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Protocols

Influence of the state of phase of lipid bilayer on the exposure of glucose residues on the surface of liposomes

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ABSTRACT

The presence of carbohydrate-binding proteins (*i.e.* lectins) on the surface of various bacterial strains and their overexpression in some tumor tissues makes the use of glycosylated liposomes a promising approach for the specific drug delivery in antibacterial and anti-cancer therapies. However, the functionalization of liposome surface with sugar moieties by glycosylated amphiphiles does not ensure the binding of sugar-coated vesicles with lectins. In fact, the composition and properties of lipid bilayer play a pivotal role in the exposure of sugar residues and in the interaction with lectins. The influence of the length of the hydrophilic spacer that links the sugar to liposome surface and of the presence of saturated or unsaturated phospholipids in the lipid bilayer on the ability of glucosylated liposomes to interact with a model lectin, Concanavalin A, was investigated. Our results demonstrate that both the chain length and the prensece of unsaturation, parameters that strongly affect the fluidity of the lipid bilayer, affect agglutination. In particular, agglutination is favored when liposomes are in the gel phase within a defined range of temperature. Moreover, the obtained results confirm that the length of the PEG spacer, that influences both lipid organization and the exposure of sugar moieties to the bulk, plays a crucial role in liposome/lectin interaction

1. INTRODUCTION

Liposomal drug delivery systems are largely investigated because they are biocompatible, can entrap both hydrophilic and hydrophobic drugs, promote their internalization into cells and improve their pharmacokinetic and pharmacodynamic profiles [1]. In the administration of a drug there is generally a compromise between therapeutic efficacy and side effects. The decoration of liposome surface by specific ligands allows selective targeting to certain cells or organelles, thus reducing the distribution of the active principle to non-targeted tissues and its side effects. Decorating liposome surface with sugar moieties is one of the approaches used to efficiently target the lipid carriers to bacterial and cancer cells whose surfaces are rich of lectins [2,3], a class of proteins devoid of enzymatic activity that specifically bind to carbohydrates [4]. The presence of sugar residues on liposome surface should thus increase their specificity and therapeutic efficacy as drug delivery systems in antibacterial and anti-cancer therapy.

The occurrence of interaction between sugar-coated liposomes and lectins has been largely investigated *in vitro* through the agglutination assay, a process that involves the aggregation of glycosylated vesicles upon their specific binding with the protein. In fact, since lectins contain typically more than one sugar-binding site, their interaction with glycosylated liposomes results in an easily detectable agglomeration of lipid nanoparticles and protein in solution. It is well known that the extent of agglutination depends on sugar surface density [5,6] and on the length of the spacer [7–9] connecting the sugar residue with the hydrophobic part of the glycolipid, however, the mere presence of sugar moieties on vesicle surface might not be sufficient to guarantee the interaction with lectins. In fact, even subtle modifications of lipid components can dramatically affect physicochemical properties of liposomes [10,11] and their ability to interact with the biological *milieu* [12–14]. In particular, phospholipid components play a key

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role in determining the overall characteristics [15] and functionality of lipid membranes [16–19]. The effect of lipid chain length on the behaviour of liposomes incorporating a glycophorin in lectin-mediated agglutination indicated the control of lipid-phase on sugar-lectin interaction and highlighted the importance of saturated phospholipid components. However other parameters, such as the conformation of the protein, might be affected by lipid phase and temperature in such a complex system [19].

Here we report an investigation aimed at clarifying the influence of the structure of liposome components on the capability of surface decorating glucose moieties to interact with Concanavalin A (Con A), a soybean lectin that contains four binding sites for glucose. Liposomes were formulated with one of two glucosylated amphiphiles, **1** and **2**, differing for the length of the polyoxyethylene spacer that links the glucose moiety to the quaternary nitrogen, and a saturated (dimyristoyl-*sn*-glycero-phosphocholine, DMPC, or dipalmitoyl-*sn*-glycero-phosphocholine, DPPC) or unsaturated (palmitoyloleoyl-*sn*-glycero-phosphocholine, POPC, or dioleoyl-*sn*-glycero-phosphocholine, DOPC) phosphatidylcholine (PC, Chart 1).

This study aims at correlating the physicochemical properties of the formulations to their composition to find out the parameters and conditions that might guarantee a high extent of agglutination. At this aim, experiments of liposome agglutination mediated by Con A, monitored by optical density (OD) measurements, were carried out for all the investigated formulations. The same lipid formulations were also investigated as monolayers at the air/water interface by Langmuir compression isotherms, to better understand the physicochemical parameters responsible for the different extent of agglutination observed for the various liposomes.

2. EXPERIMENTAL SECTION

2.1. Materials

Glucosylated amphiphiles **1** and **2** were prepared as previously described [20,21]. DOPC, DMPC, DPPC and POPC were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Con A from *Canavalina ensiformis* (Jack bean), phosphate buffer solution (PBS, 0.01 M phosphate buffer 0.0027 M KCl, 0.137 M NaCl, pH 7.4) and all reagents employed for the synthesis of **1** and **2** were purchased from Sigma-Aldrich.

2.2. Methods

2.2.1. Liposome preparation

Lipid films were prepared on the inside wall of a round-bottom flask by evaporation of solutions containing the proper amount of PC (dissolved in $CHCl_3$) and **1**(**2**) (dissolved in MeOH) at 95:5 molar ratio. The obtained films were stored overnight under reduced pressure (0.4 mbar), then PBS was added to obtain a 1 mM lipid dispersion for DLS and agglutination experiments. The solutions were heated at 50 °C and vortex-mixed, then the aqueous suspensions were freeze-thawed six times from liquid nitrogen to 50 °C and finally extruded 10 times through a 100 nm polycarbonate membrane (Whatman Nucleopore). The extrusions were carried out above the main transition temperature (Tm) of liposomes using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

2.2.2. DLS experiments

DLS measurements were performed on freshly prepared liposomes and a week after their preparation using a Malvern NanoZetaSizer, equipped with a 5 mW HeNe laser (wavelength = 632.8 nm) and a digital logarithmic correlator. The normalized intensity autocorrelation functions were measured at an angle of 173° at 25.0 ± 0.1 °C. The autocorrelation functions were analyzed by using the cumulant fit. The first cumulant was used to obtain the apparent diffusion coefficient D of the particles, further converted into apparent hydrodynamic diameters, D_h, by using the Stokes-Einstein relationship $D_h = k_BT/3\pi\eta D$, where k_BT is the thermal energy and η the solvent viscosity.

2.2.3. Agglutination experiments

The agglutination of PC/**1**(**2**) (95:5) liposomes in the presence of Con A was determined by the time-dependent changes of specific turbidity of a 3 mL sample of 0.83 mM liposomes in PBS in a 1 cm quartz cell upon addition of Con A (0.33 mg/mL final concentration) in PBS. Scans were carried out at 25 °C on a Cary 300 UV−vis double beam spectrophotometer (Varian Australia PTY Ltd., Mulgrave, Vic., Australia), at 525 nm, immediately after mixing and every minute for 600 min. In the case of DMPC/**1**(**2**) liposomes agglutination experiments were also carried out at 15, 20, 25, 30, 45 °C whereas in the case of DPPC/**1**(**2**) liposomes at 25, 35, 40, 45 °C. Agglutination experiments were replicated on three different samples.

2.2.4. Langmuir trough measurements

The Langmuir trough technique was used to study PC/**1**(**2**) monolayers at 95:5 molar ratio at the air/water interface. PBS was used as subphase. The minitrough was thermostated by a 25 °C bath. A volume of 25 μL of lipid solution (0.8 mg/1 mL dissolved in $CHCl_3$) was spread over the aqueous subphase using a Hamilton microsyringe. After the deposition, the solvent was allowed to evaporate for 5 minutes at an initial pressure lower than 1 mN/m before starting three cycles of compression/expansion up to surface pressure (π) = 4mN/m to stabilize the monolayer. As precaution to prevent the oxidation of unsaturated PC and to avoid changes in surface pressure due to their exposure to air, the acquisition of each isotherm was completed in ~10 minutes and all the samples were exposed to air for the same length of time. The symmetric compression/expansion cycles were performed by moving the nylon barriers at a constant rate of 25 mm/min, then the isotherms were registered closing completely the barriers (target to stop at π = 100 N/m). The reported isotherms are the average of at least three different and independent runs. The experimental error for the final concentration of each sample was estimated by means of the error propagation, being in the order of ±2.5% for the reported values. For calculation, we considered the error of the analytical balance to be $\pm 0.5 \times 10^{-4}$ g, and the error of Hamilton syringe of ±1.25% of nominal volume.

3. RESULTS AND DISCUSSION

3.1. Agglutination experiments

The increase of the dimension of particles in solution due to agglutination in the presence of Con A was investigated by OD measurement on liposomes featuring ~ 100 nm D_h. In fact, the size and the stability of all investigated formulations were evaluated by DLS measurements that confirmed that they were all monodispersed and stable for at least one week, and featuring the size imposed by the extrusion procedure (*i.e.* $D_h \approx 100$ nm). Agglutination experiments were carried out only on liposomes containing 5 mol% of glucosylated amphiphile because at 10 mol% of glycosylated lipid in DMPC [20,21] or DPPC liposomes a multimodal distribution was observed.

The sample relative to liposomes containing DOPC and POPC, *i.e.* an unsaturated PC, and either **1** or **2** did not show agglutination at 25 °C (well above the Tm, ≈−18 °C for DOPC containing liposomes and ≈ −2 °C for POPC containing liposomes) with the exception of a slow agglutination in the case of DOPC/**2** liposomes (Fig. 1). In this