Preface

This publication contains full papers of both oral and poster presentations of the symposium **"Immobilized Cells: Basics and Applications"** that was held in Noordwijkerhout, The Netherlands, 26-29 November 1995.
Industrial processes with micro-organisms are generally based on the exploitation of free, growing cells. Alternatively, immobilized cells can be used. In that case cells are attached to or entrapped in an inert support. In a continuously operated bioreactor medium containing the substrate will be supplied. Substrate will be converted into a product by the immobilized cells and product and remaining substrate disappear with the outflowing medium. The immobilized cells are retained easily in the bioreactor and as such utilized continuously. In this way the capacity of the process is independent of the growth rate of the micro-organisms involved. Especially in cases where cells grow slowly immobilized-cell processes are more advantageous than processes with suspended cells. Also for very specific situations where the presence of biomass in the product should be prevented (e.g. champagne), drugs should be dosed gradually to the medium (e.g. islets of Langerhans), phage infections in starter cultures should be prevented (e.g. lactic-acid bacteria for cheese production) and plasmids should be stabilized (for application of genetically engineered cells in a continuous mode), immobilized cells perform better than suspended cells.
The physiology of immobilized cells has been studied widely last decade. In 1990, during the symposium "Physiology of Immobilized Cells" in Wageningen, an extensive overview of this field was given. Until now, physiological knowledge did not result in wide use of such processes in practice.

Since research in the field of immobilized cells started, an enormous quantity of papers have been published. For many processes the complex physiology in a heterogeneous environment is becoming clear now. In addition it is shown that in many processes it is more efficient to use immobilized cells than suspended cells. Nevertheless, applications of immobilized cells in industrial processes are limited. For this reason it was considered essential by the organizers of the symposium to cover the path from basic physiological research to applications and bring together scientists from different disciplines from academia, industry and research institutes. For applications, physiology needs to be integrated with engineering. The goal of the symposium "Immobilized Cells: Basics and Applications" was to relate basic research to applications. Another aim was to extract guidelines for characterization of immobilized cells in view of process design and application from the contributions.

Reviews and recent developments of basic research methods essential for applications, bioreactors developed for applications and applications of immobilized-cell processes were discussed in 4 sessions (oral and poster presentations). Both academic and industrial research was presented in these sessions:

Basics 1: physics

Physical aspects of support materials in relation to fermentations or release of components are key factors in the path from basic research to applications.

Basic physical characteristics of immobilized cells and support materials such as immobilization techniques (including large scale), stability of support materials and interactions of support and media (e.g. diffusion coefficients) were discussed. This session was directed to methods for determination of these physical characteristics and to the key factors for application.

Basics 2: physiology, mass transfer and dynamic modelling

The physiology of immobilized cells and transfer of components through support materials have been studied widely. These basic aspects have been integrated in several dynamic models. This session was directed to the essential physiological and mass transfer aspects that are important for application of immobilized cells and to their incorporation in dynamic models, including methods for validation of these models.
In addition to that, physiology of immobilized and co-immobilized bacteria, fungi, yeast, lichen, micro-algae and recombinant micro-organisms was presented.

Immobilized-cell reactors

Bioreactors for cultivation of immobilized cells are different in some respects from reactors for suspended cells. Essential characteristics of such immobilized-cell reactors were discussed. In this respect scale-up of the bioreactors is an essential requisite in the trail from basic research to applications.

Applications

Some processes with immobilized cells have been applied in practice. In this session a few of these processes in food technology, environmental technology, clinical applications, production of enzymes and amino acids have been discussed in more detail. Important phases in the development of the processes were high-lighted.
Additionally, promising future applications were identified.

The manuscripts presented in these proceedings give an extensive and recent overview of the research and applications of immobilized-cell technology. We hope the manuscripts will also stimulate researchers from the biological disciplines to implement in their research strategy questions that will be raised if the processes are scaled up (**think big!**), and industry and researchers studying scale up discuss scale-up aspects in an early stage of the research with researchers from the biological disciplines (**scale-up by scaling down!**).
In addition the organizers hope that the integrated approach presented at this conference will stimulate universities to implement this strategy in their educational programmes as well. Helpful tools are formed by the guidelines for the characterization of immobilized cells!

The Editors, Wageningen and London, December 1995.

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Immobilized Cells: Basics and Applications

Why immobilize?

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Introduction

Why immobilize? - The simplest answer may be: because nature does it! While scientists are requested to be mobile it is obviously advantageous for many biocatalytic systems to be immobile. A good example of this is Yellowstone Park (USA) where one can see thick mats of microorganisms and algae at the edge of hot springs. With a continuous supply of substrates, there is obviously selective pressure to become immobile. In nature different biocatalytic systems adapt remarkably well to reaction conditions, e.g. temperature, pH and substrate concentration in the effluent of the above mentioned hot springs.
Also in the laboratory one can see - sometimes unwanted - spontaneous immobilization. In chemostate cultures, which are operated close to washout conditions, microorganisms often develop a tendency to grow on the reactor wall. If nature shows such a clear tendency towards immobilization it is obvious that one also can exploit such phenomena in technical systems.

While the use of immobilized whole cells has already been known for a long time (e.g. production of acetic acid with microorganisms immobilized on pieces of wood) technical systems using immobilized isolated enzymes were applied much later. In the fifties, the first laboratory systems were described in the literature [1-2]. The first full-scale system using immobilized enzymes went into operation in 1969. The Tanabe Company, Japan, used immobilized acylase for the kinetic racemic resolution to obtain L-methionine [3].

The Japanese were also pioneers in the field of immobilized living and dead cells (production of ethanol from glucose, production of acrylamide from acrylnitrile) [4].

Full-scale systems using immobilized higher cells came into use in the eighties. A well known example is the plant of Bayer, USA, for the production of factor VIII. Here, the cells are not immobilized on a carrier but retained by a membrane in a continuously operated system [5].

In the following, some reasons, problems, and solutions are given which may occur, if enzymes, microorganisms, or higher cells are to be immobilized. The examples used are mostly from our own developments. It is not possible here to give a comprehensive overview, since there are now dozens of systems on an industrial scale - which are seldom described in detail - and thousands of systems in laboratory scale - which are better documented.

Immobilized enzymes

Enzymes should be understood here as enzymes in dead cells, partially purified enzymes, or purified enzymes. Immobilization can be achieved by carrier fixation, encapsulation, cross linking and/or the use of membranes for catalyst retention in a continuously operated system. In recent years there has been a trend towards using "macroporous" microcarriers. Such carriers can be produced from a mixture of glass powder and salt. After moulding and sintering the inert salt fraction is washed out [6]. The pore size and the porosity can be varied within a wide range by the appropriate selection of the particle size and the volume fraction of the salt so that even the centre of the particle can be reached by (reduced) enforced flow.

In our experience, homogeneous biocatalysts immobilized in membrane reactors often have advantages in comparison to heterogeneous biocatalysts. Catalytic systems become complex if a coenzyme is needed. Since a coenzyme is a transport metabolite it does not make sense to immobilize the coenzyme on a carrier. For readily water-soluble reactants one can exploit the fact that in the case of NAD very low coenzyme concentrations are needed to saturate the enzymes involved. The enzymes are retained in the membrane reactor, while the coenzyme is continuously dosed into the system.

For substrates with intermediate water solubility, the coenzyme can be covalently bound to soluble polymer in order to be retained by a membrane together with the enzymes.

If the water solubility of the reactants is very low, one can exploit this to deliver the substrate to the water phase from an organic solvent via a membrane. After the product is formed, it leaves the water phase and is obtained from the organic phase. In this case the entire catalytic system is "immobilized" in the water phase, while the organic solvent is a continuous phase [7].

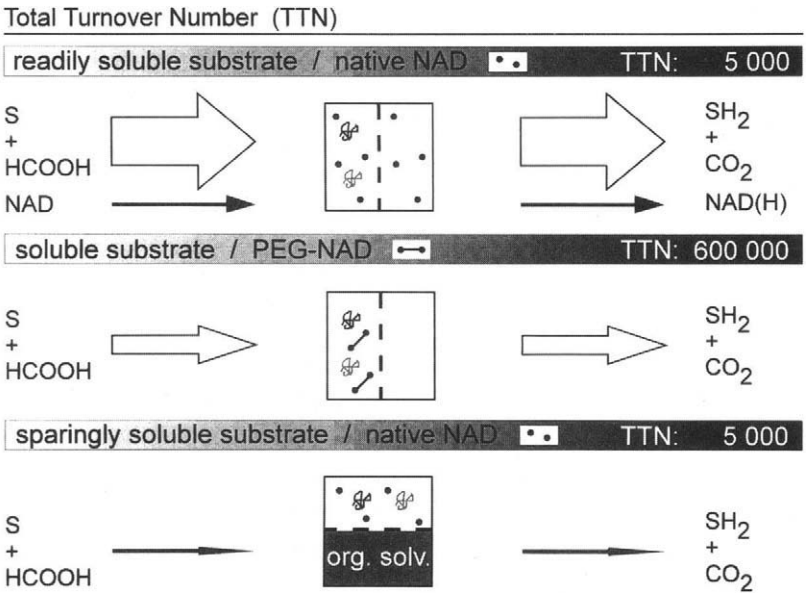


Figure 1. Continuous cofactor regeneration for reactants of different water solubility.

Microorganisms

Microorganisms are immobilized (in a dead form), if no enzyme purification is required (e.g. production of acrylamide from acrylnitrile). In other cases living, non-reproducing cells, retained by a membrane or fixed to a carrier, are used. The microbial glucose oxidase catalyzes the production of gluconic acid from glucose while the simultaneously present catalase decomposes the hydrogen peroxide formed.

Another example is the use of immobilized yeast for coenzyme-dependent reductions. Glucose is used as the hydrogen source, taking advantage of the intracellular cofactor regeneration system. The microbial system is limited with respect to other nutrients so that the glucose is mainly used for cofactor regeneration and not for cell growth.

Anaerobic microorganisms are good examples of immobilization by means of membranes or carriers. One need not be afraid of substrate limitation with respect to oxygen. Furthermore, most of the carbon source is converted to the desired product (e.g. ethanol, acetic acid, biogas). Increasing space-time yield by

increasing substrate concentration is limited by product inhibition or as in anaerobic waste water treatment, by the substrate concentrations available in practical waste waters. All these arguments strongly support the use of immobilization techniques. Nevertheless, one has to take into account the fact that in the mentioned systems cells reproduce and form carbon dioxide. Both of these "byproducts" must readily leave the reactor in order to avoid accumulation. For this purpose macroporous carriers or membranes can be used. With membranes a bleed technique has to be applied, where a minor part of the effluent leaves the reactor in an unfiltered state in order to avoid uncontrolled accumulation.

A first example of such systems is the production of ethanol from glucose by means of *Zymomonas mobilis* . Only 2.3 % (at most) of the glucose consumed by *Zymomonas mobilis* is needed for cell mass formation. That means that non-growth-coupled ethanol production has a negligible effect on ethanol selectivity.Growth (and thus ethanol formation) is strongly inhibited by ethanol. Cell growth becomes zero at ethanol concentrations above 70 g/l. With the given ethanol selectivity of ~ 0.5 g ethanol/g glucose it does not make much sense to use glucose concentrations higher than 140 g/l. One has to make a compromise between maximal product concentration and maximal space-time yield. Here, cell immobilization helps a lot, since for sensible ethanol concentrations the cell-specific ethanol production rate is comparatively low. One can nevertheless reach acceptable space-time yields by increasing the cell concentration. Both by carrier fixation as well as by the use of membranes, an at least 10-fold increase of catalyst concentration can be reached in comparison to chemostate conditions [8-9] (Figure 2).

In order to get rid of daughter cells and carbon dioxide and at least partially overcome product inhibition a two-stage fluidized-bed reactor using "macroporous" microcarriers was employed. Using an initial substrate concentration of 120 g glucose/l, an ethanol concentration of 50 g/l was reached (80 % conversion in the first stage, 99 % conversion in the second stage). Space-time yield (at 99 % conversion) was 12 g/(lxh). In comparison to chemostate the space-time yield could be increased by the factor of 2.5. Since, the biomass concentration was increased 10-fold, the effectiveness factor was only about 0.25. An important additional advantage of the two-stage fludized-bed cascade in comparison to a chemostate was the fact that this system could be operated with a non-sterilized glucose feed stock, which is a byproduct of starch hydrolysis. When this feed stock was used in a chemostate, Lactobacilli became dominant after some time (shift from ethanol production to lactic acid production). In the fluidized-bed cascade contaminating Lactobacilli were continuously washed out,

due to a monoseptic precolonization of the carrier with *Zymomonas mobilis* and the short residence time.

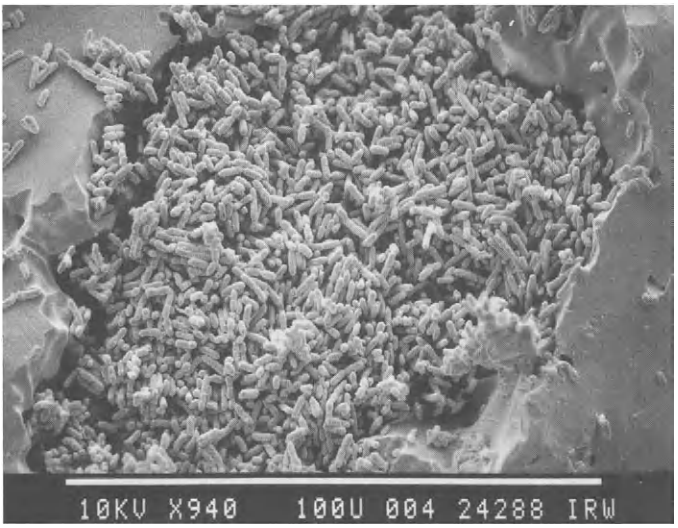
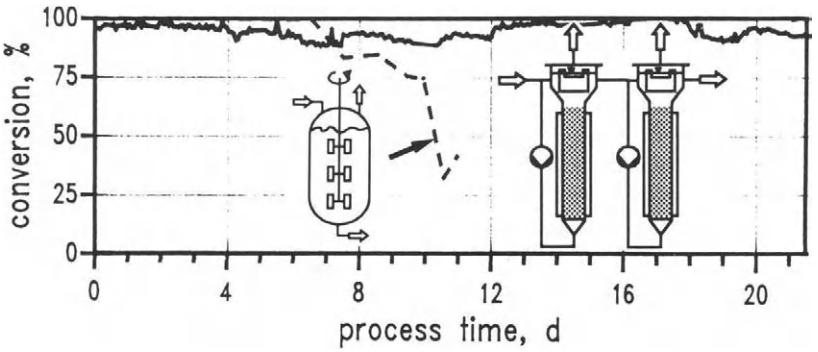


Figure 2. Scanning electron micrograph of a pore inside the macroporous glass carrier (Siran™) fully colonized by *Zymomonas mobilis*.



(120-135 g/l glucose; pH = 5.0; T = 30 °C; $V_{\text{fluidized bed reactor}}$ = 55 l: residence time 4 h)

Figure 3. Comparison of ethanol fermentation from hydrolized B starch in a chemostate and in a two-stage fluidized-bed cascade.

For a quite different purpose, the concentration of *Zymomonas mobilis* was increased in a membrane reactor. The reactor was placed into the core of the magnet of nuclear magnetic resonance machine for in-vivo NMR measurements of intracellular metabolites.

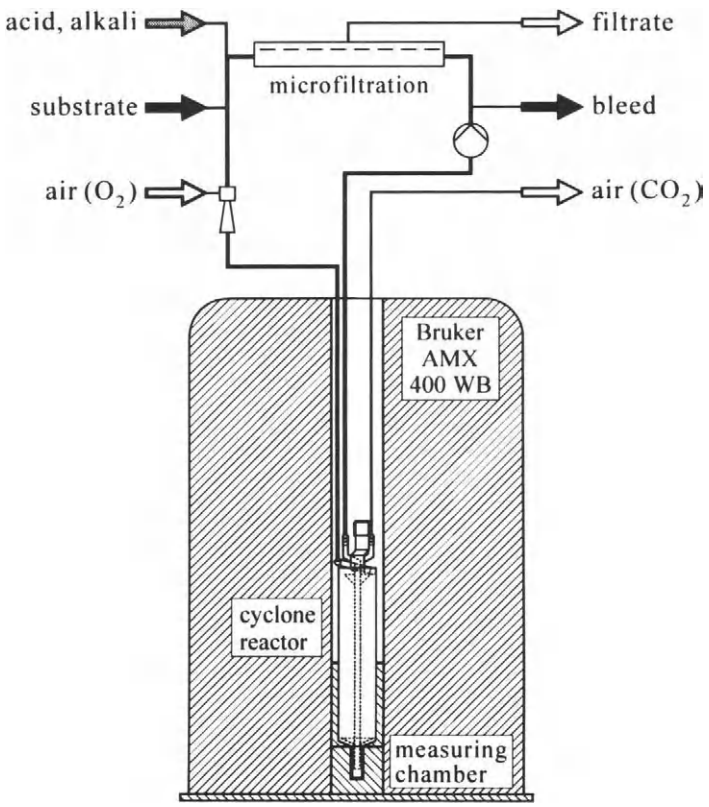


Figure 4. Chemostate with cell retention (by means of a microfiltration membrane) to increase cell density.

By decoupling the substrate and catalyst residence time in a chemostate with partial cell retention it was possible to measure intracellular metabolites under steady-state conditions with better signal/noise-ratio.

Acetogenium kivui is a homoacetate fermenting bacterium, the acetate yield is very high, but there is strong product inhibition.

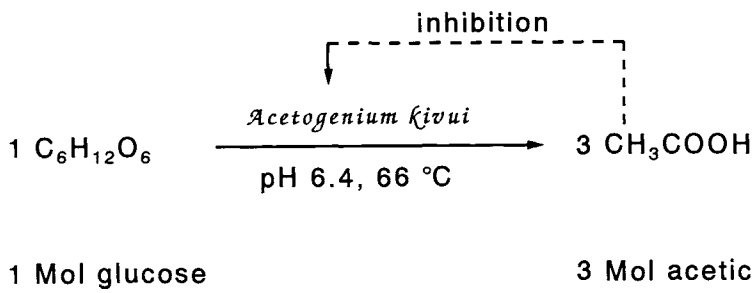


Figure 5. Reaction scheme for the homoacetate fermenting bacterium *Acetogenium kivui*.

It is advisable to use integrated product removal to prevent acetic acid from accumulating. For this purpose electrodialysis is useful. Simultaneously the acetic acid concentration can be increased in the effluent of the electrodialysis unit.

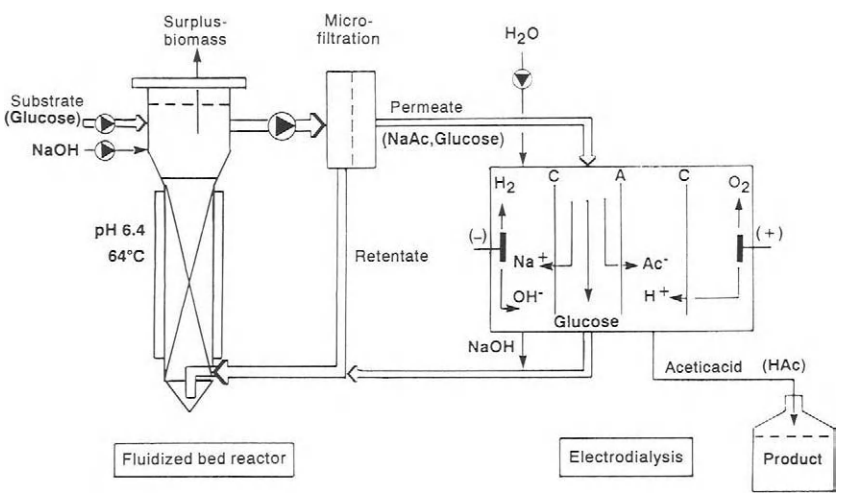


Figure 6. Integrated electrodialysis for the removal of acetic acid fermented by *Acetogenium kivui* from glucose.

A fluidized-bed reactor with carrier-fixed *Acetogenium kivui* is used. Product removal is effected from the bypass [10].
From biological waste water treament we can get a clear answer to the question "Why immobilize?". In most cases the substrate concentration is so low that a continuous fermentation, following the chemostate method, would result in a very low space-time yield. By means of sludge sedimentation and recycling the biocatalyst is immobilized within the reactor system in order to increase the catalyst concentration. While in aerobic waste water treatment sludge recycling only makes sense until the oxygen transfer becomes limiting, in the anaerobic case mass transfer with respect to the biogas produced becomes limiting only at extremely high biocatalyst concentrations. In such cases the release of biogas can

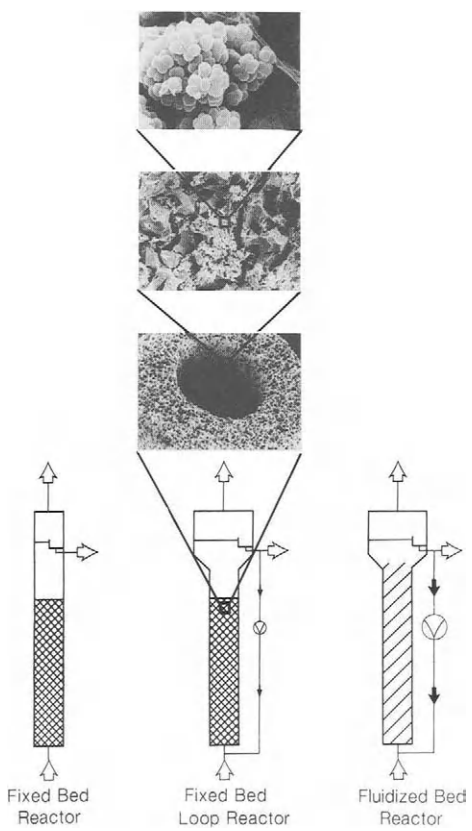


Figure 7. Fixed-bed loop reactors or fluidized-bed reactors with an external recycling loop are used in the Jülich High Perfomance Biogas Process.

be enhanced by using a fixed bed loop reactor or a fluidized-bed reactor with carrier-fixed microorganisms. Biogas is readily released due to the recycle flow. So appropriate values with respect to substrate, product and pH can be established [11]. It was possible to reduce the chemical oxygen demand of the waste water up to 250 kg per m^3 total reactor volume and day. This is achieved by a very high (readily accessible) biomass concentration within a "macroporous" microcarrier.

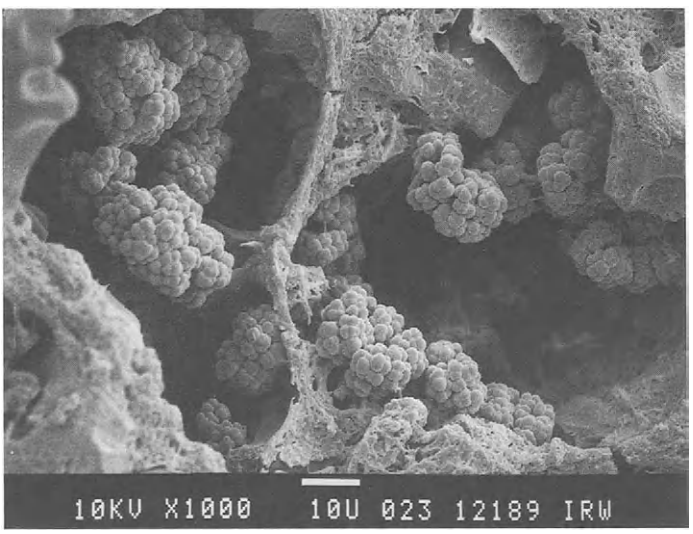


Figure 8. Scanning electron micrograph of a glass carrier colonized with *Methanosarcina barkeri* for anaerobic waste water treatment.

The advantages of the process are: no energy-intensive oxygen supply, biogas formation, which can replace natural gas and small amounts of exit sludge. The process has been commerialized by several licensees. Glass carriers are used in reactors with volumes up to 170 m^3, volcanic stone is used as a carrier in reactors up to 1200 m^3.

Mammalian cells

With respect to the question "Why immobilize?", higher cells are of special interest because some of them (anchorage-dependent cells) can only grow if they

are attached to surfaces. A special technique has been developed for such cells (T-flasks, roller bottles, non-porous microcarriers). Such systems are characterized by the formation of a confluent monolayer of cells. Other higher cells (e.g. hybridoma cells) have mostly been cultivated up to now in suspension culture. Nevertheless, the basic reasons for immobilization are the same although higher cells show some special features. They can produce cytocines and adhesion factors. To keep them "happy", sometimes one has to supplement the medium with such compounds (being one of the reasons for the use of serum in the medium composition). The cell-specific productivity of such factors already reaches a maximum at comparatively low substrate concentrations. On the other hand, it is well known that high substrate concentrations can lead to inhibitory or even toxic product levels (ammonia, lactic acid). Under these circumstances immobilization is especially useful, since a high flow of a medium with low concentration can be used avoiding cell washout. High cell density is good for a high concentration of supporting factors (adhesion factors, growth factors), low medium concentration is good for low concentrations of inhibitory or toxic products.

Since higher cells have a diameter of about 10 μm and sediment rapidly, they can be kept in a reactor system not only by carrier fixation but also by sedimentation (centrifugation) or by means of appropriate filters (e.g. hollow fibre membranes, spin filters).

Most of the products obtained from higher cells (monoclonal antibodies, enzymes, pharmaproteins) are of high molecular weight. So there is always a danger of product retention in continuously operated systems if too "tight" filters/membranes/carriers are used. This is one of the reasons why we think that macroporous microcarriers are especially useful in this field. Using this technique release of product and daughter cells is easily achieved. Macroporous microcarriers also have the advantage of providing a large internal surface, while at the same time concentration gradients within the carrier can be minimized. A high density of such carriers can best be achieved in fluidized-bed reactors. But here a scale-up problem arises. A medium rising through a fluidized-bed will soon be depleted with respect to oxygen. A solution to this problem is bubble-free oxygen supply via reactor-integrated silicon tubes (cross flow mode of oxygen supply). The diffusion of carbon dioxide in silicon is even higher than for oxygen so that carbon dioxide release is not a problem. Bubble-free aeration has the additional advantage that sensitive cells in suspension are not damaged and that flotation of proteins is avoided [12].

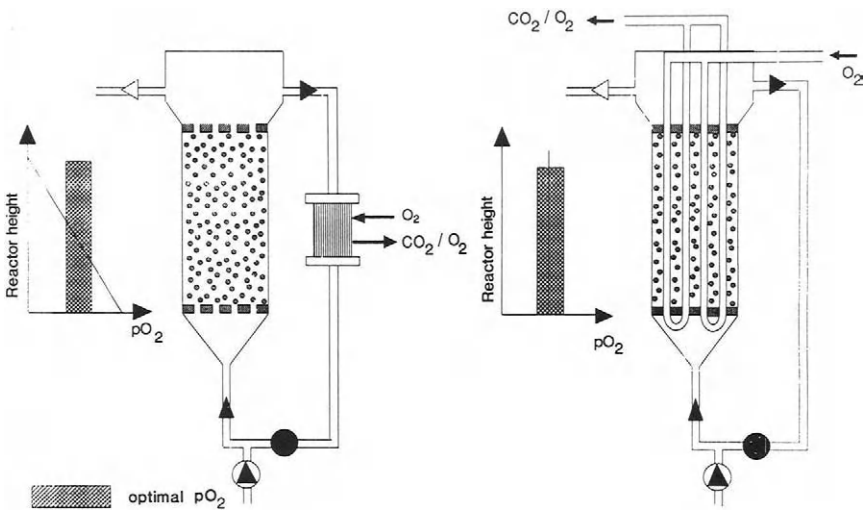


Figure 9. Comparison of bubble-free aeration with oxygen supplementation in a recycle loop and in a reactor-integrated mode.

The cross-flow mode of oxygen supply within the fluidized bed also allows an upward stream, which is just sufficient to fluidize the particles (high particle/cell density, no washout of small particles, low shear stress)

The surface of the carrier must be "bycompatible". In our own experiments we mostly used Siran™-beads with a diameter of about 0.5 mm (Schott AG, Mainz, Germany). These carriers are made from borosilicate glass following a method as described above. After washing them properly with nitric acid, quite a number of mammalian cells colonize these carriers spontaneously. In other cases, a surface modification is useful. For this purpose, coating the carriers with gelatine and afterwards "bathing" these coated carriers in serum proved to be effective [13]. Probably, fibronectins are taken up from the serum and function as adhesion factors for mammalian cells.

The following scanning electron micrographs show beads densely colonized by hybridoma cells, Chinese hamster ovary cells, and baby hamster kidney cells. Cell densities up to 10^8 cells/ml have been reached (Figure 10).

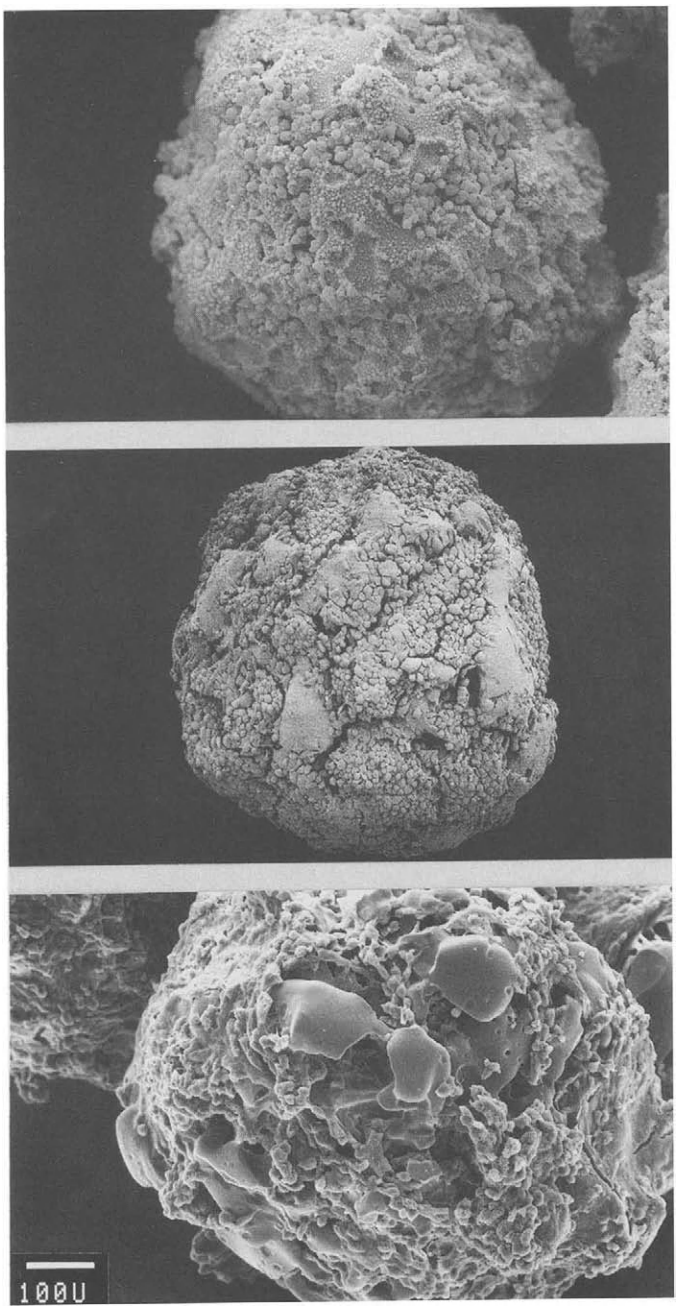


Figure 10. Scanning electron micrographs of macroporous Siran™-beads colonized by hybridoma cells (top), Chinese hamster ovary cells (middle) and baby hamster kidney cells (bottom).

The beads were used for the production of monoclonal antibodies (hybridoma cells), glycosyltransferases (CHO cells) and antithrombin III (BHK cells). In fluidized beds with recycle loop residence times as low as 2 h were adequate. In-situ measurement of cell density was possible using the fact that the capacity of a capacitor is influenced by living cells present between the plates of a capacitor. Thus it could also be shown that between 90 % and 99 % of the cells are within the carrier. The carrier material itself does not disturb the capacity measurement, since its capacity is constant. All cells were cultivated under steady-state conditions (constant levels of substrates including oxygen, products, pH etc.). This feature might prove in future to be very useful for improving reproducibility with respect to product formation. Batch and fed-batch techniques always have the disadvantage that the cells "see" changing physiological conditions during a batch.

Outlook

Immobilization of higher cells will become more and more important in the field of medical biotechnology. It has been shown that the behaviour of suspension cultures can be quite different in comparison to "organlike" cultures (with high cell density). We cultivated cancer cells in a fluidized-bed reactor under steady-state conditions (for cytotoxicity testing) and stroma and stem cells (for in-vivo cell propagation). Other possible candidates are hepathocytes, skin cells, cartilage cells and maybe T cells.

During evolution immobilization was perfected more and more. If there was a continuous supply of nutrients, even simple cells developed a tendency to immobilize on surfaces. Another technique is attachment to each other (pelletisation). "Immobilized" higher systems reached higher and higher volume-specific physiological activities until cell differentiation could be developed. Finally this cell differentiation even allowed life outside water. Obviously, this is only possible if a lot of cells attach to each other. One can also say that the gathering and thus immobilizing of a lot of cells is not only needed for cell differentiation but also that this immobilization - to each other - is the secret of becoming mobile.

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R.H. Wijffels, R.M. Buitelaar, C. Bucke and J. Tramper (Eds)
Immobilized Cells: Basics and Applications

Alginate gels - Some structure-function correlations relevant to their use as immobilization matrix for cells.

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Introduction

In the last two decades advanced use of polysaccharides for gel-entrapment or encapsulation of cells has become a challenging method for the biotechnologist. Of the many proposed techniques the use of alginate gel beads stands out so far as the most promising and versatile method. This immobilization procedure can be carried out in a single step process under very mild conditions and is therefore compatible with most viable cells. The possible uses for such systems in industry, medicine, and agriculture are numerous, ranging from production of bulk chemicals to cell transplantation [1]. Large scale applications have, however, been hampered by mechanical, chemical a well as biological instability. For improving the functionality of the alginate gels it is essential to recognize that alginate is a collective term for a family of polymers with a wide range in chemical composition, sequential structure, molecular size and, hence, in their functional properties. In the present paper we will report on how features such as, charge, porosity, swelling behaviour, long time stability and gel strength depend on the chemical structure and the molecular size of the alginate molecule, and that these properties further can be modified by controlling the kinetics of the gel-formation.

Immobilization

Immobilization of cells by entrapping them in a hydrogel is generally carried out by mixing the cells with a water-soluble polymer, and subsequent gelling of the polymer by adding cross-linking agents. For alginate, gelling is induced by adding cations such as calcium or strontium. By dripping the alginate-cell mixture into a solution containing multivalent cations, the droplets will instantaneously form gel-spheres by ionotropic gelation, entrapping the cells within a threedimentional lattice of ionically crosslinked polymer (figure 1). Entrapment of cells in alginate gels was first described by Hackel *et al.* [2], who used aluminium ions, and by Kierstan and Bucke [3], who used calcium ions. The technique had, in principle, been applied for many years by the food industry for making such products as artificial berries and caviar.

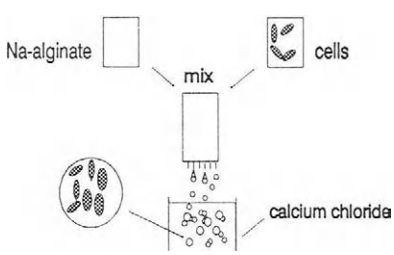

Figure 1 Immobilization of cell in alginate gel beads.

Limitations

Gel-entrapment in alginate, as the technique is known today, has some limitations due both to the inherent nature of the alginate molecule itself, as a biodegradable polydisperse material, and to the nature of the gel as a reversible ionic network. As a consequence of the latter, substances with high affinity for calcium ions such as phosphate or citrate will sequester the crosslinking calcium ions and consequently destabilize the gel. Since the calcium ions can be exchanged with other cations, the gel will also be destabilized by high concentrations of non-gelling ions, such as sodium and magnesium. In some specific applications toxicological and immunological aspects are of significance. Although alginate fulfils the requirements for additives in food and pharmaceuticals, some alginates contain small amounts of poly-phenols which might be harmful to sensitive cells. When gels are used for cell-transplantation, the alginate must also be biocompatible and free from pyrogen and immunogenic materials such as proteins and complex carbohydrates.

Which type of alginate should be chosen?

There is of course no such thing as an ideal alginate bead to meet the requirements of all immobilized cell systems. However, alginate gel beads should ideally have high mechanical and chemical stability, controllable swelling properties, a defined pore size, and a narrow pore-size distribution and a low content of toxic, pyrogenic and immunogenic contaminants. These criteria may be met by selecting the alginates according to composition, sequential structure, molecular weight and purity, and by controlling the kinetics of the gel-formation.

Alginate chemistry

Alginate was first described by the British chemist E. E. C. Stanford in 1881. Its occurrence in nature is mainly limited to the marine brown algae (Phaeophyta), although exocellular polymeric material resembling alginates from brown algae are also produced by soil bacteria such as *Azotobacter vinelandii* and several species of *Pseudomonas*. Alginate exists in the brown algae, as the most abundant polysaccharide comprising up to 40% of dry matter. It is located in the intercellular matrix as a gel containing sodium, calcium, magnesium, strontium and barium ions and its main function is believed to be skeletal, giving both strength and flexibility to the algal tissue. Because of its ability to retain water, and its gelling, viscosifying and stabilizing properties, alginate is widely used industrially and the

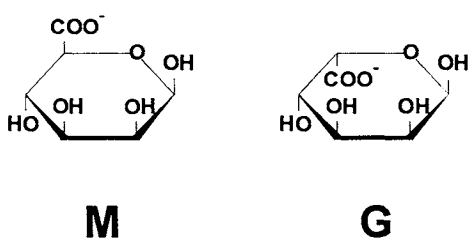

Figure 2a) The monomer composition in alginate. M: β-D-mannuronate, G: α-L-guluronate.

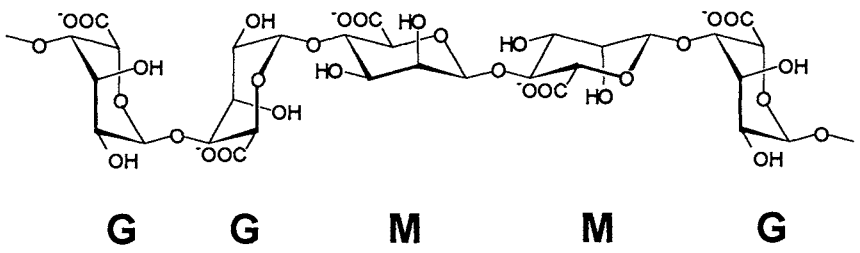

Figure 2b) The alginate chain. Chair conformation.

technical application of alginate forms the basis of the exploitation of brown seaweeds in the western hemisphere. In molecular terms alginate is a family of unbranched binary copolymers of 1-4 linked ß-D-mannuronic acid (M) and α-L-guluronic acid (G) (illustrated in Figure 2a) and b)), of widely varying composition and sequential structure depending on the organism and tissue it is isolated from. The monomers are arranged in a block-wise pattern along the chain with homo-polymeric regions of M and G termed M- and G-blocks respectively, interspaced with regions of alternating structure (MG-block). It is well established that alginates do not have any regular repeating unit and that, except in some bacterially derived polymers, the distribution of the monomers along the polymer chain cannot be described by Bernoullian statistics. Accordingly, the sequential structure is not determined by the monomer composition (monad frequencies) alone, but by measurements of diad, triad and higher order frequencies. The four diad (nearest

Table 1
Composition and sequence parameters of algal alginates

Source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,M}$
Laminaria japonica	0.35	0.65	0.18	0.48	0.17
L. digitata	0.41	0.59	0.25	0.43	0.16
L. hyperborea					
Blade	0.55	0.45	0.38	0.28	0.17
Stipe	0.68	0.32	0.56	0.20	0.12
Outer cortex	0.75	0.25	0.66	0.16	0.09
Lessonia nigrescens	0.38	0.62	0.19	0.43	0.19
Ecklonia maxima	0.45	0.55	0.22	0.32	0.32
Macrocystis pyrifera	0.39	0.61	0.16	0.38	0.23
Durvillea antarctica	0.29	0.71	0.15	0.57	0.14
Ascophyllum nodosum					
Fruiting body	0.10	0.90	0.04	0.84	0.06
Old tissue	0.36	0.64	0.16	0.44	0.20

Source: ref. [4]

neighbour) frequencies F_{GG}, F_{GM}, F_{MG}, and F_{MM}, and the 8 possible triad frequencies F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}, F_{MMM}, F_{MMG}, F_{GMM} and F_{GMG} can be measured by n.m.r. techniques [5][6]. Knowledge of the diad and triad frequencies then allows calculation of average block-length; $\overline{N}_G = F_G/F_{MG}$, and $\overline{N}_M = F_M/F_{MG}$. For blocks consisting of at least two contiguous units, $\overline{N}_{G>1}=(F_G-F_{MGM})/F_{MGG}$, and $\overline{N}_{m>1}= (F_M-F_{GMG})/F_{MMG}$. Both the composition and block-structure varies in different types of alginates. Commercial alginates are produced mainly from *Laminaria hyperborea*, *Macrocystis pyrifera*, and *Ascophyllum nodosum*, and to a lesser extent from *L. digitata*, *L. japonica*, *Ecklonia maxima* and *Lesonia nigrescens*. The composition and sequential structure may, however, vary with seasonal variations and growing conditions. The highest content of guluronic acid residues is usually found in alginate prepared from stipes of old *L. hyperborea* plants. Table 1 summarizes the composition and some sequential parameters of the most common types of alginates.

Functional Properties

The interest in the sequential structure of alginates comes mainly from the strong correlation between structure and the functional properties which are of both biological and industrial significance. Although the intrinsic inflexibility of the alginate molecules in solution increases in the order MG<MM<GG, the viscosity is mainly a function of the molecular size. In contrast the selectivity for binding of cations and the gel-forming properties varies widely depending on composition and sequence. Divalent cations such as calcium, strontium and barium, bind selectively to the G-blocks in a highly cooperative manner, where the size of the cooperative units is reported to be more than 20 monomers. The characteristic affinities are a property exclusive to polyguluronate, while polymannuronate is almost without selectivity. The affinity of alginates for alkaline earth metals increase in the order $Mg^{2+}<<Ca^{2+}<Sr^{2+}<Ba^{2+}$, a property unique for alginates compared to other polyanions. In the divalent/divalent ion-exchange equilibrium

$$Alg_2Me_I + Me_{II}^{2+} \rightleftharpoons Alg_2Me_{II} + Me_I^{2+}$$

the selectivity coefficient is defined by

$$k_{Me_I^{2+}}^{Me_{II}^{2+}} = \frac{X_{Me_{II}^{2+}} C_{Me_I^{2+}}}{X_{Me_I^{2+}} C_{Me_{II}^{2+}}}$$

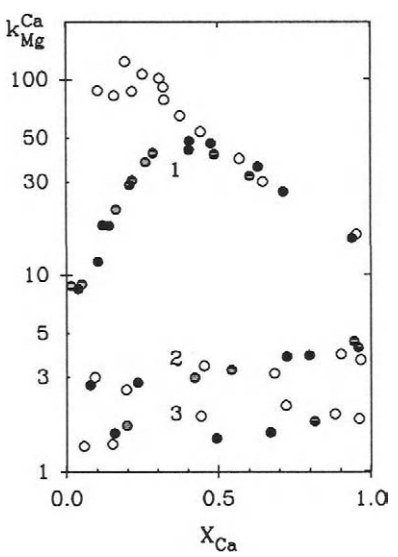

Figure 3 Cooperativity in binding of divalent ions to G-blocks in alginates: Selectivity coefficient as a function of ionic composition in the gel for different alginate fragments. Curve 1: Fragment with 90 % G, DP_n=50; curve 2: "alternating" fragment, 38% G, DP_n= 20; curve 3: fragment with 10% G, DP_n=26. ●: Dialysis of fragments in their sodium form; ○: dialysis first against 0.2M $CaCl_2$, then against a mixture of $CaCl_2$ and $MgCl_2$. Total concentration of $CaCl_2$+$MgCl_2$=0.2M. Data taken from ref. [11].

where $X_{Me^{2+}}$ is the mole fraction of metal ions bound to the alginate ($X_{Me_I^{2+}}+X_{Me_{II}^{2+}}=1$) and $C_{Me_I^{2+}}$ and $C_{Me_{II}^{2+}}$ are the molar concentrations of ions in solution at equilibrium.

From experiments involving equilibrium dialysis of alginate [7][8][9][10][11], it is shown that the selectivity for alkaline earth metals and transition elements increases markedly with increasing content of α-L-guluronate residues in the chains, and that polymannuronate blocks and alternating blocks were almost without selectivity. This is illustrated in figures 3 and 4.

The high selectivity between similar ions as the alkaline earth metals indicates that the mode of binding can not be by non-specific electrostatic binding only, but that some chelation caused by structural features in the G-blocks has to be responsible for the selectivity. This characteristic property is explained by the so-called "egg-box" model [12], based upon the linkage conformations of the guluronate residues (see figure 5). The selectivity of alginates for multivalent cations is also dependent on the ionic composition of the alginate gel, as the affinity towards a specific ion increases with increasing content of the ion in the gel (co-operativity) [10], as seen in figure 3. Thus, a Ca-alginate gel has a markedly higher affinity towards Ca^{2+} ions than has the Na-alginate solution. This has been theoretically explained [10][13] by a near-neighbour auto-cooperative process (Ising model), and it may be physically explained by the entropically unfavourable binding of the first divalent ion between two G-blocks and the more favourable binding of the next ions in the one-dimensional "egg-box" (zipper mechanism).

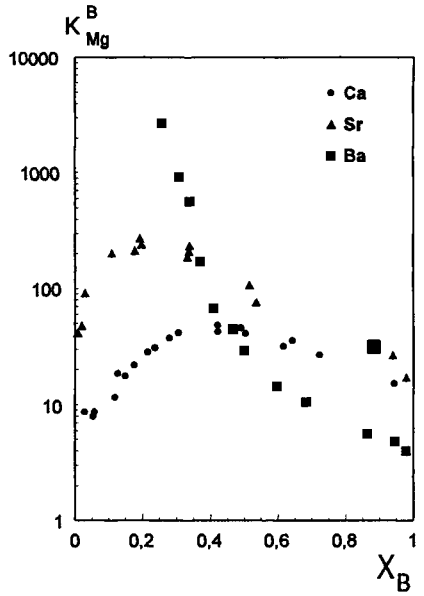

Figure 4 Selectivity coefficient K^B_{Mg} as function of molar fraction X of different earth alkaline ions B for alginate fragments with 90% guluronic acid. (Data taken from ref. [11].

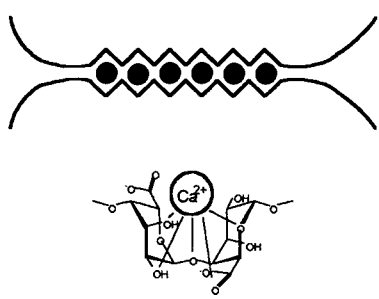

Figure 5 The egg-box model for binding of divalent cations to alginate (adapted from ref. 12) and the probable chelation of ions by the GG sequence.

Gel strength

In general, alginates rich in guluronate residues form strong, brittle gels, while M-rich alginates form softer, more elastic gels. Figure 6 shows the correlation between the average G-block length and compression modulus for Ca-alginate gelbeads. The elastic modulus of an

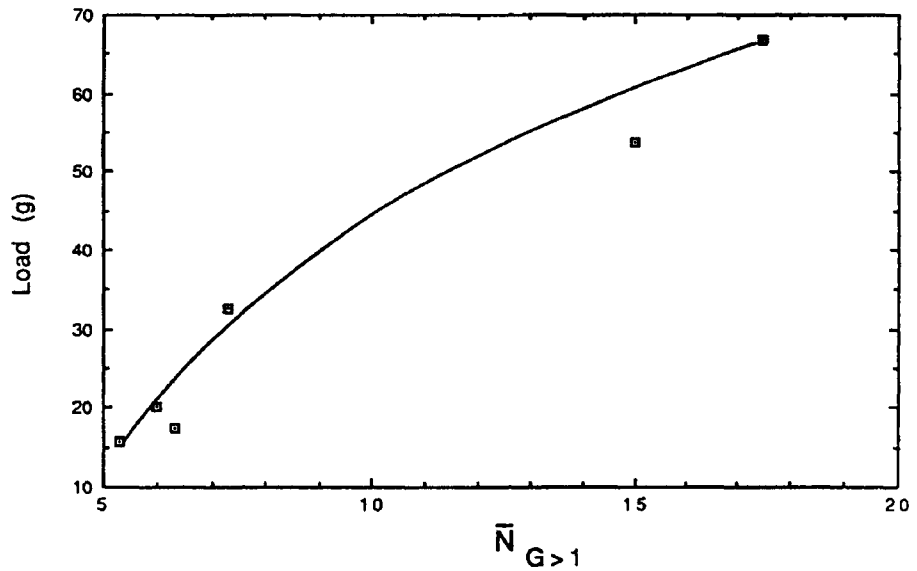

Figure 6 Gel strength of Ca-alginate gel beads (4.5%) versus the average length og G-blocks ($\bar{N}_{G>1}$)

alginate gel must depend on the number and the strength of the crosslinks and as the length and stiffness of the chains between crosslinks. The modulus also depends on the crosslinking ions the gel strength increases with the affinity between polymer and crosslinking cations [10][11][14]. It is however still unclear whether this increase in modulus is caused by a higher strength of the junction zones, or if it is caused by a larger number of junction zones.

Stability of the alginate molecule

For many uses, it is important to be aware of the factors which determine and limit the stability of aqueous alginate solutions and the chemical reactions being responsible for the degradation. The molecular weight of alginate in solution may be severely reduced over a short period of time under conditions favouring degradation. The glycosidic linkages are susceptible to both acid and alkaline degradation and oxidation by free radicals. Because alginates are natural products, they may be digested by many microorganisms. Extracellular alginate lyases capable of breaking the glycosidic linkages by a β-elimination reaction [15], are isolated from different marine and soil bacteria [16].

For alginates in the range of molecular weights giving viscosity of interest as immobilization material, the degree of chain scission, α (=1/DP_n), will increase linearly with time as a pseudo zero order reaction [17]. The rate of this increase will be proportional to the concentration of catalysts like OH^- and H^+ or oxidizing agents like ·OH. Due to the inverse relationship between DP_n and α, any physical property depending directly on molecular weight will be more severely affected by chain breaks the higher the molecular weight of the starting material.

A convenient way to characterize alginate degradation is to present it as a change in 1/[η] with

time. At constant catalytic activity, there is a linear relationship between $1/[\eta]$ and time for an extended period. This is because of the reciprocal relationship between α and the molecular weight and because the exponent of the Mark-Houwink equation is close to unity for alginates. Figure 7 shows $\Delta 1/[\eta]$ after 5 hours in solutions of *L. digitata* alginate at different pH values and at 68 °C [18]. It can be seen that the degradation is at its minimum around neutrality and increases in either direction. The increased instability at pH values less than 5 can be attributed to proton catalysed hydrolysis, whereas the reaction responsible for the degradation at pH 10 and above is the β-alkoxy-elimination. As may be seen from Figure 7, there is also a significant degradation even at pH values around neutrality. Most brown algae contain varying amounts of phenolic compounds which are extracted together with the alginate and are contained as a contamination in most alginates. The degradation effect of phenolic compounds belongs to the class of ORD reactions (Oxidative-Reductive Depolymerization). This effect is a result of auto-oxidation of reducing compounds. This process is favoured by the dissolved oxygen and by the presence of reducing compounds, such as polyphenols. It is thus important to know that the phenolic content of a commercial alginate varies with the algal source from which the alginate is isolated (Highest in *A. nodosum* and lowest in *L. digitata*) and the isolation procedure. It is also possible to remove most of the phenolics with simple purification procedures [19].

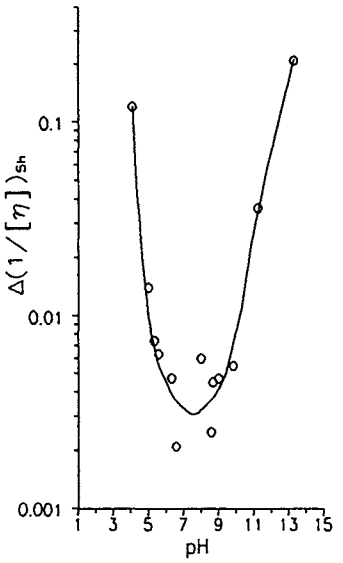

Figure 7 Degradation of *L. digitata* alginate taken as decrease in intrinsic viscosity after 5 hours at different pH. Data taken from ref. [18].

Sterilization

The sterilization of alginate solutions and powder is troublesome; almost any sterilization process causes depolymerization, loss of viscosity and possibly also reduced gel strength. It has been shown [20] that sterilization processes as diverse as heating (autoclaving), ethylene oxide treatment and γ-irradiation all lead to polymer breakdown. Heating of an alginate solution will inevitably enhance polymer breakdown because this process increases the reaction rate of all the depolymerization processes outlined above. For immobilization purposes, we have earlier recommended sterile-filtering rather than autoclaving to preserve the alginate solution's original viscosity [21]. This procedure also removes some contaminants such as proteins and polyphenols [19] and the final solution forms highly transparent beads.

Alginate gel beads

The correlation between molecular weight and molecular weight distribution (MWD), composition and sequense and functional properties of alginate gel beads are summarized in figures 8a) and b). The molecular size will mainly affect the viscosity and hence the filterability.

Below a critical value, depending on the concentration, the gel-forming abilities are reduced. The critical molecular weight depends on the concentration of alginate as should be expected from the theory of polymer coil overlap. Above the critical value the mechanical properties and swelling is governed by the monomeric composition, and block-structure. By increasing the content of G, the gels become mechanically stronger, and with enhanced stability in the presence of anti gelling ions (Na^+, Mg^{2+}) and calcium sequesters. High-G gels exhibit high porosity, low shrinkage during gel-formation, and do not swell after drying [22]. By increasing the M content, the gels become softer and more elastic; they shrink more and their porosity will be reduced. They would swell after drying and are more easy to dissolve in the presence of EDTA or phosphate ions than high-G material. More surprisingly, composition and sequence also have impact on the immunological properties of alginate. We have recently shown that alginate molecules themselves are potent stimulator of cytokine (TNF-α, IL-1 and IL-6) production in exposed monocytes [23].

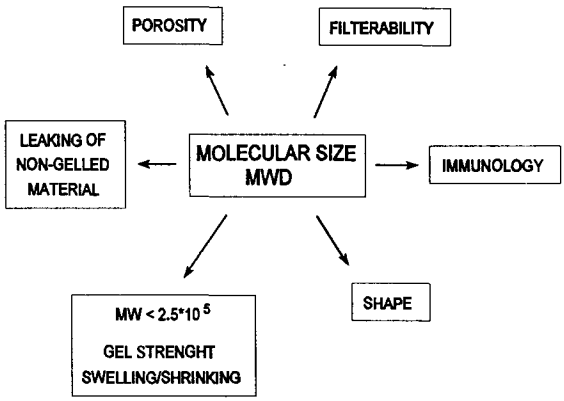

Figure 8a Correlation between molecular size and molecular weight distribution (MWD) and functional properties of alginates.

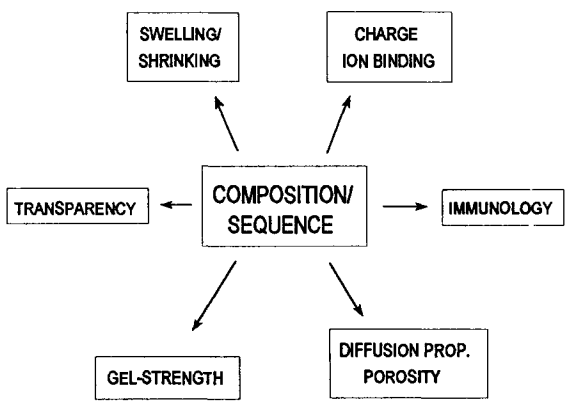

Figure 8b Correlation between composition and sequence and functional properties of alginates.

The size of the beads depends upon factors such as the viscosity of the sodium alginate solution, and the type and concentration of alginate. The size can be controlled easily by applying a coaxial air stream [24] to blow off the droplets. This technique normally produces gel spheres ranging from 700-5000 μm in diameter. Smaller beads (100-300 μm) require more sophisticated equipment where droplets are formed by connecting the syringe to a vibration devise [25], or by electrostatic pulses [26]. Even smaller beads (<5 μm) can be formed by spraying alginate through a nozzle, provided the surface tension in the calcium chloride gelling bath is reduced by adding a surfactant [27].

Chemical stability - swelling

The major limitation of the use of calcium alginate as cell immobilization matrix is its sensitivity towards chelating compounds, such as phosphate, citrate and lactate or non-gelling cations such

as sodium or magnesium ions. Various ways to overcome this have been suggested. The simplest is to keep the gel beads in a medium containing a few millimole per litres of free calcium and to keep the sodium calcium ratio less than 25:1 for high-G alginate and 3:1 for high-M alginate. However, alginate gels can also be stabilized by replacing calcium ions with other divalent cations having a higher affinity for alginate. The affinity series for various divalent cations is: $Pb^{2+}>Cu^{2+}>Cd^{2+}>Ba^{2+}>Sr^{2+}> Ca^{2+}>Co^{2+}$, Ni^{2+}, $Zn^{2+}>Mn^{2+}$.

A common approach for stabilizing alginate gels are covalent crosslinking. Various techniques have been applied, including direct cross-linking of the carboxyl groups, or covalent grafting of alginate with synthetic polymers. This gives gels with improved stability and mechanical strength, but in most cases the coupling conditions are to harsh for fragile cells.

Porosity

Diffusion characteristics are essential for the use of alginate gels as immobilization matrix. It is therefore important to know the pore sizes and the pore size distribution. Self-diffusion of small molecules seems to be very little affected by the alginate gel matrix whereas transport of molecules by convection is restricted by the gel network. The self-diffusion of small molecules such as glucose, ethanol and lactate has been reported to be as high as about 90 % of the diffusion rate in water [28]. The diffusion rate depends, however, on the cell load of the beads. For larger molecules such as proteins diffusional resistance occurs, although even large proteins with $MW>3{\cdot}10^5$ will leak out of the gel beads with rate dependent on their molecular size [22][29]. The highest diffusion rates of proteins, indicating the most open pore structure, are found in beads made from high-G alginates. This may partly be related to the lower shrinkage of these types of gels, but even when this is corrected the highest diffusion rates are found in high-G alginate. A tentative model is given in figure 9.

The porosity of alginate gels has been studied by various techniques including electron-micrograph, gel permeation chromatography and diffusion studies. These studies reveal that the alginate gels core is macro-porous with pores ranging from 5-200nm, while the network on the bead surface is more narrow 5-16nm [30]. Evidence for a non-uniform distribution of polymer in the gel beads will be discussed below. Since the alginate matrix is negatively charged, electrostatic interaction between proteins or any other charged species and alginate must be taken into consideration. Most proteins are negatively charged at pH 7 and will not easily diffuse into the gel matrix. On the other hand, when immobilized in the gel, they tend to leak out more rapidly than should be expected from their free molecular diffusion.

Several of the procedures for stabilizing the alginate gels mentioned above will also have some influence on the porosity. Exchange of calcium with strontium or barium reduces the porosity slightly, but their main effect on porosity is due to stabilization against swelling. Larger effects are achieved by letting the alginate beads react with a polycation. Formation of polyanion-polycation membranes with polypeptides or

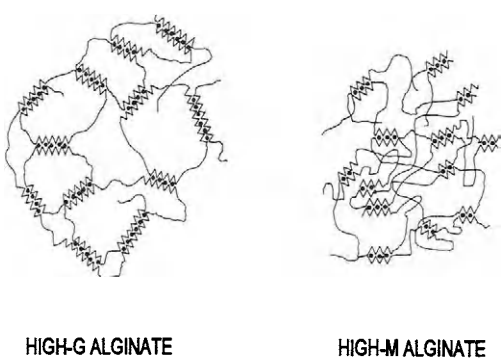

Figure 9 Tentative model of alginate gel network in gels made from alginates with different composition and sequense.

chitosan has been used for generating certain cut off values for proteins. By controlling the molecular weight of the polycations certain pore-sizes below certain cut-off values have been obtained [31]. The influence of the polycation on the pore-size distribution is, however, not clear.

The porosity of calcium alginate gel may significantly be reduced by partially drying of the beads. Provided the beads are made out of an alginate rich in guluronic acid, the beads will reswell only slightly in water, and the increased alginate concentration will reduce the average pore size.

Gelling kinetics

Alginate gels are often regarded as non-equilibrium gels. After a cation has induced random dimerization of G-blocks, the resulting egg-box structure does not necessarily correspond to the most stable conformation. Because of the high activation energy of reopening, the structure is kinetically trapped in the initial conformation. As a result, one can see quite a dramatic difference in gel strength between Nature's own gels in the algae and the artificially prepared gels [13]. Most probably, a large degree of the G-blocks do not overlap to form the energetically most favourable and strongest network structure in ordinary laboratory or industrially prepared gels. Also laboratory produced gels made from the same alginate material, varying only the gelling kinetics can give dramatic differences in functional properties. A good example is the formation of inhomogeneous gels.

Gel homogeneity

Alginate gels prepared by the dialysis method often exhibit a concentration inhomogeneity in that the polymer concentration is considerably lower in the centre of the gel than at the edges. When divalent metal ions diffuse into an alginate solution, the rapid ion-binding and formation of network produce an inwardly moving gelling zone. Alginate will diffuse from the centre of the gel towards this gelling zone, leading to a depletion of alginate in the centre. A theory providing a qualitative explanation of experimental data may be found in the literature [32]. The polymer gradient is essentially governed by the relative diffusion rate between the soluble alginate

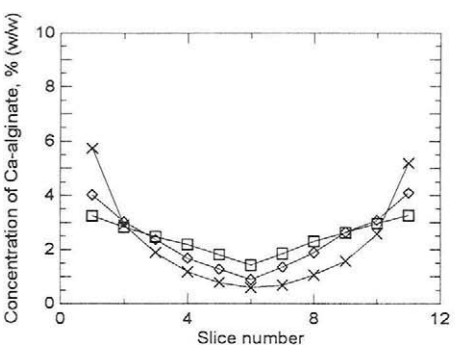

Figure 10a Concentration of alginate as function of slice number for alginate gel sylinders made from high-G alginate gelled in $CaCl_2$ of various concentrations. ×: 0.02M, ◇: 0.05M and □: 0.1M.

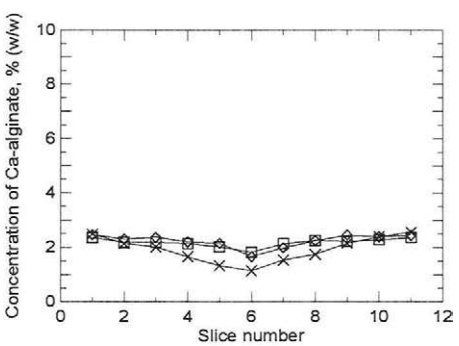

Figure 10b Concentration of alginate as function of slice number for alginate gel sylinders made from high-G alginate gelled in a mixture of 0.45% NaCl and $CaCl_2$ of various concentrations; ×: 0.02M, ◇: 0.05M and □: 0.1M.

molecules and calcium ions. The homogeneity of the gel can thus be controlled by a careful selection of molecular weight and concentration of alginate together with the concentration of gelling and absence non-gelling ions. In general, low molecular weight alginate, low concentration of gelling ions and non-gelling ions give the highest inhomogeneity. This is illustrated in figure 10 a) and b). Homogeneous alginate gels can also be made by internal release of calcium from Ca:EDTA or from Ca:Citrate or Ca $(CO_3)_2$ in the presence of a slow acidifier like glucono-δ-lactone (GDL) [33].

Conclusions

Cells entrapped in phycocolloid gels have many potential applications in Biotechnology, ranging from biocatalysts in fermentation to artificial seeds in agriculture and carrier materials for transplantation of living tissue. However, since the gelling material is a heterogeneous group of polymers, with diverse functional properties, their success as immobilization matrices will depend on a proper choice of material and methodology for each application. In addition to seaweeds as raw material for alginates of widely different chemical composition, one should bear in mind that alginates are also microbial polysaccharides with a well understood biosynthetic pathway. Both future production of special alginate qualities by fermentation is possible, as well as enzymatic modification of seaweed alginates to give tailor-made alginates for certain applications. The genes coding for the mannuronan C-5 epimerases in *Azotobacter vinelandii* have recently been sequenced and cloned in our laboratory [34][35]. These enzymes convert M to G in the polymer chain. This opens up the possibilities for large production of the enzyme and future use in modulating the functional properties of alginates.

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Immobilized Cells: Basics and Applications

Determination of biofilm diffusion coefficients using micro-electrodes

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Introduction

The structure of a biofilm imposes a diffusive resistance for the transport of metabolites. As a consequence, concentration profiles will develop which affect the local microbial reaction rate. Eventually, this leads to severe mass transfer limitations and results in less effective, partially penetrated biofilms. Therefore, characterization of the mass transfer properties of biofilms is considered essential for modelling and scale-up of microbial conversions [1]. Except for biofilms with a very open and loose structure, the mass transfer inside a biofilm is predominantly diffusive and can be characterized with a single parameter: an effective diffusion coefficient.

Various experimental approaches may be used to obtain a biofilm diffusion coefficient. If the conversion rate is known at different substrate concentrations, the diffusion coefficient can be calculated using a diffusion-reaction model. The results of such experiments display large differences, ascribed to biofilm inhomogenities and unknown local kinetics [2].

Knowledge of the local reaction rate is not required if the flux or the transient uptake rate of a non-reacting compound is measured. To this end the biofilm must be inactivated or a non-consumable tracer has to be used. Both methods exhibit drawbacks; the inactivation procedure might alter the biofilm properties, while the resemblance of a non-consumable tracer with relevant metabolites is often disputable. Literature values show considerable variation, connected to these experimental difficulties [3].

The aim of this experimental work was to characterize the influence of the bacterial fraction on the biofilm diffusion coefficient. Experiments with oxygen and glucose were performed in well defined model systems to circumvent the experimental problems mentioned above. An artificial biofilm consisting of agar, containing inert polystyrene particles of the same size as bacteria, was investigated and the obstruction effect of these particles was compared to that exerted by immobilized bacteria. Diffusion experiments were performed with a micro-electrode positioned in the middle of a spherical biofilm and the transient response on a concentration step in the well mixed bulk was monitored. This technique also enabled the measurement of diffusion in active biofilms. Steady-state experiments were performed in a classical diffusion cell. From the results obtained, the permeability of the bacteria was estimated and influences of the inactivation method applied were quantified.

Theory

In a biofilm, the cells are connected to a polymer matrix and surrounded by a continuous liquid phase with the same properties as the bulk liquid. If the bacteria and the polymers are considered impermeable they form solid obstacles (volume fraction ϕ) which affect diffusive transport of metabolites in two ways. The available liquid volume is reduced to a fraction $(1-\phi)$ by exclusion. Furthermore, the pathlength of the diffusive molecules is increased by steric hindrance, referred to as obstruction or tortuosity.

In the literature there is some confusion about the diffusion coefficient measured by different methods. Using a classical diffusion cell, the (pseudo) steady-state flux through a flat film is measured which is determined by both the tortuosity and the porosity. Consequently, the effective diffusion coefficient (D_{eff}) is measured. In case of non steady-state methods like, for example, the transient uptake rate of a quantified amount of gelbeads, the response on a stepwise concentration change in the bulk is monitored. The diffusion coefficient is calculated from the pace at which the new equilibrium is reached. As this depends on the tortuosity only, a transient diffusion coefficient (D) is obtained which equals $D_{eff}/(1-\phi)$. If the solid phase is permeable, the difference between the two methods is more complex [4].

Several models have been developed to relate the effective diffusion coefficient to the structural parameters of the heterogeneous medium. For a solid phase consisting of a suspension of impermeable spheres, homogeneously distributed in a continuous liquid phase (diffusion coefficient D_{aq}) and steady-state conditions, Maxwell deduced [5]:

$$D_{eff} = \frac{(1-\phi)}{(1+\phi/2)} \cdot D_{aq} \tag{1}$$

This model was verified by experiments and Monte-Carlo simulations [6]. In case of permeable objects, with a diffusion coefficient D_s, this formula is extended to:

$$D_{eff} = \frac{2/mD_s + 1/D_{aq} - 2\phi(1/mD_s - 1/D_{aq})}{2/mD_s + 1/D_{aq} + \phi(1/mD_s - 1/D_{aq})} \cdot D_{aq} \tag{2}$$

with m as the partition coefficient of the tracer component between the solid and the liquid phase.

For a fine polymer structure with a chain thickness comparable to the molecular jump distance, Mackie and Meares found from statistical considerations [7]:

$$D_{eff} = \frac{(1-\phi)^3}{(1+\phi)^2} \cdot D_{aq} \tag{3}$$

Their experimental data on electrolyte diffusion in a resin membrane correlated suprisingly well with the model predictions. The equation is applicable only, if the molecular diameter is small compared to the average distance in the polymer network.

Experimental

Organism and culture conditions. *Micrococcus luteus* (ATCC 4698) was cultivated in a shake flask at 30°C with a rich medium (pH 7) containing: 50.5 mM glucose, 85.6 mM NaCl, 5 mM K_2HPO_4, 0.5 % w/w yeast extract and 0.5 % w/w peptone. The bacteria were harvested after two days growing.

Immobilization procedure. The cell suspension was centrifuged at 11,300 g and 5°C, during 10 minutes. The cells were washed with a 50 mM K_2HPO_4 buffer (pH 7). The dry weight of a cell suspension was determined after centrifuging (10 min, 11.300 g) and drying (12 h, 100°C) and the cell volume was calculated assuming 0.2 gr dw/ml bacteria [8].

Agar (4 % w/v; Merck, extra pure) was mixed with the same buffer and sterilized at 120°C for 2 minutes. The agar solution and the diluted bacterial suspension were vigorously mixed at 50°C. The liquid agar/bacteria mixture was trickled in paraffin oil of 4°C to obtain spherical beads with a diameter of 3-4 mm. Flat films were made by pouring the agar in a horizontal mould (6.0×10^{-2} m diameter) and cooling with ice.

Inactivation procedure. The immobilized bacteria were inactivated by incubating the agar beads or slabs, during 15 h, in 50 mM phosphate buffer (pH 7) with 0.2 % w/v $HgCl_2$. Thereafter, the beads were washed (2×2 h.)with buffer without $HgCl_2$.

Artificial biofilm. Monodisperse polystyrene beads (5 w/v %) with a diameter of 0.9 μm, suspended in a 1:1 ethanol/water mixture (kindly donated by Dr. Eshuis from Eindhoven University of Technology) were concentrated by sedimentation. Under vigorous stirring, the agar was added directly to the polystyrene-water-ethanol mixture and heated slowly to 98 °C. After evaporation of the ethanol and dissolving of the agar, the mixture was cooled to 55 °C and treated as above.

Micro-electrodes. The glucose electrode consisted of a glucose oxidase coated platinum micro-electrode (tip diameter 10 μm). Preparation and use are described elsewhere [9]. The sensitivity was 50 pA/mmol at 30 °C and the response time (t_{90}) was less than 5 s. The combined oxygen electrode (tip diameter 8 μm) contained a glass coated platinum wire and a reference electrode and has been described by Revsbech and Ward [10]. The sensitivity was 2 nA/mmol at 30 °C and the response time (t_{90}) was less than 2 s.

Transient diffusion measurements. Micro-electrode measurements were performed in a rectangular vessel, filled with 1×10^{-4} m^3 50 mM phosphate buffer (pH 7). The micro-electrode was positioned in the centre of a fixed, submerged gelbead with a motor-driven micro-manipulator. Mixing was accomplished by bubbling air, nitrogen or oxygen through the solution (8.3×10^{-6} m^3/s); while the temperature of the solution was kept at 30.0°C. The experimental set-up was placed in a Faraday cage to reduce electromagnetic interference from the surroundings. The response in the middle of the bead, after a stepwise concentration change in the bulk liquid, was recorded. For glucose, the concentration was increased from 0 mM to 1 mM, while the oxygen concentration step was always taken larger than 0.2 mM. The diffusion coefficients were obtained

by fitting the experimental curve with the solution of the partial differential equation describing diffusion in a sphere [11].

Diffusion cell. The flux of glucose and oxygen through a biofilm was measured in a classical diffusion cell consisting of two well-mixed compartments (60×10^{-6} m^3), separated by a flat slab with a thickness d of 2 mm. The cell was completely surrounded by a water jacket and kept at 30.0°C. Initially both compartments were filled with 50 mM phosphate buffer (pH 7). An experiment was started after a concentration change in one of the compartments. After a Fourier time of $D_{eff} t/d^2 = 0.85$ (pseudo) steady-state was established and the concentration change in one compartment was monitored. The concentration in the other compartment was calculated from a mass balance (glucose) or assumed constant (oxygen). The diffusion coefficient was determined from the time dependent concentration difference according to Axelsson et al. [12].

For a glucose experiment the concentration in one compartment was changed to 0.03M and 8 samples of 0.25×10^{-3} m^{-3} were taken with 15 min intervals. The concentration was measured spectrofotometrically with a commercial enzyme kit (SIGMA diagnostics). For an oxygen experiment, the cell was filled with nitrogen saturated buffer and the experiment was started with aeration of one compartment. The oxygen concentration in the other compartment was monitored with a macro-electrode.

Partition coefficients. The partition coefficient of oxygen between the liquid phase and the bacterial fraction or the polystyrene fraction were determined in an 11 ml vessel at 30.0°C. Agar beads or membrane pieces of $3 \times 3 \times 2$ mm^3 with a total volume of 3 ml and a known volume of bacteria or polystyrene was mixed with 5 ml buffer and flushed with nitrogen gas. Then the vessel was filled completely with 3 ml air saturated buffer and sealed. After equilibrium was reached the oxygen concentration was measured with a macro-electrode and the partition coefficient was calculated. The glucose partition coefficients were measured similarly; concentrations were determined enzymatically.

Results

The influence of the polymer matrix, *i.e.* agar, on the diffusion coefficient was determined with micro-electrodes and a typical experiment is given in Fig 1. The diffusion coefficients of oxygen and glucose decreased with increasing agar contents (Fig. 2). The variance of the oxygen and glucose measurements was 3% and 5%, respectively, expressed as a 95% confidence interval. The density of the agar matrix was established at 1400 kg/m^3 and was used to compare the results with the model of Mackie and Meares.

The obstruction effect of non-reactive particles of the same size as bacteria was investigated in agar gels (1.5% w/v) containing different fractions of polystyrene (Fig. 3). The decrease of the diffusion coefficient of glucose with an increasing fraction of polystyrene was in good accordance with the model of Maxwell, while

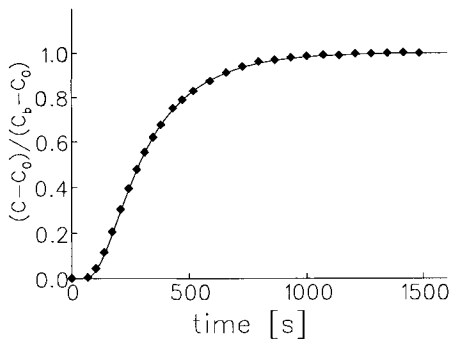

Fig. 1. *Oxygen response measured in the centre of a gelbead (agar 2% w/v, radius 2.252 mm). Solid line: theoretical relation with* $D = 2.45 \times 10^{-9}\ m^2 s^{-1}$.

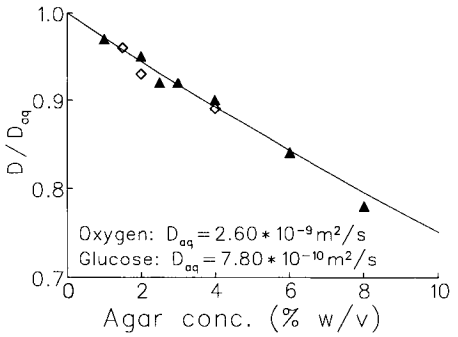

Fig. 2. *Normalized diffusion coefficients for glucose* [◊] *and oxygen* [▲] *determined with micro-electrodes; drawn line: eq. 3.*

the decrease of the diffusion coefficient of oxygen was much larger than predicted. The partition coefficient of oxygen between the liquid phase and polystyrene appeared to be high (m=6), this uptake was reversible. Polystyrene was found to be impermeable for glucose (m=0). Due to the uptake of oxygen in the polystyrene a larger amount of oxygen has to diffuse in the gelbead before equilibrium is reached. Subsequently, the transient response is delayed. The apparent diffusion coefficient obtained (D_{app}) can be related to the effective diffusion coefficient after a proper extension of the microscopic mass balance and assuming instantaneous local equilibrium:

$$D_{app} = \frac{D_{eff}}{1 + (m-1)\cdot\phi} \tag{4}$$

The obstruction effect of real bacteria was investigated in agar, containing different fractions *M. luteus*. Diffusion coefficients in active biofilms can be measured directly with micro-electrodes, if the substrate concentrations are sufficiently high. Then zero-order activity will prevail in the entire bead and external concentration steps are transmitted unattenuated into the bead. The Monod constant (K_m) of *M. luteus* for glucose amounted to 0.33 mM, concentration dependent activity for oxygen was not observed (K_m«0.05 mM). Only the diffusion of oxygen in active biofilms was measured, as the glucose electrode was just linear upto 4 mM [9]. The results were compared to glucose and oxygen measurements in inactivated

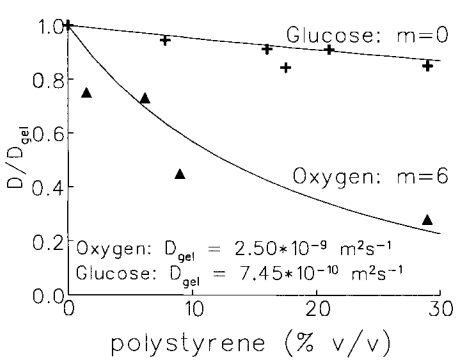

Fig. 3. *Glucose* [+] *and oxygen* [▲] *diffusion compared to eq. 1, adapted with the partition coefficient* m *and eq. 4.*

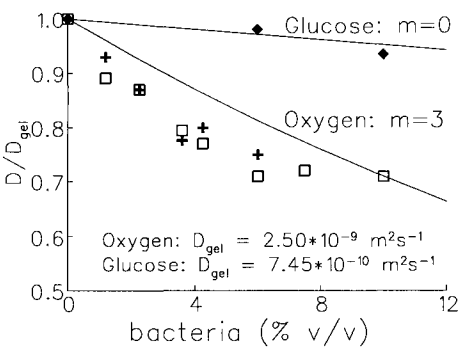

Fig. 4. *Oxygen diffusion in agar containing active bacteria (+), compared to diffusion of glucose (◆) and oxygen (□) in agar, containing inactivated bacteria.*

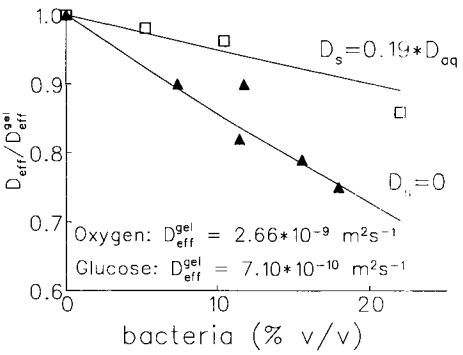

Fig. 5. *Normalized diffusion coefficients for glucose (▲) and oxygen (□) measured in the diffusion cell; drawn lines: eq.2.*

biofilms (Fig. 4).

Again, the decrease of the diffusion coefficients of oxygen and glucose showed a marked difference. Measurements of the partition coefficients between the liquid phase and inactivated bacteria revealed that oxygen dissolved (m=3) into the biomass. Just as with polystyrene, the bacteria were found to be impermeable for glucose (m=0).

Additional diffusion measurements with glucose and oxygen were performed in a diffusion cell (Fig. 5). The decrease of the effective diffusion coefficient of oxygen with increasing bacterial fraction was smaller than the one found for glucose. From these results and the established partition coefficient, the diffusion coefficient of oxygen in the bacteria was calculated with eq. 2 and amounted to 5×10^{-10} m^{2}s^{-1}.

Discussion

The diffusion coefficient in spherical particles could be determined accurately using micro-electrodes that measured the transient response inside gelbeads. In traditional transient techniques, the concentration change in the bulk liquid is monitored. This requires a high volume ratio gelbeads and liquid, otherwise the concentration differences are too small. However, large amounts of gel particles hinder an effective mixing which results in a considerable external mass transfer resistance [13]. The transient micro-electrode technique does not exhibit such disadvantages. Mixing is accomplished easily, as for an experiment only one particle is needed. The concentration change in the gel particle is directly proportional to the concentration step applied in the bulk. Furthermore, a batch of particles with a known diameter distribution is not required, as the dimension