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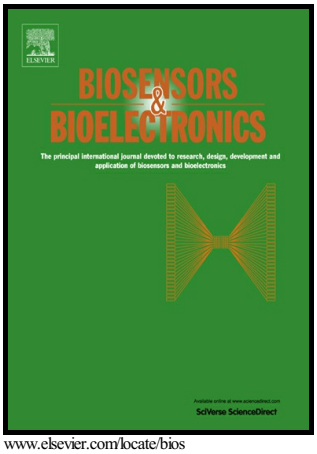

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Modified nanoporous titanium dioxide as a novel carrier for enzyme immobilization

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Abstract

ε-Poly-L-lysine (EPL)-modified mesoporous titanium dioxide (M-TiO_2) was assembled through the electrostatic attraction between EPL and M-TiO_2. Through modification, the M-TiO_2 surface tends to form multilayered and complex architectures, which can be used as artificial matrices to change the microenvironment of carriers for enzyme immobilization. The modified M-TiO_2 was characterized through scanning electron microscopy, Fourier transform infrared spectroscopy, zeta potential analysis, and thermogravimetric analysis. All of the immobilized enzymes with negative charges display strong storage stability, thermal stability, and good reusability. Results indicate that EPL can self-assemble onto the surface of M-TiO_2 and form a considerable number of active coatings. Our results also demonstrate that

this simple and novel method can be potentially used to immobilize negatively charged enzymes for biosensor applications.

Keywords: titanium dioxide, ε-poly-L-lysine, enzyme immobilization, modification

1. Introduction

As biocatalysts, enzymes are widely used in various fields, such as industrial biosynthesis, healthcare industry, and environmental protection. However, their use is currently limited because of serious disadvantages, such as high cost, instability, and difficult recovery. Enzymes loaded on a carrier, a highly functional biological machine, can effectively prolong their half-life, improve their structural instability, and increase their reusability (Ariga et al. 2013; Kim et al. 2006). Thus, immobilized enzymes provide specific advantages, such as broad optimal pH and temperature range, strong stability, and simple product purification (Kim et al. 2006). An important factor of enzyme immobilization is that carriers should create an inert and biocompatible microenvironment (Mitchell et al. 2002). In other words, enzyme immobilization aims to confine enzymes onto carriers through simple adsorption, covalent bonding, encapsulation, or sophisticated method combinations, which do not significantly affect enzyme activity while stability is improved (Kim et al. 2006; Livage et al. 2001; Shen et al. 2015).

Various materials with nanostructures have been used as carriers to immobilize enzymes and to develop enzyme-based biosensors. As inorganic host carriers, mesoporous materials have been extensively investigated (Ariga et al. 2013; Kim et al. 2006). Mesoporous materials are also influenced by vital factors, such as pore

structure, effective enzyme loading, and mass transfer resistance, which determine the stability and catalytic efficiency of immobilized enzymes (Ansari and Husain 2012). However, the high cost and difficulty in production of these mesoporous materials limit their practical industrial applications (Prakasham et al. 2007).

Mesoporous titanium dioxide (M-TiO_2), a semiconductor nanomaterial, was prepared in this study via a simple synthesis route at quite a low cost (Li et al. 2009). M-TiO_2 is a safe, environmentally friendly nanocrystalline material with narrow pore size distributions and ordered pore structures; the pore diameter of this material is approximately 10–100 nm (Magner 2013; Wei et al. 2013). Furthermore, the structure of TiO_2 can be controlled during fabrication; its structure at a nanometer scale precision should be controlled to synthesize nanoporous materials with novel functions. (Malgras et al. 2015; Wei et al. 2013). M-TiO_2 also possesses a high mechanical strength, a large surface-to-volume ratio, and an excellent physical and chemical stability; it can also be modified with various functional groups. (Lori and Nok 2004; Sadjadi et al. 2009; Tang et al. 2010; Wei et al. 2013). However, intrinsic inorganic characteristics and insufficient functional groups on the TiO_2 surface are accounted for a small amount of loaded biomolecules. Surface modification is an effective mechanism to improve the biocompatibility between TiO_2 and biomolecules; as a result, this mechanism can increase the number of biomolecules loaded on its surface. For instance, γ-glutamyltranspeptidase immobilized on silylated M-TiO_2 exhibits a loading capacity significantly superior to pure M-TiO_2 (Wang et al. 2014). Poly(sodium acrylate)-g-methoxy poly (ethylene oxide) is used to modify TiO_2 and to

improve the biocompatibility between TiO_2 and lipase. This modification has created an amphiphilic microenvironment for lipase immobilization (Wang et al. 2014). TiO_2 is a competing substrate in biosensors. For example, glucose oxidase is immobilized on the surface of carbon nanotube-modified electrode with a nanoporous TiO_2 layer, thus, a biosensor with remarkable detection sensitivity efficient electron transfer can be developed (Cui et al. 2013). A TiO_2 nanoparticle modified by Ti_3C_2 MXene nanocomposite encapsulates hemoglobin to create a mediator-free biosensor (Wang et al. 2015). Previous studies on the immobilization of enzymes on titanous and titanic complexes of poly (*N*-acryloyl-4 and 5-aminosalicylic acid) provided a basis of future studies on enzyme immobilization on TiO_2 (Kennedy and Epton 1973).

The greatest challenge in enzyme immobilization is the provision of sufficient linkages between carriers and enzymes to prevent enzymes from falling off and to avoid losing bioactivity gently (Wicklein et al. 2011). Nanomaterials have also been widely developed. Amine groups can be connected to its surfaces through chemical or plasma methods (Couturaud et al. 2013; Mévellec et al. 2007). Trypsin and BSA are immobilized onto polystyrene and α-chymotrypsin is immobilized onto polypropylene by using chemical or plasma methods to create amine surfaces (Boulares-Pender et al. 2009; De Jesús Martínez-Gómez et al. 2010). Self-assembly, which is an environmentally friendly method, has also been extensively explored. Under normal conditions, self-assembly is dominated by electrostatic adsorption, hydrogen bonding, and Van der Waals force (V. Klitzing 2006). For example, chitosan/γ-poly-glutamic acid polyelectrolyte multilayer films have been investigated to examine

cell–biomaterial interactions in tissue regeneration (Antunes et al. 2011). Moreover, self-assembled CpG oligonucleotide-conjugated gold nanoparticles (AuNPs) have been used to develop an AuNP-based polyvalent immunostimulatory nanoagent (Wei et al. 2012). Indeed, in order to increase the amount of enzymes loading on the carriers, high-molecular-weight polymer self-assembles onto the surface of mesoporous materials exhibiting an interest for biotechnological applications, especially polyionic biological molecules. M-TiO_2 is a useful carrier to attach positively charged biological materials because its surface is negatively charged at physiological pH (Wang et al. 2014; Wei et al. 2013).

In this study, ε-poly-L-lysine (EPL), which is a new type of polymer, was used to modify M-TiO_2 via self-assembly. EPL is an L-lysine linear homopolymer consisting of a unique structure that links α-carboxylic acid groups and ε-amino groups. This homopolymer is cationic at physiological pH because of its side chains (free amino groups) with positive charges (Xia et al. 2013; Zhang et al. 2012). EPL is a potential biological adhesive material because of its electrostatic interaction between enzymes and M-TiO_2. We proposed that this simple method can promote the amount of enzymes loaded on the modified carrier and can increase enzyme recovery. With these properties, this technique is an optimum candidate for enzyme immobilization.

To demonstrate that M-TiO_2 can immobilize negatively charged enzymes on its surface, we used six kinds of enzymes, namely, sucrose isomerase (SIase), laccase, lipase, neutral protease, urease, and alkaline protease, as model enzymes in immobilization experiments. Layer-by-layer (LBL) self-assembly, a highly versatile

method, can be used to fabricate the controlled layered structures of component materials; this method uses very simple and rapid procedures and thus has been extensively explored (Ariga et al. 2014). In our study, the LBL self-assembly technique was applied to modify the structure of M-TiO_2 and to immobilize enzymes. M-TiO_2 was prepared by grafting EPL at physiological pH. EPL-M-TiO_2, which is a functionalized product, was then used as a carrier to immobilize enzymes in phosphate buffer solution. Moreover, this method does not use organic solvents, which denature proteins; with this characteristic, this method is suitable for biomaterial applications. The structural characteristics of EPL-M-TiO_2 were examined using several methods. We investigated the enzymatic properties, storage stability, thermal stability, and reusability of immobilized enzymes, which are vital factors influencing biosensor sensitivity.

2 Materials and methods

2.1 Materials

Pure SIase was obtained from recombinant *Escherichia coli* (pET22b-*pal*I) strain, as described previously (Li et al. 2011). Laccase, lipase, neutral protease, urease, and alkaline protease were purchased from Novozyme Co., Ltd. EPL, whose molecular weight is approximately 2.5–3.5 kDa, was synthesized by *Streptomyces albulus* PD-1 strain. M-TiO_2 was provided by Lu (He et al. 2004). Other chemicals and reagents used in this study were obtained from Sigma Co., Ltd.

2.2. Surface modification of M-TiO_2

M-TiO_2 was immersed in deionized water and subject to ultrasound treatment for 30 min to remove / and was rinsed thrice with 50 mM phosphate buffer (buffer A; pH 6.0). The M-TiO_2 powder was then separated through vacuum suction filtration. EPL and M-TiO_2 were immersed in buffer A at 4 °C to alter the charge and pore structure of M-TiO_2. The EPL-modified M-TiO_2 powder was subsequently washed with deionized water to remove the unbounded EPL and was dried in a freeze-drying machine.

2.3. Properties of free and immobilized enzymes

The effect of temperature on free and immobilized SIase and urease, laccase and lipase, and neutral protease and alkaline protease was investigated at 10–60 °C, 20–70 °C, and 30–80 °C, respectively. To determine the optimal pH, we performed the enzyme assay at different pH values. To determine their storage stability, we stored the free and immobilized enzymes at 4 °C for 2 months. The thermal stability of alkaline protease was studied by incubation them at 65 °C for 325 min. The thermal stability of the other enzymes was studied by incubating them at 50 °C for 325 min. The kinetic parameters of the immobilized and free enzymes were determined by measuring the rate of reaction with an increasing sucrose concentration (1 μM–10 mM). The Michaelis–Menten constant (K_m) and maximum reaction velocity (v_{max}) were calculated using the Lineweaver–Burk equation.

2.4. Reusability of immobilized enzymes

The reusability of immobilized enzymes was studied by determining the activity of residual enzyme in catalytic reaction. The immobilized enzymes were used

repeatedly, washed with buffer A thrice after each batch of reaction. Sixteen batches of the reaction were performed.

3. Results and discussion

3.1. Mechanism of M-TiO_2 modification and immobilization of enzyme on EPL-M-TiO_2

Improving the amount of enzyme loading on the surface of carriers is crucial for the industrial applications of immobilized enzymes. To increase the enzyme loading, we modified the M-TiO_2 with EPL. The surface modification dramatically changed the surface characteristics of M-TiO_2. Given that EPL is abundant with free amino groups, the negative charges on the surface of M-TiO_2 can be shielded by positive charges of the EPL (Fig. 1). This phenomenon caused the surface of M-TiO_2 to bear positive charges, rendering the M-TiO_2 to immobilize negatively charged enzymes via electrostatic adsorption on its surface, as well as to increase enzyme loading. The principle of this method of enzyme immobilization is based on the properties of the materials and enzyme self-assembly. EPL serves as connecting arm between the negatively charged M-TiO_2 surface and the negatively charged enzymes. In addition, hydrogen bonding and Van der Waals force interactions exist among M-TiO_2, EPL, and enzymes.

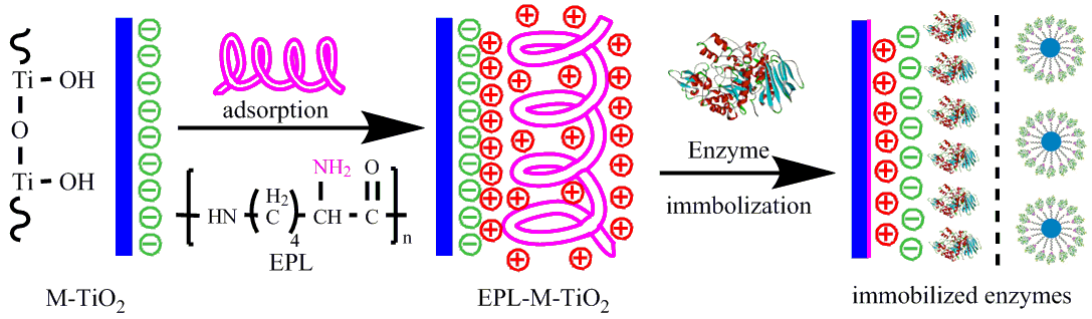


Fig. 1 Schematic diagram illustrating the process of modification of M-TiO_2 and enzymes immobilized on carriers.

3.2. Characterization of M-TiO_2 and EPL-M-TiO_2

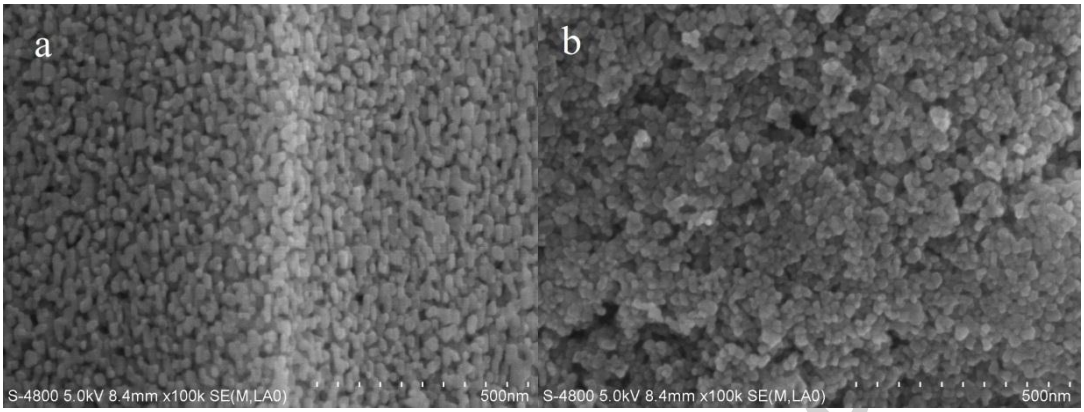


Fig. 2 SEM images of M-TiO_2 (a) and EPL-M-TiO_2 (b)

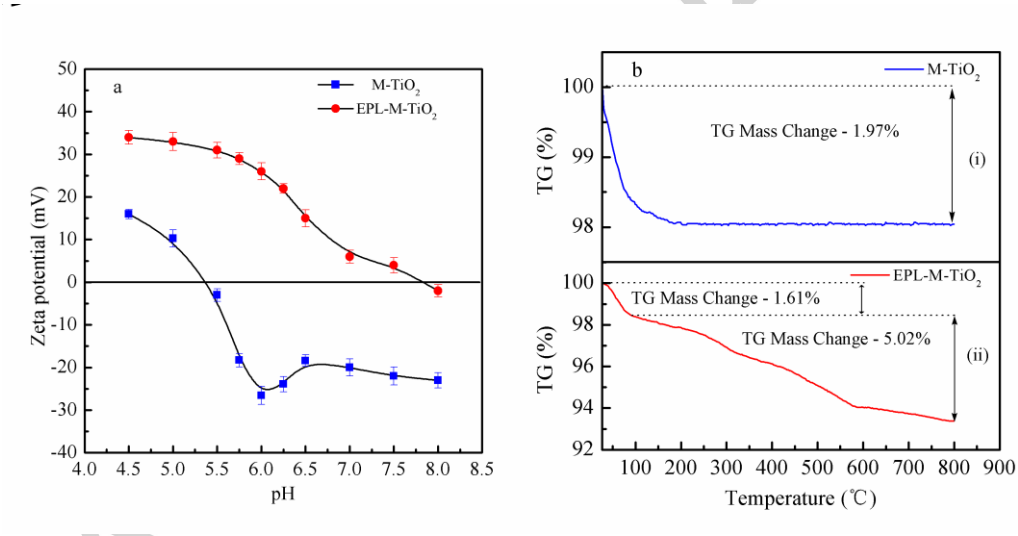


Fig. 3 The zeta potential value (a) of M-TiO_2 (●) and EPL-M-TiO_2 (■) in different pH values; thermogravimetric analysis curves (b) of M-TiO_2 (i) and EPL-M-TiO_2 (ii).

The crystal form of M-TiO_2 is clearly anatase, which is reflected in sharpening of all its diffraction peaks [Fig. S1 in the Supplementary information (SI)]. The FTIR spectrum of the modified M-TiO_2 is depicted in Fig. S2 in SI. The peak at 460 cm^{-1}

indicates Ti–O–Ti stretching, whereas that at 3419 cm^{-1} is attributed to the hydroxyl groups of M-TiO_2 (Wang et al. 2014). Moreover, the bands at 1668 and 3150 cm^{-1} can be attributed to the bending vibration of free amino groups and the N–H stretching vibration, respectively (Rao et al. 2012). The two other peaks at 1100 and 620 cm^{-1} indicate the C–H stretching vibration and the out-of-plane bending vibration of C=O, respectively (Liang et al. 2014; Shima and Saka 1977). The bending vibration of C–N–H also occurs at 1537 cm^{-1}. The analysis of FTIR thus determines that EPL founds an effective functionalized on the surface of native M-TiO_2. The EPL-modified surface of M-TiO_2 was further investigated using SEM (Fig. 2), which reveals that the micrometer-sized flakes intensive configuration on the picture; moreover, compared with native materials, aggregation occurred in EPL-M-TiO_2, possibly resulting from the layer-by-layer self-assembly of M-TiO_2 and EPL. Thus, both M-TiO_2 and EPL-M-TiO_2 displayed highly ordered porous structures with semblable distributions. Studies have shown that the surface of different oxide carriers exhibits a specific pH value in the presence of water, thereby affecting enzymatic activity (Foresti et al. 2010). ZP value of a carrier in different pH values can reflect its chemical characteristics and the charge condition of its surface. The charge status of carriers can be modified using materials with carboxyl or amino groups (Lei et al. 2002). The hydroxyl groups of TiO_2 interact with the amino groups in the form of ionic bond, hydrogen bond, and other molecular interactions (Ukaji et al. 2007). As a consequence of the large number of unbonded hydroxyl groups on its surface, zero ZP of M-TiO_2 was approximately pH 5.3 (Fig. 3a). After M-TiO_2 was modified with EPL,

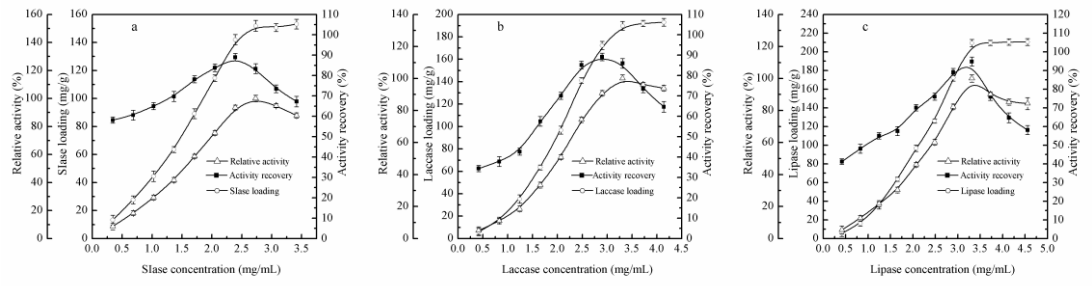
a notable shift in ZP value indicates that the surface characteristics of M-TiO_2 changed dramatically. This finding can be attributed to the fact that the surface of M-TiO_2 carries positive charges owing to the presence of EPL. Consequently, the zero ZP of M-TiO_2 increased to approximately pH 7.8, which may increase enzyme loading, and maintain the activity and structure of enzymes. TGA of M-TiO_2 and EPL-M-TiO_2 was also implemented to determine the amount of EPL absorbed on M-TiO_2 surface (Fig. 3b). From 25 °C to 105 °C, the TGA curves of M-TiO_2 and EPL-M-TiO_2 reveal a weight loss of 1.97% and 1.61%, respectively, resulting from water evaporation. A clear weight loss curve from 105 °C to 800 °C was observed in EPL-M-TiO_2 but not in M-TiO_2 [Fig. 3b(i)], which can be attributed to the decomposition of loaded EPL. Furthermore, the TGA result demonstrated that approximately 5.02% EPL was absorbed on the total weight of carriers after M-TiO_2 modification.

3.3. Determination of the parameters of pore structure

The mesoporous materials, exhibiting pore diameters of 8–20 nm, are quite suitable for enzyme immobilization (Chao et al. 2013). As a carrier of immobilized enzyme, M-TiO_2 is one of the nanoporous materials; its pore structure plays a vital role in enzyme immobilization (Magner 2013). When the pore size of materials matches that of the enzyme molecules, a high enzymatic stability can be achieved (Takahashi et al. 2000). The absorption isotherm curves (Fig. S3a and S3b, in SI) were obviously type IV-like isotherms, indicating the excellent mesoporosity of the carriers. Moreover, the pore size distribution plots (Fig. S3c, in SI) demonstrated that

the average pore diameter of M-TiO_2 and EPL-M-TiO_2 had the mesoporosity of a quite narrow distribution of their pore size. The special values for surface area, the average pore volume, and pore diameter were summarized in Table S1 in SI. The surface area of M-TiO_2 increased from 71.69 m^2/g to 74.03 m^2/g, whereas the pore volume decreased from 0.247 cm^3/g to 0.158 cm^3/g after modification. Following modification with trimethoxysilane, the pore volume of M-TiO_2 decreased from 0.37 cm^3/g to 0.31 cm^3/g, and the mercaptopropyl groups constituted up to approximately 3.5% of the total weight of the supports (Wei et al. 2013). Compared with the results of the two different modification methods, the unobvious change in pore volume of the mercaptopropyl-modified M-TiO_2 may result from the low amount of mercaptopropyl groups loaded onto the M-TiO_2. These results suggest that the loading quantity of EPL on the surface of the modified carrier is a direct result of both the surface area and pore size of the EPL-M-TiO_2. Following carrier modification, pore diameter decreases and surface area increases (Takimoto et al. 2008; Zhao et al. 2013). Given their large surface area and porous structure, the modified carriers are potentially great matrices for enzyme immobilization.

3.4 Enzyme immobilization



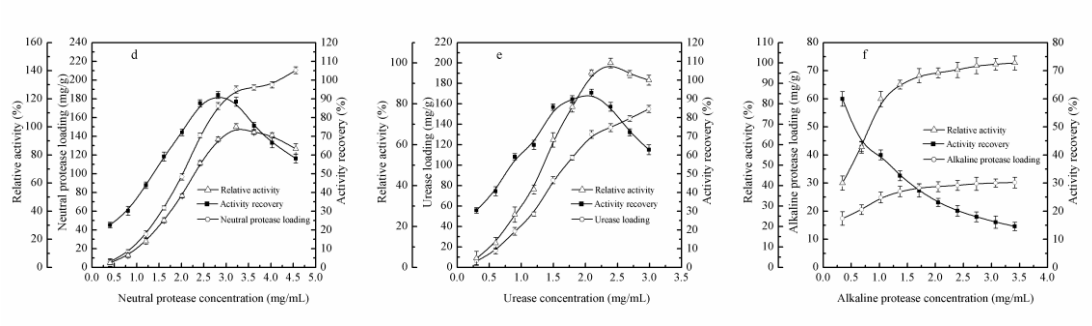


Fig. 4 Effect of the initial enzyme concentration on the activity recovery (■), the enzyme loading (○) and the relative activity (△) of immobilized enzymes. SIase (a), laccase (b), lipase (c), neutral protease (d), urease (e) and alkaline protease (f).

Effective enzyme loading is one of the important characteristics of mesoporous materials. Modified M-TiO_2 was used to immobilize enzymes (SIase, laccase, lipase, neutral protease, urease, and alkaline protease) in different initial concentrations of enzyme solution. The immobilization capacity of the modified M-TiO_2 was evaluated by studying its enzyme loading. Fig. 4 shows that the amount of enzyme (SIase, laccase, lipase, neutral protease, or urease) immobilized on the modified M-TiO_2 increased with increasing enzyme concentration. It is similar for the trend of the activity recovery, relative activity and enzyme loading of the above five kinds of enzymes with negative charges (shown in Fig. S4 in SI) in the process of enzyme immobilization. This trend is similar to that of activity recovery, relative activity, and enzyme loading of the five negatively charged enzymes mentioned above (Fig. S4 in SI) during enzyme immobilization. For example, when the initial concentration of SIase was greater than 2.7 mg/mL, the SIase loading remained at 151.2 mg/g. Thus, a novel phenomenon can be seen in Fig. 4a, wherein at SIase concentration of more than 2.4 mg/mL, the increase in SIase loading was not obvious and the relative

activity of the immobilized SIase decreased slightly. Moreover, a steep decline was observed in the activity recovery of the immobilized SIase. The excessive enzyme loading is likely to lead to the agglomeration of enzyme proteins onto the surface of EPL-M-TiO_2. Space steric effect can also restrain transmission and diffusion of the molecules of products and substrate, which can reduce enzyme activity (Liu et al. 2012). In our study, 2.4 mg/mL (2.9, 3.3, 2.8, and 2.1 mg/mL) was chosen as the optimal SIase (laccase, lipase, neutral protease, and urease, respectively) concentration for immobilized operation. Table S2 shows the enzyme loading at the highest activity recovery. The immobilized lipase displayed the largest enzyme loading (209.3 mg/g) and activity recovery (94.8%). Except for immobilized alkaline protease, the enzyme loading and activity recovery of the other immobilized enzymes were greater than 130.0 mg/g and 89.0%, respectively. The laccase immobilized on core-shell magnetic nanoparticles exhibited enzyme loading of 39.25 mg/g and an activity recovery of approximately 43.28%. In addition, the enzyme loading of lipase immobilized on silica-coated Zn dust was 63.5 mg/g, and the maximum adsorption value of urease immobilized on metal-chelated cryogel was 11.30 mg/g. By comparing these carriers, we speculate that EPL-M-TiO_2, which consists a surface exhibiting an excellent biocompatibility, may provide a hydrophilic microenvironment for enzyme loading and for retention of enzyme bioactivity (Deng et al. 2015; Uygun et al. 2013; Xie and Zang 2016). The positive results obtained in the negatively charged enzymes can be attributed to the modification of the M-TiO_2 surface with EPL, which renders abundant amino groups that effectively bind with

enzymes. This study found that the electrostatic adsorption and hydrogen bond may play an important role in the interactions between the carriers and enzymes. Fig. 4f shows that the amount of alkaline protease loading on the surface of EPL-M-TiO_2 and the activity of immobilized enzyme increased gradually with increasing initial enzyme concentration, whereas the activity recovery decreased precipitously. When the alkaline protease concentration was greater than 2.4 mg/mL, enzyme loading was basically stable at 29.2 mg/g, indicating a relatively balanced state between the enzyme and the carrier. Considering the results of enzyme loading, we chose 1.7 mg/mL of enzyme solution for immobilization. However, this concentration demonstrated a considerably low enzyme activity recovery (27.3%). Thus, 0.34 mg/mL alkaline protease solution was selected to immobilize alkaline protease on the modified M-TiO_2, and results reveal an activity recovery of approximately 60.0% and an alkaline protease loading of approximately 17.4 mg/g.

3.5. Characterization of free and immobilized enzymes

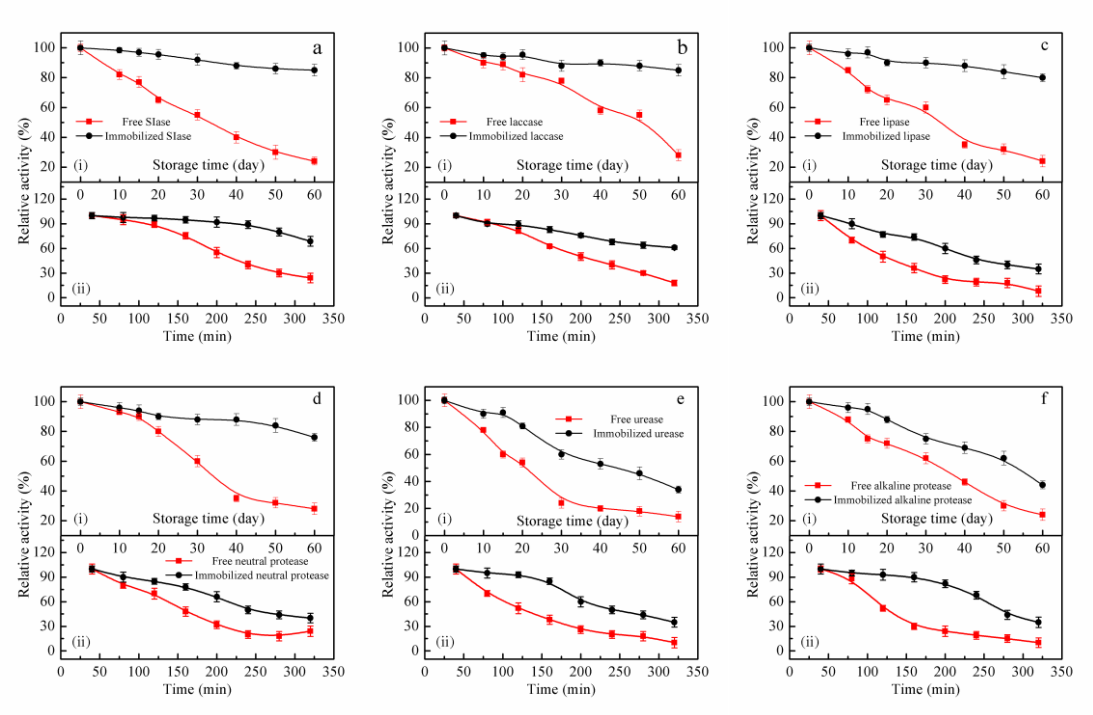


Fig. 5 Storage stability (i) and thermal stability (ii) of the free and immobilized enzymes. SIase (a), laccase (b), lipase (c), neutral protease (d), urease (e) and alkaline protease (f).

Immobilization is always accompanied by changes in activity, optimum temperature, pH, affinity for its substrate, and stability. The extent of these changes depends on the supports, enzymes, and immobilization conditions. Fig. S5 in SI shows that the optimum temperature of immobilized SIase, laccase, neutral protease, and alkaline protease enzymes remained the same, whereas that of urease and lipase increased, which is possibly and solely caused by the change in electrostatic potential in their surface. As Fig. S6 in SI shown that the loss of activity was lower in immobilized enzyme than in free enzyme, and the immobilized enzymes were more active with noticeable broadening of their optimum pH range than the free enzymes. Furthermore, charge interaction is an important factor in determining the stability of enzymes in

mesoporous materials (Han et al. 2002). When the charges of the enzymes are opposite that of the surface of carriers, enzyme adsorption is accelerated and the enzyme environment is rendered relatively stable. However, when enzymes and carriers carry the same charges, both the amount of enzyme loading on carriers and the stability of immobilized enzymes is unsatisfactory because of electrostatic repulsion between enzymes and carrier (Kim et al. 2006). Apart from good enzyme loading and great enzyme properties, improved stability is another an important factor for biosensor construction. Strong stability is one of the significant parameters of an immobilized enzyme. Fig. 5 shows the effect of storage stability of free and immobilized enzymes. The storage stability of immobilized SIase (laccase, lipase, and neutral protease) was much better than that of the free enzyme. After 2 months of storage at 4 °C, the relative activity of immobilized SIase (laccase, lipase, and neutral protease) was retained at approximately 84%, whereas that of the free enzymes was lower than 28%. By contrast, the relative activity of the immobilized urease and alkaline protease is less than 40%. Although the storage stability of immobilized urease and alkaline protease is worse than that of the immobilized SIase (laccase, lipase, and neutral protease), the relative activity of the former is higher than that of free enzymes (lower than 22%). In addition, thermal stability was measured by incubating the free and immobilized enzymes at the same temperature, and the relative activity was tested as described above. After 3 h, the immobilized and free SIase retained approximately 90% and 60% of their original activity, respectively (Fig. 5a), and the immobilized and free alkaline protease retained approximately 88% and

28%, respectively (Fig. 5f). The thermal stability of all the immobilized enzymes is superior to that of the free enzymes, which is commonly considered as the enzyme immobilization caused by increasing enzyme rigidity. Fig. 3a shows that the significant changes in ZP of M-TiO_2 occurred at pH 6.0, where the values of M-TiO_2 before and after modification were −26.6 and 26.1 mV, respectively. The remarkable change in ZP is not only beneficial to enzyme combination but is also conducive to the stability of enzyme immobilization. The much smaller pore structure of EPL-M-TiO_2 (Table S1 in SI), which can protect the active site and structure of the enzyme, provides a suitable environment for enzyme immobilization (Orman et al. 2010). This finding is attributed to the multipoint bond formation, such as hydrogen bond, Van der Waals force, hydrophobic interactions, and salt bond, between the modified carrier and enzymes (Ansari and Husain 2012). The EPL-functionalized M-TiO_2 obviously offers certain advantages, such as providing appropriate pore size and optimizing the surface of the carrier, and thus efficiently immobilizes enzymes with high catalytic activity in a stable manner. Galactosyltransferase and trypsase are immobilized by carriers with arms, whose activity and thermal and storage stability were greatly enhanced (Demers and Wong 1985; Nouaimi et al. 2001). Moreover, papain, trypsase, and alkaline phosphatase immobilized on carriers with hydrophilic arms are more stable (Hasegawa et al. 1990; Jayakumari and Pillai 1991). Thus, the good stability of immobilized enzymes is mainly attributed to the hydrophilic arms around the enzyme, which artificially creates a hydrophilic environment, and to the electrostatic interaction between enzymes and carrier. Therefore, we speculate that the

thermal stability of enzyme increases to a great extent because the hydrophilic arm creates a suitable microenvironment. Furthermore, the pore size of the carriers plays an important role in the stability of the enzyme. In a word, the hydrophilic EPL in the channel of carrier not only changes the pore structure, but also plays the role of a connecting arm. The strong stability of immobilized enzymes is the result of the interactions of the enzyme molecule interactions with the microenvironment. Table S3 in SI shows that K_m values of immobilized SIase, lipase, and neutral protease are smaller than that of the free enzymes, indicating that these immobilized enzymes demonstrate a better affinity to their substrates compared with the free enzymes. The lipase immobilized on HPMC-CHY matrix displays the same phenomenon, whereas K_m of neutral protease loaded on *N*-succinyl chitosan is greater than that of the free enzyme (Badgujar and Bhanage 2015; Li et al. 2006). In addition, K_m of other immobilized enzymes was greater than that of free enzymes. The interaction between the carrier and enzyme can be obtained by comparing K_m and v_{max} of an enzyme both in the immobilized and free states. Compared with that of the free enzymes, v_{max} values of laccase immobilized on core-shell magnetic nanoparticles, lipase immobilized on HPMC-CHY matrix, and alkaline protease immobilized on amino-functionalized magnetic nanoparticles decrease at various degrees (Badgujar and Bhanage 2015; Deng et al. 2015; Hu et al. 2015). The change in K_m and v_{max} of the immobilized enzymes is often associated with the steric hindrance of the active site by carriers or with diffusional limitation to substrate and product transport near

the surface of carrier. Thus, the other conclusion can be drawn is that the change in K_m is related to the property of the enzyme itself.

3.6. Reusability of the immobilized enzymes

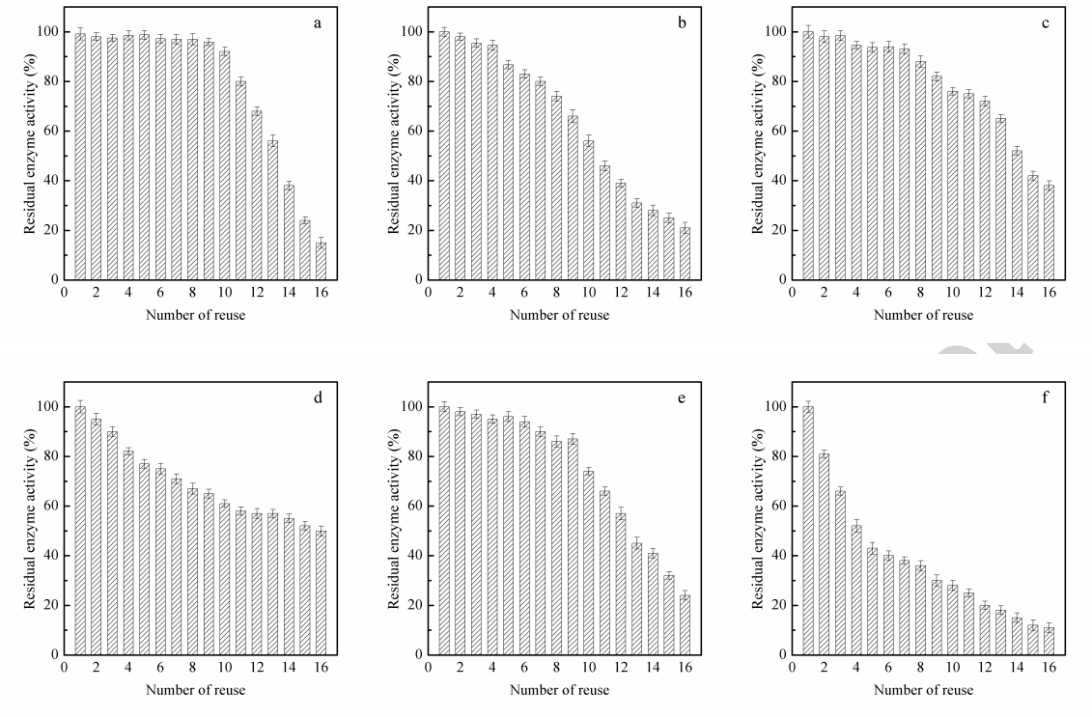


Fig. 6 Reusability of immobilized SIase (a), laccase (b), lipase (c), neutral protease (d), urease (e) and alkaline protease (f).

Besides the strong storage and thermal stability, reusability, which determines the potential of enzymes for actual industrial applications, is another important characteristic of immobilized enzymes (Hu et al. 2015). Fig. 6f shows that with increasing number of batch reaction, the residual activity of the immobilized alkaline protease decreased rapidly, demonstrating that enzymes fall off from the carrier during enzyme catalysis resulting from frequent shock. Another reason is that electrostatic potential surface of alkaline protease tends to zero; thus, the enzyme and the carrier cannot link together firmly through electrostatic adsorption. In addition, the optimum

pH value (we used 8.0) of alkaline protease was higher than that of the other enzymes and thus caused a huge impact on ZP value (Fig. 3a), weakening the interaction force between EPL and M-TiO_2. The other immobilized enzymes exhibited quite a good reusability. For instance, the immobilized SIase retained 90% of the initial activity and the immobilized neutral protease also retained 60% after reuse in 10 batches. However, the activity of the immobilized SIase and urease declined rapidly after 10 batches. The EPL solution autoclaved at 120 °C for 20 min or boiled at 100 °C for 30 min is also not degraded (Shih et al. 2006). In addition, EPL can effectively inhibit the growth of microorganisms on the surface of the carrier owing to its strong antimicrobial activity, which can protect enzymes from being degraded by protease secreted by microorganisms (Bo et al. 2014; Li et al. 2014). We infer that the activity decreases during batch reaction not because of EPL degradation, which weakens the interaction force between EPL and the carrier. The chief reason for this phenomenon is the degradation of the enzyme itself and the enzyme leakage from the carriers during washing and repeated use. The reusability of laccase immobilized on Fe_3O_4@SiO_2 and neutral protease immobilized on chitosaneous hydrogel is similar to that of immobilized neutral protease in this work; the relative activity of immobilized urease (50%) using sodium alginate was lower than that of immobilized urease (68%) in this study after eight cycles and the reusability of alkaline protease immobilized on amino-functionalized magnetic nanoparticles was better than that of immobilized alkaline protease in this study. (Danial et al. 2015; Deng et al. 2015; Hu et al. 2015; Li et al. 2006). The relatively high reusability of the immobilized enzymes in our study

can be attributed to EPL self-assembly, plays an important role in applying enzyme immobilization through a layer of positive EPL film coating on the surface of M-TiO_2. Compared with other immobilization methods, this simple immobilization method offers certain promising advantages. The enzyme loading, the features of immobilized enzymes, and the conductivity of carriers are the key factors in constructing biosensors (Wang et al. 2015). We maintained that the characteristics of the enzymes immobilized on the surface of modified M-TiO_2 are completely consistent with those of the enzyme-based biosensors and that modified M-TiO_2 show promising potentials to be used as the carrier model in constructing electrochemical biosensors.

4. Conclusions

M-TiO_2 was successfully modified by EPL through electrostatic adsorption. Immobilized enzymes exhibit great operational stability, storage stability, thermal stability, and good reusability. This study described a novel self-assembly technique to immobilize enzymes on the surface of EPL-M-TiO_2. An optimum enzyme with negative charges was loaded on the surface of the modified M-TiO_2. Therefore, EPL-M-TiO_2, a modified porous material, is expected to apply in more enzymes. This study also demonstrated that the modified M-TiO_2 can be used as a biocompatible carrier to immobilize enzymes and to construct enzyme-based biosensors.

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Highlights

- Mesoporous titanium dioxide was modified by <epsilon>-poly-L-lysine successfully.
- The modified carrier could immobilize enzymes with negative charge.
- Immobilized enzymes show great stability and superior reusability.