Enzyme encapsulation in microparticles composed of polymerized ionic liquids for highly active and reusable biocatalysts

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Horseradish peroxidase (HRP) is encapsulated in polymerized ionic liquid microparticles (pIL-MP), which are prepared by polymerization of 1-vinyl-3-ethylimidazolium bromide in the presence of the crosslinker *N,N′*-methylenebis(acrylamide) in a concentrated water-in-oil (W/O) emulsion. pIL-MP encapsulating HRP chemically-modified with comb-shaped polyethylene glycol (PM_{13}-HRP) exhibit excellent activity for guaiacol oxidation in an aqueous solution. The PM_{13}-HRP in pIL-MP shows more than 2-fold higher activity than that of the enzyme encapsulated in a polyacrylamide microparticle. The catalytic activity declines with an increase in the crosslinker concentration of the pIL-MP, probably due to suppression of substrate diffusion. The activity of PM_{13}-HRP in pIL-MP depends on the external environment of the gel (*i.e.* pH and temperature). The pIL-MP are easily recovered from the reaction mixture by centrifugation, which makes it possible to recycle the biocatalyst for repeated oxidation reactions.

Introduction

Over the last decade, the utility of ionic liquids (ILs) has been successfully demonstrated in organic synthesis,[1] separation,[2] analytical chemistry[3] and bioprocesses[4–7] as alternative solvents in place of conventional organic solvents. One of the most attractive features of ILs is their controllable solvent properties. The physicochemical properties of ILs can be controlled by changing the combination of cations and anions or by appending functional groups to IL molecules.[8] When ILs are used as reaction media in biocatalytic reactions, they provide a specific and desirable reaction environment displaying contradictory solvent properties, *e.g.* high polarity and hydrophobicity. In these solvents, polar substrates are soluble just as in water, yet synthetic reactions are also possible due to limited water content, analogous to hydrophobic organic solvents. Ionic liquids have been actively utilized as reaction media in biocatalysis using diverse types of enzymes and whole-cell biocatalysts.[4]

Enzyme immobilization is a key technique in the preparation of robust biocatalysts that successfully catalyze numerous reactions in industrial bioprocesses. By immobilizing biocatalysts on solid supports or in synthetic or natural polymer matrices, the biocatalysts acquire long-term stability in aqueous or non-aqueous media, reusability for repeated reaction cycles, and ease of product separation.[9] Since enzymes are often inactivated by chemical modification in covalent immobilization processes, physical immobilization such as adsorption or entrapment in host supports is desirable. A number of studies have been conducted on extensive exploration of reliable methods and suitable support materials for enzyme immobilization.[10]

Recently, researchers have employed ILs as a component in enzyme immobilization. In these systems enzymes or proteins are incorporated in ILs that are supported on host matrices such as cellulose,[11] chitosan,[12] gelatin,[13] or silica-gel.[14] ILs themselves can be functionalized by chemical modification of, mainly, their cationic components to obtain "task-specific ILs".[15] ILs bearing a vinyl group in the cationic component can be directly polymerized by free radical polymerization.[16] The polymerized ILs could be used as support materials for enzyme immobilization, offering an IL-like microenvironment for the immobilized enzymes. López-Ruiz *et al.* have recently reported an amperometric glucose biosensor based on polymerized IL microparticles (pIL-MP) containing glucose oxidase.[17]

Our previous work demonstrated that enzymes are dramatically activated and stabilized in ILs by chemical modification with comb-shaped poly(ethylene glycol) (PM_{13}).[7] The PM_{13} modification should be effective for activation and stabilization of enzymes that are encapsulated in microparticles with a highly ionic nature, such as pIL-MP. In the present study, we have examined the encapsulation of PM_{13}-modified horseradish peroxidase (PM_{13}-HRP) in pIL-MP, and demonstrated the availability and the potential of the enzyme-loaded pIL-MP as a novel functional biocomposite material.

Results and discussion

Preparation and characterization of pIL-MP incorporating HRP

One of the most fascinating features of ILs is the potential for addition of novel functionality to IL solvents by chemical modification. To obtain an IL polymer, we employed a polymerizable IL bearing a vinyl group in the cationic component of the molecule. Vinyl-derivatized IL, 1-vinyl-3-ethylimidazolium bromide ([Veim][Br]), was synthesized by reaction of 1-vinylimidazole

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and bromoethane.[16c] In the preparation of pIL-MP, we used polymerization in a concentrated water-in-oil (W/O) emulsion, which was previously developed by López-Cabarcos *et al.* for the preparation of polyacrylamide microparticles.[18] A schematic diagram of the preparation of pIL-MP is shown in Fig. 1. The monomer [Veim][Br], crosslinker *N,N′*-methylenebis(acrylamide), initiator ammonium persulfate (APS) and enzyme were dissolved in the dispersed aqueous phase of the emulsion. Polymerization of [Veim][Br] was initiated by addition of *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED) to the emulsion. After polymerization, elastic gel particles were formed and recovered by centrifugation. The gel particles swollen with water were converted to a white powder after lyophilization.

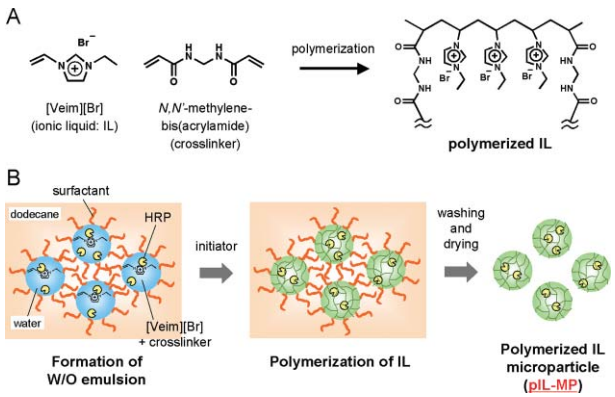

Fig. 1 Schematic diagram of (A) polymerization of ionic liquid [Veim][Br] in the presence of crosslinker *N,N′*-methylenebis(acrylamide); (B) preparation of pIL-MP containing HRP using a W/O concentrated emulsion.

The morphology of freeze-dried pIL-MP encapsulating PM_{13}-HRP, and that material swollen with water, were observed by scanning electron microscopy (SEM) and optical microscopy, respectively (Fig. 2). The SEM image revealed that freeze-dried pIL-MP were in the form of ellipsoidal and collapsed particles with a rough surface (Fig. 2B). After washing the pIL-MP with water, the abundant water associated with the pIL-MP was removed during freeze-drying, leading to shrinking of the gel to form creased particles. It was assumed that the ellipsoidal shape of the pIL-MP could be ascribed to high shear stress during polymerization of [Veim][Br] in highly viscous emulsion. From the optical microscopy images shown in Fig. 2D, it is apparent that after rehydration the pIL-MP were spherical with a slightly fuzzy rim, probably indicating extensive hydration of the gel particles in the aqueous dispersion. The pIL-MP have a number of ionized groups (imidazolium cations) in the side chains of the polymer, and were expected to be fully hydrated as a highly swollen gel in the form of spherical particles.

Enzymatic activity of pIL-MP containing HRP

The activity of HRP encapsulated in microparticles was examined *via* oxidation of guaiacol by H_2O_2 in an aqueous solution in which the microparticles were dispersed. The effects of PM_{13} modification of the encapsulated HRP and the host support materials on enzymatic activity were investigated (Fig. 3). Our previous study demonstrated that enzymes are significantly activated in ILs by chemical modification with PM_{13}.[7] We thus examined

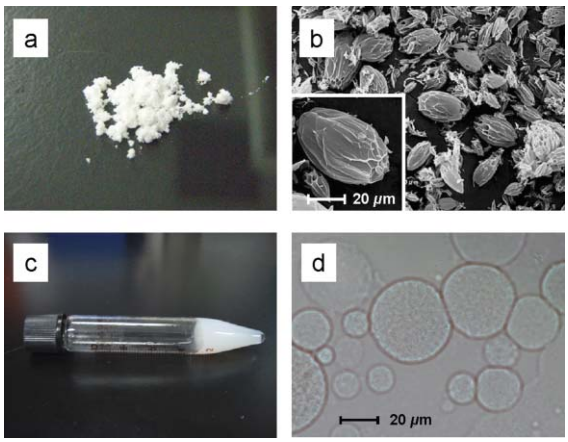

Fig. 2 Morphology of pIL-MP encapsulating PM_{13}-HRP under dried and wet conditions. (a) pIL-MP after freeze-drying; (b) SEM image of (a); (c) pIL-MP saturated with water; (d) optical microscope image of (c) dispersed in water.

the activity of pIL-MP encapsulating either native HRP or PM_{13}-HRP. The activities of the two pIL-MPs are presented in Fig. 3A, together with that of pIL-MP in the absence of HRP (empty pIL-MP). While pIL-MP alone does not oxidise guaiacol, the pIL-MP incorporating native HRP showed oxidative activity, and 3-fold higher activity was observed for pIL-MP encapsulating PM_{13}-HRP. Enzymes are known to be severely deactivated in halide-containing ILs such as [Bmim][Cl].[19] The concentration of [Veim][Br] (1.23 M) during the preparation of pIL-MP in the present study seems to be relatively high and could inactivate the enzyme. Radical species generated during the polymerization also seem to be injurious. Modification of HRP with PM_{13} bearing a high density of PEG would be effective for the protection of the enzyme from such harsh conditions and maintaining the enzyme activity. PM_{13}-modification was shown to be also valid for the preparation of a bioactive pIL-MP.

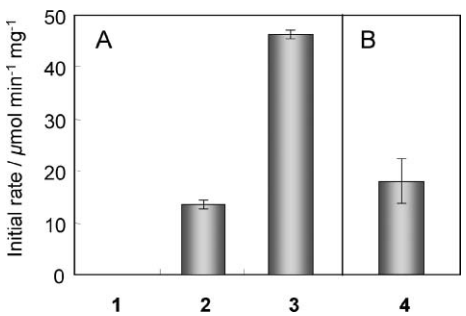

Fig. 3 Oxidation activity of native HRP and PM_{13}-HRP encapsulated in (A) pIL-MP and (B) polyacrylamide microparticles. 1, empty pIL-MP; 2, native HRP in pIL-MP; 3, PM_{13}-HRP in pIL-MP; 4, PM_{13}-HRP in polyacrylamide microparticles. Reaction conditions: 5 mM guaiacol, 1.5 mM H_2O_2, 40 μg ml^{-1} pIL-MP.

In the immobilization of enzymes, host matrices providing three-dimensional polymer networks to entrap enzymes normally have a significant impact on the catalytic activity of the immobilized enzyme. To investigate the effect of the polymer matrix, we prepared polyacrylamide gel particles containing PM_{13}-HRP in the same manner as for pIL-MP. Fig. 3B shows the activity of

PM_{13}-HRP encapsulated in polyacrylamide microparticles. More than 2-fold higher activity was obtained in pIL-MP compared with that in polyacrylamide microparticles. There seem to be several possible factors contributing to the substantial increase in catalytic efficacy in pIL-MP. One factor might be the difference in substrate diffusion. While polyacrylamide gel is a "hard gel" packed with neutral polymer, pIL-MP is a "soft gel" with a very high water content due to the numerous ionized groups of the polymer matrix. The difference in the internal conditions of each gel would have a profound influence on the mobility of a substrate in the microparticles. The substrate could diffuse to the active sites more rapidly in pIL-MP than in polyacrylamide gel, resulting in an increased reaction rate. Another possible reason for the high activity in pIL-MP is the distribution of guaiacol substrate in the gel. Ionic liquid polymer possesses a number of imidazolium cation units in the side chains of the polymer, which can probably accumulate the guaiacol *via* hydrophobic or π-π interactions. The substrate could then permeate into pIL-MP more smoothly than compared with polyacrylamide microparticles, resulting in an increase in the local concentration of substrate. However, further comprehensive elucidation of the mechanism for the enhanced activity in pIL-MP is needed, including an examination of the possibility of structural and functional alteration of the HRP by multivalent interactions with the polycationic matrix in pIL-MP.

Effect of pIL-MP concentration and degree of crosslinking on reaction rate

The initial rate of the oxidation reaction increased in proportion to the concentration of pIL-MP in the reaction mixture (Fig. 4). This suggests that guaiacol oxidation occurred by the action of PM_{13}-HRP encapsulated in pIL-MP. We prepared pIL-MP with different concentrations of the crosslinker *N,N′*-methylenebis-(acrylamide) from 1 to 8 wt%. At 1 wt% crosslinker, microparticles were not formed. The characterization of the pIL-MP obtained is described in Table 1. The pIL-MP prepared with low concentrations of crosslinker absorbed a large amount of water, which gradually decreased with increasing crosslinker concentration. The enzymatic activity of the pIL-MP decreased with increasing crosslinker concentration (Fig. 5). This trend can be ascribed to the suppression of substrate diffusion. In more highly crosslinked microparticles diffusion of the substrate is restricted by the smaller pores or cavities in the gel, leading to a decrease in enzyme activity. Although the highest activity was observed at 2 wt% crosslinker, we selected pIL-MP with 3.5 wt% crosslinker as the optimum biocatalyst, taking into consideration yield, protein loading and physical strength of the microparticles.

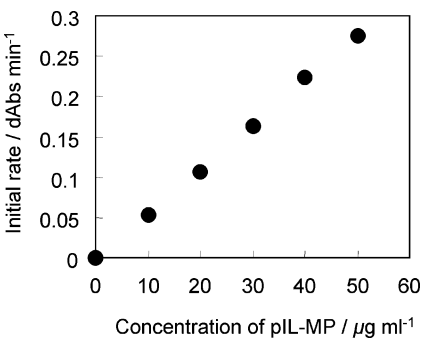

Fig. 4 Reaction rate of guaiacol oxidation by pIL-MP encapsulating PM_{13}-HRP with different particle concentrations. The concentration range of the pIL-MP was 0 to 50 μg ml^{-1}.

Table 1 Characterization of pIL-MP prepared with different concentrations of crosslinker *N,N′*-methylenebis(acrylamide)

Crosslinker (wt%)	1.0	2.0	3.5	5.0	6.5	8.0
Yield of pIL-MP (mg)	–	64	77	75	102	68
Protein content in pIL-MP (wt%)[a]	–	0.33	0.43	0.41	0.42	0.37
Water absorption (mg per mg of pIL-MP)[b]	–	56.0	25.3	11.2	8.3	7.2

[a] Protein content of pIL-MP based on BCA assay.[20] [b] Maximum absorption of water per mg of pIL-MP.

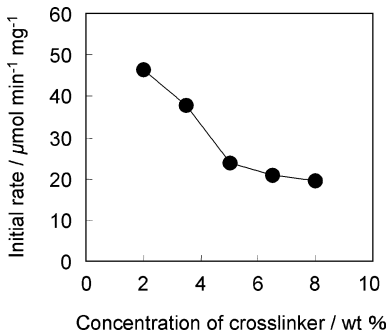

Fig. 5 Effect of crosslinking on enzyme activity of PM_{13}-HRP in pIL-MP.

Influence of reaction conditions on biocatalytic performance of PM_{13}-HRP in pIL-MP

To elucidate the catalytic performance of PM_{13}-HRP immobilized in pIL-MP, we investigated the effect of some reaction parameters on the activity in an aqueous solution, in comparison with the catalytic behavior of free PM_{13}-HRP (not immobilized) (Fig. 6). The influence of pH on activity is depicted in Fig. 6A. PM_{13}-HRP encapsulated in pIL-MP showed pH-dependence with a bell-shaped curve with a maximum around pH 7, which is similar to that of free PM_{13}-HRP. While the pIL-MP were prepared using phosphate buffer at pH 7, the activity of the PM_{13}-HRP encapsulated in the particles depends on the pH of external aqueous solutions during the reaction. This suggests that external water molecules permeate through the microparticle gels and determine the microenvironment pH in the gel.

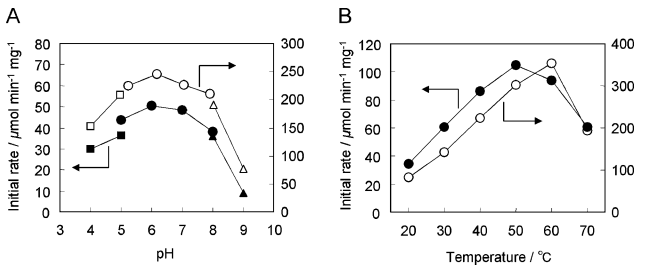

Fig. 6 Influence of some reaction parameters on the activities of free PM_{13}-HRP (open symbols) and PM_{13}-HRP in pIL-MP (closed symbols): (A) dependence on pH of external aqueous solutions (squares: acetate buffer, circles: phosphate buffer, triangles: tris-HCl buffer); (B) dependence on reaction temperature.

It is well known that the thermal stability of enzymes is improved by immobilization in host materials.[9,10] Hydrogels are also useful for improving thermostability due to multipoint interactions between protein molecules and polymer matrices (*e.g.* polyelectrolytes).[10] The effect of reaction temperature on the activity of PM_{13}-HRP is shown in Fig. 6B. The highest activity was obtained at 60 °C for the free PM_{13}-HRP, while for PM_{13}-HRP immobilized in pIL-MP the highest activity was at 50 °C. The difference in optimum temperature for these two biocatalysts indicates that a slight decrease in the thermal stability of PM_{13}-HRP results from immobilization in pIL-MP. This could presumably be caused by interaction of the enzyme with the polycationic matrix in the gel despite substantial modification of the enzyme with PEG.

Recycling of pIL-MP

Immobilization of biocatalysts on solid supports or other host materials often causes a reduction of biocatalytic performance. Indeed, in the present system the activity of PM_{13}-HRP was decreased to around one-third compared with the free form by encapsulation in pIL-MP, as can be seen in Fig. 6. The decrease in activity can be attributed to reduced mass transfer of a substrate in a microparticle gel composed of a three-dimensional polymer network. On the other hand, immobilized biocatalysts are attractive in that they can be recovered from reaction mixtures and reused. For this reason we examined the recyclability of PM_{13}-HRP-loaded pIL-MP in consecutive oxidation reactions. A glass centrifugation tube was used for the successful recovery of pIL-MP from reaction mixtures. Fig. 7 indicates that PM_{13}-HRP-loaded pIL-MP can be reused after recovering the catalyst by simple centrifugation. A slight decrease in activity was found in the initial recycling steps, possibly due to slight leakage of enzyme that was loosely immobilized on the surface of the pIL-MP. The overall recyclability of the catalyst is greatly advantageous for industrial bioprocesses.

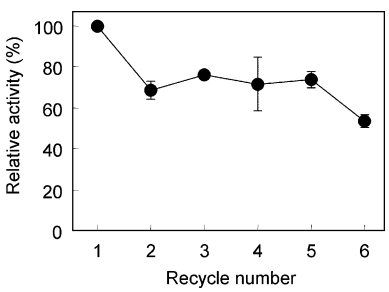

Fig. 7 Recycling of pIL-MP encapsulating PM_{13}-HRP in repeated oxidation reactions.

Conclusions

Horseradish peroxidase was encapsulated in microparticles composed of polymerized ionic liquid. PM_{13}-HRP encapsulated in pIL-MP successfully catalyzes the oxidation of guaiacol and shows activity depending on the external aqueous pH and the reaction temperatures. PM_{13}-HRP in pIL-MP exhibits higher activity than in conventional polyacrylamide microparticles, and is easily recycled by centrifugation from reaction mixtures. The pIL-MP investigated is expected to be an excellent host material for immobilizing both native and chemically-modified enzymes, and will open a new route to fabrication of functional biocatalysts and biochemical sensors.

Experimental

Materials

1-Vinylimidazole was purchased from Tokyo Chemical Industry, and bromoethane and HRP (E.C. 1.11.1.7, 100 units mg^{-1}) were from Wako Pure Chemical Industries. *N,N′*-Methylenebis(acrylamide), ammonium persulfate (APS), *N,N,N,N*-tetramethylethylenediamine (TEMED) and Span 80 (Kishida Chemical) were purchased from Kishida Chemical. Thirty percent acrylamide/bis solution (containing 29 wt% of acrylamide and 1 wt% of *N,N′*-methylenebis(acrylamide)) was obtained from Biorad and used after diluting appropriately with deionised water. Comb-shaped poly(ethylene glycol) (PM_{13}; SUNBRIGHT AM-1510K, Mw: 15,000–20,000) was purchased from NOF Co. and used without further purification. All other chemicals were of reagent grade.

Synthesis of polymerizable ionic liquid [Veim][Br]

1-Vinyl-3-ethylimidazolium bromide ([Veim][Br]) was synthesized following the procedure described in the literature[16c] with minor modification. Bromoethane (33 g, 0.3 mol) was added dropwise to 1-vinylimidazole (28 g, 0.3 mol) with vigorous stirring in an ice-bath, followed by heating in a water bath at 40 °C for 2 h. The resulting white solid, [Veim][Br], was washed with ethyl acetate several times to remove unreacted materials then dried using a rotary evaporator, followed by freeze-drying for 24 h. The product was stored in a desiccator over silica gel to avoid water absorption due to its hygroscopicity. Yield: 50 g, 82%. ^{1}H NMR (300 MHz; DMSO-d_6; TMS): $\delta = 9.53$ (s, 1H, N*CH*N), 8.21 (s, 1H, N*CH*C), 7.69 (s, 1H, C*CH*N), 7.30 (dd, 1H, CH_2*CH*N), 5.96 (dd, 1H, *HC*HCHN), 5.42 (dd, 1H, H*CH*CHN), 4.23 (q, 2H, NCH_2C), 1.45 (t, 3H, CH_2CH_3, 7.4 Hz). Found: C, 41.24; H, 5.51; N, 13.68. $C_7H_{11}N_2Br$ requires C, 41.38; H, 5.42; N, 13.79%.

Modification of HRP with PM_{13}

Chemical modification of HRP with comb-shaped PEG (PM_{13}), which has multivalent reactive sites (*i.e.* carboxylic acid anhydride) to react with the amino groups of the protein molecule, was performed according to our previous report.[7] The resulting PM_{13}-modified HRP (PM_{13}-HRP) was a light brown powder whose protein content was estimated by bicinchoninic acid (BCA) assay.[20]

Encapsulation of HRP in microparticles composed of polymerized [Veim][Br]

Polymerized ionic liquid microparticles (pIL-MP) incorporating HRP were prepared according to the literature,[18] using a W/O concentrated emulsion. The dispersed aqueous phase contained monomer [Veim][Br] (0.375 g), crosslinker *N,N′*-methylenebis(acrylamide) with concentration from 1 to 8 wt% for [Veim][Br], initiator APS (7.5 mg), and HRP or PM_{13}-HRP (0.75 mg protein based on BCA assay) in 0.1 M sodium phosphate

buffer (1.5 ml) at pH 7. The continuous organic phase (0.3 ml) comprised *n*-dodecane (225 μl) and Span 80 (75 μl). The W/O concentrated emulsion was prepared by dropwise addition of the aqueous phase to the organic phase with vigorous magnetic stirring. Polymerization of [Veim][Br] was started by adding TEMED (19 μl) to the emulsion after purging with nitrogen to remove residual oxygen. Reaction for 1 h at room temperature gave a gel-like emulsion from which polymer particles were precipitated by adding 10 ml of acetone to the reaction mixture, followed by centrifugation (2,500 rpm, 10 min) in a glass centrifuge tube. The precipitated microparticles were washed three times with deionized water and the resulting microparticle gel was freeze-dried for 24 h to obtain white powder. Microparticles incorporating PM_{13}-HRP were prepared in the manner described above using PM_{13}-HRP with the same protein concentration as in native HRP. To estimate the loading of the protein in the microparticles, pIL-MP containing HRP or PM_{13}-HRP were suspended in deionized water ([pIL-MP] = 20 mg ml^{-1}) then subjected to BCA assay.[20]

Encapsulation of HRP in polyacrylamide microparticles

Polyacrylamide microparticles incorporating PM_{13}-HRP were prepared by a method analogous to the preparation of pIL-MP. A 30% acrylamide/bis solution was diluted with deionized water to an appropriate concentration and used as the acrylamide monomer solution. PM_{13}-HRP (containing 0.75 mg of protein) and APS (3.5 mg) were dissolved in 1.5 ml of the diluted acrylamide solution. The resulting solution was added to 0.3 ml of organic solvent (225 μl of dodecane and 75 μl of Span 80) to form a W/O concentrated emulsion. Polymerization was started by adding TEMED (19 μl) to the emulsion, and conducted with stirring in a water-bath at 25 °C for 1 h. The polymer product was isolated by precipitation with freezing methanol then centrifugation (2,500 rpm, 10 min) in a glass centrifuge tube. Subsequent procedures were the same as for the preparation of pIL-MP.

Characterization of microparticles

The morphology of the pIL-MP in wet and dry conditions was investigated by microscopy. The gel microparticles swollen with water were observed using an optical microscope (Olympus IX70), and freeze-dried microparticles were examined by scanning electron microscopy (SEM; Shimadzu SS-550).

The water absorption capacities of pIL-MP prepared with a range of crosslinker concentrations (2–8 wt%) were measured as follows. Each pIL-MP (10 mg) was dispersed in deionized water (1 ml) and allowed to stand for 2 h to saturate the particles with water. The supernatant was separated by centrifugation, and the weight of pIL-MP measured to estimate absorption of water.

Activity of HRP encapsulated in microparticles

The peroxidase activities of HRP encapsulated in the microparticles were measured using a guaiacol/H_2O_2 assay.[21] A typical procedure is described below. To a 3 ml optical cuvette, 0.12 ml of microparticle dispersion (1 mg ml^{-1}) in 0.1 M phosphate buffer, 0.3 ml of 50 mM guaiacol, and 2.28 ml of 0.1 M phosphate buffer were added. Reaction was initiated by adding 0.3 ml of 15 mM H_2O_2 to the cuvette with stirring at 25 °C, and the oxidation of guaiacol was monitored by a UV–vis spectrophotometer (JASCO V-570) at 470 nm (ε_{470} = 26000 M^{-1} cm^{-1}). The activity was corrected by dividing the initial reaction rate by protein content for each pIL-MP.

Recycling test

Recycling of the pIL-MP encapsulating PM_{13}-HRP was examined as follows. Oxidation was performed in a glass centrifuge tube using 0.4 mg ml^{-1} of pIL-MP, 2.78 mM guaiacol, and 0.83 mM H_2O_2. After reaction for 2 min, the reaction mixture was centrifuged (2,500 rpm, 2 min) to isolate the PM_{13}-HRP-loaded pIL-MP. The supernatant was diluted with 0.5 ml of acetone, and the concentration of the products in the supernatant was measured by a UV–vis spectrophotometer at 470 nm. Fresh substrate solution was then added to the recovered pIL-MP in the glass tube, and subsequent oxidation reactions were conducted to evaluate the relative activity of pIL-MP with recycle number.

Acknowledgements

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