Lipidic composite vesicles based on poly(NIPAM), chitosan or hyaluronan: behaviour under stresses

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Lipidic composite vesicles based on poly(NIPAM), chitosan or hyaluronan: behaviour under stresses

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ABSTRACT

Giant Unilamellar Vesicles (GUVs) consisting in self-closed lipid bilayers of 0.5-100 µm diameter are considered as oversimplified models of cells because of their biological membrane and micrometric size while Large Unilamellar Vesicles (LUVs) of 100-500 nm diameter have applications in drug delivery. To improve structural and mechanical properties of these vesicles, we have developed two categories of composite polymer-vesicles. The first category is prepared by encapsulating solutions or networks of poly (N-isopropyl-acrylamide) (polyNIPAM) chains. PolyNIPAM exhibiting a Low Critical Solution Temperature (LCST) at 32°C, composite NIPAM-vesicles are thermo-responsive. The second category of vesicles is obtained by adsorption of polyelectrolytes (chitosan or hyaluronan or both layer-by-layer) on their outer surface. Chitosan and hyaluronan are respectively positively and negatively charged polymers; both are biocompatible and allow to tune the net charge of the vesicles. All these composite vesicles hold promise as passive mechanical models of cells and as drug carriers because of their improved structural and mechanical properties and enhanced resistance to various mechanical or chemical stresses if compared to unmodified vesicles. Poly (NIPAM) vesicles present the additional advantage to be potential thermo-responsive drug carriers, collapsing reversibly at 32°C with a release of 98% of their internal solution.

Keywords: *Liposomes; Cell models; Polyelectrolyte adsorption; NIPAM; Thermo-responsive material; Volume transition*

INTRODUCTION

Vesicles (often referred as liposomes) consisting of a self-closed phospholipidic membrane enclosing water are used as oversimplified models of cells or drug carriers when they exhibit micrometric or sub-micrometric sizes respectively. Nevertheless their very poor mechanical properties and resistance to various stresses such as salt or pH shocks limit their relevance to mimic real cells and their efficiency as drug carriers.

Living cells are very complex systems in which biological and mechanical properties are reciprocally regulated. Nevertheless, it appears that the first steps of many biological processes are mainly controlled by their mechanical properties. It is then of interest to develop biomimetic polymeric "capsules" with adjustable mechanical properties that can be considered as "passive mechanical models" of cells. This approach may allow decephering biological processes by studying the response of such models in biological situations (adhesion to a substrate, movement in a flow…) as a function of their mechanical parameters. Preparation of such composite polymeric viscoelatic vesicles at the sub-micrometric scale may allow getting drug carriers with improved in vivo resistance. The first step to develop mechanical models of cells consists of modifying internal media of liposomes by encapsulating a polymer system [1-7] to model the viscoelastic properties of the skeleton of cells [8-11]. The second step is to improve the mechanical and structural properties of liposome membranes and among the possible ways to achieve this aim, coating membranes with various polymers is especially relevant from a biological point of view since various macromolecules such as proteins and glycoconjugates interact with lipid bilayers to form the complex membrane of living cells [12]. In addition, dealing with liposome applications for drug delivery, decoration of the membrane by a polymer may improve their structural stability, biocompatibility and drug delivery efficiency [13]. Within this context, besides neutral poly (ethylene glycol), which in particular increases in vivo liposome circulation time, chitosan, a cationic polymer was recently used to enhance liposomes biocompatibility [14], biodegradability [15] and mucoadhesivity [16]. Anionic polysaccharides such as heparin which reduces the thrombogenicity of biomaterial surface [17] or hyaluronan (HA) which confers specific targetting character to coated liposomes for cancer therapy are also of interest [18]. Additionally, HA is biodegradable and also a constituent of the extra-cellular matrix involved in the regulation of cell activity [19].

Using thermo-responsive polymer such as polyNIPAM may allow preparing composite liposomes with thermo-tunable structural and mechanical properties. Following this approach, we have firstly enclosed thermo-mechanical polyNIPAM viscoelastic systems into the internal media of vesicles and secondly coated their membrane with two different biocompatible polyelectrolytes: the positively charged chitosan and the negatively charged hyaluronan and with alternate layers of each one.

In this paper, we present the various composite polymer-vesicles developed and show their potential interest as drug carriers.

EPERIMENTAL

Preparation of the composite liposomes

GUVs are prepared from 1-2 dioleoyl sn-glycero 3-phosphocholine lipids (DOPC) or from a mixture of 99% DOPC and 1% of fluorescent-labeled (rhodamine or fluoresceine) DOPC for fluorescence experiments, using the standard electro-formation method [20] : in brief a solution of lipids (1 mg/mL in chloroform) is spread onto two conductive glass plates, the solvent is removed and the electro-formation chamber is formed by the two plates facing each other and separated by a Teflon spacer. The solution to be enclosed in the vesicle is injected in the chamber. GUVs are formed by application of an alternative potential between the two plates. LUVs are then obtained by extrusion through a 0.2 mm filter of the electroformed GUVs, their diameter is of the order of 200 ±10 nm. Preparation of polyNIPAM elastic vesicles (so called gel-vesicles) is relatively difficult: a 'pre-gel' solution (water + monomers + crosslinker + photo-initiator + possible drugs) is injected in the electro-formation chamber. Polymerization is induced by Ultra Violet-irradiation and the challenge is to prevent gelation of the external solution, which would trap unusable gel-filled vesicles in the external macroscopic gel. This is possible by a selective control of the kinetic, which allows preparing gel-vesicles easy to handle and moving freely in the external solution. Preparation of polyNIPAM viscous vesicles (so-called sol-vesicles) is easier, the method is the same as for gel-vesicles except that the solution injected in the electro-formation chamber does not contain any crosslinker; we get sol-vesicles encapsulating a polyNIPAM solution and freely moving in the same solution. Polyelectrolyte-coated vesicles are obtained by incubation of vesicles in chitosan and hyaluronan solutions at the

desired pH. Chitosans (linear β(1-4) random copolymer of D-glucosamine and N-acetyl-D-glucosamine) with different weight-average molecular weights (Mw) and degrees of acetylation (DA) and hyaluronic acids (hyaluronan; linear alternated copolymer of β(1-3) D-glucuronic acid and β (1-3) N-acetyl-D-glucosamine) with different Mw (from 1.14to104 to 1.8□106) are used. For fluorescence experiments, chitosan and hyaluronan are respectively labeled with fluoresceine and rhodamine. For experimental details on the preparation of NIPAM-vesicles, see [7] and of polyelectrolyte coated vesicles see [21] and references therein.

Characterization of liposomes

Various techniques are used to characterize the micrometric (GUVs) and sub-micrometric (LUVs) composite vesicles. Direct observation is performed on GUVs using various optical microscopy illumination techniques, for polyNIPAM systems, see [7], and for polyelectrolyte systems, see [21]. Zeta potential and polydispersity are determined on LUVs and GUVs by using a Zetasizer (Nano ZS90, Malvern, UK). Isothermal titration microcalorimetry (ITC) measurements are realized at 20 °C using a Microcal VP-ITC titration microcalorimeter (Northampton, MA). Mechanical properties of the vesicles were determined on GUVs by hydrodynamic nanotubes extrusion (see ref [22][23]), micropipette suction (see ref [24]) and by Atomic Force Microscopy in the colloid probe mode using a Molecular Force Probe (MFP) 3D AFM (Asylum Co, Santa Barbara, USA) mounted of an inverted microscope.

RESULTS AND DISCUSSION

PolyNIPAM systems

By encapsulating systems of free or covalently crosslinked polyNIPAM chains we have respectively obtained viscous and elastic vesicles so called 'sol- and gel-vesicles'. We are able to tune in a controlled way the NIPAM vesicles internal viscosities and Young moduli from 0.001 to 1 Pa.s, and from 0.5 to 25 kPa respectively, which is pertinent to mimic mechanical properties of internal media of some real cells [8,9,10,11]. Mechanical resistance of the membrane to an applied stress was tested for unmodified and polyNIPAM sol and gel-vesicles using the micropipet aspiration technique. The membrane tension σ is determined from the imposed micropipet suction pressure ΔP by the Laplace law , where Rp and Rv are respectively the internal micropipet and vesicle radii [25].

$$\sigma = \frac{\Delta P\, Rp}{2\left(1 - Rp / Rv\right)}$$

For unmodified GUVs and sol-GUVs , we found that the membrane is disrupted at tensions of the order of 0.7±0.4 mN/m. In the case of gel-GUVs, pressures as high as ΔP = 1000 Pa. can be applied without disrupting the membrane, which corresponds to a tension σ = 15 mN/m, well above the disruption tension measured for unmodified and sol-GUVs. This indicates that the two membrane leaflets are likely to be strongly coupled to the internal polymer network. Saturated DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) membranes exhibit the same strong coupling with poly(NIPAM) gel, which shows that this coupling is not completely due to the presence of covalent bonds between the NIPAM network and the lipid bilayer.

Sol or gel-vesicles are thermo-mechanical responsive because polyNIPAM exhibits a Low Critical Solution Temperature (LCST) at Tc= 32°C (see Figure 1): above this temperature, polyNIPAM chains collapse leading either to small aggregates formation in dilute solutions (local demixion of the solution) or to a global volume transition of all the chains in the case of highly concentrated solutions (strongly entangled polyNIPAM chains) or covalent gels (covalently crosslinked polymer). Because of the strong interaction polyNIPAM gel / membrane, gel-vesicles behave like homogeneous elastic beads and undergo a sharp global volume transition when the temperature is raised up to 32°C, releasing 98% of the internal solution. This transition is fully reversible and the membrane remains undamaged after few collapse-swelling cycles. This critical temperature can be varied by glucose concentration of the external solution where the vesicles are suspended of by chemical modification of NIPAM. Gel-vesicles are then promising as drug carriers with temperature controlled release.

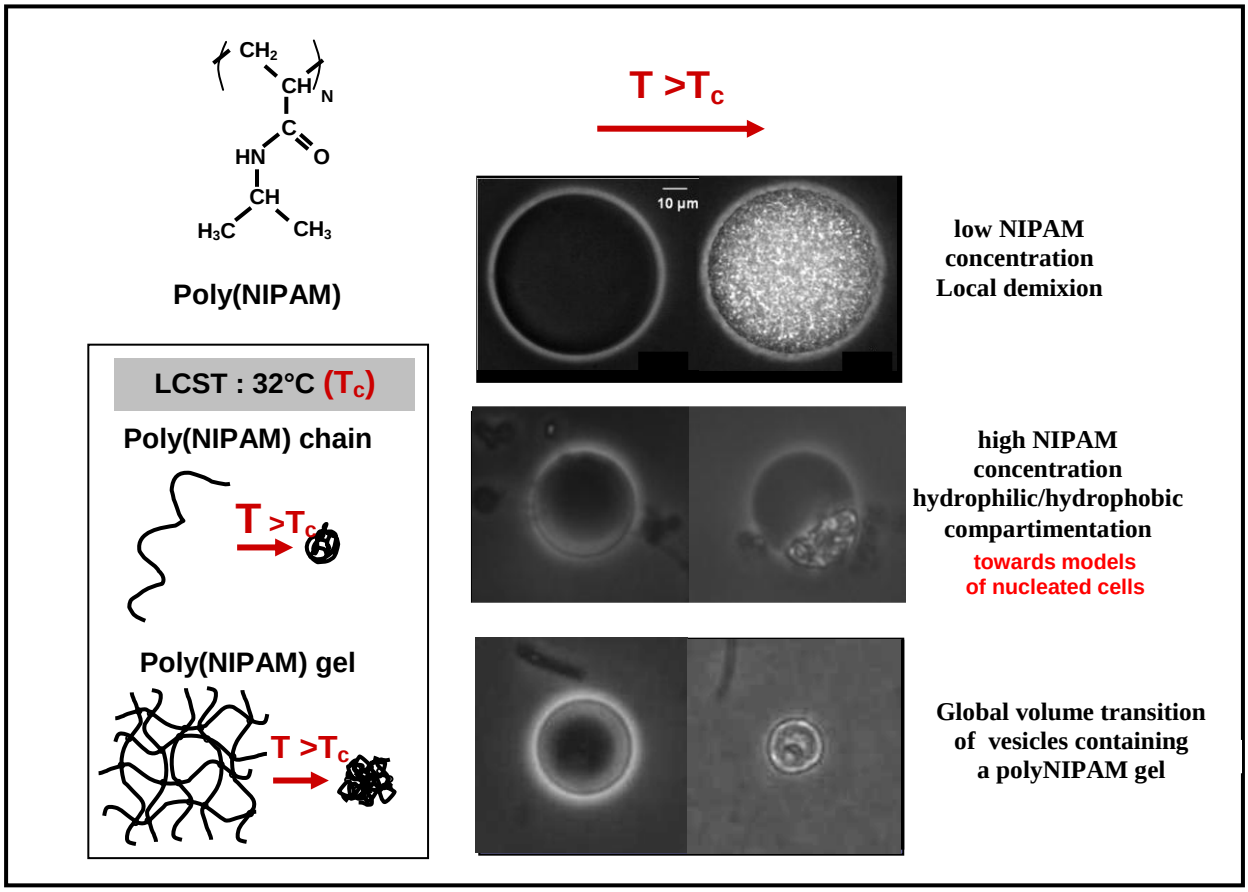

Fig.1. Thermoresponsive polyNIPAM and thermal transitions of the various kinds of NIPAM-Vesicles

To summarize, the composite vesicles exhibit three distinct behaviors as the temperature is increased up to 32°C depending on their preparation conditions : i) a global volume transition of the vesicle (decrease of 98% in the vesicle volume) ii) a volume transition of the poly(NIPAM) inner medium while the vesicle retains its original volume (model of nucleated cells), iii) a phase micro-separation of the poly(NIPAM) inner medium, the volume of the vesicle remaining unchanged.

Chitosan and hyaluoronan coated vesicles

By coupling studies on DOPC LUVs and GUVs, we demonstrate the electrostatic origin of the interactions polyelectrolyte/lipid membrane and probe the influence of the relative membrane/polyelectrolyte charge (varied by pH) on these interactions. Because of the zwitterionic nature of DOPC lipids, polyelectrolyte adsorbs whatever the respective global sign of the membrane and the polyelectrolyte (both tunable by pH) but the amount of adsorbed polymer is higher in the case of opposite charge signs of the membrane and of the polymer. A patch-like structure of the coated membrane is proposed, where bare domains alternate with charged polyelectrolyte coated domains. Using polyelectrolytes with different molecular weights, it was shown that the conformation and consequently the amount of adsorbed polymer strongly depends on chemical structure of the polyelectrolytes: adsorption of chitosan is independent on the molecular weight and its conformation is concluded to be flat on the surface; amount of hyaluronan adsorbed increases when its molecular weight increases and it is concluded that it forms loops at the interface. Polyelectrolyte-coated vesicles are more stable than bare vesicles even in extreme pH conditions (1.5< pH<11) [26, 27] and relatively high salt concentrations (up to 10-2M).

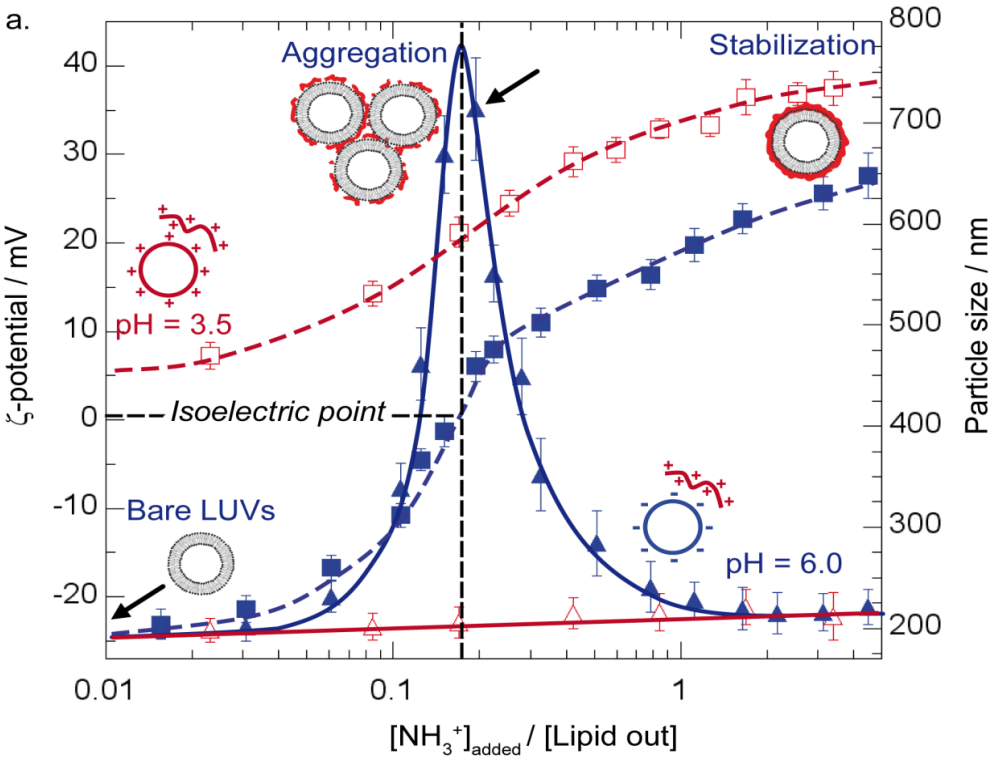

Fig.2. Formation of finite aggregates of chitosan coated vesicles.

Mechanical properties of the membrane are modified by coating leading to specific behaviours such as a stabilization of the composite vesicles against different external stresses (pH, salt, osmotic deflation). An interesting point is that depending on the concentration in polyelectrolyte of the solution where the vesicles are suspended, they can form aggregates with a defined number of aggregation at the isoelectric point leading to potential multi-compartment carriers [28]. The results obtained with chitosan are given in Figure 2.

Overcharging in excess of chitosan allows the formation of well dispersed positively charged vesicles. Adsorption of polyelectrolyte is important to stabilize the lipidic film but also to tune the global charge of the composite vesicle to control adhesion of surface.

CONCLUSION

We have developed polymer composite vesicles, which are promising as both mechanical models of cells and drug carriers because they present improved structural and mechanical properties and enhanced resistance to various stresses of biological interest (pH, salt shocks, osmotic pressure, point acting forces…). They are obtained either by encapsulating polyNIPAM viscoelastic systems or coating their external membranes with biocompatible polyelectrolytes. NIPAM-gel-vesicles are of high interest in the context of drug delivery because they behave like homogeneous elastic beads with a sharp volume transition inducing a release of 98% of their internal solution. Covering the membrane of such NIPAM-gel-vesicles with targeting molecules and increasing its transition temperature from 32°C to 37-40°C by NIPAM substitution may allow getting smart drug carriers able to target a specific organ and liberate encapsulated drugs in response to temperature changes. As far as polyelectrolyte-vesicles are concerned, they also hold promise as potential multi-compartment carriers because of their ability to form well-defined finite aggregates when suspended in external polyelectrolyte solution at specific concentration.

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