Highly active horseradish peroxidase immobilized in 1-butyl-3-methylimidazolium tetrafluoroborate room-temperature ionic liquid based sol–gel host materials†

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Dramatically enhanced activity and excellent thermal stability of horseradish peroxidase were obtained by immobilizing it in a 1-butyl-3-methylimidazolium tetrafluoroborate room-temperature ionic liquid based sol–gel matrix.

In recent years, the immobilization of enzymes or proteins in solid-state host materials has been extensively studied.[1] Compared with soluble enzymes, immobilized enzymes have the same functionality but usually have higher thermal, storage, and operational stability, easy separation from products and reusability. Sol–gel derived silica glasses are most popularly used for the encapsulation of biomolecules due to their porosity, transparency, chemical stability and convenient preparation, and much work has been carried out on the development of biocatalysis[2] and sensors.[3] Despite their good properties, because of the slow diffusion rate of the substrate and the destruction of alcohol formed in the sol–gel process, lower activity of the immobilized enzymes was usually obtained and was a serious obstacle for their application.[1a,4,5]

Room-temperature ionic liquids (ILs), which are composed entirely of ions and are liquids at ambient or even far below ambient temperature, have attracted much attention as novel environmentally benign solvents due to their negligible vapor pressure, wide potential window, good solubility and conductivity, *etc.*, and have been widely used in organic synthesis, liquid–liquid extraction, electrochemistry and inorganic synthesis.[6] As alternative solvents, ILs are of great interest and have potential applications in biocatalysis and much work has been carried out.[7,8] Moreover, improved stability of the enzymes has been obtained in the IL solvents as compared to that in conventional organic solvents.[6e]

In this work, horseradish peroxidase (HRP) was selected as a model and was for the first time encapsulated into an IL based silica gel matrix by using 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIM^+BF_4^-$) IL as a template solvent for the matrix *via* a simple sol–gel method. Compared with that in the conventional silica matrix without IL, dramatically enhanced activity and excellent thermal stability of HRP immobilized within the IL based matrix were obtained.

The silica sol–gel materials were synthesized through a sol–gel reaction of tetraethyl orthosilicate (TEOS) in the presence of $BMIM^+BF_4^-$. First, 2 ml TEOS as precursors and 1 ml $BMIM^+BF_4^-$ were mixed with 1.0 ml water under mild magnetic stirring and 0.05 ml of 0.1 M HCl was added. A homogeneous solution was obtained after 3 h. The transparent HRP–IL@GEL (gel containing HRP prepared with IL) without any cracks was produced by mixing 2 mg mL^{-1} HRP (in phosphate buffer, pH 6.86) with the sols in equivalent volume and stored for two weeks at ambient temperature. A HRP@GEL (gel containing HRP prepared in the absence of IL) was also prepared in a similar procedure for comparison. (The details of the preparation, characterization of the samples and their activity assays are available as ESI†).

The specific activity of the HRP–IL@GEL was assayed to be of 465 U per gram HRP in our experiments. For comparison, the specific activity of the HRP@GEL was also measured, and was only 15.5 U per gram, which was similar to those reported previously.[1d] It is worth noting that the specific activity of HRP–IL@GEL was about 30-fold higher than that of HRP@GEL though optimization experiments were not presented. The specific activity of immobilized enzymes was usually affected markedly by both the limited mass transport and the destruction of the organic solvent such as ethanol.[5a,5b] Fig. 1 shows the TEM image of the pore morphology and structure of the matrix of HRP–IL@GEL after the removal of the IL by calcination. Apart from the bulk pore structure, numerous worm-like interconnected channels and pores around 3 nm were obvious. The specific surface area and

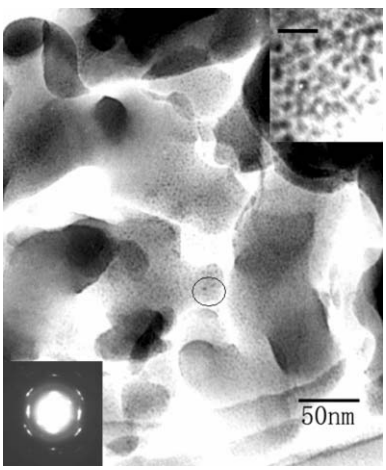

Fig. 1 TEM images of HRP–IL@GEL samples after calcination at 500 °C. The pores of the matrix are visible as the lighter worm-like channels. The scale bar is 50 nm. Insert images are the corresponding electron diffraction pattern (bottom left) and the magnification of the image in the circle (top right, the scale bar is 10 nm).

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b4/b417680d/

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pore volume of the HRP–IL@GEL calcined at 500 °C were 350 $m^2\ g^{-1}$ and 0.43 $cm^3\ g^{-1}$ by BET measurement, respectively, and the BJH average diameter was calculated to be about 4.9 nm (Fig. S3 in ESI†). It was thought that the 3D pore structure would facilitate the internal diffusion of the substrate so that a higher specific activity of HRP–IL@GEL was obtained. On the other hand, as compared with the detrimental effect of alcohol on the activity of the enzyme, IL showed good compatibility with biomolecules and it was thought that enzymes and even whole cells are active in various ILs.[6e] Laszlo *et al.* have reported the catalysis of hemin activated by an electron acceptor in IL solutions and it was found that the activity of hemin increased with the enhanced amount of IL in a methanol–IL system.[7d] It indicated that the negative effect of some organic solvents such as ethanol could be lessened, or even eliminated, by the existence of IL. The ubiquitous interconnected channels in the silica bulk shown in the inset of Fig. 1 also indicated that the enzyme was enwrapped by IL during the sol–gel immobilization process, which could protect the enzyme from being destroyed by ethanol for its significant properties of polarity, non-coordinating, weak nucleophilicity and so on,[7d] resulting in the high activity of the immobilized enzyme.

Fig. 2 shows the typical kinetic data obtained for HRP–IL@GEL in colorimetric analysis. A good linear relationship ($1/V_0$ *vs.* $1/S_0$, inset of Fig. 2) indicated that the enzymatic reactions followed Michaelis–Menten kinetics. The Michaelis constant (K_M) of HRP–IL@GEL was calculated to be 4.87 mM, which was slightly smaller than that of the HRP@GEL and larger than that of free HRP in solution because of the weaker binding of the substrate to the enzyme and the slower mass transfer rate. The catalytic constant (k_{cat}) of HRP–IL@GEL was 1.725 s^{-1}, which was larger than that of HRP@GEL due to the high activity of HRP–IL@GEL. The catalytic efficiency (k_{cat}/K_M) of the HRP immobilized in the IL based matrix was $3.5 \times 10^2\ M^{-1}s^{-1}$.

The thermal stability of HRP–IL@GEL was also investigated as shown in Fig. 3. In this study, almost no loss of the activity of

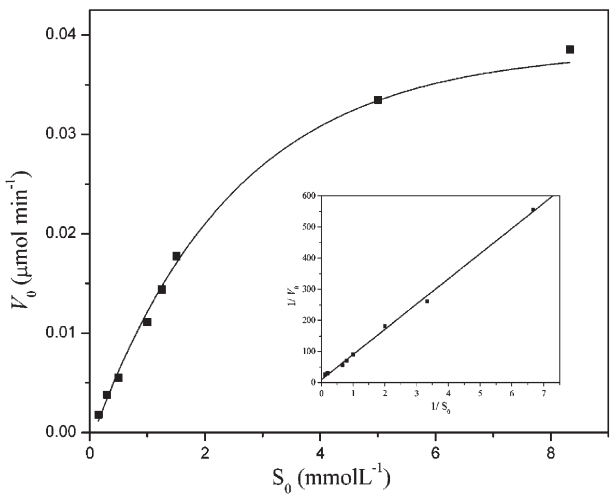

Fig. 2 Typical kinetic data of HRP–IL@GEL, the initial rate (V_0) of HRP–IL@GEL catalyzed oxidation of guaiacol as a function of substrate concentration (S_0). The measurements were made in phosphate buffer solution (pH 6.86) with 1.5 mM of H_2O_2, 5.7 mg of HRP–IL@GEL and guaiacol with various concentrations at ambient temperature.

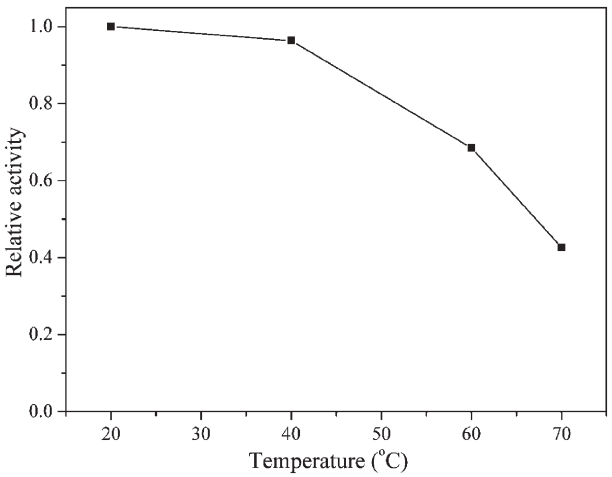

Fig. 3 The thermal stability of HRP–IL@GEL. Each sample was treated at the stated temperature for 30 min. The activity assays were carried out in phosphate buffer solution (pH 6.86) with 1.5 mM of H_2O_2, 1 mM of guaiacol and 5.7 mg of HRP–IL@GEL at ambient temperature.

HRP–IL@GEL was observed at temperatures below 40 °C. The HRP–IL@GEL retained about 68% of the apparent activity after thermal treatment at 60 °C, which was significantly higher than that of free HRP.[5b] The significantly improved thermal stability of encapsulated HRP can be attributed to the reduced deactivating or denaturing thermal motion of the protein molecules in the confined space of the host matrix during the thermal treatment process.[5b,9] For the free enzymes, irreversible intermolecular aggregates could be formed for the thermally unfolded proteins. Matrix cages and unfolding were limited. Recently, some authors have also reported the increased stability of enzymes in IL compared with that in some organic solvents.[7c,7e,7f,10] It was thought that the hydrogen bond and the electrostatic interaction between IL and enzyme resulted in a high kinetic barrier for the unfolding of the enzyme, thus the rigid structure of the enzyme was protected from being destroyed.[1c,5c,7e] Further work should be carried out to investigate thoroughly the mechanism for the high activity and good thermal stability of the enzyme immobilized in the IL based matrix.

In summary, dramatically enhanced activity of HRP was obtained by immobilizing HRP in a transparent and crackless $BMIM^+BF_4^-$ IL based silica sol–gel matrix and its specific activity was about 30-fold greater than that in silica gel without IL prepared by conventional sol–gel methods. Furthermore, excellent thermal stability of HRP immobilized in IL based matrix was also obtained. It was thought that $BMIM^+BF_4^-$ would act both as a template for the formation of the mesoporous matrix that improved the mass transfer and as a stabilizer to protect the activity of the immobilized HRP from being destroyed by the alcohol formed in the sol–gel process. The IL based bio-gel is promising for application in the fields of biocatalysis, bioanalysis, biosensor and bioelectronics *etc.*, as well as providing an alternative way for us to utilize ILs in biocatalysis.

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Notes and references

1 (*a*) I. Gill and A. Ballesteros, *J. Am. Chem. Soc.*, 1998, **120**, 8587; (*b*) B. C. Dave, B. Dunn, J. S. Valentine and J. I. Zink, *Anal. Chem.*, 1994, **66**, 1120A; (*c*) C. Lei, Y. Shin, J. Liu and E. J. Ackerman, *J. Am. Chem. Soc.*, 2002, **127**, 11242; (*d*) K. Smith, N. J. Silvernail, K. R. Rodgers, T. E. Elgren, M. Castro and R. M. Parker, *J. Am. Chem. Soc.*, 2002, **124**, 4247.
2 J. F. Diaz and K. J. Balkus, *J. Mol. Catal. B*, 1996, **2**, 115.
3 U. Kunzelmann and H. Bottcher, *Sensors Actuators B*, 1997, **38**, 222.
4 (*a*) R. B. Bhathia, C. F. Brinker, A. K. Gupta and A. K. Singh, *Chem. Mater.*, 2000, **12**, 2434; (*b*) M. L. Ferrer, F. D. Monte and D. Levy, *Chem. Mater.*, 2002, **14**, 3619.
5 (*a*) C. T. Kresge, M. E. Leonowicz, W. J. Roth, J. C. Vartuli and J. S. Beck, *Nature*, 1992, **359**, 710; (*b*) Y. Wei, H. Dong, J. Xu and Q. Feng, *ChemPhysChem*, 2002, **9**, 802; (*c*) Y. J. Han, G. D. Stucky and A. Bulter, *J. Am. Chem. Soc.*, 1999, **121**, 9897.
6 (*a*) T. Welton, *Chem. Rev.*, 1999, **99**, 2071; (*b*) J. Dupont, R. F. Souza and P. A. Z. Suarez, *Chem. Rev.*, 2002, **102**, 3667; (*c*) Y. Zhou, J. H. Schattka and M. Antonietti, *Nano Lett.*, 2004, **4**, 477; (*d*) D. R. MacFarlane, J. Huang and M. Forsyth, *Nature*, 1999, **402**, 792; (*e*) P. Wasserscheid and T. Welton, *Ionic Liquids in Synthesis*, Wiley-VCH, Weinheim, 2003; (*f*) Y. Liu, M. Wang, Z. Li, H. Liu, P. He and J. H. Li, *Langmuir*, DOI: 10.1021/la0475829, in press; (*g*) J. H. Li, Y. Shen, Y. Zhang and Y. Liu, *Chem. Commun.*, 2005, 360; (*h*) Z. Li, H. Liu, Y. Liu, P. He and J. H. Li, *J. Phys. Chem. B*, 2004, **108**, 17512; (*i*) P. He, H. Liu, Z. Li, Y. liu, X. Xu and J. H. Li, *Langmuir*, 2004, **20**, 10260.
7 (*a*) U. Kragl, M. Eckstern and N. Kaftzik, *Curr. Opin. Biotechnol.*, 2002, **13**, 565; (*b*) F. V. R. Rantwijk, R. M. Lau and R. A. Sheldon, *Trends Biotechnol.*, 2003, **21**, 131; (*c*) P. L. Lozano, T. D. Diego, D. Carrie, M. Vaultier and J. L. Iborra, *Biotechnol. Bioeng.*, 2001, **75**, 563; (*d*) J. A. Laszlo and D. L. Compton, *J. Mol. Catal. B: Enzym.*, 2002, **18**, 109; (*e*) M. Persson and U. T. Bornscheuer, *J. Mol. Catal. B: Enzym.*, 2003, **22**, 21; (*f*) P. Lozano, T. D. Diego, D. Carrie, M. Vaultier and J. Iborra, *Biotechnol. Lett.*, 2001, **23**, 1529.
8 (*a*) D. L. Compton and J. A. Laszlo, *J. Electroanal. Chem.*, 2003, **553**, 187; (*b*) G. Hinckley, V. V. Mozhaev, C. Budde and Y. L. Khmelnitsky, *Biotechnol. Lett.*, 2002, **24**, 2083; (*c*) K. Okrasa, E. G. Jampel and M. Therisod, *Tetrahedron: Asymmetry*, 2003, **14**, 2487.
9 (*a*) D. Avnir, A. Braun, O. Lev and M. Ottolengh, *Chem. Mater.*, 1994, **6**, 1605; (*b*) C. Shen and N. M. Kostic, *J. Am. Chem. Soc.*, 1997, **119**, 1304.
10 S. N. Baker, T. M. McCleskey, S. Pandey and G. A. Baker, *Chem. Commun.*, 2004, 940.

