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Enzyme immobilisation using SBA-15 mesoporous molecular sieves with functionalised surfaces

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Abstract

Functionalised hexagonal mesoporous SBA-15-type molecular sieves with pore sizes in the range 51–56 Å have been prepared using non-ionic block copolymers and used for immobilisation of the enzyme trypsin. Thiol, chloride, amine, and carboxylic acid functional groups were attached by siloxypropane tethers to the siliceous surface of SBA-15 via two methods, post-synthesis grafting and in situ synthesis. Phenylsiloxane groups were also incorporated using these two methods. The resulting solids were rendered porous and used to immobilise trypsin, giving variable but in general higher retention of the enzyme molecules than was observed on unfunctionalised, purely siliceous SBA-15. The resulting supported enzyme catalysts were shown to be active and stable catalysts for the hydrolysis of *N*-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA). The solids prepared by supporting the enzyme on thiol-functionalised SBA-15 prepared by in situ synthesis were found to be the most promising. Trypsin supported on thiol-functionalised SBA-15 was shown to be recyclable.

Keywords: Mesoporous molecular sieve; SBA-15; Functionalisation; Trypsin; Enzyme immobilisation

1. Introduction

Immobilisation of enzymes on solid supports permits highly selective catalysis to be performed using materials that are chemically and mechanically robust and readily separated from reaction mixtures [1,2]. Enzyme stability may be enhanced due to reduced autolysis in the case of protease enzymes, and more generally reduced protein aggregation, as a result of separation of enzyme molecules adsorbed on the surface. In principle, the regular mesoporous silicas formed by liquid crystal templating routes that have been the subject of intense recent study offer attractive supports for a range of catalytic complexes [3,4]. Once the template molecules are removed they possess pores which may have a range of topologies and connectivities, the dimensions of which can be from 20 to 300 Å and beyond [5–7]. The pores are well-defined, with narrow distributions of pore diameter. The internal surfaces of these pores, which can achieve some $1000\,m^2\,g^{-1}$, are lined with silanol hydroxyls. These possess affinities suitable for the physical adsorption of molecules, for example through hydrogen bonding, and may also be used as reactive points for the attachment of tethering functional groups.

In our own work, we have previously examined the efficacy of a range of pure silica mesoporous molecular sieves in immobilising the enzyme trypsin by physical adsorption [8]. Trypsin was chosen as a suitable enzyme for studies of this kind because it is a globular

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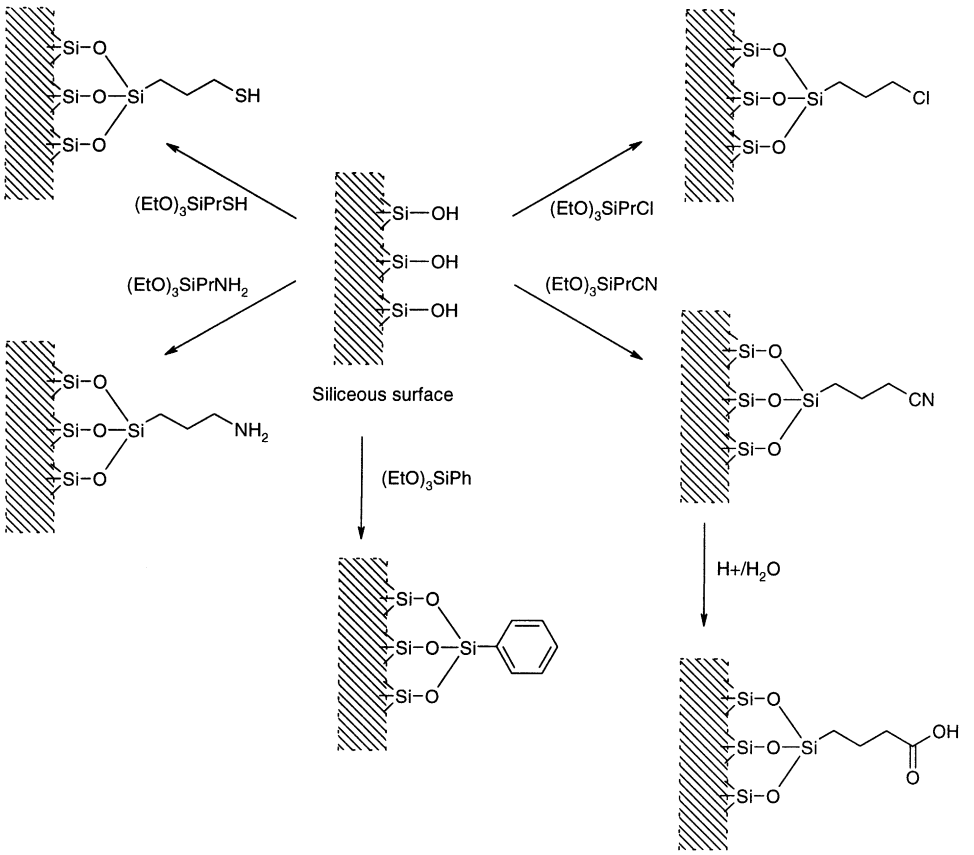
enzyme of a suitable size (38 Å) and molecular mass (23,000) to be incorporated into the pores of mesoporous solids [9]. The enzyme's activity for amide hydrolysis is readily assessed colorimetrically, since the hydrolysis product of BAPNA, *p*-nitroaniline, is strongly absorbing in the visible region (405 nm) [10,11]. The active site of trypsin consists of aspartic acid, histidine and serine residues, and the surface of the enzyme possesses –S–S– groups, amines and thiol groups, which present suitable sites for attachment via both physical and chemical bonds [12]. Cubic MCM-48 (25 Å), hexagonal MCM-41 (32 Å) and SBA-15 (59 Å) were studied. MCM-48 was found to be unsuitable due to its small pore size, but enzymes supported on siliceous MCM-41 and SBA-15 were found to be active. However, there was very significant (35–52%) leaching observed from the support under the reaction conditions and it was, therefore, concluded that the interaction between the enzyme and the inorganic surface was not strong enough [8].

One approach to reducing the degree of leaching of enzymes from mesoporous molecular sieves is to partially reduce the pore openings at the external surface by silanation, thereby 'trapping' the enzyme molecules within the pores but still allowing reactant and product molecules the ability to diffuse in and out of the pores [13,14]. We have chosen here an alternative approach, that is using organic functionalisation of the internal surface to strengthen interaction of the enzymes with the pore walls. In addition, we have concentrated on the largest pore mesoporous solid, SBA-15 [5], for further study, because it should permit easier access of reactant molecules to the active sites of the enzyme and transport of products out of the pores.



Scheme 1. Preparation of post-synthesis functionalised SBA-15 materials.

The functional groups required for such modification are likely to depend strongly on the chemical composition and structure of the enzyme being studied [15]. Recent studies have shown that numerous functional groups, including amines, chlorides, thiols, carboxylic acids and phenyl may be attached successfully to the surface of mesoporous molecular sieves via tethering alkyl chains [16–19]. These groups subsequently provide different interactions between the surfaces of the support and the enzyme molecules. By examining the functional groups on the surface of an enzyme molecule, a suitable "counter-functional group" on the surface of the support could provide strong interaction for immobilisation.

Two routes for functionalising the surface of mesoporous molecular sieves have been reported. In situ functionalisation involves the addition of the target species, usually as the triethoxysilane $(EtO)_3Si–X$, where X is the functional group, during the preparation of the gel [20–22]. The product from this route may not be calcined thermally as the functional group is expected to decompose at high temperature. Therefore, the extraction of the block copolymer template using an organic solvent, ethanol for example, is necessary. The porosity of the molecular sieves will be determined by the degree of template removal that is achieved. Post-synthesis functionalisation involves the grafting of the functional group onto a calcined sample [23–29]. Once again, triethoxysilane with a suitable functional group is usually the reagent for this type of experiment (see Scheme 1). In this case, the support possesses a more ordered structure with no templates inside the pores. However, the procedure requires an extra step compared with in situ preparation and also requires three silanol hydroxyls in a suitable configuration for optimal binding. Furthermore, the diameter of the pores may be reduced due to the coating of an extra layer of Si–X species. Such pore size reduction could be significant in those mesoporous molecular sieves with pore diameter <40 Å, such as MCM-41.

In our previous studies, a serious leaching problem was found following enzyme immobilisation using pure siliceous mesoporous molecular sieves by the centrifugation method. A certain amount of unattached enzyme molecules was also separated out from the supernatant. This free enzyme could be active during activity assessment of the immobilised enzyme and would, therefore, affect the accuracy of the assessment. To avoid this, *ultrafiltration* has been used in this study to separate the free enzyme from the solid. In this way, the measured activity will be due to the immobilised enzyme only.

In this report, the suitability of surface functionalised SBA-15 for enzyme immobilisation was examined, with focus on the effect of the chemistry of the support surface on the enzyme immobilisation.

2. Experimental

2.1. Preparation of pure silica SBA-15 mesoporous molecular sieves

Pure siliceous SBA-15 was prepared using a reported procedure [5]. Pluronic P123 triblock copolymer ($EO_{20}–PO_{70}–EO_{20}$, BASF) was used as surfactant template. The molar composition of the gel was 1 SiO_2:0.017 P123:2.9 HCl:202.6 H_2O. The surfactant Pluronic P123 was dissolved in the mixture of water and HCl at 40°C. Tetraethylorthosilicate (98%, Aldrich) was added to the surfactant solution and the mixture stirred for 24 h. The mixture was then transferred to a Teflon bottle and heated at 100°C for 2 days. The white solid was filtered, washed with distilled water, air-dried and calcined at 550°C for 4 h under flowing nitrogen. The calcination procedure was repeated under flowing oxygen.

2.2. In situ functionalisation of mesoporous molecular sieves

Using the same procedure as for the preparation of SBA-15, 5 mol% of $(EtO)_3Si–X$ (phenyltriethoxysilane $(EtO)_3Si–Ph$, (98%, Aldrich), 3-aminopropyltriethoxysilane $(EtO)_3Si–PrNH_2$, (98%, Aldrich), (3-chloropropyl)triethoxysilane $(EtO)_3Si–PrCl$, (98%, Aldrich), mercaptotriethoxysilane, $(EtO)_3Si–PrSH$, (95%, Avocado) and 4-(triethoxysilyl)butyronitrile $(EtO)_3Si–PrCN$, (98%, Aldrich)) were used. This corresponds to 0.8 mmol g^{-1} of the tethering groups on the silica support. For a typical reaction, a similar procedure to the synthesis of siliceous SBA-15 was performed and triethoxysilane was introduced together with TEOS. The samples were named as *is*X-SBA-15, where X is the functional group. After the completion of hydrothermal treatment at 100°C for 2 days, the white solid was filtered, washed and

air-dried. The surfactant template was removed by refluxing with ethanol (2 g of sample in $3 \times 100\,cm^3$ EtOH) for 8 h. In the case of propylnitrile functionalised solid, the nitrile group is expected to react further to give the carboxylic acid group.

2.3. Post-synthesis functionalisation of SBA-15

Triethoxysilanes with suitable functional groups were used. Generally, 1.0 g of calcined SBA-15 was suspended in 30 ml of 1,4-dioxane (99%, Avocado). Then 4 mmol triethoxysilane was added per gram of silica support and the reaction mixture was heated to reflux for 24 h (see Scheme 1). The white solid was filtered off, washed with diethyl ether ($3 \times 20\,cm^3$, BDH) and dried under vacuum. The samples were then named *ps*R-SBA-15 where R is the functional group on the surface. For *ps*PrCOOH-SBA-15, the sample of *ps*PrCN-SBA-15 was refluxed in 1 M HCl solution for 4 h.

2.4. Characterisation of mesoporous materials

Calcined SBA-15 was characterised using powder X-ray diffraction (Philips X'Pert System diffractometer using Cu Kα radiation, $\alpha = 1.5418$ Å) and the nitrogen adsorption isotherm at $-196^\circ C$ (Micromeritics ASAP2010 instrument, in collaboration with University of Edinburgh). Surface modified samples were studied using ^{13}C solid state MAS NMR (Bruker MSL500 instrument 125 MHz for ^{13}C) and CHNS elemental analysis (Carlo Elba 1108 instrument). A JEOL 200 kV microscope was used to record the TEM micrographs of *is*PrSH-SBA-15 and *ps*PrSH-SBA-15 materials. TGA (TA instrument) was performed to measure the organic content of the solid samples.

2.5. Immobilisation of enzymes and ultrafiltration

Trypsin from bovine pancreas (Sigma, EC 3.4.21.4) was used. Solid support (0.25 g) was suspended in $5.0\,cm^3$ of 5.2 μM trypsin solution ($120\,mg\,dm^{-3}$, 2.4 mg per gram of support) in 50 mM Tris–HCl buffer (pH = 6.0, Sigma) for 2 h at 4°C under stirring. The immobilisation of trypsin had previously been found to be optimum at pH = 6.0 [9]. The supernatant was first separated from the solid materials by centrifugation. The protein content of the supernatant was measured using the Bradford assay [30]. The amount of trypsin immobilised was, therefore, calculated by subtracting this figure from the blank. The solid was eventually washed with Tris–HCl buffer (pH = 6.0) using the technique of ultrafiltration. Ultrafiltration has been commonly used in protein separations. Here, the ultrafiltration was used to remove unsupported enzyme from solid-supported enzyme. Thus, the catalytic activity of the supported enzyme should not be affected by any unsupported enzyme, which may possess a high activity. An Amicon ultrafiltration kit with a YM100 type membrane disc (cut-off limit at MW = 100,000) was used. The solid was washed with $3 \times 10\,cm^3$ Tris–HCl buffer solution (pH = 6.0).

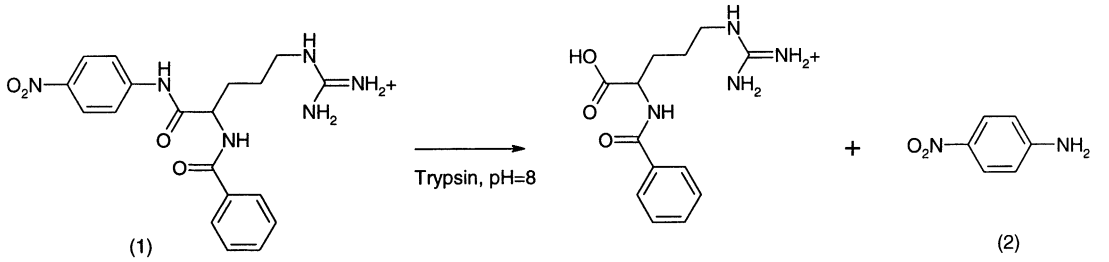
2.6. Assessment of leaching

The ease with which the trypsin leaches out of these composite solids was assessed using the procedure given below. The trypsin-solid samples prepared above were re-suspended by stirring in a pH = 8.0 Tris–HCl buffer solution ($5\,cm^3$) for 2 h. The pH was chosen to be the same as that used in catalytic activity testing. The suspended solids were permitted to settle for 2 h. The solution left above the solid was again tested using the Bradford assay to indicate the amount of trypsin that had been leached from the solids.

2.7. Catalytic measurements

Hydrolysis of *N*-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA, Sigma) was used to assess the activity of the immobilised trypsin [10,11]. A 1 mM BAPNA solution was prepared by dissolving 21.7 mg BAPNA in $1\,cm^3$ DMSO (99%, Fluka), then diluting with Tris–HCl at pH = 8 buffer. For a 0.25 g immobilised enzyme sample, $20\,cm^3$ of 1 mM BAPNA solution (pH = 8) was added and stirred at 25°C. The absorbance of the supernatant at 405 nm, which was due to the formation of *p*-nitroaniline (see Scheme 2), was taken every 15 min. The amount of nitroaniline in the supernatant was calibrated using aqueous *p*-nitroaniline solution. Measurements were also made using free trypsin, using 600 μg of the enzyme (the same as used originally in each supported enzyme preparation).

In each case, the catalytic performance was calculated as μmoles nitroaniline produced per micro gram



Scheme 2. BAPNA (1) hydrolysis by tripsin at pH = 8.0. The yellow colour of the supernatant is due to the formation of *p*-nitroaniline (2).

of protein. Initial rates were calculated over the first 15 min of reaction.

3. Results and discussion

3.1. Characterisation of pure siliceous SBA-15

The XRD pattern of calcined SBA-15 shows two peaks at $2\theta = 1.6$ and 1.82°, which correspond to the (1 1 0) and (2 0 0) reflections. A further shoulder at $2\theta = 2.34^\circ$ (2 1 0) was also observed. The (1 0 0) diffraction peak was not recorded in the 2θ range $>1.5^\circ$ accessible to the diffractometer. The (2 0 0) d-spacing of the calcined SBA-15 was calculated to be 48.0 Å. A similar XRD diffraction pattern was observed for uncalcined and extracted SBA-15 samples.

From the nitrogen adsorption experiment, the BET surface area of the calcined SBA-15 was found to be 918 $m^2\,g^{-1}$ (BJH pore diameter: 56.3 Å; mesopore volume: 1.15 $cm^3\,g^{-1}$). A comparison with functionalised SBA-15 samples will be given later.

Besides calcination, solvent extraction is another successful method for removal of template molecules from mesostructured solids. This is particularly useful for thermally sensitive samples, such as the in situ functionalised SBA-15 prepared in this study. Since the organic functional groups on the surface are thermally unstable, removal of surfactant template by calcination is not viable [31]. Extraction with solvent is one of the commonest methods to remove the template in such a situation, although achieving 100% extraction is difficult. The amount of template in as-prepared and extracted SBA-15 was evaluated using TGA under flowing oxygen. More than 70% of the template was removed from as-synthesised SBA-15 after three extractions and no significant improvement was observed on the fourth extraction. Based on this result, all the in situ functionalised samples were extracted with ethanol three times for optimal template removal.

3.2. Characterisation of functionalised SBA-15

The XRD and TEM studies confirm the solids to be SBA-15 materials with hexagonal arrays of pores. The post-synthesis functionalised sample (*ps*PrSH-SBA-15) possesses a well-defined hexagonal structure (Fig. 1a). The in situ functionalised sample (*is*PrSH-SBA-15) possesses a structure with less well-ordered hexagonal array of pores (Fig. 1b), but the structure is still clearly visible over a shorter length scale.

The BET surface area and pore diameter of thiol-functionalised SBA-15 samples were measured and found to be similar (for *ps*PrSH-SBA-15, BET surface area: 685 $m^2\,g^{-1}$; BJH pore diameter: 56.3 Å; mesopore volume: 0.97 $cm^3\,g^{-1}$ and for *is*PrSH-SBA-15, BET surface area: 643 $m^2\,g^{-1}$; BJH pore diameter: 51.2 Å; mesopore volume: 0.93 $cm^3\,g^{-1}$). Nitrogen adsorption–desorption isotherms of unfunctionalised (SBA-15) and in situ functionalised (*is*PrSH-SBA-15) materials are depicted in Fig. 2. When compared with calcined SBA-15, the BET surface area of *is*PrSH-SBA-15 was decreased by ca. 25% while the mesopore volume decreased by 20% (similar results were obtained from *is*PrSH-SBA-15). The lower surface and pore volume of *is*PrSH-SBA-15 could be caused by incomplete template removal.The ^{13}C CP MAS NMR of in situ functionalised SBA-15 (Table 1) showed resonances from the added organosiloxane groups, plus unremoved block copolymer template and residual solvent. A band of peaks at 67–77 ppm is assigned

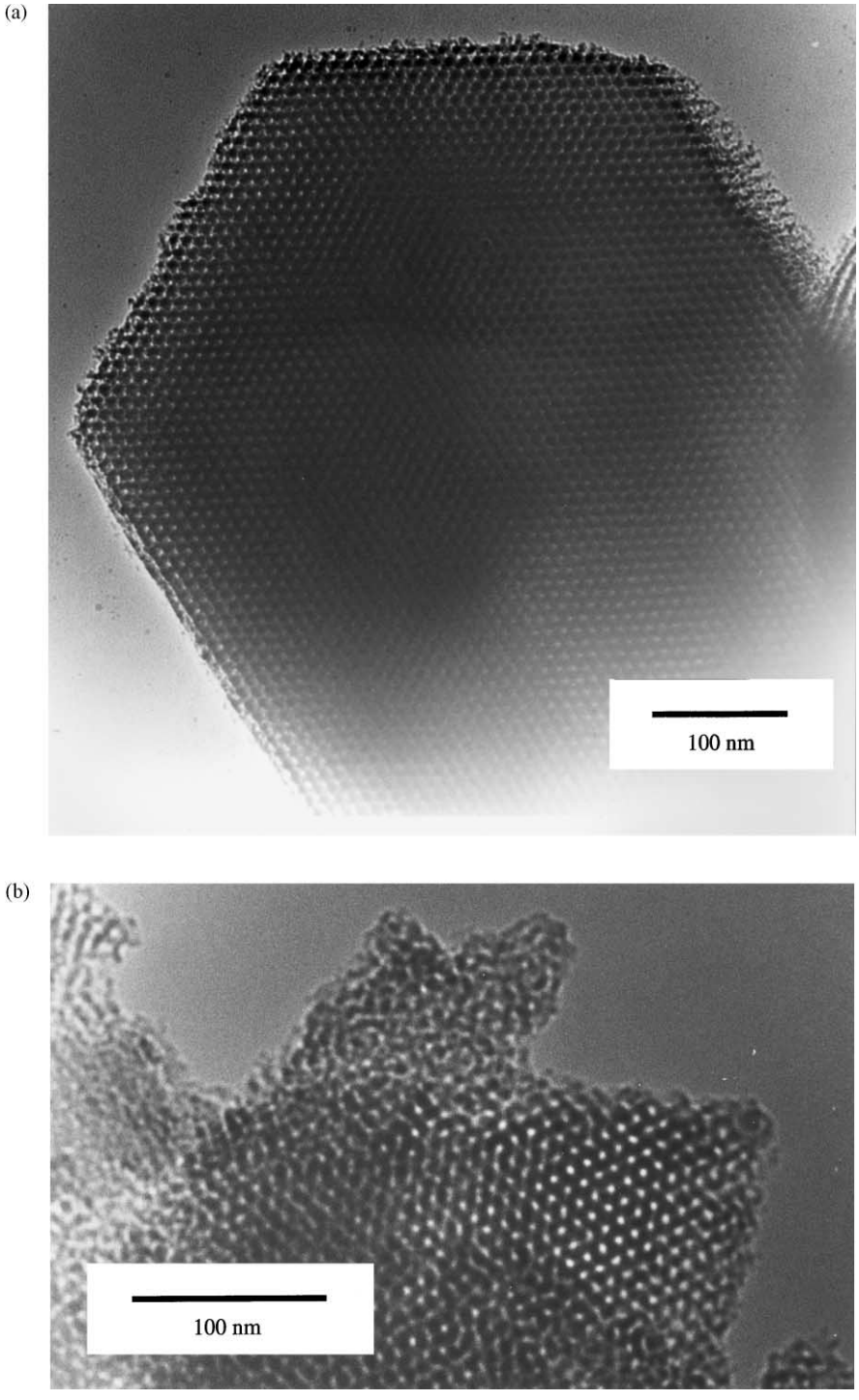


Fig. 1. TEM images for (a) *ps*PrSH-SBA-15 and (b) *is*PrSH-SBA-15. A highly ordered hexagonal array of mesopores was observed from the image of *ps*PrSH-SBA-15 material while a less ordered array of pores was observed from the image of *is*PrSH-SBA-15 material.

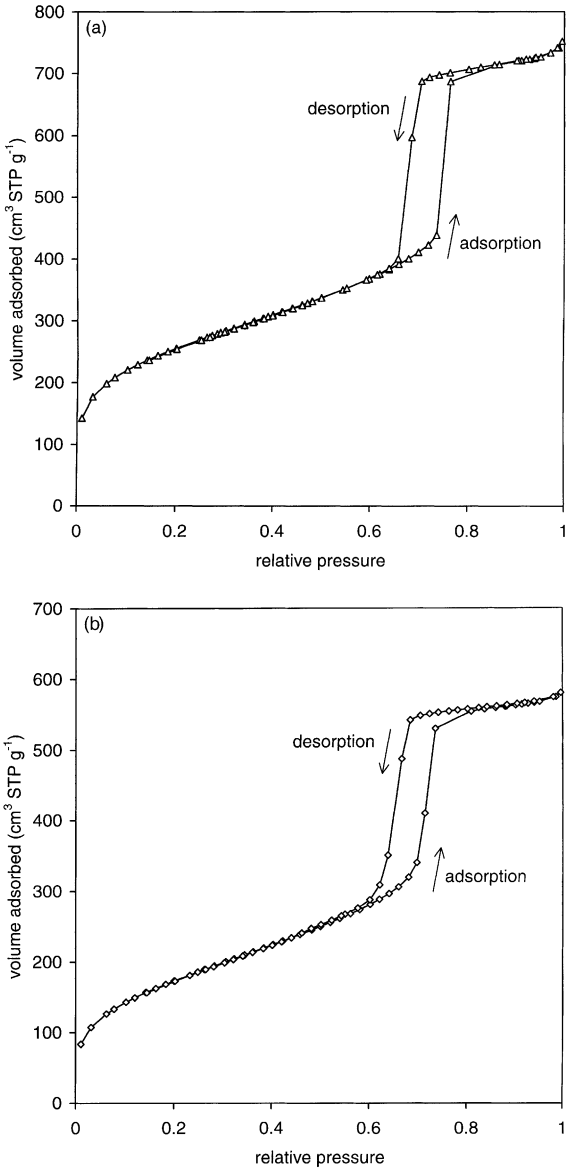


Fig. 2. Nitrogen adsorption isotherms for the (a) unfunctionalised SBA-15 and (b) in situ functionalised *is*PrSH-SBA-15 materials. All these materials show a typical type IV adsorption–desorption isotherm which corresponds to the mesostructure of these materials.

to ether groups of the EO–PO–EO surfactant residue and peaks at ca. 16 ppm (CH_3–) and ca. 59 ppm (–CH_2–OH) are from the ethanol, either formed during hydrolysis of ethoxysilane or as the solvent residue. A peak at 9.5–10.5 in each of the propylsiloxane tethered samples is ascribed to the methylene carbons bonded to silicon atoms (–CH_2–Si–) and a peak in the range 26–27.5 ppm to the second carbon on the propyl tether. The third carbon, adjacent to the different functional groups, is also observed. In the case of PrCOOH-SBA-15 materials, ^{13}C NMR showed the existence of hydrocarbon chain but the carboxylic carbon was not observed, presumably because it was not significantly enhanced by cross polarisation from protons. Examples of ^{13}C spectra from propyl thiol and propyl chloride in situ functionalised solids are given in Fig. 3. For phenyl functionalised surfaces a band of peaks between 127 and 134 ppm is observed. ^{13}C MAS NMR of selected post-synthesis functionalised SBA-15 are in general of lower quality than those of in situ functionalised samples. They show similar resonances due to added functional groups (Table 3) as well as resonances due to residual solvents, such as dioxane (66.5 ppm), the solvent used during functionalisation, and ether (58 ppm), the solvent used in washing the samples. These results indicate in a qualitative way the inclusion of –Cl, –SH, –NH_2 and –Ph groups on the SBA-15 surface.

Quantification of functional group loads in SBA-15 samples was performed for thiol and amine functionalised SBA-15 samples using elemental analysis (CHNS). This was complicated by the existence of (a) the template residue (polyethers) inside all in situ samples; (b) residual solvent (dioxane) trapped inside the pores of post-synthesis functionalised samples; and (c) the silanol groups (Si–OH) on the SBA-15 surface. For the samples with N or S atoms (PrSH-SBA-15 and $PrNH_2$-SBA-15), CHNS elemental anaysis is, therefore, useful for quantification of the amount of functional group on the sample surface. From the results of CHNS elemental analysis, *ps*PrSH-SBA-15 contains 1.57% w/w of sulphur (0.52 mmol g^{-1}), with 1.86% w/w for *is*PrSH-SBA-15 (0.64 mmol g^{-1}). This indicates that the post-synthesis functionalisation was not complete, only showing 18% reaction. In situ synthesis results in incorporation of more than 90% of the siloxane present in the original gel. For amine-functionalised samples, *ps*$PrNH_2$-SBA-15 contains 2.21% w/w of nitrogen (1.64 mmol g^{-1}), with 0.64% w/w for *is*$PrNH_2$-SBA-15 (0.52 mmol g^{-1}). Similar to the thiols, amine functionalisation proceeded more efficiently for in situ synthesis (74%) than for post-synthesis grafting (58%). With the use of the post-synthesis grafting route, amine

Table 1
Peak assignments for ^{13}C MAS NMR spectra of functionalised SBA-15[a]

Functionalised SBA-15	Chemical shift (ppm)							
	Tether groups –Si–$C^{(1)}H_2$–$C^{(2)}H_2$–$C^{(3)}H_2$–X			Functional group	Block copolymer template	Solvent		
	$C^{(1)}$	$C^{(2)}$	$C^{(3)}$			Ethanol	Dioxane	Ether
*ps*PrSH-SBA-15	10.5	27.3	27.3	–	–	–	66.3	58.4
*ps*PrCl-SBA-15	9.5	26.6	47.6	–	–	–	67.1	59.7
*ps*PrNH_2-SBA-15	8.9	22.7	43.0	–	–	–	–	–
*is*PrSH-SBA-15	10.5	27.5	27.5	–	70.5–76.2	16.1, 58.4	–	–
*is*PrCl-SBA-15	9.5	26.3	46.6	–	70.6–76.1	16.3, 59.8	–	–
*is*PrNH_2-SBA-15	9.2	21.2	43.0	–	70.0–76.1	15.9, 61.0	–	–
*is*PrCOOH-SBA-15	10.5	19.3	35.5	Not observed	67.3–76.1	15.9	–	–
*is*Ph-SBA-15	–	–	–	127.0, 129.2, 134.0 (phenyl group)	67.5–72.0	16.0, 57.5	–	–

[a] $C^{(1)}$, $C^{(2)}$, and $C^{(3)}$ are the carbon atoms on the propyl chains –Si–$C^{(1)}H_2$–$C^{(2)}H_2$–$C^{(3)}H_2$–X, where X is the functional group.

functionalisation showed a higher conversion than thiol functionalisation because the reaction was catalysed by the presence of the basic amine. Increasing the reaction temperature could increase the conversion but would require that a solvent with a higher boiling point be used. This would cause difficulties in subsequent solvent removal.

3.3. Enzyme immobilisation and leaching

In our previous work, we have observed that the use of purely siliceous mesoporous molecular sieves to immobilise enzymes results in considerable leaching during reaction. This is due to the weak interaction between the enzyme molecule and the siliceous surface of the molecular sieve. The use of functionalised silica gel to immobilise enzymes has been previously reported [15]. The functionalisation increased the interaction between the enzyme molecule and the support surface. Our own results also show the benefit of functionalisation in decreasing the amount of leaching.

Table 2 shows the immobilising and leaching behaviour of trypsin on SBA-15 with different functional groups. In contrast to calcined SBA-15, as-prepared SBA-15, which is non-porous, did not adsorb any trypsin. This indicates that most of the trypsin should have settled inside pores of SBA-15. However, as we reported before, over 50% of the supported trypsin was found to leach out in a Tris–HCl buffer solution (pH = 8.0). Post-synthesis functionalisation produces SBA-15 materials with a wide range of retention characteristics for the trypsin. Whereas no improvement was observed for phenyl-functionalised solids (55% leaching) and only moderate improvement for amine and thiol (20 and 16% leaching, respectively), strong retention was observed for acid and chloride functionalised solids (11 and 0% leaching respectively). Whilst the phenyl groups did not show any strong interaction, the other solids all exhibited stronger enzyme–surface interactions than the parent material.

The in situ samples, *is*PrSH-SBA-15 and *is*PrCl-SBA-15 stood out in the leach test, only 1% of leaching being recorded in each case. This must be due to the chemical interaction between the surface of the enzyme molecule and the functional groups on the molecular sieves. Alkyl chloride groups were thought to be able to react with amine groups, which are available on the surface of the trypsin molecule. The –S–S– bonds on the surface of a trypsin molecule are available for interaction with the thiol groups on the molecular sieve surface. This is potentially useful for immobilisation and separation as many enzymes and proteins have thiol groups or –S–S– bonds on their surface.

In our experiments, a relatively low loading (ca. 0.2% w/w) of enzyme was used compared with other reports on enzyme immobilisation (where loadings of ca. 20% w/w were used) because of the unique structure of these mesoporous molecular sieves. Since the

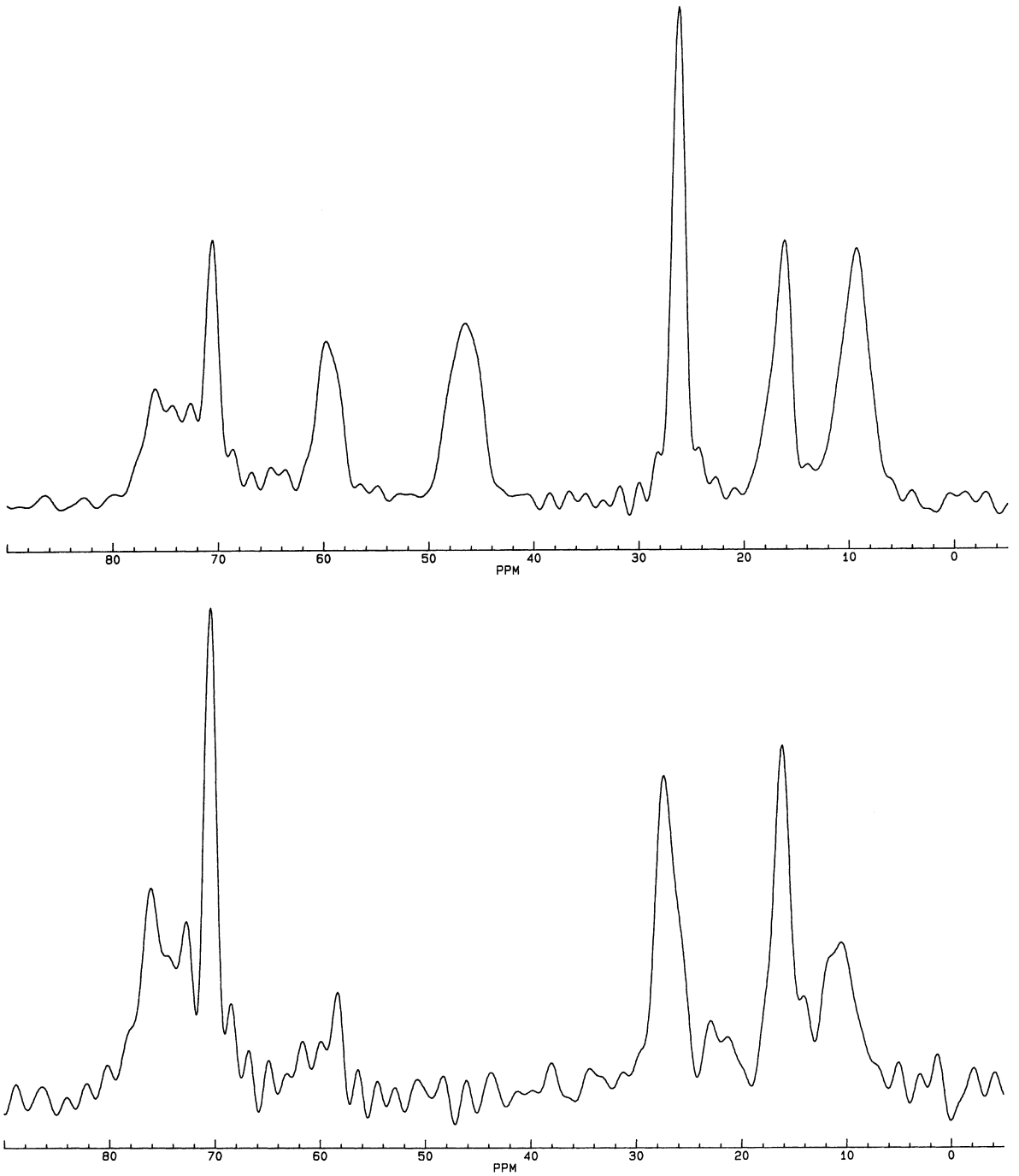


Fig. 3. ^{13}C solid state MAS NMR for (top) in situ functionalised *is*PrCl-SBA-15 and (bottom) in situ functionalised *is*PrSH-SBA-15.

Table 2
The percentage of trypsin found in the supernatant solution after immobilisation process and the percentage of trypsin leached from support during the leaching test

Support	Residue non-adsorbed (%)	Leached from support (%)
Uncalcined SBA-15	100	–
Calcined SBA-15	0[a]	52
SBA-15 (extracted with EtOH)	6	48
Post-synthesis		
*ps*PrSH-SBA-15	27	16
*ps*PrCl-SBA-15	6	0[a]
*ps*PrNH$_2$-SBA-15	18	20
*ps*PrCOOH-SBA-15	3	11
*ps*Ph-SBA-15	12	55
In situ		
*is*PrSH-SBA-15	10	1
*is*PrCl-SBA-15	7	1
*is*PrNH$_2$-SBA-15	22	25
*is*PrCOOH-SBA-15	30	2
*is*Ph-SBA-15	9	19

[a] Under the measurable limit.

pore diameter of these materials are relatively small (ca. 6 nm), high loading could inhibit substrate and product diffusion and reduce specific activities.

3.4. Catalytic activities of immobilised enzymes

Table 3 shows the initial activities of the SBA-15 samples with supported trypsin, expressed as production of nitroaniline per mg of supported protein. These are compared with the specific activities of free trypsin.

Neither siliceous SBA-15 alone, nor SBA-15 functionalised with thiol, showed any activity for the hydrolysis reaction, so all measured conversion was due to enzymes in the samples. As expected, free enzyme solutions showed the highest specific activity, but in the best examples, supported trypsin shows activities very close to those of the free enzyme.

There is a wide-range of different specific activities when different functionalised groups are used to immobilise the enzymes. In addition, the specific activities observed for samples where the tethers are attached post-synthesis are in general lower than those where the tethers are included in situ during preparation.

Table 3
Initial rate of BAPNA hydrolysis and immobilisation and catalysis efficiencies of supported and free trypsin

Catalysts/supports	Initial rate for BAPNA hydrolysis (μmoles of *p*-nitro-aniline per mg protein per min)	Immobilisation and catalysis efficiency[b]
Free trypsin	0.46	1.00
Calcined SBA-15 (recycled)	0.25 (0.39)	0.26
SBA-15, extracted with EtOH	0.33	0.35
Post-synthesis		
*ps*PrSH-SBA-15	0.05[a]	0.06
*ps*PrCl-SBA-15	0.11	0.22
*ps*PrNH$_2$-SBA-15	0.03	0.04
*ps*PrCOOH-SBA-15	0.08	0.15
*ps*Ph-SBA-15	0.20	0.17
In situ		
*is*PrSH-SBA-15 (recycled)	0.44[a] (0.29)	0.84
*is*PrCl-SBA-15	0.14	0.28
*is*PrNH$_2$-SBA-15	0.02	0.03
*is*PrCOOH-SBA-15	0.39	0.59
*is*Ph-SBA-15	0.27	0.43

[a] An average value from three repeated experiments.

[b] Immobilisation and catalysis efficiency was calculated as the fraction of protein retained on the solid multiplied by the relative specific activity compared with free trypsin.

For the SBA-15 supports prepared by inclusion of siloxane functional groups during synthesis, high specific activities are obtained for thiol- and carboxylic acid-functionalised surfaces, which approach those of the free enzyme. Phenyl- and chloride-functionalised supports show reduced values, and amine-functionalised supports result in negligible observed conversion. The different specific activities of these in situ functionalised samples are thought to be due to the differences in chemistry among the tethering groups. In particular, the amine groups appear to inhibit catalytic activity strongly. Among these samples, the thiol-functionalised SBA-15 was the best for supporting trypsin, in terms of both catalytic activity and degree of leaching (1%). The combined immobilisation and catalytic efficiency of supporting trypsin on this solid (immobilisation efficiency × relative specific catalytic activity) is 84% of that of the free enzyme. We attribute this to interaction between thiol

groups on the surface of the solid with either –SH or –S–S– groups on the outside of the enzyme. The carboxylic acid-functionalised solid also showed high specific activity and high enzyme retention.

For the supports prepared by post-synthetic 'grafting' of tethers, the activities are low in all cases, in particular, the thiol- and acid-functionalised solids show much poorer activity when prepared in this way. Although there are textural differences in the SBA-15 material prepared by the two methods (post-synthetic grafting was performed on more highly ordered solids) the reason for the much lower activity in post-synthesised samples is thought rather to lie in the chemistry of the tethering groups. In particular, repeat preparations of post-synthesis thiol-modified SBA-15 gave very low catalytic activities. This may be attributed to the interaction of either incompletely reacted triethoxylsilanes or residual solvent with the active sites of the trypsin.

To assess the degree of leaching of enzyme from these functionalised supports, the *is*PrSH-SBA-15 with supported enzyme was examined. 0.25 g of the sample was suspended in 20 cm^3 1 mM BAPNA (pH = 8.0) solution at 25°C under conditions typical of the measurements of catalytic activity. After 15 min of the reaction the solid was removed by filtration. Any further reaction in this solution must be due to catalysis by free trypsin. In the experiment, no further conversion was observed. Fig. 4 compares the result of this experiment with the repeat runs of BAPNA hydrolysis over trypsin on *is*PrSH-SBA-15 where the catalyst was left suspended in the solution. This indicates that negligible leaching of the enzyme from this support occurs under the conditions of the catalytic reaction.

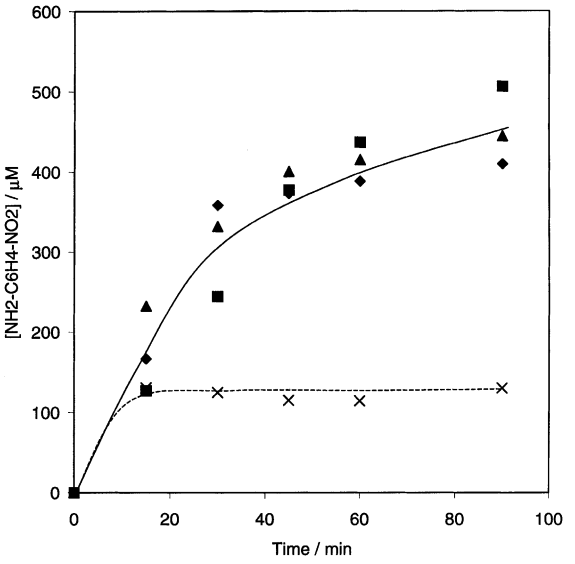


Fig. 4. Hydrolysis activity of BAPNA catalysed by trypsin immobilised on in situ functionalised *is*PrSH-SBA-15 expressed as the production of the hydrolysis product, *p*-nitroaniline ((◆), (■), (●)). This is compared with the results of an experiment where the solid catalyst was removed from the BAPNA solution after 15 min, and the rate of production of *p*-nitroaniline was subsequently followed (×).

3.5. Recovery and reusability of catalysts

In general, enzymes are difficult to recover and re-use. Recovery and reusability of immobilised enzymes are, therefore, important aspects of this study. Two samples, trypsin on unfunctionalised SBA-15 and *is*PrSH-SBA-15, were chosen to be recovered and re-used in the hydrolysis test. After the first test, these samples were filtered, washed with 2 × 10 ml of buffer (pH = 8) and dried at 4°C for a week. The catalytic activities of these two re-used samples are given in Table 3. Re-used trypsin supported on *is*PrSH-SBA-15 showed an initial activity 66% of the fresh catalyst. The initial activity from reusing the trypsin supported on unfuctionalised SBA-15 was found to be higher than that on the fresh catalyst. This was attributed to leaching of trypsin from the solid to give free enzyme, which was seen to have the highest specific activity. This encouraging result indicates the possibility of re-using enzymes immobilised on mesoporous materials.

4. Summary and conclusions

These results indicate that the functionalisation of the internal surfaces of mesoporous solids permit enzyme immobilisation via enzyme–support interactions that are considerably enhanced compared to those with unfunctionalised silica surfaces. The strength of these interactions, and therefore, the extent of leaching observed under reaction conditions is found to depend strongly on the nature of the functional groups attached to the surface. Retention of up to 90% of the trypsin is observed in the most favourable cases.

Catalytic testing of the most promising composite supported enzyme solids shows that strong attachment does not adversely affect the enzyme's ability to hydrolyse BAPNA. The method of functionalisation is found to be critical – thiol, chloride and acid functionalised solids are more active when the functional group have been incorporated during the synthesis of the mesoporous molecular sieve rather than by post-synthetic treatment. Studies also show the nature of the functional group is critical. Although amine-functionalised solids show enhanced leaching characteristics, they strongly reduce the observed catalytic activity of trypsin. The most promising catalyst in this study is a trypsin-thiol functionalised SBA-15 catalyst where the functionalisation is performed in situ. This solid, which shows negligible leaching in both pre-catalytic and catalytic assays, achieves a catalytic activity per gram of original protein 84% of that measured for the free enzyme.

Re-use of the catalysts is also possible, even after a period of a number of days. Trypsin supported on the thiol-functionalised solid described above shows an initial activity some two-thirds of that displayed by the fresh catalyst.

We are currently extending this work to examine systems when the activity and specificity of enzymatic catalysis can be combined with features of shape selectivity that are achievable for large molecules when using well defined mesoporous supports.

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