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Urease Encapsulation in Nanoorganized Microshells

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ABSTRACT

Stable hollow polyelectrolyte capsules were produced by means of the layer-by-layer assembling of poly(allylamine), PAH, and poly(styrenesulfonate), PSS, on melamine formaldehyde microcores followed by the core decomposition at low pH. These capsules are nonpermeable for urease in water and become permeable in a water/ethanol mixture. The capsules were loaded with urease in water/ethanol mixture and then resuspended in water. The urease molecules are kept in the capsule, whereas the small urea molecules rapidly diffuse through the capsule wall providing a substrate for the biocatalytic reaction.

A thin film assembly by means of alternate adsorption of oppositely charged linear polyions was introduced in the nineties by Decher et al.[1] The basis of this method involves resaturation of polyion adsorption, resulting in the reversal of the terminal surface charge of the film after deposition of each layer. The method provides the possibility of designing ultrathin multilayer films with a precision better than one nanometer of defined molecular composition. The assembly process elaborated for planar solid supports was adapted for nano- and microtemplates (colloid particles with sizes of 0.1 to 5 microns, e.g., latex spheres, lipid tubules, microcrystals, biological cells, and other colloids).[2−12] In this process, a polycation solution is added to the suspension of colloid particles, and after adsorption saturation, the particles are separated from free polycations in solution. Then, a polyanion layer is deposited. In the same manner, one can deposit any number of polyion layers on the shell. Recently, a multifiltration procedure for separation of modified particles from unreacted polyions was introduced,[8] which allowed producing larger amounts of shelled particles, as compared with an earlier procedure based on separation of colloids and polyions by centrifugation.[2−5] After the shells are formed, one can dissolve the core particles to obtain empty capsules with a layer thickness tuned in the range from 5 to 50 nm and with needed composition.[2−3,8] The permeability properties of polyion capsules can be varied by shell composition and layer number.[9,13] As shown in ref 12, the wall permeability for dextran and albumin depends on pH: at low pH the capsule walls were open, and at pH higher than 8 they were closed. By varying the pH in the capsule suspension in the presence of the macromolecules, the encapsulation was performed. An operation with opening−closing capsule walls composed of polyion multilayers is based on Rubner's recent finding[14] that varying solution pH can induce charge disbalance in polycation−polyanion complexation in the multilayer, resulting in opening of ca. 100-nm pores.

In this work, as a part of our efforts to create unique and complex colloids with tailored enzymatic activity, we report the assembly of polyion capsules loaded with urease. In traditional bioreactors, urease was immobilized by covalent bonding or with acrylamide gel on different substrates (glass beads or glass wool, nylon netting, nitrocellulose).[15−20] Nanocomposites containing urease multilayers on 470-nm latex were recently prepared.[21]

We encapsulated urease in microshells assembled via layer-by-layer assembly of linear polycations and polyanions on a 5-μm diameter template. Hollow polyelectrolyte capsules were fabricated at pH 6.5 by alternate adsorption of four bilayers of sodium poly(styrenesulfonate) (PSS) (Aldrich, Mw 70,000)/poly(allylamine) hydrochloride (PAH) (Aldrich, Mw 50,000), onto 5-μm diameter melamine formaldehyde (MF) particles (Microparticles Gmbh, Berlin), using sequential adsorption with the filtration method.[4,22] The latex templates were dissolved, and the obtained microcapsules were loaded with urease.

A confocal fluorescence image[23] in Figure 1 (left) illustrates that FITC-labeled urease with concentration 10 mg/mL is excluded by the polyelectrolyte shells. The interior of

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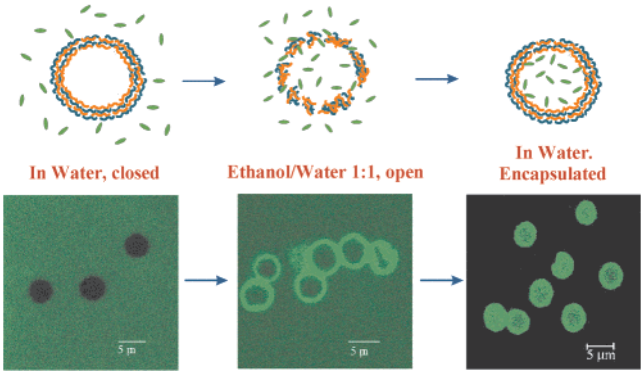

Figure 1. Permeation and encapsulation of urease-FITC into polyion multilayer capsules. Left, in water; middle, in water/ethanol mixture 1:1; right, the capsule with encapsulated urease again in the water. Top, scheme; bottom, confocal fluorescence images of the capsules.

the capsules remains dark and outside the capsule the background containing FITC-urease is fluorescent. Hollow polyelectrolyte capsules are not permeable for compounds with large molecular weight, such as proteins.[9,12] This observation indicates the closed state of the capsules. Figure 1 (center) shows a confocal fluorescence image of labeled urease with capsules after addition of ethanol (1:1 water ethanol mixture). In this case, the fluorescence coming from the interior of the capsule is the same as the outside background fluorescence. This points at penetration of protein into the capsules and indicates an open state of the capsule wall.

The transition between the open and closed state of the polyelectrolyte multilayer capsules introduced by adding ethanol in aqueous solution is reversible. When the capsules are transferred into water, the polyion shells become closed for urease molecules. The reversible reorganization of polyions in water and water/ethanol mixture resulting in a permeability change was utilized for urease encapsulation as follows: The macromolecules were first exposed to polyion capsules in a water/ethanol mixture, then ethanol was removed after centrifugation and the capsules were resuspended in water.[24] After that, the polyion capsule walls were closed and the urease was captured inside, as illustrated in Figure 1 (right). The interior of the capsule is bright and constant over time, and there is no fluorescence signal from the solution. Thus, urease filled the capsules. The images did not change with time, indicating that urease is preserved inside the capsules. The fluorescence near the inner capsule surface is brighter than in the center of the capsule. This is due to adsorption of proteins onto adhesive polyion multilayers, as was observed earlier for dextran-loaded capsules.[12−13]

The mechanism of reversible permeability changes in the polyion multilayers is not yet understood. Probably, it is related to segregation of the polyion network in water/ethanol media. Such segregation might lead to defects in the shell, and pores might be open big enough to pass 5-nm diameter urease globules through the wall. Returning capsules into pure water causes a relaxation of the polyion walls to a closed structure.

Figure 2 a,b gives TEM images (JEOL-2000 FX instrument, 200 kV) of empty and loaded capsules. An empty capsule looks like a folded thin shell, but a loaded capsule looks like a filled balloon, similar to the optical image (Figure 1). We succeeded in filling the enzyme inside the capsule, but we cannot quantify the enzyme content. To estimate the weight of encapsulated urease, we weighed an equal number of empty and loaded capsules with a quartz crystal microbalance, QCM.[25] QCM frequency shifts are proportional to the mass attached to an electrode (ΔF), and the ratio $\Delta F_{\text{loaded}}/\Delta F_{\text{empty}}$ is proportional to the mass ratio of loaded and empty capsules dried on the QCM electrodes. This ratio was found to be ca. 1.1, indicating that the loaded capsule is 10% heavier than the empty one. If we assume a similar hydration of the dried shells, we can estimate the mass of the urease

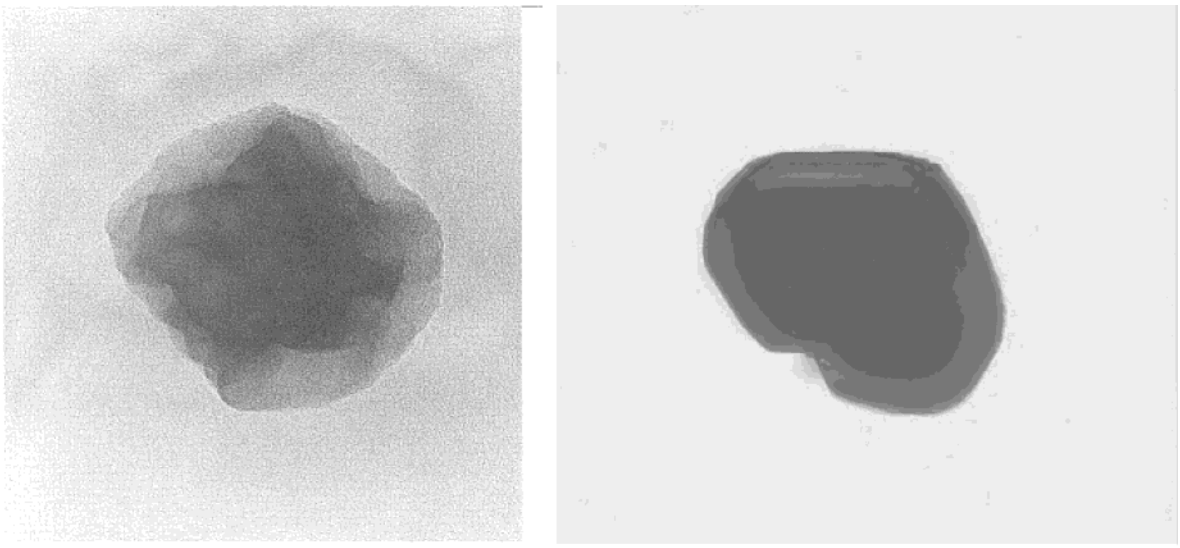

Figure 2. TEM images: empty (a) and urease loaded (b) microcapsules, magnification: × 300 K.

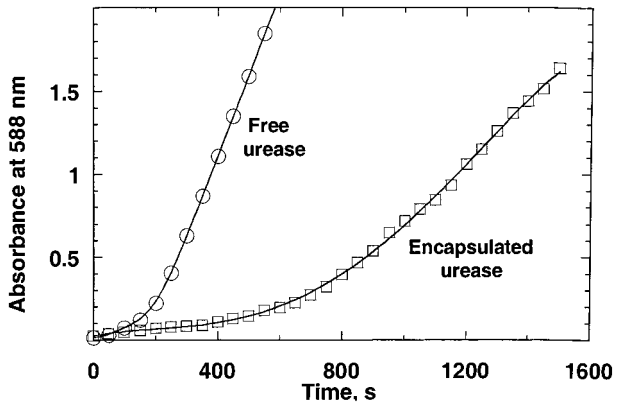

Figure 3. Absorbance at 588 nm by 2.9 mL of enzymatic activity assay solution after addition of 0.1 mL of urease-loaded microcapsule solution and the same test for addition of 0.05 mL of 0.02 mg/mL free urease.

kept inside the capsule. These estimations are approximate and are based on the assumption that the wall thickness is much smaller that the diameter of the shell. The mass of the capsule is $m = 4\pi r^2 h\rho = 1.38 \times 10^{-12}$ g, where $r = 2.5 \times 10^{-4}$ cm is the capsule radius, $h = 16 \times 10^{-7}$ cm is the wall thickness,[1−3] and the polyion density $r = 1.1$ g/cm^3. The mass of the urease loaded in one capsule of the volume of 6.3×10^{-11} cm^3 is ca. 1.4×10^{-13} g, which corresponds to 170,000 urease molecules or the concentration of 2.1 mg/mL. This urease concentration is close to the initial concentration used for the loading from water/ethanol solution.

A colorimetric assay based on the hydrolysis of urea was used for the activity control of free and immobilized urease, as monitored by the pH-sensitive dye bromcresol purple.[29] Figure 3 gives a comparison of the catalytic activity of the urease loaded into the capsules and free urease. For this analysis, 0.1 mL of loaded capsules containing 2×10^{-6} g urease were added to 2.9 mL of the assay solution. In the second experiment, 1×10^{-6} g of free urease was added. The solution pH increase due to ammonia production during the enzymatic reaction was monitored by the pH sensitive dye, bromcresol purple. The absorbance of this dye at 588 nm increases linearly with pH in the range of 5.8 to 7.5. The urease enzyme activity was determined as an increment of the ammonium production with time.[17−19] For the increment calculations, we used the curve parts with linear increment. For free urease, this linear increment began after 200 s incubation, and for encapsulated enzyme we obtained a linear slope in the region beginning from 700 s. Probably, a delay of about 500 s is needed either to reach an equilibrium concentration of urea inside the capsule (0.08 mg/mL) or to enable reaction of the products with PAH in the shell wall. The activity increment for 2×10^{-6} g encapsulated urease was 2×10^{-3} abs units/s, and for 1×10^{-6} g of free urease it was 8×10^{-3} abs units/s. The activities normalized to the enzyme mass are 10^3 abs units/(s·g) for encapsulated urease and 8×10^3 abs units/(s·g) for free urease. Thus, urease encapsulated inside the polyion shell preserved 13% of its activity as compared with free enzyme. This is a reasonable decrease due to the substrate diffusion difficulties in penetrating into the capsules. For comparison, the bioactivity for urease immobilized with polycations on latex dropped to 25% as compared with free enzyme.[21] The urease activity inside the capsules was also stable as compared with free urease: after 5 days storage at 7 °C, encapsulated urease completely preserved its activity while free urease kept at the same conditions in aqueous solution lost 45% activity. The polyion shell protects encapsulated enzymes from proteases and microbes, as was indicated in ref 26.

The concept of enzyme encapsulation in microshells by opening and closing pores can be applied to fabrication of enzymatic micro- and nanoreactors. Selective wall permeability allows for substrates and reaction products to diffuse freely through capsule walls, whereas the encapsulated enzymes are kept in the capsules. This technology provides also a possibility to encapsulate several proteins at the same time in the capsules to catalyze sequential enzymatic reactions. The loaded enzymes might be released out of the capsule after certain pH or salt treatment, which could find some application in drug delivery and sustained release systems.

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(22) Capsule preparation. PSS and PAH were used in water solutions at a concentration of 2 mg/mL with 0.5 M NaCl. For this we added 10 mL of aqueous PSS solution to 50 mL solution of 10 wt% MF-latex, allowed 20 min for adsorption saturation, removed unreacted PSS by filtration, and washed the modified latex with pure water. At the second stage, aqueous PAH was added, adsorbed, and excess polycation was filtered out. A stepwise application of this procedure resulted in formation of $(PAH/PSS)_4$ shells. Afterwards, we decreased the solution pH to pH = 1, latex-cores were dissolved, and empty shells were washed with water to remove MF-residues. Thus, the hollow capsules were produced.
(23) Confocal laser scanning microscopy. Confocal micrographs were taken with Leica TCS SP, equipped with a 100× oil immersion objective. The investigated capsule suspension was placed between glass slide and cover slip glued at the edges. Excitation wavelength 488 nm was chosen accordingly to fluorescein labels.
(24) To encapsulate urease, one part of empty capsule suspension was mixed with 2 parts of 96% ethanol, and 1 part of 10 mg/mL urease solution (U-4002, Sigma) was added. After a 10 min incubation period, the capsules were centrifuged and washed with water to remove ethanol.
(25) To control the weight of empty and loaded capsules, the quartz crystal microbalance technique (QCM, USI-System, Japan) was used.[27] In the measurements, 5 μL of empty shells or loaded capsules with equal particle concentrations were dropped onto the QCM electrode and dried. By frequency shift, we calculated the mass of capsules in the solutions. The long-term stability (several hours) of the quartz resonator frequency was within ±2 Hz. The resonators used were coated with evaporated gold electrodes (0.16 cm^2) on both faces, and their resonance frequency was 9 MHz (AT-cut). For the QCM electrodes used in this work, the following relationships between the frequency shift (ΔF, Hz) and polyion layer mass (M, g) is valid: $M = -0.87 \times 10^{-9} \cdot \Delta F$.[27−28]
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(29) Urease activity assay. A colorimetric assay based on the hydrolysis of urea was used for the activity control of free and immobilized urease, as monitored by the pH-sensitive dye bromcresol purple. A test solution containing 25 mM urea, 0.015 mM bromcresol, and 0.2 mM EDTA was adjusted to pH 5.8. This solution was placed in a UV cell and stirred. A known amount of the urease-loaded capsules was then added to this solution, and the kinetics was monitored by following the UV absorption at 588 nm. The increment of the absorbance with time was used to characterize the urease activity in the sample.[18,19]

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