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REVIEW

Carrier-free immobilized enzymes for biocatalysis

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Abstract Methods for the preparation of carrier-free insoluble enzymes are reviewed. The technology of cross-linked enzyme aggregates has now been applied to a range of synthetically useful activities. Fusion proteins are also gaining momentum because they allow a relatively selective aggregation or even a specific self-assembly of the desired enzyme activity into insoluble particles in the absence of potentially denaturing chemicals required for precipitation and cross-linking. Recycling of insoluble protein particles for multiple rounds of batchwise reaction has been demonstrated in selected biotransformations. However, for application in a fully continuous biocatalytic process, low resistance to mechanical stress and high compressibility are issues for consideration on carrier-free enzyme particles.

Keywords Biocatalysis · Carrier-free immobilization · Cross-linked enzyme aggregate · Pull-down domain · Self-assembling systems

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Introduction

Insoluble enzyme preparations are useful catalysts for industrial biotransformations. Being more easily separated and recycled than their soluble counterparts, they are conducive to continuous process development and are therefore often the preferred choice of biocatalyst for manufacturing scale process operation (Buchholz et al. 2005; Bommarius and Riebel 2005). The originally soluble enzyme is usually made insoluble by its attachment to the surface of an insoluble carrier, a process called immobilization (Cao et al. 2003; Cao 2005; Sheldon 2007a). There are innumerable variants of the general procedure, attributable to the large number of different carriers available and the various exploitable strategies for fixing the enzyme to them (Cao et al. 2003; Cao 2005). Encapsulation in organic (Buchholz et al. 2005; Cao 2005) or inorganic gels (Betancor and Luckarift 2008) constitutes a special mode of carrier-mediated enzyme immobilization. An often made observation for carrier-bound insoluble enzymes is that in comparison to the base preparation, they have reduced specific activity but have acquired extra shelf-lifetime and stability under the operational conditions. Despite notable recent efforts (Hanefeld et al. 2009), the molecular basis for gain and loss of activity and stability in immobilized enzymes is not well understood. Unfortunately, design of enzyme immobilization for optimum catalyst performance is still largely empirical (Sheldon 2007a).

There are two other main drawbacks of carrier-bound insoluble enzymes. A large amount of non-catalytic mass (≥90% of the total mass of biocatalyst) is introduced to a bioreactor operating with carrier-bound enzymes. This can be a serious problem when enzymes are employed that have a low specific activity. Furthermore, the carriers used are often considerably more expensive than the enzymes attached to them. Therefore, if one managed to precipitate the target enzyme into an insoluble particle that shows good retention of the specific activity of the soluble protein and at the same time has useful resistance to mechanical stresses, a powerful carrier-free, yet heterogeneous biocatalyst might be obtained and pitfalls with the use of solid carriers could be partly eliminated. This review summarizes recent advances in methodology for carrier-free enzyme immobilization. Reported approaches are distinguished according to the amount of biological recognition and specificity involved in the process of protein aggregate formation.

Cross-linked enzyme aggregates

A solid preparation of an enzyme can principally be obtained by precipitation or crystallisation. Cross-linking with bifunctional reagents such as glutardialdehyde is usually required to stabilize the insoluble form in suspension during further application. Cross-linked enzyme crystals and aggregates have been introduced, with variable success, to the field of biocatalysis.

The method of cross-linked enzyme crystals (CLECs), discovered in the 1960s (Quiocho and Richards 1964), has since been applied to a limited number of enzymes including ribonuclease A, subtilisin, caprboxypeptidase B, alcohol dehydrogenase, and some lipases (Cao et al. 2003). The CLECs prepared often displayed useful resistance against denaturation by heat, organic solvents and proteolysis (Sheldon 2007b). Crystal size and retention of specific activity could be tuned in some cases by variation of the conditions for crystallization and cross-linking (Margolin 1996). As shown for *Burkholderia cepacia* lipase, coating of CLECs with surfactants or β-cyclodextrin could contribute to further enhancement of shelf-life and operational stability (Rajan and Abraham 2008). The scope of the CLEC technology is currently limited by the requirement for a successful crystallisation of the (purified) target enzyme.

Insoluble catalysts were also obtained by cross-linking enzymes (CLEs) directly in solution. However, the resulting enzyme preparations were difficult to handle and mechanically unstable. The precipitation required delicate balancing of a number of process variables (protein and cross-linker concentration, pH, temperature, ionic strength, mixing and others) and was therefore generally not reproducible (Cao et al. 2003).

In recognition of the limitations of CLECs and CLEs, Cao et al. (2000) introduced, and later commercialized (http://www.cleatechnologies.com), the technology of cross-linked enzyme aggregates. The original abbreviation CLEA is now also a registered trade name. As first demonstrated for penicillin G acylase, and subsequently applied to an impressive number of enzymes (e.g. lipases, proteases, amidases, esterases, phytase, oxynitrilase), CLEAs are made by a general procedure that involves salt- or solvent-induced (micro)aggregation of the enzyme or a desired combination of several enzymes, followed by chemical cross-linking to obtain a stable all-protein precipitate. For example, ammonium sulfate, *tert*-butyl alcohol or polyethylenglycol were useful to trigger the aggregation of penicillin G acylase. The cross-linking reagents can likewise be varied according to requirements of the particular enzyme used (Mateo et al. 2004; Kubáč et al. 2008). However, glutardialdehyde was most often applied. CLEAs were generally found suitable for biocatalysis in organic solvents (Cao et al. 2000). Two or more enzymes can in principle be joined in an aggregate, yielding a so-called Combi-CLEA (Mateo et al. 2006). Preparation of a Combi-CLEA containing pectinase, xylanase and cellulase activity was a notable success (Dalal et al. 2007). Combi-CLEAs might be interesting multi-purpose catalysts. They might be also useful for multi-step biocatalytic transformations (Vafiadi et al. 2008).

While CLEAs do eliminate the need to crystallize the enzyme of interest, there remains the inherent disadvantage that the exact protocol of aggregation and cross-linking must be established for each enzyme anew, implying that a substantial effort must be devoted to the optimization of conditions, as discussed below. In addition, some purification of the

crude protein will in most cases be required prior to its precipitation.

Conditions used for aggregation and cross-linking

High-throughput experimentation facilitates optimization of the different interacting process variables (precipitant and the concentration thereof, cross-linker, etc.) that are involved in the preparation of CLEAs (Schoevaart et al. 2004). Maximum retention of specific activity of the soluble enzyme is a principal concern. However, structural properties like size, porosity and resistance to mechanical stress are also important (Yu et al. 2006). Choice of the best applicable precipitant is usually empirical but can draw on the long-standing experience in protein purification (Bommarius and Riebel 2005). Several studies underscore the importance of precipitant selection in the preparation of CLEAs (López-Serrano et al. 2002).

Despite its widespread use for cross-linking of proteins, the small and highly reactive glutardialdehyde, particularly when used in high concentrations, often causes large losses of activity in enzymes (Schoevaart et al. 2004), either due to chemical modification of groups essential for function or general denaturation induced by the derivatization. Addition of substrates or ligands during cross-linking can therefore provide useful protection. It is also possible to replace glutardialdehyde by dextran polyaldehyde, a commercial polymer of 100–200 kDa that is obtained by oxidation of dextran. Due to size exclusion, penetration of dextran polyaldehyde into the enzyme precipitate is restricted as compared to glutardialdehyde and activity retention may be improved significantly, as shown for penicillin G acylase (Mateo et al. 2004). Some enzymes that were inactivated by the use of glutardialdehyde in the CLEA process like alcohol dehydrogenase and some nitrilases and nitrile hydratases could be successfully cross-linked with dextran polyaldehyde (Kubáč et al. 2008).

Addition of bovine serum albumin (BSA) or polyethylenimine during the cross-linking step served the dual purpose of stabilizing the enzyme activity and providing additional amino groups for reaction with glutardialdehyde. CLEA preparations of a lipase, penicillin G acylase, and laccase were notably improved with respect to specific activity and resistance to elevated temperature or chemical denaturation using cross-linking in the presence of BSA (Shah et al. 2006; Cabana et al. 2007). Paucity of surface amino groups in glutaryl acylase has probably prohibited production of CLEAs using the "conventional" approach. Using polyethylenimine to provide extra sites for cross-linking, stable CLEAs of this enzyme were obtained (López-Gallego et al. 2005).

At least for some CLEAs, size clearly matters and structural features of the resulting preparation are relevant for process optimization. Yu et al. (2006) showed optimum activity for CLEAs of *Candida rugosa* lipase in particles having a diameter of about 40–50 μm. Control over CLEA size is anything but easy because the (possible) influence from essentially all process variables has to be considered. An interesting development was the preparation of dendritic CLEAs of trypsin using CO_2-expanded reverse micelles, obtained from bis(2-ethylhexyl)sulfosuccinate and isooctane (Chen et al. 2006). Under raised CO_2 pressure, trypsin precipitates in the reversed micelles and could subsequently be cross-linked with glutardialdehyde. The size of the resulting CLEAs varied between 7 and 38 nm and was adjustable by varying the water-to-surfactant ratio and the concentration of the enzyme in the reverse micelle. The specific activity of dendritic CLEAs exceeded in some cases the specific activity of conventionally prepared CLEAs.

Operational stability

Published work on CLEAs has focused mostly on biocatalytic applications in organic synthesis (Sheldon 2007b). A detailed biochemical engineering analysis of the use of CLEAs in (continuous) processes is to our knowledge lacking. Several authors have recognized the problem of weak resistance of CLEAs to mechanical stresses such as those applied by stirring (Khare et al. 1991; Kim et al. 2007). CLEAs of penicillin G acylase were encapsulated in a polymeric matrix prepared from polyvinylalcohol (Wilson et al. 2004), the so-called LentiKats (Jekel et al. 1998). The resulting enzyme preparation was remarkably stable (>50 days) under shaken conditions in buffer and also resisted incubation in aqueous dioxane (70% v/v) for several hundreds of

hours. Encapsulation resulted in a decrease in activity to 60% of the free CLEAs (Wilson et al. 2004). Entrapment into hierarchically-ordered mesoporous silica is another approach for CLEA stabilization against mechanical stress. Stability of α-chymotrypsin and lipase under rigorous shaking conditions was increased more then tenfold (Kim et al. 2007).

In summary, CLEAs have become widely known in the field of biocatalysis and have proven their usefulness for preparative organic synthesis on small scale of operation. Given suitable optimization, the technology has broad scope and is in principle applicable to any available enzyme. However, it is currently not clear if conventionally prepared CLEAs are robust enough to compete with established carrier-bound insoluble enzyme preparations used in industrial scale biotransformations, the conversion of β-lactam antibiotics for example. However, CLEAs are certainly compatible with existing technologies of encapsulation that could be used to make them more resistant to operational stress factors. Application of CLEAs in fixed or fluidized bed reactors used in continuously operated processes should be more often demonstrated in the future (Hara et al. 2007; Aytar and Bakir 2008; Vafiadi et al. 2008). CLEAs of a thermostable γ-lactamase were recently employed in a flow microreactor built from a capillary column (Hickey et al. 2009). The highly miniaturized reaction system was applied for substrate screening and enzyme kinetic characterization, and the CLEA-microreactor showed useful stability under conditions of continuous processing (Hickey et al. 2009). The microfluidic reactor containing immobilized enzymes, perhaps in the form of CLEAs, could be a generally useful tool for biocatalytic process development (Hickey et al. 2007; Thomsen and Nidetzky 2009; Schwarz et al. 2009).

Selective pull-down domains

A significant improvement of CLEA production could come from a molecular design of the aggregation process such that it now features elements of biological recognition and specificity. Not only would optimization of conditions for enzyme precipitation become unnecessary, but it should also be much easier to achieve control over retention of activity and final particle size during formation of the insoluble protein.

Fusion to a highly soluble protein such as the maltose-binding protein (Kapust and Waugh 1999) is a well known approach to improve the solubility of target proteins that are otherwise prone to aggregation (inclusion body formation) under conditions of recombinant protein production. The authors have suggested exploitation of the reverse approach for production of enzyme aggregates. Conceptually, a chimeric protein is generated that harbors the enzyme of interest fused to a very poorly soluble protein such as the cellulose-binding module of *Clostridium cellulovorans* (*Cc*CBM) (Nahálka and Nidetzky 2007). Protein production in a heterologous host such as *Escherichia coli* is carried out under conditions that induce selective pull-down of the folded chimeric protein via intermolecular self-aggregation of the *Cc*CBM. The idea that enzymes trapped in inclusion bodies can be catalytically active and therefore are not completely useless is certainly not new. In their significant studies on the dynamics of protein deposition in inclusion bodies, Villaverde and Carrió (2003) showed that retention of biological function in such aggregated proteins is actually quite common.

A first case for application of the pull-down domain was provided using the dimeric flavoenzyme D-amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAO) (Nahálka and Nidetzky 2007). Upon fusion of *Cc*CBM to the *N*-terminus of the enzyme, *Tv*DAO synthesized in *E. coli* BL21 (DE3) was targeted almost completely into intracellular protein particles. Enzyme-lacking *Cc*CBM was almost completely soluble under otherwise identical conditions for recombinant protein production, indicating that the pull-down domain fulfilled the expected solubility switch function efficiently. Aggregates of *Cc*CBM-*Tv*DAO displayed about 40% of the specific activity of the purified soluble oxidase. The high activity level in oxidase aggregates was remarkable for two reasons. First of all, it indicates that *Cc*CBM shows little interference with folding and function of its fusion partner, despite the structural complexity of *Tv*DAO. Secondly, it suggests that the overall catalytic activity deposited in the aggregates was not strongly compromised by mass transfer effects or in other words, the insoluble enzyme was readily accessible to its substrates, D-amino acid and O_2. Using SDS–PAGE, it was shown that the *Cc*CBM-*Tv*DAO preparation as isolated contained mainly the protein of interest. *Cc*CBM-*Tv*DAO displayed weak bind affinity to

microcrystalline cellulose and was substantially more stable than the soluble oxidase under conditions of D-methionine conversion in a bubble-aerated bioreactor. Encapsulation of *Cc*CBM-*Tv*DAO contributed additional stability (Nahálka and Nidetzky 2007).

Nahálka and colleagues have demonstrated the scope of *Cc*CBM as a versatile pull-down domain. They recently obtained catalytically active aggregates of a maltodextrin phosphorylase (*Pyrococcus furiosus*) (Nahálka 2008), sialic acid aldolase (SAA; *E. coli* K-12) (Nahálka et al. 2008) and a polyphosphate kinase (PPK; *Silicibacter pomeroyi*) (Nahálka and Pätoprstý 2009) with useful retention (≥83%) of the specific activity of the respective soluble enzyme. *Cc*CBM-SAA particles were further entrapped in alginate hydrogels stabilized by cross-linking with glutardialdehyde (Nahálka et al. 2008). The insoluble SAA catalyst was still reasonably active (51%) and displayed high operational stability as well as useful recyclability in a repeated batch process for neuraminic acid synthesis (20 rounds of conversion). It could be freeze-dried and re-hydrated with nearly full retention of activity. Work with *Cc*CBM-PPK demonstrates yet another important advantage of selective pull-down of the target enzyme as in this case, contaminating phosphatase activity from the *E. coli* background is efficiently removed by simple washes of the insoluble protein fraction (Nahálka 2008).

In a recent study unrelated to biocatalysis, *Cc*CBM was applied to prepare functional aggregates of different lectins (Nahálka et al. 2009). A microtiter plate assay was developed in which the insoluble lectins should be used for glycoprotein recognition in a high-throughput format. *Cc*CBM fusions of two sialic acid-binding lectins were shown to "agglutinate" (precipitate) sialylated proteins under the assay conditions, leading to a positive read-out when multivalent binding by the lectin occurred (Nahálka et al. 2009).

In summary, fusion to *Cc*CBM as pull-down domain appears to be a generally applicable method of producing insoluble, yet highly active aggregates of different enzymes. Noncovalent attachment of the *Cc*CBM-enzyme to cellulose will facilitate separation of the protein particles from bulk liquid (Nahálka and Nidetzky 2007). However, additional processing steps (encapsulation, cross-linking) may be needed to finally obtain an operationally robust biocatalyst. Because aggregation under control of a pull-down domain is unlikely to enhance the intrinsic stability of the enzyme used, there is a clear potential in applying (thermo)stable catalysts (Littlechild et al. 2007), provided that extreme process conditions (like high temperature or high salt, for example) do not cause unwanted disaggregation.

Self-assembling systems

Well-known for lipids and non-natural polymers (Graff et al. 2004), the self-assembly into insoluble ordered structures is a feature recognized for a growing number of proteins (Bayley et al. 2004; Hampp and Oesterhelt 2004; Sleytr et al. 2004). Using chimeric proteins in which the enzyme of interest is fused to a module capable of self-assembly it could be possible to obtain insoluble enzyme preparations that have a well defined, perhaps crystalline-like association state. Exploitation of self-assembly properties may allow one to achieve relatively precise control over size and shape of the resulting enzyme particles. Although of interest, encapsulation through self-assembly of another protein or peptide entity (disjoined from the target enzyme) is not considered here.

Fusion to a cell surface layer protein

Cell surface layers (S-layers) are common "protein membrane" structures of the cell envelope of archaea and bacteria (Schuster et al. 2008). They have a lattice-like appearance and are formed through a self-assembly process. Isolated protein units of natural S-layers are capable of recrystallization into precisely defined monolayers in suspension and at different interfaces. These properties of S-layer proteins have promoted a wide range of (nano)biotechnological applications (Sleytr et al. 2007). Although fusion to an S-layer protein has been explored for protein array development (Tang et al. 2008) and other analytical purposes (Sleytr et al. 2007), there are surprisingly few examples showing application of S-layer technologies to enzyme immobilization and biocatalyst development. However, a notable common feature of S-layer fusions was retention of the original function of the individual components of the chimeric protein, that is, the self-assembly properties and the respective biological activity (Sleytr et al. 2007).

A thermophilic laminarinase (LamA from *Pyrococcus furiosus*) fused to the S-layer protein SbpA from *Bacillus sphericus* was produced as an insoluble aggregate in *E. coli* that was unfortunately not characterized. However, after solubilization by chemical unfolding and renaturation, the SbpA-LamA fusion was successfully immobilized on different solid supports (Tschiggerl et al. 2008). Liposomes featuring a self-assembled fusion of an enzyme and an S-layer protein on the surface constitute an interesting approach towards potentially useful biocatalysts. The easily tuneable size of liposomes could provide flexibility in design of the immobilized enzyme preparation. Using glucose-1-phosphate thymidyltransferase fused to the *C*-terminus of the S-layer protein SgsE from *Geobacillus stearothermophilus*, Schäffer et al. (2007) also demonstrated the possibility for recycling of the insoluble, liposome-supported catalyst.

It is worth noting that production of S-layer fusion proteins in *E. coli* and other hosts (*Saccharomyces cerevisiae* or HeLa cells) appears to generally yield insoluble products resulting from intracellular self-assembly or aggregation (Blecha et al. 2005). These "inclusion bodies" might already be useful as carrier-free insoluble enzyme preparations.

Fusion to other scaffolding proteins

Proteins forming high-molecular weight assemblies have been used for display of catalytic modules on the surface of the resulting insoluble supramolecular structures. Relevant examples include the stress-responsive 145.8 kDa protein SP1 from aspen plant (*Populus tremula*) which forms a ring-shaped homododecamer (Wang et al. 2002) and is highly resistant to extreme conditions such as low and high pH, high temperature (T_m of 107°C), organic solvents and the presence of various proteases (Wang et al. 2006). A construct in which SP1 was fused to glucose oxidase (Gox) from *Aspergillus niger* was produced as an insoluble aggregate in *E. coli*. Thus precipitated oxidase showed substantially improved stability as compared to the native enzyme. The non-catalytic mass of the Gox-SP1 aggregate was only 10% (w/w) of the total particle mass. Gox-SP1 was assembled into nanotube structures containing hundreds of enzyme molecules per tube (Heyman et al. 2007).

In another, however, rather preliminary example, *Candida antarctica* lipase B was designed for protein particle formation by joining the enzyme to a capside protein of the potato virus (Carette et al. 2006). Insoluble virus protein obtained through co-expression of the fusion protein and the native capside protein in the plant *Nicotiana benthamiana* displayed esterase activity towards *p*-nitrophenyl caproate as substrate.

Fusion to an elastin-like polypeptide

Elastin-like polypeptides (ELPs) of variable length containing the pentapeptide repeat Val-Pro-Gly-Xxx-Gly where Xxx is any amino acid (Meyer and Chilkoti 1999) precipitate reversibly upon heating above the transition temperature. Numerous ELP fusions were developed with the aim of facilitating downstream processing recombinant proteins through their selective precipitation from crude cell extracts of the host organism (Meyer et al. 2001). The technology holds considerable promise for biocatalysis but has not been widely applied in the field (Fujita et al. 2009). One could imagine a general procedure in which an aggregate of ELP-enzyme is formed first and stabilized by an ensuing cross-linking step. Methods for cross-linking of elastin-like polypetides are proposed in the literature (Lim et al. 2007).

Fusion to proteins binding to polyhydroxyalkanoate granules

Polyhydroxyalkanoates (PHAs) are naturally occurring polyesters, found in various bacteria that stockpile them for energy storage (Madison and Huisman 1999). A wide range of bioplastic materials have been prepared from PHAs (Lu et al. 2009). An interesting and exploitable feature of PHAs is that the biosynthetic enzyme (PHA synthase) remains covalently attached to the emerging polyester granule. PHA synthase can be expressed in a wide range of host organisms (Rehm 2003). Fusion proteins harboring an active PHA synthase should therefore produce PHAs during their recombinant production and at the same time attach themselves to the polyester particles. Proof of concept (for biocatalysis) was obtained using a β-galactosidase-PHA synthase fusion (Peters

Table 1 Semi-quantitative comparison of methods used for preparation of carrier-free insoluble enzymes

	CLECs	CLEAs	Pull-down domains	S-layers	Silaffins
Activity retention[1]	$+\ldots+++_a$	$+\ldots+++_e$	$+_l\ldots++_m$	$+_o$	$+\ldots++_q$
Ease of reuse[2]	$+++_b$	$+_f$	$+_m\ldots+++_n$	$++_o$	$+++_r$
Activity after recycling[3]	$++_b$	$+_{g,h}\ldots+++_i$	$+++_l$	$+_o$	$+_r$
Mechanical stability[4]	$+_a$	$+++_j^7$	$++_l$	n.s.	n.s.
Thermal stability[5]	$++\ldots+++_a$	$+_f$	$++_n$	$++_p$	$+_q\ldots+++_s$
Storage stability[6]	$+_c^8$	$++_k\ldots+++_i$	$+++_l^9$	$+++_o$	$+\ldots++_s$
Production steps	Enzyme purchase/ production, crystallization, cross-linking$_d$	Enzyme purchase/ production, precipitation, cross-linking$_j$	Cloning + expression, product isolation, cross-linking, (entrapment)$_n$	Cloning + expression, product isolation, self assembly$_p$	Cloning + expression, product isolation, silification$_q$

CLECs Cross-linked Enzyme Crystals, *CLEAs* Cross-linked Enzyme Aggregates. *1* 0–75% of native enzyme: '+', 75–100%: '++', ≥100%: '+++'. *2* Centrifugation with more than 5000×*g*: '+', ≤5000×*g*: '++', ≤1500×*g*, sedimentation used or suitable for continuous-flow systems: '+++'. *3* <100% in second cycle: '+', 100% in up to ten cycles: '++', 100% in more than ten cycles: '+++'. *4* Retention of full activity under average agitation conditions for ≤1 day: '+', ≤2 days: '++', >2 days: '+++', 'n.s.': not specified. *5* Extension of half life at high temperatures, compared to native enzyme ≤5-fold: '+', ≤10-fold: '++', >10-fold '+++'. *6* Assuming exponential decay of activity during storage, decay constant $\lambda \geq 0.2$ month^{-1}: '+', $\lambda > 0.02$ month^{-1}: '++', $\lambda \leq 0.02$ month^{-1} or suitable for lyophilization: '+++'. *7* CLEAs encapsulated in LentiKats. *8* Surfactant-coated CLECs. *9* Lyophilization possible, but no storage time indicated

a Margolin (1996), *b* Haering and Schreier (1998), *c* Rajan and Abraham (2008), *d* Roy and Abraham (2006), *e* López-Serrano et al. (2002), *f* Dalal et al. (2007), *g* Cabirol et al. (2008), *h* Zhao et al. (2008), *i* Rajendhran and Gunasekaran (2007), *j* Wilson et al. (2004), *k* Aytar and Bakir (2008), *l* Nahálka et al. (2008), *m* Nahálka (2008), *n* Nahálka and Nidetzky (2007), *o* Schäffer et al. (2007), *p* Tschiggerl et al. (2008), *q* Marner et al. (2009), *r* Chien and Lee (2007), *s* Poulsen et al. (2007)

and Rehm 2006). A sandwich-type binding of β-galactosidase to a polyester particle displaying a PHA synthase fusion with anti-β-galactosidase scFv (single chain variable fragment of an antibody) was demonstrated (Grage and Rehm 2008). While potentially useful for analytical purpose, it would seem that simple procedures of attaching the enzyme of interest to PHAs are preferred in biocatalysis.

An alternative way of immobilizing enzymes on PHAs might be through fusion with a phasin. The phasins are small (14–30 kDa) amphiphilic proteins which are the main constituents of the PHA-granule associated proteome (Moldes et al. 2004; Neumann et al. 2008). Co-expression of genes encoding the relevant phasin fusion and PHA biosynthetic enzymes might provide the desired enzyme activity immobilized on PHA granules.

(Auto)encapsulation using biomimetic silica

The silaffin polypeptide from the diatom *Cylindrotheca fusiformis* induces precipitation of silica from monosilicic acid solution (Kröger et al. 2002). The repeat unit of the silaffin polypeptide, the R5 peptide (H_2N-SSKKSGSYSGSKGSKRRIL-COOH), was utilized to immobilize butyrylcholinesterase. Highly active and stable biosilica spheres were obtained (Luckarift et al. 2004). The addition of R5 peptide to a solution containing enzyme and silicic acid promoted bio-silification at room temperature and under mild chemical reaction conditions (Naik et al. 2004). Using R5 fusions, phosphodiesterase and organophosphate hydrolase peptide were recently produced as immobilized enzyme preparations in *E. coli*. Each of the fusion proteins initiated silica polycondensation, and

enzymatic activity (69–97%) was retained in the resulting silica spheres (Marner et al. 2009). An additional metal ion complexing peptide $(His)_6$-tag enabled direct recovery of the silified insoluble catalyst from crude cell extract, as demonstrated for D-amino acid oxidase (Chien and Lee 2007).

Conclusions

Table 1 compares, on a semi-quantitative basis using different criteria, the methods of carrier-free immobilization discussed in this paper. The pros and cons related to biocatalyst preparation and process operation are summarized. Among the methods available, CLEA technology is clearly the one most advanced and widely used. However, fusion proteins designed for aggregation could improve the current protocols for production of CLEAs. Encapsulation may in all cases be needed to eventually obtain mechanically stable biocatalysts.

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References

Aytar BS, Bakir U (2008) Preparation of cross-linked tyrosinase aggregates. Process Biochem 43:125–131

Bayley H, Braha O, Cheley S, Gu L-Q (2004) Engineered nanopores. In: Niemeyer CM, Mirkin CA (eds) Nanobiotechnology. Wiley-VCH, Weinheim, pp 93–112

Betancor L, Luckarift HR (2008) Bioinspired enzyme encapsulation for biocatalysis. Trends Biotechnol 26:566–572

Blecha A, Zarschler K, Sjollema KA, Veenhuis M, Rödel G (2005) Expression and cytosolic assembly of the S-layer fusion protein mSbsC-EGFP in eukaryotic cells. Microb Cell Fact 4:28

Bommarius AS, Riebel BR (2005) Biocatalysis. Wiley-VCH, Weinheim

Buchholz K, Kasche V, Bornscheuer UT (2005) Biocatalysts and enzyme technology. Wiley-VCH, Weinheim

Cabana H, Jones JP, Agathos SN (2007) Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. J Biotechnol 132:23–31

Cabirol FL, Tan PL, Tay B, Cheng S, Hanefeld U, Sheldon RA (2008) *Linum usitatissimum* hydroxynitrile lyase cross-linked enzyme aggregates: a recyclable enantioselective catalyst. Adv Synth Catal 350:2329–2338

Cao L (2005) Carrier-bound immobilized enzymes: principles, applications and design. Wiley-VCH, Weinheim

Cao L, van Rantwijk F, Sheldon RA (2000) Cross-linked enzyme aggregates: a simple and effective method for the immobilization of penicillin acylase. Org Lett 2:1361–1364

Cao L, van Langen L, Sheldon RA (2003) Immobilised enzymes: carrier-bound or carrier-free? Curr Opin Biotechnol 14:387–394

Carette N, Engelkamp H, Akpa E, Pierre SJ, Cameron NR, Christianen PCM et al (2006) A virus-based biocatalyst. Nat Nanotechnol 2:226–229

Chen J, Zhang J, Han B, Li Z, Li J, Feng X (2006) Synthesis of cross-linked enzyme aggregates (CLEAs) in CO_2-expanded micellar solutions. Colloids Surf B 48:72–76

Chien L-J, Lee C-K (2007) Biosilicification of dual-fusion enzyme immobilized on magnetic nanoparticle. Biotechnol Bioeng 100:223–230

Dalal S, Sharma A, Gupta MN (2007) A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities. Chem Cent J 1:16

Fujita Y, Mie M, Kobatake E (2009) Construction of nanoscale protein particle using temperature-sensitive elastin-like peptide and polyaspartic acid chain. Biomaterials 30:3450–3457

Graff A, Benito SM, Verbert C, Meier W (2004) Polymer nanocontainers. In: Niemeyer CM, Mirkin CA (eds) Nanobiotechnology. Wiley-VCH, Weinheim, pp 168–184

Grage K, Rehm BHA (2008) In vivo production of scFv-displaying biopolymer beads using a self-assembly-promoting fusion partner. Bioconjug Chem 19:254–262

Haering D, Schreier P (1998) Novel biocatalysts by chemical modification of known enzymes: cross-linked microcrystals of the semisynthetic peroxidase seleno-subtilisin. Angew Chem Int Ed 37:2471–2473

Hampp N, Oesterhelt D (2004) Bacteriorhodopsin and its potential in technical applications. In: Niemeyer CM, Mirkin CA (eds) Nanobiotechnology. Wiley-VCH, Weinheim, pp 146–167

Hanefeld U, Gardossi L, Magner E (2009) Understanding enzyme immobilisation. Chem Soc Rev 38:453–468

Hara P, Hanefeld U, Kanerva LT (2007) Sol–gels and cross-linked aggregates of lipase PS from *Burkholderia cepacia* and their application in dry organic solvents. J Mol Catal B 50:80–86

Heyman A, Levy I, Altman A, Shoseyov O (2007) SP1 as a novel scaffold building block for self-assembly nanofabrication of submicron enzymatic structures. Nano Lett 7:1575–1579

Hickey AM, Marle L, McCreedy T, Watts P, Greenway GM, Littlechild JA (2007) Immobilization of thermophilic enzymes in miniaturized flow reactors. Biochem Soc Trans 35:1621–1623

Hickey AM, Ngamsom B, Wiles C, Greenway GM, Watts P, Littlechild JA (2009) A microreactor for the study of biotransformations by a cross-linked γ-lactamase enzyme. Biotechnology J 4:510–516

Jekel M, Buhr A, Willke T, Vorlop K-D (1998) Neuartige Geleinschlußimmobilisate (LentiKats) in der Biotechnologie. Chem lngenieur Technik 70:438–441

Kapust RB, Waugh DS (1999) *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci 8:1668–1674

Khare SK, Vaidya S, Gupta MN (1991) Entrapment of proteins by aggregation within sephadex beads. Appl Biochem Biotechnol 27:205–216

Kim MI, Kim J, Lee J, Jia H, Na HB, Youn JK, Kwak JH, Dohnalkova A, Grate JW, Wang P, Hyeon T, Park HG, Chang HN (2007) Cross-linked enzyme aggregates in hierarchically-ordered mesoporous silica: a simple and effective method for enzyme stabilization. Biotechnol Bioeng 96:210–218

Kröger N, Lorenz S, Brunner E, Sumper M (2002) Self-assembly of highly phosphorylated silaffins and their function in biosilica morphogenesis. Science 298:584–586

Kubáč D, Kaplan O, Elišáková V, Pátek M, Vejvoda V, Slámová K, Tóthová A, Lemaire M, Gallienne E, Lutz-Wahl S, Fischer L, Kuzma M, Pelantová H, van Pelt S, Bolte J, Křen V, Martínková L (2008) Biotransformation of nitriles to amides using soluble and immobilized nitrile hydratase from *Rhodococcus erythropolis* A4. J Mol Catal B 50:107–113

Lim DW, Nettles DL, Setton LA, Chilkoti A (2007) Rapid cross-linking of elastin-like polypeptides with hydroxymethylphosphines in aqueous solution. Biomacromolecules 8:1463–1470

Littlechild JA, Guy J, Connelly S, Mallett L, Waddell S, Rye CA, Line K, Isupov M (2007) Natural methods of protein stabilization: thermostable biocatalysts. Biochem Soc Trans 35:1558–1563

López-Gallego F, Betancor L, Hidalgo A, Alonso N, Fernández-Lafuente R, Guisán JM (2005) Co-aggregation of enzymes and polyethyleneimine: a simple method to prepare stable and immobilized derivatives of glutaryl acylase. Biomacromolecules 6:1839–1842

López-Serrano P, Cao L, van Rantwijk F, Sheldon RA (2002) Cross-linked enzyme aggregates with enhanced activity: application to lipases. Biotechnol Lett 24:1379–1383

Lu J, Tappel RC, Nomura CT (2009) Mini-review: biosynthesis of poly(hydroxyalkanoates). J Macromol Sci C 49: 226–248

Luckarift HR, Spain JC, Naik RR, Stone MO (2004) Enzyme immobilization in a biomimetic silica support. Nat Biotechnol 22:211–213

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Margolin AL (1996) Novel crystalline catalysts. Trends Biotechnol 14:223–230

Marner WD, Shaikh AS, Muller SJ, Keasling JD (2009) Enzyme immobilization via silaffin-mediated autoencapsulation in a biosilica support. Biotechnol Prog 25:417–423

Mateo C, Palomo JM, van Langen LM, van Rantwijk F, Sheldon RA (2004) A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. Biotechnol Bioeng 86:273–276

Mateo C, Chmura A, Rustler S, van Rantwijk F, Stolz A, Sheldon RA (2006) Synthesis of enantiomerically pure (S)-mandelic acid using an oxynitrilase–nitrilase bienzymatic cascade: a nitrilase surprisingly shows nitrile hydratase activity. Tetrahedron 17:320–323

Meyer DE, Chilkoti A (1999) Purification of recombinant proteins by fusion with thermally-responsive polypeptides. Nat Biotechnol 17:1112–1115

Meyer DE, Trabbic-Carlson K, Chilkoti A (2001) Protein purification by fusion with an environmentally responsive elastin-like polypeptide: effect of polypeptide length on the purification of thioredoxin. Biotechnol Prog 17:720–728

Moldes C, García P, García JL, Prieto MA (2004) In vivo immobilization of fusion proteins on bioplastics by the novel tag BioF. Appl Environ Microbiol 70:3205–3212

Nahálka J (2008) Physiological aggregation of maltodextrin phosphorylase from *Pyrococcus furiosus* and its application in a process of batch starch degradation to α-D-glucose-1-phosphate. J Ind Microbiol Biotechnol 35:219–223

Nahálka J, Nidetzky B (2007) Fusion to a pull-down domain: a novel approach of producing *Trigonopsis variabilis* D-amino acid oxidase as insoluble enzyme aggregates. Biotechnol Bioeng 97:454–461

Nahálka J, Pätoprstý V (2009) Enzymatic synthesis of sialylation substrates powered by a novel polyphosphate kinase (PPK3). Org Biomol Chem 7:1778–1780

Nahálka J, Vikartovská A, Hrabárová E (2008) A cross-linked inclusion body process for sialic acid synthesis. J Biotechnol 134:146–153

Nahálka J, Mislovičová D, Kavcová H (2009) Targeting lectin activity into inclusion bodies for the characterisation of glycoproteins. Mol Biosyst 5:819–821

Naik RR, Tomczak MM, Luckarift HR, Spain JC, Stone MO (2004) Entrapment of enzymes and nanoparticles using biomimetically synthesized silica. Chem Commun 1684–1685

Neumann L, Spinozzi F, Sinibaldi R, Rustichelli F, Pötter M, Steinbüchel A (2008) Binding of the major phasin, PhaP1, from *Ralstonia eutropha* H16 to poly(3-hydroxybutyrate) granules. J Bacteriol 190:2911–2919

Peters V, Rehm BHA (2006) In vivo enzyme immobilization by use of engineered polyhydroxyalkanoate synthase. Appl Environ Microbiol 72:1777–1783

Poulsen N, Berne C, Spain J, Kröger N (2007) Silica immobilization of an enzyme through genetic engineering of the diatom *Thalassiosira pseudonana*. Angew Chem 119: 1875–1878

Quiocho FA, Richards FM (1964) Intermolecular cross-linking of a protein in the crystalline state: carboxypeptidase-A. PNAS 52:833–839

Rajan A, Abraham TE (2008) Studies on crystallization and cross-linking of lipase for biocatalysis. Bioprocess Biosyst Eng 31:87–94

Rajendhran J, Gunasekaran P (2007) Application of cross-linked enzyme aggregates of *Bacillus badius* penicillin G acylase for the production of 6-aminopenicillanic acid. Lett Appl Microbiol 44:43–49

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33

Roy JJ, Abraham TE (2006) Preparation and characterization of cross-linked enzyme crystals of laccase. J Mol Catal B 38:31–36

Schäffer C, Novotny R, Küpcü S, Zayni S, Scheberl A, Friedmann J, Sleytr UB, Messner P (2007) Novel biocatalysts based on S-layer self-assembly of *Geobacillus stearothermophilus* NRS 2004/3a: a nanobiotechnological approach. Small 3:1549–1559

Schoevaart R, Wolbers MW, Golubovic M, Ottens M, Kieboom APG, van Rantwijk F, van der Wielen LAM,

Sheldon RA (2004) Preparation, optimization and structures of cross-linked enzyme aggregates (CLEAs). Biotechnol Bioeng 87:754–762

Schuster B, Pum D, Sleytr UB (2008) S-layer stabilized lipid membranes (review). Biointerphases 3:FA3-11

Schwarz A, Thomsen MS, Nidetzky B (2009) Enzymatic synthesis of β-glucosylglycerol using a continuous-flow microreactor containing thermostable β-glycoside hydrolase CelB immobilized on coated microchannel walls. Biotechnol Bioeng 103:865–872

Shah S, Sharma A, Gupta MN (2006) Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. Anal Biochem 351:207–213

Sheldon RA (2007a) Enzyme immobilization: the quest for optimum performance. Adv Synth Catal 349:1289–1307

Sheldon RA (2007b) Cross-linked enzyme aggregates (CLEAs): stable and recyclable biocatalysts. Biochem Soc Trans 35:1583–1587

Sleytr UB, Egelseer E-M, Pum D, Schuster B (2004) S-layers. In: Niemeyer CM, Mirkin CA (eds) Nanobiotechnology. Wiley-VCH, Weinheim, pp 77–92

Sleytr UB, Huber C, Ilk N, Pum D, Schuster B, Egelseer EM (2007) S-layers as a tool kit for nanobiotechnological applications. Microbiol Lett 267:131–144

Tang J, Badelt-Lichtblau H, Ebner A, Preiner J, Kraxberger B, Gruber HJ, Sleytr UB, Ilk N, Hinterdorfer P (2008) Fabrication of highly ordered gold nanoparticle arrays templated by crystalline lattices of bacterial S-layer protein. ChemPhysChem 9:2317–2320

Thomsen MS, Nidetzky B (2009) Coated-wall microreactor for continuous biocatalytic transformations using immobilized enzymes. Biotechnol J 4:98–107

Tschiggerl H, Breitwieser A, de Roo G, Verwoerd T, Schäffer C, Sleytr UB (2008) Exploitation of the S-layer self-assembly system for site directed immobilization of enzymes demonstrated for an extremophilic laminarinase from *Pyrococcus furiosus*. J Biotechnol 133:403–411

Vafiadi C, Topakas E, Christakopoulos P (2008) Preparation of multipurpose cross-linked enzyme aggregates and their application to production of alkyl ferulates. J Mol Catal B 54:35–41

Villaverde A, Carrió MM (2003) Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnol Lett 25:1385–1395

Wang W-X, Pelah D, Alergand T, Shoseyov O, Altman A (2002) Characterization of SP1, a stress-responsive, boiling-soluble, homo-oligomeric protein from aspen. Plant Physiol 130:865–875

Wang W, Dgany O, Wolf SG, Levy I, Algom R, Pouny Y et al (2006) Aspen SP1, an exceptional thermal, protease and detergent-resistant self-assembled nano-particle. Biotechnol Bioeng 95:161–168

Wilson L, Illanes A, Pessela BCC, Abian O, Fernández-Lafuente R, Guisán JM (2004) Encapsulation of cross-linked penicillin G acylase aggregates in lentikats: evaluation of a novel biocatalyst in organic media. Biotechnol Bioeng 86(5):558–562

Yu HW, Chen H, Wang X, Yang YY, Ching CB (2006) Cross-linked enzyme aggregates (CLEAs) with controlled particles: application to *Candida rugosa* lipase. J Mol Catal B 43:124–127

Zhao L, Zheng L, Gao G, Jia F, Cao S (2008) Resolution of N-(2-ethyl-6-methylphenyl) alanine via cross-linked aggregates of *Pseudomonas* sp. lipase. J Mol Catal B 54:7–12