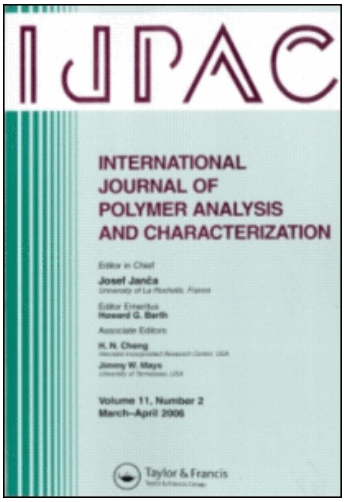

International Journal of Polymer Analysis and Characterization
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713646643

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Online publication date: 20 November 2009

To cite this Article Rinaudo, M. , Quemeneur, F. and Pépin-Donat, B.(2009) 'Stabilization of Liposomes Against Stress Using Polyelectrolytes: Interaction Mechanisms, Influence of pH, Molecular Weight, and Polyelectrolyte Structure', International Journal of Polymer Analysis and Characterization, 14: 8, 667 — 677
To link to this Article: DOI: 10.1080/10236660903289623
URL: http://dx.doi.org/10.1080/10236660903289623

International Journal of Polymer Anal. Charact., 14: 667–677, 2009

ISSN 1023-666X print
DOI: 10.1080/10236660903289623

Stabilization of Liposomes Against Stress Using Polyelectrolytes: Interaction Mechanisms, Influence of pH, Molecular Weight, and Polyelectrolyte Structure

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Abstract: This work concerns the stabilization of liposomes by biocompatibly charged polysaccharides: chitosan, a positively charged polyelectrolyte at pH < 6.0, and hyaluronan, a negatively charged polyelectrolyte at pH > 2.0. The charge density of these two biopolymers depends on pH as well as the charge density of the zwitterionic lipidic membrane. The ζ-potential measurements were performed on LUVs as a function of pH for various polyelectrolyte structures. Results allow us to conclude that the interactions between the external surface of vesicles and polyelectrolytes are mostly of electrostatic origin. Then, we address the question of the role of polyelectrolyte decoration on the stability of vesicles in acidic conditions by optical microscopy observations on GUVs. It is clear that decorated vesicles are more resistant to different external stresses than the bare ones.

Submitted 20 June 2009; accepted 27 August 2009.
Presented at the 21st International Symposium on Polymer Analysis and Characterization held 22–24 June 2009 at the Tomas Bata University in Zlin, Zlin, Czech Republic. We acknowledge financial support by the International Research Training Group 667 "Soft Matter Physics of Model Systems" and the German-French University (UFA-DFH) Saarbrücken.

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Keywords: Adsorption of polyelectrolyte; Chitosan; Hyaluronan; Liposome; Stabilization of lipidic membrane against stresses

INTRODUCTION

Adsorption of polyelectrolytes on charged surfaces plays an important role in materials science and biomedical applications.[1,2] In particular, the interactions between polyelectrolytes and charged lipid bilayers, especially self-closed bilayers named liposomes, have been extensively investigated. Such polyelectrolyte-decorated liposomes simulate biological membranes and allow the study of polymer-cell interactions.[3–5] From an application point of view, polyelectrolyte decoration of liposomes may enhance efficiency of drug formulation.[6,7]

A large range of liposome sizes is available: large unilamellar vesicles (LUVs with 100–500 nm diameter), used as protective capsules for medical applications,[8,9] and giant unilamellar vesicles (GUVs with 0.5–100 μm diameter), generally studied as oversimplified models of biological cells.[10] GUVs under study are prepared by electroformation[11] from DOPC, a zwitterionic phospholipid, in the presence of a sucrose solution, and LUVs with a 200 nm diameter are obtained by extrusion of GUVs through a calibrated filter.

The present work concerns liposome stabilization by biocompatibly charged polysaccharides: chitosan, a positively charged polyelectrolyte at pH < 6.0, and hyaluronan, a negatively charged polyelectrolyte for pH > 2.0. The charge density of both the two biopolymers and the lipidic membrane varies as a function of pH.[12–15]

EXPERIMENTAL SECTION

Materials

Lipids

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Mw = 786.15), 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (18:1–12:0 NBD PC) (Mw = 881.53), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (18:1 Liss Rhod PE) (Mw = 1301.73) were purchased from Avanti Polar Lipids and dissolved separately as received in a chloroform/methanol solution (9/1 volume ratio) at 10 mg/mL. Fluorescent labeled lipids were then mixed with DOPC in a weight ratio of 50:1 for the 18:1–12:0 NBD PC and 80:1 for

the 18:1 Liss Rhod PE to a total concentration of 1 mg/mL. Solutions were kept at −20°C until used. Sucrose, glucose, NaOH, and HCl were purchased from Sigma-Aldrich and used as received.

Highly purified 18.2 MΩ · cm water was used for the preparation of all the solutions.

Giant Unilamellar Vesicles (GUVs)

GUVs, filled with a 200 mM sucrose solution, were obtained by the electroformation process.[11] This procedure essentially leads to formation of GUVs of radii between 5 and 50 μm.

Large Unilamellar Vesicles (LUVs)

LUVs were obtained by extrusion through a 0.2 μm filter of the GUVs previously obtained by electroformation. LUVs prepared in these conditions are unilamellar.[16,17] The extruded LUVs of 200 ± 10 nm diameter encapsulating a 200 mM sucrose solution were suspended in an external 200 mM sucrose solution at pH = 3.5 or pH = 6.0. The exact lipid concentrations were determined for each LUV sample by spectrofluorometry in relation to the content in the labeled lipid involved.[13]

Polyelectrolytes

Chitosan (Chit) is a linear random copolymer of D-glucosamine and N-acetyl-D-glucosamine with a molecular weight M_w of 5×10^5 and a degree of acetylation (DA) of 0.2, purchased from Kitomer (Marinard, Canada). Hyaluronic acid (hyaluronan, HA) is a linearly alternated copolymer of D-glucuronic acid and N-acetyl-D-glucosamine purchased from ARD (Pomacle, France). Its weight-average molecular weight Mw is 6.63×10^5. The chemical structure of these two polysaccharides is given in Figure 1. In order to observe the membrane decoration by polyelectrolyte, we have labeled chitosan with fluorescein (Chit-Fluo)[18] and hyaluronan with rhodamine (HA-Rhod).[19]

The solution of anionic polyelectrolyte (HA) was prepared at 0.4 g/L by dissolving the polymer in an aqueous solution of 200 mM sucrose at pH = 6.0, while solubilization of cationic chitosan (Chit) requires addition of a stoichiometric amount of HCl on the basis of $-NH_2$ content in the chitosan (final pH around 3.5). The solutions of polyelectrolyte were stirred for one night at room temperature, until complete solubilization. The solutions of polyelectrolyte were diluted for vesicle incubation at 0.1 or 0.01 g/L in a solution of 200 mM sucrose at pH = 3.5 or 6.0 and directly used.

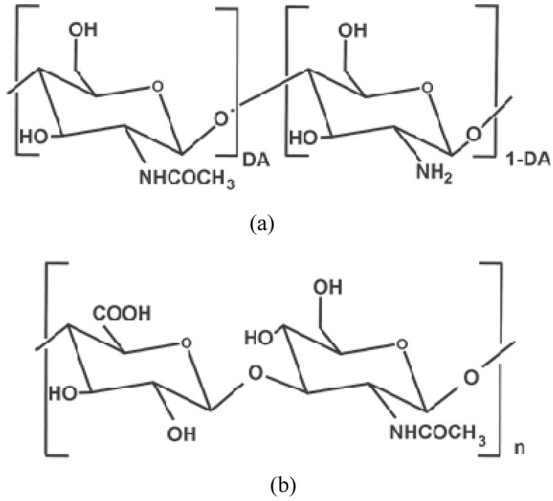

Figure 1. Schematic representation of polyelectrolyte repeat unit structure: (a) chitosan and (b) hyaluronan.

Methods

ζ-Potential and Dimension Measurements on LUVs

ζ-potential and dimension measurements on LUVs were performed at 20°C with a commercial Zetasizer (Zetasizer NanoZS, Malvern, France). The ζ-potential values were determined using the Smoluchowski equation relating the ionic mobilities with the surface charge and averaged over 10 repeated measurements. Particle sizes were controlled in situ by light-scattering measurement: extruded LUVs exhibit a 200 ± 10 nm diameter. For each ζ-potential measurement, the following protocol was repeated: a given volume of polyelectrolyte solution tested was added to the liposome suspension; after homogenization by stirring and 30 min incubation, 1 mL of this mixture was injected in the Zetasizer Nano Cell; ζ-potentials and average sizes of the dispersed liposomes were then measured. After each measurement, the whole solution was 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 polyelectrolyte solution.

Microscopy Observations

In order to avoid interaction between GUVs or polyelectrolytes with the glass substrate, the glass was covered by a hydrophilic polymer,

poly(ethylene glycol) (PEG), grafted covalently onto the glass according to the protocol given by Zhang et al.[20] The GUVs were transferred to a viewing cell filled with a 200 mM glucose solution. The lower density of the surrounding glucose solution causes the sucrose-filled vesicles to sediment to the bottom of the cell and creates a variation of optical index between the inner and outer media of vesicles required for phase-contrast microscopy observation.

We found that, whatever the polyelectrolyte, decorated GUVs always exhibit some loose interactions with the glass plate, probably due to a weak adhesion even after passivation of the surface with PEG.[20] In the absence of glass treatment, however, chitosan- and hyaluronan-decorated vesicles sediment, adhere, and finally burst.

Phase-contrast microscopy observations were performed using a phase-contrast inverted microscope (Olympus CKX41) equipped with 10× and 20× objectives and an AVT MarlinF080B numerical camera (Imasys, Suresnes, France).

Confocal microscopy observations were performed with a UltraView LCI Nipkow Disk scanner (PerkinElmer GmbH, Rodgau-Jügesheim, Germany) attached to a Zeiss Axiovert 200 microscope (Zeiss GmbH, Heidelberg, Germany) equipped with a C-Apochromat 63X, 1.2 NA water immersion objective. GUV observations were made at 488 nm excitation using 500 LP emission filters for the chitosan probed with fluorescein (Chit-Fluo) or the 18:1–12:0 NBD PC lipids and at 568 nm excitation and 600/45BP emission filters for the HA labeled with rhodamine (HA-Rhod) or the 18:1 Liss Rhod PE lipids. Fluorescence acquisitions at these two excitation wavelengths were made successively.

RESULTS AND DISCUSSION

Polyelectrolyte-Lipid Membrane Interaction

Adsorption of each of the two polyelectrolytes on the surface of the zwitterionic GUV membrane is directly observed by fluorescent confocal microscopy at pH = 6.0 (see Figure 2): as expected, positive chitosan adsorbs on a negatively charged membrane (a); the striking point is that it is also the case for negative hyaluronan (b). It was previously demonstrated by confocal microscopy that this decoration by both polyelectrolytes is stable over a large range of pH (from pH = 1.0 up to pH = 10.0) whatever the respective net charge of the lipidic membrane and polyelectrolyte.[19]

At the sub-micrometric scale, the role of polyelectrolyte structure and pH on ζ-potential measurements of LUVs is studied. Upon progressive additions of chitosan in a LUV suspension (at the initial

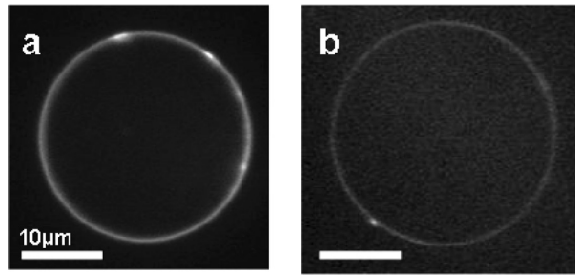

Figure 2. Confocal microscopy observations of chitosan (a) and hyaluronan (b) decoration of DOPC GUVs at pH = 6.0.

pH of 3.5 or pH = 6.0) (see Figure 3), we observed a large variation of the ζ-potential from −23 to +30 mV at pH = 6.0 and from +5 to +45 mV at pH = 3.5. Results allow concluding that interactions between the external surface of the zwitterionic lipid bilayer and the polyelectrolyte are mostly of electrostatic origin.

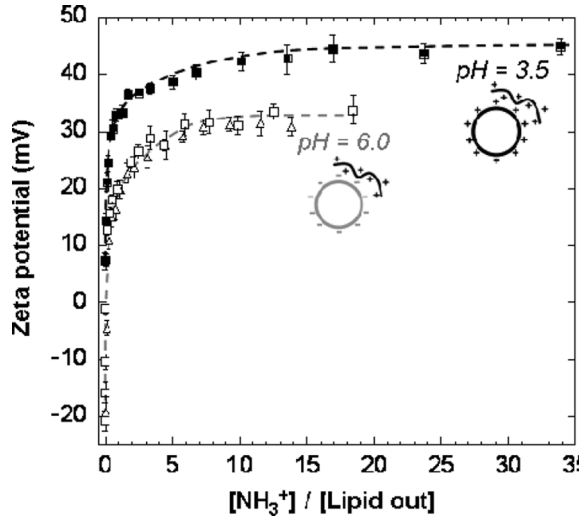

Figure 3. Variation of ζ-potential of the LUVs in the presence of chitosan as a function of the added amount of polyelectrolyte, at pH = 6.0 and 3.5, expressed by the ratio of concentrations between the ionized groups (NH_3^+) and the accessible lipids of the external leaflets of the membrane. Data were obtained with chitosan (Mw = 5×10^5, DA = 0.2) at pH = 6.0 (open square) and at pH = 3.5 (solid square) and chitosan labeled with fluorescein (Chit-Fluo, Mw = 5×10^5) at pH = 6.0 (open triangle).

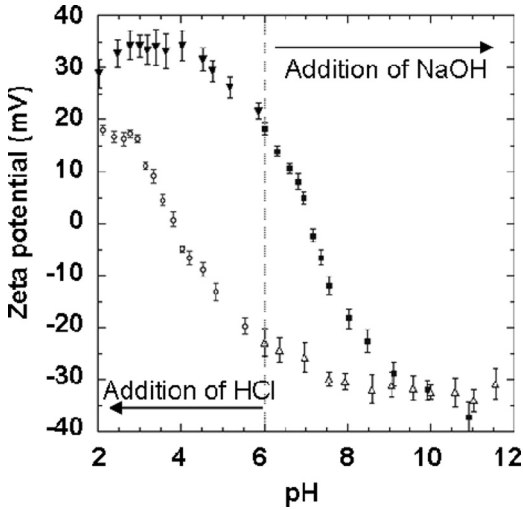

Figure 4. Variation of the ζ-potential as a function of the measured pH for bare LUVs (◯ and △) and for chitosan-decorated LUVs (decoration at pH = 6.0) (▼ and ■). Reproduced from Quemeneur et al.[13] with the permission of the American Chemical Society. Copyright 2009.

In Figure 3, the data obtained with the fluorescent chitosan are given for pH = 6.0; they confirm that the fluorescent probe does not modify the physical behavior of chitosan.

In addition, the same ζ-potential variation is obtained with different chitosans of variable molecular weights and DA when the chitosan amount added is expressed as a ratio of $-NH_3^+$/accessible lipid.[21] These results reveal the existence of a strong interaction between chitosan and lipid membrane and allow concluding that chitosan probably adsorbs flat on the vesicle surface.

In Figure 4, the ζ-potential of bare vesicles is compared with that of chitosan-coated vesicles in a large domain of pH values ranging from 2.0 to 12.0. It shows that the isoelectric point is obtained for pH values of 4.0 and 7.2, for bare and decorated vesicles respectively. In addition, at pH = 2.0, chitosan, even when it is highly positively charged, largely influences the ζ-potential of positively charged vesicles. At higher pH, the ζ-potential turns to negative for the two types of vesicles. In this pH domain, chitosan becomes insoluble in aqueous medium due to its zero charge; then, it is important to determine if chitosan still interacts with the vesicle membrane. The remaining chitosan decoration was directly observed using fluorescent chitosan on GUVs in confocal microscopy performed at pH = 10.0.[19]

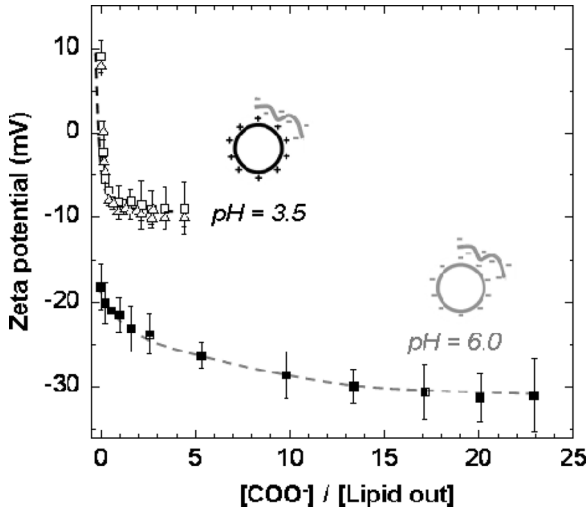

Figure 5. Variation of ζ-potential of the LUVs in the presence of hyaluronan as a function of the added amount of polyelectrolyte at pH = 6.0 and 3.5 expressed by the ratio of concentrations between the ionized groups (COO^-) over the accessible lipids of the external leaflets of the membrane. Data were obtained with hyaluronan ($Mw = 6.63 \times 10^5$) at pH = 6.0 (solid square) and at pH = 3.5 (open square) and hyaluronan labeled with rhodamine (HA-Rhod, $Mw = 6.63 \times 10^5$) at pH = 3.5 (open triangle).

Experiments on adsorption of hyaluronan are also performed at the same two pH values. The main results are given in Figure 5. At pH = 6.0, negatively charged vesicles interact with negatively charged hyaluronan and the ζ-potential becomes highly negative and stabilizes the dispersion of vesicles. At pH = 3.5, the vesicles are positively charged but strong interaction with hyaluronan causes the charge inversion for a small ratio [COO-]/[lipid out] = 0.03. Then, whatever the pH, in excess of hyaluronan, the coated vesicles become negatively charged.

From the data obtained for ζ-potential measurements on LUV suspensions, it is shown that adsorption of polyelectrolytes with different net charge allows controlling the net charge of zwitterionic lipid vesicles. Dealing with chitosan, its adsorption gives a positive vesicle in acidic conditions up to pH = 7.2; for pH > 7.2, chitosan-decorated vesicle becomes negative (we stress that chitosan remains adsorbed on the external surface up to pH = 10.0). In the presence of a small amount of HA ([COO-]/[lipid out] > 0.03), the HA-decorated vesicle is negatively charged for all pH ≥ 3.5.

In the case of hyaluronan adsorption, it is shown that ζ-potential evolution is largely dependent on the molecular weight: the higher the molecular weight, the higher the negative charge of the coated vesicles. From these results, it may be concluded that hyaluronan is adsorbed by a train-and-loop mechanism.[21]

Stability of Lipid Membranes in the Presence of Polyelectrolyte

The role of polyelectrolyte decoration on the stability of vesicles is now examined. Therefore we directly observed the behavior of GUVs by optical microscopy under external stresses. Using fluorescent-labeled polyelectrolytes, we observed that decoration is stable over a large range of pH values ($2.0 < pH < 10.0$) as mentioned before and that the decorated vesicles are more resistant to different external stresses such as pH, salt, and osmotic shocks than the bare vesicles.[12,13,19] For example, the resistance of decorated vesicles in acidic conditions is illustrated in Figure 6: the bare vesicles observed at $pH = 2.0$ immediately burst, while down to $pH = 1.5$ both chitosan- and hyaluronan-decorated vesicles remain stable and exhibit only a progressive spherical osmotic deflation. A small fraction of decorated-vesicles bursts after some 30 min,

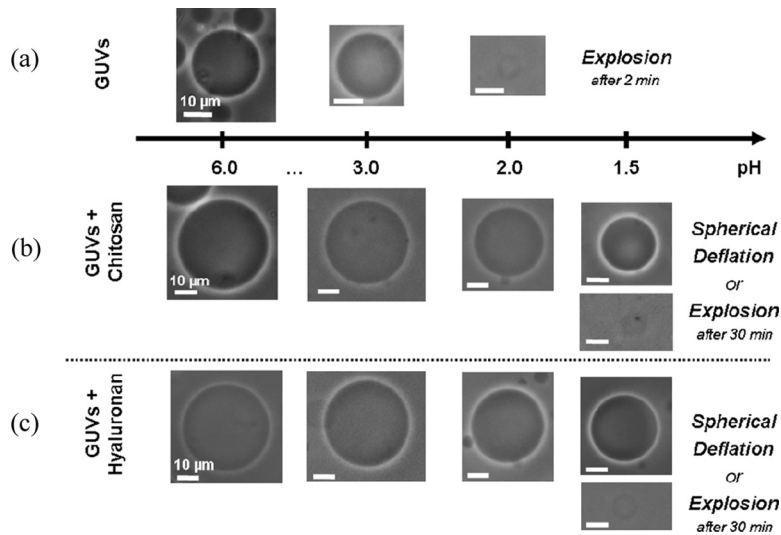

Figure 6. Behavior of non-decorated GUVs (a) and chitosan-decorated (b) or hyaluronan-decorated (c) GUVs as a function of pH, in acidic conditions. The scale bars represent 10 µm.

which is explained in terms of decoration heterogeneity (differences in coverage degree from one vesicle to another).

These original results allow concluding that, for the two polyelectrolytes with different structures and polarities, in a large range of pH values, the adsorption stabilizes the vesicles, forming a polyelectrolyte-complexed interface.

CONCLUSION

Polyelectrolyte decoration and pH adjustment allow tuning in a controlled way the net charge of the coated vesicles from positive to negative. Decoration by both biocompatible chitosan and hyaluronan dramatically enhance resistance of vesicles towards external stresses such as pH, salt shocks, and osmotic pressure. It also allows controlling the interaction of vesicles with charged surfaces or polymers. This holds promise to extend the conditions for liposome applications, especially in the pharmaceutical or biomedical domains.

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