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Review

Advances in Enzyme Immobilisation

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Abstract

Improvements in current carrier-based immobilisation strategies have been developed using hetero-functionalised supports that enhance the binding efficacy and stability through multipoint attachment. New commercial resins (Sepabeads®) exhibit improved protein binding capacity. Novel methods of enzyme self immobilisation have been developed (CLEC, CLEA, Spherezyme), as well as carrier materials (Dendrispheres), encapsulation (PEI Microspheres), and entrapment.

Apart from retention, recovery and stabilisation, other advantages to enzyme immobilisation have emerged, such as enhanced enzyme activity, modification of substrate selectivity and enantioselectivity, and multi-enzyme reactions. These advances promise to enhance the roles of immobilisation enzymes in industry, while opening the door for novel applications.

Keywords

Biocatalyst, biocatalysis, enzyme, immobilisation, immobilization,

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Introduction

Biocatalytic process economics can be enhanced by enzyme reuse and the improvement in enzyme stability afforded by immobilisation. The capacity to retain or recover enzymes also allows biocatalyst separation from product, thereby permitting continuous processes, and prevents carry-through of protein or activity to subsequent process steps (Polizzi et al. 2007). Immobilisation can also improve enzyme performance under optimal process reaction conditions (e.g. acidity, alkalinity, organic solvents, and elevated temperatures), a requirement that has often retarded enzyme application in industrial chemical synthesis (Bommarius and Riebel 2004).

In spite of the long history and obvious advantages of enzyme immobilisation (Katchalski-Katzir and Kraemer 2000), Straathof et al. (2002) estimated that only 20% of biocatalytic processes involve immobilised enzymes. However, over the last few years a number of interesting new developments have been reported in the literature and patent applications (Spahn and Minteer 2008), indicating that enzyme immobilisation has entered an exciting new phase.

Types of immobilisation

Entrapment:

Enzyme entrapment (Fig. 1 A) is typically achieved using a polymer network such as an organic polymer or sol-gel and is usually performed *in situ* (Sheldon 2007a). Entrapment protects enzymes by preventing direct contact with the environment, thereby minimising the effects of gas bubbles, mechanical sheer

and hydrophobic solvents, but has the drawback of mass transfer limitations and low enzyme loading (Lalonde and Margolin 2002).

Figure 1

A common method of entrapment is through use of silica sol-gel matrices formed by hydrolytic polymerisation. Reetz and Jaeger (1998) use alkylsilane precursors ($RSi(OCH_3)_3$) or combinations of $RSi(OCH_3)_3$ and $Si(OCH_3)_4$ to provide heterogeneous biocatalysts with a sol-gel entrapped *Pseudomonas aeruginosa* lipase. By adjusting the polymerisation conditions the polymer porosity, network structure, surface functionalities, and particle size can all be modified. In particular the method of drying, the solvent surface tension, and polymer composition of the sol-gel allows for modulation of porosity. These gels are referred to, in order of decreasing density, as xerogels (air dried), ambigels (more hydrophobic, and hence dry with attenuated capillary stress and limited shrinkage), and aerogels (supercritical drying, with negligible shrinkage) (Pierre 2004).

Santos et al. (2008a, 2008b) have investigated polysiloxane (POS)-polyvinyl alcohol (PVA) hybrid matrices for *Candida antarctica* lipase B (CaL-B) immobilisation, and demonstrated that the percentage of PVA in the sol-gel can significantly influence the physical properties of the particle, such as hardness and surface area.

Bruns and Tiller (2005) entrapped horseradish peroxidise and chloroperoxidase in a nanophase-separated amphiphilic network consisting of a co-polymer of poly(2-hydroxyethyl acrylate) (PHEA) and bifunctional poly(dimethylsiloxane) (PDMS). Initial enzyme loading occurred in aqueous media, where the hydrophilic polymer (PHEA) network swelled to allow uptake of the enzyme. The polymer was then when placed in organic medium (*n*-heptane) in which the hydrophilic network shrank and the separate but interpenetrating hydrophobic polymer network expanded, effectively trapping the enzyme. This particle was therefore suitable for biocatalytic application in organic solvents.

Not all entrapment polymers are silicon-based. For example Lee and Huang (2008) used epoxide activated hydrogels to immobilise trypsin, using co-polymers *N*-isopropylacrylamide (NIPAAm), glycidyl methyacrylate (GMA) and *N*, *N*-dimethyl acrylamide (DMA; while Temiño et al. (2005) used PVA to immobilise and stabilise a dehydrogenase for use in organic solvents.

Encapsulation

Similar to entrapment, encapsulation protects the enzyme from the external environment but has limited application for the biocatalysis of large substrates as they are prone to mass transfer limitations (Lalonde and Margolin 2002).

Zhang et al. (2008, 2009) have recently developed an elegant method of layered enzyme entrapment and encapsulation (Fig. 2) in which β-glucuronidase was mixed with carboxymethyl cellulose and $CaCl_2$. The solution was subsequently extruded through a needle into a 1% (w/v) alginate solution. The resultant soft

capsules provided an enzyme-compatible environment. The capsules were then reacted with protamine (a small arginine-rich protein) which, being too large to enter the capsules, ionically associated with the surface alginate. The protamine was then used to precipitate silicates at the surface to form a hard silicate shell for the particle, preventing compression or swelling.

Another recent development has been the ingenious use of an emulsion containing an aqueous solution of polyethyleneimine (PEI) to encapsulate *Trametes versicolor* laccase (Kouisni and Rochefort 2008) (Fig. 3). The method uses a cross-linking agent (sebacoyl chloride) that is soluble in organic solvents (Rochefort et al. 2008). As the PEI (a highly branched polymer) and enzymes are only soluble in the aqueous phase, the subsequent cross-linking only occurs at the phase interface where the PEI and the cross-linking agent coincide, thereby forming spherical PEI membranes or microcapsules with the enzyme entrapped within.

Figure 2

Figure 3

Support based immobilisation

Immobilisation to a prefabricated support can provide rigidity, enabling the use of various reactor configurations for biocatalysis, such as fixed-bed reactors

(Kunamnei et al. 2008). However this method suffers from dilution of volumetric and specific activity as carriers can account for 90-99% of the mass or volume of the catalyst (Sheldon 2007a; Lalonde and Margolin 2002).

Adsorption is relatively simple and inexpensive method of immobilisation, and does not chemically modify the enzyme, but it has limitations as the enzyme tends to leach out, especially in aqueous solvents. This can result in difficulties in process design and down stream processing. Hence the method is best suited to immobilisation of lipases for use in organic solvents, such as commercial preparations of immobilised *Candida antarctica* lipase B (CaL-B), which include Novozyme 435 (Novozymes) and Chirazyme (Roche Molecular Biochemicals). Macroporus acrylic polymer resins such as Amberlite XAD-7 (Takaç and Bakkal 2007) can be used for enzyme adsorption, while CaL-B immobilised on VP OC1600 (Bayer) is widely used for the production of speciality chemicals (Miletić et al. 2009). Alternatively silica-based materials such as modified aerogels (Gao et al. 2009) or celite can be used. Chaplin et al. (2002) immobilised *Pseudomonas fluorescens* lipase on celite for the resolution of menthol from an eight diastereomer mix in an organic solvent based reaction. Adsorption is regularly used in large scale processes, particularly where the enzyme is inexpensive (Lalonde and Margolin 2002).

Ionic binding is another simple non-covalent immobilisation technique. Enzymes can be bound to polysaccharide biopolymers such as, dextran, agarose and chitosan. These polymer supports may be functionalised with a variety of chemical groups to achieve ionic interaction, including quaternary ammonium, diethylaminoethyl and carboxymethyl derivatives. This method has been applied commercially for glucose isomerase production of high fructose syrup (Lalonde

and Margolin 2002). Alternatively functionalised macroporus acrylic polymer resins such as Amberlite™ FPC3500 (cationic) or FPA54 (anionic) can be used. Binding is reversible, and although advantageous for re-use of the support, protein leaching is a potential problem.

The most interesting recent developments are in the area of immobilisation through covalent binding. Here the ε-amino group of lysine is typically (but not exclusively) used as the point of covalent attachment. Lysine is a relatively common amino acid in proteins, frequently located on the protein surface, is of above average reactivity, and provides good bond stability (Křenkova and Foret 2004). Epoxide groups are typically used on the support for linkage as they are relatively stable, can bind lysine, and react with protein under very mild conditions (Mateo et al. 2007c). After immobilisation the residual groups are quenched with a primary amine containing chemical such as tris(hydroxymethyl)aminomethane (Tris) to prevent further non-specific reactions (Křenkova and Foret 2004). The optimal immobilisation support would often have short spacer arms and a high density of reactive groups required for multi-point attachment, thereby providing rigidity to the enzyme (Mateo et al. 2007b,c).

The commercial support Eupergit® (Evonik, previously Degussa), a macroporus sphere (170 µm average diameter) activated with epoxides and made of a co-polymer of *N*,*N*'-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide, has long been used for enzyme covalent immobilisation, including commercial applications (Katchalski-Katzir and Kraemer 2000). Eupergit C has allowed for 15-100% retention of activity for a wide range of enzymes (Mateo et al. 2007c; Boller et al. 2002; Bulawayo et al. 2007).

Recently Sepabeads (Resindion) have begun to supersede Eupergit. Sepabead FP-EP consists of polymethylacrylate-based resin activated with epoxide functional groups for protein binding and has surface fissures providing a much greater enzyme binding surface (and hence specific catalyst loading). The fissures also provide many of the protective advantages of entrapment such as protection from gas bubbles and shear (Mateo 2007b).

The enzyme activity can be strongly influenced by the mode of immobilisation and carrier properties (Bommarius and Riebel 2004; Lalonde and Margolin 2002), with chemical modification resulting in a degree of enzyme inactivation. For example, Sepabead EP failed to immobilise *Solanum tuberosum* epoxide hydrolase, while Sepabead EP-NH_2 (which present a proportion of epoxy groups modified by ethylene diamine) bound the enzyme but gave poor residual activity. Sepabead EP-Cu (wherein a proportion of epoxy groups are modified with iminodiacetic acid and $CuSO_4$) gave full immobilisation and good activity (70%), while dextran modified glyoxyl-agarose beads gave 95% activity towards styrene oxide (Mateo et al. 2007a).

Although short linking groups assist in stability through increased rigidity, it is not always optimal for activity as it can result in steric hindrance. Inclusion of hydrophobic spacer arms (1,6 diamino hexane) during glutaraldehyde immobilisation on a silica support improved lipase activity (Ozyilmaz 2009).

To enhance binding, resins with multiple reactive functional groups have been developed (Mateo et al. 2000), such as Sepabeads EC-HFA with epoxy groups on an ethylenediamine layer (Mateo et al. 2003; Mateo 2007c). The rationale for inclusion of the amine groups is that they rapidly bind the protein through ionic

interaction with protein carboxylic acids. This aligns the protein for subsequent kinetically enhanced (through closer proximity) covalent bond formation between epoxide groups and lysine residues. Heterofunctional supports have further provided rigidity to multi-subunit enzyme complexes (Bolivar et al. 2009). Moreover, these modifications provide some selectivity in the location of protein attachment, thereby moderating steric effects.

A further multifunctional support development involves a combination of resin epoxides and thiol groups, the latter to bind free cysteine thiol side groups in a protein (Grazú et al. 2003, 2005). Chemically thiolated enzyme (wherein the thiol compound acts as a leaving group) was bound to the support through a disulphide bond. The epoxy groups on the support react subsequently with proximate amines on the surface of the enzyme, a reaction that is enhanced by adjusting the reaction medium to a higher pH.

Another useful modification involves the use of metal chelate epoxy supports (Sepabead EP-Cu). This product can purify, immobilise and stabilise poly-His-tagged enzymes in a single step (Pessela et al. 2003).

The epoxide resins can also be modified for immobilisation of multimeric enzymes. Commercial epoxy resin supports Eupergit C or Sepabeads did not give viable activity retention for trimeric uridine phosphorylase or tetrameric or purine nucleoside phosphorylase. However modification of the Sepabeads FP-EC3 with PEI or with a combination of dextran aldehyde and PEI gave 78 and 100% activity retention respectively (Rocchietti et al. 2004). Dextran aldehyde coating can also prevent enzyme inactivation by gas bubbles (Betancor et al.

2005). Other polymer compositions have also been investigated (Miletić et al. 2009).

A new method has recently been developed for producing PEI based resins. The PEI dissolves in the aqueous phase of a water-in-oil emulsion. By addition of a limited quantity of water-soluble cross-linker (such as glutaraldehyde) spherical particles of cross-linked PEI are formed. The particles can be isolated from the oil phase and used for enzyme immobilisation (Fig. 3; Jordaan et al. 2009a). Although the very high density of amine groups can be used for anionic binding of proteins, they can also be used as a basis for dialdehyde covalent cross-linking of enzymes. The high amine density and the open fibre network allows for extremely high enzyme loads of >30% g/g.

Apart from binding to individual particles, attachment to fibres is an option. Enzyme immobilisation on electrospun polymer nanofibers has been recently reviewed by Wang et al. 2009. After the initial round of binding to the functional groups on the fibre, enzyme loading can be increased by cross-linking additional enzyme to the bound enzyme (e.g. using glutaraldehyde) to yield enzyme aggregates coating the fibre (Kim et al. 2008).

Nanotechnology is of interest for enzyme immobilisation, but for industrial biotechnology immobilised enzyme nano-particles could be difficult to handle and recover by centrifugation or filtration. However magnetic nano-particles allow for facile recovery (Betancor et al. 2005; Prakasham et al. 2007). Another interesting concept is to link the enzyme first to a polymer, and then subsequently cross-link the polymers. This would allow for specific spacer arm lengths to be used. Kim and Grate (2003) used this concept, generating single

enzyme nano-particles (SEN) which were later used to provide covalently entrapped enzymes through cross-linking.

Porous surfaces limit the accessibility of large molecules such as DNA carbohydrate polymers, and proteins, and hence Luarent et al. (2008) have reviewed the increasing impact of solid surface supports for such applications. Yu and Liang (2009), with the aim of improving peroxidase loading, have taken advantage of the self assembly of polyelectrolyte (PE) coated polystyrene particles to construct three dimensional colloidal crystal arrays (CCA) and hollow colloidal crystal arrays (HCCA) with very large surface areas. Similarly micro-reactors and micro-fluidic devices are currently of interest in chemistry and enzyme immobilisation in such reactors is being studied (Křenkova and Foret 2004).

Self immobilisation

As mentioned above the use of solid supports for enzyme immobilisation can reduce the specific and volumetric activity of the biocatalyst by a factor of 10 or more. Carrier-free enzyme immobilisation is possible using bifunctional cross-linkers such as glutaraldehyde to bind enzymes to each other without resorting to a support. Physically stronger biocatalysts can be produced by cross-linking when the enzymes are in close proximity, such as protein crystals. Cross-linked Enzyme Crystals (CLEC; St Clair and Navia 1992; Fig. 4) were commercialised by Altus Biologics (Margolin 1996). Particle size varied from 1–100 µm and had high mechanical stability (partly attributable to the innate stability provided by crystallisation), and could function in organic solvents (Roy and Abraham, 2004).

As the crystals contain only one enzyme, this method ensures that no contaminating activities were present.

Unfortunately CLEC formation requires extensive protein purification and method development, and although broadly applicable, it only works for crystallisable enzymes. The crystal formation also means only one enzyme type can be incorporated into the particle. Although they functioned well, the drawback was cost (Brady et al. 2004). However, there is still interest in this area (Abraham and Bindhu 2009) and future advances in biotechnology may yet allow for renewed commercial application.

A less-expensive method of enhancing enzyme proximity for cross-linking is by simply precipitating the protein and cross-linking the aggregates to form particles of about 50-100 µm diameter (López-Serrano et al. 2002; Kaul et al. 2007). These cross-linked enzyme aggregates (CLEA) were developed in Sheldon's laboratory (Cao et al. 2001; Sheldon et al. 2005; Fig 4) and commercialised by CLEA Technologies (Netherlands). Examples of enzymes immobilised this way include nitrile hydratase (Kubáč et al. 2008) lipase (López-Serrano et al. 2002), nitrilase (Kaul et al. 2007), penicillin acylase (Pchelintsev et al. 2009), amino acylase (Bode et al. 2002) and others (Sheldon 2007b).

Through subtle modification of the cross-linking conditions the properties of a CLEA can be adjusted significantly. Cross-linkers (such as glutaraldehyde, glutaraldehyde- ethylene diamine polymers, or dextran aldehyde) may be selected for optimal activity of a specific enzyme (Kaul et al. 2007). Pchelintsev et al. (2009) found that variation in the duration of the precipitation step prior to cross-linking influenced the activity and microstructure of penicillin acylase

CLEA. Wilson et al. (2009) discovered that excess cross-linking agent reduced the enzyme conversion yield, productivity and stability, while Majumder et al. (2008) also noted that the degree of cross-linking influenced enantioselectivity.

Multimeric enzymes can disassociate, which would lead to leaching problems and loss of activity with carrier-based immobilisation where perhaps only one of the monomers was bound. Wilson et al. (2004) demonstrated that multimeric enzymes, such as tetrameric catalases, can be immobilised using the CLEA method and retain significant activity with negligible loss of protein under denaturing conditions of surfactant (SDS) and temperature. Incorporation of PEI into the CLEA (as a cross-linker) also appears to reduce oxygen related enzyme inactivation in sensitive enzymes such as nitrilase (Mateo et al. 2006).

CLEAs may require physical support to increase rigidity for some applications. Wilson et al., 2002 immobilised CLEAs by entrapment within rigid Lentikats^{TM} (polyvinyl alcohol hydrogel). Alternatively Lee et al. 2005 used mesocellular mesoporous silica particles wherein enzyme was added at high loads into the pores of silica particles and then cross-linked *in situ* to form CLEAs. The aggregates were too large to exit through the pores and could not leach out into the medium (Kim et al. 2008).

Recently a new variation on the theme of self-immobilisation has been developed. By forming a water-in-oil emulsion of dissolved lipase and surfactant in a mineral oil, addition of a bifunctional cross-linker generated spherical carrier-free enzyme particles (Spherezymes) (Fig. 3 and 4). In this process the lipase tends to migrate to the phase boundary and orientates the hydrophobic face associated with the active site towards the oil phase. The enzyme is

subsequently cross-linked to form permanent spherical enzyme particles, and the solvent removed. This method was found to provide hyper-activated lipases that were functional in both aqueous and organic solvents (Moolman et al. 2005; Brady et al. 2007). The general method could also be used to produce solid particles of other classes of enzyme, such as laccase (Jordaan et al 2009b, personal communication) and combinations of enzymes.

Spontaneous Self immobilisation

Some enzymes form large aggregates spontaneously. Sewell's laboratory has demonstrated that some nitrilases form linear polymeric bodies of considerable mass that can be recovered easier than the single monomers (Thuku et al. 2009). Alternatively macro-proteins can be used to function as a scaffold for other enzymes. An elegant technology developed by Heyman et al. (2007a) used a 12.4 kDa protein (SP1) that spontaneously assembles into homododecamer rings of 148.8 kDa, which may in turn form macro-molecular stacks. A gene for glucose oxidase was fused in frame into the SP1 gene and the resultant protein self assembled into active multi-enzyme nanotube particles supporting hundreds of glucose oxidase molecules. Heyman et al. (2007b) subsequently made the technology more sophisticated by fusing cohesin to the to the SP1 molecule. This allowed them to bind a cellulose enzyme from *Thermobifida fusca* which had been fused to dockerin. The beauty of this system is that potentially any enzyme could be fused with a dockerin and bound to the structure. The system had a high activity to support ratio.

Another method of self-assembling immobilisation involves the use of cellulose binding domains. By fusing other genes with the cellulose binding domain, it is in principle possible to bind any enzyme to cellulose (an inexpensive support). Hwang et al. (2004) fused a lipase from *Bacillus stearothermophilus* to the cellulose binding domain fragment of a cellulose gene from *Trichoderma hazianum*. This fused gene was then expressed and the lipase active protein was bound to Avicel (microcrystalline cellulose). Similarly Nahálka and Gemeiner (2006) fused the cellulose-binding domain from *Clostridium cellulovorans* (a mesophile) with thermostable enzymes from *Pyrococcus furiosus* (either glycerol kinase, phosphomanno-mutase, or GCD-mannose phosphorylase). These enzymes bound to cellulose at moderate temperatures (30-40ºC), but detached at 80-90ºC. Hence the enzymes could be used as free enzymes during the reaction but became immobilised, and therefore easily separated from the reaction mixture, once the reaction was completed and cooled.

Ho et al. (2008) used a method involving methyl methacrylate, a catalyst, and enzyme that spontaneously forms enzyme coated poly(methyl methacrylate) nano-particles at 80ºC due to a reaction involving free radicals.

Emerging new advantages of immobilisation

Enhanced performance

Enzymes tend to form aggregates in organic solvents and hence tend to be poorly accessible for the substrate. Immobilisation of enzymes has improved

their activity in organic solvents a hundred fold (Khalaf et al. 1996; Sheldon 2007a).

The high density of enzymes available through immobilisation may provide advantages. Heyman et al. (2007b) attributed higher specific activity of a cellulase to the high density of the enzyme in the immobilisation form, possibly due to cooperative effects on the substrate.

Hyper-activation for substrates has also been noted. Lipases have two conformations, open and closed (Aloulou et al. 2006; Palomo, 2008). The open form is considerably more active and is induced by activation at the interface of hydrophobic and hydrophilic phases. Locking lipases in the open conformation during immobilisation has been achieved on supports (Mateo 2007b) CLEA (López-Serrano et al. 2002; Sheldon 2007b) and Spherezymes (Brady et al. 2008).

Due to advances in molecular biology it is now also possible to modify enzymes (through directed evolution or site directed mutagenesis) to enhance immobilisation, such as generation of additional binding residues (Mateo et al. 2007c) or adaptation to a particular support (Ansorge-Schumacher et al. 2006).

Modification of substrate selectivity

Directed immobilisation involves the selection of the point of attachment through specific interactions between functional groups on the support and the enzyme, as permitted through increasing knowledge of enzyme structure. This can influence enzyme orientation and therefore activity (Palomo 2008). Modulation

of enzyme substrate selectivity, as well as a decrease of enzyme inhibition, has been achieved by selection of the immobilisation technique (Mateo et al. 2007b).

Substrate imprinting can modify the substrate preference of an enzyme when immobilising enzymes (a process known as cross-linked imprinting or CLIP). Kaulpiboon et al. 2007 used an enzyme substrate to fix the structure of cyclodextrin glycosyltransferases during rigidification by immobilisation, thereby enhancing the synthetic capability of the enzyme relative to its hydrolytic activity.

In particular, modulation of enzyme enantioselectivity is of immense interest in biotechnology for the formation of single enantiomer compounds as 54% of drug molecules are chiral and need to be of an enantiomeric purity of 99.5% (Ran et al. 2008). Typically this has been attempted by genetic engineering (May et al. 2000). However, improvement and even reversal of enantioselectivity has been achieved simply through immobilisation (Palomo 2008). Cabrera et al. (2009) demonstrated that the stereoselectivity of CaL-B lipase for hydrolysis of racemic 2-*O*-butyryl-2-phenylacetic acid changes both quantitatively (% enantiomeric excess (ee)) and qualitatively (*R* or *S* enantiomer product) when bound hydrophobically to different supports, shifting from >99% ee (*S*) on Lewatit to 95% (*R*) on octyl agarose. Although most of the success in this type of modulation of enantioselectivity has been achieved with lipases, Wang et al. (2008) also found that the esterase of *Kelbsiella oxytoca* provided a much higher enantiomeric ratio in the hydrolysis of (*R*,*S*)-ethyl mandelate when the enzyme was immobilised on Eupergit C 250 L.

Lastly, the enzyme optima for pH and temperature may change with immobilisation. Increases in temperature optima for an immobilised enzyme are

usually a function of the improved thermostability of immobilised enzymes. The observed shifts in enzyme pH optima after immobilisation are perhaps less clear, but may be a function of changes in the micro-environment (Cabrera et al. 2009).

Multi-step reactions and selective compartmentalisation

Immobilisation also permits multi-enzyme reactions, artificial metabolic pathways and chemo-enzymatic cascade processes through compartmentalisation of the individual catalysts (Sheldon 2007a). For example Brazeau et al. (2008) have developed a multi-enzyme pathway, immobilised on Eupergit C, for the synthesis of the monatin. Co-immobilisation of coupled enzyme systems can enhance activity (Betancor et al. 2006), such as where nitrobenzene nitroreductase and glucose-6-phosphate dehydrogenase were co-encapsulated in silica particles, wherein the G6PD allowed regeneration of NADPH. St. Clair et al. (2000) also demonstrated that the CLEC could also retain co-factors for redox reactions.

Selective compartmentalisation of enzymes during immobilisation could provide advantages. van Dongen et al. (2009) used a co-polymer of isocyanopeptides and styrene to form porous polymersomes with azido-derivatised horseradish peroxidise anchored to the membrane surface, hydrophobic CaL-B located in the bilayer membrane, and hydrophilic glucose oxidase located in the polymersome lumen. The three enzymes were able to perform a demonstration three sequential step reaction using glucose acetate as the initial substrate (which was subsequently deactylated by the lipase and oxidised by the glucose oxidase), yielding peroxide that was subsequently used by the peroxidise to oxidise ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)). Similar

uses of compartmentalisation and multiple enzymes (laccase and lipase) have been achieved using Spherezymes (Brady et al. 2008).

Conclusions

The proliferation of novel enzyme immobilisation methods provides a whole range of new technical possibilities. Some of these are due to methods that permit more targeted, multipoint and faster binding. Other methods, such as self immobilisation techniques, are providing higher volumetric and specific activity. Another development is the addition of structure to immobilisation, which was previously homogeneous, pointing the way to interesting new developments. These new methods can allow for reaction conditions optimal for a specific enzyme and for a particular substrate target.

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Figure Legends

Figure 1: Enzyme immobilisation strategies: entrapment (A), encapsulation (B), solid support (C) and self immobilisation (D). Enzymes are represented by circles.

Figure 2: The multi-layered encapsulation method of Zhang et al. 2008.

Figure 3: Comparison of some emulsion based enzyme immobilisation methods.

Figure 4: Comparison of some carrier-free enzyme self-immobilisation methods.

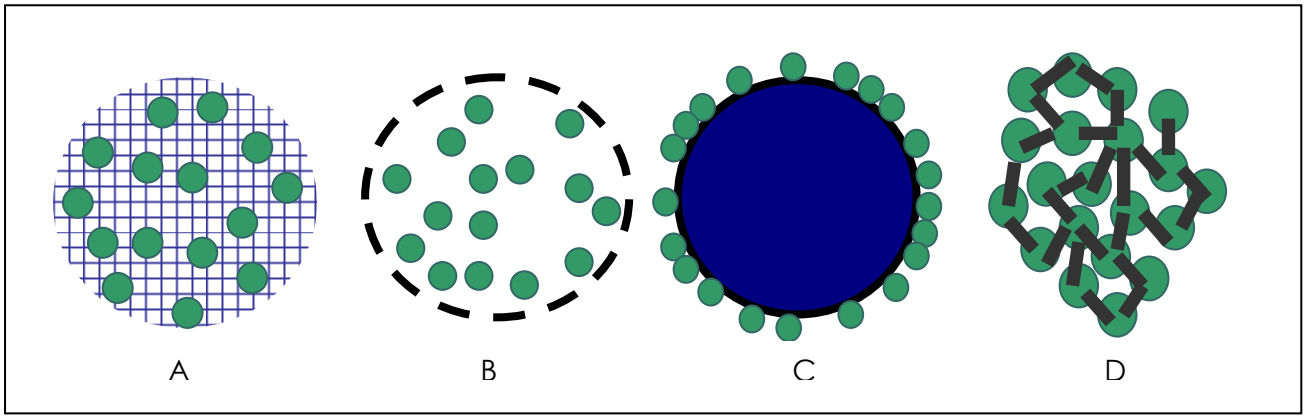

Fig 1

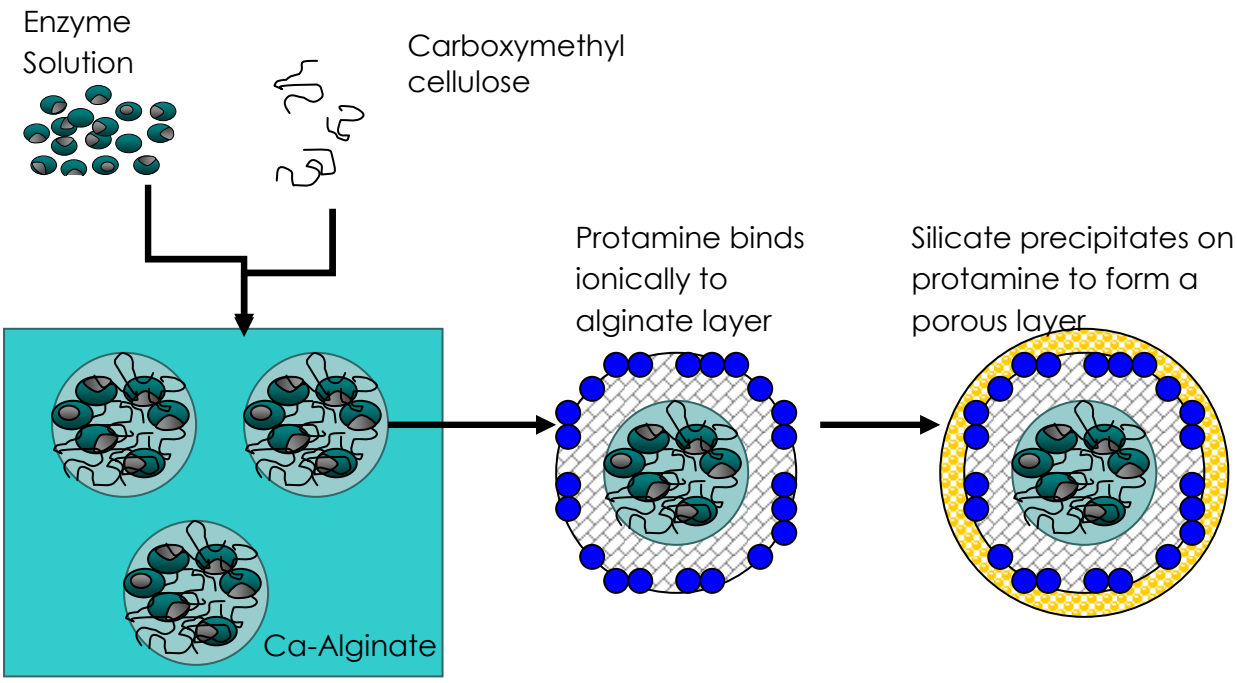
Enzyme
Solution
Carboxymethyl
cellulose
Protamine binds
ionically to
alginate layer
Silicate precipitates on
protamine to form a
porous layer
Ca-Alginate

Fig 2

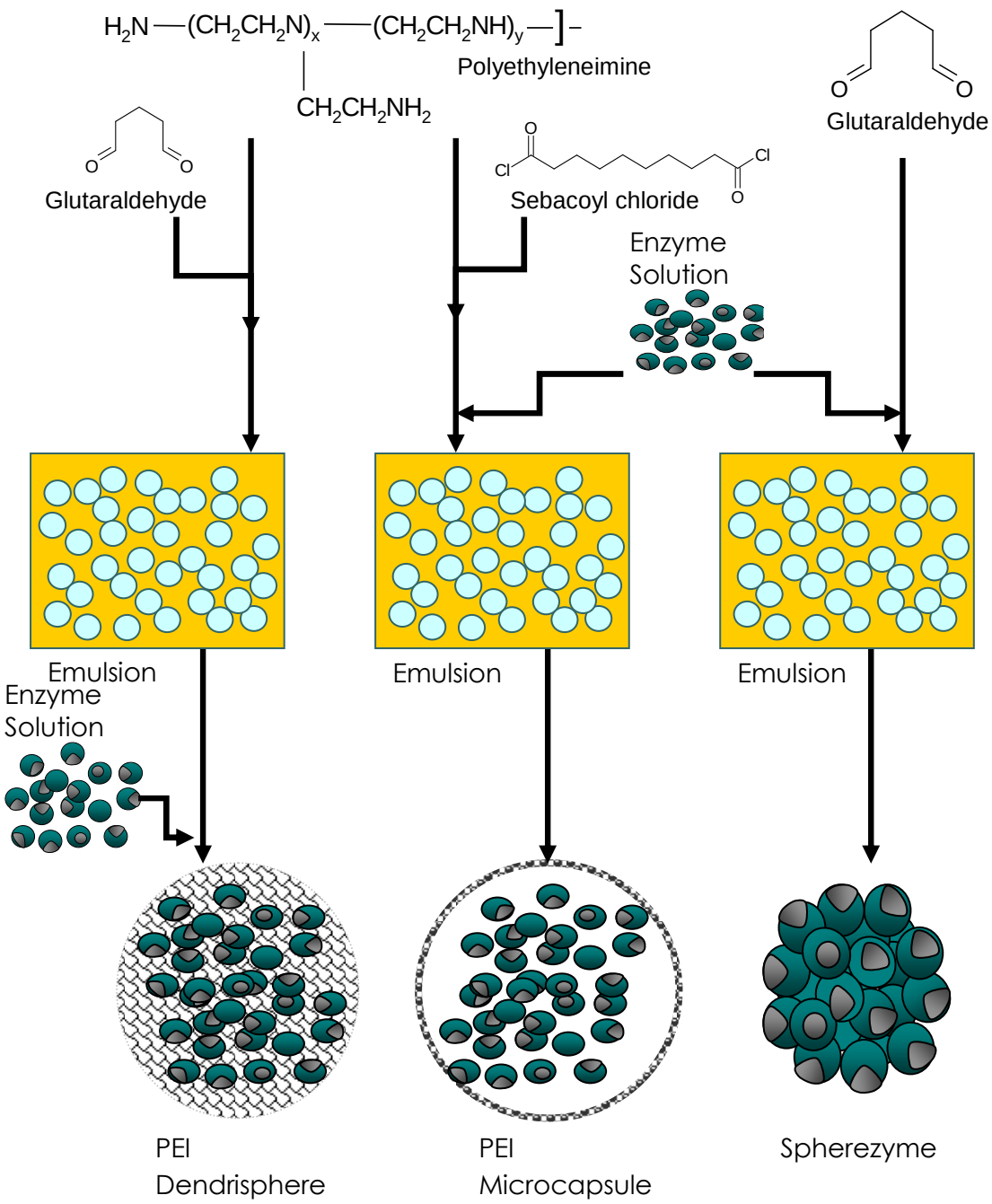
Polyethyleneimine
Glutaraldehyde
Glutaraldehyde
Sebacoyl chloride
Enzyme Solution
Emulsion
Emulsion
Emulsion
Enzyme Solution
PEI Dendrisphere
PEI Microcapsule
Spherezyme

Fig 3

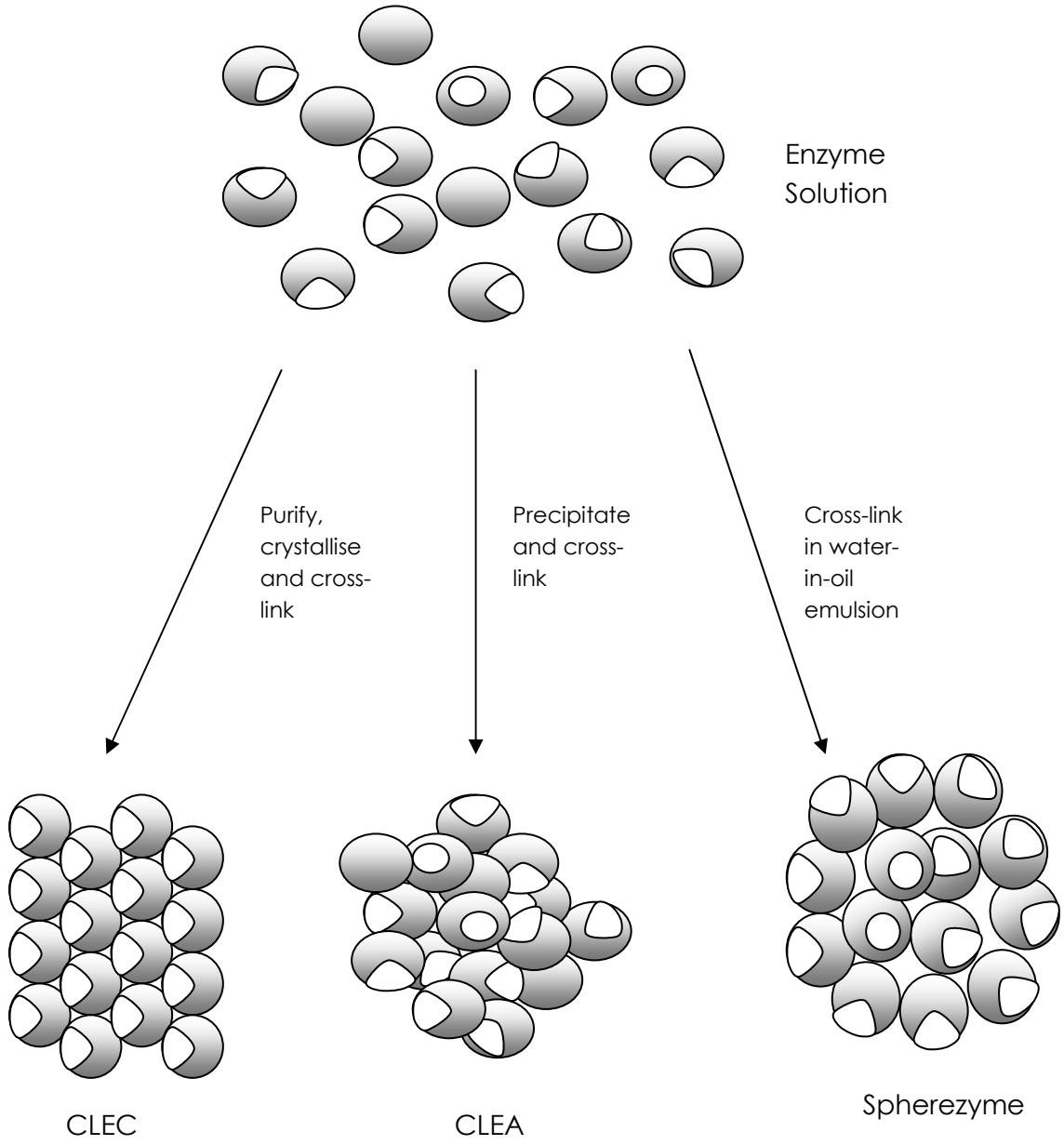
Enzyme Solution
Purify, crystallise and cross-link
Precipitate and cross-link
Cross-link in water-in-oil emulsion
CLEC
CLEA
Spherezyme

Fig 4