Biotechnology and Food Science

Review article

Immobilization techniques and biopolymer carriers

Elżbieta Górecka,* Magdalena Jastrzębska

Institute of Fermentation Technology and Microbiology, Technical University of Lodz, 90-924, Lodz, Poland

*elzbieta.fornal@gmail.com

Abstract: *Various immobilization methods are expected to have a bright future in the field of pharmacy, medicine or industry. Production of immobilized enzymes mainly by CLEAs introduces a whole new level of biotransformations, where biocatalysts are highly purified and active. Improvement of CLEAs technology will certainly develop food and pharmaceutical industry. Another immobilization path is affinity binding, also focused on enzymes. Preparation of generic protocols for enzyme attachment will increase the sensitivity of this technology, as well as its simplicity. Biocatalyst re-usage will significantly decrease the costs. Immobilization in nanodelivery systems may shortly dominate the field of medical sciences. The vast problem of civilization diseases is the difficulty in medicaments administration, either due to the size of the drug or the inaccessibility of the treated site. Nanodelivery systems will overcome this issue, as they are able to carry drugs directly to their site of action. Cancer, Alzheimer's disease or autoimmune disorders will become controllable. Finally, other immobilization methods are being engineered in order to provide systems with better interactions with GI wall. Carriers will be adjusted for the highest selectivity for mucosal wall, whereas viral and bacterial mechanisms of uptake will be incorporated into biocatalyst to ameliorate the physiology of absorption.*

Keywords: *immobilization methods, biosynthesis pathways, biopolymers, alginate, chitosan.*

Introduction – immobilization methods

Definition

Immobilization is a general term describing a wide variety of the cell or the particle attachment or entrapment [1]. It can be applied to basically all types of biocatalysts including enzymes, cellular organelles, animal and plant cells [2]. Currently, different kinds of immobilization have found wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries [2].

The huge variety of possible applications was a catalyst for the improvement of the existing methods and the development of new methods of immobilization. Especially, the insoluble immobilized enzymes were sought after by the different branches of the industry since second half of the 20th century, as there are several

benefits of using them in comparison to the soluble ones. One of those advantages, is a possibility to reuse such enzymes in a high-scale processing, which reduces production costs, even if the price of the immobilized enzyme per mass unit is higher than its counterparts.

In the case of the cells immobilization, field of their application spreads from food industry to biomedical sciences. Microorganisms retained on a carrier can be used in continuous and semi-continuous production processes (biosynthesis of vitamins, amino acids, organic acids, production of monoclonal antibodies, recovery of heavy metals, whole cell enzymatic reaction and ethanol fermentation), allowing for significant cost decrease, as the biocatalyst does not need to be refilled [3-5]. Besides, entrapment or encapsulation creates a protective barrier around the immobilized microbes, ensuring their prolonged viability during not only processing but also storage. This fact is crucial for probiotics, which are often stored in temperatures close to 0°C or frozen [6]. Finally, immobilized cells and tissue may be used in transplantations instead of whole organs. This allows to avoid complicated surgery and limits the use of immunosuppression drugs, as immune cells and antibodies are prevented from entering the microcapsule [7].

Support selection

Apart from the method itself, the support selection is one of the crucial decisions to be made in the course of preparation of the immobilization process. Selection criteria differ among one another, depending on the biocatalyst of interest, but there are still few basic features that must be considered. Material used as a carrier should have chemical, physical and biological stability during processing, as well as in the reaction conditions; sufficient mechanical strength, especially for its utilization in reactors and industry; should be nontoxic both for the immobilized cell/bioparticle, as well for the product; also should have adequate function groups for binding biocatalyst and high loading capacity. Profitability of the material application and its processing costs always have to be taken upon consideration.

Other criteria, such as physical characteristics (porosity, swelling, compression, material and mean particle behavior), as well as possibility for microbial growth, biodegradability, solubility, are more application specific [8].

Adsorption

Adsorption is the elementary and probably the simplest method of reversible immobilization. It is most commonly used for attachment of cells (eg. for continuous beer maturation), however enzymes adsorbed on different carriers are also found in various biotechnological processes (Figure 1).

Adsorption is based on weak forces, however still enabling an efficient binding process. Usually, in bonds formation several forces are involved: van der Waals forces, ionic and hydrophobic interactions and hydrogen bonds [7,9,10]. Sometimes also affinity binding is included in this group [7].

While the method is easy in preparation, costs are low and reloading of the support is possible, it has many disadvantages and very few applications. Mainly, adsorption of the cells is used for exploratory work over short periods of time [10]. Due to weak interactions between the support and the biocatalyst there is a very high rate of leakage, binding is unstable, there is no possibility to control the loading, so the reproducibility is also low [9,11]. Even though, in most cases the cell productivity is not affected, it is very susceptible to changes in ambient conditions such as pH, temperature, ionic strength. The listed factors are responsible for leakage or even complete desorption of the catalyst [7,10,11].

A wide range of both organic and inorganic materials can be used as a support. Among most common, we can find carboxymethyl-cellulose, starch, collagen, modified sepharose, ion exchange resins, active charcoal, silica gel, clay, aluminum oxide, titanium, diatomaceous earth, hydroxyapatite, ceramic, celite or treated porous glass.

Disulphide bonding

Disulphide binding, is generally applied for enzyme immobilization, and may be seen as a variation of covalent bonding, as there are stable covalent bonds formed between activated support and free thiol group (for example on cysteine) in the biocatalyst. However, those bonds can be easily broken using suitable agent under mild conditions, what classifies this method as a reversible immobilization. Dithiothreitil (DTT) is the most popular agent for disulphide bond decomposition (Figure 1).

An additional advantage of this immobilization method is the possibility to influence the reactivity of thiol groups by alteration of pH [7]. Support material can be chemically activated using such agents as maleimide or iodoacetyl to activate silica [12] or by photonic induction [13].

Chelation or metal binding

Chelation or metal binding (Figure 1) is used mostly as the chromatography method, when there is a requirement for an easy regeneration of the support without reducing the immobilization yield, but at the same time there is a very low level of leakage as binding is relatively strong [14]. It is based on the ability of the side chains of certain amino acids (histidine, tryptophan, tyrosine, cysteine and phenylalanine) to substitute weakly bonded ligands in the metal ions that have been immobilized by chelating group covalently bound to a solid support [14,15].

Chelation utility comes mainly from the method being quite universal as there is a wide selection of chelating anions [14]. Reversibility of this method can be attained in two ways: either by exposing surface to a stronger chelator like ethylodiaminetetraacetic acid (EDTA) or by using excess of competitor ligand [15]. Supports used are organic materials, usually cellulose, chitin, alginic acid and silica-based carriers, that were activated by binding to their nucleophilic groups by coordinate or covalent bonds or a transition metal salts or hydroxides [7].

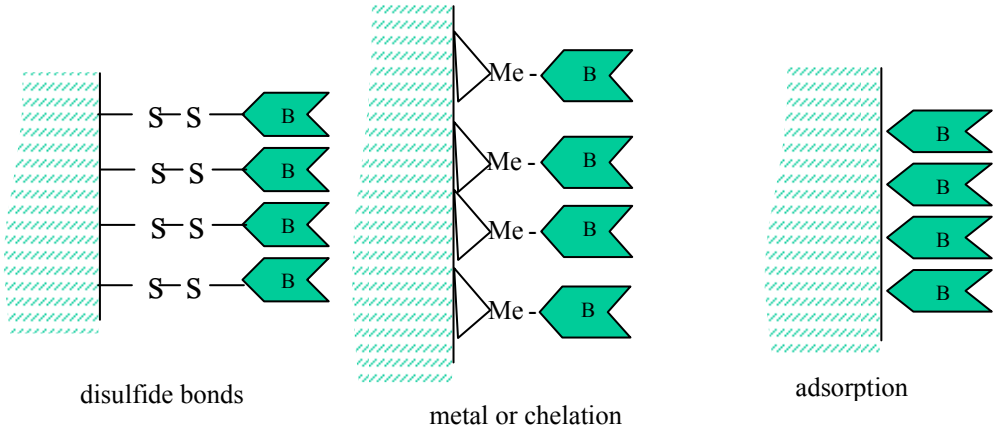

Figure 1. Reversible methods of immobilization (B – biocatalyst) [7]

Covalent bonding

Covalent bonding falls into the irreversible immobilization category. It is one of the most widely used methods for enzymes immobilization (Figure 2). Popularity of this approach is mainly connected with the stability of the bonds formed between the enzyme and the support, which prevents enzyme release into the environment [7,9]. What is more, the unlimited contact between the enzyme and the substrate due to the lack of any barrier between them and the localization of the enzyme on the surface of the support, together with an increased stability of the biocatalyst are additional advantages [9]. In covalent bonding, the most important factor determining its usability, is the direction of the enzyme binding. It was found that to achieve the highest activity level the active center amino acids must not be involved in binding with the support. There is a wide variety of ways derived from this method depending on the type of a matrix and substrate. Depending on active groups present in molecule that is to be immobilized, there are two possible ways of coupling with the support. It is possible to either add reactive function groups to polymer without its modifications, or to modify support matrix to generate activated groups. In both cases, it is desirable to generate electrophilic groups on the support, that will react with strong nucleophiles on the protein.

This method usually involves binding via the side chains of lysine (ε-amino group), cysteine (thiol group), aspartic and glutamic acids (carboxylic group) [7]. Matrices usually include: agarose, cellulose, poly(vinyl chloride), ion exchange resins and porous glass.

Despite all advantages that this method presents when applied to enzymes, it is rarely applied for immobilization of cells. It is caused mainly by the fact that agents used for covalent bonds formation are usually cytotoxic and it is difficult to find conditions when cells can be immobilized without any damage [9].

There are few reports of successful covalent binding of the cells and most of them concern yeast. In 1977 Navarro and Durand published an article describing

a successful way of covalent binding of *Saccharomyces carlsbergensis* on porous silica beads. [16] Two years later there was another publication concerning yeast (*Saccharmyces cerevisiae, Saccharomyces amurcea*) and bacteria (*Sarratia marcescents*) immobilization with this method on borosilicate glass and zirconia ceramics [17].

Entrapment

Entrapment is an irreversible method, where immobilized particles or cells are entrapped in a support matrix or inside fibers (Figure 2).

Drawbacks of this type of immobilization are usually connected with the costs of immobilization, diffusion limitations, deactivation during immobilization and abrasion of support material during usage. Another disadvantage is low loading capacity as biocatalysts have to be incorporated into the support matrix. This aspect creates the problem of choosing the best support material. It probably is the most important parameter, as immobilized particle-size to support material pore-size ratio is a deciding factor for the usability of ready probes. When pores are to small, adsorption can be done only on external surface, what excludes high load of particles. On the other hand, when the pores are too big material is leaking, what also decreases the loading [18].

There are also many studies aiming at the control of the particle arrangement in gel matrix. Among them we can find trials performed with ultrasound standing waves, which do not disrupt the integrity and the cell viability of yeast, mammalian cells or erythrocytes [19].

It is possible to use the following polymers as a matrix: alginate, carrageenan, collagen, polyacrylamide, gelatin, silicon rubber, polyurethane, polyvinyl alcohol with styrylpyridium groups [1,7].

Encapsulation

Encapsulation is another irreversible immobilization method (Figure 2), similar to entrapment. In this process, biocatalysts are restricted by the membrane walls (usually in a form of a capsule), but free-floating within the core space [20,21].

The membrane itself is semi-permeable, allowing for free flow of substrates and nutrients (when cells are used as a biocatalyst), yet keeping the biocatalyst inside. The factor determining this phenomenon is the proper pore size of the membrane, attuned to the size of core material. This limited access to the microcapsule interior is one of the main advantages of microencapsulation, for it protects the biocatalyst from the harsh environmental conditions. As most immobilization method, it prevents biocatalyst leakage, increasing the process efficiency as a result [3]. Furthermore, no chemical modifications of the core material are required for the immobilization, so the activity remains intact. Finally, microencapsulation is suitable for microorganisms, living cells as well as for multi-enzyme systems for sequential enzymatic reactions, as several different biocatalyst may be incorporated into the capsule, providing they have no negative influence on each other [21].

However, as every technology, microencapsulation has some disadvantages. The most severe one is the necessity for a very strict pore size control, which is especially difficult in the case of small biocatalysts like some enzymes or bacteriophages. What is more, this technique is not available for biocatalyst with a size similar to their reaction product, as it would result in a leakage of both (when the size is smaller than the membrane pores) or burst of the capsule (when they are both accumulated within the membrane) [20].

The biopolymers useful for encapsulation are: alginic acid (alginate), chitosan, maltodextrin and cellulose. However, double-layer microcapsules built of two different polymers are also very popular. The most common are coated with chitosan, poly-L-lysine (PLL), polyvinyl acetate (PVA), gelatin, boric acid and κ-carrageenan [21].

Cross-linking

Cross-linking is an irreversible method of enzymes immobilization that does not require a support. This factor decreases costs, at the same increasing time specific and volumetric activity of biocatalysts (Figure 2).

There are two main methods of cross-linking. First one is Cross-Linking Enzyme Aggregates (CLEA) and the other Cross-Linking Enzyme Crystals (CLEC). Both are derived from a method developed in 1960s involving the usage of glutaraldehyde, which reacts with NH2 groups on protein surface (CLE). This first method had some very serious drawbacks, like low mechanical stability, low reproducibility and low activity retention.

Therefore, several improvements were implemented. The first refinement was using glutaraldehyde to crystallize enzymes. It was proved that in CLEC method enzymes are much more resistant to denaturation by heat or organic solvents and to proteolysis. Also their operational stability together with easier recycling and controllable size gave this method advantage over CLE [7,22].

The main disadvantage of this method is the necessity for very high purification of enzyme and the need to crystallize it, what is laborious and time consuming. As a result another improvement emerged – CLEA, which allowed to work in aqueous solutions. Basics of CLEA are that by addition of salts, organic solvents or nonionic polymers the aggregates of enzymes are formed which maintain their activity, but their stability is increased. It was found to be cheaper and easier than CLEC and, what is more important, has a wider range of applications. Also there is a possibility to perform so called *combi*-CLEA, where there are different types of enzymes present in aggregates [23]. However, there is a problem with the size of obtained aggregates (diameter no greater than 10μm), because substrate particle may lay in the same size range in a heterogeneous reaction system. This can be a serious drawback for enzymes recovery in continuous processes [24].

Main disadvantage of both CLEC and CLEA methods are constraints of diffusion when aggregate or crystal size is increased. This is the reason for introduction of new methods based on self-immobilization of enzymes in an

emulsion. This way we can retain activity while controlling size and incorporating the macrostructure by adjusting the speed of mixing [23].

An important factor is to use proper cross-linking agent. Usually, it is glutaraldehyde, as it is inexpensive and readily available in commercial quantities, nevertheless, it was found unsuitable for immobilization of some enzymes, for example nitrilases, where sometimes low retention or even no retention was observed. In this case, dextran polysaccharide can be used as cross-linking agent [22]. Other cross-linking reagents used are for example *bis*-isocyanate, *bis*-diazobenzidine, diazonium salts and functionally inert proteins, such as Bovine Serum Albumin or ovoalbumin.

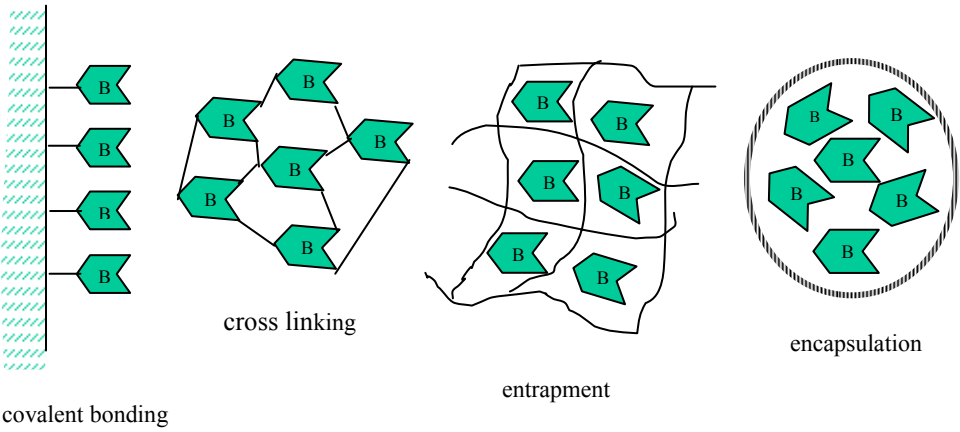

Figure 2. Irreversible immobilization methods (B – biocatalyst) [7]

Characteristics of biopolymers as the carriers for immobilization of enzymes and cells

Alginate

Alginate is a naturally occurring polymer extracted on industrial scale from various species of brown algae (*Phaeophyceae*), including *Ascophyllum*, *Laminaria*, *Lessonia*, *Ecklonia*, *Durvillaea* and *Macrocystis*. The choice of species is based on their abundance in the given part of the world and does not affect the quality of the polymer itself. Furthermore, some species of *Pseudomonas* and *Azotobacter* produce alginate as an exopolysaccharide by means of various extracellular epimerases. Although the biosynthetic pathway has been well explored for both genera, the polymerization itself and transport mechanism are still a matter of research. Therefore, only the process of synthesis by brown algae is presented in this paper.

One investigated pathway originates from the glucose metabolism, where fructose-6-phosphate instead of 1,6-fructosebisphosphate is converted into mannose-6-phosphate by phosphomannose isomerase (Figure 3). As a result,

β-D-mannuronic acid is obtained, which is one of the two monomers of nascent alginate [25].

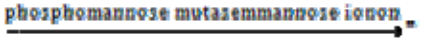

Figure 3. Biosynthetic pathway of alginic acid

Furthermore, brown algae as well as *Pseudomonas* and *Azotobacter* species synthesize various epimerases which convert β-D-mannuronic acid into α-L-guluronic acid – the second alginate monomer. The best described (Figure 4) are seven homologous 5-C-epimerases from *Azotobacter vinelandii*: AlgE1-7 [26,27].

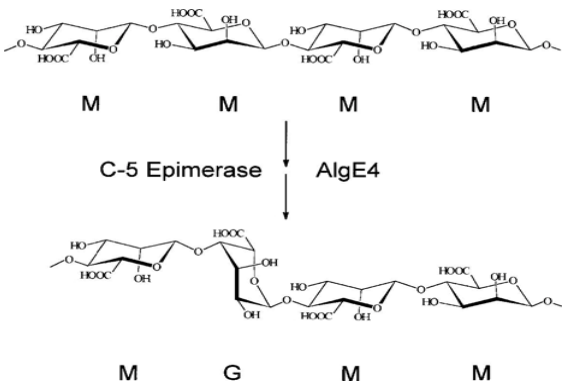

Figure 4. Enzymatic conversion of mannuronate into guluronate [26]

Since alginate is a non-repeating copolymer, it was suggested that epimerases act in a random manner. The resulting polymer consists of blocks of monomers combined in three ways: homoblocks of guluronic acid residues (GGGGGG), homoblocks of mannuronic acid residues (MMMMM) and heteroblocks of interchanging M and G (MGMGMG). What is more, each chain does not usually include more than 20 units.

Both monomers belong to the uronic acids family, which are generally derived from sugar molecules. In each case the hydroxyl group is oxidized yielding a carboxylic acid.

Chitosan

Chitosan (Figure 5) is a naturally occurring polymer, derived from chitin and as well as chitin it is isolated from fungal cell walls and arthropod exoskeletons (lobsters, shrimps, crabs and insects). On industrial scale, the polymer is obtained from crustaceans – particularly their shell wastes. Chitin is composed of 5000-8000 residues of highly ordered β-(1,4)-N-acetyl-D-glucosamine (2-acetamido-2-deoxy-δ-glucose). Since *in vivo*, one residue out of ten is linked to a protein through its nitrogen atom, isolated chitin already has a 10% degree of deacetylation. Chitosan is its alkalline deacetylated form with various degrees of deacetylation (40-98%), characteristic for each strain [20,25,28].

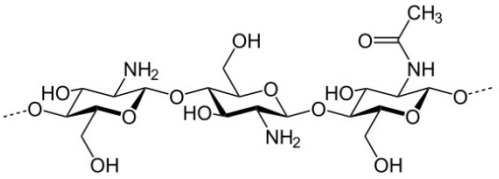

Figure 5. Chitosan chain [www.pl.wikipedia.org]

Again, the biosynthetic pathway originates from glucose phosphorylated to glucose-6-P and further isomerased to fructose-6-P (Figure 6).

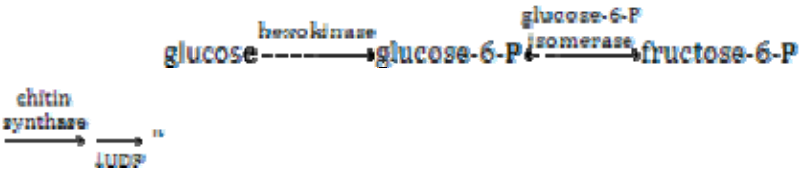

Figure 6. Biosynthetic pathway of chitosan

Chitosan is produced simultaneously with chitin itself, only in this case chitin synthase action is accompanied with a deacetylase removing acetyl groups from the developing chain.

Carrageenan

Carrageenan is an alternating copolymer of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-D-galactose. Galactose residues are modified by the presence of sulfate ester substituents at different carbons of the pyran ring. Basing on the location and number of those substituents, six fractions of carrageenans are distinguished (Figure 7). μ-fraction, δ-fraction and ν-fraction are biological precursors of the remaining types, as enzymatic modifications at the end of the synthesis process convert all three of them into κ, λ and ι. K-form gels in the presence of cations, particularly K^+, although some research suggest gelation in the presence of Na^+ as well [29]. Gel formation process is reversible and temperature dependent. Potassium ions promote aggregation of chains in desired conformation as the polymer hardens on cooling. I-carrageenan also

forms thermo-reversible gels, only with Ca^{2+}, whereas λ-carrageenan is soluble in the presence of all cations [20,25].

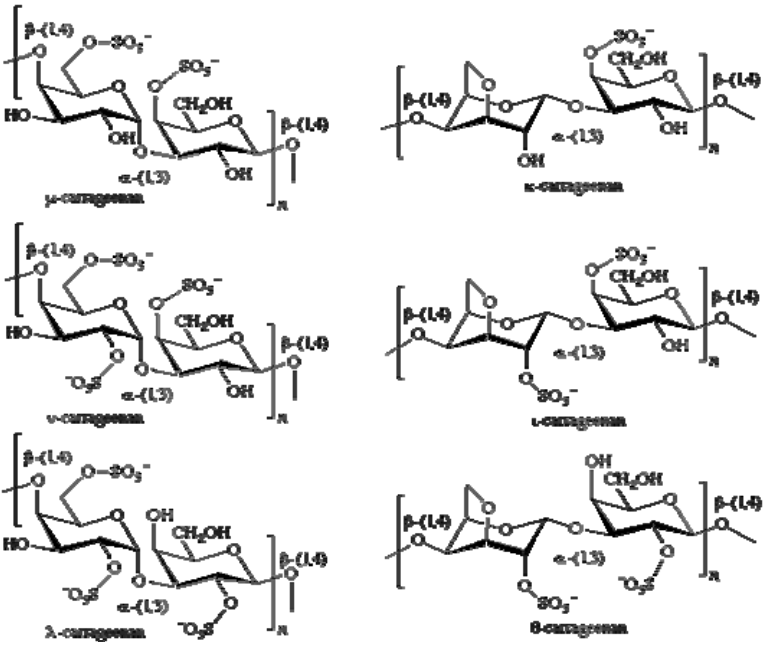

Figure 7. Fractions of carrageenan [www.enzyme-database.org]

Carrageenan is isolated from several species of red algae (*Rhodophyta*): *Eucheuma*, *Chondrus*, *Gigartina* and *Iridea*. Some of the mentioned species produce chosen fractions more eagerly than the other, what facilitates the purification process. For instance, κ-carrageenan is the main type in *E. cottonii*, ι-carrageenan in *E. spinosum* and λ in *G. acicularis* [30].

There is hardly no information on the biosynthesis pathway of carrageenans, for the genetics of the red algae still remains unexplored.

Cellulose

Cellulose (Figure 8) is the most widely-spread natural polymer, comprised of 1,4-linked β-D-glucopyranosyl chains, additionally bound by hydrogen bonds (approximately 36 glucan chains per fibril). Such structure provides the longest known fibrils, composed of 8000-15000 glucose residues 3nm thick. Due to its mechanical resistance, it is a component of plant cell walls and in some cases also bacterial cell walls (eg. *Acetobacter sp.*) [31]. Since cellulose has a highly conserved structure, the biogenesis scheme presented applies to all organisms (both microorganisms and higher organisms).

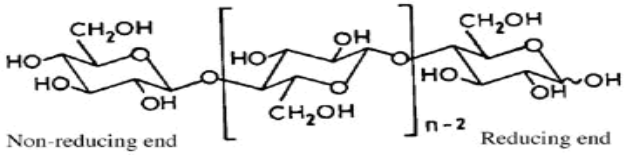

Figure 8. Cellulose chain [www.chempolymerproject.wikispaces.com]

In bacterial cells, cellulose is produced by cellulose synthase which catalyses polymerization of glucose residues, as presented in Figure 9. Therefore, the biosynthetic pathway also originates from glucose.

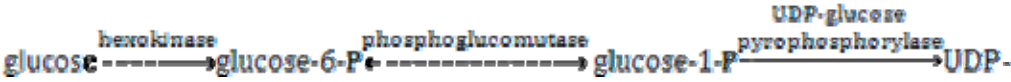

Figure 9. Biosynthetic pathway of cellulose

Maltodextrin

Maltodextrin (Figure 10) is a polysaccharide derived from starch by its partial hydrolysis via different amylases and disproportionation enzymes (Figure 11). It is composed of 3 to 19 D-glucose residues linked by α-1,4-glycosidic bonds throughout the chain. The dextrin equivalent varies from 3 to 20. As starch itself, maltodextrins are present in plant and microbial cells [31].

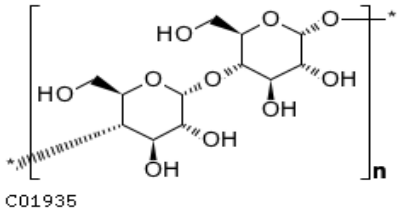

Figure 10. Maltodextrin chain [www.genome.jp]

The polymer is easily soluble in water solutions and can undergo spray-drying without being damaged in the process. Therefore, it is suitable as a coating material in microencapsulation by spray-drying.

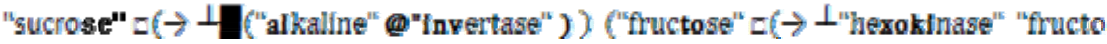

Figure 11. Biosynthetic pathway of maltodextrin

Collagen/gelatin

Biosynthetic pathway of collagen is very complex, incorporating several genes found on variety of chromosomes (Figure 12).

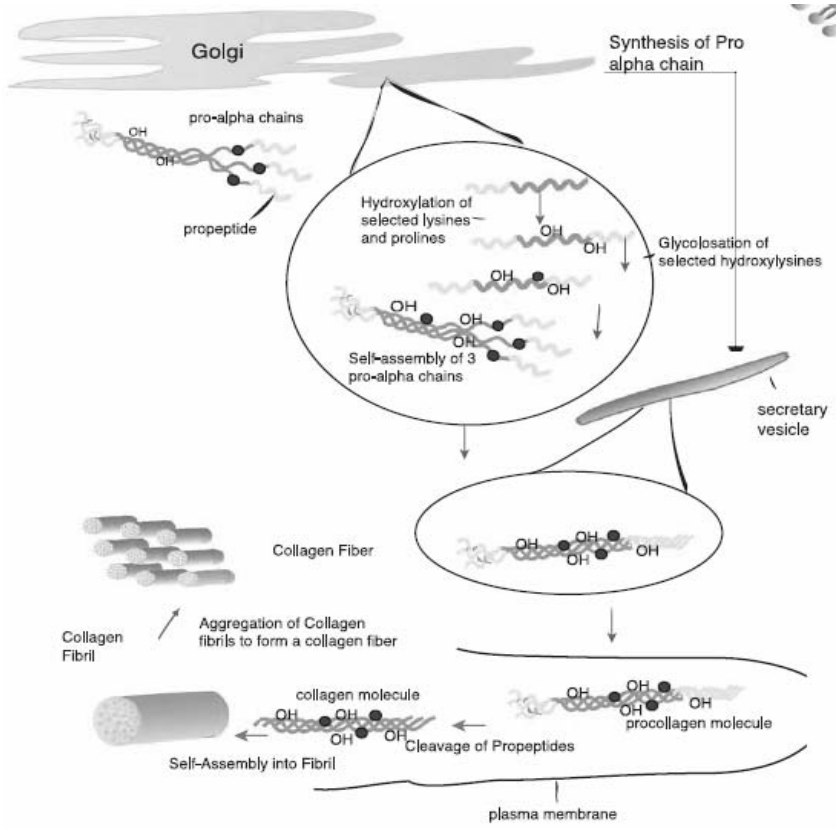

Figure 12. Biosynthetic pathway of collagen [25]

The production of collagen occurs once pro-α-chain mRNA is available and attaches to ribosomes on rough endoplasmic reticulum. In translation process pro-α-chains of collagen are formed with several peptides integrated with its chain that increase its solubility for transport to the Golgi Apparatus via microtubules. Chains are packed to form pro-collagen and secreted outside the cell. The extension peptides are removed by pro-collagen proteinases and collagen is assembled into fibrils [25].

Gelatin is obtained from collagen by extraction with water or acid solution at proper temperatures.

Alginate – main immobilization techniques for enzymes, organelles and cells

Physical gelation entrapment

The biocatalyst particles are mixed with the polymer solution. Entrapment in alginate occurs as a result of the salts addition – salt-set gels. Physical gelation produces macroporous gels, however chemical particles, enzymes or cells are additionally bound by hydrogen bonds or another type of non-covalent linkage. Besides, such immobilization contributes to the increase of the entrapped enzyme stability as a result of its structure rigidification [21,32-34].

Entrapment-coating/cross-linking

Coating and cross-linking are, next to covalent bonding, variations of traditional entrapment via physical gelation.

Covalent entrapment is used to bind soluble biocatalysts to the support with covalent bonds. However, only synthetic, so-called smart polymers are engaged in matrix formation, therefore this method is not further described in this paper [21].

An alternative to covalent entrapment, which may interfere in the case of enzyme with active center, is coating. This technique produces an additional polymer layer around an existing bead, reducing the pore size and as a result preventing biocatalyst leaching. Unfortunately, the observed diffusion constraint is also responsible for decrease in the enzyme or cell activity. Alginate is most often utilized as the primary bead and coated with other polymers, enumerated in the following sections [35-37].

Another technique, which reduces biocatalyst leakage, is cross-linking. It is performed by addition of cross-linking agent, of which the most common is glutaraldehyde and genipin [38]. However, it can only be applied for enzymes. Cross-linking of cells is extremely troublesome, for the whole system would be broken during microorganisms division.

Encapsulation – Interfacial processes for the formation of solid shell around the liquid droplet of the enzymes, chemical particles or cells

Interfacial processes are based on polymerization of the support monomers on the interface between the biocatalyst droplets and the continuous organic phase. Depending on the membrane solidification method, this process has been divided into several subtypes.

Internal gelation

In the internal gelation process, the support polymer is dispersed in the continuous organic phase with the addition of a surfactant and the biocatalyst. Furthermore, the cross-linking agent – in this method a polycation solution – is mixed with the emulsion. As a result, homogeneous microcapsules with larger pores are obtained. The most commonly used polymer is sodium alginate and the hardening cations are calcium and zinc ions. Microspheres produced in this manner are relatively weak and susceptible to deformation under harsh conditions, therefore further coating or hardening should be applied [39-42].

Interfacial cross-linking

In this process, one water-soluble monomer is mixed with the biocatalyst in the continuous organic phase with surfactants. In the second step another water-

soluble monomer is added to the mixture, which causes polymerization of both at the droplet interface, containing the biocatalyst inside. Alginates are usually the first polymer, further solidified with chitosan, poly-L-lysine or gelatin [43,44].

Encapsulation – Phase inversion methods for the preparation

Solidification of the membrane in phase inversion techniques is obtained via change of solubility of the encapsulation system. As a result of solvent evaporation or extraction, the support is precipitated in a form of insoluble microcapsules [45].

Coacervation

The coacervation process is based on the desolvatation phenomenon, induced by various factors. From the choice of those factors, originated the classification of this process into simple coacervation and complex coacervation [46,47]. Simple coacervation is induced via the addition of salts, solvents or the action of temperature or pH. Whereas complex coacervation occurs after the addition of an oppositely charged polymer [43,48,49].

In each case, the process can be divided into 3 steps:

- Formation of immiscible chemical phases
- Deposition of liquid coating around the core
- Rigidification of the coating.

Alginate is, after cellulose derivatives, the second most common polymer used for this purpose [21,50].

Liquid drying

Spray-drying is a process which allows to produce microcapsules from large volumes of biocatalyst mixed with a support polymer solution. The mixture is atomized in a chamber while heated up to 200°C. Ready-made Ca-alginate microspheres can be spray-dried for long-time storage, however the drying conditions must be very carefully chosen, as membrane is often disrupted in the process and biocatalyst leakage or even inactivation occurs [51,52].

Encapsulation – Template leaching

This set of methods is built on a combination of encapsulation by one of the above processes with another immobilization technique. The main objective for the use of such an advanced system is obvious – to limit the biocatalyst leakage. Besides, the activity and system selectivity is enhanced. Furthermore, the problem may be approached at two different angles – one is focused on the pore-size decrease (coating) and the second on the biocatalyst size increase (cross-linking).

Encapsulation and coating

Coating is based on the formation of a second polymeric layer around the existing capsule in order to reduce the pore size. The second membrane may be an oppositely charged polymer molecule – like poly-L-lysine, gelatin and chitosan used for alginate capsules coating. If required, the third layer is formed – for instance another alginate membrane around poly-L-lysine or chitosan [44].

Encapsulation and cross-linking

As in the case of entrapment and cross-linking, the main point of encapsulation and cross-linking is to increase the biocatalyst size. Hence, cross-linking agents are incorporated into the system. Most commonly-used are glutaraldehyde, genipin and other [53]. As mentioned before, cross-linking is only applicable for enzymes, not for cells.

Chitosan – main immobilization techniques

Physical gelation entrapment

The principle of physical gelation by means of chitosan occurs by other polymer solution addition. The most popular methods include salt-set gelation (described in "Alginate – main immobilization techniques" section) with use of tripolyphosphate and symplex (polyelectrolyte-polyelectrolyte complex) with the use of κ-carrageenan, PVP and hexametapolyphosphate. The enumerated techniques display the most mechanically durable matrices with low swelling capacity [21,34,54,55].

Entrapment-coating/cross-linking:

Chitosan is a popular coating material in both entrapment-coating and encapsulation-coating. Its amine groups bind strongly to alginate carboxylic groups, forming a thermally and mechanically stable beads [56,57].

In case of cross-linking, chitosan may serve as the primary matrix, produced by salt-set gelation or symplex, with entrapped enzyme submitted for further cross-linking [21].

Encapsulation – Interfacial processes for the formation of solid shell around the liquid droplet of the enzymes

Internal gelation

Chitosan is a less popular material for internal gelation, however the method for its preparation is available. Chitosan in aqueous solution with the biocatalyst is pressed into the continuous organic phase (petroleum ether or paraffin) to form an emulsion and further hardened with glutaraldehyde [38,58].

Interfacial cross-linking

When chitosan is used as the primary polymer in the interfacial cross-linking process, the second polymer could be carrageenan or tripolyphosphate (TPP). However, chitosan is more often utilized as the secondary polymer for alginates [38,43].

Encapsulation – Phase inversion (liquid drying)

Spray-drying of chitosan microspheres represents the same problems as Ca-alginate capsules. The drying conditions may easily damage the shell and eliminate the biocatalyst activity. However, when the process is working correctly, the biocatalyst is suitable for transport and storage at a broad temperature range [51,52,59].

Template leaching

Encapsulation and coating

In the encapsulation-coating technique, chitosan is used as both the primary and secondary layer. Chitosan microcapsules can be coated with κ-carrageenan or tripolyphosphate [38,60]. However, CS strongly binds alginate, forming a tight second membrane [5,6,60,61].

Encapsulation and cross-linking

Chitosan microspheres are proved to prolong the residence time of microorganisms, due to their protonated carboxylic groups strongly binding the negatively charged cell surface [58]. Anyhow, for enzymes encapsulation cross-linking may be required. In this case, typical cross-likers are utilized – genipin and glutaraldehyde.

Immobilization techniques with other polymers

Physical gelation entrapment

Cellulose is engaged in the polyelectrolyte complex (PEC) physical gelation as its acetate salt. The gelation method itself is called complexation and is built on cellulose acetate-TiO_2 gel fibers. In the case of enzymes entrapped in this manner it was proved to show higher enantioselectivity and resistance to altering reaction conditions [21,62-64].

A synthetic cellulose derivative – hydroxypropyl methylcellulose acetate succinate is prone to pH-sensitive gel formation. For this compound the transition occurs at pH 4.0. However, this method is relatively unpopular, as covalent immobilization is much more efficient [21,65,66].

The last group of physical gelation techniques is temperature-induced. Usually, the most common is cold-set gelation with the use of gelatin or PVA (polyvinyl acetate). Solubility of both polymers is susceptible to temperature change, therefore, the biocatalyst can be mixed with the liquid-state polymer and subsequently cooled until solidification. The resulting matrix is an open-pore structure with biocatalyst bound by additional non-covalent bonds [21,56,67-70].

κ-Carrageenan participates in both PEC physical gelation and temperature-induced physical gelation. In the first case, it accompanies chitosan to form a polyelectrolyte-polyelectrolyte complex. Nevertheless, the properties are less satisfactory than for chitosan-alginate matrices. Carrageenan absorbs less water into the system and is disintegrating as a result instead of swelling. This feature is responsible for elevated drug release [60].

Thermoreversible gels of κ-carrageenan are formed while cooling in the presence of sodium and potassium ions. K^+ at low concentration creates a coil-to-double helix structure and Na^+ participates in aggregation of CRG through phase separation mechanism. Both ions should be used at appropriate ratio, which produce a gel of desired properties [29,71].

Interfacial processes for the formation of solid shell around the liquid droplet of the enzymes, chemical particles and cells

Interfacial encapsulation processes are rarely based on polymers like cellulose, carrageenan or maltodextrin. Only gelatin is a relatively good secondary polymer utilized in interfacial cross-linking with alginate [44].

Phase inversion

Coacervation

As previously mentioned, ethylcellulose and cellulose nitrate are the most popular biopolymers encountered in the encapsulation processes via coacervation. Those support materials present beneficial viscosity to molecular weight ratio which positively influences the encapsulation efficiency and release behavior [21,50].

Liquid drying

Maltodextrins (MDX) are the most suitable polymers for spray-drying process, as their structure is high-temperature resistant. Besides, MDX represent good encapsulated ingredients protection against oxidation, improve heat stability and shelf-life. The obtained capsules have random shape. Finally, the solubility and encapsulation properties are improved by addition of a surfactant to the mixture (eg. Tween 80) [59,72-75].

Template leaching (encapsulation and coating)

Gelatin and κ-carrageenan, next to poly-L-lysine, are popular coating polymers for alginate and chitosan microcapsules, since they do not represent satisfactory encapsulating properties [44,60,76].

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