COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Colloidal stable silica encapsulated nano-magnetic composite as a novel bio-catalyst carrier†

Xin Gao,[a] K. M. Kerry Yu,[a] Kin Y. Tam[b] and Shik Chi Tsang*[a]

[a] *Surface and Catalysis Research Centre, School of Chemistry, University of Reading, Reading, UK RG6 6AD*

[b] *AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire, UK SK10 4TG*

Received (in Cambridge, UK) 29th August 2003, Accepted 21st October 2003
First published as an Advance Article on the web 7th November 2003

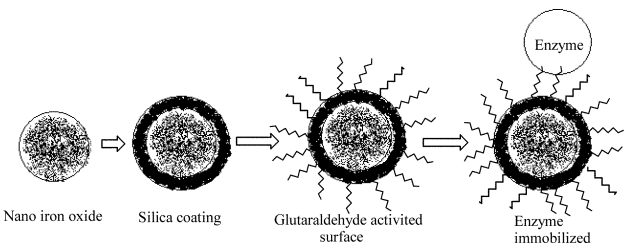
A colloidal stable silica-encapsulated magnetic nano-composite of a controlled dimension is, *for the first time*, employed to carry β-lactamase *via* chemical linkage on the silica overlayer: activity study reflects that this new type of immobilisation allows site (enzyme) isolation, accessibility as good as free enzyme and recovery & reusability upon application of magnetic separation.

To facilitate separation from product there are existing research strategies on enzyme immobilisation onto bulk solid supports, primarily *via* adsorption, ion exchange, encapsulation and covalently linking.[1] However, each reported method suffers one problem from other with regards to the ease of separation, enzyme accessibility and stability of the system. For example, enzyme encapsulation inside sol–gel silica matrix, mesoporous silica and zeolitic related porous structures can offer separation advantage but most studies showed lower specific activity than that of the free enzymes owing to the diffusion limitation.[2] It is well known that rate of transport from external medium to catalyst site (enzyme) in liquid phase is proportional to $1/L^2$ where L is the diffusion path accessing the site. As a result, enzyme activity can benefit much from the use of small solid carriers. Thus, reported work using microbeads offer a good enzyme accessibility but these composite particles of greater than 1 μm may experience significant attrition.[3] Reports on using *strong non-porous supports* of sizes (as new commercial products) of 1–10 μm with a satisfactory degree of attrition resistance are well documented. However, exploitation of enzyme carriers of even smaller size is worthwhile since a support reaching nanometric regime will give theoretically no attrition problem.[4,5] It is noted that such small composites, if used, are almost impossible to separate in a bio-reactor by conventional means (leads to plugging of filters and valves by the composite fines). Efficient separation of the suspended small solid enzyme carriers from product using an external magnetic field is therefore of immense interest. It is noted that syntheses of both coated and uncoated nanosize magnetic particles have been reported[6,7] but the uses of these small size particles as enzyme carriers are scarce. In contrast, there are a lot of reports on using micron size magnetic bodies to carry enzymes.[8,9]

Here we report a simple synthesis of silica-encapsulated nanosize iron oxide (a superparamagnet) carrying β-lactamase by water-in-oil microemulsion technique,[10] which renders the solid attached enzyme stable in a colloid form. With optimisation of the silica coated magnetic carrier and immobilisation skills it is demonstrated that, the solid anchored β-lactamase shows a comparable activity as free enzyme with no apparent enzyme accessibility problem that is commonly encountered in immobilisation. The immobilised enzyme can be recovered and re-used upon application of magnetic separation.

Typically, the synthesis was as follows:‡ cetyltrimethyl ammonium bromide (CTAB) as surfactant was added and stirred with toluene. A solution containing Fe(II) and Fe(III) species was added dropwise into the toluene. After 4 h stirring NH_3 solution was then added. As a result, a molar ratio of water to surfactant of W = 20 was altogether added to define the size of reversed micelle.[10] The solution gradually turned black but with no observation of precipitate. According to Massart's method[11] co-precipitation of Fe(II) and Fe(III) with NH_3 in water gives magnetite precipitates. Since the synthesis was carried out inside the emulsion droplets hence it is envisaged that tiny magnetic iron oxide particles must be formed as a stable colloid. At this point the tetraethylorthosilicate, TEOS was added. Formation of the silica-gel coating onto the micelle-hosted iron oxide was expected because the high pH catalysed hydrolysis/condensation of the TEOS at the water/oil interface. After aging, the colloid was repeatedly washed and redispersed in hot ethanol and toluene by magnetic means. Thermogravimetry (TG) confirmed the near quantitative removal of the surfactant and revealed a high surface coverage of –OH groups on the material ($\sim 6 \times 10^{20}$/g). §Immobilisation of β-lactamase I on the colloid solution was then carried out *via* surface functionalisation and enzyme immobilisation. First, colloid composites in toluene were allowed to mix with excess 3-aminopropyltriethoxysilane[12] and reflux overnight. The washed amine-functionalised composites (IR confirmed) were mixed with chemical linker, glutaraldehyde (in excess) in a phosphate buffer for 12 h. The extensively washed glutaraldehyde-activated composites were mixed with a purchased β-lactamase I solution. After incubation, the nano-composite carrying enzyme was magnetically separated, washed and kept in phosphate buffer at 4 °C prior to use. No noticeable change in particle size from XRD and TEM is observed. The preparation procedures of nano-composite immobilised β-lactamase I is summarised in Scheme 1.

Characteristic peak positions and relative intensities in typical XRD (in ESI†) of the collected colloid particles before the enzyme immobilisation correspond well to the expected Fe_3O_4 phase (Magnetite). From the Williamson–Hall plot, the average diameter of the iron nanoparticle calculated by XRD peak broadening is 9.94 nm. TEM images (Fig. 1) visualise a thin but Si and O rich (from EDS) coating clearly surrounding each spherical shaped core nanoparticles. This undoubtedly suggests the nano-iron oxide particles are encapsulated in thin silica matrix which appears to be amorphous and porous. The average size of these iron nanoparticle cores from the TEM images is about 9.1 nm with 3.5 nm silica coating. The fringe separation obtained from TEM lattice imaging, which is 25.35



Scheme 1 A preparation of nano-magnetic composite carrying enzyme on its external silica surface.

† Electronic Supplementary Information (ESI) available: XRD in Fig. S1; TEM in Fig. S2; SEM in Fig. S3; EDS in Tables; Magnetisation in Fig. S4; Material reusability upon mangetic separation in Fig. S5; Regression in Fig. S6. See http://www.rsc.org/suppdata/cc/b3/b310435d/

DOI: 10.1039/b310435d

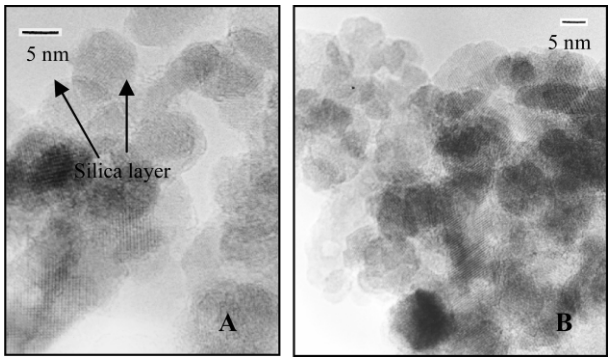


Fig. 1 TEM photographs of silica encapsulated nano-composites.

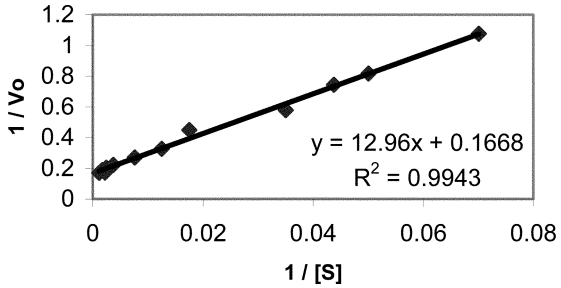


Fig. 2 A Michaelis constant study of nano-magnet immobilized β-lactamase I using the Lineweaver–Burke plot (a direct fit to Michaelis–Menten equation to obtain the constant is at the ESI for comparison).†

Å per 10 fringe separations, corresponds well to the most intense peak observed in the XRD analysis. To analyse the elemental composition, energy dispersive spectrometry (EDS) was applied and the results are presented in ESI. Average atomic ratios obtained from six selected area are: Fe : O : Si = 24.08 : 61.95 : 13.97 suggesting the individual particle is comprised of: $Fe_3O_{4.24}$·1.74 SiO_2.. Separate experiments clearly showed that the size of the oxide core and the thickness of the external coating using different amounts of starting materials can be tailored (in ESI). The magnetic properties of these silica-encapsulated nano-composites were determined by vibrating sample magnetometry (VSM). A typical saturated magnetisation of the silica-encapsulated nano-composites (in ESI†) is 76.16 $Am^2\ kg^{-1}$-Fe_3O_4 at 1 000 KA/m corresponding well with the literature value of bulk Fe_3O_4 (92 $Am^2\ kg^{-1}$) but the low remanence value of 4.5 $Am^2\ kg^{-1}$ and the low coercivity of 15.3 KA m^{-1} indicate that the particles are in superparamagnetic state as expected of nano-size Fe_3O_4.[13]

An assay evaluation of active β-lactamase I on nano-magnetic composite was studied at 25 °C. Phenoxymethylpenicillin (Penicillin V) in phosphate buffered water was used as the substrate molecule. The active enzyme content was estimated through monitoring a decrease of the substrate concentration in a given period of time (30 s) using UV-visible spectrometer at 232 nm (ε = 1,643 $M^{-1}cm^{-1}$) in an excess substrate concentration. An average turnover number, TON of 453.9 s^{-1} was recorded with our purchased enzyme in solution, which displays a comparable activity as those quoted in the literature.[14] Notice that the β-lactamase I supported on the nanomagnetic composite shows a ¶TON of 244.0 s^{-1}, which corresponds to 53.76% of active enzyme content. This value is substantially higher than those reported in the literature using carbon nano-materials as solid carriers (16.4%).[14] It is envisaged that the loss of active enzyme content is partly due to limited external surface sites available (because of the assumption made in¶ and the dynamic size of the enzyme is larger than the average silica pore size of 3.79nm) and partly to deactivation as the enzyme moieties are attached by subsurface sites. A kinetic study of the immobilized β-lactamase I was carried out over a range of initial substrate concentrations at 25 °C. The initial velocities (Vo) of penicillin V were determined *via* adding aliquots of a fixed amount of supported enzymes over various initial substrate concentrations. Fig. 2 is the Lineweaver–Burke linear plot, which is derived by inverting the Michaelis–Menten equation ($K_M = -1/x$ intercept; $V_{max} = 1/y$ intercept). Thus, the plot of 1/*Vo vs.* 1/[*S*] in Fig. 2 indeed gives a straight line with a fitting coefficient of over 0.99. The determined K_M value of 77.7 μM (V_{max} = 5.99 μMs^{-1}) matches well with the quoted value of 64 μM of free enzyme in literature.[15] The comparable Michaelis value to the free enzyme implies that this type of solid immobilised enzyme does not seem to suffer from diffusion. The rate of catalysis apparently depends on the steady state of enzyme-substrate complex formation and destruction rather than any physical mass transfer limitations (inter- and intra- particle diffusion, mixing problems, *etc*. would give a large deviated Michaelis value). Thus, the potential of using the functionalised nanomagnetic composite to carry a high enzyme content but with no significant encounter of diffusion limitation is presently revealed. The reusability of the nano-magnetic composite hosted enzyme was studied using magnetic separation. Greater than 95% activity was found on the first re-use (see details in ESI†).

In summary, immobilisation of β-lactamase onto functionalised silica encapsulated nanomagnet gives 54% retention in activity as compared to its native form but this could offer a separation advantage to the enzyme using magnetic means.

Notes and references

‡ 0.02 mol CTAB; 100 g dried toluene; a solution made up of 0.3428 g $FeCl_2$·$4H_2O$ and 0.9321 g $FeCl_3$·$6H_2O$ in 6.2 g H_2O; NH_3 (18.1 M, 1 ml), 6.9351 g TEOS; 5 days aging.

§ A colloid solution of 1.084 g nano-composites in 50 ml toluene; 5 g 3-aminopropyltriethoxysilane; 10 ml of 25% (w/v) glutaraldehyde; potassium phosphate buffer (pH = 7, containing 0.5 M NaCl); purchased β-lactamase I (5 ml containing 1.2×10^{-4}g enzyme of MW 29kD with 33 × 38 × 49 Å size[14]). All procedures were carried out in nitrogen.

¶ TON is calculated assuming that all the enzyme molecules are immobilised on the support (with excess amino groups) without the determination of enzyme quantity on the support.

1 G. F. Bickerstaff (Ed.) *Immobilisation of Enzymes and Cells*, Humana Press: Totowa, NJ, 1997.
2 (*a*) S. Braun, S. Rappoport and R. Zusman, *Mater. Lett.*, 1990, **10**, 1; (*b*) Y. Wei, J. Xu, Q. Feng, H. Dong and M. Lin, *Mater. Lett.*, 2000, **44**, 6.
3 Y. H. Lee, Y. K. Bae and E. J. Jeong, *J. Microbiol. Biotechnol.*, 1996, **6**(6), 432.
4 M. Qhobosheane, S. Santra, P. Zhang and W. Tan, *Analyst*, 2001, **126**, 1274.
5 Y. Lvov and F. Caruso, *Anal. Chem.*, 2001, **73**, 4212.
6 T. Tago, T. Hatsuta, K. Miyajima, M. Kishida, S. Tashiro and K. Wakabayashi, *J. Am. Ceram. Soc.*, 2002, **85**, 2188.
7 S. Santra, R. Tapec, N. Theodoropoulou, J. Dobson, A. Hebard and W. H. Tan, *Langmuir*, 2001, **17**, 2900.
8 P. J. Halling and P. Dunnill, *Enzyme Microb. Technol.*, 1980, **2**, 2.
9 M. Y. Arica, H. Yavuz, S. Patir and A. Denizli, *J. Mol. Catal. B*, 2000, **11**, 127.
10 K. M. K. Yu, D. Thompsett and S. C. Tsang, *Chem. Commun.*, 2003, 1522.
11 R. Massart, *IEEE Trans, Magn.*, 1981, **MAG-17**, 1247.
12 S. H. Lau, V. Caps, K. W. Yeung, K. Y. Wong and S. C. Tsang, *Microporous Mesoporous Mater.*, 1999, **32**, 279.
13 A. N. Thorpe, et al., *J. Mater. Res.*, 2000, **15**, 2488.
14 S. C. Tsang, J. J. Davis, M. L. H. Green, H. A. O. Hill, Y. C. Leung and P. J. Sadler, *J. Chem. Soc., Chem. Commun.*, 1995, **17**, 1804.
15 M. Scripcariu, S. Fleschin and V. Magearu, *Roum. Biotechnol. Lett.*, 2002, **7**, 664–665.