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Activity of *Candida rugosa* Lipase Immobilized on γ-Fe_2O_3 Magnetic Nanoparticles

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The use of nanophase materials offers many advantages due to their unique size and physical properties. Hybrid nanoscale materials are well established in various bioprocesses such as nucleic acid detachment,[1] protein separation,[2] and immobilization of enzymes.[3]

An important area of interest is the immobilization of proteins and enzymes on magnetic particles. Several magnetic particles[3,4] and magnetic supports such as microspheres of various biomaterials[5] encapsulating the magnetic particles, and copolymers[5] with magnetic particles have been used with good results. However, due to size constraints (usually 75−100 μm), these microparticles cannot be placed at specific locations that are relevant to cellular biochemical processes. Preferably, such particles would possess a very low magnetic hysteresis and high stability. The functionalized γ-Fe_2O_3 magnetic nanoparticles used as a support in this communication possess all these traits. In addition, because of the inherent structure and size of these particles, they are superparamagnetic which addresses aggregation and flocculation concerns.[6] Here, we report the stability and enzymatic activity of *Candida rugosa* lipase (E.C.3.1.1.3) immobilized on γ-Fe_2O_3 magnetic nanoparticles.

Lipases are frequently employed enzymes as they are commonly used for the synthesis of enantioenriched monomers and macromers and for polymerization reactions.[7] It is shown here that these enzymes, when immobilized on magnetic γ-Fe_2O_3 nanoparticles, can be easily separated from the reaction medium, stored, and reused with consistent results. This system offers a relatively simple technique for separating and reusing enzymes over a longer period than that for free enzymes alone and for enzymes which are immobilized by physisorption. This can be explained by the use of covalent immobilization that does not permit the loss of enzyme by desorption from the support and protects the enzyme from denaturation by constraining it to the local environment of the particle.[8] However, while the ability to stabilize and recover the enzyme is achieved by chemical immobilization, some enzymatic activity may be lost by chemical bonding since the active site is hidden or restricted from assuming the conformation needed to initiate the catalysis.[9]

For the immobilized enzyme in the present case separation is facilitated by the use of a magnet where either the substrate solution is removed while the immobilized enzyme is held in place with a magnetic field or vice versa.

γ-Fe_2O_3 nanoparticles were prepared by sonication of Fe(CO) in decalin and the subsequent annealing of the amorphous Fe_2O_3 nanoparticles.[10] The average size of the γ-Fe_2O_3 nanoparticles is 20 ± 10 nm (see Figure 2), with saturation magnetization value of 61emu/g.[10] Figure 1 presents the strategy used to immobilize *Candida rugosa* lipase on the γ-Fe_2O_3 nanoparticles. 11-Bromoundecanoic acid was covalently linked to the nanoparticle surfaces by heating the nanoparticles in acid solution in ethanol by using

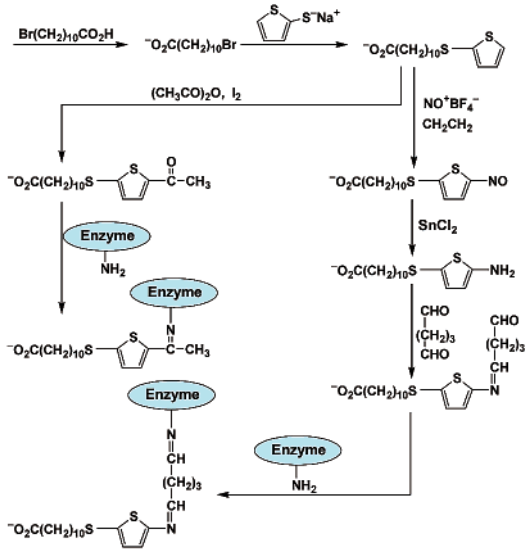

Figure 1. The strategies used to immobilize *Candida rugosa* lipase on the γ-Fe_2O_3 nanoparticles.

microwave irradiation for 10 min. Nucleophilic substitution using 2-thiophene thiolate resulted in thiophene-functionalized nanoparticles. These were either acetylated using acetic anhydride with iodine as catalyst or reacted with nitrosonium tetrafluoroborate in methylene chloride to produce the nitroso derivative. Given the chemistry of thiophene, we assume that in both cases electrophilic substitution occurred at the 5-position. In all cases full coverage of the surface area was achieved which was proven by thermogravimetrical analysis. Notice that coating of the γ-Fe_2O_3 with 11-bromoundecanoic acid decreases the magnetization of the bare γ-Fe_2O_3 on average only by 12%, resulting in nanoparticles with value still far greater than any previously reported magnetic support.

The acetylated nanoparticles were reacted directly with the enzyme, which was chemically bonded to the nanoparticle surface via a C═N bond. The nitroso-functionalized nanoparticles were reduced to the corresponding amine-functionalized nanoparticles with $SnCl_2$, and the enzyme was chemically connected using glutaraldehyde.

For the immobilization on acetylated nanoparticles, 17.3 mg of lipase (ca. 8% protein content according to protein assay) was reacted with 32.38 mg of the acetylated nanoparticle in 8 mL of phosphate buffer (10 mM, pH 7.5) under gentle shaking for 24 h at room temperature. The immobilization on amino-functionalized nanoparticles was conducted in a one- or two-pot reaction, respectively. In the one-pot approach, 30 mg of amino-fuctionalized nanoparticles was mixed with 5 μL of a 50 wt % solution of glutaraldehyde in water and 2 mL of phosphate buffer and was shaken for 1 h at 25 °C. Thereafter, 92 mg of lipase was added in

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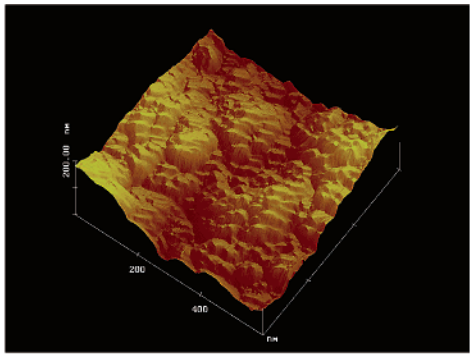

Figure 2. AFM 3D height image of the enzyme magnetic nanoparticles moieties. Average size is 20 ± 10 nm after deconvolution.[11]

4 mL of phosphate buffer and immobilized for 23 h at room temperature. In the two-pot approach, 30 mg of amino-functionalized nanoparticles was mixed with 50 μL of a 50 wt % solution of glutaraldehyde in water and 2 mL of phosphate buffer and was shaken for 3 h at 25 °C. The glutaraldehyde solution was removed by decanting the solution out while holding the particles down with a magnet, and the particles were washed three times with 4 mL of phosphate buffer; 92 mg of lipase in 4 mL of phosphate buffer was immobilized by gentle shaking at room temperature for 21 h. In all three approaches the product was separated from the supernatant by holding the particles down with a magnet, taking the solution out, and thereafter washing four times with 4 mL of phosphate buffer.

The amount of immobilized enzyme was obtained by standard BCA protein assays of the original lipase solution, the supernatants, and washing solutions after immobilization, respectively (in the case of the glutaraldehyde linker the supernatant and the wash solutions were dialyzed to prevent false results in the BCA assay). In the case of acetylated nanoparticles we obtained a loading of 5.8 μg of protein per mg of nanoparticles. For the one-pot reaction with amino-functionalized particles we obtained 55.6 μg per mg of nanoparticles and for the two-pot reaction, 22 μg per mg of nanoparticles.

Samples for AFM were prepared by spreading a small amount of the enzyme-functionalized magnetic nanoparticles on a thin layer of glue deposited on a steel disk. Imaging was performed using a Nanoscope Multimode Scanning Probe Microscope, DI, equipped with an EV scanner operating in tapping mode. Nanoprobe SPM etched silicon tips (TESP), were used for scanning. Imaging was performed in soft tapping conditions (amplitude setpoint between 1.8 and 2.000 V). To measure the particle size, the broadening effect due to the tip has been taken into account.[11]

Images show a preference for particles to self-associate, probably due to magnetic attraction, the absence of a strong interaction with the substrate, and the procedure of sample preparation (Figure 2). It is not possible to recognize discrete single enzyme nanoparticle moieties because of the aggregation between particles.

The enzymatic activity of the immobilized lipase was determined by following the ester cleavage of *p*-nitrophenol butyrate (100 mM solution) via UV spectroscopy (Molecular Devices SpectraMax Plus 384 spectrometer, at 450 nm and 25 °C). The enzymatic activity for *Candida rugosa* lipase immobilized on γ-Fe_2O_3 magnetic nanoparticles is lower than that for the free enzyme, but in all three cases the enzymatic activity was approximately equivalent (acetylated 1.1×10^{-7}, one-pot reaction 7.8×10^{-8}, two-pot reaction 1.6×10^{-7} mol/min per mg of protein, in comparison with free enzyme 2.6×10^{-5} mol/min per mg of protein). Autohydrolysis of the *p*-nitrophenolbutyrate and ester cleavage by magnetic γ-Fe_2O_3 nanoparticles without enzyme was not observed.

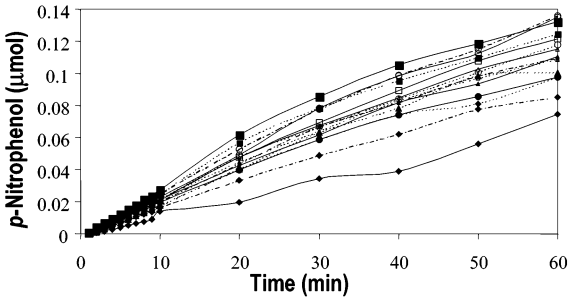

Figure 3. Enzymatic activity of *Candida rugosa* lipase immobilized on γ-Fe_2O_3 nanoparticles as a function of time (days). (—□ 1; - - -○ 2; —× 3; - - -× 4; - - -● 5; - - -△ 8; —○ 9; —▲ 10; - - -▲ 15; —Δ 16; —● 23; - - -◆ 24; - - -▲ 25; - - -◆ 26; —◆ 27).

The loss in activity is significantly higher than that reported for enzymes immobilized on micrometer-sized polymeric beads using physisorption. However, in that latter case it was observed in our laboratory that the enzyme desorbs from the beads when they are exposed to solution, suggesting that at least some of the reaction was carried out in solution and not on the surface support. This is not the case here, where the enzymes are chemically bonded to the nanoparticles.

The most significant advantage of our samples is their long-term stability. We have observed constant activity over one month in the case of lipase immobilized on acetylated nanoparticles, as can be seen in Figure 3. Our hybrid enzyme−nanoparticle composites show just ∼15% decrease in activity over that period probably due to desorption or denaturation (approximately 2% reduction was noticed over a time period of 14 days for lipase immobilized on amino-functionalized nanoparticles). These experiments were carried out by separating the nanoparticles from the solution at the end of each day, washing them with buffer solution, introducing them to freshly prepared ester solutions, and measuring the reaction kinetics. This (so-far unreported) long-term stability illustrates the advantage of attaching the enzymes chemically to the nanoparticles. Such stability might make economically viable the use of expensive enzymes and hence opens a new horizon for enzymatic catalysis in biotechnology.

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References

(1) Levison, P. R.; Dennis, J.; Badger, S. E.; Hathi, P.; Davis, M. J.; Brue, I. J.; Schimkat, D. *J. Chromatogr., A* **1998**, *816*, 107.
(2) Diettrich, O.; Millsa K.; Johnson, A. W.; Hasilik, A.; Winchester, B. G. *FEBS Lett.* **1998**, *441*, 369.
(3) Dekker, R. F. H. *Appl. Biochem. Biotechnol.* **1989**, *22*, 289.
(4) (a) Bahar, T.; Celebi, S. *Enzyme Microb. Technol.* **2000**, *26*, 28. (b) Sohn, B. H.; Cohen, R. E.; Papaefthymiou, G. C. *J. Magn. Mater.* **1998**, *182*, 216. (c) Meldrum, F. C.; Heywood, B. R.; Mann, S. *Science* **1992**, *257*, 522. (d) Ji, Z.; Pinon, D. I.; Miller, L. J. *Anal. Biochem.* **1996**, *240*, 197.
(5) (a) Horisberger, M. *Biotechnol. Bioeng.* **1976**, *18*, 1647. (b) Rusetski, A. N.; Ruuge, E. K. *J. Magn. Magn. Mater.* **1990**, *85*, 299.
(6) (a) O'Brien, S. M.; Thomas, O. R. T.; Dunnill, P. *J. Biotechnol.* **1996**, *50*, 13. (b) Tong, X.-D.; Xue Bo; Sun, Y. *Biotechnol. Prog.* **2001**, *17*, 134.
(7) Kalra, B.; Kumar, A.; Gross, R. *Chem. Rev.* **2001**, *101*, 2097.
(8) Chaplin, M. F. *Enzyme Technology*; Cambridge University Press: London, 1990, 13.
(9) (a) Tan, J. S.; Martic P. A. *J. Colloid Interface Sci.* **1990**, *136*, 415. (b) Lu, D. R.; Park, K. *J. Colloid Interface Sci.* **1991**, *144*, 271.
(10) Shafi, K. V. P. M.; Ulman, A.; Dyal, A.; Yan, X.; Yang, N.-L.; Estournes, C.; Fournes, L.; Wattiaux, A.; White, H.; Rafailovich, M. *Chem. Mater.* **2002**, *14*, 1778.
(11) Engel, A.; Schoenenberger, C.-A.; Müller, D. J. *Curr. Opin. Struct. Biol.* **1997**, *7*, 279.

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