Chitosan and hyaluronan coated liposomes for pulmonary administration of curcumin

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Abstract

Aiming at improving the nebulization performances and lung antioxidant protection of curcumin, chitosan or hyaluronan-coated liposomes were prepared and their characteristics and performances were compared with that of uncoated liposomes. Curcumin loaded liposomes displayed a diameter lower than 100 nm, the coating with both polymers led to a small increase of vesicle size around 130 nm and the zeta potential turned to positive values using chitosan while remained negative using hyaluronan. Chitosan allowed the formation of more lamellar and stiffer vesicles with a higher bilayer thickness ($d_B \sim 59$ Å) with respect to the uncoated liposomes, whereas hyaluronan allowed the interdigitation of the bilayers ($d_B \sim 47$ Å) due to the polymer intercalation between phospholipid head groups resulting in vesicles mainly organized in uncorrelated bilayers. Both polymer coatings, especially hyaluronan, greatly improved the stability of the vesicles, especially during the nebulization process, promoting the deposition of

the phytodrug in the furthest stages of the impactor in high amount (≥50%). Polymer coated vesicles were biocompatible and improved the curcumin ability to protect A549 cells from the oxidative stress induced by hydrogen peroxide, restoring healthy conditions (cell relative metabolic activity 100%). In particular, a synergic effect of curcumin and hyaluronan was observed resulting in a proliferative effect and a subsequent further enhancement of cell relative metabolic activity up to 120%.

Keywords: hydrophilic polymers, coated liposomes, oxidative stress, pulmonary delivery, A549 cells.

1. Introduction

Extensive research have shown that many lung diseases, such as cystic fibrosis, asthma, chronic obstructive pulmonary disease, tuberculosis and continuous respiratory infections, can result in chronic local inflammation causing broad lesions, changes in the architecture of the airway walls, cell modification and cancer development (Mantovani et al., 2008; Nair et al., 2010).

Synthetic drugs have been used for many years with the aim to control these pulmonary diseases, but the chronic administration is often associated to undesired side effects or drug resistance, which is responsible of therapy failure. For this reason, the use of natural and safe active ingredients, as therapeutic agents or as adjuvants of synthetic molecules, has raised large interest.

Among others, curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a bioflavonoid obtained from the rhizome of *Curcuma longa*, has shown a wide variety of promising pharmacological activities such as anti-inflammatory, anti-microbial, anti-cancer, and anti-Alzheimer as demonstrated in both preclinical and clinical studies (Naksuriya et al., 2014). The bioflavonoid is likely stopping the inflammatory cascade process by its synergic antioxidant and anti-inflammatory activity (Manju and Sreenivasan, 2011).

However, because of its chemical structure and low water solubility, curcumin bioavailability and stability are very low and its administration, regardless the route chosen, often results in poor therapeutic effect. The use of nanotechnologies to deliver natural drugs, represents a useful strategy to overcome such kind of problems. Thanks to their high biodegradability and biocompatibility, liposomes are suitable lung delivery systems that can offer advantages for local administration such as uniform deposition and high pulmonary surface distribution of the active agent.

In previous studies, curcumin was loaded in several liposomal formulations and their properties were tested by using different administration routes: i.e., the therapeutic properties of curcumin loaded liposomes in lung cancer cells were evaluated and compared with those of native and β-cyclodextrin enclosed phytodrug (Rahman et al., 2012); in another work, liposomes prepared with dimyristoylphosphatidylcholine and loading curcumin were used, and their ability to inhibit relative metabolic activity of cancer cells (70-80%) was demonstrated (Lee et al., 2014; Thangapazham et al., 2008). On the contrary, few studies focused on the preparation and evaluation of the efficacy of curcumin loaded phospholipid vesicles for lung delivery. Therefore, in this work, to fill this lack of knowledge, polymer surface-modified liposomes were designed to improve curcumin bioavailability and retention into lung tissue. The addition of suitable polymers represents a valid strategy to protect liposomes from enzymatic attack, improve their mucoadhesion properties and increase the local bioavailability of the drug (Manca et al., 2014, 2011; Taylor et al., 2010). Among the various polymers able to interact with liposome surface, chitosan possesses good mucoadhesiveness and, as already described, is able to protect and stabilize vesicles (Lemarchand et al., 2004). Surface modification of particles with chitosan has already been demonstrated to prolong their residence time in the airways in comparison with uncoated ones, thanks to ionic interactions between positive amino groups of the polymer and the negative mucus gel layer (Mura et al., 2011; Zaru et al., 2009). Besides, the negatively charged hyaluronic acid has also been extensively used to protect liposomes and enhance their

77 mucohadesive properties (Lim et al., 2000; Pritchard et al., 1996). It is a natural non-toxic,
78 biodegradable and non-immunogenic, polysaccharide composed of alternating d-glucuronic acid
79 and *N*-acetyl-d-glucosamine repeating units (Dosio et al., 2016). Hyaluronic acid is native in all
80 the extracellular matrix of human organism as well as in lung environment where it is
81 metabolized by endogenous enzymes (Oh et al., 2010). As previously reported, the hydrophilic
82 hyaluronan can improve liposome stability and drug delivery performances because of its
83 bioadhesive properties as well as its capability to act as a targeting moiety to the epithelial cells
84 expressing cluster determinant 44 (CD44) (Dufaÿ Wojcicki et al., 2012; Lemarchand et al., 2004;
85 Oh et al., 2010). Indeed, hyaluronic acid binds to the extracellular domain of CD44, a cell surface
86 molecule, providing its activation by either a conformational change in the CD44 molecule or a
87 change in the distribution of CD44 molecules on the cell surface (Lesley et al., 1992). CD44 is
88 ubiquitously expressed on leukocytes and parenchymal cells including normal endothelial,
89 epithelial and smooth muscle cells, and is widely over-expressed in inflamed and tumour
90 initiating tissues (Dosio et al., 2016; Nascimento et al., 2016). Furthermore, Teder and
91 collaborators demonstrated its presence in lung cells where it plays an essential role in the
92 resolution phase of pulmonary inflammation, (Dentener et al., 2005; Papakonstantinou and
93 Karakiulakis, 2009; Teder et al., 2002).
94 In this work, liposomes were prepared using a mixture of soybean phospholipids and curcumin,
95 and their surface was coated by adding chitosan or sodium hyaluronate. Physico-chemical
96 properties of the vesicles, such as size, surface charge, morphology and structure, were
97 thoroughly evaluated using Photon Correlation Spectroscopy (PCS), cryogenic transmission
98 electron microscopy (cryo-TEM) and Small-Angle X-ray Scattering (SAXS). Moreover, their *in*
99 *vitro* ability to be nebulized was assessed using a New Generation Impactor. The formulation
100 biocompatibility and ability to inhibit hydrogen peroxide damages were evaluated using the
101 A549 cells as model of lung epithelium.

102 **2. Materials and Methods**

2.1. Materials

A commercial mixture of soybean phospholipids (Lipoid S75, S75), containing 70% phosphatidylcholine, 9% phosphatidylethanolamine and 3% lysophosphatidylcholine, triglycerides and fatty acids, was kindly supplied by AVG (Milan, Italy) and Lipoid GmbH (Ludwigshafen, Germany). Sodium hyaluronan (HA) low molecular weight (200-400 kDa) was purchased from DSM Nutritional Products AG Branch Pentapharm (Aesch/Switzerland). Chitosan (CH) low molecular weight, curcumin (CUR) (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) and all other products of analytical grade, were purchased from Sigma (Milan, Italy).

2.2. Vesicle preparation

Curcumin (0.5 mg/ml) was dispersed in water and added to a flask containing S75 (60 mg/ml). Lipids were left swelling in the dispersion overnight and sonicated for 20 cycles (5 seconds on and 2 seconds off) using an ultrasonic disintegrator (Soniprep 150, MSE Crowley, United Kingdom). Appropriate amount of chitosan (5 mg/ml) was dissolved under stirring in acetic acid (0.1% v/v) aqueous solution, while hyaluronan (5 mg/ml) was dissolved in distilled water. Curcumin loaded liposomes (5 ml) were slowly added to the polymer dispersion (10 ml) under stirring at 25°C; the stirring was maintained for 1 hour to obtain the final coated vesicles (Zhuang et al., 2010). Liposome dispersion (5 ml) was diluted in distilled water (10 ml) to obtain the same phospholipid and drug concentration of the coated vesicles: curcumin 0.17 mg/ml, phospholipids 20.00 mg/ml, polymer 3.33 mg/ml.

Each vesicle suspension was purified from the non-incorporated drug by dialysis against distilled water at 25°C for 4 hours, using dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., Ranchio Domingo, USA).

Coated vesicle dispersions were ultra-centrifuged at 37,000 × g for 4 hours at 4°C to remove the excess of polymer and washed three times with distilled water.

2.3. Vesicle characterization

Formation and morphology of vesicles were evaluated by cryo-TEM. A drop of each sample was placed on a glow-discharged holey carbon grid and then blotted with filter paper to obtain a thin aqueous film, which was vitrified by plunging the grid (kept at 100% humidity and room temperature) into ethane maintained at its melting point, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous films were transferred to a Tecnai F20 TEM (FEI Company) using a Gatan cryotransfer (Gatan, Pleasanton, CA), and the samples were observed in a low-dose mode. Images were acquired at 200 kV at a temperature around -173°C, using low-dose imaging conditions not exceeding 20 $e^{-}/Å^{2}$, with a CCD Eagle camera (FEI Company). The average diameter and polydispersity index (PI; a measure of the size distribution width) of the particles were determined by Photon Correlation Spectroscopy using a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK). Samples were backscattered by a helium-neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility.

The lipid content of the dispersions was determined by the Stewart assay (Muzzarelli, 1998). Vesicle dispersions (10 µl) were diluted with the specific reagent consisting of a mixture of chloroform and an aqueous solution of ammonium ferrithiocyanate (0.1 N) The obtained mixtures were maintained in the dark at room temperature for 30 min and then analysed at 485 nm using a UV spectrophotometer (Spectrometer Lamba 25, Perkin Elmer, Milan, Italy). Aggregation efficiency (AE%) was calculated as the percentage of the amount of phospholipids measured in the dispersion after the purification process of the polymer (ultra-centrifugation) versus the amount initially measured (Manca et al., 2015a, 2015b).

Drug entrapment efficiency (EE%), was expressed as the percentage of the amount of curcumin found after purification by dialysis versus that initially used. Unpurified and purified vesicles were disrupted by dilution with methanol (dilution 1:500) and the curcumin content of each dispersion, was quantified by HPLC, using a chromatograph Alliance 2690 (Waters, Milano,

Italy) detector equipped with a photodiode array detector 996. Analysis was performed at 424 nm, using a column SunFire C18 (3.5 μm, 4.6x150 mm) and a mixture of acetonitrile, water and acetic acid (90:9.7:0.3, v/v), delivered at a flow rate of 1.0 ml/min.

2.4. Small-Angle X-ray Scattering (SAXS)

SAXS experiments were carried out using a S3-MICRO (Hecus X-ray systems, Graz, Austria) coupled to a GENIX-Fox 3D X-ray source (Xenocs, Grenoble, France) and a 50 focused X-ray beam with 0.1542 nm at Cu Kα-line. The q range was 0.003-0.6 $Å^{-1}$, where $q = (4 \pi \sin \theta)/\lambda$ is the modulus of the scattering wave vector, θ the scattering angle and λ the wavelength. All scattering curves, recorded at 25°C were reproduced twice, and a representative curve was selected, plotting the scattering intensity (I) as a function of the scattering vector (q). SAXS patterns were calculated using the bilayer model developed by Pabst (Pabst et al., 2003, 2000)and applied in the program GAP (Global Analysis Program).

2.5. Nebulization of formulations and aerodynamic behaviour.

The *in vitro* deposition of tested formulations was evaluated using the next generation impactor (Eur. Ph 7.2, Copley Scientific Ltd., Nottingham, United Kingdom) and the PariSX® air jet nebulizer connected to a ParyBoySX® compressor. Vesicular formulations (3ml) were placed in the nebulizer and aerosolized to dryness directly into the throat of the impactor. At the end of the experiment, the sample deposited into the different stages of the impactor was recovered with methanol and drug content was quantified by HPLC (section 2.3.). Deposition performances were evaluated calculating the total mass output (TMO%), the Fine Particle Dose (FPD), and the Fine Particle Fraction (FPF). Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) values were calculated without including the mass deposited in the induction port. The cumulative amount of particles with a diameter lower than the stated size of each stage was plotted as a percentage of recovered drug versus the cut-off diameter, and the MMAD of the particles was extrapolated from the graph (Manca et al., 2014).

2.6. Cell relative metabolic activity assay

The human basal epithelial alveolar cells (A549) were maintained at 37°C and 5% CO_2 in a humidified atmosphere and cultured in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and 10% foetal bovine serum.

Cell viability was evaluated as a function of their relative metabolic activity using the 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide (MTT) test (Manca et al., 2014). Cells ($7.5x10^3$) were seeded in 96-well plates. After 24 h, suspensions (25 µl) at different phospholipid and phytodrug dilutions to reach the appropriate concentrations (0.02-2 mg/ml and 0.15-17 µg/ml for S75 and curcumin respectively) were added and after 48 h of incubation, MTT solution (0.5 mg/ml) was added to each well. Later (2-3 h), the culture medium was removed, replaced with dimethyl sulfoxide and the absorbance of the solubilized dye was measured at 570 nm with a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.P.A, Bernareggio, Italy). Results are shown as percentage of cell relative metabolic activity in comparison with non-treated control cells (100% cell relative metabolic activity).

2.7. Effect of curcumin loaded formulations on oxidative stress damage of cells

A549 cells were seeded in 96 well plates and incubated until confluence. Untreated cells under optimal conditions and cells treated with hydrogen peroxide but without curcumin were used as negative and positive control, respectively. Cells were simultaneously treated with hydrogen peroxide (1:30000 v/v dilution) and formulations properly diluted to reach two different curcumin (17 and 8.5 µg/ml) and phospholipid (1 or 2 mg/ml) concentrations, incubated for 3 hours and successively washed with PBS. Finally, the MTT assay was used to measure cell relative metabolic activity. Results are shown as percentage of cell relative metabolic activity in comparison with non-treated cells (negative control, 100% cell relative metabolic activity).

2.8. Statistical analysis

Results are expressed as the mean ± standard deviation. Multiple comparisons of means (ANOVA) were used to substantiate statistical differences between groups, while Student's t-test

was used to compare two samples. Significance was tested at the 0.05 level of probability (p). Data analysis was carried out with the software package XLStatistic for Excel.

3. Results and Discussion

3.1. Vesicle characterization

Vesicle formation and their structure were firstly confirmed by cryo-TEM. Micrographs showed the formation of spherical and unilamellar liposomes (Figure 1A). Chitosan coating modified the vesicle structure, which appeared spherical too and formed by one or more lamellae (Figure 1B). Similar structures were obtained by Hasan et al., who found that chitosan-coated liposomes were surrounded by a contrasting band (Hasan et al., 2016). On the contrary hyaluronan coated liposomes disclosed unilamellar structures very similar to the uncoated vesicles (Figure 1C).

Mean size, polydispersity index and zeta potential of the formulations are shown in Table 1, which also contains data of empty vesicles to evaluate the effect of curcumin incorporation on these parameters. Mean diameter of empty liposomes was ~90 nm and samples showed a good homogeneity (polydispersity index ~ 0.18). When curcumin was incorporated into the vesicles, their size did not change significantly but the polydispersity increased and the zeta potential became less negative indicating a contribution of the flavonoid on the bilayer surface (Castangia et al., 2014; Hung et al., 2008; Manca et al., 2015a, 2015b). The addition of chitosan coating led to an increase of the vesicle mean diameter in empty (~115 nm) and especially in curcumin-loaded vesicles (~132 nm) clearly due to the polymer layer deposition on vesicle surface, as previously found by Morton et al. and Bishop et al. when coating liposomal carriers or ~~to~~ gold nanoparticles, respectively (Bishop et al., 2016, 2015; Morton et al., 2014). Zeta potential of liposomes was strongly negative (-62 mV, Table 2) because S75 is a mixture of phosphatidylcholine, negatively charged phospholipids and fatty acids. At pH~6 the negative phosphate residues of the phospholipids are distributed on the membrane surface leading to a negative potential value (Abramović et al., 2008). The positively charged molecules of polymeric chitosan can easily interact with the negative phosphate groups of phosphatidylcholine allowing

232 stable, electrostatic interactions. The inversion of the zeta potential of chitosan liposomes from
233 negative to highly positive values (+70 mV) confirmed the presence of the positive polymer
234 coating on the liposome surface, while the addition of curcumin partially reduced the zeta
235 potential of the loaded chitosan liposomes (+35 mV). This highly positive zeta potential value is
236 indicative of vesicle stability against aggregation processes, due to the electrostatic repulsion
237 among the particles.
238 The addition of hyaluronan to empty liposomes did not change vesicle size and only allowed a
239 slight increase of sample polydispersity and a decrease of zeta potential that became less
240 negative. The addition of curcumin to hyaluronan liposomes caused an increase of vesicle size,
241 the polydispersity index remained alike while zeta potential, as for empty coated liposome,
242 became less negative (-20 mV). The surface charge is an important parameter, which affects the
243 stability of vesicles in dispersion and should be always strongly negative or positive (Akhtar et
244 al., 2012).Usually a zeta potential around -20 mV is still enough to ensure the system stability as
245 previously reported (Manca et al., 2015a). Even though hyaluronan is negatively charged, it can
246 interact with zwitterionic phosphatidylcholine heads. Indeed, previous studies demonstrated that
247 the orientation and conformation of the choline head groups on the bilayer surface are not
248 constant since they can be affected by binding or adsorption of charged molecules (Scherer and
249 Seelig, 1989).
250 Curcumin entrapment efficiency of liposomes (96%) was slightly higher than that of coated
251 liposomes (~85%) probably because of a loss of the drug during the ultracentrifugation process
252 (table 1). In addition, the amount of phospholipids in the vesicle dispersions was measured before
253 and after the purification process (ultra-centrifugation), using a colorimetric assay, thus,
254 obtaining the amount of phospholipids actually aggregated into vesicles. For each formulation
255 the efficiency of phospholipid aggregation was calculated as percentage and it was very high
256 (92%) for liposomes confirming the good ability of phosphatidylcholine to form closed bilayer
257 vesicles also in the presence of curcumin. The aggregation efficiency of coated liposomes was

lower (~80%) than that of liposomes probably because a little amount (10%) of small and uncoated vesicles was still present in the supernatant after centrifugation (table 1).

SAXS analysis is a powerful complementary tool to evaluate the physicochemical bilayer modifications caused at molecular level by host chemicals, thus, allowing to collect detailed information on bilayer structure and internal spaces occupied by the two portions of phospholipids inside them. Empty and curcumin-loaded liposomes and polymer-coated liposomes were analysed by SAXS to evaluate the drug and polymer effect on vesicle assembling and features, which can affect the drug *in vivo* release (Gómez Gaete et al., 2008). Data were obtained using the fitting procedure elaborated by Pabst (Pabst et al., 2003), which provides electron density profiles of lamellar membrane. Such theoretical model should be used when the power-law diffuse scattering under the Bragg peaks is detectable, allowing to obtain important information on membrane fluctuations (Fernandez et al., 2008). Scattering curves of intensity versus wave vector (q) for samples are shown in Figure 2. Empty and curcumin loaded liposome patterns presented only a first order reflection peak (q ~ 0.10 $Å^{-1}$) broadened and unsharpened indicating the presence of unilamellar, soft vesicles having a bilayer thickness (d_B) ~52.4Å (table 2). Chitosan liposomes displayed a first order diffraction peak at the same q value (0.10 $Å^{-1}$), but more sharpened and, hence, indicative of more lamellar (oligolamellar) and more stiff structures with a higher bilayer thickness d_B~ 59 Å and a definite repetition distance, d ~62 Å. These changes on diffraction peak reflect an improvement in the structural ordering of the lamellae. Therefore, the vesicle structure changed from unilamellar to partial oligolamellar with an increase of the bilayer thickness, stiffness and vesicle size due to the formation of the polymer layer on the bilayer surface. The system stiffness increased because oligolamellar vesicles are more rigid than unilamellar vesicles for the formation of different close concentric lamellae. Moreover, the presence of an electric layer surrounding a membrane is theoretically expected to increase bending and rigidity of the structure (Mertins and Dimova, 2013). The increase of membrane thickness (d_B), observed in chitosan liposomes, seems to be due to a stronger

interaction of the chitosan with the polar regions of the phospholipid bilayer, with a consequent separation of the apolar chains and enlargement of their width (z_H) while the polar heads (σ_H) remained constant.

Diffraction peak of hyaluronan coated liposomes was much flattened and broad indicating that the vesicles are mainly organized in uncorrelated bilayers having thickness (d_B) lower than that of uncoated liposomes. A decrease in intensity could come from a reduction of the bilayer electron density contrast due to interference between scattering units or to a change in symmetry (size) of the scattering objects, or to a high polydispersity of the sample (Riske et al., 2001). Bilayer thickness (d_B) reduction suggests that hydrophobic segments of hyaluronic acid may partially insert between the lipidic domain of the bilayer as previously reported by Gomez Gaete et al for DPPC-hyaluronic acid microparticles (Gómez Gaete et al., 2008). This polar heads enlargement favoured the lipid chains shift to the opposite bilayer polar heads resulting in a decrease of the apolar chain width (z_H). The results of SAXS analysis showed a minor influence of curcumin on the structural parameters probably indicating its superficial position in the bilayer and also due to its low concentration (Castangia et al., 2014).

3.2. Nebulization Studies

Formulations were nebulized for 15 minutes using the PariSX® air jet nebulizer connected to the next generation impactor in order to evaluate *in vitro* curcumin deposition and particle aerodynamic diameter. Indeed, the nebulizer content was almost completely aerosolized, the total mass output (TMO) was ~100% for liposomes (97±5%), hyaluronan liposomes (98±2%) as well as for curcumin dispersion (95±4%), while it was lower for chitosan liposomes (87±8%) probably due to the adhesive properties of chitosan on the apparatus surface. The amount (FPD) and the percentage (FPF) of curcumin deposited in the lower stages of the impactor, were measured (Table 3). The highest drug deposition was obtained using hyaluronan coated liposomes (~130 µg and 93%) which reached values ~4-6 fold higher than those of drug dispersions and ~2 fold higher than that of liposomes. The drug deposition provided by chitosan coated liposomes was

slightly higher than that of liposomes and always 2-3 fold higher than that of the drug dispersion. However, using this formulation almost 50% of curcumin reached the furthest stages of the impactor mimicking the deepest part of the respiratory tree.

It is well known that the process of jet nebulization involves repeated cycles of aerosol drop formation and their recapture in the nebulizer reservoir before the formulation leaves the device. During this process, considerable shearing forces are applied to the samples, leading to development of dispersion drops and their fragmentation, subsequent aggregation, fusion and formation of new larger drops (Manca et al., 2015b; Melis et al., 2016). Carrier stability against these shearing forces plays a key role for their suitable use for pulmonary administration because it affects the final aerodynamic diameter (MMAD) of the aerosolized particles (Abu-Dahab et al., 2001). Curcumin dispersion and liposomes had a large MMAD (~5 μm), which did not allow the particles to reach the latter stages of the impactor, suggesting that only a small part of the free drug could reach the deep airways. Using liposomes, the shearing forces may cause the rupture of the vesicles and curcumin leakage, which, similarly to the dispersion, may undergo the aggregation of the individual molecules during the nebulization process, with the consequent formation of stable and large agglomerates unable to be nebulized (Manca et al., 2014). Differently, coated vesicles showed a good aptitude to be nebulized because the hydrophilic polymers had a remarkable effect on the physical properties and stability of the vesicles (Manca et al., 2011). In particular, the polymer coating could improve vesicle resistance during aerosolization reducing their rupture, facilitating structure re-assembling and, finally, promoting the delivery of high amount of curcumin to the lowest stages of the impactor. The MMDA of both coated liposomes was ~3 μm, lower than that of liposomes, confirming the polymer ability to improve vesicle stability and nebulization performances.

3.3. *In vitro* biocompatibility and antioxidant activity

Cytotoxic effect of samples on A549 cells was assayed for 48h by testing different dilutions (corresponding to the following concentrations: S75 0.02-2 mg/ml; drug 0.17-17 μg/ml), using

the MTT assay (Manca et al., 2014). Empty formulations did not cause any reduction in cell relative metabolic activity (≥100%) at all the tested dilutions (Figure 3). Furthermore, empty hyaluronan coated liposomes induced a slight increase in cell relative metabolic activity up to ~113% thanks to hyaluronic acid ability to improve cell migration and proliferation (Manca et al., 2015; Manuskiatti and Maibach, 1996). The cell incubation with curcumin loaded vesicles led to a small decrease in cell relative metabolic activity, which was always ≥ 80% even at the highest sample concentration (S75 2 mg/ml; drug 17 µg/ml) (Figure 3).

Increased levels of reactive oxygen species provided by hydrogen peroxide may damage cellular processes even leading to cell death. Curcumin, as a potent antioxidant, hampers the formation of these reactive oxygen species protecting the cells (Bar-Sela et al., 2010; Jurenka, 2009; Strimpakos and Sharma, 2008). The carrier ability to modulate curcumin antioxidant activity and to protect cells from hydrogen peroxide damages was tested for 3 h using A549 and the effect provided by the flavonoid dispersion was used as reference. The addition of hydrogen peroxide to the cells (untreated cells) led to a reduction (~40%) of cell relative metabolic activity and the simultaneous treatment with curcumin dispersion did not improve cell survival ($p>0.05$). On the contrary, the vesicles promoted the antioxidant activity of curcumin within the cells, while protecting them even at the lowest curcumin concentration (8.5 µg/ml). Hydrogen peroxide stressed-cells treated with curcumin loaded vesicles showed the same cell relative metabolic activity of the unstressed cells or even higher, up to 120% using hyaluronan coated liposomes. This result is probably related to the synergic effect of curcumin and hyaluronic acid, the first performing its antioxidant activity and the last stimulating cell proliferation (Castangia et al., 2015; Manca et al., 2015a; Manuskiatti and Maibach, 1996). It has also been suggested that, due to the interaction with CD44 receptor, hyaluronan may activate pathways involved in the regulation of cellular redox status and intracellular ROS generation (Halicka et al., 2009; Ye et al., 2012).

4. Conclusions

The combination of nanotechnologies and natural drugs represents a smart strategy to ameliorate the treatment of pulmonary diseases or prevent chronic inflammation. Aiming to this, curcumin loaded liposomes were modified by coating their surface with chitosan or hyaluronan to protect both phytodrug and vesicles and improve curcumin local efficacy. Positively charged, oligolamellar and stiff vesicles were obtained using the first polymer and negatively charged, unilamellar and less stiff vesicles with the second one. Overall results disclosed that vesicles coated with hyaluronan appeared more appropriate to improve lung deposition and to effectively protect A549 cells from the oxidative stress induced by hydrogen peroxide. Curcumin loaded hyaluronan coated liposomes may represent a potential and safe delivery system for the local treatment of different lung diseases able to prevent chronic inflammatory conditions.

Figure 1. Cryo-TEM micrographs of **(A)** curcumin loaded liposomes, **(B)** chitosan coated liposomes and **(C)** hyaluronan coated liposomes. Arrows indicate the structure of the vesicles.

Figure 2. Representative SAXS patterns of empty and curcumin (CUR) loaded liposomes, chitosan (CH) coated liposomes and hyaluronan (HA) coated liposomes.

Figure 3. Cell relative metabolic activity (MTT assay) of A549 cells incubated with empty and curcumin (CUR) loaded liposomes, chitosan (CH) coated liposomes and hyaluronan (HA) coated liposomes, as a function of their concentration (A) and after simultaneous exposure to hydrogen peroxide and curcumin loaded vesicles. Each independent experiment was repeated three times. Results are expressed as mean values ± standard deviation (n=9).

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