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Biocatalysis

Highly Efficient Phase Boundary Biocatalysis with Enzymogel Nanoparticles**

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Abstract: *The enzymogel nanoparticle made of a magnetic core and polymer brush shell demonstrates a novel type of remote controlled phase-boundary biocatalysis that involves remotely directed binding to and engulfing insoluble substrates, high mobility, and stability of the catalytic centers. The mobile enzymes reside in the polymer brush scaffold and shuttle between the enzymogel interior and surface of the engulfed substrate in the bioconversion process. Biocatalytic activity of the mobile enzymes is preserved in the enzymogel while the brush-like architecture favors the efficient interfacial interaction when the enzymogel spreads over the substrate and extends substantially the reaction area as compared with rigid particles.*

The major difference between enzymatic biocatalysis in living organisms and manmade phase-boundary catalysis is the dynamics of the catalytic centers.[1] Limitations of solid-phase catalysis are due to poor mobility of the catalytic centers resulting in a net loss of biocatalytic activity as compared with soluble enzymes that can more readily reach the surface of insoluble substrates and adjust spatial orientation at the phase boundary.[2] Enzymatic catalysis is vital for the regulation of chemical reactions in cells. Nature provides numerous examples of compartmentalized enzymatic reactions that are used in different metabolic pathways and localized in different cellular compartments where the organelle's internal environment is optimized to maintain the highest level of enzyme activity and selective transport of reactants and synthesized products. The enzymes are confined in compartments, although they are in motion which is vital for their biological functions to secure access to spatially organized catalytic sites in the folded protein molecules.[1] Intracellular enzymatic catalysis is controlled within the cellular membrane through regulation of pH, ionic strength, and the presence of inhibitors through gated transport across the membrane. Remarkably, natural living systems demonstrate other examples of enzymatic catalysis when enzymes are secreted and released by cells and organisms to the cell environment. Extracellular biocatalytic activity of secreted enzymes in vitro is strongly affected by the environmental conditions. Typically, cell-secreted enzymes are effectively lost after a limited period of time because of diffusion, degradation, or nonproductive binding. The lost enzymes are replaced by new secreted proteins. However, in industrial technologies initial enzyme costs are high enough to warrant recovery and reuse of enzymes but such processes are often too costly.[3]

Here we have developed a biocatalytic enzymogel which is designed as a core–shell nanoparticle with a superparamagnetic core and a polymer brush scaffold loaded with enzymes. The enzymogel demonstrates a novel type of phase-boundary biocatalysis with a unique combination of properties including a) biocatalysis involving enzymes in the particle, b) stimuli-triggered release of enzymes to the nanoparticle's surrounding and extracapsular biocatalysis, c) biocatalytic conversion of substrates contacting the enzymogel when the enzymes act to bridge the particle and the substrate and engulf the substrate, and d) stimuli-triggered reattachment of released enzymes for their reuse. The enzymogel nanoparticle mimics catabolism of either internal or extracapsular substrates. This versatility turns the particle into a universal biocatalytic "supercapsule" for a number of applications such as synthesis of biofuels; the storage, delivery and reuse of enzymes in biotechnology; and in multifunctional biomaterials when enzymogel nanoparticles could be remotely guided to the surface of drug-loaded containers or temporal implants (absorbable sutures) and degrade them upon contact.

A few approaches to solve the problem of enzyme delivery and recycling have been developed to date: 1) immobilization of enzymes on solid nonporous supports and 2) encapsulation of enzymes using semipermeable capsules. Immobilization of enzymes on supports may provide a simple way to recover and reuse them which will significantly reduce costs for many industrial processes of biotechnology.[2] Enzymes can be immobilized using methods either fixing their interaction with a carrier (physical or covalent binding to support), or carrier-free cross-linking methods; both approaches bring a history of advantages and disadvantages widely reported in literature.[4] In many cases, however, immobilization may result in changing the enzyme structure

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and altering their activity, specificity, and selectivity.[5] Attachment to the carrier by strong covalent binding can irreversibly deactivate enzymes or lower the catalytic performance.[6] Physical adsorption of enzyme molecules may also lower enzyme activity resulting from considerable changes in the enzyme structure both on flat[7] and colloidal supports.[8] Therefore, carrier surface modification in order to prevent a direct contact between the support and the immobilized enzyme is being explored.[9] Specifically, an approach has been recently reported that would preserve enzymatic activity after immobilization on support by attachment (covalent or physical) of enzymes to a soft polymer network (gel lattice).[10] It also includes stimulus-responsive polymer networks that undergo conformational changes in response to changing environment pH, temperature, and the ionic strength.[11] The immobilization (encapsulation) of enzymes in gels can protect enzymes from structural alterations and subsequent deactivation, but the process suffers from a low enzyme loading as well as low enzyme diffusivity in the host matrix.[12]

One of the most efficient ways to ensure higher loading and mobility of enzyme molecules with polymer-based carriers is to attach them to soft, flexible polyelectrolyte brushes—arrays of polymer chains end-tethered to colloidal particles at high grafting density.[13] It has been shown that the ionic strength in the system is the decisive parameter in enzyme adsorption of polyelectrolyte brushes grafted onto solid supports.[13d,e] The native secondary structure[14] and the activity[12] of the adsorbed enzyme molecules are preserved, thus making colloidal particles decorated with densely tethered polyelectrolyte chains promising carriers for enzyme recovery and reuse by a gradual release of enzymes from the brush after increasing the ionic strength.[15] Another recent study has demonstrated that protein molecules adsorbed on polyelectrolyte brushes are not "frozen" in terms of protein dynamics, but undergo changes probably because of the presence of repulsive electrostatic interactions.[16] Although a variety of immobilization methods using polymer networks have been designed and tested, it is still challenging to develop stable, efficient, and economically feasible hybrid biocatalytic systems for repeated enzyme recovery and recycling without activity loss for biotechnological and medical applications.

The proposed design of enzymogel nanoparticles includes a core–shell structure with an inorganic core and a polymer brush shell (Figure 1 a). A typical example is represented by a 100 ± 10 nm silica core with a 30 ± 5 nm (in the dry state) poly(acrylic acid) (PAA) brush. To enable magnetic separation of the enzymogel particles, the core was made of one or few 15 nm γ-Fe_2O_3 superparamagnetic particles that were enveloped by a silica shell. The PAA brush was synthesized by surface-initiated polymerization of *tert*-butylacrylate and the subsequent hydrolysis of poly(*tert*-butylacrylate). In an aqueous environment in a pH range from 5 to 7 the enzymogel particle has a homogeneous, swollen, negatively charged PAA brush which is clearly observable in cryo-TEM images (Figure 1 b). The PAA shell shrinks at pH 4.5 but still maintains a net negative charge (Figure 1 c). PAA is a weak polyelectrolyte with a monomer unit pK_a of 4.5 and is

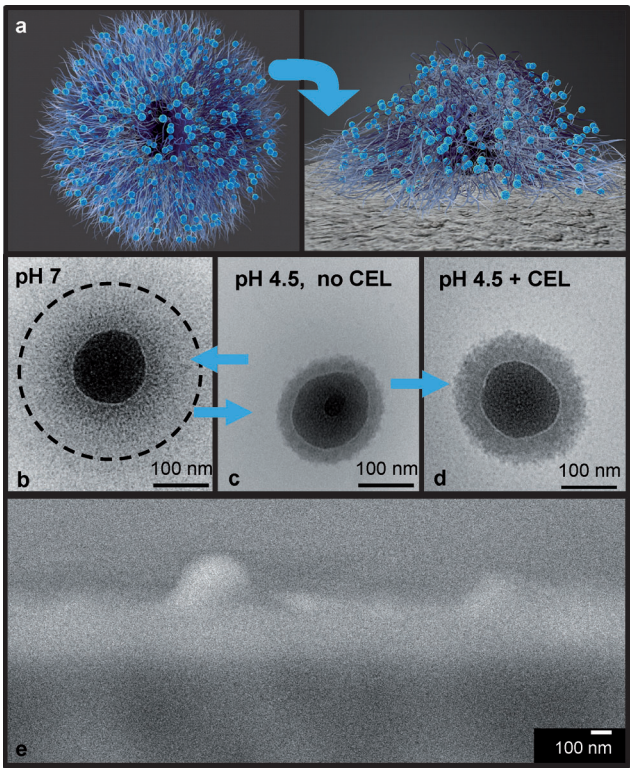

Figure 1. An enzymogel nanoparticle: schematic a) of the particle that consists of an γ-iron oxide core, a silica shell, and a PAA brush loaded with enzymes: the particle with spherical symmetry in solution and interacting with a solid substrate; cryo-TEM images of the particle with a 120 nm in diameter silica core and b) swollen and c) shrunken PAA brush at pH 7 and pH 4.5, respectively. d) The brush is uniformly loaded with CEL enzymes at pH 4.5. e) The spreading of the brush over the substrate surface is shown in the SEM image.

negatively charged in a pH range of 4 to 8 which is optimal for the highest enzymatic activity for most enzymes. The enzymogel can be loaded with positively charged enzymes in this pH range. In our working example, we use cellulase (CEL) enzymes to cleave cellulose molecules and convert them into glucose for biofuel or biochemical production.[17] CEL is typically isoelectric at pH 4.9 and, thus, attains a moderate positive charge at pH 4.5. CEL was loaded into the enzymogel particles at pH 4.5 (Figure 1 d). The loading of the enzyme is reversible and a major portion of it can be released at pH 7 and a high ionic strength. Adsorption and desorption kinetics in a typical CEL concentration range[18] were monitored with in situ ellipsometry on the surface of a reference sample of the PAA brush grafted to the Si-wafer substrate and using the Bradford assay, particle size analysis methods and thermogravimetric analysis for adsorption/desorption of CEL by the enzymogel. The amount of the attached enzymes can reach about 300 % of the PAA by weight (see the Supporting Information for details). Desorption reaches about 80 % of the adsorbed amount and depends on the ionic strength and pH (see the Supporting Information). The pH-triggered uptake/release is reproducible.

The enzymogel loaded with CEL demonstrates a high affinity of CEL to the PAA brush. CEL resides in the brush

even after multiple washing in buffer at pH 4.5. Limited leakage (about 5%) of CEL was observed after rinsing of the fully loaded enzymogel particles (see the Supporting Information). About the same amount of CEL was exchanged by dye-labeled CEL indicating a high affinity and quasi-irreversible adsorption of enzymes by the enzymogel under the specified conditions.

An important aspect of enzyme dynamics in the enzymogel particle was discovered from experiments with a fluorescent dye-labeled CEL (FL-CEL). We applied fluorescence recovery after photobleaching experiments to estimate mobility of CEL in the enzymogel (see the Supporting Information). The in this experiment estimated CEL diffusion coefficient in the PAA brush at pH 4.5 is 1.3×10^{-10} cm^2 s^{-1}, similar to the diffusion of the CEL adsorbed on cellulose.[19] Thus, the enzymogel demonstrates a unique combination of very high affinity to the enzyme and mobility of the attached enzymes.

This combination of the high affinity and mobility of CEL in the enzymogel yields multiple benefits for the use of the enzymogel particles with a high loading capacity accompanied by unchanged biocatalytic activity and mobility of the enzymes. We explain this behavior using the properties of the polyelectrolyte brushes and the ampholytic character of the enzymes. The high affinity is caused by counterion release because of the formation of the polyelectrolyte complex which provides a substantial gain of entropic energy and increases the activation barrier for the exchange between molecules in the enzymogel and in the solution. At the same time, the activation energy for translocation of enzymes between PAA segments is decreased due to the ampholytic nature of proteins which simultaneously carry similar and opposite charges to the charge of PAA. The more detailed mechanisms are the subject of further research.

The brush assembled on the magnetic-core nanoparticles was used to hydrolyze cellulose by attracting the enzymes from the solution at pH 4.5 and releasing them into the bioreactor at pH 7 which demonstrated the stimuli-responsive behavior of the enzymogel. The encapsulated CEL retains its biocatalytic activity in the brush and the released CEL retains its biocatalytic activity at the original level. The most remarkable property of the enzymogel is that CEL remains active even when residing in the brush. In Figure 2a, biocatalytic activity of CEL in six different systems is compared. The systems are divided into two groups based on a cellulose substrate used: (I–V) hydrolysis of insoluble cellulose (filter paper standard) and (VI–VIII hydrolysis of semi-soluble colloidal dispersion of α-cellulose (MM = 9000 g mol^{-1}).

In all the systems, protein-basis concentrations of CEL and cellulose in buffer solutions at pH 4.8 are the same. In the reference experiments (II and VII), free (unattached) CEL demonstrates a reference level of activity that is higher for colloidal cellulose (VII) than for filter paper (II). The CEL released from the enzymogel retains its activity at the same level (IV). The grafted CEL in both examples (I and VI) is much less active. The difference between VII and VI is likely due to the loss of CEL activity because of grafting, while the difference between I and VI shows the loss of efficiency because of a poor interfacial contact between the filter paper substrate and the grafted enzymes. CEL in the brush demonstrates nearly unchanged level of activity (III and VIII) for both types of cellulose substrates and bioconversion can be repeated in several cycles if the enzymogel is reused (V). The fermentation with multiple cycling of release and capture of enzymes in and from the biomass is less efficient (see the Supporting Information) because of possible denaturing of enzymes due to alternations in pH.

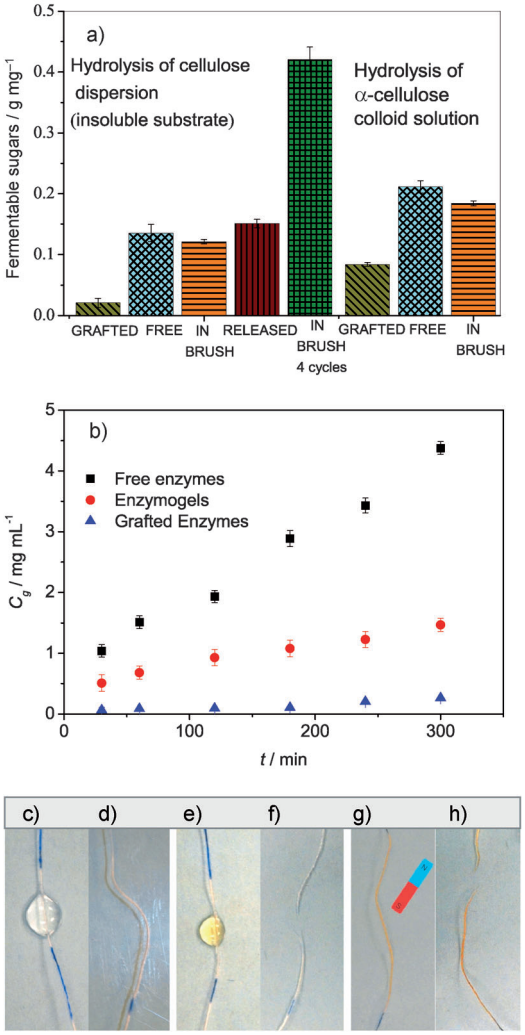

Figure 2. Biocatalytic activity of CEL (40 μg mL^{-1}) in buffer solutions at pH 5.0: a) for insoluble (I–V) and semisoluble coolidal (VI–VIII) cellulose (5 mg mL^{-1}) presented as amount of synthesized fermentable sugars per CEL (g mg^{-1}) for CEL dissolved in the buffer (II,VII), CEL grafted to 200 nm silica particles (I,VI), CEL in the PAA brush in the enzymogel nanoparticles (III V and VIII), CEL released from the PAA brush (IV), and after four cycles of the reuse of the same enzymogel particles with a magnetic core (V). b) Kinetics of glucose production shown as glucose concentration (C_g) versus time (t) using free enzymes in solution (squares), grafted enzymes (triangles), and the enzymogel (circles). Degradation of a 500 μm thick cotton floss by deposition of CEL using c) a free CEL enzyme solution, e) the enzymogel, g) and magnetic field-directed deposition d) when no rupture of the floss was observed for the free enzyme in 14 h, f) rupture was documented for the enzymogel-treated floss in 2.5 h and h) for the magnetic field-localized enzymogel in 1 h.

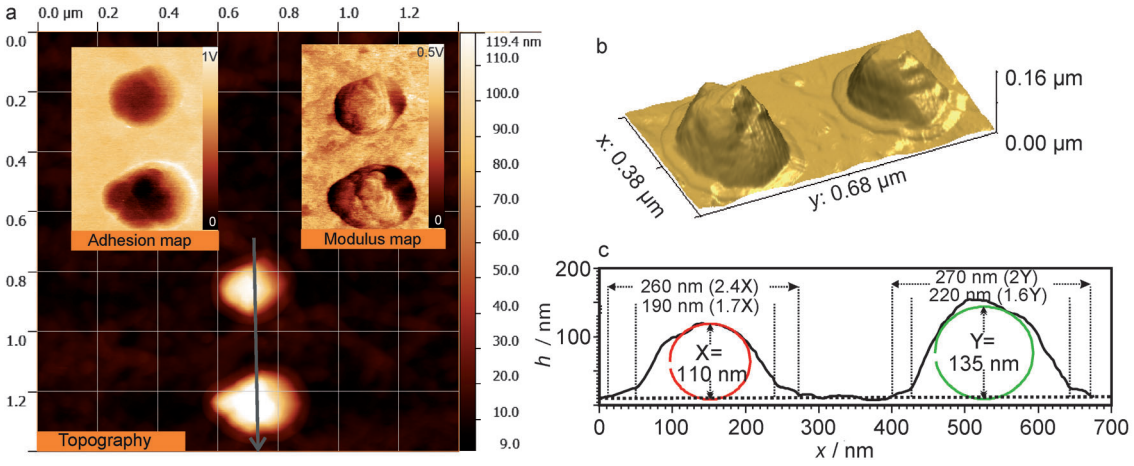

Figure 3. AFM imaging of the CEL-loaded enzymogel particles in 10 mM citrate buffer on the surface of a cellophane film using a) topography, modulus, and adhesion contrasts; 3D topographical images of the particles and their topographical cross-sectional profiles (b and c).

The results provide evidence that the enzymogel nanoparticles catalyze hydrolysis of cellulose using a new type of phase boundary catalysis (cases III and VIII); the particles are adsorbed on the surface of cellulose fibers and cleave the cellulose chains by enzymes shuttling between the PAA brush interior and the brush–cellulose interface. This shuttling is responsible for the high efficiency of cellulose conversion which is much higher than we would expect assuming that only CEL located on the exterior of the enzymogel particle could be involved in biocatalysis. The almost unchanged level of CEL activity proves that most enzyme molecules in the enzymogel are involved in the biocatlytic reactions (it is likely that only some fraction of CEL is entrapped at the particle-brush interface that causes a slightly lower sugar yield as compared with free CEL). By comparing the kinetics of glucose production using free CEL, grafted CEL and enzymogel (Figure 2b), we estimated the mean diameter of contact of the enzymogel particles with the cellulose substrate. The estimated value is about 140 nm (see the Supporting Information). Such contact area is possible if the CEL loaded brush spreads over the surface of the substrate. The latter was proved with in situ AFM experiments when single enzymogel nanoparticles were examined on the surface of a cellophane film (a model of cellulose substrate). The images of single adsorbed-on-the-substrate nanoparticles using topographical, adhesion and mechanical modulus contrasts are shown in Figure 3a. The spreading of the enzymogel over the substrate is clearly visible on the 3D topography images and topographical cross-sections (Figure 3b and c) and on the SEM image (Figure 1e). A simple estimation (see the Supporting Information) shows that the reaction contact area of an enzymogel nanoparticle due to the spreading (engulfing) is about an order of magnitude greater than for a solid particle of the same diameter with a traditional method of surface immobilization (grafting) of enzymes. The latter is proved in the reference experiment (Figure 2b, triangles).

The enzymogel nanoparticle provides a unique opportunity for industrial enzyme recovery. Because of the high biocatalytic activity of the enzymes that reside in the enzymogel there is no need to release and extract enzymes. In other words, the enzymogel can be used for biomass conversion as integral moieties. After conversion of the cellulosic biomass, the enzymogel nanoparticles can be magnetically extracted and transferred into a freshly loaded bioreactor for reuse. The experiments demonstrated that this methodology provides an about four-fold increase in glucose per enzyme when compared with the traditional one-way use of CEL for cellulose conversion (Figure 2a, V).

Numerous possible applications including biomedical technologies are illustrated using experiments with a cotton floss (Figure 2c–h). A droplet of a free CEL solution was deposited on the floss (Figure 2c) and allowed to dry. In the second experiment, the droplet of enzymogel solution was deposited on the floss and dried. In both cases the concentration of CEL was the same. Then, the floss was immersed in buffer pH 5.5 solution. In 2.5 h, the floss treated with the enzymogel was broken while the one treated with a free enzyme was intact after 14 h. If at the same concentration of CEL the enzymogel was collected on the floss with a magnet, the degradation experiment demonstrated an even faster floss failure after 1 h (Figure 2g and h). Thus, application of the enzymogel using a magnetic field can be explored for localized highly efficient bioconversion processes. The possibility of a remote control of the localized biocatalysis opens a new avenue for applications in drug delivery and absorbable implants.

Enzymogel nanoparticles readily adsorb on cellulose substrates like cotton and filter paper when both the substrates and enzymogel are negatively charged in water at pH5. The cellulose surface is only weakly charged since there are no strong acidic functional groups on the surface. Thermal fluctuations overcome the weak long-range repulsive forces and enzymogel particles strongly interact with the cellulose surface by hydrogen bonds between hydroxyl groups of cellulose and carboxyl groups of PAA brushes.

In summary, this work introduces novel phase boundary biocatalysis when encapsulated enzyme retains its mobility in a soft hydrophilic polymer carrier and is capable of hydrolyzing insoluble substrates using highly dynamic behavior of the enzyme and their high affinity to the polymer brush of the

enzymogel and to the substrate. The enzyme remains encapsulated in the polymer brush and catalyzes the hydrolysis of the insoluble substrates attached to the enzymogel. The fermentation processes described in the article are illustrated with video files (see Supporting Information).

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