Biocatalytic Nanoreactors

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Polymersome Colloidosomes for Enzyme Catalysis in a Biphasic System**

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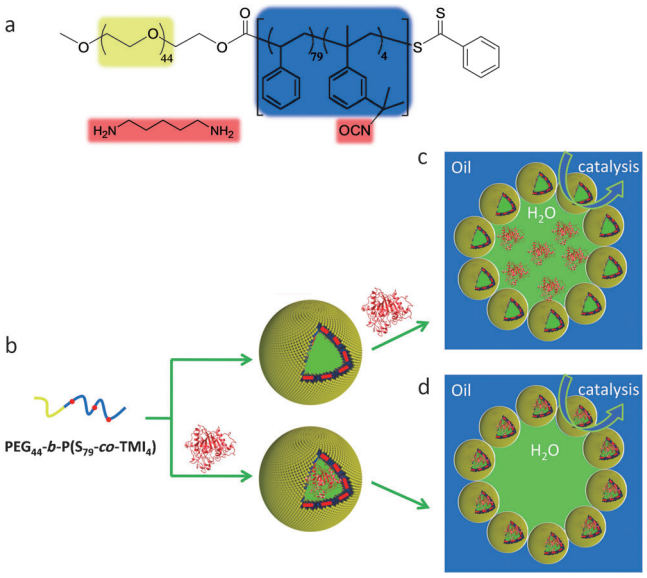
Enzymes are potent biocatalysts for a broad variety of organic reactions, providing important molecules in industry with high chemo-, regio-, and stereoselectivity[1] under mild and sustainable conditions.[2] However, widespread application of biocatalytic processes is hampered by the fact that in many cases biphasic conditions are required; biocatalysts are often only active in water, whereas the substrates are often only soluble in organic solvents. Prolonged exposure of the biocatalyst to an organic solvent and vigorous shearing and mixing to enable sufficient mass transfer often lead to its denaturation.[3] A simple and versatile method for solving this problem is to create an emulsion between an aqueous enzymatic solution and an organic medium containing the substrate. However, the necessary amphiphilic molecules, whether they are small surfactants or block polymers,[4] can also severely affect enzymatic activity and furthermore present a challenge with respect to workup and purification.[5]

Pickering emulsions, which are emulsions stabilized by colloidal nanoparticles, have proven to be useful replacements for the traditional surfactant-based emulsions.[6] The high stability of Pickering emulsions results from the permanent absorption of nanoparticles at the water/oil interface, which is in contrast to the dynamic absorption and desorption of surfactants.[6,7] Furthermore, using Pickering emulsions can avoid problems such as difficult separation during workup and foam formation, which accompany the use of more traditional emulsions. Based on these advantages, Pickering emulsions are good candidates for application in biphasic enzymatic transformations, especially as nanoparticles can be used that do not deactivate enzymes but protect them effectively from the organic medium. Up to now, only Wu et al. have applied inert silica nanoparticles to stabilize water/heptane emulsions with the encapsulation of catalytically active enzymes inside the water phase.[8] However, a more versatile system could be created if Pickering emulsions were stabilized by hollow nanoparticles such as polymersomes,[9] which themselves could be used for enzyme compartmentalization.

Polymersomes, self-assembled vesicles from amphiphilic block copolymers with a size range of 200–500 nm, can be treated as a special type of hollow colloidal nanoparticles.[10] In our group and others, polymersomes have been demonstrated to be converted into powerful and efficient bionanoreactors by encapsulation and attachment of enzymes.[11] Using polymersomes as colloidal nanoparticles to stabilize a Pickering emulsion is a promising way to achieve biphasic enzymatic transformations. The polymersome Pickering emulsion naturally provides multiple compartments for loading enzymes in different ways and maximizes the contact area at the interface between two phases. Here we present for the first time an enzyme-filled Pickering emulsion that is stabilized by polymersomes (Scheme 1). By loading a model lipase in the aqueous phase or inside the lumen of the polymer vesicles we show here that this colloidosome architecture can be efficiently and repeatedly applied in biphasic enzymatic transformations.

As a first prerequisite for the development of a polymersome-based Pickering emulsion, the polymersomes should be stable in the organic solvent applied. In order to avoid polymersome dissolution, covalently crosslinked polymer-



Scheme 1. a) The chemical structure of the block copolymer of which the polymersome is constructed. b) Representation of the crosslinking process to prepare stabilized polymersomes. c,d) Schematic representation of a Pickering emulsion with the enzyme in the water phase (c), or with the enzyme inside the polymersome lumen (d).

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somes were designed to achieve long-term stability. Despite abundant research in crosslinked micellar structures,[12] examples of crosslinked polymersomes have been quite rare up to now.[9,13] In our study, we opted to use the crosslinkable polymersome building block poly(ethylene glycol)-*b*-poly(styrene-*co*-3-isopropenyl-α,α-dimethylbenzylisocyanate) (PEG-*b*-P(S-*co*-TMI); Scheme 1), which was based on a crosslinked micellar system developed by Stenzel et al.[14a] This block copolymer was obtained by reversible addition–fragmentation chain-transfer (RAFT) polymerization of styrene and TMI employing a PEG_{44} chain-transfer agent. The inherent isocyanate moiety of TMI provides an anchor inside the hydrophobic domain of the polymersomes for crosslinking with an external crosslinking agent.[14] This approach provides versatile control over the crosslinking process. We envisioned that a TMI/styrene ratio of 5:95 would be ideal to retain the bilayer membrane and still get a fully crosslinked system.

The polymersomes were prepared by the cosolvent method.[15] The block copolymer was dissolved in THF, which is a good solvent for both segments. To induce self-assembly of the amphiphiles, ultrapure water, as a precipitant for polystyrene, was slowly added to the THF until a content of 50 vol% was reached. Then, an excess of 1,5-pentanediamine as the crosslinker was added to the mixture until all isocyanate groups had completely reacted. The crosslinking process was monitored by Fourier transform infrared (FTIR) spectroscopy. Urea peaks at 1542 and 1658 cm^{-1} were detected after the coupling reaction, whereas the vibration for the isocyanate moiety at 2256 cm^{-1} disappeared (Figure S1 in the Supporting Information).[16] After dialysis of the cloudy suspension against water, the crosslinked polymersomes were characterized by cryogenic scanning electron microscopy (Cryo-SEM; Figure S2a), dry SEM (Figure S2b), and transmission electron microscopy (TEM; Figure S2c). The polymersomes had the expected spherical morphology as shown in the Cryo-SEM image. The internal composition of some vesicles, which could be observed from the cross section, was in agreement with the typical spherical hollow structure. The thickness of the polymersome bilayer was estimated to be 40–50 nm. The average diameters of the spherical objects as determined by dynamic light scattering (DLS) were in the range of 250–450 nm.

The water in organic solvent Pickering emulsion, stabilized by the crosslinked polymersomes was formed by homogenizing the water and organic solvents, such as toluene, chloroform, and heptane (digital pictures shown in Figure S3 in the Supporting Information) in the presence of the polymersomes. Typically, 25 μL of concentrated aqueous polymersome solution (15 mg mL^{-1}) was mixed with 200 μL of toluene, and homogenized for 5 min to form the stable polymersome Pickering emulsion. For ease of characterization, the polymersomes were labeled with the dye rhodamine B. Thus, the polymersome Pickering emulsion was observed as round spherical droplets by confocal laser scanning microscopy (CLSM) (Figure 1a). The droplet size was in the range of 20–50 μm. The interface between water and toluene consisted of the crosslinked polymersomes, which were observed as red dots in the magnified image (inset, Figure 1a). In order to clearly distinguish the water and toluene phases in the Pickering emulsion, fluorescein isothiocyanate labeled dextran (FITC-Dex, 4.4 kDa) and Nile red (NR) were dissolved in water and toluene, respectively. With green emission (FITC-Dex) in the droplet and red emission (NR) outside of the droplet, the water-in-toluene Pickering emulsion could be clearly identified by the merged CLSM image (Figure 1b, single-channel images can be seen in Figure S4).

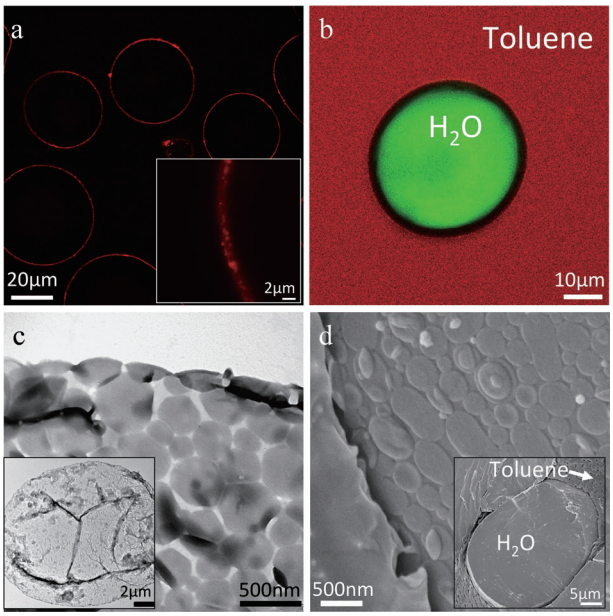

Figure 1. a,b) CLSM images of polymersome Pickering emulsions (water in toluene). a) Polymersomes are stained by rhodamine B; the inset is a magnified image. b) FITC-Dex (4.4 kDa) and Nile red were dissolved in water and toluene, respectively. c,d) TEM and Cryo-SEM images of polymersome Pickering emulsions; the insets are overviews at lower magnification.

TEM images revealed that the surface of the intact Pickering emulsion droplet consisted of closely packed polymersomes, which were collapsed as a result of the drying process (Figure 1c and inset). Cross-sectional Cryo-SEM images clearly showed polymersomes at the interface of water-in-toluene Pickering emulsions (Figure 1d and inset). Although the crosslinked polymersomes were not spherical particles anymore because of to the swelling effect of toluene on the shell of the polymersomes, the disklike morphology confirmed the stability of the crosslinked polymersomes against good organic solvents. In addition, it is worth mentioning that the polymersome Pickering emulsion could be stored unchanged for at least 200 days, as was confirmed by SEM (Figure S5 in the Supporting Information). The high stability is a result of the irreversible attachment of polymersomes at the interface between water and toluene, which is different from surfactant molecules which adsorb and desorb in a relatively dynamic manner.[6]

The structure specificity of the polymersome Pickering emulsion provides excellent possibilities for compartmentalization. Two different strategies were used to introduce enzymes, more specifically the lipase *Candida antarctica*

lipase B (CalB),[17] into this system. The first approach was to directly dissolve the enzyme in the water phase followed by formation of the Pickering emulsion. The second approach was to first encapsulate the enzyme into the lumen of the polymersomes, again followed by formation of the Pickering emulsion. For the latter approach, typically the enzyme encapsulation process involved sequential injection of 0.5 mL of CalB solution and 1.5 mL of phosphate-buffered saline (PBS) solution into 2 mL of the solution of the block copolymer in THF. After crosslinking and dialysis against PBS buffer to remove free CalB, THF, and excess crosslinker, the incorporation efficiency of the enzyme in the polymersomes was determined to be 16% by inductively coupled plasma mass spectrometry (ICP-MS).[11c,d] Prior to the enzymatic activity studies with CalB, the distribution and diffusion of CalB in both types of Pickering emulsions were determined by imaging rhodamine-labeled CalB with CLSM (Figure S6 in the Supporting Information). In the first case, CalB was not only dispersed in the water phase but also to some extent concentrated at the interface on account of the amphiphilic properties of the lipase (Figure S6a). In the second case, CalB was located only at the interface which indicated the success of encapsulating the enzyme in the lumen of the polymersomes (Figure S6b). Even 24 h after formation of the Pickering emulsion, there was no diffusion of CalB to the toluene phase in either case.

The catalytic performance of the two types of CalB-loaded Pickering emulsions was first investigated with the esterification of 1-hexanol and hexanoic acid using toluene as the organic phase. Two other experiments were carried out as controls. A polymersome Pickering emulsion without CalB served as a negative control, and a biphasic water/toluene system containing free CalB was used as a benchmark. Substrates were introduced into the toluene phase to initiate the enzymatic reaction. It was shown that the conversion of the reaction reached 80–90% at equilibrium with both Pickering emulsions, while less than 25% conversion after 24 h was observed for the biphasic system (Figure 2a). Since hexanol and hexanoic acid are more hydrophilic substrates than hexyl hexanoate, their accessibility to CalB at the Pickering emulsion interface is higher; consequently, the equilibrium is shifted more toward esterification than is the case for traditional homogenous catalysis systems (Figure S7).[17] After normalizing, the specific activity of CalB in the water phase of the polymersome Pickering emulsion (25.2 U mg^{-1}) was more than 4.4 times higher than that of native CalB in the biphasic water/toluene system (5.7 U mg^{-1}; Figure 2b). In addition, the specific activity of CalB in a Pickering emulsion stabilized by commercial PS nanoparticles (average diameter 300 nm) was calculated as 22.7 U mg^{-1} (Figure S8), which demonstrated that Pickering emulsions based on both the polymersomes and traditional nanoparticles were effective for improving enzyme activity. Furthermore, the specific activity of CalB could be further improved by maintaining the concentration of the enzyme but increasing the concentration of polymersomes to 16 mg mL^{-1} (Figure 2c). The average size of the droplets decreased from 267 μm to 52 μm when the polymersome concentration increased from 4 to 16 mg mL^{-1} (Figure S9). Increasing the polymersome concentration further had almost no effect on the droplet size. Wu et al. reported that the reaction mechanism of CalB followed Michaelis–Menten kinetics.[8] Thus, it is reasonable to believe that the improvement of the enzyme's specific activity is due to the significantly increased interfacial area of the polymersome Pickering emulsion droplets, which enhances the contact between substrate and enzymes. When CalB was encapsulated in the lumen of the polymersome, the specific activity of CalB (70.8 U mg^{-1}) was even 2.8 times higher than that of CalB positioned in the water phase of the Pickering emulsion system (25.2 U mg^{-1}) (Figure 2b). Since all of the CalB in the lumen of polymersomes was distributed at the interface of the Pickering emulsion, the effective use of the enzyme was enhanced compared to the former case.

The versatility of the polymersome Pickering emulsion system was examined by applying other organic solvents and different substrates for both esterification and hydrolysis. For instance, the esterification of 1-hexanol and hexanoic acid was also performed with high conversion when toluene was replaced with heptane. The specific activity of CalB in the water phase and encapsulated in the lumen of polymersomes was calculated to be 21.1 and 60.3 U mg^{-1}, respectively

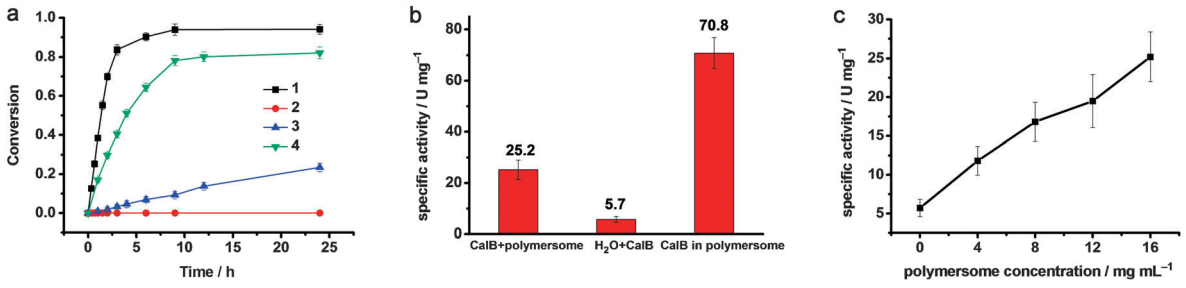

Figure 2. a) Plot of the conversion of the esterification of 1-hexanol with hexanoic acid catalysed by CalB under different conditions versus reaction time. Curve 1: CalB was dissolved in the water phase of the polymersome Pickering emulsion; Curve 2: polymersome Pickering emulsion without CalB; Curve 3: CalB in a biphasic water/toluene system; Curve 4: CalB encapsulated in the lumen of the polymersome Pickering emulsion. b) Chart of the specific activities of CalB dissolved in the water phase of the polymersome Pickering emulsion (left), CalB in a biphasic water/toluene system (middle,) and CalB encapsulated in the lumen of the polymersome Pickering emulsion (right). c) Plot of the specific activity of CalB dissolved in the water phase of the polymersome Pickering emulsion with increasing polymersome concentration; CalB concentration was kept the same; the organic solvent in all cases is toluene.

(Figure S10). Other reactions such as the esterification of benzyl alcohol and phenylacetic acid and the hydrolysis of *p*-nitrophenyl acetate all proceeded well with our polymersome Pickering emulsion systems (Figures S11 and S12).

Another apparent advantage of polymersome Pickering emulsions for biphasic enzymatic reactions is the ease of enzyme separation from substrates and products in the organic phase. Using a polymersome Pickering emulsion with CalB in the lumen of the polymersomes, the esterification of 1-hexanol and hexanoic acid was carried out repeatedly with both toluene and heptane as the organic phase. After each reaction cycle, only the organic solvents were removed and the biphasic system was rinsed several times with fresh solvents. New substrates were then introduced again to the system. During the purification process, there was almost no deactivation of CalB encapsulated in the lumen of the polymersomes. Therefore, the specific activity of CalB was maintained at 89% and 62% even after eight cycles (continuous operation for 192 h) of esterification in toluene and heptane, respectively (Figure 3). The conversion of the

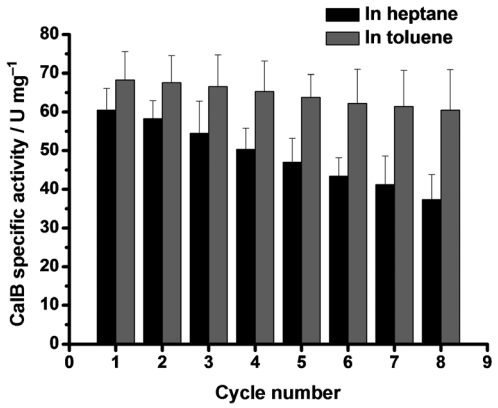

Figure 3. Chart of the specific activity of CalB encapsulated in the lumen of polymersome Pickering emulsions for cycles of esterification in heptane (black) and toluene (gray), respectively.

esterification reaction remained around 90% in all cases. The easy separation process and recyclability of CalB in polymersome Pickering emulsions (especially in the toluene case) indicates a significant improvement over the traditional surfactant-stabilized biphasic enzymatic systems.

In conclusion, the first successful construction of a polymersome-stabilized Pickering emulsion for application in biphasic enzymatic catalysis has been demonstrated. This type of Pickering emulsion was stabilized by fully packed cross-linked polymersomes at the water/oil interface. CalB, as a model enzyme, was loaded either in the water phase or in the lumen of the polymersomes of the Pickering emulsion, which highly enhanced its catalytic performance. Furthermore, the recyclability of CalB in the polymersome Pickering emulsion system could be effectively realized. Since the special structure of the polymersome Pickering emulsion naturally creates a system with different compartments, different enzymes or other catalysts can be loaded in separate spaces; this is ideal for performing cascade reactions. Overall, it is reasonable to expect that the novel polymersome Pickering emulsion system can be developed into a creative application in biphasic enzyme catalysis.

Experimental Section

Preparation of the polymersome Pickering emulsion: A 25 μL portion of the polymersome solution or the CalB-loaded polymersome solution diluted to 20 mg mL^{-1} was mixed with 200 μL of toluene. The mixture was treated with a vibration homogenizer at room temperature for 5 min. For the preparation of CalB in water, CalB was first dissolved in the polymersome solution with a concentration of 0.1 mg mL^{-1}. Similar procedures were performed with other organic solvents such as heptane.

Assessment of the catalytic performance of CalB by esterification of 1-hexanol and hexanoic acid in toluene and heptane: Typically, the substrate solution (1 mL), containing 400 mmol L^{-1} of 1-hexanol and hexanoic acid in toluene or heptane, was added to a Pickering emulsion (1 mL) containing CalB. The esterification reactions were carried out on a rotating shaker (80 rpm) at room temperature. For determining the product concentration, a 1 μL aliquot of the toluene or heptane solution was removed at different reaction times and analyzed by gas chromatography (GC). One unit of CalB activity (U) was defined as 1 μmol product produced within 1 min. The specific activity was related to the amount of the enzyme (U mg^{-1}). All reactions were repeated at least three times.

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