ELSEVIER

Enzyme and Microbial Technology 27 (2000) 319–324

ENZYME and MICROBIAL TECHNOLOGY

www.elsevier.com/locate/enzmictec

Glucose oxidase release from calcium alginate gel capsules

Ana Blandino*, Manuel Macías, Domingo Cantero

Biological and Enzymatic Reactors Research Group, Department of Chemical Engineering, Food Technology and Environmental Technologies, Faculty of Sciences, University of Cádiz (UCA), 11510 Puerto Real (Cádiz), Spain

Received 2 December 1999; received in revised form 15 February 2000; accepted 15 March 2000

Abstract

Diffusion of glucose oxidase within calcium alginate gel capsules has been assayed and the experimental data fitted to a simple semi-empirical power equation, which is used to analyse the solute release from polymeric devices. It was found that an increase in the concentration of sodium alginate and calcium chloride gives rise to a reduction in the enzyme leakage. This was verified when glucose oxidase (GOD) diffusion percentages were compared in capsules with thicknesses of the same order of magnitude but obtained under different experimental conditions. So, the use of sodium alginate and calcium chloride solutions of concentrations 0.5% w/v and 2.6% w/v, respectively, lead to a diffusion percentage of 25 ± 2. This percentage was reduced to 8 ± 3 when sodium alginate and calcium chloride concentrations were fixed at 1% w/v and 4% w/v, respectively, even though the thicknesses of the capsules were of the same order of magnitude. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Calcium alginate gel capsules; Enzyme diffusion; Gelling condition; Glucose oxidase

1. Introduction

Industrial development of enzymatic reactors requires the use of immobilized enzymes in cases in which the cost of the biocatalyst is high. To a large extent this procedure prevents enzyme losses due to washout and, at the same time, maintains high concentrations of the biocatalyst [1]. It is well known that the effective immobilization of biocatalysts can be achieved using several techniques, one of which is encapsulation within a gel matrix. This immobilization technique consists of enclosing the biocatalyst, in an aqueous solution, inside a semipermeable membrane capsule [2]. There are two main advantages inherent in this immobilization method: the particle structure allows contact between the substrate and biocatalyst to be achieved in an appropriate way and, in addition, it is possible to immobilize several enzymes at the same time [3].

Encapsulation in calcium alginate gels is characterised by the very mild conditions in which the immobilization procedure is carried out and by its low cost and ease of use [4]. Moreover, by changing the gelation conditions it is possible to control easily some of the capsule characteristics, such as the thickness [5–7] and permeability to different substrates of the gel membrane [8–12]. However, several disadvantages are also associated with this method, namely the low stability (affected by substances such as citrate, lactate, and phosphate) and the high porosity of the membrane [13,14]. The latter characteristic limits the application of calcium alginate gel capsules to high molecular weight compounds and whole cells or organelles [15–18]. As regards this problem, pore size is a critical parameter in selecting a matrix for a particular enzymatic immobilization process. Low molecular weight substrates and products can easily diffuse into or out of a matrix with large pores and large pores can also cause problems by allowing the immobilized enzyme to leak out. However, there are very few studies that take into consideration the enzyme leakage and practically none that discuss this phenomenon in terms of diffusion in porous materials [19–21]. In this context, the main objective of this work was to study the diffusion of an enzyme of high molecular weight out of calcium alginate gel capsules, obtained under different experimental conditions (i.e. at various sodium alginate and calcium chloride concentrations). Based on the results obtained, the diffusional properties of calcium alginate gel capsules are discussed in relation to the structure of the gel matrix. In

* Corresponding author. Tel.: 0034-956-83-0907; fax: 0034-956-83-7565.

0141-0229/00/$ – see front matter © 2000 Elsevier Science Inc. All rights reserved.
PII: S0141-0229(00)00204-0

addition, the experimental data for enzyme leakage were adjusted to a semiempirical equation, derived by Peppas et al., that describes the solute release from polymeric devices [22,23]. Glucose oxidase (GOD) was chosen as a model enzyme in this study.

2. Materials and methods

2.1. Experimental methods

2.1.1. Materials Materials

Alginic acid salt, obtained from brown algae (*Laminaria hyperborea*), was purchased from Fluka BioChemika (Fluka art. n° 71238, Switzerland). A medium viscosity sodium salt of carboxymethylcellulose (CMC) was obtained from Fluka BioChemika (Fluka art. n° 21902). Anhydrous calcium chloride (purissimum grade) was used as the calcium salt for capsule formation (Panreac art. n° 141219, Spain).

Glucose oxidase (β-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4.) Type X-S from *Aspergillus niger*, 128 000 U/mg solid (69% protein), was purchased from the Sigma Chemical Company (St. Louis, MO, USA, G 7141). Its molecular weight was 152 000 Da.

Calcium acetate 1-hydrate (for analysis) was used for the diffusion assays (Panreac art. 121211, Spain).

Lowry Reagent, modified for use in the determination of total protein concentration, was purchased from the Sigma Chemical Company (SIGMA, P 5656).

2.1.2. Enzyme encapsulation

Calcium alginate capsules were prepared by extrusion using a simple one-step process similar to that described by Nigam et al. [2]. Several sodium alginate solutions of different concentrations were prepared, as appropriate for the particular experiment to be performed, and were used as anionic solutions. For the preparation of the cationic solutions, CMC was dissolved in different solutions of $CaCl_2$ to give a 3% w/v CMC solution. CMC was used as a non-gelling polymer to modulate the viscosity and density of cationic solutions to ensure the spherical shape of the capsules. The enzyme was dissolved in the cationic solution to give a final concentration of 55 μg/ml.

Droplets of the cationic solution (10–20 ml of $CaCl_2$/CMC/GOD solution, depending on the number of capsules to be obtained) were dropped through a silicone tube, using a peristaltic pump, into 200 ml of sodium alginate solution. The sodium alginate solution was maintained under constant stirring (330 rev./min), using a magnetic stirrer situated at the bottom of the vessel, in order to keep the droplets from sticking together and to minimise the external mass transfer resistance. A dropping height of 10 cm was used to ensure that spherical droplets were formed. The inner diameter of the silicon tube was 1.6 mm. The total dropping time was kept lower than 1% of the residence time of the capsules in the anionic solution in order to ensure that capsules were all formed over the same period.

Once the $CaCl_2$/CMC/GOD solution had been dropped into the alginate solution, a capsular membrane formed instantaneously around each droplet due to the cross-linking of the interfacial alginate molecules by calcium cations. The gelation time, or period in which capsules were formed, was one hour in all cases. This gelation time was selected because no significant changes were observed in the membrane thickness when longer gelation times were employed, indicating complete utilisation of calcium for the cross-linking of alginate.

Before the removal of capsules the sodium alginate solution was diluted more than four-fold by adding the appropriate amount of distilled water. This dilution of the alginate solution outside the capsules reduces the possibility of capsules sticking together when they are in close contact and also helps to stop the gelation process.

After diluting the sodium alginate solution, the capsules were filtered off using a Büchner funnel. With the aim of stabilising the calcium alginate membrane, the capsules were immediately transferred to a 1.3% w/v calcium chloride solution and incubated for 15 min. Finally, the capsules were rinsed with distilled water to remove excess calcium chloride. All of the above procedures were carried out at 25°C.

Ten of the capsules were separated from the rest to perform the external diameter and membrane thickness measurements. The remainder of the capsules were used for the diffusion and encapsulation efficiency assays.

2.1.3. Diameter and membrane thickness measurements

Measurement of the external diameter of the capsules was carried out by using a caliper after the surface of the capsules had been dried with filter paper. The membrane thickness was studied by cutting the capsules in half and carrying out measurements in at least four different locations on the membrane. Use of the image processing software MIP 4 ADVANCED allowed us to measure the membrane thickness on an image of each half capsule, captured by a video camera connected to a microscope. Calibration of the system was carried out using the same software on an image of a Neubauer chamber of 0.0025 mm^2.

2.1.4. Diffusion assay

To assess glucose oxidase diffusion out of the calcium alginate capsules, a number of capsules were placed in a 100 ml Erlenmeyer flask with 50 ml of 50 mM calcium acetate buffer (pH 5.1). The number of capsules employed for these experiments was selected by maintaining a constant relationship between the total volume of all the capsules and the volume of buffer solution. The buffer solution was agitated at 600 rev./min with a Teflon bar on a magnetic stirrer. It was confirmed from preliminary experiments that the liquid film resistance around the capsule could be neglected at the

stirrer speed employed. The temperature was kept constant at 25°C.

The percentage of diffusion of glucose oxidase was calculated from the measurement of protein concentration within the core of the capsules both before and after the diffusion experiments. These measurements were carried out by placing five capsules in five test tubes, one per test tube, along with 2 ml of buffer solution in each. The capsules were then cut in half in order to dissolve their contents. The protein concentration in each sample was assessed by the Lowry technique, as modified by Peterson [24], and the mean percentage of diffusion calculated.

$$percentage\ of\ diffusion = \left(1 - \frac{[GOD]_{core,t=22h}}{[GOD]_{core,t=0h}}\right) \cdot 100 \quad (1)$$

Moreover, samples of 1 ml were periodically taken from the well-stirred buffer solution in order to analyse the protein diffusion profile, replacing the liquid removed during sampling. Each protein concentration determination was carried out in triplicate. Diffusion of glucose oxidase from the capsules was analysed during 22 h of the process.

2.1.5. Enzyme encapsulation efficiency

To assess the enzyme encapsulation efficiency, it was necessary to measure the protein concentration in the cationic solution and within the core of the capsules before the diffusion experiments. Measurements of protein concentration in the cationic solution were carried out by adding five droplets of this solution to five test tubes, one drop per test tube, each containing 2 ml of buffer solution. The protein concentration in each sample was assayed. The protein concentration within the core of the capsule was determined according to the method described above.

$$encapsulation\ efficiency = 100 \cdot \frac{[GOD]_{core,t=0h}}{[GOD]_{drop}} \quad (2)$$

2.2. Mathematical model

The data for the enzyme concentration measured in the well-stirred buffer solution were adjusted to a simple semi-empirical power equation [23]. This equation can be used to analyze the solute release from any polymeric device irrespective of its geometric shape. According to this equation, the solute release from polymeric devices can be written in terms of the Fickian diffusional release, which occurs by the usual molecular diffusion of the solute due to a chemical potential gradient, and the relaxational release, which is due to the swelling in water of hydrophilic polymers:

$$\frac{M_t}{M_\infty} = k_1 \cdot t^m + k_2 \cdot t^{2m} \quad (3)$$

In this equation the first term on the right-hand side is the Fickian contribution and the second term is the relaxational contribution; M_t/M_∞ is the fractional release of the solute; t is the release time; k_1 and k_2 are two kinetic constants with units of t^{-m} and t^{-2m}, respectively, and m is the diffusional exponent for solute release, which typically varies from 0.43 to 0.5 depending on the geometry of the device. Eq. (3) is a short-time approximation and is only valid when $M_t/M_\infty < 0.60$.

The values of the two kinetic constants (k_1 and k_2) were determined by a nonlinear regression method based on the Marquardt algorithm [25]. The coefficient m was determined graphically from the aspect ratio diameter/thickness of the capsules [23]. The total amount of enzyme in the solution after infinite time, M_∞, was determined according to the equation:

$$M_\infty = \frac{N\frac{4\pi R_e^3}{3} c_o V_L}{V_L + N\frac{4\pi R_e^3}{3}} \quad (4)$$

where R_e is the radius of the capsule, c_o is the initial enzyme concentration in the capsule, N is the number of capsules and V_L is the volume of the solution excluding the space occupied by capsules.

3. Results and discussion

3.1. Effect of gelation conditions on the percentage of diffusion of GOD

The results obtained, which demonstrate the influence of sodium alginate concentration and calcium chloride concentration on the capsule characteristics and on the percentage of diffusion of GOD, are represented in Table 1. It can be seen, that on increasing the sodium alginate concentration of the solutions used for capsule formation, the thickness of the membrane and the diameter decrease for a given calcium chloride concentration. On the other hand, on increasing the calcium chloride concentration the thickness and diameter of the capsules increase for a given sodium alginate concentration. However, the core diameter—obtained from the external diameter and membrane thickness of the capsule—was always of the same order of magnitude (5.4 $\pm$ 0.1) and was independent of the operational conditions (data not reported).

On the other hand, the use of concentrated calcium chloride solutions reduces significantly the percentage of GOD that diffuses out of the capsules. So, irrespective of the sodium alginate concentration assayed, the use of 1.3% w/v $CaCl_2$ solutions causes an enzyme leakage of around 40%; however, this percentage is considerably reduced when 4% w/v $CaCl_2$ solutions are used. In the same way, the concentration of the biopolymer solution affects the diffusion of the enzyme. It can be observed that the main restrictions to the enzyme flux occur when high concentration solutions of the bipolymer are employed.

Table 1
External diameter of capsules, thickness of gel layer capsules, encapsulation efficiency and percentage of diffusion of GOD from the capsules obtained under different gelation conditions

	[Sodium alginate] 0.5% w/v		
	$[CaCl_2]$		
	1.3% w/v	2.6% w/v	4.0% w/v
Diameter (mm)	6.4 ± 0.1	7.4 ± 0.1	8.4 ± 0.2
Thickness (mm)	0.39 ± 0.01	1.03 ± 0.03	1.50 ± 0.04
Encapsulation efficiency (%)	94 ± 4	87 ± 4	87 ± 4
Diffusion (%)	45 ± 4	25 ± 2	21 ± 5

	[Sodium alginate] 0.75% w/v		
	$[CaCl_2]$		
	1.3% w/v	2.6% w/v	4.0% w/v
Diameter (mm)	6.1 ± 0.2	7.1 ± 0.1	7.7 ± 0.05
Thickness (mm)	0.34 ± 0.01	0.86 ± 0.07	1.26 ± 0.02
Encapsulation efficiency (%)	68 ± 3	91 ± 3	85 ± 5
Diffusion (%)	49 ± 4	29 ± 3	20 ± 5

	[Sodium alginate] 1% w/v			
	$[CaCl_2]$			
	1.3% w/v	2.6% w/v	4.0% w/v	5.5% w/v
Diameter (mm)	6.0 ± 0.1	6.8 ± 0.1	7.4 ± 0.05	7.8 ± 0.05
Thickness (mm)	0.24 ± 0.01	0.61 ± 0.01	0.98 ± 0.02	1.15 ± 0.02
Encapsulation efficiency (%)	70 ± 2	71 ± 5	85 ± 4	95 ± 4
Diffusion (%)	39 ± 4	33 ± 5	8 ± 3	4 ± 2

Statistics: $\alpha = 0.05$; $n = 10$ for measurements of diameter and thickness of capsules; $n = 5$ for measurements of encapsulation efficiency and percentage of diffusion of GOD.

The enzyme encapsulation efficiency, i.e. the percentage of enzyme contained within the core capsule in relation to the initial amount employed for the capsule formation, was higher than 70% under all the experimental conditions tested.

All these results can be explained by taking into consideration the gel formation process, which is assumed to be an almost instantaneous and irreversible process that is governed by the diffusion of the two components involved in it: sodium alginate and Ca^{2+} ions. In this respect, the fact that the metallic cation has a smaller size than the polymer molecules means that it is mainly the cation that diffuses between the alginate chains, binding to unoccupied binding sites on the polymer. Thus, once the cationic solution is dropped into the alginate solution, a capsular membrane forms instantaneously around the droplet. This membrane will then grow from the flux direction of the Ca^{2+} ions. This explains the fact that the diameter of the core capsules remains constant in all the assays performed. When the mass of the calcium ions contained within the core capsule is exhausted, the gelation process finishes.

At stated previously, on increasing the sodium alginate concentration of the solutions used in the capsule formation, the thickness of the membrane decreases for a given calcium chloride concentration. This effect is presumably due to the fact that on increasing the number of biopolymer molecules per unit solution volume, in the vicinity of the core capsule, the number of binding sites for Ca^{2+} ions also increases. As a result, a more densely cross-linked gel structure will probably form and, consequently, it will have a lower thickness. This would explain the fact that the percentage of enzyme diffusion decreases with increasing alginate concentration, for a given calcium chloride concentration, due to the dense membrane in the capsules. In connection with this phenomenon, we observed qualitatively that the capsules obtained form 1.0% w/v sodium alginate solutions were more resistant, from a mechanical point of view, than those obtained from less concentrated solutions. In fact, the capsules formed from sodium alginate solutions of concentrations lower than 0.25% w/v had poorly gelled and sticky surfaces and were very fragile and difficult to handle. Therefore, it can be stated that the use of concentrated sodium alginate solutions results in a less porous gel and justifies the supposition outlined above.

On the other hand, on increasing the calcium chloride concentration, the thickness of the membrane increases for a given sodium alginate concentration. This kind of behaviour confirms that the membrane thickness increases continuously until complete consumption of the calcium ions contained in the core of the capsule has been achieved. As expected, the percentage of GOD diffusion decreases with increasing calcium chloride concentration, for a given sodium alginate concentration, due to an increase in the diffusional resistance that enzyme molecules suffer in their flow through a thicker gel membrane.

From the results obtained it seems clear that diffusional properties of calcium alginate capsules depend on gelation conditions (sodium alginate and calcium chloride concentrations). This can be verified when GOD diffusion percentages are compared in capsules with membrane thicknesses of the same order of magnitude but obtained under different experimental conditions. Thus, the use of sodium alginate and calcium chloride solutions of concentrations 0.5% w/v and 2.6% w/v, respectively, leads to the formation of capsules with a gel film thickness of 1.03 ± 0.03 mm and a diffusion percentage of 25 ± 2. However, this percentage is reduced to 8 ± 3 when sodium alginate and calcium chloride concentrations are fixed at 1% w/v and 4% w/v, respectively, even though the thickness of the capsules is of the same order of magnitude (0.98 ± 0.02).

All of these results confirm that capsules obtained from concentrated biopolymer and cation solutions present a gel network that is more densely cross-linked and, therefore, has a smaller matrix mesh size. In this way, the combination of 1% w/v sodium alginate solutions with 4% and 5.5% w/v calcium chloride solutions leads to percentage enzyme losses of 8 ± 3 and 4 ± 2, respectively. Clearly, the employment of sodium alginate solutions of concentrations

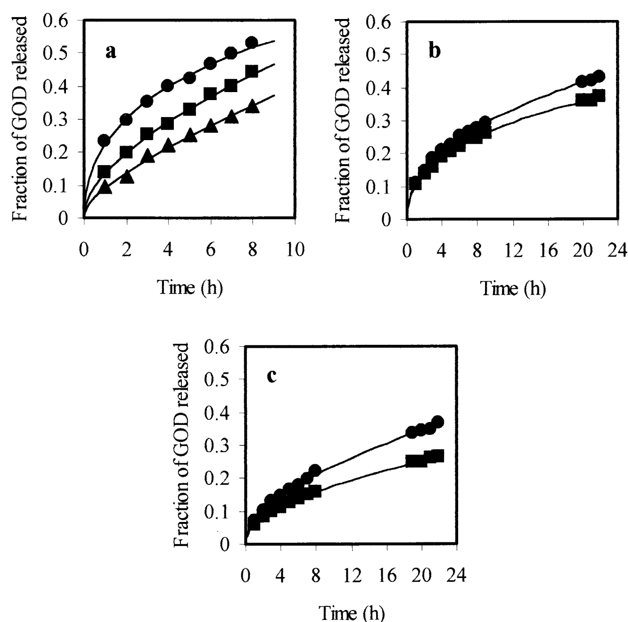

Fig. 1. Effect of calcium chloride and sodium alginate concentration on fraction of GOD released from capsules. a, 1.3% w/v $CaCl_2$; b, 2.6% w/v $CaCl_2$; c, 4% w/v $CaCl_2$. Symbols: ●, 0.5% w/v sodium alginate; ■, 0.75% w/v sodium alginate; ▲, 1.0% w/v sodium alginate.

higher than 1% w/v would have further diminished the fraction of GOD leakage. However, the technique of capsule formation limits the biopolymer solution concentration that can be employed. In this respect, it was found that the capsules stuck to each other when 1.5% w/v sodium alginate solutions were used because of the viscosity of the solution. Moreover, in these cases it was necessary to increase the CMC concentration of the cationic solutions in order to ensure the spherical shape of the capsules, with the consequent problems of dissolution.

3.2. GOD diffusion studies

With the aim of modelling the kinetics of enzyme diffusion from the capsules, the experimental data of GOD concentration, measured in the well-stirred buffer solution, were adjusted to a simple semi-empirical power equation derived by Peppas et al. [23] to describe the process of solute release from polymers. As an example, the theoretical predictions and the measured values of fraction of GOD released versus time, for a number of different gelation conditions, are shown in Fig. 1. As can be seen, the model fitted the experimental data well. Moreover, diffusion profiles of GOD were similar regardless of the alginate and $CaCl_2$ concentrations used in the preparation of the capsules. The diffusion of GOD from the capsules is characterised by a very high initial rate and, after this first stage, the diffusion rate decreases. This release profile is characteristic of reservoir systems, with an initially high release rate indicating the so-called "burst" effect. This effect probably results from the incorporation of the enzyme within the capsule membranes during their formation. This may arise because, when gelation occurs, water is expelled from the capsules because the gel contracts during crosslinking. Water migrates to the bead surface and this flux may carry enzyme molecules that will become trapped in the gel membrane of the capsules. After this first stage, the diffusion rate decreases with time because of the greater diffusional pathlength.

Table 2
Effect of sodium alginate and $CaCl_2$ concentrations on the estimates of the diffusional exponents and kinetic constants for release of GOD from calcium alginate gel capsules

[Sodium alginate]	[$CaCl_2$] 1.3% w/v			
	k_1 (h^{-m})	k_2 (h^{-m})	m	r^2
0.5% w/v	0.25	0.02	0.48	0.84
0.75% w/v	0.12	0.01	0.49	0.88
1.0% w/v	0.07	0.02	0.49	0.83
[Sodium alginate]	[$CaCl_2$] 2.6% w/v			
	k_1 (h^{-m})	k_2 (h^{-m})	m	r^2
0.5% w/v	0.11	$k_2 \ll k_1$ (n.s.)	0.47	0.83
0.75% w/v	0.11	$k_2 \ll k_1$ (n.s.)	0.47	0.89
[Sodium alginate]	[$CaCl_2$] 4% w/v			
	k_1 (h^{-m})	k_2 (h^{-m})	m	r^2
0.5% w/v	0.07	$k_2 \ll k_1$ (n.s.)	0.46	0.92
0.75% w/v	0.06	$k_2 \ll k_1$ (n.s.)	0.46	0.93

n.s.: non-significant value.

It can be seen that the rate of enzyme release at a given time and $CaCl_2$ concentration depends on the sodium alginate concentration and thus on capsule membrane thickness and degree of crosslinking. On increasing the alginate concentration, the number of apparent cross-linking points increased, resulting in the decreased mesh size within the gel, a factor that affects GOD leakage.

The kinetic constants that were determined and the diffusional exponent in the capsules, as a function of sodium alginate and $CaCl_2$ concentration, are given in Table 2. As shown in Table 2, the values for the kinetic constant k_1 are always higher than for k_2, indicating that the diffusional mechanism predominates. Hence, the molecular relaxation in the polymer contributes to a small extent to the enzyme diffusion. Once again the dependency that exits between the diffusion of GOD and the capsule characteristics was confirmed. In this respect, the employment of more concentrated biopolymer and cation solutions reduces the percentage of leakage of GOD from the capsules and, therefore, the value of k_1 decreases. This suggests the possibility of tailoring the diffusional properties of the capsules by altering appropriate formulation conditions such as sodium alginate and calcium ion concentrations.

References

[1] Gemeiner P. Introduction to enzyme engineering. In: Enzyme engineering. Immobilized biosystems. Gemeiner P (Ed.) Ellis Horwood, NY: Ellis Horwood Series in Biochemistry and Biotechnology, 1992. pp. 9–12.

[2] Nigam SC, Tsao I-F, Sakoda A, Wang HY. Techniques for preparing hydrogel membrane capsules. Biotechnol Tech 1988;2:271–6.

[3] Chang TMS, McIntosh FC, Mason FG. Semipermeable microcapsules: preparation and properties. Can J Physiol Pharmacol 1966;44: 115.

[4] Christenson L, Dionne K, Lysaught M. Biomedical application of immobilised cells. In: Fundamentals of animal cell encapsulation. Goosen MFA (Ed.) CRC Press, Boca Raton, MFA Goosen, 1993, 7–41.

[5] Blandino A, Macias M, Cantero D. Formation of calcium alginate gel capsules: influence of sodium alginate and $CaCl_2$ concentration on gelation kinetics. J Biosci Bioeng 1999;88:686–9.

[6] Yamagiwa K, Shimizu Y, Kozawa T, Onodera M, Ohkawa A. Formation of calcium-alginate gel coating on biocatalyst immobilization carrier. J Chem Eng Jpn 1992;25:723–28.

[7] King GA, Daugulis AJ, Faulkner P, Goosen MFA. Alginate-polylysine microcapsules of controlled membrane molecular weight cut off for mammalian cell culture engineering. Biotechnol Progr 1987;3:231–40.

[8] Yamagiwa K, Kozawa T, Ohkawa A. Effects of alginate composition and gelling conditions on diffusional and mechanical properties of calcium-alginate gel beads. J Chem Eng Jpn 1995;28:462–7.

[9] Martinsen A, Storrø I, Skjak–Braek G. Alginate as immobilization material: III. Diffusional properties. Biotechnol Bioeng 1992;39: 186–94.

[10] Hulst AC, Hens HJH, Buitelaar RM, Tramper J. Determination of the effective diffusion coefficient of oxygen in gel materials in relation to gel concentration. Biotechnol Tech 1989;3:199–204.

[11] Aslani P, Kennedy RA. Effect of gelation conditions and dissolution media on the release of paracetamol from alginate gel beads. J Microencapsul 1996;13:601–4.

[12] Aslani P, Kennedy RA. Studies on diffusion in alginate gels. I. Effect of cross-linking with calcium or zinc ions on diffusion of acetaminophen. J Control Release 1996;42:75–82.

[13] Shoichet MS, Li RH, White ML, Winn SR. Stability of hydrogels used in cell encapsulation: an in vitro comparison of alginate and agarose. Biotechnol Bioeng 1996;50:374–81.

[14] Martinsen A, Skjak–Braek G, Smidsrød, O. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng 1989;33:79–89.

[15] Lee GM, Palsson BO. Simplified method of making alginate-polylysine microcapsules for hybridoma cell culture using RPMI 1640 medium. Biotechnol Tech 1990;4:341–44.

[16] Lloyd–George I, Chang TMS. Characterization of free and alginate-polylysine-alginate microencapsulated *Erwinia herbicola* for the conversion of ammonia, pyruvate, and phenol into L-tyrosine. Biotechnol Bioeng 1995;48:706–14.

[17] Tay LF, Khoh LK, Loh CS, Khor E. Alginate-Chitosan coacervation in production of artificial seeds. Biotechnol Bioeng 1993;42: 449–54.

[18] Yamagiwa K, Shimizu Y, Kozawa T, Onodera M, Akira O. Ethanol production by encapsulated and immobilized yeast. Biotechnol Tech 1994;8:271–4.

[19] Longo MA, Novella IS, Garcia LA, Díaz M. Diffusion of proteases in calcium alginate beads. Enzyme Microb Tech 1992;14:586–90.

[20] Leckband D, Langer R. An approach for the stable immobilization of proteins. Biotechnol Bioeng 1991;37:227–37.

[21] Hertzberg S, Kvittingen L, Anthonsen T, Skjak–Braek, G. Alginate as immobilization matrix and stabilizing agent in a two-phase liquid system: application in lipase-catalysed reactions. Enzyme Microb Technol 1992;14:42–7.

[22] Peppas NA, Sahlin JL. A simple equation for the description of solute release. III. Coupling of diffusion and relaxation. Int J Pharm 1989; 57:169–72.

[23] Peppas NA. Analysis of Fickian and non-Fickian drug release from polymers. Pharm Acta Helv 1985;60:110–11.

[24] Peterson GL. A simplification of the protein assay method of Lowry et al. that is more generally applicable. Anal Biochem 1977;83:346–56.

[25] Marquardt D. An algorithm for least-squares estimation of non linear parameters. J Soc Ind Appl Math 1963;11:431–41.