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Layersome: Development and optimization of stable liposomes as drug delivery system

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Abstract

This paper describes the development of stable drug delivery systems named layersomes. The layersomes are conventional liposomes coated with one or multiple layers of biocompatible polyelectrolytes in order to stabilise their structure. The formulation strategy is based on an alternative coating procedure of positive poly(lysine) (pLL) and negative poly(glutamic acid) (pGA) polypeptides on initially charged small unilamellar liposomes (SUVs). The size distribution and the zeta potential of the final entity depend on the number of polyelectrolyte layers and the charge of the last coating layer. Morphological studies were achieved by flux cytometry and cryo electron microscopy. Release studies of encapsulated hydrophilic 5(6)-carboxyfluorescein (5,6CF) in the presence of Triton® or ethanol showed an increased membrane resistance of the layersomes compared to classical SUVs. Finally, encapsulation of piroxicam (PX) was performed with success.

Keywords: Stable liposomes; Layersomes; Coating; Charged polymers

The layer-by-layer self-assembly of polycations and polyanions has been largely applied to coat solid surfaces and consequently modulate their physicochemical properties (Decher, 1997). The major drawback of liposomes is their instability during storage or in biological media (Ruysschaert et al., 2004; Sulkowski et al., 2005), which is related to surface properties. Different strategies were investigated to increase nanoparticle stability. One of them consists in the modification of their external surface by optimisation of the lipid composition of the membrane (Kokkona et al., 2000), by incorporation of bioadhesive molecule (chitosan) in their membrane (Thongborisute et al., 2006), by the polymerization of a two-dimensional network in the hydrophobic core of the membrane, by coating with nonporous silica (Begu et al., 2007) or by the addition of surface active polymers (Ruysschaert et al., 2004). Another generation of liposomes, named "stealth liposomes" was developed in order to evade interception by the immune system after administration. The layer-by-layer coating concept is one of the strategies used for the preparation (Sukhorukov et al., 2005) or the stabilization (Mobed and Chang, 1998) of nanosystems. Thus, liposomes coated by sodium poly(styrene sulfonate) (Radtchenko et al., 2002) or pLL (Volodkin et al., 2007) were developed improving their stability. Actually, films made of polypeptides and natural polysaccharides are of great interest due to their ability to create biomimetic architectures (Boulmedais et al., 2004; Richert et al., 2004; Zhang et al., 2005). In particular, films constituted by the alternate adsorption of PLL and PGA have already shown interesting biological properties (protein adsorption, cell adhesion, biocompatibility...) which can be controlled depending on the outermost layer, the presence of proteins or the number of layers (Richert et al., 2002; Schultz et al., 2005). Considering these results, the present paper describes the development of multilayer liposomes obtained by the layer-by-layer deposition of pGA and pLL to take advantages of their favourable biological properties.

As starting material, large unilamellar liposomes composed of phosphatidylcholine, phosphatidylglycerol and cholesterol (solubilized in chloroform/methanol 9/1 (v/v), 80/20/50 molar ratio, respectively) were prepared using a solvent evaporation process followed by a rehydration with a 10 mM Hepes buffer (pH 7.4) containing 15 mM NaCl to reach a final lipid con-

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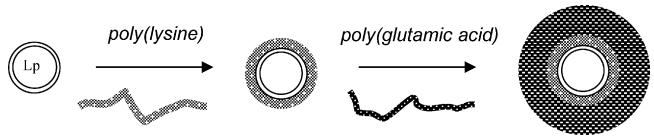

Fig. 1. Schematic representation of layer-by-layer preparation of layersomes.

centration of 10 μmol/mL. The consecutive sonication of this solution led to SUVs that were used as standard liposomes (Lps) references. The Lps displayed an average diameter of 100 ± 15 nm (Coulter Hialeah, FL) and a zeta potential of −60 ± 10 mV (Zetamaster 3000, Malvern Instrument, Orsay, France) (Fig. 2A). The next step consisted in the alternative deposition of positively and negatively charged biocompatible polypeptides: pLL or pGA, respectively (Fig. 1) (Gangloff et al., 2006). The layer-by-layer process, described previously for multilayer polyelectrolyte films (Decher, 1997), was applied to SUVs to produce the layersomes. Briefly, polycation and polyanion solutions were prepared at 0.5 and 1.0 mg/mL, respectively. Diluted liposomes suspension (0.67 μmol of phospholipids/mL) was first slowly dropped into the pLL solution in equal volumes during 20 min at room temperature under magnetic stirring. As described by Michel and collaborators a buffer with low ionic strength was used to avoid a too strong screening of the repulsive electrostatic forces between liposomes and polypeptide (Michel et al., 2004). A final dialysis eliminated the polymer in excess (10 mL of previous liposomes, in 2 × 2 L of Hepes 10 mM, NaCl 150 mM, 12 h, membranes of MWCO = 100,000 Da (Spectra/Por, Spectrum Laboratories, Rancho Dominguez, Canada)). The polymer deposition spontaneously occurred due to electrostatic interactions between the negative charges of phosphatidylglycerol and positive charges of polylysine. As detailed Fig. 2A, these Lp-pLL exhibited positive zeta potential (+60 ± 10 mV) with a slightly increased average diameter (127 ± 22 nm) compared to primarily Lp. The second layer with pGA was obtained under the same process conditions. Finally, the Lp-pLL-pGA liposomes exhibited a larger diameter of 250 ± 54 nm with a negative zeta potential (−45 ± 10 mV). These results are consistent with a layer-by-layer coating of liposomes. In order to confirm the association of pGA to the Lp-pLL, a fluorescent pGA was synthesised in our laboratory. When incubated with the pGA-fluorescein the liposomes were identical in size to

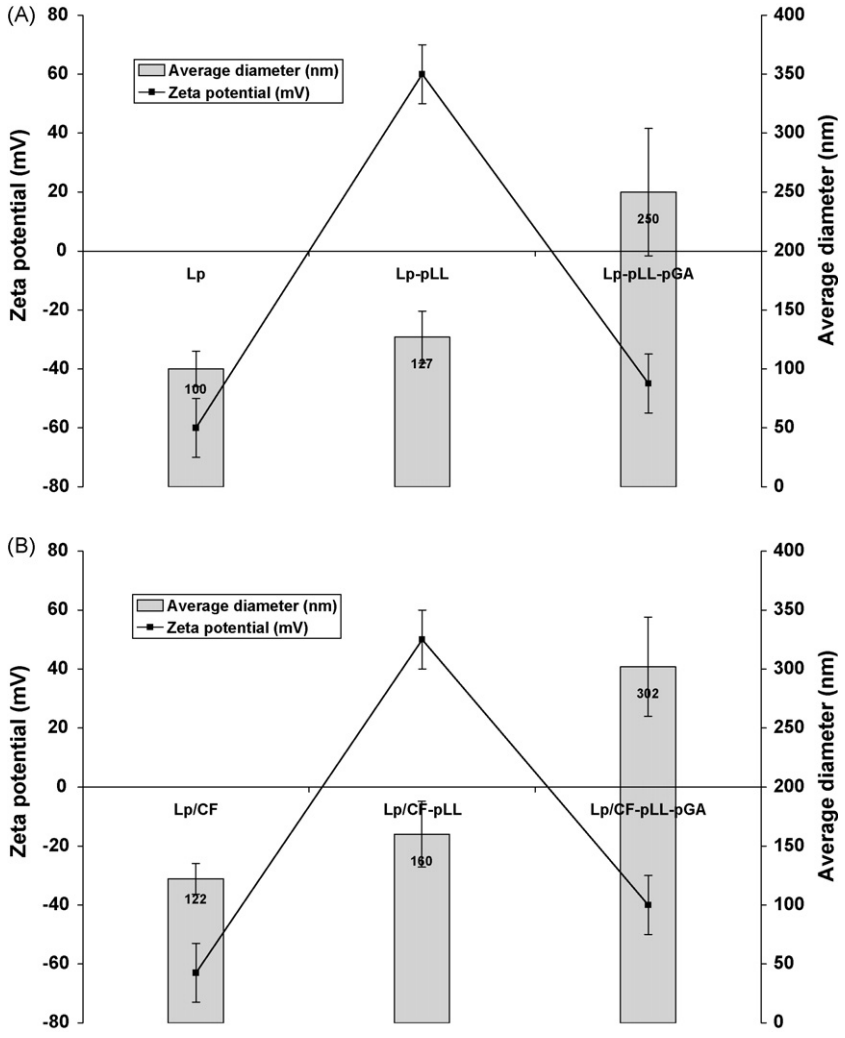

Fig. 2. Evolution of average diameter and zeta potential of liposomes with or without polypeptide coating, incorporating (B) or not (A) 5,6CF.

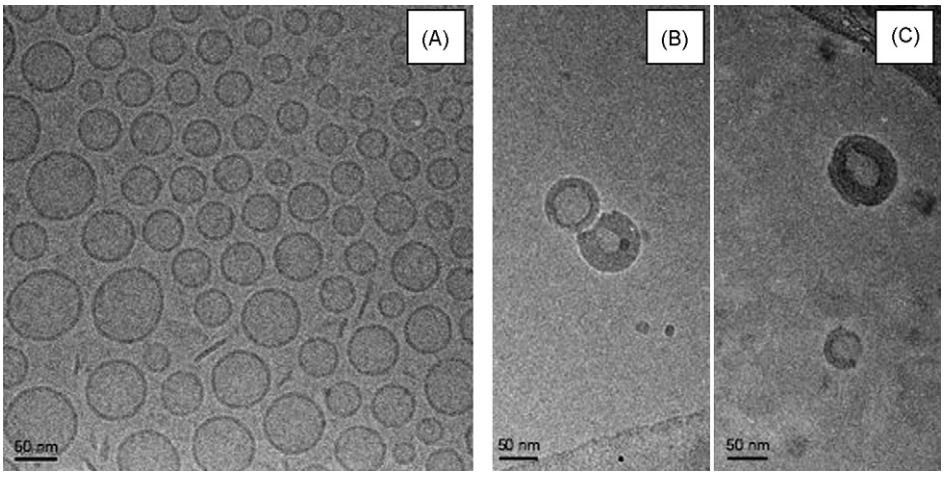

Fig. 3. Cryo electron micrographs of frozen hydrated reference liposomes (Lp) (A) and layersomes (Lp-PLL) (B and C). Images were acquired on a FEI Tecnai 20 transmission electron microscope equipped with a field emission gun and operating at 200 kV, at a magnification of 45,000×, on a Pelletier cooled CCD camera.

those formed with pGA but their fluorescence, as determined by flux cytometry (FACStar and cell sorter (BD Biosciences, CA)), was increased. These results demonstrate that the fluorescent polypeptide associates with the pLL-coated liposomes.

Cryo electron microscopy of frozen hydrated of layersome suspensions at a concentration of 1 mg/mL was used to characterize the morphology of the final assemblies obtained without (Fig. 3A) or with pLL (Fig. 3B). The starting liposomes (Lp) showed a typical spherical structure with distinct bilayered phospholipid membrane surrounding an aqueous core. With pLL-coated liposomes, similar structures were observed with thicker membranes and larger average diameters (Fig. 3B). Furthermore, some structures appeared partially coated by multilamellar membrane (Fig. 3C). Similar objects were revealed by cryo electron microscopy of cationic lipid vesicles coated with DNA (Huebner et al., 1999). These structures were attributed to polyanion-induced membrane fusion resulting in alternated deposits of broken liposome membranes upon addition of DNA.

The membrane permeability was determined after encapsulation of a small hydrophilic dye (5,6CF) and measure of its release from liposomes. The dry phospholipid was hydrated in Hepes buffer in the presence of 40 mM 5,6CF (concentration of 5,6CF for which fluorescence is quenched). After sonication, the non-entrapped dye was separated from the liposomes by gel filtration on a Sephadex G75 exclusion column and the amount of CF co-migrating with the liposomes was quantified by visible spectroscopy ($\lambda = 490$ nm, $\varepsilon = 85{,}000$). The three liposome formulations described above (Lp/CF, Lp/CF-pLL, Lp/CF-pLL-pGA) had similar average diameters and zeta potentials as non-fluorescent liposomes (Fig. 2B) and were able to encapsulate the same quantities of dye (between 32.4 and 38.7×10^{-6} μmol/L in the final suspension).

Permeability experiments were performed by measuring the fluorescence of the 5,6CF released from the liposomes (700 μL) upon addition of 200 μL of either a 10% aqueous solution of ethylic alcohol, pure ethylic alcohol or a 0.1% aqueous solution of Triton X100. The 5,6CF release was expressed with the ratio $R = (F_{max} - F_0)/F_{max}$ in which F_{max} represents the maximal fluorescence value after the addition of alcohol or Triton X100 solutions and F_0 was the initial fluorescence of the liposome suspensions.

The membrane of the reference liposomes was easily destabilized by ethanol 10%, ethanol or Triton (R values of 0.34, 0.69 and 0.75, respectively). However, with a single pLL layer, only highly concentrated alcohol ($R = 0.45$) and Triton ($R = 0.54$) were able to release the encapsulated dye. Finally, only liposomes coated with both pLL and pGA were stable in all three conditions with R values of 0. These data are in agreement with the one obtained by Ge and collaborators who stabilized dimyristoylphosphatidic acid vesicles by two layers of synthetic polyelectrolyte (poly(sodium styrene sulfonate)) and poly(allylamine hydrochloride) (Ge et al., 2003).

The last part of our work consisted in encapsulation of piroxicam inside the lipid bilayer of layersomes. Piroxicam is a non-steroidal anti-inflammatory drug, negatively charged at physiological pH, therefore presenting a weak water solubility (Banerjee and Sarkar, 2002). We consequently incorporated a cationic lipid (stearylamine) into the liposome membrane in order to facilitate its incorporation. Piroxicam was diluted in chloroform (10 mg/mL) and added to a chloroform solution of PC, stearylamine and cholesterol (PC/SA/Chol, 60/30/10). In contrast to previous formulations, the zeta potential of these liposomes was positive (+51 mV) due to the positive charge of stearylamine. Consequently the first polyelectrolyte used for coating was pGA. All the formulated liposomes (Lp/PX, Lp-PX-pGA and Lp-PX-pGA-pLL) encapsulated piroxicam with an identical final concentration (42 μg of PX/mL) measured following Nagaralli et al. (2002). Interestingly, the polypeptide shell did not compromise the encapsulation effectiveness.

In conclusion, the layer-by-layer strategy was successfully applied to sequentially coat charged liposomes. This surface modification stabilized the structure of the liposomes and led to stable drug delivery systems: the layersomes. Our approach offers proof-of-concept of a general strategy that could be applied with other kind of polymers on liposomes. Oral adminis-

tration or their incorporation in biomaterials are among potential fields of application.

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