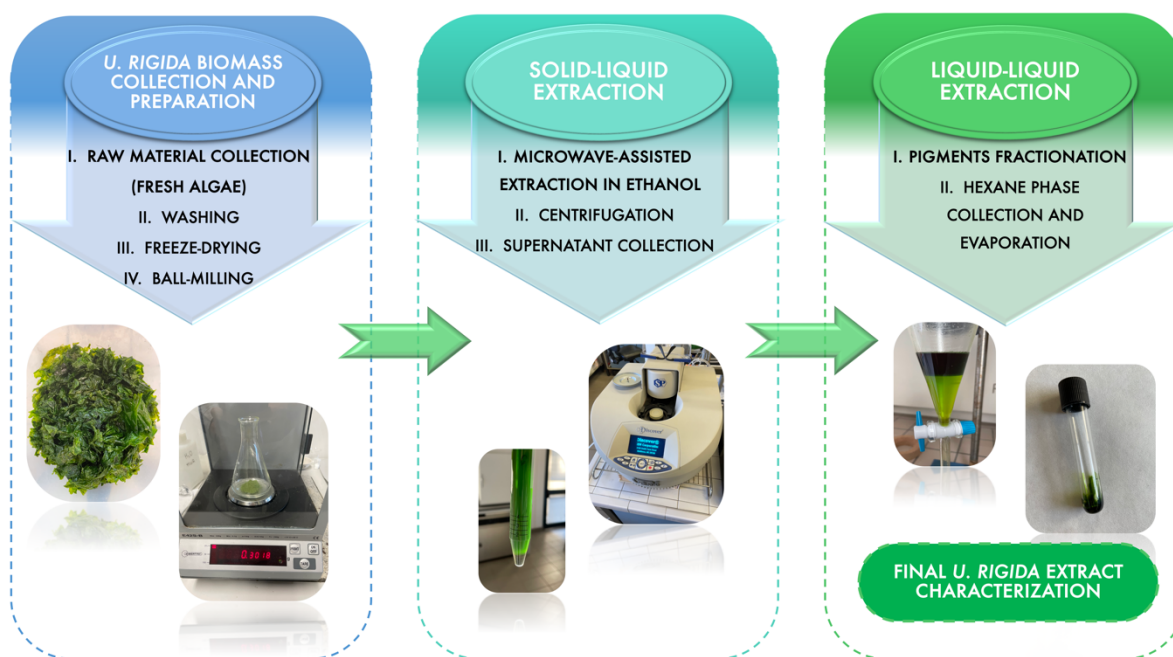


Figure 4.2. Schematic representation of the photoactive extract development from the biomass of green marine algae, *U. rigida*. (**Paper II**)

Hence, in this study, MAE with ethanol was chosen for its high efficiency, rapid processing time, and eco-friendly nature. After centrifugation, the ethanol solution was further processed to isolate a fraction abundant in chlorophylls. The final dark-green hydrophobic extract was obtained from *U. rigida* marine algae with the average yield of $(3.5 \pm 0.3)\%$ of the dry algae weight (DW), and then analyzed to determine the pigments composition. The presence of chlorophyll a in the extract was confirmed by HPLC-MS qualitative analysis (**Figure 4.3**).

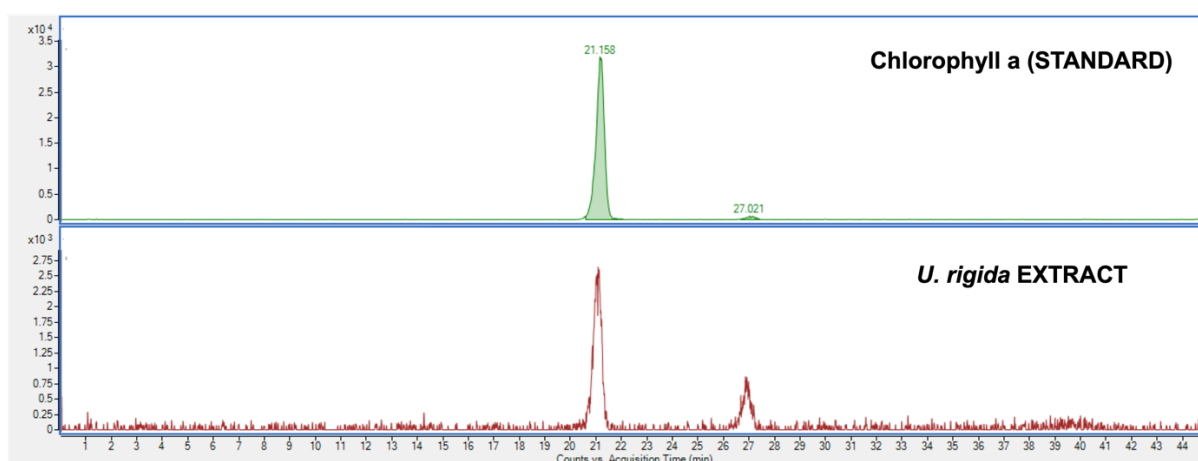


Figure 4.3. HPLC-MS qualitative analysis of the chlorophyll present in the developed *U. rigida* extract. (**Paper II**)

Given the ability of algal pigments to absorb light in the UV-Vis range, a quantitative HPLC analysis was performed using a diode array detector (HPLC-DAD). By comparing the UV-Vis spectra (**Figure 4.4**) and retention times of analytical standards with those of the sample peaks, the analysis confirmed the presence of chlorophyll a as the major component. Additionally, smaller amounts of chlorophyll b and the carotenoid, lutein were also detected. Specifically, it was found that the *U. rigida* extract was composed of chlorophyll a and chlorophyll b at concentrations of $(28 \pm 4)\%$ and $(2.6 \pm 0.5)\%$, respectively, which corresponds to $(0.98 \pm 0.09)\%$ and $(0.093 \pm 0.008)\%$ of the dry algae weight. The low amount of lutein $(0.92 \pm 0.01)\%$ found in the extract is probably related to the residue after L-L fractionation.

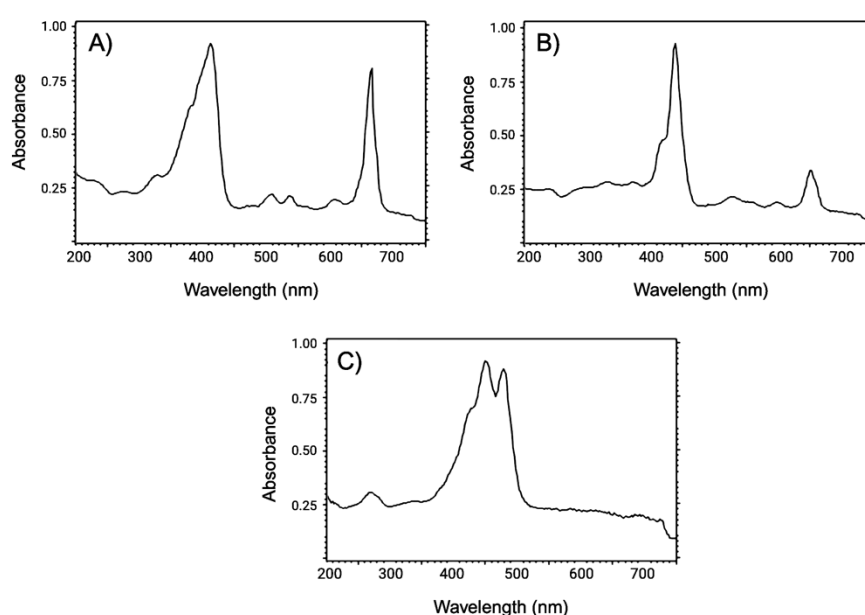


Figure 4.4. UV-Vis absorbance spectra of the analytical standards for the quantified pigments in *U. rigida* extract: chlorophyll a (A), chlorophyll b (B), lutein (C). (**Paper II**)

4.2. Extract encapsulation in two types of cubosomes for PDT – a comparative study

Then, the developed chlorophylls-rich *U. rigida* extract was encapsulated in two types of MO-based cubosome nanocarriers for comparison purposes, with the final concentration of 0.375 mg/mL, and high encapsulation efficiencies, namely $(99.2 \pm 0.5)\%$ in the standard CUB stabilized with PF108 and $(97.3 \pm 0.9)\%$ in Pluronic-free TS-CUB, optimized in the previous study (see Chapter 3). The final wt% compositions of the formulations were MO/PF108/W=3.3/0.3/96.4 (CUB) and MO/Sp80/TC/W=3.5/0.2/0.1/96.2 (TS-CUB). Both types of nanocarriers presented high capacity of accommodating a similar amount of hydrophobic cargo within the lipid bilayer, as typically seen in similar cubosome

formulations [2–4], including the study reported in Chapter 3. Hence, no significant differences were observed between standard Pluronic-based CUB and novel TS-CUB formulations' loading efficiencies.

4.2.1. Physicochemical characterization

For the physicochemical analysis of the developed nanosystems loaded with algae extract, classical scattering techniques were applied (SAXS, DLS, ELS), along with microscopic NPs visualization by cryo-TEM.

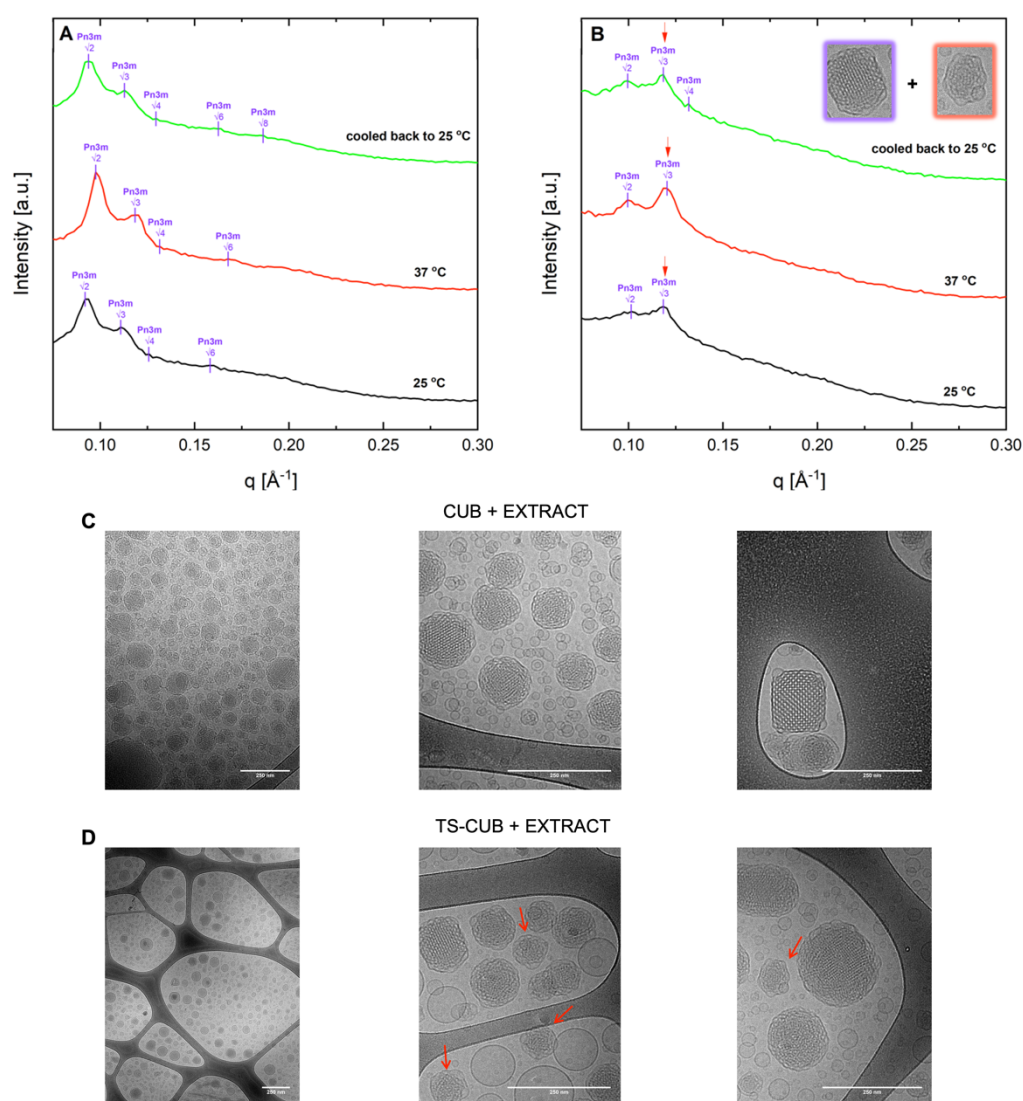


Figure 4.5. SAXS patterns obtained at 25 °C, 37 °C and then after cooling back to 25 °C of CUB+extract (A) and TS-CUB+extract (B), along with the cryo-TEM images of these formulations (blotted on the grid at 25 °C) (C) and (D), respectively. Red arrows indicate the possible presence of an L_3 phase with the sponge NPs marked. (Paper II)

Firstly, SAXS was used to assess the phase behavior and internal structure of the NPs containing the extract at 25 °C, as well as at body temperature, 37 °C, followed by cooling back to 25 °C to evaluate any structural hysteresis. These specific conditions were selected to mimic storage and physiological environment, for potential future application *in vivo*. **Figures 4.5A,B** present the corresponding results, while the structural parameters, such as the lattice parameter (a) and water channel radius (r_w) derived from the scattering patterns, are summarized in **Table 4.1**. Moreover, cryo-TEM analysis, conducted to examine the morphology, confirmed the nanostructure of the studied particles in the CUB+extract and TS-CUB+extract formulations, as shown in **Figures 4.5C,D**.

Table 4.1. LLC phase, lattice parameter (a), and water channel radius (r_w) of the investigated CUB+extract and TS-CUB+extract formulations at 25 °C, 37 °C, and after cooling back to 25 °C. Data are reported as an average value $\pm$ SD. (**Paper II**)

Sample	Temperature (°C)	Phase	a (Å)	r_w (Å)
CUB+extract	25	Pn3m	98 ± 1	21.3 ± 0.6
	37	Pn3m	93 ± 2	19.2 ± 0.8
	back to 25	Pn3m	96 ± 1	20.4 ± 0.4
TS-CUB+extract	25	Pn3m	90 ± 3	18 ± 1
		L ₃	-	15.5
	37	Pn3m	89.9 ± 0.8	18.1 ± 0.3
		L ₃	-	15.0
	back to 25	Pn3m	92 ± 3	19 ± 1
		L ₃	-	16.0

The CUB loaded with *U. rigida* extract (**Figure 4.5A**) was characterized by the presence of the single Pn3m phase, suggesting that the extract may interact with the lipid (MO) bilayer. Indeed, standard cubosomes stabilized with Pluronics typically display the simultaneous presence of the Pn3m and Im3m phases [5]. Specifically, the Pn3m phase represents the native MO structure in aqueous environments at this concentration, while the Im3m phase arises from the interaction between the block copolymer's hydrophobic segments and the lipid bilayer. In case of CUB+extract the first two diffraction peaks can be assigned to the Pn3m phase,

however the remaining peaks appear at the noise level, likely due to the presence of other particles, such as vesicles, presented on the cryo-TEM images (**Figure 4.5C**) (described in more detail in the paragraph referred to the cryo-TEM results). Therefore, combining SAXS with cryo-TEM results allowed for the confirmation that the two prominent peaks correspond to the Pn3m cubic structure. Moreover, the calculated lattice parameter values and the water channel radii align well with previously reported ones for similar formulations [6,7]. Upon heating to 37 °C, the Pn3m phase remains stable, with only a slight shift of the peaks toward higher q values, resulting in the slight decrease of the structural parameters, likely related to thermal-induced rearrangement of the MO hydrophobic tails [8,9]. Then, when the sample is cooled back to 25 °C, the Pn3m phase persists, although the peak positions do not fully return to their original values, showing minor hysteresis, explained by the longer time of equilibration required, usually more than 1 hour, for the Pn3m phase rearrangement and fully return to its initial structure after thermal cycling. In case of the TS-CUB+extract (**Figure 4.5B**), the formulation displays less distinct SAXS peaks with lower intensity, along with a broader, more pronounced diffuse band, commonly linked to the presence of the polydisperse vesicles within the nanosystem [10], as further confirmed by cryo-TEM images (**Figure 4.5D**) (described in more detail in the next paragraph). Previous studies, presented in Chapter 3, have shown that the unloaded (empty) TS-CUB formulation primarily exhibits a pure Pn3m phase. When the extract is loaded within, the two prominent peaks assigned to the Pn3m phase are visible. Nevertheless, the second peak is more intense than the first, which is unusual since peak intensity typically decreases with the scattering order due to the probabilistic nature of scattering events [11]. This anomaly could be explained by the specific orientations (alignments) of the crystalline planes enhancing certain scattering signals, however, it also raises the possibility of the coexistence of two different phases. Based on the phase diagram of MO with additives like Sp80 in water [12], an L_3 (sponge) phase may form under similar conditions. Given that this study refers to a comparable lipid composition with Sp80 included, it is plausible that the system phase behaviour is close to the boundary between the Pn3m and L_3 phases. Moreover, this interpretation is further supported by cryo-TEM images of the TS-CUB+extract formulation presented in **Figure 4.5D**, which, apart from the cubosomes and vesicles, also show structures resembling sponge-like particles (highlighted with red arrows), suggesting the presence of an L_3 phase. Furthermore, it is important to notice that Sp80 can affect the packing parameter of the building lipids like monoolein (MO), potentially leading to the formation of such more disordered L_3 structure, the so-called “melted cubic phase” [12]. This phase typically presents two diffusive bands [13,14], with the first associated with

interparticle (cell–cell) interactions, and the second related to the thickness of lipid bilayer. Therefore, the peaks observed at $q = 0.12 \text{ \AA}^{-1}$ and highlighted with red arrows in **Figure 4.5B**, may be assigned as the first of the L_3 phase, corresponding to the interparticle interactions. Upon heating to $37 \text{ }^\circ\text{C}$, the peaks related to both phases ($Pn3m$ and L_3), become more defined, while their shift to higher q values corresponds to the minor reduction in lattice parameter and water channel radius, while keeping the same nanostructure, and in the case of CUB+extract. In the case of CUB+extract formulation visualized by cryo-TEM (**Figure 4.5C**), the honeycomb-like structures characteristic of cubosomes are visible [15–17]. Additionally, the presence of small vesicles was noted, probably resulting from the influence of Pluronic, which can alter the curvature of the lipid interface [18]. As already mentioned, in the TS-CUB+extract formulation, three distinct nanoparticle types were observed (**Figure 4.5D**): a) cubosomes with a morphology and internal structure similar to those seen in the CUB+extract sample, b) vesicles of various sizes (ranging from 20 to 200 nm), likely caused by the MO interfacial curvature changes due to the presence of TC [10], and c) structures resembling sponge NPs (marked by red arrows). The presence of these sponge structures aligns with literature reports on L_3 phase dispersions with comparable compositions [14] and supports the SAXS findings discussed earlier.

Table 4.2. Apparent hydrodynamic diameter (D_h), polydispersity index (PDI) and ζ -potential of CUB+extract and TS-CUB+extract formulations at $25 \text{ }^\circ\text{C}$ and $37 \text{ }^\circ\text{C}$. Data are reported as an average value $\pm$ SD. (**Paper II**)

Parameter	Temperature ($^\circ\text{C}$)	CUB+extract	TS-CUB+extract
D_h (nm)	25	133 ± 1	114 ± 1
	37	131 ± 1	120 ± 1
PDI	25	0.128 ± 0.005	0.189 ± 0.009
	37	0.13 ± 0.01	0.19 ± 0.02
ζ -potential (mV)	25	-26 ± 3	-41 ± 2
	37	-19.1 ± 0.4	-32 ± 1

Table 4.2 presents the apparent hydrodynamic diameter (D_h), polydispersity index (PDI), and ζ -potential values for the two *U. rigida* extract-loaded nanosystems (TS-CUB and CUB), measured at two different temperatures ($25 \text{ }^\circ\text{C}$ and $37 \text{ }^\circ\text{C}$). Overall, the physicochemical parameters indicate that incorporating *U. rigida* extract alters the internal nanostructure of both

formulations (CUB and TS-CUB) by affecting MO effective packing parameter. This structural impact should be considered in the context of the developed nanosystems application, since drug release behavior is closely tied to the internal organization of the nanocarriers, particularly significant in TS-CUB+extract formulation, where, apart from cubosomes, and vesicles, the sponge particles are also present. In addition, it's worth noting that the studied formulations did not exhibit temperature sensitivity affecting their structure significantly, indicating their potential for *in vivo* application at body temperature without losing the initial nanostructure.

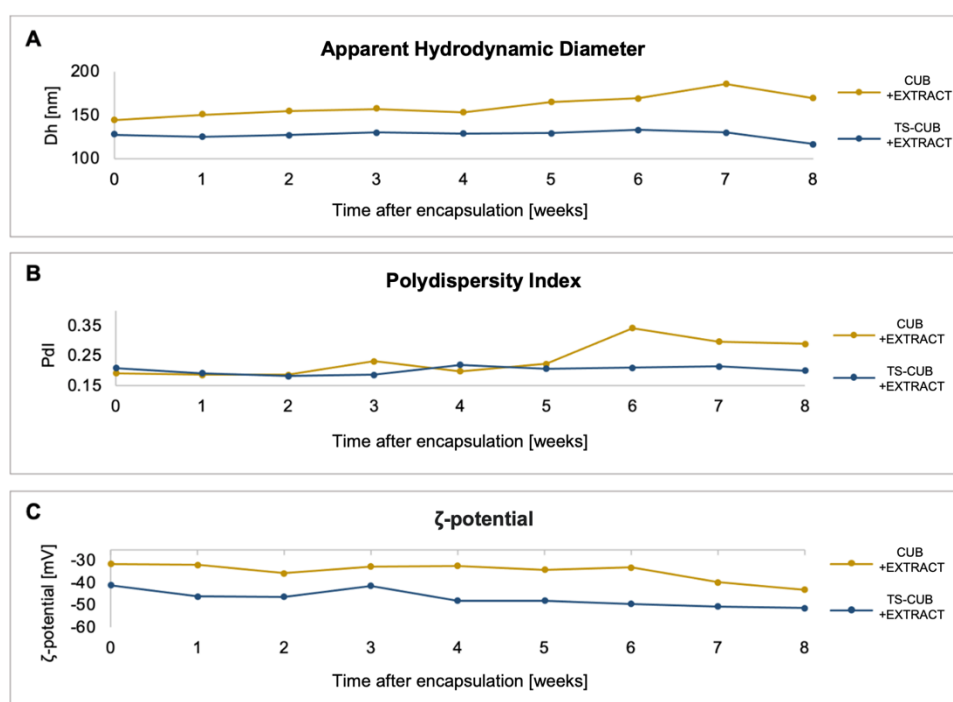


Figure 4.6. Week-by-week DLS and ELS stability experiment with the measurements of an apparent hydrodynamic diameter (D_h) (A), polydispersity index (PDI) (B), and ζ -potential (C) of the CUB+extract and TS-CUB+extract formulations at 25 °C. (Paper II)

It can be also noticed that these parameters are comparable to those observed in empty nanocarriers characterized by similar compositions [2,19], including the study described in Chapter 3. Temperature increase did not significantly affect particle size, but a noticeable decrease in the absolute ζ -potential values is observed. This change may be attributed to increased thermal motion of ions at the nanoparticle interface, leading to a less strongly charged electrical double layer. Despite the temperature change, the size distributions remained stable and consistent with those typically reported for cubosomes and sponge NPs [12,20]. Additionally, the long-term stability of the studied formulations was demonstrated over a period of 2 months lasting DLS and ELS experiments, as shown in **Figure 4.6**.

4.2.2. Biocompatibility and photodynamic activity against pancreatic cancer cells

For the preliminary photoactivity evaluation of the developed nanosystems before further PDT action investigation, ROS assay based on the photooxidation of ABMDMA by singlet oxygen ($^1\text{O}_2$) generated by the studied samples was performed. The scavenger oxidation, associated with the decrease of its absorbance, was measured in time, and the % of remaining ABMDMA was calculated to assess the level of each sample's photoactivity. **Figure 4.7** represents the assessment of ROS production, comparing CUB or TS-CUB nanocarriers loaded with *U. rigida* extract and free extract in various concentrations (0.88, 1.7, 3.4, 5.2, and 8.8 $\mu\text{g/mL}$).

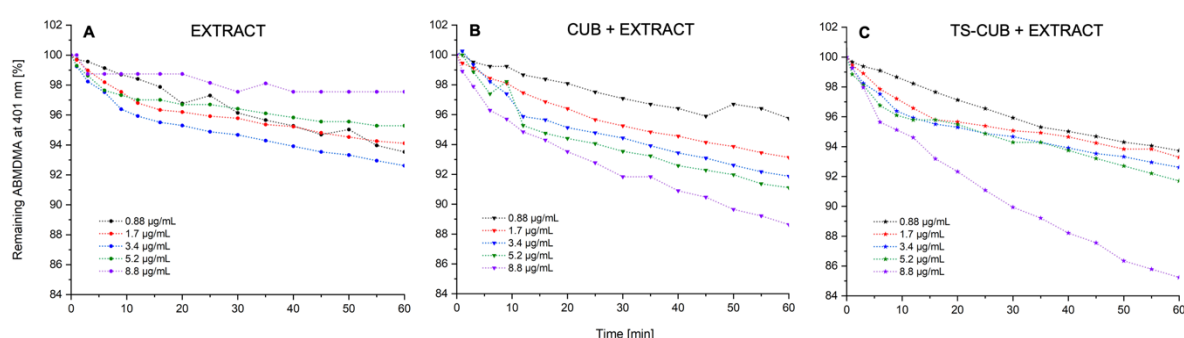


Figure 4.7. ROS assay for the preliminary photoactivity evaluation of the *U. rigida* extract (A), CUB+extract (B), and TS-CUB+extract (C) at different extract concentrations (0.88, 1.7, 3.4, 5.2 and 8.8 $\mu\text{g/mL}$) based on the measurement of ABMDMA photooxidation by $^1\text{O}_2$ produced by the studied samples. (Paper II)

The production of singlet oxygen was demonstrated by all tested samples, as indicated by the gradual decline in the percentage of remaining ABMDMA over time, confirming their photoactivity. Since the highest scavenger level drop was from 100% to $(85 \pm 2)\%$ after one hour in the case of TS-CUB+extract, this sample presented the most efficient ROS production. On the contrary, unloaded extract at the same concentration presented the weakest photoactivity, with a slight decrease to only $(98 \pm 2)\%$ of ABMDMA. Such a result might be related to the pigments degradation or aggregation in the absence of protective encapsulation. On the other hand, increasing extract concentration within both CUB and TS-CUB nanosystems consistently led to enhanced photoactivity. Similar findings have been reported in prior research involving other nanocarriers, where higher photosensitizer concentrations corresponded to greater ROS production [21,22]. Overall, the encapsulated extract exhibits greater photosensitive activity than its free unloaded counterpart, suggesting that encapsulation in LLCNPs effectively boosts ROS generation.

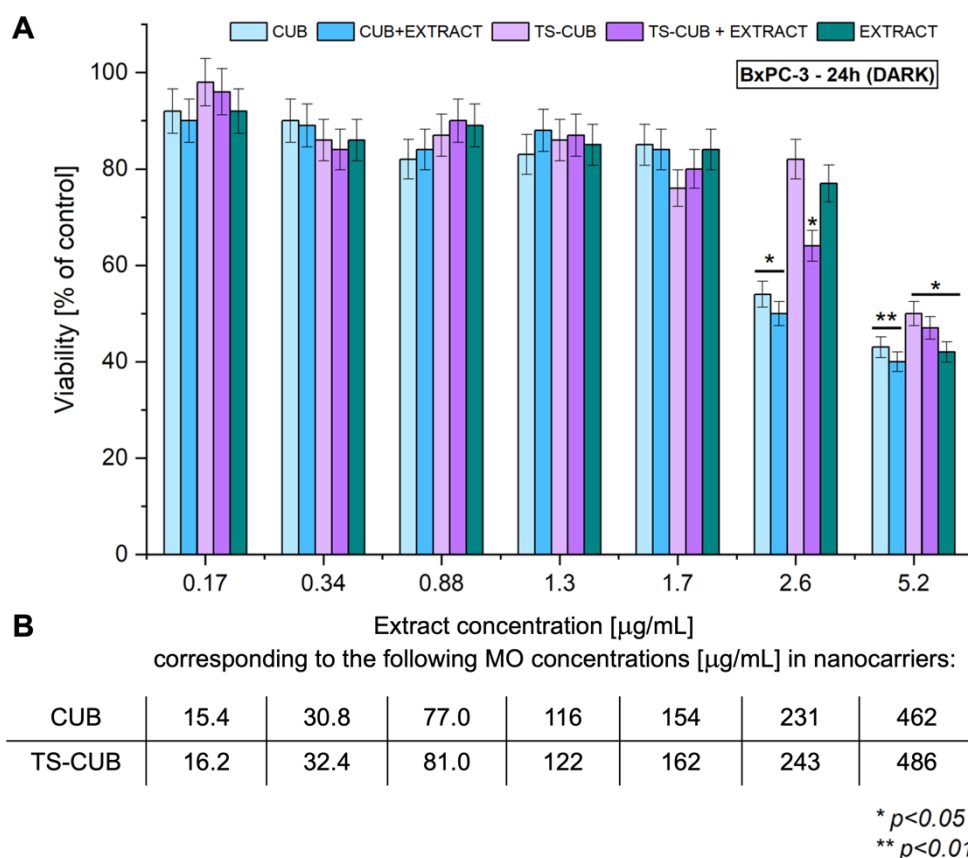


Figure 4.8. Biocompatibility assessment by MTT cytotoxicity in the dark performed on BxPC-3 cells after 24 hours of incubation with the empty and loaded with the extract CUB and TS-CUB, as well as free extract at various concentrations studied (0.17, 0.34, 0.88, 1.3, 1.7, 2.6, 5.2 $\mu\text{g/mL}$) (A). MO concentrations in nanocarriers according to the applied dilutions are displayed below the x-axis (B). The results are presented as an average value $\pm$ SD for minimum $n = 3$, * statistical ANOVA analysis: $p \leq 0.05$ and $p \leq 0.01$. (Paper II)

Since an ideal PS should present no significant cytotoxicity in the dark, as described in the Introduction (paragraph 1.1.3.2. *Photosensitizers*), the MTT cytotoxicity assay was performed in dark conditions for the formulation biocompatibility assessment before evaluation of the PDT action. To this aim, human pancreatic cancer cells (BxPC-3 cell line) were incubated for 24 hours under dark conditions with the free extract, CUB+extract and TS-CUB+extract in various concentrations of the extract (0.17, 0.34, 0.88, 1.3, 1.7, 2.6, and 5.2 $\mu\text{g/mL}$), and empty CUB and TS-CUB (diluted in the same manner as the extract-loaded nanocarriers to ensure the same MO concentrations in nanocarriers). In line with ISO 10993-5 standards, cell viability below 80% is regarded as indicative of cytotoxicity, which is further categorized as weak (80%–60%), moderate (60%–40%), and strong (below 40%) [23]. As shown in **Figure 4.8**, the data reflect the viability of BxPC-3 cells after treatment with the tested samples.

Free extract tends to decrease cell viability progressively with the increase of its concentration, with the most pronounced reduction to around 40% at 5.2 $\mu\text{g/mL}$. On the other hand, comparison between the extract-loaded CUB/TS-CUB and their empty counterparts clearly indicates that the nanocarrier composition itself also contributes to the observed cytotoxic effect. This is likely due to the potential cytotoxicity of the lipidic building block (MO) and Pluronic in the CUB composition [24,25], particularly noticeable at 2.6 $\mu\text{g/mL}$. A clear concentration-dependent trend emerges at lower extract concentrations (0.17–1.3 $\mu\text{g/mL}$), since all samples induce only minimal cytotoxicity, with cell viability remaining close to the control. However, from 1.7 $\mu\text{g/mL}$ of the extract, the cytotoxicity becomes more evident, with the most significant differences between the studied samples at 2.6 $\mu\text{g/mL}$. At this concentration, standard CUB displays moderate cytotoxicity, while the novel TS-CUB formulation presents minimal cytotoxic effect. This confirms an improved biocompatibility of the TS-CUB-based nanosystem, while replacing the stabilizer, Pluronic (PF108), with the less cytotoxic Sp80 and TC. Nevertheless, when the extract is loaded into the nanocarriers, cytotoxicity increases for CUB+extract and TS-CUB+extract, likely due to the too high concentration of the extract, as it represents a weak cytotoxic effect as well, when it is not loaded in nanocarriers. The significantly higher cytotoxicity observed after treatment with TS-CUB+extract in comparison to both the free extract at the same concentration and empty TS-CUB may be explained by improved extract delivery to the cells due to encapsulation in such nanocarriers (described deeper in the next section about cellular uptake). However, due to the comparably low levels of cell viability in case of CUB+extract and empty CUB, the high cytotoxicity could also stem from the high MO content in these formulations (231 $\mu\text{g/mL}$). It is important to highlight that current studies generally identify a cytotoxic threshold of MO in cubosomes at about 100 $\mu\text{g/mL}$ [4,15,24]. Indeed, depending on the type of stabilizer used in the formulation, this value may differ; for instance, a 25% reduction in toxicity was observed after employing a polyphosphoester analog of Pluronic F127 [26]. Additionally, the level of cytotoxicity can vary based on the resistance level of the specific cell line tested.

As already mentioned, in this study, the cytotoxicity was evaluated on the pancreatic cancer cells, which are characterized by high resilience, consistent with strongly aggressive nature of this malignancy, one of the deadliest forms of cancer with very low survival rates [27,28], which has been the focus of many research concerning understanding the mechanisms behind such high tumor resistance [27–32]. Therefore, the BxPC-3 resilience to the tested samples may explain the insignificant cytotoxic levels up to 154 $\mu\text{g/mL}$ of MO (in CUB), corresponding

to an extract concentration of 1.7 $\mu\text{g/mL}$. At the highest concentration tested (5.2 $\mu\text{g/mL}$), all formulations reduced cell viability by 50% or more, suggesting that this dose is not appropriate for therapeutic applications. This could be due to excessive cytotoxic levels of both the extract and MO within the nanocarriers. Overall, given the results described here, and the MO cytotoxicity thresholds, the optimal, biocompatible concentration selected for the further experiments on cellular uptake, and the PDT action was 1.3 $\mu\text{g/mL}$ of the *U. rigida* extract.

For the determination of the extract cellular uptake, flow cytometry was applied to count fluorescent cells related to the presence of specific fluorochromes within, in this case, the extract rich in fluorescent chlorophylls. **Figure 4.9** represents the determination of cellular uptake levels of BxPC-3 cells treated with extract-loaded (1.3 $\mu\text{g/mL}$) nanocarriers (CUB+extract, TS-CUB+extract), along with the free extract at the same concentration, and empty CUB/TS-CUB as negative controls, in comparison to the control cells. The rightward shifts demonstrated in **Figure 4.9** reflect an increased cellular uptake.

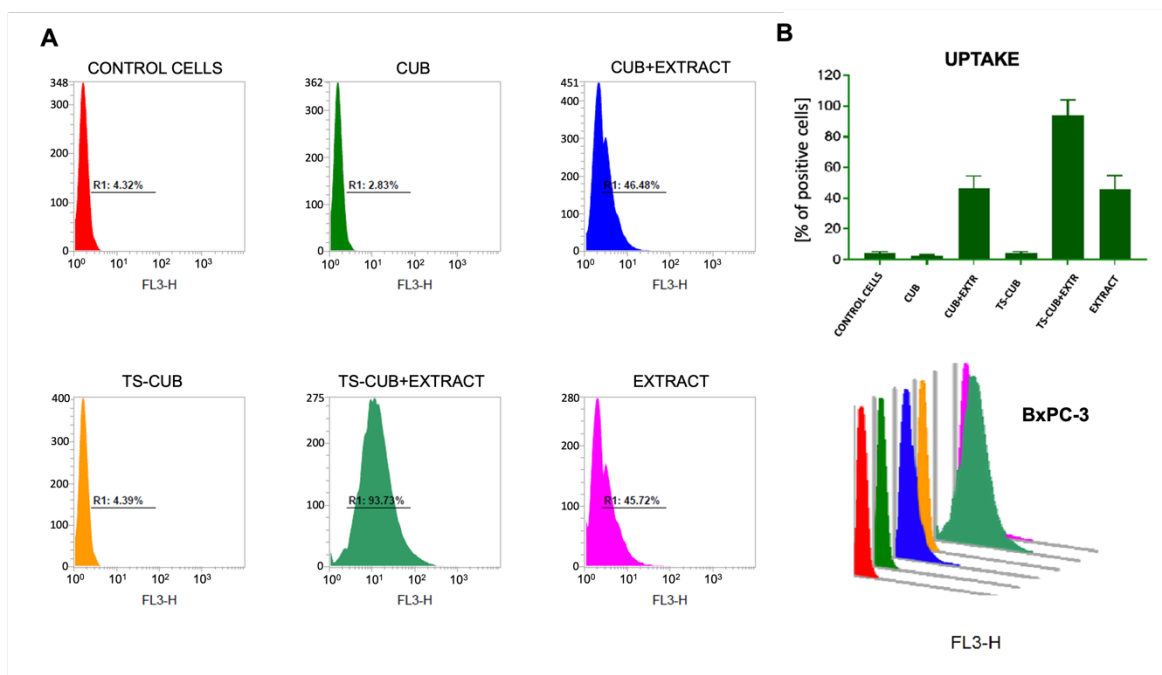


Figure 4.9. Cellular uptake determination by flow cytometry of BxPC-3 cells treated with studied samples and 24 hours of incubation: CUB+extract, TS-CUB+extract, free extract, all at the selected extract concentration of 1.3 $\mu\text{g/mL}$, along with the negative controls (empty CUB and TS-CUB), in comparison to control cells (untreated) (A); comparative cellular uptake chart of all samples (B). (Paper II)

As anticipated, low fluorescence levels in control cells and cells treated with empty cubosomes are likely associated with the cells autofluorescence occurrence [33]. However, a significant increase was observed in cells treated with the free extract and the CUB+extract formulation. An even more pronounced fluorescence enhancement was detected in cells treated with TS-CUB+extract. These findings, when compared to controls, suggest that the encapsulation in novel cubosomes significantly enhanced the uptake of *U. rigida* extract in BxPC-3 cells. The difference in uptake levels between the two extract-loaded formulations, considering their distinct compositions, indicates that TC and Sp80 may contribute to the enhanced cellular internalization. Specifically, the membrane permeability and fluidity might be influenced by these molecules, thus aiding the uptake process [34]. Smaller particles generally enhance the efficiency of endocytosis, while the high negative ζ -potential can strengthen electrostatic interactions with the positively charged regions of cellular membranes, thus potentially boosting internalization [35]. Therefore, another reason for the increased cellular uptake observed after encapsulation in TS-CUB, could be the smaller size of NPs, and an increased negative ζ -potential in comparison to CUB, resulting in facilitated transport across the tumor cell membrane. Indeed, even if these observations align with other research on this topic, additional in-depth biomolecular studies are needed to verify the specific pathways of cellular uptake involved here.

With the aim to assess the final anticancer phototoxic activity of the developed nanosystems loaded with photoactive algae extract, fluorescence bioimaging was performed to evaluate the morphology of cells, specifically, the cytoskeleton organization by F-actin staining. As shown in **Figure 4.10**, the experiments were conducted after 24 hours of incubation using the same types of samples as in previous tests – namely, CUB/TS-CUB formulations loaded with the extract (CUB+extract and TS-CUB+extract), empty CUB/TS-CUB, and the free extract at the optimized concentration of 1.3 $\mu\text{g/mL}$. The samples were evaluated under two conditions: in the dark and following red light irradiation, to assess photodynamic (PDT) activity. The cells morphology displayed a notable difference in the appearance of F-actin after exposure to TS-CUB+extract when combined with irradiation. In contrast to the other studied samples, the cytoskeleton of such cells was significantly disrupted, as evidenced by the F-actin fibers reorganization and destabilization. Moreover, under PDT conditions, comparison of cell bioimaging between treatments with the free extract and its encapsulated form clearly indicates that encapsulation within TS-CUB significantly enhances the phototoxic efficacy of the *U. rigida* extract, highlighting its potential for PDT against resistant pancreatic cancer.

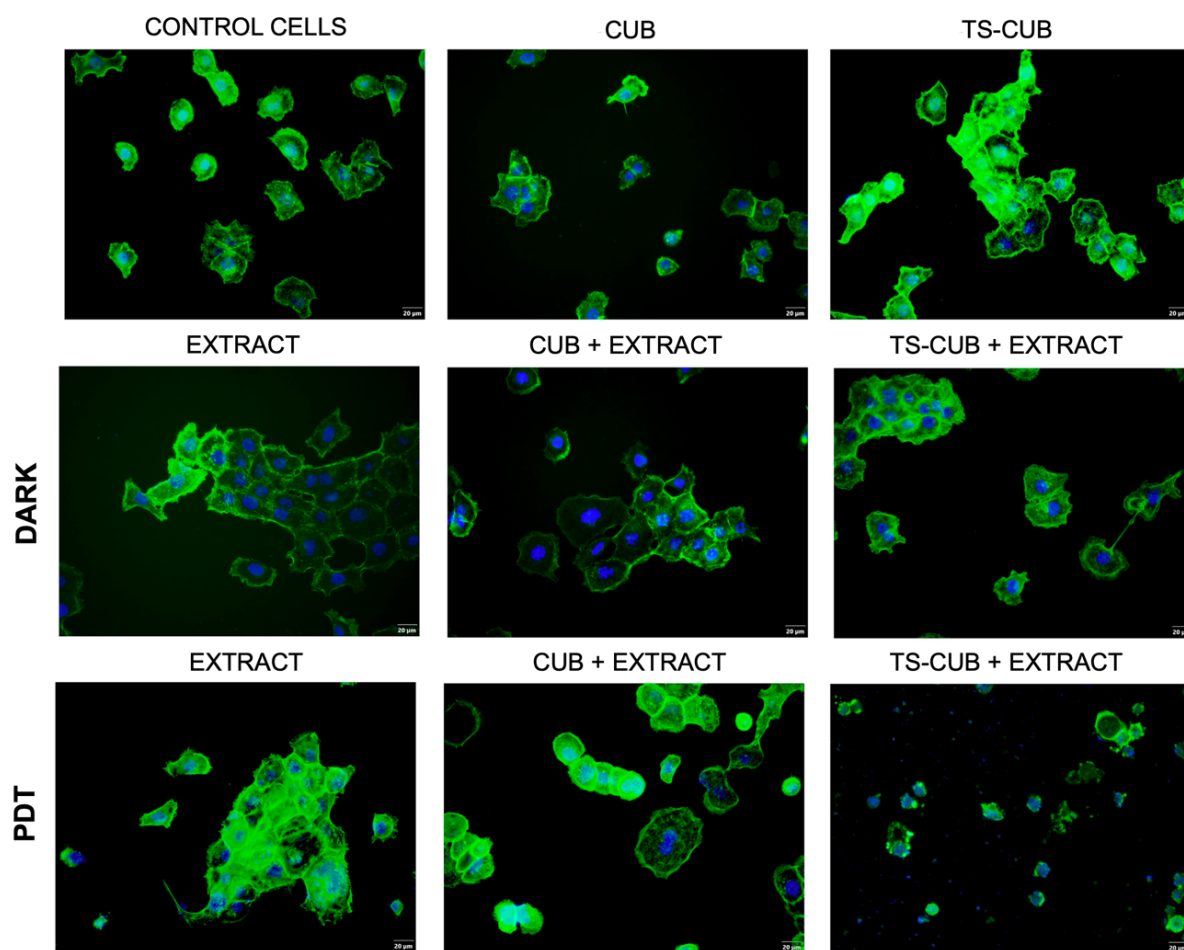


Figure 4.10. F-actin fluorescence bioimaging of human pancreatic cancer cells (BxPC-3) treated with the studied formulations: extract-loaded (CUB+extract, TS-CUB+extract), empty cubosomes (CUB, TS-CUB), and free extract, all at the extract concentration of 1.3 µg/mL, in comparison to untreated control cells. The experiments were performed in dark and after irradiation (PDT) followed by 24 hours of incubation. (**Paper II**)

Results presented here are in line with the experiments described previously in this paragraph (4.2.2. *Biocompatibility and photodynamic activity against pancreatic cancer cells*), highlighting the increased cellular uptake of this formulation, which likely enhanced extract phototoxicity. These findings also reinforce the results presented in Chapter 3 on PDT treatment of melanoma cells, which demonstrated the high effectiveness of the optimized TS-CUB nanocarriers loaded with a commercial photosensitizer [2]. Overall, the combination with PDT revealed the *in vitro* anticancer potential of the novel Pluronic-free cubosomes loaded with the photoactive algal extract against pancreatic cancer cells.

4.3. Conclusions and future perspectives

Chapter 4 presented a study highlighting the environmentally friendly use of *Ulva rigida* (green marine algae) biomass, which is rich in natural bioactive photosensitizers, specifically chlorophylls, thereby demonstrating its potential therapeutic value for PDT. Given the rising mortality rate and the urgent need for effective treatments for highly resistant pancreatic cancer, a green solution referring to the photoactive chlorophylls-rich hydrophobic extract isolated from algae and loaded in biocompatible cubosome nanocarriers for PDT, is demonstrated here as comparative research on two types of cubosome formulations (standard CUB and novel TS-CUB). Encapsulation of *U. rigida* extract in LLCNPs, able to accommodate a high amount of the hydrophobic cargo, constitutes an added advantage to this approach. Furthermore, this research demonstrated an efficient delivery of the photoactive pigments to pancreatic tumor cells, in a stable, protected, and bioactive form. Further, the physicochemical characterization of the studied nanocarriers confirmed their stable nanostructure characterized by a typical Pn3m cubic phase, and a second phase (L₃) in the case of TS-CUB+extract formulation, along with vesicles. Moreover, the structural investigation of the developed nanosystems at the body temperature (37 °C) supports their potential *in vivo* applications. Encapsulation of *U. rigida* extract in the novel TS-CUB formulation, in contrast to the free extract and standard Pluronic-based CUB, resulted in enhanced photoactivity – attributed to increased ROS generation – as well as a significant improvement in extract uptake by BxPC-3 cells. Along with the lower cytotoxic levels in the dark, such results demonstrate excellent biocompatibility of TS-CUB, in agreement with the previous biological studies on this type of cubosomes (described in Chapter 3). On the other hand, a strong anticancer photoactivity of the extract loaded in TS-CUB was confirmed by bioimaging studies after irradiation, with the notable advantage of encapsulation in such nanocarriers, by demonstrating a significant increase in the efficiency of bioactive cargo. Accordingly, the developed TS-CUB+extract formulation can be considered a promising candidate for the treatment of pancreatic cancer by PDT.

To the best of my knowledge, this research is the first to report the efficient encapsulation of the photoactive algae extract within cubosomes. As such, this work introduces a promising green nanoplatform for exploring a wide range of valuable natural bioactive compounds derived from biomass for the nanomedicine and pharmaceutical applications. Therefore, Pluronic-free TS-CUB nanocarriers were then selected as nanocarriers for the encapsulation of caulerpin, a characteristic algae pigment with potential anticancer properties, extracted from the invasive species *C. cylindracea*, as described in the next chapter.

4.4. References

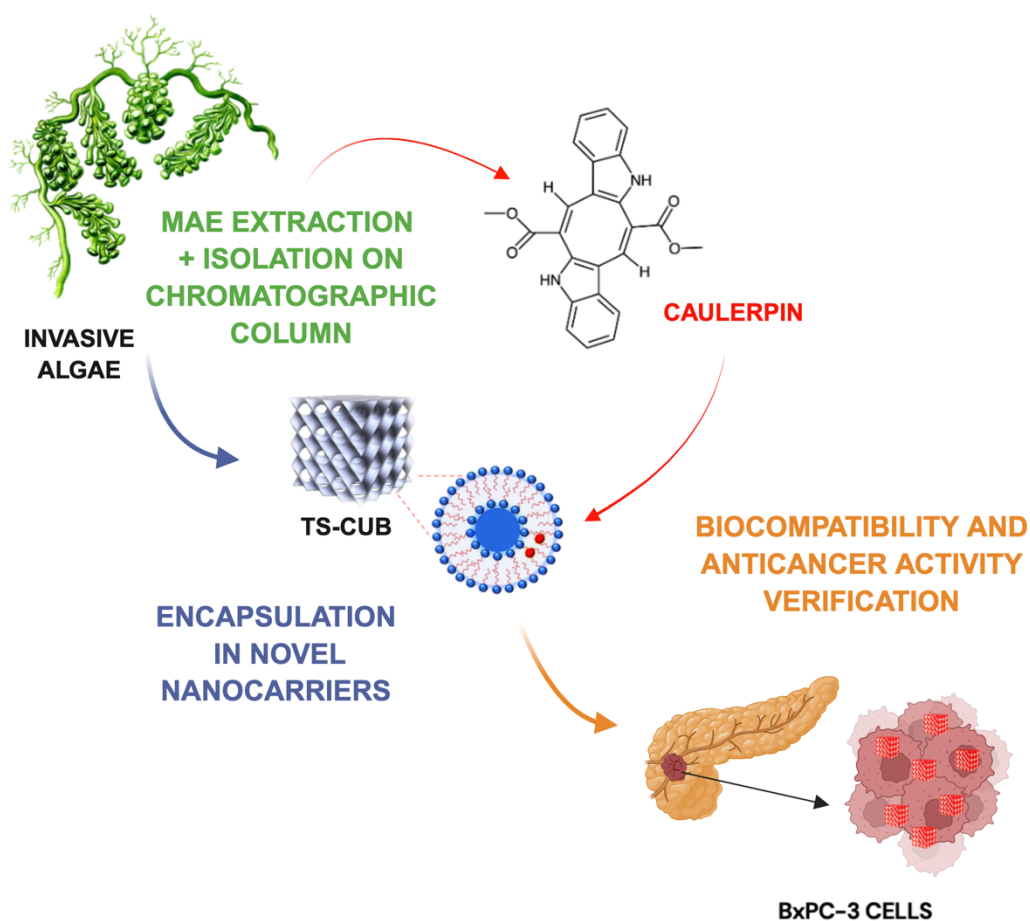
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Chapter 5 – Caulerpin as a characteristic anticancer pigment from *Caulerpa cylindracea* against pancreatic tumor cells after loading in novel cubosomes

- 5.1. Caulerpin isolation from *C. cylindracea* invasive algae biomass by green MAE extraction and chromatographic column
- 5.2. Caulerpin encapsulation in novel cubosomes for therapy against pancreatic cancer
 - 5.2.1. Physicochemical characterization
 - 5.2.2. Strong anticancer potential of caulerpin-loaded nanoformulation
- 5.3. Conclusions and future perspectives
- 5.4. References



The TS-CUB formulation, examined in the comparative study on *U. rigida* extract (see Chapter 4), demonstrated greater biocompatibility and bioactivity than the standard Pluronic-based formulation (CUB), making it a more suitable system for the delivery of algal pigments. Since TS-CUB presented promising encapsulation characteristics, in this research, the anticancer potential of another pigment, caulerpin, found in *Caulerpa* species, was studied after loading in TS-CUB (refer to **Paper III**). Particularly, this alkaloid, after isolation from the biomass of marine invasive algae known as *Caulerpa cylindracea* (described in the Introduction, paragraph 1.1.1.3. *Caulerpa cylindracea*), was tested against human pancreatic cancer cells (BxPC-3), in its free form, as well as after loading in TS-CUB. Therefore, the main goal of this study was to assess the advantage of caulerpin encapsulation in novel cubic nanocarriers for the potential anticancer treatment of the highly resistant pancreatic cancer.

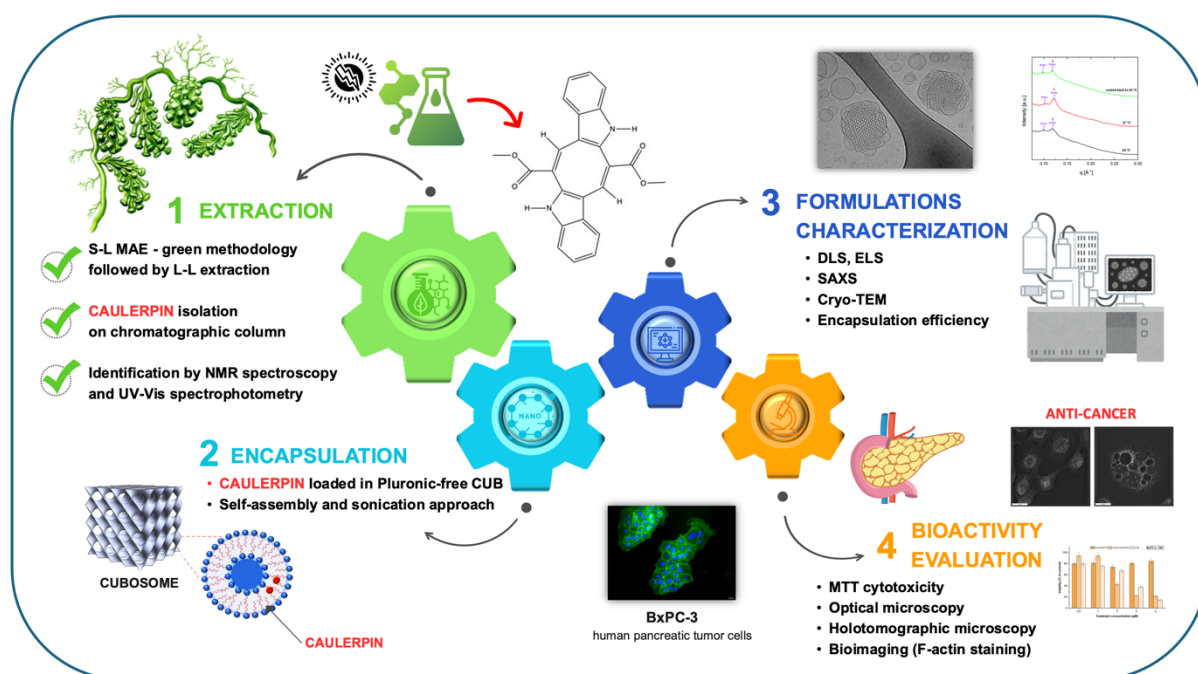


Figure 5.1 Schematic representation of the study.

For this purpose, apart from the standard physicochemical characterization of the obtained formulations by SAXS, cryo-TEM, DLS, and ELS, a series of biological tests were performed. They involved MTT cytotoxicity assay, followed by the detailed assessment of the cancer cells' morphology after treatment with caulerpin-loaded nanocarriers (TS-CUB+caulerpin) by HTM and F-actin fluorescence bioimaging (see **Figure 5.1**). Based on the obtained results, the encapsulation of caulerpin in TS-CUB was validated and shown to be advantageous, as evidenced by the significant enhancement of its anticancer activity in the encapsulated form compared to the free, non-loaded compound. An added benefit of this approach is the utilization

of invasive algal biomass, which may contribute to environmental protection and marine ecosystem stabilization, as described in more detail in the Introduction (paragraph **1.1.2.2. Invasive algae species**).

5.1. Caulerpin isolation from *C. cylindracea* invasive algae biomass by green microwave-assisted extraction and chromatographic column

Herein, after proper biomass collection and preparation, following the procedure from the previous study about *U. rigida* (see Chapter 4), caulerpin was isolated from another species of algae, namely *C. cylindracea*, an invasive seaweed. Namely, the pigment was extracted by MAE, followed by the isolation and purification by chromatographic column, as presented in **Figure 5.2**.

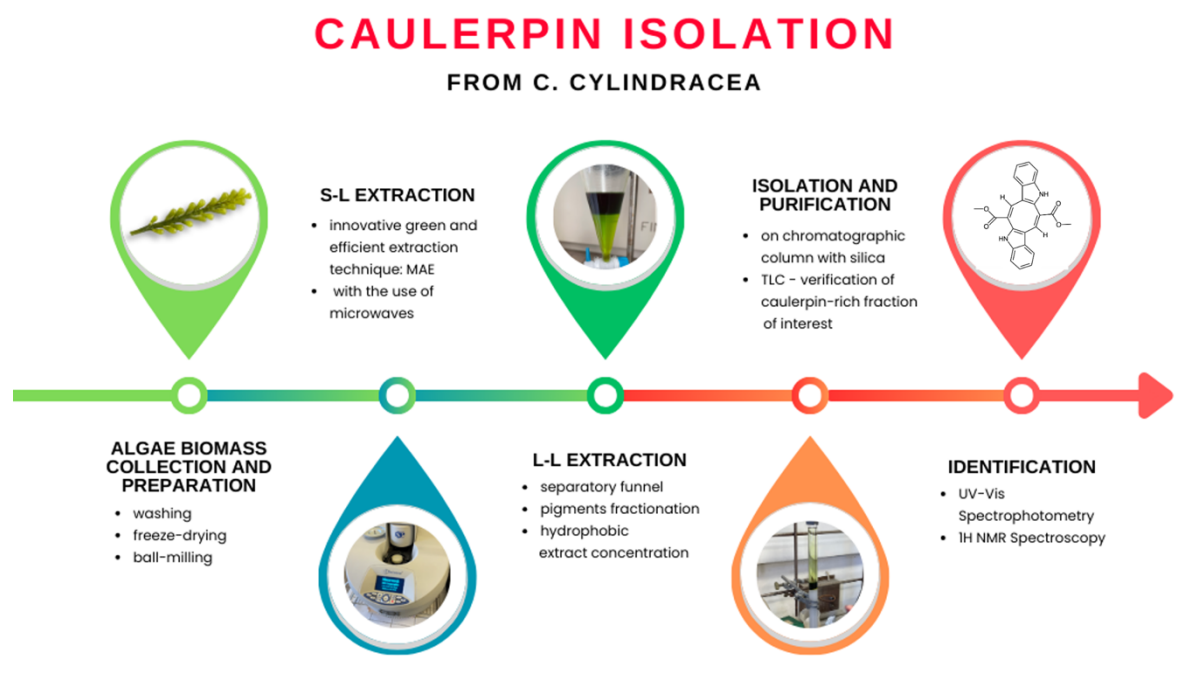


Figure 5.2. Schematic representation of caulerpin isolation from *C. cylindracea*. (**Paper III**)

In the first extraction step, a hydrophobic dark green extract from *C. cylindracea* was obtained by S-L MAE with a yield of $(3.1 \pm 0.3)\%$ DW. Following further fractionation through the separatory funnel and then purification by chromatographic column, the pure compound was isolated with a yield of $(0.47 \pm 0.02)\%$ DW, as an orange-red solid pigment. It is important to notice that the yield was notably higher than those obtained by traditional techniques reported in the literature, such as the 0.1% DW of caulerpin extracted from *C. racemosa* using maceration [1] or the 0.2% DW via traditional Soxhlet extraction [2]. Then, the isolated

compound was identified by UV-Vis spectroscopy and ^1H NMR spectroscopy. Since caulerpin is a pigment that presents strong absorbance at specific wavelengths: 222, 270, 292, and 317 nm, by comparison of the UV-Vis absorbance spectrum of the isolated compound presented in **Figure 5.3**, with the literature data [3], it was preliminarily identified as caulerpin.

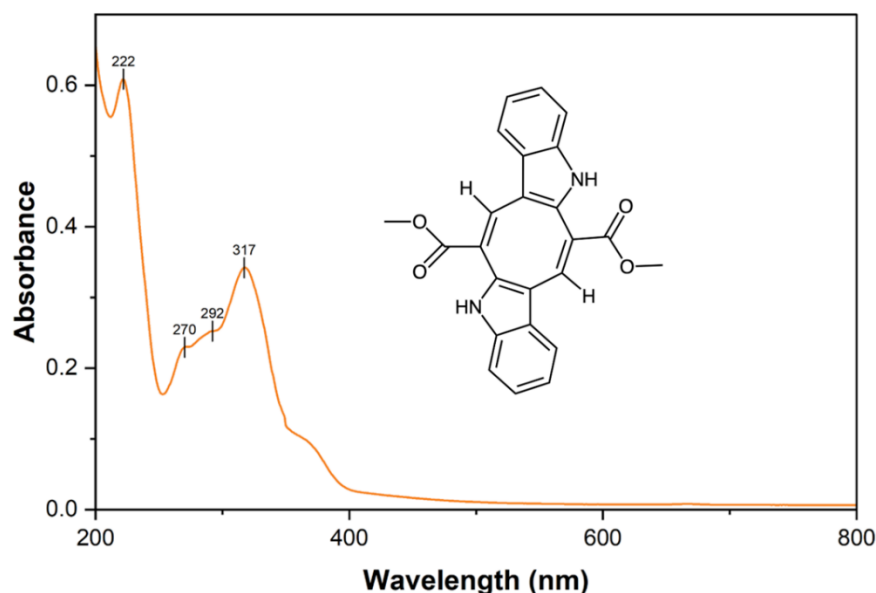


Figure 5.3. UV-Vis absorbance spectrum of the isolated product in ethanol exhibiting characteristic for caulerpin peaks, namely at 222, 270, 292 and 317 nm [3] with its chemical structure presented. (**Paper III**)

For further confirmation, the product isolated from *C. cylindracea* was then characterized by ^1H NMR spectroscopy, through its chemical structure evaluation, along with the purity verification. Therefore, **Figure 5.4** presents the ^1H NMR spectrum of the isolated compound, displaying signals that correspond to the chemical structure of caulerpin, aligning with the literature spectroscopic data for the caulerpin standard evaluated in the same solvent, CDCl_3 [4]. Namely, the signal at 9.20 ppm is attributed to 2 protons from two amino groups hydrogen-bonded to the ester carbonyls. A signal highlighted at 8.06 ppm corresponds to 2 protons attached to the cyclooctatetraene ring that links the two indole groups. Furthermore, signals between 7.07 and 7.45 ppm result from 8 protons attached to the two aromatic rings, while the signal at 7.26 ppm is related to the solvent, CDCl_3 . At 3.91 ppm, the highest signal is present and assigned to 6 protons of two methyl ester groups bonded to the central ring. The final peak corresponds to residual water. Overall, these spectroscopic characteristics confirm that the final product obtained from *C. cylindracea* is pure caulerpin.

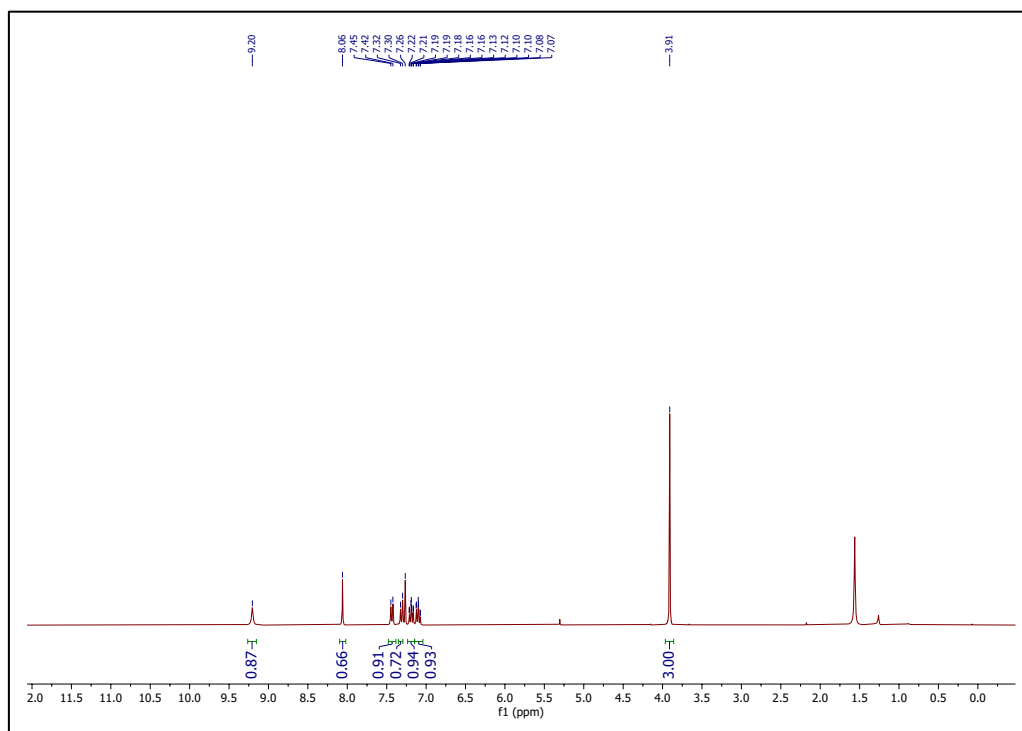


Figure 5.4. ^1H NMR spectrum of the isolated product in CDCl_3 with the signals corresponding to caulerpin's chemical structure. (**Paper III**)

5.2. Caulerpin encapsulation in novel cubosomes for therapy against pancreatic cancer

Isolated and identified pure pigment, caulerpin, was then encapsulated in TS-CUB, to enhance its bioavailability, facilitate the delivery to target tumor cells and therefore improve its efficacy in potential pancreatic cancer treatment. Briefly, the pigment was loaded in the nanocarriers with the wt% composition of MO/Sp80/TC/W=3.5/0.2/0.1/96.2 and caulerpin concentration of 0.125 mg/mL with a high encapsulation efficiency of $(92.6 \pm 0.9)\%$, in line with the previous TS-CUB-based formulations presented in Chapter 3 [5] and 4.

5.2.1. Physicochemical characterization

To assess the internal structure and LLC phase of the caulerpin-loaded TS-CUB formulation, the SAXS technique was applied. The measurements were conducted at various temperatures to observe any potential structural changes in the formulation and evaluate its behavior under storage (room temperature) and potential *in vivo* conditions (body temperature). The sample was subsequently cooled back to 25 °C to check for any structural hysteresis. Hence, **Figure 5.5A** displays the SAXS patterns of the caulerpin-loaded cubosomes (TS-CUB+caulerpin), while **Table 5.1** provides the calculated structural parameters for the predicted phases, namely, the lattice parameter (a) and water channel radius (r_w).

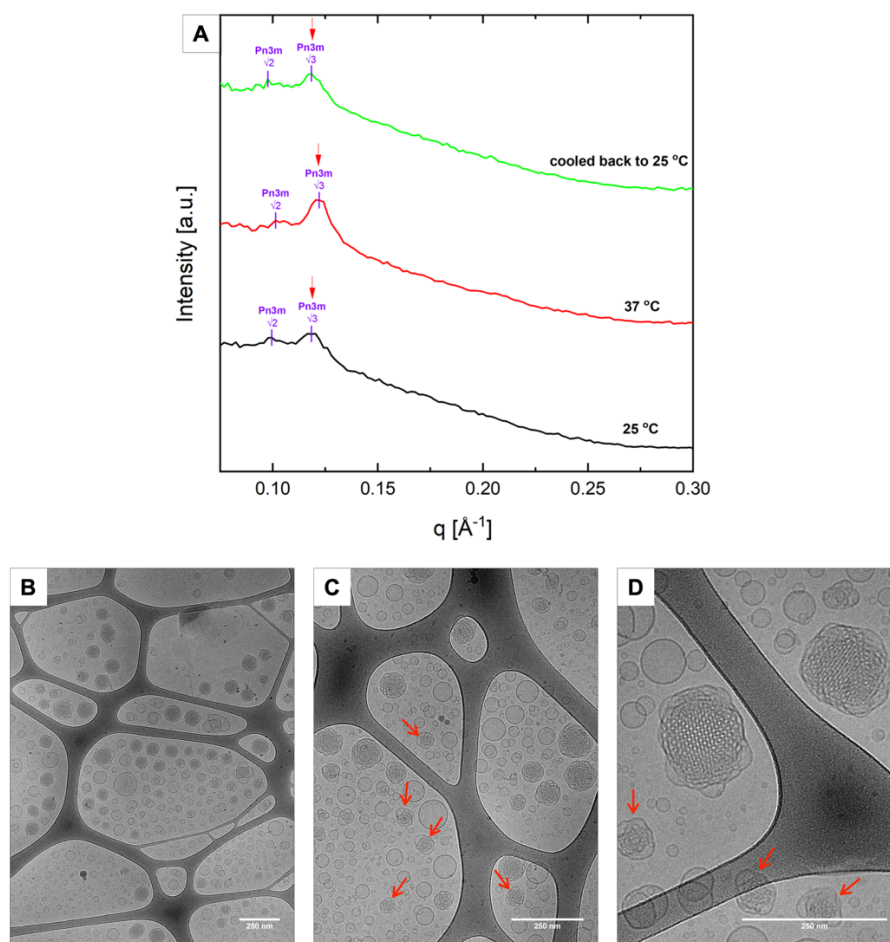


Figure 5.5. SAXS patterns of TS-CUB + caulerpin nanosystem at 25 °C, 37 °C and returned to 25 °C after the heating cycle (A), along with its cryo-TEM images (B, C, D). Red arrows highlight the possible presence of the sponge NPs with an L₃ phase. (**Paper III**)

The main peaks indicative of the Pn3m cubic phase show low intensity, accompanied by a broad and high diffusive band (**Figure 5.5A**), which is linked to the presence of other particles in the formulation, such as polydisperse vesicles. This interpretation is supported by the cryo-TEM images of the formulation presented in **Figures 5.5BC,D**, which will be discussed in more detail in the following paragraph. Nevertheless, the two visible peaks are located at positions corresponding to the Pn3m cubic phase with a lattice parameter of $90.6 \pm 1.9 \text{ \AA}$ at 25 °C, a typical value for the Pn3m. Additionally, since scattering is probabilistic and peaks usually decrease in intensity with the order of scattering, the second peak presenting a higher signal than the first, suggests the possible presence of a second phase, in line with the results of the TS-CUB loaded with *U. rigida* extract presented in Chapter 4 and also discussed there. Thus, the first peak of the suspected L₃ phase is highlighted with a red arrow in **Figure 5.5A**.

Table 5.1. LLC phase, lattice parameter (a), and water channel radius (r_w) of TS-CUB+caulerpin at 25 °C, 37 °C and cooled back to 25 °C, as well as empty TS-CUB at 25 °C from Chapter 3 [5]. Data are reported as an average value $\pm$ SD. (**Paper III**)

Sample	Temperature (°C)	Phase	a (Å)	r_w (Å)
TS-CUB+caulerpin	25	Pn3m	91 ± 1	18.4 ± 0.7
		L ₃	-	15.5
	37	Pn3m	88 ± 1	17.5 ± 0.4
		L ₃	-	14.6
	back to 25	Pn3m	91.5 ± 0.6	18.7 ± 0.2
		L ₃	-	15.4
TS-CUB	25	Pn3m	92.1 ± 0.1	22.0 ± 0.3

Then, to examine the morphology of the NPs and confirm their internal structure, cryo-TEM measurements were conducted (**Figures 5.5BC,D**). Starting with the first one, many cubosomes exhibiting their characteristic honeycomb-like structure are present [6–8], along with some other particles, in agreement with previous studies on TS-CUB formulations (Chapter 3 and 4). Consistent with the empty TS-CUB [5], the presence of vesicles is likely due to the inclusion of Sp80 and TC in NPs composition [9]. Additionally, sponge NPs are observed in the sample (marked with red arrows), whose presence is associated to the formulation composition, as discussed in the previous paragraph concerning SAXS. The coexistence of the bicontinuous Pn3m cubic phase with the L₃ phase in the caulerpin-loaded formulation suggests that the hydrophobic cargo may influence the internal structure of the NPs. Taking into account the composition of TS-CUB, such results suggest that the pigment encapsulation along with the presence of Sp80 alter the NPs structure through changes in the effective packing parameter of the lipidic building block, MO. Such a conclusion is supported by the TS-CUB+extract formulation analyzed in the previous Chapter 4, also presenting sponge NPs after algal photoactive pigments encapsulation, in contrast to the empty TS-CUB investigated in Chapter 3 [5]. Furthermore, as presented in **Table 5.1**, when the temperature is raised to 37 °C, a slight shift towards higher q values is observed, resulting in a slight decrease of the lattice parameter (a) and water channel radius (r_w) of the NPs, in line with the behavior of the TS-CUB+extract. As already mentioned in Chapter 4, the shift at 37 °C is explained by the increased thermal motion of the MO hydrophobic tails, causing an expansion of its hydrophobic region. Consequently, the lattice parameter and water channel radius decrease while the general nanostructure, in this case, remains unchanged [10,11]. Moreover, heating to 37 °C results

in a more prominent first peak of the L_3 phase and a broader first peak of the Pn3m phase, suggesting an increased transition of the bicontinuous Pn3m cubic phase to the L_3 phase as the temperature rises. However, these observations are speculative, based on the analysis of a single peak. Further investigations at higher resolutions would be required for a more thorough assessment. In general, highlighting the potential of TS-CUB+caulerpin for *in vivo* applications, the structure of NPs in the formulation remains stable at the human body temperature of 37 °C. Furthermore, the loaded formulation was analyzed for the NPs size, expressed as the apparent size (D_h) polydispersity index (PDI), and ζ -potential, using DLS and ELS, as presented in **Table 5.2**. Such measurements were once again performed at two different temperatures to assess the sample stability. Overall, all studied parameters align with those of the empty formulation with the same composition (MO/Sp80/TC/W) presented in Chapter 3 [5]. Moreover, no noticeable changes in size, polydispersity, or ζ -potential were presented, suggesting that the increase in temperature does not impact these formulation parameters. Hence, supporting the previous SAXS experiments, the stability of the loaded formulation at 37 °C was confirmed.

Table 5.2. An apparent hydrodynamic diameter (D_h), polydispersity index (PDI) and ζ -potential of TS-CUB+caulerpin and empty TS-CUB at 25 °C and 37 °C. Data are reported as an average value $\pm$ SD. (**Paper III**)

Parameter	Temperature (°C)	TS-CUB+caulerpin	TS-CUB
D_h (nm)	25	113 $\pm$ 2	118 $\pm$ 1
	37	115.0 $\pm$ 0.3	122 $\pm$ 2
PDI	25	0.20	0.23
	37	0.19	0.25
ζ -potential (mV)	25	-35 $\pm$ 1	-35 $\pm$ 1
	37	-31 $\pm$ 2	-32 $\pm$ 1

5.2.2. Strong anticancer potential of caulerpin-loaded nanoformulation

Firstly, for the preliminary assessment of the anticancer activity, TS-CUB+caulerpin formulation was tested on the highly resistant human pancreatic cancer BxPC-3 cells along with the free caulerpin and empty TS-CUB in a time-dependent MTT cytotoxicity assay. For this purpose, a series of tests was conducted at various caulerpin concentrations (0.5, 1, 2, 3, 4 μ M) over four incubation periods: 4, 8, 24, and 48 hours (**Figure 5.6**). These cytotoxicity tests were carried out to evaluate the most effective drug amount for the subsequent bioimaging studies.

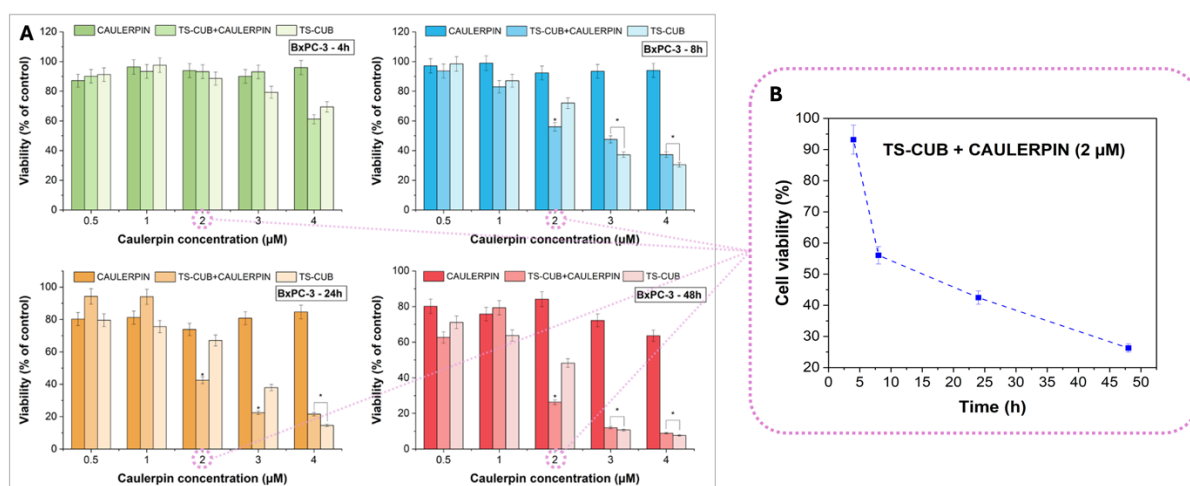


Figure 5.6. MTT cytotoxicity time-dependent experiments performed on BxPC-3 cells after 4, 8, 24 and 48 hours of incubation with free caulerpin, TS-CUB+caulerpin at various caulerpin concentrations (0.5, 1, 2, 3, 4 μM), and empty TS-CUB (diluted in the same manner as the loaded ones) with the corresponding MO concentrations of 56, 112, 223, 334 and 445 $\mu\text{g/mL}$ (A); the decrease of cell viability along with the increased incubation time for the selected 2 μM concentration (B). The results are presented as an average value $\pm$ SD for minimum $n = 3$, * statistical ANOVA analysis: $p \leq 0.05$. (**Paper III**)

As shown in **Figure 5.6A**, after 4 hours of incubation with the studied samples, particularly in the case of 4 μM of caulerpin concentration, noticeable differences between the samples were observed. TS-CUB+caulerpin led to about a 60% reduction in cell viability compared to the control. However, the empty cubosomes exhibit weak cytotoxicity at this concentration, as well as at 3 μM , suggesting that these concentrations are too high. After 4 hours, the lower doses of caulerpin showed no significant cytotoxicity in the case of all studied samples. On the other hand, after a longer incubation time (8 hours), some differences between the samples were evident even from a concentration of 2 μM . At this dose, caulerpin in its loaded form proved greater cytotoxicity (approximately 55% cell viability compared to the control) than both the empty nanocarriers and the free caulerpin. After 8 hours, in case of the higher caulerpin concentrations, the empty TS-CUB presented some toxicity as well. Moreover, after 24 hours of BxPC-3 cells with the studied samples, the differences become more evident. The TS-CUB+caulerpin formulation at 2 μM exhibited strong cytotoxicity, reducing cell viability to approximately 40%, in contrast to the free compound, which showed no significant cytotoxicity after 24 hours. This underscores the advantage of encapsulating caulerpin in TS-CUB. Although the cytotoxicity of caulerpin against pancreatic cancer cells remains underexplored, this

compound in its free form has been tested against several human cancer cell lines [12–15]. Briefly, against colorectal cancer cell lines (HCT-116 and HT-29), after 48 hours of incubation, IC₅₀ values were only observed at very high concentrations (119 and 179 μ M), respectively [13], in contrast to those studied in this work. This could explain why, even at the highest caulerpin concentration (4 μ M) and the longest incubation time (48 hours), free caulerpin presented only weak cytotoxicity (beyond 60% of cells remaining). On the other hand, after 48 hours, caulerpin loaded in cubosomes at 2 μ M, reduced cell viability to 25%, demonstrating strong cytotoxicity. However, after this incubation time, even the empty TS-CUB began to influence the viability of BxPC-3 cells significantly, with about a 50% reduction in comparison to control cells. Such a result may be associated with the cytotoxicity of MO, as described in more detail in the previous chapter (paragraph 4.2.2. *Biocompatibility and photodynamic activity against pancreatic cancer cells*). The lack of strong toxicity of empty nanocarriers after 24 hours at MO concentration of 223 μ g/mL, exceeding the reported limits, is probably related to the high resistance of BxPC-3 cells, as in the case described in Chapter 4. Nevertheless, an extended incubation time of 48 hours leads to a significant increase in cell mortality by empty nanocarriers, indicating possible significant toxicity of MO. After 72 hours of incubation with caulerpin, cytotoxicity investigations on two human ovarian carcinoma cell lines (wild-type 2008 and cisplatin-resistant C13) by Ferramosca et al., resulted in 60% of viable 2008 cells remaining, and 30% in the case of the C13 cell line [15]. Thus, the significant potential of the pigment loaded in relevant nanocarriers, such as TS-CUB, for treating pancreatic cancer is emphasized, as encapsulation notably reduces the incubation time required to achieve substantial cytotoxicity, even against the highly resistant BxPC-3 cells.

In conclusion, due to the enhanced antitumor activity by caulerpin encapsulation, along with the biocompatibility of the empty TS-CUB, 2 μ M was chosen as the optimal drug concentration for further experiments on pancreatic cancer cells. To highlight the anticancer potential of TS-CUB+caulerpin at the selected concentration, the decrease in mitochondrial activity leading to the reduction in cell viability, as the treatment time increases, is presented in **Figure 5.6B**. Given the moderate cytotoxic effect observed even with the TS-CUB after 48 hours, a 24-hour incubation with the anticancer formulation appeared to be the most suitable for BxPC-3 cells. Furthermore, to assess the morphology of BxPC-3 cells, the culture appearance was observed by an optical microscope. As presented in **Figure 5.7**, the cells were exposed to free caulerpin (2 μ M), TS-CUB+caulerpin (2 μ M), and empty TS-CUB, with control cells for comparison. The images (scaled at 500 μ m) were taken after 8 and 24 hours of the incubation with samples and reveal notable differences in the morphology of human pancreatic tumor cells, BxPC-3.

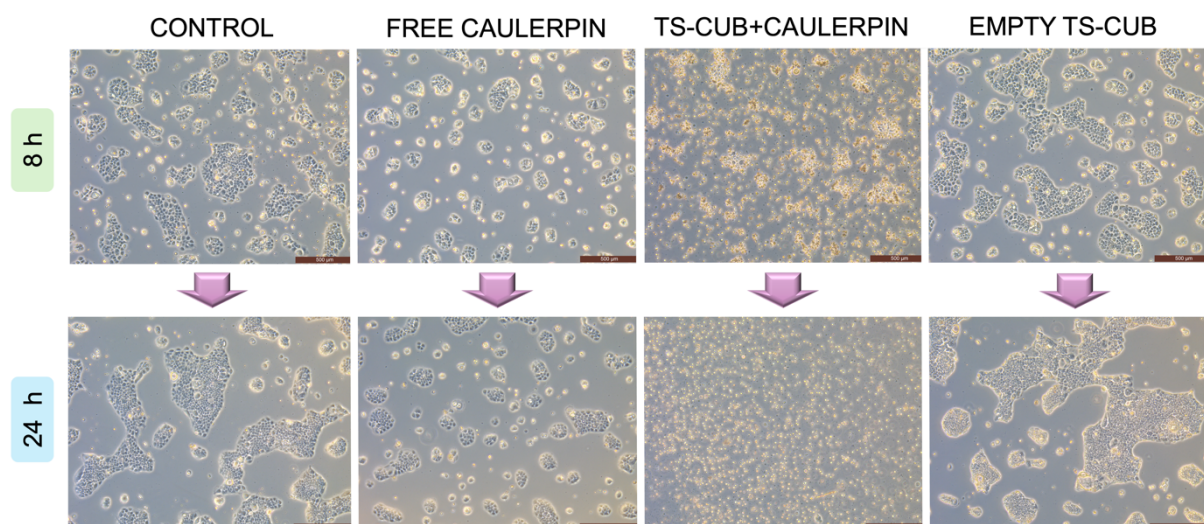


Figure 5.7. Morphological assessment of cells culture by optical microscopy of BxPC-3 cells, treated with free caulerpin (2 μ M), TS-CUB+caulerpin (2 μ M) and empty TS-CUB, in comparison to untreated control cells, with 8 and 24 hours of incubation. The scale bar represents 500 μ m. (**Paper III**)

The growth and condition of the control group appeared appropriate, as evidenced by the increased growth area and the formation of larger cell clusters. Similar observations were made for the empty nanocarriers, with comparisons of images taken after 8 and 24 hours confirming the biocompatibility of TS-CUB at the previously selected concentration, as indicated by the MTT assays. In contrast, the images presenting cells treated with TS-CUB+caulerpin suggest strong toxicity against BxPC-3 cells, since only fragments of dying cells are visible, especially after 24 hours of incubation. This conclusion arises from the indication of apoptosis, characterized by reduction in cell size due to cytoplasmic shrinkage, followed by the active degradation of cells components. Apoptotic bodies formed by separation of parts of the cytoplasm and nucleus, are then phagocytosed and degraded [16,17]. The presence of smaller clusters in comparison to the control, as well as individual small cells or their parts, in the case of the caulerpin in its non-loaded form at the same concentration (2 μ M), suggests ongoing cellular stress with the induction of apoptotic processes in the BxPC-3 cells as well. Nevertheless, despite these signs of potential anticancer effect of the alkaloid, after prolonged incubation time (24 hours), the cells continue to grow in small clusters, suggesting the metabolizing process of free caulerpin, and regeneration in the meantime. Overall, these findings highlight that caulerpin in its loaded form (TS-CUB+caulerpin) presents significantly higher anticancer potential against resistant pancreatic cancer BxPC-3 cells than the compound

in its free form at the same concentration (2 μM), demonstrating therefore the benefits of encapsulation in TS-CUB, along with the biocompatibility of empty nanocarriers.

To investigate the morphological changes in pancreatic cancer cells more thoroughly, a holotomographic microscope (HTM) was employed. As in the previous experiment with the use of an optical microscope, the BxPC-3 cells were exposed to various samples, including free caulerpin (2 μM), TS-CUB+caulerpin (2 μM), and empty TS-CUB in comparison to control cells. The supravital observation of each case was performed after three incubation times: 4, 8, and 24 hours, with the microscopic images captured and presented in the following **Figure 5.8**.

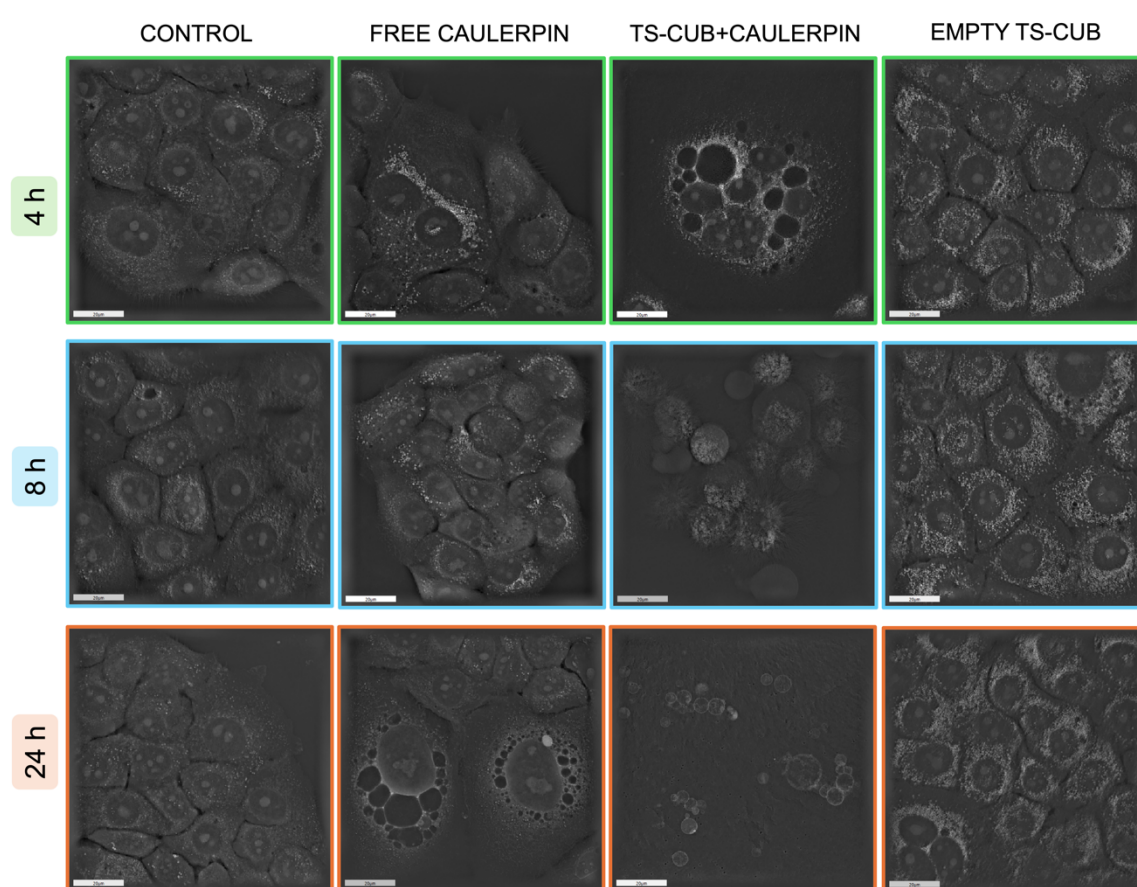


Figure 5.8. Morphological assessment of apoptotic features by HTM of BxPC-3 cells, treated with free caulerpin (2 μM), TS-CUB+caulerpin (2 μM) and empty TS-CUB, in comparison to untreated control cells, with 4, 8 and 24 hours of incubation. The scale bar represents 20 μm . (Paper III)

As described in the Experimental (chapter 2.3.7. *Holotomographic microscopy*) this innovative tool integrates holography and tomography, making it particularly useful for rapidly

distinguishing healthy cells from apoptotic ones and for monitoring both external and internal cellular morphological changes in a non-invasive, label-free manner [18,19]. Studies by Salucci et al. suggest that HTM is a reliable method for the deeper evaluation of key apoptotic hallmarks such as chromatin condensation, micronuclei, cell blebbing, and apoptotic bodies [18]. Autophagy is an essential process for cellular turnover, triggered by cellular stress, acting as a repair mechanism for damaged cells, allowing them to survive temporary periods of starvation [20]. On the other hand, the membrane-bound organelles of all animal cells (except for erythrocytes), lysosomes, play a key role in the breakdown of the material acquired through the processes of endocytosis or autophagy [21]. The degree of lysosomal destabilization under conditions of cellular stress influences whether reparative autophagy occurs or if the cell undergoes apoptosis or necrosis, the typical forms of cell death [20]. Hence, after 4 hours of the treatment with free caulerpin, cells demonstrate noticeable accumulation of lysosomes, suggesting ongoing cellular stress and potential involvement in autophagy, likely induced by caulerpin. In addition, the presence of vacuoles serves as another indicator of stressful and unfavorable conditions for cells, since the vacuolization is considered the key feature of autophagy [17]. Alternatively, Maltese and Overmeyer investigated a type of nonapoptotic cell death known as “methuosis,” characterized by large, filled with fluid, vacuoles replacing the cell’s cytoplasm [22]. In this study, vacuolization is particularly noticeable in cells treated with TS-CUB+caulerpin, after just 4 hours, in contrast to the empty TS-CUB, with no significant differences compared to the control, except for an increase in intracellular vesicles. After a prolonged time of incubation (8 hours) with free compound, cells displayed morphological features suggesting potential regeneration and indicating that 2 μ M of caulerpin in its non-loaded form does not appear to be significantly cytotoxic to BxPC-3 cells during this time, in contrast to its loaded form, supporting the previous studies. This finding can be attributed to the high resilience of pancreatic cancer cells to most traditional anticancer treatments [23], related to their ability to create a unique tumor microenvironment, composed of specific molecules involved in tumor cell protective biological processes, thus contributing to the drug resistance [24,25], as was also discussed in Chapter 4. Chand et al. described one of the mechanisms behind pancreatic cancer resistance, referred to *Hypoxia-Induced Resistance*, associated with the tumor microenvironment's lack of oxygen and nutrients, which creates selective pressure that favors the survival and proliferation of the most aggressive and resilient tumor cells, resulting in their high resistance to chemotherapy [26]. To cope with the rough stress promoted by chronic hypoxia, pancreatic cells activate a complex response by protective hypoxia-inducible factors (HIFs), the proteins specialized to regulate the biology of tumor cells [27].

Such transcriptional response plays a huge role in drug resistance, as demonstrated by Cheng et al., who proved that under hypoxic conditions, downregulating HIF-1 α or NF κ B by siRNA sensitized pancreatic cancer cells to the well-known anticancer drug, gemcitabine [28]. Nevertheless, after 8 hours, a notable difference in the morphology of BxPC-3 cells exposed to TS-CUB+caulerpin compared to other samples is demonstrated by HTM. The formation of cellular structures, such as buds or blebs, characterizes the dying cells [17]. As reported by Ziegler and Groscurth, blebbing was observed as the hallmark of the operating cell's death, along with cell shrinkage and the formation of apoptotic bodies [29]. Hence, it can be concluded that after treatment with TS-CUB+caulerpin, the process of operative apoptosis led to the formation of the round structures resembling blebs or buds, which are the portions of cytoplasm expelled by dying cells that are detaching from the substrate. In comparison to the free caulerpin, encapsulation in nanocarriers offers a clear advantage, likely by allowing the compound to penetrate the cells and enhance its anticancer effects. Liu et al. suggest that caulerpin may have an impact on the mitochondrial ROS-regulated HIF-1 activation with the expression of HIF-1 downstream target gene, by inhibiting the electron transport to mitochondrial complex III [12]. This anticancer approach, which targets the already mentioned mechanism of *Hypoxia-Induced Resistance*, may sensitize cancer cells to the potential drugs, as demonstrated by Cheng et al. [28]. Additionally, another strategy explaining caulerpin's antitumor effect against pancreatic cells is its ability to inhibit PTP1B protein and modulate AMPK enzyme, which monitors the energy status of the cell [30–32], along with the observation of Xu et al., who demonstrated that the pancreatic cancer progression is blocked by the inhibition of PTP1B through the PKM2/AMPK/mTOC1 pathway targeting [33]. The empty TS-CUB led to an increase in the intracellular vesicles secretion after 8 hours of incubation, although with no significant impact on BxPC-3 cells condition, as their morphology remains similar to the control cells. At the longest incubation time, chosen also in the previous series of MTT tests (24 hours), free caulerpin induces the formation of larger vacuoles, indicating ongoing cellular stress, although many of the healthy cells are still present. In contrast, after 24 hours of the treatment with TS-CUB+caulerpin, only remnants of cells with the expelled cytoplasm are observed, suspended in the medium (explaining the poor image sharpness). In case of the empty TS-CUB, there is a marked increase in vesicle accumulation within cells after 24 hours of incubation. However, no morphological changes related to the ongoing cells' death were observed, indicating once again the biocompatibility of empty nanocarriers in such concentration, as proved by previous experiments. This study demonstrates that, from all studied samples, the most successful treatment of the resistant pancreatic cancer was presented

by the caulerpin encapsulated in TS-CUB nanocarriers, at the bioactive cargo concentration of 2 μ M, after 24 hours of incubation with BxPC-3 cells. When treated with caulerpin in its non-loaded form, the cells present high regeneration ability by metabolizing free caulerpin, thus demonstrating their strong resistance mechanisms.

In conclusion, the HTM investigations indicate that, while caulerpin may present anticancer activity, treating pancreatic cancer with the free compound is not sufficient to achieve the desired outcome. However, when caulerpin is loaded into appropriate nanocarriers, such as TS-CUB, apart from inducing cellular stress conditions, there is also a noticeable increase in apoptotic activity in cancer cells, suggesting that the encapsulation significantly enhances caulerpin efficiency in combating the resistant pancreatic tumour cells, BxPC-3.

To validate the previously mentioned findings regarding the efficacy against pancreatic cancer of the TS-CUB formulation loaded with caulerpin, further cytoskeleton bioimaging studies were performed by F-actin staining and visualization by fluorescence microscopy. Hence, **Figure 5.9** displays the images of BxPC-3 cells exposed for 24 hours to free caulerpin (2 μ M), empty TS-CUB and loaded TS-CUB+caulerpin at the same caulerpin concentration concerning the untreated control cells. Briefly, the bioimaging of BxPC-3 cells treated with TS-CUB+caulerpin demonstrates disrupted intercellular connections, along with the cytoskeleton shrinkage of some cells, where only the nuclei remain visible, surrounded by residual cytoplasm, probably dehydrated during apoptosis [29,34], as presented by HTM studies. Moreover, a reduction in the size of nuclei is observed, likely due to the nuclear condensation in the dying cells [17]. Such observation significantly contrasts with the other images, namely the cells treated with free caulerpin and with empty nanocarriers (TS-CUB), where the cells appear in appropriate condition and maintain morphology comparable to the control cells, despite fewer clusters in those treated with empty TS-CUB. Taking into account such results, the fluorescence bioimaging of cells treated with free caulerpin supports the previous statement that BxPC-3 cells can metabolize such compound in its non-loaded form and recover in time. Moreover, when compared to caulerpin loaded in cubosomes (TS-CUB+caulerpin), the advantages of its encapsulation, by the improvement of caulerpin anticancer activity, are evident once again. The ability of TS-CUB nanocarriers to efficiently deliver the bioactive payload, followed by the penetration of cells, enables the induction of apoptosis in BxPC-3 cells, achieving the intended anticancer effect.

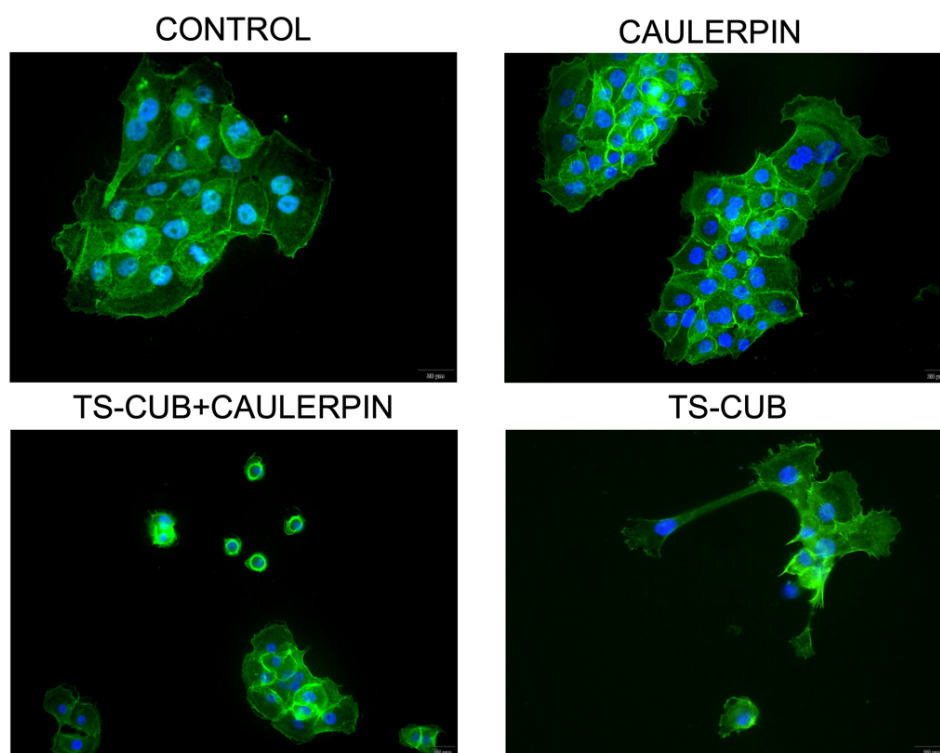


Figure 5.9. Morphological assessment of the cytoskeleton appearance by F-actin staining and fluorescence bioimaging of BxPC-3 cells, treated with free caulerpin (2 μ M), TS-CUB+caulerpin (2 μ M) and empty TS-CUB, in comparison to untreated control cells after 24 hours of incubation. The scale bar represents 20 μ m. (**Paper III**)

In conclusion, caulerpin loaded in Pluronic-free cubic LLCNPs (TS-CUB) at the optimal concentration of 2 μ M exhibits potent antitumor activity against the resistant BxPC-3 cell line.

5.3. Conclusions and future perspectives

Pancreatic tumor still remains one of the most difficult cancers to treat due to its strong resistance to traditional therapies. With an effort to develop an effective treatment, this study investigates the potential of the algal pigment, caulerpin, a bisindole alkaloid derived from *C. cylindracea*, with promising anticancer properties, although not tested against pancreatic cancer yet, to the best of my knowledge. The novel approach presented in this study integrates the principles of *Green Chemistry* with nanotechnology by using MAE to isolate pure caulerpin from the biomass of the invasive algae species, followed by the encapsulation in innovative, Pluronic-free bicontinuous cubic LLCNPs. By incorporating biocompatible surfactants (TC and Sp80) in such formulation, the cytotoxicity is minimized, as proved also in previous studies concerning TS-CUB (see Chapter 3 and 4), indicating high biocompatibility of such NPs.

Furthermore, in this study, the promising capacity of TS-CUB nanocarriers for efficient drug encapsulation with high efficiency, followed by their delivery, while protecting the cargo from degradation, and thereby enhancing its therapeutic potential, is demonstrated once again.

The physicochemical analysis of such formulation demonstrated the presence of cubosomes coexisting alongside L₃ sponge NPs and vesicles, stable even at 37 °C, indicating promising *in vivo* application. After a series of cytotoxicity experiments, the optimal concentration of caulerpin (2 µM) was selected for further studies, considering the biocompatibility of empty nanocarriers. An initial assessment of cancer cell morphology using optical microscopy was followed by a detailed morphological analysis thanks to the HTM technique, well-suited for investigating apoptotic hallmarks. Furthermore, a significant cell shrinkage and the destruction of the cytoskeleton were validated by F-actin fluorescence bioimaging, confirming the anticancer effect of the TS-CUB+caulerpin against BxPC-3 cells. Overall, this research demonstrates that caulerpin loaded in relevant cubosome nanocarriers significantly induces apoptotic processes of the human resistant pancreatic cancer cells, exhibiting its remarkable antitumor activity after the alkaloid encapsulation. The loaded caulerpin is not metabolized by BxPC-3 cells as rapidly as its free form, allowing it to effectively induce apoptosis, finally leading to the death of cancer cells. These findings suggest that the proposed formulation could serve as a new tool for overcoming the challenges associated with the high resistance of pancreatic cancer, offering an efficient treatment with the naturally derived therapeutic drug combined with the loading in relevant nanocarriers. In addition, the possibility of encapsulating pigments isolated from the widely available marine algae biomass in TS-CUB was confirmed again, with attention paid to the probable induction of the sponge NPs' appearance after the cargo loading, similarly to the formulation with *U. rigida* extract described in Chapter 4.

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Concluding remarks

This PhD thesis presents the development and thorough evaluation of a novel Pluronic-free MO-based LLCNPs, namely cubosomes (TS-CUB) and hexosomes (TS-HEX), stabilized by sorbitan monooleate and tuned with sodium taurocholate. Initial investigations demonstrated the promise of TS-CUB, when co-loaded with commercial PS, chlorin e6 and the adjuvant, coenzyme QH, in the PDT of metastatic melanoma. Due to its superior performance compared to novel hexosomes, and standard cubosomes, TS-CUB was selected as the optimal delivery nanoplatform for further exploration.

High abundance of marine algae biomass, especially invasive species such as *C. cylindracea*, combined with the effective, green, and economical way of isolating bioactive compounds by MAE, constitutes an interesting green alternative to synthetic drugs. Particularly, this work demonstrates how to harness the potential of invasive algae species. Therefore, naturally occurring pigments from green marine algae biomass were investigated for encapsulation in previously optimized cubosomes. After initial testing of the formulation on a commercial PS, an attempt was made to load the hydrophobic photoactive extract from *U. rigida* rich in chlorophylls, potent natural photosensitizers. After physicochemical evaluation of the new formulations, followed by a series of biological studies on BxPC-3 cells, in comparison with standard Pluronic-based cubosomes, the significant advantages of TS-CUB for the algal pigments encapsulation were demonstrated, which prompted its use as a nanoplatform for encapsulation of caulerpin from *C. cylindracea*, an alkaloid with potential anticancer properties. A noteworthy aspect is the appearance of sponge NPs in the nanosystem after encapsulation of algal pigments, in contrast to empty nanocarriers, which is evidence of the influence of these hydrophobic cargos on the structure of NPs present in the formulation and thus may play a role in their target application.

In general, this thesis presents two investigations on highly effective anticancer nanosystems based on novel cubosomes with algal pigments encapsulated within: TS-CUB loaded with photoactive *U. rigida* extract and TS-CUB loaded with caulerpin. The first one demonstrates the feasibility of using these nanocarriers for PDT of pancreatic cancer in combination with photoactive extract. The second one proves great advantages in induced cell death of pancreatic tumor cells by the loaded caulerpin. In both cases, noticeable advantages of encapsulation in TS-CUB were demonstrated, such as an increased cellular uptake, indicating effective

delivery of the potential drug, leading to its increased efficiency in fighting highly resistant pancreatic cancer cells. In addition, testing the formulations at 37 °C demonstrated their stability at the body temperature, suggesting further *in vivo* studies, required for their full anticancer evaluation for potential future application as the final drug against pancreatic cancer.

Overall, this thesis contributes to the advancement of LLCNPs in the fields of chemical sciences, the pharmaceutical industry, and nanomedicine. Furthermore, I believe that the formulations presented herein will serve as a versatile nanoplatform for the encapsulation of various natural bioactive compounds derived from biomass, adhering to the principles of *Green Chemistry* and *Sustainable Development*.

Annex

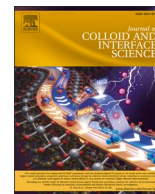
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Paper I



Regular Article

Cubosomes and hexosomes stabilized by sorbitan monooleate as biocompatible nanoplateforms against skin metastatic human melanoma

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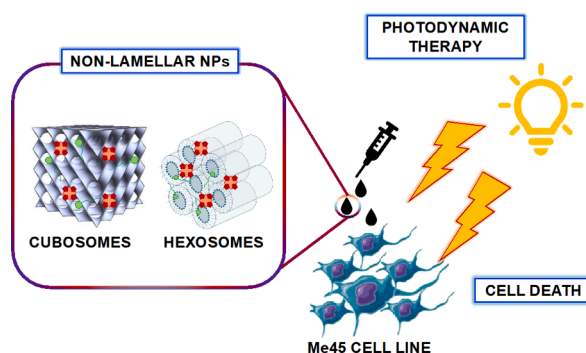
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GRAPHICAL ABSTRACT



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ABSTRACT

Nanoparticles have become versatile assets in the medical field, providing notable benefits across diverse medical arenas including controlled drug delivery, imaging, and immunological assays. Among these, non-lamellar lipid nanoparticles, notably cubosomes and hexosomes, showcase remarkable biocompatibility and stability, rendering them as optimal choices for theranostic applications. Particularly, incorporating edge activators like sodium taurocholate enhances the potential of these nanoparticles for dermal and transdermal drug delivery, overcoming the stratum corneum, a first line of defense in our skin. This study reports on the formulation of monoolein-based cubosomes and hexosomes incorporating taurocholate and stabilized by Span 80 and co-encapsulating Chlorin e6 and coenzyme QH for photodynamic therapy in skin metastatic melanoma. The formulations were optimized using small-angle X-ray scattering, and cryo-transmission electron microscopy confirmed the presence of cubosomes or hexosomes, depending on the ratio between taurocholate and Span 80.

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Furthermore, the co-loaded nanoparticles exhibited high encapsulation efficiencies for both Ce6 and the coenzyme QH. *In vitro* studies on human melanoma cells (Me45) demonstrated the biocompatibility and photodynamic activity of the loaded formulations. These findings show the possibility of formulating more biocompatible cubosomes and hexosomes for photodynamic therapy in skin cancer treatment.

1. Introduction

Nanoparticles (NPs) have emerged as promising tools in the field of medicine due to their unique properties and versatile applications, offering significant advantages in nanomedicine, including controlled drug delivery [1–3], imaging [1] and immunological assays [4]. Ongoing research [1,5–8], particularly on lipid nanoparticles [9–11], continues to explore the potential of such carriers, demonstrating their excellent biocompatibility and stability, minimizing potential adverse effects, and enhancing drug efficacy.

Among these NPs, nanoscopic dispersions of the inverse bicontinuous cubic or of the inverse hexagonal phases (known as cubosomes or hexosomes, respectively) are promising candidates in drug delivery applications [12]. Briefly, cubosomes are described as nanoparticles whose inner structure is characterized by a lipid bilayer arranged in space in a manner that it can be superimposed over a periodical minimal infinitive surface (IPMS) originating a network of two disconnected water channels. The double diamond (space group Pn3m), the double gyroid (space group Ia3d), and the primitive (space group Im3m) are the most important IMPS in lipid self-assembly, and several studies have reported on their peculiar properties as drug carriers, matrices for protein crystallization [13], and nanoreactors [14]. Conversely, the inner structure of hexosomes is constituted by elongated inverse micelles packed in a hexagonal array. These non-lamellar NPs present a higher colloidal stability and may also encapsulate a higher amount of cargo, due to the higher hydrophobic volume [15], respect to the conventional liposomes. Glycerol monooleate (commonly known as monoolein, MO) and phytantriol are the most used lipids [12,16] to produce such NPs, even though other lipid mixtures may be used to tune the size or the crystallinity [17–21]. In order to avoid coalescence and subsequent phase separation, cubosomes and hexosomes commonly require a steric stabilizer, being the block copolymers known as Pluronics the most used [8,22]. Nevertheless, Pluronics are not biodegradable *in vivo* and may induce a cytotoxic effect [23,24]. These NPs have been explored as drug carriers for various administration routes, including topical application [8]. In this perspective, dermal and transdermal delivery of bioactive compounds offer a non-invasive and safe option, particularly to circumvent first-pass metabolism which may degrade the drug [25]. The outermost layer of the skin is the stratum corneum (SC) and it serves as a critical barrier between the internal and external environment of the body [26]. Indeed, one of its primary functions is to prevent water loss from the body and to protect against external insults such as pathogens, chemicals, and UV radiation [27]. The SC consists of a “brick and mortar” structure [28], where the non-living corneocytes serve as the “bricks” embedded in a dense collagen-rich lamellar phase, akin to the “mortar”. This barrier can be surmounted by encapsulating the drug within formulations containing permeation enhancers [29,30], such as lipids, which can fluidify SC lipids, thereby facilitating the delivery of the encapsulated payload into the desired skin layers. Moreover, edge activators, often referred to as softeners of lipid membranes, may be incorporated into the formulation [31,32]. These compounds act as softeners for lipid bilayers, enhancing carrier elasticity and deformability. Examples of edge activators include bile salts (BSs) [33,34], cholesterol [35], and polyethoxylated lipids [32]. When included in MO-based formulations, even aliquots of BSs may influence the lipid effective packing parameter, and prior research has demonstrated that, when integrated into the MO structure, sodium taurocholate promotes a shift from planar to positively curved interfacial curvature due to a combination of steric and electrostatic interactions, thereby facilitating vesicle

formation [36]. Hence, incorporation of BSs into MO-based formulations should be carefully evaluated to avoid undesired transitions of the inner structure of the NPs, as different phases may affect the delivery kinetics [13,14,37]. Diffusion of the drug across the matrix of bulk phases can be a limiting factor in topical administration, as it would require long contact times. On the other hand, liquid dispersions of such phases may be applied as a topical solution, when a short contact time is required, allowing for an efficient PDT after irradiation, bypassing the systemic administration and possible cytotoxic effects [27].

Photodynamic therapy (PDT) is a technology attracting significant attention due to promising outcomes in tumor treatments [38,39]. The advantage of PDT over other chemotherapeutic approaches lies in the possibility of producing reactive oxygen species (ROS), such as the singlet oxygen $^1\text{O}_2$ [40], by activating a photosensitizer (PS) through the action of light of a proper wavelength. In this way, PDT allows delivering the phototoxic activity towards a desired target in a spatial and temporal manner [41], mitigating the severe side effects often experienced using standard therapeutical approaches. In addition, differently to chemotherapy and radiotherapy, ROS generated through PDT can stimulate anti-tumor immune responses [42]. Nevertheless, the hydrophobicity of common PS molecules and the subsequent aggregation in solution may hinder the feasibility of PDT, hence the encapsulation in lipid NPs can overcome this obstacle. Even though there are several examples of vesicular and polymeric carriers with this aim in the literature [43], the studies devoted to cubosomes are limited in number [44] and principally developed by our group [45–49].

In this context, we discuss the formulation of MO-based cubosomes and hexosomes suitable for PDT co-encapsulating Chlorin e6 (Ce6), a second-generation photosensitizer, and the reduced form of the coenzyme Q₁₀ (CoQH), a natural antioxidant added as supporting anticancer agent (Scheme 1). The nanoparticles were designed to include biocompatible and biodegradable components, in comparison to the conventional cubosomes and hexosomes formulated with Pluronics. First, sodium taurocholate (TC), a natural bile salt, was also embedded in the nanoparticles as an edge activator to facilitate, in principle, the interaction with membranes and SC-crossing in topical administration. Sorbitane monooleate (Span80, S80) was employed as steric stabilizer to tune the curvature of the interface of the NPs containing TC, yielding either cubosomes or hexosomes depending on the relative ratio between TC and S80 in the formulation. The loaded formulations were then tested *in vitro* against malignant melanoma cells, Me45, to evaluate their applicability in PDT. It is worth noting that this therapy has never been coupled to hexosomes, to the best of our knowledge.

2. Materials and methods

2.1. Chemicals and reagents

The following chemical compounds, purchased from Sigma-Aldrich (Poznan, Poland), were used to prepare cubosomes or hexosomes: sodium taurocholate hydrate (TC, $\geq 97\%$), Sorbitan monooleate, Span 80 (S80), Coenzyme QH (CoQH). Monoolein (MO, 1-monooleoylglycerol, RYLO MG 19PHARMA, glycerol monooleate; 98.1 wt%) was a kind gift from Danisco A/S, DK-7200, Grinsted, Denmark. Chlorin e6 (Ce6) from Frontier Scientific (US) was used as a second-generation photosensitizer. Other reagents and solvents were of commercial grade and were used as received. The aqueous solution used for all experiments was prepared with doubly distilled water and purified using a Milli-Q purification system (Millipore, Bedford, MA).

2.2. Preparation of sodium taurocholate/Span 80 bicontinuous cubic or hexagonal nanoparticles

The NPs were prepared by dispersing an appropriate amount of melted MO (at 40 °C) in an aqueous solution of TC and S80 using a UP100H ultrasonic processor (Hielscher Teltow, Germany – cycle 0.9, amplitude 90 %,) for about 10 min, until the dispersion appeared homogeneous. NPs loaded with C6 and CoQH were obtained by dispersing the photosensitizer and the antioxidant in the melted lipid phase with the help of an ultrasonic bath for 5 min at 40 °C before adding the aqueous solution of the stabilizers. The total sample volume was usually 4 mL. After sonication, most formulations appear slightly transparent or milky, depending on the concentration of TC in the system. The compositions of nanoparticles are reported in Table 1.

2.3. Characterization of the NPs colloidal properties

2.3.1. Analysis of the size and ζ -potential

The apparent hydrodynamic diameter of the formulations (empty and loaded) was determined by dynamic light scattering (DLS) in optically homogeneous polystyrene cells at 25 °C, using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK; DTS software) with a detection angle of 173°. Results are given as an average of three measurements, with at least ten runs (20 s each). The autocorrelation function was fitted by a second-order cumulant method and the apparent hydrodynamic diameter (Dh) was then extracted together with the polydispersity index (Pdl).

The ζ -potential (zeta) of the nanoparticles was measured by means of electrophoretic light scattering (ELS), monitoring the movement of the particles in an electric field. The measurements were conducted in a folded capillary zeta cell at 25 °C using the same Malvern Zetasizer Nano ZS apparatus. Each value was obtained as the average of three consecutive measurements, with at least twenty runs.

The measurements at pH 5 to mimic the environment of the skin were performed in the same environment by diluting the sample 1:50 in an aqueous solution acidified by adding HCl until pH 5 is reached and leaving the sample to equilibrate for 1 h.

2.3.2. Inner structure of the NPs

Small angle X-ray scattering (SAXS) profiles of the empty formulations in the optimization step were recorded following the setting already described by our group [50]. The instrument, coupled with a GeniX x-ray generator (50 kV and 1 mA) detected the signal with a 1D-PSD-50 M system (HECUS x-ray Systems, Graz, Austria) with 1024 channels. The working range of q , the scattering vector, was $0.005 \text{ \AA}^{-1} \leq q \leq 0.6 \text{ \AA}^{-1}$. Thin-walled 2 mm glass capillaries were filled with the samples and each diffraction pattern was recorded for 3 h at 25 °C.

The structure of the optimized NPs co-loaded with Ce6 and CoQH was checked using the SAXSLab instrument (JJ-Xray, Denmark) at Lund University, following an already published protocol [51,52]. The SAXS patterns were recorded for 1 h each at 25 °C.

From each maximum in the SAXS pattern, the scattering vector values $q = 4\pi \sin(\theta)/\lambda$ were taken, and the spacing of the Bragg reflection d was calculated using the following equation:

$$d = 2\pi/q \quad (1)$$

The lattice parameter, a , was calculated through Eqs. (2) and (3) for the inverse bicontinuous cubic and inverse hexagonal phase, respectively:

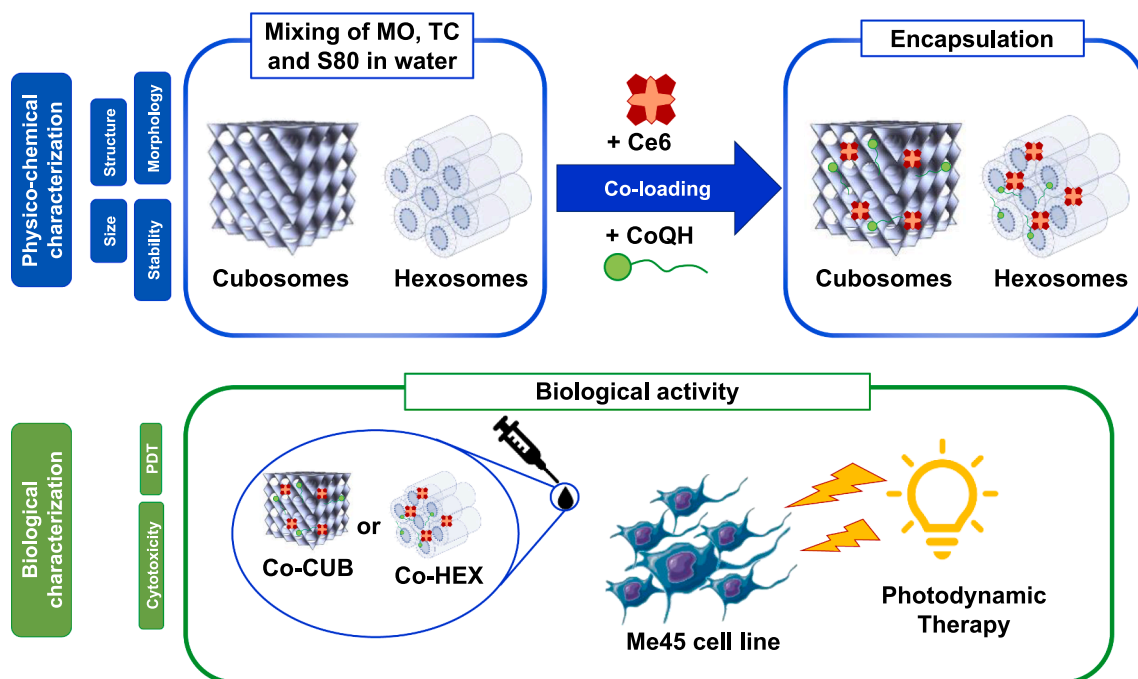
$$a = d(h^2 + k^2 + l^2)^{1/2} \quad (2)$$

$$a = 2d/(3)^{1/2}(h^2 + k^2 + l^2)^{1/2} \quad (3)$$

Where h , k and l are Miller's indexes describing the crystallographic planes of a given phase. Then, the lattice parameter was used to evaluate the water channel radius, r_w , as follows for the inverse bicontinuous cubic phase:

$$r_w = \left[(A_0 / -2\pi\chi)^{1/2} a \right] - l \quad (4)$$

where l is the lipid length value at 25 °C (17 Å) [53], a is the lattice parameter obtained from the SAXS analysis, and A_0 and χ are the surface area and the Euler characteristic of the infinite periodic minimal surface geometries (for Pn3m, $A_0 = 1.919$, $\chi = -2$). For the inverse hexagonal



Scheme 1. Schematic representation of the study involving the formulation of the cubosomes and hexosomes with MO, TC and S80 encapsulating Ce6 and CoQH, their physico-chemical (upper panel) and biological characterizations (lower panel), in the latter case against Me45 cell line.

Table 1

Composition of GMO, S80, TC and water (W) for the samples investigated (concentrations are reported as wt %), phase, lattice parameters (a) and water channel radius (r_w). The values of a and r_w for sample 2 are not defined (n.d.) given the poor resolution of the SAXS pattern.

Sample	GMO	S80	TC	W	S80:TC ratio	Phase	a (Å)	r_w (Å)
1	3.50	0.20	0.05	96.25	4:1	H _{II}	59.5 ± 0.3	30.7 ± 0.2
2	3.50	0.20	0.10	96.20	2:1	Pn3m	n.d.	n.d.
3	3.50	0.20	0.20	96.10	1:1	Vesicles	—	—
4	3.50	0.20	0.40	95.90	0.5:1	Vesicles	—	—
5	3.50	0.20	0.50	95.80	0.4:1	Vesicles	—	—
6	3.50	0.10	0.10	96.20	1:1	Vesicles	—	—
7	3.50	0.40	0.10	95.90	4:1	H _{II}	59.3 ± 0.3	30.6 ± 0.2
8	3.50	0.50	0.10	95.80	5:1	H _{II}	61.0 ± 0.2	31.5 ± 0.1

phase:

$$r_w = (a(1 - \phi)^{1/2})((3)^{1/2}/2\pi)^{1/2} \quad (5)$$

where

$$\phi = (m_{\text{lipids}}/\rho_{\text{lipids}})/((m_{\text{lipids}}/\rho_{\text{lipids}}) + (m_{\text{water}}/\rho_{\text{water}})) \quad (6)$$

and m and ρ are the mass and the density of the lipids and water, respectively.

All the equations used for the SAXS analysis are taken from the previous study on MO lipid mesophases from Kulkarni and collaborators [53].

Here, it was assumed that the length of lipids does not significantly deviate from that of MO in a pure MO bilayer. Data are reported as mean values ± standard deviation, SD, for at least two peaks highlighted in the pattern.

2.3.3. Morphological assessment

4 µL of the samples were blotted on a lacey carbon grid (Ted Pella Inc, Redding, CA, USA) at 25 °C in an environmental chamber with 90 % humidity, vitrified by rapidly inserting the grid in liquid ethane, and then stored in liquid nitrogen until the beginning of the measurement. The morphological evaluation of the cubosomes and hexosomes was done by means of Cryogenic Transmission Electron Microscopy, cryo-TEM, following a protocol and the settings already published [34], using a Jeol JEM-2200FS transmission electron microscope (JEOL, Tokyo, Japan) equipped with a field-emission electron source, a cryopole piece in the objective lens and an in-column energy filter (omega filter).

2.3.4. Co-encapsulation efficiency of Ce6 and CoQH

Evaluation of the active cargo encapsulation efficiency (EE, %) in the optimized cubosomes and hexosomes was investigated by UV–vis absorbance measurements with 1 cm path thermostatic length quartz cell (Hitachi U-5100 spectrophotometer, Mannheim, Germany) according to our previous study [49]. The cargo content was quantified at $\lambda_{\text{Ce6}} = 405$ nm and $\lambda_{\text{CoQH}} = 275$ nm after the disruption of cubosomes or hexosomes with THF:water (3:1) mixture. The EE of the active cargo was quantified as follows:

$$\text{EE (\%)} = (\text{weight of the active agent in cubosomes})/(\text{weight of the feeding compound}) \times 100 \%$$

2.4. In vitro biological response upon human melanoma cells

2.4.1. Cell cultures

Biological studies were performed on a human melanoma cell line initiated through an explant culture of malignant melanoma cells derived from a lymph node metastasis of skin melanoma (Me45). Primary human cutaneous keratinocytes (HaCaT) were applied as a normal (control) cell line. The Me45 cell line was obtained from the Oncology Centre Gliwice (Poland), where the line was derived from a 35-year-old woman's lymph node cells. The HaCaT, a spontaneously immortalized

keratinocyte cell line from the adult human skin of a 62-year-old male, was purchased from ATCC®. The cells were cultured in culture flasks (25 cm², Falcon) in DMEM (Sigma) with the addition of 10 % fetal bovine serum (FBS; Sigma-Aldrich) and 50 µg/mL penicillin and streptomycin (Sigma-Aldrich). The cultures were incubated at 37 °C and in the presence of 5 % CO₂ humidified atmosphere. The cells intended for all experiments were detached by trypsinization (Trypsin-EDTA solution, T4049, Sigma-Aldrich) and neutralized by Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich).

2.4.2. Cytocompatibility and photodynamic action protocols

The *in vitro* cytotoxicity evaluation of cubosomes and hexosomes, empty and co-loaded with Ce6 and CoQH against Me45 and HaCaT was performed using an MTT reduction assay. The selected samples were measured at three different dilutions, e.g., 1:80, 1:160, 1:300; such values correspond to 437, 219 and 117 mg/mL of MO and 8, 4 and ca. 2 µM of Ce6 for the empty and loaded formulations, respectively.

For the photocytotoxicity studies, the Me45 cells were irradiated after 4 h of their cultivation for 10 min with a total light dose of 10 J/cm² using a lamp (OPTEL Opole, Fibre Illuminator, Poland) with polarized light filtered with barrier filters (λ_{max} 600–630 nm). The energy flux rate was 10 mW/cm² at the level of the cell monolayer. MTT assay for evaluation of PDT effectiveness was performed after 4 h and 24 h post-irradiation. Mitochondrial function was expressed as a percentage of viable cells under treatment relative to untreated control cells, followed by determination of the absorbance using a multi-well scanning spectrophotometer at 570 nm (EnSpire Perkin–Elmer, Poland).

2.4.3. Immunofluorescent F-actin staining protocol and cell morphology bio-imaging

Cancer cells were seeded on round glass coverslips (φ 18 mm, Thermo Fisher Scientific) in 6-well plates (Sarstedt, Equimed, Poland), and left to adhere for 24 h, after which the cells were incubated with the nanoparticles for another 24 h. At the end of the incubations, the cells were washed twice with PBS (BioShop, EPRO, Poland), fixed for 10 min in 4 % paraformaldehyde (Polysciences, Inc., Bergstrasse, Germany), and again washed with PBS. The cytoskeleton was analysed by immunofluorescent cells' labelling of F-actin using Alexa Fluor®546 Phalloidin (Thermo Fisher). Fluorshield™ with DAPI (4,6-diamidino-2-phenylindole) was applied to visualize the nuclei and to mount the cells after excitation at 405 nm. The samples were studied on an Olympus BX53 fluorescence microscope (Evident Europe GmbH, Warsaw, Poland). For cell morphology analysis 3D Cell Explorer holotomographic microscope (HTM) was used (Nanolive SA, Sygnis, Poland). Images of the samples were taken and analysed using Steve software (Nanolive SA, Sygnis, Poland).

2.4.4. Statistical analysis

Results of the *in vitro* experiments are presented as mean values ± SD for minimum $n = 3$ and compared by two-way ANOVA for multiple comparisons and $\alpha = 0.05$. Comparisons of samples exhibiting P values ≤ 0.05 were considered as statistically significant. Results were analyzed using the commercial software GraphPad Prism 7.0.

3. Results and discussion

3.1. Optimization and characterization of the formulations

The present investigation delves into the formulation of MO-based liquid crystalline NPs known as cubosomes and hexosomes, stabilized by S80 and containing TC. These components were added to increase the biocompatibility (S80) and penetration properties (TC) in a possible topical administration of the NPs. A PS moiety (Ce6) was encapsulated for potential PDT treatment of skin metastatic melanoma, in addition to CoQH, to test the possibility of carrying an antioxidant with anticancer ability.

Since both S80 and TC can affect the MO phase behavior in water [36], a preliminary investigation was undertaken to optimize the formulations. Particularly, previous studies demonstrated that the addition of TC favors the reduction of the MO effective packing parameter, a consequent increase of the interfacial positive (towards water) curvature and, finally, a transition from cubosomes to vesicles [33,34]. The use of S80 as stabilizer is based on the idea that its hydrophobic portion is expected to reverse this lamellar forming effect, restoring the bicontinuous cubic structure or favoring inverse hexagonal nanostructures at low and at high concentrations of S80, respectively.

Therefore, samples were prepared alternatively keeping the MO/TC or the MO/S80 mass ratio (wt%) constant while changing the content of the remaining components, as reported in Table 1. A preliminary screening via SAXS was performed to explore the combined effect of TC and S80 on the structure of MO dispersions in water. According to the scattering results (Fig. S1A and S1B in SI), already at the very low content of 0.2 wt% of S80 and 0.05 wt% of TC, the native cubic bicontinuous Pn3m nanostructure is lost, and the SAXS pattern shows

the Bragg peaks characteristic for an inverse hexagonal (H_{II}) phase. A small increase in TC content provokes an evolution of the nanoparticles' inner structure, as indicated by the SAXS pattern of sample 2. Due to the very low resolution of the patterns, no Bragg peaks were observed, although the recorded signal closely recalls that is often observed in the presence of cubosomes [52,54,55]. Further additions of TC very likely cause the transition towards unilamellar vesicles and this is denoted by the broad diffuse scattering of the SAXS patterns, as already observed in similar lipid compositions [34,56,57]. Experiments performed on samples having larger S80 content clearly demonstrate the role of this surfactant in enhancing negative interfacial curvature, as evidenced by the formation of a H_{II} phase dispersion when the S80 content exceeds 0.40 wt%.

These preliminary results summarize how the combinatory effect of TC and S80 can be exploited to alter the curvature of MO bilayers with even small amounts of the surfactants.

The final assessment of the presence of cubosomes and hexosomes was conducted by visualizing the samples using cryo-TEM. Sample 2 (CUB) was selected for analysis due to the suspected presence of cubosomes, whereas sample 8 (HEX) was chosen for its superior colloidal stability observed over time compared to the other samples exhibiting H_{II} patterns through SAXS analysis. Visual inspection confirmed the stability of both samples for approximately one month.

As shown in Fig. 1A and 1B, sample 2 is composed of a large number of polydisperse vesicles and nanoparticles with the typical honeycomb inner structure of cubosomes [5]. The heterogeneity of the sample is not surprising as the presence of TC affects the self-assembly of MO, yielding a broad population of vesicles, as already reported by our group [34]. On the other hand, as already commented, the lamellar forming effect is then counterbalanced by the presence of S80 and cubosome are hence

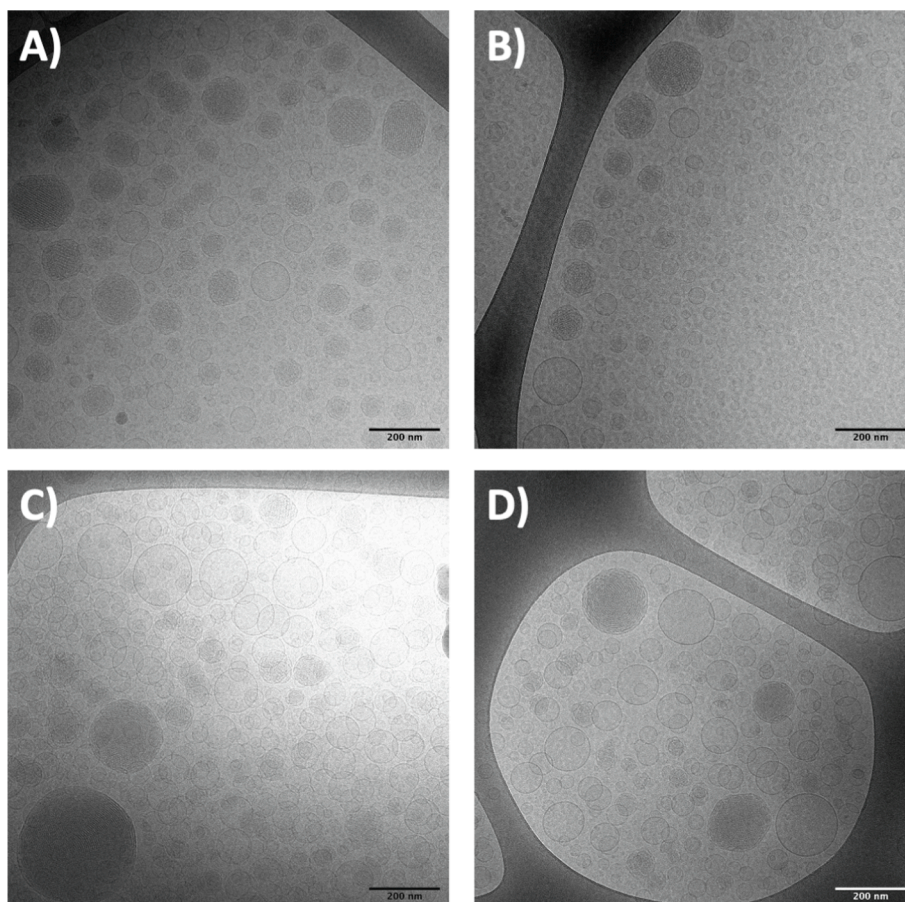


Fig. 1. Cryo-TEM micrographs of samples 2 (A and B, CUB, MO/S80/TC/W = 3.5/0.2/0.1/96.2) and 8 (C and D, HEX, MO/S80/TC/W = 3.5/0.5/0.1/95.8).

found in the sample. In line with the previous SAXS investigations, Fig. 1C and 1D prove that sample 8 is constituted by hexosomes and vesicles, but no cubosomes are visible in the images obtained. Also in this case, a large population of vesicles is present, suggesting that some heterogeneity in terms of compositions at the NPs' level is occurring.

In general, the screening via SAXS and cryo-TEM confirmed the optimal ratios between S80 and TC (S80:TC) to obtain cubosomes and hexosomes using MO as building block. Finally, DLS measurements highlighted that increasing the S80:TC ratio in the formulation induced an increase in terms of apparent hydrodynamic diameter, as shown in Fig. S1C and S1D of SI. It is important to notice that the polydispersity index of such formulations is slightly higher (ca. 0.22–0.25) than those formulated with MO and other stabilizers (ca. 0.05–0.15); [20,34,48,52,58] such fact could be explained by a large distribution of vesicles, as reported in the cryo-TEM images, that affect the overall size distribution of the NPs.

Samples 2 and 8 (from here referred to as CUB and HEX, respectively) were then used as models for the co-encapsulation of Ce6, a second-generation photosensitizer, and CoQH, an antioxidant with possible anticancer activity, to produce formulations (CoCUB and CoHEX, for cubosomes and hexosomes, respectively) suitable for PDT of skin metastatic human melanoma.

Results from the SAXS measurements are shown in Fig. 2; it is noteworthy that the higher resolution and intensity from the SAXSLab instrument enabled the revelation of five low but distinctly visible Bragg peaks, corresponding to a Pn3m phase, as shown in Fig. 3A for CUB and CoCUB. The identification of the phase was proved by the linear trend of the q values of each peak plotted against the square root of $(h^2 + k^2 + l^2)$, as shown in Fig. S3 of SI. In general, the co-encapsulation of the two hydrophobic molecules (Ce6 and CoQH) did not affect the inner structure of both formulations, as only a slight shift of the q values of the peaks related to the Pn3m and H_{II} phases towards higher q s was observed. Subsequently, the calculated lattice parameters and the water channel radius are slightly reduced as shown in Table 2, in agreement with other studies focused on the encapsulation of hydrophobic drugs [5,8].

The hydrodynamic diameter, ζ -potential and PDI values of both formulations did not vary upon co-encapsulation of Ce6 and CoQH; moreover, such figures are also in line with those reported in the literature for samples of cubosomes and hexosomes formulated with different lipid compositions [59–62]. The encapsulation efficiencies (EE), i.e., the amount of payload encapsulated within the NPs, were

evaluated using UV–Vis spectroscopy (the spectra of the empty and co-loaded formulations are reported in Fig. S4). The concentration of Ce6 and CoQH retained in the formulation reached an EE up to 95 % (Table 3), which is in line with the ability of such NPs to allocate hydrophobic molecules in the lipid palisade [51,63–66].

Taking into account the desired topical administration of the formulations, the stability of the empty and loaded carriers was tested at pH 5 to mimic the pH of the skin, as shown in Table 3. These investigations proved that there are no significant deviations from the behavior of the formulations in water after dilution at pH 5.

3.2. Bioimaging and immunofluorescence study

As a next step, the bioimaging features of the empty and loaded samples of cubosomes and hexosomes were assessed against human skin melanoma cells. For this purpose, human malignant melanoma cells derived from a lymph node metastasis of skin melanoma (Me45) were applied. Such cell line derived from a lymph node metastasis of skin melanoma is highly metastatic and resistant to conventional cancer therapies [67]. However, our previous studies proved its greater sensitivity to PDT compared to generally less aggressive primary melanoma cells (MeWo) [68], especially when we used chlorin Ce6 loaded into cubosomes [49]. Here we pose the question if the obtained formulations can be then used for PDT against a resistant cancer cell line, such as Me45. The results of the *in vitro* bioimaging tests are shown in Figs. 3 and 4.

At first, the effect of cubosomes and hexosomes empty (CUB and HEX) or co-loaded with Ce6 and CoHQ (CoCUB and CoHEX) on melanoma cell morphology was investigated. 3D cell bioimaging was performed using holo-tomographic microscopy (HTM), which enabled the study of the cellular morphology with stained nuclei post-exposure to the nanoparticles or free Ce6 (Fig. 3). Remarkably, cell exposure to the empty and loaded cubosomes formulations did not cause any observable changes in morphology, whereas the hexosomes formulations induced a slight elongation of the cell shape, reducing the cell 2D area. Interestingly, exposition to CoHEX provoked an accumulation of lysosomes, i.e., lysosomal stress response (LSR), considered to be an early marker of autophagy [69]. Depending on the intensity of such stress, the cell dies or restored to homeostasis [70]. In other studies it was also demonstrated, that self-assembled nanoparticle with Ce6 and celastrol, a tripterine isolated from the root extracts, induced autophagic cell death after the activation of the photosensitizer photodynamic reaction [69].

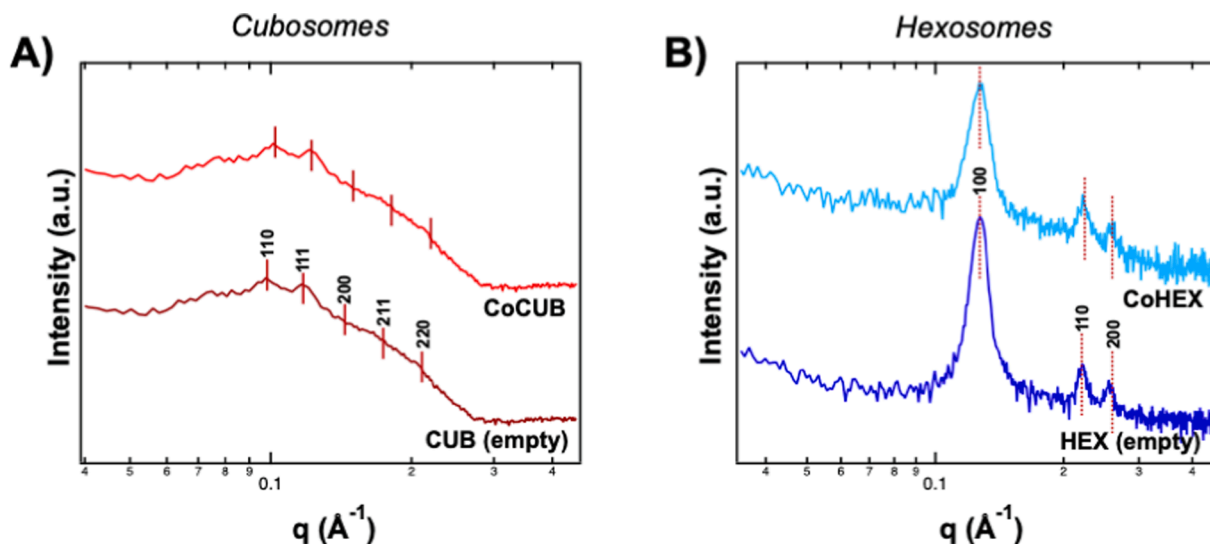


Fig. 2. SAXS patterns (log-log) of the empty and loaded cubosomes (A) and hexosomes (B) formulations for the PDT application. The position of the peaks for the Pn3m and H_{II} phases are highlighted by full and dashed red lines, respectively, with the corresponding indexing represented by the Miller's indices on top. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

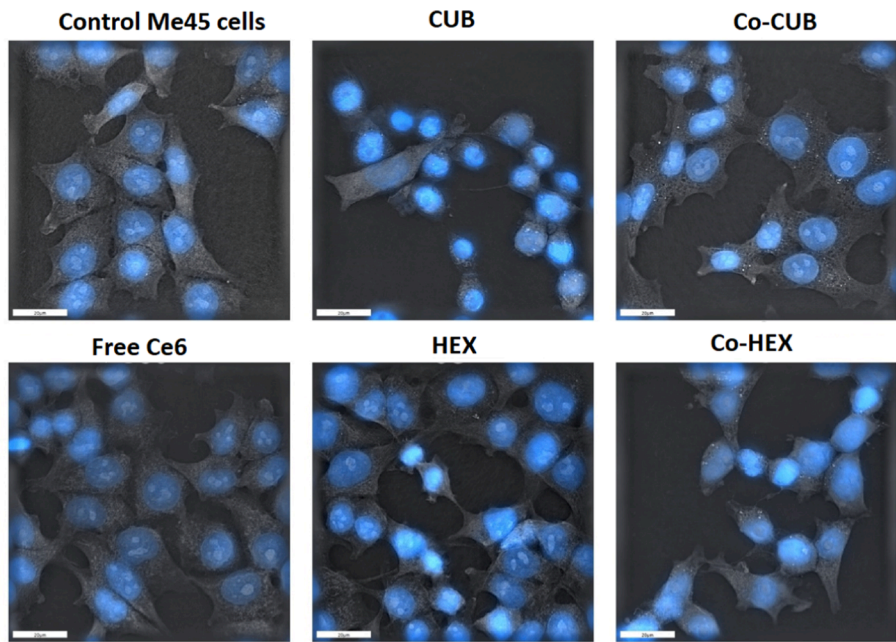


Fig. 3. Bioimaging of the human melanoma Me45 cells morphology using holotomographic microscopy, after treatment with empty and loaded (CoCUB and CoHEX) cubosomes and hexosomes. The final PS concentration was 2 μ M, corresponding to 117 mg/mL of MO. The scale bar represents 20 μ m.

Table 2
Lattice parameter (*a*) and *radii* of the water channel (*r_w*) values calculated from the SAXS patterns of the empty and loaded formulations at 25 °C.

Sample	<i>a</i> (Å)	<i>r_w</i> (Å)
CUB	92.1 ± 0.1	22.0 ± 0.3
CoCUB	90.2 ± 0.1	21.2 ± 0.2
HEX	59.5 ± 0.3	30.6 ± 0.2
CoHEX	57.2 ± 0.4	28.2 ± 0.3

Consequently, in order to analyze the impact on the structure of the cytoskeleton after exposure to the nanoparticles, F-actin staining was performed. As shown in Fig. 4, no significant reorganization of actin fibers was observed upon exposure to the cubosome formulations (empty and loaded), demonstrating no influence on the cytoskeletal dynamics. It is worth mentioning that, likewise to HTM imaging, free Ce6, and CoHEX formulation, caused some cell elongation. In the case of free Ce6, a noticeable tension of the stress fibers after exposure was observed. Cell features and changes induced by the hexosomes may be related to the higher concentration of S80 within the nanoparticles, which may affect cell viability.

In general, these findings showed that the novel cubosomes and hexosomes formulations containing S80 and TC did not have a direct impact on cellular morphology or actin cytoskeleton dynamics, which correspond to safety profiles in biomedical applications [1].

3.3. Cytocompatibility and photo-photodynamic activity

Since these nanoparticles are intended for applications in PDT, the cytotoxicity and photodynamic activity of the cubosomes and hexosomes samples were tested against human malignant melanoma cells (Me45), while their cytocompatibility was checked against primary human cutaneous keratinocytes (HaCaT), used as a control cell line. The concentration-dependent cytotoxicity of the samples was studied at three different dilutions (1:300, 1:160, and 1:80). First, the cytotoxicity of the empty formulations was assessed by incubating the different dilutions of the NPs in HaCat for 24 h in dark conditions. The results (Fig. S5) indicated that cubosomes are safe over the entire concentration range, showing cell survival at 100 % of the control. It is important to notice that the concentrations observed as viable in this study are higher than the ones reported for the MO-based cubosomes against other cell lines [8,71], hinting that the biological behavior of the cubosomes could be cell line-dependent, and not just composition-dependent. In the case of hexosomes, a higher decrease in cell viability was observed, especially when the most concentrated sample (cell viability below 50 % compared to the control). The observed phenomenon could be explained due to the already mentioned LSR, and cell elongation observed during the HTM bioimaging and F-actin staining of the cell cytoskeleton.

The less cytotoxic concentration of the nanoparticles, corresponding to 2 μ M Ce6, was selected for the following time-dependent photodynamic activity experiments provided on Me45 cells (Fig. S6). The formulations tested here can be considered as non-cytotoxic upon HaCaT cell line even in comparison to other nanocarriers described in the literature for topical administration [72]. The main goal of these

Table 3
Apparent hydrodynamic diameter (*D_h*), Polydispersity Index (*PdI*) and ζ -potential of the cubosomes and hexosomes formulations, empty and co-loaded with Ce6 and CoQH in water and at pH 5. The last two rows referred to the encapsulation efficiencies (EE%) of Ce6 and CoQH in water.

Sample	<i>D_h</i> (nm)		<i>PdI</i>		ζ -potential (mV)		EE _{Ce6} (%)	EE _{CoQH} (%)
	Water	pH 5	Water	pH 5	Water	pH 5		
CUB	114 ± 2	113 ± 2	0.21 ± 0.01	0.22 ± 0.01	−39 ± 1	−39 ± 1	–	–
CoCUB	115 ± 1	113 ± 2	0.21 ± 0.00	0.21 ± 0.01	−40 ± 1	−38 ± 1	95 ± 2	93 ± 2
HEX	132 ± 1	123 ± 1	0.27 ± 0.01	0.24 ± 0.01	−42 ± 1	−43 ± 1	–	–
CoHEX	135 ± 1	132 ± 1	0.28 ± 0.00	0.27 ± 0.01	−40 ± 1	−38 ± 0	90 ± 1	90 ± 1

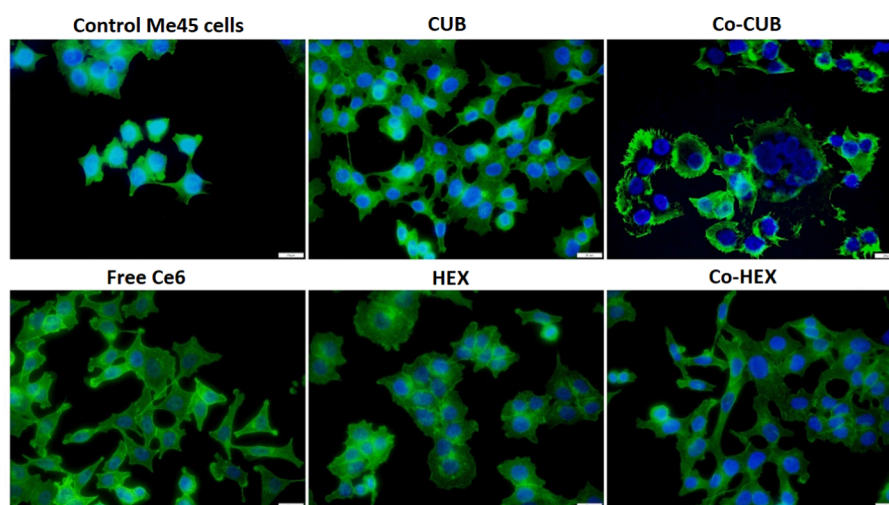


Fig. 4. Immunofluorescence F-actin staining performed on Me45 cells, after treatment with empty and loaded (CoCUB and CoHEX) cubosomes and hexosomes. The final PS concentration was 2 μ M, corresponding to 117 mg/mL of MO. The scale bar represents 20 μ m.

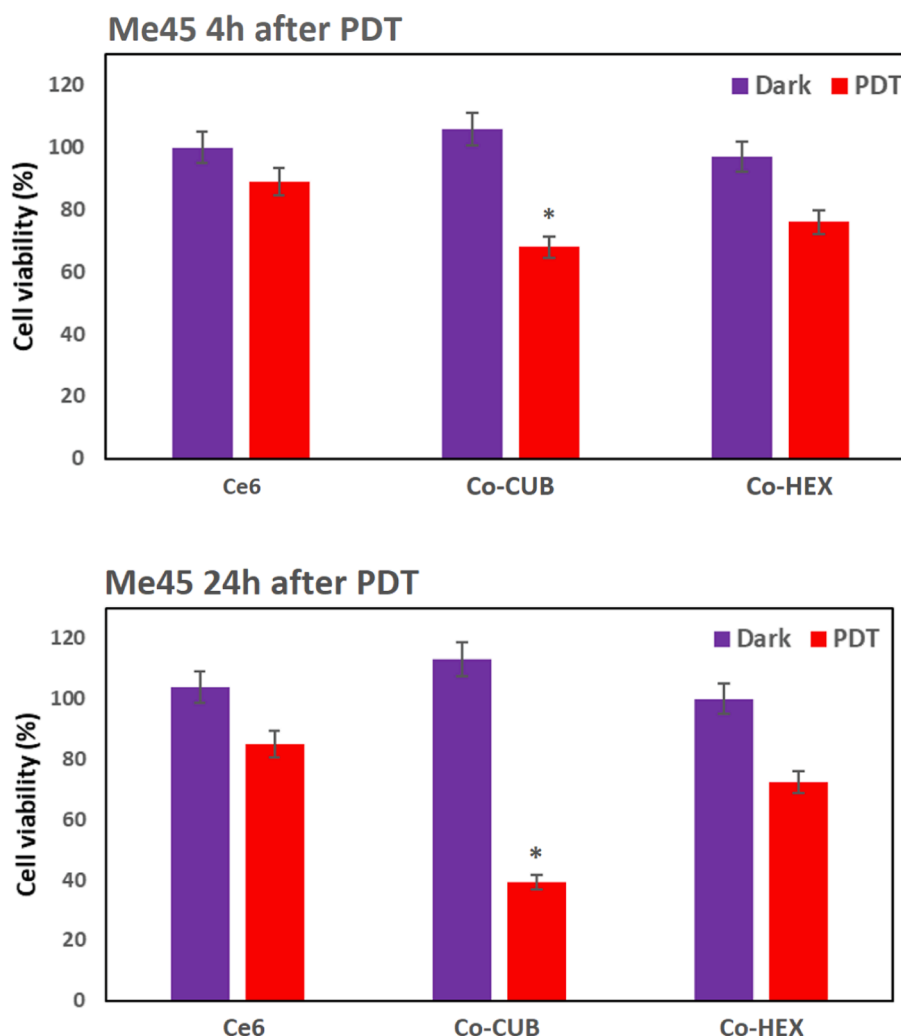


Fig. 5. Dark cytotoxicity (without irradiation) and photocytotoxicity (PDT action) of loaded cubosomes (CoCUB) and hexosomes (CoHEX) compared to free Ce6 molecules (the final PS concentration was 2 μ M) upon human melanoma Me45 cell line. The incubation was provided for 4 h (top panel) and 24 h (bottom panel) post-red light irradiation. The cell viability in each group was expressed as a percentage of control (untreated with studied nanocarriers) Me45 cancer cells. The results are represented as mean values $\pm$ SD for minimum $n = 3$, * statistical ANOVA analysis: $p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigations was to evaluate the PDT effectiveness of the nanoparticles co-loaded with Ce6 and CoQH. From Fig. 5, melanoma cancer cell exposure to red light (10 min with a total light dose of 10 J/cm^2 , λ_{max} 600–630 nm) followed by 4 h and 24 h incubation with the nanoparticles significantly decreased the mitochondrial activity, resulting in the decrease of remaining viable cells in the case of both loaded cubosomes and hexosomes in comparison to the free Ce6. The post-incubation photodynamic effect emerged as notably significant when CoCUB was administered, resulting in a decrease in cell viability to less than 65 % of the control, surpassing the impact observed for CoHEX, where the decrease in cell viability remained below 80 % of the control. Regarding the results at 24 h incubation, an enhanced photo-cytotoxic effect was observed for the cells treated with CoCUB, showing a decrease in cell survival below 40 %. In contrast, CoHEX or the free Ce6 did not significantly decrease the cell survival with additional incubation time. Such trends are in line with the outcome of the Reactive Oxygen Assay (Fig. S6 in the SI). The results indicated the lowest efficiency in ROS production for the free (non-encapsulated) photosensitizer, which proves the advantage of its encapsulation in nanocarriers and their protective effect. Also, CoCUB exhibited a higher efficiency in ROS production and photoactivity, which goes hand in hand with the cytotoxicity after irradiation.

In general, the encapsulation of Ce6 within the formulations enhanced the photodynamic effect, in accordance with other studies, that were reported for different formulations [73,74]. Available *in vivo* data recorded using micelles also proved a promising photodynamic efficiency against melanoma of Ce6 [75]. Tan et al. emphasized the promising potential of cubosomes and hexosomes, especially in the context of bioactive compounds for skin and melanoma cancer applications [62]. However, the biological features of these formulations are not investigated *in vivo*, and the *in vitro* data (especially on the hexosomes) are quite limited.

4. Conclusions

Our investigation showed a novel approach to the stabilization of non-lamellar lyotropic liquid crystalline nanoparticles, i.e., cubosomes and hexosomes formulated with MO and stabilized by S80 and including TC as an edge activator. By carefully balancing the ratio between these two additives, two candidates, one of cubosomes and one of hexosomes, were then tested as carriers suitable for PDT against skin melanoma. The samples were characterized by a mixture of lamellar and non-lamellar nanoparticles in both cases, due to the presence of TC and S80. Indeed, the former may facilitate the use of these formulations for topical administration [33,34], and S80 can stabilize the interface of the nanoparticles. Upon co-encapsulation of a PS molecule, Ce6, and an adjuvant, CoQH, the formulations were not affected in their fundamental physico-chemical parameters. We then demonstrated the higher effectiveness of CoCUB in the photodynamic treatment of skin human melanoma metastatic cells, in comparison to the CoHEX formulation. The cell viability upon incubation with the cubosomes stabilized by S80 and TC reports on the high biocompatibility of the sample. Since metastatic cells are recognized to be highly mobile and invasive, our achievements could be key in the potential application of these novel non-lamellar nanopatform for skin melanoma treatments. The ability of cubosomes and hexosomes to induce photodamaging of cancer cells seems similar to the ones of samples reported in the literature [43]. Indeed, the samples applied in this study are composed of both lamellar and non-lamellar NPs, so discerning the individual contribution of each specie is not trivial. Indeed, one may speculate that cubosomes and hexosomes may present a higher dosage of PS, hence, exerting a stronger PDT over time, respect to the vesicles. Understanding how lamellar or non-lamellar nanoparticles can perform in PDT and cancer treatment in general is an open question in the field; [15,76–78] specific investigations devoted on understanding the role of composition of the nanoparticles may be key to answering to such dilemma. Liquid

dispersions as the ones proposed in this work are suitable to be applied directly in contact with the desired target, especially when a short contact time is needed. *In vitro* dermal or transdermal release tests on skin would be needed to assess the ability of the formulations, in relation to the content of TC, to penetrate either in the superficial or deeper skin strata.

CRediT authorship contribution statement

Marco Fornasier: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karolina Krautforst:** Writing – review & editing, Formal analysis, Data curation. **Julita Kulbacka:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Data curation. **Peter Jönsson:** Writing – review & editing, Resources, Investigation, Data curation. **Sergio Murgia:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Urszula Bazylińska:** Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcis.2024.08.126>.

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SUPPORTING INFORMATION

Cubosomes and hexosomes stabilized by sorbitan monooleate as biocompatible nanoplateforms against skin metastatic human melanoma

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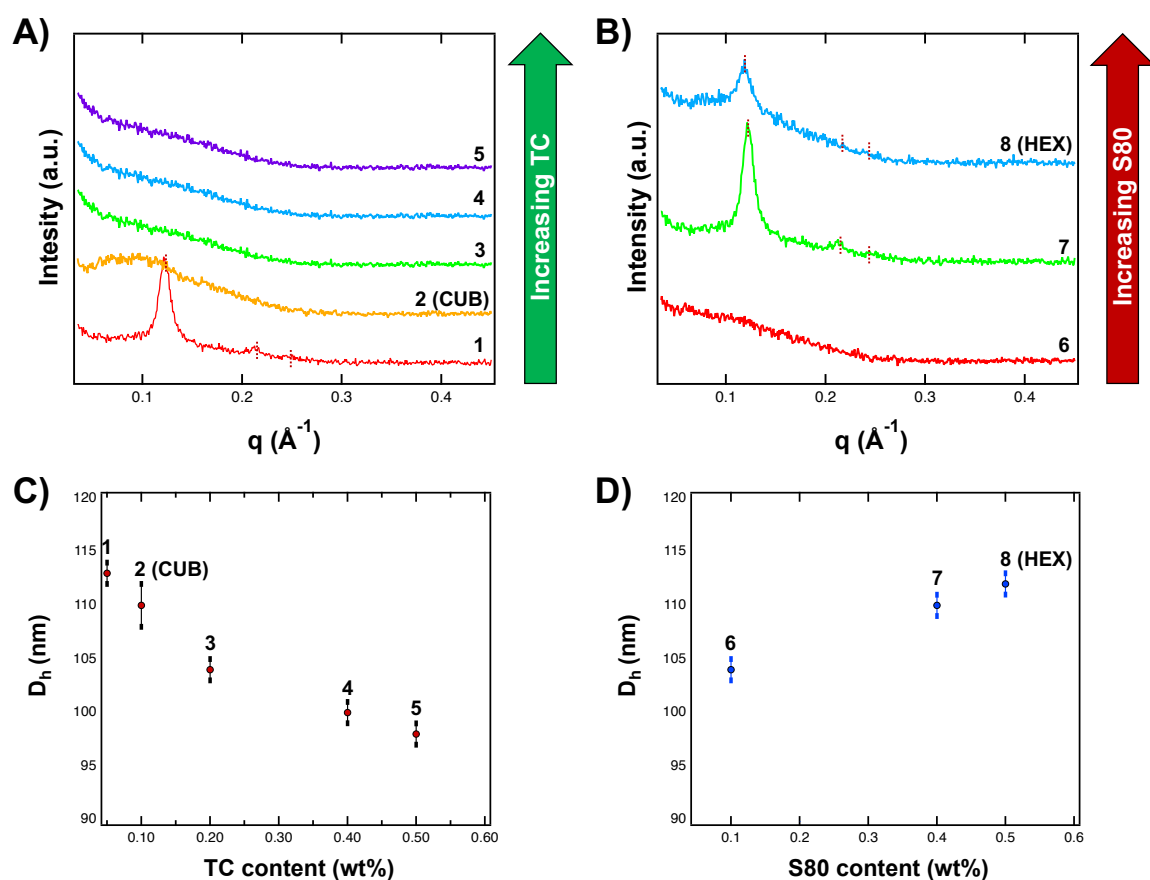


Figure S1. SAXS patterns at 25 °C of the samples varying either the TC content (A) or S80 content (B) and keeping fixed the MO:S80 and MO:TC mass ratio, respectively. The dotted line above the quasi-Bragg peaks denoted the presence of the H_{II} phase. For the compositions of the samples, see Table 1 in the main manuscript. B and C represent the apparent hydrodynamic diameter of each formulation for varying the TC and S80 content, respectively. Each sample is denoted by the same number label as in Table 1. CUB and HEX are the model formulations for cubosomes and hexosomes, respectively.

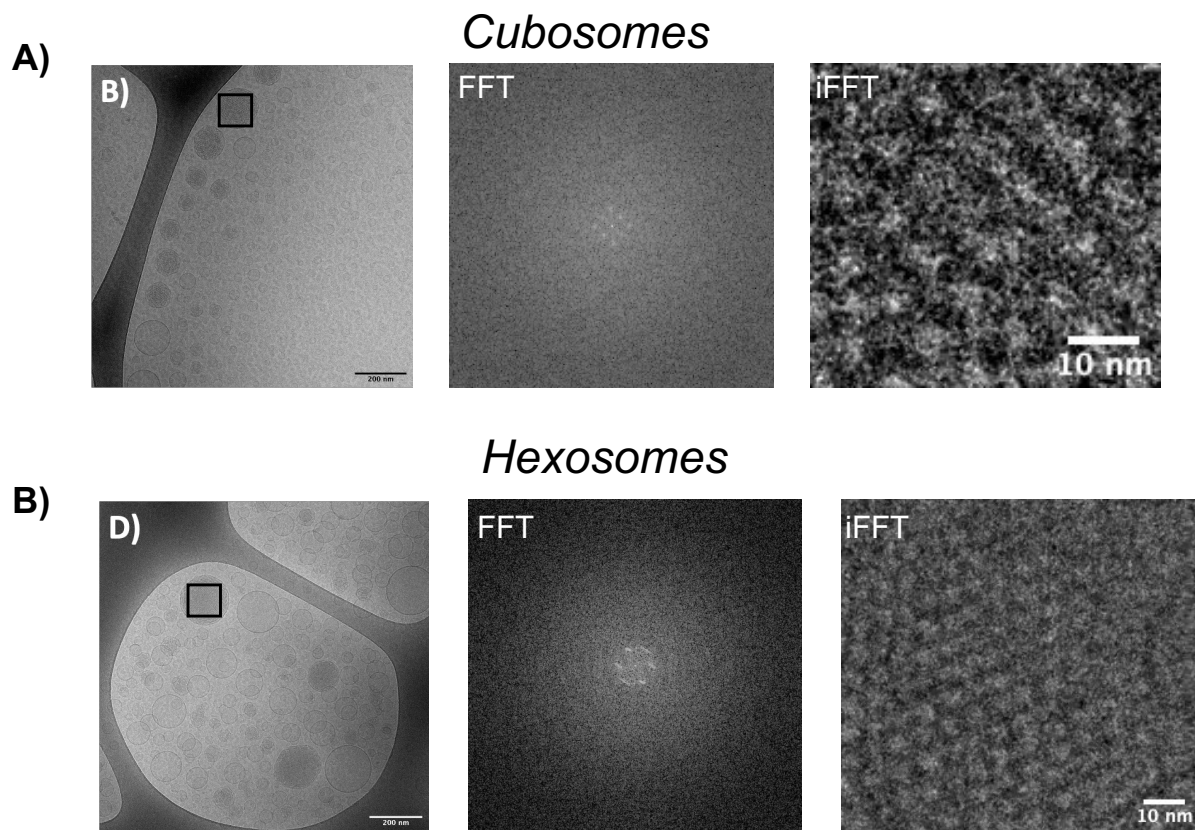


Figure S2. Selection of the cryo-TEM images presented in the main manuscript for CUB (A) and HEX (B). The middle panel is obtained by scaling the previous image and performing the Fast Fourier Transform (FFT) in the selection (black box) in the left panels, showing the 2D reflections of the crystalline planes. By applying an inverse FFT (iFFT), the right panels are obtained, representing the repetitive distances in the lattice of each phase.

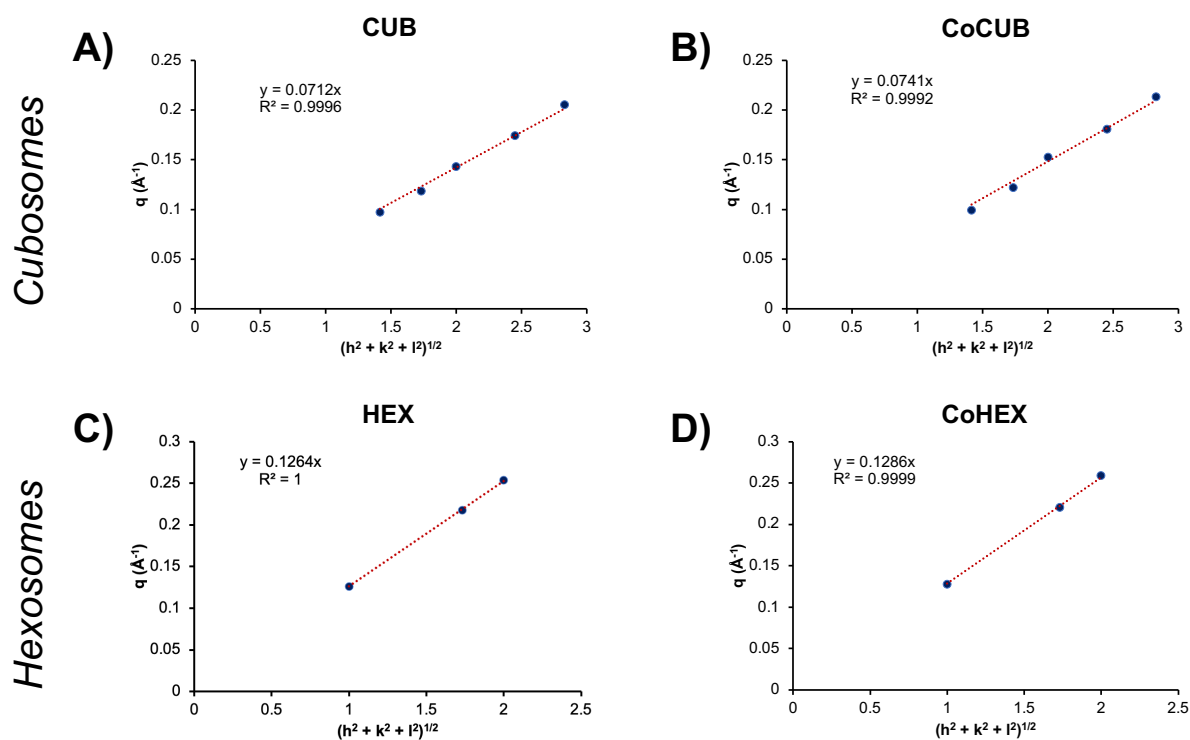


Figure S3. Plots of q values of each maximum in the SAXS pattern reported in Figure 1 versus the square root of $(h^2 + k^2 + l^2)$ where h , k and l are the Miller's indices representing the crystallographic plane of a given phase. The data were then fitted with a linear regression with intercept equal to zero, with R^2 to evaluate the quality of the fit. The good fits suggest the correct attribution of the phase in each sample.

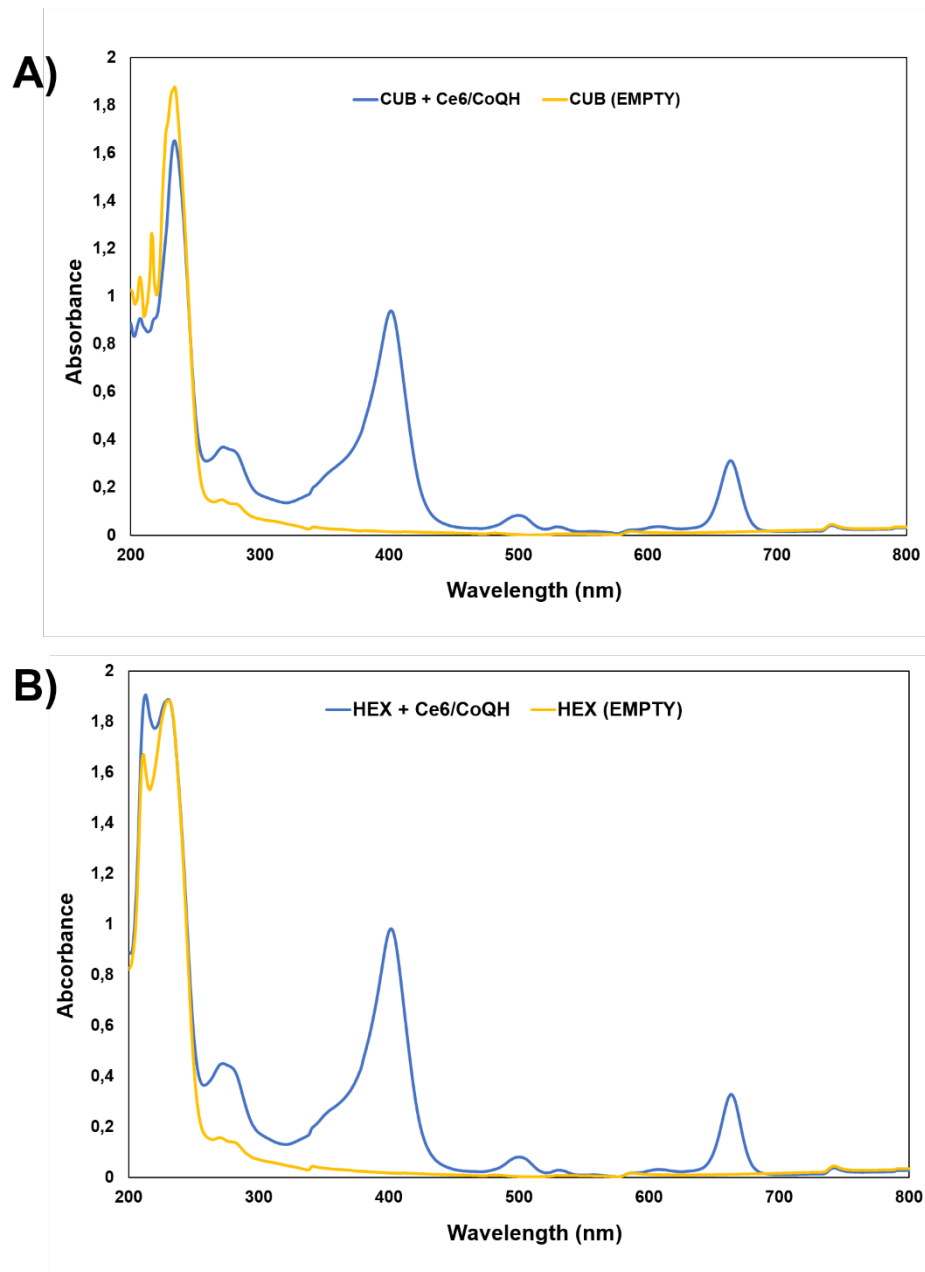


Figure. S4. UV-Vis spectra of the Ce6/CoQH co-loaded and empty A) cubosomes and B) hexosomes.

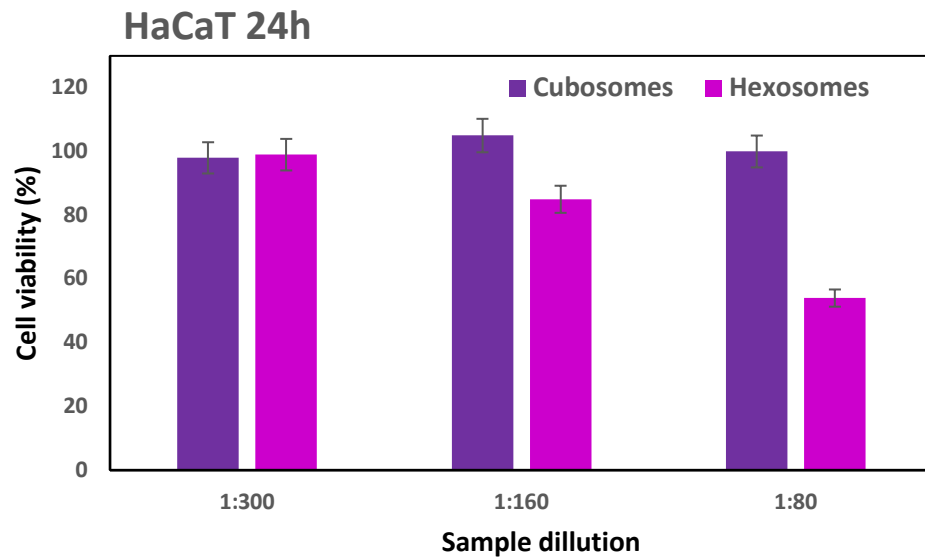


Figure S5. Cell viability upon human HaCaT keratinocyte after incubation of the empty formulations for 24 h at different dilutions. See the Material and Methods in the main manuscript for details of the concentrations and sample preparation.

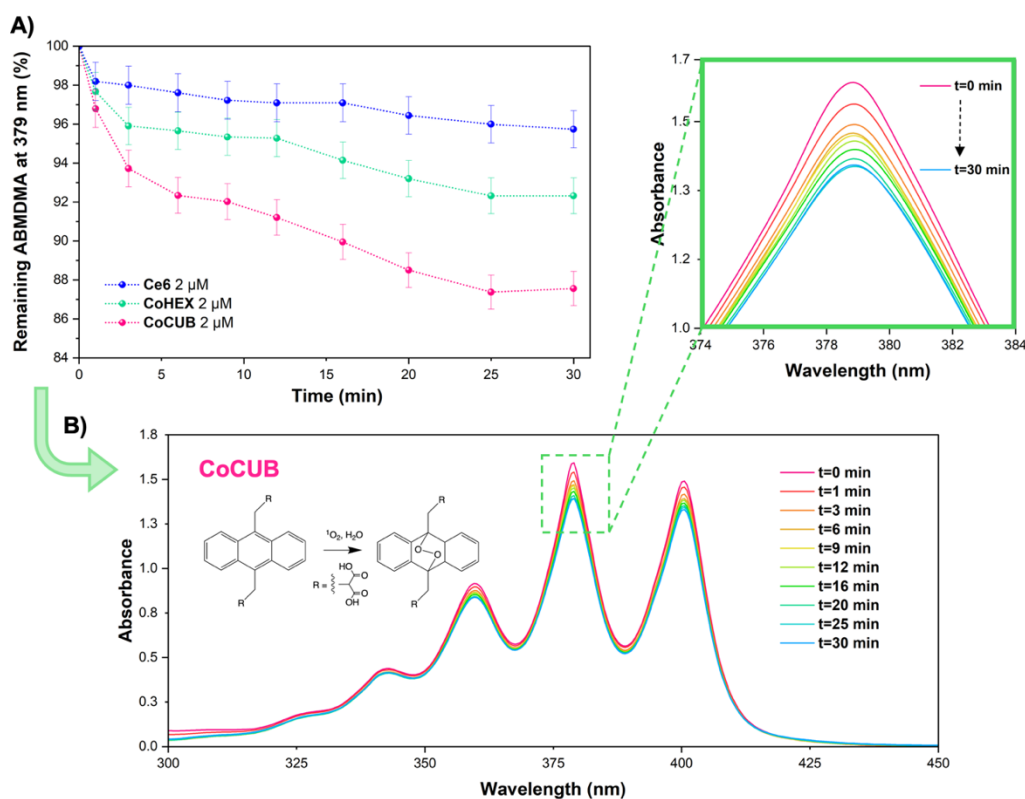


Figure S6. A) ROS production assay for the free PS (Ce6), and the loaded formulations CoCUB and CoHEX. B) Absorbance of ABMDMA over time which is reduced due to the ROS production induced by CoCUB. The insert in the top right panel shows the decrease of the peak at 379 nm within 30 min after incubation with ABMDMA.

SI1. Protocol for ROS production

Reactive Oxygen Assay tests were performed according to our previous method.[1] The test is based on the reaction of $^1\text{O}_2$ on the scavenger molecule 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA) which causes its photooxidation, thus resulting in a decrease in absorbance. Such measurements were performed using a HALO DB-20S UV-Vis Double Beam Spectrophotometer (Dynamica, UK) by evaluating the ABMDMA absorbance at 379 nm after incubation with the free Ce6, CoCUB and CoHEX after 0, 1, 3, 6, 9, 12, 16, 20, 25, 30 min (Figure S6A). The values of ABMDMA absorbance % were calculated for each sample. The Reactive Oxygen Assay was performed to evaluate ROS production (mainly the key singlet oxygen: $^1\text{O}_2$) by the samples corresponding to a concentration of 2 μ M PS, selected due to biological tests where the formulations did not exert a cytotoxic effect.

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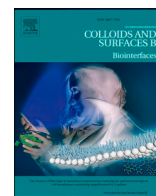
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Paper II



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Delivery of *Ulva rigida* extract by bicontinuous cubic lipid nanoplateforms for potential photodynamic therapy against pancreatic cancer

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ABSTRACT

Ulva rigida is a widely available marine algae representing a valuable biomass from which natural photosensitizers (chlorophylls) can be obtained in an environmentally friendly manner using a green microwave-assisted extraction technique. If properly loaded in biocompatible nanoformulations, such highly hydrophobic chlorophylls with photosensitizing activity may constitute effective drugs useful in photodynamic therapy (PDT) against extremely resistant pancreatic cancer cells. To permit adequate solubilization in water, prevent immune system activation, and improve pharmacokinetic properties, an extract from *Ulva rigida* biomass was encapsulated in two monoolein-based cubosome formulations differing for the dispersants used for their stabilization in water: Pluronic F108 (CUB) or a mixture of sorbitan monooleate and sodium taurocholate (TS-CUB). In both cases, high encapsulation efficiency was achieved. The formulations were investigated from a physicochemical point of view (SAXS, cryo-TEM, DLS, ELS), and the production of reactive oxygen species was evaluated. In addition, an extensive evaluation of biocompatibility and bioactivity was conducted on the human pancreatic cancer cell line BxPC-3. This assessment included an MTT cytotoxicity assay, cellular uptake analysis via flow cytometry, and cytoskeleton imaging both under dark conditions and post-irradiation to evaluate the effects of PDT. Unloaded nanoparticles were characterized by an inner bicontinuous cubic phase (Pn3m). However, after encapsulation of the *Ulva rigida* extract the presence of a sponge phase (L3) in the TS-CUB formulation was observed. Compared with CUB, TS-CUB loaded with the extract demonstrated enhanced photoactivity, superior biocompatibility, and more potent *in vitro* anticancer activity against pancreatic cancer through photodynamic therapy (PDT).

1. Introduction

Marine algae contain a wide spectrum of different bioactive molecules, due to metabolic activity adapted to different and often harsh environmental conditions [1]. Among all components of the green algae biomass, an interesting group is represented by the pigments known as

chlorophylls [2,3]. Due to their porphyrin ring structure, these hydrophobic molecules are recognized as potent natural photosensitizers (PSs). In addition, chlorophylls are highly efficient at generating reactive oxygen species (ROS) when exposed to red light [4]. This property makes them exceptionally well-suited for use in Photodynamic Therapy (PDT), a technology that requires the activation by light of a

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photosensitizing agent to produce ROS [5]. Since these ROS can selectively target and damage cancerous tissues, PDT is considered as a powerful and effective treatment option for cancer.

In this study, *Ulva rigida* C. Agardh, 1823 (hereafter *U. rigida*), a green marine alga widely distributed in many oceans and seas, also present in the Mediterranean Sea [6], is highlighted as a valuable natural source of chlorophylls. Belonging to the phylum of Chlorophyta (green algae), and known as “sea lettuce”, the genus *Ulva* includes more than 400 species worldwide, which often show large biomasses, especially in eutrophic and N-rich shallow coastal environments [7]. In those environments, especially in summer *Ulva* spp can grow exponentially and can suffocate the benthos, thus influencing negatively local fisheries and aquaculture. The *Ulva* spp. widespread presence, easy accessibility and the need to remove their large biomass to avoid any impact on the local economies make them an excellent resource for extracting photoactive compounds, ideal for use in PDT [8]. Because of their rapid growth and easiness of culturing, such algae have also been used for the production of food amendments and biofuel [9,10].

Various techniques have been developed for extracting natural compounds from biomass. However, these methods pose several challenges, being time-consuming, costly, and lacking in environmental sustainability. Consequently, there is a pressing need for more affordable, efficient, and eco-friendly extraction methods. Such advancements would enhance the practical application of these processes and add value to raw materials that are currently underutilized in significant quantities. In this context, Microwave-Assisted Extraction (MAE) is appreciated due to high extraction efficiency, short processing time, and low costs compared to traditional methods [11].

Natural photosensitizers derived from algae are gaining attention in medical and environmental applications due to their biocompatibility and potential therapeutic properties. These photosensitizers, such as chlorophylls, carotenoids, and phycobiliproteins, are naturally occurring pigments found in various algae species. They can be used in photodynamic therapy (PDT) for cancer treatment, where they generate reactive oxygen species upon light activation to target and destroy cancer cells. Algal photosensitizers offer an eco-friendly and renewable alternative to synthetic compounds, with the added benefit of being non-toxic and easily biodegradable [12,13]. Still, several factors limit the clinical use of photosensitizers (PSs). Notably, these compounds are often hydrophobic, leading to aggregation in biological biofluids, and they can degrade when exposed to biological environments. However, these challenges can be addressed by encapsulating PSs within biocompatible nanocarriers. Therefore, the special novelty of our study is focused on exploring the special potential of lipid-based liquid crystalline nanocarriers, known as cubosomes, for the encapsulation, delivery, and release of a newly obtained *Ulva rigida* extract rich in photoactive chlorophylls. Indeed, cubosomes offer a promising solution to enhance the stability and effectiveness of the chlorophyll extract for photodynamic therapy (PDT).

Cubosomes have honeycomb-like nanostructure consisting of a lipid bilayer folded in space and separating two continuous, but not-interconnected water channels [14]. Cubosome-loaded compounds are protected against degradation in solution, and their bioavailability and cellular uptake are largely improved than their free form [15]. These lipid liquid crystalline nanocarriers, moreover, are ideal candidates due to their high colloidal stability, entrapment efficiency, and ability to encapsulate hydrophobic compounds [16]. Commonly, they are stabilized by block copolymers such as Pluronics but other options have been reported to increase their biodegradability [17] and biocompatibility [18,19]. Recently, we have developed and optimized monoolein-based cubosomes and hexosome formulations stabilized by Span 80 and sodium taurocholate [20]. Such formulations, mostly with cubic phases, co-loaded with a second-generation PS and an adjuvant, showed good results in PDT applications against skin melanoma cells with minimal cytotoxicity against healthy cells. Herein, the photoactive *U. rigida* extract was encapsulated within standard Pluronic-stabilized cubosomes

(CUB) and the new type, less cytotoxic, Pluronic-free cubosomes stabilized by the combination of bile salt and a surfactant (TS-CUB), to tests its delivery, release and phototoxic activity into pancreatic tumor cells BxPC-3. Indeed, pancreatic cancer is one of the leading causes of cancer-related deaths in developed countries [21], primarily due to its complex and challenging treatment. The prognosis is often poor, with only about 9 % of patients surviving beyond five years after diagnosis [22]. Furthermore, pancreatic cancer is considered extremely resistant due to its complex and obstructive tumor microenvironment [23]. Consequently, due to the unique stability and permeability properties induced by the presence of bile salt molecules, encapsulation into the novel cubosomes may be useful as third-generation [24], combining the most promising photosensitizers with modern drug carriers, of effective photosensitizers in PDT.

2. Experimental

2.1. Materials

The molecular building block used for cubosomes preparation was glycerol monooleate (1-monooleoylglycerol, MO, RYLO MG 19 PHARMA, 98.1 wt%), kindly provided by Danisco A/S (Denmark). Other components of the cubosomes were Pluronic F108 (PF108, block copolymer, the stabilizer used in CUB), Span 80 (Sp80, sorbitan monooleate, steric stabilizer in TS-CUB), and taurocholic acid sodium salt hydrate (TC, Taurocholate, bile salt used in TS-CUB). 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA) for the evaluation of the ROS and $^1\text{O}_2$ generation efficiency were purchased from Sigma-Aldrich (Germany; New Zealand). The solvents used in the extraction process (ethanol ≥ 99.8 %, hexane ≥ 97.0 %) were purchased from Honeywell (Germany). Anhydrous sodium sulfate as a drying agent, was purchased from Sigma-Aldrich (Italy).

Acetonitrile and methanol were of HPLC grade and were purchased from Sigma-Aldrich (Italy). Orthophosphoric acid (ACS ISO, for analysis, 85 %) was purchased from Carlo Erba Reagents S.r.l. (Italy).

Water was freshly distilled and filtered through a Milli-Q apparatus (Millipore, Italy) and is referred to as water (W). For the preparation of the cubosomes, the water was filtered (before preparation) with a 0.22 μm pore size hydrophilic filter (Milli-Q system by Millipore, Germany). The analytical standards of lutein, chlorophyll a, and chlorophyll b were purchased from Sigma-Aldrich (Italy).

2.2. Biomass collection and preparation

Fresh *U. rigida* marine algae were collected from the Mediterranean Sea in Sardinia (Italy). The algae were washed with water to remove sand, salt, and epiphytes, deep-frozen at -80 °C, and freeze-dried (Benchtop, VirTis, US) for 48 h. The dry weight of the algae was 12.89 % of the fresh biomass. Then, the freeze-dried algae were milled into powder using a ball-milling technique to increase the extraction surface. For this purpose, the freeze-dried *Ulva rigida* (1.5 g) was loaded into a zirconium oxide grinding jar (35 mL) equipped with two balls ($\phi = 8$ mm). The jar was sealed and shaken for 15 min at a frequency of 20 Hz using a VWR® Beater Mixer Mill apparatus. The powdered biomass was then stored in the fridge and used in the following steps.

2.3. Extraction

The bioactive pigments (chlorophylls) were extracted from the powdered biomass as follows (Fig. 1). The extraction was performed in a two-steps process including solid-liquid microwave-assisted extraction and liquid-liquid extraction with pigments fractionation in a separatory funnel.

2.3.1. Microwave-assisted extraction

To release bioactive compounds from the cell wall into the solvent

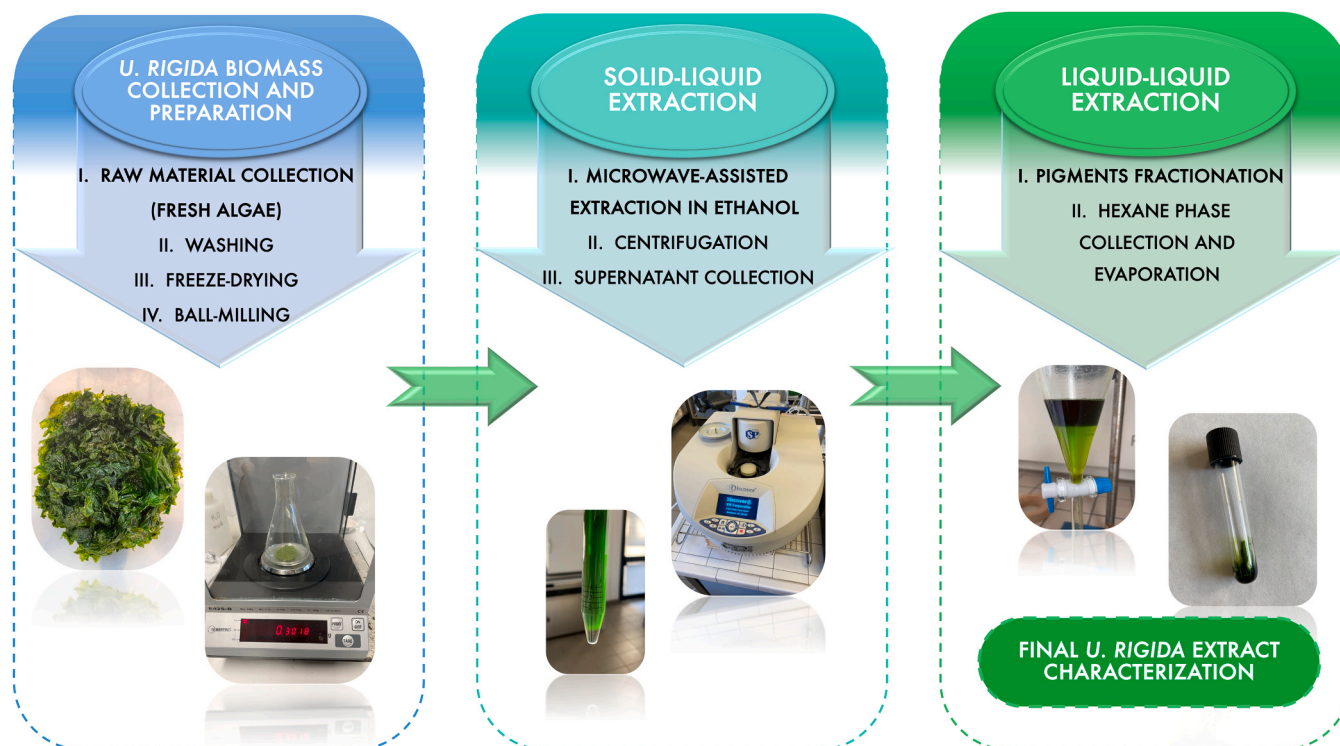


Fig. 1. General scheme of the pigments extraction process from *U. rigida* marine algae biomass.

solution, a solid-liquid extraction was performed using MAE technique (Discover SP, CEM). Ethanol was chosen as a solvent (in a solid-liquid ratio of 0.01 g of dry algae/mL of solvent) since it is a green solvent and gives high efficiency in pigments extraction from *U. rigida* [8]. The biomass was subjected to extraction at 35 °C for 1 min with a power of 300 W and a pressure of 300 PSI. After that, the solution was centrifuged (4000 rpm/10 min) (REMI R-9M, Remi Elektrotechnik Ltd., Indie) and the supernatant was fractionated by liquid-liquid extraction in a separatory funnel for the system composed of 50 % ethanolic extract, 30 % of hexane and 20 % of water, based on the high pigments extraction efficiency obtained in this way by Martins et al. in 2021 [8]. The organic hexane phase, rich in hydrophobic pigments of interest, i.e., chlorophylls, was collected, dried with anhydrous sodium sulfate (residual water remove), evaporated, and concentrated under reduced pressure in rotavapor (BÜCHI R-200, BÜCHI Labortechnik AG, Switzerland) giving the final crude *U. rigida* extract used in the next steps.

2.3.2. High-performance liquid chromatography – mass spectrometry – extract qualitative assessment

A qualitative investigation of *U. rigida* extract was performed by an ion mobility QTOF LC-MS system using a 1290 Infinity II UPLC equipped with an autosampler (G7167B), a quat pump (G7120A), a column comp (G7116B) and 6560 IM-QTOF (Agilent Technologies Inc., Palo Alto, CA, US). Before analysis, the instrument performances were tested using an Agilent tuning solution mix (G1969–85000). During the analysis, two reference masses at m/z 112.9855 and m/z 966.0007 were continuously infused into the system for constant mass correction. Positive electrospray ionization (ESI) ion mode was used to perform all the experiments with the following optimized source parameters: drying gas at 300 °C with a flow of 5 L/min, sheath gas at 350 °C at a flow rate of 12 L/min, nebulizer at 35 psi, capillary voltage set to 3500 V with a nozzle voltage of 500 V. The mass spectra were acquired by full range acquisition covering the m/z range of 100–1050.

Chromatographic separation was performed on a Kinetex EVO C18 column (150 × 2.1 mm, 1.7 µm 100 Å, Phenomenex, Castel Maggiore,

Bologna, Italy) maintained at 30 ± 1 °C. Isocratic elution of the mobile phase, with a combination of solvent A (0.1 % formic acid) 10 % and solvent B (acetonitrile + 0.1 % formic acid) 90 %, was kept for 30 min at a flow rate of 0.3 mL/min. The injection volume was 4 µL.

Data acquisition and processing were done using MassHunter Workstation Acquisition software v. B.09.00 and MassHunter Workstation Qualitative Analysis software v. 10.0 (Agilent Technologies, Santa Clara, CA, US).

2.3.3. High-performance liquid chromatography – UV-Vis spectroscopy – extract quantitative assessment

The content of pigments in *U. rigida* extract was quantified by high-performance liquid chromatography coupled with an UV-Vis detector (HPLC-UV-Vis).

The HPLC determination was performed according to a previously reported method [25]. Briefly, the analysis was performed by using an Agilent HPLC 1100 liquid chromatograph coupled with a Thermo Finnigan DAD Chromquest UV 6000 diode array detector. The chromatographic analyses were performed by injecting 10 µL of extract solubilized in methanol into a Kinetex column (5 µ, C18, 100 Å; Phenomenex, Torrance, CA, USA), and eluting with a mobile phase consisting of 0.22 M H₃PO₄ and acetonitrile mixed on a gradient mode. Three wavelengths were used: 430 nm, 470, and 660 nm for chlorophyll *b*, lutein, and chlorophyll *a* determination, respectively. Peak identification was carried out by comparing each compound's retention time and UV spectrum with the reference standard. Individual compounds were quantified using an external standard calibration method. Correlation values ranged between 0.9990 and 0.9999. The calibration curve (1–10 mg/L) was prepared in methanol from suitable dilution of standard stock solution (1000 mg/L in methanol). All analyses were conducted in triplicate and the data are reported as average value ± standard deviation (SD).

2.4. Cubosome formulations preparation and encapsulation

Cubosome formulations were prepared by following our previously

reported methodology [20]. Firstly, MO was melted at 40 °C, then the *U. rigida* extract was added and mixed with the lipid phase, until homogeneous mixing. After that, a water (W) solution of PF108 (CUB) or TC and Sp80 (TS-CUB) was added to the lipid phase and immediately sonicated by an ultrasonic processor (UP100H, Hielscher Ultrasonics, Germany) in 2 cycles (2 and 3 min) with amplitude 90 % with the pulse ON for 1 s followed by 1 s of break. The empty cubosomes were prepared in the same way without the addition of the extract. The composition (wt%) of empty and loaded formulations was MO/PF108/W= 3.3/0.3/96.4 and MO/TC/Sp80/W= 3.5/0.2/0.1/96.2 for CUB and TS-CUB, respectively.

2.5. Dialysis and encapsulation efficiency of the formulations

After encapsulation of the extract of *U. rigida*, the formulations were dialyzed to remove the excess extract into a tubing cellulose membrane (14 kDa of cutoff; Sigma Aldrich), in 2 L of water at room temperature for 2 h, changing the water after 1 h.

The values of encapsulation efficiency (EE) were evaluated via UV-Vis spectroscopy. Absorption spectra were acquired using a UV-Vis spectrophotometer (Cary Series UV-Vis-NIR Spectrophotometer, Agilent Technologies, UK) using quartz cuvette (optical path: 1 cm). In order to calculate the weight of the encapsulated extract before and after dialysis, the standard calibration curve of the extract was prepared, and the data fitted to a linear regression ($R^2 = 0.9993$). Absorption spectra of the cubosome formulations before and after dialysis were measured after dissolving in ethanol (dilution 1:15 v/v) and the EE was obtained through the following equation:

$$EE[\%] = \frac{\text{weight of the extract after dialysis}}{\text{weight of the extract before dialysis}} \times 100$$

2.6. Small-angle X-ray scattering (SAXS)

The structure of the loaded and unloaded formulations was evaluated by means of small angle X-ray scattering (SAXS). The SAXS characterization was performed following our previously published protocol [18,26]. Briefly, the samples were placed in quartz capillaries and measured at a sample to detector distance of 360 mm for around 2 h per sample at a given temperature. The inter-crystalline spacing, d , was calculated after evaluating the q positions of each peak in SAXS pattern using the following equation:

$$d = \frac{2\pi}{q_{\text{peak}}} \quad (1)$$

Then, the lattice parameter, a , describing the crystalline unit of the inverse bicontinuous cubic phases was extrapolated from equation 2:

$$a = d \cdot \sqrt{h^2 + k^2 + l^2} \quad (2)$$

Where h , k and l are the Miller's indexes. Finally, the water channel radius of the cubosomes and sponge nanoparticles was evaluated by applying equation 3 and 4, respectively:

$$r_w = (a - l) \cdot \sqrt{\frac{A_0}{-2\pi\chi}} \quad (3)$$

$$r_{w-L3} = \frac{d_{L3}}{a_{\text{cubic}}} r_{w-\text{cubic}} \quad (4)$$

where χ and A_0 are, respectively, the Euler characteristic and the surface area of the specific geometry (Pn3m, $\chi = -2$, $A_0 = 1.919$), and l is the MO hydrophobic chain length at 25 °C (17 Å) [27].

2.7. Dynamic and electrophoretic light scattering

Dynamic light scattering (DLS) measurements were performed on

the diluted (1:50 v/v) samples at 25 °C and 37 °C using a ZetaSizer Nano ZS by Malvern Instruments (Malvern, UK) (backscattering angle 173°, with a 4 mW He-Ne laser at 632.8 nm). All measurements were conducted in triplicate and the data are reported as average value $\pm$ standard deviation (SD). The apparent hydrodynamic radius (D_h) and polydispersity index (PdI) were extracted by a second-order cumulant analysis. The ζ -potential was evaluated using electrophoretic light scattering (ELS) by applying the Smoluchowski equation to the electrophoretic mobility.

2.8. Cryogenic transmission electron microscopy (cryo-TEM)

The morphology of the formulations under investigation was studied using cryogenic transmission electron microscopy following the usual protocol for liquid crystalline nanoparticles employed by our group [18]. The sample (4 μ L) was blotted on the grid at 25 °C with a relative humidity of 90 %, within 24 h from preparations and imaged using a Jeol JEM-2200FS transmission electron microscope (JEOL, Tokyo, Japan).

2.9. Reactive oxygen assay

Experiments are based on the reaction $^1\text{O}_2$ with the scavenger molecule 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA). Singlet oxygen causes ABMDMA photooxidation, resulting in a decrease of the absorbance. Spectrophotometric measurements of each sample using a UV-1650PC UV-Vis Spectrophotometer (Shimadzu, Japan) were performed for 1 h by evaluating the ABMDMA (0.15 mM) absorbance at 401 nm every 0, 1, 3, 6, 9, 12, 16, 20, 25, 30, 35, 40, 45, 50, 55, 60 min. The ABMDMA absorbance % was calculated for each sample.

2.10. Biological activity

2.10.1. Cell lines

The BxPC-3 cells (human pancreatic cancer cell lines, CRL-1687), purchased in ATCC®, were derived from a 61-year-old female in 1986. The cells were maintained in culture flasks with a surface area equal to 75 cm² (Falcon® Cell Culture Flasks) in DMEM (IITD, Wrocław, Poland) supplemented with 10 % fetal bovine serum (FBS) and 50 μ g/mL penicillin and streptomycin (Sigma-Aldrich, Poznań, Poland). The cultures were incubated in a humidified atmosphere with 5 % CO₂ at 37 °C. The cells used for all experiments were detached by trypsinization (Trypsin-EDTA solution) and neutralized with Dulbecco's Phosphate Buffered Saline (DPBS), which was purchased from Sigma-Aldrich (Poznań, Poland).

2.10.2. Cytotoxicity studies

The cytotoxicity MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell proliferation assay (Sigma-Aldrich, Poznań, Poland) was performed at different extract concentrations (0.17, 0.34, 0.88, 1.3, 1.7, 2.6, 5.2 μ g/mL) on the BxPC-3 cell line, after 24 h of incubation in dark conditions with CUB and TS-CUB loaded with the extract, as well as empty CUB and TS-CUB, and free extract. The loaded formulations were diluted to obtain a given extract concentration for the MTT test. During this process, along with the dilution of the extract inside the nanocarriers, the samples CUB/TS-CUB were also diluted, hence also the main component of each formulation, MO namely. Therefore, to compare empty and loaded nanocarriers with the same MO concentration for a given extract concentration (indicated on the x-axis in Fig. 4), all formulations were diluted in the same manner. Therefore, the extract concentrations used in the study correspond to 15.4, 30.8, 77.0, 116, 154, 231, 462 μ g/mL of the MO concentration in CUB, and 16.2, 32.4, 81.0, 122, 162, 243, 486 μ g/mL in TS-CUB. Generally, 200 μ L of cells (approximately 2×10^5 cells) were placed into 96-well plates (Sarstedt, Equimed, Wrocław, Poland). Cell viability in each test

group was expressed as percentages of the control cells (untreated with studied samples). Then, the absorbance was measured at 560 nm using a GloMax® Discover Microplate Reader (Promega) allowed us to evaluate the cell viability in each group. All experiments in this study were conducted in triplicate and the data are reported as average value $\pm$ standard deviation (SD).

2.10.3. Cellular uptake and internalization (flow cytometry)

Flow cytometric analysis was performed for the assessment of the ability of BxPC-3 cells to uptake both the formulations loaded with the extract (CUB + extract and TS-CUB + extract).

This technique was applied, due to the well-known fluorescence of the chlorophylls present in the extract, measured by flow cytometer. For this purpose, the cells (density of 4×10^5) were seeded on 24-well plates and left to adhere overnight. Each formulation was added to a final concentration of extract corresponding to 1.3 $\mu\text{g/mL}$ and the cells were incubated for 24 h at 37 °C. Empty nanocarriers (CUB and TS-CUB) were tested as negative controls, due to the lack of fluorescence ability of these samples. After incubation, followed by washing in PBS (not containing calcium and magnesium ions, IITD, Poland), cells were trypsinized and resuspended in 0.5 mL of PBS (BioShop, EPRO, Poland). The flow cytometric measurements were performed on a CyFlow Cube 6 flow cytometer (Sysmex, Poland). The fluorescence of the extract was measured with a FL-5-H detector. 10 000 events were included from each sample. Data were collected and analyzed by CyView software (Sysmex, Poland).

2.10.4. Photodynamic action and immunofluorescent F-actin staining protocol

The photodynamic action of the loaded formulations was evaluated by the immunofluorescence method [28] to evaluate the reorganization of cellular cytoskeleton organization after laser irradiation, applied for red light-activated cubosomes loaded in BxPC-3 cells. Cancer cells were seeded directly on 18 mm diameter round microscope coverslips (Thermo Fisher Scientific Inc.) in 6-well plates (Sarstedt, Equimed, Poland). The cells were left to adhere for 24 h. After this time, the cells were treated with cubosomes for 24 h with a final concentration corresponding to 1.3 $\mu\text{g/mL}$ of the extract. After incubation, samples were irradiated for 5 min using a red-light laser diode (12 mW/cm²). After washing twice with PBS (BioShop, EPRO, Poland), the cells were fixed for 10 min in 4 % v/v paraformaldehyde (Polysciences, Inc., Bergstrasse, Germany), and washed again with PBS.

The integrity of the cytoskeleton was determined by immunofluorescent labeling of F-actin cells with Phalloidin-iFluor® 488 (Sigma Aldrich, Poznan, Poland). Fluorshield™ with a fluorescent DNA-binding dye of cells grown in the culture (4,6-diamidino-2-phenylindole, DAPI) was used to visualize the nuclei and mount the cells after excitation at 405 nm. Samples were examined on an Olympus BX53 fluorescence microscope (Evident Europe GmbH, Warsaw, Poland).

2.10.5. Statistical analysis

Results of the *in vitro* experiments are presented as mean values $\pm$ SD for minimum $n = 3$ and compared by two-way ANOVA for multiple comparisons and $\alpha = 0.05$. Comparisons of samples exhibiting p values ≤ 0.05 and ≤ 0.01 were considered statistically significant and highly significant, respectively. Results were analyzed using the commercial software GraphPad Prism 7.0.

3. Results and discussion

3.1. Characterization of *U. rigida* extract and encapsulation in the nanocarriers

The extraction of an active component from a biomass is a crucial step that should be as efficient and as green as possible. Microwave-assisted extraction using ethanol was selected for its high efficiency,

short processing time, and environmentally friendly approach. This method leverages microwave energy to disrupt the cell walls of algae, releasing the desired pigments into the solvent. Subsequently, the solvent solution is processed to isolate a fraction rich in chlorophylls. The final hydrophobic dark-green extract was obtained from *U. rigida* marine algae as described in paragraph 2.3.1. and then characterized in terms of pigments composition. The average yield was $(3.5 \pm 0.3)\%$ of the dry algae weight (see section 2.2.). The qualitative HPLC-MS analysis confirmed the presence of chlorophyll in the extract (Figure S11). The comparison of UV-Vis spectrum and retention time of the analytical standard and sample peaks in HPLC-DAD analysis also highlighted the presence in the extract of chlorophyll *b* and lutein. The content of chlorophyll *a* and chlorophyll *b* in the extract quantified by HPLC-UV-Vis measurements was $(28 \pm 4)\%$ and $(2.6 \pm 0.5)\%$, respectively, corresponding to $(0.98 \pm 0.09)\%$ and $(0.093 \pm 0.008)\%$ of the dry algae weight. A small amount of lutein, $(0.92 \pm 0.01)\%$, was also detected in the extract. The UV-Vis spectra of the quantified pigments are presented in Figure S12.

Cubosome formulations loaded with *U. rigida* extract in a concentration 0.375 mg/mL were obtained with EE equal to $(99.2 \pm 0.5)\%$ in the case of standard CUB and $(97.3 \pm 0.9)\%$ for TS-CUB. No significant differences can be highlighted in this case among the conventional and the novel formulations, showing that both nanoparticles can accommodate similar amount of hydrophobic payload in the lipid palisade, as commonly observed in similar formulations [20,29,30]. Verification of the encapsulated extract localization in the hydrophobic part of the cubosome nanocarriers is shown in Figure S13.

3.2. Physico-chemical characterization of the carriers encapsulating the extracts

SAXS measurements of the formulations containing the extract were performed to evaluate the phase and inner structure at 25 °C and 37 °C and then cooling the sample down to 25 °C again to observe any structural hysteresis. Such temperatures were chosen to study the behavior of the formulations in storage and *in vivo* conditions. These results are shown in Fig. 2 and the lattice parameter (a) and water channel radius (r_w) evaluated from the patterns are presented in Table 1.

In the case of CUB loaded with the extract, only the Pn3m phase could be observed, inferring that the extract interacts with the lipid bilayer of MO. While the two first peaks may be indexed to Pn3m, the subsequent ones are presented at the level of noise, probably related to the presence of vesicles, as observed at the cryo-TEM (Fig. 2C). Nevertheless, in conjunction with cryo-TEM results, we can state that these two peaks are assigned to the Pn3m phase. Indeed, for the standard cubosomes formulated with Pluronic, a biphasic system with a Pn3m + Im3m phase is expected [31], where the former is related to the native structure of MO at this concentration of use in water and the latter is induced due to the interaction of the hydrophobic segment of the block copolymer with the lipid bilayer. The lattice parameter and water channel radius evaluated are in line with those reported in the literature with similar compositions [32,33]. When the temperature is increased to 37 °C, the phase is preserved and only a shift of the peaks at higher q values is noted, decreasing slightly the structural parameters. Cooling down the sample to 25 °C yields the same phase with a small hysteresis as the peak position of the Pn3m is not the same after the treatment. Such a phenomenon could be related to a longer equilibration time (more than 1 h) needed for the Pn3m phase to rearrange in the initial state after the heating and cooling cycle.

In general, the formulation TS-CUB encapsulating the extract shows peaks less intense and less defined, with a higher and broader diffusive band which has been associated with the presence of polydisperse vesicles in the sample [26] (as it will be explained in the next section about the morphology). As already reported by our group, the empty formulation is supposed to be a pure Pn3m. In the case of the formulation loaded with the extract, we observe two visible peaks with positions in

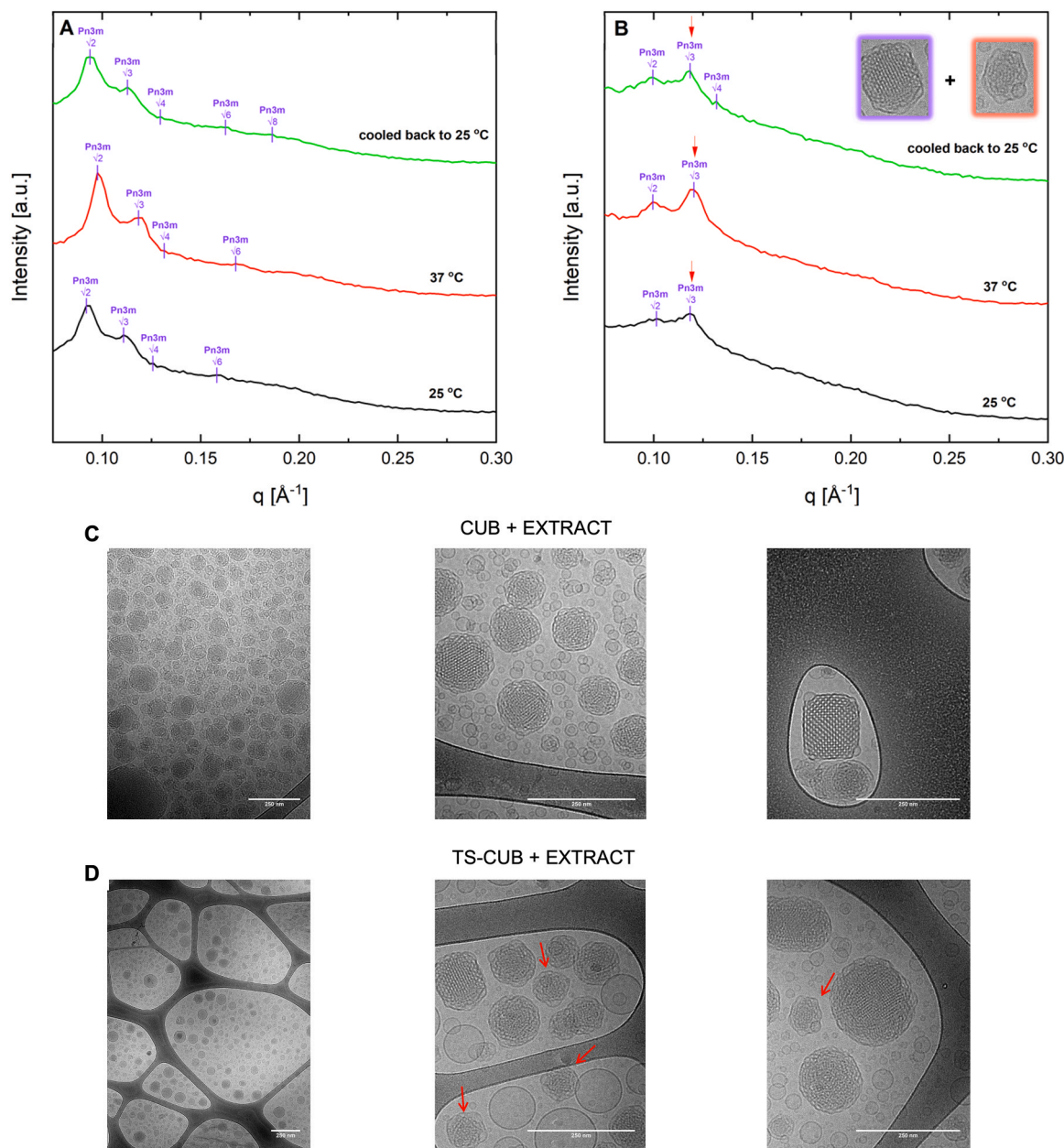


Fig. 2. SAXS diffractograms obtained at 25 °C, 37 °C and then after cooling back to 25 °C of CUB (A) and TS-CUB (B) loaded with *U. rigida* extract, along with the cryo-TEM images of these samples (C) and (D), respectively. Red arrows in (B) indicate the peak related to the possible presence of an L3 phase, followed by the observation of sponge nanoparticles in (D).

Table 1

Phase, lattice parameter (a), and water channel radius (r_w) of the investigated CUB and TS-CUB loaded with *U. rigida* extract measured at 25 °C, 37 °C, and then after cooling back to 25 °C. Data are reported as mean $\pm$ SD.

Sample	Temperature (°C)	Phase	a (Å)	r_w (Å)
CUB + extract	25	Pn3m	98 ± 1	21.3 ± 0.6
	37	Pn3m	93 ± 2	19.2 ± 0.8
	back to 25	Pn3m	96 ± 1	20.4 ± 0.4
TS-CUB + extract	25	Pn3m	90 ± 3	18 ± 1
		L3	-	15.5
	37	Pn3m	89.9 ± 0.8	18.1 ± 0.3
		L3	-	15.0
	back to 25	Pn3m	92 ± 3	19 ± 1
		L3	-	16.0

line with a Pn3m. However, a second phase may be present in the sample as the second peak is more intense than the first one, and scattering peaks normally decrease with the order of scattering as it is a probabilistic event [34]. Even though this scenario could be related to the alignments of the crystalline planes that may present scattering more intense than the first-order one, it is still probable that the sample is represented by two co-existing phases. Namely, by looking at the phase diagram of MO with other additives, such as Sp80 [35], it is possible to obtain an L3 phase. Since, in this work, we prepared the nanocarriers with similar lipid composition and Sp80 as well, an analogous phase behaviour can be predicted, namely closer to the border between the Pn3m and L3 phases. Therefore, when compared with the cryo-TEM images of this formulation (Fig. 2D), the scenario of the occurrence of the second phase (L3) is more likely to occur, because structures resembling sponge particles are visible, as indicated by the red arrows. Moreover, Sp80 may tune the lipid (commonly, MO or glycerol

monooleate) packing parameter and originate a more disordered cubic phase, commonly referred to as L3 or sponge phase [35]. Usually, a sponge phase exhibits two diffusive bands [36,37], being the first related to cell-cell interaction and the latter to the bilayer thickness. It could be speculated that the peak positioned at $q = 0.12 \text{ \AA}^{-1}$ may be linked to the cell-cell interaction in such structure. The heating to 37°C makes the peaks of the Pn3m and the first of the L3 phase more pronounced, and the shift towards higher q values means that the lattice parameter and water channel radius are decreasing in size. Overall, it can be concluded that encapsulation of *U. rigida* extract influences the nanostructure of both types of nanoparticles, by changing the effective packing parameter of MO. Given that drug release is directly linked to the internal structure of the nanocarrier, it is crucial to consider this aspect, particularly in the case of TS-CUB, due to the presence of nanoparticles characterized by an L3 phase.

As a final remark, it can be noticed that the formulations are not temperature-responsive, proving that body temperature does not affect the nanoparticles' structure.

Cryo-TEM measurements were then performed to assess the morphology of the nanoparticles (Fig. 2C and D). Starting with the CUB formulation loaded with the *U. rigida* extract, reported in Fig. 2C, the typical honeycomb-like structures of cubosomes are observed [16,38]. Small vesicles are also highlighted, very likely due to the presence of Pluronic in the sample, which affect the original curvature of the lipid interface [39]. The TS-CUB formulation loaded with the *U. rigida* extract shows three kinds of nanoparticles (Fig. 2D): (i) vesicles of different sizes in the range 20–200 nm, whose presence can be explained by the different curvature of the interface induced by the TC [26], (ii) cubosomes displaying the same morphology and inner structure as reported for the CUB sample, and (iii) sponge nanoparticles (indicated with red arrows in the images). The latter case is in line with the data reported in the literature for a sponge phase dispersion with similar compositions [37] and corroborates the SAXS data analysis of the previous paragraph.

Finally, the apparent hydrodynamic diameter (D_h), polydispersity index (PDI) as well as the ζ -potential of the two formulations loaded with *U. rigida* are reported in Table 2 at two different temperatures. Such values are similar to those of empty formulations with similar composition [40]. The increase in temperature does not have a significant impact on the size, however a drop in the absolute values of the ζ -potential is highlighted. This could be due to the higher thermal motion of the ions at the interface, yielding a lower-charged double layer surrounding the nanoparticles. The size distributions are not affected by the temperature increase and are in line with the ones of cubosomes and sponge phase nanoparticles [35,41]. Moreover, the stability in time of the given formulations was demonstrated by 2 months lasting experiment (Figure S14).

3.3. ROS production

The evaluation of ROS is shown in Fig. 3, where CUB and TS-CUB loaded with *U. rigida* extract as well as the pure extract are compared at different concentrations of the extract (0.88, 1.7, 3.4, 5.2 and $8.8 \mu\text{g/mL}$). All samples exhibit the ability to generate ROS, as the % of

remaining ABMDMA decreases with time in all cases, proving their photosensitive activity. The strongest ROS production was observed for TS-CUB loaded with extract at the highest concentration ($8.8 \mu\text{g/mL}$), since the presence of scavenger decreased from 100 % to $(85 \pm 2)\%$ after 1 h. In contrast, the weakest ROS generation was presented in the case of pure extract of the same concentration, showing $(98 \pm 2)\%$ of remaining ABMDMA.

Such counterintuitive behavior could be related to the aggregation or degradation of the pigment when not protected by encapsulation in the lipid carriers. Inversely, increasing the concentration of the extract, either encapsulated in CUB or TS-CUB, induces a stronger photoactive effect. Previous studies also reported an increase in ROS generation for other lipid nanocarriers loaded with a photosensitizer as its concentration increased [28,42]. In general, it can be stated that the activity of the extract within the formulation is higher than the one of the free forms, inferring that the encapsulation enhances the photosensitivity following the ROS production.

3.4. Biological activity

3.4.1. Cytotoxicity evaluation

The cytotoxicity MTT test on human pancreatic cancer cell line (BxPC-3) was performed at different extract concentrations (0.17, 0.34, 0.88, 1.3, 1.7, 2.6, $5.2 \mu\text{g/mL}$), after 24 h of the cells' incubation in dark conditions. According to ISO 10993-5, cell viability under 80 % is considered as cytotoxic effect, divided into weak (80 % – 60 %), moderate (60 % – 40 %), and strong (below 40 %) cytotoxicity [43]. The results presented in Fig. 4 show the viability of BxPC-3 cells treated with the studied samples of empty CUB and TS-CUB (prepared in the same manner as the loaded nanocarriers, to achieve the given extract concentration, indicated on the x-axis, as described in the Experimental section), the free *U. rigida* extract, and the CUB and TS-CUB loaded with the extract.

In case of the sample with the free extract, the cell viability tends to decrease while increasing its concentration. Pure extract shows the most significant reduction in cell viability, with a drop to approximately 40 % at $5.2 \mu\text{g/mL}$. However, when comparing the sample with loaded extract and empty cubosomes, it can be observed that the composition of empty nanocarriers also plays an important role in cytotoxicity due to the possible cytotoxic effect of MO and Pluronic [44,45], especially in case of the $2.6 \mu\text{g/mL}$. The lowest viability is observed at the highest concentration ($5.2 \mu\text{g/mL}$) for all groups. It can be noticed a concentration dependence where at lower concentrations ($0.17\text{--}1.3 \mu\text{g/mL}$), all treatments show a minimal reduction in cell viability, with values remaining close to the control level. However, as the concentration increases beyond $1.7 \mu\text{g/mL}$, the cytotoxic effect becomes more pronounced. Significant cytotoxic effects and differences between the various samples become evident at the extract concentration of $2.6 \mu\text{g/mL}$. At this dose, the new cubosomes (TS-CUB) exhibit no notable toxicity, in contrast to the standard formulation (CUB), which shows moderate cytotoxicity. This observation indicates that cytotoxicity is reduced when cubosomes are stabilized with TC and Sp80 instead of PF108, supporting the improved biocompatibility of the new formulation. Upon loading the cubosomes with the extract, an increased cytotoxic effect is observed: moderate for CUB + extract and weak for TS-CUB + extract. This effect is likely related to the extract itself, as a weak cytotoxic response is also observed for the extract in its free form at this concentration. Notably, in the case of the standard Pluronic-based CUB formulation, the cytotoxicity may also be attributed in part to the high monoolein content ($231 \mu\text{g/mL}$), since both CUB and CUB + extract show comparable levels of toxicity. In contrast, for TS-CUB + extract, the significantly lower cells viability compared to the free extract and the unloaded TS-CUB formulation may reflect the advantages of encapsulation in TS-CUB, such as an enhanced cellular delivery of the extract. The improved efficacy of this formulation will be further explored in the following section on cellular uptake. At $5.2 \mu\text{g/mL}$ all the

Table 2

Apparent hydrodynamic diameter (D_h) of the nanoparticles, Polydispersity Index (PDI) and electrophoretic mobility (ζ -potential) of CUB and TS-CUB formulations loaded with *U. rigida* extract at 25°C and 37°C . Data are reported as average value $\pm$ SD.

	T ($^\circ\text{C}$)	CUB + extract	TS-CUB + extract
D_h (nm)	25	133 ± 1	114 ± 1
	37	131 ± 1	120 ± 1
PDI	25	0.128 ± 0.005	0.189 ± 0.009
	37	0.13 ± 0.01	0.19 ± 0.02
ζ -potential (mV)	25	-26 ± 3	-41 ± 2
	37	-19.1 ± 0.4	-32 ± 1

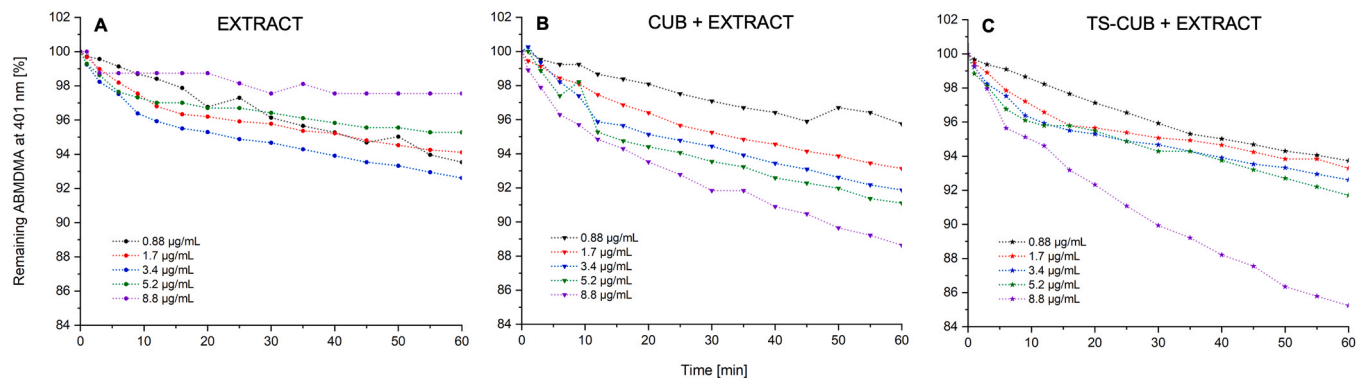
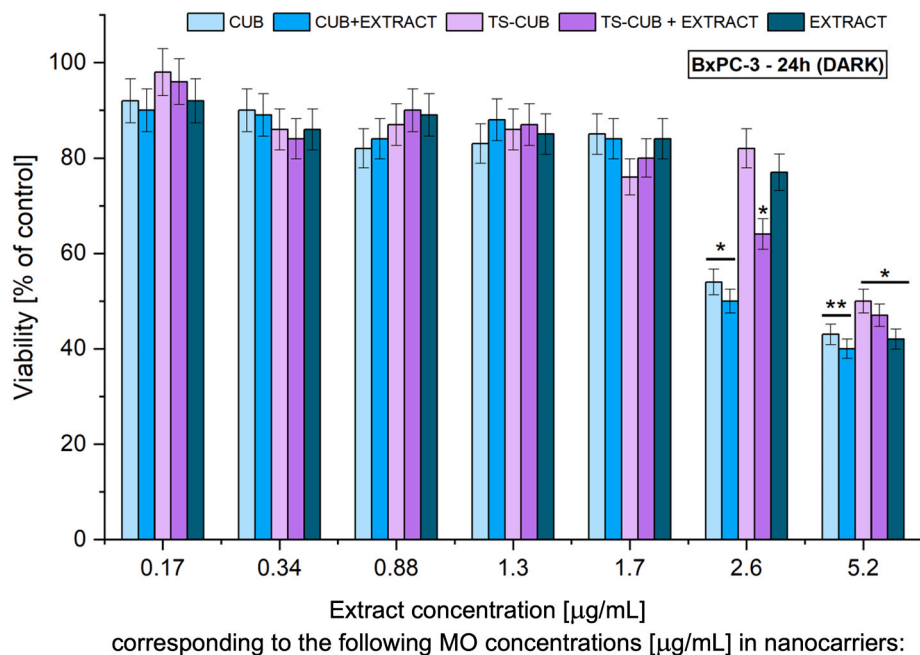


Fig. 3. Photo-oxidation of ABMDMA with the extinction of its absorbance by $^1\text{O}_2$ generated by the studied samples: *U. rigida* extract (A), CUB (B), and TS-CUB loaded with *U. rigida* extract (C) at different concentrations (0.88, 1.7, 3.4, 5.2 and 8.8 $\mu\text{g/mL}$).



CUB	15.4	30.8	77.0	116	154	231	462
TS-CUB	16.2	32.4	81.0	122	162	243	486

* $p < 0.05$
** $p < 0.01$

Fig. 4. *In vitro* cytotoxicity experiments: MTT test on BxPC-3 cells after 24 h of incubation with the investigated samples: CUB and TS-CUB loaded with *U. rigida* extract and free extract at different extract concentrations (0.17, 0.34, 0.88, 1.3, 1.7, 2.6, 5.2 $\mu\text{g/mL}$) compared to empty cubosomes, diluted in the same way as the loaded ones (to the target extract concentration), to achieve comparable MO concentrations in the formulations, indicated below the x-axis for each sample. The results are represented as mean values $\pm$ SD for minimum $n = 3$, * statistical ANOVA analysis: $p \leq 0.05$ and $p \leq 0.01$.

samples caused the death of 50 % or more cells, showing that this concentration is unsuitable for medical purposes, related probably to too high concentration of the MO in nanocarriers, as well as too high concentration of the extract itself.

It is worth noting that the literature generally agrees on a cytotoxic threshold for MO-based cubosomes of approximately 100 $\mu\text{g/mL}$ [16,30,44]. Of course, such observed cytotoxicity can vary depending on the stabilizer used (e.g., we observed a 25 % decrease in toxicity when a polyphosphoester analog of PF127 was employed) [18]. Cytotoxicity can also differ based on the specific resistance of the cell line to which the formulation is administered. Particularly, the pancreatic cancer cells utilized in this study are notably resilient, as pancreatic cancer ranks

among the most lethal cancers, with one of the lowest survival rates [46,47], and numerous studies were aimed to investigate the underlying mechanisms contributing to this resistance [23,46–49]. This fact may explain why the biocompatibility tests here presented on BxPC-3 cells under dark conditions showed no significant cytotoxic effect up to a MO concentration of 154 $\mu\text{g/mL}$, which corresponds to 1.7 $\mu\text{g/mL}$ of *U. rigida* extract. Given this cytotoxicity threshold, samples containing 1.3 $\mu\text{g/mL}$ of *U. rigida* extract were selected for the subsequent assessment of cellular uptake and photodynamic action.

3.4.2. Cellular uptake evaluation

In this study, flow cytometry was used to count cells emitting

fluorescence due to specific fluorochromes present within (in this case the fluorescent extract), with the right shifts presented in Fig. 5 indicating higher cellular uptake. Based on these experiments, Fig. 5 evaluates the cellular uptake of the algae extract by BxPC-3 cells treated with various samples. Control cells were compared with cells treated with the free extract (1.3 $\mu\text{g/mL}$), CUB, and TS-CUB, either loaded with the extract (1.3 $\mu\text{g/mL}$) or as empty carriers (negative controls).

As expected, fluorescence in control cells and cells treated with empty cubosomes was low and nearly identical, being related only to the autofluorescence emitted by cells [50]. However, a significant increase in fluorescence was observed in cells treated with the pure extract and CUB + extract. An even greater increase was noted in cells treated with TS-CUB + extract. Accordingly, comparison with the controls supports the conclusion that the enhanced cellular uptake of the extract resulted from its encapsulation in the obtained nanocarriers. The observed difference in cellular uptake between the two extract-loaded cubosome formulations, along with their different compositions, suggest that TC and Sp80 may play a role in the higher internalization efficiency of the algae extract when administering the novel cubosomes. The presence of such compounds may further modulate membrane fluidity and permeability, thereby facilitating the internalization process [51]. Another motivation for increased cellular uptake in the case of TS-CUB + extract, can be found in the smaller particle size and more negative ζ -potential of the new cubosomes compared to standard ones, which could facilitate easier transport through the tumor cellular membrane. Indeed, smaller particle size typically enables a more efficient endocytosis process, and a negative ζ -potential can promote stronger electrostatic interactions with positively charged domains on cellular membranes, potentially enhancing internalization [52]. While these suggestions are consistent with existing literature, further mechanistic studies are required to confirm the precise uptake pathways involved. In conclusion, the TS-CUB formulation delivers the photoactive *U. rigida* extract to the pancreatic tumor cells more efficiently.

3.4.3. Bioimaging of the cell morphology

The phototoxic activity was evaluated by cytoskeleton fluorescent staining before and after irradiation. In Fig. 6 is reported the bioimaging of BxPC-3 pancreatic cancer cells treated with CUB and TS-CUB formulations loaded with *U. rigida* extract (1.3 $\mu\text{g/mL}$) as well as the free extract at the same concentration, performed in dark conditions and after irradiation. The cell morphology revealed a significant difference in the F-actin appearance in the case of TS-CUB + extract after irradiation. In the case of the TC and Sp80-stabilized cubosomes loaded with the algae extract the cytoskeleton is destroyed as significant reorganization and destabilization of F-actin fibers was observed compared to the other samples, including the empty cubosomes. Comparing the cell bioimaging of free extract after PDT with its loaded form, it can be observed that encapsulation, particularly in the case of TS-CUB formulation, significantly enhances the extract's efficacy and photosensitivity.

These results are in good agreement with our previous study concerning the cytoskeleton observation after PDT [38], demonstrate the potency of our formulation in increasing the extract toxicity against pancreatic cancer cells when combined with photodynamic treatment.

4. Conclusions

Due to the increasing death rate and the urgent need for an effective drug to combat resistant pancreatic cancer, we propose a solution based on photoactive algae extracts encapsulated in modern nanocarriers, cubosomes, combined with PDT. As demonstrated in this work, this approach allows the bioactive cargo to be delivered in a stable and bioactive form to targeted cancer cells, even at body temperature (37 $^{\circ}\text{C}$), indicating potential *in vivo* applications.

The proposed formulations presented a stable structure. However, apart from the typical Pn3m cubic phase, TS-CUB exhibited possible presence of sponge nanoparticles with an L3 phase. The TS-CUB formulation showcased in this work demonstrated greater photoactivity associated with high ROS production and biocompatibility, characterized by high cellular uptake and reduced cytotoxicity under

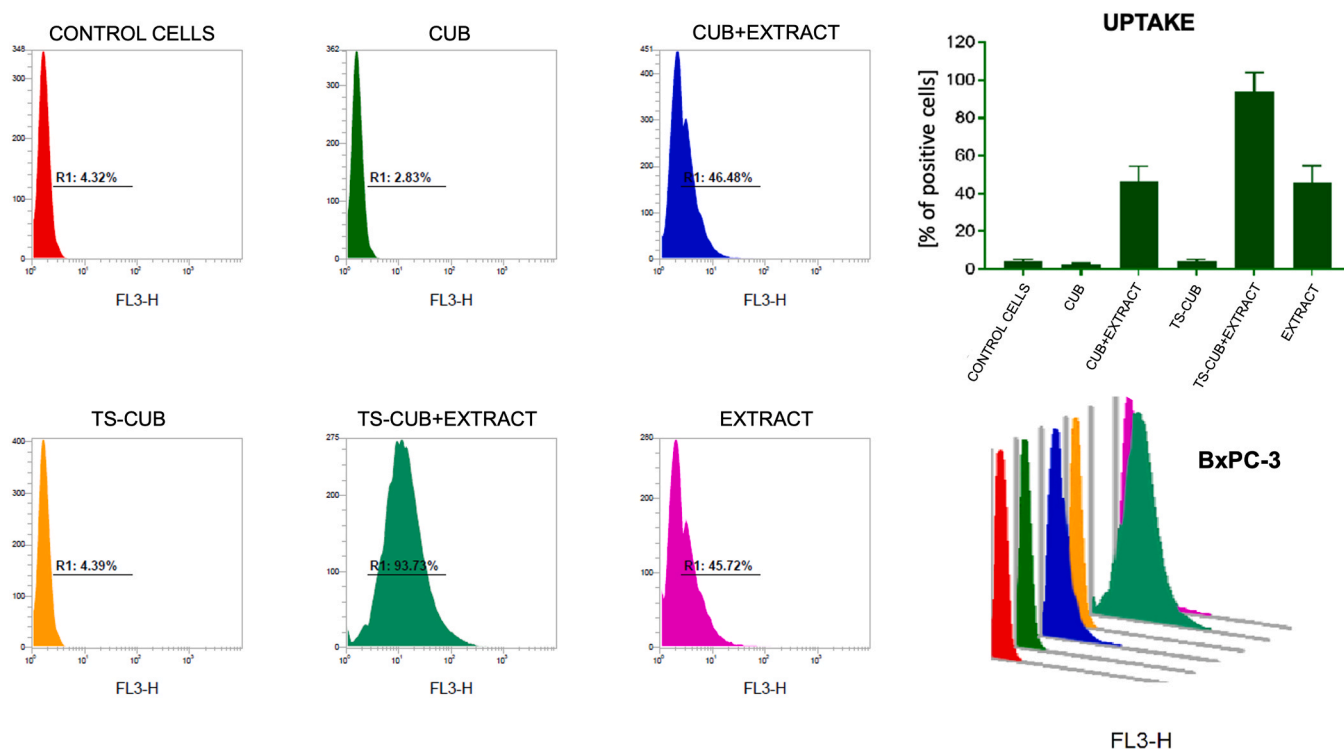


Fig. 5. Flow cytometry experiments on BxPC-3 cells – purpose of the cellular uptake of the studied samples: CUB and TS-CUB loaded with *U. rigida* extract (1.3 $\mu\text{g/mL}$) compared to free extract (1.3 $\mu\text{g/mL}$) with three negative controls (empty CUB and TS-CUB, as well as untreated control cells).

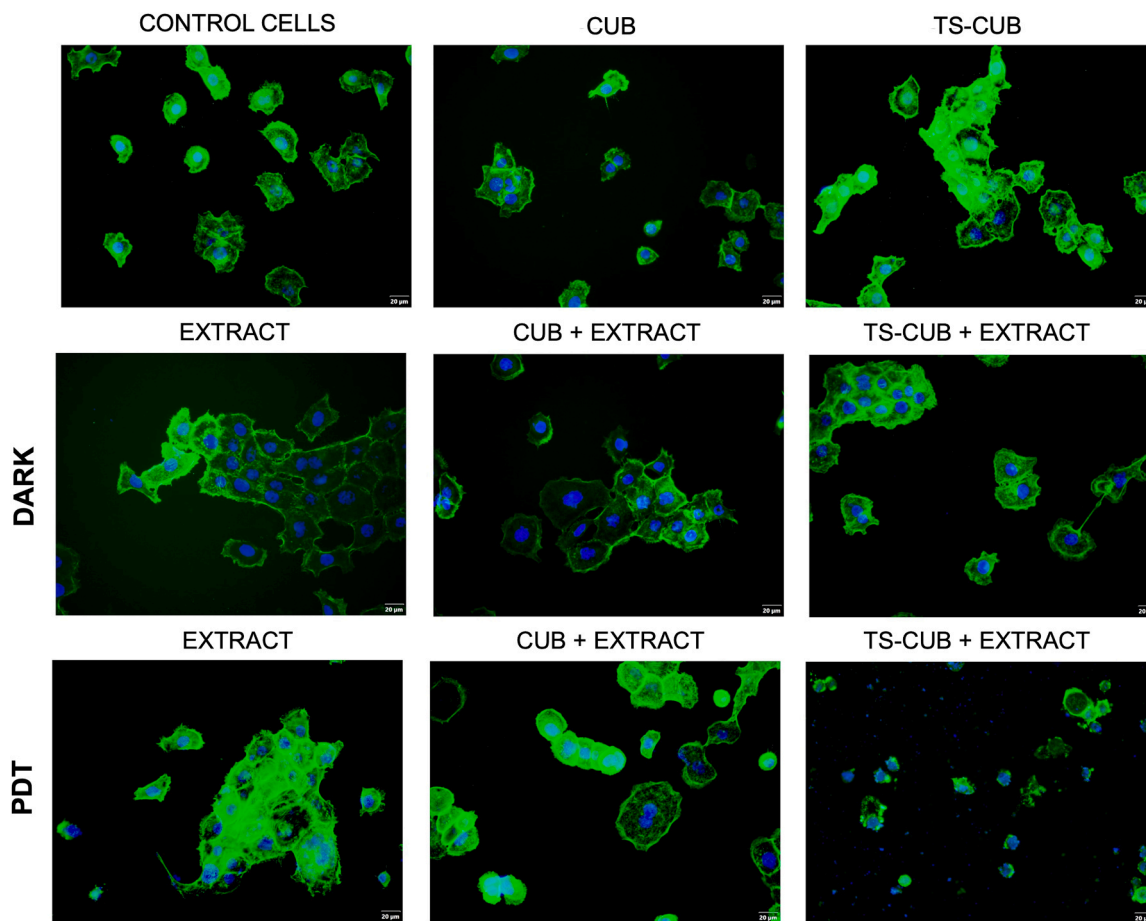


Fig. 6. Bioimaging performed on human pancreatic cancer cell line (BxPC-3) in dark conditions (DARK) as well as after irradiation (PDT); cells were treated with different samples: empty CUB and TS-CUB; extract-loaded (1.3 $\mu\text{g/mL}$) CUB and TS-CUB, as well as free extract (1.3 $\mu\text{g/mL}$).

dark conditions. Nevertheless, upon exposure to irradiation during PDT, these formulations exhibited strong photodynamic anticancer activity, making them ideal candidates for PDT in the treatment of pancreatic cancer.

This work aims to highlight the environmentally friendly development of widely available marine algae biomass, rich in bioactive natural photosensitizers with high photoactivity, enhancing its pharmaceutical value. An additional advantage of the proposed algae extract-based systems is their protection and efficient delivery via cubosome nanocarriers, which offer high stability and loading efficiency. To the best of our knowledge, this is the first work concerning the encapsulation of natural bioactive compounds obtained from marine algae in the cubosomes with increased biocompatibility, thus it may constitute a green template nanoplatform for the research on different valuable compounds obtained from biomass for applications in pharmacy and nanomedicine. In addition, this type of nanocarriers has not been thoroughly studied yet for the potential treatment of highly resistant pancreatic cancer cell line (BxPC-3), especially combined with PDT and natural photosensitizers encapsulation.

CRediT authorship contribution statement

Debora Dessi: Writing – review & editing, Software, Resources, Methodology. **Andrea Porcheddu:** Writing – review & editing, Formal analysis, Data curation. **Rita Mocci:** Writing – review & editing, Methodology, Formal analysis. **Urszula Bazylinska:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Karolina Krautforst:** Writing – review & editing, Writing – original draft,

Visualization, Software, Resources, Methodology, Investigation. **Julita Kulbacka:** Writing – review & editing, Software, Resources, Methodology, Investigation, Formal analysis. **Marco Fornasier:** Writing – review & editing, Software, Resources, Methodology, Investigation, Formal analysis. **Davide Moccia:** Writing – review & editing, Investigation, Formal analysis. **Antonio Pusceddu:** Writing – review & editing, Formal analysis, Data curation. **Giorgia Sarais:** Writing – review & editing, Formal analysis, Data curation. **Sergio Murgia:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.colsurfb.2025.114754](https://doi.org/10.1016/j.colsurfb.2025.114754).

Data availability

Data will be made available on request.

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SUPPORTING INFORMATION

Delivery of Ulva rigida Extract by Bicontinuous Cubic Lipid Nanoplatforms for Potential Photodynamic Therapy against Pancreatic Cancer

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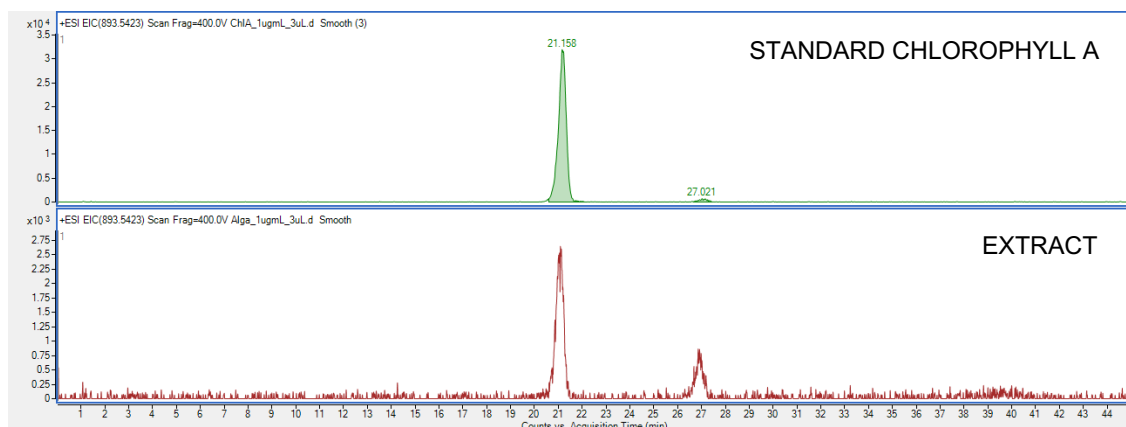


Figure S11. Qualitative HPLC-MS analysis of *U. rigida* extract containing chlorophyll a.

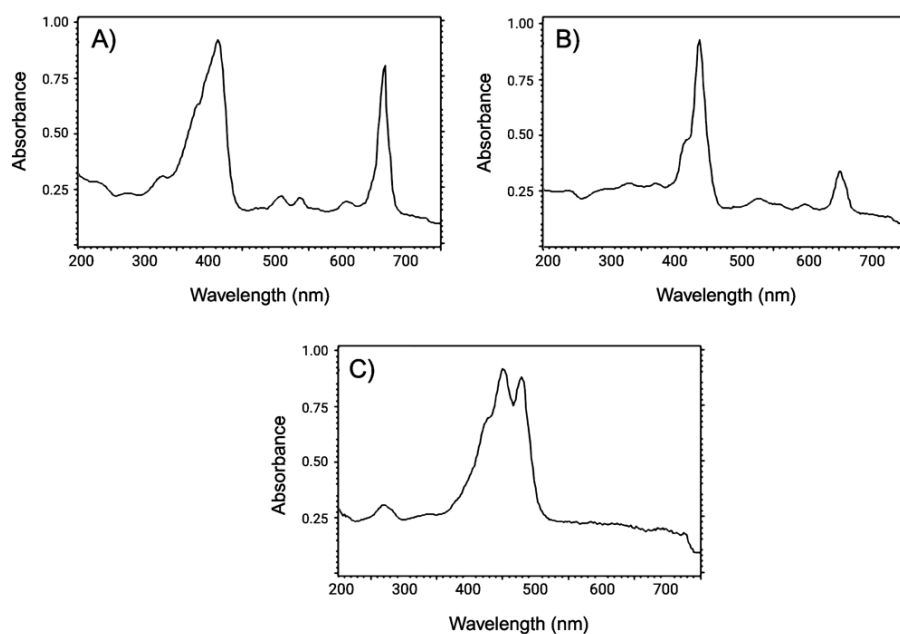


Figure S12. UV-Vis spectra of the analytical standards for the quantified pigments in *U. rigida* extract: chlorophyll a (A), chlorophyll b (B), lutein (C).

EXTRACT LOCALIZATION IN CUBOSOME - VERIFICATION

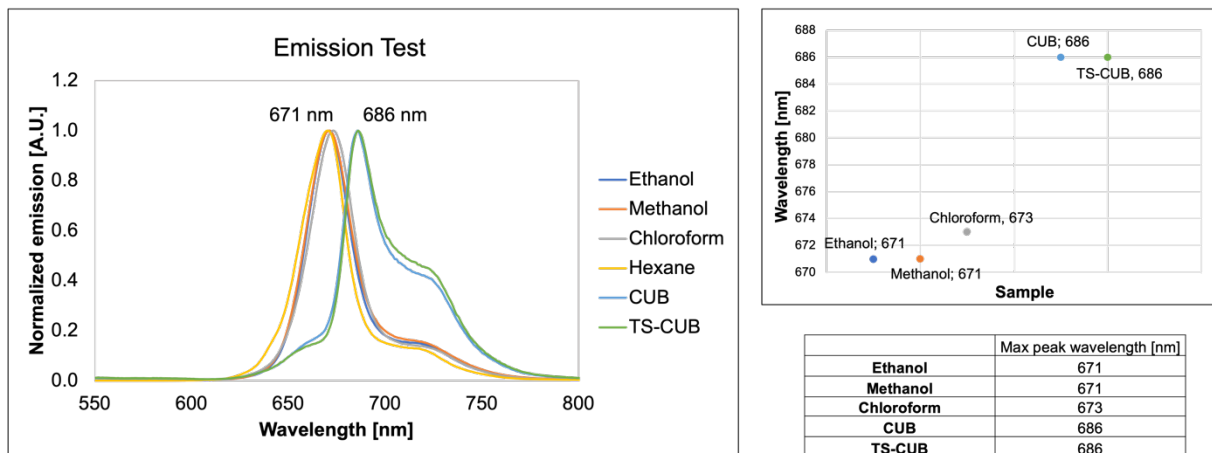


Figure S13. Fluorescence spectroscopy measurements on the extract excitation/emission wavelength in the free form (in different solvents) and loaded in the two cubosome formulations (CUB and TS-CUB).

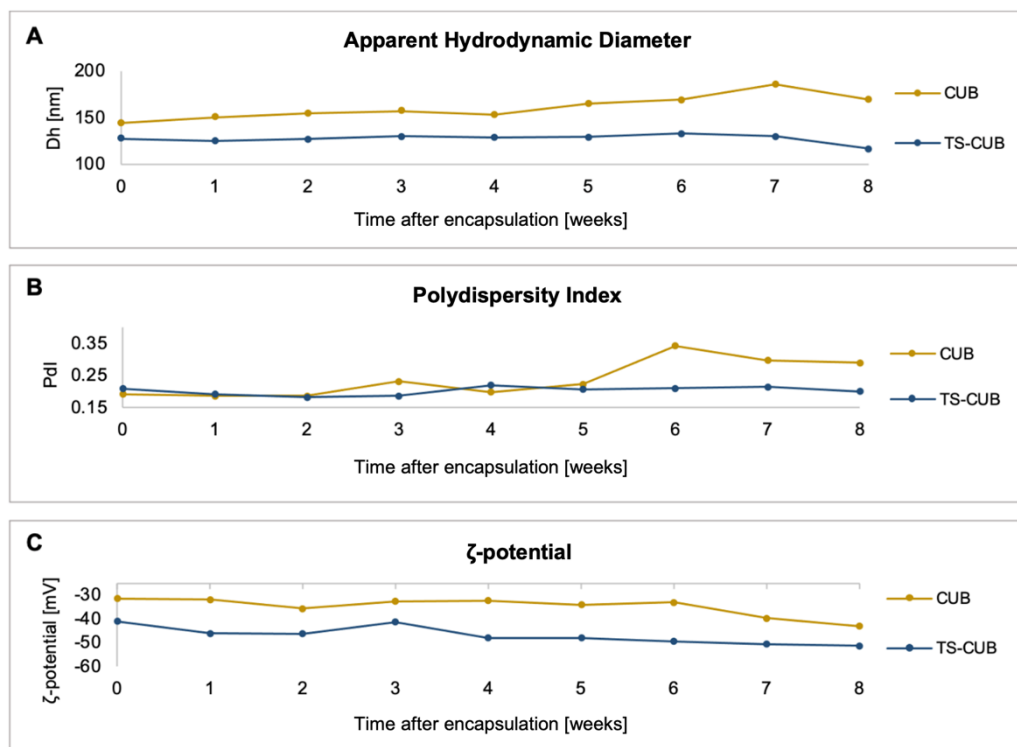


Figure S14. DLS and ELS measurements (apparent hydrodynamic diameter (D_h) of the nanoparticles (A), Polydispersity Index (PdI) (B), and electrophoretic mobility (ζ -potential) (C) of CUB and TS-CUB formulations loaded with *U. rigida* extract at 25 °C.

Paper III

Caulerpin Delivery via Pluronic-Free Cubosomes: Unlocking the Therapeutic Potential of a Pigment from an Invasive Marine Algae

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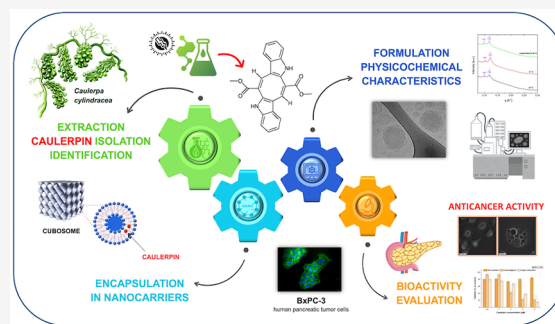
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Supporting Information

ABSTRACT: Pancreatic cancer remains one of the deadliest cancers due to its resistance to conventional therapies, necessitating the development of novel treatment strategies. This study investigates the anticancer potential of caulerpin, a bisindole alkaloid derived from the invasive marine alga *Caulerpa cylindracea*, encapsulated in biocompatible cubosomes. Caulerpin was sustainably extracted via microwave-assisted methods and formulated into lipid-based bicontinuous cubic liquid crystalline nanoparticles using Pluronic-free surfactants (sodium taurocholate and Span 80), resulting in high encapsulation efficiency and structural stability at physiological temperature (37 °C). The formulation included cubosomes coexisting with L3 sponge nanoparticles and vesicles. *In vitro* studies on BxPC-3 pancreatic cancer cells demonstrated that encapsulated caulerpin significantly outperformed the free compound, inducing marked apoptotic features such as cytoskeletal disruption and cell shrinkage, as confirmed by holotomographic microscopy and F-actin bioimaging. The enhanced therapeutic efficacy is attributed to the improved protection and sustained intracellular availability of encapsulated caulerpin, which is not rapidly metabolized as in its free form. These findings suggest that caulerpin-loaded cubosomes may represent a promising nanotechnology-based strategy for pancreatic cancer treatment.

KEYWORDS: algae, caulerpin, anticancer, cubosomes, nanocarriers, pancreatic tumor



1. INTRODUCTION

Green marine algae, genus *Caulerpa* constitutes a valuable source of different secondary metabolites like alkaloids, terpenoids, flavonoids, steroids, and tannins with potential bioactive properties against many diseases, therein anticancer activity by targeting and modulating multiple cancer cell-specific signaling pathways and cascades.^{1,2} These metabolites have been shown to affect mitochondrial health and various metabolic and stress pathways through interaction with signaling proteins such as AMPK, PTP1B, or HIF-1, as well as cell cycle progression.^{1,3} One of them, caulerpin, an orange-red pigment characteristic for *Caulerpa* species, was found in *Caulerpa cupressoides* (*C. cupressoides*), *C. paspaloides*, *C. prolifera*, *C. sertularioides*, and *C. cylindracea*.^{4–6} Caulerpin's chemical structure was first elucidated by Aguilar-Santos in 1970⁷ and later revised by Maiti and Thomson in 1977⁸ and consists of two indole groups separated by a central cyclooctatetraene ring with two methyl ester groups.^{1,9} This specific structure classifies caulerpin into the group of natural bisindole alkaloids, characterized by two indole units, more potent concerning their biological activity than their corresponding monomeric units.¹⁰ Various naturally derived medicinal products are based on indolic alkaloids, like the most studied vincamine, vincristine, or vindesine mainly due to their

activities as cerebral vasodilators, antiarrhythmics, antihypertensives, and anticancer agents.^{11,12} Caulerpin has been reported to exhibit a range of biological activities, including antinociceptive and anti-inflammatory effects,⁵ as well as antimicrobial,⁹ antiviral,¹³ and anticancer properties.^{4,14}

Pancreatic cancer is one of the deadliest malignancies due to its special complex tumor microenvironment, as well as the multitude of mechanisms and pathways responsible for the exceptional resistance of this cancer to treatment.^{15–19} Attention should also be paid to the prevention of this type of disease, as it may be associated with inflammatory processes in the pancreas caused by using certain drugs. Patients with type 2 diabetes may be particularly susceptible, since recent work by Li et al. proved that the use of antidiabetic agents from the SGLT2 inhibitor (SGLT-2i) class has been linked to cases of acute pancreatitis (AP).²⁰ AP, especially when recurrent or progressing to chronic pancreatitis, is a known risk factor for

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the development of pancreatic cancer.²¹ Therefore, monitoring patients who experience drug-induced AP (e.g., those related to SGLT-2i use) may be crucial for the early detection of neoplastic changes in the pancreas.

Xu et al. in 2019 reported that inhibition of PTP1B protein blocks pancreatic cancer progression by targeting the PKM2/AMPK/mTOC1 pathway.²² It was proved that caulerpin presents PTP1B inhibitory activity, therefore indicating its potential influence on pancreatic cancer progression.^{23,24} Moreover, caulerpin is known to induce mitochondrial dysfunctions, thereby disrupting the crucial energy balance of cells.²⁵ In 2017, Yu et al. demonstrated on SW480 and LoVo cells (colorectal cancer) that this effect is caused by metabolic reprogramming and AMPK α 1 pathway activation: caulerpin inhibits complex I of a mitochondrial electron transport chain, accompanied by the dissipation of mitochondrial membrane potential, and decreases oxygen consumption rate in cancer cells with a simultaneous surge of reactive oxygen species (ROS) and decrease in ATP production.¹⁴ In the central regulatory node of the energy metabolism and tumor progression, there is adenosine monophosphate (AMP)-activated protein kinase (AMPK), known for the detection of high ADP or AMP levels, indicating the depletion of energy stores.²⁶ Therefore, in response to the increase in the AMP/ATP ratio, high expression of AMPK energy sensor was proved to be activated by caulerpin anticancer treatment.¹⁴ On the other hand, a novel AMPK inhibitor developed recently by Schneider et al. presented an increase in the sensitivity of pancreatic cancer cells to the induction of ferroptosis—a specific type of programmed cell death dependent on iron and oxidative stress, leading to damage of cell membranes.²⁷ The unfavorable prognosis of pancreatic ductal adenocarcinoma is largely due to a hypoxia-driven tumor microenvironment regulated by carbonic anhydrase IX (CAIX), which, as demonstrated by Ghosalkar et al., can be targeted with methazolamide to enhance the sensitivity of therapy-resistant PDAC cells through inhibition of CAIX.²⁸ Moreover, Liu et al. proved that caulerpin also presents hypoxia-inducible transcription factor (HIF-1) inhibitory activity, by inhibition of the mitochondrial electron transport to complex III.³ Considering the vast amount of work on the potential anticancer activity of caulerpin, it is noteworthy that out of the many various cancer cell lines already studied, such as colorectal cancer,^{4,14} breast cancer,³ or ovarian cancer,²⁵ there is still no research on the caulerpin's activity against pancreatic cancer.

It has been proven that *C. cylindracea* Sonder 1845 is one of the most invasive species in the Mediterranean Sea,²⁹ rapidly spreading and causing many ecological changes and reducing biodiversity at a range of spatial scales.^{30–34} This alga can alter the native species' behavior, with alleged adverse repercussions even on fish growth patterns, behavior, and dynamics of the population.³⁵ Therefore, the management of the invasive algae biomass to isolate bioactive compounds could constitute an economical and widely available pharmaceutical source from natural biomass and at the same time an environmentally friendly solution, regulating the marine environment. Recent trends indicate increased application of innovative green extraction methods, including microwave-assisted extraction (MAE), characterized by increased efficiency, reduced time and lowered process costs.³⁶ Cantarino et al. proved that MAE is a suitable and efficient method for caulerpin extraction with high yield from these algae.³⁷ Conversely, current methods for caulerpin synthesis generally results in low yields (<50%) from

indole.^{38–40} These methods require at least three synthetic steps, some involving reflux conditions and the isolation of intermediates. Moreover, they rely on toxic reagents, such as POCl₃ and piperidine. In this context, extracting the bis-indole alkaloid directly from renewable biomass offers a significant advantage, enhancing both sustainability and efficiency. However, the low solubility and instability of caulerpin in aqueous media, associated with its hydrophobic nature, limit its use in the cellular environment due to reduced bioavailability.⁴¹ Therefore, there is a significant need to circumvent this problem, and encapsulation in the appropriate nanocarriers appears to be a potential solution. There are many innovative combinatorial approaches to novel anticancer treatments, especially in the field of various nanosystems. Nie et al. developed metal organic framework coated MnO₂ nanosheets for co-delivery of a survivin silencing RNA-cleaving DNase self-activated by Mn²⁺, and antineoplastic doxorubicin, with the additional ROS generation by Mn²⁺, for efficient chemo–gene combinatorial treatment of cancer proved by *in vitro* and *in vivo* studies.⁴² Furthermore, recent studies of Feng et al. on tailoring the molecular structure of doxorubicin carrier-free prodrug nanoassembly by linking with fatty alcohols have shown that the nanosystem modified with long-chain fatty alcohol improved stability but slowed the disassembly and drug release process.⁴³ On the other hand, prolonged circulation in the body and tumor accumulation were proved by hexadecanol-modified doxorubicin prodrug and significantly enhanced antitumor activity *in vivo*.⁴³

The colloidal dispersion of bicontinuous cubic lipid-based lyotropic liquid crystalline nanoparticles in water with the use of suitable surfactants and stabilizers results in nanostructured systems, referred to as “cubosomes”, having sizes ranging from 100 to 400 nm.^{44,45} These honeycomb-like structures comprise curved bicontinuous lipid bilayers organized in three dimensions, forming two internal not interconnected water channels, that can be exploited by various bioactive ingredients.^{46–48} Cubosomes are able to deliver bioactive cargo to the target disease tissue, protecting it from the degrading cellular environment and, in the case of water-insoluble compounds, preventing their aggregation.⁴⁹ Recently published works^{50–53} indicate that cubosomes are gaining utility and acceptance as anticancer drug delivery systems with possible theranostic approaches. For the potential treatment of cervical cancer, Zhang et al. proposed combinatorial cubosomes coloaded with well-known anticancer drugs: cisplatin and paclitaxel and coated with poly-*ε*-lysine for sustained drug release, with an enhanced therapeutic efficacy proved against HeLa cells.⁵² Moreover, cubosomes loaded with copper acetylacetonate as anticancer drug, were for the first time functionalized with Affimer proteins to actively target carcinoembryonic antigens on LS174T colorectal cancer cells.⁵⁴ In this way, Pramanik et al. have developed novel promising Affimer-tagged cubosomes by their first comprehensive preclinical evaluation through *in vitro* and *in vivo* experiments, proving selective accumulation in cancer cells in comparison to normal ones, minimal tissue absorption in vital organs with no significant signs of toxicity, along with the reduced tumor growth.⁵⁴ Such examples demonstrate an enormous therapeutic potential of these liquid crystalline nanocarriers to fight various types of cancer; therefore, research on them should be continuously expanded and updated.

CAULERPIN ISOLATION

FROM *C. CYLINDRACEA*



Figure 1. General scheme of the path from *C. cylindracea* algae biomass collection and preparation to pure caulerpin isolation and identification.

With the above knowledge, we aimed to assess the antitumor potential of caulerpin, isolated from *Caulerpa cylindracea* using green MAE and chromatographic methods against highly resistant pancreatic cancer cells (BxPC-3). To overcome the challenges posed by the tumor microenvironment, which often impedes drug efficacy, we propose encapsulating caulerpin within newly formulated cubosomes. These cubosomes (caulerpin@cub) offer enhanced biocompatibility and stability, achieved through an optimized surfactant mixture instead of traditional Pluronic copolymers.⁵⁵ The combination of sustainable caulerpin extraction from *C. cylindracea* biomass and its encapsulation in advanced biocompatible nanocarriers hold promise for improving the efficacy of this potential anticancer agent against extremely resistant pancreatic cancer.

2. EXPERIMENTAL SECTION

2.1. Materials. The main building block used for cubosomes preparation was glycerol monooleate (1-mono-oleoylglycerol, MO, RYLO MG 19 PHARMA, 98.1 wt %), kindly provided by Danisco A/S (Denmark). Other components of the cubosomes were Span 80 (Sp80, sorbitan monooleate, steric stabilizer) and taurocholic acid sodium salt hydrate (TC, taurocholate, bile salt) purchased from Sigma-Aldrich (Germany). The solvents used in the extraction and caulerpin isolation process (ethanol, hexane, diethyl ether, and ethyl acetate) were purchased from Honeywell (Germany). Anhydrous sodium sulfate as a drying agent was purchased from Sigma-Aldrich (Italy). The chromatographic column was performed using EcoChrome™ MP Silica gel 60 Å, particle size 0.040–0.063 mm (230–400 mesh), purchase from Merck (Germany). Cerium sulfate used in TLC analysis was purchased from Merck (Germany).

Deuterated chloroform used in the NMR analysis was purchased from Sigma-Aldrich (Germany). Water was freshly distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) and is referred to as water (W). For the preparation of the cubosomes, water was filtered (before the preparation) with a 0.22 μm pore size hydrophilic filter (Milli-Q system, Millipore, Germany).

2.2. Biomass Collection and Preparation. Fresh *C. cylindracea* thalli were collected from the Mediterranean Sea in Sardinia (Italy). The algae were washed with water to remove sand, salt, and epiphytes, deep-frozen at −80 °C, and freeze-dried (Benchtop, VirTis, US) for 48 h (−51.8 °C, 0.150 mbar). The dry weight (DW) of the algae was $4.3 \pm 0.4\%$ of the fresh biomass. Then, the freeze-dried algae were milled into a powder with the use of a ball-milling technique to increase the extraction surface. For this purpose, the freeze-dried *C. cylindracea* (1.5 g) was loaded into a zirconium oxide grinding jar (35 mL) equipped with 2 balls ($\phi = 8$ mm). The jar was sealed and shaken for 15 min at a frequency of 20 Hz using a VWR Beater Mixer Mill apparatus. The powdered biomass was then stored in the refrigerator and used in the next steps.

2.3. Caulerpin Isolation. Caulerpin was isolated from the powdered biomass of *C. cylindracea* marine algae as follows (Figure 1). The extraction was performed in a two-step process including solid–liquid microwave-assisted extraction and liquid–liquid extraction with pigment fractionation in a separatory funnel. Then, caulerpin was isolated and purified on a silica chromatographic column and identified by UV–vis spectrophotometry and ¹H NMR spectroscopy.

2.3.1. Microwave-Assisted Extraction. To release bioactive compounds from the cell wall into the solvent solution, solid–liquid extraction was performed using the MAE technique (Discover SP, CEM). Ethanol was chosen as a solvent (in a solid–liquid ratio of 0.1 g of dry algae/mL of solvent) since it is a green solvent and gives high extraction efficiency.⁵⁶ The biomass was subjected to microwave-assisted extraction with stirring at 90 °C for 7 min with a power of 200 W and pressure of 300 PSI. Extraction conditions were chosen based on literature data concerning the evaluation of the most efficient extraction methodology for caulerpin isolation.³⁷ After that, the solution was centrifuged (4000 rpm/10 min) (REMI R-9M, Remi Elektrotechnik Ltd., Indie) and the supernatant was evaporated and dissolved in diethyl ether for further liquid–liquid extraction in a separatory funnel 1:1 with distilled water. The organic diethyl ether phase, rich in hydrophobic pigments of interest, i.e., caulerpin, was collected, dried with anhydrous

sodium sulfate, and concentrated under reduced pressure in a rotavapor (BÜCHI R-200, BÜCHI Labortechnik AG, Switzerland) giving the final crude *C. cylindracea* extract used in the next steps.

2.3.2. Isolation of Caulerpin from *C. cylindracea* Extract and Purification on Chromatographic Column. Silica gel was loaded into the chromatographic column and conditioned with a mixture of hexane:ethyl acetate mixture (8:2). The crude *C. cylindracea* extract was loaded into the chromatographic column by dry-loading and by adsorption on silica gel before dissolution in diethyl ether. The column was then eluted with a solution of hexane:ethyl acetate (8:2). The fractions were collected until the caulerpin was found by TLC identification (hexane:diethyl ether 1:1) based on characteristic reaction with cerium sulfate (resulting in a red spot after gentle heating) and literature data of the R_f parameter.²⁵ Caulerpin-rich fractions of interest were collected, and the solvents were evaporated in a rotavapor. The resulting orange-red solid was further purified by crystallization from acetone and hexane.

2.3.3. Caulerpin Identification by UV–vis Spectrophotometry and ^1H NMR Spectroscopy. To identify the isolated compound, its absorbance spectrum in ethanol was recorded using a UV–vis spectrophotometer (Cary Series UV–vis–NIR spectrophotometer, Agilent Technologies, U.K.) using a quartz cuvette (optical path, 1 cm) as well as ^1H NMR spectrum in CDCl_3 to identify its chemical structure and purity. ^1H NMR measurements were carried out at 25 °C using a Bruker Avance 300 MHz (7.05 T) spectrometer at the operating frequency of 300.131 MHz. A standard BVT 3000 variable-temperature control unit with an accuracy of 0.5 °C was used.

2.4. Caulerpin Encapsulation in Cubosomes. Cubosome formulations were prepared by following a previous methodology from our group.⁵⁵ In the beginning, MO was melted at 40 °C and caulerpin added and mixed with the lipid phase until obtaining a homogeneous mixture. After that, a solution of TC and Sp80 in water was added to the lipid phase and immediately sonicated by an ultrasonic processor (UP100H, Hielscher Ultrasonics, Germany) in 2 cycles (2 and 3 min) with 90% amplitude with the pulse ON for 1 s followed by 1 s of break. The empty cubosomes were prepared in the same manner without the addition of caulerpin. The composition (wt %) of empty and loaded (caulerpin@cub) cubosomes was respectively MO:TC:Sp80:W=3.5:0.2:0.1:96.2.

2.5. Dialysis and Encapsulation Efficiency. In order to remove the excess cargo after the encapsulation in nano-carriers, caulerpin@cub formulation was dialyzed after loading it into a tubing cellulose membrane (14 kDa cutoff; Sigma-Aldrich), in 2 L of water for 2 h at room temperature, changing the water after 1 h.

The encapsulation efficiency (EE) was evaluated by UV–vis spectroscopy. The absorption spectra were acquired using a UV–vis spectrophotometer (Cary Series UV–vis–NIR spectrophotometer, Agilent Technologies, U.K.) using a quartz cuvette (optical path, 1 cm). The standard calibration curve of caulerpin was prepared to calculate the weight of the encapsulated caulerpin before and after dialysis, with the data fitted to a linear regression ($R^2 = 0.9999$). Absorption spectra of the studied cubosome formulations before and after dialysis were measured after dissolving in ethanol (dilution 1:5, v/v), and the EE was calculated through the following expression:

$$\text{EE (\%)} = \frac{\text{weight of the caulerpin after dialysis}}{\text{weight of the caulerpin before dialysis}} \times 100$$

2.6. Small-Angle X-ray Scattering (SAXS). The structure assessment of caulerpin@cub formulation was performed by small-angle X-ray scattering (SAXS) experiments at the SAXSLab instrument (JJ-Xray, Denmark), following our previously published protocol.^{57,58} For this purpose, the samples were placed in quartz capillaries and measured at a sample to detector distance of 360 mm for around 2 h per sample at a given temperature. The intercrystalline spacing (d) was calculated after evaluating the q positions of each peak in the SAXS patterns using the following equation:

$$d = \frac{2\pi}{q_{\text{peak}}} \quad (1)$$

Then, the lattice parameter (a), describing the crystalline unit of the inverse bicontinuous cubic phases, was extrapolated from eq 2:

$$a = d\sqrt{h^2 + k^2 + l^2} \quad (2)$$

where h , k , and l are the Miller's indexes. Finally, the water channel radii of the cubosome and sponge nanoparticles were evaluated by applying eqs 3 and 4, respectively:

$$r_w = (a - l)\sqrt{\frac{A_0}{-2\pi\chi}} \quad (3)$$

$$r_{w-L3} = \frac{d_{L3}}{d_{\text{cubic}}} r_{w-\text{cubic}} \quad (4)$$

where χ and A_0 are, respectively, the Euler characteristic and the surface area of the specific geometry (Pn3m, $\chi = -2$, and $A_0 = 1.919$), and l is the MO hydrophobic chain length at 25 °C (17 Å).⁵⁹

2.7. Dynamic and Electrophoretic Light Scattering (DLS, ELS). Size, polydispersity index, and ζ -potential of the nanoparticles were evaluated by dynamic and electrophoretic light scattering (DLS and ELS, respectively). All measurements were conducted in triplicate on the diluted (1:50, v/v) samples at 25 and 37 °C on a ZetaSizer Nano ZS by Malvern Instrument (Malvern, U.K.) (backscattering angle, 173°; with a 4 mW He–Ne laser at 632.8 nm). The apparent hydrodynamic radius (D_h) and polydispersity index (PDI) were extracted by a second-order cumulant analysis of the autocorrelation curves of each sample. The ζ -potential was evaluated by ELS measurement and application of the Smoluchowski equation to the electrophoretic mobility. The signal was collected at a fixed angle of 17°.

2.8. Cryogenic Transmission Electron Microscopy (cryo-TEM). The morphology of the caulerpin@cub formulation was imaged by cryogenic transmission electron microscopy (cryo-TEM). Briefly, 4 μL of the studied sample was blotted on a carbon-coated copper grid at 25 °C with a relative humidity of 90%, within 24 h from preparations, and then imaged using a JEOL JEM-2200FS transmission electron microscope (JEOL, Tokyo, Japan).

2.9. Biological Activity. **2.9.1. Cell Lines Culturing.** The BxPC-3 cells (human pancreatic cancer cell line, ATCC, CRL-1687) were maintained in culture flasks with a surface area equal to 75 cm^2 (Falcon cell culture flasks) in RPMI 1640 (Sigma-Aldrich, Poznan, Poland) supplemented with 10% fetal bovine serum (FBS) and 50 $\mu\text{g}/\text{mL}$ penicillin and streptomycin (Sigma-Aldrich, Poznan, Poland). The cultures were incubated in a humidified atmosphere with 5% CO_2 at 37

°C. The cells used for all experiments were detached by trypsinization (Trypsin–EDTA solution) and neutralized with Dulbecco's phosphate buffered saline (DPBS), which was purchased from Sigma-Aldrich (Poznan, Poland).

2.9.2. Cytotoxicity Studies. The cytotoxicity MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell proliferation assay (Sigma-Aldrich, Poznan, Poland) was performed for various caulerpin concentrations (0.5, 1, 2, 3, and 4 μ M). All formulations were uniformly diluted to ensure a fair comparison between empty and loaded nanocarriers at the same MO concentration (corresponding to 56, 112, 223, 334, and 445 μ g/mL) for each caulerpin concentration.

BxPC-3 cells were studied after 4, 8, 24, and 48 h of incubation. Generally, 200 μ L of cells (approximately 2×10^5 cells) were placed into 96-well plates (Sarstedt, Equimed, Wroclaw, Poland). Then, the absorbance was measured at 560 nm using a GloMax Discover microplate reader (Promega), which allowed evaluation of the cell viability in each group expressed as percentages of the control cells (untreated with studied samples). Each data set represents three independent biological experiments with three technical wells per condition. The data are reported as average value $\pm$ standard deviation (SD).

2.9.3. Optical Microscopy (Culture Morphology). The cell culture morphology was studied with an optical inverted microscope DMi1 (Leica Microsystems, Germany). Pictures were taken after 8 and 24 h of BxPC-3 incubation in RPMI culture medium at 37 °C. BxPC-3 cells treated with free caulerpin (2 μ M), caulerpin@cub (caulerpin content = 2 μ M), or empty cubosomes, and compared to untreated control cells. BxPC-3 cells were cultured on a special microscope glass.

2.9.4. Holotomographic Microscopy (HTM). Morphological changes of BxPC-3 tumor cells over time (4, 8, and 24 h) were assessed using a holotomographic microscope (Nanolive, Switzerland) by comparing control cells with 3 studied samples: cells treated with free caulerpin (2 μ M), caulerpin@cub (caulerpin content equal to 2 μ M), or empty cubosomes. Cells were previously detached from cell culture flasks and cultured on 35 mm² Petri dishes with special microscope glass bottom (μ -Dish 35 mm, low glass bottom, Ibbidi, Germany), by incubation for 24 h at 37 °C in RPMI culture medium. All microscopic preparations were viewed intravitaly.

2.9.5. Immunofluorescent F-Actin Staining Protocol. The integrity of the cytoskeleton was determined after 24 h of postencapsulated potential drug exposure by immunofluorescent labeling of F-actin cells with Alexa Fluor546 Phalloidin (Thermo Fisher Scientific Inc.). Fluorshield with a fluorescent DNA-binding dye of cells grown in the culture (4,6-diamidino-2-phenylindole, DAPI) was used to visualize the nuclei and mount the cells after excitation at 405 nm. Samples were examined on an Olympus BX53 fluorescence microscope (Evident Europe GmbH, Warsaw, Poland).

2.9.6. Statistical Analysis. Results of the *in vitro* experiments are presented as mean values $\pm$ SD for a minimum of 3 and compared by two-way ANOVA for multiple comparisons and $\alpha = 0.05$. Comparisons of samples exhibiting *p* values ≤ 0.05 and ≤ 0.01 were considered statistically significant and highly significant, respectively. In the case of viability assay, all results were compared to untreated control. Results were analyzed using the commercial software GraphPad Prism 7.0.

3. RESULTS AND DISCUSSION

3.1. Characterization of Caulerpin and Encapsulation in Cubosomes. This study aims to evaluate the bioactivity against human pancreatic cancer cells of an alkaloid with potential anticancer properties, known as caulerpin, characteristic of several species within the genus *Caulerpa*, a group of green algae. Here, pure caulerpin was extracted from

C. cylindracea and encapsulated within cubosomes to improve its bioavailability and potentially increase its effectiveness in pancreatic cancer treatment.

First, the hydrophobic dark green extract from *C. cylindracea* (see Section 2.3.1) was obtained with a yield of $3.1 \pm 0.3\%$ DW. Then, after further purification process on a chromatographic column, pure caulerpin was isolated in a form of orange-red solid pigment with a yield of $0.47 \pm 0.02\%$ DW. According to the literature, this value is significantly higher than those obtained through classical methods, such as 0.1% DW of caulerpin extracted from *C. racemosa* via maceration,⁶⁰ or 0.2% DW achieved by traditional Soxhlet extraction.⁶¹

Caulerpin was identified by UV–vis spectrophotometry because it is a pigment that exhibits high absorbance at characteristic ranges of wavelengths: 222, 270, 292, and 317 nm.⁸ The absorbance spectrum of the isolated compound is presented in Figure 2. The sample was then investigated by ¹H

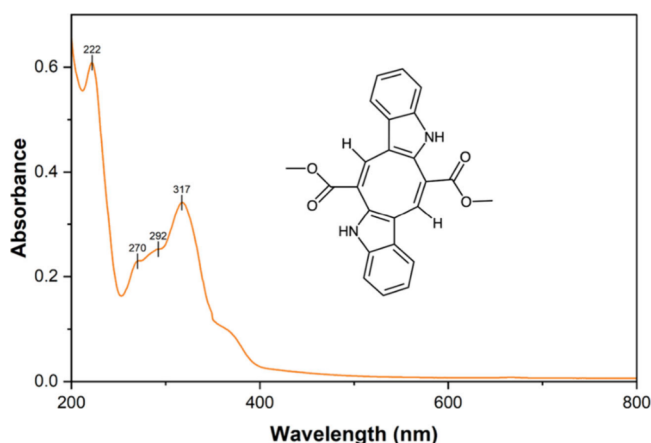


Figure 2. UV–vis absorbance spectrum of isolated caulerpin in ethanol exhibiting characteristic peaks at 222, 270, 292, and 317 nm along with the chemical structure of caulerpin.

NMR spectroscopy to further identify the product and confirm its purity (Supporting Information Figure S1). The pure caulerpin was subsequently encapsulated in cubosomes (caulerpin@cub) at a concentration of 0.125 mg/mL (EE = $92.6 \pm 0.9\%$).

3.2. Physicochemical Characterization of the Nanocarriers Loaded with Caulerpin. SAXS measurements were performed to evaluate the inner structure and phase of the formulation loaded with caulerpin and at different temperatures to observe possible changes in the structure of the formulation and evaluate their behavior in storage (room temperature) and potential *in vivo* conditions (body temperature).

The sample was then cooled to 25 °C to observe any structural hysteresis. Figure 3A presents the SAXS diffractograms of the cubosomes loaded with caulerpin, whereas Table 1 reports on the evaluated structure parameters, e.g., lattice parameter (*a*) and water channel radius (*r_w*).

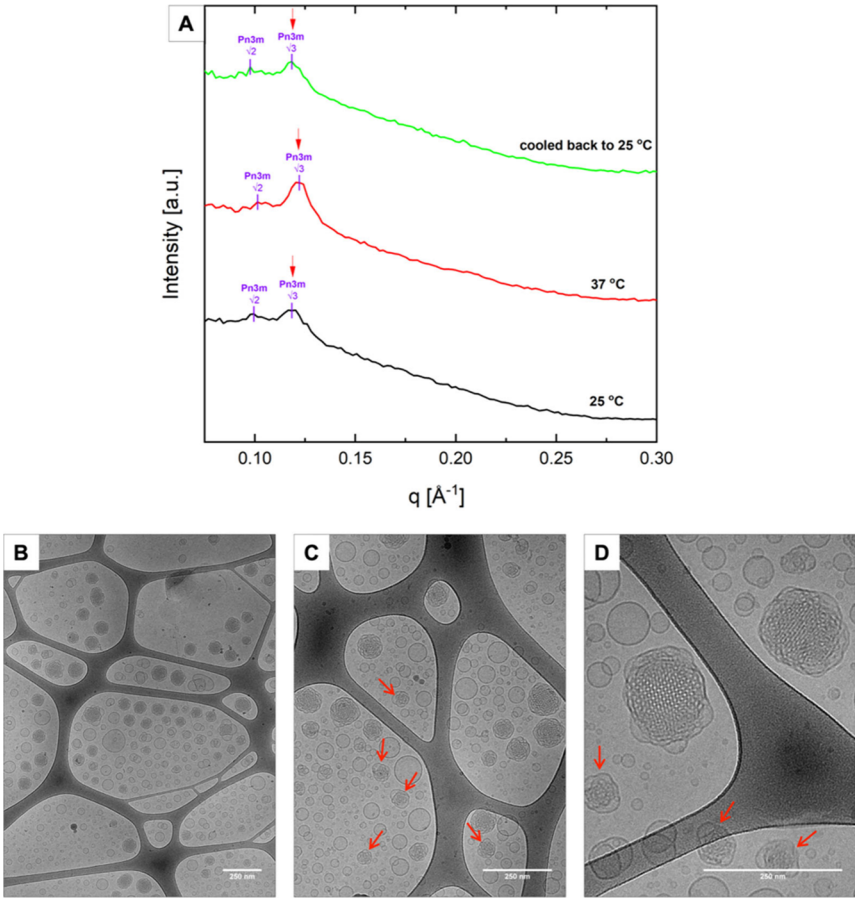


Figure 3. SAXS diffractograms at 25 °C, 37 °C after the heating cycle and returning to 25 °C (A) and cryo-TEM images (B, C, D) of the caulerpin@cub formulation. Red arrows indicate the presence of sponge phase (L3) nanoparticles in the formulation. The scale bar is 250 nm.

Table 1. Phase, Lattice Parameter (*a*), and Water Channel Radius (*r_w*) of caulerpin@cub and Empty Cubosomes at 25, 37, and Cooled Back to 25 °C^a

sample	T (°C)	phase	<i>a</i> (Å)	<i>r_w</i> (Å)
caulerpin@cub	25	Pn3m	91 ± 1	18.4 ± 0.7
		L3		15.5
	37	Pn3m	88 ± 1	17.5 ± 0.4
		L3		14.6
	back to 25	Pn3m	91.5 ± 0.6	18.7 ± 0.2
		L3		15.4
empty cubosomes ⁵⁵	25	Pn3m	92.1 ± 0.1	22.0 ± 0.3

^aData are reported as mean ± SD.

In Figure 3A the main peaks suggesting the Pn3m cubic phase present low intensity with a high and broad diffusive band related to the presence of other particles in the studied formulation, like polydisperse vesicles. This conclusion is based on the compatibility with cryo-TEM images of this formulation presented in Figures 3B–D, discussed in more detail in the next paragraph. The two visible peaks are in the position corresponding to the Pn3m phase with the lattice parameter 90.6 ± 1.9 Å at 25 °C, a value typical for this type of cubic phase. Furthermore, as scattering is a probabilistic event and normally the peaks decrease with the order of scattering, the second peak, being more intense than the first one, indicates the possible presence of a second phase. Moreover, Sp80 tunes the packing parameter of the lipid (MO), rendering a more disordered cubic phase, commonly referred to as L3 phase

(sponge phase).⁶² The first suspected peak of the L3 phase is highlighted in Figure 3A with a red arrow. Therefore, caulerpin encapsulation and the presence of Sp80 affected the structure of the nanoparticles, by changing the effective packing parameter of MO. Increasing the temperature to 37 °C causes a small shift toward higher *q* values, indicating lower values of the lattice parameter (*a*) and water channel radius (*r_w*) of caulerpin@cub, as reported in Table 1. The shift at 37 °C is attributed to increased thermal motion of the hydrophobic tails of MO, leading to an expansion of its hydrophobic region. Consequently, this results in a reduced lattice parameter and water channel radius while maintaining the same nanostructure.⁵⁹ Moreover, heating to 37 °C makes the first peak of the L3 phase more pronounced and the first peak of Pn3m broader, suggesting that a larger portion of the Pn3m phase transitions to L3 as the temperature increases. Nevertheless, these are speculations based on the analysis of one peak. Deeper investigations at higher resolutions or at a source with higher brilliance would be needed for complete assessment. Overall, the sample remains stable at the human body temperature of 37 °C, demonstrating its potential for *in vivo* applications.

In order to investigate the nanoparticles' morphology, cryo-TEM measurements were performed. Panels B–D of Figure 3 are representative images of caulerpin@cub. Starting from Figure 3B, a lot of cubosome showing their typical honeycomb-like structure are observed⁴⁷ in coexistence with other objects. In agreement with the empty formulation,⁵⁵ the presence of vesicles is probably related to Sp80 and TC in the

composition—vesicle forming ingredients.⁵⁸ Moreover, sponge nanoparticles are observed in the sample and highlighted with red arrows (Figure 3C,D). Their presence is attributed to the formulation's composition, as discussed in detail in the previous SAXS section. The coexistence of the bicontinuous cubic phase with the L3 phase in the formulation loaded with *C. cylindracea* algae pigment indicates the potential influence of hydrophobic cargo on the nanoparticles structure.

Then, caulerpin@cub was studied for the particles' size described by apparent hydrodynamic diameter (D_h), polydispersity index (Pdl), and ζ -potential by DLS and ELS measurements in two different temperatures to evaluate a sample's stability and further application *in vivo*. The results are presented in Table 2. All parameters are in line with the empty

Table 2. DLS and ELS Measurements (Apparent Hydrodynamic Diameter (D_h) of the Nanoparticles, Polydispersity Index (Pdl), and ζ -potential of caulerpin@cub and Empty Cubosomes at 25 and 37 °C^a

	T (°C)	caulerpin@cub	empty cubosomes
D_h (nm)	25	113 ± 2	118 ± 1
	37	115.0 ± 0.3	122 ± 2
Pdl	25	0.20	0.23
	37	0.19	0.25
ζ -potential (mV)	25	−35 ± 1	−35 ± 1
	37	−31 ± 2	−32 ± 1

^aData are reported as average value ± SD.

formulation characterized by the same composition (MO:TC:Sp80:W).⁵⁵ To the best of our knowledge, only a few scientific studies have investigated cubosomes loaded with compounds derived from algae.

No significant differences in size, polydispersity, or ζ -potential were observed, indicating that the temperature increase does not affect these parameters of the formulation, thereby confirming its stability for potential *in vivo* applications.

Concerning the long-term stability of the formulation (Figure S2), the apparent hydrodynamic diameter of the nanoparticles, especially up to week 7, did not show significant changes, while the Pdl value, not exceeding 0.250, confirmed a narrow particle size distribution. The measured ζ -potential, with values always in the range of −30/−40 mV can be called into play for the observed colloidal stability.⁶³

3.3. Biological Activity. **3.3.1. Anticancer Effect Evaluation.** The antitumor effect (Figure 4) of caulerpin@cub was studied by a time-dependent MTT cytotoxicity assay on human pancreatic cancer cell lines (BxPC-3). The tests were performed at different caulerpin concentrations, 0.5, 1, 2, 3, and 4 μ M corresponding to 56, 112, 223, 334, and 445 μ g/mL of MO, after 4 incubation periods, 4, 8, 24, and 48 h (Figure 4A). The series of MTT tests were performed to choose the most efficient caulerpin concentration for further internalization and bioimaging experiments.

After 4 h of incubation with samples at 4 μ M caulerpin concentration, significant differences between samples can be observed. Particularly, caulerpin@cub induced about 60% reduction of the cell's viability vs control. However, the empty cubosomes are weakly cytotoxic at this concentration as well as at 3 μ M, indicating that these concentrations are too high. A lower alkaloid quantity is not toxic for BxPC-3 after 4 h of incubation with all samples. After 8 h of incubation, significant differences between the samples emerge starting at a concentration of 2 μ M. At this concentration, caulerpin@cub exhibits higher cytotoxicity (approximately 55% cell viability vs control) compared to both empty cubosomes and the free compound. At higher concentrations, the empty cubosomes also demonstrate toxicity. MO, the lipid component used to form cubosomes, has been previously evaluated for cytotoxicity on various cell lines, with several studies reporting that significant cytotoxic effects often occur at concentrations of about 100 μ g/mL.^{53,64,65} As caulerpin was diluted in the formulation to obtain the concentrations studied by the MTT assay series, the MO was also proportionally diluted (corresponding MO concentrations are specified in the previous paragraph). The results suggest that the cytotoxicity

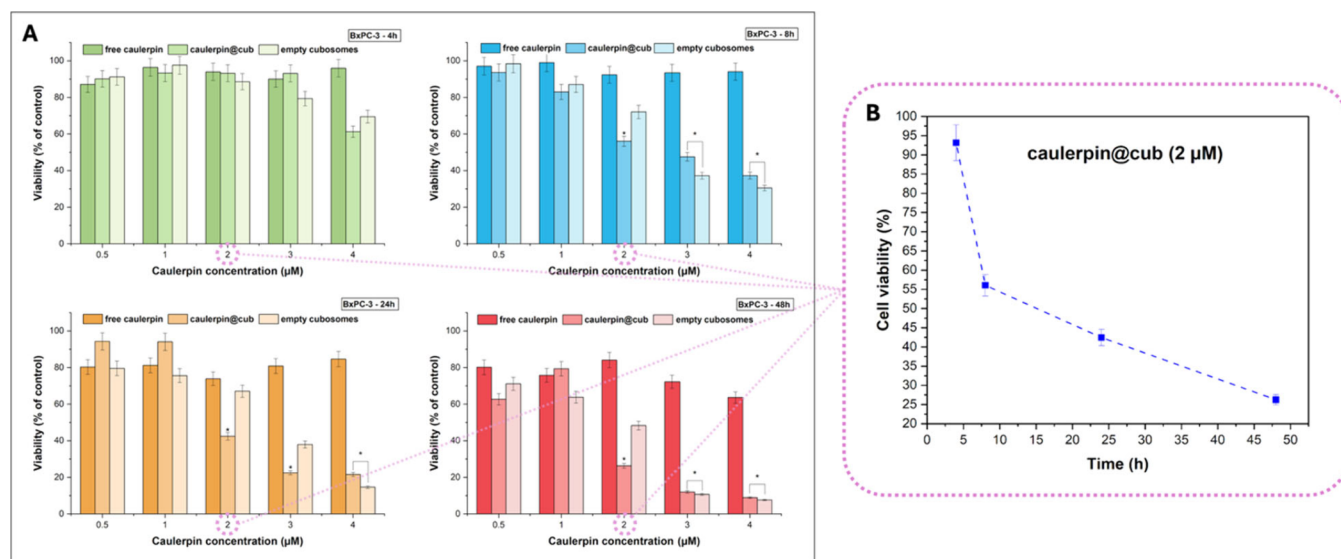


Figure 4. *In vitro* cytotoxicity experiments. MTT test on BxPC-3 cells after 4, 8, 24, and 48 h of incubation with free caulerpin, empty cubosomes, or caulerpin@cub at different alkaloid concentrations (0.5, 1, 2, 3, and 4 μ M), corresponding to 56, 112, 223, 334, and 445 μ g/mL MO (A); dependence of cell viability on incubation time for the selected concentration of 2 μ M (B). * $p \leq 0.05$.

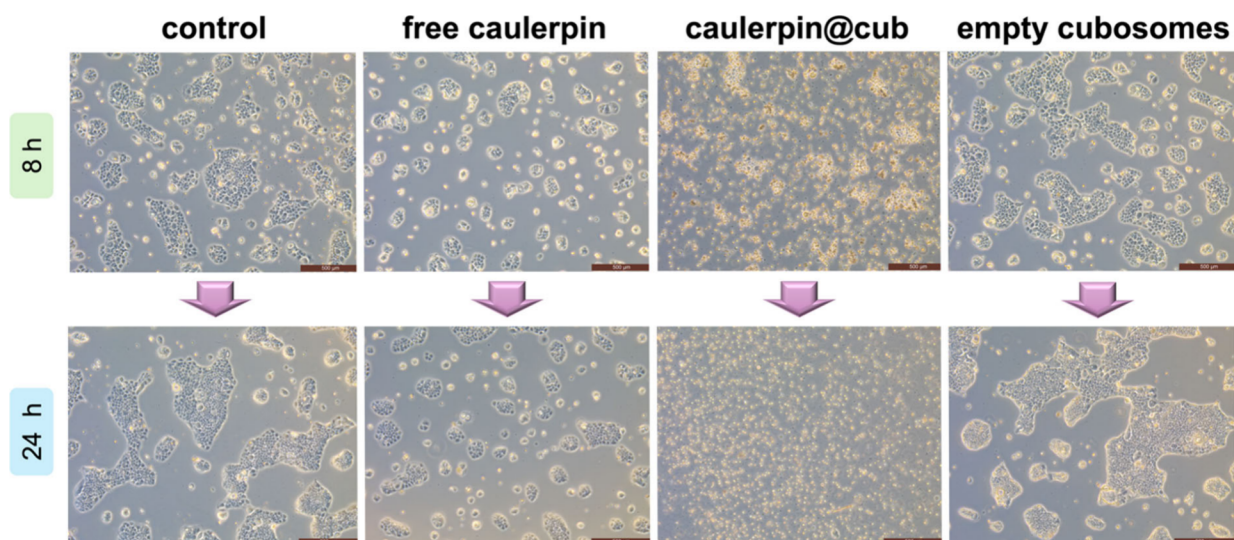


Figure 5. BxPC-3 cells morphology assessment by optical microscopy, after 8 and 24 h of incubation with different samples: free caulerpin (2 μM), caulerpin@cub (2 μM) and empty cubosomes, in comparison to untreated control cells. The scale bar represents 500 μm .

threshold of the unloaded nanocarriers in this study is even higher than previously reported, as the concentration of 112 $\mu\text{g/mL}$ MO (equivalent to 1 μM of caulerpin) still presented over 60% cell viability after 48 h, indicating only “weak” cytotoxicity. This suggests an increased biocompatibility compared to conventional Pluronic-based cubosomes, as shown in our previous work.⁵⁵ The concentration-dependent cytotoxicity becomes more pronounced at 48 h, particularly at higher MO levels (223, 334, and 445 $\mu\text{g/mL}$), which aligns with findings from several earlier studies indicating that such concentrations significantly surpass the biocompatibility limits for these nanocarriers.^{53,64,65} After 24 h, the difference becomes more pronounced, with caulerpin@cub at 2 μM showing strong cytotoxicity, reducing cell viability to about 40%. In contrast, free caulerpin does not exhibit significant toxicity after 24 h, highlighting the advantage of encapsulating caulerpin within cubosomes. Although, research on caulerpin cytotoxic effects against pancreatic cancer remains an unexplored area, caulerpin in its free form has been tested against many human cancer cell lines.^{3,4,14,25} For instance, IC_{50} after 48 h of incubation was demonstrated only at very high concentrations (119 and 179 μM) of caulerpin against colorectal cancer cell lines: HCT-116 and HT-29, respectively.⁴ This phenomenon may explain the lack of a strong cytotoxic effect (about 60% of remaining cell viability) in the case of free caulerpin tested in our study, even at the highest concentration of 4 μM and the longest incubation (48 h). After this time, caulerpin@cub at 2 μM reaches 25% (strong cytotoxicity); however, it can be observed that even the empty cubosomes start to be significantly cytotoxic to BxPC-3 (about 50% of remaining cell viability). Ferramosca et al. have investigated the cytotoxicity of caulerpin against two human ovarian carcinoma cell lines (2008, wild type, and C13, cisplatin-resistant type) after an even longer incubation time (72 h) and achieved only 60% of cell viability in the case of 2008, and just over 30% in the case of C13.²⁵ This highlights the remarkable potential of caulerpin encapsulation in cubosomes for the treatment of pancreatic cancer as it significantly reduces the incubation time required to achieve substantial cytotoxicity, even against the highly resistant BxPC-3 cell line. The work of Zhou et al. describes studies on BxPC-

3 cells and their resistant subtypes (BxPC-3-GR).⁶⁶ The IC_{50} after 48 h of incubation with gemcitabine for BxPC-3 cells was $0.024 \pm 0.003 \mu\text{M}$, while for BxPC-3-GR cells it increased to $2.68 \pm 0.104 \mu\text{M}$, indicating a significant development of drug resistance in this type of pancreatic tumor cells.⁶⁶ Furthermore, some other well-known anticancer drugs, namely, cisplatin, capecitabine, and irinotecan, presented an IC_{50} of $1.656 \pm 0.284 \mu\text{M}$, $7.890 \pm 0.911 \mu\text{M}$, and $2.530 \pm 0.082 \mu\text{M}$, respectively, after 48 h of treatment on BxPC-3 cells.⁶⁶ In our work, even at the highest tested dose (4 μM), free caulerpin showed only 60% of cell viability after 48 h, whereas after loading in nanocarriers, significant cytotoxicity (approximately 40%) was demonstrated already after 24 h at a concentration of 2 μM . Such value is comparable with the IC_{50} of the mentioned pancreatic cancer drugs (cisplatin, irinotecan), almost 4 times lower than IC_{50} of capecitabine studied by Zhou et al. on BxPC-3 cells, as well as comparable to gemcitabine studied against resistant BxPC-3 populations. Remarkably, our result of 40% cell viability in cells treated with caulerpin@cub was achieved after only 24 h of incubation—half the duration used in comparable studies—indicating its strong cytotoxic effect relative to already known pancreatic cancer drugs. Mechanistically, caulerpin targets mitochondrial complex I, PTP1B, and the AMPK/mTORC1 axis,^{14,24} pathways distinct from gemcitabine’s nucleoside-analog mechanism.⁶⁷ This orthogonal mode of action makes caulerpin@cub a potential candidate for overcoming gemcitabine resistance or combination therapy.

In summary, these results indicate that a concentration of 2 μM caulerpin was the most suitable for subsequent biological tests on BxPC-3 cells. This choice is supported by the observed influence of caulerpin encapsulation on antitumor bioactivity along with the demonstrated biocompatibility of the empty cubosomes. The optimal time of action is 24 h of incubation, because after 48 h, moderate cytotoxicity was observed also in the case of empty cubosomes. Figure 4B shows the dependence of cell viability on incubation time for the selected concentration of 2 μM in the case of caulerpin@cub, which decreases while extending the incubation time, highlighting the antitumor potential of the tested formulation.

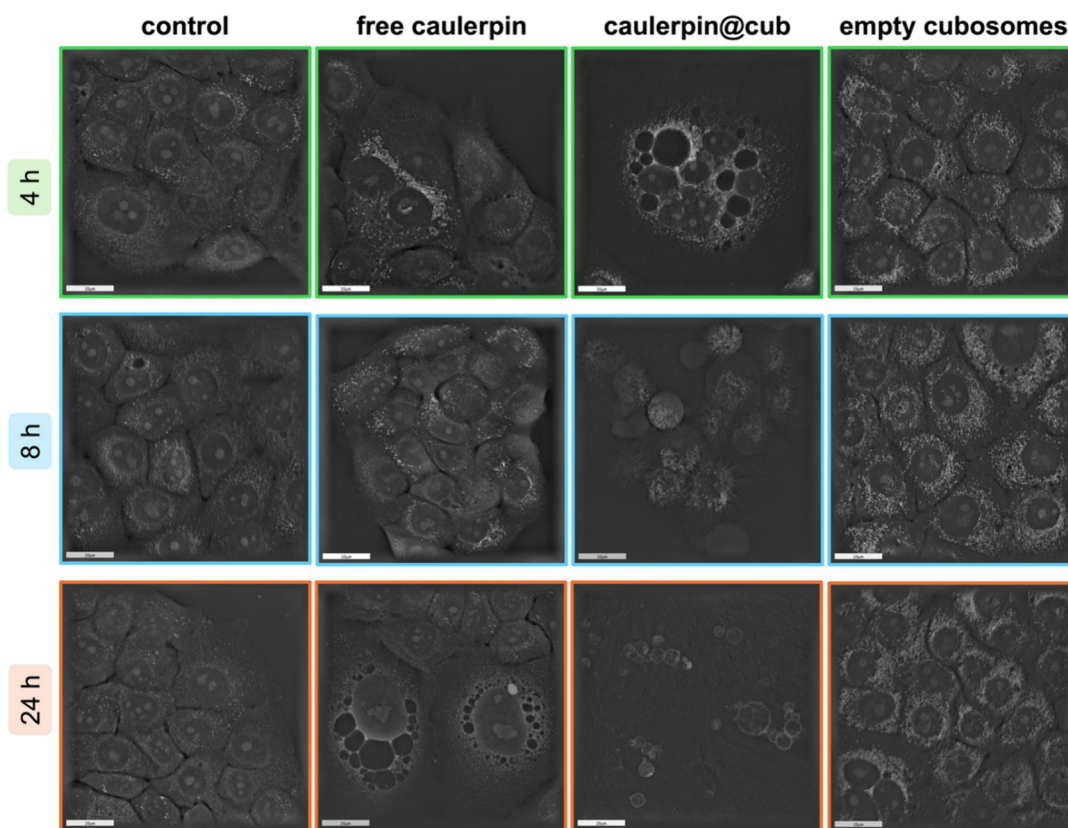


Figure 6. BxPC-3 cells morphology assessment by holotomographic microscopy, after 4, 8, and 24 h of incubation with different samples: free caulerpin (2 μ M), caulerpin@cub (2 μ M), and empty cubosomes, in comparison to untreated control cells. The scale bar represents 20 μ m.

3.3.2. Bioimaging of the Cell Morphology. For comprehensive evaluation of the BxPC-3 cells morphology and thus the influence of the tested samples on the appearance and behavior of such cells, the assessment was based on three different microscopic experiments, showing both the entire cell culture on a larger scale by optical microscopy but also specific cells, cell clusters, or apoptotic features by live cell HTM or F-actin fluorescence bioimaging at a higher magnification.

3.3.2.1. Optical Microscopy (Culture Morphology). In order to assess the culture morphology of BxPC-3 pancreatic cancer cells, a series of images were taken supravivally by optical microscope after two incubation times: 8 and 24 h. Free caulerpin (2 μ M), caulerpin@cub (2 μ M), and empty cubosomes were examined against control cells (Figure 5). The images of BxPC-3 cells, shown on a scale of 500 μ m, indicate significant differences in the morphology of human pancreatic tumor cells after incubation with selected samples. As for the control cells, in principle only the surface of their growth increases, the clusters are larger, and the condition of the cells is appropriate. Similar observations can be done by comparing the images of the empty cubosomes for 8 and 24 h, supporting their biocompatibility. On the other hand, cells treated with free caulerpin (2 μ M), present smaller clusters or single small cells or their parts are visible, which indicate the cellular stress conditions and potential induction of apoptosis by caulerpin toward BxPC-3. Indeed, during apoptosis the cell actively breaks down its components and decreases in size by forming and separating portions of the cytoplasm and nucleus into apoptotic bodies.⁶⁸ However, after longer incubation, apoptosis does not occur, and the cells continue to grow in small clusters, metabolizing caulerpin and regenerating in the

process. In contrast to the above-mentioned samples, caulerpin@cub presents strong toxicity against BxPC-3, because in the image presenting cells treated with this sample after 24 h, only remnants of dead cells are visible. After the typical apoptotic process, cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation, as well as plasma membrane blebbing, apoptotic bodies are formed, which are phagocytosed and degraded.⁶⁹ Therefore, in conclusion, these results indicate the potential effectiveness of caulerpin@cub in combating extremely resistant pancreatic cancer, additionally indicating the advantages of the encapsulation and biocompatibility of empty cubosomes.

3.3.2.2. Holotomographic Microscopy (HTM). To go deeper into the details of the morphological changes in pancreatic cancer cells, a holotomographic microscope was used. This is a valuable new tool which combines two techniques, holography and tomography, useful for quickly distinguishing healthy from apoptotic cells, as well as monitoring both external and internal morphological cellular changes supravivally, using a non-invasive, label-free method.^{70,71} According to the studies of Salucci et al., HTM seems to be a reliable method for further analyzing well-established apoptotic features, including cell blebbing, chromatin condensation, micronuclei, and apoptotic bodies.⁷⁰ BxPC-3 cells were treated with different samples: empty cubosomes, caulerpin@cub (2 μ M), and free caulerpin (2 μ M). Results relative to control cells are shown in Figure 6. The images were taken supravivally after 4, 8, and 24 h of incubation. Starting from the cells treated with free caulerpin, after 4 h the accumulation of lysosomes inside the cell is noticeable, indicating induced cellular stress and some processes related

to autophagy, probably due to the presence of caulerpin. Autophagy, in addition to being a vital process for cellular turnover, is also induced by cellular stress conditions and serves as a crucial repair mechanism for damaged cells and helps them survive periods of temporary starvation.⁷² Lysosomes are membrane-bound organelles, present in all animal cells (except erythrocytes), within which the breakdown of materials taken up by endocytosis or autophagy, takes place.⁷³ The extent of lysosomal destabilization during cellular stress conditions determines whether reparative autophagy, or typical cell death types, apoptosis or necrosis, will occur.⁷² Moreover, some vacuoles are visible there, another element indicating unfavorable and stressful conditions for cells. Vacuolization is one of the hallmarks of autophagy, which can further lead to necrosis or apoptosis.⁶⁹ In cancer cells, under the influence of stress, e.g., exposure to anticancer drugs, lysosomes enlarge and fuse with autophagosomes, forming structures resembling vacuoles, such as doxorubicin-induced autophagolysosome formation in the resistant breast cancer cells.⁷⁴ This phenomenon is consistent with previous reports, which indicate that the TFEB pathway is activated in response to stress, leading to increased lysosome biogenesis and autophagy.^{75,76} After the phase of intensive lysosomal fusion and the formation of large autophagolysosomes, cells can undergo a regeneration process, which is manifested by a decrease in the volume of these structures and restoration of homeostasis. Such reversible nature of lysosome reorganization suggests that they not only perform a degradative function but also actively participate in the adaptation and survival of cancer cells under stress conditions, e.g., drug stress.⁷⁶ Additionally, Zhitomirsky and Assaraf emphasize the role of lysosomes in cancer cell resistance to chemotherapy, indicating that lysosomes sequester drugs, inactivating them and reducing the efficacy of treatment.⁷⁷ In continuation of these findings, they showed that excessive accumulation of drugs in lysosomes can trigger lysosomal exocytosis—a process in which the contents of lysosomes, including stored drugs, are actively removed from cells, increasing their resistance to chemotherapy.⁷⁸ In addition to this, in 2014, Maltese and Overmeyer have studied a nonapoptotic cell death called “methuosis”, with characteristic displacement of the cytoplasm by large, fluid-filled vacuoles that originate from macropinosomes.⁷⁹ The vacuoles are particularly visible in the image of the caulerpin@cub sample. Cells treated with empty cubosomes after 4 h of incubation do not show any differences with the control, and only an enhanced number of intracellular vesicles is visible. After 8 h, cells incubated with free caulerpin exhibit morphological characteristics indicative of potential regeneration. This observation suggests that free caulerpin at a concentration of 2 μM is not toxic to BxPC-3 cells within this time frame. This result can be explained by the resistance of pancreatic cancer to most conventional treatment methods,¹⁹ due to its resilience related to remarkable capacity to orchestrate a special tumor protective microenvironment full of different components with huge impact on cancer cell biology, and therefore on a treatment resistance.^{18,80} One of the mechanisms of pancreatic cancer cells, described by Chand et al. as *hypoxia-induced resistance*, is linked to the nutrient- and oxygen-deprived tumor microenvironment. Hypoxia occurs when cancer cells do not receive enough oxygen, which is a result of uneven development of blood vessels within the tumor.⁸¹ This condition creates selective pressure that promotes the survival and growth of the most aggressive and

resilient pancreatic cancer cells, making them highly resistant to cytotoxic chemotherapeutic agents.¹⁷ To overcome the harsh stress imposed by chronic hypoxia, pancreatic cells orchestrate a multifaceted response by activating hypoxia-inducible factors (HIFs),⁸² special protecting proteins able to regulate tumor cell biology. It turns out that this transcriptional response is involved in drug resistance, demonstrated by Cheng et al., who proved that under hypoxic conditions siRNA inhibition of either HIF-1 α or NF κ B, has sensitized pancreatic cells to gemcitabine, a commonly used cytostatic drug.⁸³ When hypoxic cells were treated with gemcitabine at concentrations > 0.1 μM , their viability rate was significantly higher than that of normoxic. This suggests that hypoxia may contribute to chemoresistance in pancreatic cancer cells.⁸³ Under normoxic conditions for 0.5 μM gemcitabine, the BxPC-3 cells viability was shown at the level of about 60% (comparable with 0.5 μM caulerpin@cub after 48 h), in contrast to hypoxia where this value was slightly below 80% after 48 h.⁸³ However, for 1 μM gemcitabine, about 40% of viable cells were present (in normoxia) and about 70% (in hypoxia). In our study, a concentration of caulerpin twice as high was required to achieve a 40% cell viability when combined with nanocarriers; nevertheless, this effect was obtained in half the incubation time (24 h). Moreover, since caulerpin can additionally influence this process, by sensitizing cells to its action in hypoxia,^{3,83} this may constitute its additional advantage in the potential treatment of pancreatic tumor characterized by such conditions.

A significant difference with other samples can be observed in the image of cells treated with caulerpin@cub after 8 h of incubation because the process of apoptosis is operative. The round structures resembling blebs are the cytoplasm thrown out by cells that began to detach from the substrate and die. Dying cells are characterized by the formation of cellular buds, fragments, or blebs.⁶⁹ Ziegler and Groscurth have observed the shrinkage of cell, blebbing, and apoptosis bodies formation as morphological features of ongoing cell death.⁸⁴

According to Liu et al., caulerpin can disrupt mitochondrial ROS-regulated HIF-1 activation and HIF-1 downstream target gene expression by inhibiting the transport or delivery of electrons to complex III.³ As proved by Cheng et al. in 2014, this anticancer strategy based on overcoming the hypoxia-induced resistance mechanism may be responsible for sensitizing cancer cells to the action of potential drug.⁸³ Another anticancer strategy may be related to caulerpin's PTP1B inhibitory activity,^{23,24} since Xu et al. proved that inhibition of PTP1B protein blocks pancreatic cancer progression by targeting the PKM2/AMPK/mTOC1 pathway.²² Moreover, compared to the result of the free compound, a noticeable advantage of encapsulation is presented, probably by enabling the compound to reach the interior of cells and therefore increase its anticancer activity. These results show that although caulerpin may exhibit some anticancer properties, treating cells with this compound in its free form is insufficient to achieve the intended effect. After loading caulerpin in relevant nanocarriers, apart from introducing a stressful situation, an increase in apoptotic processes of cancer cells is visible, indicating a significant influence of the encapsulation on the caulerpin's effectiveness in fighting pancreatic cancer. No significant premature release of caulerpin from the tested cubosomes, demonstrated by the *in vitro* drug release test (Figure S3), highlights the potential of its efficient delivery encapsulated within the nanocarriers with

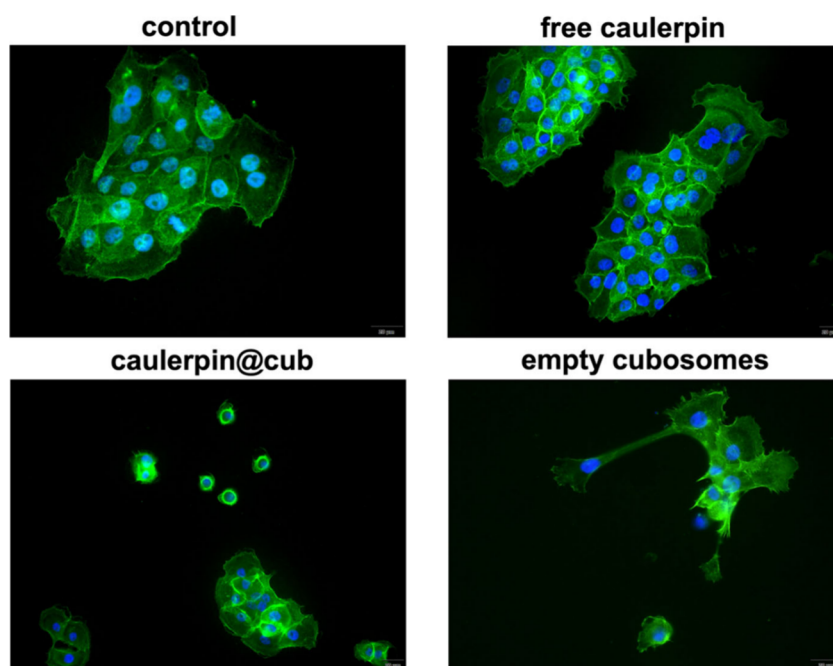


Figure 7. F-actin staining fluorescence bioimaging performed on human pancreatic cancer cell lines (BxPC-3) after 24 h of incubation. Cells were treated with different samples: free caulerpin (2 μ M), caulerpin@cub (2 μ M) and empty cubosomes, in comparison to untreated control cells. The scale bar represents 20 μ m.

the protection from undesirable off-target activity or degradation, thus explaining its enhanced effect. After 8 h, the empty cubosomes induce the secretion of even more intracellular vesicles but do not fatally affect the condition of BxPC-3 cells, their morphology being similar to the control cells. After 24 h, free caulerpin begins to induce the formation of increasingly larger vacuoles, indicating still ongoing cellular stress. However, there are still many healthy cells that are visible. In the image of cells treated with caulerpin@cub after 24 h of incubation, basically only the remains of cells and thrown out cytoplasm suspended in the medium are visible (hence the poor sharpness of the image). After 24 h of cell incubation with empty cubosomes, the accumulation of vesicles is significantly greater but still not inducing cells' death. These experiments show that the most effective against pancreatic cancer BxPC-3 cells is caulerpin (2 μ M) loaded in cubosomes after 24 h. However, in the case of free caulerpin, the cells demonstrate the ability to metabolize it and regenerate over time, indicating a high level of resistance.

3.3.2.3. Cytoskeleton F-Actin Fluorescence Staining. To confirm previously discussed results about the effectiveness of the given caulerpin@cub formulation against pancreatic cancer, we performed cytoskeleton bioimaging studies. **Figure 7** presents the fluorescence bioimaging of BxPC-3 cells cytoskeleton by F-actin staining, treated with caulerpin, caulerpin@cub, and empty cubosomes formulations, along with control. Cells were incubated with test samples for 24 h at the 2 μ M caulerpin concentration. It is important to notice that the results obtained with different imaging techniques may differ due to their unique properties and range of detection of cellular structures. While in the case of previous live HTM technique some structures are visible, after F-actin staining the cells are fixed, and the permeabilization may further remove the transient vacuoles visible by previous live cell bioimaging, so the outlines look smoother. Moreover, F-actin staining visualizes the actin cytoskeleton but does not reveal

membranous structures like lysosomes or autophagolysosomes, with the intense peripheral actin signal potentially masking internal structures and limiting the ability to assess lysosomal quantity and function without additional specific staining.

In the cell bioimaging treated with caulerpin@cub formulation, the disruption of intercellular connections can be observed, as well as the shrinkage of some of the BxPC-3 cells, and only the nuclei are visible with the residual cytoplasm, which was probably dehydrated in the process of apoptosis,^{84,85} as shown by previous HTM studies (section 3.3.2.2). In addition, there is a reduction in the nuclei size, probably related to nuclear condensation occurring during the process of cell death.⁶⁹ This observation contrasts with the other images of the control cells, free compound (caulerpin), and the empty cubosomes, where the cells look healthy and have the appropriate morphology, considering the smaller number of clusters in the case of cells treated with cubosomes sample. Bioimaging of cells treated with free caulerpin reinforces the previous conclusion that BxPC-3 cells can metabolize the free compound and regenerate. Furthermore, when compared to caulerpin@cub, the benefits of caulerpin encapsulation—such as enhanced bioactivity efficiency—are once again demonstrated. This further illustrates that it is possible to overcome the challenges posed by the highly diverse and complex pancreatic tumor microenvironment, which often traps potential drugs and hinders their effectiveness. Thanks to the ability of cubosome nanocarriers to transport the bioactive cargo and reach the interior of cells, it is possible to induce cancer cells' apoptosis. As previously mentioned, several pathways could explain such an anticancer activity of caulerpin. Available data reveal that caulerpin inhibits mitochondrial complex I, causing $\Delta\Psi_m$ loss and an early ROS release.¹⁴ Furthermore, it was proved that further prolonged AMPK activation related to mTORC1-4E-BP1 axis inhibition, induced by caulerpin, disrupted glycolysis, and glucose metabolism in colorectal cancer cells, ultimately

leading to their death.¹⁴ It was also demonstrated that caulerpin can inhibit PTP1B²⁴ and block HIF-1 stabilization under hypoxia by inhibition of the mitochondrial electron transport to complex III.³ These mechanisms are involved in the intrinsic (mitochondrial) apoptotic pathway and match the lysosomal swelling, chromatin condensation, and membrane blebbing that we report. Similar conclusions were reached in earlier biological experiments presented in this work, further supporting the thesis that the formulation based on caulerpin encapsulated in cubic liquid crystalline nanocarriers at the appropriate concentration (2 μ M) exhibits strong antitumor activity against BxPC-3 cells.

4. CONCLUSIONS

Pancreatic cancer remains one of the most challenging cancers to treat due to its high resistance to conventional therapies. With the aim to find an effective treatment method, this study explores the potential of caulerpin, a naturally occurring bisindole alkaloid derived from

C. cylindracea, which has demonstrated promising anticancer properties, but has not yet been tested against pancreatic cancer. The innovative approach presented here combines green chemistry with nanotechnology, namely, MAE to isolate pure caulerpin from *C. cylindracea* invasive algae biomass, and its encapsulation in novel biocompatible bicontinuous cubic liquid crystalline nanoparticles, cubosomes. The use of biocompatible surfactants (TC and Sp80) in the proposed formulation minimizes cytotoxicity, promoting efficient drug delivery with its protection against degradation, therefore increasing therapeutic potential. The physicochemical characteristics confirmed high encapsulation efficiency of the formulation, characterized by the presence of cubosomes coexisting with L3 sponge nanoparticles and vesicles. The formulation was proved stable even at 37 °C. A series of cytotoxicity tests allowed for the selection of the appropriate, optimal concentration of caulerpin (2 μ M) for subsequent studies. After the initial assessment of cancer cells morphology by optical microscopy, an in-depth morphological analysis was performed using the HTM technique, suitable for the investigation of apoptotic features. Moreover, the anticancer effect of the caulerpin@cub formulation against BxPC-3 cells was confirmed by F-actin bioimaging, indicating, among others, a strong cell shrinkage and destruction of the cytoskeleton. In general, this work demonstrated significant apoptotic effects on human pancreatic cancer cells of the caulerpin encapsulated within cubosomes, showing superior antitumor activity, in contrast to the free compound. Loaded caulerpin is not metabolized by cancer cells as quickly as its free form, which enables its effective action by inducing apoptotic processes and ultimately leading to cells death. These results suggest the possibility of resistance problem reduction and a more effective anticancer action.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.5c00340>.

¹H NMR spectrum of isolated caulerpin in CDCl₃ along with its description, results of the long-term formulation stability test, as well as additional experimental data and results concerning the *in vitro* drug release test (PDF)

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Notes

The authors declare no competing financial interest.

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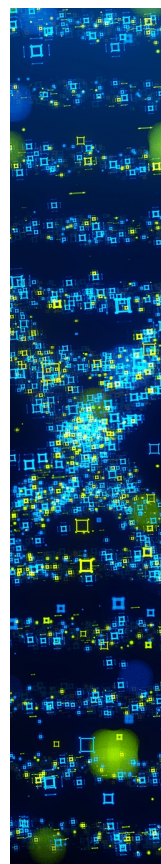
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SUPPORTING INFORMATION

Caulerpin Delivery via Pluronic-Free Cubosomes: Unlocking the Therapeutic Potential of a Pigment from an Invasive Marine Algae

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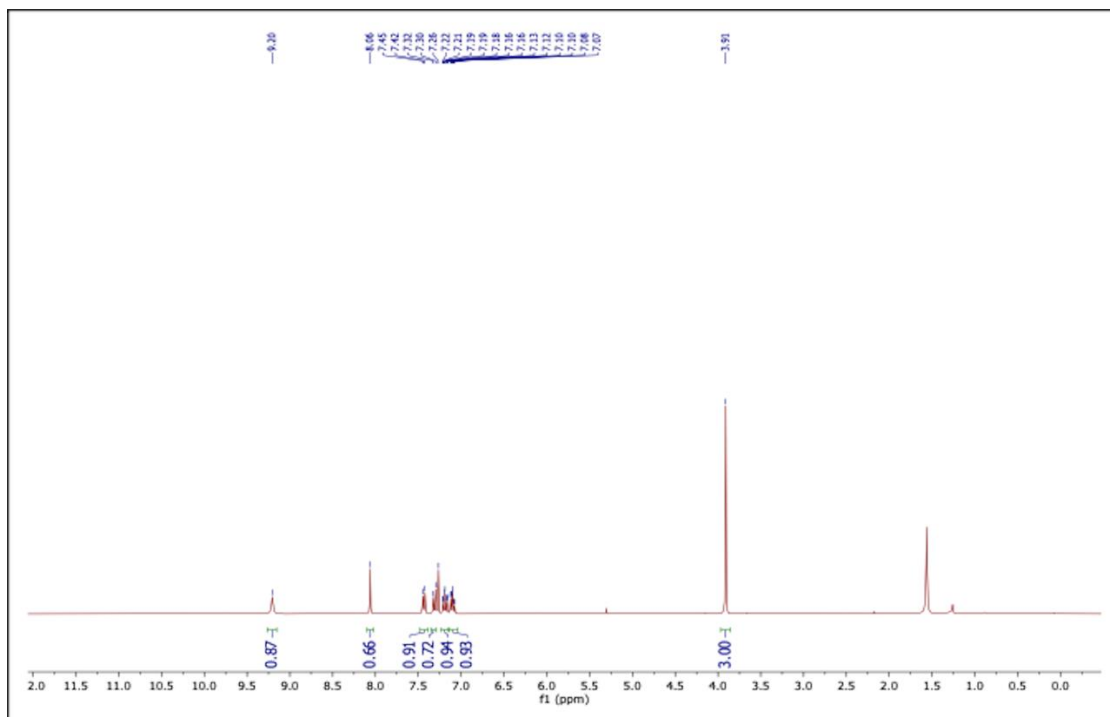


Figure S1. ^1H NMR spectrum of isolated caulerpin in CDCl_3

The ^1H NMR spectrum of the isolated compound presents the signals corresponding to caulerpin's chemical structure, correlating with the literature data of caulerpin standard in the same solvent.¹ The signal at 9.20 ppm is identified with 2 protons from 2 amino groups, hydrogen bonded to the ester carbonyls. Then, at 8.06 ppm, a signal from 2 protons bonded to the cyclooctatetraene ring connecting two indole groups is highlighted. Further, from 7.07 to 7.45 ppm, there are signals from 8 protons bonded to two aromatic rings and at 7.26 ppm, there is a signal from the solvent, CDCl_3 . At 3.91 ppm, 6 protons from 2 methyl ester groups, bonded to the central ring, present the highest signal. The last peak is identified with the residual water. Overall, these spectroscopical characteristics suggest that the final product from *C. cylindracea* is indeed pure caulerpin.

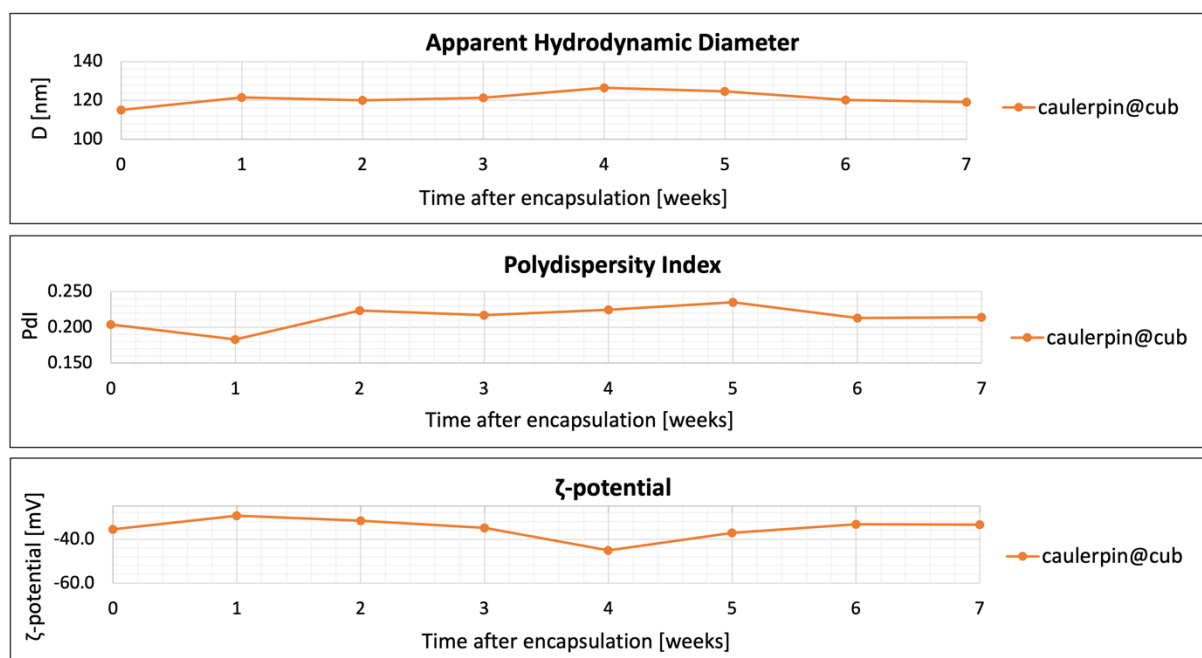


Figure S2. caulerpin@cub stability assessment by week-by-week DLS and ELS measurements.

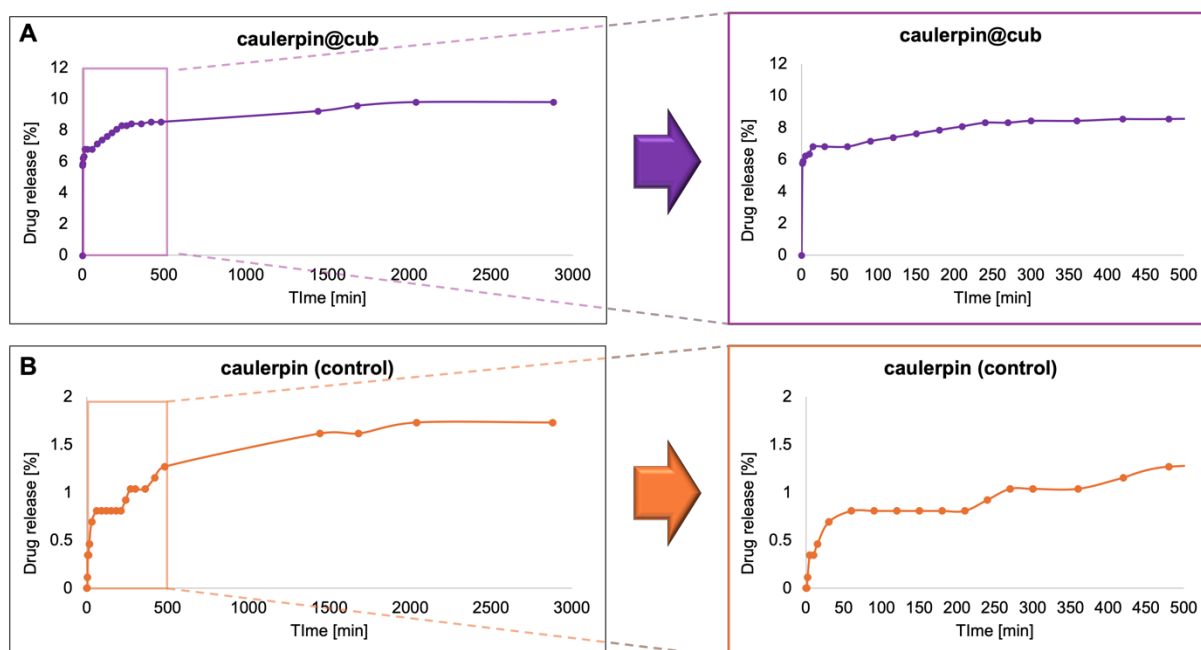


Figure S3. Encapsulated caulerpin release profiles (A) and the free pigment as a control (B).

Experimental

The release behavior of the encapsulated caulerpin was studied using UV-Vis spectroscopy combined with a dialysis technique according to the previous protocols²⁻⁴. A dialysis bag (Carl Roth, Germany) with a molecular weight cut-off of 14 kDa was used. Namely, 2 mL of the prepared cubosome formulation loaded with caulerpin was enclosed in the dialysis membrane and submerged in 25 mL of phosphate-buffered saline (PBS) at a physiological pH of 7.4, which acted as the release medium. The setup was then placed on a magnetic stirrer set to 200 rpm. To monitor drug release, 1 mL samples were taken from the external medium at regular time intervals and immediately replaced with fresh PBS of the same pH. The percentage of drug released was then calculated by comparing the amount of free caulerpin in the medium to the total drug content in the original sample using UV-Vis spectroscopy.

Discussion

The drug release profile of caulerpin at physiological pH (7.4) from the cubosomes is reported in Figure SI3. The initial amount of caulerpin detected is related to non-encapsulated drug during cubosomes formulation. Therefore, release of caulerpin over 48 hours did not exceed 4%, indicating slow and low release of this potential drug. Hydrophobic drugs can be released from carriers into the aqueous environment due to diffusion across the carrier membrane, concentration differences, and drug-water interactions. Although these drugs are less likely to pass into water, they can gradually migrate out of the carrier, especially when the carrier

structure is destabilized or due to osmotic pressure. Such release is a natural physicochemical process that can occur despite the hydrophobic nature of the drug molecule.⁴ This finding demonstrated that a small amount of free caulerpin may be released, despite its hydrophobic nature. Nevertheless, no significant premature release was observed during the test.

The drug is well encapsulated in the nanocarrier, as already proved by EE determination. Caulerpin is a hydrophobic pigment which is strongly bounded to the lipid matrix of the cubosome, thus hindering its diffusion into the aqueous environment of PBS. The premature release is avoided in this case, since it is expected that the nanocarrier will protect the drug from degradation or off-target activity before it reaches the target tumor tissue. The uptake of cubosomes by cancer cells is associated with the EPR (Enhanced Permeability and Retention) effect, which involves passive accumulation of these nanocarriers within the tumor due to the specific structure of its blood vessels characterized by leakage. The cubosomes can "leak" from the bloodstream into the tumor tissue thanks to their appropriate size to achieve this effect (~20–200 nm).^{5,6} Lyotropic liquid crystalline nanoparticles, such as cubosomes, are known for their increased bioavailability and prolonged drug action due to its sustained release.^{7–10} Longer circulation in the blood is associated with greater chance of reaching the tumor (especially with the EPR effect). Long retention of the drug in the nanocarrier extend its half-life and allow for longer action in the body, expected in case of cancer treatment. Since the cubosomes are stable at physiological pH, they do not degrade or disintegrate, therefore we believe that caulerpin will not be significantly released before the nanocarrier rich tumor cells, where will be internalized via endocytosis and due to specific tumor conditions (such as acidic microenvironment and lysosomal enzymes) the breakdown of the cubosomes lipid matrix and release the drug intracellularly will be promoted. Since the anticancer activity of caulerpin@cub was proved by series of *in vitro* tests on BxPC-3 cells, we believe that such reasoning provides potential application *in vivo*.

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