Immobilization of Penicillin G Acylase: The Key to Optimum Performance

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Abstract: Penicillin G acylase is a major industrial biocatalyst that is used in the enzymatic production of 20,000 t a^{-1} of 6-aminopenicillanic acid, the industrial β-lactam intermediate, as well as in the enzymatic production of semi-synthetic β-lactam antibiotics. Because efficient recovery and reuse of the biocatalyst is a prerequisite for a viable process, much attention has been focused on the immobilization of penicillin G acylase. Methods that have been studied and will be discussed in this review include covalent attachment to porous organic and inorganic carriers, inclusion in and attachment to biopolymer gels and carrier-free immobilization techniques. Highly active and stable preparations have been developed; mass transfer limitations in the carrier are now a major barrier to further improvement of the biocatalyst performance.

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Keywords: covalent attachment; immobilization; β-lactam antibiotics; penicillin G acylase

1 Introduction

1.1 Penicillin G Acylase, an Industrial Biocatalyst

In 1960, the discovery of penicillin G acylase (penicillin amidohydrolase E.C. 3.5.1.11) was reported independently by four industrial research groups.[1–4] Penicillin G acylase is a serine hydrolase, like the lipases and serine proteases, but its mechanism is somewhat different. It belongs to the class of the *N*-terminal nucleophile hydrolases, which have no catalytic triad but an *N*-terminal serine that is activated by a bridging water molecule (see Figure 1).[5] The biological role of penicillin G acylase is not known, but it has been suggested that penicillin G acylase may play a role in the free-living mode of the organisms to metabolize phenylacetic acid derivatives as a carbon source.[6] Otherwise, a function in a defense mechanism against natural β-lactam antibiotics could be assumed.

Penicillin G acylase has a somewhat complex substrate specificity. The acyl binding subsite is highly specific for phenylacetic acid; only small groups, such as hydroxy or amino groups, are allowed at the 2-position and

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Fred van Rantwijk (1943) studied organic chemistry at the Delft University of Technology where he remained as a staff member. He received his PhD in 1980, for work under the guidance of Professor H. van Bekkum. Since the late 1980s he has been working on the application of enzymes in organic synthesis. His particular research interests are the use of enzymes in non-natural reactions, enzyme immobilization, and transformations using multi-enzyme systems.

Agnes Kallenberg (1981) studied chemical engineering at the University of Twente, where she graduated with honors in November 2004. She did her diploma work at DSM Anti-Infectives, Delft, on immobilization of penicillin acylase for application in enzymatic β-lactam antibiotic conversion reactions, under the supervision of Prof. Dr. J. F. J. Engbersen. In March 2005 she joined Shell Chemicals in Moerdijk.

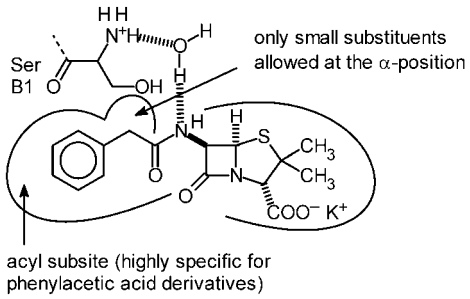

Figure 1. Substrate specificity of penicillin G acylase.

the enantiomer specificity is low. The penicillin-recognizing subsite, in contrast, is highly specific for L-amino acids but also discriminates other chiral primary amines.[7] Its substrate specificity would make penicillin G acylase a highly attractive amine resolution biocatalyst, provided that an efficient methodology for using the enzyme in anhydrous media is eventually developed.

The introduction of semi-synthetic β-lactam antibiotics, starting with ampicillin in the early 1960s, initiated a development that would make the β-lactam nucleus in the form of 6-aminopenicillanic acid (6-APA) a major pharmaceutical intermediate.[8] The newly discovered penicillin G acylase was initially employed as an industrial catalyst for the manufacture of 6-APA from penicillin G. This was long before the use of biocatalysis in the fine chemicals industry in general and the procedure was inconvenient as well as inefficient by present-day standards: the productivity was low due to the large reaction volumes and the (whole-cell) biocatalyst was discarded after use.

The solution was sought in chemistry, in agreement with the spirit of the times, and a stoichiometrically efficient, chemical deacylation process of penicillin G was ultimately developed[9] and commercialized by the Nederlandsche Gist en Spiritusfabriek (now a subsidiary of DSM); it is often referred to as the 'Delft Cleavage' (Figure 2). In spite of its now obvious shortcomings, 'Delft Cleavage' was universally favored over the microbial procedure mentioned earlier and remained in use for 15–20 years.

Over this latter period, biocatalysis was increasingly adopted by the fine chemicals industry, greatly assisted by the highly active, robust and recyclable biocatalysts that were becoming available. Against this backdrop, enzymatic procedures for the cleavage of penicillin G continued to be investigated.[10] Penicillin G acylases with improved stability were obtained by screening and, by employing recombinant DNA technology, efficient production was achieved. Combined with efficient immobilization, which made recycling of the biocatalyst possible,[11,12] dramatic reductions in enzyme costs were forthcoming.[13] There also was an awareness that the traditional procedure had reached a high degree of maturity, whereas the need for the antibiotics industry to re-

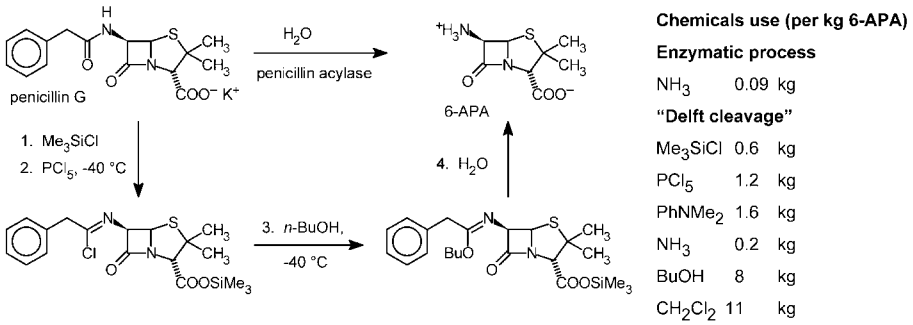

Figure 2. Chemical and enzymatic production of 6-APA compared.

duce its waste/product ratio[14] (the E factor[15]) was increasingly recognized.

An enzymatic process[16,17] was eventually introduced in the mid-1980s and its economic, environmental and operational advantages over the chemical 'Delft Cleavage' were such that the latter process has been swept from the market.[18,19] The enzymatic cleavage of penicillin G (Figure 2) is carried out in water at a slightly elevated temperature, rather than in halogenated solvent at $-40\,^{\circ}C$ and nowadays affords approx. 20,000 t a^{-1} of 6-APA in excellent yield.[20]

It has already been noted that efficient immobilization was one of the keys to the successful adoption of the enzymatic cleavage of penicillin G. Immobilization is a mixed blessing, however, because it affects the mobility of the solutes as well, resulting in mass-transfer effects that are loosely categorized as diffusion limitation. In penicillin G *hydrolysis*, diffusion limitation merely results in a reduced effectiveness of the biocatalyst but in the enzymatic *synthesis* of β-lactam antibiotics its effects are much more pernicious.

The kinetically controlled, enzymatic synthesis of β-lactam antibiotics from the β-lactam nuclei 6-APA and 7-aminodeacetoxycephalosporanic acid (7-ADCA) and appropriate side-chain donors (see Figure 3) has been studied from the 1960s onwards.[21,22] Penicillin G acylase is the catalyst of choice for such a procedure, due to its unique selectivity.

The main obstacle confronting kinetically controlled synthesis is the accompanying hydrolysis of the side-chain donor as well as the product (see Figure 4), which necessitates the use of an excess of the side-chain donor and renders the downstream processing more laborious. The synthesis/hydrolysis ratio (S/H, mol product per mol hydrolyzed side-chain donor formed) is often used as an indicator of the viability of the process.[23] Dissolved enzymes were commonly used in academic studies, but an industrial procedure would, generally speaking, employ an immobilized preparation for economic viability. In immobilized preparations, however, diffusion limitation in the enzyme carrier tends to increase hydrolysis, because it causes reactant depletion inside the biocatalyst particles whereas the product accumulates.[24] The combined effects of intrinsic acyl transfer efficiency (r_S/r_H, see Figure 4) of penicillin acylase and diffusion limitation were of such magnitude that the industrial synthesis of the β-lactam antibiotics continued to depend exclusively on chemical procedures.[8]

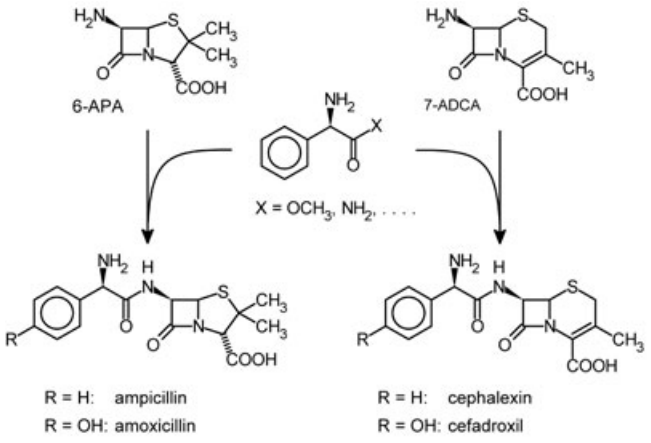

Figure 3. Penicillin G acylase catalyzed synthesis of β-lactam antibiotics. ampicillin and cephalexin.[22]

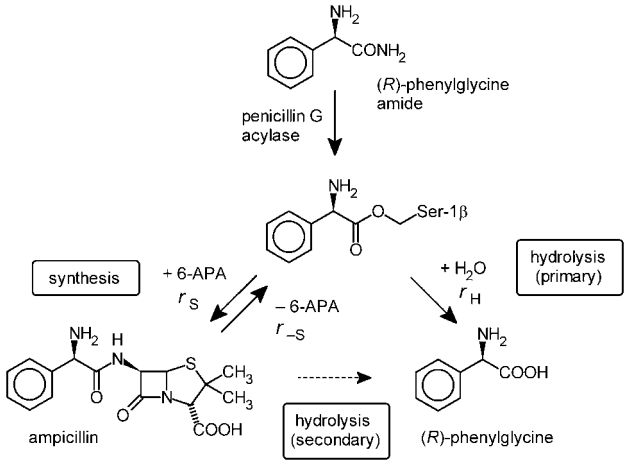

Figure 4. Kinetically controlled synthesis of ampicillin from (R)-phenylglycine amide and 6-APA and the competing hydrolysis.

A breakthrough in the enzymatic approach was finally achieved when it was discovered that performing the reaction at high, i.e., 0.3–0.5 M, concentrations dramatically improved the yield.[25] A general procedure emerged[26] in which the desired β-lactam nucleus is acylated with an excess of an activated side-chain donor, e.g., an ester or amide, at 0.2–0.8 M concentration in water at pH 7–8 at zero to ambient temperature (see Figure 3). As regards immobilization, it had already been shown in the early days of the enzymatic coupling that the magnitude of diffusion limitation effects depends on the immobilization method[24] and DSM reported that its proprietary gelatine-derived carriers perform much better in this respect than other ones.[27] On this technological basis, the enzymatic production of cephalexin was started by Chemferm (a subsidiary of DSM) in the mid-1990s. Recently, DSM has also announced the enzymatic production of amoxicillin and cefadroxil.[28]

The developments sketched above have made penicillin G acylase a major industrial biocatalyst. It is the result of 40 years of evolution, which is still continuing and has recently been accelerated, in particular in the Netherlands, by a very fruitful industrial-academic collaboration. Three distinct development areas can be recognized:

(i) *Screening and classical strain improvement.* Penicillin-recognizing enzymes are still being discovered[29] but until now none has equaled the penicillin G acylase from *E. coli*, which is itself the result of many years of strain improvement. Many subspecies of penicillin G acylase-producing *E. coli* exist, some of these are commercially available and *E. coli* ATCC 11105 is more or less regarded as the industrial standard.

(ii) *Genetic engineering studies* to improve the acyl transfer characteristics (r_S/r_H, see Figure 4) of penicillin G acylase have been performed[30,31] and it seems likely that some of the results have been adopted by the industry.

(iii) *Numerous immobilization studies,* aiming at a high activity per unit volume, operational stability and, for synthetic applications, efficient acyl transfers have been carried out. The review will focus on these latter subjects but attention will also be given to other applications, including use in anhydrous reaction media. Moreover, because of the industrial importance of penicillin G acylase most known immobilization techniques have been applied to this enzyme, i.e., it constitutes a microcosm of enzyme immobilization in general.

1.2 Enzyme Immobilization

The development of robustly immobilized enzymes is a major challenge in industrial biocatalysis and the subject has grown into an important research field.[32–34,36] Enzymes can be fixed to a carrier by physical forces, such as hydrophobic interaction, Van der Waals binding or ionic interactions. These are generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. Covalent binding of the enzyme to a support is stable, in contrast, and has generally been favored in the case of penicillin G acylase.[36] Basically, three methods of enzyme immobilization can be distinguished:[34]

(i) *Support binding,* usually by covalent attachment, to a prefabricated carrier. The carrier can be a synthetic resin, a biopolymer or an inorganic material;

(ii) *Entrapment* via *inclusion* of an enzyme in a polymer network (gel lattice) or a membrane device such as a hollow fiber or a microcapsule. The physical restraints generally are too weak, however, to prevent enzyme leakage entirely. Hence, additional covalent attachment is generally required;

(iii) *Cross-linking of enzyme aggregates or crystals*, using a bifunctional reactant, to prepare carrierless macroparticles.

The properties of immobilized enzyme preparations are determined by the properties of both the enzyme and the carrier material. The interaction between these endows an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties (see Figure 5).[34]

In this review, the different immobilization methods for penicillin G acylase are evaluated and compared with regards to activity in hydrolysis and synthesis, as appropriate, as well as (operational) stability. Two novel integrated reactor concepts, designed for increased performance of penicillin G acylase, will also be discussed.

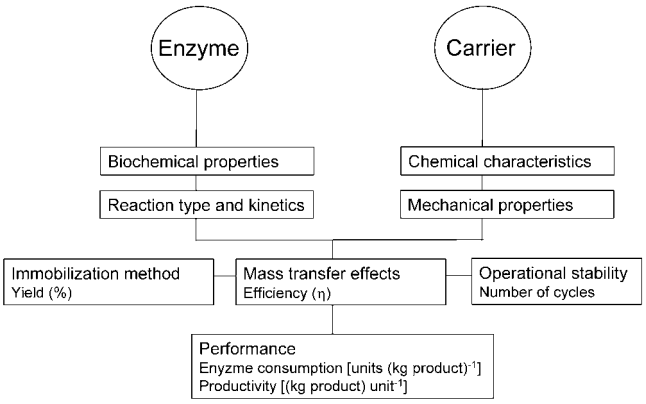

Figure 5. The properties of immobilized enzymes are determined by a combination of the properties of the enzyme and the carrier material.[34]

Some Practical Notes

The activity of penicillin G acylase is commonly measured in benzylpenicillin units (BPU) or just units (U). One BPU corresponds with an amount of enzyme that liberates one μmol of phenylacetic acid per min from penicillin G. The BPU is somewhat variable, because there is no agreement on the penicillin G concentration, the pH and the reaction temperature.[37]

Alternatively, the number of catalytically competent active sites can be measured *via* active site titration.[38,39] One μmol of penicillin G acylase from *E. coli* ATCC 11105 corresponds with 3660 BPU, which is equivalent to a catalytic turnover rate of 61 s^{-1}.[40]

2 Acrylic Resins

2.1 Eupergit C and Related Materials

Eupergit: Basic Chemistry

Eupergit® C (see Figure 6) is a macroporous copolymer of methacrylamide, glycidyl methacrylate and allyl glycidyl ether, cross-linked with *N*,*N*′-methylene-bis(methacrylamide), with average particle size 170 μ and pore diameter 25 nm.[41] Röhm, Darmstadt, Germany, developed and commercialized Eupergit® in the late 1970s as a spin-off from its long-standing enzymes and polyacrylate businesses.[42] Eupergit C is highly hydrophilic and absorbs approx. three times its weight of water.[43] Because of its structure, Eupergit is stable, both chemically and mechanically, over a pH range from 0 to 14 and does not swell or shrink even upon drastic pH changes in this range.

Enzymes are covalently attached to Eupergit C through nucleophilic attack of its free amino groups on the oxirane groups in the carrier (see Figure 6). The procedure is carried out at neutral or alkaline pH and the resulting bonds are long-term stable within a pH range of pH 1 to 12. A high salt concentration (up to 1 M phosphate), which probably exerts a 'salting out' effect, is recommended.[44] Due to the high density of oxirane groups on the surface of the beads (600 μmol per g dry Eupergit C), one enzyme molecule can form multiple bonds ('multipoint attachment'), which is considered a major contributing factor to the high operational stability of enzymes bound to Eupergit C. Any remaining oxirane groups can be blocked ('end–capped') with a variety of reagents (mercaptoethanol, ethanolamine, glycine, etc.) to prevent any undesired support-protein reactions.

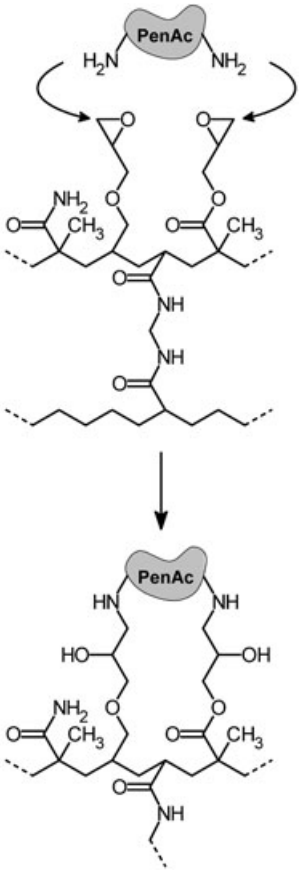

Figure 6. Structure of Eupergit C and covalent multipoint attachment of penicillin G acylase.

Immobilization of Penicillin G Acylase

Up to 1300 BPU (0.36 μmol) of penicillin G acylase are rapidly taken up per g (wet) of Eupergit C,[43] which seems to be the limit of its capacity, because residual activity is found in the supernatant when more enzyme is presented to the carrier.[40,43] Approx. 85–90% of the bound active sites are still catalytically competent, as determined by active site titration, which results in a maximum active site density of 0.3 μmol (1.8×10^7) per g carrier.[43] The *activity* yield is much lower, however, due to a reduction of the turnover rate (see Table 1).[40,43] This activity loss is ascribed to diffusion limitation in the carrier, as will be discussed later. It should be noted that these preparations were much more active than would be expected on the basis of the product information from Röhm[41] (581 *vs.* 200 BPU g^{-1}), which the authors ascribe to the use of a more purified enzyme solution.[43]

Operational Stability

Penicillin G acylase on Eupergit® C, when subjected to an operational stability test over >800 cycles, maintained approx. 60% of its starting activity.[42] The immo-

Table 1. Immobilization of *E. coli* pen G acylase on Eupergit® C with different loading.[43]

Active sites			Activity	
Presented [μmol g^{-1}][a]	Immobilized [μmol g^{-1}][a]	Turnover rate (s^{-1})	Beads (BPU g^{-1})[a]	Crushed (BPU g^{-1})[a]
0.12	0.10	54	322	299
0.23	0.20	38	465	499
0.35	0.29	32	556	833
0.47	0.30	32	581	930

[a] Wet weight.

bilization of highly purified penicillin G acylase resulted in a less stable preparation; reaction of the residual oxirane groups with dithiothreitol stabilized the latter biocatalyst, however.[42]

Diffusion Limitation in Eupergit® C

One major disadvantage of Eupergit® C and microporous supports in general is the diffusion limitation, which decreases the efficiency of the biocatalyst, due to depletion of the reactant and accumulation of the products in the carrier beads. The hydrolysis of penicillin G generates acid, moreover, which induces a pH gradient in the carrier. Since the pH affects the enzyme's activity as well as the reaction equilibrium, the pH gradient amplifies the general effects of the concentration gradient.[45–47] The pH gradients in Eupergit C have been studied by the Kasche group.[46,47] These were found to be very considerable (see Figure 7) and enzyme molecules at >30 μ from the surface hardly contributed to the overall reaction. The pH gradient can be adequately remedied by buffering the reaction medium,[36d,46] but this latter solution is unattractive in an industrial setting.

A considerable loss of activity of penicillin G acylase upon immobilization on Eupergit C was found, even with adequate buffering; the effect was seen to increase, moreover, with increasing active site density (see Table 1).[43] As would be expected, a substantially increased activity (corresponding with a turnover rate of approx. 50 s^{-1}) was observed when the diffusion pathway was shortened by crushing the biocatalyst beads to particle size <5 μ..

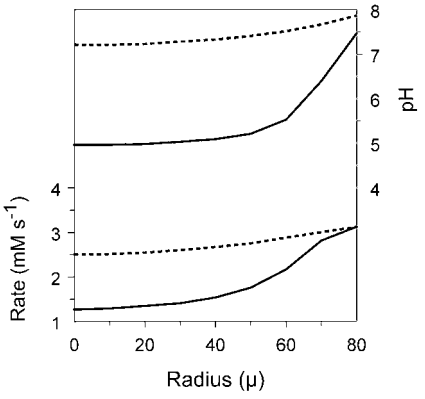

Figure 7. Penicillin G hydrolysis: reaction rate and pH gradients in the Eupergit C beads; (—) unbuffered and (---) with a 200 mM phosphate buffer.[46]

Eupergit C Immobilized Penicillin G Acylase as Synthesis Catalyst

It has already been noted that the effects of diffusion limitation are even more severe in a kinetically controlled reaction, such as the synthesis of β-lactam antibiotics, than in the equilibrium-controlled hydrolysis of penicillin G. It was accordingly found that penicillin G acylase on Eupergit C was much less efficient in the synthesis of ampicillin[47] and cephalexin,[43] as expressed in the S/H ratio, than the free enzyme. In the case of cephalexin, for example, the initial S/H ratio was found to decrease from >30 for the free enzyme to approx. 5 at an active site density of 0.2 μmol g^{-1}.[43]

Sepabeads FP-EP

Sepabeads FP-EP (Resindion, Milan, Italy) consist of a polymethacrylate-based resin functionalized with oxirane groups, similar to Eupergit C and it has very similar characteristics for binding penicillin G acylase.[48] A procedure has been reported for greatly increasing the stability of penicillin G acylase bound to Sepabeads FP-EP; it may also apply to Eupergit C. This involves the incubation of the initial immobilisate under more forcing conditions (higher pH, higher temperatures, longer incubation periods, etc.), to increase the number of enzyme-support bonds.[48] Thus, the half-life time of the immobilisate at pH 10 and 20 °C increased with a factor 18 compared to enzyme immobilized under standard conditions (pH 7 and 20 °C). It has also been reported that endcapping with an amino acid, such as glycine, instead of mercaptoethanol, resulted in a much improved stability.[48]

2.2 Amberlite XAD-7

Amberlite XAD-7 is a porous poly(methacrylic ester) resin with a specific surface area of 450 $m^2\ g^{-1}$ and an average pore diameter of 9 nm. It has been developed as a moderately hydrophilic material for column chromatography and not as a biocatalyst support. Physical adsorption of penicillin G acylase onto Amberlite XAD-7 is not stable,[36d,49] as would be expected. Hence, the resin was activated by aminoalkylation with 1,2-diaminoethane followed by derivatization with a glutaraldehyde linker (see Figure 8).

Covalent attachment of penicillin G acylase to the free aldehyde groups in the activated carrier resulted in preparations with 194 BPU g^{-1} (dry wt) activity.[49] The activity yield (based on the amount of bound enzyme) was a modest 25%. It is worth noting, however, that much more enzyme was attached when the XAD-7 beads were reduced in size. The activity increased to 400 BPU g^{-1}, corresponding with an activity yield of 33%. This latter result may be taken as an indication that the diffusion of penicillin G acylase into the 9 nm pores is extremely slow. It has similarly been found in early, related, work that penicillin G acylase had penetrated only a 25 μ layer below the surface of the XAD-7 beads in 24 h, whereas immobilization throughout the beads required a high protein concentration and a long reaction time.[36d]

The small effect of the particle size on the activity yield,[49] and, hence, the turnover rate, indicates that the transport of the reactant and products is not a major contributor to the low activity yield. This may confirm the notion that the enzyme is concentrated in a shallow layer at the beads' surface.

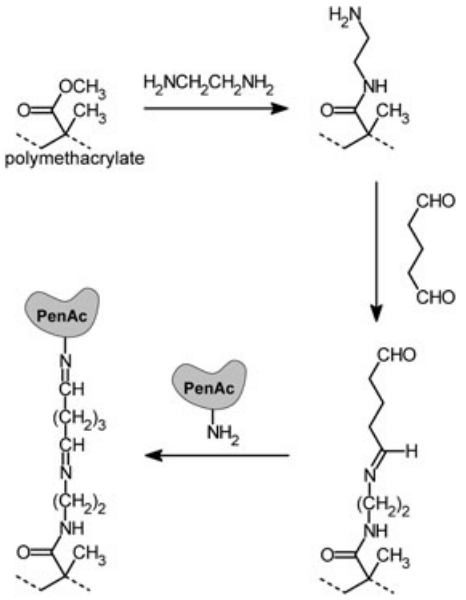

Figure 8. Activation of Amberlite XAD-7 and covalent attachment of penicillin G acylase.

A gradual activity loss upon repeated reuse of the biocatalyst was observed and attributed to chemical instability of the Schiff's base linkers. Reduction of the latter with sodium borohydride indeed extended the operational life-time of the biocatalyst to >400 h at 37 °C.[49]

3 Biopolymer Supports

3.1 Aldehyde-Agarose

Agarose is a linear polysaccharide (MW approx. 12 kD) that is obtained from seaweed and consists of alternating units of D-galactose and 3,6-anhydro-D-galactose. Aldehyde-agarose contains free aldehyde groups, to which enzymes can be covalently attached *via* Schiff's base formation with the free amino groups of lysine residues at the protein's surface.[50] Cross-linked aldehyde-agarose gels are commercially available from various sources.

The covalent attachment of penicillin G acylase, as well as various other enzymes, to cross-linked aldehyde-agarose has been extensively studied in the group of Guisán, mainly with the objective of improving its thermal stability and tolerance of organic solvents. Up to 600 BPU of penicillin G acylase can be bound per mL of gel, but the loading was deliberately limited to 10 BPU mL^{-1} in these studies, to avoid diffusion limitation in the activity tests.[51] Thus, multipoint-attachment of penicillin G acylase to aldehyde-agarose (activity yield 80%) increased its (thermal) stability by a factor of 2500. Reductive methylation of the remaining free amine groups with, successively, formaldehyde and sodium borohydride further increased the stability threefold.[51]

It was surmised that a hydrophilic nano-environment is the key to rendering enzymes resistant to polar organic solvents.[52] Such a nano-environment was created by treating penicillin G acylase, multipoint attached to glyoxyl-agarose, with a polyamine (amine-dextran[53]), followed by reaction with dextran polyaldehyde and reduction with sodium borohydride. Presumably, both steps involve further cross-linking of the enzyme and the carrier by these reagents. Thus, the enzyme molecules become tightly embedded in an environment with a high concentration of amino and hydroxy groups. It was indeed found that when these preparations were incubated in aqueous mixtures of 70% DMF or 60% dioxane followed by rehydration, 80% of the original activity was recovered.[52]

The gain in stability that has been accomplished by these multipoint attachment and cross-linking techniques is very impressive, but we note that a much sterner stability test: maintaining enzymatic *activity* in, for example, 70% DMF, still has to be performed.

3.2 Inclusion in Gelatine

The immobilization of enzymes or cells by inclusion in biopolymer gels, such as gelatin or alginate, dates back to the early days of biocatalysis. The procedure is relatively simple, does not require functionalization of the support and is regarded as biocompatible due to the mild reaction conditions. A recently published procedure for the entrapment of penicillin G acylase in gelatin [54] illustrates the principle. Permeabilized *E. coli* cells containing penicillin G acylase were suspended in an aqueous solution of gelatin and sodium alginate.[55] The resulting mixture was added dropwise to a solution of calcium chloride, which cross-linked the alginate, to form solid beads. These were subsequently washed at pH 7.8 to remove the alginate, resulting in porous gelatin beads that contained the enzyme within the protein matrix. Cross-linking with glutaraldehyde stabilized the beads and presumably also attached the enzyme to the matrix.

The resulting biocatalyst (of unstated activity) was packed into a column and a solution of penicillin G was pumped through. The initial activity was maintained during five 9-h runs with intermittent washings before the activity dropped off.[54] Hence, it would seem that the cross-links are insufficiently stable; it is also possible that the number of free amino groups in gelatin is too low for multipoint attachment.

3.3 Gelatin-Chitosan

Gist-brocades (now a subsidiary of DSM) described in the late 1980s a procedure for the inclusion of cells into cross-linked gels of gelatin and a polymer containing free amino groups, such as chitosan, alginate amine or polyethylene imine.[56] Eventually, the beads were stabilized by cross-linking with glutaraldehyde. Presumably, this is the carrier of the penicillin G hydrolysis biocatalyst, Separase G,[20] but no details have been disclosed. Later, it was claimed that the pencillin G acylase from *E. coli* performed much better in the synthesis of β-lactam antibiotics when immobilized in gelatin-chitosan than with other supports.[27] Presumably, this latter result provided a basis for the development of the in-house biocatalyst of DSM Anti-Infectives, trademarked Assemblase®, which is employed in the enzymatic synthesis of semi-synthetic β-lactam antibiotics.

Several collaborative academic-industrial studies of Assemblase have been published, which is rather an exception and warrants a discussion in some detail. The turnover rate of Assemblase in penicillin G hydrolysis, based on active site titration, is 22 s^{-1}, which is much less than that of the dissolved enzyme (61 s^{-1}) and also less than that of an Eupergit C-immobilized preparation of similar active site density (31 s^{-1}).[40]

A more detailed study focused on the distribution of penicillin G acylase within the beads, which is crucial for understanding mass transport issues.[57] The Assemblase beads were wet-sieved into size fractions and the active site density in each fraction was measured (see Figure 9). The average enzyme loading was found to decline with increasing particle diameter, which was confirmed in hydrolysis experiments. It was concluded, on the basis of these results, that the enzyme is mainly present in a shallow shell at the beads' surface.[57] This latter conclusion has recently been confirmed by immunolabeling.[58]

The performance of Assemblase in the synthesis of cephalexin from (*R*)-phenylglycine amide and 7-ADCA (see Figure 3) was investigated in detail and compared with dissolved enzyme.[59] Assemblase was a less efficient acyl transfer catalyst, as judged by the S/H ratio[23], than the dissolved enzyme, which is ascribed to diffusion limitation in the Assemblase carrier.[59] This latter effect is caused by depletion of the nucleophile, 7-ADCA, in the active site and accumulation of the product, which result in increased hydrolysis and a lower product yield (see Figure 4). The magnitude of this latter effect depended on the enzyme concentration in the carrier and the pH, as would be expected and, on the basis of a kinetic model, optimum reaction conditions were predicted and verified.[59]

The question remains why Assemblase performs, in terms of the S/H ratio, so much better in cephalexin synthesis than, for example, Eupergit C-immobilized penicillin G acylase, in spite of the ample pore diameter of the latter material. One reason could be that the enzyme is actually located in a superficial shell of the Assemblase beads. Moreover, it is probably incorrect to view the Assemblase carrier as a solid matrix; its major constituent actually is water, held together by a loosely cross-linked network of gelatin and chitosan.[60] But it should also be considered that the Assemblase-bound enzyme is only 30% effective in the hydrolysis of penicil-

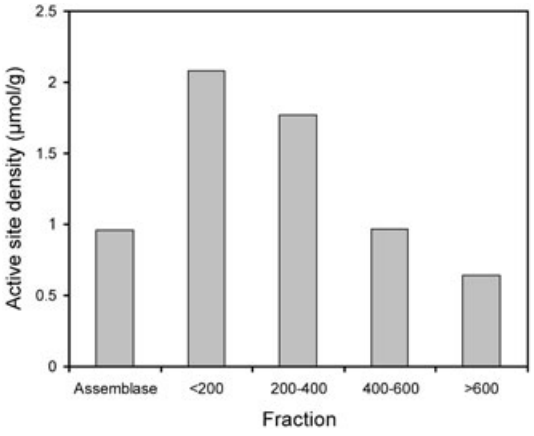

Figure 9. Active site density of Assemblase and its sieved fractions.[57]

lin G,[40] which is less than penicillin G acylase attached to intact Eupergit C beads and less than half the effectiveness of crushed Eupergit C.[43] Since the discrepancy cannot be reasonably ascribed to diffusional limitation in Assemblase, it would seem that the low effectiveness of the biocatalyst is inherent to the (proprietary) procedure employed in its preparation.

3.4 Biospecific Immobilization on Supported Concanavalin A

Concanavalin A belongs to the class of the lectins, proteins that react with specific terminal sugar residues, which are useful probes in studying carbohydrates of cell surfaces. Concanavalin A is also used as a linker in the immobilization of glycosylated enzymes.[61]

Native penicillin G acylase from *E. coli* is not glycosylated and, hence, does not interact very well with concanavalin A. A synthetic glycoconjugate of penicillin G acylase was prepared by reaction of the latter with a mannan-derived polyaldehyde, followed by reduction of the resulting Schiff's bases and the unreacted aldehyde groups.[62] The support was prepared by activating cellulose beads with chloro-1,3,5-triazine followed by covalent attachment of concanavalin A. This latter carrier, which was only very lightly (1% wt) loaded with concanavalin A, absorbed a small amount of the penicillin G acylase-mannan conjugate. The expressed activity was 18 BPU g^{-1}, corresponding with an activity yield of 74%.[62] Second layers of concanavalin A and penicillin G acylase-mannan conjugate were successively deposited on the first immobilisate (bioaffinity layering), but the gain in activity was small.[62] There was little activity loss upon storage or repeated reuse of the biocatalyst.

In short, the biospecific immobilization of penicillin G acylase has been shown to be feasible, but the procedure is complex and the activity of the resulting biocatalyst is quite modest.

4 Inorganic Supports

4.1 Functionalized Silica Supports

Silica is a very well studied material that can be prepared in wide ranges of particle size and porosity. Techniques for the stable, covalent modification of silica surfaces have been developed for application in liquid-phase chromatography and can be applied to the covalent immobilization of enzymes with little change. The particle size of chromatographic silica materials is in the range of 5–100 μ, with pore diameters of 10–20 nm. A larger particle size, which would be desirable with application as a biocatalyst support in mind, entails the problem of overly long diffusion pathways and concentration gradients. A bimodal pore structure, combining macro-sized interconnecting channels with a microporous structure, would combine a high specific area with unimpeded diffusion. Monolithic materials that meet this latter requirement have recently been developed for application in catalysis and are finding their way into biocatalysis;[63] monolithics-based HPLC columns are commercially available.

The immobilization of penicillin G acylase on activated silica supports has mainly been accomplished *via* the free amino groups of lysine residues at the enzyme's surface.[64] The enzyme has been covalently bound to activated aminopropyl silica as well as oxirane-functionalized silica (see Figure 10); this latter approach resulted in a much higher loading of the support.[64,65] Thus, 180 mg of enzyme were immobilized per g of microparticulate (5 μ) silica, expressing an activity of 1250 BPU g^{-1}.[64] These results compare well with Eupergit C (see Table **1**) and probably reflect the large specific surface area of silica as well as the small particle size. Even more protein was immobilized on monolithic silica and the recovered activity also was higher, but we note that the very high specific activity [20.8 kBPU (g protein)$^{-1}$] of the latter material that has been published[64] was not reproduced in a subsequent paper.[65]

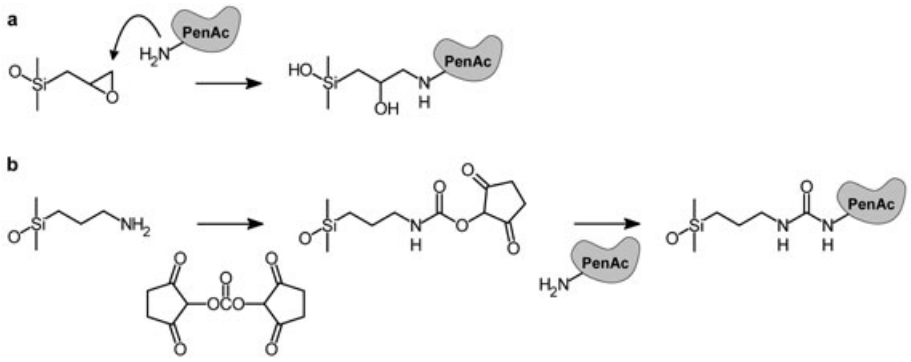

Figure 10. Synthesis scheme for immobilization of penicillin G acylase; on oxirane-functionalized silica (a) and aminopropyl silica (b).[64]

The potential of penicillin G acylase on silica was demonstrated in a chromatographic reactor set-up, which combined the hydrolysis of penicillin G with separation of the products.[64,65] The biocatalyst also showed promise as a chiral stationary phase. Summarizing, oxirane-functionalized silica is a promising support for penicillin G acylase, provided that the material can be shown to meet the industrial requirements of chemical and mechanical stability.

It would seem attractive to prepare functionalized silicas by preparing the latter from the appropriate precursor, rather than by post-synthesis modification. Thus, an aminopropyl-modified silica xerogel has been prepared by the hydrolysis of a mixture of tetraethoxysilane and aminopropyltrimethoxysilane.[66] The modest surface area of the resulting material (165 $m^2\ g^{-1}$) indicates a large pore diameter, in the order of 40 nm. The aminopropyl content – 0.3 mmol g^{-1} – was much less than would be expected on the basis of the composition of the starting mixture (1.9 mmol g^{-1}). Apparently, 85% of the aminopropyl groups are buried inside the silica matrix. Subsequent activation with glutaraldehyde and covalent immobilization of penicillin G acylase (see Figure 11) afforded a biocatalyst with a modest activity of 110 BPU g^{-1} (dry wt), although the activity yield was an impressive 80%.[66] The thermal stability at 60 °C ($t_{0.5}=37$ min) compared well with that of penicillin G acylase on Eupergit C ($t_{0.5}=10$ min).

4.2 Entrapment in a Silica Matrix

In contrast with immobilization on a silica *surface*, enzymes can also be entrapped within a silica *matrix*. This latter procedure was pioneered by Reetz, who showed it to be effective and efficient with lipases.[67] When applied to penicillin G acylase, the activity recovery was a poor 10%, which the authors ascribed to diffusion limitation in the silica matrix.[68] We note that penicillin G acylase is inherently more sensitive to diffusion limitation than, for example, a lipase. Moreover, the procedure has not been designed for fast mass transport, because this issue is of little relevance considering the generally quite low turnover rate of enzymes in organic solvents.

4.3 Immobilization on Molecular Sieves

Delaminated Zeolites

The pores in zeolites are too small (0.5–1.2 nm) to admit penicillin G acylase or any other protein but exfoliation of lamellar zeolites, to yield microcrystalline silica sheets that cluster into meso- and macroporous aggregates, is a potential solution to this latter problem.[69] Because they are composed of platelets, these delaminated zeolites reconcile a high surface area ($>600\ m^2\ g^{-1}$) with a large pore size; something that cannot be accomplished with traditional microporous materials. The large pore size and the regular distribution of silanol groups, as well as the easy recovery and recycle of the delaminated support by calcination, stimulated interest into the possible application of delaminated zeolites as enzyme support.

Experiments have been performed with the delaminated pure silica ITQ-6,[70] which was activated *via* modification with 3-aminopropyl(triethoxy)silane followed by treatment with glutaraldehyde.[71] Penicillin G acylase was covalently attached by reacting the free amino groups of penicillin G acylase with the anchored aldehyde functionality. Finally, the imine bonds were stabilized by reduction to secondary amines with sodium borohydride (see Figure 12).[71]

The activity of the resulting preparation was 2440 BPU g^{-1}, which is one of the highest ever reported for a carrier-bound penicillin G acylase and can be ascribed to the highly accessible structure. The enzyme also gained stability upon covalent immobilization, as the half-life time at pH 8 and 46 °C increased from 3 h for the free enzyme to 18 h for the immobilized one. We note that chemical instability of the carrier could con-

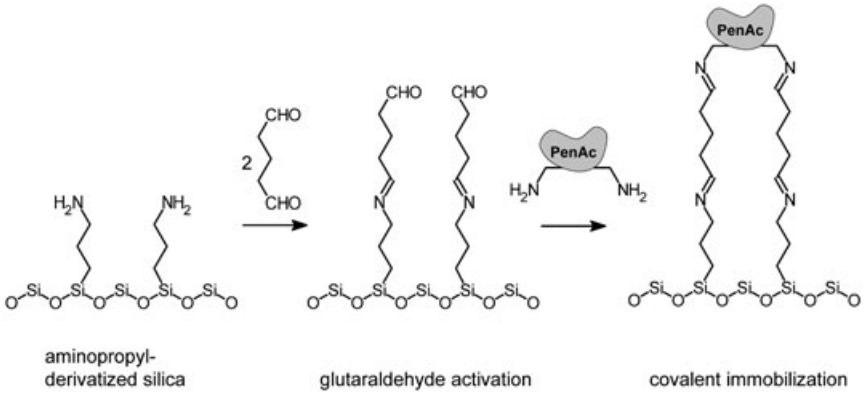

Figure 11. Activation of aminopropylsilica and covalent multipoint attachment of penicillin G acylase.[66]

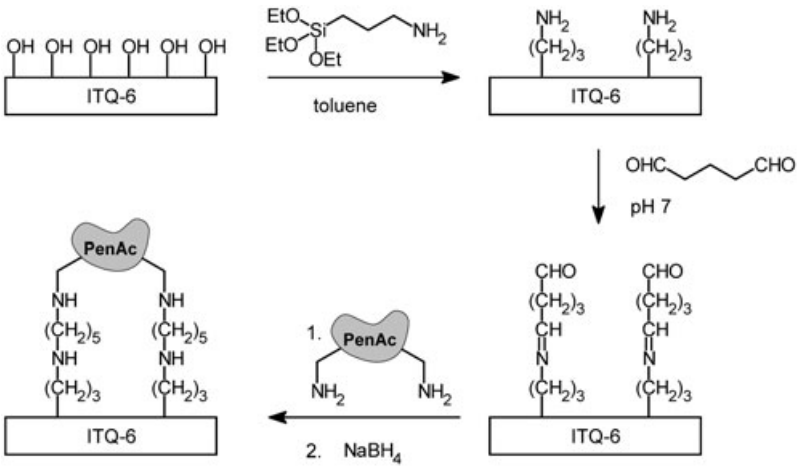

Figure 12. Covalent immobilization of penicillin G acylase on the delaminated zeolite ITQ-6.[71]

tribute to the activity loss, since O–Si–C bonds are known to be unstable at pH > 7.

In conclusion, covalent immobilization of penicillin G acylase on ITQ-6 yields a highly active biocatalyst that could be attractive for synthetic applications because of its large pore size. The practical value of the technique also depends critically on the mechanical stability of the aggregates, which has not yet been investigated.

Mesoporous Molecular Sieves

The MCM class of molecular sieves represents a semi-crystalline material (pure silica or silica-alumina) that characteristically has a regular arrangement of tubular pores with a 1.5–10 nm diameter depending on the size of the surfactant vesicles that act as templates in the synthesis of MCM molecular sieves.[72] The best-known representative, MCM-41, has a 4.1 nm pore diameter when synthesized; it is reduced to approx. 3–3.5 nm upon calcination.[73] Pure silica MCM-41 has some weak cation exchange capability and the number of ion-exchange sites increases upon incorporation of alumina.

Absorption of penicillin G acylase into MCM-41 was observed,[74] although the pore size of 3.3 nm is rather small in relation to the molecular dimensions of the enzyme ($7 \times 5 \times 5$ nm).[5] More penicillin G acylase seems to be absorbed upon incorporation of Al_2O_3 into the carrier, because the activity increased from 364 BPU g^{-1} at Si/Al = ∞ to 511 BPU g^{-1} at Si/Al = 25.[74] Hence, the authors concluded that the enzyme is mainly bound *via* coulombic interaction with anionic sites in the molecular sieve. Approx. 30% of the initial activity was lost upon repeated reuse of the biocatalyst, although such a loss of activity was not observed upon washing with pure water.[74] We would surmise that there is some exchange of penicillin G acylase with cations in the (buffered) reaction medium.

Activation of a pure silica MCM-41 with glutaraldehyde, followed by covalent attachment of penicillin G acylase, resulted in a preparation with a much lower activity (90 BPU g^{-1}), which is ascribed to the reduction of the pore diameter to 1.6 nm, caused by the modification with glutaraldehyde.[74] Contrary to expectations, activity was lost upon reuse of the covalently attached biocatalyst. Hence, it would seem that the covalent bond is not very stable.

We note that the activity of these biocatalysts is rather low, compared with the results that have been obtained using microporous silica with a comparable surface area.[64] We suspect that binding of penicillin G acylase mainly occurs in areas were the tubular pore system is irregular or has partly collapsed.

Functionalized mesoporous molecular sieves with pore diameter in the 6.9 nm range have been prepared by coprecipitation of tetraethoxysilane and various organosiloxanes.[75] Physical adsorption on bare or functionalized material resulted in biocatalysts with a modest activity (80–120 BPU g^{-1}) that rapidly leached enzyme into the medium, as would be expected. Covalent immobilization of penicillin G acylase to glutaraldehyde-activated molecular sieve resulted in a somewhat better activity (167 BPU g^{-1}) but much lower than can be accomplished with microporous silica, as described above.[76]

4.4 Celite Rods

Celite® is a trade name for diatomite, a silica material that consists of the skeletons of diatoms (a family of single-cell algae). Celite R-640 rods constitute a porous material that is based on diatomite; its surface area is a modest 65 m^2 g^{-1} and the mean pore diameter is 200 nm (about 8 times larger than Eupergit C). Celite R-640 differs from Celite powder in its capacity to adsorb (more than 90% by weight) and release water and

act as a water buffer.[77] This latter characteristic could find its origin in the clay binder used in the manufacture of the rods.

Up to 300 BPU of penicillin G acylase were absorbed per g of Celite R-640.[77] This rather low capacity of the carrier presumably is a consequence of its low specific surface area. The activity recovery was low, approx. 45 BPU g^{-1}, which corresponds with an activity yield of 17%.[77] The procedure is noteworthy, nevertheless, because the preparation maintained a useful activity in toluene, provided that a water activity > 0.4 was maintained. The Celite carrier has a beneficial role by acting as a water buffer. The potential of penicillin G acylase on Celite R-640 was demonstrated in the *N*-acylation of tyrosine ethyl ester in toluene medium, one of the first examples of successful use of penicillin G acylase in an organic solvent.[77]

4.5 Layered Double Hydroxides Pillared by Glutamate Ions

Layered double hydroxides (LDH) or hydrotalcite-like materials consist of cationic sheets separated by anionic interlayers. Their composition corresponds with the general formula $[M_{1-x}^{II}M_x^{III}(OH)_2]^{x+}(Y^{n-})_{x/n} \cdot z\ H_2O$, where the identities of the divalent M^{II} and trivalent M^{III} cations, the interlayer Y^{n-} and the stoichiometric coefficient x can be varied widely.[78] An LDH that was suitable for the covalent immobilization of enzymes was synthesized from magnesium/aluminum hydroxide that contained carbonate as the interlayer anion. The material, when dissolved into aqueous glutamic acid, reprecipitated as a glutamate-pillared LDH upon the addition of base.[79] X-ray diffraction showed the presence of a crystalline layered phase with an interlayer spacing of 0.76 nm.

The glutamate-pillared LDH was functionalized with glutaraldehyde, followed by covalent immobilization of penicillin G acylase, *via* reaction of its free lysine residues with the aldehyde groups at the surface (see Figure 13). Approx. 1200 BPU were bound per g of support, with a recovered activity of 487 BPU g^{-1} (41%).[79] The resulting biocatalyst was much more thermally stable than the dissolved enzyme and showed no loss of activity after 4 h at pH 8 and 50 °C. It was reused for 10 times with little activity loss.[79]

The immobilization of pencillin G acylase on glutamate-pillared LDH is promising on account of the high activity and stability of the preparation. A major drawback is the quite complex preparation of the carrier, however, while questions regarding its particle size and mechanical stability remain unanswered.

4.6 Lipid Biocomposite Films on Inorganic Supports

Lipid films of controlled thickness, consisting of stearic acid or octadecylamine, were deposited on quartz and silicon substrates. These films assumed a bilayered structure, in which lipid bilayers are separated by hydrophilic channels (see Figure 14). The anionic (stearic acid) and cationic (octadecylamine) films absorbed penicillin G acylase to the same extent.[80] This latter result is somewhat unexpected, as the experiments were performed at pH 6.5, which is well below the enzyme's isoelectric point (8.1). Hence, the authors concluded that electrostatic interactions are not the main driving force for ab-

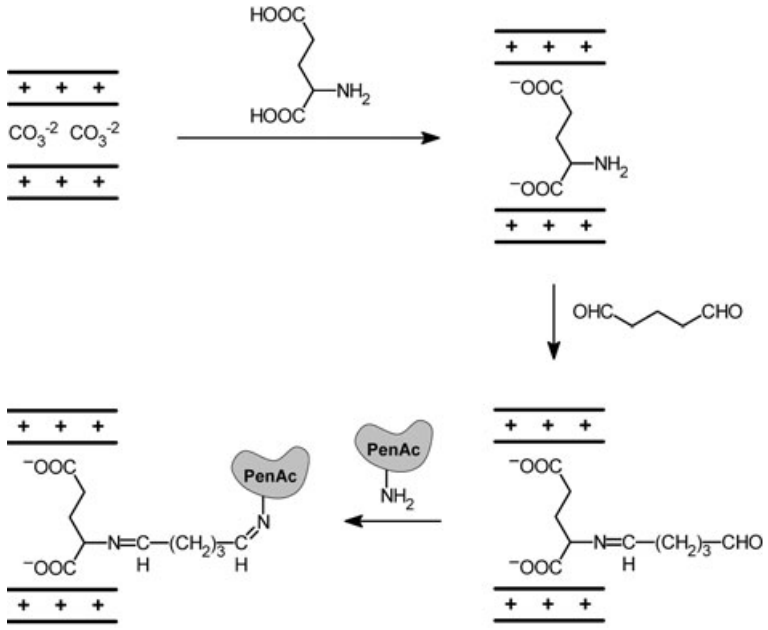

Figure 13. Immobilization of penicillin G acylase on a glutamate-pillared LDH.[79]

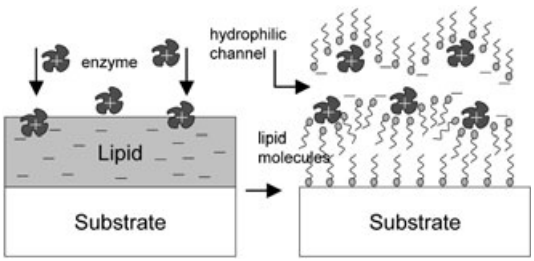

Figure 14. Schematic representation of the absorption of penicillin G acylase into a lipid bilayer film.[80]

sorption. The mass loading of the lipid film doubled when the thickness of the stearic acid layer was increased from 25 nm to 50 nm, showing that the immobilization of penicillin G acylase is not just on the surface of the film but inside the lipid bilayers.[80,81]

The absorption of the enzyme proved to be irreversible as no enzyme was released by rinsing at pH 6.5 or 7.5. The activity of the penicillin G acylase was little affected by immobilization into the lipid film and the biocatalyst could be reused for a number of times with little activity loss.

We conclude that this approach of physically absorbing pencillin G acylase into a lipid film is potentially useful, provided that it can be adapted to real-world conditions, for example, by depositing such lipid bilayers on a monolithic carrier.

5 Carrierless Immobilization

Carrierless enzyme immobilization is potentially advantageous because it saves the costs of the carrier, which surpass those of the enzyme in some cases, and avoids the handling of a large mass of inactive material in the course of the reaction. Thus, insoluble enzyme preparations have been prepared by the cross-linking of a crude cell extrudate with a bifunctional reagent. Such biocatalysts can be packed in a column but are too fragile for use in a stirred batch reactor. Insoluble, particulate biocatalysts have also been prepared by the cross-linking of spray-dried enzymes with glutaraldehyde,[82] but when applied to penicillin G acylase the activity recovery was quite low.[83] We will focus on two techniques for the carrierless immobilization of penicillin G acylase: cross-linked enzyme crystals and aggregates.[84]

5.1 Cross-Linked Enzyme Crystals

The cross-linking of enzyme crystals was first described in 1964,[85] but the potential of cross-linked enzyme crystals (CLECs) as robust biocatalysts only became apparent in the early 1990s.[86] CLECs are manufactured in a two-stage process: first a concentrated protein solution is crystallized, then the crystals are rendered permanently insoluble by cross-linking with a bifunctional reagent such as glutaraldehyde. The crystallization step has often been regarded as a major obstacle to the widespread adoption of CLECs, since the development of an adequate protocol is labor-intensive and time-consuming. It should be noted, however, that crystallization also is an efficient purification procedure, in particular on a large scale.

CLECs of penicillin G acylase (CLEC-EC®) have been commercially available for some time from Altus Biologics but the production of biocatalyst CLECs has recently been discontinued. CLECs are highly active, since they consist of pure protein and are mechanically as well as chemically highly stable.[87] According to the inventors, rapid diffusion of substrate and products can be achieved, compared to other forms of immobilized enzymes, as a consequence of the small size of the crystals (in the order of 15 μ, measured along the shortest axis) and the open channels (approx. 3.5–5.0 nm) between the individual enzyme molecules. It should be noted, however, that the pore diameter of Eupergit C is approx. five times larger (25 nm), which may be offset by the larger particle size of this latter material.

5.2 Cross-Linked Enzyme Aggregates

Alternative to crystallization, the physical aggregation of protein molecules into super-molecular structures and subsequent precipitation can be induced by the addition of salts, organic solvents or non-ionic polymers to protein solutions, without perturbation of the original three-dimensional structure of the protein. Indeed, precipitation induced by ammonium sulfate, polyethylene glycol, and some organic solvents such as alcohols, is a commonly used method of protein purification. When these solid aggregates are rendered permanently insoluble by chemical cross-linking, cross-linked enzyme aggregates (CLEAs) are obtained (Figure 15).[88]

The effect of the precipitant on the activity of penicillin G acylase CLEAs was studied.[88] Up to 80% of the original activity was recovered upon precipitation with *tert*-butyl alcohol (T-CLEA, see Figure 16). An active site titration study of the P-CLEA, which had been precipitated with poly(ethylene glycol) revealed that loss of active sites and loss of turnover rate contributed equally to the activity loss.[40] The authors claim that the aggregation step plays a crucial role in maintaining the activity and, presumably, the native conformation of the protein. This latter notion is emphasized by the low activity recovery that resulted when penicillin G acylase was cross-linked in solution, bypassing the precipitation step (CLE, see Figure 16).[88]

The specific activities of the T-CLEA (12 kBPU g^{-1}), the CLEC (16 kBPU g^{-1})[88] and penicillin G acylase on oxirane-modified monolithic silica [20.8 kBPU (g

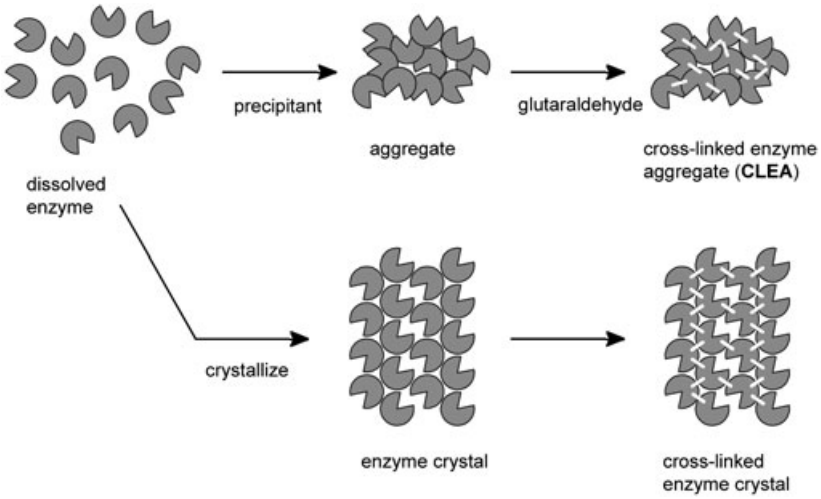

Figure 15. Comparison of the formation of CLEAs *versus* CLECs.

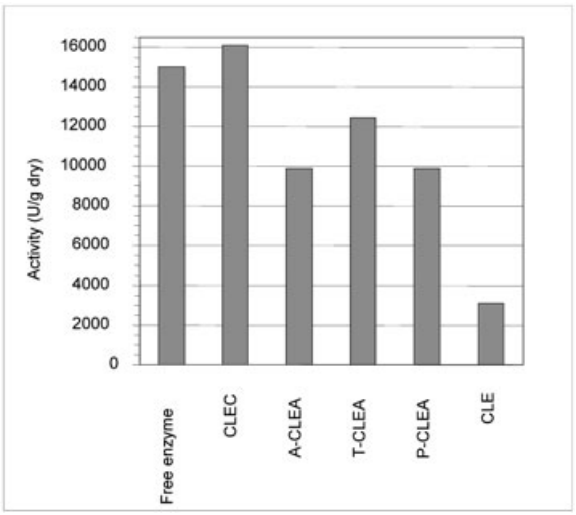

Figure 16. Penicillin G hydrolysis activity of penicillin G acylase CLEAs.[88] Precipitants: ammonium sulfate (A-CLEA), *tert*-butyl alcohol (T-CLEA), and PEG 8000 (P-CLEA); CLE was obtained by cross-linking penicillin G acylase in solution.

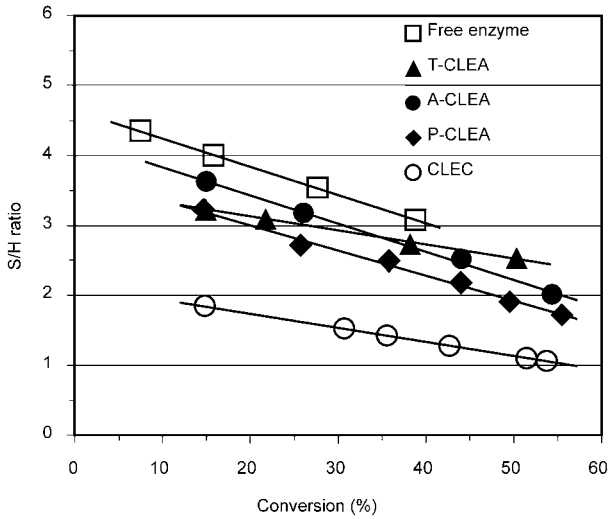

Figure 17. Synthesis/hydrolysis ratio *versus* the conversion of 6-APA in the synthesis of ampicillin catalyzed by free and different types of cross-linked penicillin G acylase.[88] Reaction conditions: 300 mM 6-APA, 500 mM (*R*)-phenylglycine amide and 20 BPU penicillin G acylase in 20 mL water, stirred at pH 7 and 20 °C.

protein)$^{-1}$][65] diverge widely, possibly because of differences in purity. The activity recovery of the T-CLEA (80%) is better than that of a crushed Eupergit C preparation (65%).[43]

Penicillin G Acylase CLEAs as Synthesis Catalysts

The synthetic performance of penicillin G acylase CLEAs was investigated in the synthesis of ampicillin from (*R*)-phenylglycine amide and 6-APA (Figure 3), in particular with regard to the competition between synthesis and hydrolysis (see Figure 4). The CLEAs mediated the reaction at an S/H ratio[23] that was close to that of the dissolved enzyme, whereas CLEC-EC afforded approximately half that value (Figure 17). Remarkably, the S/H ratio of the T-CLEA declined less as the reaction proceeded, compared with the free enzyme, A-CLEA and P-CLEA. Apparently, the structure of the enzyme is subtly changed by precipitation with *tert*-butyl alcohol. It is also noteworthy that the productivity of the T-CLEA was almost four times that of the CLEC and even higher than that of the free enzyme (see Table 2).[89]

The T-CLEA proved to be stable under the reaction conditions. Leakage of enzyme into the solution did not take place and the full initial activity was recovered to the point were ampicillin started to precipitate.[88] At

Table 2. Performance of immobilized penicillin G acylase in the synthesis of ampicillin[89]

Biocatalyst	Conversion [%]	S/H	Relative productivity
Free enzyme	88	2.0	100
CLEC-EC	72	0.7	39
T-CLEA	85	1.6	151
PGA-450	86	1.6	0.8

this point, approx. 30% of the original activity was lost, presumably because the crystallization caused some break-up of the aggregates.

Ampicillin Synthesis in Organic Media

An obvious way to prevent parasitic hydrolysis in enzymatic acyl transfer reactions is to use an organic solvent at minimum water concentration. Such conditions are not tolerated by free penicillin G acylase, however, in contrast with lipases. On the other hand, penicillin G acylase CLEAs proved to be unexpectedly resistant to hydrophilic organic solvents, such as acetonitrile, 2-ethoxyethyl ether and *tert*-amyl alcohol.[88] Ampicillin synthesis was indeed observed in such media, although at a much-reduced rate. Contrary to expectations, however, the improvement in S/H ratio in relation to the conversion was only marginal, compared with reaction in water. High (up to 86%) ampicillin yields resulted from reactions in acetonitrile medium in the presence of a CLEA of the relatively unknown penicillin G acylase from *Alcaligenes faecalis*.[90] A minimum water activity of 0.4–0.7 was required for enzymatic activity,[90] which is in the same range as required by penicillin G acylase on Celite R-640.[77]

CLEAs: Concluding Remarks

Penicillin G acylase CLEAs are highly active and stable preparations with an impressive synthetic capability. Their potential to maintain the activity of penicillin G acylase in non-aqueous media has been convincingly demonstrated, moreover. In an industrial environment, their small particle size, which renders CLEAs more difficult to filter than the traditional immobilisates, may prove an obstacle to their adoption. Consequently, current research on CLEAs is aimed at improving their filterability without seriously compromising their activity.[91] Furthermore, it is worth noting that the CLEA methodology has been successfully applied to the immobilization of a wide variety of enzymes, including lipases, esterases, nitrilases, oxynitrilases, phytase, glucose oxidase, galactose oxidase, catalase, laccase and an alcohol dehydrogenase.[91] We conclude that their ease of preparation coupled with wide applicability, including crude enzyme preparations, and high activity retention and stability will eventually lead to the widespread use of CLEAs in industrial biotransformations.

6 Emerging Techniques

6.1 Integrated Purification and Immobilization of Penicillin G Acylase

Procedures for the manufacture of industrial biocatalysts should be as simple as possible for economic reasons. Hence, the purification of the crude enzyme, which is often just the homogenized cell mass, is dispensed with whenever possible. Penicillin G acylase is a special case, however, because covalent immobilization is an absolute requirement, which makes the presence of contaminating proteins, adding to the use of expensive carrier, undesirable. Hence, there is an interest in the integrated purification and immobilization of penicillin acylase.

Aqueous two-phase partitioning is a relatively simple procedure for the enrichment of enzymes. Thus, purified penicillin G acylase was obtained from disrupted and pre-treated *E. coli* cells by partitioning in a phosphate poly(ethylene glycol) biphasic system. The enzyme was recovered from the PEG-rich top phase, in a yield of up to 79% and a purification factor of 7.8.[92] A recycle process was set up comprising enzyme removal from the top phase by immobilization, addition of fresh enzyme to the bottom phase and recombination of the two phases for the next cycle (see Figure 18).

Two types of support were used in the immobilization step: Eupergit C and aldehyde-activated agarose. The activity of the Eupergit C immobilisate was rather low (approx. 85 BPU g^{-1}) and the time that was required to absorb this modest amount of enzyme from the top phase was 50 h.[92] The presence of 12% PEG, which oth-

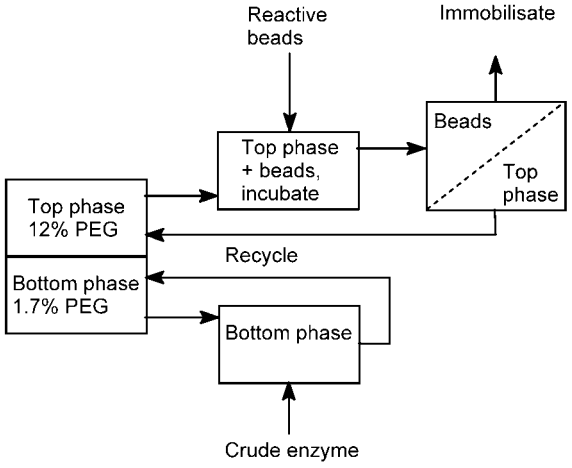

Figure 18. Integrated purification and covalent immobilization of penicillin G acylase.[92]

erwise had only a minor effect on the outcome, may retard the immobilization due to its effect on the viscosity. The low final activity could possibly be caused by remaining protein contaminants in the top phase. Aldehyde-agarose absorbed penicillin G acylase from the top phase to a final activity of approx. 50 BPU g^{-1}, not a bad result considering that its density of aldehyde groups is, at 40–50 μmol mL^{-1}, only 30% of the density of oxirane groups in Eupergit.

In conclusion, the approach, integrating purification and immobilization, is sound but improvements are required to make it practically feasible. It is also worth noting, in this context, that the CLEA methodology described above constitutes a combination of enzyme purification (by precipitation) and immobilization.

6.2 Covalent Attachment to a Thermosensitive Polymer

Poly-*N*-isopropylacrylamide (polyNIPAM) is a thermosensitive polymer: it precipitates from aqueous solutions when heated above 32 °C. Hence, conjugation of polyNIPAM with proteins provides the latter with thermosensitivity and allows their separation from aqueous solutions by small changes in temperature, pH or ionic strength.[93] Conjugation of penicillin G acylase with, for example, polyNIPAM could provide for easy separation of the biocatalyst at the end of the reaction while avoiding the mass-transfer limitations that seem an unavoidable consequence of immobilization on a carrier.

A reactive polyNIPAM derivative was synthesized by the copolymerization of NIPAM and *N*-acroyloxysuccinimide, in a 19:1 ratio, into a copolymer (see Figure 19) with a molecular mass of 39 kDa.[94] The density of activated ester functions in the copolymer was approx. 0.5 mol %. When penicillin G acylase was challenged by a 35-fold weight excess of this latter copolymer, 37% of the original activity was recovered in the conjugate. According to gel filtration chromatography, the structure of the polymer-enzyme conjugate was mainly represented by single enzyme molecules modified with many chains of copolymer.[94]

To increase the protein content in the conjugate, experiments were performed with a 3.5:1 weight ratio of copolymer and enzyme. The resulting conjugate contained 24% of the initial activity and was four times as active as the previous one. The protein content in the active polymer fractions was 13% (w/w) and, unfortunately, its response to a thermal shift was much weakened.[94] A further increase of the protein loading in the conjugate caused its response to temperature and ionic strength shifts to decline even more. Presumably, the large mass fraction of hydrophilic enzyme in the conjugate interferes with its thermoresponsitivity, which depends on balancing hydrophilic and hydrophobic interactions.

The conjugate with 13% protein content was evaluated in the synthesis of cephalexin from (*R*)-phenylglycine amide and 7-ADCA (see Figure 3). Its acyl transfer efficiency (S/H)[23] was comparable with that of the dissolved enzyme.[94]

In conclusion, the principle of covalently attaching an enzyme to a thermoresponsive polymer is sound in principle. The technique requires further development to become suitable for reduction to industrial practice. It would seem that fine-tuning of the hydrophobic moieties in the polymer, to balance the hydrophilicity of the attached enzyme, is a first requirement.

6.3 Magnetic Biocatalyst Beads

The filtering characteristics that are desirable in industrial practice put a lower limit of 0.1–0.2 mm on the particle size of immobilized catalysts, which leads to diffusion limitations. Catalyst removal using a magnetic field would be a good alternative, even in the presence of solid products, without imposing any lower limit on the particle size. Microparticulate polymers, which are magnetic due to embedded magnetite, are now commercially available and are applied in protein purification and cell separation, for example.[95]

The covalent immobilization of penicillin G acylase to various types of magnetic microparticles has recently been published.[96] The best results, out of several types of polymers, were obtained with magnetic oxirane-activated poly(vinyl alcohol) microbeads, with average particle diameter 1.8 μ and a specific surface area of 84 m^2 g^{-1}.[97] Penicillin G acylase was covalently immobilized to the oxirane groups *via* a 1,6-diaminohexane spacer and a glutaraldehyde linker. The maximum loading

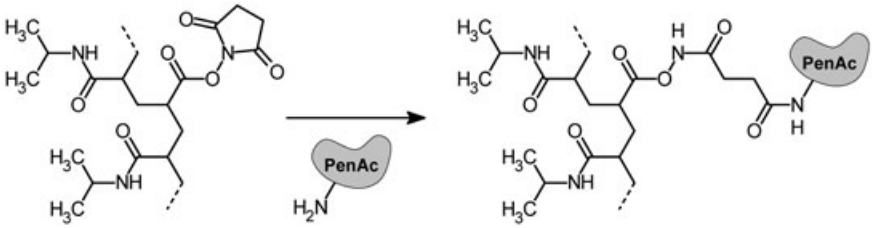

Figure 19. Attachment of pencillin G acylase to a polyNIPAM-derived thermoresponsive copolymer.[94]

was 28 mg g^{-1}, which corresponds with an expressed activity of 20 BPU g^{-1} on the basis of the published k_{cat} value of 20 s^{-1}.[96] Cephalexin was synthesized from (*R*)-phenylglycine amide and 7-ADCA in the presence of the magnetic biocatalyst with an S/H ratio of eight, which is between the values of the free enzyme and the Eupergit C-immobilized one (13 and 3, respectively).

Because kinetic resolutions are also highly sensitive to diffusion limitations, the magnetic penicillin G acylase was applied in the hydrolysis of *N*-phenylacetyl-(*R*,*S*)-phenylalanine derivative. The enantiomeric ratio (*E*) was 230, which compares rather well with dissolved enzyme ($E = 1000$) and a Eupergit C preparation ($E = 25$).[96] This latter value indicates severe diffusion limitation in the carrier.

Thus, the principle of rendering biocatalysts magnetic for easy separation has been well illustrated and proven to be sound. A major point to be improved is the low catalyst loading, which resulted in an expressed activity that is 10 times less than an average Eupergit C immobilisate.

6.4 Thermodialysis Through a Catalytic Membrane

Thermodialysis involves the application of a temperature difference over a membrane. Such conditions provoke a solvent flow from the warm to the cold side, combined with differential solute fluxes. Thermodialysis over a catalytic membrane can be used to overcome diffusion limitations, according to a basic set-up shown in Figure 20.[98]

The membrane was based on nylon, which has few free end groups for the covalent attachment of enzymes. Hence, a polymethacrylate layer was grafted onto the nylon base.[99] This latter procedure is elaborate, but has the advantage that the nano-enviroment of the immobilized enzyme molecules is decoupled from the properties of the carrier. There are indications, moreover, that enzymes immobilized on graft copolymers are more readily available for reaction than is the case with enzymes entrapped in a gel, where problems of diffusion of reactants and products can arise, especially if the products are inhibitors.[99]

The catalytic membrane was prepared from a commercially available nylon membrane, on which a methacrylic polymer was grafted by radical-initiated polymerization of the appropriate methacrylic ester.[98–100] The grafted membrane was activated *via* reaction with a 1,6-diaminohexane spacer and a glutaraldehyde linker (see Figure 21). Finally, penicillin G acylase was covalently bound to the activated membrane. These steps were separately optimized for maximum membrane-bound activity but the activity yield (based on membrane-bound enzyme) could not be improved beyond a modest 11%.[99] The activity of the membrane-bound biocatalyst decreased 50% upon the first use, but subsequently remained stable, at 43–60% of the original value, over a prolonged period of repeated use.

The thermodialysis reactor comprises two chambers that are separated by the catalytic membrane; the thermostatted reaction mixture is continuously circulated through both chambers (see Figure 20).[98,100] The reactor was applied in the synthesis of cephalexin from (*R*)-phenylglycine amide and 7-ADCA (see Figure 3). The course of the reaction was very similar to that of

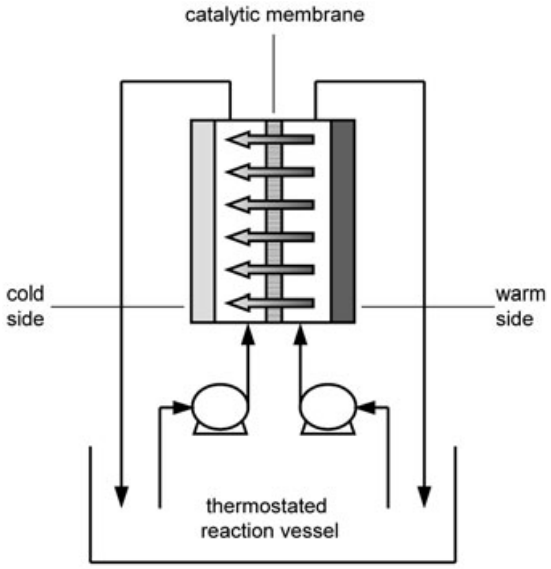

Figure 20. Schematic representation of the thermodialysis reactor. The solvent flow is from the warm to the cold side.[98]

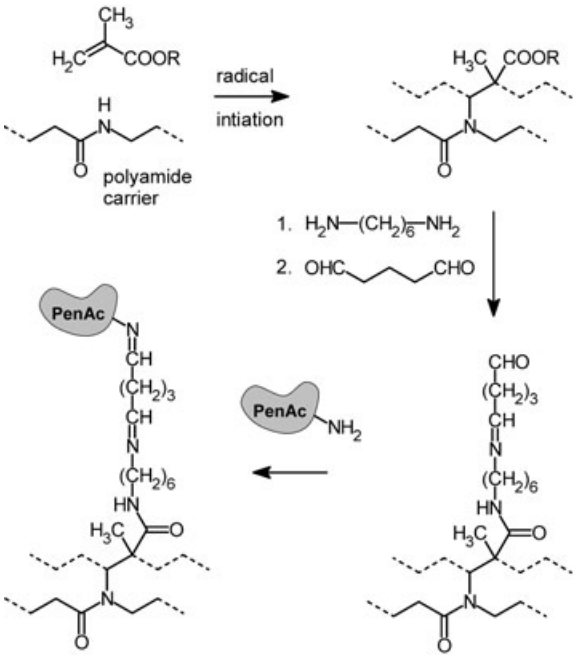

Figure 21. Activation of nylon and covalent attachment of penicillin G acylase.

an Assemblase-catalyzed one (see Section 3.3) when both chambers were at the same temperature. When a temperature gradient of $=30\,^{\circ}C$ was applied,[101] in contrast, the reaction profile resembled that of the free enzyme.[98] This latter effect is ascribed to the temperature gradient-driven flow across the membrane. In a related study, the kinetic parameters of the cephalexin synthesis were measured with and without a temperature gradient. The (apparent) Michaelis constants[102] of the membrane-bound biocatalyst were, under isothermal conditions, 3.5–4 times those of the free enzyme. This latter ratio decreased to approx 1.6 when a temperature gradient was applied,[100] which also is an indication that a temperature gradient improves the mass-transfer in the membrane.

It has become clear that the principle of the membrane thermodialysis is sound and quite promising, but some major problems still must be solved to make it a technically and economically viable option. Presumably, the activity yield of the membrane-bound enzyme (6% in the long run) can be improved. The temperature difference and, hence, the gain in performance, is limited by the tendency of 7-ADCA and cephalexin to undergo thermal degradation. It would be preferred to keep the warm chamber below $30\,^{\circ}C$, even though the contact time is short. The catalyst loading (enzyme per m^3 of reactor) is undesirably low, but can, in principle, be improved by switching to a hollow fiber module. Such a design could also allow higher temperatures, due to the reduction in residence time.[98]

6.5 A Catalytic Electro-Membrane Reactor

In the hydrolysis of penicillin G, immobilized penicillin G acylase is less, often much less, efficient than the free enzyme. A major reason is the accumulation of acid, which lowers the turnover rate of penicillin G acylase and, moreover, enhances the inhibitory effect of the phenylacetate side product. One way to influence the transport of the reactant and products in the carrier matrix is to apply an electric field.

This latter principle forms the basis of the electro-membrane reactor set-up shown in Figure 22. Penicillin G acylase was entrapped in a polyacrylamide slab by radical-initiated polymerization of a mixture of an aqueous solution of acrylamide, phosphate buffer and enzyme.[103] The resulting catalytic membrane separates the reactant chamber, through which penicillin G was circulated, from the product chamber. Both chambers were replenished by circulation *via* separate reservoirs, which were kept at pH 8. An electric field was applied *via* two electrodes, which were separated from the reactant and product chambers by ion-exchange membranes.[103]

In the absence of an electric field all mass transport is by mass gradient-driven diffusion. The reaction presumably takes place in the membrane, in a shallow zone facing the reactant chamber, similar to a conventionally immobilized biocatalyst,[43] and the products diffuse back into the reactant chamber. Upon application of a positive field the reaction rate was seen to increase (see Figure 23). A plausible explanation is the separation of the anionic products from the protons: the diffusion of the 6-APA and phenylacetate anions out of the reaction zone, back into the reactant chamber, is accelerated by the field whereas the protons are drawn towards the product chamber (see Figure 22c). Hence, the pH in the reaction zone is kept constant. A negative field (Figure 22d), in contrast, pulls the anionic compounds (penicillin G, 6-APA and phenylacetate) deeper into the membrane to-

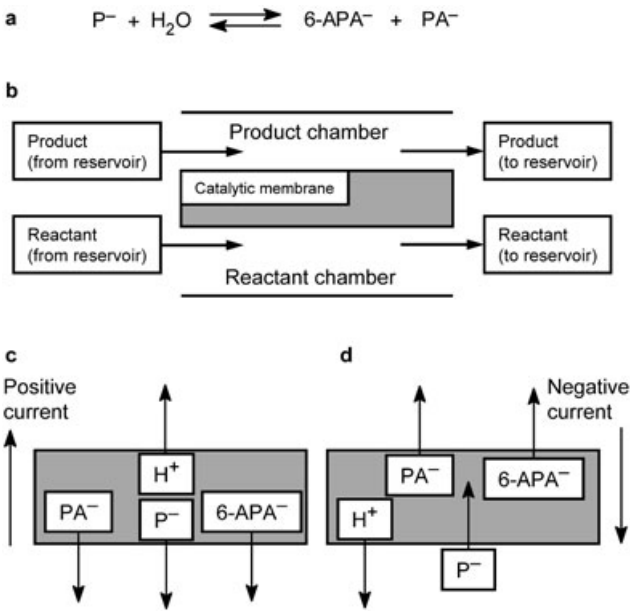

Figure 22. Principle of the electro-membrane reactor (b) and the movement of the ions under an applied positive (c) of negative (d) field. P^-, penicillin G anion; PA^-, phenylacetate anion.

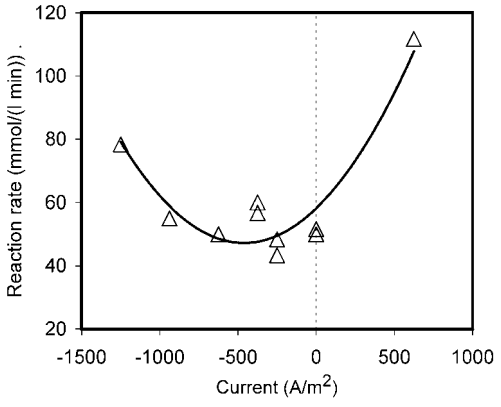

Figure 23. Rate of the penicillin G hydrolysis reaction as function of the current density.[103]

wards the product chamber. The initial result was a drop in reaction rate, which is caused, presumably, by the lengthening of the diffusion pathway. A modest gain in reaction rate was only observed at a large negative current (Figure 23). Even then, diffusion limitation remained predominant, as is shown by the small increase in productivity upon a five-fold increase of the enzyme loading in the membrane, in contrast to what was observed when a positive current was applied (see Table 3). A negative current also resulted in the partial separation of 6-APA, which accumulated mainly in the product chamber, and unconverted penicillin G, which was mainly recovered from the reactant chamber.[103] Such integration of the reaction and downstream processing is potentially interesting, although it will generally be preferred to run the reaction to complete conversion.

A potential drawback of the electro-membrane reactor is the resistive heating of the membrane and the solutions in the chambers, which may cause deactivation of penicillin G acylase as well as degradation of the β-lactam compounds. A temperature rise was observed in the reactant stream in particular, which was quite modest but rose to 50 °C when allowed to accumulate in the course of the reaction. The insertion of a cooler will obviously keep the temperature within acceptable limits.

Summarizing, the rate of penicillin G hydrolysis can be significantly enhanced by applying an electric field to the membrane-bound biocatalyst, but it still has to be demonstrated that the enzyme utilization and/or the space-time yield improve in comparison with conventional procedures. It is not clear, moreover, that the ultimate target: control of the reaction by kinetics instead of by mass transport phenomena, is within reach. It has been suggested, however, that this latter objective may be achieved by employing a multi-component reactor, for example, electrodialysis or adsorption for component separation.[104] If successful, it may be expected that the principle of the electroemembrane reactor may be much more generally applicable.

Table 3. Productivity of the immobilized penicillin G acylase in the electro-membrane reactor.[103]

Current density [A m^{-2}]	Enzyme loading [U cm^{-3}]	Relative productivity [%]
+625	150	710
+625	30	214
0	30	100
−1250	30	162
−1250	150	232

7 Conclusion and Future Outlook

The objectives of immobilization are easy recovery and reuse, high operational stability and activity per unit volume. A wide range of solutions to meet these demands has been developed and successfully demonstrated. Discussing these in a scientifically meaningful way is difficult, however, because enzyme preparations of different purity have been used and the assay conditions do rather diverge. Reporting data on active site recovery and turnover rate, which would also help to elevate the debate to a higher level, is not yet common practice, moreover. With these reservations, some representative results are summarized in Table 4.

Mass transfer limitations, which in current practice are closely linked to particle size, are now a major obstacle to further improvement. Overcoming these, without sacrificing performance, will require highly innovative approaches. The principle of the monolithic carrier, which decouples the diffusion pathway from the particle size, is a highly promising development that is nearly certain to be adopted in practice. Separation of the biocatalyst without filtration would remove all limitations to the particle size and, hence, reduce or remove diffusion limitations. Embodiments of this latter approach are magnetic microparticulate biocatalysts and attachment of enzymes to a 'smart' polymer. Finally, thermodialysis, to incite a solvent flow through a membrane-immobilized biocatalyst, deserves to be mentioned in this context.

Table 4. Immobilization of penicillin G acylase: overview.

Preparation	Particle Size [μ]	Recovery [%]		Activity [BPU g^{-1}]	Turnover rate [s^{-1}]	Ref.
		Activity	Active sites			
Eupergit-C	160	75	85	322	54	43
Eupergit-C	160	43	83	556	32	43
Assemblase®	200	n.d.	n.d.	378	22	40
Epoxy-SiO_2	5	n.d.	n.d.	1250	n.d.	64
Zeolite ITQ-6	n.d.	119	n.d.	2440	n.d.	69
Pillared clay	n.d.	41	n.d.	487	n.d.	79
CLEC	150 × 75 × 15	n.d.	n.d.	16085	n.d.	89
CLEA	45	55	73	12440	45	40

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