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Control of the interaction between membranes or vesicles: Adhesion, fusion and release of dyes

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Abstract

This paper describes the different molecular recognition systems used for inducing adhesion, fusion of vesicles as well as their physical properties. A third part concerns the control of the release of vesicles at the solid interface thanks to an optimized polyelectrolyte coating. Various chemical strategies based on the incorporation of recognition lipids are presented and compared to induce and control the interaction between membranes. The kinetics effects in the adhesion and fusion processes are demonstrated by complementary techniques such as the micropipettes, fluorimetry and reflexion interference contrast microscopy.

Keywords: Fusion; Adhesion; Vesicle release; Polyelectrolyte coating; Lipid membrane; Molecular recognition

1. Introduction

The interactions between cellular membranes are very well-controlled in many biological phenomena such as the entry of a virus into a cell, cell signaling, vesicle trafficking, cell organization into tissues. Adhesion, fusion and vesicle release appear to be highly regulated in all of these processes [1]. The complex structure of biological membranes gives a very high mechanical stability and deformability necessary to induce adhesion, fusion and release of water-soluble compounds [2]. This structure is typically represented by a lipid bilayer membrane containing proteins, an extracellular matrix and a cytoskeleton connected to the lipid membranes through protein-, peptide- or sugar-specific bonding.

Facing the complexity of biological membranes, we aimed at designing synthetic membrane systems, which allow for inducing phenomena like adhesion, fusion and controlled release of water-soluble compounds. From this point of view, the so-called liposomes or vesicles consisting of a closed lipid bilayer appear to be a very attractive system. In particular, they can adhere and fuse, and can be used for transport and delivery of their enclosed aqueous solution. The use of liposomes [3] offered numerous application possibilities in the medical field since such closed containers are highly impermeable to small hydrophilic molecules, a category to which most drugs belong to [4]. Liposomes are used for the delivery of a high amount of toxic drugs to target cells avoiding secondary effects on the healthy cells [5]. This is done by modifying the membrane to carry specific interaction site with surface receptors for the target cells. By taking advantage of their physicochemical properties as well as their chemical composition and mechanical properties, vesicles can be easily modulated to optimize the system for one given

Abbreviations: TAP, triaminopyrimidine lipid; BAR, barbituric acid lipid (see ref. [20]); PLL, poly-L-lysine; CL, cholesterol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol); EPC, egg phosphatidyl choline; POPC, (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine); POPG, (1-palmitoyl-2-oleoyl-*sn*-3-phosphatidyl-DL-glycerol)

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function such as adhesion, fusion or release [6]. In addition, supported membranes have been also successfully used to study many biological processes such as adhesion, lipid reorganization or ion transport. Supported membranes allow investigating these processes by using all the techniques relative to the surfaces [7].

The question addressed in this paper is how to control the interaction between soft surfaces such as vesicles and supported films. We first describe various molecular recognition systems, which induce a selective adhesion or fusion of vesicles. Then we introduce the physical techniques allowing analysis of the dynamics of these events in particular the micropipette technique and optical microscopy. The initial step to be completed from a chemical point of view is to synthesize fusogenic molecules which mimic the role of their biological counterparts involved in cell fusion, i.e. the so-called fusion proteins, e.g. SNAREs [8]. On the other hand, applying physical approaches and analysis to the fusion event brings understanding of the fundamental aspects of the process, and points to governing factors and driving forces involved. Besides the fundamental interest to understand the interaction of membranes brought into close contact, the control on fusion is relevant for fields like gene transfer, drug delivery and bioengineering. Membrane fusion is a vital process since it is involved in many cellular functions and stages of cell life. In addition, functional vesicular systems can be used as a building block for the preparation of self-organized assemblies and nanomaterials. Finally, the incorporation of vesicles into polyelectrolyte supported films is investigated to control the release of the adhered vesicles. The polyelectrolytes have been successfully employed to prepare, for example, bioactive films by successive deposition of oppositely charged polyelectrolytes [9] and incorporation of proteins [10] or DNA [11].

1.1. Synthetic adhesive and fusogenic vesicular systems

In a first approach, the aim was to mimic the cell adhesion process by using the well-known molecular recognition system based on the specific interaction between the integrin proteins and Arg–Gly–Asp (RGD) tripeptide [12,13]. In vitro, visualization of membrane-related processes is possible using giant vesicles [14,15], a handy model membrane system of cell-size dimensions. They are well visible under an optical microscope and thus allow direct manipulation and observation of membrane interactions. In addition, the giant vesicles that have a size in the micrometer range, i.e. cell-size, reflect the membrane properties and behavior as they are in cells.

To test the adhesion of giant vesicles with endothelial cells bearing $\alpha_V\beta_3$ integrin membranar proteins, a synthetic lipopeptide bearing a cyclic RGD pentapeptide was prepared to functionalize giant unilamellar vesicles as well as the corresponding fluorescent chalcon lipopeptide. This peptidic sequence exhibits a well-defined molecular geometry which greatly enhances the affinity for $\alpha_v\beta_3$ integrin [16]. It was found that RGD giant vesicles can adhere selectively onto a surface coated with integrin proteins or endothelial cells (see Fig. 1). When the reflection interference contrast microscopy technique (RICM) is applied to a chamber under flow, the adhesion zone can be directly visualized and the strength of the adhesion process can be estimated. Such vesicles adhere through

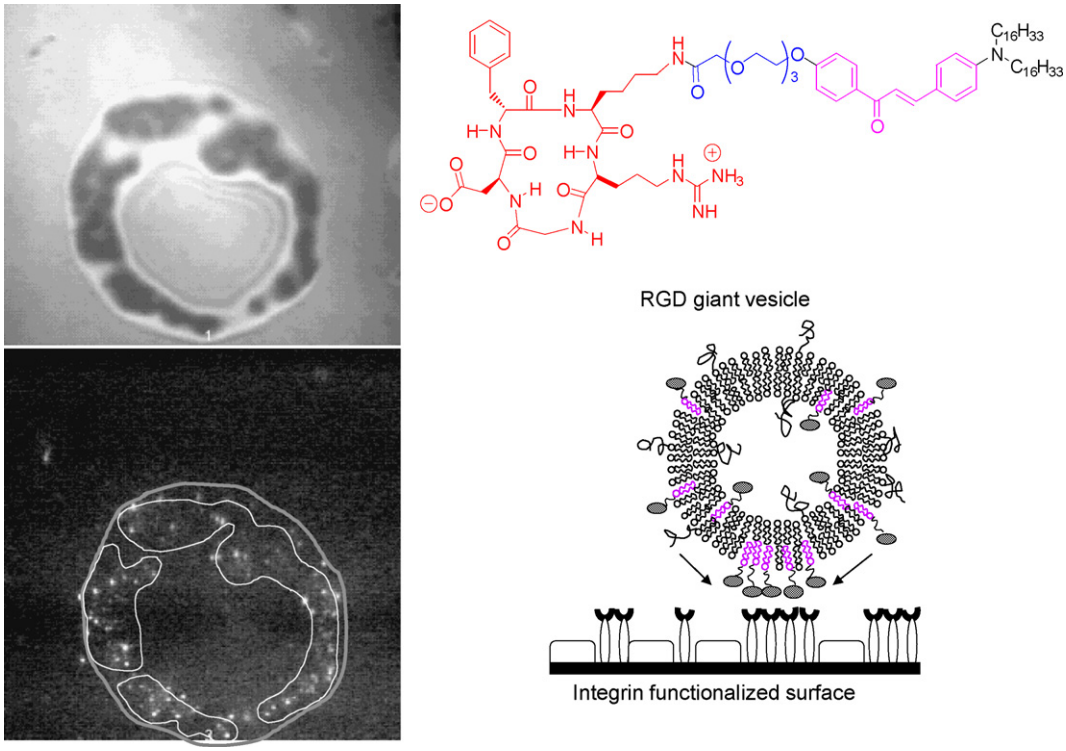

Fig. 1. Left panel: RICM (top) and fluorescent (bottom) images of an adhering RGD vesicle onto an α5β3 integrin functionalized surface showing the concentration of the fluorescent RGD ligand into the adhesion plaque. Right panel: Chemical structure of the RGD fluorescent lipopeptide (top), and a schematic illustration of the vesicle-surface interaction (bottom) (reproduced from ref. [14]).

the formation of some points of strong adhesion that appear as black zones by RICM technique [17]. Fluorescence and RICM microscopies permitted to directly visualize that the RGD fluorescent lipopeptides are concentrated in the zone of strong adhesion [18]. Therefore, these results suggested that there is a cooperative interaction between RGD ligands and the integrin proteins RGD/integrin. Nevertheless, this study illustrates also the limitation of the fluorescent labeling which does not permit to follow the RGD ligands in time because of the quenching of the fluorophore.

In a second approach, we developed the synthesis of various lipids bearing molecular recognition groups at the polar head-group. The molecular recognition between these molecular groups induces a selective interaction between two vesicle populations resulting in various processes including aggregation, lipid exchange or adhesion and fusion. Various types of interactions were tested as a driving force to bring into contact two membranes. First of all, long range electrostatic interaction [19] or electrostatically reinforced hydrogen bonding were tested [20]. Both systems induce selective contact between the two complementary vesicle populations but the adhesion was limited by the lipid exchange resulting in the neutralization of the attractive interaction and then the redispersion of the two interacting vesicles [21]. In the case of electrostatically reinforced hydrogen bonding system, the adhesion was stabilized by hydrogen bonding but still only marginally resulted the fusion of the two vesicles (see Fig. 2).

Facing this limitation due to the lipid exchange, systems based on the complexation of two or more amphiphilic ligands with divalent or trivalent ions were tested to induce fusion. We have achieved synthesizing a number of fusogenic ligands, which in presence of metal ions form coordination complexes of 2:1 ligand-to-metal ratios. These ligands L have been designed so that they contain a hydrophobic part, which preferentially partitions in lipid membranes. After addition of ions into the functionalized L vesicle suspension, two different effects are observed depending on the type of the complexation: transport of ions into the vesicle aqueous compartment (*intravesicular* complexation), or fusion of the two interacting vesicles (*intervesicular* complexation). We observed that a competition takes

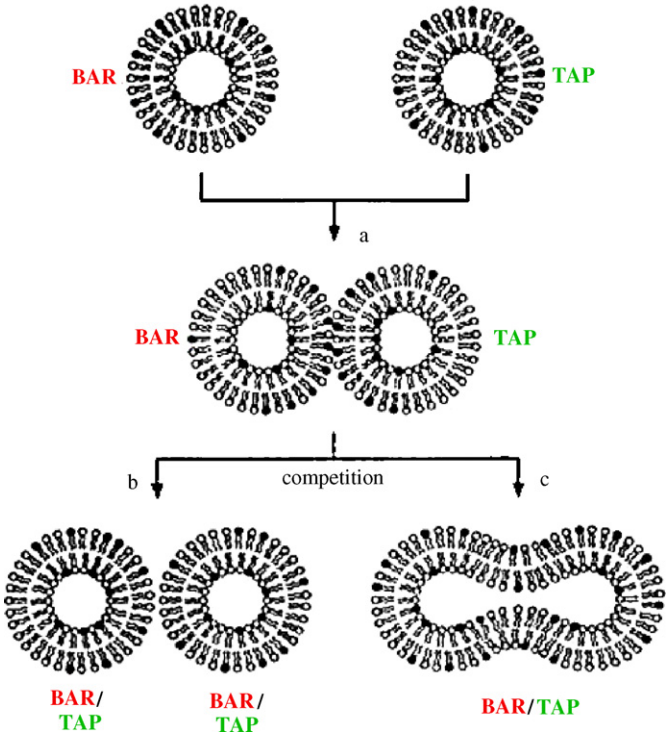

Fig. 2. Scheme of the interaction between complementary BAR and TAP vesicles: contact, adhesion, lipid exchange, redispersion and fusion. Barbituric (BAR) and triaminopyrimidine (TAP) lipids are synthetic amphiphiles incorporated into the vesicle membranes and interacting through several hydrogen bonds and electrostatically (ref. [21]).

place between the two types of complexes in the case of large unilamellar vesicles (see Fig. 3).

The formation of the complexes can be revealed by fluorescence in the case of the europium/diketonate ligand system or by UV spectroscopy in the case of nickel/bipyridine ligand. In the case of diketonate ligands, the formation of the complex resulted in the transport of lanthanide ions across the vesicle membrane. The study carried out on the small vesicles shows also that the binding constant of the complexation is reinforced at the interface of the vesicles [22].

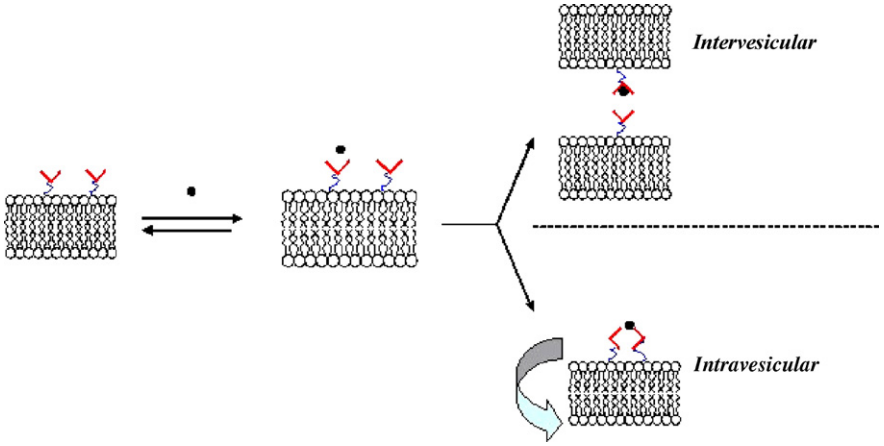

Fig. 3. Competition between intra and intervesicular complexations of vesicle membranes functionalized with amphiphilic ligands (see also ref. [22]).

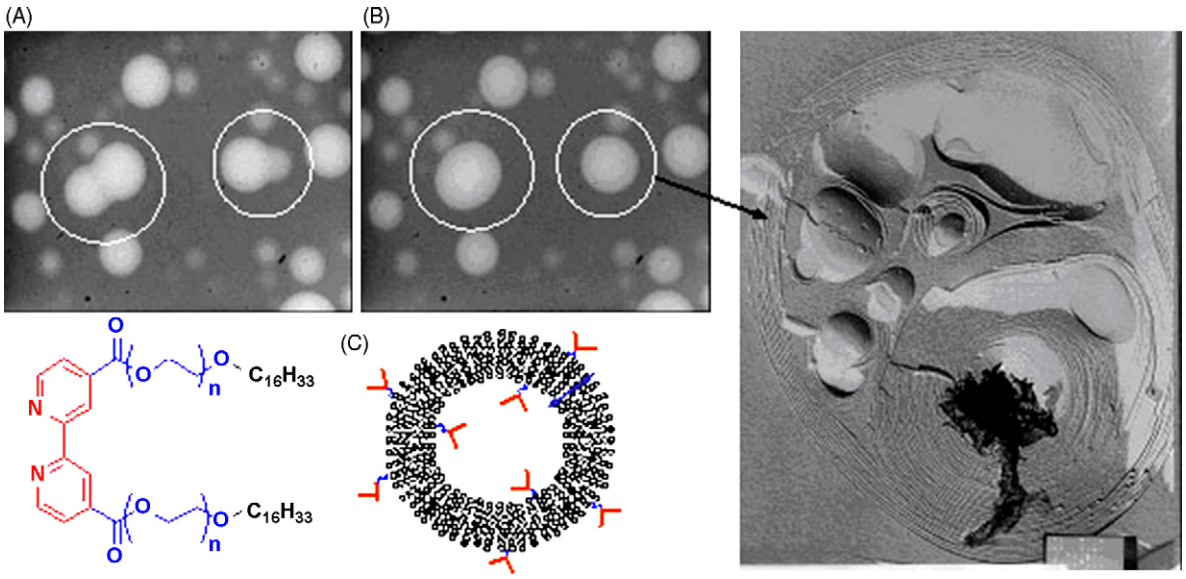

Fig. 4. (A and B) Images of fluorescence optical microscopy showing the fusion in time between giant vesicles resulting from multiple fusion induced by addition of nickel ions on a solution of large unilamellar vesicles (LUVs) of 100 nm diameter initially. The LUV were labeled by a water-soluble fluorescent Rhodamine dye. (C) Image obtained from electron microscopy showing the multilamellar structure of the giant vesicles resulting from multiple fusion of LUVs. Schematic representation of the fusogenic system (reproduced from ref. [23]).

In the case of bipyridine ligands bearing PEG spacers of different lengths, the addition of nickel ions induced the fusion of large unilamellar vesicles and resulted the formation of giant multilamellar vesicles [23] as shown in Fig. 4. The molecular mechanism of fusion involves the formation of the inter-membrane ligand-ion complex detected by UV spectroscopy, which brings together opposing membranes and induces tension on the bilayer by the formation of a multilamellar structure stabilized by the bipyridine-ion complex.

1.2. Dynamics of the adhesion and fusion processes

To study inter-membrane interactions as a first step one needs to bring two target giant vesicles together. For this purpose, experimentalists have already developed manipulation tools like micropipettes [24,25] and optical tweezers [26,27]. The second step involves local perturbation of the membranes in contact and observation of the vesicle response. This technique was successfully used to investigate the adhesion between a RGD giant vesicle and an endothelial cell [28]. In this study, the micropipette was used for breaking the adhesion with well defined forces. It was shown that the mechanical adhesion strength strongly depends on the duration of force application and reveals pronounced kinetic effects.

Recently, it has been demonstrated that a successful control on fusion can be achieved by micropipette manipulation of functionalized vesicles (see Fig. 5) [29]. As model membranes, giant unilamellar vesicles have been exploited. The vesicles were functionalized with the synthetic fusogenic ligands mentioned above [22]. Local injection of lanthanide ions in the contact zone between two vesicles (see Fig. 5) played the role of membrane perturbation and fusion initiation. The ions were observed to induce tension on the bilayers and trigger fusion. Fusion was observed with temporal resolution of 50 μs. This resolution is unprecedented since the data on direct microscopy observation of fusion reported in the literature covers fusion processes only down to about a few tens of milliseconds. The fusion dynamics as observed with high temporal resolution revealed that fusion

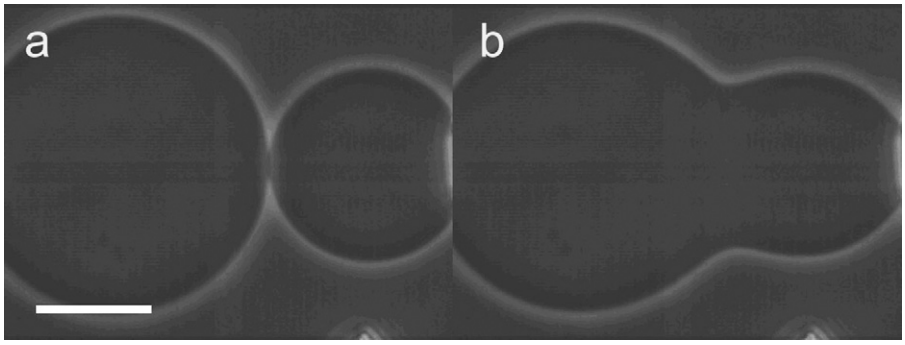

Fig. 5. Fusion of two functionalized giant unilamellar vesicles as observed with phase contrast microscopy: (a) before fusion; and (b) 60 ms after fusion. The scale bar corresponds to 20 μm. The vesicles are held by two micropipettes (the tip of the right one is visible in the first snapshot as a bright line) and a third micropipette (lower right corner) is used for the injection of a solution of lanthanide ions which trigger fusion.

is surprisingly fast. The data suggested that the fusion pore or neck formation is faster than 100 μs [29]. In addition, the time dependence extrapolation of the fusion neck diameter down to nanometer sizes gave strong evidence that fusion is completed already within about 200 ns, which compared to previous estimates, is faster by about three orders of magnitude. The latter is in agreement with simulations on fusion [30].

Similar results have been obtained using a second approach to induce fusion, namely electrofusion of vesicles [29,31]. The effect of electric fields on model membranes and giant vesicles has been extensively studied [32]. The necessary condition for fusion to occur between two vesicles in contact is that the applied electric pulse is strong enough to electroporate the two opposing membranes. Analysis of the dynamics of electrofusion revealed behavior that is very similar to that observed with ligand-mediated fusion [29].

Even though the two methods, ligand-mediated fusion and electrofusion, provide control on vesicle fusion, questions concerning the fusion mechanism and evolution still remain open. For example, it is not known whether hemifusion occurs in the early stages of fusion. Hemifusion is the event whereby the external leaflets of the two membrane bilayers mix prior to fusion of the vesicle enclosed volumes. Answering this question is for the moment hindered by experimental difficulties. In order to detect hemifusion fluorescent labeling methods are exploited. However, the fusion process is very fast (less than 100 μs see ref. [29]) while standard fluorescent dyes have low quantum yield, bleach fast and thus are 'slow' and difficult to use as a marker. A solution to this problem presents the usage of the so-called quantum dots, strongly fluorescent nano-particles which are long lived and easy to detect individually [33,34]. Our idea is to have these particles incorporated in or attached to the membrane of one of the fusing vesicles and follow the mixing of the two membranes after fusion. With this aim, we are currently working on the control of the interaction between QD and vesicle or biological membranes (Fig. 6). Eventually, more light will be thrown on the question whether hemifusion precedes fusion as an initial step. In addition, the dynamics of mixing of the two bilayers will be revealed.

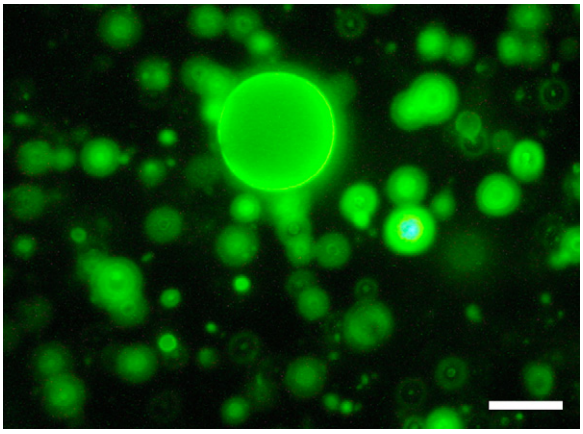

Fig. 6. QD coated with 11-mercapto undecanoic acid interacting with oppositely charged giant vesicle observed by fluorescence optical microscopy (reproduced from ref. [30]).

1.3. Engineering of the aggregation state, stability, and dye release from phospholipid vesicles by monolayer polyelectrolyte coating

The mechanical stability of the vesicles remains a major drawback for their applications as drug delivery vehicles. Depending on the temperature, the nature of the lipids and the substrate, the medium, and the vesicular diameter, most vesicles tend to adsorb on surfaces and undergo a subsequent fusion process with the substrates with a final result being the release of the encapsulated molecules [35–37]. The control of the vesicle fusion can result in the formation of a supported bilayer. Some ways have to be found to stabilize the envelope of the capsules [38]. The general strategies aiming to stabilize the vesicles have been reviewed recently [39]. Four categories of modifications can be performed:

(i) The polymerization of hydrophobic monomers which are solubilized inside the hydrophobic part of the lipidic bilayer yielding a polymer network.
(ii) The use of lipids comprising hydrophilic polymerisable groups, for instance, with an organoalkoxysilane [40].
(iii) The incorporation and subsequent polymerization of a triblock copolymer comprising polymerizable extremities [41].
(iv) The coating with a polyelectrolyte monolayer [42] or multilayer [43].

The deposition of just one polyelectrolyte layer on the surface of vesicles carrying permanent charges such as L-α phosphatidic acid, is efficient to protect them against the micellization effect of sodium dodecyl sulfate [42]. Therefore, we decided to use this strategy in view to deposit such protected and stabilized vesicles inside polyelectrolyte multilayer films [44] and thus to create surface immobilized reservoirs containing hydrophilic drugs. We have already demonstrated that the release kinetics (as measured by cyclic voltamperometry on the surface of a gold working electrode) of the encapsulated ferrocyanide ions into vesicles (made from POPC, POPG and CL) lasted over more than 10 h [45]. However, the deposition of just a monolayer of charged polymers is still a complex phenomenon: it can induce vesicle aggregation, increased membrane permeability, interleaflet exchange of charged lipids from the internal leaflet to the outer leaflet of the lipidic bilayer (the so-called flip-flop process) [46] and even total membrane disruption if the fraction of charged lipids is high [47] or the polyelectrolyte is modified with some aliphatic chains [48]. In addition, the adsorbed polyelectrolyte can be totally displaced when the polyelectrolyte coated is weakly bound to the vesicles and in contact with an oppositely charged polyelectrolyte [49].

Hence, we undertook a study aimed to investigate the best way to deposit poly-L-lysine (PLL), a biocompatible polycation, on the surface of negatively charged vesicles. We investigated both the influence of the bilayer phase state (by changing either the nature of the lipidic mixture at a given temperature or the temperature for a given lipidic composition), the order of mixing of both PLL and vesicles, the hydrodynamic conditions used to

perform the coating process as well as the chain length of the polycations. The experimental details are given in refs. [50,51].

The aggregation profile represents the change in the mean hydrodynamic diameter of the aggregates versus the ratio between the number of provided PLL molecules and the number of charged lipid molecules (either DPPG or POPG) [51]. To obtain monodispersed covered vesicles, it was found that it is more efficient to drop slowly the liposome solution (during 1 min) into the PLL solution than to use the reverse order of intermixing. In addition, the hydrodynamic conditions during the intermixing (controlled by the speed of a magnetic stirrer) allow to control the width of the aggregation zone. Increasing the rotation speed from 300 to 950 rpm allows a significant improvement of the coating process without the occurrence of carboxyfluorescein (CF) release (as checked by fluorescence spectroscopy). This is due to an increased transport rate of PLL to the surface of the liposomes and, hence, probably to a more homogenous coating without causing the formation of heterogeneous charge patches and heteroaggregation.

In the case of vesicles either in fluid (above 41 °C for the DPPC/DPPG/CL mixture) or solid state, the width of the aggregation zone increases dramatically with a decrease in the PLL molecular mass (Fig. 7A). This observation can be rationalized on the basis of zeta potential titration experiments displayed in Fig. 7B. In the region with excess of PLL (for PLL of 28 and 280 kDa the vesicles are monodisperse) the zeta potential values are equal to 36, 38, and 8 mV for PLL with viscosimetric molecular masses of 280, 28, and 2 kDa, respectively. Hence, the vesicle charge overcompensation is responsible for the stabilization mechanism and this phenomenon is more efficient for longer PLL chains, which carry more positive charges (at pH 7.4). The short PLL molecules tend to form a rigid surface layer exposing to solution less loops and tails and, as a result, provide weak charge overcompensation [51].

The maximum of the aggregation profile corresponds to the appearance of thermodynamically stable and micrometer sized aggregates (Fig. 7A), with zeta potentials close to 0 (Fig. 7B). It has to be noted that these aggregates are surprisingly monodisperse. At small PLL/DPPG ratios (but different from 0), the vesicles remain monodisperse. This observation gives some important information about the coating and aggregation processes. During the 1 min over which the PLL-vesicle intermixing takes place, the huge aggregates have no time to build up. At higher PLL/DPPG ratios corresponding to aggregation, the relative amount of huge aggregates increased with the time lag between PLL-vesicle intermixing and the instant of characterization. At the steady state of the aggregation process we observed the accumulation of highly stable aggregates of about 2 μm in diameter.

The differential scanning calorimetry (DSC) scans showed that the PLL adsorption does not significantly affect the organization of the lipids because both the main transition temperature and the shape of titration curves of the DPPC/DPPG/CL vesicles are not affected by PLL coating (data not shown, see ref. [51]).

When now considering the release of the encapsulated CF at 25 °C, it appears that the dye retention is very high (Fig. 8A, traces 1–3) for the vesicles made from synthetic lipids (DPPC/DPPG/CL mixture) both in the native and PLL covered state (for the monodisperse particles as well as for the aggregates). However, the dye is released from the vesicles made from the POPC/POPG/CL mixture of lipids (which are in the fluid state at 25 °C), Fig. 8B. In the case where the DPPC/DPPG/CL vesicles (native or PLL-coated) are heated above the main phase transition temperature for a prolonged duration, all the encapsulated dye is released (Fig. 8A, traces 6 and 7). However, when the main transition temperature is crossed for only 10 min, a burst but a non quantitative dye release is found (Fig. 8A, traces 4 and 5).

In this study, we optimized the experimental conditions for coating negatively charged phospholipid vesicles with a biocompatible polycation (PLL). Long-chain PLL are required to ensure hydrodynamic conditions allowing fast transport of PLL to the vesicle surface and thus obtaining monodisperse and fully PLL-coated vesicles. The PLL coated vesicles in the solid state are highly impermeable to encapsulated CF. The dye can be released as a burst just by crossing transiently the main phase transition temperature. This offers promising perspectives when these DPPC/DPPG vesicles (with entrapped drugs) will

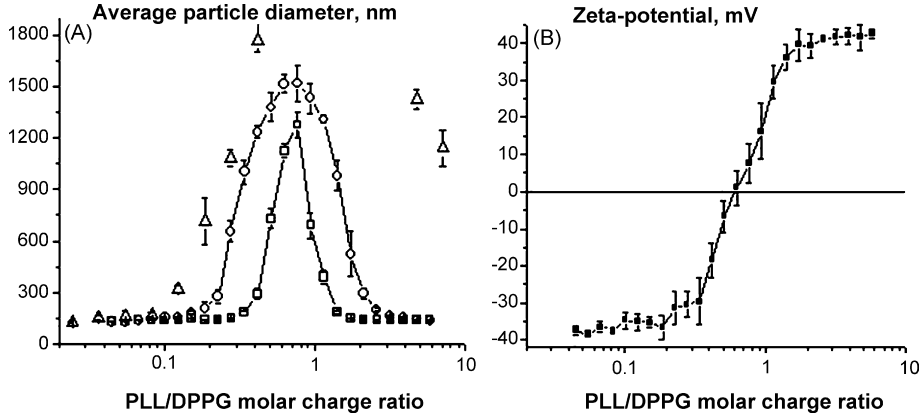

Fig. 7. (A) Average diameter of liposome-PLL complexes formed using PLL with molecular masses of 280 kDa (□), 28 kDa (◯), and 2 kDa (△) as a function of molar charge ratio PLL/DPPG. Diameter of the native DPPC-liposomes is 129 ± 2 nm. (B) Zeta-potential of complexes prepared with PLL of 280 kDa as a function of the molar charge ratio PLL/DPPG. All experiments were carried out at 25 °C.

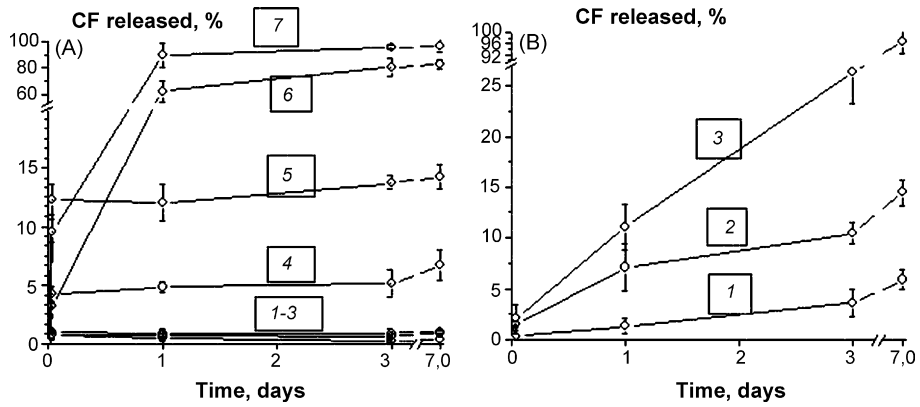

Fig. 8. (A) Release profiles of CF-loaded DPPC/DPPG liposomes at 25 °C: native liposomes (1), single PLL-covered (2), and vesicle aggregates (3). Release profiles at 54 °C: native liposomes (6), single PLL-covered (7). To obtain the release profiles upon crossing the transition temperature, the native (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 liposomes (1), single PLL-covered (2), and vesicle aggregates (3). To prepare single PLL-covered liposomes and liposome aggregates the 280 and 2 kDa PLL were, respectively, used.

be encapsulated in polyelectrolyte multilayers. The release of active molecules can be tunable by a controlled temperature pulse. Such a pulse could be obtained, for instance, by additionally doping the multilayer with magnetic nano-particles and the application of a subsequent magnetic field. Such stimuli-sensitive multilayer films are the subject of our current research.

2. Conclusion

As a conclusion the molecular recognition between complementary chemical groups was successfully used to induce selective adhesion and fusion of vesicles. The adhesion process requires not only an attractive interaction at long distance to bring into contact the membranes but also a stabilizing interaction at short distance that increases locally the tension of the membrane or induces a lipid reorganization such as a multilamellar order as observed in the case of bipyridine ligands. This interaction can be initiated by hydrogen bonds or ligand-ion complexation. In addition, our results show that these processes strongly depend on the kinetic of the binding and the lipid reorganization. In the case of the biomimetic RGD/integrin vesicular system, the observed selective adhesion is reinforced by RGD lipid migration towards the adhesion zone where multiple RGD/integrin complexes are formed. Therefore, a microsegregation was observed at a given time. In the case of adhesion or vesicle fusion, amphiphilic molecules interacting by complementary molecular groups (electrostatic or hydrogen bonding) the initial attractive interaction is efficient to bring into contact membranes but the interaction is limited by lipid exchange and kinetics of the binding. In the case of adhesion and fusion, the kinetic effects are due to the lipid lateral diffusion within the membrane and the lipid exchange between two membranes. Both factors are directly related to the fluidity of the membranes. Therefore, the fusion process was only observed when the binding or complexation induced an irreversible stabilizing reorganization of the lipids as observed for bipyridine or diketonate ligands.

Finally, the coating of the vesicle with polyelectrolytes can be modulated by an electrostatic attractive interaction depending on the polymer size and the kinetics of the polymer adsorption. The preservation of the coated vesicles is strongly affected by the mixing conditions and polymer/lipid ratio. Permeabilization of lipid membranes is dramatically increased upon crossing the main transition temperature. For polylysine-coated vesicles the layer of the biopolymer is located exclusively on the vesicle surface and does not induce any changes upon adsorption on the surface of "solid" liposomes, however it has a strong effect on the membrane integrity of "fluid" vesicles due to the lipid mobility. The results demonstrate that the protective polymer layer offers the possibility to tailor properties of the resulting liposome–polyelectrolyte complexes and to control the release of a dye initially encapsulated in the vesicle compartment.

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