Journal of Chromatography, 586 (1991) 27–33
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 568

Protein adsorption capacity of a porous phenylalanine-containing membrane based on a polyethylene matrix

Min Kim, Kyoichi Saito* and Shintaro Furusaki

Department of Chemical Engineering, Faculty of Engineering, University of Tokyo, Hongo, Tokyo 113 (Japan)

Takanobu Sugo and Isao Ishigaki

Japan Atomic Energy Research Institute, Takasaki Radiation Chemistry Research Establishment, Takasaki, Gunma 370-12 (Japan)

(First received July 30th, 1990; revised manuscript received June 17th, 1991)

ABSTRACT

A porous hollow-fibre membrane containing L-phenylalanine as a pseudo-biospecific ligand has been prepared by radiation-induc grafting of glycidyl methacrylate onto polyethylene microfiltration hollow fibre, followed by coupling of the produced epoxide gro with L-phenylalanine. The remaining epoxide group was hydrolysed into a diol group with sulphuric acid. The L-phenylalanine and t diol group acted in a complementary way as a pseudo-biospecific ligand and a hydrophilic group, respectively. The adsorption capaci of bovine gamma globulin on the resulting porous adsorbent could be determined by the specific surface area of the adsorbent when t ligand was in excess over the protein.

INTRODUCTION

When one molecule of an affinity pair is immobilized on a solid support, the resulting material is called an affinity adsorbent capable of separating the other molecule specifically from a complex solution. The separation technique using the affinity adsorbents has been applied to the purification and concentration of diluted and valuable proteins from bioprocess fluids [1], and to the removal of undesirable proteins from human plasma [2]. Of various kinds of affinity adsorbent, those based on agarose gel beads have been widely used on a laboratory scale. Since agarose retains water at the ratio of 40 g of water per gram of dry agarose, wet agarose gel is highly hydrophilic. Thus, agarose gel has the advantage that proteins will not adsorb on it non-selectively. A great number of studies have been reported concerning agarose-based affinity chromatography [3,4]. However, the relationship between protein adsorption capacity and the ligand density or specific surface area of the agarose-based affini adsorbents is not defined, because of the difficulti of varying the ligand density over a wide range ar determining the specific surface area in the wet sta for the agarose gel.

We have suggested a novel method of introdu ing functional groups into a porous polyethyle hollow-fibre membrane [5,6]. This method is effe tive in that the functionality can be easily intr duced into the porous matrix at a given density l selecting the reaction conditions of preparation.

The objectives of our study are two-fold: (1) prepare the porous adsorbent containing a hydr phobic amino acid as a pseudo-biospecific ligan and (2) to correlate the protein adsorption capaci of the porous adsorbent with its ligand density a specific surface area. In this study, L-phenylalani and bovine gamma globulin were selected as liga and protein, respectively. L-Phenylalanine has be used as a ligand for the isolation of tRNA ligase and chorismate mutase/prephenate dehydratase

0021-9673/91/$03.50

from *E. coli*, and for the purification of human IgA from serum [9].

EXPERIMENTAL

Preparation of L-*phenylalanine-containing membrane*

Fig. 1 shows the preparation process for the adsorbent containing L-phenylalanine as a pseudo-biospecific ligand. Commercially available porous polyethylene hollow fibre (Asahi Chemical Industry) was used as the trunk polymer for grafting. The inner and outer diameters of the hollow fibre were 0.62 and 1.24 mm, respectively. Reagent-grade glycidyl methacrylate (GMA) was purchased from Tokyo Kasei Industry and was used without further purification. L-Phenylalanine (L-Phe) was purchased from Wako Pure Chemical Industry.

The hollow fibre was irradiated by an electron beam at ambient temperature in a nitrogen atmosphere. Immediately after irradiation, the hollow fibre was exposed to a vapour of deaerated GMA, which reacted with the trapped radicals in the trunk polymer. Details of GMA grafting have been described in previous publications [5,6]. The amount of GMA introduced into the starting hollow fibre was defined as the degree of GMA grafting:

$$\text{degree of GMA grafting (d.g.)} = 100[(W_1 - W_0)/W_0] \quad (1)$$

where W_0 and W_1 are the weights of starting and GMA-grafted hollow fibre, respectively.

The epoxide group of the grafted branches was coupled with L-Phe. L-Phe was dissolved in deionized water, and the pH was adjusted to 13 with sodium hydroxide. The GMA-grafted hollow fibre was immersed in the L-Phe solution at 353 K. After a predetermined time, the hollow fibre was removed and washed repeatedly with water. Subsequently, the remaining epoxide group in the hollow fibre was converted into a diol group by immersing the hollow fibre in 1 *M* sulphuric acid at 353 K for 2 h. The fibre was washed several times with water and then dried under reduced pressure. The coupling efficiency of epoxide group with L-Phe, X_c, and the ligand density were defined as follows:

$$W_2 = W_0 + (W_1 - W_0)[(142+165)X_c/100 + (142+18)(1-X_c/100)]/142$$

$$\text{coupling efficiency } (X_c) = 100[142(W_2 - W_0)/(W_1 - W_0) - 160]/147 \quad (2)$$

$$\text{ligand density} = [(W_1 - W_0)/142]X_c/100/W_2 \quad (3)$$

where W_2 is the weight of L-Phe-containing hollow fibre, and the factors 165, 142 and 18 are the molecular masses of L-Phe, GMA and water, respectively. The resulting hollow fibre was designated as a Phe-C fibre, where C denotes capillary.

To evaluate the amount of protein adsorbed non-selectively on the Phe-C fibre, a diol-group-containing hollow fibre was prepared by hydrolysing the GMA-grafted hollow fibre with sulphuric acid. The resulting hollow fibre is referred to as a GMA-H-C fibre.

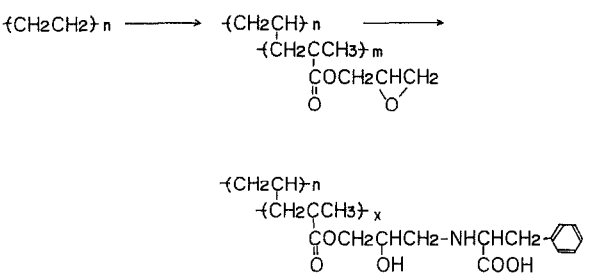

Fig. 1. Preparation of the adsorbent with L-Phe as a pseudo-biospecific ligand. The epoxide group in the GMA-grafted hollow fibre was coupled with L-Phe.

Properties of the Phe-C fibre

After the Phe-C fibre had been dried, its specific surface area was determined according to the BET method. Two drying methods for the fibres were compared: drying under reduced pressure and freeze-drying.

Because a microfiltration hollow-fibre membrane is used as the trunk polymer, the Phe-C fibre is still permeable to water. The water flux was determined using the apparatus described in ref. 10. To prevent a decrease in permeability caused by particle accumulation, water prepared by passage through an ultrafiltration (UF) module was used as the feed solution. The UF water permeated through the membrane from the inside to the outside. The pure water flux (PWF) was calculated by dividing the flow-rate by the inner surface area of the hollow fibre. The PWFs of the hollow fibres with and without methanol treatment were determined. Metha-

nol treatment indicates that, before the flux measurement, the fibre was immersed for 10 min in methanol, which was then replaced with water. The filtration pressure was kept at $1.0 \cdot 10^5$ Pa. The feed solution was kept at 303 K.

Adsorption and elution of bovine gamma globulin

Bovine gamma globulin (BGG) was purchased from Sigma (No. G5009, Cohn Fraction II, III) and used as received. BGG was dissolved in a buffer (3.3 *M* NaCl, 0.01 *M* Tris–HCl, pH 8.0). This buffer can facilitate the hydrophobic interaction and inversely depress the electrostatic binding between the protein and the polymer surface [11]. Adsorption isotherms were determined in a batchwise method. A fixed amount of the Phe-C fibre was immersed in the BGG solution. The mixture was incubated at 303 K for 24 h to allow the system to reach equilibrium. The amount of protein adsorbed on the fibre was calculated from the decrease in BGG concentration in the solution. The Langmuir isotherm closely fitted the adsorption data, and then the saturation capacity was determined. BGG was determined by UV spectroscopy.

Next, the BGG adsorbed onto the Phe-C fibre was eluted by immersing the fibre in 0.01 *M* Tris–HCl buffer containing 1–2 *M* NaCl, and in a mixture of 50% v/v ethylene glycol and 1 *M* NaCl for 24 h. Elution was done at the same volume ratio of fibre to eluate. The amount of BGG in each eluate was determined. The elution ratio, *i.e.* the ratio of the eluted amount to the initially adsorbed amount was calculated.

RESULTS AND DISCUSSION

Preparation of the Phe-C fibre

L-Phe was introduced into the hollow fibre by reaction of the epoxide group in the GMA-grafted hollow fibre with the amino group of L-Phe. Fig. 2 shows the coupling efficiency as a function of the reaction time. The coupling efficiency levelled off after 24 h. By varying the degree of GMA grafting between 10% and 200%, the final coupling efficiency and ligand density were measured. Fig. 3 shows that the final coupling efficiency was *ca.* 13%, irrespective of the degree of GMA grafting. The corresponding ligand density ranged from 0.15 to 0.6 mmol per dry gram of the Phe-C fibre. Moreover,

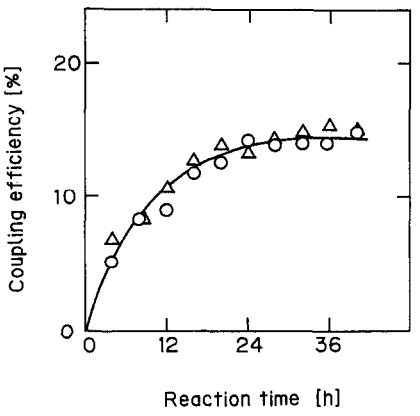

Fig. 2. Coupling efficiency as a function of reaction time. The GMA-grafted hollow fibres with 80% (○) and 130% (△) degree of GMA grafting were immersed in the L-Phe solution with pH initially adjusted to 13. Temperature: 353 K.

the ligand density per wet volume of the fibre, 0.05–0.28 mmol/ml, was calculated by multiplying each apparent density of the fibre. The ligand density of the Phe-C fibre was an order of magnitude higher than that of commercial affinity beads based on agarose, *e.g.* 0.01 mmol/ml [12]. The method suggested in this study can provide the polymeric support with a desired ligand density by selecting either the degree of GMA grafting or the coupling efficiency in preparation.

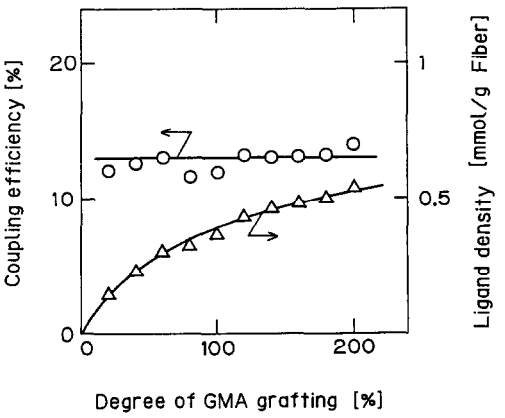

Fig. 3. Coupling efficiency as a function of degree of GMA grafting. The coupling efficiency and ligand density were calculated from eqns. 2 and 3, respectively.

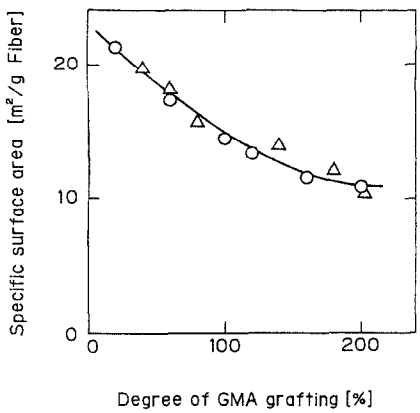

Fig. 4. Specific surface area of Phe-C fibre as a function of degree of GMA grafting. The GMA-grafted hollow fibres were coupled with L-Phe at a coupling efficiency of 13%. After the Phe-C fibre was vacuum-dried (○) or freeze-dried (△), its specific surface area was determined.

Properties of the Phe-C fibre

Fig. 4 shows the specific surface area of the hollow fibres. The specific surface area of the starting hollow fibre was 23 m^2/g. The specific surface area of the Phe-C fibre decreased as the degree of GMA grafting increased. This decrease is due to the formation of the grafted branches on the surface of the pores with relatively smaller diameters [13].

The pure water flux is shown in Fig. 5 as a function of the degree of GMA grafting, where the symbol (M) indicates the hollow fibre pretreated with methanol. Methanol filled the pores of the fibres and then was replaced with water. The PWF of the Phe-C fibre decreased gradually with an increasing degree of GMA grafting. When the degree of GMA grafting reached *ca.* 100%, the PWFs of the fibre with and without methanol treatment coincided. The epoxide group of the GMA-grafted fibre was coupled with L-Phe to reach the final coupling efficiency of 13%. Some of the uncoupled epoxide group were hydrolysed into a diol group during coupling, and some remained as epoxide. Since the remaining epoxide was hydrolysed to the diol group with sulphuric acid quantitatively, the density of the diol group increased with an increase in the degree of GMA grafting (d.g.) under a constant coupling efficiency:

$$\text{density of diol group} = [(W_1 - W_0)(1 - X_c/100)/142]/W_2 \quad (4)$$

$$= \frac{(\text{d.g.})(1 - X_c/100)/100}{142 + (\text{d.g.})(147X_c/100 + 160)/100}$$

As a result, a degree of GMA grafting of *ca.* 100% was regarded as the point at which the polyethylene-based hollow fibre was satisfactorily hydrophilised from the standpoint of the water flux.

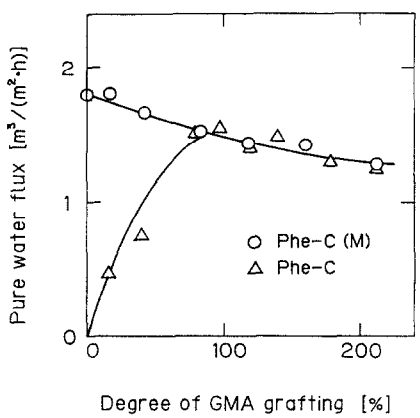

Fig. 5. Pure water flux as a function of degree of GMA grafting. Pure water flux was compared between the Phe-C fibres with and without methanol treatment. The symbol (M) indicates that the dried Phe-C fibre was immersed in methanol for 10 min and then replaced with water.

Non-selective adsorption of BGG

A negligible amount of protein adsorbed non-selectively on the adsorbents is one of the important

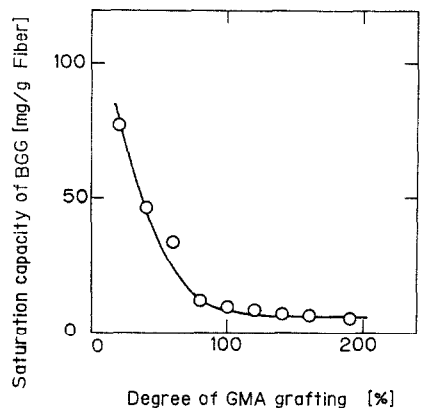

Fig. 6. Saturation capacity of BGG on the diol-group-containing fibre. The GMA-H-C fibre was prepared by hydrolysis of the GMA-grafted fibre then equilibrated with the BGG solution buffered with 0.01 *M* Tris–HCl and 3.3 *M* NaCl (pH 8).

requisites. Using exclusively diol-group-containing hollow fibre (GMA-H-C fibre), which was prepared by hydrolysing the GMA-grafted hollow fibre with sulphuric acid, non-selective adsorption isotherms of BGG were measured. The saturation capacities of GMA-H-C fibres with different degrees of GMA grafting can be obtained from the Langmuir plot of the corresponding isotherm, and they are plotted in Fig. 6 as a function of the degree of GMA grafting. The saturation capacity of BGG on the GMA-H-C fibre decreased as the degree of GMA grafting increased. When the degree of GMA grafting exceeded *ca.* 100%, it decreased to a constant value. The dependence of non-selective saturation capacity on the degree of GMA grafting was in excellent accordance with that of the water flux. Satisfactory hydrophilization of the hollow fibre suggests that the diol-group-containing grafted branches cover the polyethylene matrix entirely and retain water on themselves. Hydrated water on the polymer surface will reduce the hydrophobic interaction between the protein and the polymer surface. This effect has also been observed during the design of a biocompatible film modified with hydrophilic monomers, such as 2-hydroxylethyl methacrylate (HEMA) [14,15].

Specific adsorption of BGG

The amount of L-Phe immobilized on the hollow fibre could be varied by changing the reaction time during the coupling of the GMA-grafted hollow fibre with a constant degree of GMA grafting (d.g. = 70%, 110% and 150%). Fig. 7 shows an example of adsorption isotherms for Phe-C fibres containing an identical amount of the grafted branches and different ligand densities. Fig. 8 indicates that the saturation capacity exhibited a constant value irrespective of the density of the Phe ligand. In this range of the ligand density, the molar ratio, R_a, of the L-Phe ligand to BGG (assumed molecular mass 156 000) adsorbed specifically on it was of the order of 10^3; in other words, the ligand was far in excess of what is required to bind BGG.

$$R_a = \text{(ligand density)/(molar conc. of protein adsorbed at saturation)} \quad (5)$$

However, the density of the diol group obtained from the degree of GMA grafting of at least 100% is necessary to minimize the amount of BGG adsorbed non-selectively, as discussed above. Although an excess ligand density is reported to interfere with the affinity interaction [4], this phenomenon was not observed in this affinity system.

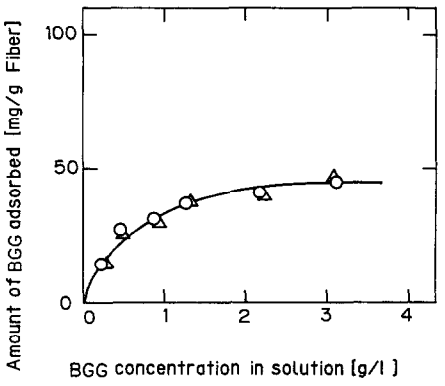

Fig. 7. Adsorption isotherms of Phe-C fibres with different ligand densities. The Phe-C fibres, which contained an identical amount of graft branches (110%) and different ligand densities of 0.2 (△) and 0.55 (○) mmol/g fibre, were equilibrated with the BGG solution.

In order to examine the possibility and extent of protein multi-point attachment on the Phe-C fibre with increasing ligand density, BGG was eluted from the Phe-C fibre with the Tris–HCl buffer containing various concentrations of NaCl. Decreasing salt concentration weakens the hydrophobic interaction between the Phe-C fibre and BGG [16]. As

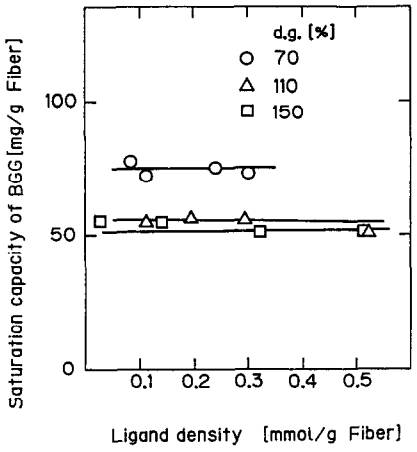

Fig. 8. Saturation capacity of BGG on Phe-C fibre as a function of ligand density. The ligand density ranged from 0.02 to 0.52 mmol/g for GMA grafting varying from 70% to 150%, and coupling efficiency ranging from 3% to 13%.

TABLE I

ELUTION RATIO FOR DIFFERENT ELUENTS AT VARIOUS LIGAND DENSITIES

Ligand density (mmol/g fibre)	Eluent		
	0.01 *M* Tris–HCl + 1 *M* NaCl	0.01 *M* Tris–HCl + 2 *M* NaCl	50 v/v% EG[a] + 1 *M* NaCl
0.101	79%	79%	100%
0.185	81%	80%	100%
0.463	82%	73%	94%

[a] Ethylene glycol.

shown in Table I, no significant difference in the elution ratio was observed in the range of the ligand density of 0.101–0.463 mmol/g. The mixture of 50 v/v% ethylene glycol and 1 *M* NaCl eluted BGG quantitatively. This indicates that the bond energy is almost constant, irrespective of the ligand density. However, it is difficult to explain the absence of multi-point attachment to the ligand.

The amount of BGG adsorbed non-selectively on the Phe-C fibre was *ca.* 10% of the amount adsorbed specifically (Fig. 6). Fig. 9 shows that the saturation capacity of BGG can be determined from the specific surface area of the porous hollow fibre when the ligand is in excess of BGG. The gamma globulin is a mixture of IgG and IgA. The predominant component, IgG, has a molecular mass of 150 000 and consists of four peptide chains: two identical heavy chains and two identical light chains. These chains are linked by strong disulphide bonds into a Y- or T-shaped structure with hinge-like flexible arms. Thus an IgG molecule would expand and contract significantly with the variation of the ionization of the ionizable groups in the molecule. When the molecule is fully charged, either at low pH or high pH, the arms can be completely extended because of charge repulsion. Where there is no intermolecular charge interaction, the arms would probably collapse, owing to an intermolecular attractive hydrophobic interaction [17]. Since the bottom end of the Y or T (the Fc portion) is much more hydrophobic than the tips of the Y or T (the $F(ab')_2$ portions) of the IgG molecules, end-on adsorption of IgG on the Phe-C is certainly favoured. There are thus two possible extreme conformations: arm-collapsed with end-on adsorption, and arm-extended with end-on adsorption. The theoretical saturation capacity, q_s, can be calculated from the following:

$$q_s = a_v M/(aN_A) \quad (6)$$

where a_v and a are the specific surface area and the cross-sectional area occupied by an IgG molecule, and M and N_A are the molecular mass of BGG and the Avogadro number. Theoretical lines for arm-collapsed and arm-extended adsorption are depicted in Fig. 9 by inserting the corresponding estimated projection area per molecule, 3900 and 10 700 Å^2. Our experimental results were situated between the two extreme conformations.

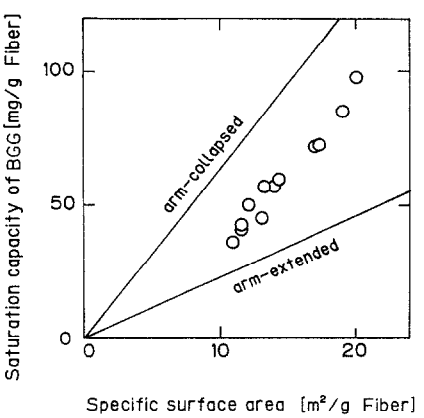

Fig. 9. Saturation capacity of BGG on Phe-C fibre as a function of specific surface area. The theoretical lines were determined from eqn. 6 for two extreme orientations: arm-collapsed and arm-extended adsorptions.

CONCLUSION

A porous hollow-fibre membrane containing L-Phe as a pseudo-biospecific ligand has been prepared by radiation-induced grafting of GMA onto a polyethylene microfiltration hollow-fibre membrane, and thereafter by coupling of the produced epoxide group with L-Phe. The remaining epoxide group was hydrolysed to a diol group with sulphuric acid.

The density of the diol group obtained from the degree of GMA grafting of at least 100% was necessary to minimize the non-selective adsorption of BGG onto the Phe-containing adsorbent based on polyethylene.

The molar ratio of the Phe ligand to BGG ranged up to 10^3. The excess ligand did not interfere with the ordinary adsorption.

The adsorption capacity for BGG of the resulting porous adsorbent could be determined by the specific surface area of the adsorbent when the ligand was in excess of BGG.

ACKNOWLEDGEMENT

The authors thank Kazuo Toyomoto of the Industrial Membrane Division of Asahi Chemical Industry Co., Ltd. for his help in providing the starting hollow fibre.

REFERENCES

1 E. Hochuli, *J. Chromatogr.*, 444 (1988) 293.
2 Z. Yamazaki, F. Kanai, Y. Idezuki, N. Yamawaki, K. Inagaki and N. Tsuda, *Jpn. J. Artif. Organs*, 16(1987) 1203.
3 P. D. G. Dean, W. S. Johnson and F. A. Middle (Editors), *Affinity Chromatography*, IRL Press, Oxford, 1985.
4 W. H. Scouten, *Affinity Chromatography*, Wiley, New York, 1981, p. 89.
5 K. Saito, T. Kaga, H. Yamagishi, S. Furusaki, T. Sugo and J. Okamoto, *J. Membrane Sci.*, 43 (1989) 131.
6 K. Saito, M. Ito, H. Yamagishi, S. Furusaki, T. Sugo and J. Okamoto, *Ind. Eng. Chem. Res.*, 28 (1989) 1808.
7 P. I. Forrester and R. L. Hancock, *Can. J. Biochem.*, 51 (1973) 231.
8 M. H. Gething, B. E. Davidson and T. A. A. Dopheide, *Eur. J. Biochem.*, 71 (1976) 317.
9 G. J. Doellgast and A. G. Plaut, *Immunochemistry*, 13 (1976) 135.
10 M. Kim, K. Saito, S. Furusaki, T. Sugo and J. Okamoto, *J. Membrane Sci.*, 56 (1991) 289.
11 B. H. J. Hofstee, *Biochem. Biophys. Res. Com.*, 63 (1975) 618.
12 Sigma Chemical Co., *Catalogue*, St. Louis, 1990, p. 1453.
13 H. Yamagishi, K. Saito, S. Furusaki, T. Sugo and J. Okamoto, *Nippon Kagaku Kaishi*, (1988) 212.
14 D. Cohn, A. S. Hoffman and B. D. Ratner, *J. Appl. Polym. Sci.*, 29 (1984) 2645.
15 B. Jansen and G. Ellinghorst, *J. Biomed. Mater. Res.*, 19 (1985) 1085.
16 J. Porath, L. Sundberg, N. Fornstedt and I. Olsson, *Nature (London)*, 245 (1973) 465.
17 B. Bagchi and S. M. Birnbaum, *J. Colloid Interface Sci.*, 83 (1981) 460.