Articles

Fabrication and Characterization of Human Serum Albumin and L-α-Dimyristoylphosphatidic Acid Microcapsules Based on Template Technique

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Biogenic microcapsules comprised of L-α-dimyristoyl phosphatitic acid (DMPA)/human serum albumin (HSA) were prepared by stepwise adsorption of DMPA/HSA multilayers onto charged colloids and subsequent dissolution of the colloidal cores. The properties of both lipid/protein modified particles and the hollow capsules were characterized by microelectrophoresis, scanning electron microscopy, transmission electron microscopy, confocal laser scanning microscopy, and scanning force microscopy, respectively. Single particle light scattering together with small-angle X-ray scattering measurements revealed that DMPA forms a bilayer structure on the HSA surface. A combination of HSA and DMPA on hollow capsules is proposed as a biomimetic membrane model.

Introduction

It is well known that most molecular recognition and transduction processes in biological systems occur at native cell surfaces, and the biological function of the receptors is regulated by soluble active substances. Both functional understanding of molecular recognition processes and their use in screening for effective compounds are very important in basic life science research and drug discovery. With the progress of biosensors and screening assays, as well as the emergence of combinatorial libraries of compounds, there is a strong demand to develop novel, highly sensitive, and fast assays for ligand−receptor vesicles or capsules.[1,2] Therefore, lipid/protein capsules will present a connection between biological systems and a broad variety of physical detection devices with high surface sensitivity.

A complex film of lipid and protein can be constructed at a curved and planar surface to form multilayers by self-assembly at the air/water interface or liquid/liquid interface.[3−5] The layer-by-layer (LbL) technique based on electrostatic forces creates a controllable approach to fabricate polyelectrolyte multilayers and microcapsules which have a defined wall thickness and size.[6−10] With this method, a variety of polyions including synthetic and natural materials have been used to construct polyelectrolyte multilayer microcapsules, which provide hollow capsules with special functions, such as catalysts, reaction cages, and drug carriers.[11−13] We have demonstrated in our previous work that human serum albumin (HSA) and lipid multilayer films can be directly formed on a drug crystal surface based on the LbL technique for a sustained release.[14] However, the detailed structure as well as the stability of the multilayer film of HSA assembled with the lipids are unknown. In the present work we fabricated the multilayer of HSA and a phospholipid (L-α-dimyristoyl phosphatitic acid, DMPA) on the colloidal particle surface through the LbL assembly, followed by core removal to obtain HSA/DMPA hollow capsules. The detailed wall structures of the capsules were analyzed by various techniques. It indicates that the well-defined lipid bilayer formed on the HSA surface will provide the possibility to incorporate membrane specific components such as channels and receptors into the capsule's wall for specific recognition.

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HSA is the most abundant protein in human blood and consists of 585 amino acids with a molecular weight of 66.5 kDa, stabilized by 17 disulfide bridges and contains a single free sulfydryl.[15−18] The location of the disulfide bridges appears to confer some rigidity within each subdomain, but the lack of interdomain disulfide bridges allows significant modifications in the shape of HSA in response to changes in pH.[16] It is known that the electrostatic interaction is an important driving force in fabricating protein/lipid complex films. Thus the HSA/DMPA multilayer growth on colloids should result in the formation of hollow capsules after the removal of the cores.

Experimental Section

Materials. The chemicals employed in this work, HSA (lyophilized powder protein, approximately 95% by biuret 66 500 Da), DMPA, and 6-carboxyfluorescein (6-CF) were purchased from Sigma. Poly(sodium 4-styrenesulfonate) PSS, $M_w = 70\,000$, and poly(allylamine hydrochloride) PAH, $M_w = 70\,000$, were purchased from Aldrich. PAH was used as received, whereas PSS was dialyzed against Milli-Q water (cutoff 14 000) and lyophilized before use. Positively charged melamine formaldehyde (MF) particles and negatively charged polystyrene (PS) particles were used as templates for preparing the multilayers and hollow capsules and were provided by Microparticles GmbH, Berlin. The water used in all experiments was prepared in a three-stage Millipore Milli-Q Plus purification system and had a resistivity higher than 18.2 Mμ cm.

Protein/Lipid Multilayer Formation. HSA was separately dissolved in citric acid buffer solutions at pH 3.8 and 3.0 with a concentration of 1 mg/mL. In this case, HSA is positively charged since it has an isoelectric point of 4.8. The lipid solution was prepared in the following way: DMPA was dissolved in a mixed solvent (chloroform:methanol = 1:1), then the solvent was evaporated at 30°C, and afterward a certain amount of water was added up to a final lipid concentration of 1 mg/mL, then the lipid aqueous solution was sonicated for 5 min. Multilayers on MF particles were fabricated as follows: 1 mL of DMPA solution was added to the MF particle suspension allowing adsorption for 2 h. Then the suspension was centrifuged followed by three washings in water in order to remove the excess unabsorbed DMPA in the solution. Then 1 mL of HSA in a pH of 3.8 or 3.0 buffer solution was added to the suspension, respectively, and the above step was repeated until the desired layers were absorbed. Finally the hollow capsules were obtained after the following step, the coated MF particles were exposed to 0.1 M HCl for 5 min; three centrifugation and Millipore water washing cycles were conducted to remove the dissolved MF oligomers from the solution. HSA/DMPA multilayers were deposited onto PS particles which were coated with three precursor polyelectrolyte layers (PAH/PSS/PAH) using the same method as described for the ζ potential measurement. The first layer is PAH because of the negatively charged surface of PS particles; 1 mg/mL PAH and PSS solution (0.5 M NaCl) were used in this procedure.

Electrophoretic Mobility Measurements. DMPA/HSA multilayer growth on PS particles was followed by measuring the electrophoretic mobility of the coated particles using a Malvern Zetasizer 4, by taking the average of five measurements at the stationary level. The mobilities (u) were converted to the ζ potential using the Smoluchowski relation $\zeta = u\eta/\epsilon$, where η and ϵ are the viscosity and dielectric permeability of the solution, respectively. All measurements were performed in air-equilibrated pure water and buffer solution without added electrolyte. The precision of the measurement is about ± 3 mV.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) Measurements. SEM observation was performed on KYKY-2800 scanning electron microscope at an accelerating voltage of 20 kV. The colloid particles were redispersed in 2.0 mL of water by sonification and air dried before measurement. TEM observation was carried out with a Philips TECNAI 20 transmission electron microscope operated at 120 kV. TEM samples were prepared by depositing a diluted suspension onto a carbon-coated copper grid. The extra solution was blotted off, and the grid was allowed to air dry for 3 min.

Single Particle Light Scattering (SPLS) Measurements. PS particles covered with lipid and protein layers were employed for SPLS measurements, which were conducted on a home-built photometer equipped with an argon laser, Innova 305 from Coherent. The principle of SPLS measurement has been described in the literature.[19] The Rayleigh−Debye−Gans theory was used to obtain a particle size distribution in the dispersion solution and further for the calculation of the shell thickness.

Confocal Laser Scanning Microscopy (CLSM). CLSM images were taken with the Leica TCS NT confocal system (Leica, Germany), equipped with a 100 × oil immersion objective with numerical aperture of 1.4. 6-Carboxyfluorescein (6-CF) was used as the fluorescence probe. The capsule solution and 6-CF were mixed on a glass cover slip. The images of capsules in aqueous environment were acquired immediately. The images in the dry state were taken after the water had evaporated.

Scanning Force Microscopy (SFM). SFM images were recorded in air at room temperature using a Nanoscope III Multimode SFM (Digital Instrument Inc., Santa Barbara, CA). The samples were prepared by applying a drop of the capsule solution onto freshly cleaved mica. SFM images were processed by using Nanoscope software and Image PC software (Version beta 2, Scion Corp.).

Circular Dichroism (CD) Measurements. CD spectra of HSA in different pH solution were recorded by a JASCO J- 715 spectrophotometer at room temperature over a wavelength of 190−260 nm. All measurements in solution were recorded in a 1 mm path length quartz cell. Each spectrum is the average of eight experiments. The secondary structure was estimated by SELCON. The estimations have an accuracy of 3%, with the usual accuracy range of protein conformation analysis performed by CD.[20]

Small-Angle X-ray Scattering (SAXS) Measurements. SAXS were carried out with a Nonius rotating anode ($U = 40$ kV, $I = 100$ mA, $\lambda = 0.154$ nm) using image plates. With the image plates placed at a distance of 40 cm from the sample, a scattering vector range of $s = 0.07-1.6$ nm^{-1} was available. 2D diffraction patterns were transformed into 1D radial averages.

Results and Discussion

1. Characterization of HSA/DMPA Multilayers Assembled onto Colloids. Scheme 1 displays the assembly of the oppositely charged HSA and DMPA adsorbed alternately onto MF. It is analogous to the assembly procedure for polystyrene PS particles. Electrophoretic measurements reveal that the surface charge changes during the alternate assembly of HSA and DMPA multilayers. The ζ potential

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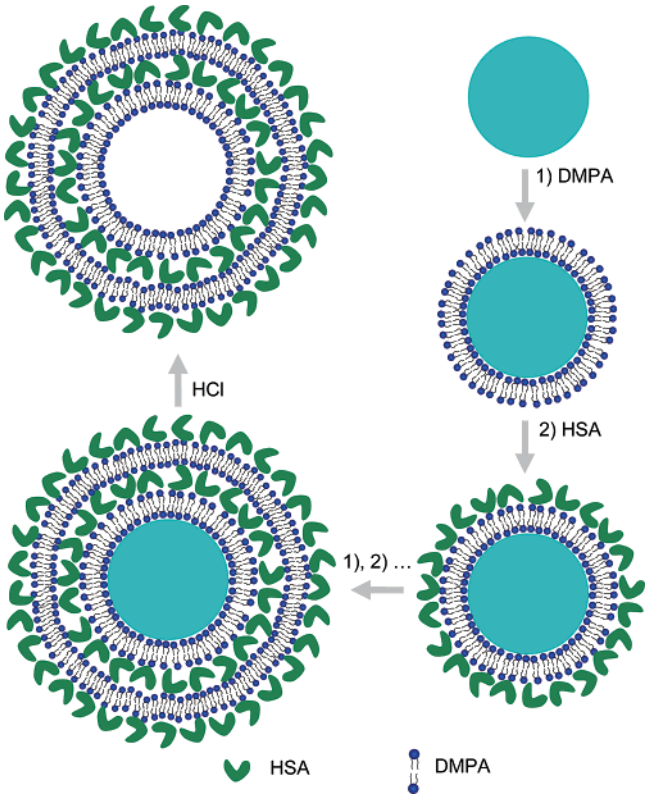
Scheme 1. Schematic Illustration of the Preparation of Hollow HSA/DMPA Multilayer Capsules Based on MF or PS Particles



in Figure 1 was calculated from the mobility measured after deposition of each layer. It varies as expected for PS particles coated with precursor polyelectrolyte layers and in a non-predicted way for coating with HSA/DMPA multilayers. The presence of DMPA as the outermost layer on the particle surface always causes a negative sign. Figure 1a shows that the presence of HSA induces a sign reversal of the ζ potentials when the HSA was adsorbed in the pH 3.8 buffer solutions. The charge of the particle was also regularly reversed after adsorbing HSA layers at pH 3.0 (Figure 1b). But a slightly higher ζ potential value can be observed at pH 3.0 than at pH 3.8. This is reasonable since HSA is more positively charged when decreasing the pH value of the solution. Finally pH 3.8 was chosen to fabricate HSA/DMPA multilayer hollow capsules because it is a moderate condition for biomaterials. The regularly alternating value of ζ potentials proves the successful assembly of HSA/ DMPA multilayers on PS particles.[21,22] It indicates that the electrostatic interaction is the main force that enables multilayer growth.

The morphology of the DMPA/HSA-coated MF particles was examined by both SEM and TEM. Figure 2 shows the SEM images of MF particles, which is uncoated (Figure 2a) and coated with a $(DMPA/HSA)_5$ multilayer (Figure 2b). Compared to the uncoated MF particle, the DMPA/HSA-coated MF particle has a rougher surface due to the coverage by the DMPA/HSA multilayer. The TEM image (inset Figure 2b) of a selected core−shell sphere displays the coating of the 10-layer composite film (arrows).

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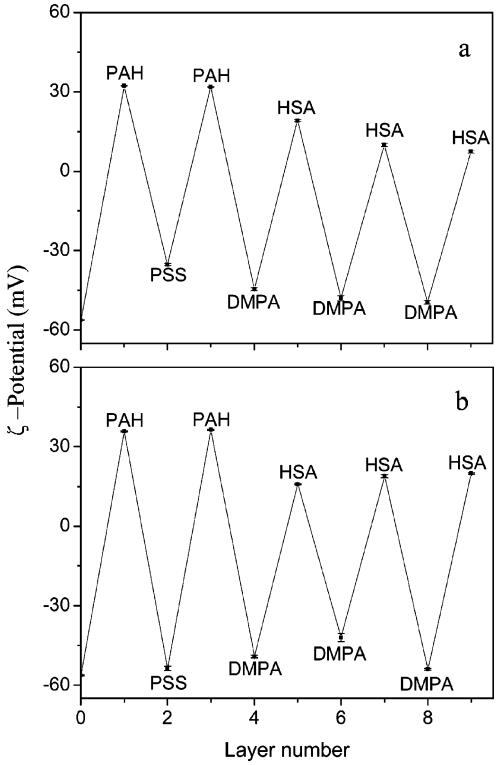

Figure 1. ζ potential as a function of layer number for polystyrene latex particles coated with HSA/DMPA multilayers. The pH value of the HSA solution is 3.8 (a) and 3.0 (b). The blue short lines are error bars.

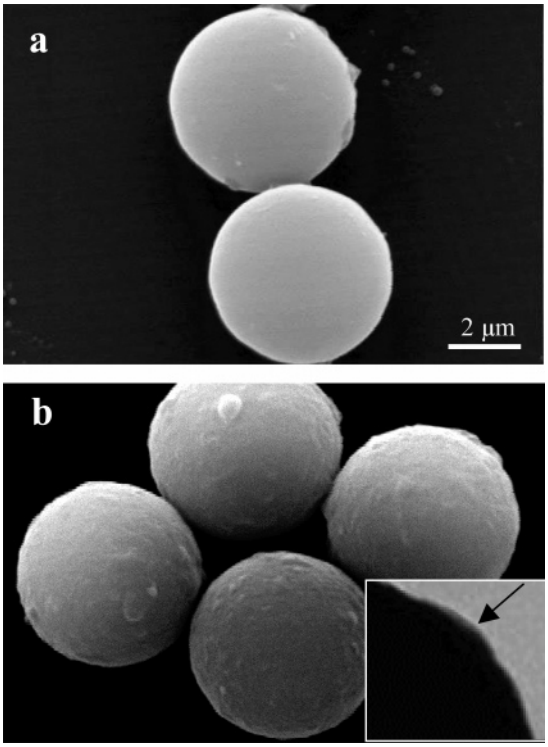

Figure 2. SEM images of MF colloid particles. (a) Bare MF particles; (b) MF particles coated with 10 layers of DMPA/HSA (inset showing the magnification TEM image of a coated MF particle).

2. Preparations and Characterization of HSA/DMPA Hollow Capsules. Multilayer-coated MF particles were exposed to 0.1 M HCl in order to remove the core templates. The dissolved MF permeates through the HSA/DMPA multilayers upon dissolution and washing. The removal of

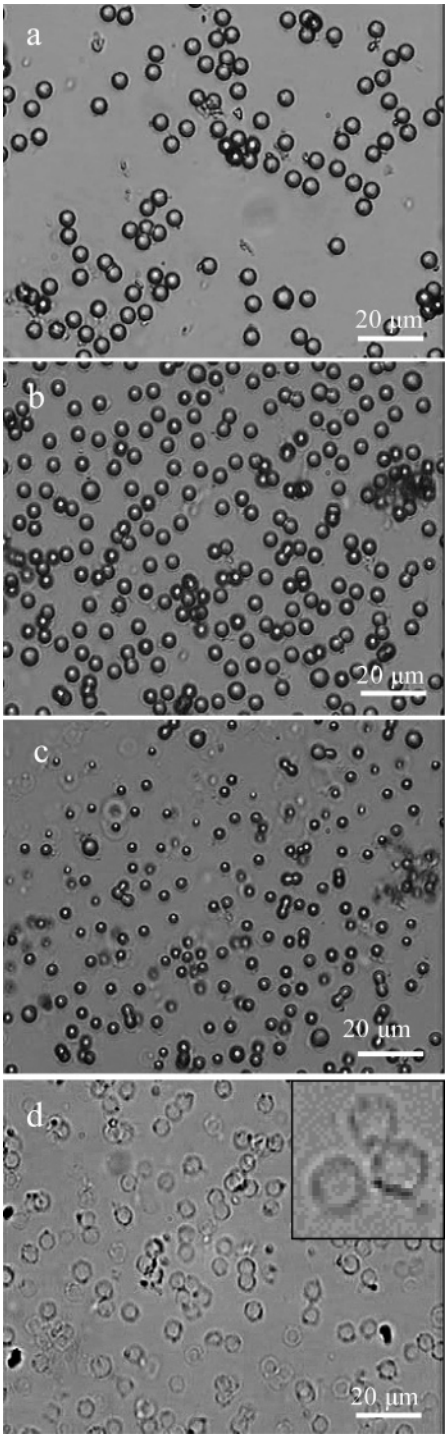

Figure 3. Microscopy images showing the removal process of MF cores in pH = 1.0 HCl solution: (a) $(DMPA/HSA)_5$-coated MF particles dispersed in water; (b) early stage of core dissolution; (c) later stage of core dissolution; (d) hollow $(DMPA/HSA)_5$ capsules (inner is enlarged image).

MF cores was observed by optical microscopy (Figure 3). Before the addition of HCl, the MF particles appear as a monodispersed system with spherical shape (Figure 3a). With the addition of HCl solution, MF cores began to shrink and the HSA/DMPA shell around the cores became visible due to the decomposition of the MF (parts b and c of Figure 3). Finally, the hollow capsules can be observed (Figure 3d). CLSM images showed that 80% of the DMPA/HSA capsules were continuous (Figure 4a). The capsules collapsed to form folds after the water was evaporated as shown in Figure 4b.

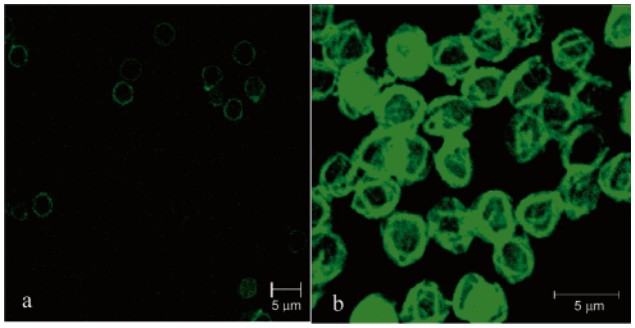

Figure 4. CLSM images of DMPA/HSA capsules. (a) 10 layers of DMPA/HSA, some broken capsules can be identified. (b) Dry state of a.

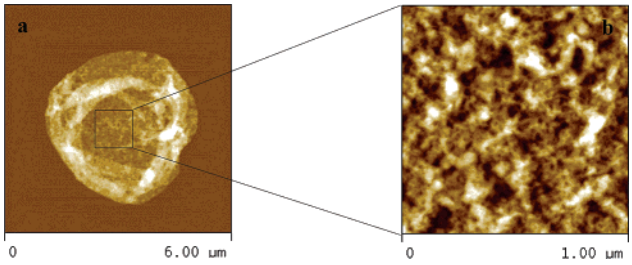

Figure 5. (a) SFM top-view image of a $(DMPA/HSA)_5$ capsule. (b) Magnified image of a part as indicated by the box in a.

Some broken capsules can be observed in Figure 4a, which is considered as a result of the osmotic pressure difference created by the dissolution of the cores. On the other hand, the nonlinear structure of HSA is presumably the main factor for the capsule's stability.[18] The stability of the capsules also can be improved by using smaller cores as previously reported for polyelectrolyte capsules.[23]

The morphology of the dried DMPA/HSA capsule was examined by SFM. The result shows that the capsules collapse due to the evaporation of the aqueous content. Some creases and folds were clearly visible in Figure 5a. On further magnification of the part of the capsules that does not include any folds, as indicated in Figure 5a, Figure 5b shows that the surface texture of the collapsed capsule is quite rough. The selected area, 0.5 μm × 0.5 μm, in the fold-free region provides a roughness measured perpendicular to the surface as ~5 ± 0.5 nm. Such a high roughness may be due to the inhomogeneity of the outermost HSA layer.

3. HSA/DMPA Multilayers Structure. The secondary structure of HSA is dominated by a higher content of α-helix. It exhibits two minima at 208 and 222 nm wavelength in CD spectra.[17] To prove that the structure of HSA will not be significantly changed at the selected pH range, we carried out CD measurements. Figure 6 shows the CD spectra of HSA at different pH values. The selected pH values of 3.8 and 3.0 (below the isoelectric point of 4.8) represent the actual adsorbing solution. One notes that, even at pH 1.0 where the MF core was removed, there is no obvious variation of the α-helix conformation corresponding to the two minima peaks in the above different pH conditions. The quantitative analysis in terms of secondary structure of HSA is given as an inset in Figure 6. Compared with the content of the secondary structure at the isoelectric point (pH 4.8),

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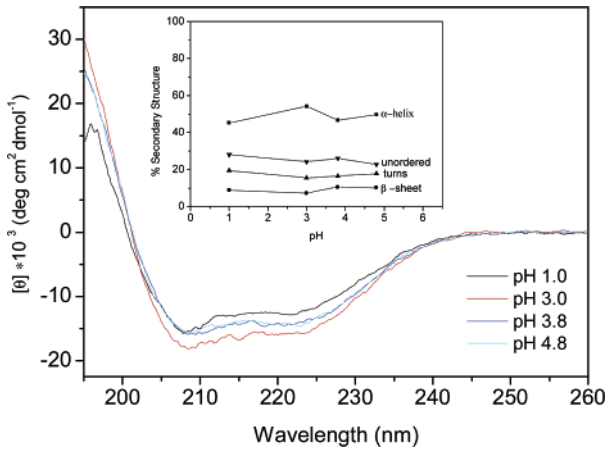

Figure 6. CD spectra of 0.1 mg/mL HSA in pH 1.0, 3.0, 3.8, and 4.8 buffer solutions. The inset shows the secondary structure fractions in different pH solution.

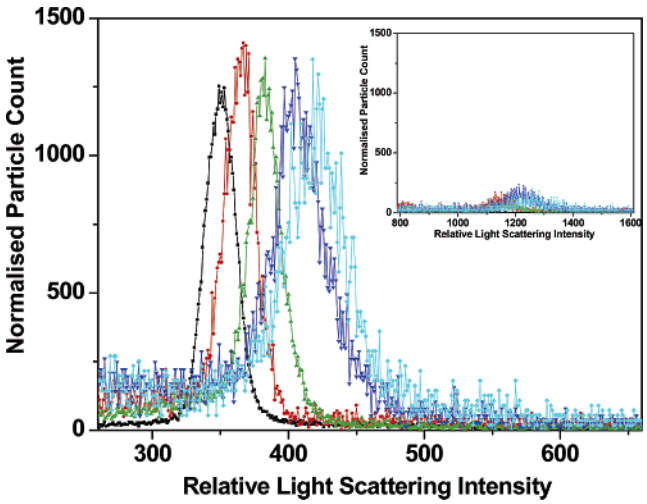

Figure 7. Normalized light-scattering intensity distribution of uncoated PS latex particles (black) and PS latex particles with PAH (red), DMPA (green), HSA (blue), DMPA (cyan), corresponding to 0, 3, 4, 5, and 6 layers, respectively. The inset shows the aggregation peak at the higher intensity part of the normalized light scattering intensity distributions.

(46.7% α-helix, 10.2% β-sheet, 17.8% turns, and 22.9% unordered), the content of α-helix, β-sheet, turns, and unordered varies by less than 5% with the different pH. It indicates the absence of changes of the HSA configuration at the pH value range of the hollow capsule fabrication.

Single particle light scattering measurements were performed to determine the thickness of the HSA/DMPA layers assembled on PS latex particles. It also provides the possibility to assess the situation of the particle aggregation after HSA and DMPA deposition. Figure 7 shows the distribution of scattering intensities, corresponding to the size changes for the uncoated and differently coated particles. One clearly observes the shift in the peak maximum corresponding to the expected increase in coating thickness. The growth of the HSA and DMPA layer on the PS particle was thus confirmed. It is noted that the width of the distribution of the coated particles was merely changed due to the alternate adsorption of the HSA and DMPA, indicating that each particle has received the same amount of deposit. The inset of Figure 7 shows an aggregate peak near 1200 a.u. scattering intensity. This peak is increasing considerably after coating with HSA, probably due to a rather low surface charge, which is usually needed for electrostatic stabilization.

Table 1. Thickness of Different Multilayer Films Coated onto PS Particles

layers	total film thickness d (±0.5 nm)
PAH/PSS/PAH	4.0
PAH/PSS/PAH /DMPA	9.6
PAH/PSS/PAH/DMPA/HSA	14.9
PAH/PSS/PAH/DMPA/HSA/DMPA	20.2

By use of the Rayleigh−Debye−Gans theory with the layer refractive index (n), the thickness of the coated layers can be determined.[24] For this system, we performed model calculations by using different refractive indices of each layer. These are known as $n = 1.59$ for the PS core and $n = 1.47$ for the polyelectrolyte, and for lipid and protein a value of 1.43 appears reasonable.[25] Thickness of different multilayer films coated onto PS particles is listed in Table 1. From the thickness difference of measured layers ($d_{PAH/PSS/PAH/DMPA} - d_{PAH/PSS/\ PAH}$), we estimate that the thickness of the lipid is about 5.6 nm, as expected for a bilayer (each lipid single layer is about 2.5 nm).[26,27] Similarly, the thickness of the HSA layer is estimated as 5.3 nm, which is a reasonable value considering the HSA dimension variation (2.7−12 nm) with change of pH values.[28,29] The protein layer will not be strictly one protein layer as part of the protein may penetrate the lipid moiety, and also the HSA layer might contain some water.

To further prove the DMPA bilayer structure formed on the HSA surface, we carried out measurements of SAXS on the PS particle surface with assembled HSA and DMPA multilayers. From the two reflection peaks presented in the small-angle region of Figure 8, one can deduce that there is

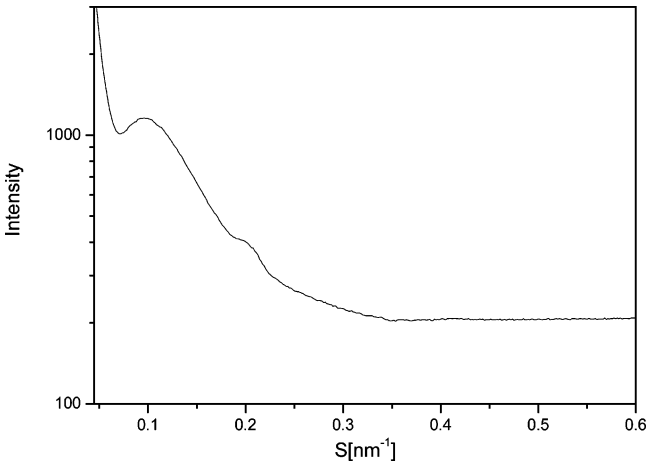

Figure 8. SAXS curve of PS particles coated with $(HSA/DMPA)_4$.

an ordered layer structure with a period of 10 nm. This means that the d-spacing of each HSA/DMPA layer is 10 nm,[30,31]

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which is consistent with the value obtained from the SPLS and SFM measurement. The thickness of the DMPA layer is indeed about 5 nm, confirming that DMPA forms a bilayer structure on the HSA surface.

Conclusions

In conclusion, we have successfully constructed HSA/DMPA multilayer microcapsules by alternating adsorption of HSA and DMPA on colloidal particles followed by the removal of cores. We have demonstrated that DMPA forms a bilayer structure on the HSA layer surface. The electrostatic interaction is the driving force for the multilayer assembly. By comparison with polycation/polyanion or protein/polyion capsules, such microcapsules comprised of protein and lipid will provide possibilities to incorporate recognition units or channels in the wall to mimic biological membranes.

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