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Gelation of the internal core of liposomes as a strategy for stabilization and modified drug delivery I. Physico-chemistry study

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Abstract

Since the application of nanotechnology to drug delivery, both polymer-based and lipid-based nanocarriers have demonstrated clinical benefits, improving both drug efficacy and safety. However, to further address the challenges of the drug delivery field, hybrid lipid-polymer nanocomposites have been designed to merge the beneficial features of both polymer-based and lipid-based delivery systems in a single nanocarrier. Within this scenario, this work is aimed at developing novel hybrid vesicles following the recent strategy of modifying the internal structure of liposomes. Specifically, polyethylene glycol-dimethacrylate (PEG-DMA, molecular weight 750 or 4,000), was entrapped within unilamellar liposomes made of hydrogenated soybean phosphatidylcholine/cholesterol, and photo-crosslinked, in order to transform the aqueous inner core of liposomes into a soft and elastic hydrogel. After appropriate optimization of the preparation and gelation procedures, the primary objective of this work was to analyze the effect of the molecular weight of PEG-DMA on the main properties of these Gel-in-Liposome (GiL) systems. Indeed, by varying the molecular weight of PEG-DMA also its hydrophilic/lipophilic balance was modified and different arrangements of the polymer within the structure of liposomes as well as different interaction with their membrane were obtained. Both polymers were found in the inner core of the liposomes, however, the more hydrophobic PEG_{750}-DMA also formed localized clusters within the liposome membrane, whereas the more hydrophilic PEG_{4000}-DMA formed a polymeric corona on the vesicle surface. Preliminary cytotoxicity studies were also performed to evaluate the biological safety of these GiL systems and their suitability as innovative materials drug delivery application.

Keywords: hydrogels; hybrid nanocarriers; gelled-core liposomes; membrane properties; drug delivery systems.

1. Introduction

In the field of nanomedicine, phospholipid-based systems (i.e. liposomes) are certainly the most investigated platform for drug delivery, due to their wide range of recognized advantages including good biocompatibility and biodegradability, low antigenicity and the unique ability to deliver both hydrophilic and hydrophobic compounds. For these features, in the 1970s liposomes were proposed as interesting and appealing drug delivery vehicles (Elbayoumi and Torchilin, 2010; Allen and Cullis, 2013). In spite of all these advantages, investigations carried out over the years, highlighted some drawbacks of liposomes as drug delivery systems, mainly related with physical and chemical stability issues. Some limitations come from the inability of the membrane of liposomes to withstand mechanical stresses and to provide different controlled release profiles without leakage. Even if these issues had not forbidden clinical use of liposomes for drug delivery, it is evident that their overcoming should further empower their application in pharmaceutical field. For this reason, various strategies were developed in order to obtain more efficient lipid-based carriers with improved drug delivery performance. One way of great interest to approach these problems is to confer tougher mechanical properties to lipid vesicles introducing a soft and elastic polymeric network into their internal core. Gel core liposomes are an effort to make stabilized liposomes with all the advantages of a liposomal carrier with the elimination of the drawback of physical instability of conventional liposomes. Indeed, the inner polymeric network serves the function of skeleton and provides mechanical support to vesicle, mimicking the elastic protein network of the cytoskeleton in cells. It also acts as a diffusion barrier for sustained drug release, together with the external phospholipid membrane. Therefore, such assemblies may be viewed as advanced vesicular drug carriers that join together peculiarities of both polymeric drug delivery systems and liposomes in a single nanocarrier (Raemdonck et al., 2014). For all these reasons, the conversion of the inner aqueous core of liposomes into a polymeric gel may represent a key strategy to obtain promising new polymeric and liposomal nanometric systems with improved stability and sustained drug release properties.

The first attempt of filling lipid vesicles with a hydrogel was reported by Torchilin et al. (1987). Starting from this pioneering work, just few other works have been published on the preparation of gel core liposomes. Moreover, these works were aimed at the physical crosslinking of polymers incorporated in the aqueous core of liposomes, induced by pH, temperature or specific ions (Tiwari et al., 2009; El-Refaie et al., 2015a, 2015b; Ahmed et al., 2019; Kawar and Abdelkader, 2019). Less work has been done on the preparation of gel core liposomes by chemical crosslinking of encapsulated monomers or polymers inside phospholipid vesicles. In this sense, some attempts can be found in the literature, but none with the aim of studying the obtained gel core liposomes as new drug delivery systems. Indeed, chemical crosslinking has been used to convert the gel-forming solution in the aqueous core of giant unilamellar vesicles to produce better *in-vitro* models of cells (Viallat et al., 2004; Campillo et al., 2009; Liu et al., 2013). Alternatively, liposomes have been just used as templates to fabricate homogeneous chemical nanohydrogels to be employed after removal of the external phospholipid membrane (Kazakov et al., 2002; Van Thienen et al., 2005). In these works, suitable concentrations of pre-gel components were entrapped in closed lipid bilayers and an opportune crosslinking technique of the gel-forming solution was used to generate a chemical hydrogel. Starting from these findings, our research group

recently proposed a Gel-in-Liposome (GiL) system obtained through UV-initiated free radical polymerization of polyethylene glycol-dimethacrylate (PEG-DMA), previously encapsulated in unilamellar phospholipid vesicles (Petralito et al., 2014). In that work, we demonstrated that the crosslinked hydrogel serves as a mechanical support that: i) imparts greater mechanical stability to the membrane, and ii) affects the permeability properties of the external lipid bilayer, thus resulting in modified release of encapsulated molecules, as compared to conventional liposomes. Those results were achieved employing a relatively small PEG-DMA (Mn 750, PEG_{750}-DMA), that is characterized by limited water solubility, likely due to the relatively high volume fraction of the hydrophobic methacrylate unit (Lin-Gibson et al., 2005). It is therefore of interest to obtain information on the effect of the molecular mass of PEG-DMA on the properties of GiL systems, since it is well reported that the chain length of PEG-DMA deeply influences structure and properties of the derived hydrogels (Lin-Gibson et al., 2005; Killion et al., 2011). The macromolecular monomers present in the liquid core, their molecular weight, as well as, their interaction with the phospholipid membrane may affect the subsequent GiL structure and its behavior as drug delivery system. In particular, depending on the chain length, PEG-DMA may show a different distribution within the GiL system, it may also show diverse interaction with the phospholipid membrane and, after crosslinking, it may generate different networks. Based on these considerations, we now pursued the aim of investigating the effect of a longer PEG-DMA, with higher hydrophilic properties compared to PEG_{750}-DMA on the main features of GiL systems, in order to better understand the behavior of these delivery vehicles and optimize their drug release profile. To this end, PEG-DMA at two different molecular weights (750 and 4,000), was first encapsulated within unilamellar vesicles made of hydrogenated soybean phosphatidylcholine and cholesterol and then it was converted into a hydrogel by UV-initiated free radical polymerization. An accurate and optimized experimental set-up was used to evaluate the properties of the newly produced GiLs, comparing them to Conventional Liposomes (CL). Hydrodynamic diameter and polydispersity index were evaluated by dynamic light scattering (DLS) experiments, whereas the ζ-potential was measured by electrophoretic light scattering (ELS) experiments. Liposome morphology was observed with transmission electron microscopy (TEM). The interaction of PEG-DMA with HSPC/Chol membrane was investigated by differential scanning calorimetry (DSC) and further by mechanical destabilization studies with TX-100. Release experiments of 5-(6) carboxyfluorescein [5-(6) CF] were performed in vertical Franz Diffusion Cell to better characterize diffusion properties of these lipid membrane/hydrogel structures. Preliminary cytotoxicity studies were also performed.

2. Experimental section

2.1 Materials

Hydrogenated soybean phosphatidylcholine (HSPC), Phospholipon® 90H from Lipoid GmbH, was kindly gifted by AVG srl (Italy); 5-(6) carboxyfluorescein [5-(6) CF] was obtained from Kodak (Rochester, NY). Cholesterol (Chol), 1,2-dichloroethane, chloroform, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), polyethylene glycol-dimethacrylate (PEG-DMA) MW 750, 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure® 2959), Triton X-100™(TX-100), Sephadex® G-

50 medium grade, dialysis tubing cellulose membrane (cut-off 12,000-14,000 Da), 1-methyl-2-pyrrolidone, sodium hydroxide (NaOH), iron (III) nitrate, ammonium thiocyanate, uranyl acetate, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin solution, trypsin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (St. Louis MO, USA). Eagle's Minimum Essential Medium (EMEM), sodium pyruvate and sodium bicarbonate were obtained from Unimed Scientifica srl (Italy). Bidistilled water, hydrochloric acid 37% (HCl) and ethanol were supplied by Carlo Erba Reagents srl (Italy). Polycarbonate membrane filters Whatman® (800, 400 and 200 nm) - Cyclopore Track Etched Membrane were purchased from Sigma Aldrich (St. Louis MO, USA).

5-(6) CF stock solutions were made by dissolving 5-(6) CF powder in few drops of 1N NaOH solution, followed by the addition of HEPES buffer (10 mM, pH=7.4) up to the appropriate volume.

PEG_{4000}-DMA was synthesized by esterification of PEG (MW 4,000) with methacrylic anhydride (MA), following a previously optimized procedure with microwave irradiation (Pacelli et al., 2014).

2.2 Preparation of GiL systems

Conventional thin film hydration method followed by extrusion was used to prepare Gel-in-Liposomes (GiL) as described in literature (Petralito et al., 2014). Briefly, 40 mg of HSPC and 4 mg of Chol (1.0:0.1 weight ratio) were dissolved in the minimum volume of chloroform (3 ml) and the organic solution was poured into a round bottom flask. The organic solvent was evaporated under reduced pressure at T=60°C (above the gel-liquid crystalline transition temperature of the lipids, T_m) to form a lipid film, which was further dried under high vacuum to remove traces of the organic solvent. The resulting lipid film was hydrated with 5 ml of HEPES buffer solution (10 mM, pH=7.4) containing a mixture of PEG_{750}-DMA or PEG_{4000}-DMA, Irgacure 2959 (25 µl of 20% w/v solution in 1-methyl-2-pyrrolidone) and the hydrophilic fluorescent marker 5-(6) CF (20 mM). The hydration process was carried out in a water bath at T=60°C, above the T_m of the lipids. A final HSPC concentration of 10 mM was obtained. The mixture was repeatedly extruded at T=60°C, through polycarbonate membranes of decreasing pore size using a thermobarrel Extruder, Lipex™ Biomembrane (Canada). The extrusion was repeated until a homogeneous size distribution was achieved (2 times through 800 nm membranes, 2 times through 400 nm membranes and finally 6 times through 200 nm membranes). Finally, liposomes were purified by size exclusion chromatography (SEC) using a Sephadex G-50 gel filtration column. The purification step was carried out with the aim of removing all the non-entrapped material from the vesicular structures. Following the same procedure without the addition of PEG-DMA, conventional HSPC/Chol liposomes were prepared and used as a control (CL samples). All liposome formulations were stored at T=4°C and used within two weeks from preparation.

2.3 Reticulation of the liposome internal core

Liposomes formulations containing PEG_{750}-DMA or PEG_{4000}-DMA were UV irradiated in a Helios Italquartz Photochemical Multirays Reactor (Italy), equipped with ten, 14 W medium pressure mercury lamps G15T8-E LAWTRONICS (λ_{max}=310 nm), for 30 min. Following this procedure, the crosslinking of the polymers inside the lipid vesicles led to the formation of GiL_{750UV}

and GiL_{4000UV} samples.

Crosslinking of PEG-DMA and consequent formation of nanogels was confirmed by dynamic light scattering (DLS) measurements, carried out after removal of the lipid bilayer, obtained by the addition of 150 µl of 30% (w/v) TX-100. After this operation, PEG-DMA nanogels were also recovered by SEC carried out on a Sephadex G-50 gel filtration column and observed for morphology with a transmission electron microscopy (TEM). Similar experiments were also carried out on non-crosslinked GiL and CL samples.

The UV stability of 5-(6) CF, over the 30 min irradiation time, was verified and reported in the Supplementary material. To this end, solutions with different concentration of the marker were irradiated under the same conditions used for the photopolymerization of PEG-DMA in the two GiL systems. At the end of the exposure, the emission fluorescence emitted by the marker at 512 nm was recorded, after excitation at 492 nm. The fluorescence spectrum was compared to the one of the not irradiated control solution. The stability study was carried out both in the presence and in the absence of the photoinitiator Irgacure 2959.

2.4 Physicochemical characterization of liposomes

2.4.1 Dynamic light scattering and ζ- potential measurements

Particle size distribution and ζ-potential were measured with a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). Hydrodynamic diameter and polydispersity index were evaluated by dynamic light scattering (DLS) experiments, whereas ζ-potential was measured by electrophoretic light scattering (ELS) experiments. The DLS and ELS techniques used a photon correlator spectrometer equipped with a 4 mW He/Ne laser source operating at 633 nm. All measurements were performed at a scattering angle of 90°, at T=25°C. Liposome samples were diluted in 10 mM HEPES buffer (pH=7.4) until a count-rate of around 200 kcps was obtained to avoid interfering multiple scattering phenomena. Size, polydispersity index and ζ-potential values of the liposome formulations are the mean of three different preparation batches ± SD and all the analyses were performed at least in triplicate.

2.4.2 Transmission electron microscopy

Morphology of GiL, GiL_{UV} and CL samples was observed with TEM performed on a Zeiss EM 10 microscope operating at 60 kV, equipped with a Soft Imaging System CCD camera AMT DEBEN XR80 (DEBEN U.K.). TEM observations were carried out after negative staining of the samples. To this end, diluted suspensions of liposomes or nanogels were dropped onto 200-mesh Formvar® copper grids (Ted Pella Inc., USA), then stained with uranyl acetate (1% w/v) for 1 min. The excess of the uranyl acetate solution was removed placing grids onto absorbent paper disk in a Petri dish. Grids were dried at room temperature in a covered Petri dish, for almost 30 min before observation.

2.4.3 Determination of the amount of phospholipids structured in vesicles

The amount of phospholipids structured to form liposomal vesicles in the CL formulation, was determined using a colorimetric assay reported in literature (Yoshida et al., 1980). The analysis is based on the formation of a hydrophobic thiocyanate iron-phospholipid complex. Briefly, aliquots (0.4 ml) of CL samples in 50% v/v of ethanol were mixed with 1 ml of thiocyanate iron reagent, previously prepared by solubilizing 0.97 g iron (III) nitrate and 15.2 g of ammonium thiocyanate in 100 ml of water. The obtained solution was acidified with 0.6 ml of 0.17 N HCl. The thiocyanate iron-phospholipid complex was extracted with 3 ml of 1,2-dichloroethane by vigorous shaking for 2 min in a vortex-type mixer. The mixture was centrifuged for 5 min at 2000 rpm and the absorbance of the organic phase was read at λ=470 nm in a Lambda™ 25 spectrophotometer (Perkin Elmer, Waltham, USA). The calibration curve was obtained with several solutions of known HSPC concentration.

The amount of phospholipids structured in GiL_{750} and GiL_{4000} samples, was indirectly evaluated by means of an empirical correlation between the phospholipid amount found in the CL samples and the optical properties of the GiL_{750} and GiL_{4000} samples. The correlation between these two parameters was possible as it was first verified that the polymer did not interfere with the optical density (OD) measurements and that the average diameter of the lipid-polymer hybrid constructs was similar to that of the CL samples. The OD of the CL samples at different concentration, in the range 20-200 nmol, was measured at λ=436 and 600 nm and the OD values were correlated with the phospholipid amount evaluated as reported above.

2.5 Loading of 5-(6) CF in GiL systems

The amount of 5-(6) CF entrapped in the internal aqueous compartment of the lipid vesicles was determined measuring the fluorescence emitted by the marker at 512 nm, after excitation at 492 nm. To this end, purified GiL and CL samples were incubated with TX-100 for vesicles lysis, then the fluorescence values were measured and the concentrations of 5-(6) CF were determined with the standard curve $y = 5.65\times10\text{-}8x + 21.5$ (R^2=0.999), obtained in the range of concentrations from 10^{-7} to 10^{-6} mol/l. The entrapment efficiency was expressed as captured volume that is defined as volume (µl) of entrapped 5-(6) CF per mg of lipid. All experiments were performed in triplicate and the results are the mean of three different preparation batches ± standard deviation.

2.6 Analysis of the thermotropic behavior of GiL by differential scanning calorimetry

Calorimetric measurements were performed using a DSC131 (Setaram, France) scanning calorimeter. Typical analyses were carried out under nitrogen flow (20 ml/min) by setting an initial isotherm at 30°C for 300 seconds, a subsequent heating ramp from 30 to 70°C or from 30 to 170°C and a final isotherm at 70 or 170°C for 300 seconds, respectively for the measurements performed in hydrated and anhydrous conditions. Samples for the DSC analyses were obtained solubilizing HSPC, PEG_{750}-DMA (or PEG_{4000}-DMA) and cholesterol in the minimum volume of chloroform (3 ml) into a round bottom flask. Samples without cholesterol were also prepared and analyzed. The organic solvent was evaporated under reduced pressure at T=60°C to form a lipid film, which was further dried under high vacuum to remove any trace of the solvent. Aliquots (5 mg) of each dried film were gently scraped from the flask and weighed in sealable aluminium pans. For DSC under standard hydrated conditions, thermograms were recorded at scan rate of 5°C/min. For DSC under anhydrous conditions, several heating/cooling cycles were run before thermogram recording at scan rate of 5°C/min. The samples were submitted to heating/cooling cycles until identical

thermograms were obtained in two subsequent runs. An empty aluminium pan was used as reference. All the analysis were performed at least in triplicate.

2.7 Interaction process of TX-100 with liposomes

The ability of the surfactant TX-100 to interact with GiL structures was studied and compared with CL samples, in order to test the stability of the hybrid nanocarriers. The systems were equilibrated for 10 min at T=37.0±0.5°C, then aliquots of 3 ml of GiL_{750} and GiL_{4000} were stepwise mixed with 50 µl of TX-100 (0.1% w/v), in order to have sub-lytic concentrations of the surfactant and the fluorescence emitted by 5-(6) CF was continuously monitored over 30 min. At the end of the analysis, a lytic concentration of TX-100 was added to the suspensions, to determine the maximum content of 5-(6) CF in each sample. The percentage of 5-(6) CF, released from the different samples, following the mechanical destabilization operated by TX-100, was calculated using Equation 1.

$$5-(6)\ CF\ released\ (\%) = \frac{(I_t - I_0)}{(I_{max} - I_0)} \times 100 \quad (1)$$

where I_0 is the initial fluorescence intensity of 5-(6) CF loaded; I_t is the fluorescence intensity measured at time t; $I_{\max}$ is the fluorescence intensity after the complete lysis of the vesicles with TX-100.
All the experiments were performed at least in triplicate.

2.8 *In vitro* release of 5-(6) CF

Release studies of 5-(6) CF were carried out using a vertical Franz diffusion cell. The cylindrical donor compartment had a cross section S of 1 cm^2 and was separated from the receptor chamber by a dialysis membrane (12,000-14,000 Da) having an area of 1 cm^2 and thickness δ_m of 48 µm. The donor chamber was loaded with 1 ml of sample (GiL_{750}, GiL_{4000}, GiL_{750UV}, GiL_{4000UV} or CL) whereas the receptor chamber was filled with 4 ml of sonicated 10 mM HEPES buffer (pH=7.4) maintained under constant stirring (400 rpm). Aliquots of 150 µL were withdrawn at fixed time points and replaced with equal volumes of fresh buffer at the same temperature. All experiments were performed at T=60.0±0.5°C (above the T_m of the lipids) to have a faster diffusion of 5(6)-CF. The samples collected from the receptor chamber were analyzed after an appropriate dilution measuring the fluorescence emitted by the marker. Release studies were carried out in triplicate and the results reported as mean values ± standard deviation.

2.9 *In vitro* evaluation of cytocompatibility

2.9.1 Cell culture

The human fibroblast cell line (WI-38) was purchased from American Type Culture Collection (ATCC® CCL-75™ – Milan, Italy). The cells were grown in EMEM, supplemented with FBS (10% v/v), L-glutamine, sodium pyruvate, sodium bicarbonate, HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml). Fibroblasts were sub-cultured 1:3 in 25 cm^2 flasks, maintained at 37°C and 5% of CO_2 in a humidified environment, and the medium was renewed two times a week, as recommended by the

supplier. For the experiments, cells were seeded in 24-well plates at a density of $3x10^4$ cells/well. After 72 h, cells (about 80% confluent) were exposed to different concentrations of GiL_{750UV} and GiL_{4000UV} for 24, 48 and 96 h.

2.9.2 Cytotoxicity test

The MTT assay (index of mitochondrial function) was used for cytotoxicity evaluation. The test is based on the conversion of the tetrazolium salt MTT to an insoluble purple formazan by mitochondrial dehydrogenase enzymes of living cells. MTT was performed as previously described (Vitalone et al., 2011). Briefly, 5 mg/ml of MTT was added to each well and, after 80 min of incubation period, the medium was removed and 250 µl of DMSO was added. The 24-well plate was shaken for 5 min, and the absorbance of the converted dye was measured by a molecular device kinetic microplate reader (Bio-Rad, Hercules, USA), at a wavelength of 595 nm. All data were reported as mean ± standard deviation of two independent experiments, carried out using six replicates. Statistical comparison was performed using the Student's t-test and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Preparation and characterization of GiL_{750} and GiL_{4000}

The aim of this study was to prepare liposomal carriers characterized by a gelled core able to modify the release rate of an encapsulated drug and the stability of the vesicle structure.

Table 1. Composition of the hybrid vesicles. The amount of both HSPC and Chol were kept constant at 40 mg and 4 mg respectively, whereas PEG-DMA was varied. The effect of HSPC/PEG-DMA weight ratio on the formation of GiL_{750} and GiL_{4000} is reported. Vesicles formation was evaluated by DLS measurements. Samples showing bimodal size distribution with a peak larger than 1 µm were considered aggregated.

PEG-DMA (mg)	PEG-DMA (MW)	HSPC/PEG-DMA (w/w)	Note
20	750	1.0:0.5	Vesicles
40	750	1.0:1.0	Vesicles
50	750	1.0:1.25	Vesicles
80	750	1.0:2.0	Vesicles
20	4,000	1.0:0.5	Vesicles
40	4,000	1.0:1.0	Vesicles
50	4,000	1.0:1.25	Aggregates
80	4,000	1.0:2.0	Aggregates

In order to reach these objectives, PEG-DMA at two different molecular weights (750 and 4,000), was used as gel-forming material to prepare GiL carriers via UV-initiated free radical polymerization of the polymer entrapped in the

vesicle core. The entrapment of the polymer was achieved during the formation stage of the liposomes, obtained via thin lipid-film hydration followed by extrusion and SEC purification.

Table 1 shows the amounts of PEG_{750}-DMA or PEG_{4000}-DMA used to prepare the different hybrid vesicles. The amount of both HSPC and Chol were kept constant, whereas PEG-DMA:HSPC weight ratio was varied from 0 to 2.0, by varying the amount of PEG-DMA. The 1.0:1.0 polymer:lipid weight ratio resulted the maximum ratio able to form vesicles with both polymers. While with PEG_{750}-DMA higher amounts of the polymer, up to HSPC/PEG-DMA 1.0:2.0 weight ratio, still resulted in vesicle formation, the employment of higher amounts of PEG_{4000}-DMA hindered the supramolecular organization of HSPC and cholesterol causing the formation of macroscopic aggregates. Therefore, a 1.0:1.0 weight ratio was used to investigate the effect of the molecular weight and hydrophilicity of the polymer on the main properties of HSPC/Chol vesicles.

Working at this specific weight ratio, after extrusion and SEC purification, the produced unilamellar samples, regardless of PEG-DMA molecular weight, shown monomodal size distribution, with polydispersity index lower than 0.10 and hydrodynamic diameter of around 200 nm, very similar to CL samples (Table 2).

Table 2. Composition and physical-chemical characterization of liposomes embedding not polymerized PEG_{750}-DMA or PEG_{4000}-DMA and conventional liposomes.

Sample	HSPC (mg)	Chol (mg)	PEG-DMA (MW)	PEG-DMA (mg)	Hydrodynamic diameter (nm)	PdI	Conductivity (nS/cm)	ζ -potential (mV)
CL	40	4	-	0	192.1 ± 8.3	0.11 ± 0.01	0.347 ± 0.007	-9.5 ± 1.4
GiL_{4000}	40	4	4,000	40	206.3 ± 3.5	0.09 ± 0.09	0.384 ± 0.008	-13.1 ± 0.9
GiL_{750}	40	4	750	40	201.7 ± 17.2	0.05 ± 0.02	0.310 ± 0.003	-16.1 ± 3.3

The amount of structured lipid, in both cases, was about 80 %, which is only slightly lower than the value obtained for conventional HSPC/Chol liposomes (~90 %).

Irrespective of the polymer characteristics, ζ-potential values of SEC-purified GiL vesicles were more electronegative with respect to the value obtained for control HSPC/Chol liposomes (Table 2). These results account for diverse surface characteristics of GiL_{4000}, GiL_{750} and CL structures that may be explained considering the different hydrophilic/lipophilic properties of PEG_{750}-DMA and PEG_{4000}-DMA. In fact, the length of the polyethylene glycol chain may have determined a different interaction of the polymer with the phospholipids and so a different arrangement within the hybrid GiL structures.

The different surface properties of the GiL systems compared to CL ones, may be responsible for the different colloidal stability observed after storage of these formulations at 4°C. The average size, size distribution and ζ-potential of the GiL formulations remained unaltered for at least 14 days, when stored at 4 °C. Furthermore, the presence of the polymer allows delaying the flocculation process that generally occurs in HSPC/Chol liposomal suspensions, during the storage at 4 °C.

As evident in Figure S1, the presence of the polymer avoids the phase separation of the colloidal dispersion, while the conventional liposomes exhibited an evident flocculation. Since the colloidal stability of nanocarriers can be usually enhanced through the addition of polymers, it is possible that a small amount of PEG-DMA surrounds liposomes and avoids their interaction.

Indeed, the presence of the polymer allowed delaying the flocculation process that generally occurs in HSPC/Chol liposomal suspensions during storage (Figure S1).

Therefore, to confirm the hypothesis of the different arrangement of PEG_{750}-DMA and PEG_{4000}-DMA within HSPC/Chol liposomes, further investigations were carried out.

3.2 Interaction of PEG-DMA with HSPC/Chol membrane

If the two polymers have a different distribution within the vesicle structure, then they should affect in a different way the thermal properties of the bilayer because of the different interactions with the phospholipids. Therefore, to confirm the hypothesis of the different arrangement of PEG_{750}-DMA and PEG_{4000}-DMA within GiL structures, DSC measurements were performed in order to evaluate the effect of the polymer on the thermal properties of the bilayer.

The thermograms of conventional and PEG modified HSPC/Chol mixtures recorded on hydrated samples are reported in Figure 1A.

The liquid-ordered L_0 phase, associated with HSPC/Chol mixtures (1.0:0.1 weight ratio), contains fast diffusing lipids, which have the acyl chains in the *all trans* conformation, and it is similar to bilayers in the gel phase. Both PEG_{4000}-DMA and PEG_{750}-DMA produced a broadening and a decrease in the enthalpy associated to the HSPC/Chol transition temperature, when combined in a 1.0:1.0 weight ratio with the lipids and analyzed under standard conditions (Figure 1A). Consistent with these results, it seems evident that a partial interaction of the gel-forming precursors with the bilayer may occur during the vesicles formation. Bilayer-polymer interaction can result in surface adsorption or insertion into the lipid bilayer (Cohen et al., 1991; Tabbakhian and Rogers, 2012). These processes may involve a shift in the T_m and/or a modification in the enthalpy associated to this transition (ΔH_m). Usually, a reduction of the ΔH accounts for insertion of the polymer into the lipid membrane, which might be explained by a decrease of van der Waals interactions between lipid acyl chains after polymer insertion. The cooperation between phospholipid molecules in the bilayers during the transition process is crucial concerning the sharpness of peak of the main phase transition. The gel to liquid-crystalline transition of pure phospholipids appears as a sharp peak, due to favorable van der Waals interactions of lipid acyl chain, resulting in high cooperativeness of the molecules. Any additive able to penetrate in some extent in a phospholipid bilayer perturbs the packing of lipid molecules, therefore the degree of cooperativity decreases and the endothermic peak of the transition broadens and shifts toward lower temperature (Demetzos, 2008).

When PEG-DMA was added to HSPC/Chol mixtures, the interaction of the hydrophobic chains of the phospholipids becomes less cooperative and the corresponding calorimetric peak decreases and broadens.

These findings suggest that, irrespective from the length of the PEG chain, PEG-DMA is able to interact with the bilayer structure leading to a different rearrangement of the lipids in the final structure. Anyway, DSC performed under these standard

conditions fails to give insights on the distribution of the polymer within the system, due to the presence of water molecules and their effect on the membrane organization. In fact, it is known that, in fully hydrated membranes, the water molecules have a high influence on the properties of the bilayer as they increase the head-group spacing of the lipids, thus reducing the van der Waals interactions between their hydrocarbon chains.

This effect may have partially masked the contribution of PEG-DMA on the thermotropic behavior of the system. On the other hand, it is known that in the absence of water molecules between the phospholipid head groups, a close packing is obtained which raises the transition temperature well beyond that of fully hydrated bilayers. Therefore, under these conditions, the ability of PEG-DMA to interfere with the phospholipid packing may be better observed, particularly a shift of the T_m. For this reason, DSC was run also upon dehydration of the mixtures, in order to obtain deeper understanding of the distribution of the gel-forming material in the hybrid structure and to provide a better qualitative comparison between HSPC/Chol/PEG_{750}-DMA and HSPC/Chol/PEG_{4000}-DMA thermotropic behavior. The thermograms recorded under anhydrous conditions are reported in Figure 1B.

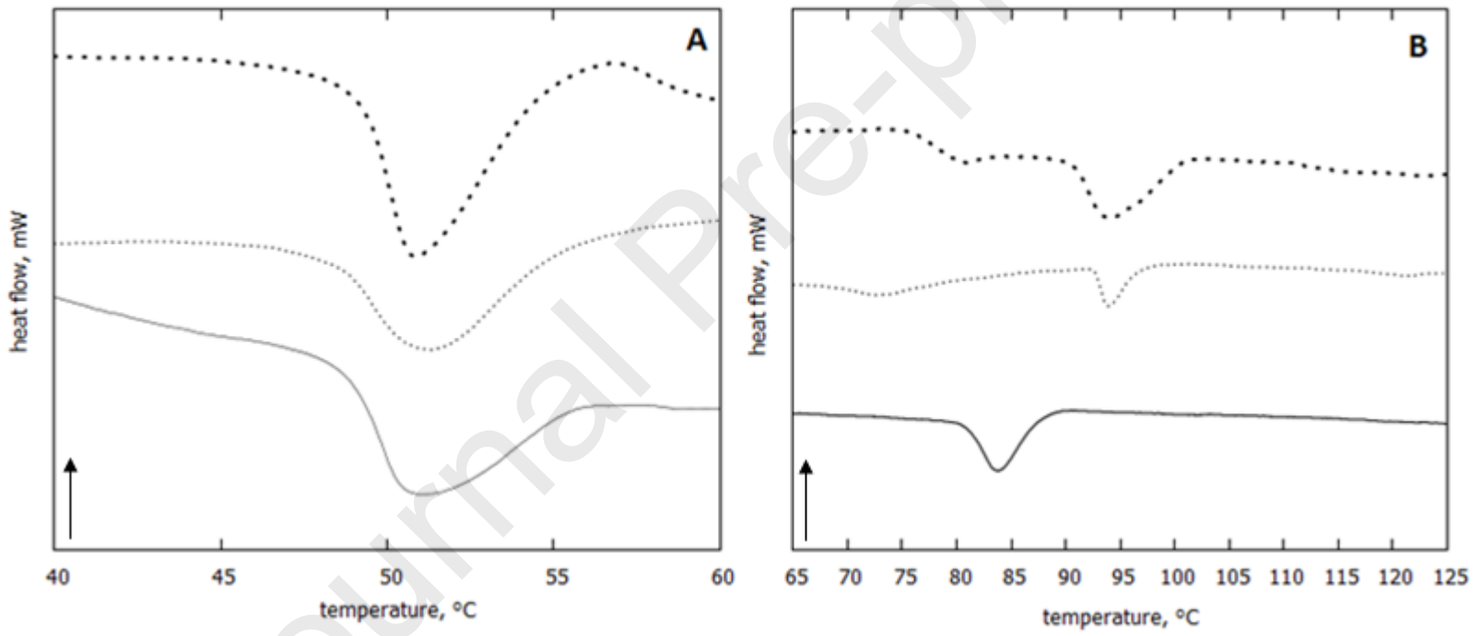

Figure 1. Effect of the presence of PEG-DMA and its molecular weight on the thermotropic phase behavior of **(A)** hydrated and **(B)** anhydrous HSPC/Chol mixtures. HSPC/Chol (...), HSPC/Chol/PEG_{750}-DMA (——), HSPC/Chol/PEG_{4000}-DMA (·····) Thermograms were recorded under nitrogen flow at scan rate 5°C/min. The arrow indicates the direction of an exothermic process.

The obtained results show significant differences in the distribution of the two polymers within the anhydrous structure which may be explained considering their different hydrophilic/hydrophobic balance. The effect was particularly evident when DSC analysis was carried out excluding cholesterol from the samples (Figure S2A and S2B). PEG_{4000}-DMA seemed to be localized in the vesicular aqueous core and/or adsorbed on the surface of the vesicles as a corona; whereas the more lipophilic PEG_{750}-

DMA, may be partly embedded in the bilayer compartment. In fact, in the dry state, PEG_{750}-DMA reduced the T_m of the HSPC/Chol mixture, while PEG_{4000}-DMA did not. We suggest that this effect of PEG_{750}-DMA on the T_m could be explained by an increased spacing between phospholipid molecules due to polymer intercalation, which results in reduced van der Waals interactions among the hydrocarbon chains of the phospholipids and, consequently, in reduced T_m values.

These results suggest that a change in the length of the polyoxyethylene chain of PEG-DMA could bring to different effect on the main properties of the bilayer, such as permeability, due to the different ability of the polymer to integrate within and interact with the HSPC/Chol membrane.

To corroborate the results obtained from DSC analysis and to have a deeper understanding of how the polymer in the hybrid structures can modify the properties of conventional vesicles, interaction studies of GiL and CL samples with the non-ionic surfactant TX-100 were carried out. In specific, information about the structural properties of the GiL system were obtained using sub-lytic concentrations of the detergent. In these conditions, the surfactant can penetrate into the membrane without destroying the vesicles and, in this way, the structure of liposomes can be preserved and phospholipids retain the architecture of a closed bilayer. However, consequent to surfactant insertion, permeability of the membrane increases and determines leakage of molecules entrapped into liposomes. Diffusion across phospholipid membranes mainly depends on their lipid composition, but it may also be influenced by the presence of non-lipid components within the bilayer, such as PEG-DMA in GiL systems. In particular, if PEG-DMA definitely assume different arrangements within the lipid vesicle depending on its molecular weight, then PEG_{750}-DMA and PEG_{4000}-DMA should offer a different resistance to TX-100 penetration into the outer monolayer of the membrane with a consequent different release of the liposomal content, upon exposure to the surfactant. In light of this consideration, monitoring the release of 5-(6) CF over time in presence of sub-lytic concentrations of TX-100 should be a suitable way to study the influence of the polymer on the physical properties of the membrane in the hybrid system.

The results from these interaction studies are reported in Figure 2.

No significant release of the fluorescent marker was detected before detergent addition for both CL and GiL systems, whereas 5-(6) CF was released upon addition, in a stepwise manner, of sub-lytic concentrations of TX-100 to the liposome suspensions. In particular, it can be observed that for CL sample, around 10% of the marker was released from the vesicles after the first addition of TX-100. The effect of the surfactant is almost instantaneous, with the fluorescence intensity and the marker concentration, rapidly reaching a constant value. The presence of the polymer modifies the amount of marker released after TX-100 addition.

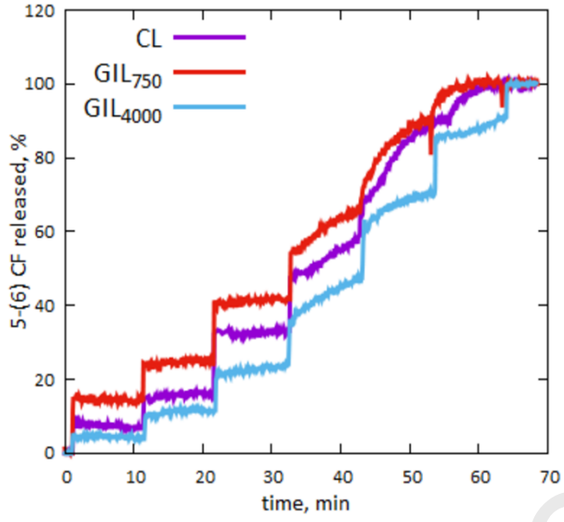

Figure 2. Mechanical destabilization studies of CL, GiL_{750} and GiL_{4000}, induced by sub-lytic concentrations of the non-ionic surfactant TX-100.

In specific, a lower percentage of 5-(6) CF was released from GiL_{4000} compared to CL samples, whereas a higher percentage of 5-(6) CF was released from GiL_{750}. Similar results were obtained with further additions of TX-100. To explain these results, we assume that the perturbation of the bilayer induced by the non–ionic surfactant can be influenced by the different ability of PEG_{750} and PEG_{4000} to interact with the lipids (phospholipids and cholesterol) composing the membrane. In fact, considering that the polymers inside the liposomes are not irradiated, we can argue that the internal diffusion of the marker was not influenced by both GiL_{750} and GiL_{4000}, so that the observed differences between the release curves after the same amount of detergent addition must be due to the modification of structural and mechanical properties of phospholipid bilayers, in other words to the different bilayer resistance. The presence of the polymer may affect the packing of the lipids and consequently the permeability of the membrane, with a final effect potentially dependent on the different balance between the hydrophilic and hydrophobic portions of PEG_{750}-DMA and PEG_{4000}-DMA.

At the same surfactant concentration, the amount of 5-(6) CF released from the GiL_{4000} structures was lower than CL and GiL_{750}, probably for the presence of some hydrophilic PEG_{4000}-DMA surrounding the vesicles surface, as depicted in Figure 3. This superficial polymeric corona may hamper the penetration of the surfactant into the bilayers, and hinder the TX-100-induced destabilization, thus resulting in a slower release of the entrapped dye. According to this hypothesis, the addition of PEG_{4000}-DMA to HSPC/Chol did not produced significant thermotropic variations in the DSC analysis (Figure 1B), therefore, specific mixing interactions between these components can be reasonably excluded. For this reason, it can be assumed that PEG_{4000}-DMA most likely spread on the external surface of GiL_{4000} and in their inner core. A similar arrangement was reported in the literature for a PEG monomethacrylate (Kostarelos et al., 2005)

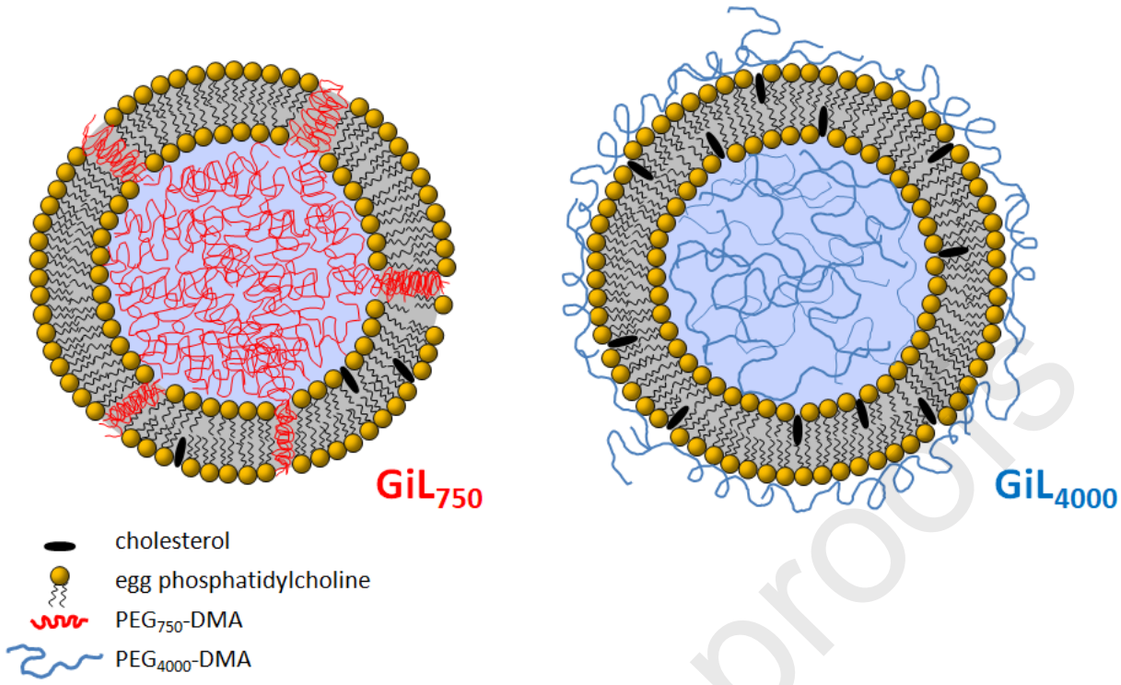

Figure 3. Schematization of the different arrangements of PEG_{750}-DMA and PEG_{4000}-DMA in GiL_{750} and GiL_{4000} structures.

Overall, these results suggest that the addition of non-lipid components to liposomes may be an interesting strategy for the modification of structural and mechanical properties of phospholipidic bilayers.

An opposite behavior was observed for PEG_{750}-DMA-modified liposomes. The presence of this polymer enhanced the release of 5-(6) CF after TX-100 addition. Considering the predominant hydrophobic nature of PEG_{750}-DMA and the results from DSC analysis, some PEG_{750}-DMA may have formed localized clusters within the liposome membrane. In fact, DSC analysis demonstrated that the calorimetric parameters of the main phase transition of HSPC/Chol mixed with PEG_{750}-DMA were lower than those measured for pure HSPC/Chol mixture under dry conditions (Figure 1B), therefore, the interaction of the polymer with the lipids may have definitely altered the membrane structural properties of the hybrid vesicles. Considering that the calorimetric parameters of the main phase transition are related to the acyl chains of phospholipids, it can be supposed that some PEG_{750}-DMA was incorporated into the hydrophobic region of GiL_{750} membrane as depicted in Figure 3. For this reason, it is probable that, in the presence of this polymer, a non-homogeneous distribution of the molecules of TX-100 was obtained within the phospholipid bilayer. Considering that the dimensions of GiL_{750} vesicles are similar to CL ones and assuming the formation of PEG_{750}-DMA clusters within the membrane, therefore the presence of lipid-rich regions (free of polymer) and polymer-rich regions within the bilayer of GiL_{750} may be supposed. If TX-100 preferably penetrate the lipid-rich domains, then, in these regions of the membrane, the detergent/lipid ratio may reach higher values than that obtained for CL systems. In this way, a higher increase of the local permeability of the membrane can be obtained, which is responsible for the higher release of the liposome payload.

3.3 Photo-polymerization of PEG-DMA in GiL structures

After SEC purification of the vesicles, PEG-DMA was photo-polymerized by UV irradiation for 30 min. According to DLS and

TEM results, the polymerization step did not alter the integrity as well as size and morphology of the vesicles. In particular, TEM analysis carried out on conventional and PEG-DMA modified liposomes before and after UV irradiation, did not evidence any morphological change due to the polymer introduction and its subsequent polymerization (Figure 4), in comparison with conventional vesicles.

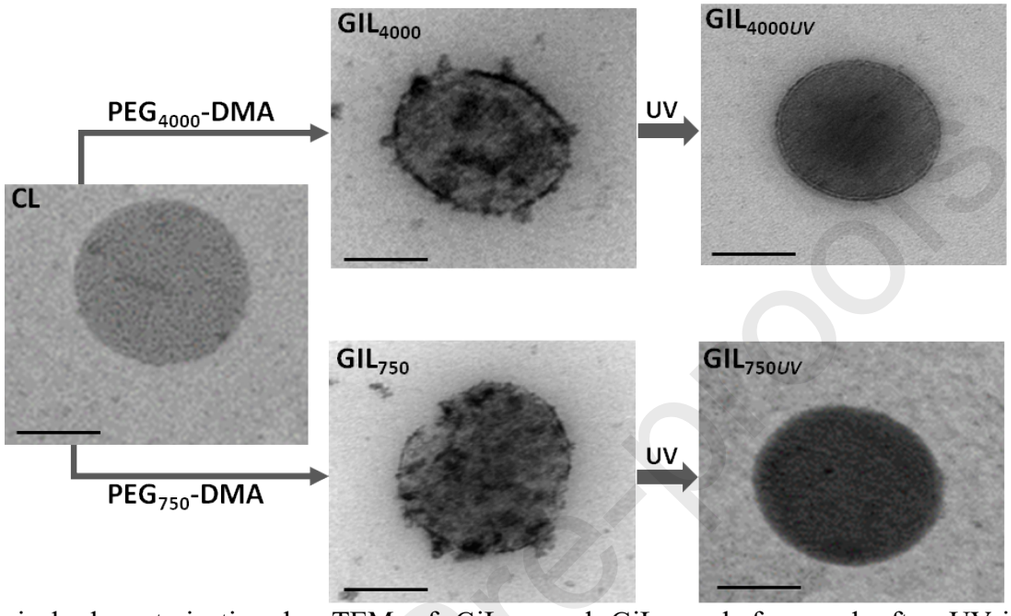

Figure 4. Morphological characterization by TEM of GiL_{750} and GiL_{4000}, before and after UV-induced free radical polymerization, to evaluate the effect of the presence of PEG_{750}-DMA and PEG_{400}-DMA on vesicles morphology (scale bar=100nm).

Moreover, TEM images show a morphological difference between irradiated and non-irradiated hybrid structures, suggesting that UV exposure can effectively lead to the crosslinking reaction of the methacrylic groups of PEG_{750}-DMA and PEG_{4000}-DMA, with consequent polymer crosslinking and formation of a gelled core.

In order to confirm the crosslinking reaction of PEG-DMA and the formation of a gel within the core of GiL samples, the lipid bilayers of the vesicles were removed with the non-ionic detergent TX-100. To this end, lytic concentration of TX-100 were added to GiL_{750} and GiL_{4000} before and after UV irradiation (Figure S3 and S4). Before UV crosslinking, the addition of TX-100 resulted, as expected, in complete vesicles destruction, as indicated by the disappearance of the peak at around 200 nm, and the formation of mixed micelles (peak at ~10 nm), whereas PEG-DMA was diluted in the external bulk.

Both systems (GiL_{750} and GiL_{4000}) show the same behavior and behave like CL samples. Instead, after UV exposure, the treatment with TX-100 resulted in a colloidal dispersion characterized by a bimodal size distribution, where it can be observed a peak at around 10 nm of the mixed micelles due to removal of the lipid bilayer and a peak at around 200 nm, which can be attributed to the nanogel formed into the liposomes core following PEG-DMA crosslinking (Table S1).

To further confirm PEG-DMA crosslinking and gel formation, the nanogels made of PEG_{750}-DMA and PEG_{4000}-DMA were recovered, after TX-100 treatment, by SEC purification and analyzed by DLS and TEM, for dimension and morphology respectively. TEM images show a spherical shape for the nanohydrogels with a mean diameter of about 210 nm, slightly larger

than the template liposomes, maybe due to gel swelling after the bilayer removal (Figure 5).

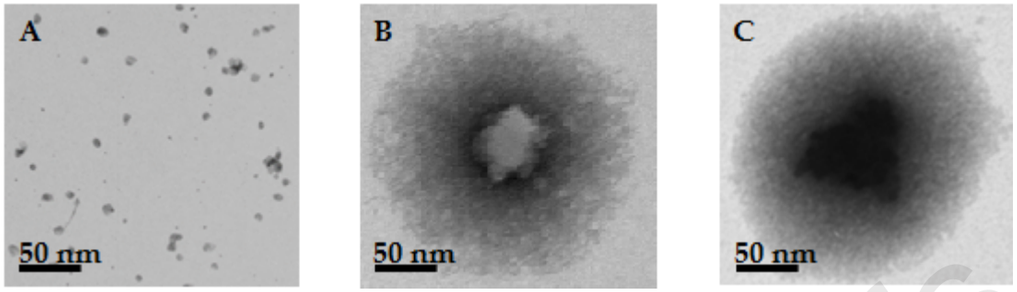

Figure 5. Morphological characterization by TEM of mixed micelles (A), PEG_{750}-DMA_{UV} nanohydrogel (B) and PEG_{4000}-DMA_{UV} nanohydrogel (C), obtained after treatment with lytic concentration of TX-100, respectively from CL, GiL_{750UV} and GiL_{4000UV}.

3.4 Analysis of the release data from GiLs

GiL_{750} and GiL_{4000} were loaded with a fluorescent hydrophilic marker, able to mimic the behavior of a hydrophilic drug, in order to evaluate the potential use of these hybrid vesicles for drug delivery applications.

Since PEG-DMA was crosslinked by UV-induced free radical polymerization, first of all a photostability study was carried out exposing 5-(6) CF to the UV radiation. The graph reported in Figure S5 suggests that the irradiation did not alter the fluorescent properties of the marker, also in the presence of the radical photoinitiator Irgacure 2959, therefore confirming the possibility of a direct loading of 5-(6) CF in GiL systems.

The actual loading of the marker in polymer-modified (GiL) and control (CL) liposomes was evaluated by fluorescence spectroscopy. CL samples liposomes showed a trapped volume of 2.11±0.28 µl of 5-(6) CF/mg HSPC. Instead, the values of the actual loading of GiL samples, regardless of the molecular weight of the polymer, resulted slightly lower respect to CL samples (GiL_{750}: µl/mg 1.59 ± 0.19 - GiL_{4000}: 1.58 ± 0.19 µl/mg), most likely for the co-entrapment of the polymer in the aqueous core of the liposomes. In fact, previous studies carried out with PEG_{750}-DMA at 1.0:20.0 lipid:polymer weight ratio hinder the passive loading of the fluorescent probe during the hydration step of the lipid thin-film, and required an active loading of the marker into preformed liposomes (Petralito et al., 2014). On the contrary, working at 1.0:1.0 lipid:polymer weight ratio it was possible to obtain the passive loading of the marker, with both PEG_{750}-DMA and PEG_{4000}-DMA. These results are particularly interesting because, while the active loading is a well-established loading technique, it cannot be always employed, since it can be successfully applied only to ionizable molecules. Usually, active loading of non-ionizable drugs into preformed liposomes results in poor entrapment efficiencies, therefore, a passive loading, in those cases, could be the only chance for a successful entrapment.

These results suggest that both PEG-DMA can be used to convert the liquid aqueous core of liposomes into a gel, even if the resulting hybrid systems may possess different properties, such as a different resistance to the release of a loaded molecule by virtue of the different ability of PEG_{750}-DMA and PEG_{4000}-DMA to interact with the phospholipid bilayer, as well as, the

different degree of crosslinking of the corresponding gels. For this reason, release studies of 5-(6) CF were carried out in order to evaluate the effect of PEG-DMA gel to the marker diffusion.

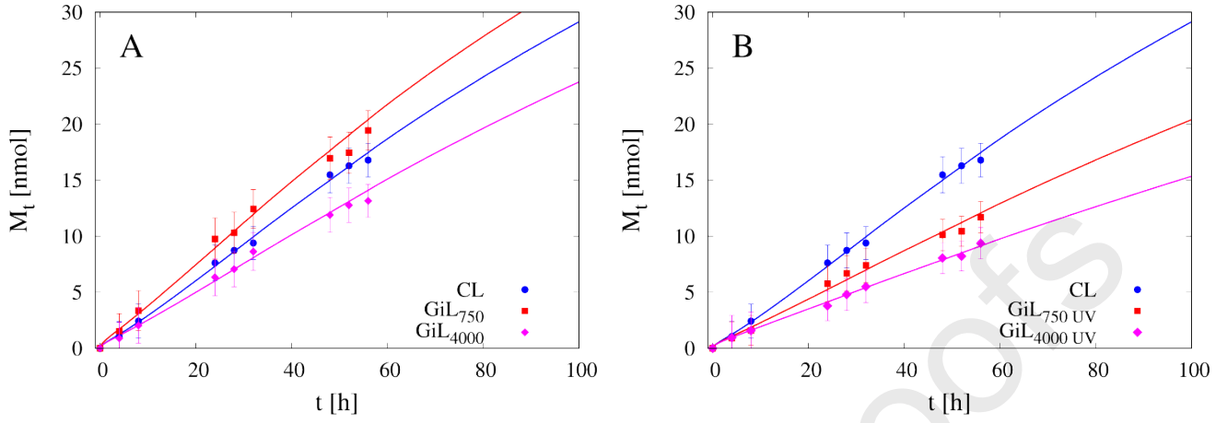

Figure 6. Experimental release data of 5-(6) CF from **(A)** CL, GiL_{750} and GiL_{4000}; **(B)** CL, GiL_{750UV} and GiL_{4000UV}.

The results of the release studies are reported in Figures 6 A-B as the total amount of marker released, up to time *t*, as a function of time, i.e. M_t vs *t* for CL, GiL_{750}, GiL_{4000}, GiL_{750UV} and GiL_{4000UV}. Figures 6A shows 5-(6) CF release data from CL and GiL systems not UV-irradiated. It can be observed that the presence of the polymer modified the release rate of 5-(6) CF. In particular, the introduction of PEG_{750}-DMA in HSPC/Chol vesicles increased the release rate of the fluorescent marker, whereas PEG_{4000}-DMA reduced it. These results can be explained by considering the structures of the GiL systems proposed in Figure 3. The presence of clusters of PEG_{750}-DMA within the bilayer can reduce the resistance offered by vesicle membrane towards the diffusion of 5-(6) CF, whereas the external corona of PEG_{4000}-DMA can hinder the transport process of the marker to the external bulk.

After UV-irradiation, the gelation of the inner core is responsible of a new added resistance to drug transport in GiLs (and absent in CL) and the release curves for GiL_{4000UV} and GiL_{750UV} became slower than that for CL. This implies that, after irradiation, the internal transport resistance to marker release became controlling for GiLs and adds to the double-layer resistance. By reasonably assuming that (1) the double-layer resistance was not altered by UV irradiation and (2) the internal resistance for GiLs was only slightly affected by the molecular weight of PEG-DMA, it is predictable, as actually experimentally found, that the release curve for GiL_{4000UV} was slower than that for GiL_{750UV} which in turn was slower than that for CL. These results, further supporting the structure proposed for GiL_{750} and GiL_{4000}, found a quantitative explanation in Petralito et al. (2020) where a theoretical analysis of experimental release data is presented in order to better understand all the complex transport phenomena involving marker/drug diffusion inside the liposome internal core (pre and after gelation) and through the hybrid liposome membrane.

3.5 Cytotoxicity studies

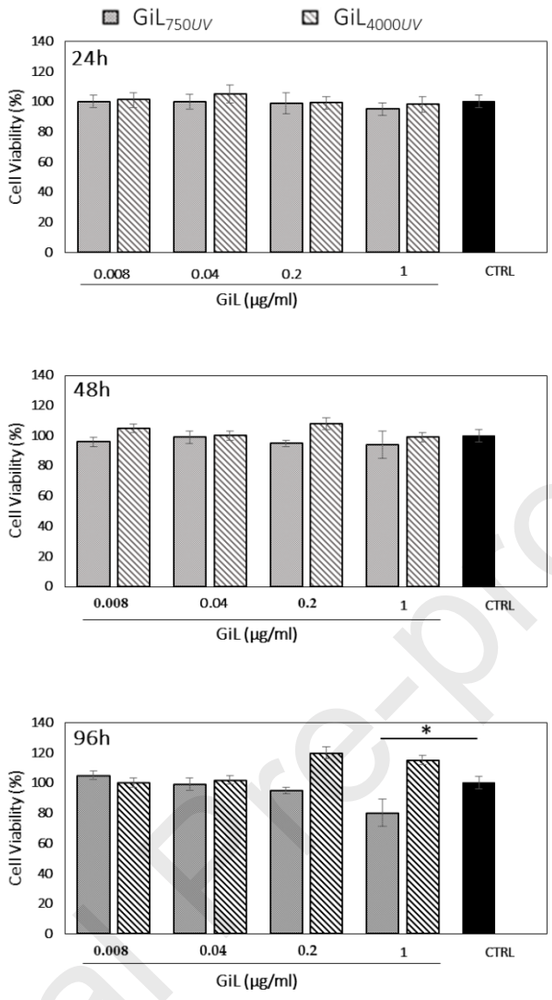

Figure 7. MTT assay performed on WI-38 human fibroblasts at 3 different time points: 24 - 48 - 96 h. Cells were kept in contact with different concentrations of GiL_{750UV} and GiL_{4000UV}, ranging from 0.008 to 1 µg/ml. CL samples were used as control (* $p<0.05$).

The effect of GiL_{750UV} and GiL_{4000UV} on the mitochondrial function of human fibroblasts, WI-38, was assessed by the MTT reduction test.

According to the results reported in Figure 7 the hybrid vesicles did not induce any inhibition of WI-38 proliferation, over all the range of concentrations tested, with the only exception of the highest concentration of GiL_{750} and only after 96 h of exposure. However, even if slightly statistically significant ($p=0.04$), that decrease could be considered not biologically relevant as the reduction in cell viability was less than 22%.

4. Conclusions

In this work, PEG-DMA was successfully incorporated in unilamellar HSPC/Chol liposomes and used to modify the liquid aqueous core of the vesicles into a hydrogel, as a result of its UV-induced free radical polymerization. It was found that the

arrangement of PEG-DMA within the liposomes as well as its effect on their main properties depend on the molecular weight of the polymer. In fact, the more hydrophobic PEG_{750}-DMA partly interfered with the phospholipid packing forming localized clusters within the liposome membrane. The presence of non-crosslinked PEG_{750}-DMA domains produced an enhancement of the permeability of the bilayer, which offered a lower resistance towards the diffusion of the entrapped fluorescent marker 5-(6) CF, compared to control HSPC/Chol liposomes. Nevertheless, the membrane-permeabilizing effect of PEG_{750}-DMA was nullified after the polymer crosslinking and the consequent gel formation, evidencing an increase of the internal resistance to the marker diffusion.

When the length of the polyethylene glycol chain of PEG-DMA was extended a different arrangement of the polymer within HSPC/Chol vesicles was obtained. Because of its greater hydrophilicity, PEG_{4000}-DMA was found inside the liposome core, but also partially adsorbed on the external surface of the vesicles, where it formed a polymeric corona. This arrangement was responsible for an increase of the resistance to the marker diffusion, observed even in the non-crosslinked system, and resulting in a slower release of the entrapped 5-(6) CF compared to control liposomes. Also for GiL_{4000UV}, the internal transport resistance became greater, and actually controlling, after UV-induced polymerization of PEG_{4000}-DMA.

Overall these results suggest that, irrespective of the molecular weight of PEG-DMA, its combination with phospholipid vesicles appears an interesting strategy to develop stable drug delivery systems. In Petralito et al. (2020) a detailed transport model is proposed, aimed at describing the entire 5-(6) CF diffusive pathway from the vesicles inner, through the double-layer membrane, into the buffer solution in the donor chamber of the Franz Cell and from there to the accepting chamber, where withdrawals are performed to evaluate the released drug concentration. The model permits to give a quantitative estimate of the diffusional resistances offered by the inner core (liquid or gelled) and by the double-layer membrane for CLs and different GiLs systems.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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References

Ahmed, S., Corvis, Y., Gahoual, R., Euan, A., Lai-Kuen, R., Couillaud, B.M., Seguin, J., Alhareth, K., Mignet, N., 2019. Conception of nanosized hybrid liposome/poloxamer particles to thicken the interior core of liposomes and delay hydrophilic drug delivery. Int. J. Pharm. 567, 118488. https://doi.org/10.1016/j.ijpharm.2019.118488

Allen, T.M., Cullis, P.R., 2013. Liposomal drug delivery systems: from concept to clinical applications. Adv. Drug Deliv. Rev. 65, 36-48. https://doi.org/10.1016/j.addr.2012.09.037

Campillo, C.C., Schroder, A.P., Marques, C.M., Pépin-Donat, B., 2009. Composite gel-filled giant vesicles: membrane homogeneity and mechanical properties. Mater. Sci. Eng. C 29, 393-397. https://doi.org/10.1016/j.msec.2008.08.001

Cohen, S., Bañó, M.C., Chow, M., Langer, R., 1991. Lipid-alginate interactions render changes in phospholipid bilayer permeability. Biochim. Biophys. Acta Biomembr. 1063, 95-102. https://doi.org/10.1016/0005-2736(91)90358-F

Demetzos, C., 2008. Differential scanning calorimetry (DSC): a tool to study the thermal behavior of lipid bilayers and liposomal stability. J. Liposome Res. 18, 159–173. https://doi.org/10.1080/08982100802310261

Elbayoumi, T.A., Torchilin, V.P., 2010. Current trends in liposome research. Methods Mol. Biol. 605, 1-27. https://doi.org/10.1007/978-1-60327-360-2_1

El-Refaie, W.M., Elnaggar, Y.S., El-Massik, M.A., Abdallah, O.Y., 2015a. Novel curcumin-loaded gel-core hyaluosomes with promising burn-wound healing potential: development, in-vitro appraisal and in-vivo studies. Int. J. Pharm. 486, 88-98. https://doi.org/10.1016/j.ijpharm.2015.03.052

El-Refaie, W.M., Elnaggar, Y.S., El-Massik, M.A., Abdallah, O.Y. 2015b. Novel self-assembled, gel-core hyaluosomes for non-invasive management of osteoarthritis: in-vitro optimization, ex-vivo and in-vivo permeation. Pharm. Res. 32, 2901-2911. https://doi.org/10.1007/s11095-015-1672-8

Kawar, D., Abdelkader, H., 2019. Hyaluronic acid gel-core liposomes (hyaluosomes) enhance skin permeation of ketoprofen. Pharm. Dev. Technol. 24, 947-953. https://doi.org/10.1080/10837450.2019.1572761

Kazakov, S., Kaholek, M., Teraoka, I., Levon, K., 2002. UV-induced gelation on nanometer scale using liposome reactor. Macromolecules 35, 1911-1920. https://doi.org/10.1021/ma011644+

Killion, J.A., Geever, L.M., Devine, D.M., Kennedy, J.E., Higginbotham, C.L., 2011. Mechanical properties and thermal behaviour of PEGDMA hydrogels for potential bone regeneration application. J. Mech. Behav. Biomed. Mater. 4, 1219-1227. https://doi.org/10.1016/j.jmbbm.2011.04.004

Kostarelos, K., Emfietzoglouc, D., Tadros, T.F., 2005. Light-sensitive fusion between polymer-coated liposomes following physical anchoring of polymerisable polymers onto lipid bilayers by self-assembly. Faraday Discuss. 128, 379–388. https://doi.org/10.1039/b403407d

Lin-Gibson, S., Jones, R.L., Washburn, N.R., Horkay, F., 2005. Structure−property relationships of photopolymerizable poly(ethylene glycol) dimethacrylate hydrogels. Macromolecules 38, 2897-2902. https://doi.org/10.1021/ma0487002

Liu, J., Lu, N., Li, J., Weng, Y., Yuan, B., Yang, K., Ma, Y., 2013. Influence of surface chemistry on particle internalization into giant unilamellar vesicles. Langmuir 29, 8039-8045. https://doi.org/10.1021/la4015652

Pacelli, S., Paolicelli, P., Pepi, F., Garzoli, S., Polini, A., Tita, B., Vitalone, A., Casadei, M.A., 2014. Gellan gum and polyethylene glycol dimethacrylate double network hydrogels with improved mechanical properties. J. Polym. Res. 21, 1-13. https://doi.org/10.1007/s10965-014-0409-4

Petralito, S., Spera, R., Pacelli, S., Relucenti, M., Familiari, G., Vitalone, A., Paolicelli, P., Casadei, M.A., 2014. Design and development of PEG-DMA gel-in-liposomes as a new tool for drug delivery. React. Funct. Polym. 77, 30-38. https://doi.org/10.1016/j.reactfunctpolym.2014.02.002

Petralito, S., Paolicelli, P., Nardoni, M., Tedesco, A., Trilli, J., Cesa, S., Casadei, M.A., Adrover, A.,2020. Gelation of the internal core of liposomes as a strategy for stabilization and modified drug delivery II. Theoretical analysis and modelling of *in-vitro* release experiments. Submitted to Int. J. Pharm. (IJP-S-20-00970R1)

Raemdonck, K., Braeckmans, K., Demeester, J., De Smedt, S.C., 2014. Merging the best of both worlds: hybrid lipid-enveloped matrix nanocomposites in drug delivery. Chem. Soc. Rev. 43, 444-472. https://doi.org/10.1039/C3CS60299K

Tabbakhian, M., Rogers, J.A., 2012. Interaction of insulin, cholesterol-derivatized mannan, and carboxymethyl chitin with liposomes: a differential scanning calorimetry study. Res. Pharm. Sci. 7, 43-50.

Tiwari, S., Goyal, A.K., Khatri, K., Mishra, N., Vyas, S.P., 2009. Gel core liposomes: an advanced carrier for improved vaccine delivery. J. Microencapsul. 26, 75-82. https://doi.org/10.1080/02652040802170897

Torchilin, V.P., Klibanov, A.L., Ivanov, N.N., Ringsdorf, H., Schlarb, B., 1987. Polymerization of liposome-encapsulated hydrophilic monomers. Macromol. Rapid Commun. 8, 457-460. https://doi.org/10.1002/marc.1987.030080907

Van Thienen, T.G., Lucas, B., Flesch, F.M., van Nostrum, C.F., Demeester, J., De Smedt, S.C., 2005. On the synthesis and characterization of biodegradable dextran nanogels with tunable degradation properties. Macromolecules 38, 8503–8511. https://doi.org/10.1021/ma050822m

Viallat, A., Dalous, J., Abkarian, M., 2004. Giant lipid vesicles filled with a gel: shape instability induced by osmotic shrinkage. Biophys. J. 86, 2179–2187. https://doi.org/10.1016/S0006-3495(04)74277-0

Vitalone, A., Di Giacomo, S., Di Sotto, A., Franchitto, A., Mammola, C.L., Mariani, P., Mastrangelo, S., Mazzanti, G., 2011. Cassia angustifolia extract is not hepatotoxic in an in vitro and in vivo study. Pharmacology 88, 252-259. https://doi.org/10.1159/000331858

Yoshida, Y., Furuya, E., Tagawa, K., 1980. Direct colorimetric method for the determination of phospholipids with dithiocyanatoiron reagent. J. Biochem. 88, 463-468. https://doi.org/10.1093/oxfordjournals.jbchem.a132993

Gelation of the internal core of liposomes as a strategy for stabilization and modified drug delivery I. Experimental investigation and optimization.

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- Stefania Cesa: formal analysis and visualization;
- Michela Relucenti and Roberto Matassa: investigation;
- Annabella Vitalone: investigation and data curation;
- Alessandra Adrover: supervision, validation and writing - review & editing;
- Maria Antonietta Casadei: funding acquisition and supervision;
- all authors provided critical feedback, discussed the results and commented on the manuscript.

Gelation of the internal core of liposomes as a strategy for stabilization and modified drug delivery I. Experimental investigation and optimization.

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- PEG-DMA was incorporated in unilamellar liposomes
- PEG-DMA was used to modify the aqueous core of liposomes in a hydrogel
- The arrangement of PEG-DMA within the vesicles depends on its molecular weight
- The effect of PEG-DMA on the properties of liposomes depends on its molecular weight.
- Polymer inside the vesicles modifies the release properties