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Engineering Aspects of Carriers for Immobilized Biocatalysts

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Introduction

Many biocatalysts occur naturally attached to the cell membranes or, at least, entrapped within the cells. This most likely led to the idea that even isolated enzymes may work better when immobilized on a solid carrier. Another reason, though, can be much easier down-stream separation of products and their easier work-up in a technology that uses heterogeneous catalysts.

The legendary textbook example of an 'ancient' enzyme immobilization is the adsorption of invertase and proteolytic enzymes on inorganic supports, including charcoal, described in the year 1908 (Michaelis and Ehrenreich, 1908). However, this was too early to be recognized by both the scientific and industrial community as a break-through, and has been largely forgotten. Fast industrial growth after World War II also opened doors to exploration of novel technologies. One of many new research directions aimed at the heterogenization of homogeneous catalysts to combine the high efficiency and unique selectivity of homogeneous catalysts with ease of handling and simpler technology typical for the heterogeneous catalysts. No wonder that, among others, the most powerful and most selective natural catalysts, the enzymes, also became a very popular target for this effort.

An enzyme immobilization rush resulting in a huge number of papers, patents, reviews (for example Keyes and Albert, 1986; Linhard, 1987; Kennedy and Cabral, 1987; Kennedy, Melo and Jumel, 1989; Woodley, 1992) and monographs (for example Wingard, 1972; Zaborsky, 1973; Chibata, 1978; Wiseman, 1985; Rosevear, Kennedy and Cabral, 1987; Veliky and McLean, 1994) started in the mid-1950s and has continued ever since. The four volumes of Methods in Enzymology (Mosbach, 1976, 1987) are the most authoritative references so far.

At present, the application of immobilized biocatalysts is well established in manufacturing of commodity products such as 6-aminopenicillanic acid, high-fructose corn syrups, and L-amino acids (Mosbach, 1987; Marconi, 1989), but it can also be traced in analytical applications such as medical diagnostics and therapy (Carr and

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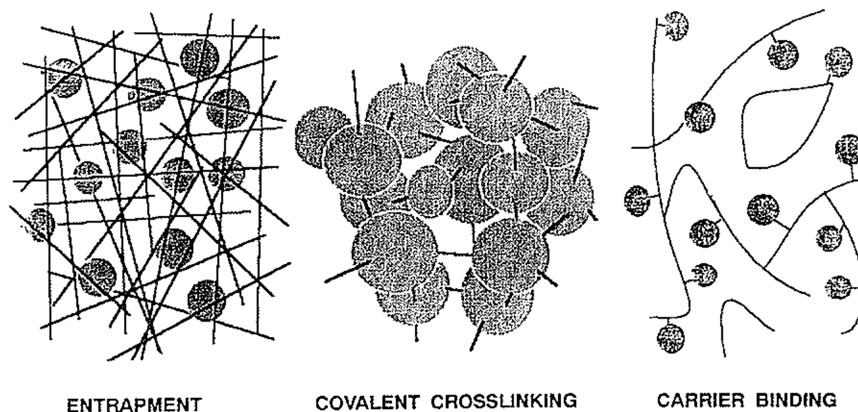


Figure 1. Schematic representation of immobilization techniques.

Bowers, 1980; Kennedy, Melo and Jumel, 1989; Marconi, 1989), biosensors (Suzuki and Karube, 1981; Guilbault, 1985; Fernandez-Romero and Castro, 1993), chromatographic packings and detectors (Domenici *et al.*, 1990; Klein, 1991; Turkova, 1993; Haginaka *et al.*, 1994; Marko-Varga, Johansson and Gorton, 1994), in electronics (Shulga *et al.*, 1994), in on-line solid phase reactors (Alebic-Kolbah and Wainer, 1993; Calatayud and Garcia, 1993; Shan, McKelvie and Hart, 1993), in organic synthesis (Woodley, 1992) and in many others.

Methods for immobilization of biocatalysts

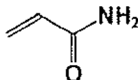
Despite the enormous literature describing the immobilization of biocatalysts, only a few basic concepts were used that can be classified as entrapping, crosslinking and carrier-binding (Chibata, 1978). A simplified graphical representation of these techniques is shown in *Figure 1*.

ENTRAPMENT

In contrast to other immobilization methods, the entrapped biocatalyst moieties are not covalently linked together or to any solid support but are included within an insoluble, highly hydrophilic matrix that physically hinders leakage of the active species. The biocatalyst is typically admixed to a solution of monomers or a soluble polymer and the mixture is converted to a gel.

The advantages of entrapping are simplicity of the immobilization method, low cost, possibility of immobilization of more than one enzyme at any level of purity or within a mixture of cells, high yields of immobilization and easy recovery of reaction products. These advantages make the method attractive for industrial processes (Mosbach, 1987; Marconi, 1989).

The most popular polymer for entrapping appears to be polyacrylamide gel, first used by Bernfeld (Bernfeld and Wang, 1963), that is typically prepared by copolymerization of acrylamide (I) with small amounts of methylene-bis-acrylamide (III).



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Besides crosslinked polyacrylamide, some other hydrophilic polymers were used, such as poly(acrylic acid), poly (vinyl alcohol), poly(vinylpyrrolidone), poly(2-hydroxyethyl methacrylate) (Chibata, 1978; Mosbach, 1987), but these, in contrast to the acrylamide based gels, did not find any large-scale application. The formation of the matrix by a free-radical polymerization may diminish the activity particularly due to the negative effect of free radicals and reaction heat released during large scale batch polymerizations. Therefore gentler techniques were developed for entrapment, techniques that retain almost all the activity of the original biocatalyst (Mosbach, 1987) and involve natural polymers like κ-carrageenan, alginate or agar. Enhanced stability and activity was achieved by entrapment in lyotropic liquid crystals (Voss and Miethe, 1992).

Hydrophilic polymers such as poly(N-isopropylacrylamide) characterized by the lower critical dissolution temperature (LCDT) seem to be a useful alternative to the classical polyacrylamide gels. They dissolve in water at a low temperature while they are completely insoluble at temperatures above the LCDT. If such a polymer is cross-linked to a gel, it swells and opens the pores at a low temperature and allows the enzyme molecule to penetrate the bulk phase. After the temperature is increased to the working level, the gel shrinks, and the pores close and entrap the enzyme (DeRossi *et al.*, 1991).

The entrapment method is best suited for immobilization of whole cells. The enzymes are relatively small and the mesh size of the matrix must be even smaller. This dramatically decreases the diffusion rate of substrates and products within the matrix and the overall activity is low. A general drawback of the entrapped catalysts is their poor mechanical strength. The catalyst particles are soft and easily deformed, and that limits their use in large columns.

Free enzyme solutions can also be entrapped within a polymeric envelope with a thin wall that allows permeation of low molecular weight substrates and products while keeping the enzyme in an optimal environment and preventing it from leaving. This special category of immobilization is called *encapsulation*. The immobilized catalysts may have different shapes and sizes starting from liposomes and vesicles with a size of about 50 nm over the micron sized hollow fibers up to tubes and membranes, the size of which is not limited at all (Chibata, 1978; Inloes *et al.*, 1983). This method was particularly suited for the development of the concept of artificial cells (Chang, 1972). The encapsulation of various enzymes into an array of uniform microcapsules provides excellent activities and is very promising for use in bioreactors (Parthasarathy and Martin, 1994).

COVALENT CROSSLINKING

Both proteins and whole cells contain many reactive groups that can be used either directly or after an additional activation step for a reaction with at least bifunctional low molecular weight compounds. These crosslinking agents form bridges that link the enzymes or cells together, resulting in stable macroscopic

aggregates. The crosslinking reactions can be both condensations and addition polymerizations.

The number of condensation agents is rather high. However, bifunctional glutaraldehyde, that reacts easily with amino groups, is used most often, owing to both simplicity of work and commercial availability. The condensation technique is frequently used for immobilization of cells (Vojtisek and Jirku, 1981) while it is less preferred for isolated enzymes (Carasik and Carrol, 1983).

For crosslinking by a polymerization reaction, the enzyme has to be 'vinylated', that is, the enzyme is provided with polymerizable double bonds, for example by reaction with glycidyl methacrylate (D'Angiuro *et al.*, 1978). The next step is copolymerization of the enzyme 'macromonomer' with another monomer typically initiated by a free-radical initiator.

CARRIER BINDING

As may be understood from the title of this chapter, the carrier is crucial for this type of immobilization. The biocatalyst is attached to a surface of the carrier either by a non-covalent or a covalent bond. Immobilization through non-covalent adsorption relies on electrostatic, hydrophobic and dipole-dipole interactions, and hydrogen bonds. The chemical bonds typical of covalent immobilization are much stronger. The type of binding depends very much on the carrier itself. Since carrier binding is most frequently used in biotechnology, this review covers the immobilization with a special focus on the engineering aspects of the carriers.

Classification of carriers

Many materials such as clay, bricks, synthetic organic and inorganic polymers, naturally occurring materials, corn cobs, etc, have been used as carriers during the last four decades. There are also many different classifications of carriers described throughout the literature. None of these classifications is ideal. The carriers have too many facets to be fitted into any rigid criteria of permanent validity. The classification, if necessary, must be flexible and related to use. For example, the carriers may be categorized according to their surface chemistry into those bearing hydroxyl groups, epoxide groups, amino groups, etc. Other classifications may rely upon the nature of the carrier (organic, inorganic, natural, synthetic) or upon the immobilization reaction. In this chapter, we concentrate on those properties that are closely related to design of a viable biotechnology process.

Sometimes, there is a tendency, to point out the 'all star' carriers that comply with all requirements of the particular technology. It has to be emphasized that no one carrier is ideal and any choice must be a result of an optimal combination of both positive and negative features taken carefully into account.

The criteria we are going to use for reviewing the carriers are as follows: shape, stability in the working medium, resistance to external and internal mechanical forces, mass transfer, bulk and surface chemistry, polarity, reusability, environmental impact and cost considerations. We are aware of the fact that this classification is also inaccurate and that the chosen categories overlap each other.

PHYSICAL SHAPE

Each technology requires the immobilized biocatalysts to possess a particular shape. Some technologies such as stirred tanks are quite flexible and the shape is not that important while others are very shape-dependent such as membrane reactors.

Irregular particles. Simple grinding of large blocks of hard materials is often used in manufacturing irregular particles. Soft polymers such as polyacrylamide gels are disintegrated by cutting with a blade or passing through a metallic mesh. These materials are generally cheaper than those with better defined shapes as the technology is simpler. However, the sharp edges of rigid materials are fragile and shatter while forming fines that wash out and plug the columns, erode the pump pistons and even damage the products.

Beads. Regular shapes such as beads, membranes and flat surfaces are preferred, even though the preparation is more sophisticated. The only variable that defines the shape of a bead is the diameter. The required size of the carrier depends on the process in which it is supposed to operate and varies within several orders of magnitude from a few nanometers up to millimeters. The designed size of the support defines the method of its preparation.

Suspension processes start by dispersing one liquid phase in another liquid phase in a form of small droplets. These are precursors of the final solid beads. Dispersion achieved by simple stirring is a random process. Continued stirring results in a two-phase system in which coalescence and redispersion reach a dynamic equilibrium and leads to droplets of different sizes. The size depends on viscosity of the continuous phase, interfacial tension, phase ratio, and intensity of stirring (energy input) that is a function of shape of both stirrer and reactor, and of the stirring speed (Brooks, 1990; Yuan, Kalfas and Ray, 1991). Therefore, products of a suspension process consist of particles having a broad size distribution. This has to be narrowed for some applications by size fractionation such as sieving. The suspension process is used generally for preparation of beads, regardless of the chemical nature of the material. For example, silica, dextran or glycidyl methacrylate beads are prepared in a suspension. The difference is in the chemistry that changes the original droplets of a liquid phase to a solid phase.

Spraying of one phase into the other is a less common method for preparation of suspensions, and was used to get beads from thermoreversible polymer solutions (Mosbach, 1976) or from acrylamide based monomers (Woodward *et al.*, 1982). The method is based on solidification of droplets descending from a capillary or from a set of capillaries located at the top of a tube filled with an immiscible liquid. The solidification occurs while the droplets pass the bulk liquid phase and the final beads are collected at the bottom. Similar techniques employ forcing of the monomer phase through pores of a tubular glass membrane into the aqueous phase followed by a polymerization (Omi *et al.*, 1994). The advantage of these rather special processes is a relatively narrow particle size distribution of the resulting beads. This, however, hardly outweighs the complexity of the equipment and the low productivity.

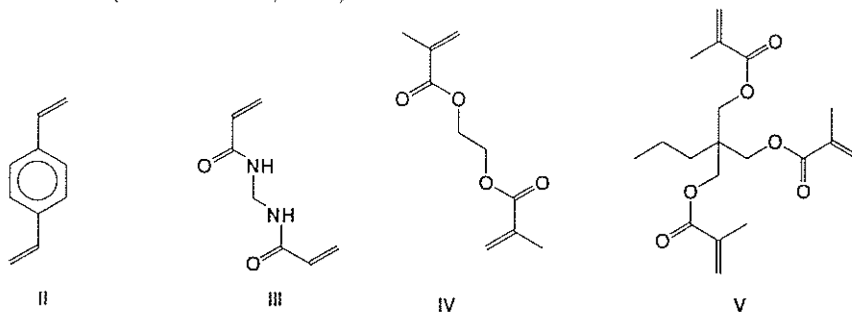
The beads used as a carrier have to be nearly insoluble in all liquids used during both

manufacturing and biotechnology processes. The obvious method of achieving this goal is crosslinking. Two basic methods are available for preparation of beaded crosslinked carriers suitable for immobilization: *crosslinking of soluble macromolecules and crosslinking polymerization.*

Crosslinking of existing polymers is very common for preparation of polymer-based carriers for enzyme immobilization. The most famous examples of this class of materials produced by Pharmacia (Uppsala) are based on dextran and agarose (Sephadex, Sepharose), natural polysaccharides crosslinked to different degrees by epichlorohydrin or by hydrophilic polymer chains. The product line ranges from soft gels to rigid beads (Mosbach, 1976; Arshady, 1991a).

A process similar to the subsequent chemical crosslinking by bifunctional reagents is a sol-gel transition. Some naturally occurring polysaccharides or proteins are insoluble in water at ambient temperature due to non-covalent interactions between their chains which successfully replace the chemical crosslinks. These materials readily dissolve at increased temperature and the hot solution is dispersed in an immiscible liquid and the system cooled to produce beads swollen with water. Typical examples of thermoreversible gels are agarose, starch, or gelatin (Hupkes and Tilburg, 1976; Kuu and Polack, 1983).

Cellulose is also a polysaccharide that is insoluble in any individual solvent, even at a high temperature, due to the crosslinking by many hydrogen bonds within its crystalline structure. Cellulose xantogenate (viscose) produced by a reaction of cellulose with carbon disulfide is a liquid. Therefore, it can be dispersed in an immiscible medium and the droplets solidified at a higher temperature when the viscose decomposes back to the original cellulose. Porous, mechanically strong beads are formed (Baldrian *et al.*, 1978).



In contrast to the previous techniques, the *crosslinking polymerization* in a suspension starts from low molecular weight monomers that are able to form long chains upon the polymerization reaction (Brooks, 1990; Yuan, Kalfas and Ray, 1991). The monomer mixture contains a polyvinyl crosslinking monomer such as divinylbenzene (II), methylene-bis-acrylamide (III), ethylene dimethacylate (IV) and trimethylolpropane trimethacrylate (V). The product of crosslinking polymerization is an insoluble network that swells to an extent that depends on the amount of crosslinking agent present in the mixture.

An *emulsion polymerization* provides beads with a size in submicron range. The concept of the emulsion polymerization is simple. Molecules of a surfactant (soap) aggregate in water to form colloidal moieties called micelles. A monomer added to the micellar system dissolves partly inside the micelles where the polymerization takes

place initiated by a water-soluble free-radical initiator. The stirring efficiency is less important that in suspension polymerization. The product of emulsion polymerization, called latex, is a stabilized colloid dispersion of tiny polymer particles in water. These cannot be obtained as dry matter without loss of the particulate character (Poehlein, 1986). The latex particles can also be used as 'seeds' and swell with new monomers. The subsequent polymerization results in much larger, stable and size monodisperse beads (Ugelstad *et al.*, 1985). Latex particles have not often been used as carriers (Kawaguchi, Koiwai and Ohtsuka, 1988; Okubo, Kondo and Takahashi, 1993) though an immobilization on the surface of very small non-porous beads provides for much faster mass transfer. On the other hand, such small particles cannot be used in column processes and even in stirred tanks their separation from the reaction mixture may represent a serious problem.

Membranes. Membranes are thin barriers that allow preferential passage of certain substances between two bulk phases located on either side of the membrane. Manufacturing of classical membranes uses unit processes such as extrusion of melts or casting. They have been used in biotechnology for years as an important part of the downstream processing (Wang, Sinskey and Butterworth, 1970) and in membrane reactors (Carr and Bowers, 1980; Vieth, 1988). Membranes have also been used as a carrier for covalent immobilization.

The number of polymers typically used to manufacture membranes is very limited. Typical examples are polyolefines such as polyethylene and polypropylene, polysulfone, poly(oxyphenylene) and polyamides. None of these contain any reactive groups suited for a covalent attachment of the biocatalysts, as they are intentionally made as inert as possible. Therefore, an activation step is required prior to the immobilization to provide the surface with some reactive groups. For example, the polyolefines are modified by a plasma-induced grafting (Wang and Hsiue, 1993), the polyamides are partly hydrolyzed (Da Silva *et al.*, 1991), the polysulfones are functionalized by a Friedel-Crafts reactions (Ansorge and Staude, 1983). Though the immobilization techniques are not simple and use of 'classical' membranes carrying an immobilized enzyme for a large scale production is unlikely, the shape of this carrier is the most advantageous for construction of devices such as enzyme electrodes.

New materials introduced recently by 3M Company, the Empore discs, formed by particulate sorbent embedded in a Teflon web, that combine the positive properties of both beads and membranes, seem to be also promising for immobilization of biological catalysts.

Continuous tubular porous carriers. Recently, carriers in non-traditional shapes emerged that can be used for both liquid chromatography and immobilization of enzymes. For example, a paper-like cellulose rod consisting of a tightly rolled fine paper was activated using methods typical for polysaccharide supports and α-amylase or antibodies immobilized with a high yield (Kennedy and Paterson, 1993). Rods of macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) contain epoxide groups directly suitable for binding biologically active molecules (Svec and Fréchet, 1992).

Fibers. Unlike membranes, the outer surface of very fine fibers is relatively large. Therefore, some attempts were made to use for example poly(vinyl alcohol) (DeRossi, 1991) or poly(ethylene-*co*-acrylic acid) (Emi, Murase and Hayashi, 1994) fibers for immobilization of enzymes and cells. Since all the biocatalyst is exposed at the surface, no diffusional restriction of activity occurs and the conjugates exhibit very good kinetics. In contrast to latex particles, handling of a bunch of fibers, cloth or knitted fabric is much simpler.

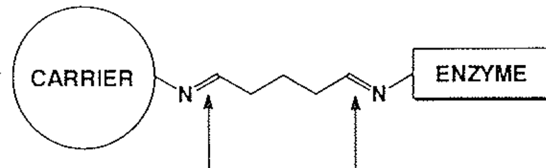
Stability of carriers in the working medium

The immobilized biocatalysts are used in various media such as aqueous solutions differing in acidity (basicity), ionic strength and chemical composition, or in organic solvents. Quite harsh conditions are used for cleaning and for regeneration of the carriers after the catalytic activity is exhausted. Therefore, the carrier has to be sufficiently stable.

Chemical stability of the immobilized biocatalysts may become a serious limiting factor for use of certain carriers. All natural and many synthetic polymers include heteroatoms in the main chain or in the crosslinks that can be subjected to chemical attack and cleavage. This process may even result in a total dissolution of the carrier. For example, the amide bonds -CO-NH-, which are a part of the polyamide main chain and methylene-bis-acrylamide crosslinks, and the ester bond -CO-O- of polyesters or ester-based crosslinking agents are hydrolytically cleavable. On the other hand, the hydrocarbon -C-C- or ether -C-O-C- bonds are very stable and such supports cannot decompose at typical reaction conditions.

The majority of enzymatic reactions proceed at pH values ranging from 3 to 10. This pH range typically does not damage the organic carriers that are stable despite the presence of labile bonds mentioned above. However, aqueous solutions with a high pH are lethal for some inorganic supports. For example, silica already begins to dissolve at pH 8.

Attention should also be paid to the stability of the link between the carrier and the immobilized enzyme, which can be rather labile and react with some components of the reaction medium while liberating the catalyst. For example, immobilization on a carrier containing primary amino groups via glutaraldehyde results in two weak imine bonds pointed with the arrows:



In order to improve the stability of this conjugate, the imino groups are reduced by sodium borohydride to stable amino groups. The post-immobilization stabilization works well with aminated organic supports. The inorganic carriers are typically provided with amino groups by a reaction with 3-aminopropyltrimethoxysilane. The Si-O-Si siloxane bond between the attached activation reagent and the matrix is not stable at low pH and the enzyme leaks. Another example of a procedure resulting in chemically unstable binding is the widespread cyanogen bromide activation of

polysaccharide carriers. The coupling procedure results in a charged surface and the leakage of the immobilized biocatalyst due to hydrolysis of the isourea derivative is substantial. The alginate gels are 'crosslinked' by an electrostatic interaction with a bivalent metal ion such as calcium. The alginate gels are used for entrapment of whole cells. Therefore, the media used in the process must not contain monovalent metal ions or protons that could exchange the Ca(II) ions and dissolve the matrix.

Several new activating methods were developed during the last two decades that increase considerably the stability of the conjugates (Mosbach, 1987; Kennedy, Melo and Jumel, 1989).

Thermal stability is typically a small problem for the carriers that do not change their properties even at a temperature well over 100°C. Some care has to be taken for thermoreversible gels as they can dissolve at an elevated temperature. The inorganic carriers are extremely heat stable. Their properties do not change even at a temperature of several hundred degrees centigrade, at which organic materials already burn.

Biological stability of the biocatalyst itself is often more important than that of the carrier. For example, some micro-organisms produce extracellular proteinases that destroy the immobilized enzyme and render the conjugate inactive (Messing, 1975). Some carriers based on natural polysaccharides are also good nutrients for micro-organisms that digest the carrier and release the originally immobilized species into solution. The synthetic polymers and inorganic supports resist microbial attack very well.

Resistance of carriers to external and internal mechanical forces

It is impossible to define any general limit for the forces that the carriers must adopt since they depend on the technology in which the immobilized biocatalyst is used. For example, hollow fibers and tubes have to resist the pressure provided by the liquid that is being pumped through, membranes should withstand normal force, etc.

Particulate supports are employed in both packed bed and stirred tank reactors. The packed bed is actually a tube filled with particles of the immobilized biocatalyst and the substrate solution is pumped through the bed under a pressure necessary to overcome the flow resistance of the column. The back pressure is proportional to the flow rate, size of the particles and to the length of the bed and tends to deform the support. In a down-flow operation, the force representing the weight of the bed above a particle also contributes to the deformation. The size uniformity of particles is advantageous as the pressure drop in a packed column is also proportional to the particle size distribution. The narrower the distribution, the lower the back pressure. Uniformly sized carriers may be packed in higher columns or a less powerful pump may be used to achieve an equal flow rate (Chibata, 1978).

In contrast to the rigid supports, the easily deformed soft carriers cannot be used in high packed beds. However, they are well suited for batch and continuous stirred tank reactors where the immobilized catalyst is dispersed in the medium by gentle stirring. The shear force is much smaller and no pressure vector is operative. Therefore, the stress is limited and the mechanical strength is less important.

Hybrid supports (pore-matrix composites) combine positive features of both gels and hard porous materials. The soft gel that provides for fast mass transfer is placed

into pores of a rigid matrix that cares for the mechanical stability (Arshady, 1991; Petro and Berek, 1992; Boschetti, 1994). The only disadvantage is low binding capacity, because the hard matrix does not contribute to the immobilization.

A fluidized bed reactor combines some features of both packed bed and stirred tank. Immobilized catalyst is held in the fluidized state by a stream of processed liquid. In this case, the catalyst has to resist the abrasion that occurs when the particles collide. Recently, Pharmacia (Uppsala) launched new silica-agarose composite sorbents with enhanced particle density under the trade name Streamline designed for use in expanded beds.

In addition to the external forces discussed above, an internal stress within the carrier can also occur on account of both swelling and osmotic pressure. These internal forces depend on the characteristics of the surrounding medium such as pH value and ionic strength. Swelling is known to be very powerful and can easily shatter even very tough materials. Ion exchangers are a typical example of supports that need special care, as they already change their size by swelling upon the exchange of the counter ions.

Mass transfer in carriers

Mass transfer plays an important role in the kinetics of any reaction that proceeds in a multiphase system. Immobilized biocatalysts represent such a system in which the substrate is transported from the bulk phase to the active site linked to a solid surface and the product of the reaction is released back to the bulk phase. Convection and diffusion are considered to be the most important mass transfer mechanisms: which one becomes the reaction rate controlling step depends entirely on the porosity of the support.

Diffusion appears to be less important if the catalyst is immobilized only on the outer surface of a non-porous solid (Gemeiner, Stefuca and Bales, 1993). The reaction rate (enzymatic activity) is governed by the mass transfer within the bulk of the medium surrounding the particles that is promoted by stirring and through the thin unstirred film of a liquid adhering to the surface of the carrier. However, the surface per weight or volume unit for a typical non-porous carrier is small and can accommodate only very limited amounts of enzyme molecules or even whole cells. The surface/volume ratio is more advantageous for very small particles such as those prepared by an emulsion polymerization (Vanag *et al.*, 1989) or fibers (DeRossi, 1991). However, with decreasing size of the carrier, the technical complexity of its recovery increases.

Porous carriers are preferred. Their porosity can be permanent or it can be achieved by swelling. Slightly crosslinked polymers are not porous in the dry state. The structure 'opens' by swelling and the interior of the carrier becomes accessible for the fast penetration of even large molecules. The extent of crosslinking determines the 'mesh size' of the swollen support (Arshady, 1991a). This category of carriers involves, for example, hydrogels based on natural polysaccharides and will be discussed in detail later.

Highly crosslinked rigid polymers and inorganic carriers do not swell. In contrast to the swollen gels, the rigidity and porosity of the carrier is achieved by a polymerization in presence of inert components (Arshady, 1991a, b). Polymers manufactured in this

way are called macroporous and remain porous even in the dry state (Seidl *et al.*, 1967; Guyot and Bartolin, 1982). Extensive studies led to the development of matrices with improved accessibility and capacity (Guyot *et al.*, 1991).

The porous properties and the morphology of the porous inorganic particles depend on the method of their preparation and that, in turn, is affected by the chemical nature of the material. The porous particles prepared by a polycondensation of hydrated salts of inorganic acids followed by a heat ageing, such as silica, titania, zirconia, exhibit morphological features similar to that of macroporous polymers (Unger and Trudinger, 1989; Arshady, 1991a).

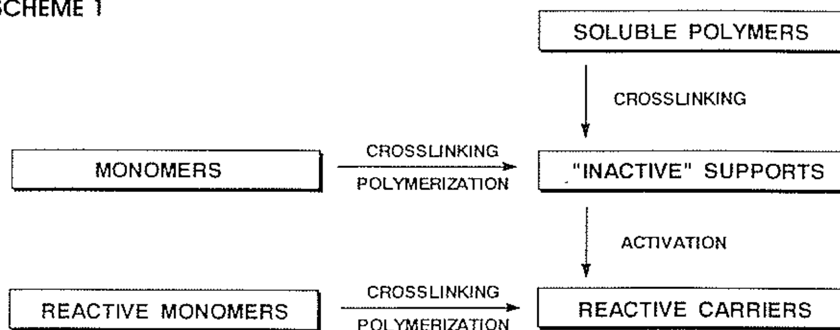
The preparation of porous glass is based on the fact that certain borosilicate glass compositions exist which, after heat treatment (500–700°C), can be leached to form a porous framework. In contrast to the globular morphology of both polymer and inorganic macroporous carriers, the morphology of the controlled pore glass is different and resembles tortuous capillaries. The pore size distribution is very narrow (Messing, 1975).

Bulk and surface chemistry of carriers

Segregation between bulk and surface chemistry is relevant as both can vary remarkably. Activated silica particles are typical examples of a carrier with an extremely different chemical composition of the pure silicon dioxide bulk material and of the surface activated with organic groups. At the other extreme are poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads prepared directly by a crosslinking polymerization of both monomers. This carrier contains the same chemistry at the surface and in bulk. The bulk of a carrier has a special function in maintaining good mechanical properties and its chemistry in relation to the immobilized catalyst is less important because it need not come in contact with bioactive molecules as it is not exposed on the surface. On the other hand, the chemistry of the surface controls reactivity of the carrier and its ability to bind the catalyst, while it does not contribute to the overall stability of the matrix.

The examples shown above suggest that the difference or identity of both bulk and

SCHEME 1



surface chemistry depends on the preparation method. If the carrier is produced in a single reaction step, for example by a crosslinking suspension polymerization, the beads are chemically homogeneous. If an activation step, that concerns only the

surface, is involved in the preparation of the reactive carrier, the chemical composition of the material is heterogeneous and, consequently, the discrimination between bulk and surface is correct. However, if the activation involves all the bulk, the carrier is again chemically homogeneous.

Scheme 1 shows ways available for preparation of a support with reactivity sufficient for enzyme immobilization.

The upper right corner represents a group of soluble polymers comprising native and modified natural polymers (starch, dextran, agarose, cellulose and its derivatives, gelatin). These are generally not suitable carriers for an immediate binding but they serve as an excellent starting substance for preparation of a particulate material. The crosslinking of these soluble macromolecules results in supports that still do not contain any reactive groups able to attach an enzyme. These 'inactive supports' are also produced directly from small monomer molecules such as 2-hydroxyethyl methacrylate, vinylpyrrolidone, maleic anhydride, acrylamide and its derivatives, styrene, etc. (Coupek, Krivakova and Pokorny, 1973; Specht and Brendel, 1977; Gerig and Loehr, 1980; Schulte and Horser, 1982; Handa *et al.*, 1983; Kolarz *et al.*, 1989; Marconi, 1989; Patel, Rodrigues and Cohen, 1990) by a copolymerization with crosslinking monomers such as II–V.

The goal is to obtain reactive carriers. There are two ways to achieve this goal: chemical modification (activation) of the 'inactive' carriers prepared in previous steps and a direct copolymerization of reactive monomers.

Activation may require several consecutive reaction steps leading to an active group which converts the 'inactive' support to a real carrier. There are many examples of activations, such as reaction of the agarose gels with bromcyan, chlorocarbonates and carbonyldiimidazole, diazotization of amino groups linked to a polymer matrix, reaction of 2-hydroxyethyl methacrylate copolymers with epichlorohydrin, reaction of silica with 3-aminopropyltrimethoxysilane followed by activation with glutaraldehyde, etc. (Mosbach, 1976, 1987).

Corresponding carriers are also accessible directly in a single step by a copolymerization of reactive monomers such as 4-fluorostyrene or 3-fluoromethacrylanilide (Manecke and Schlunsen, 1976), 2,3-epoxypropyl methacrylate (Svec *et al.*, 1975; Kramer *et al.*, 1975), 4-iodobutyl methacrylate (Brown and Joyeau, 1973), vanilin methacrylate (Brown and Joyeau, 1974), acrolein (Tarhan and Pekin, 1983), etc.

It is obvious that the preparation of reactive carriers by copolymerization requires more sophisticated monomers. Each new monomer also demands development of specific polymerization conditions in order to produce a carrier with optimal properties. The cost of these advantages is in the chemical homogeneity of the beads that contain only the required functionalities, and a higher consumption of the valuable reactive monomer, most of which is buried inside the matrix. The major part of the active groups is not exposed on the surface and, therefore, not available for the reaction between the carrier and a biocatalyst.

The approach involving chemical reactions of a polymer typically amplifies the chemical heterogeneity. The carrier contains not only the expected reactive groups but also the starting groups together with various other groups remaining from intermediate or parallel reactions. None of these groups can be removed because they are all an integral part of the polymer network. An optimization of the reaction conditions can

suppress the chemical heterogeneity but never fully prevent it. Although the chemical heterogeneity of the surface groups is a drawback of this approach, the activation of 'inactive' supports is the most frequently used method for the preparation of reactive carriers because a variety of activated polymeric carriers is obtained from a single 'inactive' support while keeping the other properties unchanged. Moreover, the modification may proceed in a site-selective fashion so that the major part of the reactive groups will be located only on the surface of pores large enough to accommodate the biocatalyst (Smigol, Svec and Fréchet, 1993).

Scheme 1 also illustrates that at least one polymerization of monomers or the crosslinking of a soluble polymer followed by an activation has to be done in order to prepare a reactive carrier.

The surface chemistry is simple when the enzyme is to be bound non-covalently. The first industrial process exploited an enzyme adsorbed by an ion-exchange interaction on DEAE Sephadex for separation of D and L-amino acids (Chibata, 1978). A binding of that kind vastly simplifies the regeneration of the carrier.

So far, the types of active groups of the reactive carrier were not defined or limited. Their choice must take into account the counterpart groups available in enzyme molecules or cells. The genetic code comprises 20 amino acids but only some of them carry groups suitable for immobilization reactions. For example, ε-amino groups of lysine and arginine and the N-terminal amino group, carboxyl groups of aspartic and glutamic acid and the C-terminal carboxyl, phenolic hydroxyl groups of tyrosine and aliphatic hydroxyl groups of serine and threonine, imidazole group of histidine and indole group of tryptophan can safely be used for reactions. Hydroxyls and amino groups are most frequent in proteins. The average content of serine and threoine in proteins is 7.8% and 6.5%, respectively, while the average content of lysine is 7.0% (Means and Feeney, 1971). The nucleophilicity of aliphatic hydroxyl groups is poor and that makes the amino groups the most favorable point of attachment in the immobilization chemistry.

The chemical nature of groups typically used for an immobilization reaction still does not say anything about the topography of the immobilization reaction. The activity of the conjugate is dramatically decreased when the active site is sterically hindered by attachment through a group in close vicinity to the active center. This can be avoided by oriented immobilization that employs very specific interaction between an antibody immobilized through the heavy Fc chain and the chosen part of the bioactive molecule (Turkova, 1993; Turkova *et al.*, 1994). Oriented immobilization is, so far, important for studies in basic enzymology rather than for any practical use. However, the progress in engineering of catalytical antibodies with almost any specificity, and improvements in production techniques may make this approach accessible even for applied biotechnology (Schulz and Lerner, 1993; Janda *et al.*, 1990).

The chemistry of the support material has also an important effect on the stability of the bound enzyme. The immobilization restricts the unfolding of the enzyme (Mosbach, 1976). The carrier additionally creates a specific microenvironment that might in either way change the stability and activity. The choice of the carrier is crucial in the emerging applications of immobilized enzymes in non-aqueous media (Tramper *et al.*, 1992). For example, proteolytic enzymes immobilized on a polymer containing carbohydrate moieties were found to be active in polar organic solvents such as

tetrahydrofuran, dioxane and acetonitrile that were detrimental for the native enzymes (Wang *et al.*, 1992).

Polarity of carriers

All the life on the Earth is based on reactions in aqueous media. This implies that the immobilized biocatalyst should also be located in a hydrophilic environment, part of which is provided by the carrier itself. Indeed, the immobilizations described in the literature favor hydrophilic supports. They also allow for better mass transport of water-soluble substrates toward the immobilized active sites which catalyze the transformation. Even the organic syntheses catalyzed by immobilized enzymes, antibodies and cells require some water to be present in the immediate vicinity of the catalysts. For these, tailoring of carriers with balanced hydrophilicity/hydrophobicity is essential (Tramper *et al.*, 1992).

The hydrophilic surface also decreases the extent of non-specific interactions of a hydrophobic character which may involve the active site and make it inaccessible for substrates. They can also contribute to the irreversible conformational changes of the catalyst molecule and substantially decrease its activity.

Carriers based on natural polysaccharides consist of hydrophilic saccharide repeating units containing many hydroxyl groups. The hydrophilicity of a synthetic carrier is typically achieved by polymerization of monomers that contain hydrophilic groups such as ether -C-O-C-, amide -CO-NH- and hydroxyl groups -OH. Also, the ionized groups typical for ion-exchangers, such as carboxyl $-COO^-$, amino $-NH_2$, -NHR, $-NR_2$, $-N^+R_3$ and sulfo groups $-SO_3^-$ are very hydrophilic. The electrostatic interaction with the opposite charged groups of an enzyme can be advantageously utilized for the immobilization but it also can induce non-specific interactions with the consequences shown above.

Reusability of carriers

Although the stability of an immobilized enzyme has been reported to be improved as compared to a soluble enzyme, the activity of the immobilized enzyme decreases during both storage and operation (Chibata, 1978). Major factors that influence the loss of activity of an immobilized catalyst involve denaturation, bacterial contamination, leakage of enzyme, incorrect operation, etc. After the activity decreases to a limit that makes the catalyst useless, the catalyst has to be discharged from the technology and either wasted or reprocessed. The route taken is determined by the immobilization chemistry and properties of the support.

Typically, biocatalysts immobilized by entrapment and covalent crosslinking cannot be reprocessed. Some carrier-bonded conjugates also do not provide the option of the removal of the inactive biocatalyst, new activation and immobilization. For example, the secondary amino group obtained by the reaction of a protein with the epoxide group of a carrier is very strong. It would be feasible to hydrolyze the protein and remove it from the support except for the amino acid that has been involved in the binding, but there is no easy way to restore the initial epoxide functionality of the carrier.

It is generally easy to reuse a carrier on which the enzyme is immobilized by a weak non-covalent bond. The hydrophobic and electrostatic interactions are eliminated by

a change of the ionic strength or pH of the surrounding medium. A new portion of fresh enzyme can be immobilized immediately after washing out the inactive protein with a buffer solution and restoring the original charge. High leakage of enzyme during operation and storage is, however, the penalty paid for easy regeneration.

Inorganic carriers are readily regenerated by a pyrolyzing process, as they are stable even at very high temperature. The immobilized enzyme is literally burned off in a furnace at a temperature above 400°C in the presence of air or oxygen. After cooling, the carrier is generally ready for a new activation and immobilization of a fresh enzyme (Messing, 1975). This makes the inorganic carriers very tempting but their pH instability limits must also be taken into consideration.

Environmental impact of carriers

Besides other environmental issues typical for any biotechnology, utilization of immobilized biocatalysts adds one more: How to get rid of the exhausted catalyst? Some of them, such as polysaccharides, polyesters and polyamides, are biodegradable and can be simply composted. Others can be reprocessed and the problem involves the inactive biocatalyst only. But there is still a large group of biocatalysts immobilized on carriers that are not biodegradable, that produce toxic products when burned or that pollute water when dissolved. Such immobilized biocatalysts would be best avoided in planning for a viable biotechnology.

Cost considerations

The industrial use of immobilized enzymes and cells is an evident confirmation that the costs of the technology are no higher than those of conventional processes (Chibata, 1978; Marconi, 1989). The economy of any process depends on many different factors such as cost of labor, energy, inputs, overhead, etc. It is not the aim of this review to discuss all of them in detail and only the carrier itself is brought into focus.

It is clear from the previous discussion that a universal carrier with exclusively positive features definitely does not exist. A carrier has to be used that represents the best possible compromise for the particular technology. It is also obvious that for highly sophisticated sensors containing tiny amounts of a delicate enzyme, the cost of the carrier may be neglected. On the other hand, immobilized glucose isomerase for large scale production of high fructose syrups or immobilized penicillin acylase for production of 6-aminopenicillanic acid must compete with traditional well-established technologies based on soluble enzymes or cells. Hence, the cost of the carrier has to be compensated by another advantage such as lower labor and production costs.

Supports produced in large quantities will definitely be cheaper than the same carriers prepared on laboratory scale. The overall cost of the immobilized enzyme can be substantially reduced by employing a regenerable carrier or immobilization technique.

Economy continues to be the crucial point in the deciding whether the immobilized enzyme or cells will be used in a technology. This, despite speculation on the great potential of immobilized biocatalysts and overzealous enthusiasm, is also why only a few industrial processes are yet accomplished.

Future trends

Despite the large number of papers dealing with the immobilization of biocatalysts, the number of applications, though growing continuously, has not reached its upper limits. Future development requires joint efforts of molecular biology, genetic engineering and materials science in order to accomplish preparation of heterogenized biocatalysts with properties completely suited for industrial use in new applications that could challenge or even outperform the standard technologies. An enormous potential is also to be expected in 'small' applications, that are however big in their importance, such as analytical methods, enzyme electrodes, diagnostics, artificial cells, active prostheses and many others.

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