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Influence of Polyelectrolyte Chemical Structure on their Interaction with Lipid Membrane of Zwitterionic Liposomes

Francois Quemeneur,[†] Marguerite Rinaudo,[‡] and Brigitte Pépin-Donat*[,†]

Laboratoire Electronique Moléculaire Organique et Hybride/UMR 5819 SPrAM (CEA-CNRS-UJF)/INAC/ CEA-Grenoble, 38054 Grenoble Cedex 9, France, and Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), affiliated with Joseph Fourier University, BP53, 38041 Grenoble Cedex 9, France

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In this paper we extend our previous experimental work on interaction between polyelectrolytes and liposomes. First, the adsorption of chitosan and alkylated chitosan (cationic polyelectrolytes) with different alkyl chain lengths on lipid membranes of liposomes is examined. The amount of both chitosans adsorbed remains the same even if more alkylated polysaccharide has to be added to get saturation if compared with unmodified chitosan. It is demonstrated that alkyl chains do not specifically interact with the lipid bilayer and that electrostatic interaction mechanism governs the chitosan adsorption. The difference observed between unmodified and alkylated chitosans behavior to reach the plateau can be interpreted in terms of a competition between electrostatic polyelectrolyte adsorption on lipid bilayer and hydrophobic autoassociation in solution (which depends on the alkyl chain length). Second, interaction of liposomes with hyaluronan (HA) and alkylated hyaluronan (anionic polyelectrolytes) is analyzed. The same types of results as discussed for chitosan are obtained, but in this case, autoassociation of alkylated HA only occurs in the presence of salt excess. Finally, a first positive layer of chitosan is adsorbed on the lipid membrane, followed by a second negative layer of HA at three different pHs. This kind of multilayer decoration allows the control of the net charge of the composite vesicles. A general conclusion is that whatever the pH and, consequently, the initial charge of the liposomes, chitosan adsorption gives positively charged composite systems, which upon addition of hyaluronan, give rise to negatively charged composite vesicles.

I. Introduction

Adsorption of polyelectrolytes on charged surfaces plays an important role in materials science[1–5] and biomedical applications.[6–9] In particular, interactions between polyelectrolytes and charged lipid bilayers have been extensively investigated under theoretical[10–16] and experimental approaches.[17–22] Electrostatic coupling of polyelectrolytes to lipid bilayers produces materials with specific properties and enhanced stabilities when used in various devices, such as chemical sensors[23] or drug carriers. Many factors can affect polyelectrolyte adsorption on a lipid bilayer: (i) the initial bilayer properties (chemical structure of the lipids, autoassociated configuration), (ii) polyelectrolyte properties (chemical structure, charge density, molecular weight (Mw), solution concentration), and (iii) equilibrium solution properties (pH and ionic strength).[24,25] The combined effects of these factors control polymer−polymer or polymer−lipid bilayer interactions.

Liposomes consist of self-closed phospholipid bilayers (or multilayers).[26] Their diameters range between a few nanometers and hundred micrometers. Three main kinds of liposomes are distinguished: small unilamellar vesicles (SUVs; 20−100 nm), large unilamellar vesicles (LUVs; 100−500 nm), both usually used as protective capsules,[27–29] and giant unilamellar vesicles (GUVs; 0.5−100 μm), generally studied as oversimplified models of biological cells.[30] Interaction of liposomes with macromolecules is of relevance to simulate intercellular, polymer−cell,[31–34] and liposome−cell interactions[35–39] and is of interest in the domain of soft-matter physics.[40] Furthermore, liposomes decorated with polyelectrolytes are extensively used as drug carriers[41,42] and it is demonstrated that coating liposomes with polyelectrolytes enhance their therapeutic activity[43–46] and circulation lifetime[47,48] in an intravenous environment. For example, complexes of liposomes with DNA have recently received much interest as nonviral gene delivery vehicles[49–52] for a variety of biomedical applications.

In our previous works[53,54] we have quantified chitosan (a linear biocompatible cationic polyelectrolyte) adsorption on both large and giant vesicles. We have demonstrated that chitosan−lipid bilayer interaction enhances vesicles stability with regard to various stresses (for example, pH and salt shocks).

However, it seems of interest to further study the changes in liposome structural and physical properties resulting from their interaction with polyions relaying with the structure of the polyelectrolytes. Actually, it is reported that polyelectrolyte adsorption affects membrane behavior: permeability,[55–57] fusion,[58] phase transformations,[59–61] and stabilization.[62,63] It is the reason why, in the present paper, we first focus on the effect of alkyl chain lengths grafted on chitosan on the mechanism of its adsorption. Then, we show that hyaluronan (HA, an anionic polyelectrolyte) and a HA-alkylated derivative can also be adsorbed on the same DOPC lipid bilayer. The two polysaccharides investigated (HA and chitosan) were chosen for their good biocompatibility recognized for biomedical applications.[64] Finally, we report the formation of the chitosan/hyaluronan complexes on the lipid bilayers. The advantage of the use of HA as external layer is to confer better biocompatibility to the decorated vesicles.

* To whom correspondence should be addressed. Tel.: 00 33 4 38 78 38 06. Fax: 00 33 4 38 78 51 13. E-mail: brigitte.pepin-donat@cea.fr.

[†] Laboratoire Electronique Moléculaire Organique et Hybride/UMR 5819 SPrAM (CEA-CNRS-UJF)/INAC/CEA-Grenoble.

[‡] Centre de Recherches sur les Macromolécules Végétales.

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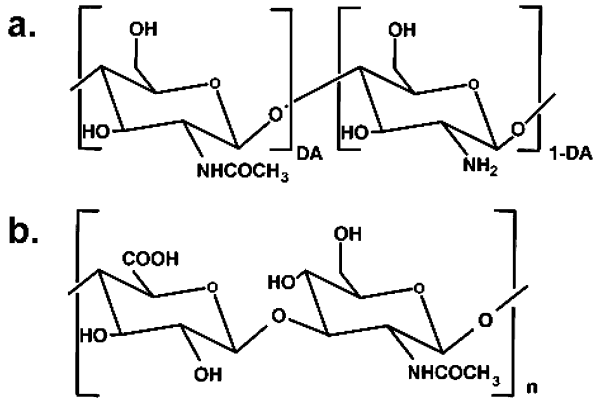

Figure 1. Structure of the polyelectrolytes. Repeated units of chitosan (a) and hyaluronic acid (b).

Among the different techniques used to modify surfaces, the deposition of polyelectrolyte multilayers (PEM) has attracted great attention due to its easy handling and large potentiality in terms of new original materials.[65] Actually based on the alternate adsorption of polycations and polyanions,[66] this technique allows to built-up films with tunable properties by adjusting several parameters such as the chemical nature of the polyelectrolytes, pH and ionic strength, or post-treatment of the film.[67,68] Our objective is to associate such polyelectrolyte complexes with lipid membranes to develop a new attractive class of composite polymer−lipid membranes.

II. Materials and Methods

Materials. *Lipids.* 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC; Mw = 786.15) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt; 18:1 Liss Rhod PE; Mw = 1301.73) are purchased from Avanti Polar Lipids, dissolved as received in a chloroform/methanol solution (9/1 volume ratio) at 10 mg/mL and mixed in a weight ratio of 80:1 to a total concentration of 2 mg/mL. Solutions were kept at −20 °C until used.

Purified Water. Highly purified 18.2 MΩ·cm water is used for the preparation of all the solutions.

Sucrose, HCl, and NaCl. Sucrose, HCl, and NaCl are purchased from Sigma-Aldrich and used as received.

Large Unilamellar Vesicles (LUVs; for ζ-Potential Measurements). GUVs obtained by electroformation process[69] are extruded through a 0.2 μm filter.[53] LUVs prepared in these conditions are unilamellar.[70,71] The extruded LUVs of 200 ± 10 nm diameter encapsulate a 200 mM sucrose solution and are suspended in an external 200 mM sucrose solution containing NaCl and HCl at controlled concentrations allowing reaching desired pH and salt conditions.

Initial Polyelectrolytes. Chitosan (Chit; it is a linear random copolymer of D-glucosamine and N-acetyl-D-glucosamine) is obtained from Far East crab shells by Mullagaliev[72] with a degree of acetylation (DA) equal to 5% and a weight-average molecular weight Mw equal to 2.25×10^5. Hyaluronic acid (hyaluronan, HA; it is a linear alternated copolymer of D-glucuronic acid and N-acetyl-D-glucosamine) is purchased from ARD (Pomacle, France). Its weight-average molecular weight, Mw, is equal to 7.08×10^5. The chemical structures are presented in the Figure 1.

Alkylated Polyelectrolytes. The initial polyelectrolytes (Chit and HA) are chemically modified to introduce hydrophobic alkyl groups randomly along their backbones. Alkylated chitosans are prepared by a direct reductive amination reaction,[73] using the different aldehydes.[72] The chitosan derivatives obtained exhibit a low degree of alkylation of 5% but with different alkyl chain lengths: C6, C10, and C12. The reference of the polymers will indicate the length of the alkyl chain (*n* carbon chain) as Chit-Cn. Alkylated hyaluronan is prepared by reacting aldehydic chain (1-decanal: C10) with the adipic dihydrazide derivative (HA-ADH). The alkylated hyaluronan (HA-C10) used has a degree of alkylation of 8%; it was provided by ARD (Pomacle, France).[74,75]

The solutions of anionic polyelectrolytes (HA and HA-C10) are prepared at 0.4 g/L by dissolving the polymer in 200 mM sucrose at pH = 6.0, while solubilization of cationic chitosans (Chit and Chit-Cn) requires addition of a stoechiometric amount of HCl on the basis of -NH_2 content in the chitosan (final pH around 3.5). The solutions of polyelectrolyte are stirred for one night at room temperature, until complete solubilization. The solutions of polyelectrolyte are diluted for vesicles incubation at 0.04 g/L in a solution of 200 mM sucrose at chosen pH and NaCl concentrations and directly used.

β-Cyclodextrins (β-CD). β-CD are kindly provided by Roquette (Lestrem, France). Cyclodextrins are cyclic oligosaccharides composed of a hydrophobic cavity able to complex hydrophobic substances and a hydrophilic outer character leading to their water solubility. They are commonly used to complex alkyl chains and surfactants.[76]

Methods. ζ-Potential (ζ) and dimension measurements on LUVs are performed at 20 °C with a commercial Zeta-sizer (Zetasizer NanoZS, Malvern, France). The ζ-values are determined using the Smoluchowski relation relating the ionic mobilities with the surface charge and averaged over 10 repeated measurements. The particle sizes are controlled in situ by light scattering measurement. For each ζ-measurement, the following protocol is repeated: a given volume of polyelectrolyte solution tested is added to the liposome suspension; after homogenization, we inject 1 mL of this mixture in the Zetasizer Nano Cell; the ζ of the dispersed liposomes is measured. After each measurement the whole solution is collected from the Zetasizer Nano cell and reintroduced into the bulk solution (to keep a nearly constant volume of solution) before the addition of the next volume of chitosan solution. Those steps are repeated as many times as necessary. The lipid concentrations are measured by spectrofluorometry for each sample.

The ζ is defined as the electrostatic potential at a specific distance of the particle surface; this distance is named "slipping plan". In our previous work,[54] we have showed that the variation of ζ can be related to the amount of polyelectrolytes fixed on the liposomes.

Thereafter, we define a normalized ζ as the difference between the measured ζ and the value of the ζ of the initial lipid membrane (i.e., LUVs in absence of adsorbed polymer) to compare the different polyelectrolytes.

Fluorescence Measurements. As the lipid concentration cannot be directly deduced from the amount of lipids involved in GUVs electroformation, we measure the fluorescence intensity of labeled lipids (rhodamine: excitation/emission 557/587 nm) in the initial solution of LUVs obtained by extrusion of GUVs[54] to evaluate the amount of lipid involved in liposomes in suspension. Fluorescence experiments are performed at 20 °C with a Perkin-Elmer Luminescence Spectrometer LS50B. We assume that the amount of lipids included in the external leaflet of the lipid membrane represents one-half of the total lipids.

III. Results and Discussion

A. Adsorption of Amphiphilic Chitosan Derivatives. To further study the mechanism of interactions between polyelectrolytes and lipid membranes, we have investigated the role of the alkylation of chitosan on its interaction on the lipid bilayer. In the following, we address the question of the role of the alkyl chains length and self-aggregation in solution on the adsorption of alkylated chitosans on liposomes.

1. Influence of the Alkyl Chain Length. First we investigate the effect of the alkyl chain length grafted on chitosan on the ζ variation. Measurements are performed with the same protocol for the four samples (homogenization after injection of the chitosan or Chit-Cn, incubation during 30 min before the first measurement). At pH = 3.5, the chitosan is positively charged (amino groups of the chitosan are fully protonated[77]) and the

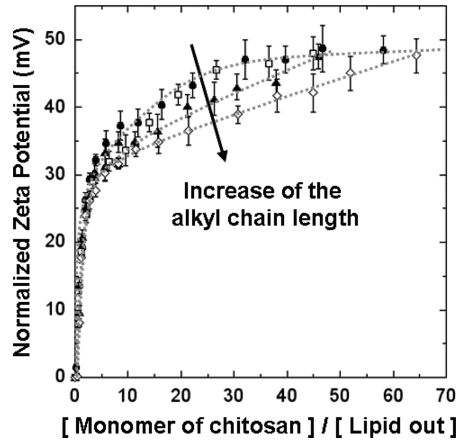

Figure 2. Influence of alkyl chain length on normalized ζ-potentials of LUVs at pH = 3.5 vs the ratio of chitosan monomer per accessible lipids of the membrane (*R*), for chitosan modified with different alkyl chain lengths: unmodified chitosan (Mw = 2.25 × 10^5; ●), Chit-C6 (□), Chit-C10 (▲), and Chit-C12 (◊). Dotted lines are added to guide the eye and have no physical meaning.

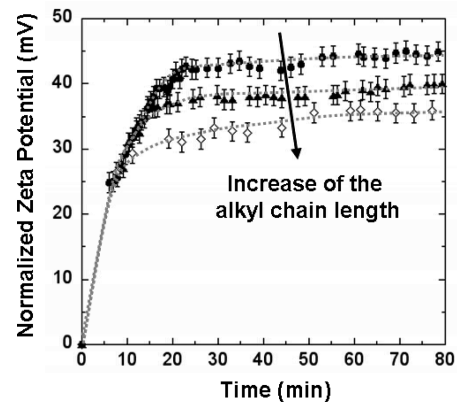

Figure 3. Influence of alkyl chain length on normalized ζ-potential of LUVs at pH = 3.5 vs time: unmodified chitosan (Mw = 2.25 × 10^5; ●), Chit-C10 (▲), and Chit-C12 (◊). The ratio *R* of chitosan monomers per accessible lipids of the membrane is equal to 20. Dotted lines are added to guide the eye and have no physical meaning.

lipid membrane is positively charged (ζ around + 5 mV).[54] Then, at low ionic concentration, the electrostatic repulsions between polycationic chains are stronger than the hydrophobic attraction between their alkylated grafted chains.

Figure 2 presents the evolution of the ζ of liposomes during progressive addition of chitosan (Mw = 2.25 × 10^5) and modified chitosans (Chit-Cn; alkyl chains: C6, C10 and C12) at pH = 3.5; ζ is normalized and expressed as a function of the parameter *R* equal to the ratio of chitosan monomers over DOPC groups concentration in the external layer of LUVs.

Upon addition of the different polyelectrolytes (Chit and Chit-Cn) in the LUVs suspension, the ζ variations display the same general trend: a sharp increase for *R* lower than 3, then a smooth variation before reaching a constant value equal to + 48 mV in each case in large excess of polymers ($R = R_{lim}$). Nevertheless, it appears that the value of R_{lim} necessary to reach the plateau depends on the alkyl chain length. For Chit-C6, the variation fits well with that observed for the unmodified chitosan, while, for Chit-C10 and Chit-C12, R_{lim} is higher than that of unmodified chitosan and increases with the alkyl chain lengths. In the case of Chit-C12, R_{limit} is larger than 60, which corresponds to around 3 alkyl chains per lipid of the external leaflet.

We have already demonstrated that ζ is related to the amount of chitosan adsorbed on liposomes;[54] our new data demonstrate that the maximum degree of decoration is independent of alkyl chain lengths grafted on chitosan. Our interpretation is that alkyl chains do not specifically interact with the lipid bilayer: electrostatic interaction mechanism governs the chitosan adsorption. The delay observed with alkylated chitosans to reach the plateau can be interpreted in two ways: (i) in terms of competition between electrostatic polyelectrolyte adsorption on lipid bilayer and hydrophobic autoassociation in solution (which depends on the alkyl chain length), and (ii) due to the presence of alkyl chains; the rate of diffusion of modified chitosan close to the lipid bilayer is decreased and the intermediate points in Figure 2 are obtained out of equilibrium.

2. Kinetic Effect on ζ-Measurements. To check the validity of the second assumption (out of equilibrium ζ-measurement), we have observed the variation of the ζ as a function of time. For these experiments, chitosan-decorated LUVs are incubated at pH = 3.5 for a ratio of $R \sim 20$ (cf. Figure 2). The variation of the normalized ζ versus time for the different chitosans is given in Figure 3.

After the injection, we observe that the ζ increases with the same trend for the different derivatives, before reaching a constant value after 30 min, for all the chitosans either alkylated or unmodified. We note that the plateau values are in agreement with the measurements presented in Figure 2 for the ratio $R \sim 20$ (+42 mV for the unmodified chitosan, +38 mV for the Chit-C10, and +35 mV for the Chit-C12). Taking into account that all the experimental measurements of Figure 2 were performed after 30 min of incubation, we can conclude that all the points reported in Figure 2 correspond to measurements performed at equilibrium and that only the competition between adsorption of the polyelectrolytes on the lipid membranes and autoassociation of the alkylated derivatives in solution is responsible for the delay to reach the plateau in ζ. Such association between alkylated chitosan, depending on alkyl chain length, was already reported in the literature.[72] In this paper, it is observed that the longer the alkyl chains are, the more important the hydrophobic autoassociations are. Moreover, the authors demonstrate that addition of cyclodextrins (where inclusion of hydrophobic alkyl chains can occur) suppresses a large part of the alkyl/alkyl associated domains while salt addition favors hydrophobic association due to the screening of electrostatic interchain repulsions. The same observations were made by Creuzet et al.[75] on alkylated hyaluronic acid. Taking these results into account, we now study the influence of cyclodextrin and salt additions to further confirm the influence of alkylated chitosan autoassociation on ζ-variation.

3. Influence of β-CDs on Alkylated Chitosan−Lipid Interaction. To evaluate the competitive effect of autoassociation of the alkyl chains in solution and adsorption of the amphiphilic polyelectrolytes on the lipid membrane, we tried in a first step to reduce the hydrophobic autoassociation by inclusion of the alkyl chains in cyclodextrin cavities. For this purpose, we prepared a solution of Chit-C12 at pH = 3.5 containing stoichiometric β-CD amount with respect to alkyl chains. In Figure 4, we compare the variation of the ζ of liposomes in presence of Chit-C12 and β-CDs, with the results previously obtained for unmodified chitosan and Chit-C12 in absence of β-CDs (previously reported in Figure 2).

With the β-CDs/Chit-C12 complex, we observe a large variation of the ζ, which reaches a constant value of +48 mV equal to that obtained for unmodified chitosan for a ratio $R = 25$. In absence of cyclodextrins, the same plateau value is reached for Chit-C12, but for $R = 65$. These results show that the addition of β-CDs reduces the amount of monomeric units

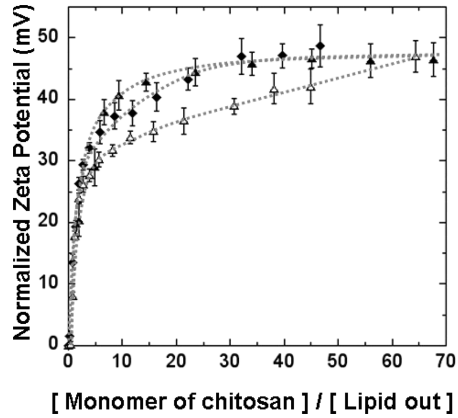

Figure 4. Normalized ζ-potentials of LUVs at pH = 3.5 as a function of the ratio *R* of chitosan monomer per accessible lipids of the membrane, for different chitosan systems: unmodified chitosan (Mw = 2.25 × 10^5; ♦), Chit-C12 (△), and Chit-C12 in presence of β-cyclodextrins (▲). Dotted lines are added to guide the eye and have no physical meaning.

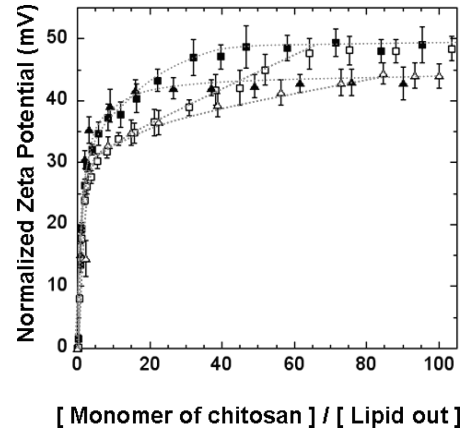

Figure 5. Normalized ζ-potentials of LUVs at pH = 3.5 vs the ratio of chitosan monomer per accessible lipids of the membrane, for different chitosan systems: unmodified chitosan (Mw = 2.25 × 10^5; ■) and Chit-C12 (□) in the absence of NaCl, initial chitosan (Mw = 2.25 × 10^5; ▲), and Chit-C12 (△) in presence of NaCl 5 mM. Dotted lines are added to guide the eye and have no physical meaning.

necessary to reach the plateau. In the literature, it is reported that β-CDs can extract lipids from the membrane, which results in its dissolution.[78] Therefore, we have checked that the presence of cyclodextrins (in our experimental conditions) neither affects ζ values nor leads to a decrease in the liposome diameter (constant value of 200 ± 10 nm), and we observe that liposomes are not dissolved by β-CDs added in a stoichiometric amount (one β-CD per chitosan alkyl chains), even for a large ratio *R* corresponding to around 0.5 β-CDs per accessible lipid head.

The striking result of this study is that the same maximum amount of chitosan is adsorbed on liposomes whatever the conditions (unmodified chitosan, chit-C12 or chit-C12/β-CD), which leads us to assume that the alkyl chain do not incorporate into the lipid membrane and that the only role of alkyl chains is to cause a delay before reaching the maximum decoration, which remains constant. In other words, aggregation of alkylated chitosan in solution is only responsible for the larger R_{lim} required to reach the ζ-plateau for Chit-C10 and C12 (Figures 2 and 4). These results are in good agreement with our previous conclusion claiming that the main mechanism of interaction between chitosan and phospholipid bilayer are of electrostatic origin.[53,54] Our result can be regarded as conflicting with that of the literature, which shows that chitosan-derived polymer with alkyl chains can dissolve DPPC liposomes. But in this case, chitosan is prepared in pure water (low cationic charge at pH ∼ 6.0−8.0) and added in a very large amount (up to 20 alkyl chain per lipid);[79] in our case, the conditions are completely different: pH = 3.5 and the maximum of alkyl chains added per lipid is equal to 3.

4. Influence of Salts on Alkylated Chitosan−Lipid Interaction. In a second step, to complete the study of the competition between the autoassociation of the alkylated derivatives in solution and the adsorption on the liposomes, we tried to increase the alkyl/alkyl association by screening the electrostatic interchain repulsions by salt addition. Therefore, we prepared solutions of liposomes and of chitosans (unmodified chitosan and Chit-C12) with NaCl 5 mM at pH = 3.5. Figure 5 shows the variation of the ζ of liposomes as a function of the amount of added chitosans in absence and presence of NaCl.

In the presence of 5 mM NaCl, we observe for both chitosans, large variations of the normalized ζ, which reaches the same constant value of +44 mV at *R* = 20 and *R* = 85 for unmodified chitosan and the Chit-C12, respectively. In comparison, in absence of NaCl, the plateau of +48 mV was reached for *R* = 25 and 65 for the unmodified chitosan and the Chit-C12, respectively.

This result demonstrates that NaCl, by screening the electrostatic repulsions, reinforces the autoassociation of alkyl chains in solution and consequently is responsible for the increase in R_{lim} for the alkylated chitosan.

Both studies of β-cyclodextrin and salt addition on adsorption of chitosan and alkylated derivatives point out the influence of hydrophobic aggregation in solution but seem to exclude the anchoring of the alkylated chain in the investigated zwitterionic lipid bilayer. This hypothesis is in agreement with the suggested conclusion of a published paper[80] in which the thermal transition of neutral membrane (DMPC, zwitterionic lipid) is reported to be unmodified upon alkylated chitosan interaction. We also demonstrate that the degree of decoration, corresponding to the plateau value in ζ, is independent of the alkyl chain lengths confirming thus the electrostatic origin of the adsorption; in accordance with our previous works,[54] the fraction of liposome surface covered by chitosan is found to be lower than 25% (assuming a monolayer of chitosan and same order of magnitude for the chitosan monomeric unit and the lipid polar head areas).

To complete the study of interactions between polyelectrolytes and lipid membranes, we now focus on the adsorption of an anionic polyelectrolyte, the hyaluronan. In the following sections, we address the question of the role of charge density and ionic strength on the adsorption of initial hyaluronan (HA) and alkylated hyaluronan (HA-C10) on the zwitterionic membrane of liposomes.

B. Hyaluronan Adsorption: Role of pH and Alkylation. To study the influence of the charge density of hyaluronan on its adsorption on LUVs membrane, two series of experiments at two distinct and controlled pH values (pH = 3.5 and pH = 6.0) were performed. Therefore, we prepared initial solutions of HA and vesicles at fixed pH values (3.5 and 6.0) and observed the variation of the liposome ζ as a function of the added HA at these fixed pH. At pH = 6.0, the hyaluronan is highly negatively charged (90% of carboxyl groups are ionized), while at pH = 3.5, hyaluronan has a lower negative charge density (only 25% of the carboxyl groups are ionized).[81]

Figure 6 presents the variation of ζ as a function of added HA expressed by the ratio *X* of ionized carboxylic groups (COO^-) per lipids of the external leaflet.

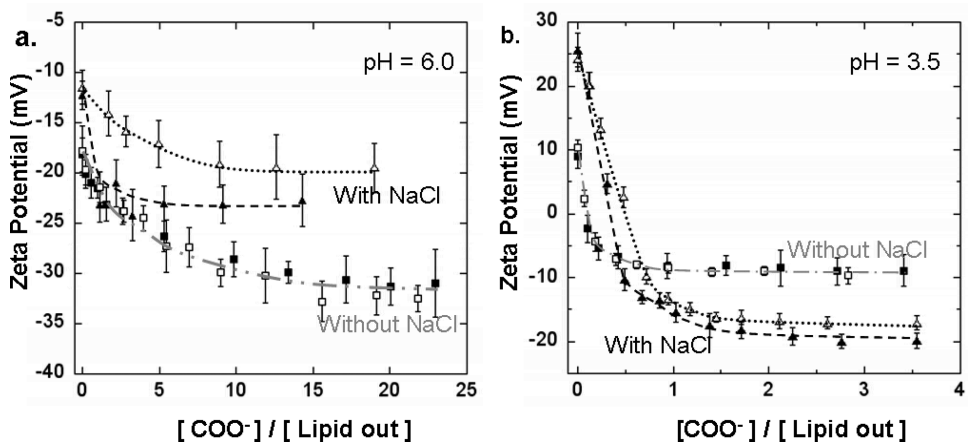

Figure 6. ζ-Potentials of LUVs at pH = 6.0 (a) and pH = 3.5 (b), in the presence of hyaluronic acid (HA; Mw = 7.08×10^5), as a function of the ratio X of the amount of ionized HA carboxyl groups over accessible lipids of the membrane. The data were obtained without NaCl for the unmodified hyaluronan (HA; ■) and the HA-C10 (□) and in presence of 5 mM NaCl for the unmodified hyaluronan (HA; ▲) and the HA-C10 (△). Dotted lines are added to guide the eye and have no physical meaning.

At pH = 6.0 (Figure 6a, solid squares (■)), we observe that ζ of the naked LUVs equals −17 mV and reaches a constant value of −32 mV in the presence of an excess of HA, while at pH = 3.5 (Figure 6b, solid squares (■)), the initial ζ equals +8 mV and goes to a constant value of −8 mV. As we detailed in our previous papers,[53,54] the differences in the initial ζ-values at both pH values (3.5 and 6.0) are attributed to the zwitterionic character of the membrane. At pH = 3.5, the membrane is positively charged due to the influence of the quaternary ammonium and to the repression of the phosphate (and eventually the carboxylate) dissociation. At pH = 6.0, the negative charge dominates.

In the absence of added salt, we observe the variation of ζ as a function of addition of both hyaluronan (HA) and alkylated hyaluronan (HA-C10). Results are the same for these two polyelectrolytes. The total variation of ζ is nearly the same at both pH values (16 mV at pH = 3.5 and 15 mV for pH = 6.0). The curve at pH = 3.5 goes to a plateau corresponding to ratio $X_{\text{limit}} = [\text{COO}^-]/[\text{lipid}] \sim 0.5$, whereas at pH = 6.0, it decreases smoothly and tends to a plateau for a ratio $X_{\text{limit}} \sim 15$. The difference in X_{limit} can be interpreted in terms of electrostatic interactions: it is attributed to a stronger affinity at pH = 3.5 than at pH = 6.0 between the negatively charged hyaluronan and the zwitterionic lipid membrane (positively charged at pH = 3.5 and negatively charged at pH = 6.0). The nearly constant value of $\Delta\zeta$ at both pH values is interpreted in terms of an equal amount of HA carboxylic charges adsorbed on the membrane for unmodified and alkylated polymers.

To observe the role of salt concentration as previously examined for chitosan, ζ-measurements were performed in a 5 mM NaCl solution. The same trends are observed for HA and chitosan (see Figures 5 and 6). For unmodified HA, at pH = 6.0 (Figure 6a, solid triangles (▲)), we observe that the ζ of the naked LUVs equals −12 mV (we have already observed that the initial ζ of liposome at pH = 6.0 is sensitive to the presence of salt[53]) and reaches a constant value of −23 mV more rapidly than in the absence of salt ($X_{\text{limit}} = [\text{COO}^-]/[\text{lipid}] \sim 2.5$) but nearly the same $\Delta\zeta$ is obtained, corresponding to nearly the same amount of HA adsorbed than in absence of salt. The screening effect of salt depressed the electrostatic repulsions between negative liposome and negative polyelectrolyte and favors the polyelectrolyte adsorption (X_{limit} lower). For alkylated HA at pH = 6.0 (Figure 6a, open triangles (△)), the ζ goes more smoothly to nearly the same constant value, and we interpret this result in the same way as for alkylated chitosan: charge screening by salt leads to induce autoassociation of the alkylated polymer free in solution.

For unmodified HA in 5 mM NaCl solution, at pH = 3.5 (Figure 6b, solid triangles (▲)), the initial ζ equals +25 mV and goes to a constant value of −20 mV for a larger X_{limit} ratio than in the absence of an excess of salt ($X_{\text{limit}} \sim 1.5$). The large $\Delta\zeta$ value indicates that more HA carboxylic charges are adsorbed than in the absence of salt. For the alkylated HA (Figure 6b, open triangles (△)), we observed a small increase of X_{limit}, which is interpreted in terms of competition between hydrophobic interaction of alkylated grafted chains in solution and electrostatic adsorption on the lipid membrane, as it was the case for alkylated chitosan (see Figure 5).

A tentative quantification of these results is proposed in the following: the variation of ζ[54] being assumed to be directly related to the amount of charges adsorbed on the membrane (corresponding to the COO^- groups of the hyaluronan), we can estimate the fraction of liposomes surface covered by hyaluronan. Considering the same area for the hyaluronan monomeric unit and lipid polar head, and taking into account that only 25% of the carboxylic HA groups are ionized at pH = 3.5, the degree of decoration at this pH is calculated to be 16 times higher than at pH = 6.0.

The main conclusion is that the negatively charged polyelectrolyte (HA) is adsorbed by the DOPC lipid membrane whatever the value of the pH giving rise to a negatively charged particle while with the positively charged chitosan, the particles were always positively charged.

C. Polyelectrolyte Complex Formation on the Lipid Membrane. The adsorption of successive layers of positively charged and negatively charged polyelectrolytes on the membrane of liposomes is now being investigated. Considering our previous data, the first polymer adsorbed was chitosan, followed by adsorption of hyaluronan to improve the stability. Hyaluronan, a polysaccharide largely present in human tissues, is used as an external layer to ensure an optimized compatibility in the body, in case of eventual biomedical application.[82]

To characterize this polyelectrolyte complex formation on the lipid membrane, ζ-measurements were performed at three distinct and controlled pH values (pH = 3.5, 4.7, and 6.0). Before the addition of chitosan, we have controlled the charge of the lipid membrane at the different pHs. The ζ of the naked LUVs equal, respectively, 20 mV at pH = 6.0, 5 mV at pH =

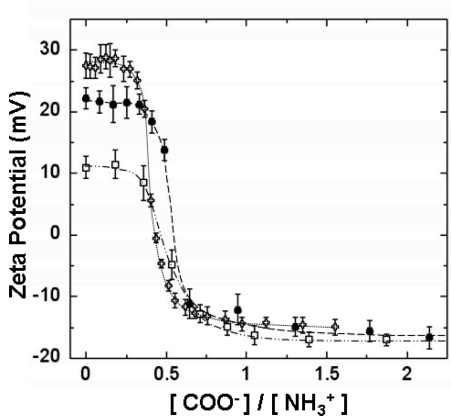

Figure 7. ζ-Potentials of chitosan decorated LUVs (Mw chitosan = 2.25×10^5) in the presence of hyaluronic acid (HA; Mw = 7.08×10^5) as a function of the ratio of the amount of ionized HA carboxyl groups over the amount of chitosan protonated amino groups. The data were obtained at pH = 3.5 (open cross), pH = 4.7 (●) and at pH = 6.0 (□) for chitosan-decorated LUVs incubated at a ratio R of chitosan monomer per accessible lipids of the membrane ~5. Dotted line is added to guide the eye and have no physical meaning.

4.7, and +5 mV at pH = 3.5. The membrane is thus negatively charged at pH = 6.0 and 4.7, while at pH = 3.5 it is positively charged, as shown previously.[54]

Then the naked LUVs were first incubated in a chitosan solution at a ratio R = [chitosan]/[lipid] ~ 5 to form the first layer of chitosan on the liposomes. This ratio corresponds to a significant degree of decoration by chitosan with a low concentration of free polyelectrolyte in solution (monomeric concentration lower than 3×10^{-5} M, whatever the pH).[54]

After chitosan addition, the ζ of the decorated LUVs go to positive values, respectively, +11 mV at pH = 6.0, +22 mV at pH = 4.7, and +28 mV at pH = 3.5. Figure 7 shows variation of ζ of the chitosan-decorated LUVs as a function of the amount of HA added for the three different pHs. The protocol used for the polyelectrolyte complex formation is similar to that adopted for single polymer addition (see ζ-measurements in Materials and Methods).

Upon addition of HA, we first observed that the ζ-value remains nearly constant until approximately a ratio $\Phi = [COO^-]/[NH_3^+] \sim 0.3$ and then decreases sharply to finally reach the same constant value of −16 mV for $\Phi_{limit} = [COO^-]/[NH_3^+] \sim 1$ for the three different pHs.

The initial positive plateau is explained by the formation of a complex between added HA and chitosan remaining free in solution (nonadsorbed on the liposomes). When the "neutralization" of the free chitosan is completed, added HA can adsorb on the chitosan-decorated liposomes, leading to a decrease in ζ before reaching a negative plateau.

IV. Conclusion

It is demonstrated that alkylation of chitosan (a cationic polysaccharide) does not modify the degree of coverage of liposomes by the unmodified polyelectrolyte; in addition, alkyl chains do not interact with the lipid membrane and do not lead to solubilization of the membrane. It is also shown that a competition exists in the systems between electrostatic adsorption and autoassociation of alkylated chains in solution. This association is repressed in the presence of β-cyclodextrin, but is promoted by the addition of salt. Second, it is demonstrated that, whatever the initial pH (3.5 and 6.0) and the net charge of the liposome, negatively charged polyelectrolytes (unmodified and alkylated hyaluronan) can adsorb and give rise to a negatively charged composite vesicle. Alkylated hyaluronan gives the same adsorption as unmodified HA in the absence of salt. At pH = 3.5, for both HA, the degree of coverage of the membrane is largely increased in relation to the initial positive net charge of the liposomes. At pH = 3.5 and 6.0, in presence of excess salt, hydrophobic autoassociation of alkyl chains leads to a higher amount of HA-C10 necessary to get the plateau than for HA. This behavior is similar to that observed with alkylated chitosan.

We would like to stress that, whatever the pH and consequently the initial charge of the liposomes, chitosan adsorption gives positively charged composite systems, which upon addition of hyaluronan, give rise to negatively charged composite vesicles. The charge of these vesicles can be tuned in a controlled way by adsorption of successive polyelectrolyte layers leading to the modification of their interaction between artificial surface and living systems.

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