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Development of a microemulsion for encapsulation and delivery of gallic acid. The role of chitosan

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ABSTRACT

A novel water-in-oil (W/O) microemulsion based on natural oils, namely extra virgin olive oil (EVOO) and sunflower oil (SO), in the presence of non-ionic surfactants was successfully formulated. The novel microemulsion was used as a carrier for gallic acid (GA) to assure its protection and efficacy upon nasal administration. The work presents evidence that this microemulsion can be used as a nasal formulation for the delivery of polar antioxidants, especially, after incorporation of chitosan (CH) in its aqueous phase. The structure of the system was studied by Small Angle X-ray Scattering (SAXS), Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR) spectroscopy techniques. By the addition of CH, the diameter of the microemulsion remained unaltered at 47 nm whereas after the incorporation of GA, micelles with 51 nm diameter were detected. The dynamic properties of the surfactant monolayer were affected by both the incorporation of CH and GA. Moreover, the antioxidant activity of the latter remained unaltered (99 %). RPMI 2650 cell line was used as the *in vitro* model for cell viability and for GA nasal epithelial transport studies after microemulsion administration. The results suggested that the nasal epithelial permeation of GA was enhanced, 3 h post administration, by the presence of 0.2 % v/v microemulsion in the culture medium. However, the concentration of the transported antioxidant in the presence of CH was higher indicating the polymer's effect on the transport of the GA. The study revealed that nasal administration of hydrophilic antioxidants could be used as an alternative route besides oral administration.

1. Introduction

The delivery of bioactive compounds with drug-like activities to their targets is a challenging field, as the carrier needs to protect the hosted molecules while overcoming the limitations of the selected route of administration. Nanotechnology based carriers such as nanoparticles, liposomes, nanoemulsions, microemulsions and others [1–9] have already been successfully used for the delivery of bioactive compounds offering a protective environment and higher solubility for the encapsulated compound with user compliance at once [10]. Microemulsions are thermodynamically stable, transparent nanodispersions of oil, surfactant, co-surfactant (occasionally) and water [11]. In the literature, there are many examples of microemulsions successfully used for the delivery of bioactive compounds for the treatment of pathological conditions [12,13]. Nonetheless, the high concentration of emulsifiers and the occurrence of phase separation in physiological conditions are the drawbacks that these formulations may exhibit.

Administration of bioactive compounds hosted in nanosystems can be achieved by different routes such as oral, dermal, ocular, nasal [14–17] *etc.* Oral administration is the most studied one but exhibits many disadvantages regarding the first-pass effect, the slow onset of action and the degradation processes [18,19]. As a result, different approaches must be used such as the development of capsules and coatings for targeting the complex gastrointestinal tract [20]. A recent study of our group showed the relative low transport of the antioxidant hydroxytyrosol, encapsulated in microemulsions, across intestinal epithelium [21], indicating the need of an alternative administration route and modification of the used nanocarriers. The nasal route has gained attention, since it was proven to be an "entrance" for both systemic and brain delivery [22]. It is a non-invasive route, with high vascularization, high systemic drug absorption, able to avoid first-pass hepatic metabolism while offering the possibility for direct brain delivery [23]. Moreover, is an ideal route for treating chronic diseases due to the compliance of the patients, substituting oral administration [24]. Many

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drugs can be found in the market as nasal formulations such as sumatriptan [25], zolmitriptan [26], oxytocin [27] among others.

Microemulsions, as liquid-in-liquid colloidal systems with a wide range of applications, have been already studied in nasal delivery [28,29] but the majority of the reported systems include high co-surfactant concentration, which may lead to side effects such as irritation and allergy. In addition, the literature focuses on O/W systems due to the low bioavailability of the hydrophobic and the high molecular weight synthetic drugs. However, in nasal cavity, hydrophilic compounds are also facing absorption problems and the development of appropriate W/O systems is of paramount importance.

In the present study we focused on the formulation of an adequate novel system with natural oils in the absence of co-surfactants in order to avoid any undesirable side effects. Non-ionic surfactants were exclusively used for the formulation and, as far to our knowledge, it is the first time that distilled monoglycerides (DMG) were used in a microemulsion for nasal application. In addition, chitosan (CH) was used as penetration enhancer, after its incorporation in the dispersed phase of the system and its structural effect was studied by Small Angle X-ray Scattering (SAXS), Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR).

Gallic acid (GA) was used in the present study as a model bioactive compound to prove the effectiveness of the novel system in nasal administration, especially due to its therapeutic potency and low bioavailability after oral administration [30]. GA is a natural phenolic compound mostly found in grapes and black tea [31,32] and it has been in the center of interest for its antioxidant, antimicrobial, anti-inflammatory and even antidepressant properties [33–36]. Interestingly, it has been reported that regular consumption of GA may inhibit the amyloid fibril formation which characterizes many protein misfolding diseases [37]. However, oral administration of GA to animals showed low bioavailability with low maximum drug concentration in plasma leading to the need of alternative route of administration [38].

The low metabolic environment of nasal mucosa and the leaky nature of the epithelium make this route ideal for antioxidant delivery [39]. Moreover, the use of mucoadhesive ingredients in the formulation can increase the residence time in the mucosal epithelium overcoming the rapid mucociliary clearance mechanism. In recent years, the natural polymer, CH has attracted interest as a mucus adherent agent in nasal delivery systems [40]. CH is produced by deacetylation of chitin found in crustacean shells [41]. Its positive charge helps to interact with the negatively charged mucin and offers prolonged residence time while acts as a penetration-enhancer by opening the tight junctions [42–45]. The human nasal epithelial cell line RPMI 2650, derived from septum carcinoma, was used in order to evaluate *in vitro* the proposed microemulsion as a nasal drug delivery system. This cell line has been extensively used in the field of nasal administration as it closely resembles normal human cells with respect to karyotype and mucus production [46]. Interestingly, the most adequate conditions for this cell culture in order to construct a cell layer for drug absorption studies, is the air-liquid interface conditions (ALI) [47,48].

The aim of the present study was the development of a new biocompatible W/O microemulsion based on non-toxic ingredients for the effective encapsulation of GA and its potential use as nasal carrier. Our objective was the formulation of a non-toxic carrier, in the absence of any co-surfactant molecule, which effectively protects the antioxidant until its release while increasing its transport with the use of a natural mucopenetrating agent.

2. Materials and methods

2.1. Chemicals

5-Doxyl-stearic acid (5-DSA), galvinoxyl free radical, gallic (GA) and protocatechuic (PC, IS) acids were purchased from Sigma-Aldrich, Germany. Polyoxyethylene sorbitan monoolate (Tween 80™) was obtained from Sharlau, Spain. Distilled monoglycerides of vegetable fatty acids (DMG 0295) were a kind gift from Palsgaard, Denmark. Chitosan (viscosity 200–600 mPa.s, 0.1 % in 0.5 % Acetic acid, 20 °C; Deacetylation value: 80 %, CH) was purchased from TCI, Belgium. Extra virgin olive oil (EVOO) and sunflower oil (SO) were commercial products purchased from a local market. High-purity water was obtained from a Millipore Milli Q Plus water purification system.

2.2. Cell line

The cell line RPMI 2650 (CCL-30) was kindly provided by Dr. Fabio Sonvico (University of Parma, Parma, Italy). Cells between passage 16–30 were grown in 75 cm^2 flasks in complete Minimum Essential Medium (MEM) containing 10 % (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acid (NEAA) solution and maintained in a humidified atmosphere of 95 % air 5% CO_2 at 37 °C. Cells were sub-cultured according to the ATCC protocol. MEM, FBS, 100 U/mL penicillin and 100 mg/mL streptomycin, phosphate-buffered saline (PBS) and trypsin-EDTA were purchased from Gibco (Invitrogen Corporation, Life Technologies, UK). 12-well ThinCert™ polycarbonate inserts, 0.4 μm pore size and growth surface 4.67 cm^2 were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany).

2.3. Formulation and phase behavior study

W/O microemulsion consisting of EVOO and SO as the oil (continuous) phase, Tween 80 and DMG as biocompatible surfactants and ultra-pure water as the aqueous (dispersed) phase were prepared by adding the aqueous phase to a mixture of oil and surfactants. Gentle shaking led to an optically transparent system. For the preparation of a mucoadhesive microemulsion the ultra-pure water was replaced by a CH solution (0.1 % CH in 1% acetic acid solution). For the determination of the systems' monophasic areas the corresponding pseudo-ternary phase diagrams were constructed as described previously [49]. In the present study for the encapsulation of GA in the microemulsions an aliquot of GA was added in the mixture of surfactants and the non-polar solvents. For all experiments, the concentration of GA was kept constant in the microemulsion at 2.8 mM.

2.4. Structural study

2.4.1. Small angle X-ray scattering (SAXS)

Small angle X-ray Scattering (SAXS) experiments, for microemulsions in the presence and absence CH solution, were carried out on a Nano-inXider vertical SAXS/WAXS system of Xenocs SA, France equipped with a Cu kα source and a set of two detectors for continuous SAXS/WAXS measurements. The wavelength of the X-ray radiation was 0.154 nm and the sample-to-detector distance was 937.5 mm in SAXS. The exposure time for each scattering frame was 600 s (in the VHR mode) in a full vacuum environment using Low Noise Flow cell sample holder, enabling injection of various samples without changing experimental conditions.

2.4.2. Dynamic Light Scattering (DLS)

A Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (UK) equipped with a He-Ne laser (632.8 nm) using a non-invasive back scatter (NIBS) technology was used for the DLS study of the microemulsions. A scattering angle of 173° and a quartz type cuvette were used. Each sample was measured after filtering through 0.45 μm cellulose for dust-free conditions at 25 °C. Data were processed using the Malvern Zetasizer Nano software.

2.4.3. Electron paramagnetic resonance (EPR)-membrane dynamics

EPR spectra, were recorded at constant room temperature (25 °C), using a Bruker EMX EPR spectrometer operating at the X-Band, Bruker USA with the use of a WG-813 Q-Wilmand (Buena, NJ) Suprasil flat cell.

Spin probe technique was used to obtain information about the interfacial properties of the surfactant monolayer in the studied microemulsions. In order to obtain the desired concentration of the spin probe ([5-DSA] = 1.2×10^{-4} M) in the microemulsions the formulations were added to vials where the appropriate amount of the spin probe had been formerly deposited. Experimental results where expressed by means of rotational correlation time (τ_R) reflecting the mobility of the probe, and the order parameter (S) expressing the rigidity of the surfactant membrane. In the present study, the parameters were calculated for empty and GA-loaded microemulsions in the absence and the presence of CH. In addition, the parameters τ_R and S were determined for a reference microemulsion with 1% acetic acid solution as aqueous phase. Simulations for all the spectra were performed with home-written programs in MATLAB (The MathWorks) employing the Easy Spin toolbox for EPR spectroscopy [50].

Viscosity measurements were conducted with the use of a DV-I Prime Digital Viscometer (Brookfield Engineering Laboratories, USA), equipped with a cone spindle (CPA-40Z). Experiments were performed in triplicate for each sample under constant temperature (25 °C).

2.5. Antioxidant activity assessment

For the study of the antioxidant activity of the novel microemulsion the stable galvinoxyl free radical was used in the EPR spectrometer mentioned above. The experimental procedure followed is described in a previous study of our group [51]. In the present investigation, 0.1 mL of each W/O microemulsion was added to 0.9 mL of galvinoxyl (0.25 mM) solution in isooctane. EPR spectra were recorded at room temperature for 30 min.

2.6. Biological assessment

2.6.1. Cell proliferation assay

Cell proliferation was assessed 48 h after treatment by the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay according to the manufacturer's standard protocol. MTT stock solution (5 mg/mL) was added to each culture being assayed to equal one tenth of the original culture volume and incubated for 3 h. At the end of the incubation the solution was removed and the converted dye was solubilized in isopropanol: DMSO solution in ratio 1 to 1 in order to dilute the insoluble purple formazan. Absorbance of converted dye was measured at 570 nm and Eq. 1 was used to determine the cell viability.

$$\text{Cell viability}\ (\%) = \frac{(OD\ of\ treated\ cells)}{(OD\ of\ control)} \text{x}\,100 \quad (1)$$

Where, OD is the optical density.

2.6.2. In vitro nasal transport studies

For permeability experiments, cells were seeded on 12-well ThinCertrs™ permeable polyester filters of 0.4 μm pore size. In order to establish the ALI model, cell suspension was seeded onto the filters at a seeding concentration of 4×10^5 cells/cm^2 per filter. The media on the apical compartment were removed 8 days post-seeding. Media in the basolateral chamber were replaced every two days. Cell layers were allowed to grow and differentiate under ALI conditions up to 21 days. Transepithelial electrical resistance (TEER) was measured as described previously [21].

On the 21 st day post seeding, monolayers were incubated in the apical compartment containing a GA aqueous solution, empty or loaded microemulsions at 0.2 % v/v in HBSS. The apical compartment received 0.6 mL of medium while the basolateral compartment received 1.2 mL. The basolateral media were collected at different time intervals of incubation and were immediately analyzed by LC–MS/MS for the detection and quantification of GA. A calibration curve of GA in the transport medium was constructed with different GA concentrations ranging from 0.05 to 1 μg/mL (Fig. S1). PC (internal standard, IS) concentration was kept constant at 0.1 μg/mL. In addition, as oxidation phenomena occur during the incubation time, the oxidation profile of GA during the studied time points was also measured (Fig. S2).

2.7. Quantitative analysis

For the quantification of the transported GA, analysis was performed with the LC–MS/MS based on a variation of the method by Basu et al. [52]. Separation was performed with an Agilent Eclipse Plus C-18 column (50 mm × 2.1 mm inner diameter, 3.5 um particle size) with a RRLC in-line filter kit (2.1 mm, 0.2 μm filter) (Agilent, USA). Electrospray Ionization (ESI) operating in negative mode was used for both GA and IS. More details for chromatographic conditions and mass spectrometry analysis can be found in supplementary material. (Section S1)

For sample preparation, 100 μL of the basolateral side were transferred into a 1.5 mL Eppendorf tube and 450 μL of ice-cold acetonitrile were added. The sample was then centrifuged for 10 min in 10.000 rpm at 4 °C for protein precipitation. The supernatant was transferred into a clean HPLC appropriate vial and evaporated to dryness. The pellet was redissolved in 100 μL of H_20: ACN (1:1) and 10 μL IS solution (1 μg/mL) were individually added in the sample. The mixture was vortexed and a 5 μL aliquot was injected into HPLC–MS/MS for analysis.

3. Results and discussion

3.1. Formulation and phase behavior study

Phase behavior of multi-component systems such as microemulsions can be studied by pseudo-ternary phase diagrams. In order to formulate a system adequate for nasal delivery ingredients of non-toxic origin were chosen. EVOO and SO were used as biocompatible components of the continuous phase. The use of EVOO can give an added value to the final formulation due to its anti-inflammatory, antioxidant and neuroprotective properties [53,54]. In the present study, DMG and Tween 80 were used, as biocompatible surfactants, in equal amounts. As Constantinides et al. have reported, the use of a combination of two amphiphilic molecules with totally different HLB values is able to create a thermodynamically stable nanodispersion [55]. In the literature, Tween 80 has been extensively studied in nasal applications due to its membrane penetration efficacy and its positive results in the absorption of hormones and drugs [56]. Also, recently, this non-ionic surfactant was reported to increase drug systemic absorption and passive transport to the Blood Brain Barrier (BBB) after intranasal administration [57]. In order to avoid the involvement of another synthetic surfactant the Generally Recognized as Safe (GRAS) DMG was used. DMG is a mixture of mono- and di- glycerides of fatty acids with low-HLB with application in food industry and oral delivery systems [58]. Water was the dispersed phase of the system while CH solution was used for the construction of a system with mucoadhesive/mucopenetrating properties. Fig. 1 represents the phase diagram of the two systems, where a narrow isotropic region corresponding to the monophasic area (1φ) and a broad multi-phase region can be observed. As the ratio of emulsifiers was increased, increasing amounts of the dispersed phase were incorporated in the system expanding the boundaries of the monophasic region. Interestingly, an almost identical monophasic area was obtained when water was replaced with CH solution but longer period of time was needed for the systems to reach equilibrium. The incorporation of CH in a microemulsion system could extend easily the formulation's properties by creating a final effective carrier. To strengthen the biocompatibility of the system a relatively low surfactant concentration was selected. More specifically, the system was formulated with 23.4 % w/w of surfactants, a low ratio comparatively to other nanosized formulations [59,60]. Additionally, natural oils are a challenging option for the formulation of new systems without the use of co-surfactants, due to their increased humidity and the complex structure [61]. Finally,

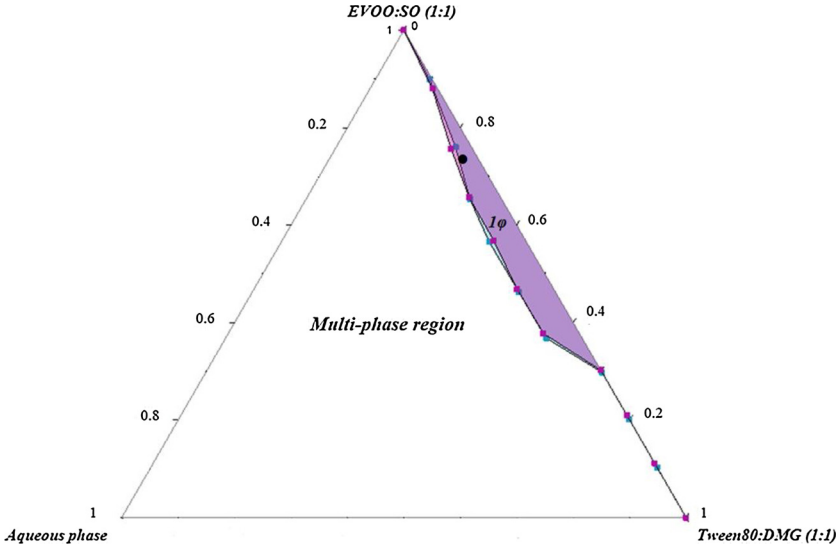

Fig. 1. Pseudo-ternary phase diagram of the system consisted of EVOO/SO/Tween 80/DMG/ aqueous phase. Water and CH solution were used as dispersed phases of the systems. The monophasic region of the system containing water is defined by the light blue (. • .) while the one formulated with CH solution by the purple line (. • .). Temperature was kept constant at 25 °C. Point (•) corresponds to the described microemulsion composition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the composition of the microemulsion chosen for further studies was: EVOO: SO (1:1) 72.4 % w/w, Tween80: DMG (1:1) 23.4 % w/w and 4.2 % w/w aqueous phase.

3.2. Structural study

3.2.1. Small angle X-ray scattering (SAXS)

SAXS is an analytical and non-destructive method for investigating nanostructures in liquids and solids. SAXS is able to probe the colloidal length scales of 10–1000 Å [62] and therefore is an appropriate method for determining the size and the structure of colloidal systems such as microemulsions. In the present study, the method was used in order to clarify the possible alterations that may be provoked in the nanodispersion's structure after the incorporation of the CH polymer in the dispersed phase. Fig. 2a represents the intensity profile of microemulsions in the absence (closed circles) and presence (open circles) of CH. Interestingly, the intensity profile for both cases is similar in the majority of q region. However, in lower q values (0.007–0.02 $Å^{-1}$) the intensity of the microemulsion with the CH solution is higher than the classic microemulsion. Interestingly, the slope of both SAXS spectra at q range 0.011-0.012 $Å^{-1}$ is similar indicating a characteristic size of approximately 55 nm for both systems, giving an insight into the contour length of the elongated micelles. Looking at the high q region of the spectra obtained by WAXS (Fig. 2b) the curves are overlapping. This result indicates that the observed alteration in the mentioned low q values is significant and related to the impact of CH molecules on the reverse micelles. It is assumed that the scattering pattern does not change probably due to the low concentration of incorporated CH (0.1 % w/w in the aqueous solution). However, as this concentration was satisfactory regarding the *in vitro* studies no further structural studies were conducted. These indicative results clearly show that the reverse micelles do not have the classic SAXS pattern of spherical conformation.

This is an anticipated result due to the nature of the surfactant molecules Tween 80 and DMG. It is well known that the Critical Packing Parameter (cpp) of Tween 80 is below 0.2 [63] creating spherical micelles whereas DMG is able to create a wide range of structures such as lamellar, cubic and reversed hexagonal depending on temperature and other factors [64]. However, as the scope of the present study was not the exhaustive examination of the structure of the system, we focused on how the GA and CH affected the size and the interfacial properties of the surfactant layer of the system.

3.2.2. Dynamic Light Scattering (DLS)

In the case of DLS, if the particles are not spherical the hydrodynamic radius is often taken as the apparent hydrodynamic radius or equivalent sphere radius [65]. As observed from the SAXS study (Fig. 2) the microemulsions in the present study do not have a spherical conformation. Nevertheless, DLS can be used in order identify any alterations after the addition of different compounds regarding the apparent hydrodynamic radius. As a result, measurements were carried out to

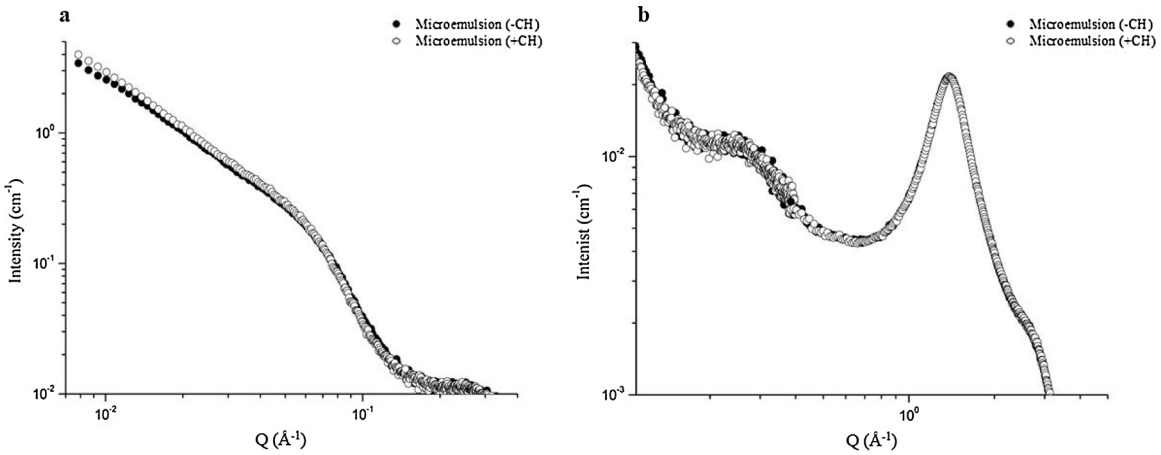

Fig. 2. SAXS/WAXS curve of microemulsion EVOO/SO/DMG/Tween80/H2O without (closed circle) and with CH (open circle), with detail on two different length scales (a) SAXS region length scales ~100–1 nm and (b) WAXS region length scale ~ 1–0.1 nm.

Table 1
Droplet diameter (nm) and polydispersity index (PdI) of the empty and GA-loaded microemulsions.

	Diameter (nm)	PdI
Empty (- *CH*)	46.7 ± 0.6	0.101 ± 0.01
Loaded (- *CH*)	50.1 ± 0.8	0.110 ± 0.02
Empty (+ *CH*)	47.4 ± 1.4	0.109 ± 0.01
Loaded (+ *CH*)	52.5 ± 0.9	0.120 ± 0.02

evaluate the apparent hydrodynamic diameter and the polydispersity index (PdI) of the dispersed aqueous phase. Table 1 shows the diameter and the PdI of the studied systems. As it can be observed, when GA was added in the microemulsion the diameter slightly increased whereas the PdI remained unaltered. This behavior could be explained by the small molecular size of GA in combination with its partition coefficient. A similar increase in the diameter of the micelles has been also reported by our group in a system consisting of Mygliol 810/Isopropyl myristate/Lecithin/Glycerol/Ethanol/Water [33]. The incorporation of CH solution (+CH) in the aqueous phase of the system did not affect the diameter of the microemulsion, in agreement with the SAXS results, and the addition of GA followed the same profile as in the case of the absence of CH (−CH). To conclude with, the incorporation of CH, did not provoke changes in the size of the system's dispersed phase but the encapsulation of GA resulted in small changes in the diameter of the aqueous droplets.

3.2.3. Electron paramagnetic resonance (EPR)-membrane dynamics

In order to obtain information about microemulsion's surfactant membrane, the use of the EPR spin probe technique was applied. The success of this technique derives from the ability of the environment close to the probe to influence the spin probe's EPR spectrum [66]. This technique is essential for the identification of the systems' membrane rigidity and the localization of an encapsulated molecule-drug, information fundamental for the prediction of the carrier's delivery efficacy.

In the present study, the encapsulation of GA and CH induced alterations in the surfactant monolayer that reflect on the increased τ_R values of the system as can be seen in Table 2 and Fig. S3. The restrictive motion of the N–O moiety of the 5-DSA indicates the interference of both additives in the surfactant layer of the system. In a previous study, the encapsulation of GA has induced the same alterations [67]. In a different system studied by our group [68] applying a molecular dynamics approach, indicated that GA does not participate in the surfactant membrane. Nevertheless, in the first case [67] the presence of Tween 40 and ethanol increased the solubility of the GA in contrast to the latter case where the microemulsion system was consisted of surfactants with low HLB values, environment limiting the solubility of GA. These apparently controversial results indicate that the nature of the system's ingredients affect the localization of the bioactive compound regarding its solubility. In the present study, Tween 80 increased the solubility of GA and as a result, the bioactive appears to be in higher concentration closer to the polar heads of the surfactant where Tween 80 and water coexist.

The pH of the microenvironment may influence the state of the fatty acid-based spin probe [69]. In the case of the CH-free systems the 5-DSA exists in its ionized form whereas in the case of CH-loaded systems the pH decreased as a consequence of the addition of acetic acid. This change results in a different localization of the 5-DSA in the interface closer to the surfactants' chains. For this reason, for the microemulsions containing CH, we used as a reference system a microemulsion formed with 1 % acetic acid solution. The increased τ_R value of 5-DSA in the system with 1 % acetic acid in comparison with the one with ultra-pure water, reflects a more packed environment, closer to the surfactants chains. The rigidity of the membrane seems also to be increased in the localization area of the 5-DSA. On the other hand, comparing the parameters for the system with acetic acid solution and the respective one with CH, the interaction of CH with the surfactant membrane is confirmed by the decreased mobility of the spin probe.

To conclude with, the addition of GA and CH induced changes in the spin probe's movement indicating their involvement in the interface. The participation of both molecules in the interface is fundamental for two reasons: i) the CH molecules interact with the mucus of the nasal epithelium exploiting its mucoadhesive properties and ii) the encapsulated-GA is more likely to be released. From the values presented in Table 2 we can see a slightly altered rigidity of the membrane. No alteration in viscosities was detected in all cases (Table S2).

Table 2
Rotational correlation time (τ_R) and order parameter (S) of 5-DSA in the studied systems.

	τ_R (ns)	S
Empty (- CH)	4.15 ± 0.01	0.32 ± 0.01
Loaded (- CH)	4.31 ± ≤0.01	0.34 ± 0.01
Empty (acetic acid)	4.55 ± 0.02	0.36 ± ≤ 0.01
Empty (+CH)	4.23 ± 0.02	0.34 ± 0.02
Loaded (+ CH)	4.50 ± 0.01	0.35 ± 0.01

3.3. Antioxidant activity assessment

Studying the antioxidant profile of the system with the encapsulated GA is of high importance as it proves the retention of GA's activity after the encapsulation. In addition, the antioxidant capacity of the novel system is crucial as it can serve as a useful radical scavenger in nasal cavity especial in cases of inflammation responses (polyposis) [70]. As Gao et al. [71] have studied, the topical application of natural oils can reduce inflammation by activating intracellular antioxidant pathways or by simple scavenging the reactive oxygen species (ROS). In the present study, the scavenging activity of the systems was studied by following the intensity of the EPR signal of galvinoxyl *versus* time. Table 3 shows the effect of the incubation time on the percentage of free radical's inhibition. The GA loaded microemulsion shows a high antioxidant activity as it is able to scavenge the total amount of the free radical after 10 min of reaction. Additionally, the empty microemulsion also presents a high antioxidant capacity calculated at 69 %, at the first minute of reaction. EVOO is known for its high concentration in antioxidants such as hydroxytyrosol, caffeic acid, syringic acid *etc.* [72] The addition of CH in the aqueous phase of the system affects neither the total antioxidant activity of the microemulsion nor the activity of the GA making the system appropriate for the co-existence of a hydrophilic antioxidant and the polymer with the mucopenetrating properties. Fig. S4 shows the EPR spectra of galvinoxyl stable free radical after 1 min incubation with the systems of Table 3.

3.4. Biological assessment

3.4.1. Cell proliferation assay

Cell proliferation assay, in the RPMI 2650 culture, was performed

Table 3
% Scavenging activity of empty and loaded system in the absence (−CH) and presence (+CH) of CH towards galvinoxyl free radical *versus* time.

	% Scavenging activity			
Time (min)	Empty (-CH)	Loaded (-CH)	Empty (+CH)	Loaded (+CH)
1	69.2 ± 5.2	92.2 ± 0.8	62.1 ± 4.3	93.4 ± 0.8
5	67.4 ± 6.1	97.3 ± 0.9	69.1 ± 3.2	96.7 ± 0.7
10	81.1 ± 5.5	98.6 ± 0.1	73.7 ± 3.1	98.5 ± 0.1
15	86.2 ± 6.8	99.1 ± 0.1	76.7 ± 3.0	99.1 ± 0.2
20	87.6 ± 6.5	99.1 ± 0.1	76.4 ± 3.1	99.1 ± 0.2

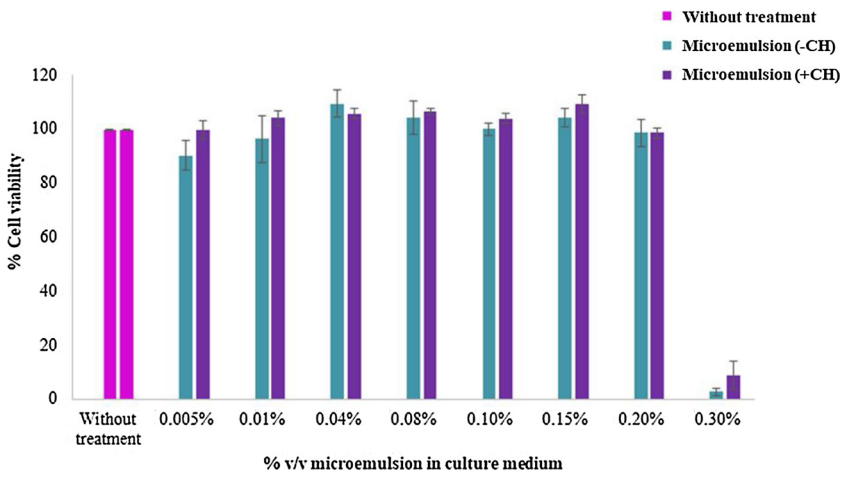

Fig. 3. Effect of the empty microemulsions in cell viability, in a range of concentrations 0.005 %–0.3 % v/v in the culture media of the RPMI 2650. The incubation period of the microemulsion in the culture media was 48 h. Each column represents the mean ± SD. Green bars represent the system without CH and the purple the system with CH in the aqueous phase.

using the MTT assay. Cells were treated with the empty microemulsions at a range ratio of 0.005 % to 0.3 % v/v in the culture medium. As can be seen from Fig. 3 the nanocarriers did not exhibit any cytotoxic effect up to the concentration of 0.2 % v/v ratio. In the case of 0.3 % v/v the cytoxicity was extremely increased which can be explained by the presence of the high concentration of surfactants and especially of Tween 80 in the cell culture medium, as others have also observed. As Kürti et al. [73] have mentioned, after a 4-h MTT dye conversion cell viability test, Tween 80 above the concentration of 5 mg/mL significantly reduced the viability of RPMI 2650 cells in a dose-dependent way, confirming our findings. In comparison to the most studied Caco-2 cell line, RPMI 2650 is more sensitive to Tween 80, as literature indicates [74]. The cytotoxicity of a microemulsion with Tween 80 and DMG in the co-culture Caco-2/TC7 HT29-MTX has been previously tested and indicated that even in higher concentrations of the surfactant in the microemulsion system the co-culture can tolerate higher microemulsion concentrations. However, the leaky nature of the mucosal epithelium balanced the surfactant sensitivity of the cell culture especially in the presence of carefully selected excipients minimizing the quantities needed. The cytotoxicity profile between microemulsion without and with CH towards the RPMI 2650 cells did not change confirming its biocompatible nature. As a result, the formulated microemulsion can be safely used as local or systemic nasal delivery systems in the mentioned concentrations.

3.4.2. *In vitro nasal transport studies*

The problem of low nasal permeability rates for polar molecules, including low molecular weight drugs and compounds is reflected in their low bioavailability [74]. In general, polar compounds with low molecular weight (MW < 1000) are able to be transported through the nasal epithelial barrier transcellularly or paracellularly. To confirm the potential permeability enhancement effect of the formulated microemulsion, the total amount of GA that was transported across nasal mucosa model was measured. For this purpose, RPMI 2650 cells were cultivated in special filters under ALI conditions. The percentages of GA, in free and encapsulated state, that has been transported through the constructed epithelium towards time of incubation are presented in Fig. 4. A calibration curve of GA in the transport medium (HBSS) was constructed with different GA and IS concentrations ranging from 0.05 to 1 μg/mL (see Fig. S1). The nasal epithelial transport of encapsulated GA follows a similar pattern with the GA aqueous solution. However, after 3 h, the amount of the permeated GA shows substantial differences between the formulations, confirming the greater permeation in the presence of the microemulsion's ingredient. This behavior could be attributed in the presence of different penetration enhancers existing in the microemulsion, such as the phospholipids of the edible oils and the surfactants. Tween 80, fatty acids and phospholipids are some of the

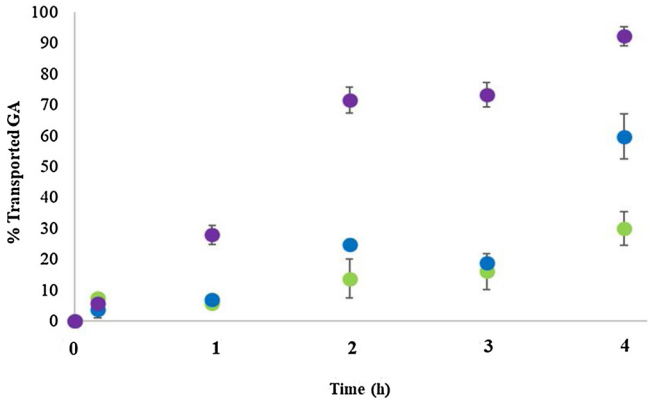

Fig. 4. Transport study of GA acid through the nasal constructed epithelial barrier in solution (●) and encapsulated in microemulsion in the absence (●) and the presence (●) of CH solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

most representative ingredients which affect the permeability of the epithelial cell layer by modifying the phospholipid layer, membrane proteins or the outer layer of the mucosa. Some of these enhancers also have an effect on the tight junctions and work as enzymatic inhibitors [24]. In addition, the altered permeation profile between the 3rd and the 4th hour may be attributed to the ability of the Tween 80 to be diffused and reach the tight junctions. This interesting result, amplify the use of nasal route as the most appropriate route for hydrophilic antioxidants encapsulated in microemulsions in comparison to the oral administration. In the latter case, the ingredients of the microemulsion system and especially the surfactants, did not possess any enhancing effect on the transport of hydroxytyrosol, behavior attributed to the interactions of the DMG and the molecule itself with the mucus layer. However, this behavior is not reproduced here as the microemulsion system increased the permeability of GA 3 h post administration. This may be attributed to the different properties of the mucus layer produced by the nasal and intestinal cell lines. The nasal mucus layer is generally described as less viscous and thicker than the intestinal, environment that facilitates the nasal transport of GA [75].

In the case of the microemulsion with CH, the profile changes with a significant increase of antioxidant's transport through nasal epithelium. It has been previously reported, that CH increases cell permeability by affecting the tight junctions [75] a fact which is confirmed by the lower TEER values (Fig. 5) after the system's administration. In addition, CH in acidic environment is positively charged and its amine groups interact with the mucus layer, affecting thus, the adhesiveness in the mucosal epithelium. The last years, CH has been incorporated in

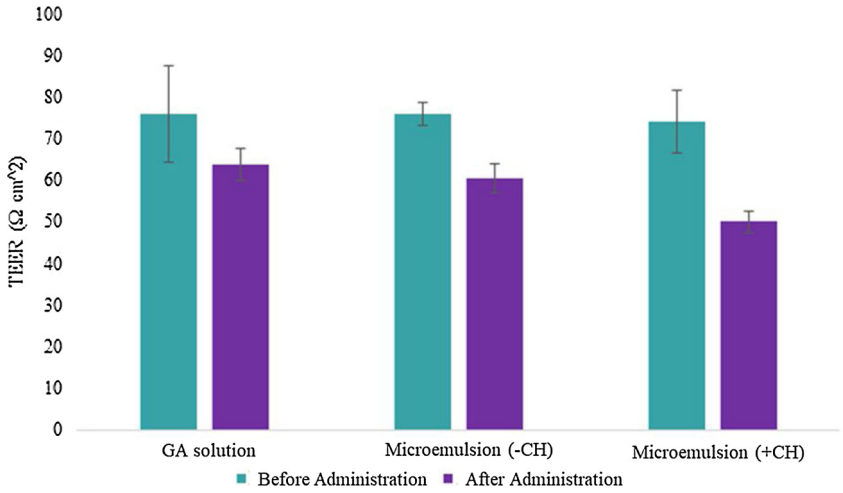

Fig. 5. Transepithelial electrical resistance TEER values before and after the 4 h apical incubation of the nasal cell lines with free and encapsulated GA. The measurements were taken after 15 min of equilibration with HBSS (transport media) in room temperature.

different systems from simple solutions to complex nanoparticles in order to increase the bioavailability of drugs and proteins [43]. It is known that induces changes in cellular actin which is one of the major factors affecting the paracellular transport across epithelia Also, it has been reported that interacts with the epithelial membrane and opening the tight junctions while Tween 80 is able to penetrate in the opened gaps. Thus, the tight junction proteins such as occludin ae naked and may collapse indicating a synergistic effect [76]. As recently described, by means of SAXS, CH also affects the integrity of the mucus layer by binding with glycoproteins and creating channels for the easier diffusion of the transported molecule [45]. As a result, the encapsulation of CH in the microemulsion systems and its subsequent release in the mucus layer facilitates the transport of GA. This strategy could be more efficient in delivery of compounds in comparison to CH nanoparticles where the carrier could strongly interact with the mucins, leading to the entrapment of the system in the complex glycoprotein layer. As can be seen from Fig. 4 the presence of different enhancers in the culture medium provoked drastic changes in the nasal transport of GA. Surfactants, less drastically, alter the GA distribution whereas CH increases its concentration in the basolateral compartment immediately [77].

Concluding this study, it should be considered that the formulation of appropriate biocompatible carriers such as microemulsions for administration of bioactive compounds is a strategy for both their protection and their efficient epithelial transport. Thus, the proposed microemulsion, composed of edible oils and biocompatible surfactants, could be used as carrier of GA for pharmaceutical applications. The ability to form a biocompatible microemulsion system with penetration enhancers which can be easily modified with the addition of biopolymers is important. The structural investigation of the proposed system revealed the participation of both GA and CH in the surfactant layer of the system which subsequently may affect its availability after nasal administration. The system showed high antioxidant activity and is ideal for topical nasal inflammation conditions. The *in vitro* results of the present work also provide strong evidence about the biocompatibility of the used oils and surfactants but also about their effect on the nasal absorption of small polar compounds. Further experimentation with the proposed system would be useful by introducing different polyphenols and derivatives of CH with pharmaceutical interest. A possible application of the GA loaded microemulsion could be a nasal spray for increasing systemic concentration of antioxidants while having a topical anti-inflammatory activity.

4. Conclusions

A non-toxic W/O microemulsion was successfully formulated to serve as a nasal carrier for hydrophilic antioxidants. The present system combines: exclusively natural oils, penetration (Tween 80) and mucoadhesion enhancers (CH) in a thermodynamically stable system with relatively low surfactant concentration. GA was successfully encapsulated while retained its antioxidant activity. The structural study revealed the participation of both molecules (GA and CH) in the surfactant monolayer of the system. The system's cytotoxicity was measured with the classic MTT method in the RPMI 2650 cell line and the system did not exhibit cytotoxic effect, in the absence and in the presence of CH, up to the threshold of 0.2 % v/v. Our study confirmed that CH, even in low concentration, increased the GA permeability through nasal epithelium while affecting the tight junctions of the cell layer.

Overall, this study demonstrates that biocompatible microemulsion systems can be used in order to protect the sensitive antioxidant molecules, until the time of administration. Moreover, their ingredients are able to affect, in a positive way, the permeability profile of the bioactive molecules through constructed epithelia making it a promising absorption enhancing system for nasal delivery of antioxidants. Even though, RPMI 2650 is one of the most studied cell lines for nasal *in vitro* assays, the study, especially of cytotoxicity must be conducted with the use of primary physiological cell lines in order to ensure that the microemulsion will not affect the normal cells. The differences observed regarding the intestinal and nasal absorption of antioxidants encapsulated in microemulsions indicate that the mucosal epithelium of nose is more sensitive to microemulsion's ingredients in contrast to the intestinal, making the nasal administration appropriate for the delivery of those compounds.

CRediT authorship contribution statement

Evgenia Mitsou: Conceptualization, Methodology, Writing - original draft, Investigation, Visualization. **Vasiliki Pletsa:** Writing - review & editing, Supervision. **George T. Sotiroudis:** Formal analysis, Investigation. **Pierre Panine:** Formal analysis, Investigation. **Maria Zoumpanioti:** Writing - review & editing, Supervision. **Aristotelis Xenakis:** Conceptualization, Writing - review & editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2020.110974.

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