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Hydrolysis of Magnetic Amylase-Imprinted Poly(ethylene-*co*-vinyl alcohol) Composite Nanoparticles

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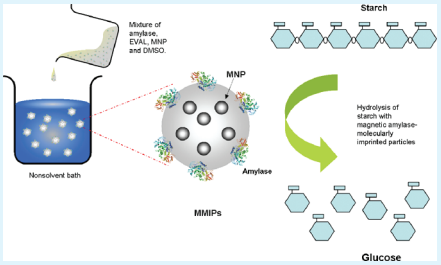
ABSTRACT: Molecularly imprinted polymers (MIPs) have frequently been employed as recognition elements in sensing applications, or for the controlled delivery of small molecule drugs. An equally important but less well studied application is the use of MIPs in the binding and immobilization of active enzymes. In this study, magnetic MIPs (MMIPs) recognizing the enzyme amylase were prepared using phase inversion of poly(ethylene-*co*-vinyl alcohol) (EVAL) solutions with 27−44 mol % ethylene in the presence of amylase. The size distribution, specific surface area, magnetization, and composition were characterized by dynamic light scattering (DLS), Brunauer−Emmett−Teller (BET) analysis, superconducting quantum interference devices (SQUID), and X-ray diffraction (XRD), respectively. The mean size of MMIPs was ∼100 nm and the magnetization was 14.8 emu/g. The activities of both bound template and rebound enzyme was established by measuring glucose production via starch hydrolysis, at different temperatures, for MIPs with different compositions (wt % EVALs and mol % ethylene). The highest hydrolysis activity of MMIPs (obtained with 32 mol % ethylene) was found to be 1545.2 U/g. Finally, compared to the conventional catalysis process, MMIPs have the advantages of high surface area, suspension, easy removal from reaction, and rapid reload of enzyme. The good activity of amylase MMIPs persists after 50 cycles of starch hydrolysis.



KEYWORDS: *molecular imprinting, amylase, poly(ethylene-co-vinyl alcohol), magnetic nanoparticles, hydrolysis*

1. INTRODUCTION

Molecularly imprinted polymers (MIPs) can be used for biosensing,[1,2] bioseparation,[3] and drug delivery.[4] During the past decade, successful imprinting of proteins has made MIPs useful for recognition of large molecules, as reviewed by Turner et al.[5] and Bossi et al.[6] Typically, natural antibodies are then employed for quantification of protein readsorption into MIPs via the enzyme-linked immunosorbent assay (ELISA).[7] Because antibodies generally recognize structural features (epitopes), it is reasonable to suppose that many structural features of target proteins remain intact on MIP binding. Thus, imprinted proteins may, in many cases, retain some of their biological activity, such as catalytic activity in the case of bound enzymes. The retention of activity is by no means assured, however, as the imprinting process may induce protein denaturing. For instance, that lysozyme is denatured by dimethyl sulfoxide (DMSO) was recently reported.[8] Hayden et al. developed surface imprinting of trypsin on quartz crystal microbalance (QCM) chips for the detection of native and denatured trypsin.[9] Thus, there is considerable interest in identifying MIP/enzyme systems in which catalytic activity is retained.

Production of glucose using amylase is a critically important bioresource technology. Immobilization of amylase has been investigated to reduce amylase use in industrial applications. Conventionally, conjugation and entrapment of amylase with biomaterials reduces the activities of the enzyme. Renewal of amylase may be required for continuous operation of glucose production. Numerous approaches have been developed for immobilizing amylase using temperature-sensitive membranes,[10] a natural polymer,[11] or the materials chitin, chitosan,[12,13] hydroxyapatite, tannin-chitosan, tannin-spharose, and cellulose fibers, glass beads,[14] zirconia,[15,16] and recently, alginate.[12,17] Magnetic chitosan beads have been synthesized and used to improve hydrolysis of starch by bound amylase.[13] Because of the micrometer size (e.g., 150−300 μm) of the magnetic chitosan beads, magnetically driven mixing is able to reduce concentration gradients and increase the rate of hydrolysis.

Incorporation of magnetic nanoparticles into MIPs has been reported in numerous studies with large molecule targets. Several model proteins, such as albumin,[18,19] lysozyme,[20] hemoglobin,[21] and ribonuclease,[22] have been imprinted on the surface of magnetic MIPs. Interfering adsorption by nontarget proteins, and the reusability of MIPs are addressed in most reports; however, the activities of bound targets has not generally been explored. The process of molecular imprinting may cause protein denaturation and loss of function, either because of the solvents used, or possibly because strong intermolecular forces between the monomers or polymers and the template. In spite of this, epitope recognition is still

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generally possible with imprinted proteins; thus, it is reasonable to expect imprinted proteins to retain some catalytic activity.

In this work, the imprinting of amylase with EVAL is presented and the hydrolysis of starch under different catalytic and imprinting conditions are compared. Magnetic particles were also encapsulated into MIPs to enable the rapid separation of bound enzyme and reactants and products.

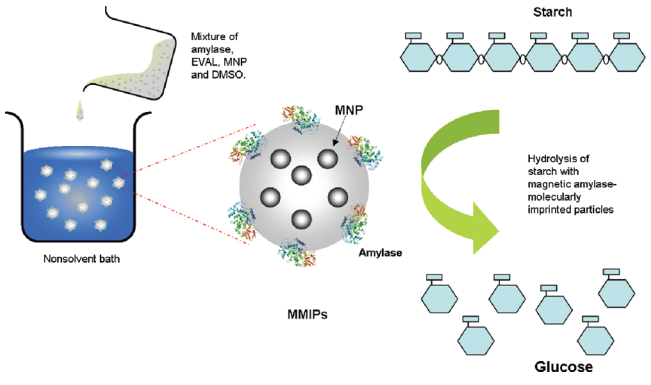
2. EXPERIMENTAL SECTION

2.1. Reagents. α-Amylase (EC 3.2.1.1, Cat. No. 10080, 43.6 U/mg) from hog pancreas was purchased from Fluka Biochemika (Buchs, Switzerland). Poly(ethylene-*co*vinyl alcohol), EVAL, with ethylene 27, 32, 38, and 44 mol % (product no. 414077, 414093, 414085, 414107) were from Sigma-Aldrich Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO, product # 161954) was purchased from Panreac (Barcelona, Spain) and used as the solvent to dissolve EVAL polymer particles in the concentration of 1 wt %. Absolute ethyl alcohol was from J. T. Baker (ACS grade, NJ). Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich Co. (St. Louis, MO) and used for the removal of target molecules. All chemicals were used as received unless otherwise mentioned.

2.2. Formation of Magnetic Amylase-Imprinted Poly(ethylene-*co*ethylene alcohol) Composite Nanoparticles. The synthesis of molecularly imprinted EVAL nanoparticles can be found elsewhere[19] and included four steps: (1) Synthesis of magnetic nanoparticles (MNPs) by the Massart method, which is simply coprecipitation of a mixture of iron(III) chloride 6-hydrate and iron(II) sulfate 7-hydrate by sodium hydroxide. This magnetite was mixed with oleic acid for better dispersion and repeatedly washed while being adsorbed on a magnetic plate, and then freeze-dried overnight. (2) Dissolving amylase in 1 mg/mL in 20 mL DMSO and adding granular EVAL to the protein/DMSO solution to form clear EVAL solution (EVAL/DMSO = 1.0 wt % unless otherwise mentioned), and 25 mg of magnetic particles were then added. (3) The dispersion of 0.5 mL EVAL solution in 10 mL nonsolvent (e.g., deionized water/isopropanol = 2/3 in weight);[23] and then (4) removal of the template molecule by washing in 10 mL 0.1 wt % SDS solution and deionized water for 15 min three times, separating the MIPs magnetically after each washing. The nonimprinted polymers (NIPs) were prepared identically, except that the template protein was omitted.

2.3. Glucose Production with Magnetic Amylase-Imprinted EVAL Composite Particles. To measure enzymatic activity, glucose production by imprinted and nonimprinted particles was assayed (as shown in Scheme 1). Five hundred milligrams of particles were incubated with 10 mL of starch solution in a 20 mL vial. Starch hydrolysis was carried out at 60 °C for 90 min (unless otherwise mentioned). A magnetic field was applied at the end of hydrolysis to separate glucose/starch solution from magnetic amylase-imprinted polymeric particles for 1 min. Eight hundred microliters of the glucose/starch solution sample was also stored in an eppendorf microcentrifuge tube at 4 °C and analyzed with ARCHITECT *ci* 8200 system (Abbott Laboratories, Illinois) at E-Da Hospital. (This integrated platform combines immunoassay and clinical chemistry, running up to 200 immunoassay tests and up to 1200 clinical chemistry tests an hour.)

Scheme 1. Synthesis and Hydrolysis of Magnetic Amylase-Imprinted Poly(ethylene-*co*-vinyl alcohol) Composite Nanoparticles



MIP particles were tested before washing, when template amylase molecules were still bound; after washing; and after rebinding amylase. The rebinding of amylase to the magnetic molecularly imprinted composite (ca. 15 mg) was performed with 1 mL of 1 mg/mL amylase in phosphate buffered saline (PBS). The rebinding duration was 30 min to give the highest adsorption on MIPs and then MIPs were separated using a magnetic plate. A UV/vis spectrophotometer (Lambda 40, PerkinElmer, Wellesley MA) was then used to measure the concentration decrease in the stock solution, determined by absorption at 265 nm for amylase.

2.4. Size Distribution and Atomic Force Microscopy Image of Magnetic Amylase-Imprinted EVAL Composite Particles. Amylase-imprinted polymers particles with different imprinting template concentrations and nonimprinted polymer particles were monitored by a particle sizer (90Plus, Brookhaven Instruments Co., NY). The measurement of the particle size distribution was based on dynamic light scattering (DLS) at 25 °C with 3 min duration data collection at the 90° detection angle. The average count rate of the background was 15 kcps and that of each measurement was between 20−500 kcps. The scattering laser power of this instrument is the standard 35 mW. The CONTIN algorithm was used to analyze data.

Atomic force microscopy of the magnetic molecularly imprinted polymers was also performed using with NT-MDT Solver P47H-PRO AFM, (Moscow, Russia). Images were made in air (room temperature (ca. 27 °C) and 87% relative humidity) using the tapping mode with scan rate 0.75 Hz. The cantilever was a SiO_2 probe (model: TGS1, NT-MDT, Moscow, Russia) with 2 nm probe tip size and 144 kHz resonant frequency.

2.5. Surface Area, Magnetization and X-ray Diffraction Analysis of Magnetic Amylase-Imprinted Poly(ethylene-*co*-ethylene alcohol) Composite Nanoparticles. The magnetic nanoparticles, amylase-imprinted magnetic EVAL composite nanoparticles (before and after removal of template) were freeze-dried for the surface area, magnetization, and crystalline structure analyses. Nitrogen adsorption measurements were performed with a NOVA 1000e (Quantachrome Instruments, FL), and Brunauer−Emmett−Teller (BET) analysis was performed with the Autosorb program (Quantachrome Instruments, Florida). BET analysis was carried out for N_2 relative vapor pressure of 0.05−0.3 at 77 K. Superconducting quantum interference devices (SQUID) are very sensitive magnetometers used to measure extremely small magnetic fields, based on superconducting loops containing Josephson junctions. The magnetization of magnetic and amylase-imprinted particles was monitored with a the magnetic property measurement MPMS XL-7 system (Quantum Design, San Diego, CA) at 298 K in ±15000 G. X-ray diffraction analysis (D8 Advance XRD, Bruker, German) was used to determine the crystalline structure of the α-Fe_2O_3 encapsulated MIP nanoparticles with Cu Kα radiation λ = 1.5406 Å.

3. RESULTS AND DISCUSSION

MIPs with bound amylase can efficiently hydrolyze starch to glucose, Figure 1. The template amylase enzyme remains functional when it is imprinted into the EVAL polymer ("before washing"), and amylase that is rebound by the MIPs has an even higher activity. The variations in glucose production using different ethylene mole % of amylase-imprinted poly(ethylene-*co*-vinyl alcohol) composite particles are similar for both initially imprinted template and readsorbed enzymes, as shown by the nearly parallel curves. Glucose production via starch hydrolysis with amylase-imprinted EVAL particles was roughly 23.8 ± 1.9 − 54.8 ± 0.4 mg/dL for the

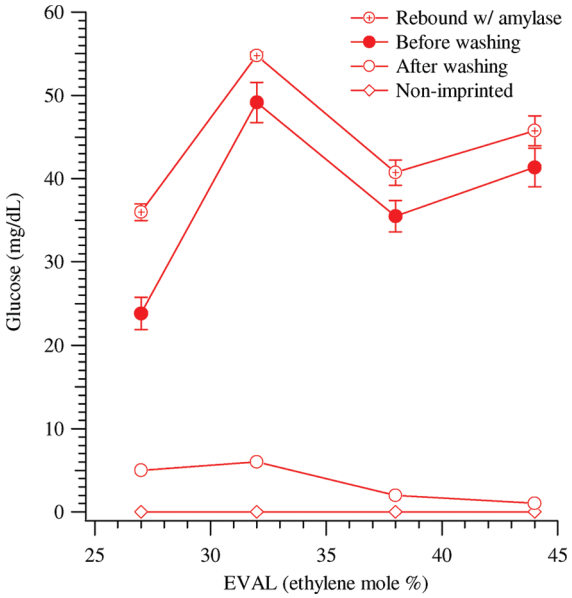


Figure 1. Glucose production with magnetic amylase-imprinted composite magnetic MIPs with different mol % ethylene contents: (before washing (●); after washing (○); after rebinding (⊕); and nonimprinted controls (◊)).

compositions of EVAL studied, i.e. from 27 to 44 mol % ethylene. Notably, the highest glucose production was 49.2 ± 2.4 to 54.8 ± 0.4 mg/dL with EVAL with 32 mol % ethylene. Glucose production with EVAL containing 32 mol % ethylene was about roughly 1.5- to 2-fold higher than that with 27 mol % ethylene. Interestingly, the efficiency of amylase-imprinted EVAL particles was enhanced by 10.7−51.0% after amylase rebinding; this may indicate that rebound amylase is less constrained or confined than the original template molecules, and is thus able to retain higher activity. The nonspecific adsorption of amylase on NIPs[24] may also provide some small production of glucose, but without the reusability and stability at higher temperature achieved by the MIPs.

Figure 2 shows the magnetization, an atomic force microscopy image, and the size distribution of magnetic amylase-imprinted EVAL particles, measured with dynamic light scattering (DLS). Figure 2a plots the size distributions of composite particles using EVALs containing different mole ratios of ethylene, measured before template removal using DLS. The average sizes of particles were roughly 125−167 nm before template removal; after template removal, the particle sizes were found to be 86−114 nm. The mean sizes of particles made with different EVAL compositions were fairly close; the differences in their activities are thus not likely caused by major morphological change in the size or shape of the nanoparticles. The atomic force microscopy image (Figure 2b) indicates that the magnetic amylase-imprinted EVAL particles (containing 32 mol % ethylene) after template removal were <100 nm in size. Due to the smaller size of MNPs, the specific surface area is larger. The surface area of magnetic nanoparticles (MNPs) and MIP-coated magnetic nanoparticles (MNPs) are 16.94 and 12.83 m^2/g, respectively, by the BET analysis. Although the freeze-drying process may affect the polymeric microstructure, the coating of MIPs on MNPs reduced the specific surface area of particles (or magnetic particle aggregates). The zeta potential of the MIPs before and after template removal were 3.54 ± 1.56 and −4.6 ± 1.62 mV, respectively. The MIP particles were placed in water or PBS and then titrated with amylase; the zeta potential is shown in Figure 2c. The zeta potential of MIPs in DI water increased more rapidly than in PBS, indicating that the ionic strength[25] and pH may be related to the deposition of amylase on MIPs.

Figure 2d shows the magnetization of the magnetic nanoparticles and the synthesized composite particles. The superparamagnetic property of the magnetic nanoparticles was retained in the composite particles, even though the magnetization was somewhat reduced. The amylase-imprinted EVAL composite particles showed a magnetization reduced by about 25% (from 20.4 to 14.8 emu/g under a 15000 G magnetic field), compared to the magnetic nanoparticles (MNP) alone. The X-ray diffraction (XRD) peaks of the three samples correspond to (311), (511), and (440), revealing that face-centered cubic Fe_3O_4 was encapsulated in EVAL nanoparticles (JCPDS card # 19−629).

The roles of template concentration and EVAL/DMSO weight ratio during MIP synthesis were also explored, Figure 3a; the effects of hydrolysis temperature and substrate concentration are reported in panels b and c in Figure 3. The 10 mL hydrolysis solution contains: 2.5 mg/mL magnetic amylase-imprinted particles (imprinted with 1 mg/mL of amylase in 1 wt % EVAL/DMSO solution), at an initial starch concentration of 8 mg/mL at 60 °C. Each point in Figure 3a shows the performance (glucose production) from a different MIP preparation, with template still bound, all measured under the same conditions (MIP and starch concentrations) as described in materials and methods. In general, increasing the EVAL/DMSO ratio had a beneficial effect on glucose production, though the reasons for this are not clear. It may be that higher EVAL concentrations lead to better template binding, perhaps owing to a tighter packing of matrix polymer around the template enzyme. Higher template concentration increased glucose production, as expected. However, the increase was much lower than proportionate: a 300-fold increase in template concentration gave <10-fold increase in glucose production. This apparent saturation in activity could simply reflect the finite number of binding sites that can be formed in a given number of MIP particles. We note, however, that a template concentration of 1 mg/mL and EVAL/DMSO wt ratio of 1% still gives an EVAL:template weight ratio of more than 10; it is perhaps surprising that site saturation could occur so dramatically with a still-large excess of EVAL. It should be noted, however, that template may well distribute nonuniformly in the precipitating MIP particles. In particular, if the template molecules are surface active (with respect to the EVAL/nonsolvent), then site crowding would occur at very low template concentrations.

Temperature also altered the activity of amylase-imprinted EVAL particles (Figure 3(b)) with bound template. The highest activity of amylase was at 60 °C, and then decreased rapidly at higher temperatures. The peak activity occurred at a same temperature, compared to activity reported for the free enzyme.[26] This suggests that the binding to the MIP does not reduce the activity of amylase enzyme. Figure 3c shows the kinetics of starch hydrolysis by the amylase-MIPs, at two different substrate concentrations, using EVAL with 32 mol % ethylene. In these measurements, the concentration of amylase MIPs was 25 mg/mL and the reaction temperature was at 60 °C. The rate of production of glucose was independent of starch concentration at short times, as expected when the enzyme is saturated with excess substrate. After ca. 30 min, the

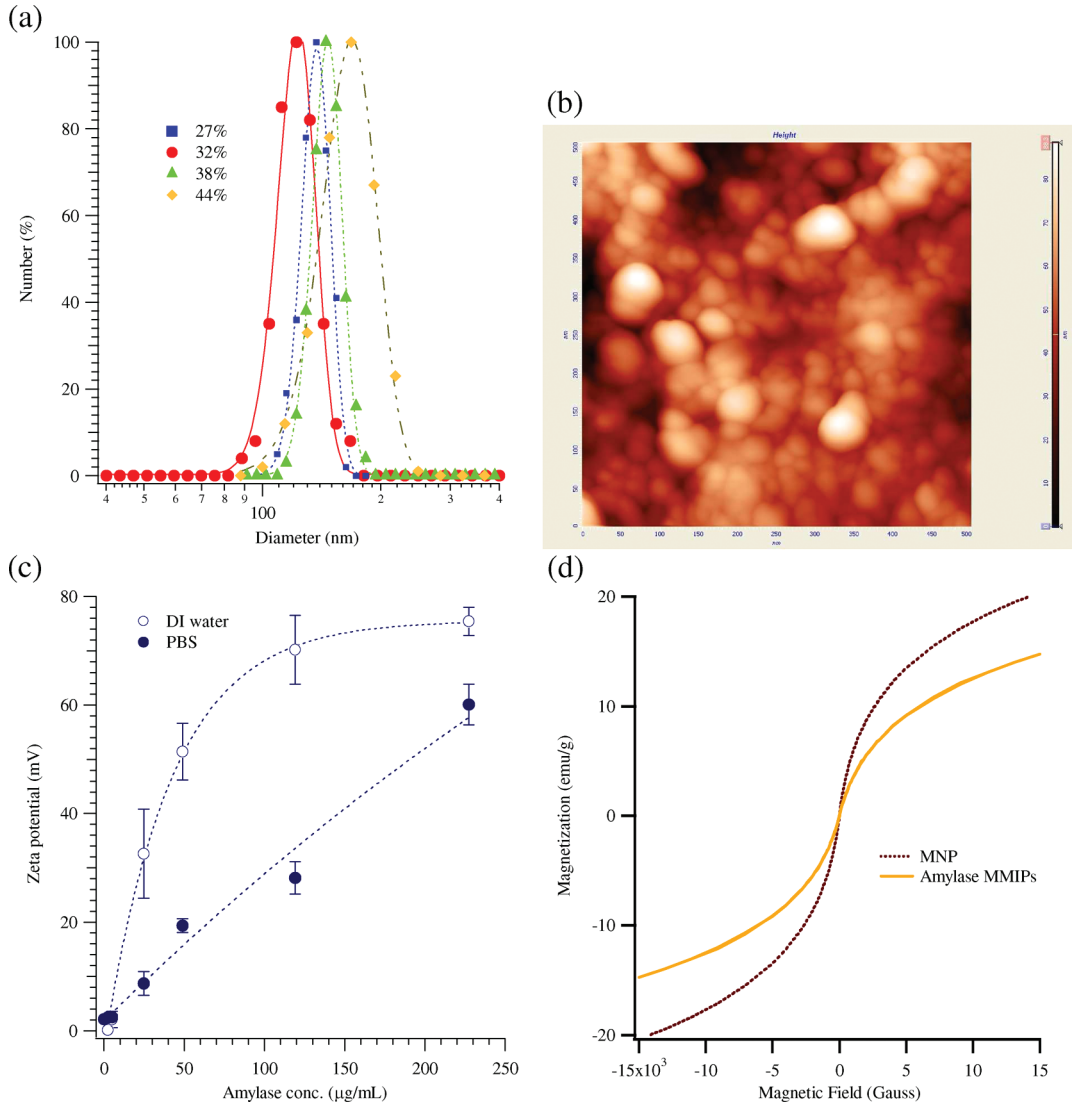


Figure 2. (a) Size distribution with magnetic amylase-imprinted composite particles; (b) atomic force microscopy image and (c) zeta potential titration of magnetic amylase-imprinted EVAL (containing 32 mol % ethylene) particles. The image size of the AFM is 500 × 500 nm^2 and the maximum height is 90 nm. (d) Magnetization measurement of magnetic particles when the amylase imprinting concentration is 0.1 mg/mL.

production of glucose from the lower concentration starch solution begins to show significant slowing, while glucose production from the higher concentration substrate is still increasing rapidly (though not as rapidly as from the initial hydrolysis.) The exact kinetics of hydrolysis of starch by amylase is exceptionally complex, owing to the structural and chemical complexity of the starch substrate.[27] Lines shown are therefore guides to the eye and are not intended to represent a specific hydrolysis model. We note that the hydrolysis activity of the amylase-MIPs (826 ± 4 mg/dL) is comparable to literature reports of hydrolysis under the same conditions (400 mg enzyme-immobilized magnetic chitosan beads at 25 °C in 10 mg/mL starch for 90 min).[13]

The activity of a 1 mg/mL amylase solution and 2.5 mg/mL magnetic amylase-imprinted particles determined using the ARCHITECT *ci* 8200 system were 32187 and 3863 U/L, respectively. Therefore, the activity of the immobilized amylase was equivalent to a free enzyme concentration of 0.12 mg/mL; this gives a weight ratio of enzyme:MIP of 48 mg/g. The actual readsorption of amylase on MIPs was measured using UV/vis spectroscopy, and found to be 63.5 ± 1.5 mg/g. Thus, the rebound amylase has an activity that is about 80% of the activity of the free enzyme.

Lastly, the reusability of the amylase-MIPs was studied, Figure 4. Starch hydrolysis was carried out at 60 °C for 90 min, with 0.5 g of MMIP particles incubated with 10 mL of starch solution in a 20 mL vial. As the performance of MMIPs before washing is nearly as high as after washing and rebinding amylase, the MMIPs without template removal were used for these hydrolysis measurements. For the measurements shown, a starch solution was added to MMIPs; hydrolysis was measured; MMIPs were then separated using a magnetic plate, and the cycle was repeated for 50 iterations, showing only a small drop in hydrolysis activity (17%).

4. CONCLUSIONS

Using MIPs as enzyme carriers enhances physical adsorption and is suitable for catalytic applications requiring high specific activity. Compared to the conventional separation process, magnetic nanoparticles have the advantages of suspension and easy removal. In this study, the optimum (highest imprinting effectiveness) ethylene mole ratio of commercially available

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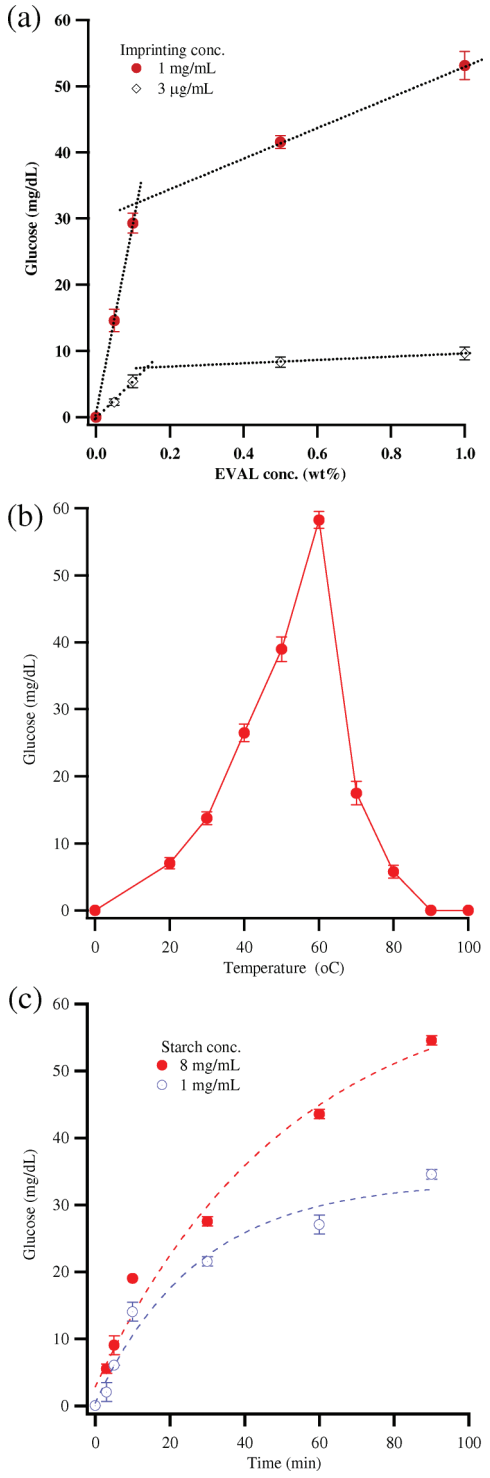

Figure 3. Glucose production is affected by (a) imprinting template concentration, polymer (EVAL) concentration; (b) reaction temperature; and (c) starch concentration and reaction duration. The hydrolysis solution contains 2.5 mg/mL magnetic amylase-imprinted particles (imprinted with 1 mg/mL of amylase in 1 wt % EVAL/DMSO solution), 10 mL at an initial starch concentration of 8 mg/mL at 60 °C.

EVALs was found to be 32 mol % for amylase imprinting. The optimal ethylene mole ratio did not correlate with particle size, suggesting that other factors, such as recognition site chemistry, play a dominant role in imprinting effectiveness and/or activity of bound enzyme. The temperature dependence of amylase

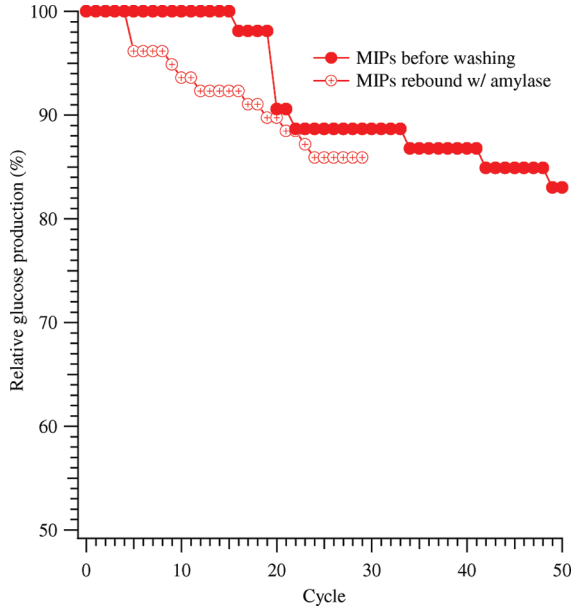

Figure 4. Reusability of magnetic amylase-imprinted EVAL composite nanoparticles (before washing (●); after rebinding (⊕)) after repeated cycles of starch hydrolysis. The standard deviation is less than 3% for each point.

activity on amylase-imprinted polymeric particles is the same as that reported in literature for free amylase,[26] indicating a relatively weak interaction between amylase and the MIPs; such weak interactions probably allow the bound enzyme to function with a specific activity that is comparable to (80%) of that of the free enzyme. Finally, glucose production using magnetic amylase-imprinted EVAL particles is comparable with that in literature.[13] The reusability and suspension of amylase-imprinted EVAL particles was even better in this study than previously reported, with good activity remaining after 50 cycles of starch hydrolysis.

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Notes

The authors declare no competing financial interest.

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