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ARTICLE

Encapsulation of enzymes in silica nanocapsules formed by an amphiphilic precursor polymer in water

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In this work we report a fully aqueous and highly efficient method for the microencapsulation of enzymes in silica nanocapsules during their formation process. In this approach, enzymes are first enclosed into unilamellar vesicles self-assembled by an amphiphilic precursor polymer – poly(ethylene glycol) substituted hyperbranched polyethoxysiloxane (PEG-PEOS) – in water. After subsequent condensation under basic conditions enzyme-loaded silica nanocapsules are obtained. Due to the significant volume shrinkage during the PEG-PEOS conversion, the encapsulation efficiency is very high, i.e. by adding only 5 wt.-% PEG-PEOS almost 50 % of the enzyme from the solution is encapsulated. As compared with the free enzyme, the encapsulated by this means protease preserves almost 40% of its activity, exhibits significantly enhanced stability against the change of environmental conditions, and can be repeatedly regenerated without a significant activity loss.

Introduction

Microencapsulation technologies that allow enclosing tiny particles or droplets in a membrane-like physical barrier have been of great interest for a long time due to their relevance to medicine, agriculture, food and cosmetic industries.[1-3] Commonly employed systems include liposomes, polymer particles and emulsion droplets, and the encapsulated substances range from drugs, pesticides, fragrances to biomolecules like enzymes. Enzymes are important biocatalysts for a broad variety of organic reactions with extraordinary high activity and high level of region-, chemo- and stereo-selectivity under mild and sustainable conditions.[4-6] They find numerous applications in various fields such as chemical synthesis, food, textile, cosmetics, medicine, etc. Numerous efforts have been devoted to enzyme immobilization driven by the benefits with respect to enhanced stability, reusability and prevention of enzyme contamination in products.[7] Compared with other enzyme immobilization techniques like adsorption and covalent binding, in the encapsulation approach the chemical structure and conformation of enzymes are not modified, many enzymes can be immobilized simultaneously and the preparation conditions are usually mild. Since the enzyme molecules are physically confined in the capsules, it is desirable that they cannot diffuse through the membrane barrier, whereas the substrates and products may pass freely.

Organic polymers are the most commonly used shell materials for the preparation of encapsulated enzymes.[7] Conventionally, aqueous solutions of enzymes are encapsulated in a membrane utilizing the concept of interfacial processes like interfacial polymerization,[8] coacervation,[9] complexation,[10] etc. Layer-by-layer deposition is another well-known approach.[11] Usually the capsules are first prepared by the sequential adsorption of oppositely charged polyelectrolytes onto sacrificial particles, which are subsequently removed, and then loaded with enzymes by simple incubation in a medium where the capsules are permeable.[12] On the other hand, enzyme crystals themselves can be used as templates to deposit alternating polyelectrolyte layers to form polymer capsules enclosing enzymes in the core.[13] Silica is a promising alternative for encapsulation applications due to its chemical inertness, mechanical stability, biocompatibility, easy functionalization and optical transparency.[14] As compared with organic polymer based systems, the silica capsules exhibit much higher resistance to temperature, organic solvents, highly aggressive chemical substances and microbial attacks. Moreover, due to the intrinsic brittleness of the pure silica, the encapsulated substances can be released by breaking the capsules under mechanical force. Silica has been widely used for enzyme immobilization and in most of the cases enzymes are entrapped in sol-gel silica matrices[15, 16] or mesoporous silica structures.[17-19] Silica hollow spheres (capsules) have a unique hollow core structure with a high storage capacity as well as a water reservoir for maintaining the activity of encapsulated biological substances. However, it is a big challenge to incorporate enzymes into silica capsules, since most of them are prepared by removing the core materials using either calcination or chemical etching,[20] and the post-loading of high molecular weight compounds is hindered because of the non-swellable nature of

silica. It was reported that the sol-gel process in water-in-oil emulsions allowed encapsulating enzymes in silica capsules,[21-23] however, a large amount of organic solvent that could deactivate the enzymes should be used.

Recently we have developed a new self-templating method for the preparation of silica nanocapsules.[24] It is based on the self-assembly of an amphiphilic silica precursor polymer – poly(ethylene glycol) substituted hyperbranched polyethoxysiloxane[25] (PEG-PEOS) – in water to unilamellar vesicles, which are then converted to pure silica nanocapsules under basic conditions. Not only the mechanism of the silica capsule formation, i.e. substances can be encapsulated during the process of the silica capsule formation, but also the biocompatible ingredients and fully aqueous reaction make this approach promising for the encapsulation of fragile biomolecules like enzymes. Since a high pH value (pH = 11) is required to prepare the silica nanocapsules, the substances should be resistant to this medium so that they do not lose activity during the encapsulation process. In this work we use protease from *Bacillus sp.* (EC. 3.4.21.14), which is stable at high pH and catalyzes the hydrolysis of peptide bonds in proteins,[26] as a model enzyme of high molecular weight for the encapsulation experiments, and address the encapsulation efficiency as well as the properties of the encapsulated enzyme.

Experimental

Materials

Tetraethoxysilane (TEOS, 99%, VWR), acetic anhydride (98%, Merck), tetraethyl orthotitanate (95%, Merck), poly(ethylene glycol) monomethyl ether (PEG, average molecular weight 350, Fluka), protease from *Bacillus sp*. (Sigma-Aldrich), sodium dodecyl sulfate (SDS, ACS reagent, ⩾99.0%, Sigma-Aldrich), ammonium hydroxide solution (ACS reagent, 28.0-30.0 % NH_3 basis, Sigma-Aldrich), and Tris-HCl (PharmaGrade, Sigma) were used as received. Oligopeptide Suc-Ala-Ala-Pro-Phe-AMC was purchased from Bachem. Deionized water of Milli-Q grade was used for all experiments. PEG-PEOS, where 10 % ethoxy groups were substituted by poly(ethylene glycol) monomethyl ether with molecular weight of 350, was prepared according to a literature procedure.[24] The product has a brutto formula $SiO_{1.11}(OEt)_{1.60}(PEG)_{0.18}$. The degree of branching determined by ^{29}Si NMR spectroscopy[25] was 0.45, and the number and weight average molecular weight was measured by means of size-exclusion chromatography in chloroform calibrated with polystyrene standards to be 2800 and 4800, respectively.

Synthetic procedures

Encapsulation of protease in silica nanocapsules. Under magnetic stirring at 500 rpm PEG-PEOS (0.5 g) was dispersed in an aqueous solution (8.5 g) containing 2.125 mg protease. After 20 min, the ammonia aqueous solution (1 mL) was added, and the stirring was continued for another 24 h. Afterwards, the nanocapsules were isolated by centrifugation at 10000 rpm, and washed 3 times with 0.1 M Tris-HCl buffer of pH 8.5, and then re-dispersed in this buffer solution.

Analytic methods

Protein content measurements. Protein content was measured on a Thermo Scientific Multiskan MK3 spectrometer using BCA protein assay kit.

Enzyme assays. Enzyme activity assays were carried out in 96-well microtiter plates (black flat bottomed). An enzyme solution (10 μL) was diluted by a Tris-HCl buffer (40 μL, 0.1 M, pH 8.5), and then a substrate solution (50 μL, 200 μM Suc-Ala-Ala-Pro-Phe-AMC in 0.1M Trish-HCl buffer, pH 8.5) was added. The enzymatic activity was evaluated at room temperature by monitoring the increase of fluorescence intensity within 15 min using a multi-mode microplate luminescence reader (BioTek Synergy 2, USA, excitation: 360 nm, emission: 450 nm,). The specific activity of enzyme in this work is defined as the amount of free 7-amino-4-methylcoumarin (AMC) produced by 1.0 mg of enzyme per min.

Enzyme stability tests. The enzyme activity was measured at different temperature in order to assess the thermal stability of the encapsulated enzyme. Prior to the measurements the enzyme solutions in Tris-HCl buffer were incubated at different temperature (25, 40, 60 and 80°C) for 1h. The pH stability of the enzyme was studied by dispersing the enzyme in Britton-Robinson buffer of different pH values, and the activity measurements were carried out at 25°C. The influence of the surfactant (SDS) concentration on the enzyme stability was investigated by adding different amounts of SDS into the enzyme solutions and incubating the resulting solutions for 30 min at 25°C before the enzyme assays.

Enzyme reusability tests. For the reusability tests of the encapsulated enzyme, the silica nanocapsules encapsulating protease were isolated after 1h catalytic reaction at 25 °C by centrifugation at 10000 rpm. The nanocapsules were then washed three times with the Tris-HCl buffer (0.1 M, pH 8.5) and re-dispersed in this buffer for the next test cycle. The initial activity measured during the first assay was taken as 100%.

Field-emission scanning electron microscopy (FE-SEM). FE-SEM measurements were carried out on a Hitachi S-4800 microscope. The samples were prepared by placing a drop of an aqueous dispersion of silica nanocapsules on a silicon wafer substrate. Before being placed into the specimen holder, the samples were air-dried under ambient conditions and sputter-coated with gold prior to examination. Elemental analysis of freeze-dried silica capsules was accomplished by energy dispersive spectroscopy (EDS, Brucker Quantax 400) attached to FE-SEM.

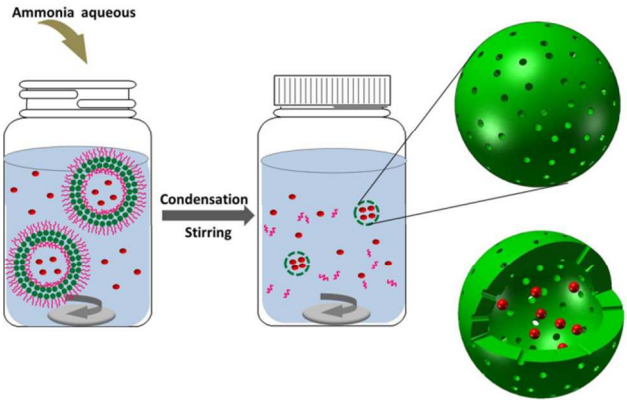
Transmission electron microscopy (TEM). TEM measurements were performed on a Hitachi H800 microscope. 2 μL of a diluted aqueous dispersion of silica capsules were placed on a formvar coated copper grid and air-dried at room temperature.

Dynamic light scattering (DLS). Hydrodynamic diameter of capsules was measured with the zetasizer Nano ZS (Malvern Instruments) with noninvasive back-scatter technology at a scattering angle of 173°.

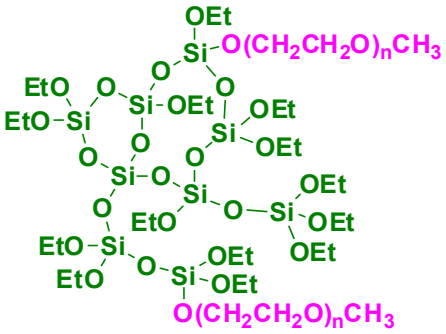
Nitrogen adsorption-desorption measurements. Nitrogen adsorption–desorption isotherms were recorded at 77 K on a Tristar II analyzer (Micromeritics, USA). Pore-size distribution curves of silica-capsules were calculated on the basis of the adsorption branch of nitrogen isotherms using the Barrett-Joyner-Halenda (BJH) method.

Results and discussions

The process for the encapsulation of enzymes in silica nanocapsules is illustrated in Scheme 1. As demonstrated in our previous study, the size of the silica nanocapsules formed by the amphiphilic silica precursor polymer, PEG-PEOS (Scheme 1b), decreases with the increase of dispersing energy.[24] In this work, PEG-PEOS is dispersed in the aqueous solution of protease under gentle stirring in order to obtain relatively big capsules. In this way part of the enzyme is enclosed in the unilamellar vesicles formed by this polymer. After adding an aqueous solution of ammonia, the vesicles are converted into silica nancapsules loaded with enzyme macromolecules. The resulting capsules are then isolated by centrifugation.



(a)



(b)

Scheme 1. Encapsulation of enzymes in silica nanocapsules (a) formed by amphiphilic precursor polymers, PEG-PEOS (b), in water.

After re-dispersion in the Tris-HCl buffer, the hydrodynamic diameter of the silica capsules was measured by DLS. A monomodal size distribution with a mean diameter of 233 nm and polydispersity index of 0.091 was observed (Fig.1). Figure 2 displays the electron micrographs of the silica nanocapsules exhibiting a near-perfect spherical morphology and a mean diameter of 180 nm, which is in a good agreement with the DLS data. The TEM image (Fig.2b) shows clearly a hollow structure with a mean shell thickness of 30 nm. As compared with nanocapsules without encapsulated enzyme formed under similar reaction conditions, neither size nor morphology is altered, indicating that the presence of protease does not influence the formation of silica nanocapsules. Furthermore, the high mechanical strength of the capsule shell was confirmed by the fact that no broken capsules were detected in the electron micrographs.

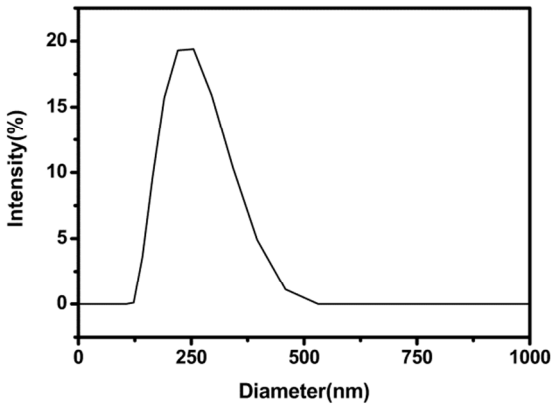


Fig.1 Hydrodynamic diameter distribution of silica nanocapsules encapsulating protease in 0.1 M Tris-HCl buffer of pH 8.5.

To confirm the loading of protease, elemental analysis of freeze-dried silica capsules with and without protease loading was carried out by means of EDS. The results of the EDS study are presented in Table 1. It can be clearly seen that the silica nanocapsules consist of pure SiO_2, and carbon and nitrogen elements were detected in the silica capsules loaded with protease. The encapsulated enzyme amount can, however, not be measured precisely by this means. To estimate the weight of the encapsulated protease, the enzyme content in the combined supernatant was determined spectroscopically using BCA protein assay kit. It was found that 48 % protease in the solution was encapsulated into the silica nanocapsules, and the loading of the enzyme in the centrifuged and freeze-dried capsule was 12 mg/g. The density of the sol-gel silica is 1.65 g/cm^3, the mean capsule size and wall thickness determined by electron microscopy are 180 nm and 30 nm, respectively, so the mean mass of a silica capsule is calculated to be 3.5 x 10^{-15} g. Therefore, one capsule should contain 4.2 x 10^{-14} mg protease. The molar mass of the protease is 27300; it is thus found that the average number of the protease molecules in each capsule is 900. Considering that the concentration of PEG-PEOS in the enzyme solution is only 5 wt.-% and the silica content of PEG-PEOS is 33 %, the encapsulation efficiency is surprisingly high. We believe that this must be the result of significant shrinkage of the capsules upon conversion to silica (Scheme 1), which was evidenced previously by DLS measurements,[24] leading to the concentrating of the enzyme inside the capsules.

Table 1 Elemental analysis data obtained via energy dispersive spectroscopy on silica capsules.

Element	Count (wt.-%)	
	Pure silica capsules	Protease loaded silica capsules
Si	41.39	40.71
O	58.61	56.14
C	-	1.23
N	-	1.91

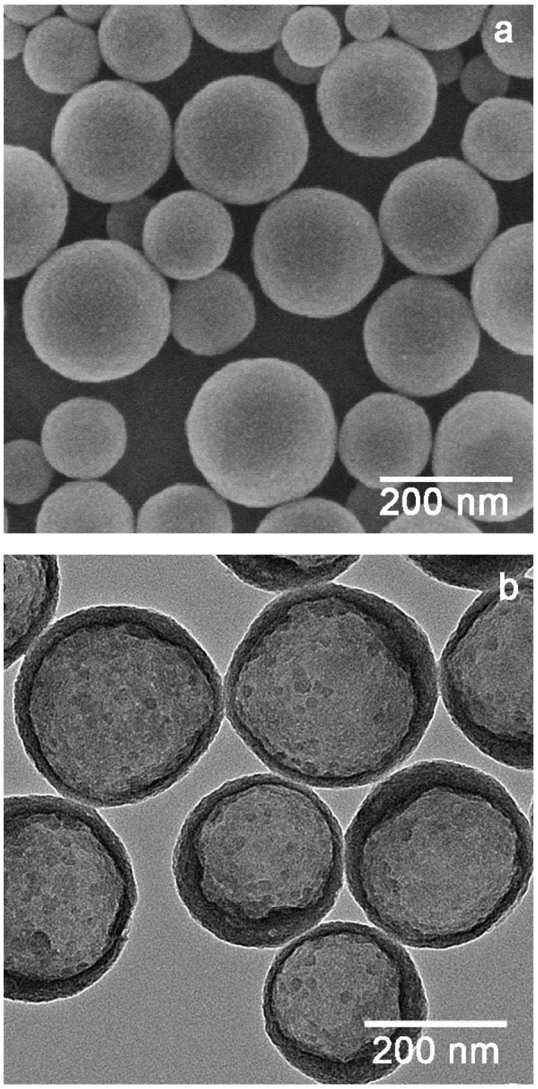


Fig.2 FE-SEM (a) and TEM (b) images of silica nanocapsules loaded with protease.

It is a question whether the protease encapsulated in the silica capsules is still catalytically active. For this it is important that the substrates can diffuse across the capsule shell through the pores. The porosity of the capsule shell was studied by nitrogen adsorption-desorption measurements. The silica capsules show a type IV nitrogen adsorption isotherm with a type H3 hysteresis loop[27] (Fig.3, inset), which was reported for silica nanorattles[28] and silica nanocapsules[29-31] with a mesoporous shell. The pore size distribution (Fig.3) was calculated with the BJH method. The sample exhibits a narrow mesopore size distribution with a peak diameter of 3.8 nm. The mesoporous silica shell is obtained possibly due to the formation of partly multilayered vesicle structure, in which PEG fragments are incorporated. Small molecules can certainly diffuse through these pores, but large enzyme macromolecules should be kept inside the capsules.

The enzyme assays were carried out using Suc-Ala-Ala-Pro-Phe-AMC oligopeptide as substrate.[32-35] In this system AMC becomes fluorescent, when it is freed after the peptide bond is hydrolysed. According to the activity assays, the protease encapsulated in the silica nanocapsules exhibits at 25 °C in 0.1 M Tris-HCl buffer of pH 8.5 a catalytic activity of 55.27±7.15 $U \cdot mg^{-1} \cdot min^{-1}$, meanwhile the activity of the free protease under the same conditions is 142.82±5.82 $U \cdot mg^{-1} \cdot min^{-1}$, and it remains unchanged after stirring in the same medium as for the encapsulation experiments (i.e. pH 11 with ammonia). Thus, protease encapsulated inside the silica nanocapsules preserves almost 40 % of its activity as compared with the free enzyme. The lower activity is typical for the encapsulated enzyme due to the retarded diffusion of the substrate and product through the capsules shell.[7]

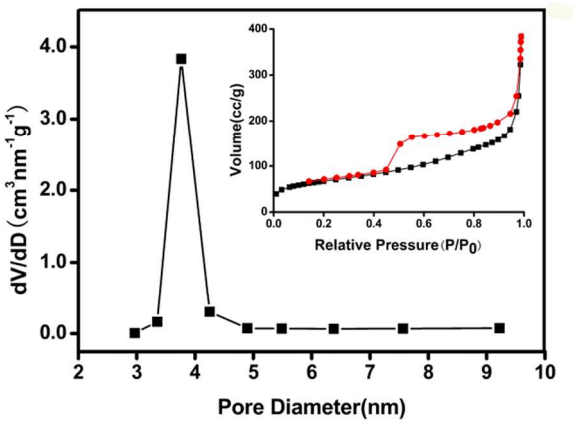


Fig.3 Pore size distribution curve of silica nanocapsules calculated from the nitrogen adsorption-desorption isotherm (inset) using BJH method.

Enzymes are generally very sensitive towards the change of environmental conditions. The protease encapsulated in the silica nanocapsules was studied regarding their stability against temperature and pH change. Fig.4 summaries the enzyme activity measured after incubating for 1h at different temperature and pH values. It can be seen from Fig.4a that the free protease loses quickly the activity upon heating. At 80 °C it preserves only 10 % of the activity of room temperature. At the same time, the encapsulated enzyme shows a much higher thermal stability and the activity declines only for about 20 % after heating at 80 °C for 1 h. The pH value of the environment has a strong influence on the conformation and hence the activity of the enzymes. As shown in Fig.4b, the highest enzyme activity is observed at pH 10 for both free and encapsulated protease, in contrast to the enzyme encapsulated using a sol-gel process.[36] This feature implies that the encapsulated protease retains its original conformation in the lumen of the silica nanocapsules without any interaction with the wall.[37] The encapsulation in silica improves significantly the stability of enzyme against the pH alteration. As can be seen in Fig.4b, upon decrease of the pH value from 10 to 7, the encapsulated protease loses only about 20 % of its activity, at the same time, the activity of the free enzyme declines for about 45 %.

Proteases are well-established ingredients in most household laundry detergents.[38] Moreover, they are often used in cell lysis and DNA extraction in the presence of surfactants.[39, 40] Some surfactants, for example SDS, are chaotropic agents that disrupt and denature the structure of protease and reduce the enzymatic activity. In this work the activity of the encapsulated protease in the presence of SDS was also investigated. Fig.5 shows the activity of both free and encapsulated protease incubated for 1 h in the media with different concentrations of SDS. At 0.5 %

SDS concentration the activity of the encapsulated protease is 84 % of that measured in the absence of SDS. In the case of free protease the activity decreases for 83 % at 0.5 % SDS concentration. When the concentration of SDS increases to 3 %, the encapsulated enzyme still preserves 46 % of the initial activity, at the same time, only 12 % of the activity remains for the free enzyme. As a conclusion, the encapsulated protease is much more stable in the presence of SDS than the free one.

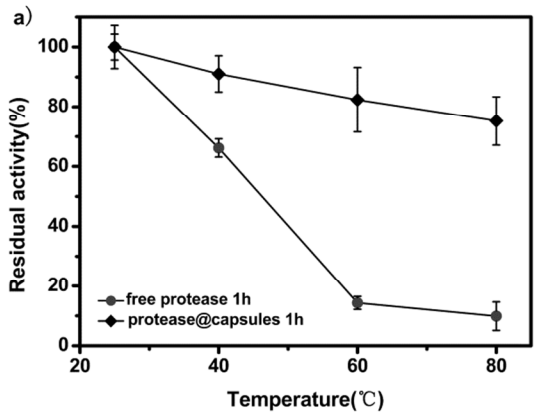


Fig.4 Residual activity of free and encapsulated protease *versus* temperature (a) and pH (b).

As shown above, the encapsulation of enzymes in silica nanocapsules can significantly improve their stability against the change of environmental conditions. This is certainly due to the presence of an efficient barrier consisting of silica surrounding the enzyme solution, retarding the material and energy exchange across the capsule shell. Due to the small pore size, it becomes even not possible for big macromolecules to pass through. Fig.6 presents the residual activity of the encapsulated protease during eight regeneration cycles. It can be seen that during the first couple of cycles almost no loss of activity is observed, even after 8 recovery cycles more than 50 % of the initial activity is still preserved. It seems that the silica shell indeed prevents the leakage of the protease molecules from the capsules. Furthermore, it also indicates that the silica nanocapsules have a good mechanical strength, which allows keeping their integrity during the centrifugation and continuous stirring processes as confirmed by the electron microscopy investigation. The activity loss during the recovery might be contributed to the loss of the capsules during the centrifugation and washing process due to the small capsule size.

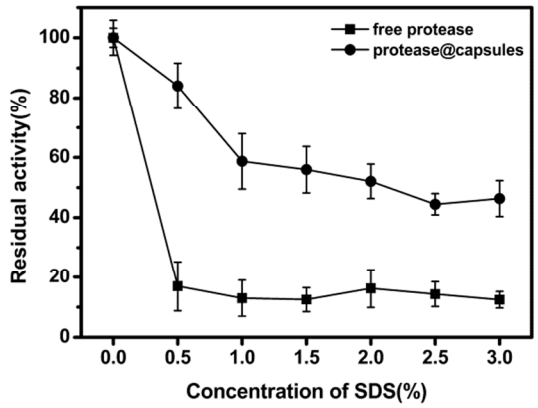


Fig.5 Residual activity of free and encapsulated protease at 25 °C and pH 8.5 *versus* SDS concentration.

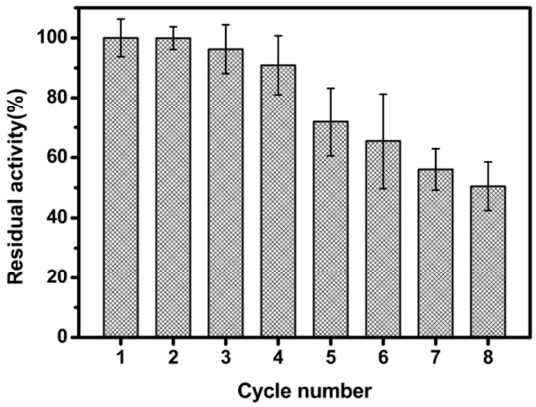


Fig.6 Residual activity of protease encapsulated in silica nanocapsules during regeneration cycles.

Conclusions

In this work protease was successfully encapsulated in 100-300 nm big silica nanocapsules by adding an amphiphilic silica precursor polymer, PEG-PEOS, to the aqueous enzyme solution during gentle stirring. The capsules consist of a mesoporous shell with a mean pore diameter of 3.8 nm, which allows the material exchange across the shell, but meanwhile prevents the leakage of the encapsulated enzyme. Importantly, the encapsulation efficiency is very high, i.e. by adding only 5 wt.-% PEG-PEOS almost 50 % of the enzyme from the solution is encapsulated. As compared with the free protease, the one encapsulated in the silica capsules exhibits 40 % lower catalytic activity, but shows significantly enhanced stability against the change of temperature, pH and the concentration of chaotropic surfactants. Furthermore, the enzyme-loaded capsules can be

repeatedly regenerated without a significant activity loss. These novel silica nanocapsules provide promising platforms for development of biosensors, drugs and enzyme reactors.

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Notes and references

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Synopsis

Enzymes are encapsulated in silica nanocapsules during their formation, i.e. they are first enclosed in unilamellar vesicles formed by an amphiphilic silica precursor polymer in water, and the enzyme-loaded silica nanocapsules are then obtained via subsequent basic condensation.