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Electrostatic and covalent immobilisation of enzymes on ITQ-6 delaminated zeolitic materials

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ITQ-6, a delaminated material derived from the lamellar precursor of the Ferrierite zeolite, is an excellent support for immobilisation of enzymes; this has been proved by supporting two different enzymes, β-galactosidase from *Aspergillus Oryzae* and penicillin G acylase from *Escherichia Coli*, by electrostatic and covalent interactions, respectively; the adequacy and versatility of the ITQ-6 support and the high stability and catalytic activity of the resultant supported enzymes are shown, with ITQ-6 clearly superior to other already well optimised laboratory and industrial supports for enzymes.

It is well known that enzymes are the most active and selective catalysts for many reactions of commercial interest. However, their low thermal and/or solvent stability and difficult recycling are the major drawbacks for a broader industrial application. In order to improve the stability and performance of enzymes as catalysts, they have been supported in a variety of solids which include organic gels, resins, silicas[1–5] or more recently mesoporous molecular sieves such as MCM-41 or SBA-15.[6–8] Inorganic supports have the advantage of allowing the regeneration of the carrier when the enzyme has been deactivated. Moreover, in the case of ordered mesoporous materials a good selectivity to some enzymes can be obtained by varying the pore diameter and functional groups of the support.[8] Unfortunately, owing to the limited stability in aqueous media of some of these materials and the large steric volume of many enzymes which can result in the blocking of the channels, their possibilities as useful catalysts will be limited. Recently, a series of delaminated zeolites have been obtained as crystalline materials with a well defined external surface area of >600 m^2 g^{-1}, easily accessible to very large molecules, and containing regularly distributed silanol groups.[9,10] By taking advantage of these remarkable properties, it appears that this type of material can offer new opportunities as supports for enzymes.

Generally speaking, with conventional supports, enzyme immobilisation *via* covalent bonding has the advantage of forming strong and stable linkages that result in very robust enzyme supported catalysts. However, the irreversible covalent binding has the drawback that enzyme and support are disposed as waste after deactivation.[1–5,11–13] However, as shown here, when a delaminated zeolite, ITQ-6[10] is used as support, this can be easily recovered and recycled simply by calcination.

There are other cases in where the reversible electrostatic immobilisation of enzymes on pre-existing supports could be of industrial interest. If the enzyme becomes deactivated and can not be further used in the industrial reactor, it can be desorbed and the support can be recovered free of protein, and so available for a new immobilisation of a fresh solution of soluble enzyme.[14] Again, the surface of a delayered zeolite, such as ITQ-6,[10] appears to be highly suitable for activation and use as a reversible enzyme support.

ITQ-6, a delaminated material derived from the lamellar precursor of the Ferrierite zeolite,[10] is an excellent support for immobilisation of enzymes. This has been proved by supporting two different enzymes, β-galactosidase from *Aspergillus Oryzae* and penicillin G acylase from *Escherichia Coli*, by electrostatic and covalent interactions, respectively. The suitability and versatility of the ITQ-6 support and the high stability and catalytic activity of the resultant supported enzymes represent an alternative to other well optimised laboratory and industrial supports for enzymes.

Pure silica ITQ-6, with a specific surface area of 600 m^2 g^{-1} and 0.75 cm^3 g^{-1} pore volume was prepared by a procedure reported previously.[10]

ITQ-6 was functionalised by reacting a solution of 3-aminopropyltriethoxysilane (APS) in toluene with dehydrated ITQ-6 samples resulting in aminopropyl functionalised ITQ-6, denoted N-ITQ-6. When this material (1.0 g) was contacted with a solution of β-galactosidase enzyme (60 mg) from *Aspergillus Oryzae* (MW = 105 kD, pI < 6) to achieve its electrostatic immobilisation on the N-ITQ-6 carrier, it was found that the adsorption of the enzyme was > 99% within 1 h. The amount of adsorbed enzyme was determined from the activity of the resulting solution, after removal of the solid, for the hydrolysis of *o*-nitrophenyl-β-D-galactopiranoside (ONPG) using a 100 mM acetate buffer at pH = 4.5 and 298 K. Hydrolysis of ONPG was monitored by the appearance of a characteristic band of the hydrolysis product at 405 nm. In order to check that the negligible activity of the liquid phase is due to the adsorption of the enzyme onto the solid and not to the deactivation of the enzyme, the solid was recovered and used to catalyse the hydrolysis of ONPG. It is remarkable that the activity of the electrostatically supported enzyme was the same as the activity of the free enzyme before adsorption (the absorbances at 405 nm of the reaction mixtures after 7 min of reaction were 0.43 and 0.41 for the supported and free enzymes, respectively), indicating that all the enzyme has been immobilised, and more importantly, it remains active and accessible to the reactants upon immobilisation. No activity was found when the support, prior of adsorption of the enzyme, was used as catalyst. Also, no leaching of the supported enzyme was detected as can be concluded from the zero activity observed in the reaction media after filtering off the enzyme containing solid.

The immobilised enzyme is highly stable even in contact with solutions of relatively high ionic strength. Specifically, 98% of the immobilised enzyme is still retained on the N-ITQ-6 support in 0.30 M NaCl medium, as determined from the enzyme activity of the resulting solution after removal of the supported enzyme. This level of enzyme retention is much higher than observed for most traditional supports, and it is at least as high as that found for the best performance highly optimised supports (Fig. 1). When desired, the enzymes can be desorbed from N-ITQ-6 by increasing the NaCl concentration of the eluent solution to 1 M. This allows the regeneration of the carrier after the enzyme deactivation in the same reactor or in a fixed bed. At this point the support is ready to be recharged again with enzyme simply by contacting again the regenerated N-ITQ-6 carrier with a fresh solution of the enzyme in the appropriate conditions.

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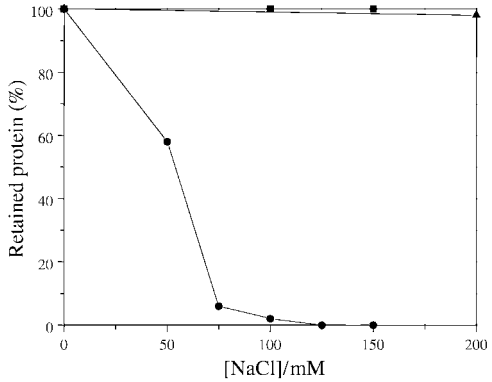
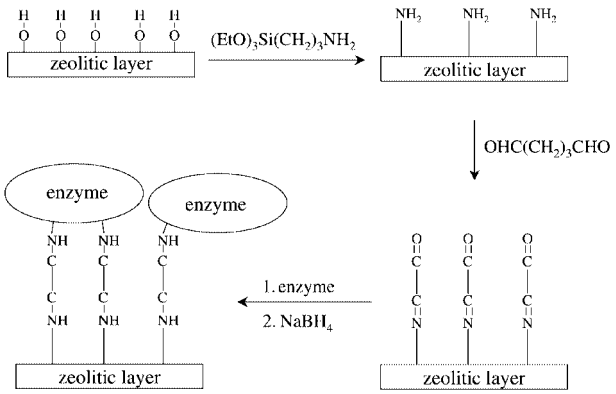


Fig. 1 Desorption of β-galactosidase from *A. Oryzae* adsorbed on diverse supports by increasing ionic strength. (▲) N-ITQ-6; (■) PEI–glyoxil–agarose composite (from ref. 14); (●) DEAE–agarose (from ref. 14).

Up to now, enzymes have been supported into mesoporous molecular sieves *via* electrostatic bonding.[6–8] However, for ITQ-6, the enzymes can also be easily supported by covalent bonding resulting in extremely stable catalysts. To do this, a portion of N-ITQ-6 was reacted with a solution of glutaraldehyde at pH = 7 yielding to a material denoted G-ITQ-6. This solid contains imine and aldehyde functional groups and now is ready for covalently immobilising the enzymes by reacting the amino groups of the enzyme with the anchored aldehyde groups at pH = 8, forming imine bonds which are further reduced with sodium borohydride to secondary amines (Scheme 1).



Scheme 1 Covalent immobilisation of enzymes on delaminated mesoporous materials.

Following the above procedure, 2050 IU of a highly voluminous enzyme, penicillin G acylase (PGA, MW = 86000) was immobilised. The catalytic activity and accessibility under reaction conditions of the covalently bonded enzyme were studied by performing the hydrolysis of penicillin G at pH = 8 and 298 K. The progress of the reaction was followed by continuous titration of the released protons with a 25 mM NaOH solution using an automatic burette. In this way, the activity of the supported enzyme was found to be 2440 IU g^{-1} of G-ITQ-6 indicating that the supported enzyme is very active and accessible to the penicillin G reactant.

Moreover, we have proved that the enzyme covalently bonded to the G-ITQ-6 support displays higher stability by comparing its deactivation rate with that of the free enzyme and also with PGA chemically bonded to amorphous silica, which has been widely used as an inorganic carrier of enzymes[15] and particularly for PGA.[16] In order to perform this experiment, aliquots of the covalently supported enzymes on both ITQ-6 and amorphous silica supports (200 IU g^{-1} support) or equivalent amounts of free enzyme were incubated in a 100 mM phosphate buffer at pH = 8 and 319 K for different times. Then, the activity for hydrolysis of penicillin G was measured and compared to that obtained for the non-incubated enzyme. The

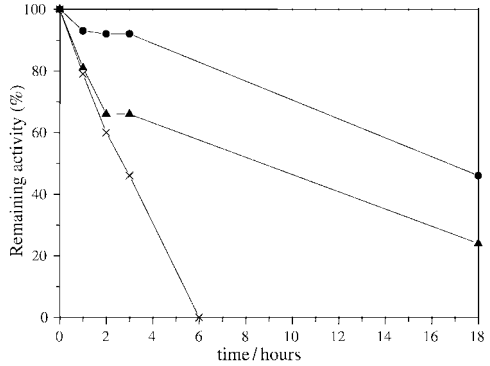


Fig. 2 Deactivation of penicillin G acylase at 319 K and pH = 8 in 100 mM phosphate buffer. (×) Free enzyme remaining activity; (▲) glutaraldehyde-modified silica immobilised enzyme remaining activity; (●) G-ITQ-6 immobilised enzyme remaining activity. Penicillin G hydrolysis reaction was carried out at 298 K and pH = 8, using 25 mM NaOH solution as a titrant.

results shown in Fig. 2 indicate that the stability of the enzyme is substantially improved when it is covalently bonded to the surface of the G-ITQ-6, in such a way that, while the activity of the unsupported enzyme was zero after 6 h of incubation, the remaining activity of the supported enzyme was close to 90%. This value was also clearly higher than that found for the enzyme supported on amorphous silica (60%), The increase in the enzyme stability suggests a multipoint attachment between the enzyme and the supports, perhaps involving the most reactive groups of the protein surface. Moreover, it is reasonable to suppose that a well structured surface, such as that found on ITQ-6, may facilitate the interactions with the enzyme and the dispersion forces might have a positive effect on the enzyme stability.

In conclusion, ITQ-6, owing to its well structured high external surface area and by regularly distributed silanol groups, opens new possibilities for supporting and stabilising many enzymes and using them for reaction with large substrates. It has been proved that the enzymes can be adequately supported by either ionic or covalent bonding whilst retaining full activity.

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