Lipid vesicular gels for topical administration of antioxidants

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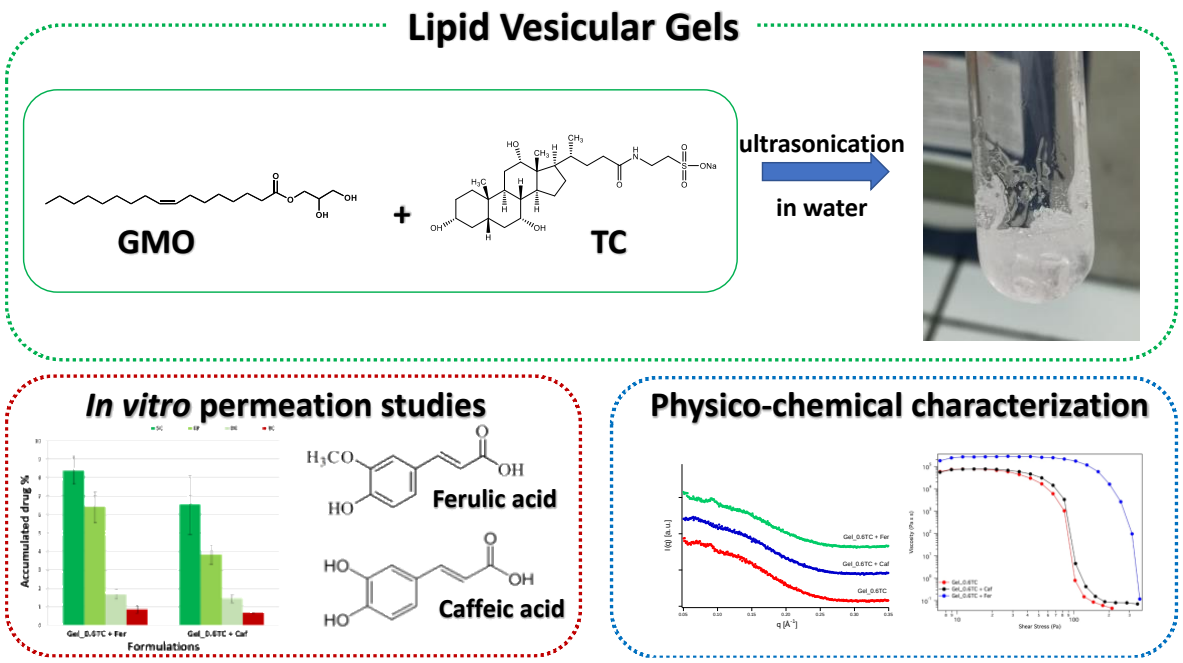
19 **Graphical Abstract**



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Abstract

The application of a formulation on the skin represents an effective way to deliver bio-active molecules for therapeutical purposes. Moreover, the outermost skin layer, the *stratum corneum*, can be overcome by employing chemical permeation enhancers and edge activators as components. Several lipids can be considered as permeation enhancers, such as the ubiquitous monoolein, one of the most used building blocks for the preparation of lipid liquid crystalline nanoparticles which are applied as drug carriers for nanomedicine applications. Recent papers highlighted how bile salts can affect the phase behavior of monoolein to obtain drug carriers suitable for topical administration, given their role as edge activators into the formulation.

Herein, the encapsulation of natural antioxidants (caffeic acid and ferulic acid) into lipid vesicular gels (LVGs) made by monoolein and sodium taurocholate (TC) in water was studied to produce formulations suitable for topical application. TC induce a bicontinuous cubic to multilamellar phase transition for monoolein in water at the given concentrations, and by increasing its content into the formulations, unilamellar LVGs are formed. The encapsulation of the two antioxidants did not affect significantly the structure of the gels. The rheological studies showed that ferulic acid has a structuring effect on the lipid matrix, in comparison with the empty dispersion and the one containing caffeic acid.

These gels were then tested *in vitro* on new-born pig skin to evaluate their efficacy as drug carriers for topical administration, showing few differences in the penetration mechanism of the two formulations.

KEYWORDS

Unilamellar vesicles; monoolein; bile salts; sodium taurocholate; skin.

1. Introduction

Monoolein is a ubiquitous lipid that is recognized as a safe component in many formulations. Its phase behaviour is characterized by a rich polymorphism. Depending on the water content and the temperature,[1] several liquid crystalline phases can be formed, such as lamellar, inverse hexagonal and inverse bicontinuous cubic phases. An interesting feature of these bulk phases is that they can be dispersed in aqueous solution *via* bottom-up or top-down approaches to obtain lipid liquid crystalline nanoparticles (LLCNPs).[2] They display the same inner structure of the corresponding bulk, but they are much less viscous, an appealing character for biomedical applications. Indeed, LLCNPs such as vesicles,[3–6] hexosomes[7–9] and cubosomes[10–13] have been suggested in nanomedicine as drug and imaging probes carriers. Given the resemblance with cell membranes, the dispersions of the lamellar phase have been widely characterized as drug carriers. Their peculiar architecture allows loading with both hydrophilic and hydrophobic payloads in the aqueous core or in the lipid bilayer, respectively. Several types of vesicles were prepared and tested, using various surfactants to improve their physico-chemical and biological performances for both topical[14] and systemic administrations.[15] In the former case, many papers reported the ability of niosomes,[16,17] ethosomes[18,19] and transferosomes[14,20] to overcome the outermost layer of the skin, the stratum corneum (SC).

The dermal/transdermal delivery of a bio-active compound is a non-invasive and safe route, especially in order to avoid the first-pass metabolism.[21] When a molecular dispersion of the drug is applied on the skin, the SC restricts the diffusion of the drug through it. SC is composed by a brick and mortar structure,[22] where the brick is represented by non-living corneocytes immersed in a dense collagen-rich lamellar phase, which represents the mortar. The alternation of hydrophilic and hydrophobic layers hinders the diffusion of molecules, hence affecting the therapeutic outcome. However, this challenging barrier can be overcome by encapsulating the drug within formulations containing permeation enhancers, such as lipids, since they are able to fluidify the lipids in the SC, thus permitting the delivery of the encapsulated payload in the deeper skin *strata*. In addition to permeation enhancers, edge activators can be added to the formulation. They act as softener for lipid bilayers, increasing the elasticity and the deformability of the carrier. Molecules such as bile salts,[9,23] cholesterol[24] and polyethoxylated lipids[25] are recognized as edge activators.

Among the different payloads which can be encapsulated, antioxidants have been quite exploited due to their pharmaceutical versatility, working as adjuvants, additives and drugs. Hydroxycinnamic acids such as caffeic (Caf) and ferulic (Fer) acid are secondary metabolites

and occur widely in the plant kingdom.[26,27] They exhibit a strong antioxidant activity, that has been tested both *in vitro* and *in vivo*. Caf and its derivatives can directly trap free radicals or scavenge them through several complex reactions. This feature is suitable to formulate gels and cream with anti-photoaging effects. Therefore, Caf and Fer have been encapsulated in several liposomial formulations for topical administration.[28]

Due to the aforehand discussed reasons, lipid vesicular gels (LVGs)[29,30] made with a well-known permeation enhancer (MO) and an edge activator (sodium taurocholate, TC) were here prepared in order to deliver caffeic acid and ferulic acid through the skin. This paper covers the physico-chemical characterization of the unloaded and loaded gels (SAXS and Rheology) and the *in vitro* permeation tests on new-born pig skin in order to assess the applicability of these formulations for local administration of antioxidants.

2. Material and Methods

2.1. Chemicals

Monoolein (GMO, glycerol-monooleate, 98.1 %) was kindly provided by Danisco Ingredients (Denmark). Sodium taurocholate (TC, TLC quality, purity ≥ 97.0 %), caffeic acid (HPLC quality, purity ≥ 98.0 %) and ferulic acid (HPLC quality, purity ≥ 98.0 %) were purchased from Sigma-Aldrich.

In order to prepare the formulations, ultrapure water was used after filtration through a Milli-Q system (Millipore). The compositions of the samples are given as % w/w.

2.2. Lipid Vesicular Gels preparation (LVGs)

LVGs were prepared by dispersing the molten MO in an aqueous solution of TC *via* ultrasonication using a UP100H ultrasonic processor developed by Hiescher for 25 minutes (amplitude of the sonication 90 %, 1 s of pulse ON followed by 1 s of break). Slightly milky or clear gels were obtained depending on the concentration of TC.

Samples containing caffeic acid (Caf) and ferulic acid (Fer) were prepared using the same protocol by dispersing the antioxidants into the molten GMO with the help of a vortex until the mixture appeared homogeneous, before adding the aqueous solution of the bile salt. The gels containing Fer and Caf appeared slightly milky and clear, respectively.

2.3. Small angle X-ray scattering measurements

The structure of the LVGs was investigated by means of small angle X-ray scattering measurements, using a S3-MICRO SWAXS Camera from HECUS X-ray Systems (Graz,

Austria). The X-ray source (GeniX) produced a Cu Kα 1.542 Å, working at 30 kV and 0.4 mV. The scattering was detected by a 1D-PSD-50 system from HECUS X-ray Systems (Graz, Austria) containing 1024 channels (width 54.0 μm). The gels were placed on a stainless stell sample sandwich-type holder, using a polymeric film as windows (Bratfolie, Kalle). The sample-detector distance was kept constant for each experiment (235 mm), giving a working q range $0.003 \leq q \leq 0.6$ Å^{-1}.

The scattering patterns of the LVGs were acquired in the temperature range 25 – 50 °C and the temperature was kept constant via a Peltier instrument. The chamber was kept under vacuum during the SAXS experiments to avoid scattering from air. The q scale was calibrated using silver behenate as a standard.

2.4. Oscillatory rheology measurements

Oscillatory shear measurements were performed by using a plate-plate geometry (20 mm diameter, 300 μm gap) with a TA Instruments Discovery Hybrid Rheometer working in controlled shear stress mode. The dependence of the storage modulus (G') and loss modulus (G'') as a function of the angular frequency was investigated in the linear viscoelastic regime of deformations (LVR; strain 0.4 %) in the frequency range 10^{-2} – 10^{2} Hz at a temperature of (25.00 ± 0.01) °C. For each sample investigated, the LVR was determined through a preliminary amplitude sweep experiment (strain range: 0.001-20 %; oscillation frequency: 1 Hz).

The dependence of G' and G'' on the imposed oscillation frequency was obtained from the phase lag between the applied shear stress and the related flow.

2.5. *In vitro* penetration and permeation studies

The ability of LVGs to modulate the antioxidants penetration and/or permeation through the skin was evaluated using skin from new-born pigs. The experiments were performed in Franz vertical cells, exhibiting a diffusion area of 0.785 cm^2. The skin of one-day-old Goland–Pietrain hybrid pigs (~1.2 kg), died of natural causes and provided by a local slaughterhouse, was excised and stored at −80 °C until the day of the experiment. Skin specimens (n = 6 per formulation) were pre-equilibrated with saline (NaCl 0.9 % w/v) at 25 °C, then sandwiched between the donor and the receptor compartments. The receptor was filled with 5.5 mL of saline solution (NaCl 0.9 w/v %), continuously stirred and thermostated at (37 ± 1) °C, to emulate in vivo conditions. 200 mg of each LVG formulations were placed onto the skin

surface. At regular intervals, up to 8 h, the receiving solution was entirely withdrawn, replaced with fresh saline to ensure sink conditions and analysed by HPLC for Caf and Fer content. After 8 h, the skin surface was gently washed with 1 mL of distilled water and then dried with filter paper. The stratum corneum was removed by stripping with adhesive tape Tesa® AG (Hamburg, Germany). Each piece of adhesive tape was firmly pressed on the skin surface and rapidly pulled off with one stroke. Epidermis was separated from dermis with a surgical scalpel. Skin *strata* were cut, placed each in a flask with methanol and sonicated for 2 min to extract the accumulated drug. The tape and tissue suspensions were filtered out and assayed for drug content by HPLC.

2.6. HPLC quantification of the antioxidants

Caf or Fer content was quantified at 321 nm using a chromatograph Alliance 2690 (Waters, Italy). The column was a XSelect HSS T3 (3.5 μm, 4.6 × 100 mm, Waters). The mobile phase was a mixture of acetonitrile, water and acetic acid (95.35:4.5:0.15 v/v), delivered at a flow rate of 0.5 mL/min. A standard calibration curve (R^2 of 0.999) was built up by using working, standard solutions (1–100 ng/μL).

2.7. Statistical analysis of data

Results are expressed as the mean ± standard deviation (SD). Multiple comparisons of means (one-way ANOVA with post-hoc Tukey HSD test) were used to substantiate statistical differences between the datasets, while Student's t-test was used to compare two samples. Data analysis was carried out with the software package XLStatistic for Microsoft Excel. Significance was tested at 0.05 level of probability (p).

3. Results and Discussion

SAXS investigations

In order to design a formulation suitable for topical administration of caffeic and ferulic acid, LVGs were obtained by mixing a chemical permeation enhancer (MO) with an aqueous solution of an edge activator (TC). The MO/W phase diagram[1] at high water dilution (higher than 50 % of water, close to room temperature) predicts the existence of a bicontinuous cubic Pn3m phase coexisting with excess of water. However, it was shown that TC affects the MO phase behavior inducing a cubic-to-lamellar phase transition already at very low concentration

(0.02 wt % for MO concentration equal to 3.3 wt %), thereby allowing the formation of a stable unilamellar vesicles dispersion in the presence of Pluronic F108, used as stabilizer.[9] Indeed, the presence of TC molecules affects (reduces) the effective packing parameter of MO and, consequently, the interfacial curvature of the system, enabling the formation of vesicles. Once the *volume* fraction of the dispersed phase overcomes the critical value of 0.494,[30]the vesicles become so densely packed that they can form very viscous LVGs. Here, with the addition of a small amount of TC to induce the cubic-to-lamellar phase transition of the dispersion, and a concentration of MO equal to 14.6 wt %, LVGs were formulated to provide the system with the necessary higher viscosity suitable for topical administration of natural antioxidants.

The compositions of the different LVGs samples are reported in Table 1.

Table 1. Composition of the LVGs samples expressed as the total amount of lipids and antioxidants (Antiox) dispersed in water (W).

LVGs	MO (wt %)	TC (wt %)	Antiox (wt %)	W (wt%)
Gel_0.6TC	14.6	0.6	-	84.8
Gel_1TC	14.6	1	-	84.4
Gel_1.5TC	14.6	1.5	-	83.9
Gel_2TC	14.6	2	-	83.4
Gel_0.6TC + Caf	14.6	0.6	0.2	84.6
Gel_0.6TC + Fer	14.6	0.6	0.2	84.6

After visual inspection, the samples appeared stable up to 7-8 weeks at least, without observing any phase separation. First, the effect of TC on MO/W dispersion was evaluated in the 0.6-2 wt % range of the bile salt.

As shown in the Fig. 1, all the samples exhibit a broad diffusive band, as commonly observed in vesicles dispersions. Depending on the number of double layers characterizing the vesicles, small quasi-Bragg peaks related to the lamellarity of the system are imposed over the broad band. The lamellarity, i.e., the number of *lamellae* which divide in sub-compartments the inner region of the vesicle, represents an important feature to be considered since it is correlated to the intrinsic stability of the system, to the drug encapsulation efficiency, and to its release.[3,31]

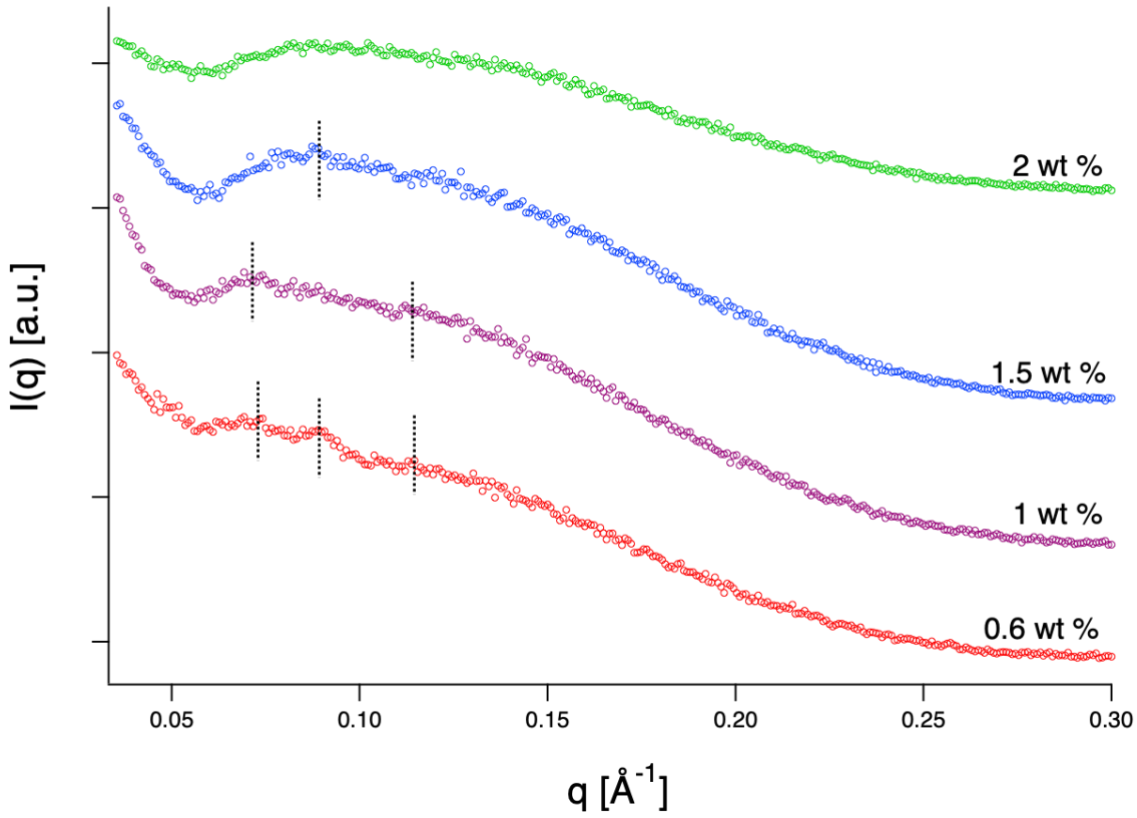
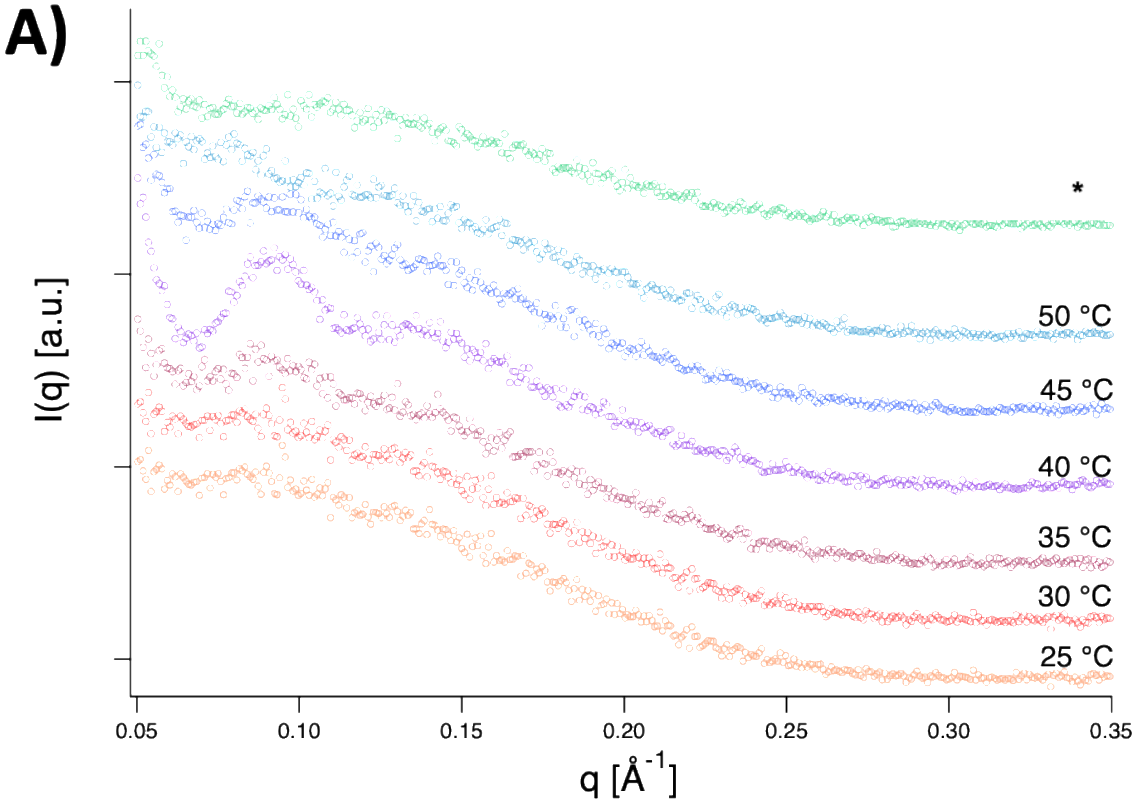


Figure 1. SAXS diffractograms of the LVGs samples at different TC content (wt %) acquired at 25 °C. The dashed lines highlight the presence of quasi-Brag peaks related to the multilamellarity of the system.

According to the experimental evidence, showing the disappearance of the quasi-Bragg peaks related to the Pn3m inverse bicontinuous cubic phase, the lamellarity of the system decreases by increasing the TC concentration into the formulation, starting from a multilamellar (Gel_0.6TC) to an unilamellar system (Gel_2TC). The presence of a multilamellar system at low TC content is not surprising: the MO phase behavior is affected by the presence of the bile salt, but the BS:MO molar ratio is not enough to give unilamellar vesicles dispersions, as shown in already reported studies.[9,32] When the BS concentration is increased in the formulation, the lamellar forming effect leads to a multilamellar-to-unilamellar structural transition, as already mentioned.

Since the temperature of the skin varies depending on the body districts, it can play a role on its physico-chemical properties, and on the efficacy of the treatment. Therefore, the thermo-responsive behavior of the Gel_0.6TC was investigated in the range 25-50 °C. Fig. 2A shows that the quasi-Bragg peaks are more pronounced in the temperature range 35-40 °C. Apparently, above 40 °C the lamellarity of the system decreases, possibly due to a lower bilayer

rigidity that, in turn, favors the unilamellar arrangement. No hysteresis was observed when the sample was cooled down again to 25 °C. The almost perfect overlap between the two scattering curves (before and after the heating cycle) represents a sign of a reversible physical transformation into the formulation.



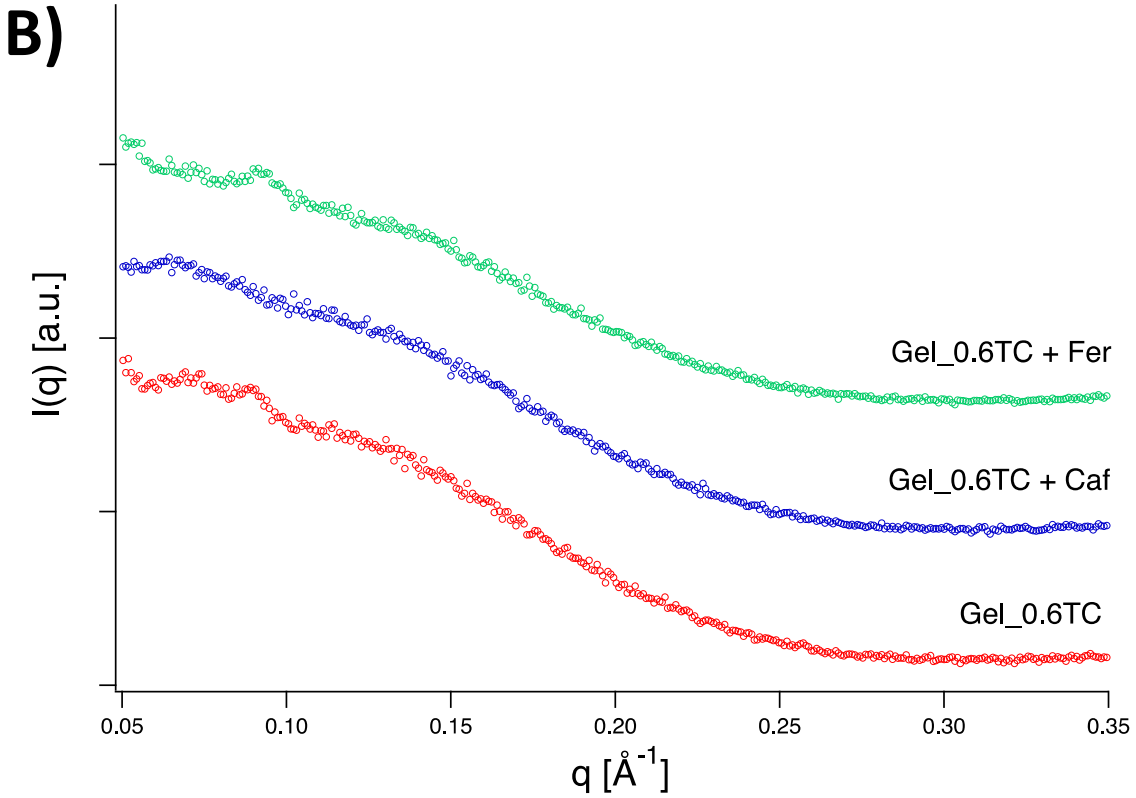


Figure 2. A) Thermo-responsive behavior of the LVG Gel_0.6TC in the range 25-50 °C and then back to 25 °C (described by the presence of *). B) Effect of the encapsulation of the antioxidants on the SAXS profile of the LVG Gel_0.6TC at 25 °C.

Considering these features, the sample Gel_0.6TC was loaded with the natural antioxidants caffeic acid (Caf) and ferulic acid (Fer) at a concentration of 2 mg g^{-1}.

The gel loaded with Fer appeared slightly milkier than the unloaded LVG, whereas the one containing Caf was yellowish. The SAXS patterns of these samples are reported in Fig. 2B, using the empty formulation as a reference. In general, this comparison proved that the presence of these two additives did not affect significantly the gel structure.

Rheological features of the LVGs

Given the macroscopical gel appearance, samples were characterized by means of rheology. The flow curves of the samples Gel_0.6TC, Gel_0.6TC + Caf and Gel_0.6TC + Fer are reported in Fig. 3.

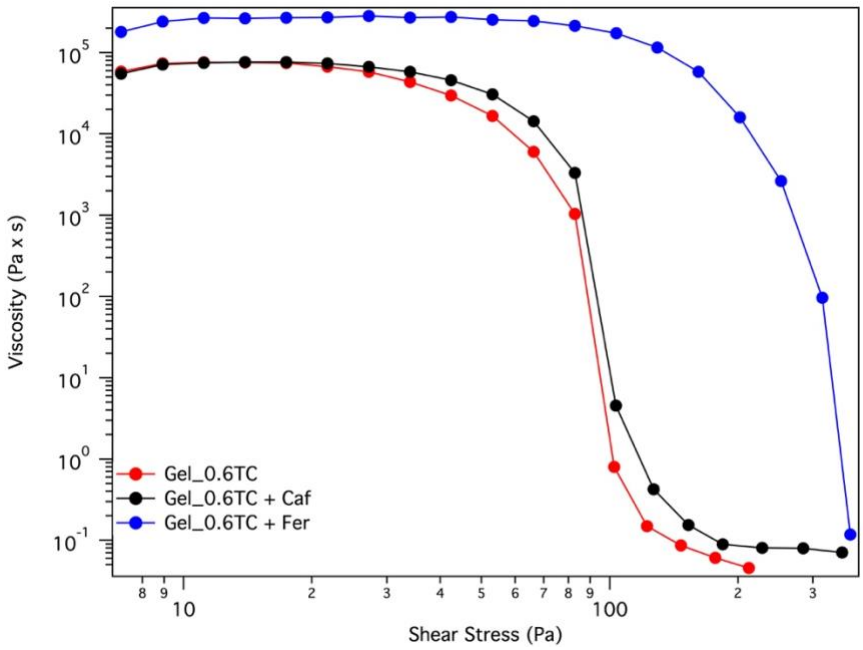


Figure 3. Flow curves of the empty Gel_0.6TC (red), Gel_0.6TC + Caf (black) and Gel_0.6TC + Fer (blue).

All the investigated samples are characterized by a Newtonian behavior in the low shear stress regime while for higher perturbations they exhibit a shear-thinning behavior as indicated by rapid decrease of the viscosity occurring at high shear stress. As indicated in Figure 4, Gel_0.6TC and Gel_0.6TC + Caf exhibit almost the same viscosity value in the whole investigated shear stress range indicating that the loading of caffeic acid does not affect meaningfully the structure of the gel system. Nevertheless, Gel_0.6TC + Fer sample has a higher viscosity over the whole investigated shear stress range; this effect could be ascribed to the structuring effect played by the ferulic acid into the gel matrix, which induces a harder and more compact structure to the LVG in comparison to the empty gel.

The flow curves were fitted using the Cross model (equation 1.):

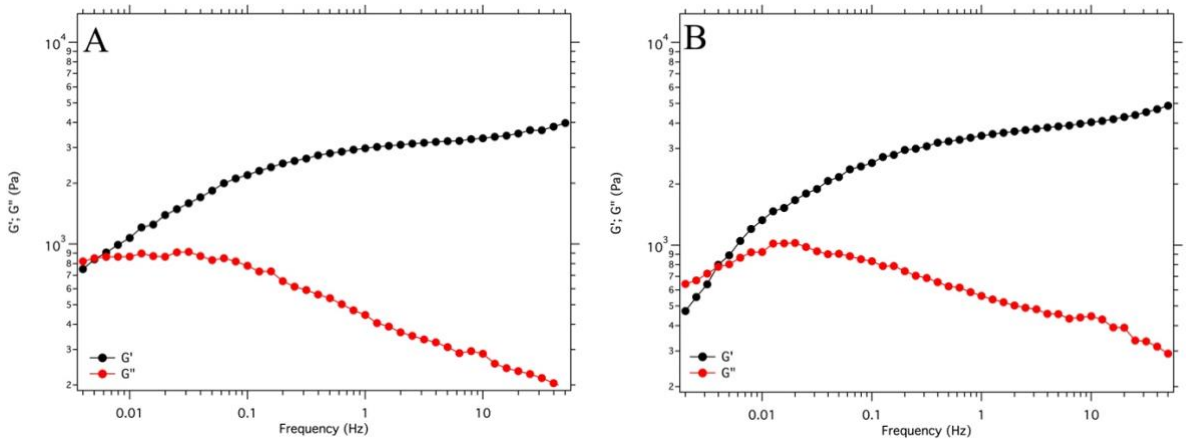
$$\eta = \eta_\infty + \frac{(\eta_0 - \eta_\infty)}{1 + (C\dot{\gamma})^m} \qquad Eq.1$$

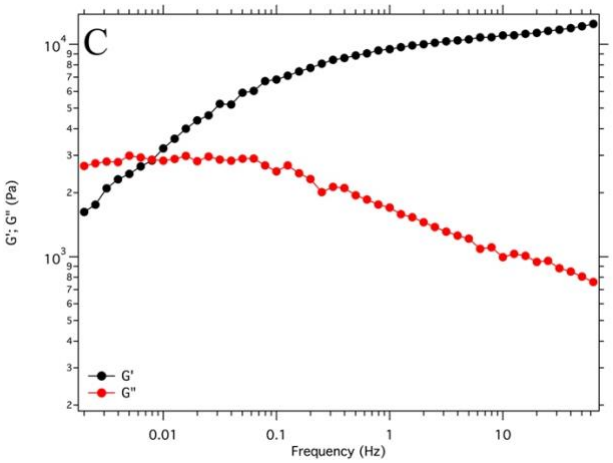
where η_∞ is the viscosity at plateau for high shear stress (in the studied case η_∞= 0 since the curves did not reach a plateau at high shear stress values); η_0 is the viscosity at low shear rate at the plateau and it represents an intrinsic viscosity of the fluid; m is the Cross rate constant

that yields the velocity gradient dependence of the rate in the shear thinning region; *C* is the Cross time constant (called also consistency of the material) that has the dimensions of time. 1/C indicates a critical shear rate that corresponds to the onset shear rate for shear thinning.

In Fig. SI1, the fittings of the flow curves are shown; the outputs data obtained from the fitting are listed in Tab SI1. The data listed in Table SI1 clearly indicate that the encapsulation of caffeic acid did not affect in a significant way the rheological features of the system Gel_0.6TC since the pure and the doped gel show almost identical parameters. On the other hand, the formulation containing ferulic acid exhibits a higher η_0, highlighting the structuring effect of the antioxidant into the LVG. The values of the Cross-rate constant are similar for all the samples, indicating a similar shear rate-dependence of the viscosity in the shear thinning region.

Furthermore, the viscoelastic properties of the LVGs before and after the loading caffeic acid and ferulic acid, were studied via oscillatory rheology by means of frequency sweep experiments. All the measurements were carried out into the LVR previously identified through amplitude sweep tests (see Figure SI1). In all the frequency sweep experiments, the strain value was fixed to 0.05 % (Fig. 4).





290 **Figure 4**. Frequency sweep curves for Gel_0.6TC (A), Gel_0.6TC + Caf (B) and Gel_0.6TC
291 + Fer (C).
292
293 Figure 4 shows that all the samples have a similar behavior, typical of viscoelastic fluids. At
294 high oscillatory frequencies, the elastic contribution prevails since *G' > G"* while below the
295 crossover frequencies, the samples exhibit a viscous behavior (*G" > G'*). Gel_0.6TC + Fer is
296 characterized by an asymptotic value of *G'* at high frequency, that corresponds to the intrinsic
297 elastic modulus of the system, that is almost five times higher than the two other gels. This
298 finding confirms the structuring effect that Fer has on the Gel_0.6TC system.
299 Since the dynamic of the investigated gels cannot be described by a single element Maxwell
300 model, the best approach to study the typical relaxation time of the investigated gels is to
301 consider the relaxation time spectrum $H(\tau)$ that depends upon G' and G'' as indicated by the
302 following equations 2 and 3:
303

304 $$G'(\omega) = G_0 + \int_0^\infty H(\tau) \frac{(\omega\tau)^2}{1+(\omega\tau)^2} \frac{d\tau}{\tau} \qquad Eq.2$$

305 $$G''(\omega) = G_0 + \int_0^\infty H(\tau) \frac{\omega\tau}{1+(\omega\tau)^2} \frac{d\tau}{\tau} \qquad Eq.3$$

306
307 Where $H(\tau)$ can be obtained following different algorithms for the inversion of the equation
308 (1) and (2).[33]
309

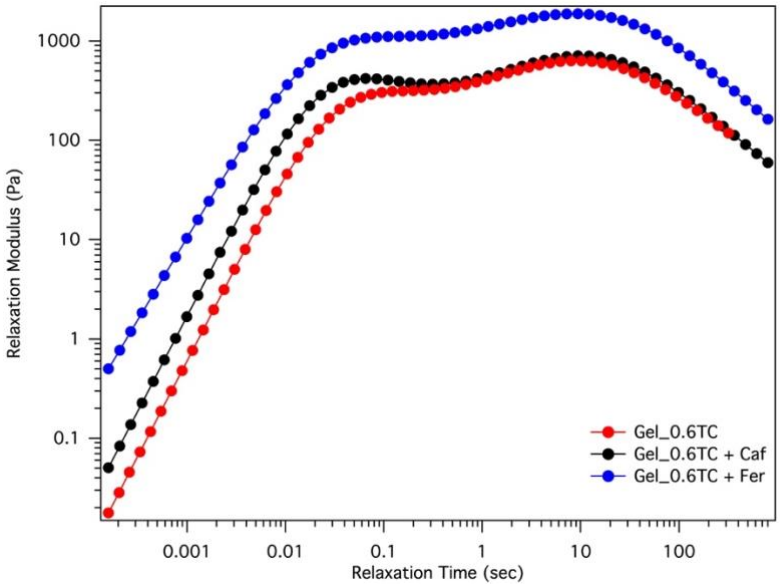


Figure 5. Relaxation plots of the Gel_0.6TC (red), Gel_0.6TC + Caf (black) and Gel_0.6TC + Fer (blue).

The relaxation mechanical spectra of the investigated LVGs are reported in Figure 5 and provide information about the distribution of the relaxation times; the profile of the curves is similar and indicates that the encapsulation of the two antioxidants in the lipid matrix do not affect the relaxation mechanism that remains almost unchanged, as indicated by the persistence of the position of the peaks. Nevertheless, the loading of ferulic acid slightly rises the relaxation modulus, confirming again the structuring character of this additive and the higher compactness of the Gel_0.6TC+Fer.

In vitro penetration studies on skin

Caf and Fer delivery into and through new-born pig skin was evaluated *in vitro* under non-occlusive conditions, using vertical Franz diffusion cells. These investigations showed the ability of the LVGs to facilitate the accumulation of both antioxidants within the different skin *strata*. As shown in Fig. 6A, the accumulation profile of the two molecules in the three layers is very similar: the largest amount of the drug was found in the outermost layers of the skin, stratum corneum (SC, 8.40% for Gel_0.6TC + Fer and 6.55% for Gel_0.6TC + Caf) and epidermis (EP, 6.40% for Gel_0.6TC + Fer and 3.83% for Gel_0.6TC + Caf) while significantly lower concentrations were found in the dermis (D, 1.69% for Gel_0.6TC + Fer and 1.45% for Gel_0.6TC+ Caf) and in the receptor compartment (0.93% for Gel_0.6TC + Fer and 0.66% for Gel_0.6TC + Caf) after 8 hours of treatment.

Nevertheless, some differences emerged by comparing the two antioxidants' profiles: the amount of Fer found in the uppermost layers of the skin (SC + EP) is greater than that of caffeic acid accumulated in the same layers (about 15 % Gel_0.6TC + Fer and about 10% Gel_0.6TC + Caf), and we found about 67 % more ferulic acid in the epidermis than caffeic acid. Moreover, as can be observed in Figure 6A, after just two hours of treatment, ferulic acid is found three-fold higher than caffeic acid in the receptor compartment. As well known, the absorption of molecules through the skin is affected by physicochemical features of the molecule itself, and its *logP* value can provide useful information and predict the behavior. Our results are perfectly in accordance with what was previously reported in the literature. Particularly, Saija *et al* tested percutaneous diffusion of Fer and Caf in saturated aqueous solutions and explained the higher skin absorption of Fer due to its higher lipophilicity, with respect to caffeic acid.[34,35] Moreover, Matri-Mestres *et al.* found limited percutaneous penetration of Caf, even when applied on the skin with penetration enhancers such as a mixture of transcutol and propylene glycol.[36]

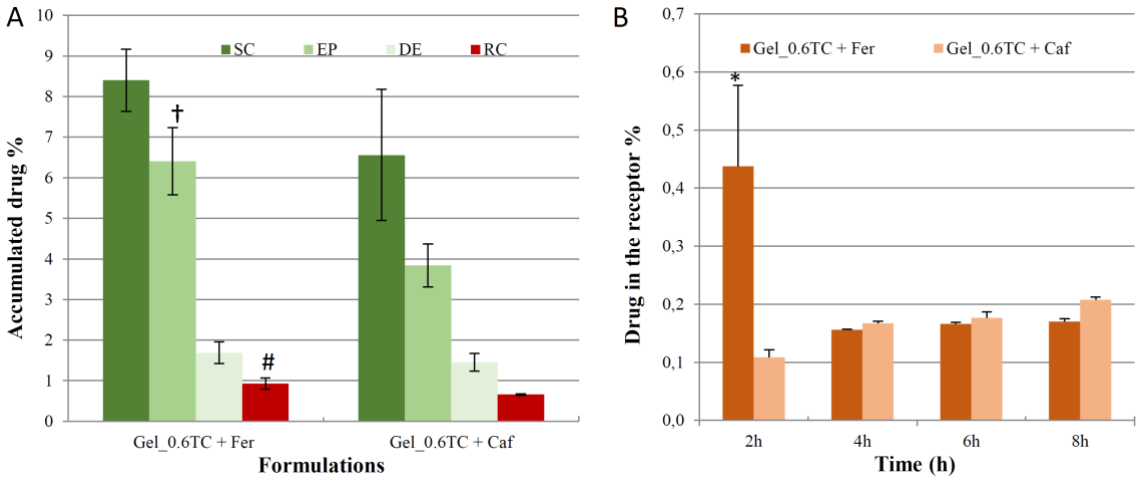


Figure 6. A) Amount of Fer and Caf retained into and permeated through the skin layers after 8 h treatment with Gel_0.6TC + Fer and Gel_0.6TC + Caf. SC, stratum corneum; EP, epidermis; D, dermis; and RC, receptor compartment. B) Amount of Fer and Caf detected in the receptor compartment at different time points after the application of Gel_0.6TC + Fer and Gel_0.6TC + Caf. The amount is expressed as the percentage of the dose applied on the skin. Results are reported as means ± standard deviations of at least 6 independent determinations. Symbols represent significance of differences between Fer and Caf in EP, † $P < 0.05$, between Fer and Caf in RC, # $P < 0.05$, and between Fer and Caf in RC after 2h, * $P < 0.05$.

In conclusion, Caf is mostly retained in the gel and then released more slowly once applied to the skin. Conversely, given its higher lipophilicity [34,35,37], and thanks to the presence of the permeation enhancers TC and MO, Fer is able to permeate all layers and reach the receptor compartment faster than Caf. However, the differences between Fer and Caf loaded LVGs that emerged from the rheological and SAXS studies suggest a different interaction between the antioxidants and the gel matrix, which can subsequently affect the release and accumulation/permeation of the investigated active compunds in/through the skin.

4. Conclusion

The application of a formulation on the skin represents an easy and non-invasive way to administrate drugs. In this way, the harsh gastrointestinal environment, which can affect the biodistribution and stability of the formulation, can be bypassed. The *stratum corneum* is indeed a challenging obstacle but the inclusion of permeation enhancers and edge activators in the carriers can improve the effectiveness and the quality of the therapy.

Therefore, LVGs were formulated with monoolein and sodium taurocholate to deliver caffeic and ferulic acid. The SAXS investigations highlighted that the presence of TC induces a phase transition from inverse bicontinuous cubic Pn3m, the native structure of GMO at these concentrations, to multilamellar vesicles dispersions. The increasing concentration of TC decreases the lamellarity of the vesicles, whereas the number of lamellae increases in the range 35 – 40 °C for the sample at 0.6 % wt of TC. The encapsulation of the antioxidants did not affect the inner structure of LVG at low TC content (0.6 wt %). The LVGs unloaded and loaded with Caf and Fer were studied by means of oscillatory measurements, displaying the shear-thinning behaviour of the formulations and the structuring effect of Fer on the lipid matrix in comparison with the empty LVG and the one loaded with Caf. The skin permeation tests showed the dermal release of both antioxidants, highlighting the applicability of these formulations for topical administration.

These findings exhibited the effect of LVGs composed with MO and TC, recognized as safe and used in the cosmetical and pharmaceuticals industries, for local application of bio-actives. Indeed, the interactions of these components with keratinocytes could be evaluated to understand the nature of the permeation phenomena. Some studies showed the applicability of NMR spectroscopy both in solid and liquid state for this purpose.[38] Moreover, formulating a mechanism of penetration of these carriers could represent a future project so to improve and enhance the experimental design for future applications.

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