Protein separation using membrane-encapsulated soluble ligand conjugates

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A new approach for isolating and recovering biological macromolecules using membrane-encapsulated soluble ligand conjugates was investigated. Membrane-encapsulated solid adsorbents have been successfully developed and employed in our laboratory to isolate and purify proteins and enzymes directly from culture broths. This new concept also makes it possible to use soluble ligand conjugates instead of solid adsorbents inside membrane capsules. In this work, model membrane-encapsulated soluble and insoluble ligands comprising Blue Dextran and Blue Sepharose entrapped within calcium alginate membranes were studied to compare adsorption characteristics such as capacities and rates. Experimental results suggest that membrane-encapsulated soluble ligands may be expected to result in higher overall adsorption capacity compared to membrane-encapsulated solid adsorbents with comparable adsorption rates.

Keywords: Bioseparation; affinity adsorption; membrane-encapsulated ligand; soluble ligand conjugate

Introduction

Currently a number of techniques are being commercialized to produce valuable therapeutic proteins from different host systems. Regardless of which host is used for protein synthesis, considerable engineering problems are faced in isolating and purifying biological molecules from crude protein mixtures. A trace amount of synthesized product is usually present in a colloidal suspension contaminated with a large number of proteins and other macromolecules. Conventional solids separation methods such as centrifugation and filtration are often inefficient in removing colloidal solids from these viscous broths. Most primary isolation steps are nonspecific, relying mainly on differences in molecular size, charge, or hydrophobic interactions. These lead to lower product yields in subsequent isolation and purification steps.

Affinity interactions based on biological recognition offer a powerful tool for separating bioproducts. Ideally, the use of affinity separation in early stages of primary isolation may result in a significant increase in process yield because of increased selectivity. *Table 1* shows some examples of affinity-based isolation methods that can potentially be used at an early stage of a bioseparation process. Besides direct batch adsorption using small solid affinity adsorbents,[1] two other processes, which combine biospecific adsorption with aqueous two-phase extraction, have been suggested in the literature. One uses solid affinity adsorbents,[2,3] and the other one uses water-soluble polymer–ligand conjugates,[4-7] in combination with aqueous two-phase extraction. In the first case, solid adsorbent particles with the adsorbed bioproduct can be partitioned into the upper polyethylene glycol (PEG)-rich phase, while contaminants such as cell debris move to the lower dextran-rich phase. An alternative is to covalently bond affinity ligands to one of the polymers used in the aqueous two-phase extraction, such as PEG, to form one of the separating phases. The desired product can then be preferentially pulled into the upper PEG-rich phase, while most of the contaminant proteins and cell debris are partitioned into the lower dextran-rich phase. However, problems associated with aqueous two-phase extraction, such as reagent costs and large-scale processing of viscous fluids, have to be addressed for broader applications. For example, new, lost-cost polymers specially designed for aqueous two-phase systems have been studied.[8]

Membrane-encapsulated semi-specific and specific solid affinity adsorbents have been proposed and investigated in our laboratory. The advantages and disadvantages of using membrane-encapsulated adsorbents are discussed elsewhere.[1,9-10] Briefly, the hydrophilic capsule membrane can be used to prevent adhesion of

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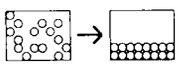
Received 7 December 1988; revised 28 March 1989

Table 1 Affinity-based isolation methods at whole broth stage

Process	Form of affinity ligand	Recovery of ligands	Problems
Direct batch adsorption	Solid adsorbent particles	Sedimentation	•Fouling •Difficulty in recovery of fine adsorbent particles
Aqueous two-phase partitioning with carrier adsorbents	Solid adsorbent particles	Two-phase extraction  PEG Salt/dextran	•High cost of reagents •Limited range of operating physicochemical conditions
Aqueous two-phase affinity partitioning	Soluble polymer conjugates	Two-phase extraction  PEG Dextran	•Difficulty in separation of bioproduct from phase forming polymer
Batch adsorption using membrane-encapsulated ligands	Soluble polymer conjugates / Solid adsorbent particles		•Encapsulation Technology? •Adsorption rate and capacity?

colloidal solids and nonspecific adsorption of macromolecular contaminants to the affinity adsorbents. Also, encapsulated adsorbents can be designed large enough to allow easy recovery from culture broths. The problem of creating an additional intramembrane diffusion resistance may be minimized by using soluble polymer–ligand conjugates instead of solid adsorbents within the membrane capsules, as illustrated in *Figure 1*. Ligands covalently immobilized onto various macromolecular water-soluble polymers can be encapsulated within different semipermeable membranes. The capsule membrane's characteristics can be tailored to prevent the soluble ligands from leaking out of the capsules and yet allow the desired bioproduct easily to diffuse into the capsules and be bound to the immobilized soluble ligands.

In this study, experiments were carried out to demonstrate this concept and to compare the adsorption properties of a membrane-encapsulated insoluble ligand versus a soluble ligand. Blue Dextran and Blue Sepharose were used as model affinity and/or semi-affinity ligands in this study. We intend to prove the feasibility of using membrane-encapsulated soluble ligands as a new bioseparation tool and evaluate its characteristics.

Materials and methods

Materials

The following chemicals were used: dextran (MW = 42,600), HEPES, human serum albumin (HuSA), Reactive Blue 2-Sepharose (Blue Sepharose), Blue Dextran from Sigma (St. Louis, MO), calcium chloride from Baker (Phillipsburg, NJ), and sodium alginate (Kelco Gel LV) from Kelco (Chicago, IL).

Model experimental system

Two kinds of commercially available ligand–carrier conjugates that have the same group-specific ligand, Reactive Blue 2, were used in these experiments. One is Blue Dextran, which is a water-soluble conjugate composed of the ligand and water-soluble polymer dextran with a molecular weight of approximately 2×10^6. The other is Blue Sepharose, which is an insoluble solid adsorbent with a particle diameter of 80–200 μm. These two were encapsulated within calcium alginate membranes. Human serum albumin (HuSA) can be specifically bound to this ligand.[11–13] The molecular weight of HuSA is 66,000 and the diffusion coefficient of this protein in water, D_O, is 6.1×10^{-7} cm^2 s^{-1}.

Preparation of Ca-alginate beads

Calcium alginate beads were used to measure the effective diffusivities of HuSA in calcium alginate gel by batch diffusion experiments, as described in the literature.[14] Sodium alginate solutions (0.5%, 1%, 2%, and 4%; w/v) were prepared using deionized water. Droplets of these solutions were introduced into a well-stirred 0.5% (w/v) calcium chloride solution. The calcium alginate beads were instantaneously formed and cured in the 0.5% (w/v) calcium chloride solution until use. The beads were placed in a 25 mM HEPES buffer (pH 7.0) containing 5 mM calcium chloride for 3 h prior to the diffusion experiments.

Encapsulation of ligands in Ca-alginate membrane

The basic approach to encapsulating various bioactive materials, including affinity ligands, has been developed in our laboratory and is described in the literature.[15] A precisely measured amount of Blue Sepharose was suspended in a solution containing 0.5% (w/v) calcium chloride and 20% (w/v) dextran. Dextran serves as a viscosity enhancer to ensure a spherical shape for the capsules.[15] Most of the dextran diffuses out of the capsule after the encapsulation is completed. Similarly, a precisely weighed amount of Blue Dextran was dissolved in a solution containing 0.5% (w/v) calcium chloride and 20% (w/v) dextran. Droplets of this prepared suspension were dropped through a hypodermic needle into a rapidly stirred 0.5% (w/v) sodium

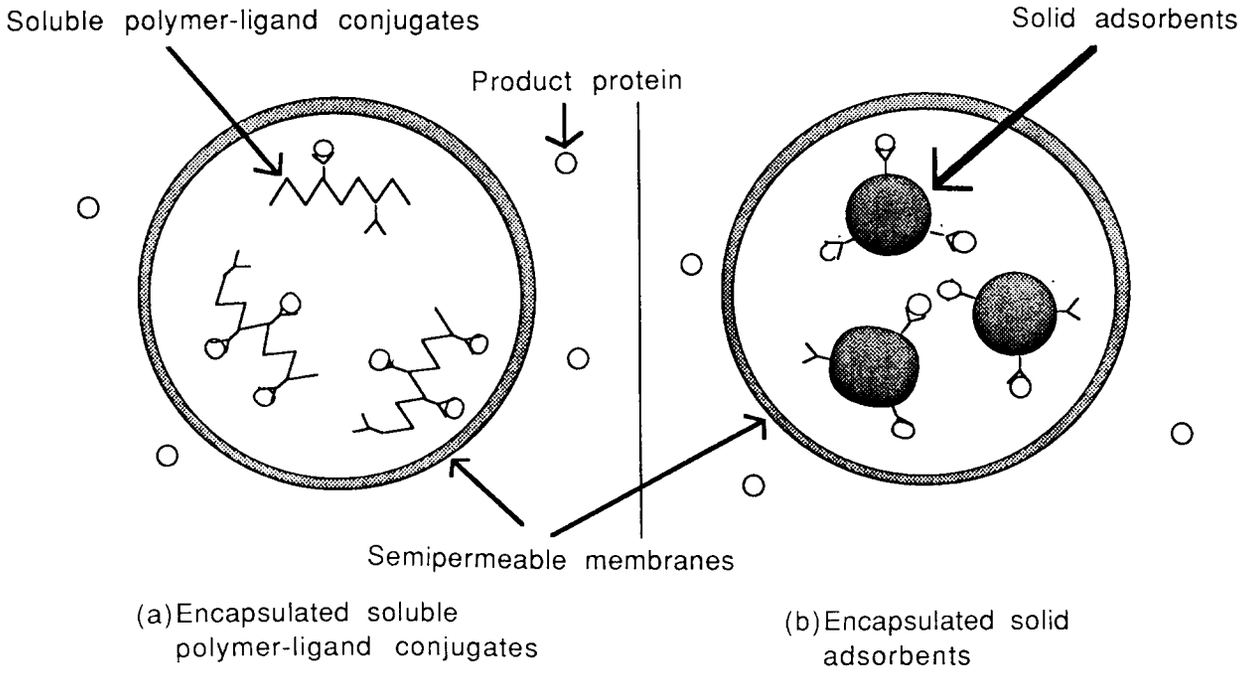


Figure 1 Bioproduct recovery using membrane-encapsulated ligands

alginate solution. Capsular membranes were formed instantaneously around the droplets and wrapped the suspension containing Blue Dextran due to rapid cross-linking of alginate molecules by calcium cations at the interface. The Blue Dextran capsules formed were allowed to stand in the alginate solution for 1 min. After that, the sodium alginate solution was diluted five-fold with deionized water and the capsules were recovered from the solution. The capsules were then washed twice with deionized water and stored in a 0.5% (w/v) calcium chloride solution overnight before use.

Diffusion of HuSA into Ca-alginate beads

One or two milliliters of calcium alginate beads prepared as described earlier were completely freed of excess buffer solution by filtering with a fine mesh and then placed in 5 ml of 1 mg ml^{-1} HuSA solution prepared using the same HEPES buffer solution described before. This buffer solution was used in all the experiments in this work. The suspension was kept mixed on a rotator. Temperature was maintained at 20°C. HuSA concentration was determined by absorbance at 280 nm.

Adsorption of HuSA onto encapsulated ligands

Adsorption equilibrium and rate data were obtained by batch adsorption experiments. Capsules containing various soluble or insoluble ligands prepared as described previously were equilibrated in 25 mM HEPES buffer containing 5 mM $CaCl_2$ (pH 7.0) for 3 h before use. Between 0.1 and 2 ml of the capsules were placed in 5 ml of 1 mg ml^{-1} HuSA solution for batch adsorption experiments.

Results and discussion

Preparation of the encapsulated ligands

The effective diffusivity, D_e, of proteins in calcium alginate gels is significantly dependent on gel concentration and also varies with proteins of different molecular size and charge.[14,16-18] D_e may also vary with the preparation procedures and the type of sodium alginate used. For a given sodium alginate concentration, the HuSA concentration changes with respect to time during batch diffusion is shown in *Figure 2*, where C is the HuSA concentration, C_O is the initial concentration, and C^* is defined by equation (1):

$$C^* = V_s C_O/(V_s + V_b) \tag{1}$$

where V_s and V_b represent the volumes of a HuSA solution and calcium alginate beads used, respectively.

A solid line in *Figure 2* represents the theoretial profile of diffusion from a well-stirred solution of limited volume into a sphere,[19] which is given by equation (2) with $D_e = D_O = 6.1 \times 10^{-7}$ cm^2 s^{-1}, where D_O is the bulk diffusivity of HuSA.

$$\frac{C}{C_O} = \frac{\alpha}{1+\alpha}\left\{1 + \sum_{n=1}^{\infty} \frac{6(1+\alpha)\exp(-D_e q_n^2 t/R_b^2)}{9 + 9\alpha + q_n^2\alpha^2}\right\} \tag{2}$$

where $\alpha = V_s/V_b$ and q_n terms are the roots of equation (3)

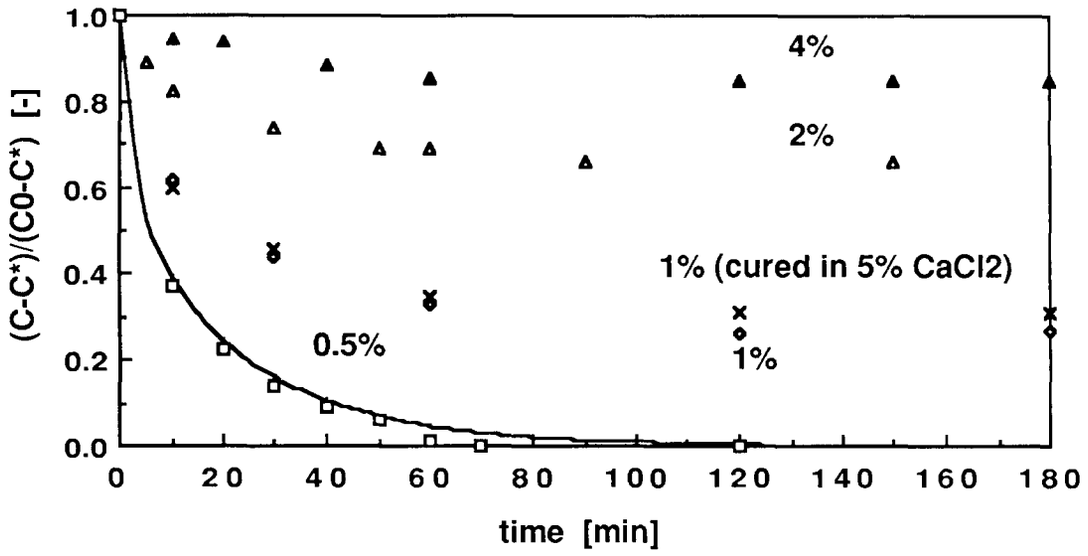


Figure 2 Diffusion of HuSA into Ca-alginate beads

$$\tan q_n = 3q_n/(3 + \alpha q_n^2) \quad (3)$$

The mean radius of beads, R_b, was determined from equation (4):

$$V_b = 4\pi R_b^3 N/3 \quad (4)$$

where N is the number of beads used.

Experimental data for HuSA diffusion into 0.5% (w/v) calcium alginate are successfully fitted by this profile, suggesting that D_e of HuSA in 0.5% calcium alginate can be considered to be equivalent to D_O. When affinity ligands are encapsulated within the capsule membranes, it is desirable to make the membrane loose enough to allow the passage of the desired protein with minimum diffusion resistance. Because of this, Blue Sepharose and Blue Dextran were initially encapsulated within calcium alginate gel membranes prepared from 0.5% (w/v) sodium alginate solutions.

Capsule size could be controlled to a diameter from 0.04 to 0.4 cm using a microdroplet generator similar to the one used by Klein *et al.*[16] Examples of membrane-encapsulated Blue Sepharose and Blue Dextran are shown in *Figures 3a* and *b* respectively.

Adsorption capacity

The adsorption equilibrium of HuSA on Blue Sepharose and Blue Dextran in 25 mM HEPES buffer containing 5 mM $CaCl_2$ (pH 7.0) is shown in *Figure 4*, on the basis of dry carrier weight. Equilibrium data were successfully correlated by the Langmuir isotherm:

$$q = Kq^*C/(1 + KC) \quad (5)$$

where q is the amount adsorbed in equilibrium with the concentration C, q^* is the saturated amount adsorbed, and K is the Langmuir constant. The parameters, q^* and K, were determined using the Langmuir plots. The resultant correlation curves are shown in *Figure 4*, with the estimated parameters given in the figure caption.

The effective ligand density of Blue Dextran on a dry weight basis is approximately one order of magnitude higher than that of Blue Sepharose. Higher ligand density in solid supports may be restricted because of other problems, such as steric hindrance of the immobilized ligands in the gel matrices, which in turn reduces the effective binding capacity.

Overall adsorption capacity based on capsule volume is compared in *Table 2*. The upper limit values of the carrier concentration in the capsule core are estimated values determined from the experiments. In a series of encapsulation studies, it was found that encapsulation of Blue Sepharose and Blue Dextran with higher concentrations than these was difficult to achieve experimentally. These results suggest that soluble ligands in capsules can be used as an alternative to obtain a higher overall adsorption capacity compared to solid adsorbents.

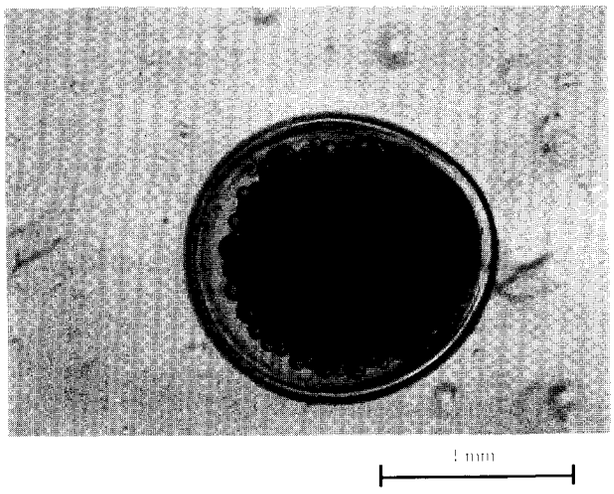
Adsorption rate

The adsorption rate was evaluated in terms of the apparent overall effective diffusivity in the capsule core, $D_{e,app}$. A theoretical model describing mass transfer of HuSA from a bulk solution to encapsulated ligands was used to evaluate $D_{e,app}$ from these experimental data. Since the details of this model have been described elsewhere,[19,20] only the basic equations and boundary conditions will be cited in this paper.

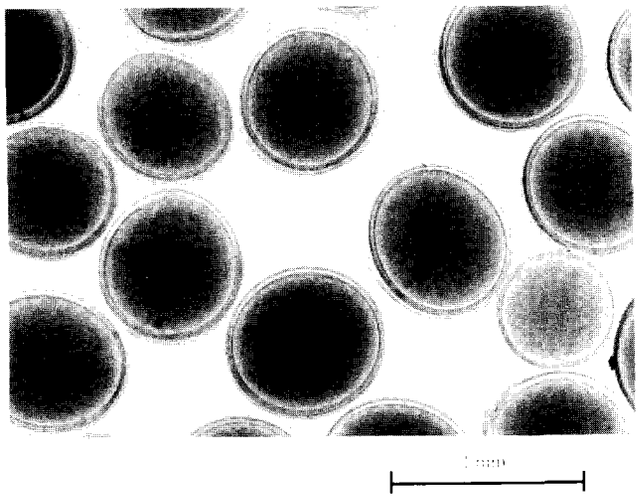
The mass balance inside the capsule core is:

$$\frac{D_{e,app}}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C_i}{\partial r}\right) = \varepsilon\frac{\partial C_i}{\partial t} + \rho_c\frac{\partial q}{\partial t} \quad (6)$$

The mass balance in the capsule membrane is:



(a)



(b)

Figure 3 (a) Encapsulated Blue Sepharose. (b) Encapsulated Blue Dextran

$$\frac{D_e}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C_i}{\partial r}\right) = \varepsilon\frac{\partial C_i}{\partial t} \tag{7}$$

Boundary conditions are:

$$r = 0;\ \frac{\partial C_i}{\partial r} = 0 \tag{8}$$

$$r = r_c\ D_{e,app}\frac{\partial C_i}{\partial r} = D_e\frac{\partial C_i}{\partial r} \tag{9}$$

$$r = R_c\ C_i = C \tag{10}$$

The assumptions made were: (1) the capsule core consisting of Blue Sepharose particles in suspension is approximated as an evenly dispersed phase; (2) the encapsulated Blue Dextran solution is considered to be immobile inside the membrane capsule; (3) the molecular diffusion of Blue Dextran is negligible in comparison with that of HuSA. Although the model employed here as a first approximation may not be adequate to describe the exact mass transfer mechanisms, it should still give us a rough estimate of the overall effective diffusivities of these two kinds of encapsulated ligand systems for comparison.

Table 2 Comparison of adsorption capacity

	Blue Sepharose (insoluble)	Blue Dextran (soluble)
q^*[mg-HuSA/g-dry carrier]	75	833
Upper limit of carrier concentration in the capsule core [g dry carrier ml^{-1} capsule]	0.150	0.075
Overall adsorption capacity [mg HuSA ml^{-1} capsule]	10	60

Figures 5 and *6* show experimental data and fitting curves of bulk concentration changes when encapsulated Blue Sepharose and Blue Dextran are used, respectively. The resultant values of $D_{e,app}$ are given in figure captions, along with the values of capsule radius R_c, capsule core radius r_c, Blue Sepharose and Blue Dextran concentration in capsule cores on the dry weight basis ρ_c, and adsorption capacity of the capsules. The $D_{e,app}$ values obtained in these two systems were about the same order of magnitude and they were approximately one order of magnitude smaller than the bulk diffusivity. This is primarily due to the support gel matrix in the case of Blue Sepharose and the highly viscous nature of the polymer–ligand conjugate solution for Blue Dextran. Possible reasons for the inadequacy of the model predictions in comparison with experimental data are likely due to some of the simplifying assumptions made for this kinetic model. More detailed modelling work is underway in our laboratory

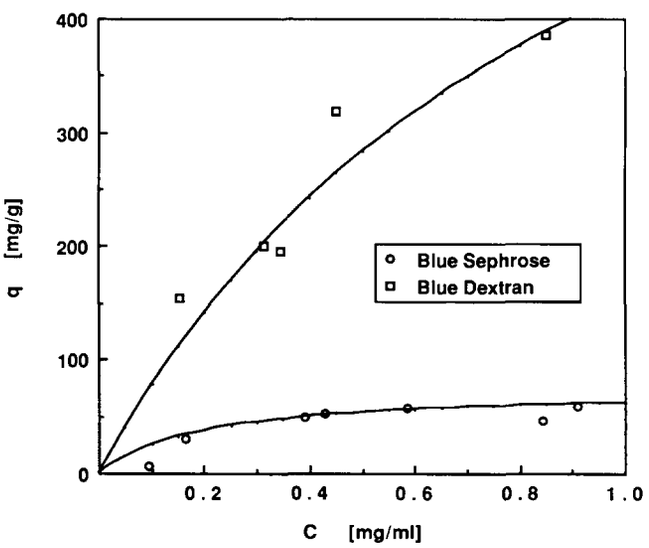


Figure 4 Adsorption equilibrium of HuSA on encapsulated Blue Sepharose and Blue Dextran. Blue Sepharose: K = 5.15 ml mg^{-1}, q^* = 74.6 mg g^{-1}. Blue Dextran: K = 1.03 ml mg^{-1}, q^* = 833 mg g^{-1}

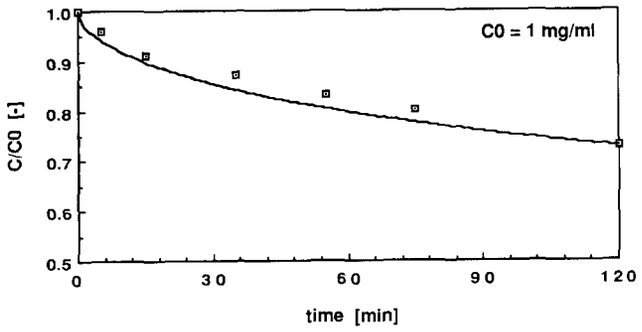


Figure 5 Batch adsorption profile of HuSA on encapsulated Blue Sepharose. R_c = 0.072 cm, r_c = 0.064 cm, ρ_c = 0.113 g ml^{-1}. Adsorption capacity = 8.5 mg ml^{-1}. $D_{e,app}$ = 7.9 × 10^{-8} cm^2 s^{-1}

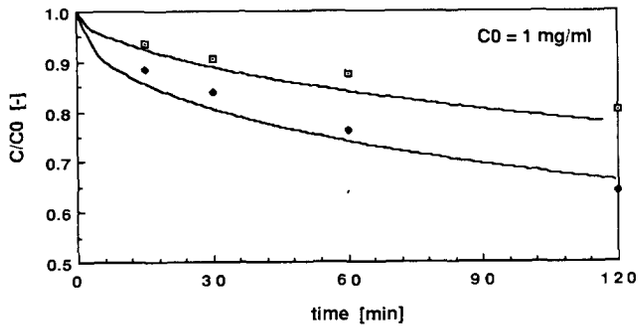


Figure 6 Batch adsorption profile of HuSA on encapsulated Blue Dextran. R_c = 0.094 cm, r_c = 0.086 cm, ρ_c = 0.025 g ml^{-1}. Adsorption capacity = 20.8 mg ml^{-1}. $D_{e,app}$ = 4.0 × 10^{-8} cm^2 s^{-1}. R_c = 0.104 cm, r_c = 0.096 cm, ρ_c = 0.010 g ml^{-1}. Adsorption capacity = 8.3 mg ml^{-1}. $D_{e,app}$ = 6.1 × 10^{-8} cm^2 s^{-1}

to delineate and identify these differences and possible methods to improve the kinetic models.

Conclusions

A new approach for isolating and recovering biological molecules, such as proteins and polypeptides, using membrane-encapsulated water-soluble ligand conjugates was studied using a simplified encapsulation technique. These new membrane-encapsulated soluble polymeric conjugates were compared with the use of solid adsorbents in terms of the adsorption capacity and rate. Blue Sepharose particles and Blue Dextran solutions were successfully encapsulated within a calcium alginate hydrogel membrane having well-defined characteristics. Experimental data suggest that the use of soluble ligands can be expected to result in a higher overall adsorption capacity compared to solid adsorbents. The adsorption rate of the membrane-encapsulated soluble ligands is only comparable to that of encapsulated insoluble ligands because of diffusional restriction due to the highly viscous nature of the soluble polymer–ligand conjugate solution.

Acknowledgement

We acknowledge the financial support of the National Science Foundation for this study.

Nomenclature

C	concentration of HuSA, mg ml^{-1}
C_i	HuSA concentration in capsule and bead, mg ml^{-1}
C_O	initial concentration, mg ml^{-1}
C^*	defined by equation (1) mg ml^{-1}
D_e	effective diffusivity in calcium alginate gel, cm^2 s^{-1}
$D_{e,app}$	apparent overall effective diffusivity in the capsule core, cm^2 s^{-1}
D_O	diffusivity in water, cm^2 s^{-1}
K	Langmuir constant, ml mg^{-1}
N	number of beads, –
q	amount adsorbed, mg g^{-1}
q_n	nth root of equation (3), –
q^*	saturation amount adsorbed, mg g^{-1}
r	radial distance, cm
r_c	capsule core radius, cm
R_c	capsule radius, cm
R_b	bead radius, cm
t	time, s
V_s	volume of protein solution, ml
V_b	total volume of beads, ml
α	ratio of solution volume to bead volume, –
ρ_c	ligand carrier concentration in capsule core, g ml^{-1}

References

1 Wang, H. Y. and Sobnosky, K. *ACS Symp Ser.* 1986, **271,** 123
2 Hedman, P. and Gustafsson, J. G. *Anal. Biochem.* 1984, **138,** 411–415
3 Frej, A-K., Gustafsson, J-G. and Hedman, P. *Biochem. Bioeng.* 1986, **28,** 133–137
4 Flanagan, S. D. and Barondes, S. H. *J. Biol. Chem.* 1975, **250,** 1484–1489
5 Kula, M. R., Johansson, G. and Buckmann, A. F. *Biochem. Soc. Trans.* 1979, **7,** 1–5
6 Kopperschlager, G. and Johansson, G. *Anal. Biochem.* 1982, **124,** 117–124
7 Harris, J. M., Case, M. G. and Hovanes, B. A. *Ind. Eng. Chem. Prod. Res. Dev.* 1984, **23,** 86–88
8 Tjerneld, F., Johansson, G. and Joelsson, M. *Biotech. Bioeng.* 1987, **30,** 809–816
9 Nigam, S. C., Sakoda, A. and Wang, H. Y. *Biotech. Progress* 1988, **4,** 166–172
10 Sakoda, A. and Wang, H. Y. *Biotechnol. Bioeng.* 1989, **34,** 1098–1103
11 Angal, S. and Dean, P. D. G. *Biochem. J.* 1977, **167,** 301–303
12 Leatherbarrow, R. J. and Dean, P. D. G. *Biochem. J.* 1980, **189,** 27–34
13 Lagercrantz, C. and Larsson, T. *Biochem. J.* 1983, **213,** 387–390
14 Tanaka, H., Matsumura, M. and Veliky, I. A. *Biotech. Bioeng* 1984, **26,** 53–58
15 Nigam, S. C., Tsao, I-Fu, Sakoda, A. and Wang, H. Y. *Biotech. Techniques* 1988, **2,** 271–276
16 Klein, J., Stock, J. and Vorlop, K. D. *Eur. J. Appl. Microb. Biotech.* 1983, **18,** 86–91
17 Hannoun, B. J. M. and Stephanopoulos, G. *Biotech. Bioeng.* 1986, **28,** 829–835
18 Itamunoala, G. F. *Biotech. Prog.* 1987, **3,** 115–120
19 Crank, J. in *Mathematics of Diffusion* Clarendon, Oxford, 1956, Chapter 6 (p. 93)
20 Nigam, S. C. Ph.D. Thesis University of Michigan, 1988