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IMMOBILIZED CELLS *and* ORGANELLES

Volume I

Bo Mattiasson

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Immobilized Cells and Organelles

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PREFACE

Besides giving some insight into basic technology (immobilization procedures, etc.) these volumes also sum up the current know-how in the area and try to predict some future trends.

Cells and organelles are small units for biochemical synthetic purposes, often the smallest practically feasible unit since they contain coenzyme regenerating system, ordered enzyme sequences, etc.

The term "immobilized cells" covers everything from dead cells with a single active enzyme species to cells proliferating on or within a three dimensional polymer matrix. The practical handling of these structures make them useful in various applications, e.g., large-scale production of biomolecules, biodegration, analysis, etc.

In recent years immobilization techniques have become very mild so that, besides microorganisms, plant and animal cells can also be immobilized. When immobilized enzymes first appeared a bright future for the technology was predicted. During the last years some severe limitations have appeared, e.g., the problems to develop a practically feasible technology for coenzyme regeneration, the lack of methods to arrange enzyme molecules in ordered clusters to perform multistep enzyme catalyzed reactions. To all these problems immobilized cells and organelles offer exciting and promising alternatives.

Is it possible to manipulate the total metabolism by immobilization of the cell? This question is not fully answered yet, but strong evidence shows that changes occur.

Immobilized animal cells grown on microparticles of weak ion-exchangers have changed the field of cell-culture dramatically. This technique now makes it possible to exploit mammalian cell lines in biotechnology.

Bo Mattiasson

THE EDITOR

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Chapter 1

INTRODUCTION

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The area of immobilized cells and organelles has expanded very fast. Many new techniques for the preparation of immobilized cells have been developed during the last decade, especially during the last 5 years. Concomitantly with this availability of methods for obtaining suitable preparations, a rather broad spectrum of applications have been investigated. The preparations used have been based on cells with different metabolic and physical characteristics: cell fragments, organelles, cell homogenates, dead cells, permeabilized cells, resting cells, starving cells, viable cells, and mixed cell cultures. Most of the applications have utilized the immobilized cells as biocatalysts, but reports also exist on the use of immobilized cells as affinity adsorbents.

Along with the development in immobilization technology, more and more gentle immobilization procedures have evolved, so that today it seems possible to immobilize almost any cell structure and keep the cell viable.

Along with these very exciting positive findings, some observations have been made which indicate that immobilization technology, even if associated with many advantages over conventional fermentation technology, also has some drawbacks; e.g., the increased difficulty for delivering a good oxygen supply to dense cell preparations, cell growth within the support, and, in some cases, changed metabolic patterns. To partly reduce these drawbacks of the conventional immobilization methods, some alternative immobilization techniques have also been developed during the last years.

The aim of these volumes is to present the state of the art concerning immobilized cells and organelles, the technology, applications, and some speculations for future developments.

The first part of the volume is, in principle, devoted to immobilization procedures for different biocatalytic entities. The second volume covers the applications. Besides having a chapter on immobilization technology as such, specific chapters cover immobilization of plant cells and mammalian cells as well as organelles. Furthermore, the alternative immobilization techniques are also covered in separate chapters, for instance, immobilization of mammalian cells behind ultrafiltration membranes, and the use of soluble, temporarily immobilized cells, in aqueous two-phase systems, is also presented. The different applications are further discussed in chapters dealing with immobilized dead cells, immobilized viable cells, aerobic cells, anaerobic cells, and mixed cultures. One chapter is also devoted to the technical considerations of importance when using immobilized cells in large-scale processes. Analytical applications of immobilized cells are covered in one chapter and the use of immobilized cells and cell membranes as affinity sorbents is presented in another.

Chapter 2

IMMOBILIZATION METHODS

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I. INTRODUCTION

There are a great number of alternative immobilization procedures to choose from when immobilization of cells is desired. Many excellent reviews and books on immobilization procedures pertaining to enzymes have been published.[1-4] Most of these published techniques for soluble proteins have also been found to be suitable for cells and organelles. The topic of immobilization methods is vast enough to fill an entire volume in itself. The intention with which this chapter is written is to give a comparative overview of the available methods. It is, however, understood that such a chapter within the available space cannot be fully comprehensive. The area has been described in more detail by some recent review articles.[5-7]

In principle, six different types of immobilization methods can be distinguished:

- Covalent coupling, including cross-linking
- Adsorption
- Affinity (biospecific) immobilization
- Entrapment in a three-dimensional polymer network
- Confinement in a liquid-liquid emulsion
- Capture behind semipermeable membranes

Some of the methods are long ago established and their applications are discussed in several of the chapters, whereas others are rather new and are currently undergoing development. One shall bear in mind that this subgrouping of methods is by no means absolute, since in some cases it may be difficult to classify a certain method as belonging to a specific group. Each of the first four techniques will be presented in the following manner: a few examples will be discussed in some detail and then a more extensive list of references and reports on other applications will be presented and briefly discussed. The last two techniques are described in separate chapters in this volume.

II. COVALENT COUPLING

This approach is perhaps the most popular one to use when immobilizing enzymes, but in the case of cells rather few systems using this technique have been reported.

The procedure is, in principle, applicable in all cases when cells are to be immobilized. However, several disadvantages are apparent from a practical point of view. Living cells, for example, are characterized by their ability to divide in the immobilized state. Covalently bound preparations would then be expected to leak a substantial number of cells. The rather few reports in this area are focused on the use of dead cells, or at least cells that are to be utilized for single step catalytic conversions only.

The method of co-cross-linking proteins to form aggregates has been successfully applied in enzyme systems. Petre et al.[8] applied the same technique for immobilization of *Escherichia coli* cells to create membranes. The procedure used was the following: 1.25 mℓ of a pH 6.8 0.02 *M* phosphate buffer solution containing 6 mg/mℓ glutaraldehyde, 60 mg/mℓ bovine serum albumin (Sigma Chemical Corp.), and 10 mg/mℓ lyophilized *E. coli* were mixed and then spread on a glass plate. After drying for 2 hr, the film produced was rinsed with distilled water.

Such co-cross-linking can, of course, also be used for producing particulate preparations. NOVO[9] has taken a step further by utilizing cell homogenates as the sole protein components in the final preparation (see Chapter 7). Navarro and Durand[10] studied the immobilization of *Saccharomyces cerevisiae* to porous glass. The cells were either adsorbed to the untreated beads or coupled to chemically modified porous glass beads. The glass support was derivatized with γ-amino propyltrimethoxysilan[11] and

then activated by treatment with glutaraldehyde. Coupling proceeded in citrate phosphate buffer 0.02 *M* pH 5 for 1 hr at room temperature. In a comparative study of the covalently bound and adsorbed cells, some interesting differences were observed (see Chapter 1, Volume II). Among the arsenal of coupling methods available only a few have actually been tested on cells.

Micrococcus luteus was coupled to carboxymethylcellulose using carbodiimide as coupling agent.[12] Since it is known that carbodiimide may be deliterious to enzyme activity, a two-step procedure was used. First the carboxyl-groups on the support were activated with carbodiimide thereby forming a reactive *O*-acylisourea. In a subsequent step, cells were added and immobilization took place.

The cells were used to convert L-histidine to urocanic acid. The authors found the histidine ammonia lyase to have a high stability and a very low content of cells in the effluent. Furthermore, periodic streaking of this effluent on nutrient agar plates gave no growth, indicating that the immobilized cells were not viable (see Chapter 1, Volume II). The authors state that the method is applicable to many systems, but that the coupling reagents are too expensive.

In another project *Zygosaccharomyces lactis* was studied when covalently linked to hydroxyalkyl methacrylate gel. By modifying the gel first with 1,6-diaminohexan and then with glutaraldehyde it was possible to bind the cells.[13] The gel containing amino groups was treated with glutaraldehyde (final concentration 5%) for 1 hr before being washed with water to remove remaining free glutaraldehyde (indicated by 2,4-dinitrophenylhydrazine). Afterwards, the gel was stored in 0.05 *M* phosphate buffer pH 7.0. Coupling proceeded in the same buffer, 0.5 g suction-dried gel was suspended in 5 m*l* of a cell suspension (10^6 cells/m*l*) and was allowed to react for several days with stirring.

By using the same coupling procedure, *Saccharomyces cerevisiae* was bound to hydroxyalkylmethacrylate.[14] In spite of an active cell-proliferation, very low cell counts were found in the effluent. Studies with electron microscopy revealed that the organism when immobilized, had changed its pattern of cell proliferation, the result being long chains of cells instead of free individual cells (see Chapter 1, Volume II). In still another report, a film reactor was designed by filling a tube with Raschig rings pretreated with gelatin and finally with glutaraldehyde.[15] Cells of *Saccharomyces cerevisiae* were added and subsequently immobilized. The method of using gelatinous hydrous metal oxides[16-18] to covalently couple cells can be considered to lie on the periphery of "classical" covalent coupling methods. This immobilization process can be envisaged to involve the replacement of hydroxyl groups on the surface of the metal hydroxide with suitable ligands from the enzyme or cell, resulting in the formation of partial covalent bonds. The principle is illustrated in Figure 1.

The immobilization procedure described in the above references was the following: Samples of metal hydroxides were prepared from their tetrachlorides. Titanium (IV) chloride 150g/*l* in 150 g HCl/*l* was neutralized to pH 7.0 by slow addition of 2.0 *M* ammonium hydroxide. The metal hydroxide thus formed was washed with saline solution to remove ammonium ions and then used for immobilization purposes. On addition of the metal hydroxide to a cell suspension, aggregation of the cells take place. After 5 min a precipitate is formed which can be recovered by centrifugation and utilized. The method is easy to follow, but a substantial loss of cells in the effluent together with a leakage of metal ion in the eluent stream has been reported.

III. AFFINITY (BIOSPECIFIC) IMMOBILIZATION

From affinity chromatography the binding reaction has been adopted. Whereby an immobilized preparation is achieved under "noneluting" conditions. In the case of

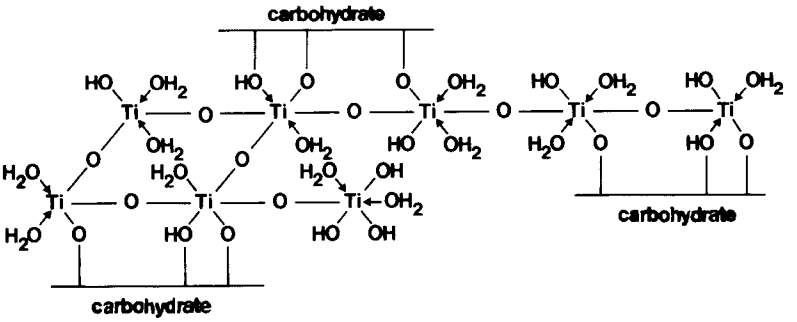

FIGURE 1. Schematic presentation of the coupling obtained by using hydrous titanium oxides. (From Kennedy, J. F., *Immobilized Microbial Cells*, American Chemical Society, Washington, D.C., 1979, 117. With permission.)

cells, the support used for affinity immobilization must contain a structure capable of interacting with structures on the cell surface. Affinity immobilization involves no drastic reactions and no real exposure to chemicals except for the adsorbent material. The method is very mild and may thus be especially useful when labile structures are handled. Future details are given in Chapter 5, Volume II but can also be found in papers on affinity chromatography of cells.[19,20] An example of the use of this technique is the following: immobilization of *Saccharomyces cerevisiae* to concanavalin-A Sepharose. The gel is suspended in a suitable buffer, e.g., Tris-HCl 0.1 *M*, pH 7.4 containing 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$, and 1 m*M* $MgCl_2$. The cell suspension is introduced and mixed with the sorbent. After extensive mixing for 10 to 20 min, excess cells are washed off and the immobilized cell preparation is ready to use.[21] This approach was used by Schmeer et al.[22] when designing a new semiartificial organ for blood treatment. Red blood cells were disrupted and glutaminase or urease was introduced before the membranes were resealed. These "ghosts" containing enzymes were immobilized on Concanavalin A Sephadex G-25. The final preparation was packed in a column for treatment of blood. It can be expected that when gentle methods of immobilization are needed, the relatively unexplored method of affinity immobilization will be a good candidate to consider.

IV. ADSORPTION

The influence by the immobilization method on the economy of the total process is often of fundamental importance, especially in large-scale processes with cheap substrates and products. This means that if a method is to be competitive, there must be a cheap procedure for immobilization. In these cases adsorption seems to be a very promising technique to use.

Adsorption is, in principle, a reversible process. This means that the support may be recovered after the catalyst is denatured. This has successfully been adopted in enzyme processes, e.g., the enzymatic resolution of racemic mixtures of amino acids.[23] An important difference between adsorbing enzymes and cells is that the latter are bound via multipoint attachment and therefore stick much stronger to the sorbent. This leads to a more efficient adsorption process, but also to a more difficult desorption process.[24,25] Furthermore, the interaction between a cell and a solid surface is a rather complicated process. From studies on mammalian cells it is known that the cells may adhere to the surface reversibly for a short time period but that this reversibility is lost as time goes on. The primary interaction between the cell and the solid surface must somehow induce a secondary, irreversible interaction.[26] Similar results have been

Table 1
ADSORPTION

Support material	Cell	Product	Ref.
Cellex-E	*Azotobacter vinelandii*	—	28
Basic, anionic ion exchanger	*Saccharomyces cerevisiae*	Ethanol	29
Wood chips	*Saccharomyces cerevisiae*	Ethanol	30
Large-surface area ceramic support	*Acetobacter*	Acetic acid	32
Cellulose (modified)	*Aspergillus oryzae*	Glucose + fructose from sucrose	31
Porous glass	*Saccharomyces carlsbergensis*	Beer	10
Fritted glass	*Escherichia coli*	Biomass	33
Fritted glass	*Serratia marcescens*	Biomass	33
Porous glass	*Saccharomyces cerevisiae*	Ethanol	10
Controlled pore glass	Mixed culture	Methane	32
Glass fiber pads	*Zymomonas mobilis*	Ethanol	33
Granular activated carbon	Mixed culture	Waste water treatment	36
Anthracite	*Pseudomonas* sp.	Phenol degradation	37

found for microorganisms, but not much data is available yet. The area of adsorbed cells has been described in terms of natural ecosystems[27] as well as old fermentation systems.

Since adsorption seems to be an important first step toward blood clotting when treating blood in extra corporal shunts or when introducing foreign material in the body, much effort has been put into understanding the adsorption process. The ultimate goal of this research is to design material with specific adsorptive abilities.

In this section we will discuss the immobilization of cells for use in biotechnological processes. Since mammalian cells are covered in Chapter 3, they will not be mentioned here.

The adsorption process can be studied with respect to the properties of the support or those of the cells. Important factors when discussing cell properties are cell wall composition, charge, and age. The relationship between volume of the cell and surface area whereby the cell adsorbs is, of course, also of importance. The properties of the support are of utmost importance for the adsorption process. Composition of the carrier, surface charge, surface area, and pore size are some of the factors that have to be taken into account. A broad spectrum of support materials are at hand. Some of the different supports that have been tested are compiled in Table 1 together with the organism studied in each case.

As can be seen from the table, quite a broad spectrum of different supports have been used to date. In some cases, i.e., ion exchangers, it is rather easy to predict whether cells will adsorb or not. In other cases, however, it may be more difficult. An interesting approach that was used in forming the first critical adhesion step has been presented by Hollo et al.[38] When trying to adsorb *Pseudomonas aeruginosa* to polyethylene they found that cells adsorbed when no carbon source was in the medium.

The plasticizer in the polymer used was utilized by the cells instead and they were therefore attracted chemotactically to the solid surface. Even if adsorption of cells to solid surfaces is no longer a novelty, there is still a need for research in order to clarify the conditions needed to achieve an optimal interaction. The great advantage with adsorption methods is in their operational simplicity. Cell loading is often carried out by simply pumping a cell suspension through a bed of adsorbent. Due to the mild conditions used, viable cell preparations can be obtained. One disadvantage with adsorption is the risk of desorption. This latter possibility, however, can be turned into an advantage by allowing one to desorb the cell preparation when its activity is declining. The matrix can thus be recycled.

V. ENTRAPMENT

Entrapment methods are based on the confinement of the cells in a three-dimensional gel lattice. The cells are free within their compartments and the pores in the material allow substrate and product to diffuse to and from the cells. Entrapment is by far the most frequently used method in laboratory experiments and there are also some examples of industrial processes based on entrapped cells. By nature, the method of entrapment has restricted the use of substrates to those having a low molecular weight. Similar restrictions must also apply to the products formed. However, there are methods now available whereby even large molecules can be utilized. In the case of immobilized enzymes one advantage often mentioned is that the catalyst, when encased in a gel-lattice, is protected from macromolecular inhibitors, e.g., hydrolytic enzymes. This may also be said to be valid for cells. Bacteriophages can also be included among potential "inhibitors". A great problem with entrapped living cells lies in their ability to divide and eventually break the support. This will further be discussed in Chapter 1, Volume II.

A. Entrapment in Synthetic Polymers

Several polymers have been utilized for the entrapment of cells. A crucial point to consider when choosing a polymer is that the monomers as well as the reaction conditions must not be deliterious to the cells. As is discussed in separate chapters, different requirements must be fulfilled if the cells are to be fully alive or, on the other hand, if only one or a few enzyme reactions need to be in operation, in which case nonviable cells would also be of use.

The most commonly used synthetic polymers are the acryl polymers. Table 2 summarizes some of the different organisms used and the biological reactions which were studied in the immobilized state.

Entrapment in an acrylic polymer is a rather simple procedure. The basic scheme for the preparation of a block polymer is given in Figure 2. Further steps that are needed to create spherical beads, etc., will be discussed later in this chapter.

1. A Typical Entrapment Procedure Using Acrylamide

Entrapment of *Saccharomyces cerevisiae* in 15% (w/w) polyacrylamide (93):

Washed cells, 5 g wet weight, were suspended in 5 m*l* ice-cold water and mixed with 10 m*l* ice-cold freshly prepared monomer solution in 0.2 *M* potassium phosphate buffer pH 7.0 containing 2.85 g acrylamide, 0.15 g *N,N*-methylene-bis-acrylamide, 20 μl tetramethylethylenediamine and 10 mg ammonium persulfate. The reaction mixture was immediately poured onto a glass plate equipped with stainless steel spacers (0.7 mm). Another glassplate was placed on top of the liquid pool. Polymerization started within 1 min and was considered complete after 1 hr. The gel sheet obtained was passed through a stainless steel mesh yielding gel grains of cubic shape, with approximate dimensions of $0.7 \times 0.7 \times 0.7$ mm.

Table 2
EXAMPLES ON CELLS IMMOBILIZED IN POLYACRYLAMIDE

Organisms	Reaction product	Ref.
Acetobacter suboxydans	Dihydroxyaceton	39
Achromobacter aceris	NADP	40
Achromobacter butyri	Glucose-6-phosphate	41
Achromobacter guttatus		42
Achromobacter liquidum	Urocanic acid	43
Arthrobacter globiformis	Prednisolone	44
Arthrobacter simplex	Prednisolone	45
		46
Bacillus subtilis	Production of α-amylase	47
Bacillus sp	Production of	48
	bacitracin	49
Brassica campestris (chloroplasts)	N_2-fixation	50
Brevibacterium ammoniagenes	Coenzyme A	51
Brevibacterium	*NADP*	52
ammoniagenes		53
Brevibacterium, ammoniagenes	L-malic acid	54
Pseudomonas testosteroni	Steroid transformation	55
Candida lipolytica	Citric acid	56
Candida tropicalis	Oxidation of phenol	57
Clostridium butyricum	Hydrogen	58
Clostridia	Hydrogen	59
Corynebacterium glutamicum	Glutamic acid	60
Curvularia lunata	Cortisol	61
Escherichia coli	6-APA	62
Escherichia coli	β-lactamase	63
Escherichia coli	L-asparatic	64
	acid	65
Escherichia coli	Hydrolysis of lactose	66
Escherichia coli	Tryptophan	67
Escherichia freundii	Glucose-phosphate	68
Gluconobacter melonogeneus + *Pseudomonas syringae*	2-keto L-gulonic acid	69
Gluconobacter melonogeneus	L-sorbosone	70
Hanensula polymorpha	Oxidation of methanol	71
Kluyvera citrophila	Penicillin acylase	72
Kluyveromyces lactis	Hydrolysis of lactose	66
Lactobacillius bulgaricus	Hydrolysis of lactose	66
Mycobacterium globiforme	Reduction of steroids	73
Mycobacterium rubrum		74
Nocardia corallina	Propylenoxide	75
Nocardia sp.	Steroid transformation	76
Penicillium chrysogenum	Penicillin G	77
Pseudomonas putida	Degradation of benzene	78
Pseudomonas testosteroni	Steroidtransformation	55
Rhizobium	Innoculant for legumes	79
Saccharomyces cerevisiae	Glutathione	80
Saccharomyces cerevisiae	Ethanol	81
Saccharomyces cerevisiae	Dehydrogenations	82
Saccharomyces cerevisiae	Sucrose hydrolysis	83
Saccharomyces cerevisiae + glucose oxidase	Fructose and gluconic acid	84
Saccharomyces cerevisiae	H_2O_2-decomposition	85
Silk glands	Silk protein	86
		87