Stabilization of Liposomes by Polyelectrolytes: Mechanism of Interaction and Role of Experimental Conditions

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Summary: ζ-potential measurements on LUVs allow to evidence the influence of pH, ionic salt concentration, and polyelectrolyte charge on the interaction between polyelectrolyte (chitosan and hyaluronan) and zwitterionic lipid membrane. First, chitosan adsorption is studied: adsorption is independent on the chitosan molecular weight and corresponds to a maximum degree of decoration of 40% in surface coverage. From the dependence with pH and independence with MW, it is concluded that electrostatic interactions are responsible of chitosan adsorption which occurs flat on the external surface of the liposomes. The vesicles become positively charged in the presence of around two repeat units of chitosan added per lipid accessible polar head in acid medium down to pH = 7.2. Direct optical microscopy observations of GUVs shows a stabilization of the composite liposomes under different external stresses (pH and salt shocks) which confirms the strong electrostatic interaction between the chitosan and the lipid membrane. It is also demonstrated that the liposomes are stabilized by chitosan adsorption in a very wide range of pH ($2.0 < pH < 12.0$).

Then, hyaluronan (HA), a negatively charged polyelectrolyte, is added to vesicles; the vesicles turn rapidly negatively charged in presence of adsorbed HA Finally, we demonstrated that hyaluronan adsorbs on positively charged chitosan-decorated liposomes at $pH < 7.0$ leading to charge inversion in the liposome decorated by the chitosan-hyaluronan bilayer. Our results demonstrate the adsorption of positive and/or negative polyelectrolyte at the surface of lipidic vesicles as well as their role on vesicle stabilization and charge control.

Keywords: chitosan; composite vesicles; hyaluronan; interaction lipid-polyelectrolytes; lipid membrane

Introduction

Adsorption of polyelectrolytes on charged surfaces plays an important role in materials science and biomedical applications.[1–9] In particular interactions between polyelectrolytes and charged lipid bilayers, especially self-closed bilayers named liposomes, have been extensively investigated to simulate intercellular and polymer-cell interactions,[10–12] as well as to enhance efficiency in drug formulation in pharmaceutical realm.[13,14]

A large range of liposomes sizes is available: Large Unilamellar Vesicles (LUVs with 100–500 nm diameter), used as protective capsules for medical applications,[15–16] or Giant Unilamellar Vesicles (GUVs with 0.5–100 μm diameter) generally studied as oversimplified models of biological cells.[17] GUVs are prepared by electroformation.[18] from DOPC, a zwitterionic phospholipid, in presence of a

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sucrose solution and LUVs with a 200 nm diameter are obtained by extrusion of GUVs through a calibrated filter.[19–21]

This work concerns the stabilization of liposomes by two different pseudo-natural polyelectrolytes: the chitosan, a positively charged polyelectrolyte and the hyaluronan, a negatively charged polyelectrolyte; the charge density of these two biocompatible polysaccharides varies as a function of pH.

In our approach, we have combined studies of LUVs and GUVs to probe the mechanisms of interaction of the polyelectrolytes with the zwitterionic lipid membrane and to detect the influence of various external stresses on the composite liposomes structure.

Experimental Part

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 $-20\,^{\circ}C$ until used.

Sucrose, Glucose, HCl and NaCl are purchased from Sigma-Aldrich and used as received. Highly purified 18.2MΩ.cm water is used for the preparation of all the solutions.

Giant Unilamellar Vesicles (GUVs), filled with a 200 mM sucrose solution, are obtained by electroformation process.[18] This procedure has a high yield of GUVs of radii between 5 and 50 μm.

Large Unilamellar Vesicles (LUVs) (for zeta potential measurements) are obtained by extrusion of the suspension of GUVs through a 0.2 μm filter. LUVs prepared in these conditions are unilamellar[22,23] with diameters of 200 ± 10 nm diameter. The extruded LUVs are suspended in an external 200 mM sucrose solution containing NaCl and HCl at controlled concentrations allowing to reach desired pH and salt conditions.

Polyelectrolytes. Chitosan (Chit; it is a linear random copolymer of D-glucosamine and N-acetyl-D-glucosamine) with a weight-average molecular weight Mw equal to 5×10^4 and a degree of acetylation (DA) equal to 4.1% is provided by Primex. Chitosan ($Mw = 10^5$ and DA = 20%) is purchased from Sigma-Aldrich. Chitosan ($Mw = 2.25 \times 10^5$ and DA = 5%) is obtained from Far East crab shells by Mullagaliev.[24] Another sample of chitosan ($Mw = 5 \times 10^5$ and DA = 19.5%) is purchased from Kitomer (Marinard, Canada).

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 of the two types of polymers are presented in Figure 1.

The solutions of anionic polyelectrolyte (HA) are prepared at 0.4 g/l by dissolving the polymer in 200 mM sucrose at pH = 6.0 while solubilization of cationic chitosan (Chit) requires addition of a stoechiometric amount of HCl on the basis of -NH_2 content

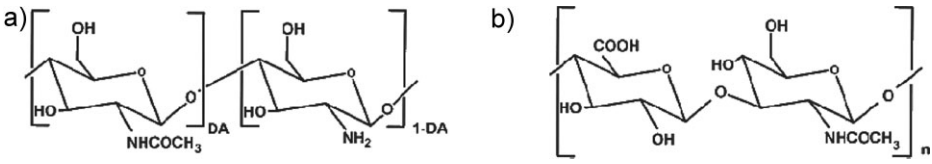

Figure 1.
Repeat unit of the two polyelectrolytes studied: (a) chitosan, positively charged polymer in acidic pH (pH < 6.0); (b) hyaluronan, anionic polymer at pH > 2.0.

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.

ζ– potential and size measurements on LUVs are performed at 20 °C with a commercial zetasizer (Zetasizer NanoZS, Malvern, France). The ζ–potential values are determined using the Smoluchowski relation which links the ionic mobilities to the surface charge. For each ζ –potential 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 apparatus and measure the ζ–potential of dispersed liposomes. 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. The lipid concentrations are measured by spectrofluorometry for each sample.[20]

Adhesion. Poly (L-lysine) coated glass slides are dried under argon flow and Secure-Seal hybridization chambers (purchased from Sigma) are stuck onto their surfaces in order to obtain observation chambers of 500 μL. The suspension of giant vesicles treated with chitosan is injected in the chamber (filled with a solution of 200 mM glucose) and, after sedimentation, the contact of the vesicles was observed in contact with the surface.[19]

Optical observations of GUVs are made using a phase contrast inverted microscope (Olympus CKX41) and a numerical camera (AVT MarlinF080B).

Results and Discussion

Chitosan- Vesicle Interaction

To highlight the decoration of the lipid membrane by the chitosan, we have first compared the behavior of bare and decorated vesicles in the presence of a poly (L-lysine)-treated substrate (positively charged in acidic medium). Bare vesicles, negatively charged at pH ~ 6.0, sediment, adhere on the surface and immediately burst, due to the increase in the membrane tension (see Figure 2a). On the contrary, chitosan decorated vesicles do not burst even after 30 min (Figure 2b) and, under gentle flow, start to move demonstrating the absence of adhesion on the positively charged substrate. These results demonstrate strong interaction between positively charged chitosan and zwitterionic DOPC vesicles. The absence of adhesion can be attributed to electrostatic repulsion between positively charged substrate and chitosan decorated-GUVs.

It is now of interest to study the role of various stresses, such as pH and salt shocks, on the revealed interaction between chitosan and vesicles.

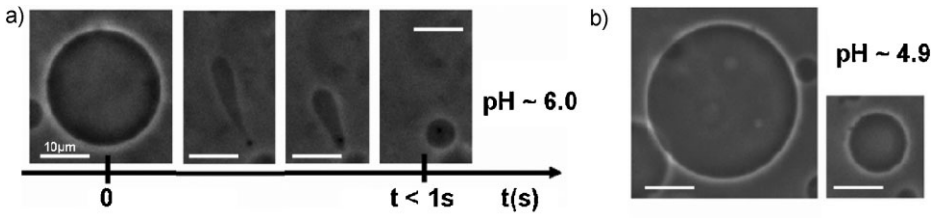

Figure 2.
Behavior of DOPC GUVs in presence of a glass substrate treated with poly (L-lysine): (a) bursting of a bare DOPC GUV entering in contact with the charged surface; (b) 2 chitosan decorated GUVs observed 30 min after sedimentation. The scale bars represent 10 μm.

Influence of pH, Salt and Chitosan on the ζ-Potential of LUVs

First, we study the effect of the pH on the ζ-potential variation of bare LUVs between pH = 6.1 and 2.0 by addition of HCl. The measured ζ-potential is negative (−25 mV) at the initial pH = 6.1. Such a negative potential for DOPC LUVs was already reported in the literature.[25] When pH is decreased, the ζ-potential increases to zero at pH = 4.0 and becomes positive; it reaches finally a constant value of +16 mV at pH = 2.9 (see Figure 3a).

When H_30^+ concentration increases, dissociation of the phosphate acid group of the lipid polar head is repressed; then, the positive contribution of the quaternary amino group becomes predominant. The large variation of ζ-potential is observed in a range of proton molar concentration up to 5×10^{-4} M, which is negligible with respect to the 200 mM concentration of the external sucrose; then its influence on the osmotic pressure remains negligible.

The role of NaCl (as a model of salt added to modify the ionic concentration) is completely different from that of HCl (see Figure 3a), which is much more efficient to modify ζ-potential going even to charge inversion. In addition for HCl and NaCl concentrations up to 5×10^{-3} M, the dimension of liposomes remains unchanged (diameter around 200 nm), which confirms that liposomes do not burst nor change their dimensions.

NaCl is able to screen the electrostatic attraction between positively and negatively charged groups of each polar head.[26] This kind of mechanism is also involved in salt effect on zwitterion solubility.[27,28]

Then, progressive additions of chitosan in a LUVs suspension are performed (see Figure 3b). During the experiment because of the addition of successive amounts of chitosan solution (at the approximate initial pH of 3.5) on the initial liposome solution (at pH = 6.1), the pH of the observed suspension is found to vary between 5.8 and 3.7 thus allowing a perfect solubility of chitosan in all the experiments.[29,30] We observe a large variation of the ζ-potential with increasing the amount of added

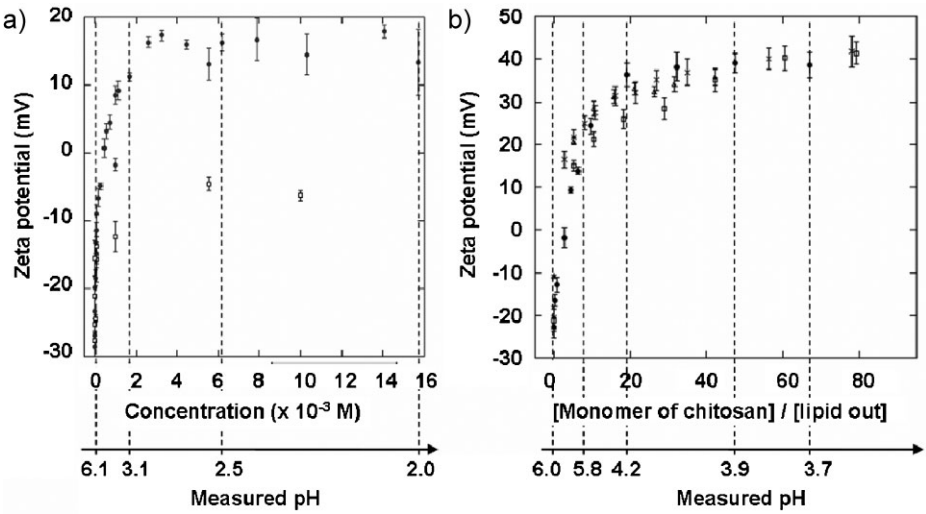

Figure 3.

Variation of ζ-potential of DOPC LUVs as a function of: (a) added (●) [HCl] and (□) [NaCl] and (b) the ratio of chitosan monomer per accessible lipids of the membrane, for four different molecular weight chitosans: (Δ) $Mw = 5 \times 10^4$, (×) $Mw = 10^5$,(●) $Mw = 2.25 \times 10^5$, (□) $Mw = 5 \times 10^5$. A pH scale is added to further characterize HCl addition. Figure 3a reproduced from [18] with the permission of the American Chemical Society. Copyright 2008.

chitosan: from −23 mV to +42 mV (see Figure 3b). In addition, with different chitosan of variable molecular weights and DA, it is shown that the same variation is obtained. Taking into account that ζ-potential of bare liposomes only varies from −26 mV to +5 mV for pH ranging between 5.8 and 3.9, it is clear that the large variations observed can neither be attributed to the pH nor to the ionic concentration variation upon addition of chitosan. These results also reveal the existence of a strong interaction between chitosan and lipid membrane.

At low chitosan concentrations (around 2 partially protonated glucosamine[30] per accessible polar heads of lipids), ζ-potential reaches zero and then becomes positive going to a plateau in large excess of chitosan. As a similar behavior was observed for polymers with different molecular weights ($Mw = 5 \times 10^4$ up to 5×10^5), it is concluded that the interaction consists in an adsorption of chitosan molecules flat on the membrane surface. The total amount of chitosan added is expressed in repeat unit added per lipid head in the external leaflet. This type of representation which allows to relate ζ-potential variation to chitosan added will be used to determine the isotherm adsorption.

Influence of Chitosan Decoration on GUVs Behavior Under pH and Salt Stresses

We now focus on the study of the influence of chitosan decoration on vesicle behavior under pH and salt stresses at the macroscopic scale. For that purpose, we vary the pH and salt concentration by controlled addition of respectively HCl and NaCl in GUVs suspensions, and observe structure modification of the bare and of the chitosan decorated GUVs (Figures 4 and 5 respectively) by optical microscopy.

As far as bare vesicles are concerned, we observe the same behavior of the vesicles as a function of [HCl] and [NaCl] concentration up to 10^{-2} M. This indicates that no difference is observable between salt and pH effects on the stability of giant vesicles while on LUVs, ζ-potential shows different trends at concentrations $>10^{-3}$ M (Figure 3a).

Bursting of bare vesicles at higher concentrations is observed: for $pH < 3.0$, membrane undergoes large thermal fluctuations and all the vesicles burst after 2 minutes at $pH \sim 2.0$. In this range of pH, dissociation of phosphate group is repressed and the lipid becomes positively charged due the presence of the quaternary ammonium. Instability of the membrane may be ascribed to repulsion between the positive charges of head groups. In addition

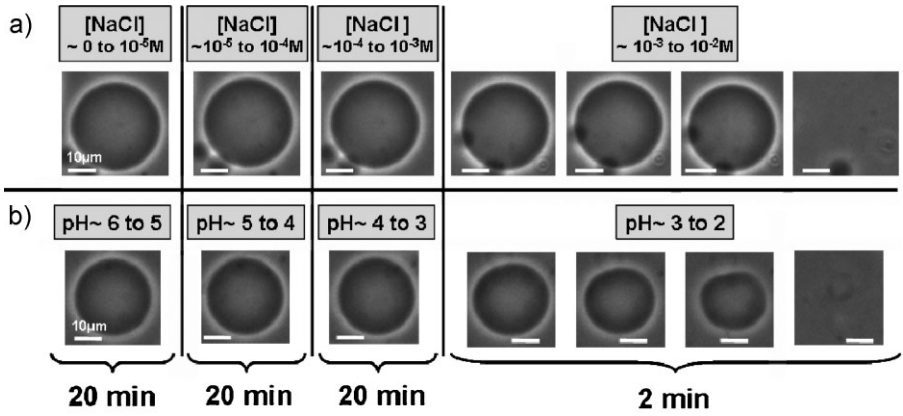

Figure 4.
Evolution of bare GUVs, initially at pH = 6.0, as a function of time, for different concentration of NaCl (up to 10^{-2} M) (a) or different pH values (b). Scale bars represent 10 μm.

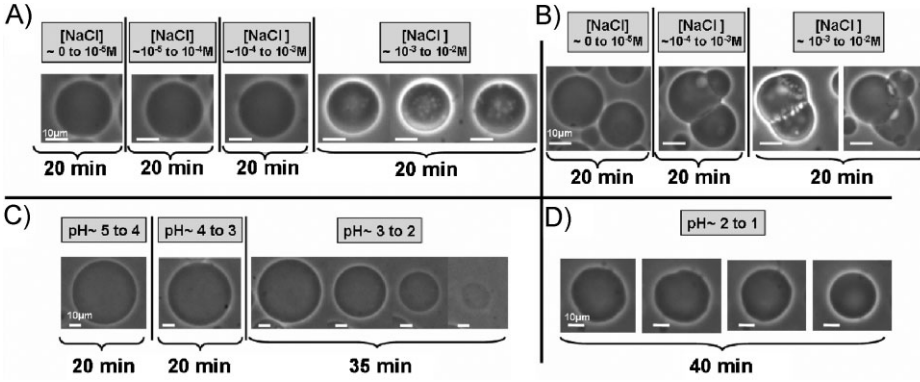

Figure 5.
Decorated DOPC GUVs as a function of time for different NaCl concentrations (up to 10^{-2}M) at initial pH = 5.0; (A) Single vesicle; (B) aggregate of vesicles; or different pH values: (C) GUVs with chitosan burst at pH = 2.0 after 35 minutes; (D) GUVs which were not tracked during the previous pH shocks, resist at pH = 1.0. The scale bars represent 10 µm.

a contribution of osmotic pressure may also contribute to disruption of the membrane; the vesicles burst at NaCl $\sim 10^{-2}$ M which may be attributed to combined effects of external osmotic pressure and ionic screening effect.

These experiments are then repeated with chitosan decorated vesicles (see Figure 5) strictly in the same conditions. Concerning the behavior of the decorated vesicles in presence of NaCl (Figure 5A and B), results are fully different if compared to that obtained as a function of pH (Figure 5C and D). No bursting occurs but we can observe aggregation (see Figure 5B) of vesicles when they are close together in the observation chamber. Addition of NaCl causes membrane aggregation; such effects of monovalent salt are reported in the literature[31] and attributed to a screening charge effect.

Above pH = 3.0, we observe the same GUVs stability as for bare vesicle. At $3.0 < pH < 2.0$, large fluctuations occur too but it takes more than 30 minutes for some of the vesicles to burst. Taking into account the strictly similar experimental conditions used for bare and decorated vesicles, the higher delay before bursting observed for chitosan decorated GUVs may be ascribed to the stabilization resulting from the presence of chitosan at surface. While some of the decorated GUVS burst at $3.0 < pH < 2.0$ (see Figure 5C), a large percentage of them never burst, even at $2.0 < pH < 1.0$. They firstly exhibit thermal fluctuations and then get stabilized under the shape of a sphere of smaller diameter in agreement with the expected osmotic deflation. These results show further evidence of the vesicles decoration. While some of the vesicles burst at pH around 2.0, as described just before, we observe that a large percentage of them remains stable at pH around 1.0 (see Figure 5D). This difference in stability may be attributed to a polydispersity in decoration degree, the highest ones corresponding to the highest stability.

We can note that no aggregation is observed by decreasing the pH likely due to electrostatic repulsion between the highly charged vesicles.

To complete this study, it seems interesting to extend the domain of pH covered to alkaline regime. From an application point of view, it is clear that the stabilization of LUVs and GUVs by chitosan in a range of pH occurring in physiological conditions should be interesting and especially in the case of pH values from pH $\sim$ 2.0 in the stomach, skin (pH 5.2 to 7.0), blood pH $\sim$ 7.4 up to pH $\sim$ 8.0 for intestine and cerebrospinal liquid.

For this experiment, chitosan decorated LUVs are incubated at pH = 6.0 for a ratio $[NH_3^+]/[lipid] \sim 3$ ($[NH_3^+]$ is calculated from the degree of protonation of chitosan at this pH; 40% of amino groups are protonated[29]). Then the pH of bare and decorated vesicles is adjusted by progressive addition of HCl or NaOH to cover the pH range from 2.0 to 12.0 (see Figure 6).

In acidic conditions, when pH decreases, ζ-potential passes through 0 at pH = 4.0, becomes positive before reaching a final value of around +16 mV for pH < 3.0 for bare liposomes. This variation reflects the repression of phosphate dissociation. Considering coated vesicles, the trend is very different: at pH = 6.0, ζ-potential equals + 18 mV (instead of - 22 mV for bare 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 bare vesicles in relationship with adsorption of positively charged chitosan. At pH in neutral to basic medium, ζ-potential variations of LUVs with or without chitosan are studied. For bare LUVs, the ζ-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 6). For decorated vesicles, when pH is increased by addition of NaOH, the ζ-potential goes to zero at pH around 7.2 then becomes negative and finally reaches the value (−30 mV) of the bare LUVs at pH = 9.0 (see Figure 6). We stress that for pH > 7.0, negative contributions of the phosphate acid and of the carboxyl groups (produced by the apolar chain oxidation) in the phospholipids become predominant. In addition, for chitosan coated–LUVs, repression of the chitosan protonation occurs. Two hypotheses are conceivable: chitosan desorption or remaining decoration with the uncharged chitosan at pH > 9.0. To solve this problem, complementary experiments are developed.

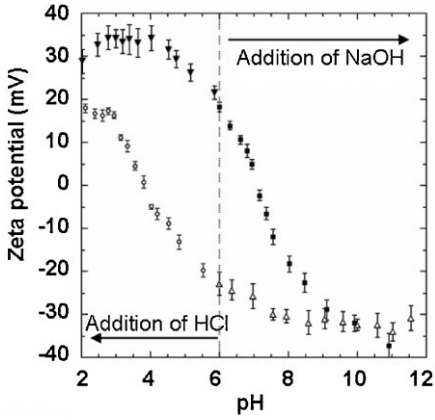

Figure 6.
Variation of the zeta potential as a function of the measured pH for bare LUVs (○ and Δ) and for chitosan-decorated LUVs (decoration at pH = 6.0) (▼ and ■). Reproduced from[19] with the permission of the American Chemical Society. Copyright 2008.

Comparative experiments are performed on bare GUVs (Figure 7A, B) and on GUVs decorated with chitosan (decoration is performed at pH 6.0) (Figure 7C.). In both cases, from pH = 6.0 (pictures a. Figure 7A, B and C) to pH = 10.0 (pictures b, Figure 7 A, B and C), vesicles remain spherical whatever their initial size (from 5 to 50 μm) and keep their initial diameter. We stress that, in these experimental conditions, the osmotic shock is negligible. At pH > 10.0, the osmotic shock is no more negligible. Behaviors of bare and decorated vesicles become different. As far as bare 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).[32,33] Such behavior was previously observed in the same pH conditions.[34]

In the case of decorated vesicles, GUVs remain spherical and their diameter decreases with increasing the amount of NaOH added for pH > 10.0. The variation of the diameter is in good agreement with the osmotic pressure variation (Figure 7C).[35] The chitosan-decorated vesicles (Figure 7C f.) remain stable after 2 hours (Figure 7C g.).

The difference in behavior between bare and decorated vesicles in neutral and basic conditions (up to pH = 11.0) demonstrates

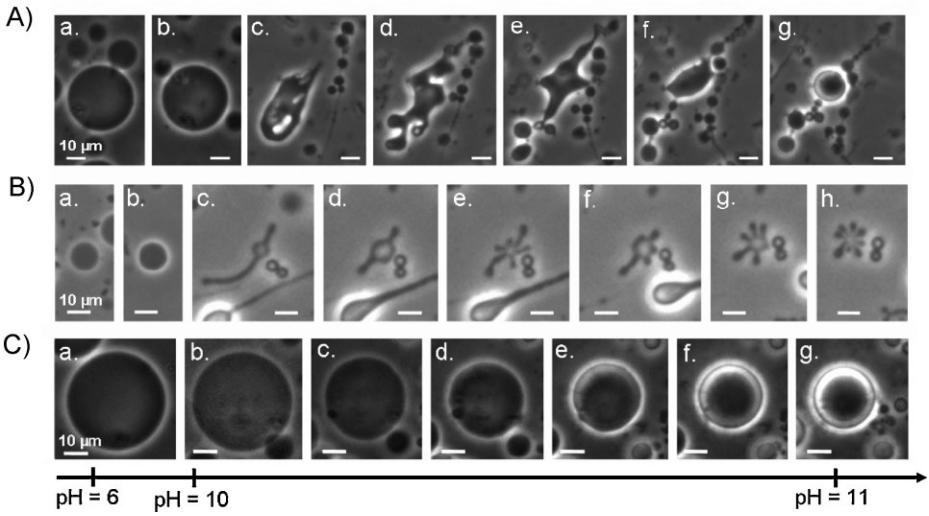

Figure 7.
Behaviors of bare GUVs (sequences A and B) and chitosan decorated GUVs (sequence C) as a function of pH ($6 < pH < 11$, produced by NaOH shocks). Delay between each picture is 10 seconds. The scale bars represent 10 µm. Reproduced from[19] with the permission of the American Chemical Society. Copyright 2008.

that chitosan remains adsorbed (at least partially) on liposomes. This was confirmed by fluorescent microscopy observation of vesicles decorated with fluorescent chitosan.

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

Adsorption and Mechanism of Interaction

In order to determine the exact amount of chitosan adsorbed (and consequently the amount of free chitosan in solution) in given experimental conditions, the pH of the suspension was fixed at two controlled pH values: 6.0 and 3.5; in these conditions, the net charge of the different constituents is fixed in the suspension. The results are given in Figure 8.

Both chitosan and lipid exact concentrations are required to establish the isotherm of adsorption. As far as lipids are concerned, 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.[20] Dealing with chitosan, it is necessary to determine both its concentration free in solution and its quantity adsorbed on the membrane. As no separation of LUVs from supernatant was possible, we assume that when we add very small amounts of chitosan solution prepared at $pH = 6.0$ (chitosan is partially positively charged, 40% of protonated amino groups[29]) chitosan is fully adsorbed on the liposomes which are negatively charged; then, the variation of ζ-potential is directly related to the amount of NH_3^+ chitosan adsorbed on the membrane, allowing to draw the resulting isotherm of adsorption at $pH = 6.0$.

Figure 8a confirms that the interaction is independent on the molecular weight of chitosan. At $pH = 3.5$, the chitosan is fully positively charged but the lipids are now positively charged; in these conditions the amount of chitosan adsorbed is determined from the variation of ζ-potential using the calibration relationship between ζ-potential variation and adsorbed NH_3^+ chitosan established at $pH = 6.0$. The lower difference ($\Delta\zeta$) between the ζ-potentials of the

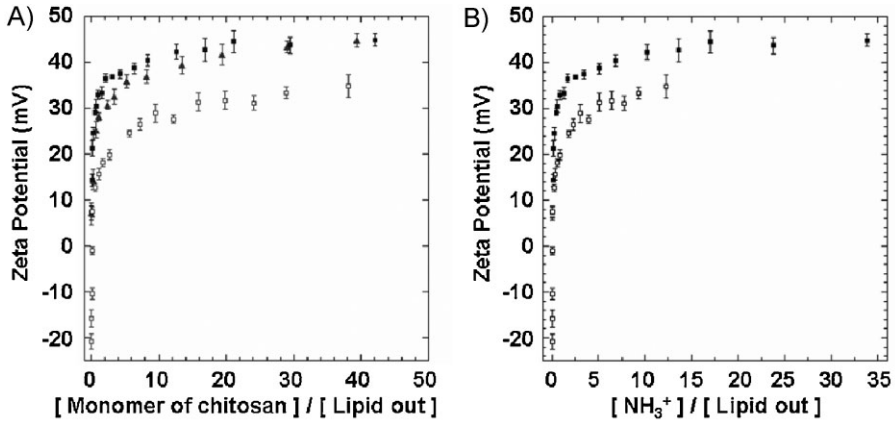

Figure 8.
ζ-potential of the LUVs (DOPC/18:1 LR) in the presence of chitosan as a function of the ratio of: (a) amount of chitosan repeat units or (b) amount of chitosan protonated amino groups over the accessible lipids on the external leaflets of the membrane. Data were obtained with chitosan Mw = 5×10^4 at pH = 3.5 (▲), and with chitosan Mw = 5×10^5 for at pH = 3.5 (■) and at pH = 6.0 (□). Reproduced from[20] with the permission of the American Chemical Society. Copyright 2008.

bare (initial value) and of the fully decorated liposome (plateau value) is lower at pH = 3.5 than at pH = 6.0.

This shows that interaction between chitosan and liposomes is looser at pH = 3.5 but does exist even if chitosan and membrane are both positively charged. Knowing the amount of charged amino groups fixed on the membrane surface, we can calculate the quantity of chitosan adsorbed taking into account both protonation (controlled by pH[30]) and acetylation degrees. The adsorption isotherms obtained at pH = 3.5 and 6.0 are discussed later (see Figure 10a), in which the chitosan adsorbed (in repeat unit) is expressed as a function of the chitosan free in solution, at equilibrium.

Hyaluronan-Vesicle Interaction

To complete the study of the interaction between polyelectrolytes and lipid membranes, we now focus on the adsorption of an anionic polyelectrolyte, the hyaluronan. We investigate the role of charge density and ionic strength on the adsorption of initial hyaluronan (HA) on the zwitterionic membrane of liposomes.

Influence of pH, Salt and Hyaluronan on the ζ-Potential of LUVs

To study the influence of the charge density of hyaluronan on its adsorption on LUVs membrane, experiments at two pH values (pH = 3.5 and pH = 6.0) are performed. 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).[36]

In Figure 9, the variation of ζ-potential as a function of added HA expressed by the ratio of ionized carboxylic groups (COO^-) per lipids of the external leaflet in absence and presence of salt (NaCl).

At pH = 6.0, in absence of salt (Figure 9A, ■), we observe that ζ-potential of the bare LUVs equals −18 mV and goes to a constant value of −31 mV in the presence of an excess of HA, while at pH = 3.5 (Figure 9B, ■), the initial ζ-potential equals +9 mV and goes to a constant value of −9 mV. 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. In absence of added salt, the total variations of

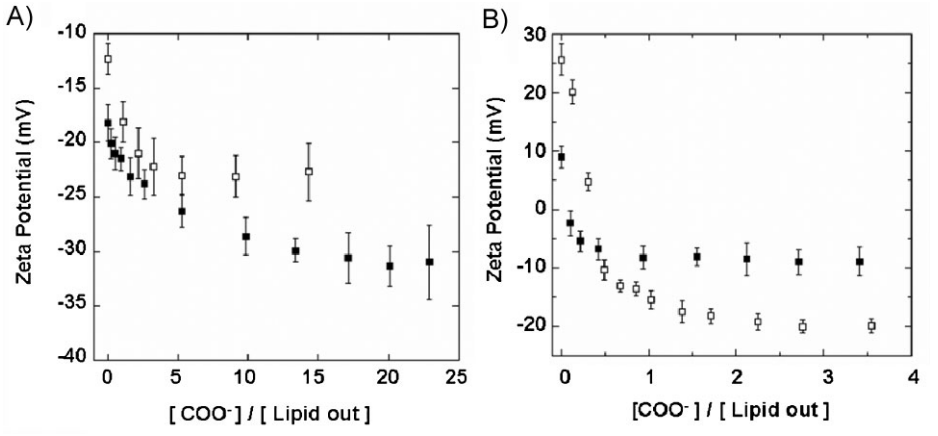

Figure 9.
Variation of the zeta potential upon addition of HA in absence (■) or presence of external salt (5mM NaCl) (□). A: pH = 6.0; B: pH = 3.5

ζ-potential (Δζ) are equals to 18 mV at pH = 3.5 and 13 mV for pH = 6.0. The curve at pH = 3.5 goes to a plateau corresponding to ratio $X_{limit} = [COO^-]/[Lipid] \sim 0.5$, whereas at pH = 6.0, it decreases smoothly and tends to a plateau for a ratio $X_{limit} \sim 15$. The difference in X_{limit} and Δζ can be interpreted in terms of electrostatic interactions: at pH = 3.5, the affinity is stronger than at pH = 6.0; at pH = 3.5, the negatively charged hyaluronan interacts with the positively charged zwitterionic lipid membrane; at pH = 6.0, both are negatively charged.

In order to observe the role of salt concentration as previously examined for chitosan, ζ-potential measurements were performed in a 5 mM NaCl solution. For HA, at pH = 6.0 (Figure 9a, □), we observe that the ζ-potential of the bare LUVs equals −12 mV (we have already observed that the initial ζ-potential of liposome at pH = 6.0 is sensitive to the presence of salt; see Figure 3a, □) and reaches a constant value

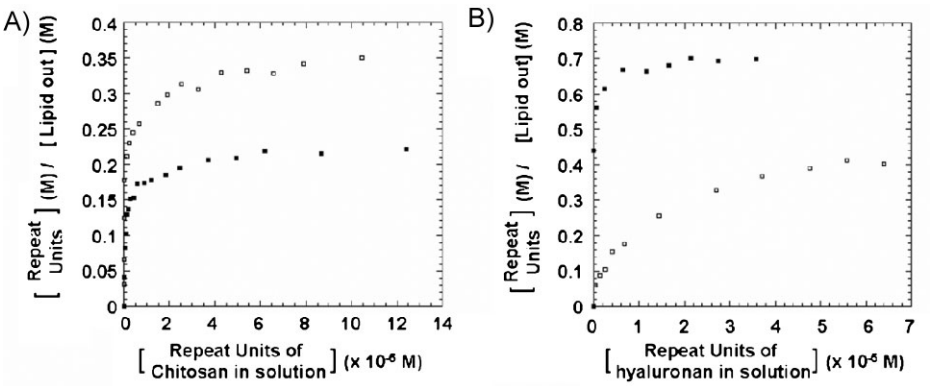

Figure 10.
Adsorption isotherms of chitosan (in $\times 10^{-6}$M) (a) and HA (in $\times 10^{-5}$M) (b) on liposomes expressed as repeat units adsorbed per lipid polar head (in the external leaflet) versus the repeat unit concentration of free polyelectrolyte in solution at equilibrium for pH = 3.5 (■) and pH = 6.0 (□).

of −23 mV more rapidly than in absence of salt ($X_{limit} = [COO^-]$ / [Lipid] ~ 2.5). The screening effect of salt depressed the electrostatic repulsions between negative liposome and negative polyelectrolyte and favors the polyelectrolyte adsorption (X_{limit} lower). For HA in 5 mM NaCl solution, at pH = 3.5 (Figure 9B, □), the initial ζ-potential 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_{limit} ~ 1.5). The large Δζ value indicates that more HA carboxylic charges are adsorbed than in absence of salt.

A tentative quantification of these results is proposed as previously proposed for chitosan[20]: we also determine the calibration relationship between the variation of ζ-potential and the amount of polyelectrolyte strongly adsorbed on the membrane in the low HA concentration zone; then, we can estimate the fraction of carboxylic charges fixed on liposome surface and the degree of decoration by hyaluronan, taking into account that there is one ionic charge at maximum per HA repeat unit. These data can be compared with that obtained for chitosan in the next paragraph.

Adsorption Isotherm of Hyaluronan; Comparison with Chitosan

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 for pH < 7.0.

The fraction of liposome surface covered by chitosan reaches an upper value of 40% at pH = 6.0 (assuming same order of magnitude for chitosan repeat unit (one sugar unit) and lipid polar head areas) where the maximum electrostatic adsorption occurs (chitosan is positively charged and membrane negative). The very low chitosan concentration at equilibrium confirms the strong interaction between chitosan and vesicle surface and allows to justify the stabilizing role of chitosan on the lipid membrane of GUVs under salt, pH and glucose stresses.[19,20] For HA, the amount of repeat units adsorbed is larger than for chitosan with a stronger affinity at pH = 3.5 (membrane is positively charged and hyaluronan is negative); the liposome surface decoration (two sugar units per HA repeat unit) rate reaches 140% at pH = 3.5.This high degree of coverage might suggest that loops are formed by HA at the surface in relation with its high molecular weight. The vesicle decoration by hyaluronan is important in view of applications in the biomedical domain due to the large biocompatibility of HA which may be considered as an interesting protecting polymer.[36]

Evidence of Chitosan-HA Interaction at the Interface of LUVs

The adsorption of successive layers of positively charged and negatively charged polyelectrolyte on the membrane of liposomes was also investigated. Considering previous data, the first polymer adsorbed was chitosan followed by adsorption of hyaluronan in order to improve the stability of the complex system. Hyaluronan, a polysaccharide largely present in human tissues, is used as external layer in order to ensure an optimized compatibility in the body, in case of eventual biomedical applications.[37]

In order to characterize this polyelectrolyte complex formation on the lipid membrane, zeta potential 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 pH. The initial lipid membrane is negatively charged at pH = 6.0 and 4.7, while at pH = 3.5, it is positively charged, as show previously.

Then, LUVs were first incubated in a chitosan solution at a ratio [monomer of Chitosan] / [Lipid] ~ 5 to form the first layer of chitosan on the liposomes. This ratio corresponds to a significant degree of decoration by chitosan without too much polyelectrolytes free in solution, as deter-

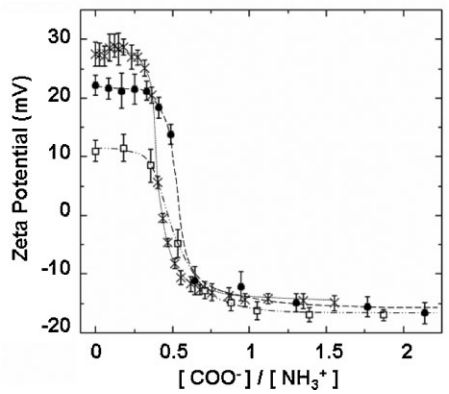

Figure 11.
ζ-potentials of chitosan decorated-LUVs (Mw chitosan = 2.25×10^5) in the presence of hyaluronic acid (HA, Mw = 7.08×10^5) as a function of the ratio of the amount of ionized HA carboxyl groups over the amount of chitosan protonated amino groups. The data were obtained at pH = 3.5 (×), pH = 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. Reproduced from[21] with the permission of the American Chemical Society. Copyright 2008.

mined from the isotherm. The zeta potentials of the chitosan 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 11 shows variation of zeta potential of the chitosan-decorated LUVs as function of the amount of HA added for the three different pH.

Upon addition of HA, we first observed that the zeta potential 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 pH. The initial positive plateau may be explained by the formation of a complex between added HA and chitosan free in solution (non-adsorbed 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 zeta potential before reaching a negative plateau. Additional experiments are needed to evidence the stability of the polyelectrolyte complex-vesicles assembly formed; for that purpose, GUVs observation in the same conditions is in progress in our laboratory.

Conclusion

ζ-potential measurements on LUV first allow us to determine the influence of pH, ionic salt concentration, and polyelectrolyte charge on the interaction between polyelectrolyte and lipid membrane. First, chitosan adsorption isotherm gives a maximum degree of decoration of 40% in surface coverage which may implies heterogeneity in the physical properties of the membrane. Our results lead us to conclude that electrostatic interactions are responsible for the polyelectrolyte adsorption, which is assumed to occur flat on the external surface of the liposomes in relation with the independence of adsorption upon MW. The vesicles are positively charged in the presence of chitosan in acid medium and down to pH = 7.2

Direct optical microscopy observations of GUVs shows a stabilization of the composite liposomes under different external stresses (pH and salt shocks) according to the degree of coverage which confirms the strong electrostatic interaction between polyelectrolytes and lipid membrane. The liposomes are stabilized by chitosan adsorption in a very wide range of pH ($2.0 < pH < 12.0$).

When a negatively charged polyelectrolyte is added to vesicles, vesicles turn rapidly negatively charged in presence of adsorbed HA whatever the pH tested in presence or absence of NaCl. The degree of coverage with hyaluronan is found to be four times that obtained with chitosan. Complementary experiments on GUVs are now in progress to test the stabilization of GUVs against different stresses (salt, pH, adhesion, osmotic pressure) in the presence of HA to further compare chitosan and hyaluronan effects on vesicle stabilization.

Finally, we demonstrate that hyaluronan adsorbs on positively charged chitosan-decorated liposomes leading to charge inversion in the liposome now decorated with a chitosan-hyaluronan bilayer. This opens the way to polyelectrolyte multilayer formation at the surface of vesicles opening the way to address the question of their role on the stabilization and charge control of vesicles.

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