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Colloidal nanodispersions for the topical delivery of Ibuprofen: Structure, dynamics and bioperformances

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ABSTRACT

Colloidal liquid-in-liquid nanodispersions such as micro- and nanoemulsions were developed, characterized and compared as potential carriers for the topical administration of ibuprofen. Both colloidal systems were based on water as the continuous phase, limonene as the dispersed phase and a mixture of pharmaceutically acceptable surfactants (Pluronic® L-35, Labrasol®, Tween 80). To improve their properties regarding penetration efficacy, an aqueous solution of chitosan was used as continuous phase in both systems. Micro- and nanoemulsions were structurally studied applying Dynamic Light Scattering (DLS), Electron Paramagnetic Resonance (EPR) spectroscopy and viscometry. Microemulsions with mean droplet diameter of 41 nm and PdI < 0.3 were obtained in the absence and presence of either chitosan or ibuprofen. Nanoemulsions were developed by high-pressure homogenization using the same ingredients at different concentrations. Unlike thermodynamically stable microemulsions, nanoemulsions showed storage stability for 2 months, higher droplet size (174 nm) and lower PdI (<0.15). In the presence of Ibuprofen droplet size and stability of the nanoemulsions were not affected. EPR spectroscopy revealed ibuprofen's location in the oil cores and gave information about the rigidity of the surfactants' monolayer. In both cases an outer compact configuration of the interfacial layer and a more flexible inner one was observed. The cytotoxicity of both systems towards human melanoma cell line WM 164 was relatively low. Interestingly, ibuprofen was released more promptly from the microemulsions (prospectively, systemic exposure increase), however the *ex vivo* studies, regarding skin uptake and penetration, revealed that the nanoemulsions are more appropriate as nanocarriers for the topical administration of ibuprofen.

1. Introduction

There is increasing interest worldwide in the development of biocompatible and safe nanoscale systems for the encapsulation, protection, and delivery of bioactive compounds. In this respect, colloidal liquid-in-liquid nanodispersions could be considered as an efficient alternative to overcome limitations related to direct usage of bioactive compounds, especially hydrophobic ones, offering scaling-up potentials, low toxicity and also greater encapsulation potency [1–5].

Colloidal delivery systems based on microemulsions and nanoemulsions have been the centre of discussion for many years due to their broad range of application from drug delivery [6–9], to encapsulation of food ingredients [10–14] and biocatalysis [15–17]. Both systems are formed by mixing of two immiscible phases, i.e., oil and water in the presence of surfactant. As well established in the literature, microemulsions are optically isotropic, thermodynamically stable and spontaneously formed colloidal systems with droplet sizes usually ranging from 5 to 50 nm. On the other hand, nanoemulsions, need external energy for their formulation, they are kinetically stable and the size of the dispersed droplets ranges from 50 to 500 nm [18]. These nanoscale colloidal systems have been proven to be effective carriers for various drugs due to their composition and structure. Surfactant molecules enhance the solubility of poorly soluble compounds and also can act as penetration enhancers. The performance of both micro- and nanoemulsions could be improved following two different approaches: (i) with

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the addition of other components in order to obtain a desired property (e.g. polymers for mucoadhesive carriers) or by (ii) their incorporation in other formulations such as gels (e.g. microemulsion based organogels-MBGs) [19–21]. Despite their many advantages, micro- and nanoemulsions also have some disadvantages. To formulate microemulsions, high surfactant concentrations are often required which might induce toxicity and irritancy effects. On the other hand, nanoemulsions are only kinetically stable making them less favourable for industrial applications while their content in surfactants is significantly lower. Despite numerous studies on these systems used as drug carriers, to our knowledge the literature lacks of a study comparing their efficiency as carriers of the same drug.

To shed light to this, the present study aims at comparing two colloidal nanodispersions (micro- and nanoemulsions) composed of the same ingredients at different proportions, towards the topical delivery of a model drug such as ibuprofen.

Ibuprofen, 2-(4-isobutylphenyl) propanoic acid, is a non-steroidal anti-inflammatory drug (NSAID) synthesized in the 1960s and still widely used [22]. It has multifunctional activity with anti-inflammatory, analgesic and antipyretic activities [23]. Depending on the cause of treatment ibuprofen has been used for headaches, migraine, osteoarthritis and soft tissue disorders. Its absorption after oral administration is sufficient, however, other routes of administration are also used such as intravenous [24], topical [25], nasal [26] and rectal [27]. Ibuprofen exhibits adverse effects as almost all drugs. Those with the highest frequency include problems regarding the gastrointestinal tract (GIT), kidney and coagulation system [28]. Topical administration of ibuprofen is used in various cases such as in patients with osteoarthritis and in athletes with severe local pain [29]. As a result, the development of various formulations for the topical application of ibuprofen has been examined during the past years by developing appropriately designed solutions [30], gels [31] and patches [32]. All formulations applied to the skin are referred as topical formulations. In dermal delivery, the drug is applied to the skin and the drug is localized into the epidermis in contrast to the transdermal delivery where the bioactive compound penetrates the skin barrier and is able to enter the systemic circulation. The main advantages of topical administration, except the circumvention of the GIT side effects, is the self-administration, low cost and ease of application especially for patients with swallowing problems [33].

In this study we have formulated micro- and nanoemulsions composed of water, Labrasol®, Tween 80, Pluronic® L-35 and limonene at different weight ratios. The surfactants were chosen because of their low irritancy, being thus accepted in pharmaceutical applications. Furthermore, Pluronic® was added in the surfactants' mixture as it has been reported to induce a faster absorption of ibuprofen [34]. The choice of limonene was based on the fact that it has been widely used as a penetration enhancer for topical and transdermal delivery of different compounds [35]. In an attempt to improve their properties regarding penetration efficacy, an aqueous solution of chitosan was used as continuous phase in the final colloidal nanodispersions.

Consequently, both micro- and nanoemulsions were structurally characterized using different techniques, such as electron paramagnetic resonance (EPR) spectroscopy, dynamic light scattering (DLS) and viscometry. The effect of composition and emulsification process on physical properties (droplet size, polydispersity index, stability, membrane dynamics, viscosity) were determined.

The cytotoxicity of micro- and nanoemulsions was investigated using the human melanoma cell line WM 164 as *in vitro* model for the determination of cell viability. Moreover, release and *ex vivo* skin permeation assessments were conducted in order to assess the bioperformance of the formulations. The overall conclusion will lead to a correlation of the physical properties of the system with their application in the topical delivery of ibuprofen.

2. Materials and methods

2.1. Materials

S(+)-2-(4-isobutylphenyl)propionic acid (ibuprofen) was provided from Fluka, Germany. Polyoxyethylene sorbitan monoolate (Tween 80) was obtained from Sharlau, Spain. Labrasol® ALF (Caprylocaproyl Polyoxyl-8 glycerides), oral grade, was kindly donated from Gattefossé, France. Limonene was obtained from Alfa-Aesar, Karlsruhe, Germany. 5-Doxyl stearic acid (5-DSA), 16-doxyl stearic acid (16-DSA) and Poly(ethylene glycol)-block-poly (propylene glycol)-block-poly(ethylene glycol) (Pluronic® L-35) were provided from Sigma-Aldrich, Germany. Chitosan (viscosity 200–600 mPa.s, 0.1% in 0.5% acetic acid, 20 °C; Deacetylation value: 80%) was purchased from TCI, Belgium. High-purity water was obtained from Millipore Milli Q Plus water purification system. All other chemicals used in the study were of analytical grade. Absolute ethanol, 2-propanol, methanol and acetonitrile (HPLC grade) were obtained by Thermo Fisher Scientific (Kandel, Germany). Porcine ears were kindly donated by a local provider in Serbia.

2.2. Formulation of microemulsions and pseudo-ternary phase diagram

O/W microemulsion (M) consisting of limonene as the dispersed phase, Labrasol®, Pluronic® L-35 and Tween 80 as surfactants and water as the continuous phase were prepared by adding the oil phase to a solution of water and surfactants in the appropriate amounts. In the case of chitosan microemulsion (CM), the aqueous continuous phase was replaced by a chitosan solution (0.1% chitosan in 1% acetic acid). For the determination of the systems' monophasic area the corresponding pseudo-ternary phase diagrams were constructed and plotted with the use of the ProSim software (ProSim Ternary Diagram – version: 1.0.3), by ProSim, Cedex, France. The final composition of the microemulsion selected for the encapsulation of ibuprofen was 69.4% w/w aqueous phase, 27.6% w/w surfactants' mixture (Pluronic® L-35/Labrasol®/Tween80, 2:1:1) and 3% w/w limonene. The aqueous phase was ultra-pure water in the case of the M system and chitosan solution in the case of the CM one.

2.3. Formulation of nanoemulsions

O/W nanoemulsions were developed by a two-step emulsification process using the same ingredients as the above described (O/W) microemulsions (2.2). Initially coarse emulsions were obtained by mixing the ingredients at ambient temperature with mechanical stirring for 30 min. The coarse-emulsions were then homogenized with a high-pressure homogenizer (Panda PLUS1000, GEA-Niro Soavi, Parma, Italy). Pressure of 800 bars and 10 homogenization cycles were applied. The final temperature of the formulation reached approximately 50 °C. In correspondence to microemulsions, nanoemulsions will be listed as N (Nanoemulsion) while CN (Chitosan Nanoemulsion) will refer to nanoemulsions with chitosan in the continuous phase. The final composition of the nanoemulsion selected for the encapsulation of ibuprofen was 87% w/w aqueous phase, 8% w/w surfactants' mixture (Pluronic® L-35/Labrasol®/Tween80, 2:1:1) and 5% w/w limonene.

2.4. Encapsulation of ibuprofen

O/W microemulsions (M) and chitosan microemulsions (CM) loaded with ibuprofen were prepared as follows: Initially ibuprofen was solubilized in limonene to result a 154 mM solution. Then,

this solution was mixed with the other components to result O/W microemulsions as described above (Section 2.2). Ibuprofen concentration in loaded microemulsions (MI) and loaded chitosan microemulsions (CMI) was 5.5 mM.

O/W nanoemulsions (N) and chitosan nanoemulsions (CN) loaded with ibuprofen were prepared as follows: The appropriate amount of ibuprofen was dissolved in limonene at room temperature and then was homogenized with the other ingredients in a two-step procedure as mentioned in Section 2.3. Ibuprofen concentration in loaded nanoemulsions (NI) and loaded chitosan nanoemulsions (CMI) was 5.5 mM.

2.5. Dynamic light scattering (DLS) and viscosity measurements

Dynamic light scattering (DLS) was used to evaluate the size and size distribution of the dispersed oil droplets in the colloidal dispersions. Measurements were carried out with a Zetasizer Nano ZS (ZEN 3600) analyzer from Malvern Instruments, (UK) equipped with a He-Ne laser (632.8 nm) using a non-invasive back scatter (NIBS) technology. Samples were prepared and measured in dust-free conditions at 25 °C. All measurements were performed in triplicate and data were processed using the Malvern Zetasizer Nano software. Empty and drug-loaded micro- and nanoemulsions were evaluated in terms of mean droplet diameter and polydispersity index (PdI). Since nanoemulsions are non-equilibrium systems with a spontaneous tendency to separate, they were also tested by DLS for their stability versus time.

Viscosity measurements of the micro- and nanoemulsions were performed using a DV-I Prime Digital Viscometer (Brookfield Engineering Laboratories, USA), equipped with cone spindle (CPA-40Z). The temperature was kept constant at 25 °C and the samples were examined at a shear rate of 150 s^{-1}. Experiments were carried out in triplicate and the results were presented as average ± S.D.

2.6. Electron paramagnetic resonance (EPR) spectroscopy

Electron paramagnetic resonance (EPR) spin-probing spectroscopy was used to investigate structural changes of the surfactants' layer in the colloidal dispersions. EPR spectra were recorded at room temperature, using a Bruker EMX EPR spectrometer operating at the X-band with the use of a WG-813 Q-Wilmand (Buena, NJ) Suprasil flat cell. Two doxyl stearic acid derivatives (5-DSA and 16-DSA) labelled at different positions of the fatty acid aliphatic chain (C5 and C16), thus reflecting the dynamics of the membrane at different depths were used. These interface-located fatty acid spin probes give EPR spectra reflecting the influence of the various constituents on the fluidity and structure of the membrane. Experimental results were expressed by means of rotational correlation time (τ_R) and order parameter (S). The detailed equations and the physical meaning of those parameters have been reported in previous studies [36]. The final concentration of the spin probes in the all systems was 1.2×10^{-4} M.

2.7. Cell culture and cell proliferation assay

The human melanoma cell line WM 164 (BRAFV600E, p53Y220C; Wistar Institute Melanoma Research Centre, https://wistar.org/) was generously provided by Dr. G. Skretas (National Hellenic Research Foundation, Athens, Greece). The cells were grown in Dulbecco′s Modified Eagle′s Medium (DMEM) containing glucose 4.5 g/L, L-glutamine and pyruvate, supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco-Life Technologies, Grand Island, NY, USA), at 37 °C in a humidified incubator with 5% CO_2. All cell culture plasticware was supplied by Corning Costar (Lowell, MA, USA). Phosphate-buffered saline (PBS) were purchased from Gibco- Life Technologies (Grand Island, NY, USA) and dimethyl sulfoxide (DMSO) suitable for cell culture and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Munich, Germany).

Inhibition of cell proliferation was assessed 48 h after treatment by the MTT 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 period the solution was discarded 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 the converted dye was measured at 570 nm. Eq. (1) was used to determine the cell viability:

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

where OD is the optical density.

2.8. In vitro release

In-vitro release studies were conducted using the Franz diffusion cell approach. Release protocol was applied using Franz diffusion cells manufactured by PermeGear Inc, USA. Each cell consisted of a donor compartment and a receptor one separated by a dialysis membrane (Visking dialysis tubing 36/32, 27 mm, MWCO 12,000–14,000, Serva, Heidelberg, Germany). Each cell had an effective diffusion surface area of 0.785 cm^2 (1 cm diameter orifice). Prior to use, the membrane was allowed to hydrate for 24 h in phosphate buffered saline (PBS) and ethanol mixture (3:2). Ethanol was added to the PBS as it is necessary for ibuprofen's solubility. The receptor compartments were water-jacketed and the water supply was connected to a thermostatically controlled water bath (Haake DC10, Karlsruhe, Germany). The receptor chambers were filled with 5.2 mL of the PBS buffer/ethanol solution and stirred with a magnetic stirrer bar. The temperature was maintained at 32 ± 0.5 °C. Membrane permeation experiments were started by placing 1.2 mL of each drug-loaded nanodispersion in the donor compartment of the cell. The membrane release profiles of ibuprofen were obtained by plotting the cumulative amount of released ibuprofen per surface unit as a function of time. The nature of the method, due to osmotic phenomena, allows the participation of the receiver's phase content to the upper compartment of the apparatus, however during the experiment no optical alterations in the system were observed. The release study was performed for 24 h and samples were taken from receptor chamber for analysis at different time intervals and analyzed with UV spectroscopy at 221 nm. The released amounts of ibuprofen in the receiver solution (PBS/ethanol, 3:2) at different sampling times were calculated by using the following standard curves: y = 43.3x, R^2 = 0.998, (Fig. S2). The linearity was proved in the concentration range 0–0.02 mg/mL.

2.9. Ex vivo permeation assessment

Modified Franz diffusion cells, different from the ones described in 2.8, with a surface of 2.01 cm^2 and 12 mL volume of the receptor compartment were purchased from Gauer Glas, Germany. Fresh porcine ears obtained immediately after slaughter were washed under cold running water, blotted dry with the soft tissue, wrapped in the aluminum foil and stored at −20 °C until use. Porcine ears were kindly donated by a slaughterhouse in Serbia, as the *ex vivo* experiments were performed in the Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia.

The release trend of ibuprofen from the proposed nanocarriers was determined by calculating the % percentage of drug in the receiver compartment during time. To prepare porcine ear skin,

after thawing at room temperature, hairs were shortened with electric trimmer to obtain a clear surface without destroying hair follicles. The full-thickness skin was removed from the cartilage using a scalpel and punched to the discs with a diameter of 25 mm. Concurrently, the *trans*-epidermal water loss (TEWL) (Tewameter® TM 210; Courage Khazaka, Koln, Germany) was measured, to ensure the absence of skin barrier damage.

The temperature in Franz diffusion cell water bath remained constant at 32 °C, which is the mean temperature of porcine ear skin surface. Receptor chamber was filled with a degassed, pre-heated mixture of 80% PBS 1X solution – 20% ethanol, and continuously stirred with a magnetic stirrer at 500 rpm. Skin was placed between donor and receptor chamber and left to equilibrate for 30 min. Each of the investigated systems was added to the donor compartment (0.5 mL), covering the skin, and then Parafilm™ was placed over, to avoid evaporation. Permeation study was performed for 30 h. After the end of the permeation study, ibuprofen that remained in the skin was extracted and its quantity was determined. Skin samples were cleaned using PBS solution and cut in small pieces to increase the surface of extraction. Extraction was performed using methanol and samples were left to shake for 24 h. Then, samples were put in an ultrasound bath for 15 min centrifuged at 3000 rpm for 15–30 min. Supernatant was stored at 4 ℃ until LC-MS/MS analysis and skin was discarded [37].

Concentration of ibuprofen was determined using LC-MS/MS both in skin extracts and in the receptor samples. The method is selective for the determination of ibuprofen since there are no other peaks at the retention time that corresponds to the retention time of ibuprofen. The linearity was proved in the range 51–10,200 ng/mL (r = 0.9995), LOQ is 51 ng/mL, whereas LOD is 17 ng/mL. The column was a Zorbax Eclipse XDB C18 (150 mm × 4.6 mm, 5 μm particle size). Mobile phase was methanol: 20 mM ammonium-acetate = 90:10 (v/v), flow rate was 0.6 mL min-1, column temperature was set to 35 °C and injection volume was 10 μL. Ibuprofen was detected and quantified in negative HESI mode (m/z = 205.1–161.1).

3. Results and discussion

3.1. Formulation of microemulsions and pseudo-ternary phase diagrams

Microemulsions are prepared by the phase titration method and can be portrayed with the help of phase diagrams. In Fig. 1 the pseudo-ternary phase diagrams of M and CM microemulsions are presented. As can be observed, the monophasic area of both systems is relatively wide. This could be attributed to the nature of the dispersed phase which is an essential oil typically used in U-type microemulsions. Dilutable microemulsions, also called U-type, can yield large isotropic regions extending from the oil-rich to the water-rich region, without any phase separation [38]. Limonene enables the formulation of wider monophasic regions as compared to hydrocarbons and triglyceride that are commonly used as oil phase. Interestingly, the addition of limonene in the systems with high surfactant concentration (more than 70% w/w) led to a monophasic system of high viscosity, indicating the presence of a colloidal gel which cannot be included in the microemulsion region but is included in the pseudo-ternary phase diagram as part of the monophasic area. Pluronic® L-35 has been used as a gelling agent in non-ionic surfactants-based microemulsions for topical application creating viscoelastic temperature-sensitive gels. The increase of Pluronic® concentration over the phase diagram creates the gel-like structures. Since it is of high importance for topical formulations to keep the surfactants' concentration low the system 69.4% w/w aqueous phase, 27.6% w/w surfactants and 3% w/w limonene was chosen for further investigation. The system was chosen due to its relatively low viscosity, low surfactant concentration and its ability to incorporate a sufficient amount of limonene for the encapsulation of ibuprofen. The systems used for further study M and CM are pointed out on the pseudo-ternary phase diagram with the use of letter A. From the phase diagram we can also observe that the extent of the monophasic area was not affected by the presence of chitosan in the aqueous phase. A similar behavior has been previously reported for a microemulsion based on olive oil and biocompatible surfactants [39].

3.2. Formulation of nanoemulsions

A series of O/W nanoemulsions containing water, limonene and a mixture of surfactants (Pluronic® L-35/Labrasol®/Tween 80, 2:1:1) were prepared by the two-step emulsification procedure. The purpose of the study was to investigate the emulsifying capacity of surfactants' mixture and then determine the amounts of oil and water leading to the formation of more stable nanoemulsions. After many trials, nanoemulsions consisted of 87% w/w aqueous phase, 8% w/w surfactants' mixture and 5% w/w limonene were selected for the encapsulation of ibuprofen.

3.3. Dynamic light scattering (DLS) – characterization and physical stability

3.3.1. Microemulsions

O/W microemulsions containing 69.4% w/w aqueous phase, 27.6% w/w surfactants and 3% w/w limonene were studied by DLS to determine their dimensions, the polydispersity index and how they have been affected by the encapsulation of ibuprofen. As can be seen in Table 1, the solubilization of ibuprofen in the oil phase and the addition of chitosan in the aqueous phase did not affect either size or polydispersity which is in agreement with results that have been previously reported [39,40].

3.3.2. Nanoemulsions

O/W nanoemulsions containing 87% w/w aqueous phase, 8% w/w surfactants and 5% w/w limonene were analyzed by DLS for droplet size and size distribution, the day of their preparation and over time at constant temperature. Immediately after preparation, oil droplets of 173.8 ± 4.1 nm diameter and 0.11 ± 0.01 PdI were measured after 10 homogenization circles. The nanoemulsions were studied for at least 60 days and remained stable throughout this time (Fig. S1). After this period of time no alterations were observed regarding the size of the oil droplet but the PdI was significantly increased, in the case of N, indicating the effect of destabilization phenomena.

As reported in various studies, chitosan is able to increase the stability of nanoemulsions however in our case the polymer's presence might have affected the polydispersity. According to Calero et al. increased chitosan concentrations in O/W emulsions create a well-developed fine-strand-type network which leads to increased emulsions stability [41].

Ibuprofen addition did not affect neither the droplet size nor the stability of the nanoemulsions as also indicated in previous studies from other research groups [42,43]. This could be possibly attributed to the strong lipophilic character of the drug and its localization in the oil phase.

Finally, the viscosity of all micro- and nanoemulsions was measured and the values are shown in Table 1. It can be concluded that the increased surfactant concentration (M, MI) lead to increased viscosity values, which can be further increased by the addition of polymers such as chitosan in the continuous phase of the systems (MC, CMI). Similar increase upon chitosan addition in the aqueous phase was also observed in the case of empty and loaded nanoemulsions. Indicatively we report the increase of the viscosity from 1.8 ± 0.1

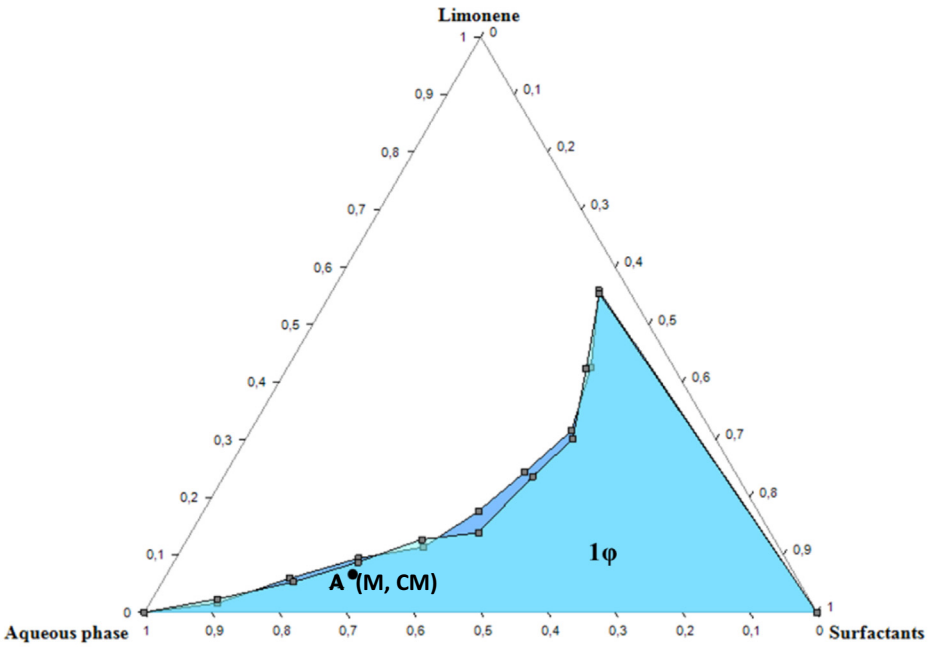

Fig. 1. Pseudo-ternary phase diagram of the system consisted of limonene, Pluronic® L-35, Labrasol®, Tween 80 (2:1:1) and aqueous phase at 25 °C. Composition is in weight ratios. Dark blue: monophasic region (1φ) for the system with water as aqueous phase. Light blue: monophasic region (1φ) for the system with chitosan solution. Clear area corresponds to the multi-phase region. Point A (M, CM) corresponds to the following composition: 69.4% w/w aqueous phase, 27.6% w/w surfactants and 3% w/w limonene. M describes the microemulsion with distilled water as the aqueous phase while CM the microemulsion with chitosan solution.

Table 1
Particle size (d, nm), polydispersity index (PdI) and viscosity of empty and drug-loaded micro- and nanoemulsions, in the absence and in the presence of chitosan. The final concentration of ibuprofen was 5.5 mM. The measurements were conducted at 25 °C.

	Empty				**Loaded**		
	Diameter (nm)	PdI	Viscosity (cP)		Diameter (nm)	PdI	Viscosity (cP)
M	41.5 ± 1.2	0.29 ± 0.02	12.6 ± 0.7	**MI**	42.1 ± 0.7	0.29 ± 0.01	12.8 ± 0.8
MC	40.8 ± 0.9	0.27 ± 0.01	37.8 ± 2.5	**CMI**	41.3 ± 1.2	0.29 ± 0.01	37.3 ± 2.1
N	173.8 ± 4.1	0.11 ± 0.01	1.8 ± 0.1	**NI**	167.8 ± 4.5	0.15 ± 0.01	1.9 ± 0.1
NC	188.4 ± 76.1	0.10 ± 0.01	4.2 ± 0.2	**CNI**	177.9 ± 4.1	0.10 ± 0.01	4.2 ± 0.1

cP to 4.2 ± 0.2 cP for the empty (N, CN) and from 1.9 ± 0.1 cP to 4.2 ± 0.1 cP for the drug-loaded nanoemulsions (NI, CNI).

3.4. Electron paramagnetic resonance (EPR) spectroscopy

The spin probing approach was applied in the present study to investigate the localization of ibuprofen in both micro- and nanoemulsions. Moreover, a correlation of the interface properties of each system with the release of ibuprofen could be also obtained. In the present study we used two doxyl stearic acid spin probes, namely 5-DSA and 16-DSA. The paramagnetic moiety of the first spin probe is localized in the part of the surfactant monolayer that is closer to the polar head groups while the second one is able to anchor in a deeper part of the same monolayer closer to the dispersed oil. As a result, any alteration in the rotational correlation time (τ_R) or in the order parameter S of the spin probes reflects the participation of the molecule of interest in the corresponding depth of the monolayer. In Table 2 the two parameters (τ_R and S) obtained from EPR spectra (Figs. S3 and S4) for the microemulsions and nanoemulsions in the absence and the presence of chitosan or ibuprofen have been summarized.

3.4.1. Microemulsions

In the case of microemulsions the τ_R values for the 5-DSA are higher than 3 ns indicating that the movement of the spin probe belongs to the slow motion regime. For that reason, spectral simulations were performed with the use of MATLAB (The Math-Works) employing the Easy Spin toolbox for EPR spectroscopy [44]. The higher 5-DSA values for both τ_R, and S values, in comparison to the corresponding ones of 16-DSA, indicate that the 5-DSA is located in a region where the surfactants are organized in a more tight way in contrast to the more flexible environment near the oil phase of the system (16-DSA location). The most important is that EPR spectral characteristics of both 5- and 16-DSA have not been altered upon the addition of chitosan or/and ibuprofen. This indicates that the drug does not participate in the surfactant monolayer where the spin probes are located but has been preferably located in the oil cores.

3.4.2. Nanoemulsions

In the case of nanoemulsions, the same experimental procedure was followed to evaluate the effect of ibuprofen and chitosan addition on EPR spectral characteristics. Firstly, in comparison with the microemulsions, the τ_R values of both spin probes were lower. This is an anticipated result because of the lower surfactant concentration and the more relaxed state of the surfactant monolayer separating the oil form the aqueous phase of the colloidal nanodispersions. In addition, it was confirmed that ibuprofen and chitosan did not affect the properties of the surfactants' monolayer. This can be explained by the presence of the same ingredients

Table 2
Rotational correlation time (τ_R) and order parameter S (S) for different spin probes (5-DSA, 16-DSA) in empty and loaded micro- and nanoemulsions, in the presence and absence of chitosan. Three independent experiments were performed for each system, values are given as means ± SD.

	5-DSA		16-DSA	
	τ_R (ns)	S	τ_R (ns)	S
M	3.88 ± 0.28	0.30 ± 0.04	0.34 ± 0.01	0.03 ± 0.01
MI	3.85 ± 0.14	0.31 ± 0.02	0.36 ± 0.01	0.03 ± 0.01
MC	4.03 ± 0.15	0.32 ± 0.02	0.36 ± 0.01	0.03 ± 0.01
MIC	3.81 ± 0.19	0.34 ± 0.01	0.37 ± 0.02	0.03 ± 0.01
N	1.62 ± 0.02	0.09 ± 0.01	0.20 ± 0.02	0.03 ± 0.01
NI	1.60 ± 0.03	0.08 ± 0.01	0.19 ± 0.01	0.04 ± 0.01
NC	1.60 ± 0.01	0.09 ± 0.01	0.21 ± 0.01	0.04 ± 0.01
NIC	1.52 ± 0.07	0.08 ± 0.01	0.18 ± 0.03	0.04 ± 0.01

ensuring the same solubility of the added compounds and their localization in the oil phase for the ibuprofen and in the aqueous phase for chitosan. Furthermore, by comparing the order parameter S of the two colloidal systems, it has to be underlined that the outer part of the surfactant monolayer is more tight in the case of microemulsions (0.30 ± 0.04) than in nanoemulsions (0.09 ± 0.01). However, this was not encountered in the case of 16-DSA indicating that the inner part of the monolayer and close to the oil phase is equally flexible.

3.5. Cell proliferation assay

The use of cell viability assays is a fundamental step in the course of the evaluation of a compound's and/or carrier's safety in terms of their use in eukaryotic cells. It is the first step to identify the concentration range, or a specific concentration, which can be used in further studies in order to fully exploit the properties of a nanocarrier. MTT assay is a quick and reliable colorimetric technique to assess inhibition of cell proliferation, comparable to several other analogous techniques [45]. Here the MTT assay was used to determine the concentration of the colloidal system adequate for further *in vitro* experiments in terms of cytotoxicity. For that reason, the MTT assay was used to compare the cytotoxic effects of micro- and nanoemulsions in the WM 164 melanoma cell line.

Both colloidal nanodispersions are well tolerated during the 48 h time period by the WM164 cells in a range of concentration 0.001%-0.1% v/v in the culture media (Fig. 2). In general, the viability tests are conducted mostly in the range of 0.001–1% v/v of the system in the culture media. Although there are some isolated reports on the lack of toxicity at higher concentrations, the reasonable range to design a toxicological study with colloidal nanodispersions seems to be below 1% v/v, which is in agreement with our findings. This is a promising result as it offers the ability to use the systems at adequate concentrations without inducing cell death. However, comparing the microemulsions to nanoemulsions, at corresponding concentrations, it is evident that the cells can better tolerate the nanoemulsion system. As the ingredients are the same, their concentration seems to play a crucial role in the cytotoxicity profile. The nanoemulsions can be used safely up to 0.1% v/v in the culture medium however the microemulsions are well-tolerated below that threshold. In particular, cell viability assay revealed that the proposed microemulsions (M, CM) when administered at a final ratio of 0.1% v/v in the culture medium did not inhibit cell proliferation. Chitosan did not alter significantly the cytotoxic profile of the systems at the used concentration; therefore, it is an appropriate adduct. In conclusion, the nanoemulsions could be safely used towards the WM 164 cell line at slightly increased concentration in comparison to the microemulsion without affecting cell viability. This makes the nanoemulsions appropriate carriers for increased concentrations of the encapsulated compound.

3.6. In vitro release study

To evaluate and compare the ability of the proposed systems to serve as carriers for topical administration of ibuprofen, release studies were conducted with the use of Franz-type diffusion cells. The two compartments containing the nanodispersions and the receiver solution, respectively, were separated by a synthetic cellulose membrane. The only variable was the medium used as carrier of the drug.

In general, an increase of ibuprofen in the receptor chambers with time was observed in all cases and a lag phase of around 2 h was observed only in the case of nanoemulsions. Ibuprofen release occurred, from all systems, according to the zero order kinetic described with a linear relationship between the cumulative amount of the released drug per unit area and time. The release rate of the drug from the microemulsions was higher in comparison to the corresponding ones from nanoemulsions as can be seen in Table S1 and Fig. 3. In addition, chitosan in the aqueous phase of the systems seems to play an important role as rheological modifier, in the release of the encapsulated ibuprofen molecules and their penetration in the receiver part of the Franz cells. Chitosan did not induce alterations in the diameter of the dispersed oil droplets or the rigidity of the interfacial layer of surfactants (cf. DLS and EPR findings). However, chitosan has altered the viscosity of the overall system. In particular the addition of chitosan in the microemulsions altered the viscosity from 12.6 ± 0.7 cP (M) to 37.8 ± 2.5 cP (CM) and from 1.8 ± 0.1 cP (N) to 4.2 ± 0.2 cP (CN). This increase affects the release of the drug and its diffusion as it has been reported in many cases of different systems and drugs [46]. In the present case, the rigidity of the membrane and the viscosity appear to affect less the release profile of the drug. In agreement with previous studies the properties of the surfactant monolayer did not affect the release of the encapsulated drug [47]. In our case, the larger interfacial area of the microemulsions, compared to that of nanoemulsions, strongly attributes to the increased release of ibuprofen from the oil droplets [48]. Overall, it can be concluded that the release of an encapsulated compound from micro- and nanoemulsions is correlated with the available surface area.

3.7. Ex vivo assay

Ex vivo permeation protocol was performed using modified Franz diffusion cell and porcine ears were chosen as the model biological membrane. As the *ex vivo* permeation study was performed under infinite dosing, permeation parameters were presented in addition with the total ibuprofen quantity recovered from the skin. Ibuprofen was quantified using liquid chromatography coupled to mass spectrometry (LC-MS/MS) at different time intervals and ibuprofen was detected in all samples taken from receptor's compartment, indicating that the drug, when applied via the tested vehicles, is likely to cross the skin, therefore increasing the chances for systemic bioavailability.

Elaborating the experimental data, the first order model was the most suitable to describe the permeation profile of ibuprofen through skin [49]. In order to fully characterize drug permeation process from the selected formulations and also the permeation profiles, values of the following parameters were also calculated and presented in the Table 3; total amount of drug permeated through skin 30 h after the treatment (Q, $\mu g/cm^2$); steady-state flux (Jss) which corresponds to the amount of bioactive compound that will flow through a surface unit during time; and permeability coefficient (Kp), which corresponds to a measure of the rate at

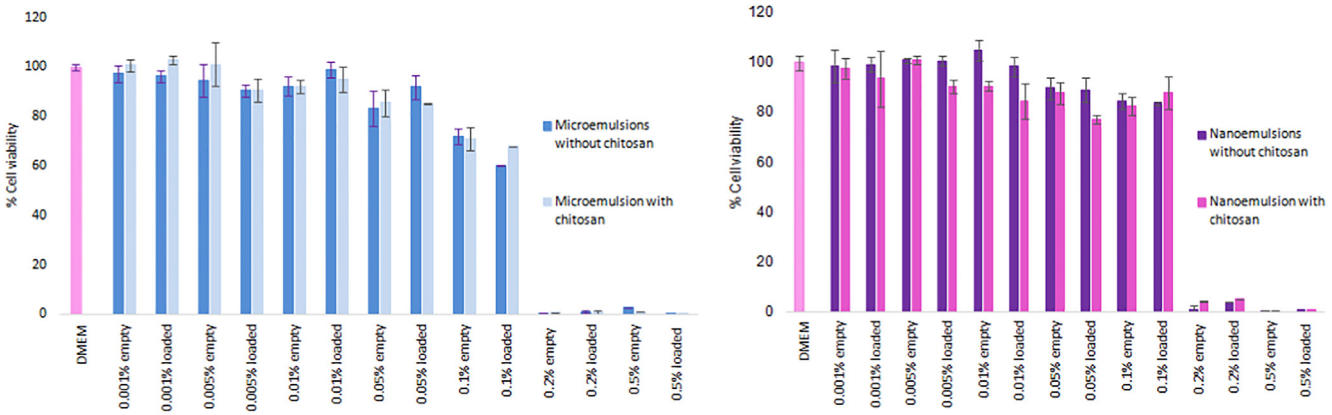

Fig. 2. Effect of the empty micro- and nanoemulsions on WM 164 cell viability, in a range of concentrations 0.001%–0.5% v/v in the culture media. The incubation period was 48 h. All values are expressed as the mean ± SD of at least two independent experiments.

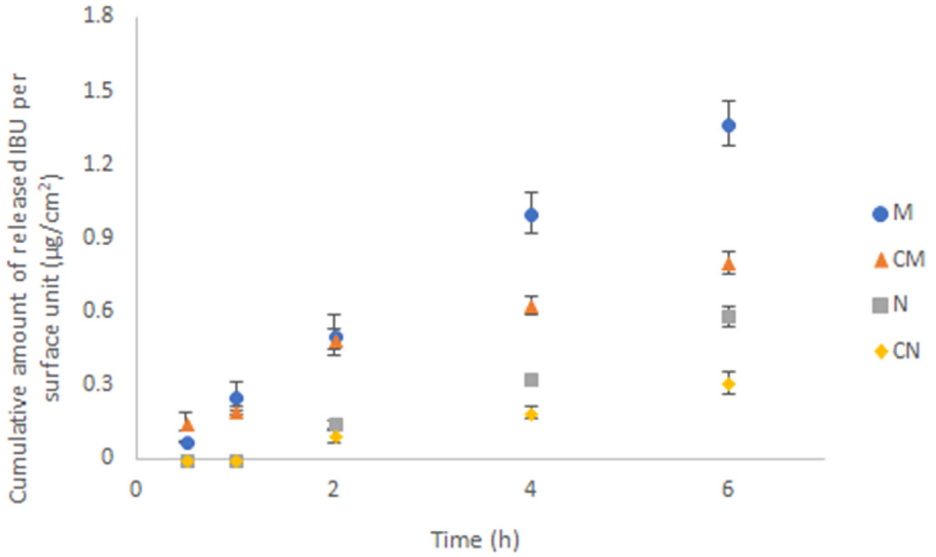

Fig. 3. In vitro release of ibuprofen from micro- and nanoemulsions determined across the synthetic dialysis membrane (MWCO 12,000–14,000). The receiver solution was PBS: ethanol (3:2) and the temperature was maintained at 32 ± 0.5 °C.

which a bioactive molecule crosses a biological membrane. Steady-state flux (Jss) of the bioactive compound through the skin membrane was determined from the linear part of the slope of the total quantity of ibuprofen that permeated through the skin versus time. C_0 corresponds to the initial concentration of the encapsulated Ibuprofen, in each case. Permeability coefficient (Kp) was calculated according to Eq. (2) [50].

$$k_p = \frac{J_{ss}}{C_o} \quad (2)$$

As reported in various studies, when ibuprofen is encapsulated in nanoemulsions, it seems to penetrate through full-thickness skin in a time-dependent manner, in accordance to the results that were here obtained. In particular Salim et al., developed Tween 80-based nanoemulsions, loaded with ibuprofen, which were applied to skin samples. As observed, ibuprofen was released in the receptor compartment, in increased concentrations, up to 8 h upon administration [51].

As observed from Table 3 in cases of both microemulsions and nanoemulsions composed of limonene, Pluronic®, Tween 80, Labrasol® and either water or chitosan solution the quantity of ibuprofen that was distributed topically was relatively high compared to the quantity of ibuprofen detected in the receptor medium. More specifically, considering the case of nanoemulsions, the experimental data indicated a 3-fold increase of permeability coefficient (Kp) and an almost comparable increase of total quantity of ibuprofen that penetrated full-thickness skin when nanoemulsion without chitosan (NI) was applied, compared to the case of nanoemulsion with chitosan (CNI). In contrast, in the literature, it has been reported that nanosystems containing chitosan possess mucoadhesive properties, resulting in enhanced drug penetration in topical application and especially in skin delivery [52].

As observed in our experimental data, when the aqueous phase of nanoemulsion was replaced with chitosan solution, ibuprofen's penetration was not enhanced, as expected. Varshosaz et al., reported a hydrophobic interaction between Pluronic and chitosan that was dependent on temperature. In particular, they observed decrease of skin permeation of ciprofloxacin when gels containing Pluronic and chitosan were applied to rat skin [53]. In our case, considering that the skin permeation experiments were performed at 32 °C, it is possible that a hydrophobic interaction between polypropylene oxide (PPO) units of Pluronic and chitosan occurred, delaying ibuprofen's release. Barradas et al., extended the study and evaluated the skin permeation of psoralen using nanoemul-

Table 3
Ex vivo skin penetration data for the different investigated colloidal systems after 30 h of application. Three independent experiments of duplicates, were performed for each system, values of total quantity of Ibuprofen are given as means ± SD.

Formulation	Jss (μg/cm^2 h^{-1})	C_0 (μg/mL)	Kp	Total Quantity in receptor medium (μg/cm^2)
MI	0.771	37,100	$2.08 + 10^{-5}$	70.53 ± 5.80
CMI	1.143	37,100	$3.08 + 10^{-5}$	34.61 ± 12.57
NI	6.375	37,100	$17.20 \cdot 10^{-5}$	153.45 ± 7.55
CNI	2.089	37,100	$5.63 \cdot 10^{-5}$	53.05 ± 15.99

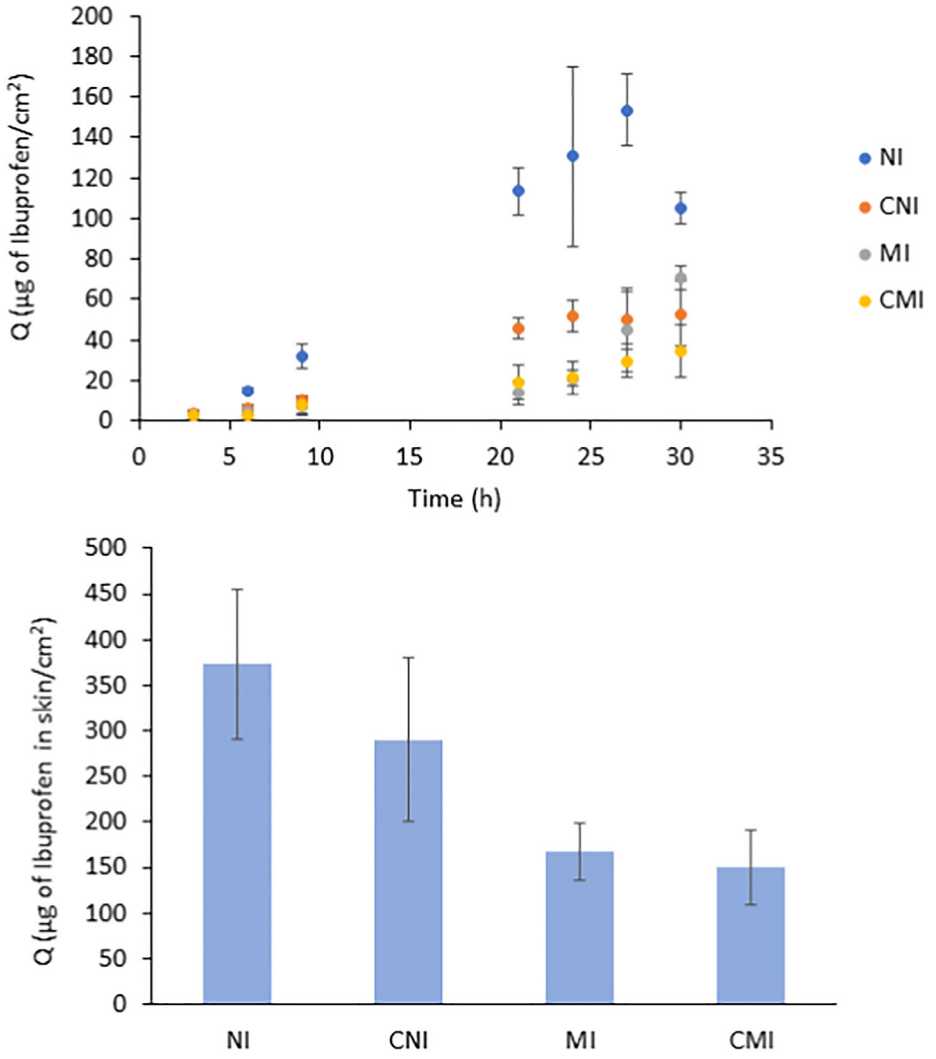

Fig. 4. Cumulative amount of Ibuprofen permeating per unit area and amount of Ibuprofen deposited in the full-thickness skin (μg/cm^2) for loaded systems (MI, CMI, NI, CNI) 30 h post administration in porcine ear skin.

sions that contained different surfactants (Pluronic F68, Cremophor RH40) and also chitosan as penetration enhancer. In the case of nanoemulsion that contained Pluronic F68 and chitosan, results indicated reduced skin permeation, in accordance to the obtained results through the ex vivo approach [54].

Interesting results were obtained when microemulsions composed of the same ingredients were applied. More specifically, the experimental data indicated a 1.5-fold increase of permeability coefficient (Kp) when microemulsion without chitosan (MI) was applied, compared to the case of microemulsion with chitosan (CMI). Moreover, an almost 2-fold decrease of total quantity of ibuprofen that penetrated full-thickness skin was observed when microemulsion without chitosan was applied compared to the case of microemulsion with chitosan. The results by combining both the cumulative amount of Ibuprofen permeating per unit area, the amount of ibuprofen deposited in the full-thickness skin and the values of Kp, indicated that the degree of ibuprofen's permeation through microemulsion with chitosan was more prolonged, as compared to microemulsion without chitosan, in agreement with previous studies (Fig. 4) [55].

According to the obtained data (Table 3, Fig. S5), nanoemulsions provide more efficient ibuprofen's permeation compared to the corresponding microemulsions. In our case, there were two different factors that affected the degree of ibuprofen's permeation, namely the ratio of ingredients and the size of droplets. Some studies reported that the aqueous phase also contributes to skin hydration as it promotes the widening of channels in the keratin layer and distortion of lipid bilayer [56]. In this case we expect that sys-

tems with high water content (nanoemulsions) will further enhance the permeation of ibuprofen. In most of the cases, the decrease of droplet's size leads to more efficient permeation of the bioactive compound [57]. In our study, results revealed that the application of nanoemulsions increased both the percentage of ibuprofen's permeation but also the value of permeation coefficient (Kp). Moreover, in other studies it was shown that skin permeation of ketoprofen, was furtherly enhanced by increasing the water content and in parallel decreasing the surfactant content, followed by increase of droplet size [58]. In addition, the increased concentration of limonene in nanoemulsion systems may lead to the increased skin uptake of ibuprofen as terpene compounds (especially monoterpenes) have reported to exhibit penetration enhancement effect due to their interaction with the lipids of stratum corneum [59].

Having in mind that the presented experiments were performed with infinite dose and in a closed system (*ex vivo* permeation experiment with Franz cells), and due to high concentration of ibuprofen reached in the receiver compartment, some interactions with the skin may have happened in the case of NI formulation, resulting in concentration decrease after the 27th hour (Fig. 4). Most probably, the sink conditions were lost after 27 h of the experiment. Such phenomenon has already been reported in the literature, as well as in the current guidelines for *in vitro* skin absorption testing. Moreover, as the experimental setting involves biological membranes, higher variability is generally expected [60,61].

4. Summary and conclusions

In the present study two O/W colloidal nanodispersions, a microemulsion and a nanoemulsion, were developed and studied as delivery vehicles for the topical administration of ibuprofen. The two nanodispersions were formulated using the same ingredients: limonene as the dispersed oil phase, Pluronic® L-35, Tween 80 and Labrasol® (2:1:1) as surfactants and either water or chitosan solution as aqueous continuous phase. Both systems were able to incorporate in their oil phase sufficient amounts of ibuprofen and were structurally characterized by means of DLS and EPR. The nanoemulsions (in the presence and absence of chitosan) showed a good stability profile for 2 months while their droplet size was not altered after the addition of the drug. EPR spectroscopy revealed that the outer part of the surfactant monolayer (closer to the aqueous phase) in both nanodispersions was more compact in correlation to the inner part. Moreover, microemulsions exhibited increased compactness in the area close to the surfactants' polar head groups while their inner phase, the area closer to the oil phase, was not substantially different in comparison with the corresponding regions of the studied nanoemulsions. This result was the indicator that the ibuprofen's higher release rate from the microemulsion system cannot be correlated with the compactness of the surfactant monolayer but to factors such as the increased surfactant concentration and formulation's viscosity. Cytotoxicity studies revealed that both systems are able to be used for topical administration, however, as expected, the increased surfactant concentration in the microemulsion minimizes the concentration range in which it can be used. Release studies revealed that ibuprofen can be released easier from the dispersed phase of microemulsion systems, a result which is in disagreement with skin penetration experiments. In particular *ex vivo* studies revealed that both nanodispersions favor the topical delivery of ibuprofen while the hydration level and the droplet size can be correlated to the effective penetration of the formulations through skin. Skin uptake in the case of nanoemulsions was increased. In general, chitosan can serve as a penetration enhancer but the existence of possible interactions with the systems' ingredients must be taken into account.

Overall, the low surfactant concentration, the higher tolerance of the cell culture in terms of cytotoxicity and the increased skin uptake make the proposed nanoemulsion system a more effective carrier regarding the topical administration of ibuprofen. Microemulsion is able to provide better storage conditions for the encapsulated compound due to its thermodynamic stability and an increased release rate from the droplets but further studies must be conducted in order to reveal how the surfactant content may affect the topical administration of lipophilic compounds.

CRediT authorship contribution statement

I. Theochari: Investigation, Data curation, Writing - original draft. **E. Mitsou:** Investigation, Data curation, Writing - original draft. **I. Nikolic:** Investigation, Data curation, Writing - review & editing. **T. Ilic:** Investigation, Data curation, Writing - review & editing. **V . Dobricic:** Investigation. **V . Pletsa:** Supervision, Writing - review & editing. **S. Savic:** Supervision, Funding acquisition, Writing - review & editing. **A. Xenakis:** Supervision, Conceptualization, Project administration, Writing - review & editing. **V. Papadimitriou:** Supervision Conceptualization, Project administration, Writing – original draft, Writing – review & editing.

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 material

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