Stabilization of drug-loaded liposomes by polypeptide surface coating. Particle aggregation control and release study

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1. Introduction

Liposomes have been used for medical applications as drug delivery systems [1]. Biodegradability and low permeability to small molecules make liposomes excellent reservoirs for drug loading/release. However, liposomes are generally rather unstable and can release an active entrapped compound into the biological fluids until the expected target has been reached. In addition, liposomes are cleared from the blood stream by the reticuloendothelial system, a process which dramatically reduces the half life time of the circulating vesicles [2]. Hence, more and more engineering of liposome surfaces is needed to optimize target delivery of therapeutics. Two main directions towards improved engineering include an improvement/control of the vesicle stability and functionalisation of the liposome surface to allow specific interaction with receptors at the target cells [3]. The ability to modulate the properties of vesicles by means of entrapped polymers is a way to control their stability [4].

Recently, we have shown that adsorption of polylysine (PLL) can stabilize unilamellar liposomes against disruption [5]. However, polypeptides and polyelectrolytes have many different effects on lipid bilayers, including an enhanced membrane permeability, membrane fusion, phase transformations, and total membrane disruption [6-9]. Establishment of a reproducible preparation procedure of polycation-stabilized vesicles with an improved stability and integrity are important to be studied for a further development of polyion-stabilized liposomes.

The main aim of the present research is to develop stabilization of liposomes by PLL surface coating with the following requirements: the coated vesicles should be stable (non-aggregated) and must retain encapsulated material for a long time. Formation of liposome-PLL complexes and study of dye release are described. Vesicles composed of synthetic lipids and natural ones were employed. We perform a multiparameter study on the influence of the lipid composition, the temperature, and the molecular weight of PLL to the release kinetics of an encapsulated dye.

2. Materials and Methods

The used lipids 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC), 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) (DPPG) were purchased from Avanti Polar Lipids (USA), whereas Cholesterol (CL), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl-DL-glycerol (POPG) were purchased from Sigma (USA). Tris(hydroxymethyl)aminomethane (TRIS, Sigma), PLL of 2, 28, and 280 kDa (Sigma), Triton X-100 (Aldrich), 5(6)-carboxyfluorescein (Fluka) were used without further purification. Throughout the study, 10 mM TRIS-buffer containing 15 mM NaCl, pH 7.4 was used and will be mentioned in the text as TRIS.

2.1. Vesicle Preparation

Unilamellar liposomes, namely DPPC/DPPG- and POPC/POPG-liposomes, loaded with CF were prepared by mechanical extrusion. The liposomes were composed of DPPC, DPPG, CL and POPC, POPG, CL respectively (components ratio 80/10/10, w/w). Unentrapped CF was removed

by gel-permeation chromatography with Sephadex G-50. Lipid concentration was determined using the Böttcher method [10].

2.3. Interaction of PLL with CF-loaded vesicles

To prepare PLL_liposome complexes, the liposome solution (0.35 mg/ml lipid concentration) was heated to 25 or 54 ^{o}C and dropped at a constant rate during one minute into the agitated PLL solution (950 rpm, maintained at the same temperature). The following methods were used for native and PLL-complexed liposomes. Dynamic light scattering measurements were performed using an HPPS 500 apparatus (Malvern Instruments, UK). Electrophoretic mobility was measured using a Malvern Zetasizer 4 (Malvern, UK). DSC measurements were performed using the Micro-Calorimetry System (MCS DSC, MicroCal Inc., Northampton, MA, USA) at a temperature ranging from 15 to 60 ^{o}C with a scan rate of 0.5°C/min.

2.4. CF release study

The CF content in the liposomes was determined after micellisation of the CF-loaded vesicles by treatment with Triton X-100. The CF released from liposomes was determined by analysis of the solution obtained after filtration of liposome suspension through ultrafree microcentrifuge filters (Sigma, USA, molecular cut-off 100 kDa). CF release was studied from native vesicles, the liposomes covered with 280 kDa PLL, and aggregates of the liposomes prepared by mixing of the vesicles with 2 kDa PLL. The molar ratio PLL/DPPG was equal to 5 in the both latest cases. The samples were stored at 25 ^{o}C or 54 ^{o}C (below and above the phase transition temperature). One hour, 1, 3, and 7 days after storage the filtrated supernatants were analysed.

3. Results and Discussion

It is found that upon complexation, the surface of DPPC/DPPG-liposomes can be covered well with PLL molecules or liposomes can aggregate as shown in Figure 1***A***. Typical complexation profiles (average diameter of complexes formed at 25 ^{o}C versus the charge molar ratio PLL/DPPG) for PLL of 2, 28, and 280 kDa are presented in Figure 2***A***. With increase of PLL concentration, the average size of the particles in the system, starting from a value close to the native vesicle diameter (129 nm), increased to values exceeding 1 μm due to formation of aggregates, and then decreases again towards the single particle diameter of 130-140 nm (well-covered PLL-stabilized liposomes).

The reason for liposome aggregation is a non-homogeneous overcompensation of surface charge of the liposomes by the PLL molecules. Detailed study on the mechanism of aggregate formation and structure is presented in our separate paper [11]. The electrophoretic mobility for PLL-vesicle complexes made with 280 kDa PLL demonstrates charge inversion when the negative value of the ξ-potential is changed to positive (Figure 2***B***) that stabilizes the liposomes against aggregation. The form and width of the complexation profiles are not changed substantially when the complexation is done for lipids in the fluid state [11]. At the same time molar ratio PLL/DPPG at the maxima of the complexation profiles is increased from about 0.7 to about 1 upon complexation in the solid and fluid states, respectively, because of lipid migration to outer leaflet in the fluid state. With decrease of PLL molecular weight the area of aggregation becomes wider. Positively charged PLL (pK_a of PLL is close to 10) adsorbs onto the solid surface as rigid and extended molecules due to electrostatic repulsion between charged side chains [12]. With increase of molecular weight the number of tails and loops of the PLL molecules exposed to solution is increased. This imparts larger overcharging to the vesicles and, as a result, better stabilization of PLL-coated vesicles against aggregation due to lower potential of short-range attraction forces. Thus, ξ-potential of complexes formed in excess of PLL at 25 ^{o}C was found to be 36, 38, and 8 mV for PLL of molecular weight 280, 28, and 2 kDa, respectively. The hydrodynamic thicknesses of 280 kDa and 28 kDa PLL layers covering the vesicle surface were found to be 11 and 7 nm, respectively.

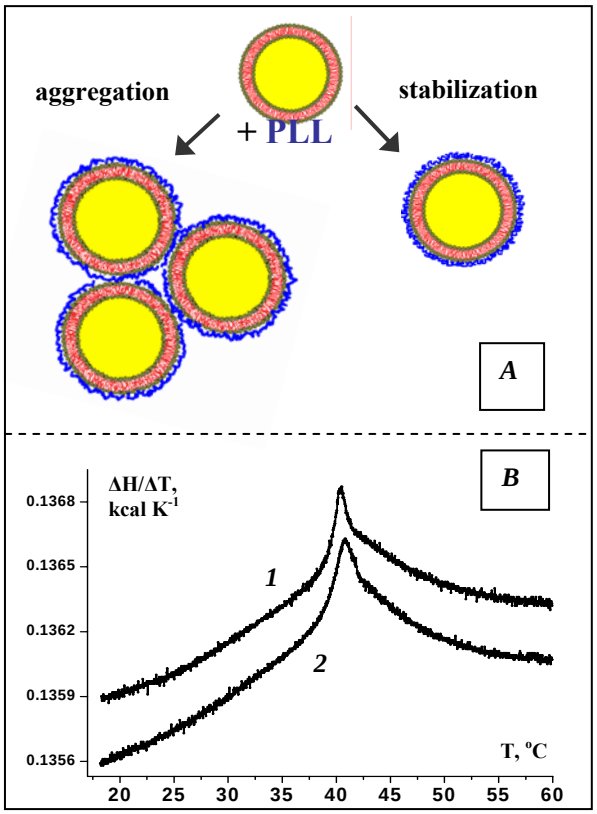

Figure 1. ***A*** **- Scheme of PLL-liposome complexation.** ***B*** **- DSC heating curves of uncoated (*1*) and PLL-coated liposomes (*2*).**

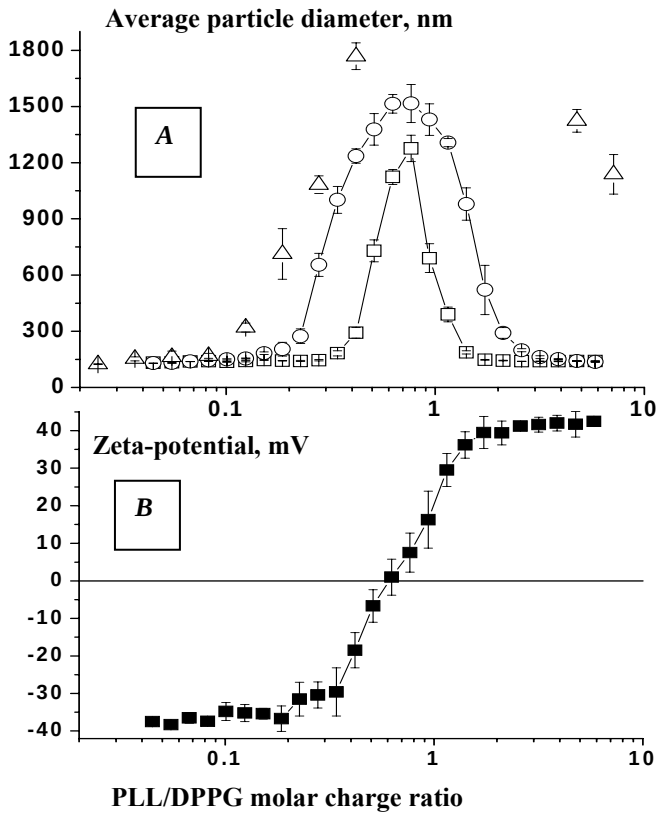

Figure 2. ***A*** **- average diameter of liposome-PLL complexes formed using PLL of 280 (□), 28 (O), and 2 (Δ) kDa as a function of molar charge ratio PLL/DPPG.** ***B*** **- zeta-potential of the complexes prepared with PLL of 280 kDa.**

PLL covering has no effect on DCS curves indicating that a layer of PLL is located exclusively on the vesicle surface and is not incorporated into the membrane but the PLL-lipid interaction suffices to stabilize the vesicles against aggregation (Figure 1, *B*).

The membrane integrity of native liposomes and liposomes coated with PLL was investigated by studying the release of CF out of the vesicle interior. The permeability of the liposome membrane depends strongly on the phase state of the liposomes. Vesicles from synthetic lipids in the solid state did not release CF after up to 7 days of storage, but in the fluid state more than half of the entrapped dye was released already after 1 day of storage (Figure 3***A***; *1* and *6*, respectively). PLL coverage/aggregation does not cause permeabilization of liposomes in the solid state (Figure 3***A***; *2*, *3*), but increases the permeability of "fluid" vesicles made from either native (Figure 3***B***; *2*, 3) or synthetic lipids (Figure 3***A***; 7). Crossing the transition temperature results in intensive CF leakage because of the appearance of leaky interfacial domains between the coexisting solid and liquid phases (Figure 3A; *4*, *5*). Detailed study on vesicle behaviour and PLL interaction is presented in our separate work [13].

Thus, a preparation procedure of stable PLL-covered negatively charged liposomes is established and it is suggested that a careful manipulation of the PLL molecular mass, PLL/lipid ratio, T, etc. allows one to tailor properties of the resulting complexes to be used for particular applications. The effect of PLL coverage on the liposome membrane permeability is studied.

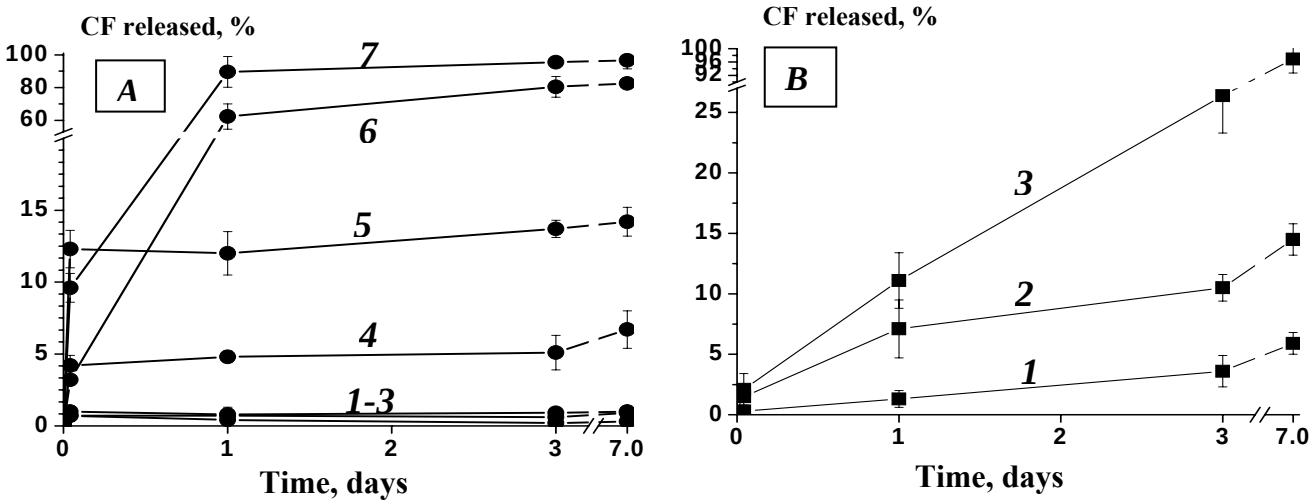

Figure 3. *A* - Release profiles for CF-loaded DPPC/DPPG liposomes at 25°C: native (*1*), single PLL-covered (*2*), and aggregates (*3*). Release profiles at 54°C: native (*6*), single PLL-covered (*7*). To obtain the profiles upon crossing the transition temperature, the native (*4*) and PLL-covered liposomes (*5*) were heated to 54°C for 10 min and cooled down to 25°C.. *B* - Release profiles for CF-loaded POPC/POPG-liposomes: native (*1*), single PLL-covered (*2*), and aggregates (*3*).

4. Conclusion

This work describes complexation between negatively charged liposomes and the cationic polypeptide PLL and describes an effect of PLL on liposome permeability. The structure of the resulting complexes can be changed from isolated polyion-coated (stabilized) liposomes to vesicle aggregates. Fabrication of stable single PLL-covered liposomes depends on a variety of parameters: PLL/liposome ratio and PLL molecular mass. Order and speed of the initial solution intermixing are also important factors revealed in our separate research [11]. Permeabilization of lipid membranes is dramatically increased upon crossing the main transition temperature. PLL coating does not induce any changes by adsorption on the surface of "solid" liposomes, however it has a strong effect on the membrane integrity of "fluid" liposomes due to the lipid mobility. DCS analysis has revealed that a layer of PLL is located exclusively on the vesicle surface. The polycation-stabilized vesicles represent a new class of carriers that could be used for drug delivery and controlled release.

5. References

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