Understanding enzyme immobilisation

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Enzymes are versatile catalysts in the laboratory and on an industrial scale. To broaden their applicability in the laboratory and to ensure their (re)use in manufacturing the stability of enzymes can often require improvement. Immobilisation can address the issue of enzymatic instability. Immobilisation can also help to enable the employment of enzymes in different solvents, at extremes of pH and temperature and exceptionally high substrate concentrations. At the same time substrate-specificity, enantioselectivity and reactivity can be modified. However, most often the molecular and physical–chemical bases of these phenomena have not been elucidated yet. This *tutorial review* focuses on the understanding of enzyme immobilisation.

1. Introduction

A catalyst enhances the rate of approach toward the equilibrium of a reaction without being substantially consumed during the reaction. By definition a catalyst must be recyclable. If a catalyst, be it an enzyme or any other type of catalyst, is dissolved in the reaction medium it is often difficult to retain, let alone reuse it. Immobilising a catalyst can be a straightforward route to enable the recycling of a catalyst.[1,2]

Enzymes are a particularly versatile class of catalysts. They are very effective and precise (bio)-catalysts that perform and

regulate processes in living matter. They often display high regio- and chemoselectivity while operating under mild conditions. The unique potential of enzymes is still far from being fully explored, indeed estimates generally agree that <1% of the micro-organisms in the environment have been cultivated to date and had their enzymes identified. Although many enzymes remain to be discovered, a vast number that catalyse a huge array of reactions have been identified and characterised and are in principle, available for use as catalysts for any reactions that nature utilises to sustain life. Many enzymes are commercially available. Prices can vary significantly, depending on the degree of difficulty in isolating the enzyme and/or if it is readily available from recombinant sources. Most of the enzymes that are currently used in organic synthesis and in industry can be produced on a large scale. Some are sufficiently cheap to be used in washing powders and other bulk applications.[3,10]

The surge in practical utilisation of biocatalysts is driven by their versatility, regio-, chemo- and enantioselectivity while

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operating under mild conditions. These features need to be translated into environmentally compatible processes. The common perception is however, that enzymes are sensitive, unstable and have to be used in water, features that are not ideal for a catalyst and undesirable in most syntheses. In many cases a way to avoid at least part of these complaints is to immobilise enzymes. For industrial scale applications, immobilisation is generally considered favourable since it allows for continuous processes.[1–10] On immobilisation, enzymes are often stabilised, and thus less sensitive toward their environment. Even processes employing insoluble enzymes suspended in hydrophobic organic media require immobilisation to optimize enzyme dispersion to improve accessibility for the substrates, as well as to avoid the aggregation of the hydrophilic protein particles. In addition, immobilisation ensures that these biocatalysts can be readily recycled. The anchoring of an enzyme onto a solid insoluble support should be straightforward and cost efficient. It has to be emphasised that enzyme immobilisation can help in the utilisation of the enzyme, but that this has to be proven separately for every case studied. The parameters determining the success or failure of immobilisation and the methodology behind it are the topic of this review.

2. General considerations

Immobilisation of an enzyme entails the interaction of two species, the enzyme and the carrier (Table 1).[5,7–9] The surface properties of both are therefore important. In the case of the enzyme, polar groups (*e.g.* amino groups on lysine and acid groups on glutamic acid), apolar surface areas or sugar moieties can influence the properties of the surface. The carrier can be prepared to match either of these surface properties of the enzyme. An essential requirement for any carrier is the need to have a large surface area. This can be achieved with small particle size materials, though this can make separation difficult, or with highly porous materials with pores of sufficiently large dimensions that do not limit diffusion of the substrates. Moreover the material needs to be chemically and mechanically stable. Alternatively the carrier can encapsulate the enzyme.[4,6] In this case the pores of the carrier have to be of sufficient size to guarantee unhindered diffusion of the

Table 1 Some of the parameters to be taken into account when planning the immobilisation of a biocatalyst and relevant sections of this review

Enzyme	Section
Size of the enzyme	7
Conformational flexibility required by the mechanism	5, 8
Isoelectric point	4, 8
Surface functional groups/charge density	4, 8
Glycosylation	3, 5, 8
Stability under immobilisation conditions	6, 7
Presence of hydrophobic regions	3, 8
Presence of hydrophilic regions	3, 8
Additives in the enzymatic preparation	5, 6
Carrier	
Organic or inorganic	3, 4, 5,
Hydrophobic or hydrophilic	3, 5, 9
Surface charges	4
Surface functionalisation	5
Chemical and mechanical stability	3, 4, 5, 6, 7
Surface area	3, 4, 5, 6, 7, 9
Porosity	9
Particle size	3, 4, 5, 6, 7, 9
Specific factors related to the reaction system	
Reaction medium	9
Diffusion limitations	9
Enzyme inhibition	9
Precipitation of products	9
Viscosity of the mixture	5, 9
Reaction thermodynamics	9
Non-specific solute-support interactions	9

substrates while ensuring that the enzyme remains locked inside. Anchoring of the enzyme to the carrier can be strengthened by covalent binding. Reactive functional groups can be introduced in the matrix of polymeric supports by choosing appropriate organic monomers.[11] In the case of silicates, surface derivatization can be achieved *via* modification of surface hydroxy groups with appropriate reagents.[12] In the most extreme case of anchoring, no carrier is used and the enzyme molecules are linked to each other, forming their own carrier.[8]

A general method that can be applied to the immobilisation of any enzyme is not available. Typically the approach used is one of trial and error, until a satisfactory system has been developed. With such an approach, a simple point to remember, though one that is not always appreciated, is that the enzyme should be stable to and during the immobilisation process.

The most frequently used immobilisation techniques fall into five categories, each with its own associated advantages and disadvantages:

1. non-covalent adsorption and deposition
2. immobilisation *via* ionic interactions
3. covalent attachment (tethering)
4. cross-linking of an enzyme
5. entrapment in a polymeric gel or capsule

Efficient immobilisation protocols should take into account the factors reported in Table 1 so that the best compromise between stability, activity, easy handling and cost is reached. At first glance this seems to imply that the immobilised enzyme will always perform worse than the native one. This is not the case. Based on an understanding of the parameters in Table 1,

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the surface properties of both enzyme and carrier can be used to advantage, obtaining enzyme preparations that outperform the native enzyme. The parameters that influence immobilisation will be critically discussed, followed by two sections that further develop and rationalise the underlying ideas. The division into five immobilisation techniques is somewhat artificial and in the literature many other divisions can be found. However in the experience of the authors, this classification has proven useful in discussing the salient points concerning enzyme immobilisation.

3. Non-covalent adsorption and deposition

The adsorption of enzymes onto carriers can proceed *via* different types of interactions. Enzymes with a large lipophilic surface area will interact well with a hydrophobic carrier. van der Waals forces and entropy changes ensure the immobilisation of the enzyme on the carrier. Sugar residues of glycosylated enzymes can ensure adsorption *via* hydrogen bonds; large hydrophilic surface areas of the enzyme will interact with a hydrophilic carrier. The advantage of immobilisation *via* entropy effects or hydrogen bonds is that the enzyme does not have to be pre-treated or chemically modified. It is even possible to use crude enzyme preparations for these immobilisations. Varying the immobilisation conditions greatly influences the results and thus might allow a straightforward manipulation of the enzyme's properties.[9]

A significant disadvantage of immobilising by adsorption is that the enzyme tends to leach readily from the carrier when used in aqueous media. This is not the case if organic solvents are used due to the intrinsic insolubility of enzymes in such media. In the case of deposition, the support is simply added to an aqueous solution of the enzyme and afterwards the biocatalyst is recovered by precipitation or by evaporating the aqueous phase. As a result, the enzyme is deposited on the solid support and no hydrophobic or entropic driving factor is involved in the immobilisation process. The majority of enzymes immobilised on Celite powder are prepared according to this procedure.[13]

3.1 van der Waals interactions/entropy changes

For efficient immobilisation *via* van der Waals interactions/entropy changes, both the carrier and the enzyme need to have large lipophilic surface areas. A range of lipophilic carriers such as EP-100 polypropylene, Accurel MP1004 polypropylene, octyl-silica and octyl-agarose are available. However, in most cases enzymes expose hydrophilic residues on their surface whereas the presence of defined hydrophobic superficial regions is typical of lipases. Lipases break down fats, therefore they are active at the interface of oil/fat and water, indeed most lipases
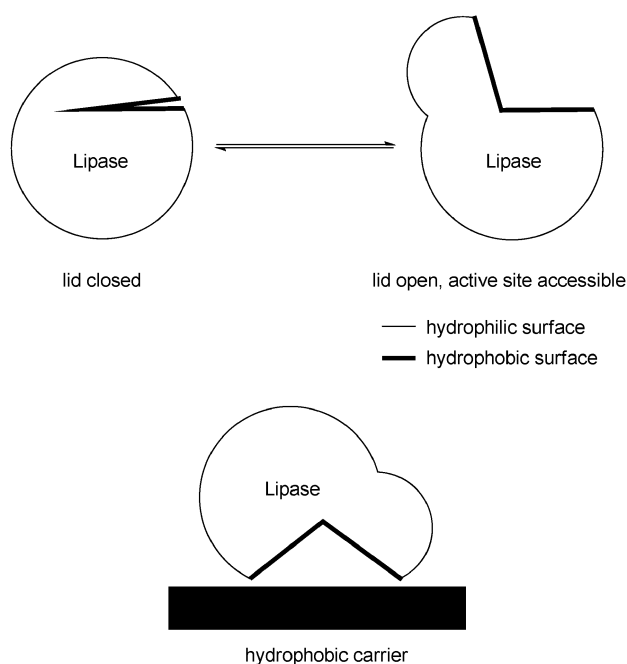

Fig. 1 Most lipases have a lid that opens upon interfacial activation. Lipases immobilised on hydrophobic carriers are presumed to be in their active conformation.

show interfacial activation. This conformational change is schematically depicted in Fig. 1. The immobilisation of lipases on hydrophobic carriers is thought to mimic this interfacial activation.[5,7,9,13–15] It has to be emphasised that there is no proof of this to date. However, experimental data from immobilisation in hydrophobic sol–gels[4] and on hydrophobic carriers demonstrate that lipases are more active under these conditions. As already discussed in section 2, the better accessibility of the lipases due to the large surface area of the carriers might also play a role.

When an enzyme is immobilised on a hydrophobic carrier the only interactions between the carrier and the enzyme are van der Waals forces. Since van der Waals forces are rather weak they are not the true driving force behind this immobilisation. Instead, it is entropy driven. One enzyme molecule displaces a large number of water molecules both from the carrier and its own surface when it is immobilised. The interaction between two materials *via* this gain in entropy is also known as hydrophobic interaction.

Adsorption of lipases on hydrophobic supports has been applied to the fermentation broth of lipase producing microorganisms. The lipase is often the only lipophilic component and it can be extracted and purified by adsorption onto hydrophobic carriers, in one single step.[16] After immobilisation

Table 2 Recycling CALB (immobilised as Novozym 435) in the kinetic resolution of *p*-chloromandelonitrile acetate

| Cycle	Ratio (%) (ee%)				
	1	2	3	4	5
(*R*)-*p*-Chloromandelonitrile acetate	98 (99)	96 (99)	98 (97)	98 (98)	98 (98)
(*S*)-*p*-Chloromandelonitrile THP-ether	86 (90)	86 (90)	88 (93)	90 (93)	91 (93)
p-Chlorobenzaldehyde	16	18	14	12	10

and removal from the broth, the lipases can be washed off the carrier indicating a disadvantage of this immobilisation method: in aqueous media leaching of the enzyme can be a major problem. If the immobilised enzyme is, however, used in hydrophobic organic solvents, no leaching occurs since the enzyme is insoluble in such solvents.

Candida antarctica lipase B (CALB) does not have a full-size lid and thus does not display interfacial activation.[5,17] Its popular commercial form "Novozym 435" consists of the lipase adsorbed on a hydrophobic macroporous polymer which is based on methyl and butyl methacrylic esters and cross-linked with divinylbenzene.[18] Novozym 435 was used for the kinetic resolution of aromatic cyanohydrin acetates in toluene, demonstrating the advantages of using an immobilised enzyme.[19] At the end of the CALB-catalysed reaction the enzyme could be filtered off and reused over five cycles without loss of activity or enantioselectivity (Table 2). The reaction mixture containing (*R*)-acetates and unprotected (*S*)-cyanohydrins could be submitted to a second transformation, the chemical protection of the (*S*)-cyanohydrins. Thus both enantiomers, protected with different protection groups and readily separable (Scheme 1) were obtained.

Due to its great stability, the same CALB preparation was applied in many dynamic kinetic resolutions (DKR). For example, good yields were achieved with Novozym 435 in the enantioselective synthesis of cyanohydrin acetates from aliphatic aldehydes (Scheme 2).[20] Alkaline NaCN established the dynamic racemic formation and degradation of the cyanohydrins while Novozym 435 induced the chirality in the acylation step.

Similarly Novozym 435 can be combined with transition metal catalysed racemisation reactions to constitute other DKRs (Scheme 3).[21] In these cases the reaction conditions are often quite extreme (elevated temperatures and oxidising or reducing conditions), but immobilised CALB was stable in such conditions.[22]

CALB has also been adsorbed on Accurel. On this extremely hydrophobic carrier the stereoselectivity and activity of CALB was even better than when immobilised as
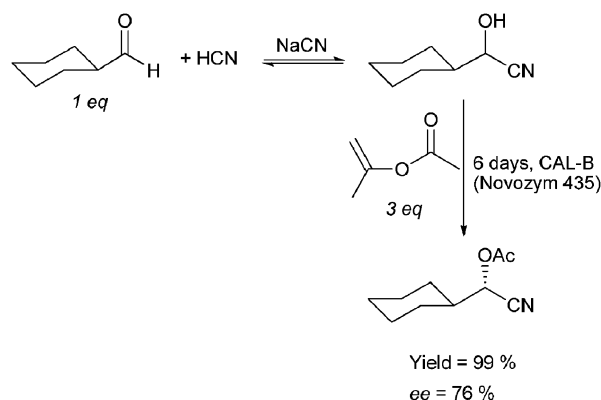
Scheme 2 Novozym 435 catalyses the enantioselective step in the DKR of aliphatic cyanohydrin acetates. Acetone cyanohydrin functions as a cyanide source in this reaction.
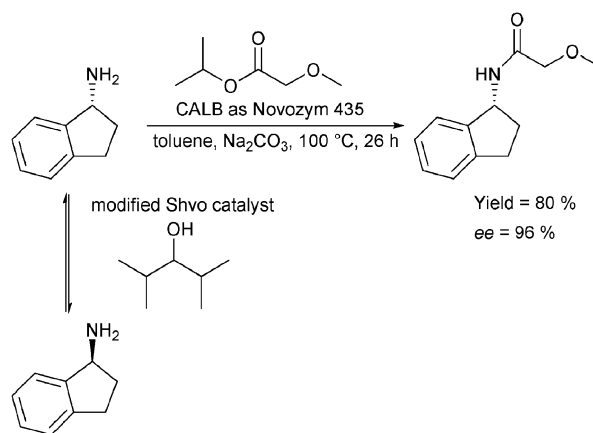
Scheme 3 Ruthenium and CALB catalysed DKR of racemic amines.

Novozym 435. However, diffusion effects were the cause for this alteration in activity and not conformational changes. This emphasises that there often is more than one reason why
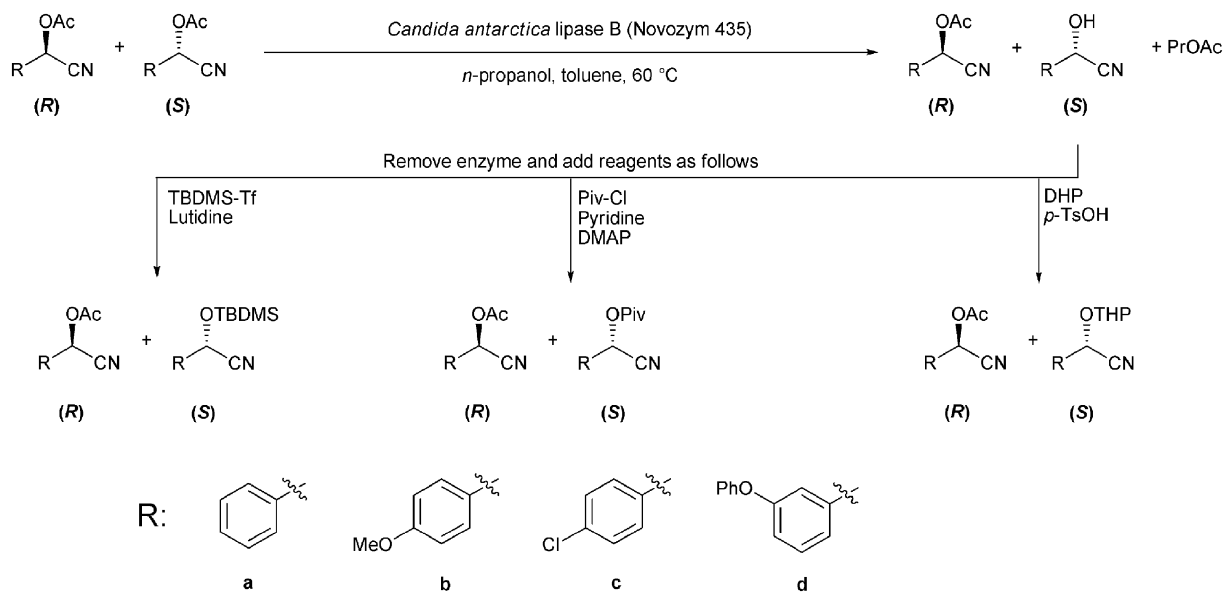
Scheme 1 CALB immobilised on a hydrophobic carrier can be used and recycled in organic solvents.

an enzyme performs better/worse when modifying the carrier (see also section 9).[23] More recently Novozym 435 and free CALB were shown to have identical stereoselectivity in the transesterification of secondary alcohols.[24] In the resolution of flurbiprofen *via* esterification of octanol in toluene a different enantioselectivity was observed, most probably caused by the adsorption of the racemic acid (flurbiprofen) onto the polymeric carrier and its progressive partition into the organic solution throughout the reaction. This again proves that great care has to be taken when evaluating the reasons for the perceived "improvements" of the enzyme.[25]

3.2 Hydrogen bonds

In most cases hydrophilic amino acid residues prevail on the surface of enzymes. In addition, enzymes may be glycosylated, further increasing the hydrophilicity of the protein. Therefore they can easily form hydrogen bonds and thus can be immobilised on hydrophilic carriers (cellulose, lignine, Avicel, Celite, porous glass, clay, silica gel).[7,9,10] A particularly popular carrier is Celite (diatomaceous earth), the silicate skeletons of diatoms. The structure and properties of Celite can vary significantly as a function of the production process. Some types of Celite are commercially available in the form of rods or beads with diameters of several mm. They are produced after being re-calcined in the presence of templates that induce porosity in the material and dramatically increase its capacity to adsorb protein onto the enlarged inner surface as well as retain water inside the pores. Therefore care has to be taken to employ the appropriate type of Celite for a specific application. As will be explained in section 9, the ability of porous Celite to bind water can be used to control the water activity in organic solvents.[26] Many commercial lipase preparations are based on Celite powder, which has a very limited capacity to adsorb water, as a carrier. This immobilisation is actually a deposition which is straightforward to perform by simply co-precipitating the protein and the Celite powder.[13,27,28] Often additives such as sugars, PEG or albumin are included into the formulation, in order to stabilise the enzyme. It is thought that these polar compounds protect enzymes against the negative influence of organic solvents and favourably influence the distribution of water in the microenvironment of the enzyme.

Lipases immobilised/deposited on Celite have been extensively used in dry organic solvents, without the risk of leaching. *Burkholderia cepacia* (also named *Pseudomonas cepacia*) lipase (BCL) was immobilised on Hyflo super-cel Celite in the presence of sucrose.[27] This immobilised enzyme was the key to the first enantioselective synthesis of cyanohydrin acetates *via* a DKR; many other examples followed, always with lipases on Celite, immobilised in the presence of sugars. This was in contrast to the above described successful DKR of aliphatic cyanohydrin acetates with Novozym 435.[20] For the enantioselective synthesis of mandelonitrile acetate *via* DKR, Novozym 435 did not perform well. Instead of catalysing the formation of mandelonitrile acetate it hydrolysed part of the acyl donor isopropenyl acetate. The acetic acid formed neutralised the base that catalysed the racemic dynamic formation of the cyanohydrin and the overall reaction came
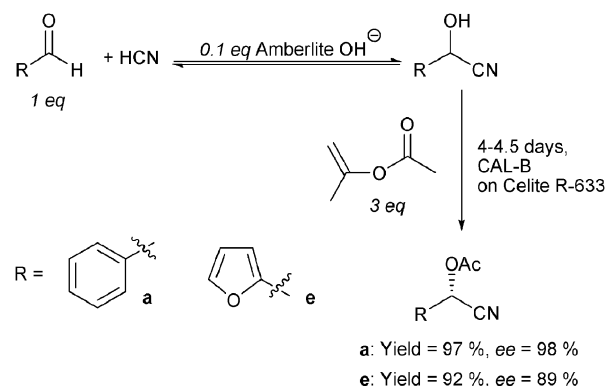
Scheme 4 CALB on Celite R-633 is an efficient and enantioselective catalyst for the DKR of aromatic cyanohydrin acetates. Acetone cyanohydrin functions as a cyanide source in this reaction.

to a halt. This hydrolysis was due to water attached to the hydrophobic carrier which was released into the reaction mixture, a detrimental example of partition (section 9). When the hydrophobic carrier was replaced by the porous silicate Celite R-633, CALB proved to be an excellent catalyst for this DKR starting from aromatic aldehydes (Scheme 4).[29] This porous Celite has a high water adsorption capacity, retaining water inside the pores, and thus maintaining the water activity of the system at low values. A direct comparison of both enzyme preparations under optimised conditions, demonstrated that CALB immobilised on Celite was the catalyst of choice when starting from aromatic aldehydes (Fig. 2). On examining the surface polarity properties of CALB, it is likely that the orientation of the enzyme on hydrophobic and hydrophilic carriers will be very different (Fig. 3).[30] In addition proteins undergo conformational changes when immobilised. These changes are dependent on the carrier[31] and therefore it would be surprising if an enzyme would behave in the same way when immobilised on different carriers. Although direct evidence of conformational changes for CALB is not available these factors should be taken into account when immobilising enzymes.
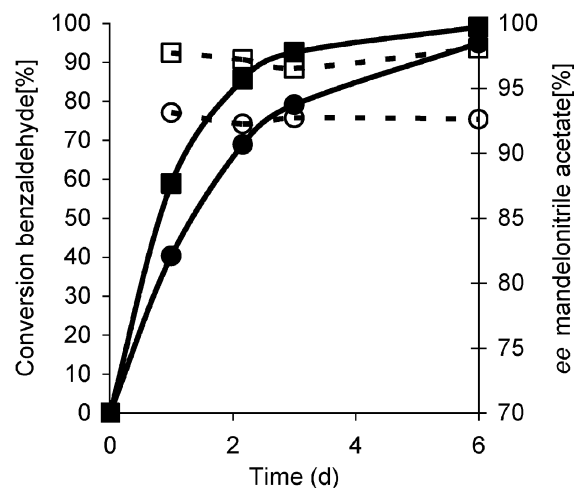
Fig. 2 Enantioselective synthesis of mandelonitrile acetate *via* DKR. CALB on Celite (conv.: ■; *ee*: □) and Novozym 435 (conv.: ●; *ee*: ○). Reprinted with permission from ref. 29. Copyright 2004, Elsevier.
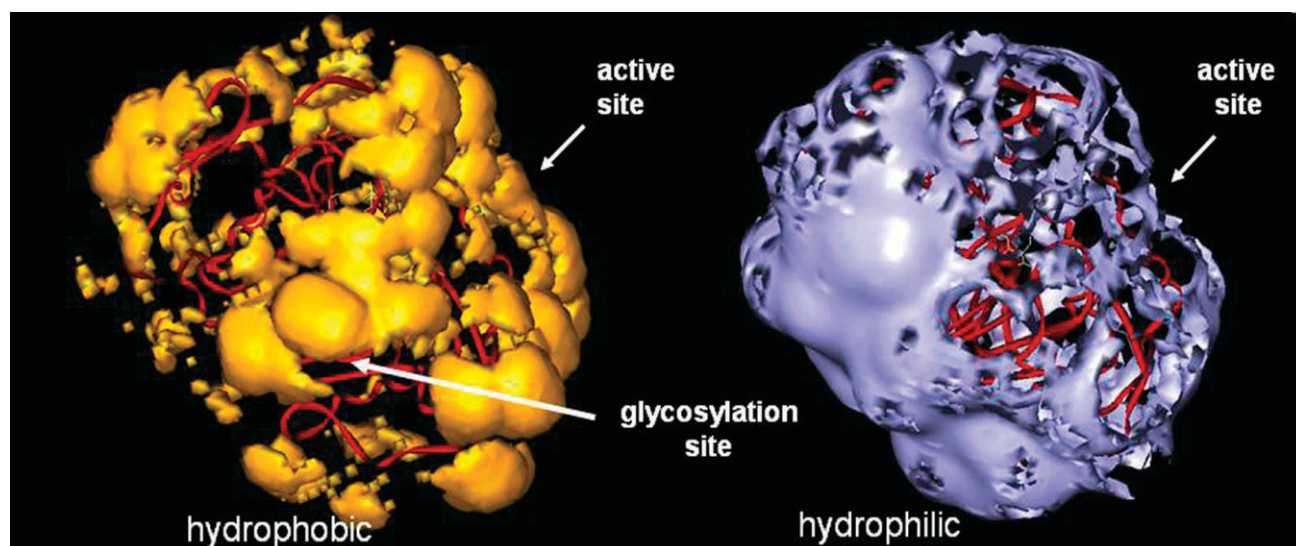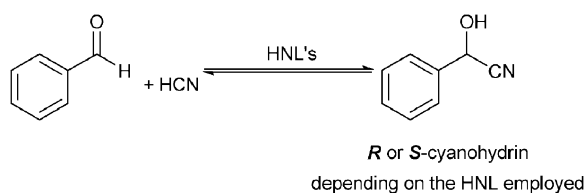
Fig. 3 Hydrophobic and hydrophilic surface areas of CALB might influence its immobilisation. Reproduced with permission from ref. 30. Copyright 2007, Wiley-VCH Verlag GmbH&Co. KGaA.

R or *S*-cyanohydrin
depending on the HNL employed

Scheme 5 HNLs catalyse the enantioselective formation of cyanohydrins.

Hydroxynitrile lyase (Oxynitrilase) from *Hevea brasiliensis* (*Hb*HNL) was immobilised on Celite. It displayed higher activity and slightly higher enantioselectivity than when immobilised on Avicel (microcrystalline cellulose) or hydrophobic polyamide Accurel EP 700. When *Hb*HNL on Celite was stored in dry organic solvents, it showed good to excellent stability. However, it was completely inactive when used in dry solvents.[28,32] Structurally different HNLs from *Prunus amygdalus*, *Manihot esculanta* and *sorghum bicolor*, were used as catalysts (Scheme 5). Each enzyme was active and selective in organic solvents only in the presence of significant water concentrations that led to the formation of a water layer, *i.e.* in a biphasic system.

4. Immobilisation *via* ionic interactions

Depending on the pH of the solution and the isoelectric point the surface of the enzyme may bear charges.[9,12] Using widely available modelling systems, the surface charge and charge distribution of an enzyme can be readily calculated and displayed.[33]

Essentially any ion exchanger can act as carrier in immobilisation *via* ionic and strongly polar interactions. Depending on the predominant charge on the enzyme (Fig. 4), the ion exchanger needs to be negatively (for instance carboxylate) or positively charged (for instance protonated amino groups).[9] The anion-exchanger polyethylenimine (PEI, containing many amino groups and no imines) was used to immobilise CALB.
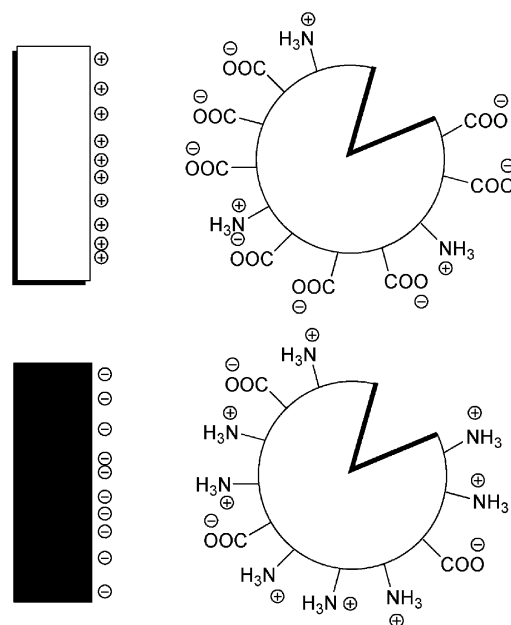

Fig. 4 Different types of ion exchangers can be used for the immobilisation of enzymes, depending on their surface charge.

Depending on the pH value and temperature chosen for the immobilisation the activity and enantioselectivity of the enzyme varied. This is most likely due to the different ionisation states of the enzyme during the immobilised process. When CALB was immobilised under conditions that were optimal for the enzyme activity it retained this optimal performance in the immobilised preparation, even when used under non-optimal conditions.[34]

The imidazole rings from histidine can act as a ligand for metal ions. In particular Cu^{2+}, Co^{2+} or Ni^{2+} have been employed to bind enzymes.[35] The enzymes need to contain easily accessible imidazole residues or a genetically introduced His tag (a short tag with six histidines, Fig. 5). The His tag has little influence on the catalytic performance of the enzymes. Benzaldehyde lyase (BAL) immobilised *via* imidazole
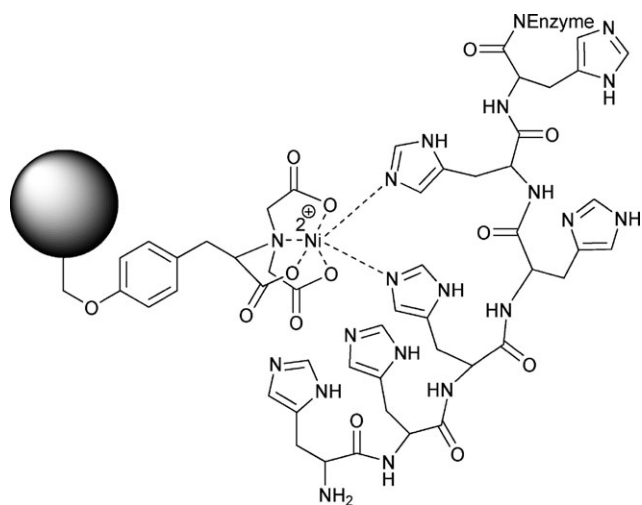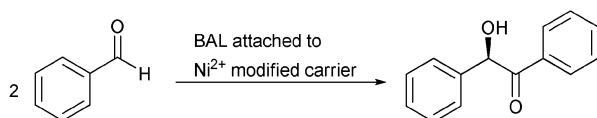
Fig. 5 Ni^{2+} attached to a carrier anchors an enzyme with a His tag to the carrier.

Scheme 6 BAL with a His tag was immobilised and catalysed the formation of benzoin.
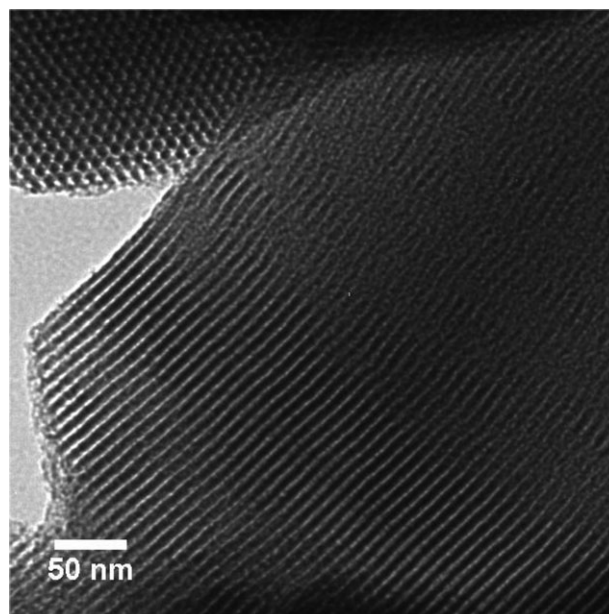

Fig. 6 TEM image of mesoporous silicate demonstrating the pore length and pore openings (top left).

materials with pore diameters which were smaller and larger than the size of the protein. In the case of the former, the protein was adsorbed (with relatively low loadings) on to

complexation of Ni^{2+} attached to a polyvinylpyrrolidinone-based matrix, could be reused several times for the formation of benzoin (Scheme 6).[36]

Mesoporous silicates (MPS) are synthesised from silane precursors using surfactant templates to yield ordered porous structures with narrow pore size distributions.[37] The diameter of the pores can be altered through appropriate selection of surfactant and the experimental conditions. Materials can be prepared with pore diameters ranging from 2 to 25 nm (Fig. 6). The large regular repeating mesoporous structures of MPS offer the possibility of adsorbing or entrapping large bio-molecules within the pores.[12] By varying the silane precursor, additional functional groups (e.g. amino, carboxylate) can be introduced into the structure to facilitate the adsorption of enzyme with retention of activity.

The ability to tailor both the surface functional groups and the diameter of the pores directly in the synthesis of the silicates opens up the possibility of shaping the structure of the materials to complement that of the enzyme. A protocol for the immobilisation of enzymes in porous materials has been described recently.[38] Adsorption of the enzymes is usually performed post synthesis and the ordered porous structure provides a sheltered or protected environment where reactions with selected substrates can proceed, with few diffusion issues. The use of trypsin immobilised in MPS as a bioreactor for the digestion of a range of proteins demonstrated that diffusion of substrate into the porous material was not rate limiting.[39] The protective environment provided by adsorption within the pores was demonstrated by the adsorption of the model redox protein cytochrome c onto
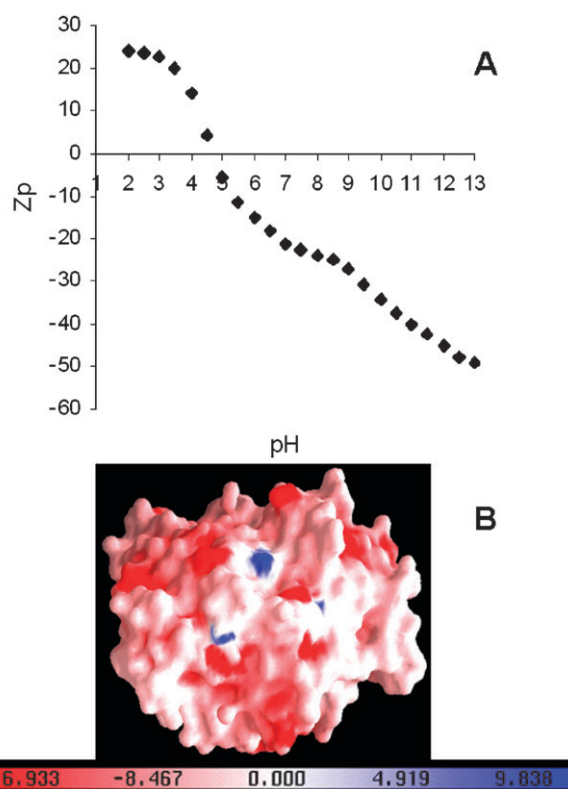
Fig. 7 Calculated (A) charge on CPO as a function of pH and (B) Poisson–Boltzmann electrostatic surface potential of CPO as calculated in GRASP at pH 7.0 (blue represents areas of positive charge and red, areas of negative charge). Reprinted with permission from ref. 41. Copyright 2007, American Chemical Society.
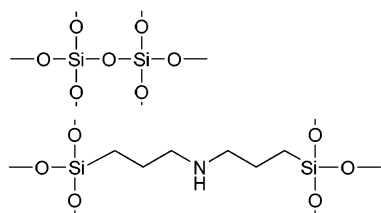
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Fig. 8 Structure of silicate and of dipropylamine modified silicates.

the external surface of the MPS. In contrast to the large pore size material, the peroxidative activity of the immobilised protein was significantly lower and less thermally stable.[40]

The immobilisation of chloroperoxidase from *Caldariomyces fumago* on to a modified MPS demonstrates how the properties of the support can be tailored to match the enzyme. Chloroperoxidase catalyses the oxidation of many different functional groups, *e.g.* the epoxidation of alkenes. Its stability, however needs to be improved. Analysis of its electrostatic surface potential (Fig. 7B) and its isoelectric point of 4.5 (Fig. 7A) indicated that it could be immobilised *via* electrostatic interactions. Taking into account the size and surface charge distribution of the enzyme (diameter 6.2 nm), tailored MPS carriers with amino groups were prepared.[41] Materials with pore diameters which were too small to accommodate
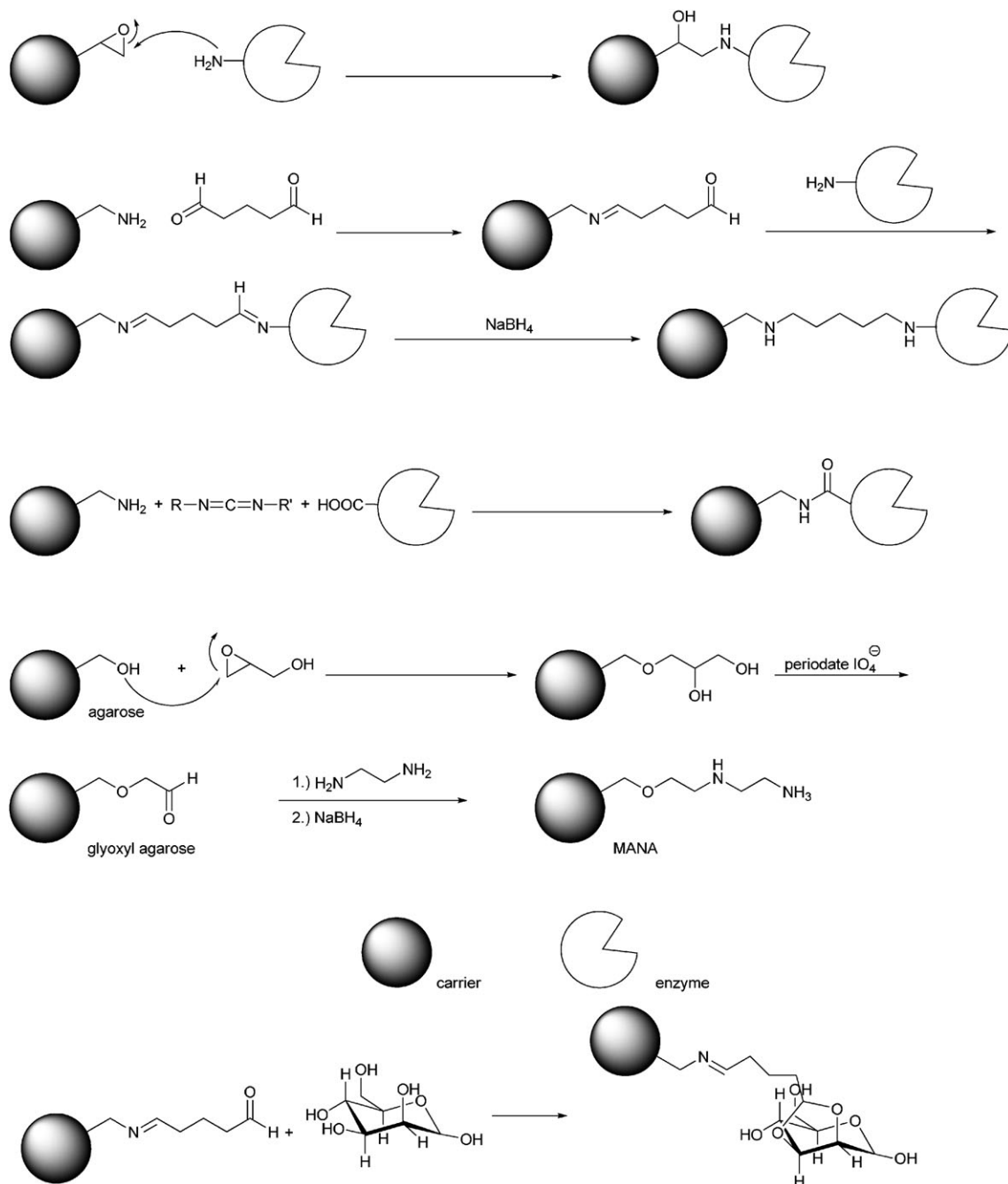

Fig. 9 Amino or acid groups on the surface of the enzyme can react with the carrier forming covalent bonds. Alternatively sugar moieties of the enzyme can be coupled to the carrier.

the enzyme did not provide a stable support. MPS with dipropylamine substituents in the silicate walls (Fig. 8) provided a stable support, adsorbing all of the enzyme from solution. The immobilised enzyme could be reused with no loss in activity over 15 cycles. Interestingly, MPS with amino groups grafted onto the silicate surface also adsorbed chloroperoxidase, but showed a steady decline in activity, with a 60% loss after 15 cycles. This loss in activity may arise from the post-synthesis grafting procedure used. For such a procedure to be successful, it is essential that the functional groups are attached in a uniform and stable manner, a process that is difficult to characterise. Incorporation of the propyl-amine functional group directly by using a modified silane precursor is a more reliable means of producing the structure required.

Ionic immobilisation is strongly dependant on the pH value and salt concentrations during immobilisation, but also during application. Similar to the leaching in aqueous media described for enzymes immobilised *via* hydrogen bonds, high salt concentrations can lead to ion exchange and washing out of the enzymes immobilised *via* ionic interactions. As already stated in Table 1 the pore size of the ion exchangers are an essential parameter that has to be part of the planning.[42]

5. Covalent binding to solid supports

Covalent binding of an enzyme to a carrier has the advantage that the enzyme is tightly fixed. Thus enzyme leaching in aqueous media is minimized and no protein contamina-tion of the product occurs. As a rule of thumb, covalent immobilisation should be preferred when working in aqueous solution and when denaturing factors exist. This is due to the fact that the formation of multiple covalent bonds between the enzyme and the carrier reduces conformational flexibility and thermal vibrations thus preventing protein unfolding and denaturation. Overall, covalently immobilised enzymes can be used in any medium whereas adsorbed enzymes should be applied in organic solvents or in pure hydrophobic reactants to avoid leaching.

A distinct disadvantage of covalent binding is that the enzyme is chemically modified. To direct such modifications is difficult but not impossible (see section 8). As might be expected, immobilisation does not occur in a uniform manner for all enzyme molecules in one batch. Nonetheless, multipoint attachment of the enzymes can be achieved linking them tightly to the carrier. This is not only limited to monomeric enzymes but also includes multimeric ones.[7,9]

Most commonly, the amino groups of the enzyme are employed for covalent immobilisation (Fig. 9). However, other functional groups on the enzyme surface can also be utilised, including sugar residues.[7,9,30] The amino group as nucleophile can attack for instance an epoxide or an aldehyde. In the case of the aldehyde the imine formed can subsequently be reduced ($NaBH_4$), ensuring irreversible immobilisation. Carbodiimides can also be used, essentially forming an amide bond to an acid group on the carrier and *vice versa*. When planning a covalent immobilisation protocol, all components of the native enzymatic preparations must be carefully considered. Most often, native enzymes are commercially
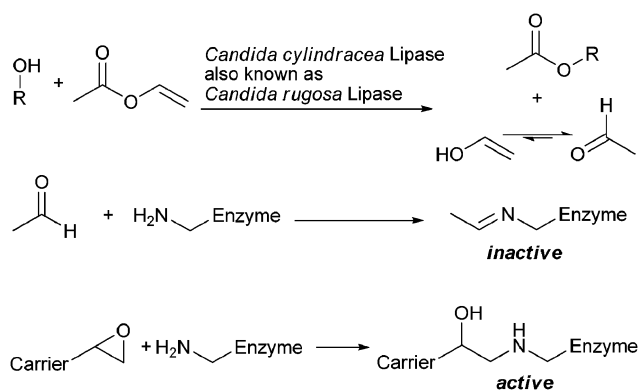

Scheme 7 *Candida rugose* lipase is protected against acetaldehyde induced deactivation when immobilised on an epoxy activated resin.

available as crude preparations containing various additives such as polyols and sugars which are commonly added as stabilisers. The actual protein content may be very low (<5%) and the interference from other reactive chemical species must always be taken into account.

As with non-covalent immobilisation, hydrophobic or hydrophilic carriers can be employed. As an additional varia-tion, reactive groups on the carrier can be attached *via* short or long spacers to the support. The reactive epoxy group has made Eupergit C and Eupergit C 250 L popular.[43] Sepabeads®, which will be discussed in more detail below, are methacrylic carriers. They can be functionalised by either epoxy or amino groups. The amino groups of the carrier can be linked to the enzyme *via* glutaraldehyde as explained above.[30] Agarose, glyoxyl agarose and the aminated glyoxyl agarose (MANA—the primary amino group is particularly nucleophilic), in addition to glutaraldehyde modified agarose or silica have over the years proven their value as carriers, too.[7]

Vinyl acetate is often used as an acyl donor in the lipase-catalysed acylation of alcohols in dry organic solvents. The side product acetaldehyde is released, which can be harmful to the enzyme, since it forms a Schiff base with the surface amino groups. *Candida rugosa* lipase (formerly named *Candida cylindracea* lipase) is particularly sensitive to this type of deactivation. When it was immobilised on an epoxy activated resin, its surface amino groups were protected and it could be repeatedly re-used (Scheme 7).[44]

Concerning the effect of polymer-enzyme spacers, longer spacers are expected to allow a wider conformational flexibility to the protein. This might be an advantage for enzymes such as lipases which are known to undergo significant conformational changes as they interact with the substrate. On the other hand, shorter spacers can confer higher thermal stability since they restrict the enzyme mobility and prevent unfolding. Supporting this, PGA from *E. coli* was shown to be more stable upon immobilisation on amino-functionalised methacrylic polymers (Sepabeads®) with shorter spacers (glutaraldehyde was used as the coupling reagent, see Fig. 9). Interestingly, the glycosylated PGA from *Pseudomonas rettgeri* (expressed in *Pichia pastoris*) showed comparable stability with both polymers due to the creation of extra covalent bonds between the sugar moiety and the polymer.[30]

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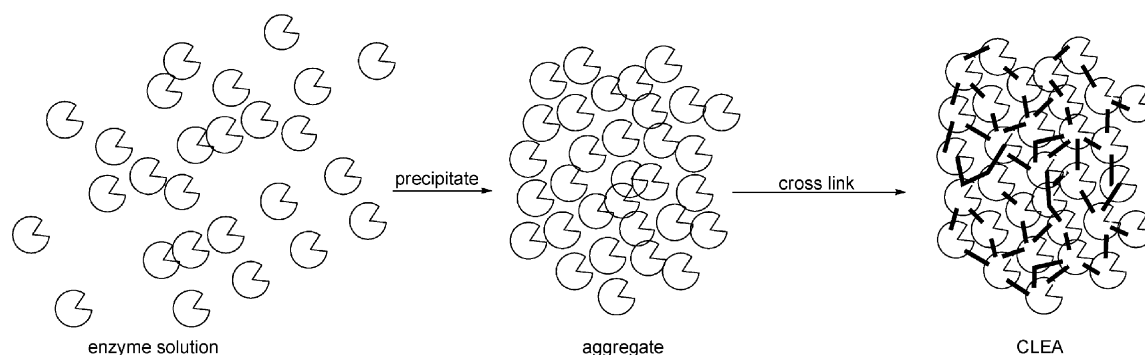
Scheme 8 Aggregation and crosslinking of an enzyme to prepare a CLEA.

6. Crosslinking of enzymes

An extreme case of covalent binding is crosslinking of enzymes by using a di-functional agent such as glutaraldehyde.[8,9] Instead of fixing the enzyme to a carrier, the enzyme here acts as its own carrier. The first step is to generate enzyme aggregates or crystals or enzymes in a spray-dried form. However, even enzymes in solution can be crosslinked. The crosslinked and thus immobilised enzyme is carrier-free, *i.e.* virtually pure enzyme is obtained eliminating the advantages and disadvantages associated with carriers.[8]

Cross Linked Enzyme Aggregates (CLEA) are prepared by first aggregating enzymes. The addition of precipitants such as acetone, ammonium sulfate, ethanol or 1,2-dimethoxyethane is followed by a crosslinker, commonly glutaraldehyde (Scheme 8). The thus obtained diimine can, but is not usually reduced. Often additives are included into the CLEAs. The lipase from *Burkholderia cepacia* (BCL) was for instance crosslinked with bovine serum albumin as protecting reagent and in the presence of dextrin.[45] When the CLEA of BCL containing dextrin was compared with the commercial Amano PS preparation, it was significantly more active. It also displayed higher activity than the xerogel of BCL (see section 7). Furthermore the BCL CLEA displayed improved enantio-selectivity. However, a catalyst should be virtually unchanged after the reaction and should be recyclable. This was not the case for the BCL CLEA. In the acylation of an alcohol with vinyl acetate in dry DIPE the CLEA rapidly lost activity (28% in the second cycle). The BCL xerogel (Fig. 10) was much more stable and could be recycled many times.[45]

7. Encapsulation

The best means of avoiding any negative influence on the structure of an enzyme is to encapsulate it. Many encapsulation methods have been developed, the sol–gel method being the most prominent and widely used technique.[4,6] Sol–gels are silica materials that are highly porous and readily prepared (Scheme 9). The sol–gel is a chemically inert glass that can be shaped in any desired way. It can be designed to be thermally and mechanically very stable, but the standard sol–gel is brittle. Sol–gels have been used extensively in the immobilisation of proteins and in particular for the development of biosensors. Although sol–gels are porous, diffusion of substrate to the enzyme can be restricted and care has to be taken to avoid this.

The synthesis of sol–gels is relatively benign for many enzymes. In the first step a tetraalkoxysilane (*e.g.* tetramethoxylsilane, TMOS) is hydrolysed *via* acid catalysis. Hydrolysis is followed by condensation and the sol is formed. This is a mixture of partially hydrolysed and partially condensed monomers. When the condensation continues the gel is formed.

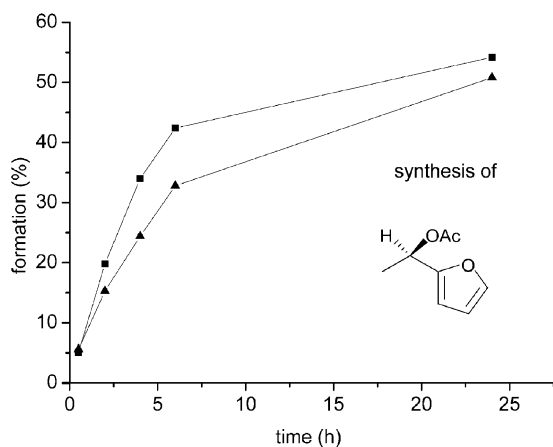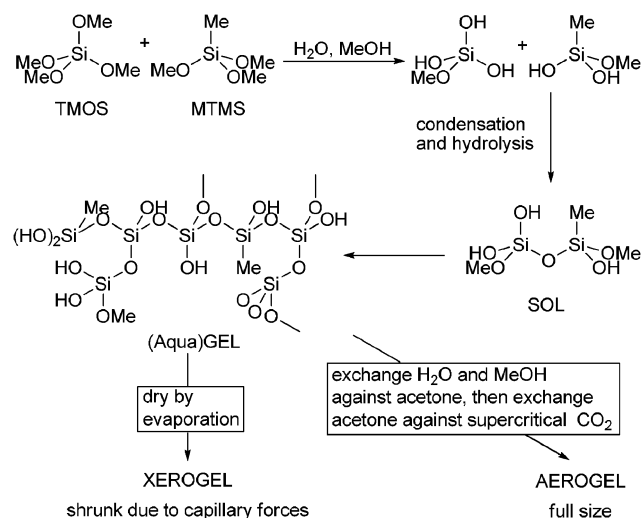
Fig. 10 BCL immobilised as lyophilised xerogel can be recycled eight times without significant loss of activity or selectivity (($\blacksquare$) cycle 1, $E = 139$, ($\blacktriangle$) cycle 8, $E = 90$).

Scheme 9 Synthesis of sol–gels.

All the pores of this gel are filled with water and alcohol, it is therefore known as aquagel. When the aquagel is dried by evaporation a xerogel is obtained. Due to the action of capillary forces during the evaporation process the aquagel shrinks and part of the structure collapses. The xerogel consequently does not have the same structure as the aquagel. To avoid such capillary action the water in the aquagel can be exchanged with acetone and then with supercritical carbon dioxide. On evaporation of the carbon dioxide the structure of the aquagel is maintained and a brittle aerogel is obtained.[4] In this manner hydrophilic aqua-, xero- and aerogels are made. By adding alkyltrialkoxysilanes (methyltrimethoxysilane, MTMS) to the synthesis mixture, sol–gels with a hydrophobic surface can be obtained. Overall the sol–gel method can generate gels with very different properties.

As discussed in preceding sections, hydrophobic surfaces can have a very positive influence on the reactivity of lipases, since they might induce interfacial activation; *i.e.* the lipase might be in its active conformation (lid open, Fig. 1). Hydrophobic sol–gels thus can activate lipases. In addition the rather brittle sol–gel can be mechanically strengthened by including porous glass beads or silica glass fibres during the sol–gel synthesis.[46,47] In this manner very active and stable lipase preparations can be obtained.

BCL has successfully been immobilised in both xerogels[45] and aerogels.[46] When BCL was immobilised in a xerogel prepared from MTMS and TMOS (Scheme 9) and subsequently lyophilised, a BCL preparation with excellent properties for acylations in dry organic solvents was obtained.[45] Residual water in dry reaction mixtures will lead to the hydrolysis of acyl donors and esters, releasing acid. This acid can significantly disturb the desired reaction; moreover due to this undesired hydrolysis more than one equivalent of acyl donor needs to be used. The target was therefore to suppress this undesired hydrolysis. Indeed, the xerogel immobilised BCL hydrolysed significantly less of the esters than Amano PS (commercial preparation of BCL) and is thus the preparation of choice in dry media. This favourable behaviour is most likely due to partitioning effects (see below). Furthermore, the xerogel could be recycled eight times with only modest loss of activity or selectivity (Fig. 10).

*Hb*HNL is structurally closely related to lipases, both have the α/β hydrolase fold structure. Given the increase in activity that was observed for lipases when immobilised in hydrophobic sol–gels, *Hb*HNL was also immobilised in a hydrophobic sol–gel. However, instead of being more active in a sol–gel, *Hb*HNL was already denatured in the sol (Scheme 9) since the methanol in the sol deactivated the enzyme. This could be avoided by removing it from the sol under vacuum before adding the enzyme. 65% of the *Hb*HNL activity was retained in the aquagel. Drying of the aquagel caused complete loss of activity, since *Hb*HNL requires a water layer to retain activity. *Hb*HNL aquagels were successfully employed in organic solvents and catalysed the formation of cyanohydrins with high enantioselectivity (reaction as in Scheme 5); the reaction being a two-phase reaction.[48] In the case of bulky substrates such as *m*-phenoxybenzaldehyde diffusion limitations were observed, a problem not uncommon in sol–gels.

Table 3 Free HNLs and their corresponding aquagels catalyse the synthesis of mandelonitrile in DIPE at r.t. Conversion percentages, *ee*'s (parentheses) and reaction times are given. Equal amounts (U) of enzymes were employed, it should however be noted that it is difficult to determine U in aquagels, due to possible diffusion limitations

*Hb*HNL		*Me*HNL		*Pa*HNL	
Free[a]	Aquagel[b]	Free[a]	Aquagel[b]	Free[a]	Aquagel[b]
4 h: 97 (97)	0.5 h: 97 (99)	4 h: 97 (98)	0.5 h: 96 (99)	4 h: 98 (97)	2 h: 97 (97)

[a] The HNL stock solution was diluted with citrate/phosphate buffer (50 mM, pH = 5.0) to a DIPE : aqueous media ratio of 5 : 1. [b] DIPE saturated with citrate/phosphate buffer (50 mM, pH = 5.0).

Table 4 Synthesis of mandelonitrile catalysed by CLEAs of different HNLs. Conversion percentages, *ee*'s (parentheses) and reaction times are given[a]

*Hb*HNL CLEA	*Me*HNL CLEA	*Pa*HNL CLEA
72 h: 55 (67)	2 h: 96 (97)	72 h: 97 (99)

[a] *Reaction conditions*: HCN (3 eq.), benzaldehyde (0.5 mmol/ml DIPE containing traces of water from the HCN solution), and the respective CLEA (6 U mmol^{-1}) were shaken at r.t.

Based on these results *Pa*HNL and *Me*HNL were also encapsulated in sol–gels. The three encapsulated HNLs were compared with the free enzymes (Table 3).[49] Both reactions were two-phase reactions, the free enzymes in a buffer layer and the aquagels filled with water. The immobilised enzymes performed better than the free enzymes; which might be due to the large surface area of the buffer layer in the aquagels when compared with the two-phase system of the native enzymes. As mentioned above, improved performance of an enzyme upon immobilisation is not always due to a higher activity of the enzyme.[18,23]

To evaluate the different immobilisation techniques, aquagel and CLEAs of *Hb*HNL, *Pa*HNL and *Me*HNL were prepared.[49] To enable direct comparison, the CLEAs were used in the presence of a buffer layer; similar to the buffer inside the aquagels. All were stable under these conditions and *Pa*HNL could be recycled ten times without loss of activity.[8] Even more interesting *Me*HNL displays remarkable stability and reactivity (Table 4, compare with Table 3) in neat organic solvents. Under these conditions the aquagel immobilised HNLs were inactive. Immobilisation as CLEA enabled the application of *Me*HNL in pure organic solvents, an application that was otherwise unattainable for these enzymes.[49]

8. Structure-based development of immobilisation

Recent research has demonstrated how the combined use of experimental and computational methods can provide rational guidelines for the selection of optimal polymeric supports as well as for the choice of immobilisation technique.

The surface polarity of lipases (from *Candida antarctica* A, and *Thermomyces lanuginosus* lipase (TLL), formerly known as *Humicola lanuginosa* lipase) and different PGAs (from *Escherichia coli* and from *Providencia rettgeri*) were analysed

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with the GRID computational method, to establish the hydrophobic and hydrophilic areas on the enzyme surface.[50]

A map of regions suitable for the establishment of interactions with different supports was created. This is of particular importance for protein orientation upon binding, since different areas will interact with different supports.

In the case of lipases which show bipolar hydrophobic/hydrophilic nature (Fig. 1 and 3) this is particularly important.

Most lipases that undergo interfacial activation need to face a hydrophobic interface to adopt the open/active conformation (Fig. 11). That means that in the case of immobilisation on a hydrophilic carrier, the lipase active site is expected to face the reaction medium and to assume a closed/inactive conformation when placed in water. When lipases are used in highly hydrophobic media (organic solvents or oils) any hydrophilic molecule (e.g. short chain alcohols in transesterification or
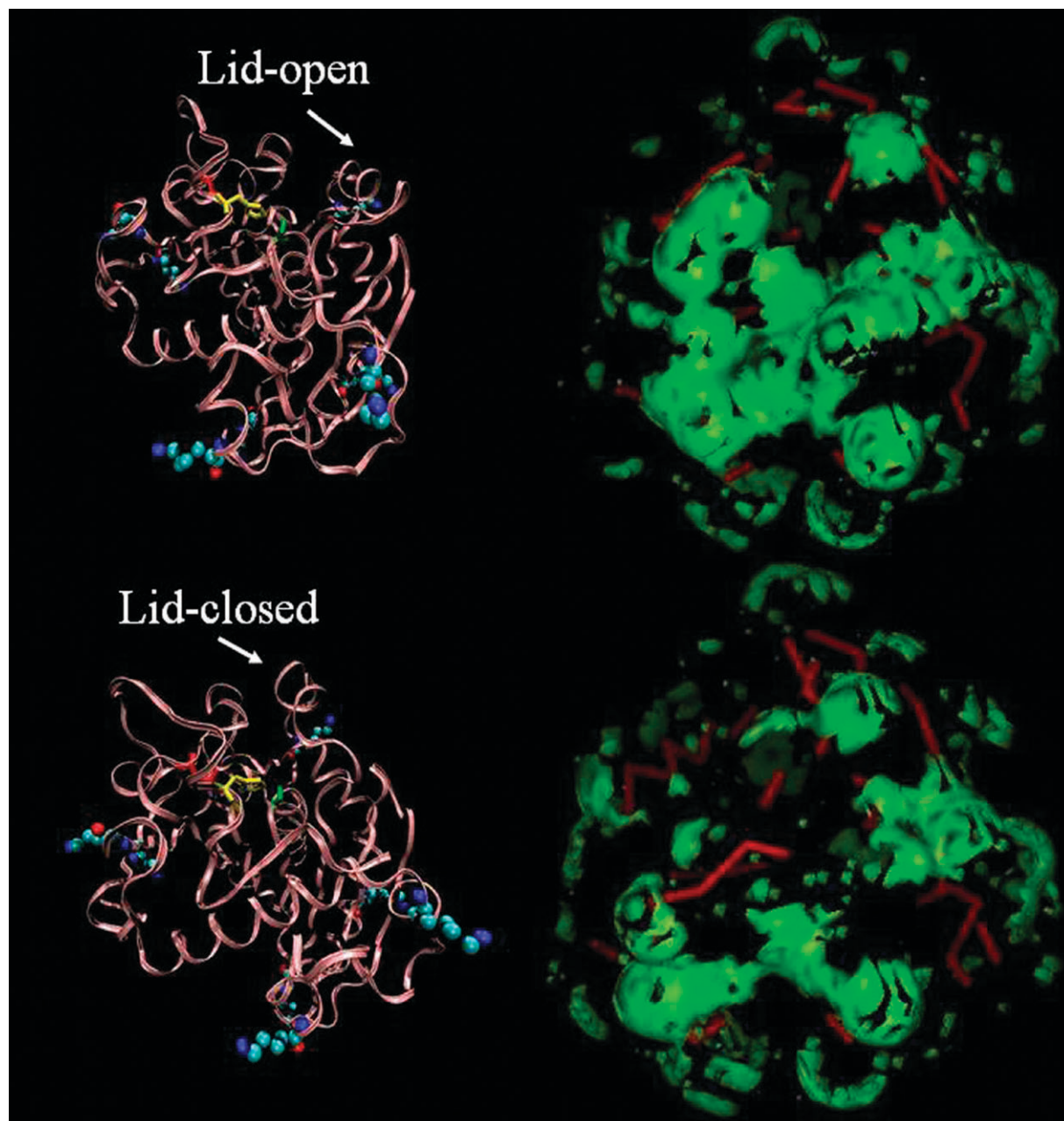

Fig. 11 Computer simulations of the structure of the lipase from *Thermomyces lanuginosus* (TLL, formerly known as *Humicola lanuginosa* lipase) in its open-active conformation (above) and the closed-inactive conformation (below). The representation on the left illustrates how the "lid" position changes and points out the catalytic triad inside the active site (green = serine; yellow = histidine; red = aspartic acid). The Lys residues on the surface are visualized using the space-filling modality and they indicate the positions that will be most probably involved in the covalent linking to functionalised supports. The simulations on the right illustrate the hydrophobic regions surrounding the opening of the active site of the enzyme (green areas) calculated with the GRID method. It is evident that the open conformation exposes a larger hydrophobic surface to accommodate hydrophobic substrates into the active site. In the closed conformation (hydrophilic environment) the hydrophobic area is hidden by the lid and a larger hydrophilic surface is exposed to water solvation.

water in esterification) is promptly adsorbed onto the carrier. This might be an advantage when the quantities are small, as described in section 3.2, but when larger quantities of water hydrate the support, then the wet particles tend to aggregate in order to minimize the surface exposed to the hydrophobic medium. This causes reduced accessibility of the biocatalyst.

In the case of enzymes such as PGA, which present a homogeneous distribution of hydrophilic zones on the surface, exploiting protein–polymer interactions for promoting favourable orientation of the protein is hardly feasible (Fig. 12).[30] However, computational analysis of the enzymes' structures concerning the location of functional groups, yields information important for covalent binding of the PGAs. In most cases, covalent binding exploits the reactivity of the nucleophilic amino groups of the lysine side chain (see section 5). Ideally, no lysine residue located in proximity of the opening of the active site should be involved in the covalent binding. In such cases alternative options such as adsorption or entrapment should be pursued.

In an analogous way, computational analysis of enzyme structures was also used as a tool for guiding the covalent immobilisation of CALB on hydrophobic Sepabeads® and to visualize the location of sugar moieties on PGA.[30] Glycosylation greatly changes the polarity of the enzyme and the sugar moieties can be used for covalent attachment. A particular enzyme may or may not be glycosylated, depending on the micro-organism source. Eukaryotic micro-organisms display *O*-glycosylation and *N*-glycosylation, the latter case being more widespread in the enzymes commonly used in biocatalysis. In most cases, *N*-glycosylation consists of 8–25 mannose units, which constitute an additional hydrophilic region

causing considerable variation of the surface properties of the enzyme. In the case of PGA it has been demonstrated that the introduction of the sugar moiety can be exploited to achieve a more active and more stable enzyme as long as a suitable carrier is selected. Computational analysis of the enzyme structure indicated that when PGA was expressed in
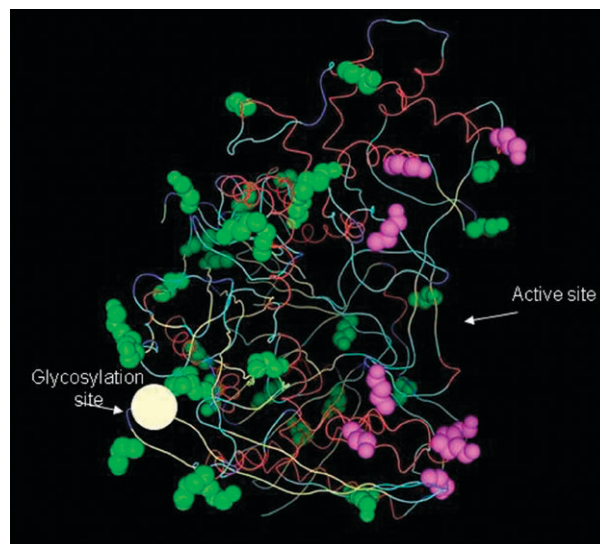

Fig. 13 Model of the structure of the PGA from *E. coli*. Lysine residues are indicated by space-filling groups. The violet residues correspond to those lysines closer to the opening of the active site. The glycosylation site (yellow) is on the opposite side. By exploiting the interactions between the glycan and the polymer a favourable orientation should be achieved.
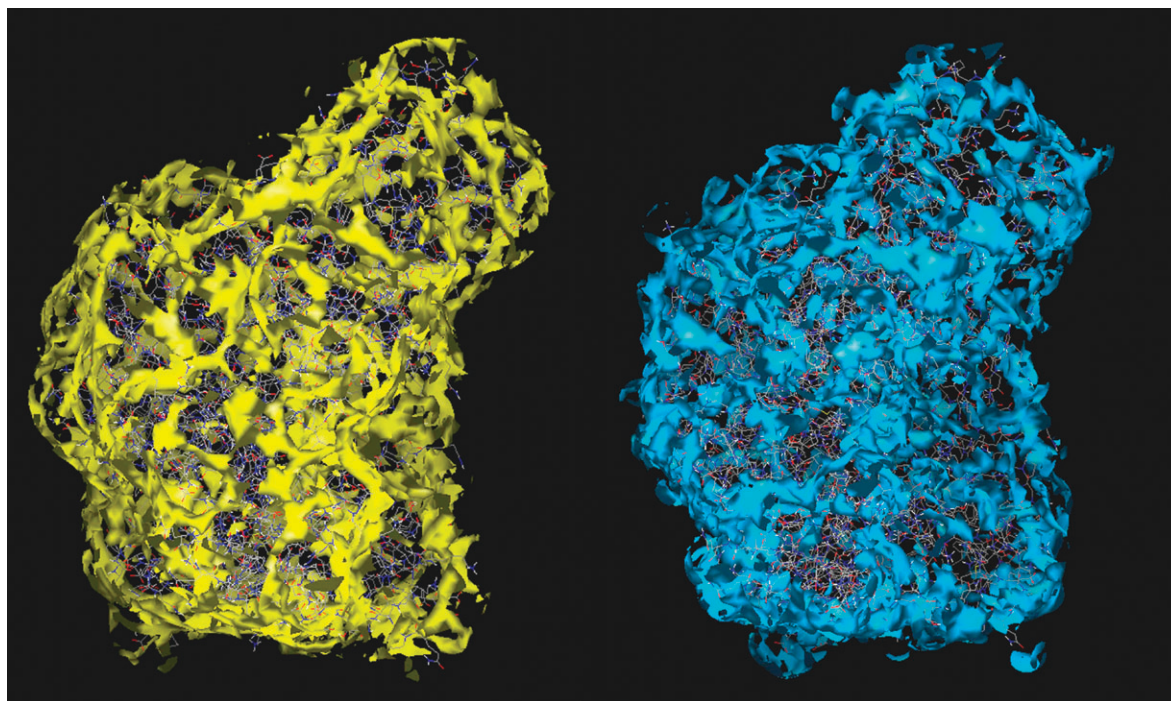

Fig. 12 Comparison of the hydrophobic areas (yellow) and the hydrophilic areas (blue) on the surface of Penicillin G amidase (PGA). A uniform distribution of the polar and hydrophobic zones on the surface of the enzyme can be noted. Reproduced with permission from ref. 30. Copyright 2007, Wiley-VCH Verlag GmbH&Co. KGaA.
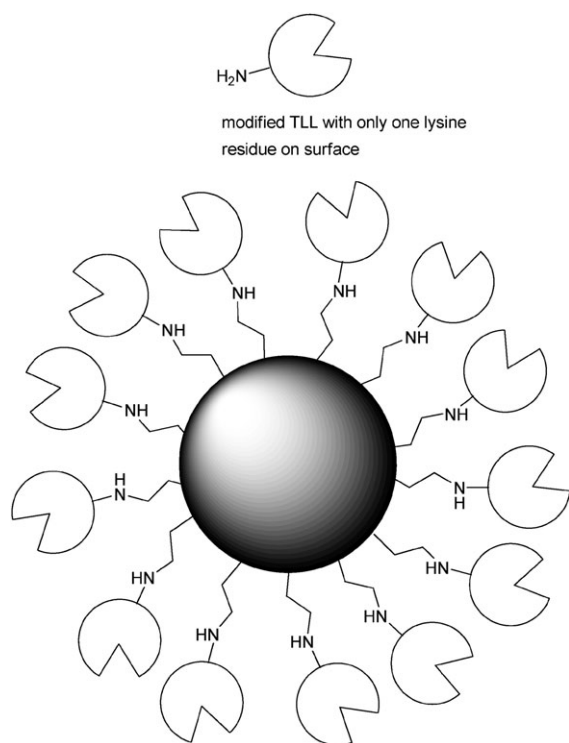
Fig. 14 Modified TLL with just one amino group on its surface, is ideally oriented when covalently immobilised.

the eukaryotic *Pichia pastoris* a sugar moiety was introduced at a location diametrically opposite to the active site (Fig. 13). By choosing a polymer (Sepabeads®) with aldehyde functionalities (obtained through pre-activation of amino groups with glutaraldehyde) and a hydrophilic nature two targets could be achieved: (i) favourable orientation of the enzyme due to hydrophilic interactions between the mannoses and the polymer, (ii) stabilisation of the protein due to the formation of cyclic acetals between the mannose units and the functionalised polymer.

Of particular interest is the recently described oriented immobilisation of lipases. The lysine residues on the surface of the enzyme were identified on the structure. Genetic engineering was utilised to remove all lysine residues close to the active site. In the case of *Thermomyces lanuginosus* lipase (TLL, Fig. 11) only one lysine residue opposite to the active

site remained. This was then utilised for the selective covalent immobilisation on Eupergit or Accurel. In this manner all the active sites of the enzyme are oriented toward the reaction mixture and higher activity of the enzyme was observed (Fig. 14).[51]

9. Partition phenomena and diffusion

Efficient biocatalysis requires that substrates and products freely diffuse inside and outside the immobilisation support. Mass transfer limitations can not only decrease the reaction rate severely, but also aggravate product inhibition or induce undesirable pH gradients. This is the case in the PGA catalysed hydrolysis of benzylpenicillin where the liberated product is the inhibitory phenylacetic acid. It should be emphasised that this well established industrial process employs biocatalysts that are expected to be reused for hundreds of cycles.[10] To minimize inhibitory effects and pH gradients inside the particles, two immobilisation methods are commonly applied on an industrial scale. The first one employs the immobilisation on methacrylic polymers with macropores (300 to 2000 Angstrom; Sepabeads®, Fig. 15) created during the polymerization process that ensure a large internal surface area and efficient mass transfer. The second method (Separase G®) consists of immobilisation of the enzyme within a loose network of highly hydrophilic polysaccharides (chitosan) that allows an efficient solvation of the enzyme and diffusion of reactants/products.

It has also been demonstrated that the reverse reaction, the PGA-catalysed synthesis of semi-synthetic beta-lactam antibiotics, is adversely affected by diffusion limitations that occur after enzyme immobilisation. Once the amide is formed and released from the active site of the enzyme, ideally the product (*e.g.* cephalexin or amoxicillin) should promptly diffuse out of the immobilisation carrier into the bulk medium (aqueous buffer). Accumulation of product inside the carrier pores will favour the hydrolytic reaction, leading to poor yields and undesired side-products. In order to distinguish between the effects due to mass transfer and the effective activity of the enzyme, titration of the active sites of PGA with the specific inhibitor (phenylmethylsulfonyl fluoride) can be performed.[52]

Partition phenomena are also of major importance in reactions that are carried out on hydrophobic substrates, which
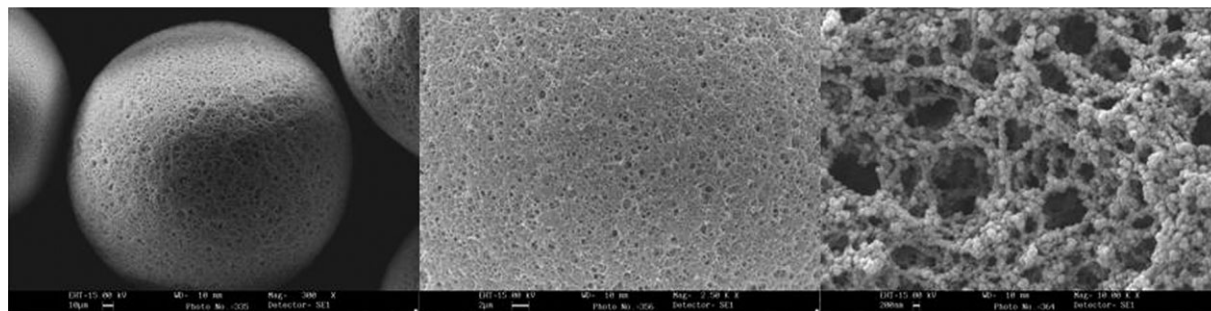

Fig. 15 Details of the structure of a methacrylic macroporous support (Sepabeads®). As the enzyme is anchored inside the pores, substrates and products are required to transfer freely from the bulk medium to the inner pores and backward. Therefore the size of the pores is crucial as well as the chemical nature of the polymer (*e.g.* hydrophilic/hydrophobic) that must favour the partition of the substrate from the reaction medium to the polymer.

Table 5 Some factors to be taken into account when planning the immobilisation of an enzyme

Immobilisation technique	Factors to be taken into account
General	Additives in the enzyme preparation that might interfere
	Stability of the enzyme under immobilisation conditions
	Stability of the carrier under operative conditions
	Protein leaching under operative conditions
	Non-specific carrier-substrates interactions
	Cost and availability of the carrier
Adsorption/deposition	
Hydrophobic organic carrier	Presence of hydrophobic regions on enzyme
	Ionic strength of the immobilisation buffer to favour protein adsorption
Hydrophilic organic carrier	Presence of hydrophilic regions on enzyme/glycosylation
Ionic interactions	pI of the enzyme
	Charged residues (type and density) on the enzyme surface
	pH and ionic strength of immobilisation buffer
Covalent binding/crosslinking	Location of the residues necessary for linking
	pH of immobilisation suitable for nucleophilic attack
	Conformational flexibility required by the catalytic mechanism
Encapsulation	Size of the enzyme
	Synthesis conditions for the polymer

Table 6 Some guidelines for the selection of the immobilisation technique. Refer also to Tables 1 and 5

Reaction system	Method of immobilisation
Dilute aqueous solution	Covalent
	Crosslinking
	Encapsulation
Dilute organic solution	Any
Concentrated, viscous organic/ inorganic mixtures	Covalent, crosslinking

will partition most favourably onto hydrophobic carriers, thus improving reaction kinetics. This is typically the case for immobilised lipases, which display remarkable differences in apparent activity as a function of the nature of the immobilisation carrier. Therefore, differences in kinetics observed between lipases immobilised on different supports are ascribable not only to conformational changes induced upon enzyme–polymer interaction (Fig. 1), by also to unequal partitioning of the substrates onto carriers which have diverse chemical natures, as well as a different accessibility of the enzymes that are anchored inside the pores.

When immobilised enzymes are used in hydrophobic media (*e.g.* non-polar organic solvents, neat oils or fatty acids and long-chain alcohols) partition of hydrophilic components onto hydrophilic carriers may severely affect the reaction. A positive effect can be obtained by exploiting the ability of a series of porous silicates to adsorb water liberated in esterification reactions, thus avoiding competing hydrolytic reactions.[53] Less desirable are unspecific adsorption phenomena that may sequestrate substrate and products and even interfere with the enantioselectivity of the process. Finally, hydrophilic molecules adsorbed on the carrier can ultimately cause particle aggregation, thus severely hampering catalysis.

10. Summary and conclusions

While immobilisation of enzymes has been largely a trial and error approach, progress in the targeted immobilisation of

enzymes is being made. Recent advances in the design of materials with tailorable pore sizes and surface functionality has enabled more precise control of the immobilisation process with retention of catalytic activity and stability. While simulation of the surface characteristics of the target enzyme can be used to aid in the design of appropriate support materials, examples of the application of molecular simulation methods for the rational development of immobilisation strategies are still limited. As the structure and mechanism of more enzymes become available, more controlled immobilisation methods will be generated. It should be noted that, even in those cases where a three dimensional structure of the enzyme is unavailable, structural models can be built up by using homology modelling methods. The examples of immobilised enzymes cited herein indicate that the choice of the immobilisation method cannot be guided simply by criteria that dictate the highest stabilisation and activity of the enzyme, but must also consider the specific configuration of the reaction that will be catalysed. In particular, successful industrial applications of biocatalysts require systems that are not only stable and active, but are low in cost and can undergo repeated re-use.

Tables 5 and 6 summarize some general recommendations that are possible to draw from all the research performed to date. But while enzyme immobilisation is slowly turning into a well understood science part of it still remains an art.

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