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Preparation and characterization of stable phospholipid - silica nanostructures loaded with quantum dots

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The structural dependence of silica-liposome hybrids on silanization conditions was investigated. Silica coatings protect liposomes against aggregation, degradation, and leakage, which is important for their application in bioimaging. Liposomes loaded with quantum dots were synthesized and attempts to obtain uniformly sized, silica-coated nanocapsules were performed.

Phospholipid vesicles or liposomes are spherically closed lipid bilayers,[1] widely used for medical and biochemical aims,[2] food and cosmetic,[3] chemical analysis.[4] Their hollow spherical structure and high loading capacity make them an attractive packaging material for encapsulation and delivery of drugs, enzymes or markers. Liposomes are also promising for biolabeling, because they can be loaded with appreciable amounts of molecules and/or nanoparticles, (e.g. quantum dots (QDs)) to amplify a total signal.[5] A major drawback of liposomes is their instability *in vivo* and during storage as well as their high sensitivity towards external influences such as variation of temperature, pH and osmotic pressure.[6] Sonication or extrusion can align the liposomes' morphology by transforming them in kinetically stable unilamellar particles and charged species could stabilize them against fusion[7]. The semi-permeability of their membrane can be controlled through coverage or templating with different manipulations, such as a polymer net [8] or silica cover.[9] Silica coverage prevents the aggregation of liposomes and the leakage of their content, increasing their stability. Although synthesis of liposome-silica derivatives is a widely used method to stabilize the lipid bilayer,[10] the dependence of morphology of the obtained vesicles on the silanization conditions is still not investigated in detail. Some articles present information about morphology of the synthesized phospholipid-silica derivatives,[8,11] but did not provide any practical information about the conditions influenced on the obtainment of these particles. To the best of our knowledge, no studies concerning the influence of the silanization agent (SA) on the shape of liposomes are available.

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Nonetheless, this information should allow to control the synthesis conditions to obtain a specifically desired morphology. The current contribution provides an overview of experimental parameters governing phospholipid-silica vesicles formation. The main goal was to understand the underlying mechanism in order to obtain submicron stable particles for their future application as biolabels. The schematic process of the silanization is presented in Fig. 1.

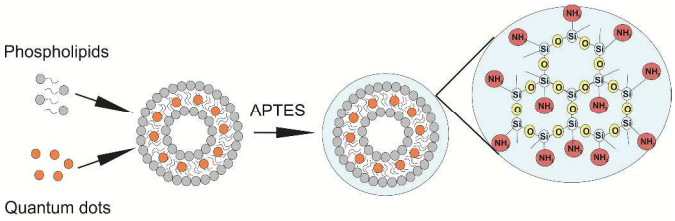

Figure 1. Scheme of the synthesis of silica-coated liposomes loaded with quantum dots, APTES - (3-aminopropyl)triethoxysilane.

Liposomes loaded with quantum dots (LQDs) were obtained by a thin-film evaporation using a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 83%) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, 17%). The technique comprised the formation of a thin phospholipid film and its subsequent hydration by buffer solution.[12] CdSe/CdS/ZnS QDs with orange emission (λ_{em}=594, quantum yield (QY)~35%, the full width at half maximum (fwhm)=38 nm) were prepared and stabilized by octadecylamine as described by Speranskaya et al.[13] Purification of the obtained LQDs from excess phospholipids and small aggregates was an important challenge. Use of the Sephadex columns[14] did not lead to complete separation of the loaded liposomes. Therefore a density gradient sedimentation was used. The gradients were obtained by placing sucrose solutions with increasing concentrations one by one vertically in the 5-mL ultracentrifuge tube. Correct sucrose gradient allowed not only segregation of different phospholipids structures, but also their pre-concentration. The optimal continuous gradient was found to be as follows: a 60% sucrose solution (1.5 mL) was placed into ultracentrifugation tube, a 30% sucrose solution (1 mL), a 20% sucrose solution (1 mL) and a 10% sucrose solution (1 mL)

were slowly added one by one to the tube without mixing. An aliquot (0.5 mL) of the liposomes solution was carefully added above all these layers avoiding mixing. The difference in density between all sucrose layers and the LQDs allows a phase separation to occur. The crucial moment is a preparation of density gradient and addition of liposomes solution. All these steps should be done slowly and carefully to obtain a well-defined phase separation between all layers. After 30-min centrifugation at 300 000 g the LQDs were located in the 30%-sucrose layer, whereas excess phospholipids could not even pass the 10%-sucrose layer (Fig. 2A). LQDs were collected and, after removal of the sucrose residue with protein concentrator tubes (9K, 20 mL, Thermo Scientific), the pellet was re-dissolved in carbonate buffer (pH~9.6) and used for further experiments. The obtained LQD solution was stored at 4 °C.

Theoretically, centrifugation time should not significantly influence on the separation process, because each particle remains stationary in sucrose fraction with an equal density. Nevertheless it is important to highlight that more than 60 min-centrifugation of LQDs at 300 000 g results in leakage of QDs out from liposomes. That could be easily indicated as decreasing fluorescence of the system (water-insoluble QDs precipitate in aqua media when they leach out of phospholipid vesicles). According to the dynamic light scattering (DLS) measurements the obtained LQDs showed a narrow size distribution around 100 nm with a polydispersity index (PDi) of 0.06. After removal of the sucrose residue with concentration tubes, LQDs were used for further silanization. The dimensions of vesicles play an important role in bioimaging due to the possible adsorption of large particles onto the surface of carriers applied in immunoassays, which might result in strong non-specific interactions. Large vesicles could also pose a problem with migration in body tissues. According to our experiments, preferably, size of label, should not exceed 200 nm.

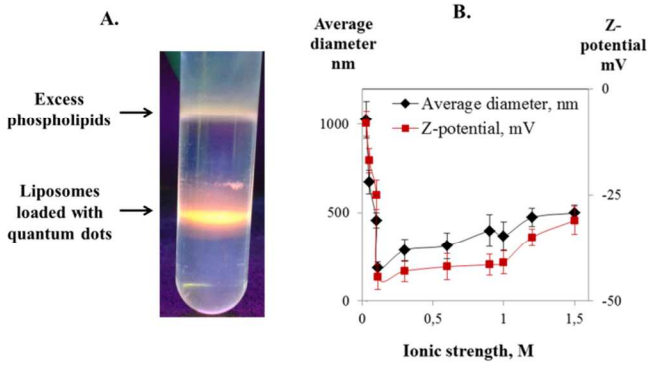

Figure 2. (A.) purification of the liposomes loaded with QDs using density gradient sedimentation (UV lamp, λ_{exc}=365 nm); (B.) dependence of the liposomes' average diameter and zeta-potential on ionic strength of working buffer during the mechanical stress experiment (5-hours shaking, pH=9, the initial average diameter~100 nm, n=5).

The stability of liposomes depends on their individual characteristics: phospholipid composition, size and type of the obtained vesicles, their surface properties and nature of the encapsulated material.[15] Packing of phospholipids in bilayers and rolling them in liposomes are based on the presence of both hydrophilic and hydrophobic groups in the phospholipid structure. Surrounding media affects the self-assembly of vesicles and their hydrodynamic radius. Choosing an optimal buffer for liposomes' self-assembly is a critical step in their development. Therefore, an influence of pH and ionic strength was investigated. Synthesis of liposomes was done varying pH values and ionic strengths of buffer solutions. Two parameters were controlled during the preparation of the vesicles: average diameter, measured by DLS, and fluorescence intensity. These were also monitored during two subsequent mechanical tests, constant shaking and ultrasonic treatment, for a period of 5 hours at room temperature. . As presented in Fig. 3 liposomes obtained in the media with pH of 7.4-9.6 were not prone to aggregation even after 5 hours of mechanical or ultrasonic treatment. Influence of ionic strength in the interval 0.03-1.5 M on the average diameter and zeta potential was studied and a higher (~2.5 times) absolute value of zeta potential (therefore, a higher stability of the obtained particles) was measured in solutions with ionic strength in the range of 0.07-0.12 M (Fig. 2B). This implies a better stability of particles in these media. Therefore, all further experiment were performed in carbonate buffer solution with pH~9 and ionic strength ~0.1 M. Loading of QDs inside phospholipids vesicles resulted in some decrease of the initial QY of QDs from 35% to 28%. The sol–gel method is the most common technique to prepare hybrid materials. Morphology of the particles obtained by sol–gel silanization using two the most frequently used SAs (tetraethyl orthosilicate (TEOS) and (3-aminopropyl) triethoxysilane (APTES)) was compared and investigated.

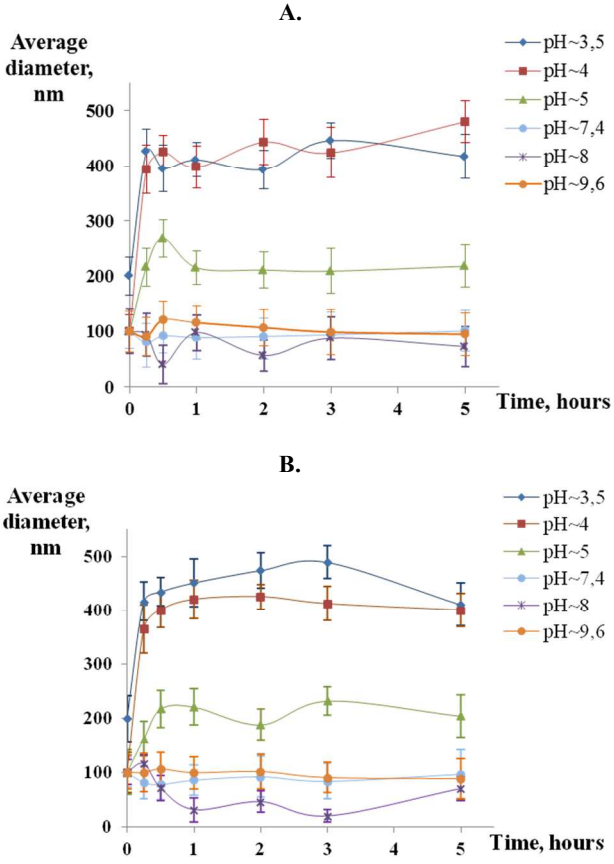

Figure 3. Dependence of the liposomes' average diameter on pH of working buffer during the constant shaking (A.) and ultrasonic treatment, (B.) (ionic strength~0.1 M, n=5).

An influence of nature of the silanization agent, the combined influence of SA/phospholipid ratio and reaction time on silica-covered liposomes loaded with QDs (SLQDs) morphology was investigated. According to literature the most used concentration of SA is 8-times molar excess under the liposomes concentration.[14,16] In

our work, molar ratios of silicon alkoxide/LQDs ranging from 2/1 to 50/1 were tested and completely opposite results were obtained for TEOS and APTES. To form the silica shell around liposomes and obtain SLQDs the techniques based on (*i*) prior hydrolysis of SA before mixing with LQDs and (*ii*) direct addition of the SA to LQDs without preliminary hydrolysis were compared. The former way included a prior hydrolysis of SA (minimum 48 hours) and its further incubation with liposomes (~72 hours). The latter process comprised a simultaneous hydrolysis and surface precipitation of silanization agent (48-72 hours). The addition of TEOS which was preliminary hydrolyzed in alkaline as well as in acidic environments (pH~3.5-9.6) resulted in gelation of the system. According to the DLS measurements size of the particles was abruptly growing with increase of the SA content within first 12 hours of silanization process. The further measurements (after 24-36-48 hours) confirmed presence of the stable aggregates with broad size distribution of which the average size exceeded several thousand nm for the particles obtained in the range of 2-50/1 of TEOS/LQDs independently on the exact ratio. Transmission electron microscopy (TEM) measurements showed that the 3D extended silica networks were formed (Fig. 4A). So, instead of silica-coated liposomes, a voluminous structure was obtained in which QDs were heterogeneously distributed. The direct addition of non-hydrolyzed TEOS in the range of 2-50/1 of TEOS/LQDs led to the formation of agglomerates, which appeared visually as white flakes. Silanization with APTES solves the issue of uncontrolled growth of silica shell as could be observed in the case of TEOS application, as APTES cannot form a structurally ordered network due to presence of the amino group in its molecule.

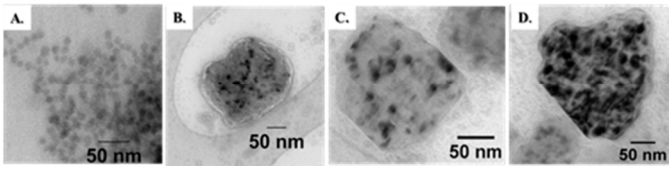

Figure 4. TEM images of silica-LQDs derivatives: after addition of TEOS (A), preliminary hydrolyzed APTES (B), non-hydrolized APTES (C), silica-coated phospholipid vesicle loaded with a higher amount of QDs (non-hydrolized APTESwas used for silanization).

Addition of the preliminary hydrolyzed APTES led to formation of the so-call "matrioshka architecture", wherein loaded liposomes were located inside of silica capsules (Fig. 4B). An addition of non-hydrolyzed APTES (25 molar excess) to the LQDs resulted into formation of relatively monodisperse SLQDs (Fig. 4C, 5) The formation of a stable sol can be explained in terms of surface stabilization and electrostatic interaction. The simulation gave an average absolute total dipole moment of DPPC of 24 D.[17] At pH~9 the LQD surface showed a quite high negative zeta-potential (up to -44 mV) due to presence of a glycerophosphoric acid residue both in DPPC and DPPE molecules. As the pK_a of the primary amino groups of DPPE is around 8.02, they did not charge at pH~9. At the same pH amino groups of APTES molecules are already protonated (pKa~10.37), thus, a targeted silanization of LQDs took place. The presence of amino groups breaches possible uniform and symmetric structure of the obtained SLQDs (Fig.5). Higher concentrations of APTES led to the formation of silica agglomerates (white flakes).
Application of a mixture of TEOS and APTES (molar ratios of TEOS/APTES were in the range of 5-1/1) as SA resulted in the formation of the silica structures similar to induced by TEOS addition, despite variation of molar ratios and different reaction condition (pH~3.5-9.6, stirring time ~12-72 hours). Silanization of the QDs-loaded liposomes resulted in an insignificant shift of fluorescence maximum (≤ 2 nm), no change in spectrum profile and relatively small decrease of the QY (from 28% to 26%). The relatively good stability of the fluorescence spectra is related to the good protection of CdSe fluorescent core from the external environment. The structure of SLQDs could be related to the big number of core/shell QDs inside phospholipids bilayer structure[18] (Fig. 4D).

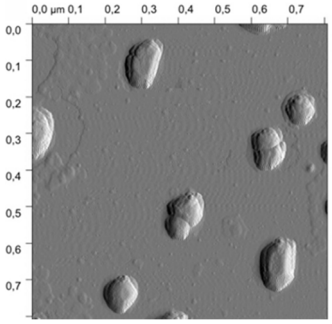

Figure 5. Topography AFM image of the obtained silica-covered liposomes loaded with quantum dots.

The particles of 100-240 nm were obtained already after 12 hours independently on reagents' ratio (in the range of 2-50/1 of SA/LQDs). Their size was not changed after 48 hours of the reaction. For drying of SLQDs sodium fluoride at 4 was added and the mixture was stirred during 48 h at room temperature in the dark. Then the sample was dried at 40 °C for 24 h.

Conclusions

The study of the interplay between the applied silanization agents, the reaction conditions and the resulting vesicle-silica morphologies allowed us to establish some interdependence for future synthesis of biomimetic vesicles of synthetic polymer particles with specified characteristics. It was shown that these regularities can advantageously be exploited for construction of more complex vesicle-silica architectures. It was proven that APTES is the optimal silanization agent for covering of QDs-loaded phospholipids vesicles. Its direct addition to the vesicles led to formation of the monodisperse silica-coated particles, whereas the application of TEOS did not give us satisfying results. The optimal molar ratio of APTES and LQDs (25/1) and reaction conditions (constant 48-hours stirring in dark) were found appropriate for the used system. An additional merit of APTES (comparing with TEOS) is presence on the surface amino groups available for bioconjugation. A further study on application of the synthesized SLQDs is the topic of a forthcoming paper.

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