Microfluidic encapsulation method to produce stable liposomes containing Iohexol

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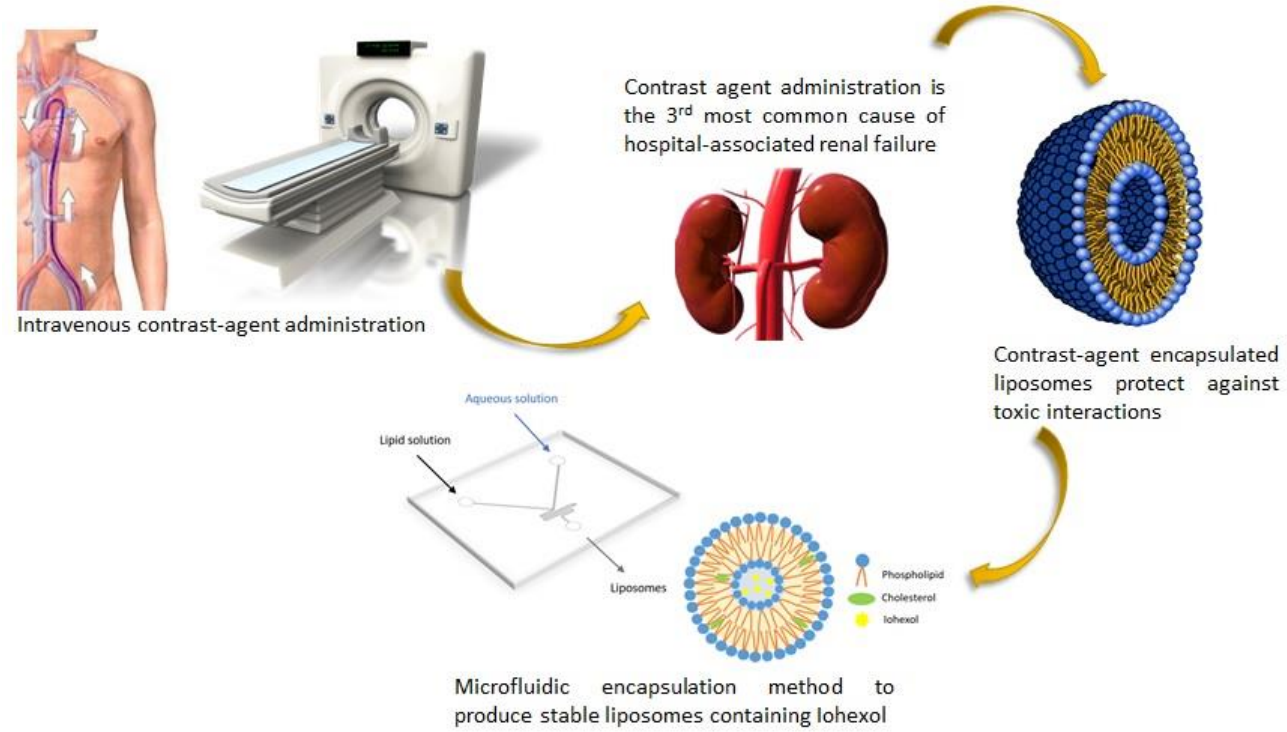
Graphical Abstract



ABSTRACT

Since the discovery of X-rays in the late 1890s, several medical imaging techniques have been developed, such as Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and Ultrasound Imaging, which are used daily to diagnose, monitor, or treat medical conditions. Some of these techniques include the use of contrast agents to enhance the contrast images, therefore, toxic effects must be considered. Among these, Contrast-Induced Nephropathy (CIN) is an acute renal failure resulting from the administration of iodinated contrast media (CM). To date, there is no definitive treatment for CIN and several prevention approaches have been evaluated. Nanoparticles (NPs) represent a promising strategy for treatment and prevention of CIN, due to their ability to deliver CM during diagnosis imaging. In this study, iohexol-containing liposomes were produced using microfluidic technique for first time. Several phosphocholine lipids (e.g. DMPC, DOPC, DPPC and DSPC) with cholesterol (2:1 ratio) were investigated and DLS, FTIR and *in vitro* release studies at 37°C were performed, with stability studies conducted on the best formulation. The microfluidic method allowed to obtain a high encapsulation efficiency (over 70%), and release profiles showed an iohexol release around or less than 0.12 mg ml^{-1} after 2 h for the majority of the formulations, which is not toxic to the kidney cells.

1. INTRODUCTION

Nephropathy is the term used to describe any disease-causing damage to the small blood vessels or blood cleaning apparatus in the kidneys, and often is an associated kidney complication of some other disease or conditions, such as immunoglobulin A (IgA) nephropathy and Contrast-induced nephropathy (CIN). There are certain situations where the interaction of a pharmaceutical agent, upon administration to a patient, should ideally be hindered or even prevented completely.

CIN is reversible acute renal failure, which is the result of unwanted interactions between radiocontrast-media (RCM) and the renal filtration system [1]. Several types of contrast media are

in use in medical imaging (X-ray attenuation: Iodine and barium; Magnetic resonance signal enhancing: gadolinium; Ultrasound scattering and frequency shift: micro-bubble contrast agents) and they can roughly be classified based on the imaging modalities where they are used. Moreover, it is the third cause of hospital acquired acute renal failure and can lead to many adverse outcomes including dialysis and increased mortality. CIN is one of the main causes of acute kidney injury (AKI) and this is associated with high healthcare costs, long hospitalization and increased morbidity and mortality [2]. Many risk factors are related to CIN and the most important is pre-existing severe renal insufficiency. Other risk factors have been reported, such as diabetes, old age, gender, hypertension or hyperuricemia, but not all of them have been rigorously confirmed [1]. Moreover, CM with high osmolarity and viscosity can increase the incidence of CIN and the injected volume of CM and the route of administration must be taken into account [3]. CIN occurs in 1.0% - 15% of all patients undergoing invasive angiographic procedures and in 50% of patients with pre-existing renal insufficiency or diabetes mellitus. The incidence is estimated to be about 7.0% - 11%, but rises up to 40% in patients with chronic kidney disease (CKD) [2, 4]. To date, there is no definitive treatment for CIN and several prevention approaches have been evaluated. Briefly, the strategies adopted for preventing toxic effects are volume expansion (hydration), administration of antioxidants such as N-Acetyl Cysteine, ascorbic acid and statins and, eventually, hemofiltration [1-3]. However, these approaches are not yet supported by clinical evidence and they require critical medical care and special treatments that are not always practical [2, 5].

For the reasons stated above, further approaches to prevent CIN should be evaluated. Under these approaches belongs also the use of Nanoparticles (NPs), which represent a promising strategy for prevention of CIN, due to their ability to deliver CM during diagnosis imaging, thus avoiding the adverse reactions associated with these compounds [6]. Accordingly, several iodine-NPs have been developed. However, there are some issues that currently limit the application of iodinated NPs *in*

vivo such as loading efficiency and burst release [7]. Furthermore, high concentrations of CM incorporated are often required to improve imaging, causing an alteration in the NPs physicochemical properties and *in vivo* behaviour [8]. Various approaches and compositions are being investigated, such as iodinated polymeric micelles, where iodine was covalently linked to the polymeric molecule to form micelles as blood pool agent (BPA) (Hallouard et al., 2010), [10, 11]. However, the major drawback of polymeric micelles is their thermodynamic instability after intravenous administration, due to dilution below the critical micelle concentration of the polymer. This causes dissociation of the micelle structure and release of the CM [12]. Polymeric NPs that have been investigated as blood pool contrast agents includes the use of poly(L-lactide) (PLA), poly (lactide-co-glycolide) (PLGA) or poly(ε-caprolactone) (PCL), thanks to their biocompatibility in terms of acceptable shelf-life and non-toxic degradation products in case of metabolization [9, 13]. Moreover, iodinated dendrimers have been developed with a PEG-core and non-ionic contrast agents covalently fixed onto the surface, but their synthesis is time-consuming [9]. Other approaches consist in the production of lipid-based NPs such as liposomes that, thanks to their structure, presenting a hydrophobic and a hydrophilic region, may incorporate both lipophilic and hydrophilic CM. Moreover, iodine may be covalently incorporated into the lipid bilayer structure with a modified phospholipid, by following examples of lipid formulation with CM as a carrier [9, 13, 14]. In addition, nanoemulsions have been developed and two formulations of 1,3-disubstituted polyiodinated triglycerides (ITG) are commercially available [9, 13].

In this study, the focus is the use of phospholipids in the production of NPs loaded with iohexol since liposomes have several properties that make them more advantageous than other drug delivery systems, such as polymeric nanocarriers. First, their phospholipidic bilayer reproduces the cell membrane structure, resulting in high biocompatibility. Furthermore, liposomes can be eliminated from the body without any toxic effects via the reticuloendothelial system, thus avoiding renal

filtration and reducing nephrotoxicity associated to the CM. Liposomes, are the most common and well-investigated nanocarriers and they may be synthetized from clinically approved components (lipids and CM) with easy preparation methods. In addition, liposomes show several advantages of high target accumulation and cellular uptake [11, 15-18].

The most commonly used method in literature for producing liposome is film hydration method given that it is simple, convenient and widely applicable, but it has several limitations such as control of the process and scalability, low encapsulation efficiency and time [18, 19]. Thus, in order to overcome these issues, many novel technologies have been applied, such as microfluidics. Microfluidics allow precise control over a fluid stream in contrast to the chaotic flows that occur in the traditional method, since it is not governed by the same laws applied at the macroscale techniques [20]. Among the microfluidics technologies, the microfluidic hydrodynamic focusing (MHF) approach has been developed by Jahn *et al.* [21] for liposome production. Typically, a stream of lipid in alcohol solution flows in the central channel of the device, while an aqueous solution flows through two lateral channels (**Fig. 1**). In this way, the stream of lipids is hydrodynamically focused by the two aqueous streams into the narrow junction of the chip. Thus, liposomes are formed due to the diffusion of different molecular species at the liquid interface between the alcohol and the aqueous phases: the two phases diffuse one into the other, causing the lipids' precipitation to form micelles first and liposomes after. The easy control and adjustment of the flow rate ratio (FRR) between the lipid and aqueous phase streams and the total flow rate (TFR), allow producing uniformly dispersed liposomes. In particular, it has been reported that the mean diameter is directly related to lipid concentration and inversely related to FRR. In addition, the device geometry has been reported to affect the liposome characteristics [22, 23]. In conclusion, microfluidic methods allow reaching a higher control over the physical properties of the final NPs: MHF allows obtaining homogeneous vesicles in terms of size and lamellarity in one-step, unlike film hydration method

that requires additional processing steps, such as extrusion or ultrasonication, resulting in non-uniform vesicles. Furthermore, this technique shows advantages, such as higher encapsulation efficiency and reproducibility, even in large a scale for production of personalized products [23].

The aim of this proof of concept research was to produce liposomes for the prevention of CIN by microfluidics. As CM, iohexol was selected, since it is a small water-soluble contrast agent that is currently used for several radiographic procedures. The first goal, and novelty, was to obtain lipid CM-loaded NPs using microfluidics, since to the best of our knowledge, there are no reports in the literature. Formulation efforts were focused on obtaining a non-leakage of the iohexol in order to avoid the toxic effects associated to the CM, such as CIN. Furthermore, it the achievement of a good encapsulation efficiency was also intended.

2. MATERIALS AND METHODS

2.1 Materials

The synthetic lipids (**Fig. 2**) 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, iohexol (MW 821.14 Da, Water Solubility 0.796 mg/mL, logP -3.05), tablets of phosphate-buffered saline (PBS, pH 7.4) and ethanol ≥99.8% were all obtained from Sigma-Aldrich.

2.2 Preparation of liposomes by Microfluidics

Liposomes were prepared using a microfluidic micro-mixer, which through hydrodynamic flow enables nanoprecipitation of lipids. The Dolomite Microfluidics System was used, connected to a Microfluidic cartridge with dimensions of 52 mm thick and 36 mm height with moulded channels of 300 µm in width and 130 µm in height with staggered herringbone structure. The mixing chips consist of two stream inlets that merge into a micro-channel (**Fig. 3**). Lipids (DMPC, DOPC, DPPC, DSPC) in combination with cholesterol (2:1 ratio) were dissolved in ethanol at a concentration of 5

mg/mL; the 2:1 ratio was chosen as has been previously reported to be the most stable ratio for the specific lipid formulations [24]. The lipid solution was introduced in one of the inlet of the microfluidics micro-mixer and the aqueous buffer (PBS, pH 7.4) or the iohexol solution (0.714 mg/mL in water) in the other one. Both fluids were delivered into the chip inlets with two pressure pumps with respective fluidic connections using FEP tubing of OD 1/16 and ID 250 µm. The TFR and the FRR were controlled using two Mitos Flow Rate Sensors (0.2-5 mL min^{-1}). TFR of 1 mL min^{-1} and FRR (lipid:aqueous phase) 3:1 were chosen, since this combination gave the best results (data not included).

2.3. *Dynamic light scattering (DLS)*

The size distribution (mean diameter and PDI) of the liposomes was measured by Dynamic Light Scattering (DLS) on a NanoBrook Omni (Brookhaven Instruments, Holtsville, NY, USA). Each sample was measured three times at 25°C with a fixed angle of 90° in a dilution of 1:100 using PBS pH 7.4. Moreover, the same instrument was used to measure the ζ-potential. For each formulation three cycles of ζ-potential measurements were performed. Size and ζ distribution was measured in multiple samples from each batch.

2.4. Encapsulation efficiency and Release studies

The most common method for study the release from NPs is dynamic dialysis [25]. Two release studies were conducted for each formulation. Prior to the analysis, the dialysis tube (Dialysis tubing cellulose membrane, Avg. flat width 10 mm, 0.4 in., MWCO 14,000, Sigma-Aldrich) was placed in boiling water for 30 min and thoroughly rinsed with water. A volume of 1 mL of each liposome suspension was added into the dialysis tube with both ends tied and the tube was suspended in PBS 10 mL at pH 7.4 and kept at 37.0±0.5°C for removal of unencapsulated CM for 1 h [26]. Then, 10 mL of PBS was removed and kept for encapsulation efficiency analysis. The PBS was replaced with fresh PBS pre-equilibrated at 37.0±0.5°C and CM release was analysed by extraction of 500 µL aliquots of

the PBS at intervals of 15 and 30 min and 1, 2, 4, 6, 24 h. The supernatant was replaced with fresh PBS pre-equilibrated at 37°C at each time point, in order to satisfy sink conditions and keeping the volume constant. The amount of drug released at each time point was determined by UV-vis spectrophotometer (FLUOstar Omega, BMG LABTECH).

The concentration of non-encapsulated or released CM was determined with the aid of a calibration curve of iohexol in purified water (R^2 =1) at wavelength of maximum absorbance of iohexol (245 nm). The Equation used is: Iohexol Concentration (mg/mL) = (Absorbance – 0.0043) / 20.897.

The encapsulation efficiency was calculated using eq. 1:

$$\% EE = ((Ci - Cn.e.) / Ci) \times 100 \quad (1)$$

where Ci is the initial concentration of iohexol added during preparation and Cn.e. is the concentration of iohexol non-encapsulated.

Release curves were drawn according to the cumulative drug release and plotted vs time using eq. 2.

$$\% \text{ Cumulative Iohexol Release}_t = ((Ct / Ce) / v) \times 100 \quad (2)$$

where Ct is the concentration released at time t and in each previous time point, Ce is the concentration of iohexol encapsulated (Ce = Ci – Cn.e.) and v is the volume of the aliquot of each sample (0.5 mL).

2.5. Fourier transform infrared spectroscopy (FTIR)

All the excipients, including the lipids (DMPC, DOPC, DPPC and DSPC), cholesterol and iohexol were analysed separately with infrared (IR). The liposome suspensions were scanned in an inert atmosphere over a wave range of 4000-650 cm^{-1} over 32 scans at a resolution of 4 cm-1 and an interval of 1 cm^{-1}. All FTIR spectra were recorded on a Spectrum Two (PerkinElmer) FT-IR spectrometer using a MIRacle (PIKE Technologies) ATR accessory. The background was subtracted from each spectrum.

184 **2.6. Stability studies**

185 The stability studies were conducted for four weeks after the preparation of the lipid
186 nanoformulation. The liposome batch was divided in two aliquots and kept at 5.0±0.5°C and at
187 37.0±0.5°C. Particle size and ζ-potential were measured at day 0, day 3 and after 1, 2, 3 and 4 weeks
188 from the preparation of liposomes.

189 **2.7 Statistical analysis**

190 All experiments were performed with calculation of means and standard deviations. Two-way
191 analysis of variance (ANOVA) was used for multiple comparisons. Significance was acknowledged
192 for p values lower than 0.05.

193 **3. RESULTS**

194 **3.1. Particle Sizing and Encapsulation efficiency**

195 Several phosphocholine lipids (e.g. DMPC, DOPC, DPPC and DSPC) were investigated using a
196 combination of 2:1 ratio with cholesterol since it is the most frequently and most stable
197 lipid:cholesterol ratio used in literature [24]. TFR 1 mL min^{-1} and FRR (lipid:aqueous phase) 3:1 were
198 settled, shown good particle size and polydispersity index (PDI; **Table 1**). Results in terms of particle
199 size and encapsulation efficiency are shown in **Fig. 4**. Empty liposomes were also produced, and
200 particle size, ζ-potential, and PDI were recorded (**Table 1**).

201 **3.2. Release Studies**

202 Release profiles of DMPC and DSPC formulations showed a leakage of iohexol less than 20% after 2
203 h, while DOPC and DPPC formulations released 22.43 ± 0.64% and 28.14 ± 6.57%, respectively (**Fig.**
204 **5a**). After 24 h, around 50% of iohexol was released by all the formulations, except for the DMPC
205 formulation that showed a lower leakage of 25.69 ± 1.26% (**Fig. 5b**). After 24 h the studies were
206 stopped, given that was most important to observe the release after 2 h, which is the elimination
207 half-life of the CM [27]. Moreover, in all cases the release after 6 h was followed by a plateau phase.

3.3. FTIR Studies

FTIR was performed for the iohexol-loading liposomes, since the resulting spectrum acts as a fingerprint for the compounds. All the excipients such as cholesterol, iohexol and lipids (DMPC, DOPC, DPPC and DSPC) were analysed separately. In all the liposome spectra (**Fig. 6**) the broad band detected at about 3300 cm^{-1} is related to O-H stretching of the hydroxyl groups present in cholesterol and in iohexol molecules. The band at about 2900 cm^{-1} represents C-H stretching, while the weak band detected at around 1700 cm^{-1} only in DOPC:Chol (2:1) formulation spectrum, represents the stretching of carbonyl groups of the esters present in the lipid. The presence of iohexol into the liposome is confirmed by the weak band at around 1600 cm^{-1} that represents C=O stretching of the amidic groups. Finally, the strong band at about 1000 cm^{-1} represents the stretching of the C-O bond [28]. Moreover, the lipid structure was not affected by the presence of the Iohexol since all the liposome spectra exhibited the peaks related to the groups of the lipid molecules analysed separately (data not included).

3.4. Stability studies

Since the DOPC:Chol formulation showed the smallest particle size and good results in terms of iohexol encapsulation efficiency and release, it was submitted to stability studies for four weeks at 5±0.5°C and 37±0.5°C, with no statistical differences between the two temperatures (**Fig. 7**). At day 0, it showed a mean diameter of 178.11 ± 2.43 nm and ζ-potential value -8.08 ± 14.71 mV. The formulation stored at 5°C showed a good stability profile after 28 days, with small-reduced size (142.88 ± 0.45 nm). The formulation kept at 37±0.5°C, follow the patent of the 5°C, except at 14 days that show a decreased in size (126.79 ± 3.88 nm) and increased in ζ-potential absolute value (-9.05 ± 1.4 mV), however after 4 weeks it showed a mean diameter of 132.78 ± 2.66 nm.

4. DISCUSSION

The microfluidic method allowed obtaining a high encapsulation efficiency, more than 70% for all the formulations. To our knowledge, any studies about iodinated NPs production by microfluidics have not been reported in literature. However, these results confirm the advantages of microfluidic technique since in the literature, the highest encapsulation efficiency that was reported is no more than 25% for iohexol-containing liposomes prepared with the extrusion method using DPPC, cholesterol and PEG2000-DSPE [15, 29, 30]. Furthermore, a drug loading of 30% was obtained for multilamellar liposomes (MLVs), given that a larger size allows for a higher drug loading capacity [31]. Moreover, DPPC, cholesterol and PEG, have been used for preparing liposomes loaded with iodixanol, which is a non-ionic dimer CM more hydrophilic than the iohexol. Even in this case, the extrusion method has been used and an encapsulation efficiency of only 25% was observed [32, 33]. In conclusion, these results confirmed that the encapsulation properties depend closely on the experimental conditions [12] and that microfluidics allows to overcome limitations of batch methods in terms of encapsulation efficiency.

Moreover, a mean diameter < 200 nm (177.64 ± 0.97 nm), that is fundamental to avoid a rapid RES uptake, was obtained for the DOPC:Chol formulation with an encapsulation efficiency of 68.45 ± 3.41%. The release profile showed a leakage of 22.42 ± 0.64% after 2 h that should be rapidly eliminated *in vivo*, since the elimination half-life of the free iohexol in patients with normal renal function is 1 - 2 h [27]. Stability studies of the DOPC:Chol formulation showed that it might be stored at 5°C for 1 month without significant changes in particle size.

5. CONCLUSIONS

In this study, we formulated iohexol-loading liposomes with high encapsulation efficiency and low release. The microfluidic method allows preparing formulations under a well-controlled process with reduced time and solvent waste, confirming the benefits of this technique. Microfluidics will

play a crucial role in the dawn of personalized medicine and in our case, for patients that cannot receive CM due to pre-existing severe renal insufficiency and other risk factors of CIN.

6. FUTURE DIRECTIONS

As for future perspectives, in order to improve the release profile and to achieve specific target tissue, the surface of the NPs may be modified to include polyethylene glycol (PEG), which is a hydrophilic compound that can also prolong time circulation and stability of liposomes [30, 34, 35]. In fact, the use of a contrast agent with a long vascular residence time can be necessary in long scanning time procedures. Eventually, thanks to the microfluidic technique, simple scalability may be carried out for the production of personalized products. In fact, microfluidics offers easy scale-up maintaining high resolution and sensitivity, decreasing cost production and time and, for these reasons, microfluidics has the potential to become widely used because it is economical, reproducible and, furthermore, can be integrated with other technologies [36, 37]. In particular, it is possible to parallelize individual setup units to increase the total output of the systems, while maintaining the advantages of microfluidics without altering the final characteristics of the NPs [38]. Such parallel approaches have already been studied for emulsion droplet production and polymeric NPs [39]. However, the scale-up is still an active area of research for chemical engineering since it requires not only architecture and materials able to support higher internal pressure, but also that the system setup can ensure the same operating conditions during the process [39, 40].

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Table & Figure Captions

Table 1: Mean particle size, polydispersity index (PDI) and ζ-potential of different liposome formulations without iohexol.

Figure 1: Schematic representation of liposome formation by microfluidic hydrodynamic focusing (MHF) method.

Figure 2: Chemical structures of: a) 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), b) 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), c) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), d) 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), e) Cholesterol and f) Iohexol.

Figure 3: Representation of microfluidic chip for the production of lipid nanoformulations.

Figure 4: Mean particle size (bars, left axis) (n=3, p value=0.0007, PDI DMPC:Chol 0.293, PDI DOPC:Chol 0.261, DPPC:Chol 0.300, DSPC:Chol 0.220) and percentage encapsulation efficiency (dots, right axis) (n=2, p value=0.01) of different liposome formulations.

Figure 5: Cumulative drug release studies over time, of different liposome formulations: a) in the first 2h, and b) after 24 hours (n=2, p value=0.04).

Figure 6: FTIR spectra of different liposome formulations: a) DMPC:Chol, b) DOPC:Chol, c) DPPC:Chol, and d) DSPC:Chol.

Figure 7: a) Mean particle size (p value 5°C=0.00000002, p value 37°C=0.0000009) and b) ζ-potential (p value 5°C=0.07, p value 37°C=0.06) of DOPC:Chol loading iohexol formulation (n=3).

400 **Table 1:**

Formulation	Mean diameter / nm	PDI	ζ-potential / mV
DMPC:Chol	435.02 ± 28.44	0.310	-2.33 ± 7.01
DOPC:Chol	132.25 ± 4.29	0.240	-27.76 ± 5.78
DPPC:Chol	416.31 ± 20.91	0.258	-1.91 ± 8.65
DSPC:Chol	671.42 ± 102.13	0.222	-10.60 ± 6.28

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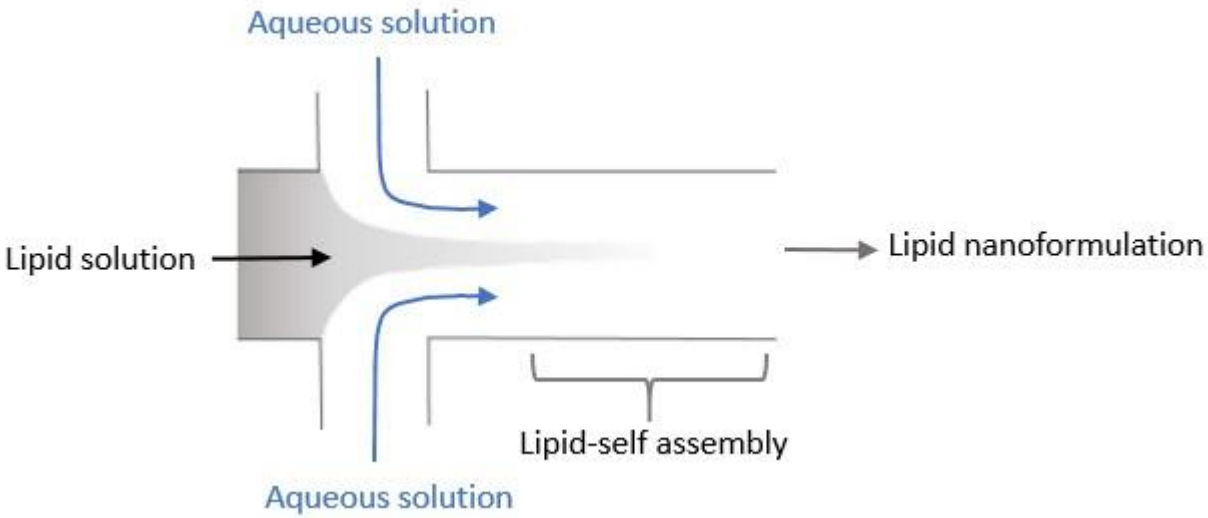
403 **Figure 1:**



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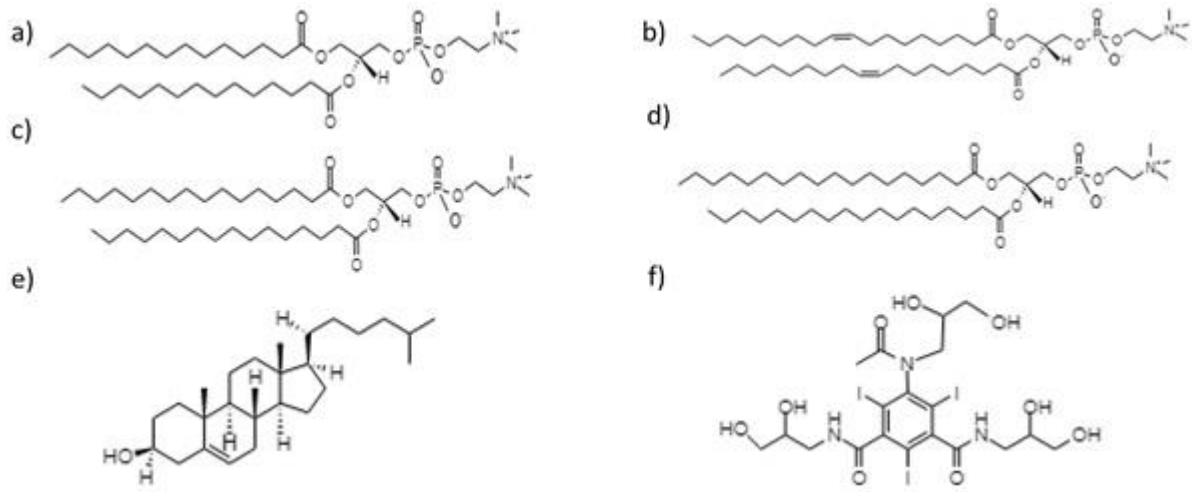

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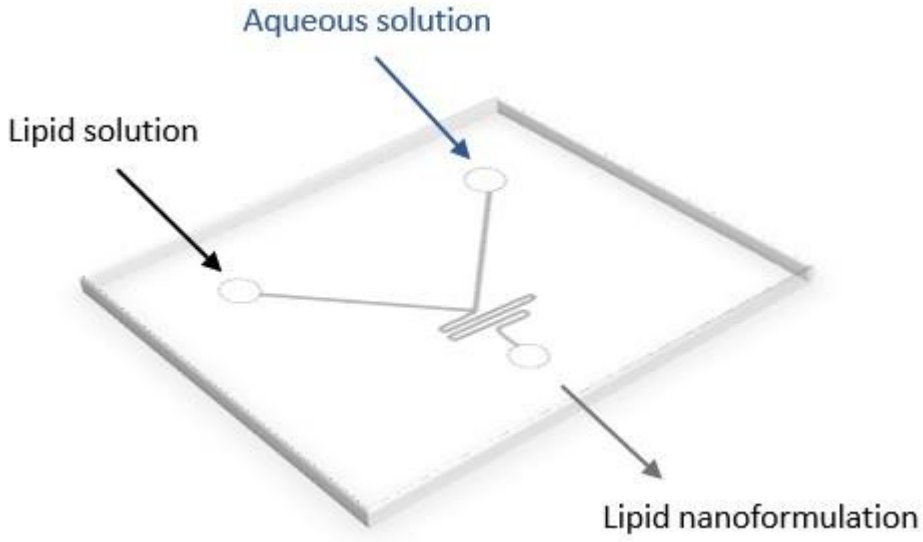

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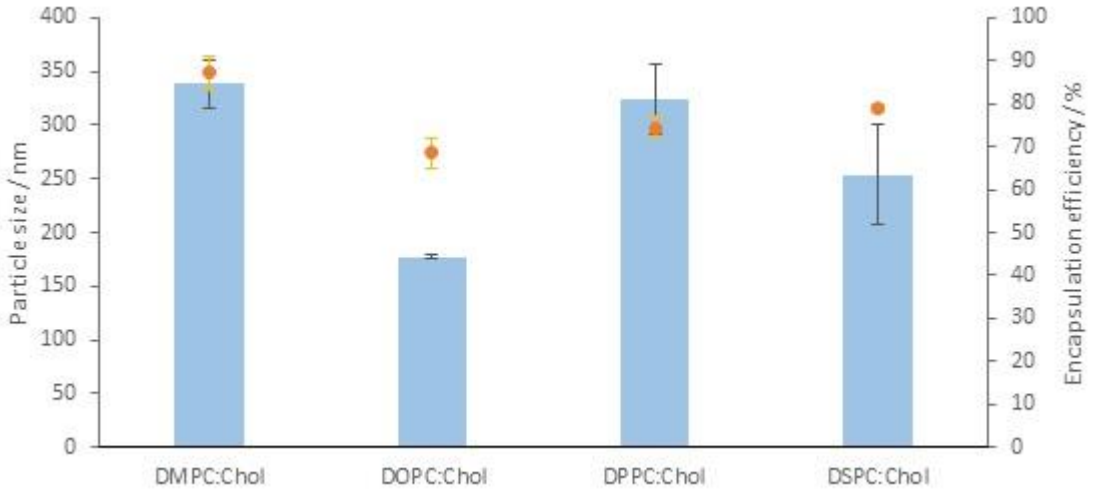

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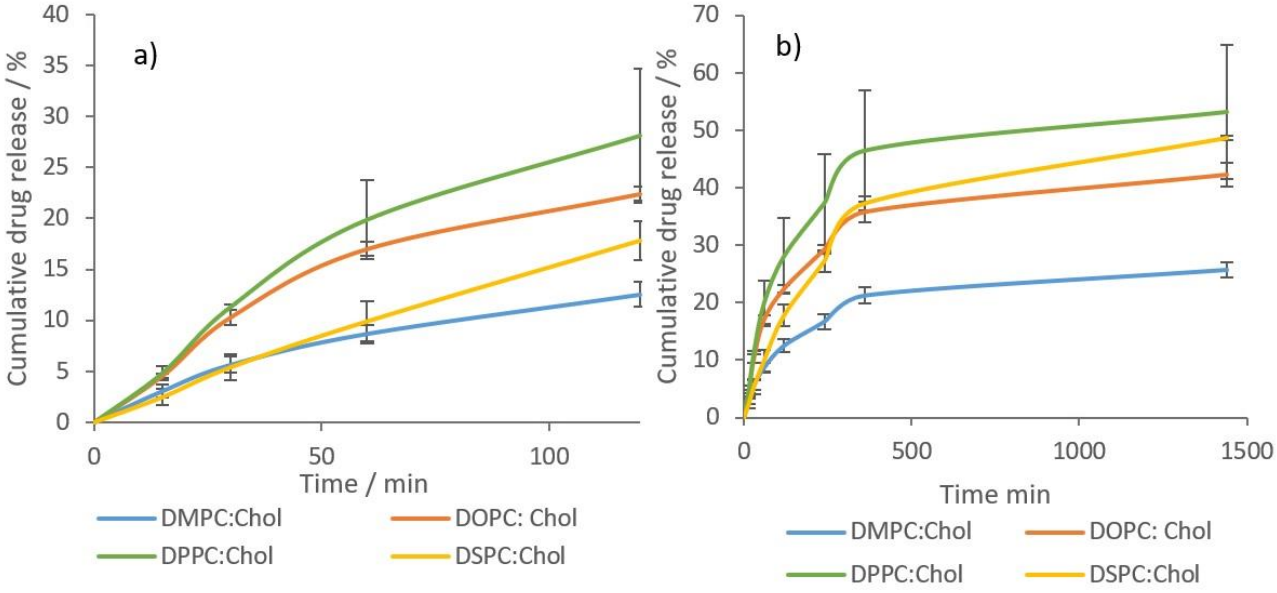

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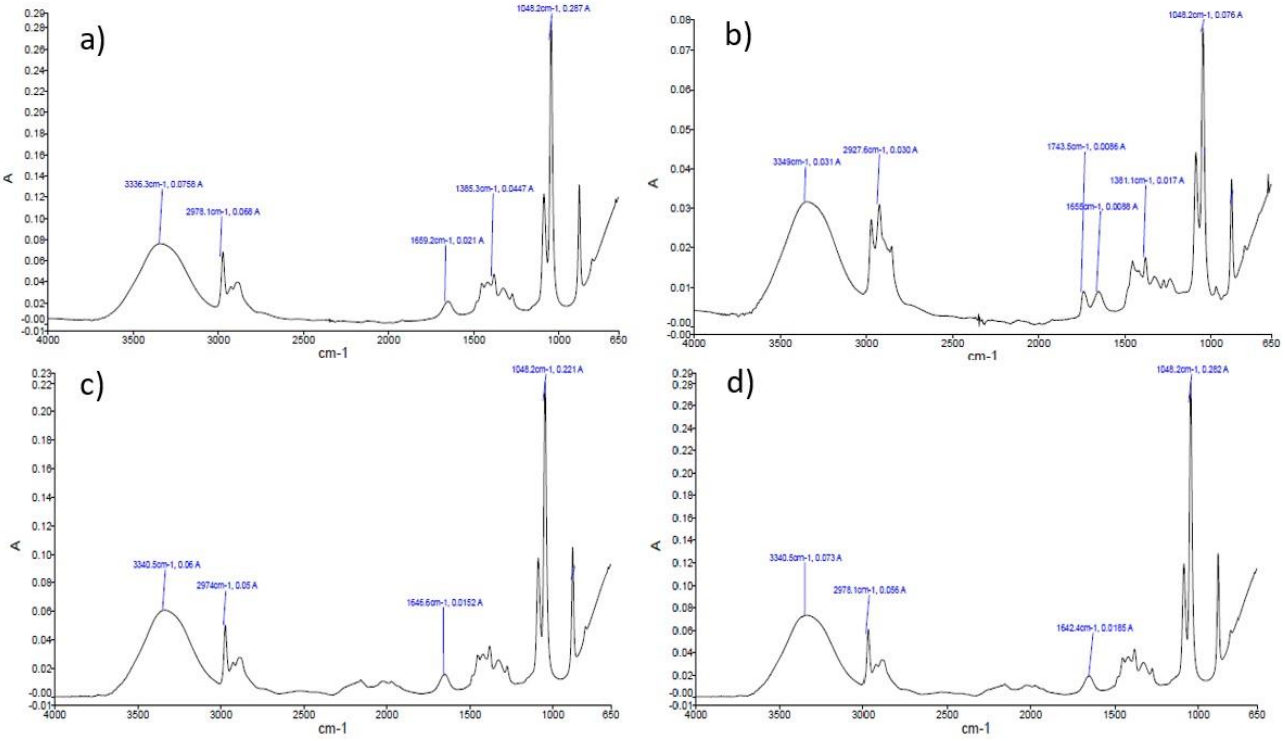
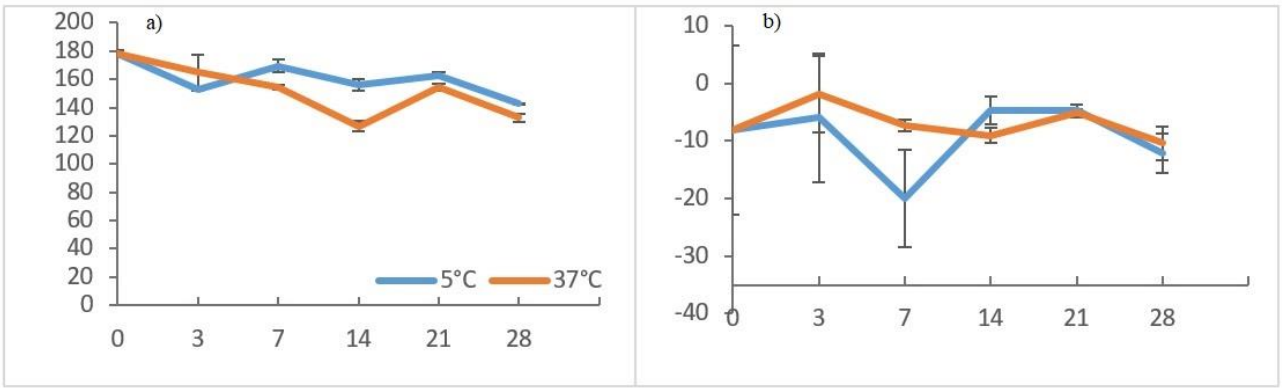
Figure 6:


a)
3336.3cm-1, 0.0758 A
2978.1cm-1, 0.068 A
1659.2cm-1, 0.021 A
1385.3cm-1, 0.0447 A
1048.2cm-1, 0.267 A
b)
3349cm-1, 0.031 A
2927.6cm-1, 0.030 A
1743.5cm-1, 0.0086 A
1655cm-1, 0.0088 A
1381.1cm-1, 0.017 A
1048.2cm-1, 0.076 A
c)
3340.5cm-1, 0.06 A
2974cm-1, 0.05 A
1646.6cm-1, 0.0152 A
1048.2cm-1, 0.221 A
d)
3340.5cm-1, 0.073 A
2978.1cm-1, 0.056 A
1642.4cm-1, 0.0185 A
1048.2cm-1, 0.282 A
A
cm-1

421 **Figure 7:**



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