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Influence of Molecular Weight and pH on Adsorption of Chitosan at the Surface of Large and Giant Vesicles

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

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

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This paper describes the mechanisms of adsorption of chitosan, a positively charged polyelectrolyte, on the DOPC lipid membrane of large and giant unilamellar vesicles (respectively, LUVs and GUVs). We observe that the variation of the zeta potential of LUVs as a function of chitosan concentration is independent on the chitosan molecular weight (Mw). This result is interpreted in terms of electrostatic interactions, which induce a flat adsorption of the chitosan on the surface of the membrane. The role of electrostatic interactions is further studied by observing the variation of the zeta potential as a function of the chitosan concentration for two different charge densities tuned by the pH. Results show a stronger chitosan−membrane affinity at pH 6 (lipids are negatively charged, and 40% chitosan amino groups are protonated) than at pH 3.4 (100% of protonated amino groups but zwitterionic lipids are positively charged) which confirms that adsorption is of electrostatic origin. Then, we investigate the stability of decorated LUVs and GUVs in a large range of pH ($6.0 < pH < 12.0$) in order to complete a previous study made in acidic conditions [Quemeneur et al. *Biomacromolecules* **2007,** *8*, 2512−2519]. A comparative study of the variation of the zeta potential as a function of the pH ($2.0 < pH < 12.0$) reveals a difference in behavior between naked and chitosan-decorated LUVs. This result is further confirmed by a comparative observation by optical microscopy of naked and chitosan-decorated GUVs in basic conditions ($6.0 < pH < 12.0$): at pH > 10.0, in the absence of chitosan, the vesicles present complex shapes, contrary to the chitosan-decorated vesicles which remain spherical, confirming thus that chitosan remains adsorbed on vesicles in basic conditions up to pH = 12.0. These results, in addition with our previous data, show that the chitosan-decorated vesicles are stable over a very broad range of pH ($2.0 < pH < 12.0$), which holds promise for their in vivo applications. Finally, the quantification of the chitosan adsorption on a LUV membrane is performed by zeta potential and fluorescence measurements. The fraction of membrane surface covered by chitosan is estimated to be lower than 40 %, which corresponds to the formation of a flat layer of chitosan on the membrane surface on an electrostatic basis.

I. Introduction

The development of appropriate colloidal systems to deliver active macromolecules is an important challenge in pharmaceutical or cosmetic realms. Nanoparticles[2,3] and specifically liposomes[4–6] are often studied as protective capsules and used as efficient drug carriers for a large panel of compounds like vaccines, diagnostic agents, or proteins.[7–16] Liposomes are made of self-closed phospholipid bilayers or multilayers. Their diameters range between a few nanometers and hundreds of 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, and giant unilamellar vesicles (GUVs) (0.5–100 μm), generally studied as oversimplified models of biological cells.[17] Liposomes used as protective capsules have to be stable in the biological environment and resistant to external constraints before reaching the target.

One approach to stabilize liposomes consists in preparing composite vesicles by associating their lipid membranes with different polymers. Actually numerous studies show that the "decoration" of liposomes with polymers affects their resistance to the in vivo environment, as well as their adsorption and specific targeting properties: for example, LUVs covered or grafted with polyethylene glycol are demonstrated to be furtive and stable in the intravenous environment.[9,18,19] Among SUV and LUV preparations, one consists in the extrusion of GUVs; therefore, it is also of interest to study GUV behavior upon decoration by macromolecules for protective capsule applications. GUVs present the great advantage to be observable by optical microscopy. Theoretical and experimental studies on GUVs upon interaction with anchored and/or adsorbed polymers highlight modifications in their membranes properties[20–22] and in their behaviors under external stresses.[23,24]

LUVs and GUVs were recently decorated with chitosan.[1] Chitosan is a copolymer of β-(1→4)-D-glucosamine and β-(1→4)-*N*-acetyl-D-glucosamine; it has been commonly used in cosmetics,[25,26] biotechnology,[27] and medicine[28–30] because of its intrinsic properties such as biocompatibility,[31,32] biodegradability,[33–37] and bioadhesivity[38–42] and its polycationic nature in acidic solutions.[43,44] The positively charged backbone of chitosan (controlled by the pH) plays a crucial role in its interaction with other macromolecules[45,46] or with negatively charged[47–50] or neutral[51–54] phospholipids of biomembranes. These composite vesicles were demonstrated to be strongly resistant to salt shocks and acidic pH.[1] In this paper, we first study the influence of the molecular weight of chitosan, of the

* Corresponding author. E-mail: Brigitte.pepin-donat@cea.fr. Tel.: 00 33 4 38 78 38 06. Fax: 00 33 4 38 78 51 13.
† Laboratoire d'Electronique Moléculaire Organique et Hybride.
‡ Centre de Recherches sur les Macromolecules Végétales.

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fluorescent labeling (of both chitosan and lipids), and of the pH on adsorption of chitosan. Then, we investigate the stability of the composite LUVs and GUVs in basic conditions ($6.0 < pH < 12.0$) to complete our first study.[1] Finally, we quantify chitosan adsorption on a LUV membrane by fluorescent studies.

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 are kept at −20 °C until used.

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

Sucrose and D-(+)-Glucose are purchased from Sigma-Aldrich and used as received.

Giant Unilamellar Vesicles (GUVs) are prepared by electroformation.[55] Approximately, 80 μL of the lipid mixture solution of DOPC/18:1 Liss Rhod PE (2 mg/mL) is spread on two indium tin oxide coated glass plates (5 cm × 5 cm). Lipid-coated glass plates are dried in a vacuum chamber for at least 2 h to remove the solvent.

Subsequently, the glass plates are placed in a swelling chamber with the coated sides facing each other. Both plates are held at a distance of 1 mm by a thin Teflon spacer. The vesicles are swollen in 3 mL of a 200 mM sucrose solution. After filling the chamber, a sinusoidal AC voltage of 1.5 V amplitude and 10 Hz frequency is applied for 4 h at room temperature. This procedure has a high yield of GUVs of radii between 5 and 50 μm.

Large Unilamellar Vesicles (LUVs) (For Zeta Potential Measurements). The GUVs obtained from the electroformation are extruded on a 0.2 μm filter and then diluted in a filtered solution of 200 mM sucrose. It was established that LUVs prepared in these conditions are unilamellar.[56,57] The extruded LUVs of 200 ± 10 nm in diameter encapsulate a 200 mM solution of sucrose and are suspended in an external sucrose and HCl mixture.

Chitosan. (Mw = 50 000 and degree of acetylation DA = 4.1%) from shrimps is provided by Primex. Chitosan (Mw = 100 000 and DA = 20%) is purchased from Sigma-Aldrich. Another sample (Mw = 500 000 and DA = 19.5%) is purchased from Kitomer (Marinard, Canada). The solutions of chitosan are prepared at 1 g/L by dissolving the polymer in 10 mL of 200 mM sucrose. To obtain complete solubilization of the polyelectrolyte, we add the stoechiometric amount of HCl on the basis of NH_2 content (final pH around 3.4).[1] The solution is placed under constant stirring for 1 night at room temperature, until complete solubilization occurs. The solutions of chitosan are diluted for vesicle incubation at 0.05 g/L in a solution of 200 mM sucrose at pH 3.4 or 6 and directly used.

Chitosan Labelling. In order to both quantify chitosan adsorption on LUVs and observe GUVs by fluorescence microscopy, we have labeled chitosan with a fluorescent probe.[58] A 1 g portion of chitosan (Mw = 500 000) is dispersed in 40 mL of distilled water and dissolved by addition of 60 mL of 0.1 M HCl; 25 mL of dried methanol are added before introduction of 50 mL of a solution of MeOH containing 52 mg of fluorescein isothiocyanate (FITC from Fluka). After being stirred during 3 h at ambient temperature, the mixture is diluted with water up to a final volume of 600 mL. The solution is neutralized with a 0.1 M NaOH solution to pH ∼7. The fluorescent chitosan precipitate is filtrated, washed with an ethanol/water 70/30 (volume ratio) mixture until the filtrate is clear, and finally dried at ambient temperature. The fraction of substituted monomeric units is determined by comparing its fluorescence in dilute acidic solution (stoichiometric amount of HCl or 0.3 M acetic acid as solvent) with a reference (a dilute solution of FITC in water of controlled concentration). Under these conditions, one over 60 sugar units (<2%) is labeled. This low percentage is chosen in order to minimize physical property changes of chitosan and LUV or GUV assemblies through labelling.

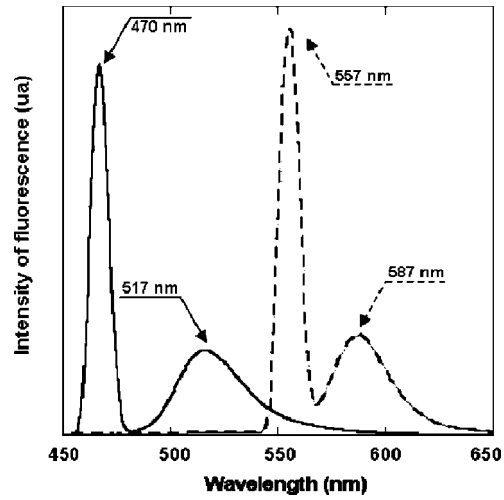

Figure 1. Intensity of fluorescence of the chitosan (solid line) and of the 18:1 Liss Rhod PE (dashed line) as function of the wavelength.

GUVs Incubated in Chitosan Solution (For Direct Observation by Optical Microscopy). Unlike Henriksen et al.[33,34] who add the liposome suspension into the chitosan solution, we choose to add progressively chitosan in the vesicle solution and incubate for 1 h at room temperature.

Methods. *Zeta potential and Size Measurements of LUVs* are performed at 20 °C with a commercial Zeta-sizer (Zetasizer NanoZS, Malvern, France). The zeta potential values are determined using the Smoluchowski relation relating the ionic mobilities with the surface charge. Then, the zeta potential measured will be considered have been directly related to the average net charge of the chitosan−lipid assembly. Values of zeta potential are averaged over ten repeated measurements and given with a precision of ±5 mV. The particle radii are controlled in situ by light scattering. For each zeta potential measurement, the following protocol is repeated: a given volume of the chitosan solution is added to the liposome suspension. After homogenization, we control the pH value of the incubation solution and inject 1 mL of this mixture in the Zetasizer Nano cell; the zeta potential of the solution is measured after 2000 s, which we have checked to correspond to equilibrium conditions and consequently to stable zeta potential values. After each measurement, the whole solution is collected from the Zetasizer Nano cell and reintroduced into the bulk solution (to keep a nearly a constant volume of solution) before the addition of the next volume of chitosan solution. Those steps are repeated as many times as necessary. Zeta potential measurements are performed in a 200 mM sucrose solution and the lipid concentrations are measured by spectrofluorometry for each sample. Protonated amino group concentrations are determined taking into account both pH values and acetylation degrees.

Fluorescence Measurements are performed at 20°C with a Perkin Elmer luminescence spectrometer LS50B. To distinguish between chitosan and lipid fluorescence contributions, the fluorescent probes are selected with different excitation and emission wavelengths (see the spectra of Figure 1): FITC (Excitation/emission 470/517 nm) for the chitosan and rhodamine (Excitation/emission 557/587 nm) for the lipid. These spectra allow the calibration curves for chitosan and lipids to be drawn independently (Figure 2).

Fluorescence intensity of the lipids is measured in the initial solution of LUVs obtained by extrusion of GUVs. Due to the weak concentrations of chitosan used especially for the first additions when the zeta potential exhibits a strong variation, it is necessary to measure the fluorescence intensity with accuracy.

Optical Observations of GUVs are made using a phase contrast inverted microscope (Olympus CKX41) and a numerical camera (AVT

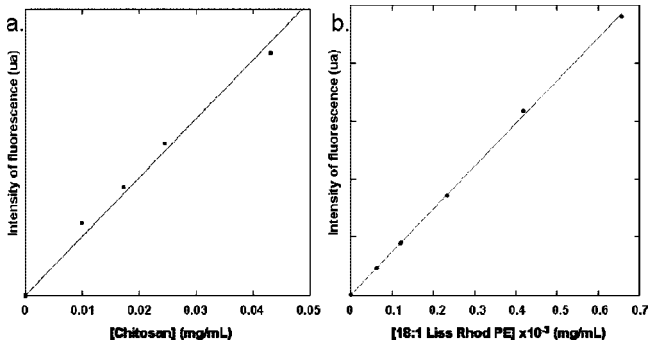

Figure 2. Calibration curves of chitosan and lipids labeled with fluorescent probes. (a) Intensity of fluorescence as function of the fluorescent chitosan concentration. (b) Intensity of fluorescence as a function of the fluorescent lipid (18:1 Liss Rhod PE) concentration.

MarlinF080B). Image analysis is performed using NIH Image J 1.36b software (freely available at http://rsb.info.nih.gov/ij/).

Isotherm of Adsorption. Both chitosan and lipid exact concentrations are required to establish the isotherm of adsorption. As far as lipids are concerned, we stress that their concentration cannot be directly deduced from the amount of lipids involved in GUVs' electroformation. Therefore, we determine by in situ fluorescence measurements the number of lipids involved in the membranes of LUVs assuming that no free lipids are present in solution. Dealing with chitosan, it is necessary to determine both its concentration free in solution and its quantity adsorbed on the membrane. This requires the separation of the decorated liposomes from the solution. We tested three methods of separation: First of all, we tried to centrifuge the LUV solutions with speeds up to 50 000*g* but we did not succeed in separating LUVs from the supernatant. Then, assuming that the decoration of GUVs was the same as that of LUVs, we tried to centrifuge solutions of decorated GUVs for a speed range between 2 and 10*g*, but the vesicles burst before separation. Finally, we tried to sedimentate decorated GUVs (containing sucrose) in an external solution of glucose at the same molar concentration, but we never succeeded in obtaining a supernatant fully free of GUVs. Therefore, in order to overcome this difficulty, we decided to use the following method: we assume that when we add very small amounts of chitosan solution prepared at pH 6, chitosan is partially positively charged[59] (40% of protonated amino groups) and consequently fully adsorbed on the liposomes which are negatively charged. Then, we admit that the variation of zeta potential is directly related to the amount of NH_3^+ chitosan adsorbed on the membrane, allowing the resulting isotherm of adsorption at pH = 6.0 to be drawn. At pH 3.4, the chitosan is fully positively charged[59] but the lipids are now positively charged; in these conditions, the amount of chitosan adsorbed is determined from the variation of zeta potential using the calibration relating zeta potential and adsorbed NH_3^+ chitosan established at pH 6.0. Knowing the amount of charged amino groups fixed on the membrane surface, we can calculate the quantity of chitosan adsorbed taking into account both the protonation (controlled by pH) and acetylation degrees.

III. Results and Discussion

A. Zeta Potential.

1. Influence of the Molecular Weight. In order to further study the mechanisms of adsorption of chitosan on the membrane of the vesicles, we have observed the effect of its molecular weight (Mw) on zeta potential variation. Because of the addition of successive amounts of chitosan solution (at 0.05 g/L and an initial pH of around 3.4) on the initial LUV solution (at pH = 6.0), the pH of the observed solution was found to vary between 6.0 and 3.6 thus allowing a perfect solubility of chitosan in all the experiments.[59,60] However, we demonstrated in a previous paper[1] that the large variation of zeta potential is neither due to the pH nor ionic concentration variations upon addition of the polyelectrolyte. Figure 3 presents the evolution of the zeta potential corresponding to three different molecular weights of chitosan (Mw = 50 000, 100 000, and 500 000) as function of the added quantity of chitosan expressed by the ratio [monomer of chitosan]/[lipid out]; "lipids out" represents the lipids included in the external leaflet and considered as half of the total lipids.

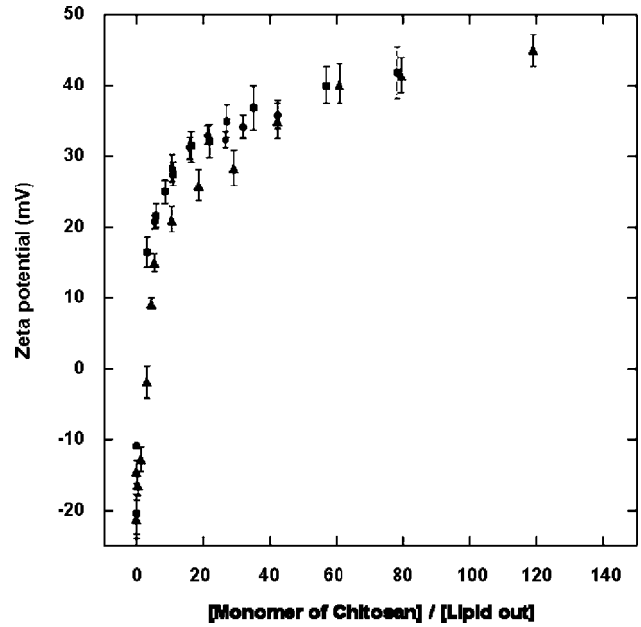

Figure 3. Zeta potentials of LUVs as a function of the ratio [monomer of chitosan]/[lipid out] for chitosan of different molecular weights: Mw = 50 000 (circles), Mw = 100 000 (squares), and Mw = 500 000 (triangles).

The zeta potential value is negative (−20 mV) at the initial pH = 6.0 (due the dissociation of phosphates and eventually carboxylates resulting from lipid oxidation during the electroformation process[61]) and becomes positive when the amount of chitosan increases, reaching a constant value of +45 mV (Figure 3). From these results, it is found that varying the molecular weight of chitosan between 50 000 and 500 000 has no influence on zeta potential variation. The adsorption mechanism remains probably the same and likely consists in a flat adsorption on the surface.

2. Role of pH on Electrostatic Behavior. In order to study the influence of the charge density of chitosan (which depends on pH) on its adsorption on a membrane of LUVs, we performed two experiments at two distinct and controlled pH values (pH = 3.4 and pH = 6.0). Therefore, we prepared two series of solutions (a solution of chitosan and a solution of vesicles) at fixed pH values (3.4 and 6.0) and studied the variation of the zeta potential as a function of the added amount of chitosan for these two fixed pH values (Figure 4).

At pH = 6.0, we observe that the initial zeta potential equals −20 mV for the nondecorated vesicles and reaches a constant value +34 mV upon chitosan addition.

At pH = 3.4, the initial zeta potential equals +9 mV for the nondecorated vesicles due to both the influence of quaternary ammonium and the repression of phosphate (and eventually carboxylate) dissociation and goes to a constant value of +45 mV. In addition, the data given in Figure 4a shows that the zeta potential is independent of the molecular weight (comparison between Mw = 50 000 and 500 000).

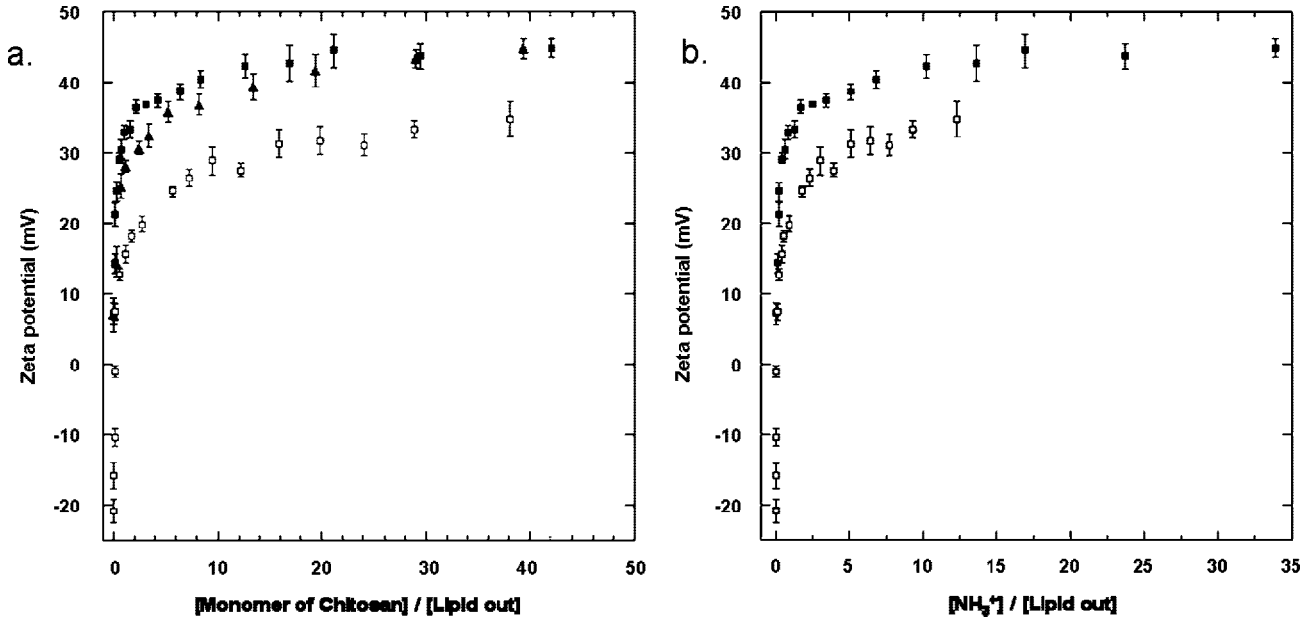

Figure 4. Zeta potentials of the LUVs (DOPC/18:1 LR) in the presence of chitosan as a function of the ratio of the following: (a) the amount of chitosan monomeric units or (b) amount of chitosan protonated amino groups over the accessible lipids on the external leaflets of the membrane. Data were obtained with chitosan Mw = 50 000 at pH = 3.4 (solid triangles) and with chitosan Mw = 500 000 for at pH = 3.4 (solid squares) and at pH = 6.0 (open squares).

We note that at pH values equal to 3.4 or 6.0, the value of the zeta potential for the LUVs studied here (let us recall that the membrane contains 2% fluorescent lipids) is the same as for LUVs only made with DOPC (determined in a previous study[1]). This shows that the addition of labeled lipids does not modify the charge of the membrane.

A difference between the final values of zeta potential obtained at pH = 3.4 and pH = 6.0 is observed, which is difficult to interpret but suggests that less positive chitosan NH_3^+ groups are adsorbed at pH = 3.4. At this pH, chitosan is positively charged (each amino group is protonated) but the membrane is also positively charged, while at pH = 6.0, the chitosan is partially charged[59] (40% of the amino groups are protonated as estimated from titration curve) but the membrane is negatively charged.

As suggested in our previous work,[1] electrostatic interactions dominate in the positively charged chitosan and liposome assembly. In order to check this hypothesis, the zeta potential variation was related to the NH_3^+ concentration (determined taking into account the degree of protonation and the degree of acetylation) as shown in Figure 4b. The curve at pH = 6.0 goes to a plateau corresponding to a ratio of $[NH_3^+]/[\text{lipid}] \sim 5$, whereas at pH = 3.4, it increases more slowly to a plateau for a ratio $\sim$ 10. Our interpretation is based on electrostatic considerations: the difference is attributed to a stronger affinity at pH 6.0 than at pH 3.4 between chitosan (positively charged) and the zwitterionic lipid membrane (becoming positive at pH = 3.4). Moreover, the interactions being independent on the molecular weight, it confirms that whatever the pH, the chitosan adsorbs flat on the membrane surface.

3. Role of pH on Coated LUV and GUV Behavior. In order to observe the stability of the chitosan decoration in a broad range of pH, we observe the influence of pH on zeta potential of naked and coated liposomes (prepared at pH = 6.0; Figure 5). For this experiment, chitosan-decorated LUVs are incubated at pH 6.0 for a ratio of $[NH_3^+]/[\text{lipid}] \sim 3$ (see Figure 4). Then, the pH is adjusted by progressive addition of HCl or NaOH to cover the pH range from 2.0 to 12.0.

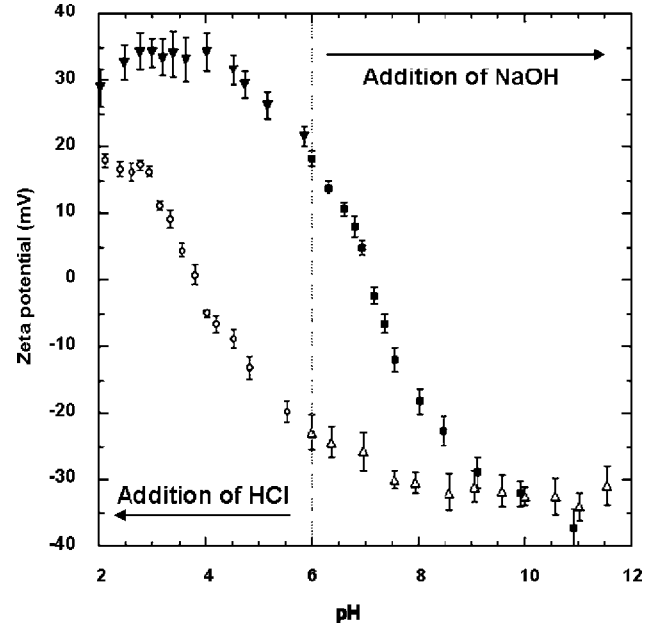

Figure 5. Variation of the zeta potential as a function of the measured pH for "naked" LUVs (open circles and triangles) and for chitosan-decorated LUVs (decoration at pH = 6.0; solid triangles and squares).

In acidic conditions, when pH decreases, the zeta potential passes through 0 at pH = 4.0 and becomes positive before reaching a final value of around +20 mV for pH < 3.0 for naked liposomes. This variation reflects the repression of phosphate dissociation. Considering coated vesicles, the trend is very different: at pH = 6.0, the zeta potential equals +20 mV (instead of −25 mV for naked vesicles) reaching a final constant value of +35 mV at pH < 3.0; this value results from conditions leading to fully protonated chitosan. It is larger than that of naked vesicles in relationship with the adsorption of positively charged chitosan. The behavior of LUVs in acidic medium was studied in our previous paper[1] and will not be further developed.

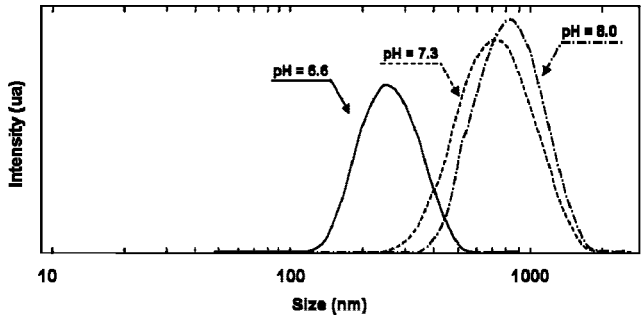

Figure 6. Variation of chitosan-coated LUV sizes upon pH from 6.6 to 8.0.

We now focus on the role of pH in neutral to basic medium on zeta potential variation of LUVs with or without chitosan. LUVs are subjected to successive shocks of pH in basic medium. For naked LUVs, the initial zeta potential equals −22 mV at pH = 6.0 and decreases when the pH increases, reaching a constant value of −32 mV at pH ≥ 7.5 (Figure 5). For decorated vesicles, when pH is increased by addition of NaOH, the zeta potential goes to zero at pH values around 7.1, becomes negative, and finally reaches the value (−30 mV) of the bare LUVs at pH = 9.0 (see Figure 5).

In the case of naked LUVs, the slight reduction of the zeta potential observed in basic conditions is interpreted as the complete neutralization of the phosphate groups of the phosphatidylcholine heads. For LUVs decorated with chitosan, we explain the decrease in the zeta potential by the repression of the chitosan protonation; the negative contributions of the phosphate acid and of the carboxyl groups (produced by the apolar chain oxidation) in the phospholipids become then predominant. In addition to these electrophoretic mobility measurements, we recorded the size of LUVs for different pH values (Figure 6).

We observed that the average diameter of naked LUVs remains constant (200 ± 20 nm) in the range of pH from 6.0 to 12.0. Concerning chitosan-decorated LUVs, we observed that, at pH 6.6, the average diameter is about 250 nm but when the pH increases to 7.3 and then to 8.0, the LUV diameter reaches a value of 800 nm. This increase in the size of LUVs can be interpreted by an aggregation process induced by the decrease of the chitosan charge density. Such aggregation was reported in our previous work[1] in the presence of NaCl and interpreted in terms of the salt charge screening effect.

These results do not enable us to conclude on a possible desorption of the chitosan or aggregation on the membrane upon the pH increase. To discriminate between these two assumptions, the influence of pH on GUV stability is studied and developed now. In order to directly detect the structural consequences of the addition NaOH at the micrometric scale, we observed the behavior of GUVs by phase-contrast microscopy as a function of the pH. The amounts of added NaOH in 200 mM glucose solution are chosen to progressively vary the pH between 6.0 and 11.0. We wait several minutes after injection in order to obtain a homogeneous concentration in the chamber. All the experiments are strictly performed under the same conditions, and vesicles are observed at the same distance from the injection zone.

Comparative experiments are performed on naked GUVs (Figure 7A and B.) and on GUVs decorated with chitosan (decoration is performed at pH 6; Figure 7C). In both cases, from pH = 6.0 (parts a in Figure 7A−C) to pH = 10.0 (parts b in Figure 7A−C), vesicles remain spherical whatever their initial size (from 5 to 50 μm) and keep their initial diameter. We stress that, under these experimental conditions, the osmotic shock is negligible.

At pH > 10.0, the osmotic shock is no more negligible. The behaviors of naked and decorated vesicles become different. As far as naked vesicles are concerned, one observes either the presence of complex shapes such as pearl necklaces and buds (Figure 7A) or shapes predicted by the ADE model (Figure 7B).[62,63] Such behavior was previously observed under the same pH conditions;[64] in their work, Lee and al. claim that at pH = 10.1 the curvature of a zwitterionic membrane is sensitive to the pH gradient and affects the shape of the deflated vesicle.

In the case of decorated vesicles, they remain spherical and their diameter decreases with an increasing amount of NaOH added for pH > 10.0. The variation of the diameter is in good agreement with the osmotic pressure variation (Figure 7C).[65] The chitosan-decorated vesicles (Figure 7C part f) remain stable after 2 h (Figure 7C part g).

The difference in behavior between naked and decorated vesicles in neutral and basic conditions (up to pH = 11.0) demonstrates that chitosan remains adsorbed (at least partially) on liposomes.

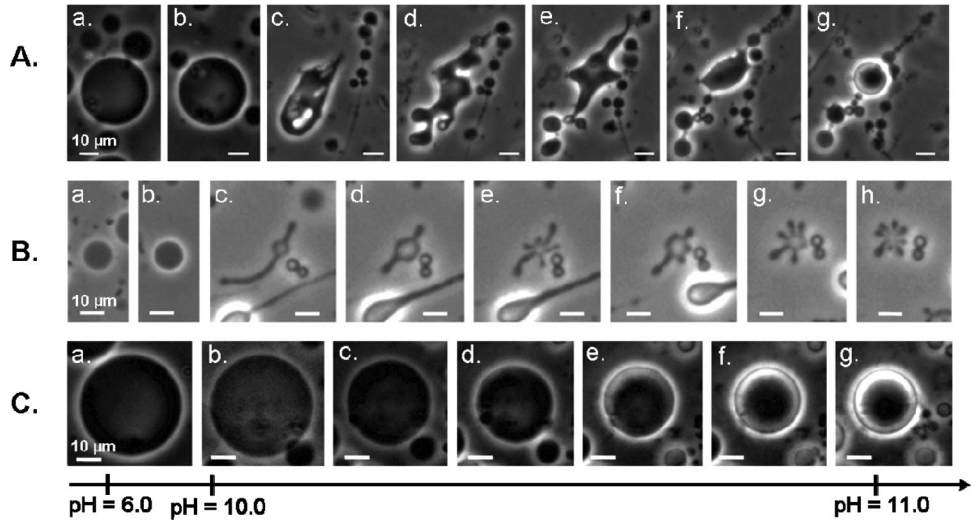

Figure 7. Behaviors of naked GUVs (sequences A and B) and chitosan-decorated GUVs (sequence C) as a function of pH (6.0 < pH < 11.0, produced by NaOH shocks). The delay between each picture is 10 s. The scale bars represent 10 μm.

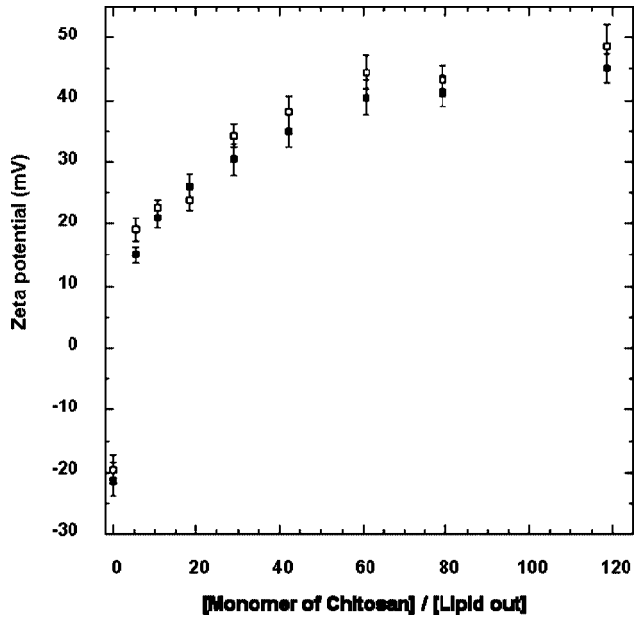

Figure 8. Zeta potential as a function of the ratio of [monomer of chitosan]/[lipid out] for chitosan (Mw = 500 000) fluorescent (open squares) and nonfluorescent (solid squares).

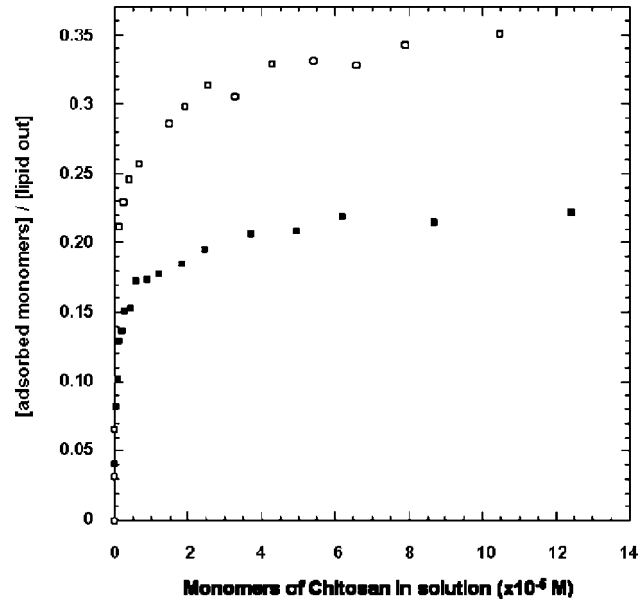

Figure 9. Adsorption isotherms of chitosan on liposomes expressed as the monomeric unit adsorbed per lipid polar head (in the external leaflet) versus the monomeric concentration of free chitosan in solution at equilibrium for pH = 3.4 (solid squares) and pH = 6.0 (open squares).

To conclude on LUV zeta potential studies and GUV optical observations, we have confirmed that chitosan is adsorbed on liposomes, whatever the pH from 1.0 and 11.0, as is easily interpreted in terms of the electrostatic mechanism.

In addition, it is demonstrated that, at pH = 3.4 as well as at pH = 6.0, the interactions are independent of the molecular weight. These data suggest that the chitosan is adsorbed flat on the surface. From an application point of view, it is clear that chitosan stabilizes LUVs and GUVs towards pH values occurring in physiological conditions and especially covering the pH values from pH ~ 2.0 in the stomach, skin (pH 5.2−7.0), blood pH ~ 7.4, and up to pH ~ 8.0 for the intestine and cerebrospinal liquid.

B. Quantitative Study of Chitosan Adsorption. The use of fluorescence measurements allows us to draw the adsorption isotherm on LUVs as explained in the experimental section. The lipid amount is determined in the initial solution. The lipid and chitosan amounts are calculated using the calibration curves given in Figure 2.

Before using the fluorescent intensity, it was necessary to check that the added probe does not induce any change in zeta potential values for lipids (as indicated before) as well as for chitosan (Figure 8).

Insofar as fluorescent labelings of both liposomes and chitosan have no influence on the zeta potential measurements, we can now relate zeta potential values to the amount of adsorbed chitosan per lipid polar head in the external leaflet according to our hypothesis previously introduced (see the Materials and Methods section).

The adsorption isotherms obtained at pH = 3.4 and 6.0 are presented in Figure 9, in which the chitosan adsorbed is expressed as a function of the chitosan free in solution, at equilibrium.

The results given in Figure 9 confirm the looser interaction between chitosan and liposomes at pH = 3.4 than at pH = 6.0. The fraction of liposome surface covered by chitosan remains always lower than 0.4 (assuming same order of magnitude for the chitosan monomeric unit and lipid polar head areas) corresponding to the upper limit of electrostatic adsorption of chitosan. The very low chitosan concentration at equilibrium confirms the strong interaction between the two components and allows the justification of the stabilizing role of chitosan on the lipid membrane of GUVs under salt, pH, and glucose stresses.

IV. Conclusion

In this paper, we demonstrate that the protonated amino groups NH_3^+ of chitosan play a crucial role in the mechanism of adsorption of this positively charged polyelectrolyte on the lipid membrane of vesicles. We also observe that chitosan has a stronger affinity for the zwitterionic membrane at pH = 6.0 than at pH = 3.4 and that the adsorption is independent of the chitosan molecular weight at fixed pH. These results lead us to conclude that electrostatic interactions are responsible for the chitosan adsorption, which is assumed to occur flat on the external surface of the vesicle. This flat adsorption is further confirmed by the low degree of coverage we observe in excess chitosan.

Furthermore, these composite LUVs and GUVS are demonstrated to be stable over a very large range of pH from 2.0 to 11.0, holding thus promise for applications in various in vivo media.

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