Mixed lipid/polymer nanostructures: From advanced materials to drug delivery systems

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Abstract

The aim of this investigation was to study the alterations of the physicochemical characteristics of L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC) and dipalmitoyl phosphatidyl choline (DPPC) liposomes, caused by the incorporation of a poly (oligoethylene glycol acrylate)-b-poly(lauryl acrylate) (POEGA-PLA) block copolymer at different molar ratios. We used Dynamic and Electrophoretic Light Scattering to determine the size and the ζ-potential; imaging techniques for investigate the structure and Static Light Scattering for quantifying the fractal morphology of the prepared nanosystems *in situ*. The size of mixed nanostructures became smaller with the incorporation of the block copolymer into the lipid membrane. The size of the prepared nanosystems ranged between 50-80nm. The fractal dimension (d_f) decreased significantly with the incorporation of block copolymer into liposomal bilayers. The morphology of DPPC:POEGA-PLA mixed nanostructures (with d_f equal to 1.8) is open (more loose). On the other hand, the morphology of HSPC: POEGA-PLA (with d_f equal to 2.1) is more compact and dense. The molar ratio of the POEGA-PLA did not alter the morphology of the mixed nanostructures, expect from HSPC:POEGA-PLA system. Finally, we studied the drug loading properties of the mixed nanostructures in order to examine their properties as advanced Drug Delivery nanosystems. Copyright © 2017 VBRI Press.

Keywords: Mixed nanostructures, liposomes, polymers, drug delivery, fractal dimension.

Introduction

Nanotechnology can help in the development of multifunctional systems with targeted bioactive molecule delivery and nanosystems with simultaneous diagnostic and therapeutic properties. The most important advantages of advanced Drug Delivery Systems (aDDSs) are the ability of size, structure and shape control, as well as the ability of integrating multiple functions in a single nanodevice or nanosystem [**1-5**]. For this reason mixed/hybrid/chimeric aDDSs have been developed combining biomaterials different in nature and chemistry (i.e. surfactants and polymers, lipids and polymers etc.) in order to design effective nanodevices with multiple applications in the biomedical field. These drug carriers of nanometer size, offer the ability of increased therapeutic effectiveness, increased loading, lower toxicity against normal tissues and achieving controlled therapeutic levels for extended time [**3-5**]. Also, they can increase the lipophilic bioactive molecules solubility and increase their stability and allowing the completion of pre-clinical or clinical studies. As mentioned above, mixed lipid/polymer nanostructures are drug delivery structures comprising polymer and lipids, which exhibit complementary characteristics of both polymeric nanoparticles and liposomes, particularly in terms of their physicochemical stability and biocompatibility. These systems may exhibit higher encapsulation efficiency and controlled release propertied of the APIs. Significantly, these nanostructures have recently been demonstrated to exhibit superior in vivo cellular delivery efficacy and targeting compared to that obtained only from polymeric (i.e. polymersomes) and lipid (i.e. liposomes) nanocarriers.

Advanced Drug Delivery Systems (aDDSs) have presented fractal dimensions [**6-12**]. Liposomes, polymeric nanoparticles and micelles are in the focus of the nanotechnology field for treatment and diagnostics of chronic and incurable diseases following Mandelbrot geometry principles. Initially the liposomal aggregation fractal approach appeared in literature. The liposomal aggregation behavior in the presence of divalent cations, especially calcium was extensively studied. The liposomal fractal dimension was estimated and images of

the fractal aggregations were taken with various imaging techniques. In some occasions, a distinctive fractal dimension increase from 1.8 to 2.1 was observed when the Ca^{2+} concentration was increased [**9-11**]. This suggests that aggregations are more compact and dense according to Reaction Limited Cluster Aggregation. The liposomal aggregation study under the prism of fractal geometry was the reason of a classic theory extension describing the stability of colloidal systems where liposomes belong. The spontaneous liposomal aggregation was presented in international literature recently, due to liposomal system aging and the aggregation fractal geometry was defined. The presence of a Lateral Cluster-Cluster Aggregation could have been a possible explanation of the observed behavior since liposomes presented the phenomenon of self-similarity as liposomes preserved their fractal structure unaltered at 2.5, despite the increase of their Euclidean characteristics (liposomes hydrodynamic radius) from nanometric to micrometric scale. Also, liposomes fractal characteristics differ in statistically important degree in conditions simulating the human organism. These liposomes fractal dimension studies and other nanotechnological chimeric drug delivery systems aim towards the fullest understanding of their physicochemical characteristics in order to design innovative drug delivery systems in nanometric scale.

The goal of this study is to investigate the alterations of the physicochemical and morphological characteristics of L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC) (**Fig. 1a**) and dipalmitoylphosphatidylcholine (DPPC) (**Fig. 1a**) liposomes, caused by the incorporation of and a poly(oligoethylene glycol acrylate)-b-poly(lauryl acrylate) (POEGA-PLA) block copolymer (**Fig. 1c**) at different molar. We used Dynamic and Electrophoretic Light Scattering for determining the size and the ζ-potential of the mixed nanostructures; imaging techniques (cryo-TEM, AFM) for investigating the structure and Static Light Scattering for quantifying the fractal morphology of the prepared systems *in situ* (i.e. in liquid state). Finally, we studied the drug loading properties of the mixed nanostructures, using ibuprofen (IBU) as the hydrophobic drug, in order to examine their properties as a DDSs.

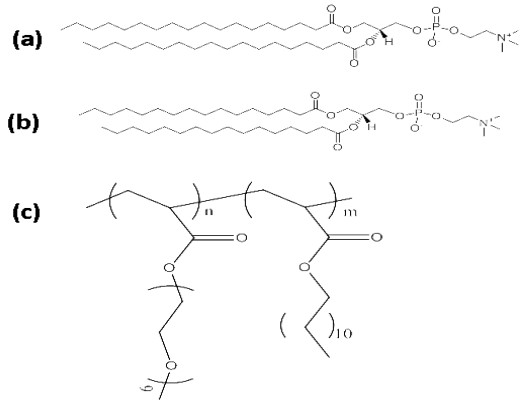

Fig. 1. The chemical structure of (a) HSPC, (b) DPPC lipids, and (c) POEGA-PLA block copolymer.

Experimental

Materials

The phospholipids used for the chimeric formulations was 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC). They were purchased from Avanti Polar Lipids Inc., (Albaster, AL, USA) and used without further purification. IBU BP/PhEur (S250 Grade) was purchased by Shasun®. Chloroform and all other reagents used were of analytical grade and purchased from Sigma–Aldrich Chemical Co. The poly(oligoethylene glycol acrylate)-b-poly(lauryl acrylate)(POEGA-PLA) block copolymer was synthesized by RAFT polymerization methodologies. The PLA block was prepared first and then used as a macromolecular chain transfer agent for the synthesis of the second POEGMA block. The molecular weight (M_w) of the diblock copolymer was 10,200 and the polydispersity index (PDI) 1.4, both values obtained by size exclusion chromatography (SEC). The copolymer contained 30wt% PLA as determined by ^{1}H-NMR spectroscopy. Its chemical structure is shown in **Fig .1** together with the structures of the lipids utilized.

Preparation of pure and mixed vesicles

Different mixed formulations have been prepared using the thin-film hydration method as described in our previous investigation [**3-5**]. Briefly, appropriate amounts of HSPC/DPPC:POEGA-PLA mixtures and DPPC/DPPC:POEGA-PLA (9:0, 9:0.01, 9:0.05, 9:0.1, 9:0.2 and 9:0.5) were dissolved in chloroform/methanol (9:1 v/v) and then transferred into a round flask connected to a rotary evaporator (Rotavapor R-114, Buchi, Switzerland). Vacuum was applied and the chimeric phospholipid/block copolymer thin film was formed by slow removal of the solvent at 50°C. The mixed film was maintained under vacuum for at least 24h in a desiccator to remove traces of solvent and subsequently it was hydrated in HPLC-grade water, by slowly stirring for 1h in a water bath above the phase transition temperature of lipids (41°C for DPPC and 52°C for HSPC). The resultant nanostructures (apparently multi lamellar vesicles, MLVs) were subjected to two, 5min and 5min sonication cycles (amplitude 70, cycle 0.7) interrupted by a 5min resting period, in a water bath, using a probe sonicator (UP 200S, dr. Hielsher GmbH, Berlin, Germany). The resultant chimeric nanostructures (tentatively assigned as small unilamellar vesicles, SUVs) were allowed to anneal for 30min.

Dynamic, static and electrophoretic scattering techniques

Physicochemical characterization of the nanostructures (by Dynamic and Electrophoretic Light Scattering) in aqueous medium is described in our previous papers [**15**, **16**].

Static light scattering has been widely used in the study of the fractal dimensions of nanoparticulate aggregates and nanoparticles. In static light scattering, a beam of light is directed into a sample and the scattered intensity is

measured as a function of the magnitude of the scattering vector q, with:

$$q = \frac{4\pi n_0}{\lambda_0}\sin(\frac{\theta}{2}) \quad (1)$$

where, n_0 is the refractive index of the dispersion medium, $\theta$is the scattering angle and λ_0 is the wavelength of the incident light. The general relation for the angular dependence of the scattered intensity, I(q) is:

$$I(q) \sim q^{-df} \quad (2)$$

where, d_f is the fractal dimension of the liposomes or aggregates with $1 \leq d_f \leq 3$ (d_f=3 corresponds to the limit of a completely compact Euclidean sphere where less compact structures are characterized by lower d_f values). The above equation is the classical result used to determine the mass fractal dimension from the negative slope of the linear region of a log-log plot of I vs. q.

Atomic force microscopy (AFM)

For AFM measurements, aqueous dispersions were spin coated onto mica surface using a standard spin coater model SPIN150, SPS-Europe B.V. (Netherland) with 400 rpm for 600 minutes and then samples were dried at room temperature for 24 hours. AFM images were obtained using a Multimode with NanoscopeIIIcontroller, Veeco (USA) AFM equipped with a piezoelectric scanner with a scan range of 10x10 μm^2. The imaging of samples was conducted in the tapping mode in ambient air conditions at a scan rate of 1 Hz using etched silicon probes (TESP, BRUKER) of nominal spring constant 42 N/m and operating at a resonant frequency of 320 kHz. All samples were imaged at room temperature. The VeecoNanoScope V531r1 program was employed to analyze the recorded images.

Cryogenic transmission electron microscopy (cryo-TEM)

Cryogenic Transmission Electron Microscopy images were obtained using a Tecnai F20 TWIN microscope (FEI Company, USA) equipped with field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Eagle 4k HS camera (FEI Company, USA) and processed with TIA software (FEI Company, USA). Specimen preparation was done by verification of the aqueous (HPLC grade water) solutions on grids with holey carbon film (Quantifoil R 2/2; Quantifoil Micro Tools GmbH, Germany). Prior to use, the grids were activated for 30 seconds in oxygen plasma using a Femto plasma cleaner (Diener Electronic, Germany). Cryo samples were prepared by applying a droplet (2.1 µL) of the solution to the grid, blotting with filter paper and immediate freezing in liquid ethane using a fully automated blotting device Vitrobot Mark IV (FEI Company, USA). After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a cryo-TEM-holder Gatan 626 (Gatan Inc., USA) and analyzed in the TEM at -178 °C. Pictures were processed using ImageJ software.

Drug loading

The chimeric formulations containing the drug ibuprofen (IBU) were prepared by dissolving IBU in the initial lipid/polymer mixture, resulting in the following molar ratios: DPPC/HSPC: POEGA-PLA 9:0:10; 9:0.01:10; 9:0.05:10; 9:0.1:10; 9:0.2:10 and 9:0.5:10. The effect of IBU incorporation in the chimeric preparations was evaluated by measuring the size, size distribution and ζ-potential of the resulting nanostructures. Mixed nanostructures incorporating IBU were frozen at -80°C overnight and were subjected to lyophilization in order to be reconstituted by chloroform and calculate the incorporation efficiency. The lyophilization was achieved overnight using a freeze drier (TELESTAR^{Q7} Cryodos-50, Spain) under the following conditions: condenser temperature from -50°C, vacuum $8.2x10^{-2}$mb. The lyophilized liposomal suspensions were stored at 4°C. Freeze-dried chimeric lipid/block copolymer vesicles were reconstituted by methanol (in order to achieve the same dispersion medium of calibration curve) to the original volume of the preparation under gentle agitation. Each sample was allowed to anneal for 30min followed by vortexing, and a relaxation period of 15min.The percentage of IBU incorporated into nanocarriers was estimated by spectrophotometry (Stat Fax® 4200, Microplate Reader, NEOGEN® Corporation), as described in the literature.

Encapsulation efficiency (EE) was calculated by using the following equation:

$$\%EE = \frac{IBU\ \ (after\ column)}{IBU\ (initial = calculated\ by\ molar\ ratio)} \times 100 \quad (1)$$

Statistical analysis

Results are shown as mean value ± standard deviation (S.D.) of three independent measurements. Statistical analysis was performed using Student's t-test and multiple comparisons were done using one-way ANOVA. P-values <0.05 were considered statistically significant. All statistical analyses were performed using "EXCELL".

Results and discussion

The physicochemical characterization of mixed nanostructures

We designed and prepared mixed lipid/block copolymer nanostructures using the thin-film hydration method. First, the prepared nanostructures were characterized by Dynamic and Electrophoretic Light Scattering Techniques and the results are presented in **Fig. 2** and **Fig. 3a, b** and **c**. The size distribution of nanostructures that will be used for biomedical purposes is very crucial, due to elimination and clearance issues associated with the in vivo behavior of these nanosystems. The size distribution of HSPC

liposomes became more homogeneous by the incorporation of POEGA-PLA block copolymer (**Fig. 2a**). The size distribution became narrower at 9:0.1 and 9:0.2 molar ratios of the prepared nanostructures (**Fig. 2a**). Similar results were obtained by the incorporation of POEGA-PLA into DPPC liposomes. The population of mixed nanostructures became narrower at 9:0.2 molar ratios (**Fig. 2a**). It should be pointed out that the size distribution of DPPC mixed nanostructures is narrower in comparison with HSPC mixed nanostructures (**Fig. 2**).

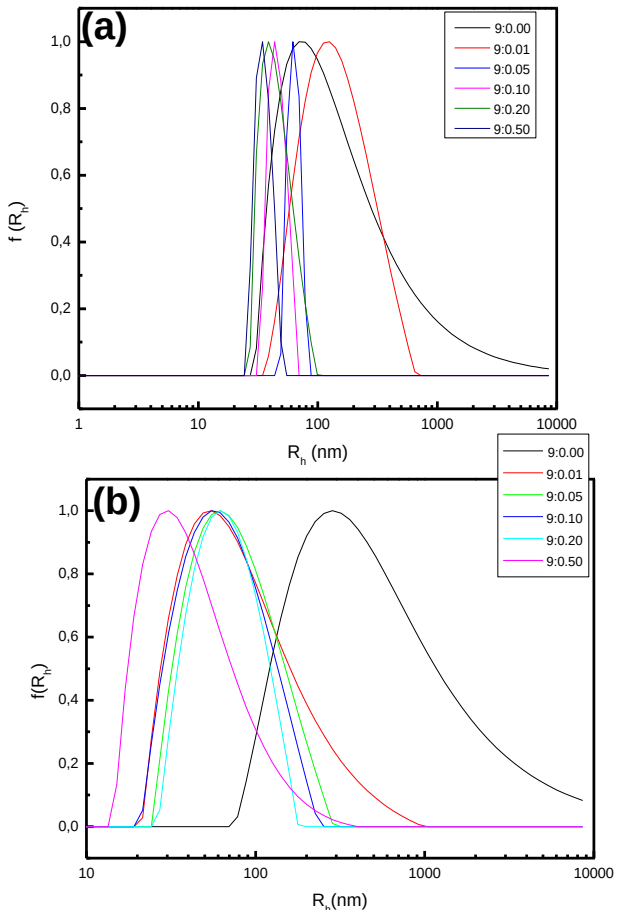

Fig. 2. Size distributions of (**a)** HSPC:POEGA-PLA and (**b)** HSPC: POEGA-PLA chimeric vesicles in HPLC-grade water.

The main transition temperature of lipid plays a key role for the cooperatively of the mixed nanostructures and the properties of the final prepared nanosystems (i.e. size, size distribution etc.). The size of the mixed nanostructures followed the same trend with the size distribution. Namely, the size of HSPC/DPPC mixed nanostructures became smaller with the incorporation of the block copolymer into the liposomal membrane. In all cases (all molar ratios), the size of the prepared systems ranged between 50nm and 80nm, indicating that the mixed systems were in the nanoscale. This phenomenology was encouraging in respect to the biomedical properties of the mixed nanostructures as drug delivery carriers (**Fig. 3a**). The scattering intensity versus the molar ratio of the two biomaterials (i.e. lipid and block copolymer) increased significantly as the block copolymer ratio increased for both lipids (**Fig. 3b**). The scattering intensity is analogous to the mass of the nanostructure and the increase of the scattering intensity values indicated that the PLA (hydrophobic) chains are incorporated into the HSPC and DPPC lipid membranes (**Fig. 3b**).

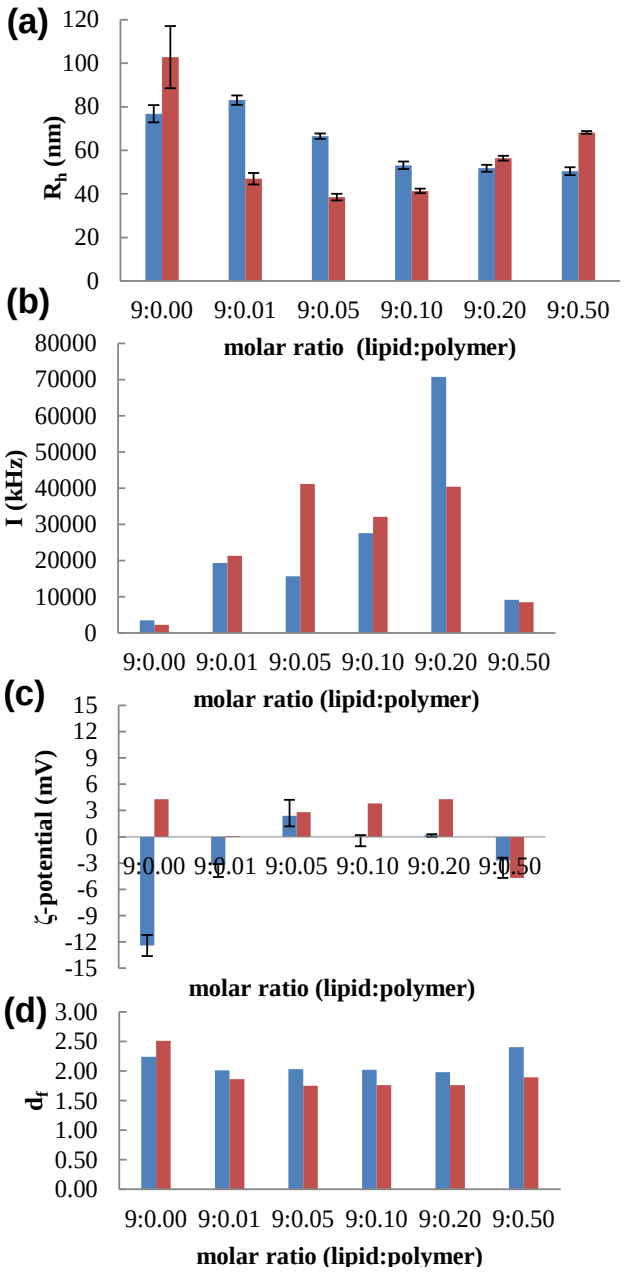

Fig. 3. The physicochemical (**a)** Hydrodynamic radius -R_h (nm), (**b)** scattering intensity- I(KHz) (**c)** ζ-potential (mV) and morphological characteristics (**d)** fractal dimension-d_f of HSPC:POEGA-PLA (blue columns) and HSPC:POEGA-PLA (red columns) chimeric vesicles in HPLC-grade water.

The incorporation of PLA chains into HSPC and DPPC lipid bilayers is a phenomenon that is driven by thermodynamic and self-assembly reasons. The preparation protocol that was used (i.e. thin-film hydration method) also played a key role for the incorporation of PLA chain into lipid membranes because

the two components were dissolved into the same solvent (co-solvent methodology). The ζ-potential values of pure HSPC and DPPC liposomes were found near zero because these lipids are zwitterionic phopsholipids (dipolar compounds). The incorporation of POEGA-PLA block polymer did not alter the surface charge of the mixed nanostructures (**Fig. 3b**).

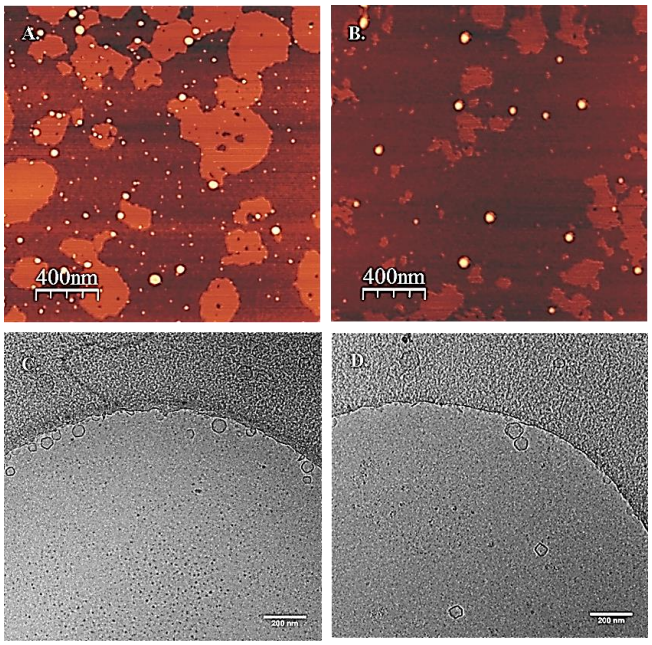

Fig. 4. AFM images (left) and Cryo-TEM micrographs (right) of chimeric vesicles DPPC:POEGMA-PLA 9:0.5 (A, B) and HSPC:POEGMA-PLA 9:0.5 (C, D).

The morphological characterization of mixed nanostructures

As mentioned above, the fractal dimension (d_f) represents a parameter for the quantification of the morphology of nanostructures in situ (i.e. in solution state). Fractal analysis has been considered as a complimentary analytical tool to evaluate the morphology of nanostructures. The d_f values of HSPC and DPPC liposomes were found near to 2.24 and 2.51, respectively from static light scattering. This observation indicates that the external morphology of liposomes is strongly dependent to the chemistry of the lipid (**Figs. 1a and 1b)**. The fractal dimension is a function of nanoparticle size and is correlated to the structural complexity of the system. For these reasons, we studied the fractal dimension of mixed nanostructures. The external structure of nanostructures changed significantly by the incorporation of polymeric guest. Namely, the incorporation of the POEGA-PLA block copolymer into lipid membranes caused alteration in the morphology of nanostructures as the d_f values indicated (**Fig. 3d**). In all cases, the d_f decreased significantly with the incorporation of block copolymer into liposomal bilayers. The morphology of DPPC:POEGA-PLA mixed nanostructures (with d_f equal to 1.8) is open (more loose). On the other hand, the morphology of HSPC: POEGA-PLA (with d_f equal to 2.1) is more compact and dense. The molar ratio of the POEGA-PLA did not alter the morphology of the mixed nanostructures, expect from HSPC:POEGA-PLA system. Fractal dimensionality of the mixed nanostructures was found to depend on the chemistry of the lipid. On the other hand, the molar ratio of the polymeric guest did not affect significantly the morphology of nanoparticles.

Having these results in mind we investigated the morphology of mixed nanostructures by cryo-TEM and AFM (**Fig. 4**). It should be noted that cryo-TEM is used to observe the structure of colloidal systems, of nano- to micrometer scale dimensions. Mixed nanostructures observed in AFM images and cryo-TEM micrographs are of circular and vesicular shapes of mainly unilamellar structures (**Fig. 4**). The size of the mixed nanostructures obtained from AFM and cryo-TEM are more or less similar and in good agreement with those measured by DLS. The shape of HSPC and DPPC mixed nanostructures are vesicular and did not exhibit

Table 1. The physicochemical characteristics of HSPC:POEGA-PLA:IBU and DSPC: POEGA-PLA:IBU chimeric vesicles.

System	Molar Ratio	R_h (nm)[1]	PDI[2]	ζ-potential	%EE[3]
HSPC:IBU	9:0:10	77.4±1.8	0.512±0.012	-10.2±1.4	84.8
HSPC:POEGA-PLA:IBU	9:0.01:10	93.1±2.1	0.334±0.018	-12.2±0.1	78.9
HSPC:POEGA-PLA:IBU	9:0.05:10	73.5±1.3	0.398±0.018	-12.4±1.8	67.2
HSPC:POEGA-PLA:IBU	9:0.1:10	53.1±1.7	0.356±0.026	-10.1±0.1	65.0
HSPC:POEGA-PLA:IBU	9:0.2:10	51.5±0.9	0.309±0.080	-14.9±4.3	61.4
HSPC:POEGA-PLA:IBU	9:0.5:10	46.1±1.5	0.310±0.010	-11.8±2.7	59.5
DPPC:IBU	9:0:10	91.4±1.4	0.492±0.007	-8.4±1.8	81.5
DPPC:POEGA-PLA:IBU	9:0.01:10	97.0±2.6	0.311±0.009	-10.1±1.4	78.2
DPPC:POEGA-PLA:IBU	9:0.05:10	88.6±1.5	0.215±0.008	-12.4±1.2	76.3
DPPC:POEGA-PLA:IBU	9:0.1:10	91.4±1.0	0.228±0.012	-13.8±1.2	56.7
DPPC:POEGA-PLA:IBU	9:0.2:10	56.4±1.1	0.159±0.017	-12.3±0.2	47.8
DPPC:POEGA-PLA:IBU	9:0.5:10	63.7±1.6	0.553±0.016	-18.2±7.4	68.5

[1]Hydrodynamic radius [2]Polydispersity index [3]Encapsulation efficiency

differences. The last one is a difference concerning the morphology of mixed nanostructure in situ/solution state (as indicated by d_f values /static light scattering) and in imaging techniques (AFM and cryo-TEM). The mixed membrane thickness (~6nm) is larger than the thickness of the pure liposomal membrane (~3-4 nm). This is additional evidence for the successful preparation of mixed nanostructures by thin-film hydration methods.

Drug loading

IBU (a non-steroid anti-inflammatory drug-NSAID) is used as a model active pharmaceutical ingredient to further investigate the loading properties of the prepared mixed lipid/block copolymer nanostructures, which are very important for drug delivery applications. IBU is practically insoluble in water but soluble in organic solvents and for this reason formulations are required in order to administer it in human body and skin. The physicochemical properties of the systems incorporating IBU were assessed **(Table 1)**. Observing the conventional liposomes, their size remained about the same, though a slight increase in PDI occurred, as well as a shift of ζ-potential to a negative value (**Table 1**). The latter is a result of IBU being negatively charged at this pH (7.4) and confirms that the nanostructures are loaded with the model Active Pharmaceutical Ingredient (API). We can conclude that IBU is incorporated into the mixed lipid/block copolymer membrane due to its hydrophobic character. For this reason, an increase of the size upon IBU loading was observed.

The drug incorporation efficiency was estimated to 80% (maximum drug loading), for conventional HSPC and DPPC liposomes (**Table 1**) and between 47 and 68% for mixed nanostructures. The incorporation efficiency is high for conventional liposomes due to the strong interaction of the NSAIDs with the phosphate region of the lipid head group. NSAIDs do not penetrate deeply into the liposomal bilayer, but are located close to the head group region [**14**, **15**]. According to the literature, at pH 7.4 (PBS), NSAIDs are negatively charged and the negatively charged groups of these drugs are therefore able to interact via electrostatic forces with the positively chargedcholine of DPPC/HSPC, which possibly alters the orientation of the lipid head group. These electrostatic interactions may be the driving force for the increased incorporation efficiencies and loading in PBS [**14**, **15**]. "Multiple interactions" and enantioselective absorption are observed between DPPC and IBU, including hydrophobic and electrostatic interactions or hydrogen bonding in a hydrophobic (lipid bilayer environment). The incorporation efficiency is lower for the mixed nanostructures due to the presence of PLA blocks into the liposomal membrane, which act as a barrier for IBU incorporation and reduce the available space within the bilayer. On the other hand, we did not observe differences in incorporation efficiency at the higher molar ratio of the POEGA-PLA. These observations may be a result of the interplay between the polymer composition and the amphiphilic character of the drug.

Conclusion

In this investigation, an innovative, nanoscale drug delivery platform was successfully designed and prepared. The components used were low and high molecular weight amphiphiles with high biocompatibility. Light Scattering techniques were used in order to study in detail the physicochemical and morphological characteristics of these aDDnSs and showed a considerable reduction of the size of mixed nanostructures, in comparison to HSPC and DPPC pure liposomes. Formation of mixed nanostructures was observed in most cases by AFM and cryo-TEM with a lipid/block copolymer membrane exhibiting kinks (raft-like nanostructures). All the mixed nanostructures have vesicular structure, as cryo-TEM images revealed, while their fractal dimensionality was found to be dependent on the composition (i.e. copolymer content). IBU, a model drug, was incorporated into the mixed lipid/block copolymer membrane due to its hydrophobic character and the encapsulation efficiency values were sufficient. An emphasis was given about the control of the membrane structure through physicochemical pathways such as playing with the molar ratio polymer/lipid components and, incorporation of APIs (model drug) which may play a role on the morphologies/fractal dimensionality of polymer/lipid mixed nanostructures. Therefore a series of experiments and mixed nanostructures can give various results depending on the size, the size distribution and the morphology of the drug delivery systems. In other words, the results may differ significantly between studies on aDDnSs used as innovative platforms in therapeutics. It can be concluded that the prepared DPPC/HSPC:POEGA-PLA mixed nanostructures could be used as drug delivery platforms and a starting point for pharmaceutical applications in order to develop aDDnSs.

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Author's contributions

All authors contributed equally to the work. Authors have no competing financial interests.

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