ARTICLE

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Investigations on Diffusion Limitations of Biocatalyzed Reactions in Amphiphilic Polymer Conetworks in Organic Solvents

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ABSTRACT: The use of enzymes as biocatalysts in organic media is an important issue in modern white biotechnology. However, their low activity and stability in those media often limits their full-scale application. Amphiphilic polymer conetworks (APCNs) have been shown to greatly activate entrapped enzymes in organic solvents. Since these nanostructured materials are not porous, the bioactivity of the conetworks is strongly limited by diffusion of substrate and product. The present manuscript describes two different APCNs as nanostructured microparticles, which showed greatly increased activities of entrapped enzymes compared to those of the already activating membranes and larger particles. We demonstrated this on the example of APCN particles based on PHEA-*l*-PDMS loaded with α-Chymotrypsin, which resulted in an up to 28,000-fold higher activity of the enzyme compared to the enzyme powder. Furthermore, lipase from *Rhizomucor miehei* entrapped in particles based on PHEA-*l*-PEtOx was tested in *n*-heptane, chloroform, and substrate. Specific activities in smaller particles were 10- to 100-fold higher in comparison to the native enzyme. The carrier activity of PHEA-*l*-PEtOx microparticles was tenfold higher with some 25–50-fold lower enzyme content compared to a commercial product.

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KEYWORDS: supported enzymes; biocatalysis; organic solvents; amphiphilic polymer conetworks; microparticles

Introduction

The synthesis of fine chemicals and pharmaceutics requires enantio- and regioselective catalysts. Enzymes are becoming a serious alternative for commonly used organometallic catalysts, which is due to the possibilities of modern biotechnology that allows the tailored design of enzymes and also their large-scale production to reasonable prices. Due to their high specificity and selectivity, low toxicity, good availability, and high activity, enzymes are superior catalysts in aqueous solution (Yuryev and Liese, 2010). However, the activity of biocatalysts in organic solvents, which greatly broadens the spectrum of substrates and also reaction types, is usually rather low, mostly due to their low solubility and stability in those media. An overview to this topic is given in several reviews (Klibanov, 2001; Rodriguez and Ferrandez-Lafuente, 2010; Schmid et al., 2001). Various approaches to increase the activity of enzymes in organic solvents are known.

The most established method is the adsorption of enzymes onto mesoporous carriers like silicates or methacrylate compounds by hydrophobic interactions (Hanefeld et al., 2009). A well-known example is the immobilization of lipase from *Candida antarctica* onto methacrylate beads, commercially available as Novozyme 435 (Bagsvaerd, Denmark). Another way is described by Sheldon et al. who developed so-called CLEA systems, where the biocatalysts exist as cross-linked enzyme aggregates (CLEA Technologies, Delft, the Netherlands) (Lopez-Serrano et al., 2002). Reetz et al. (1995) on the other hand realized the immobilization by encapsulation in sol–gel matrices, while Dordick et al. developed biocatalytic plastics with modified and copolymerized enzymes (Wang et al., 1997). Other methods known for enzyme immobilization onto carriers like polymer matrices are entrapment by complexation or in porous silica templates, covalent attachments, crosslinking, or encapsulation via prepolymer immobilization (Arika and Hasirci, 1993; Lei et al., 2002; Sheldon, 2007; Wei et al., 2001).

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We have recently discovered that amphiphilic polymer conetworks (APCNs) show great potential to activate entrapped enzymes in organic solvents. APCNs are versatile nanomaterials with the most prominent application as soft contact lenses, which allow water to diffuse through the hydrophilic acrylate phase and oxygen through the hydrophobic polydimethylsiloxane (PDMS) phase (Nicolson and Vogt, 2001). Other APCNs can even be swollen in water and perfluorinated solvents (Bruns and Tiller, 2006). Besides commercial applications many other examples of the high performance of APCNs are described in the literature, ranging from tubular networks for insulin delivery (Kennedy et al., 2000), their use as phase transfer matrix for enzymatic catalyzed conversions in organic solvents (Savin et al., 2005) and in supercritical CO_2 (Bruns et al., 2008), optical biochemical sensors for peroxide detection (Hanko et al., 2006a, b), biomimetic material for synthesis of biological membranes (Taubert et al., 2004), for release of antimicrobial surfactants (Tiller et al., 2005) for pH-sensitive drug delivery (Colinet et al., 2009; Liu et al., 2006), chemically cleavable conetworks (Rikkou-Kalourkoti and Patrickios, 2012), and for chiral separation (Tobis et al., 2010, 2011). An overview on this topic is given by Erdodi and Kennedy (2006).

The concept of APCNs as enzyme activating matrix was first shown on the example of peroxidases entrapped into a network consisting of poly(2-hydroxyethylacrylate) (PHEA) linked by PDMS (Bruns and Tiller, 2005). Later lipases and proteases were shown to greatly enhance their organic solvent activity within such a conetwork (Savin et al., 2005). The concept was always based on the necessity of the diffusion of proteins into the aqueous phase of the APCN. This usually limits the maximum loading to some 1 wt.% for rather small proteins of a limited size of up to 40 kDa (Dech et al., 2011). Unfortunately, nearly all catalytic conversions mentioned above showed strong diffusion limitations (Bruns and Tiller, 2005; Bruns et al., 2008; Savin et al., 2005). In the present study, we explore the possibility to overcome these limitations within APCNs in organic solvents by preparing small sized particles.

Experimental Section

Materials

HEA (2-hydroxyethyl acrylate), obtained from Sigma-Aldrich, St. Louis, MO has been purified by distillation and was used within 2 weeks. *N*-(3-[dimethylamino]propyl)–methacrylamide (DMAP–MAA), obtained by Sigma-Aldrich, was freshly distilled prior to use. Irgacure® 651 and 184 were kindly provided by Ciba Specialty Chemicals (now part of BASF, Basel, Switzerland). Enzymes were obtained from Sigma-Aldrich, lipase from *Rhizomucor miehei* (RmL, 30 kDa) (No. L4277) with 19,000 U/g and α-Chymotrypsin (CT, 25 kDa) (No. CHY5S) with 70,000 U/g.

The commercial RmL formulation was purified by dialysis at 4°C in an acetate buffer solution (pH 5.7, 0.1 M) against cellulose membranes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 4 days while changing the buffer solution each day and another 2 days in lower concentrated acetate buffer (pH 5.7, 0.001 M). Purified RmL was obtained in form of a beige-colored powder after lyophilization overnight.

The macromonomer MA-PDMS-MA (MW 5,200 g/mol, functionalization 92%, PDI = 1.3) was prepared according to Scherble et al. (2002). All further chemical reagents used in this work were obtained by Sigma-Aldrich in analytical grade and used without further purification. The used solvents did contain following amounts of water according to the Karl-Fischer-Titration: chloroform 19 ppm, 1-octanol 251 ppm, *n*-heptane 12 ppm.

Synthesis of Poly(2-Ethyl Oxazoline) (PEtOx) With Methacrylamide End Groups

The preparation of PEtOx as macromolecular cross-linker here followed a synthesis route, described in previous work (Dech et al., 2012). To this end, the cationic ring opening polymerization of 2-ethyl-1.3-oxazoline was initiated with *p*-dibromoxylene in an industrial microwave reactor. The polymerization was terminated with DMAP–MAA. The synthesized PEtOx showed a functionalization of some 95% and a molecular weight of 4.800 g/mol (PDI = 1.25).

Synthesis of RmL-Loaded PHEA-*l*-PEtOx APCNs

PHEA-*l*-PEtOx conetworks were synthesized as described in a previous work (Dech et al., 2012). To this end, lipase from *R. miehei* (RmL, 0.2–11 wt.%) and the photoinitiator Irgacure 651 (0.1 wt.%) were dissolved in a prepolymer solution of the polymeric cross-linker PEtOx and HEA (PEtOx:HEA = 70:30, w/w). Distilled water (up to 20 wt.%) was added until the solution was optically clear. The complete monomer mixture was than placed between two glass slides, which were covered with an adhesive poly(propylene)-tape. Polymerization was performed under UV flash light (Heraeus Kulzer, Typ Heraflash, Germany, $\lambda = 340$ nm), for 2×180 s one side and 1×180 s from other side. Finally, the formed membranes were detached from the glass slide for further applications.

PHEA-*l*-PEtOx microparticles of these membranes were obtained by milling them in a cryo mill (Spex Certiprep 6850, filled with liquid nitrogen) for 13 cycles. Each cycle was milling at 4 Hz for 6 s and a 1.5-min break.

In order to obtain a more narrow size distribution of the obtained particles, they were suspended in chloroform and 1-octanol, respectively, and passed through a metal sieve. Three sieves with a mesh size of 20, 40, and 80 μm were used. The filtrates were immediately taken for the biocatalytic assay and for particle size analysis.

Synthesis of 2-(Trimethylsilyloxy)ethyl acrylate (TMSOEA)

The synthesis of TMSOEA followed a synthesis route described by Scherble et al. (2002).

HEA (117 mL) was solved in dichloromethane (300 mL). Bis(trimethylsilyl)urea (103 g) were added in one portion. The mixture was stirred vigorously for 2 h under reflux. Urea was filtered off and the solvent was removed in vacuum. Phenothiazine (0.5 g) was added as stabilizer. The product was distilled prior to use (b.p. 34°C, 1 mbar).

PHEA-*l*-PDMS Microparticle Synthesis by Suspension Polymerization

The preparation of PHEA-*l*-PDMS (50/50, w/w) particles via suspension polymerization followed modification of the synthesis route of Savin et al. (2005). Thereby the photo initiator Irgacure 184 (9.0 mg) was dissolved in a solution of freshly distilled TMSOEA (220 μL) and telechelic methacryloxypropyl PDMS (150 μL) as macromolecular crosslinker. A tenside mixture (phosphate buffer pH 7.0, 4 mL; 1.0 wt.% Lutensol AT 25) was added followed by dispersing (40 Hz, 4 min, Vortex, Velp Scientifica, Usmate, Italy). The polymerization was then carried out with an UV polymerization lamp (Heraflash, Heraeus Kulzer, $\lambda_{max} = 340$ nm) while continuous stirring for 180 s. Separation of smaller and larger sized particles was performed via a syringe filter with metal mesh (25 μm). The obtained particles were washed in a buffer solution to remove the tenside and after this deprotected in a methanol/water (50/50) mixture for 48 h.

PHEA-*l*-PDMS Microparticle Synthesis by Aerosol Polymerization

The photo initiator Lucirin TPO (60.0 mg), freshly distilled TMSOEA (300 μL) and telechelic methacryloxypropyl PDMS (300 μL) were dissolved in 5,000 μL of freshly distilled acetone. The mixture was fogged with ultrasound for 4 min and induced into the reactor with argon as carrier gas. Polymerization was performed within the reactor for 30 min under ultraviolet light (Vitalux UV-Lampe [Osram 300 W] and 4 UV-fluorescent tubes; LT 15W/009, NARVA Lichtquellen GmbH + Co. KG, Brand-Erbisdorf, Germany). The synthesized particles were suspended in ethylacetate (30 mL) and left overnight till a pellet was formed. The supernatant was rejected and the particles were resuspended in water.

CT Immobilization Via Adsorption

Ten milligrams of CT were dissolved in 1 mL 0.1 M phosphate buffer (pH 7.8) and 100 mg PHEA-*l*-PDMS microparticles were added. The mixture was shaken at 4°C overnight. After this, the particles were separated by threefold centrifugation in phosphate-buffered solution and subsequently lyophilized overnight. The loaded amount of CT was determined by measuring the protein content in the collected CT solutions before and after the immobilization procedure using the Lowry assay.

Particles Size Analysis

A scanning electron microscope (S-4500 SEM, Hitachi, Japan) was used for the characterization of the produced microparticles. To this end, the particle suspension was spread on the sample holder and dried until complete vaporization of the solvent. The SEM images were monitored by an excitation voltage of 1 kV and 5,000-fold magnification. The particle size was determined averaging the largest diameter of 500–1,000 particles in up to 40 images.

Network Composition Analysis

Atomic force microscopy (AFM) was performed with a Veeco Dimension Icon AFM (Veeco Instruments, Plainview, NY). The measurements were performed in tapping mode using RFESP cantilevers. The images of the bulk phases were recorded on cross-sections of cryofractured membranes.

Determination of Enzymatic Activities

The activity of lipase from *R. miehei* (RmL) was measured on the example of the literature known esterification of lauric acid and 1-octanol (Bruns et al., 2008). *n*-Heptane and chloroform were used as reaction medium for the esterification at 37°C. In these media the concentration of *n*-octanol was 400 mM and that of lauric acid was 200 mM. The assay was also performed without solvent using *n*-octanol and lauric acid in a molar ratio of 2:1. Quantitative analysis of the product formation was followed by gas chromatographic measurements with tetradecane (0.01 mM) as internal standard. For this, a gas chromatograph GC Clarus 500 (Perkin Elmer, Wattham, MA, Column: CP-Sil 8 CB by Varian) with nitrogen as carrier gas was used. The injection temperature was 230°C; the FID detector temperature was 280°C. Measurements followed a temperature program of 130°C for 1 min, with a heating rate of 20 K/min up to 250°C, a split ratio of 20:1, and a flow rate of 2 mL/min.

Z_{APCN} refers to the units per weight of the enzyme loaded networks or particles, while Z_{spec} refers to the amount of protein. One unit enzyme activity is defined as the formation of 1 μmol ester per minute. In all assays, maximal 1 mg loaded lipase APCN per milliliter assay solution was used. The activities of the lyophilized powder of the purified RmL suspended in the respective assays were found to be 450 U/g in *n*-heptane, 9.5 U/g in chloroform, and 370 U/g in octanol.

The catalytic activity of CT was measured according to a procedure of Khmelnitsky et al. (1994). The enzymatic catalyzed transesterification of *N*-acetyl-phenyl-L-alanine-ethylester (APEE) to *N*-acetyl-phenyl-L-alanine-propylester (APPE) in *n*-octane at 37°C was monitored by gas chromatography with tetradecane (0.01 mM) as internal standard.

CT loaded APCN particles (70 mg) were suspended in 7 mL *n*-octane (0.01 mM TD). The reaction started by adding a solution of APEE in *n*-propanol (final concentration 1 M *n*-propanol, 30 mM APEE). The activity of the native CT powder suspended in the same assay was found to be 0.002 U/g.

Calculation of the Thiele Modulus

The Thiele moduli were calculated under the assumption of high stationary substrate concentration according to Dalvie and Baltus (1992). The k_{cat} values used were that of the highest achieved specific enzyme activity in the respective solvent and APCN and the D_{eff} values were the Diffusion coefficients of similar compounds in similar APCNs taken from previously published work. S_0 is the starting substrate concentration and [E] the enzyme concentration in the swollen APCN (Table I).

$$\phi = 0.5 \cdot d \cdot \sqrt{\frac{k_{cat} \cdot [E]}{S_0 \cdot D_{eff}}}$$

Results and Discussion

Goal of this work was to explore the potential of APCNs as activating carriers for enzymes in organic solvents by preparing small particles that might have less diffusion limitations as found in the already highly activating polymer films. We chose two systems for this investigation: CT entrapped in PHEA-*l*-PDMS and lipase from *R. miehei* (RmL) entrapped in PHEA-*l*-PEtOx.

First, the microparticle synthesis of PHEA-*l*-PDMS using emulsion polymerization according to Savin et al. (2005), who already obtained microparticles with an average size of some 80 μm, was optimized by reduction of tenside concentration and increase of dispersion time to produce smaller microparticles with more narrow size distributions. To this end, the monomer/crosslinker mixture was photopolymerized under continuous stirring and the formed microbeads were subsequently filtered off using a metal mesh filter with 25 μm pores. Thus, in contrast to previous work, particles with 40 and 20 μm in diameter could be obtained (see Fig. 1a and b). Further rigorous magnetic stirring of the 20 μm particles in *n*-heptane resulted in a mixture of rather undefined broken sphere pieces in the range of 1–20 μm. The average size was estimated from SEM images to be 5 μm.

In order to obtain even smaller particles aerosol polymerization was applied. As seen in Figure 1c the particles obtained by this procedure are indeed smaller with some 2–5 μm in diameter. Unfortunately, they could not be dispersed as single particles after the synthesis due to the high tendency of coagulation, which is most likely caused by the low degree of crosslinking and the low T_g values of both polymers. Thus the limit of the PHEA-*l*-PDMS particle size is that of the partially broken 20 μm sized spheres (broadly distributed 5 μm size).

The synthesized particles (80, 40, 20, and 5 μm) were loaded with CT by enzyme adsorption from its aqueous solution overnight. The loading of CT was some 0.7 wt.% in all cases. The catalytic activity of these particles was determined by the transesterification of APEE with 1-propanol to APPE in *n*-octane. As seen in Figure 2 there is a strong dependence of the measured specific enzyme activities and the particle size. PHEA-*l*-PDMS particles with 80 μm in diameter showed a catalytic activity of 0.3 U/g, which is a 150-fold higher activity compared to the CT powder in *n*-octane (Savin et al., 2005). With decreasing particle size, activity increases and reaches with 56 U/g a maximum at 5 μm sized particles. The smaller the particles, the shorter are the diffusion paths of substrates and products and thus the apparent enzyme activity increases. In rough estimation, the CT activity increases by factor of 3–4 with halving the particle diameters. In general, such a behavior can be explained by either external or internal diffusion resistance (Dayal and Godjevargova, 2006). In order to explore this, the Thiele moduli were calculated under assumed high stationary substrate concentration according to Dalvie and Baltus (1992) as described in the experimental part. Surprisingly, all calculated Thiele moduli were below 0.3 indicating no intrinsic diffusion resistance. Thus, it can be presumed that the diffusion limitation is caused by surface effects of the particles.

Another interesting aspect of the CT catalyzed transesterification is the apparent Michaelis constant (K_M) value with respect to the substrate APEE. While K_M of the enzyme powder is 30.1 mM, the apparent K_M value of the enzyme within the APCN is 1.8 mM. This 17-fold lower K_M allows the enzyme within the network to work faster at lower substrate concentrations. This great decrease in K_M values is rather rare for immobilized enzymes (Tiller et al., 2002).

The maximal activity of 56 U/g is a 28,000-fold higher activity compared to the free enzyme in *n*-octane. This is close

Table I. Thiele moduli of the bioactive APCNs.

Enzyme	Loading (wt.%)	Solvent	d (μm)	ϕ
CT	0.7	*n*-octane[a]	5	0.02
CT	0.7	*n*-octane[a]	20	0.06
CT	0.7	*n*-octane[a]	40	0.12
CT	0.7	*n*-octane[a]	80	0.24
RmL	0.2	$CHCl_3$[b]	11	0.01
RmL	0.2	$CHCl_3$[b]	21	0.02
RmL	0.2	$CHCl_3$[b]	22	0.02
RmL	0.2	$CHCl_3$[b]	55	0.06
RmL	0.2	1-Octanol[c]	11	0.04
RmL	3.7	1-Octanol[c]	11	0.15
RmL	0.2	1-Octanol[c,d]	265	0.85
RmL	1.8	1-Octanol[c,d]	265	2.48
RmL	3.7	1-Octanol[c,d]	265	3.55
RmL	0.2	*n*-heptane[e]	55	3.97
RmL	0.82	*n*-heptane[e]	55	4.57
RmL	3.43	*n*-heptane[e]	55	12.93
RmL	5.96	*n*-heptane[e]	55	21.17
RmL	11.08	*n*-heptane[e]	55	28.87

[a] $D_{eff} = 25\ \mu m^2/s$ (Tobis et al., 2011), network PHEA-*l*-PDMS (60 wt.% PDMS), degree of swelling $S = 1.5$ compared with 1.8 in the system used in this paper.

[b] $D_{eff} = 100\ \mu m^2/s$ (Bruns et al., 2005), network PHEA-*l*-PEtOx (70 wt.% PEtOx), $S = 2.5$.

[c] $D_{eff} = 100\ \mu m^2/s$ (Bruns et al., 2005), network PHEA-*l*-PEtOx (70 wt.% PEtOx), $S = 2.7$.

[d] Measured with membranes, d is the thickness of the swollen membrane.

[e] $D_{eff} = 0.6\ \mu m^2/s$ (Tobis et al., 2011), network PHEA-*l*-PEtOx (70 wt.% PEtOx), $S = 1.1$.

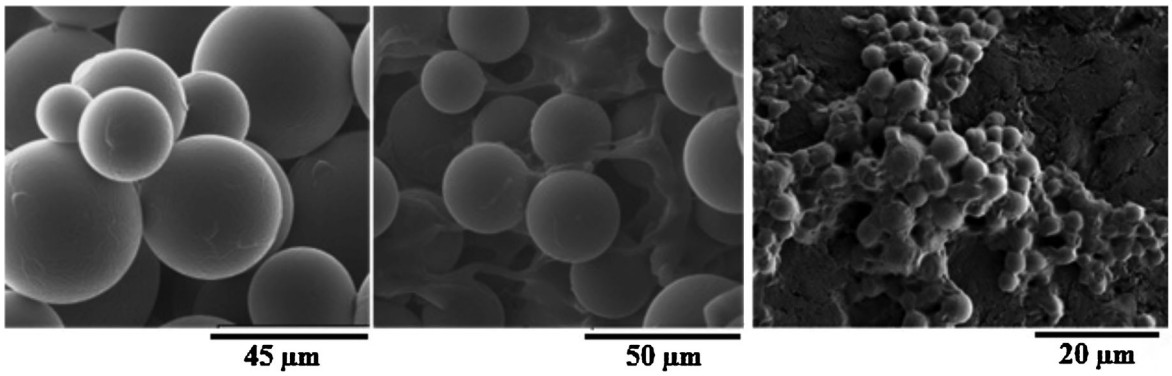

Figure 1. SEM images of PHEA-*l*-PDMS particles (**a**) prepared by vigorously stirred emulsion polymerization, (**b**) prepared by vigorously stirred emulsion polymerization and subsequent filtration through a 20-µm metal mesh, and (**c**) prepared by aerosol.

to the best activity of CT in organic media of about 65 U/g, which was obtained for CT immobilized in a sol–gel matrix (Van Unen et al., 2000). Although the activation of the entrapped CT is quite impressive, the low enzyme loading of some 0.7 wt.% does not allow to obtain high carrier activities, which are needed in the practical application of such biocatalytic materials.

In order to overcome the limited loading capacity, we have recently developed an APCN that allows entrapping enzymes by dissolving them in the monomeric mixture prior to photopolymerization (Dech et al., 2012). This APCN is based on telechelic poly(2-ethyloxazoline) (PEtOx) with methacrylate endgroups that act as crosslinker for PHEA (PHEA-*l*-PEtOx). Proteins are well soluble up to 11 wt.% in the monomeric mixture containing HEA, PEtOx and traces of water. Photopolymerization results in transparent membranes suggesting a homogeneous distribution of the enzyme under preserving the nanostructure of the APCN.

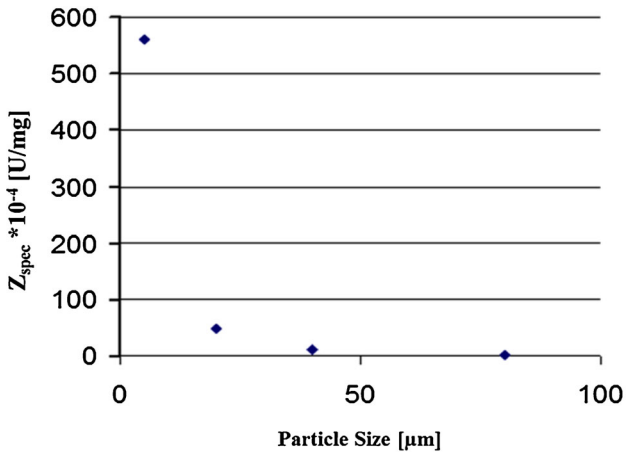

Figure 2. Specific activity of α-Chymotrypsin loaded PHEA-*l*-PDMS (50/50) particles, synthesized via suspension polymerization, in correlation to the particle size.

In order to verify this, we have prepared cross-section of a membrane with 11 wt.% RmL and investigated the nanostructure with AFM. The AFM images shown in Figure 3 indicate that no larger phase separation occurred and the enzyme containing PEtOx phases were just larger compared to the unloaded reference. Thus, even 11 wt.% content of protein still allow the formation of a fully nanophasic system.

RmL entrapped into these membranes was highly active in *n*-heptane with specific activities up to 3,450 U/g and for lipase contents of some 0.2 wt.%. Unfortunately, higher RmL loading resulted in dramatically lower specific enzyme activity. This indicates a strong diffusion limitation of the esterification reaction. Still a loading with 8.5 wt% RmL shows a carrier activity of 43 U/g. Commercial supported RmL shows an activity of some 35 U/g in *n*-heptane. We assume that the high lipase activity in the APCN was not only due to the activating polymer conetwork, but also to the fact that the membranes were particularized by the stirrer during the activity assay.

This is why we propose that further controlled particularizing might increase the APCN supported RmL activity dramatically similar to the system shown above. To this end, APCN membranes with varying RmL contents were prepared and milled in a cryo mill. Since particularizing was not successful with RmL added in commercial formulation, we decided to dialyze the enzyme formulation prior to immobilization. Cryo milling of these membranes resulted in a white powder which was further analyzed with scanning electron microscopy. After optimizing the milling procedure, particles with average size of some 50 µm with a fairly broad size distribution were obtained.

The catalytic activity of these particles in *n*-heptane was performed by measuring the rate of the often used model reaction of the esterification of lauric acid and 1-octanol (Bruns et al., 2008). Substrates as well as the products are well soluble in organic media, whereas the ester can be easily detected by gas chromatography.

As seen in Figure 4, the apparent specific activity of RmL decreases with increased enzyme load in the conetwork particles, indicating that the esterification is still diffusion

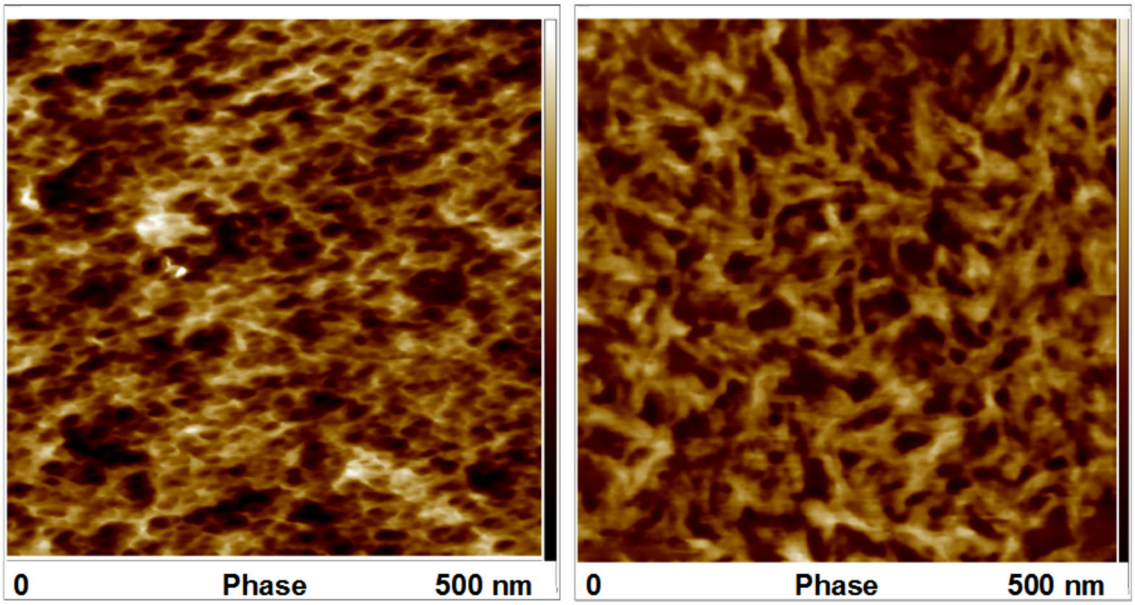

Figure 3. AFM pictures in phase, tapping mode of cryo cuts of PHEA-*l*-PEtOx (30/70) membranes, without protein (left) and with 11 wt.% of RmL (right).

controlled. The drop in the loaded particles of comparable size is from 6,040 to 940 U/g indicating a similar diffusion dependence of the reaction compared to the membranes which drop from 3,450 to 510 U/g. However, the highest carrier activity is more than twofold higher than that of the membrane. A significant increase in carrier activity (up to 100 U/g) with higher loading up to 3.7 wt.% was found. Further increasing of the loading did not result in significantly higher carrier activity. According to the Thiele moduli, the biotransformation has strong intrinsic diffusion limitations even for the lowest loaded particles.

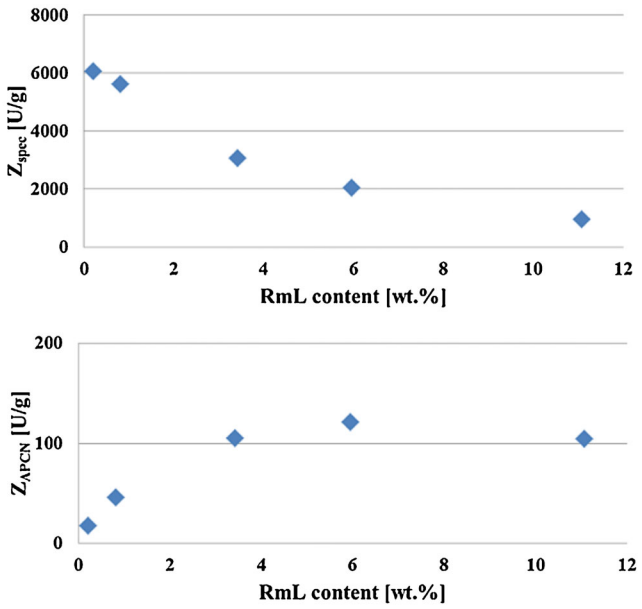

Figure 4. Specific and carrier activity of dialyzed and lyophilized RmL entrapped into PHEA-*l*-PEtOx (30/70) conetwork particles in *n*-heptane. Activity is monitored in correlation to the amount of immobilized RmL. All experiments were performed at least in duplicate and standard deviation was in a range of 5–25%.

Another important aspect of biocatalysts is their operating stability. In order to explore this, the enzymatic activity in *n*-heptane of RmL loaded PHEA-*l*-PEtOx particles (0.2 wt.% enzyme) with a specific activity of 4,890 U/g was followed over 4 days. As seen in Figure 5, the activity was undiminished over this period.

During this time, every active center catalyzed a conversion of nearly 1 million molecules and 1 g enzyme would have produced some 9,500 kg of ester. With respect to the carrier 1 g loaded conetwork would have formed more than 100 g ester.

Although the carrier as well as the specific activities are significantly increased by milling, the broad distribution of the particle size does not circumvent the diffusion conditioned limitations. Therefore, we sifted the particles through metal sieves of different mesh sizes. Unfortunately, particle suspensions in *n*-heptane, toluene, THF, and water, respectively, as well as dry particles could not be processed through the filters. The only processable suspensions were that in chloroform and 1-octanol, respectively. Particle size analysis was performed with SEM. Sieved and dried particles were analyzed by measuring the largest diameter of about 1,000 particles in total, resulting in an average diameter of 11 ± 2.5 µm for the 20 µm mesh size, 22 ± 6.6 µm for the

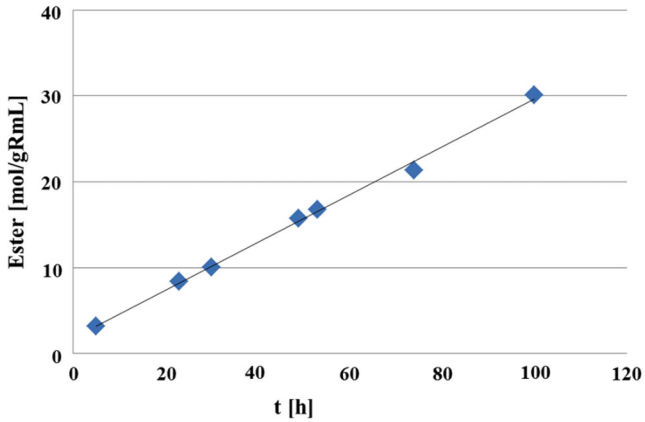

Figure 5. Ester formation catalyzed by PHEA-*l*-PEtOx$_{4.8}$ (30/70) microparticles with 0.2 wt.% RmL (Zspec 4,890 U/g) in *n*-heptane for a reaction period of 4 days.

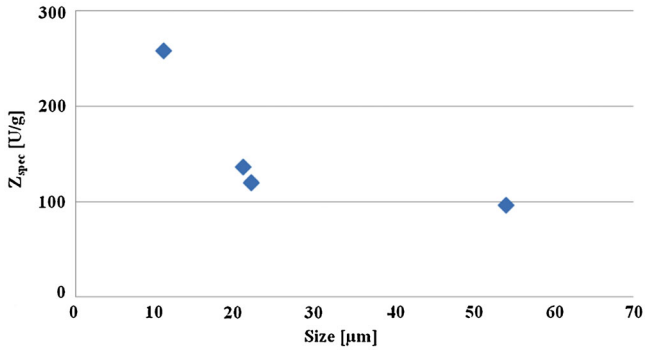

Figure 7. Specific activities (U/g enzyme) of RmL immobilized into PHEA-*l*-PEtOx (30/70) conetwork particles (RmL content 0.2 wt.%) in correlation to the average particles size (volumetric average). Assay was performed in chloroform.

40 µm mesh size, and 21 ± 5 µm for the 80 µm mesh size. The unsieved particles have a size of 55 ± 20 µm. The SEM images of the resulting particles from the chloroform filtrate are exemplarily shown in Figure 6.

Since drying of the particles after sieving turned out to be very challenging, because the dried particles formed large coagulates that were not resuspendable, the assay was performed with the particles suspended in the sieving solvents, chloroform and 1-octanol, respectively.

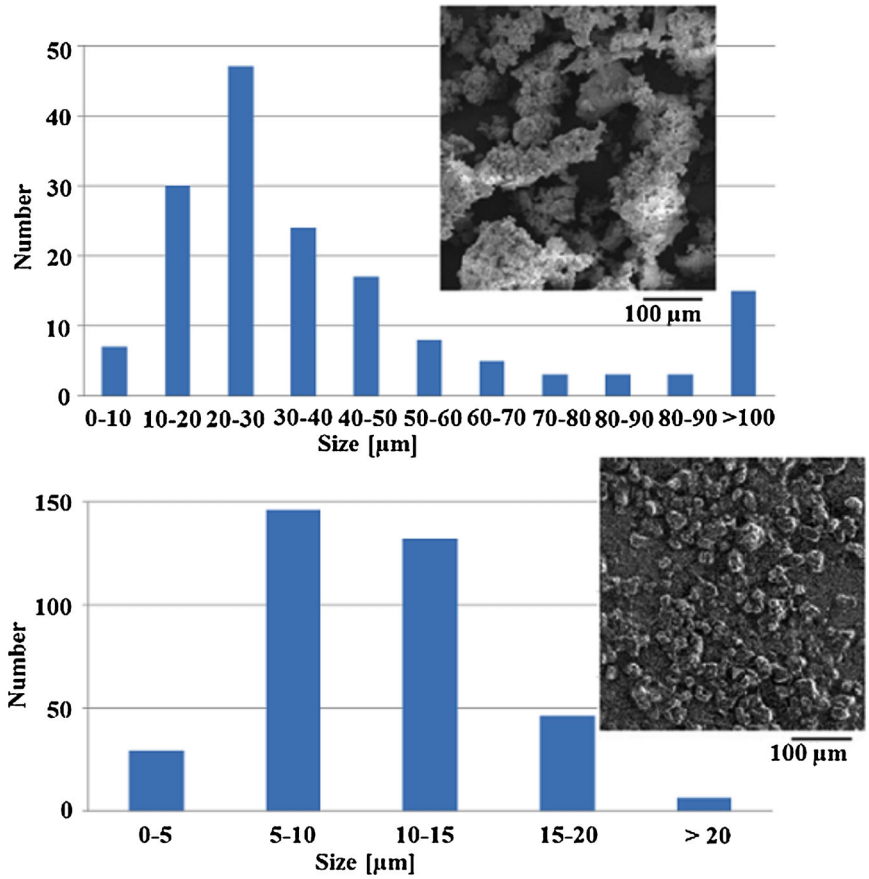

Figure 6. Size distribution and SEM images of unsieved (upper) and sieved (lower) PHEA-*l*-PEtOx$_{4.8}$ (30/70) particles. The sieve had a mesh size of 20 µm.

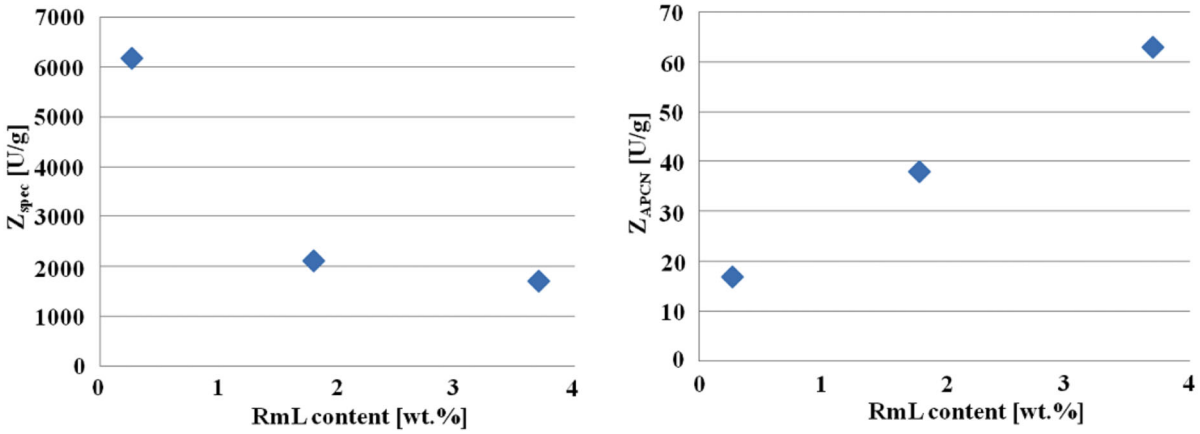

Figure 8. Specific (left) and carrier (right) activity of dialyzed and lyophilized RmL immobilized into PHEA-*l*-PEtOx (30/70) conetwork membranes without solvent. Activity is monitored in correlation to the amount of immobilized RmL. Standard deviation was in a range of 5–25%.

In comparison to the activity in n-heptane, RmL entrapped in unsieved PHEA-*l*-PEtOx particles shows a 60 times lower a specific activity of some 100 U/g and a carrier activity of 0.2 U/g, respectively in chloroform (see Fig. 7).

The lipase activity increases with decreasing of particle size. RmL entrapped into 20 µm sized particles for example shows a specific activity of 136 ± 12 U/g. Further decrease in particle size to 10 µm resulted in a specific activity up to 258 ± 67 U/g. The trend shows a less pronounced diffusion control compared to the same system in *n*-heptane and the CT loaded PHEA-*l*-PDMS conetworks. We believe this is due to the significantly higher swelling of the networks in chloroform (degree of swelling 2.5 compared with 1.1 in *n*-heptane) and the generally 10 times lower specific activity. This is supported by the low Thiele moduli, which are between 0.01 and 0.06 indicating no intrinsic diffusion limitation.

Although, the overall RmL activity is significantly lower compared to *n*-heptane as solvent, the entrapment of RmL into PHEA-*l*-PEtOx (30/70) polymer particles still activates the enzyme in this solvent by a factor of 27 compared to the specific activity of the purified RmL powder.

1-Octanol as sieving solvent is an interesting alternative to chloroform, because it is one substrate of the RmL catalyzed esterification with lauric acid which was used as activity assay. The esterification in 1-octanol represents the most likely industrial scenario, because the reaction is formally carried out in bulk that is without solvent.

Initially, the reaction was carried out with RmL-loaded membranes. As seen in Figure 8 the specific activity of the lowest RmL-loaded membrane is higher without solvent than in *n*-heptane.

This indicates that a PHEA-*l*-PEtOx network is very well suited as activating carrier matrix for RmL-catalyzed reactions. Unfortunately, the reaction is still strongly diffusion controlled, though the membranes are much higher swollen than in *n*-heptane. The degree of swelling in 1-octanol is 2.7 for PHEA-*l*-PEtOx (30/70) conetworks. This is supported by the Thiele moduli of 0.85 for the lowest enzyme loading and 3.55 for the highest enzyme content, which indicate intrinsic diffusion resistance.

The activity of the milled and sieved particles with the smallest obtainable diameter of 10 µm increases with a factor 7 for a RmL content of 0.2 wt.% (Table II). The increase of the highest loaded particles of the same size was less dramatic being 1.5-fold higher than for the respective membrane. This shows that the reaction is still diffusion controlled, even in particles as small as 10 µm in diameter. Since the Thiele moduli are below 0.2 for the highest enzyme loading, the biotransformation is obviously limited by surface transport effects.

Nevertheless, RmL supported in PHEA-*l*-PEtOx conetwork is highly catalytically active even in the substrate mixture without additional solvent. In this system, the supported biocatalyst shows a more than 100 times higher specific activity compared to the native enzyme (370 U/g). The commercially available product of immobilized RmL (Sigma-Aldrich, No. 52001) is based on the methacrylate containing copolymer Immobead 150 (ChiralVision, Leiden, the Netherlands). It shows a carrier activity of 9.6 U/g in the substrate mixture. Taking into account that the RmL-loading of the Immobead 150 is between 5 and 10 wt.%, the RmL-loaded PHEA-*l*-PEtOx microparticles show nearly tenfold higher carrier activity with some 25–50-fold lower enzyme

Table II. Specific and conetwork particle activity of dialyzed and lyophilized RmL immobilized into PHEA-*l*-PEtOx (30/70) conetwork particles without solvent.

RmL content (wt.%)	Z_{spec} (U/g)	Z_{APCN} (U/g)
0.2	42,310 ± 7,358	89 ± 15
3.7	2,400	89

content compared to the commercial product. A recent work of Cruz et al. (2011) describes the lipase immobilization onto fumed silica particles, where the catalytic activity in hexane was just twofold higher than commercially available Novozyme 435. In a work of Bayramoglu et al. (2005) for example the immobilization of *Candida rugosa* lipase on microspheres containing epoxy groups with spacer arms, a 1.5-fold activation could be obtained. In respect to this, the described CLEA system, which is an important enzyme immobilization method, shows some outstanding properties due to stability of immobilized lipase with up to 25% of activity recovery (Kartal et al., 2011). Compared to those commercially available products like immobilized RmL, Novozyme 435 and the systems discussed in recent studies, the here presented results illustrate the promising properties of PEtOx-based microparticles as activating matrix for enzymatic conversions in organic media.

Conclusion

In the past, we have shown that APCNs are excellent activating carriers for biocatalysts in organic solvents. Although great activations could be achieved, the reactions were generally diffusion controlled and thus the full potential of these networks was not reached.

In this study, we investigated potential increase of the activity of different enzymes in different APCNs by preparing microsized particles from these materials.

Suspension polymerization and aerosol polymerization were used to synthesize PHEA-*l*-PDMS microparticles of different compositions in a diameter range of 5–80 μm. The activity of supported CT in these microparticles in *n*-heptane was strongly increasing with decreasing particle diameter. The highest specific activity was 56 U/g for particles with a diameter of 5 μm. The activity of CT increases by a factor of 3–4 with halving the particle diameters.

Another particle system consisting of PHEA-*l*-PEtOx (30/70) in a diameter range of 10 to above 50 μm, loaded with RmL was tested in different organic solvents. In all solvents, smaller particles showed 10- to 100-fold higher specific activities compared to the native enzyme. This was even true for the production relevant reaction mixture without additional solvent. In the latter case, the RmL-loaded PHEA-*l*-PEtOx microparticles show nearly tenfold carrier activity with some 25–50-fold lower enzyme content compared to a commercial product.

Even the smallest particles showed a diffusion limitation of the reaction possibly by surface limited mass transport, which indicates that the here presented improvement of activity by particularization is still not the highest achievable activation of the APCN supported enzymes. Future investigations are directed towards the application of techniques to further increase the surface/bulk ratio and thus the activity of the biocatalytic APCNs.

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