Biotechnology

DOI: 10.1002/anie.201206931

Microgel-Stabilized Smart Emulsions for Biocatalysis**

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Biocatalysis is an important technique to produce enantiopure substances owing to its inherent enantioselectivity and mild reaction conditions. However, many substrates of interest are poorly soluble in water, whereas enzymes typically prefer an aqueous environment. So enzyme-catalyzed reactions are often performed in biphasic aqueous–organic reaction systems to achieve high substrate concentration and enhance productivity.[1–5]

However, enzyme structure and flexibility may be affected by the organic–aqueous interface of such biphasic reaction systems, thereby leading to a reduced enzyme activity and selectivity. Furthermore, the transport of the substrate through the organic–aqueous interface in biphasic reaction systems may constitute the rate-determining step.[6–8]

To enhance the efficiency of two-phase enzymatic reactions one needs 1) to stabilize the enzyme against denaturation at the interface, and 2) to increase the area between organic and aqueous phase, for example, by formation of an emulsion. However, the emulsion finally needs to be broken under moderate conditions to separate the organic phase containing the product and to allow for recycling of the enzyme in the aqueous phase.

Stimuli-responsive emulsifiers that allow controlled stabilization and destabilization of emulsions could strongly enhance the opportunities for enzyme-catalyzed reactions in biphasic reaction media. "Smart" microgels respond to changes in external stimuli, such as temperature and pH value, by changing their size, softness, and hydrophobicity.[9] Recently, it has been shown that poly(*N*-isopropylacrylamide) (PNIPAM)-based microgels form emulsions, the stability of which depends on temperature and/or pH value.[10–19] Chemical composition and morphology of, for example, core–shell architectures can be varied to tailor the properties of responsive microgels.[20–25]

Microgel-stabilized emulsions, also called "Mickering" emulsions, on first sight resemble Pickering emulsions that are stabilized by rigid colloidal particles. However, microgels are soft gel particles that deform at an oil–water interface,[13,15,26] and it was shown that the internal structure of microgel particles is important for its emulsion stabilization properties.[17] Furthermore it was recently observed that strongly hydrated microgels protrude only little into the oil phase.[26] Microgels might provide further advantages for biocatalysis, because it is known that enzyme immobilization inside gels can enhance enzyme stability and thus recyclability.[27,28] The adsorption of the microgel to the oil–water interface will locate the immobilized enzyme in close proximity to the oil phase while still preventing direct contact. However, it is unknown whether microgel particles that adsorb at the oil–water interface affect the enzyme stability and the substrate/product transfer between oil and water phase. Furthermore, enzymes typically require specific reaction conditions as for example, pH value, ionic strength, temperature, and it is not clear how these conditions affect the interfacial stabilization properties of responsive microgels.

Herein we present a conceptually novel approach: a two-phase system is emulsified wherein the organic (oil) phase contains dissolved substrate while the aqueous phase contains biocatalyst and microgel (Figure 1 A). After emulsification the microgels are located at the droplet surface (as schematically shown in Figure 1 B) and the enzyme converts the substrate to the reaction product. Finally, the emulsion is broken by increasing the temperature above the volume phase transition temperature (VPTT) of the microgels, thereby leading to macroscopic phase separation. The organic phase now contains the product of the enzyme reaction and

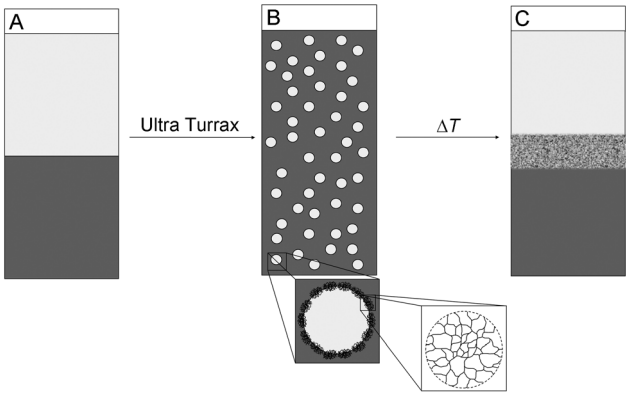

Figure 1. Our concept is to start with a two phase system (A), emulsify it using an Ultra Turrax homogenizer, stabilize the emulsion droplets with responsive microgels (B), and afterwards use the sensitivity of the microgels for breaking the emulsion (C). In the beginning (A) the organic phase contains substrate and the aqueous phase enzyme and microgel. The enzyme reaction takes place in the emulsion (B). Finally the emulsion is broken (C). The organic phase contains the reaction product; the microgels flocculate in the upper region of the aqueous phase.

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[**] This work was supported by the excellence initiative of the German federal and state government and by the Deutsche Forschungsgemeinschaft within the Sonderforschungsbereich 985. We thank Thomas Tigges for analysing emulsion stability.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201206931.

can easily be separated while the aqueous phase still contains the biocatalyst and the now flocculated microgel (Figure 1C). The flocculated microgel can be redissolved in the aqueous phase upon cooling.

The enzyme-specific reaction conditions impose severe boundary conditions for the emulsion system: 1) The emulsion needs to be prepared under moderate shear conditions, because the enzyme is shear-sensitive. 2) The emulsion must be stable at the enzyme reaction temperature and needs to be breakable at temperatures that are sufficiently low to avoid enzyme denaturation. 3) Furthermore, the reaction requires a buffer with a defined pH value and ionic strength that affect the swelling and interfacial properties of the microgels. Thus, the emulsion must be producible, stable, and breakable under very specific conditions defined by the enzymatic reaction.

Herein we report on the proof of concept to perform an enzyme-catalyzed reaction in a reversible microgel-stabilized emulsion. Here, we investigate three different types of microgels (Figure 2) with respect to their potential as stimuli-sensitive emulsion stabilizers for an enzyme-catalyzed

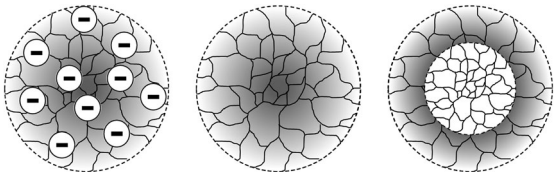

Figure 2. Three different microgel types were used to stabilize emulsions. Left: negatively charged poly(*N*-isopropylacrylamide-*co*-methacrylic acid) microgel (P(NiPAM-*co*-MAA)); middle: uncharged poly(*N*-isopropylacrylamide-*co*-*N*-isopropylmethacrylamide) microgel (P(NiPAM-*co*-NiPMAM)); right: uncharged poly-*N*-isopropylacrylamide-poly-*N*-isopropylmethacrylamide core–shell microgel (PNiPAM-PNiPMAM).

reaction: First, we chose negatively charged poly(*N*-isopropylacrylamide-*co*-methacrylic acid) microgels (P(NiPAM-*co*-MAA)), which are known to be good emulsion stabilizers depending on temperature and pH value.[12,15,18] However, electrostatic interaction between microgel and enzyme might interfere with emulsion stability and enzyme activity. Thus, we prepared two uncharged microgels: a poly(*N*-isopropylacrylamide-*co*-*N*-isopropylmethacrylamide) copolymer microgel (P(NiPAM-*co*-NiPMAM)) and a core–shell microgel prepared with a NiPAM core and a NiPMAM shell (PNiPAM-PNiPMAM).

These three switchable microgels are used to stabilize the biphasic emulsion system for reducing acetophenone to (*R*)-phenylethanol catalyzed by alcohol dehydrogenase from *Lactobacillus brevis* as model reaction. The substrate acetophenone as well as the product (*R*)-phenylethanol are both soluble in methyl-*tert*-butyl ether (MtBE), whereas the enzyme is dissolved in the buffer triethanolamine hydrochloride (TEA·HCl).[4,29] The coupled reaction of 2-propanol to acetone is used to regenerate the cofactor NADPH. Cosubstrate and coproduct are soluble in both organic and aqueous phase.[4,29] The entire reaction scheme is shown in Figure S1 in the Supporting Information.

To obtain microgels suitable for enzyme-catalyzed reactions we first synthesized three microgels (see Table S1 in the Supporting Information for details) and analyzed their temperature-dependent swelling in the buffer solution by using dynamic light scattering (DLS) (Figure 3). All three

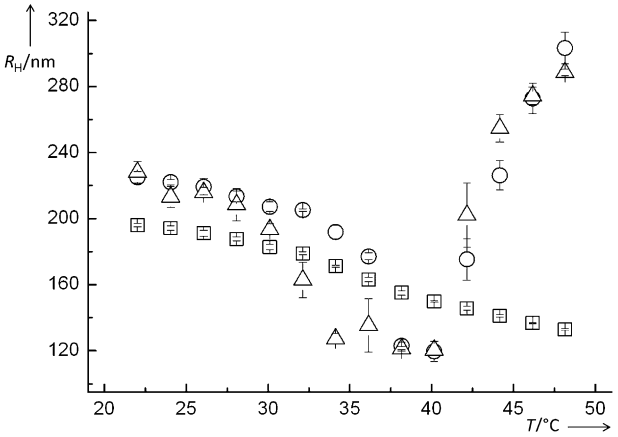

Figure 3. Temperature-dependent hydrodynamic radius (R_H) of the three microgels, measured with DLS in TEA·HCl (0.05 M; pH 7). The two uncharged microgels, P(NiPAM-*co*-NiPMAM) and PNiPAM-PNiPMAM, reversibly flocculated at elevated temperatures. □: charged microgel; ○: uncharged microgel; △: uncharged core–shell microgel.

microgels have a hydrodynamic radius in the range of 200 nm in the swollen state at low temperatures. At high temperatures differences between the charged P(NiPAM-*co*-MAA) and the two uncharged microgels are found.

The charged P(NiPAM-*co*-MAA) microgel (5.7 mass% content of MAA as determined by titration; see Figure S2 in the Supporting Information) is colloidally stable in the buffer TEA·HCl over the entire temperature range. The incorporation of dissociable MAA groups leads to electrostatic stabilization of the microgels. For this reason the temperature-induced collapse is small and occurs over a broad temperature range. The two uncharged microgels reveal a stronger volume change upon heating and they flocculate above 40 °C. These transitions are fully reversible: all microgels reswell and are not aggregated at low temperatures. The temperature-dependent size of the microgels in water is shown in Figure S3 in the Supporting Information.

The temperature sensitivity of the three microgels fits well to the temperature range in which the biocatalyst is stable.[4,29] We prepared water-in-oil emulsions with these three different microgels with an oil/water ratio of 1:1 (Figure 4). The aqueous TEA·HCl solution contains 1 wt% microgel.

In these emulsions the model enzyme reactions, reduction of acetophenone to 1-(*R*)-phenylethanol, was performed. After one hour at 30 °C[4] the emulsions were broken by increasing the temperature above the VPTT of the microgel. The emulsions stabilized with the two uncharged microgels break after 10 min at 50 °C, emulsions stabilized by the charged microgel break after 15 min at 55 °C. The broken emulsions consist of three phases, the top phase is clear MtBE, the bottom phase is the buffer phase TEA·HCl, and the flocculated microgel is located in between (Figure 4C).

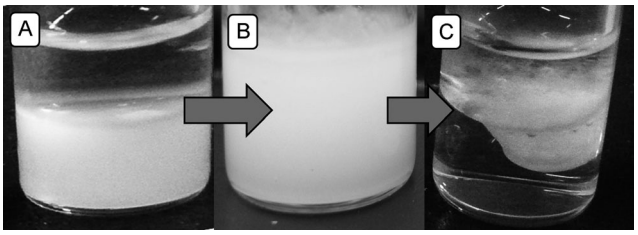

Figure 4. Stable emulsion (B) prepared of a two-phase system (A) consisting of TEA·HCl with dissolved charged P(NiPAM-*co*-NiPMAM) (lower milky phase) and MtBE (upper clear phase). The emulsion was prepared with an Ultra Turrax T25 homogenizer (1 min, 8000 rpm, room temperature). After increasing the temperature, the microgels shrink and the emulsion breaks (C). Top: oil phase, bottom: aqueous phase and flocculated microgel.

The density of the flocculated microgels depends on the microgel's composition and architecture and on the stirring conditions (see Figure S4 in the Supporting Information for further examples).

The important feature to notice is that the microgels are able to 1) stabilize emulsions under conditions required by the enzyme and 2) allow breaking the emulsions efficiently such that the oil phase, which contains the reaction product, can easily be separated.

We analyzed the organic as well as the aqueous phase of the enzyme reaction system by gas chromatography (Figure 5 A, B). The ratio of product peak area (right peak) to substrate peak area (left peak) is the same for all three microgels, thus we find the same product concentration in the oil phase independent of microgel type.

The distribution of AcPh and PhEtOH between organic and aqueous phase is expected to be given by their partition coefficients (AcPh 66.9; PhEtOH 32.1).[4] Therefore, the organic phase contains both compounds in a higher concentration than the aqueous phase (please note the different scale of the ordinates). From the partition coefficient one could expect that the amount of PhEtOH in the organic phase is about 32 times higher than the amount in the aqueous phase. However, the PhEtOH amount we find in the organic is 1/3 of the expected value. In other words, the product concentration in the aqueous phase is higher than expected from the partition coefficients. This could have different reasons: the two phases could be not yet equilibrated owing to high enzyme activity. Secondly, the microgels could influence partitioning of substrate and product between water and oil such that the partition coefficients determined in the simple two-phase system are not valid when microgels are present.

Next we varied the reaction time. Figure 5 C demonstrates the continuous product formation with time (1 h, 6 h, and 24 h reaction time), and the enzyme is still active after 24 h.

The observation of product peaks proves the feasibility of the reaction that is, the microgels do not inhibit the enzyme reaction, and substrate as well as product can pass through the microgel-covered droplet surface. This conforms to recent results showing that microgel layers on droplet surfaces include voids.[13,15,26]

Figure 5 shows that the same yield is obtained independent of the type of microgel. This is surprising, because

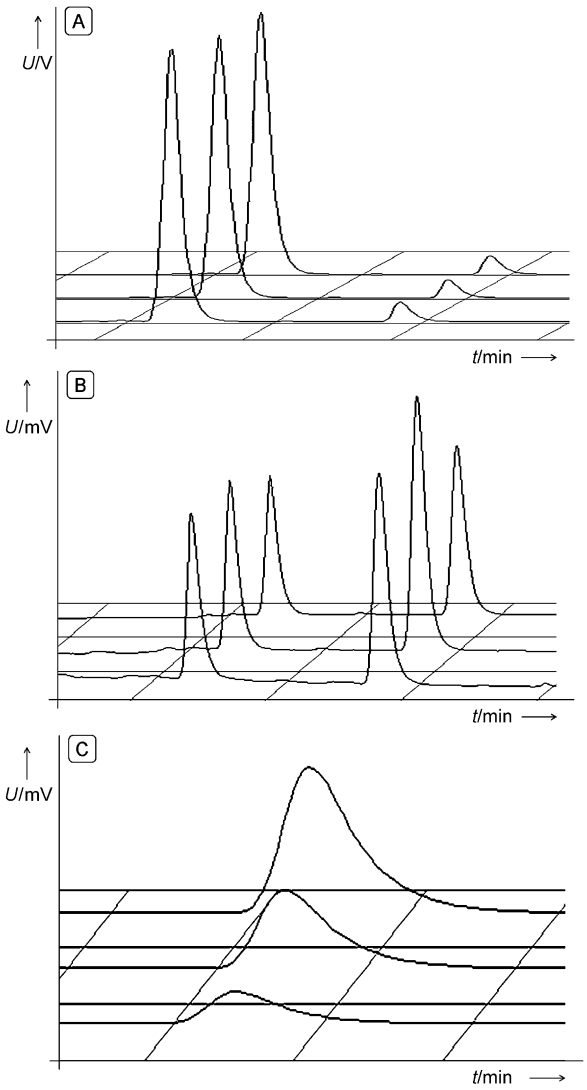

Figure 5. Gas chromatograms of the organic phase (A, C) and the aqueous phase (B). Data from three microgels (MGs) are shown in (A) and (B): charged MG: front line; uncharged MG: middle line; uncharged core–shell MG: backmost line; acetophenone: left peak, phenylethanol: right peak. The amount of product is the same for all three microgels. Diagram (C) shows the 1-phenylethanol peak in the organic phase after different reaction times (charged MG); 1 h: front line; 6 h: middle line; 24 h: backmost line. The amount of product increases with time, thus indicating that the enzyme stays active. U = detector signal in volts.

generally it is expected that charges or hydrophobic moieties introduced into the microgel may influence the distribution of the enzyme between the bulk aqueous phase, microgel surface, and the interior of the microgels and thereby affect enzyme stability and activity.[28,30–32] Similarly, the same parameters may influence the partition behavior of substrate and product as well as the microgel's behavior at the oil–water interface, that is, emulsion stabilization, which again will affect the efficiency of the reaction system. Obviously, a detailed quantitative analysis is required to fully understand the space–time yield. Such analysis needs to consider various aspects: 1) droplet size distribution, 2) localization of the

enzyme, 3) partitioning of substrate and product between organic and aqueous phase in the presence of microgels. These studies are in progress but are beyond the scope of this contribution. They will be reported in the future.

However, the three microgels reveal a different performance with respect to responsive emulsion stabilization. Although all microgels stabilize emulsions at 30 °C in TEA·HCl buffer solution, the emulsions behave differently at elevated temperatures. Emulsions stabilized with the charged P(NiPAM-*co*-MAA) microgel require stirring at 55 °C for 15 min to be broken. Emulsions stabilized by the uncharged random and core–shell microgels break already after stirring for 10 min at 50 °C, which is favorable for enzyme stability. From this point of view the two uncharged microgels are preferred compared to the charged one.

The overall performance of the uncharged microgels is very similar. However, the core–shell microgel requires more complex synthesis processes than the random copolymer microgel. Thus the latter can be considered being the most suitable microgel for the present application scenario.

To conclude we demonstrate a proof of concept that "smart" emulsions prepared by stimuli-sensitive microgels provide unique opportunities for biocatalysis in two-phase systems. Microgels can be tailored such that they enable reversible stabilization and breakage of emulsions under conditions that meet the requirements of an enzymatic reaction and allow simple product separation as well as recycling of biocatalyst and emulsifier.

Furthermore microgels allow for even more complex compartmentalization, as for example, Janus-type,[33] nanophase-separated internal structure[34] or multi-shell particles.[35] Thus microgels provide a unique toolbox for tailoring interfacial properties and microgel–enzyme interaction independently. Therefore various applications in biocatalysis are envisioned.

Experimental Section

Microgel synthesis and characterization can be found in the Supporting Information. To prepare the emulsions MtBE was used as oil and TEA·HCl solution as aqueous phase; the o/w ratio was 1:1 in all cases. TEA·HCl (0.05 M; pH 7) containing the microgel was added and mixed with an Ultra Turrax T-25 with a 10 mm head (8000 rpm, 1 min, room temperature). The (*R*)-selective NADPH-dependent alcohol dehydrogenase from *Lactobacillus brevis* was used as biocatalyst. The chosen model substrate for this reaction was acetophenone; NADPH was regenerated by substrate-coupled cofactor regeneration using 2-propanol as cosubstrate for the same enzyme. Additional information on microgel synthesis and characterization, emulsion preparation, and phase separation as well as on GC analysis and experimental setups can be found in the Supporting Information.

Received: August 27, 2012
Revised: November 5, 2012
Published online: November 26, 2012

Keywords: biocatalysis · emulsions · enzyme catalysis · microgels · nanomaterials

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