Enzyme-Catalyzed Polymerization of Phenols within Polyelectrolyte Microcapsules

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ABSTRACT: Enzyme-catalyzed synthesis of a phenolic polymer within layer-by-layer (LbL) constructed polyelectrolyte microcapsules has been introduced. This approach is based on selective permeability of capsule walls for monomer molecules, while biocatalyst and forming polymeric chains cannot leave capsules interior because of high molecular weight. Horseradish peroxidase was encapsulated into four bilayer poly(styrenesulfonate)/poly(allylamine hydrochloride) capsules with an average diameter of 5 μm using pH-driven pore opening. 4-(2-Aminoethyl)phenol hydrochloride (tyramine) was used as a monomer giving easily detectable fluorescent polymeric products after addition of hydrogen peroxide into the system. Filling of the capsules with polymer was confirmed by different methods (AFM, quartz crystal microbalance, and confocal microscopy).

Introduction

Chemical synthesis in micron and submicron confined volumes is a research area of intensive investigation. Examples of confined media include fluid systems such as reversed micellar systems,[1−3] solid systems such as mesoporous materials,[4] and microporous crystalline zeolite cages[5] where organic and inorganic synthesis can be conducted with unique reactivity and selectivity characteristics. A novel medium for synthesis is the microcapsules obtained by layer-by-layer (LbL) assemblies of oppositely charged polyelectrolytes, proteins, and nanoparticles that allow the creation of thin multilayered films with nanometer thickness.[6−9] The fabrication of LbL assemblies on extremely small solid cores results in the formation of micron and submicron capsules after dissolution of the interior material[10] (Figure 1). Until now, several different approaches to encapsulation of proteins inside polyelectrolyte capsules or in polyelectrolyte walls have been proposed.[11−16] Urease,[11,14] peroxidase,[12] α-chymotrypsin,[13] and glucose oxidase[15] have been encapsulated, and the retention of encapsulated enzyme activity has been proven. It has been shown that the capsules loaded with protein possess good stability, with no leakage of encapsulated enzyme during their storage.[17] The polyelectrolyte capsules provide interesting possibilities to design catalyst-rich microvolumes in a bulk aqueous phase. Unique permeability properties of the capsule walls allow the retention of proteins and polymers, but low molecular weight substrates can easily permeate in and out through the walls, making these microcapsules excellent carriers for biocatalytic reactions.[18,19]

Recently, synthesis of inorganic materials inside the capsules and/or within capsule walls were introduced.[20−24] These include the synthesis of nanosized magnetite (Fe_3O_4)[20] and various magnetic ferrites.[21] More sophisticated synthesis of insoluble carbonate precipitates using production of carboxyl ion via urease-catalyzed decomposition of urea also has been demonstrated.[15] Enzyme-catalyzed synthesis in polyelectrolyte capsules offers possibilities to mimic the processes occurring in living organels (such as cells), and a large variety of enzymes and their substrates open a wide area for enzymatically catalyzed synthesis.[25]

Peroxidase is among the most promising enzymes to be encapsulated. The area of peroxidase applications includes enzyme-catalyzed synthesis of polyphenols and polyanilines,[1−3,26−30] numerous enzymatic assays,[31] and other reactions where generation of radicals is enzyme-dependent.[32] Among the substrates for peroxidase, there are several water or organic solvent soluble phenols and anilines. In addition, the monomers can be modified by introducing different side groups with specific properties including selective binding of organic ligands and drugs.[28,29]

The mechanism of the horseradish peroxidase (HRP)-catalyzed reaction of phenol and aniline polymerization is well-studied.[33,34] The enzyme transforms through multiple oxidation states during the catalytic steps of substrate reacting with hydrogen peroxide to form highly reactive free radicals. Phenoxyl coupling through condensation leads to chain growth. The polymeric chains can also bind tyrosine moieties in HRP.[35]The objective of this work is to use horseradish peroxidase encapsulated inside polyelectrolyte LbL capsules as a catalytic system for a synthesis of phenolic polymers confined within the microcapsules.

Experimental Section

Materials. Poly(sodium 4-styrenesulfonate) (PSS, MW 70 000) and poly(allylamine hydrochloride) (PAH, MW 70 000) were purchased from Aldrich. Peroxidase from horseradish (HRP, 250−330 units/mg solid), fluorescein isothiocyanate (FITC) and FITC-labeled peroxidase (HRP-FITC, 200 units/mg solid), hydrogen peroxide (H_2O_2, 35 wt %), hydrochloric acid (HCl, 36 wt %), and 4-(2-aminoethyl)phenol hydrochloride (tyramine) were obtained from Sigma. Monodisperse particles (diameter ~ 5.06 ± 0.16 μm) of weakly polymerized melamine formaldehyde (MF) were obtained as 10% solution in water from Microparticle GmbH, Germany.

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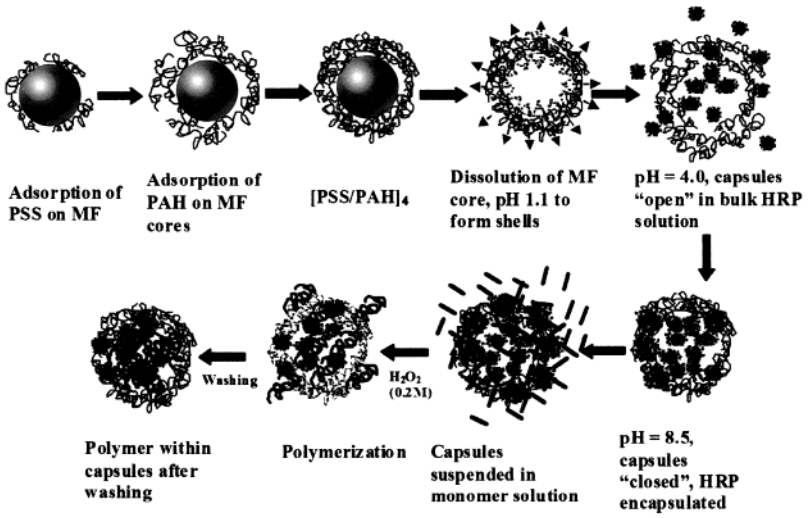
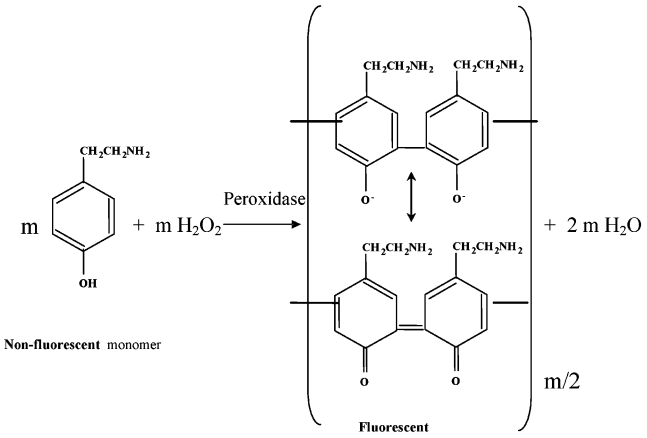


Figure 1. Schematic representation of capsules formation via LbL assembly, encapsulation of peroxidase (HRP) within capsules, and polymer formation in situ capsule.

Scheme 1. Horseradish Peroxidase Catalyzed Polymerization of Tyramine



Preparation of Microcapsules. Deposition of polyelectrolyte multilayers on MF cores was accomplished using the procedure elucidated elsewhere.[10,12] Typically, PAH and PSS solutions (0.2 mL of 3 mg/mL in 0.5 M NaCl) were alternately added to 1.5 mL diluted to 1% suspension of MF particles at pH 6.5. Each polyelectrolyte layer was adsorbed for 15 min, and then three intermediate washings with deionized (DI) water were made before addition of the next layer to remove polyelectrolytes remaining in supernatant solution. The assembly of the layers on MF cores was confirmed by monitoring alternate changes of electrophoretic potential (ξ-potential) after deposition of every layer using a Brookhaven Instruments Corp. zeta potential analyzer. For the measurement, 20 μL of the sample was diluted to 1.5 mL with DI water.

After deposition of eight alternate polyelectrolyte layers, the MF cores were dissolved by treatment with HCl (pH $<$ 1.0). The capsules obtained were washed with DI water until the capsule suspension was maintained at pH 5.5. The concentration of the sample used for further experiments was 1.5×10^8 capsules/mL.

Encapsulation of Horseradish Peroxidase in Microcapsules. $(PSS/PAH)_4$ capsules suspension, volume 200 μL, was mixed with 200 μL of HRP solution (3 mg/mL, pH 4.0), and the mixture was kept at room temperature for 24 h. Horseradish peroxidase is stable under these conditions.[8,13] At the next step, the pH of the mixture was adjusted to 8.5, and after 1 h, three washing−centrifugation cycles were carried out to remove the residual peroxidase that was not encapsulated. Confocal microscopy (a Leica DMRE2, Germany) was used to verify encapsulation of FITC-labeled peroxidase.

Polymerization in Situ. Equal volumes (200 mL) of the suspension of capsules with HRP encapsulated within and tyramine (4−60 mg/mL) solutions were mixed at pH 8.5. After 1 h, the polymerization was started by the addition of hydrogen peroxide to a final concentration of 0.2 M. The reaction mixture was kept for 24 h at room temperature (25 °C), following which traces of monomer and polymeric chains with relatively low molecular weight were removed by washing with DI water.

The masses of hollow capsules with peroxidase and the polymer synthesized inside were estimated as described elsewhere.[20−23] The mass of dry capsules in definite volume of suspension was determined by QCM weighing, and an amount of them in 1 mL was calculated from confocal laser scanning microscopy images using a transmission mode.

A specimen of free polymer in solution was prepared by mixing 400 μL of tyramine (3 mg/mL), 100 μL of HRP (3 mg/mL), and 10 μL of hydrogen peroxide (35 wt %) at pH 8.5 in order to compare the properties of polymer formed within microcapsules and that in free solution.

Characterization of Capsules. The size, integrity, and loading amount of the enzyme and polymer into a capsule were characterized using a confocal microscope. The loading was estimated as $((F_{min} - F_0)/(F_{max} - F_0)) \times 100\%$, where F_{max}, F_{min}, and F_0 are the intensities of signal from walls and interior of the capsule and that outside the capsule obtained from a profile of fluorescence along the capsule cross section. Fluorescently labeled peroxidase (HRP-FITC) was used to visualize the distribution of the encapsulated protein. For visualization of morphology and distribution of polymer within a capsule FITC was added to the capsule suspension.

When nonlabeled peroxidase (HRP) was used, the polymer of tyramine formed in situ is the only product which can give fluorescence. The emission and excitation characteristics of the polymer formed in solution and inside the capsules were studied using a fluorescence spectrometer (Photon Technology International Inc.). Although the intensity of polymer fluorescence at the excitation−emission conditions available with the confocal microscope was rather weak, it was sufficient to confirm formation of the polymer and to characterize its distribution within the capsule interior.

The atomic force microscopy (AFM) images of hollow capsules, capsules filled with peroxidase, and those after polymerization of tyramine inside were obtained with a Q-Scope 250 Quesant instrument with intermittent-contact mode. Samples for AFM were prepared by applying 5 μL of capsule suspension on fresh cleaved mica surface followed by drying of the samples at room temperature.

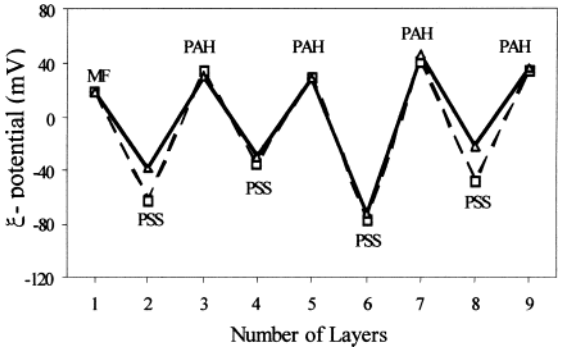


Figure 2. ζ-potential of polyelectrolyte-coated MF particles vs number of adsorbed layers. The dashed and solid lines represent data of two independent experiments on assembling $(PSS/PAH)_4$ shell on MF cores.

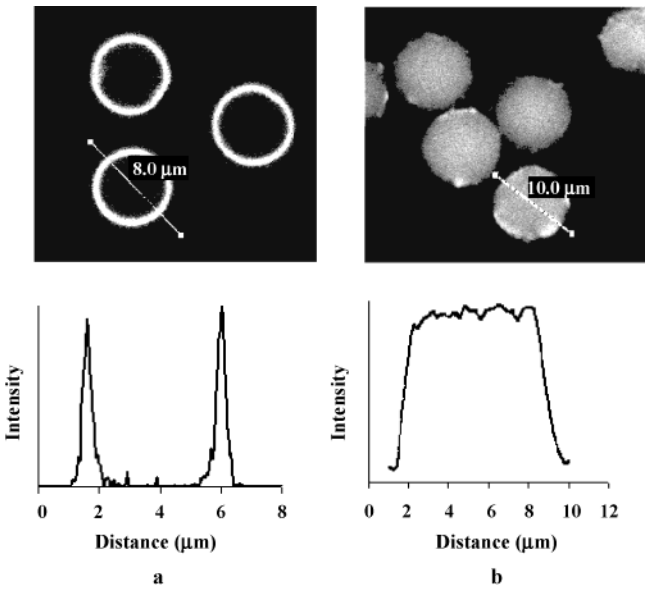


Figure 3. Confocal images of hollow $(PSS/PAH)_4$ capsules obtained after core dissolution (FITC was added to the suspension to visualized the capsules) (a) and capsules loaded with peroxidase labeled with FITC (b), as well as corresponding intensity profiles across the equatorial plane the capsules.

Results and Discussion

Four PSS/PAH bilayers were assembled on MF cores as indicated in the Experimental Section. Figure 2 shows the alternation of ξ-potential of the particles with the adsorption of each polyelectrolyte layer. After adsorption of anionic PSS a surface potential became −40 mV, after consequent deposition of cationic PAH it became +40 mV, and so on until the shell of eight polyelectrolyte layers with PAH outermost is formed. The assembly is repeatable and stable and follows the general scheme of alternate adsorption of oppositely charged linear polyelectrolytes.[6−8] The capsules obtained after core dissolution (Figure 3a) have a diameter of about 4.5 μm and are a little bit shrunken compared with the parent polyelectrolyte shell on MF cores (5.0 μm diameter).

An encapsulation of peroxidase (HRP) in the capsules was the first step toward achieving bio/polymerization in situ in the capsules. When the pH of the bulk HRP solution with the capsules suspended was adjusted to 4.0, an increase in shell wall permeability was developed.[16] This allows permeation of the protein into the capsules. When the pH is subsequently adjusted to 8.5, the capsule walls close again, leaving peroxidase trapped mostly within the capsules and less in the walls.[16] Figure 3b shows the capsules after encapsulation of FITC-labeled HRP. The confocal intensity profiles of both empty and filled capsules taken across their equatorial plane confirm almost uniform filling of the microcapsules with peroxidase (Figure 3).

Polymerization in Situ Capsules. Because of their low molecular weight, tyramine and H_2O_2 permeate freely into the capsules already loaded with peroxidase (HRP). Polymer formation occurs both in the capsule interior and in the walls where all the three reagents (tyramine, hydrogen peroxide, and biocatalyst-HRP) presented. We assume that the polymer with relatively long polymer chains remains inside the capsules due to the selective permeability of capsule walls.[20−24] The capsule walls are not permeable for macromolecules with molecular weight higher than 4000, which corresponds in our case to polymerization degree of 24. A small amount of polymer may be formed in the solution surrounding the capsules due to presence of trace amounts of the enzyme released from capsules. The microcapsules with the polymer synthesized inside were washed several times with DI water to remove traces of monomer, H_2O_2, peroxidase, and oligomers in supernatant solution.

Peroxidase-catalyzed polymerization of tyramine produces highly fluorescent polymers in accordance with the literature on fluorescent polymers formed by the enzymatic coupling of phenolic species and their derivatives.[3,36−38] The fluorescent spectrum of the polymer products formed with biocatalysis in solution by mixing of monomer and initiator is shown in Figure 4a. The excitation and emission wavelengths for the polymer are 320 and 420 nm, respectively, which are in good agreement with that (326 and 410 nm) available for this system in the literature.[36,37] In microconfined volumes when the polymer was formed inside the polyelectrolyte capsules the fluorescence spectrum changes drastically (Figure 4b). No prominent maximum but extended decline of emission with increasing the wavelength was found (while exciting at 320 nm). A tail of emission in the visible range of spectra was found, which allowed using confocal microscopy for visualization of polymer microparticles. It has to be mentioned that synthesis in submicron volumes often gives materials with a structure different from that in bulk.[3,20−22] In this case, phenol polymer was formed in the presence of the enzyme and in vicinity of a charged polyelectrolyte surface. Subtle structural variations of the polymer and its environment have a significant effect on polymer luminescence (for example, polyaniline formed in the presence of poly(styrenesulfonate) exhibits a 200 nm red shift in absorption maxima[30]).

Figure 5 shows confocal images and intensity profiles for the capsules filled with polymer synthesized at various concentrations of monomer. To observe the effect of monomer concentration on capsule loading, polymerization was performed at tyramine concentrations in the range 2−30 mg/mL. One can see that the relative fluorescence intensity is more pronounced at the walls of the capsules as compared with the capsule interior. This may be due to (1) higher partial adsorption of the catalyst (HRP) within the capsule walls, (2) adsorption of the polymer formed inside capsule onto the walls, and (3) different structure and optical characteristics of the polymer formed in polyelectrolyte layer. As the mono-

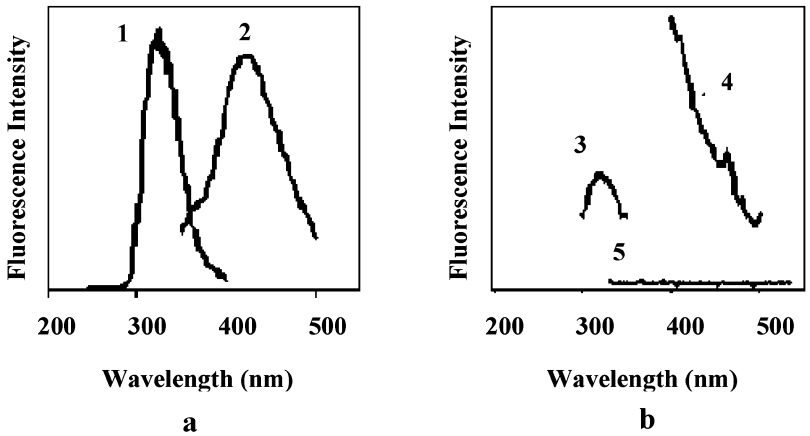


Figure 4. (a) Excitation (1, while emitted at 420 nm) and emission (2, while excited at 320 nm) spectra of polymer formed in bulk solution. (b) Excitation (3) and emission (4) spectra of shells with polymer formed inside as well as emission spectrum for empty shells (5) excited at 320 nm.

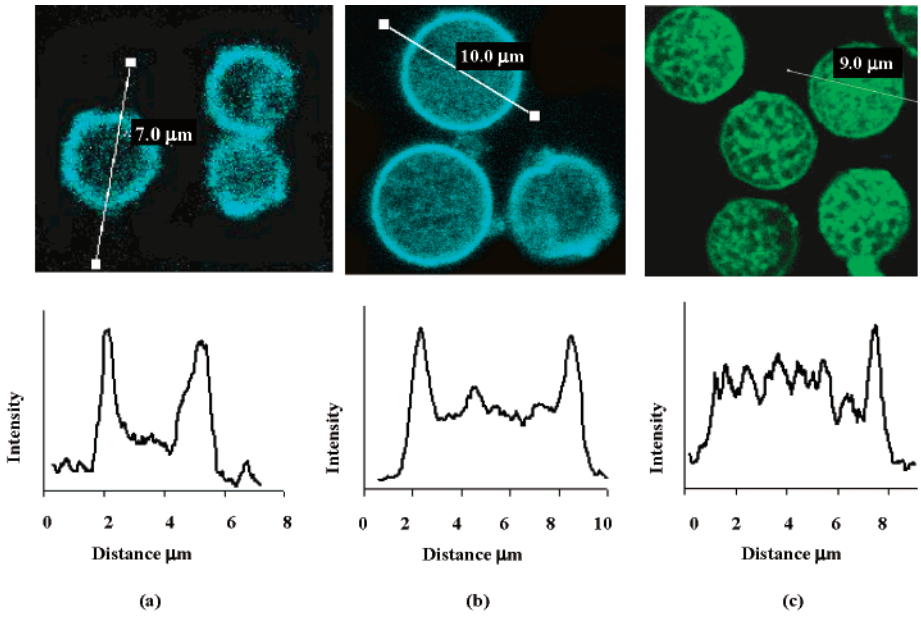


Figure 5. Confocal images of capsules with polymer formed in situ at different initial concentration of monomer: a, 2 mg/mL; b, 30 mg/mL and corresponding intensity profiles; c, the same as (b) but additionally stained with FITC.

mer concentration is increased, the ratio of the fluorescence intensity at the wall to that in the interior also increases. It is therefore likely that the amount of polymer on the microcapsule walls increases with monomer concentration. Brighter spots of 400 nm diameter are visible in the capsule cross-sectional image (Figure 5b), which indicates polymer nucleation.

To better understand the morphology of the material formed in the capsules, fluorescein isothiocyanate (FITC) was used to stain the capsules filled with the polymer (Figure 5c). The polymer distribution appears to be in the form of nuclei inside the capsules. The noticeable formation of these nuclei began 40−50 min after addition of H_2O_2 to the reaction mixture. No patterns were observed if only tyramine was added to the capsules loaded with peroxidase. The dimensions of the islets are about 400 nm in diameter, and their appearance can be related to the increasing hydrophobicity of material within the capsules. This is a consequence of the formation of the hydrophobic phenolic polymer, which prevents delivery of the monomers to the reaction zone. It should be mentioned that peroxidase acts both as the catalyst and as a template for the polymerization and that the polymer is formed at tyrosine sites of HRP.[35]

Characterization via AFM. The AFM image of hollow capsule (Figure 6) shows that the total height of the folded dried four-bilayer capsule is about 20 nm. The thickness of a capsule with peroxidase encapsulated inside increased to 40 nm, indicating that extra material is included inside. Considering the shape of dried capsule as a cylinder and taking density of solid residue of polymer and protein as ca. 1.1 g/cm^3, the mass of hollow capsule can be estimated as 0.5×10^{-12} g, and that of the HRP loaded in the capsule is ca. 0.7×10^{-12} g. Confocal microscopy gives the diameter of such capsule in solution of about 7.0 μm and its volume is 1.8×10^{-10} cm^3. Therefore, the concentration of peroxidase inside the capsule is ca. 4.1 mg/mL. It is larger than an initial peroxidase concentration used for the loading (1.5 mg/mL) and indicates accumulation of the protein inside the capsules. Similar protein accumulation was observed for polyelectrolyte capsule loading with hemoglobin and glucose oxidase when loading with initial 0.5 mg/mL resulted in 25−30 mg/mL protein

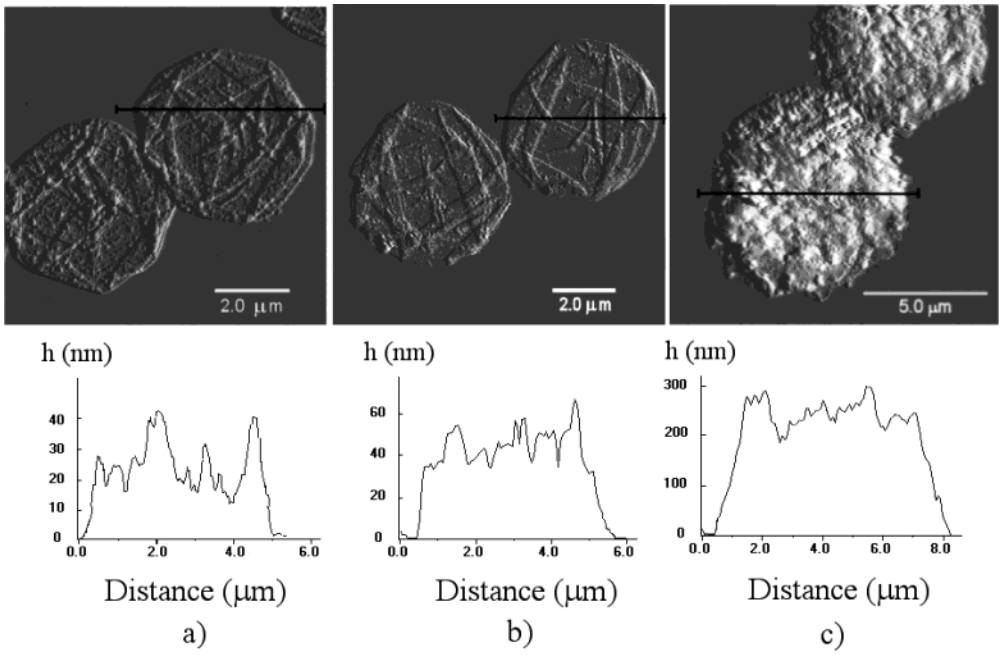


Figure 6. AFM images of capsules and height profiles of one of the capsules: (a) hollow, (b) filled with peroxidase, and (c) after polymerization of tyramine inside the capsules.

concentration in the capsule.[15] A mechanism of such protein concentration increase inside the capsules is not clear; it may be connected with asymmetrical diffusion in and out of the capsules due to asymmetry in the capsule wall having negative inner surface (PSS) and positive outermost (PAH).

Let us consider AFM image of dried polymer-loaded capsule (Figure 6c). The diameter of the capsule filled with polymer is 7.5 μm, its cross section is 44.2×10^{-8} cm^2, and the height in dry state is 240 nm (i.e., much more than thickness of empty capsules, Figure 6a). Therefore, the volume of dried capsule containing polymer is 10.6×10^{-12} cm^3, and its mass is 11.7×10^{-12} g (if density of solid residue is considered as 1.1 g/cm^3). One can calculate that the mass of polymer formed in the capsule is 10.4×10^{-12} g; this value is 1 order of magnitude higher than the amount of peroxidase loaded in one capsule, which is evidence of occurring polymerization. This conclusion is also confirmed by the results obtained by using QCM and confocal microscopy. The evaluated mass of dry capsule increases from ca. $\sim 10^{-12}$ g for empty and HRP loaded capsules to $>10^{-11}$ g after polymerization.

In the case of 30 mg/mL initial tyramine concentration the amount of monomer in one 7.5 μm diameter capsule should reach 6.6×10^{-12} g, assuming equilibrium with bulk concentration. In comparison with the polymer mass calculated within a microcapsule (10.4×10^{-12} g), it appears that additional tyramine participates in the polymerization reaction diffusing into capsules from the surrounding solution during the polymerization.

Different explanations can be proposed for the fact that the amount of the polymer remaining inside the capsules is smaller than amount of bulk monomer available for polymerization. All capsules occupy less than 1.5% of total volume of the solution, meaning that more tyramine could be polymerized. One of the reasons for the deceleration of the reaction on this stage may be in blocking peroxidase catalytic centers with formed polymers.[33,34] Indirect experimental evidence for this can be the formation of islets with increased density within capsules composed probably from HRP−polymer complexes. Among other reasons the sealing of the walls with newly formed polymer resulting in preventing penetration of tyramine molecules inside the capsules can be considered. Permeability of the walls for oligomers with molecular weight lower than several thousand could also result in losing some polymeric materials.

Conclusions

Horseradish peroxidase loaded capsules formed via layer-by-layer assembly of polyelectrolytes have been successfully employed in synthesizing polymer within the microcapsules. The general approach is based on selective permeability of the capsule walls, which are penetrable for monomers while the polymeric chains are unable to leave the capsule interior. The formation of biocatalyst-rich microvolumes in a continuous aqueous phase by encapsulation of the enzyme within the capsules of selected sizes is demonstrated in this work. Capsule diameter may be taken in the range from 100 nm to tens of microns depending on template-soluble cores. Through biocatalysis, it is possible to synthesize functional materials in the microcapsule (e.g., luminescent polymers) and to modify permeation properties of the microcapsule walls by depositing polymers in the walls. The wide variety of enzymes and monomers that can be used open up limitless possibilities for construction of new types of micro- and nanocomposites with desirable properties in a biologically friendly environment.

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