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DNA-immobilized polyhydroxyethylmethacrylate microbeads for affinity sorption of human immunoglobulin G and anti-DNA antibodies

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Abstract

Polyhydroxymethacrylate (PHEMA) microbeads were prepared by a suspension polymerization technique and activated by CNBr in an alkaline medium (pH 11.5). DNA molecules were immobilized onto CNBr-activated PHEMA beads. The amount of immobilized DNA was controlled by changing the medium pH and the initial concentrations of CNBr and DNA. The maximum DNA immobilization was observed at pH 5.0. Non-specific adsorption on the plain PHEMA microbeads was less than 0.1 mg/g. Much higher values, up to 2.75 mg/g, were achieved with the CNBr-activated PHEMA microbeads. Human immunoglobulin G (HIgG) adsorption onto PHEMA microbeads containing different amounts of DNA on their surfaces from aqueous solutions containing different amounts of HIgG at different pH values was investigated. The maximum HIgG adsorption was observed at pH 7.0. Non-specific HIgG adsorption onto the plain PHEMA microbeads was low (about 0.167 mg/g). Higher adsorption values, up to 7.5 mg/g, were obtained with the DNA-PHEMA beads. HIgG and anti-DNA antibody removal from the blood plasma obtained from a healthy donor and a patient with systemic lupus erythematosus (SLE) were also investigated. The maximum amounts of HIgG adsorbed from aqueous solution and human plasma onto the DNA-PHEMA microbeads were 7.35 and 23.46 mg/g, respectively. Anti-DNA antibody adsorption value was 40 mg/g.

1. Introduction

Identification, purification and separation of complex biomolecules may be achieved by a number of traditional techniques, such as size-exclusion chromatography, high-performance liquid chromatography, chromatofocusing, electrophoresis, centrifugation, etc., which rely on differences in the physical properties (e.g. size, charge and hydrophobicity) of the molecules to be separated [1].

Affinity sorption is a relatively new alternative technique for separation of almost any biomolecule based on highly specific biological recognition [2,3]. Recently, affinity sorption has been applied with increasing frequency as an effective therapeutic method in clinical medicine, in which toxins from patient blood are removed by using biospecific sorbents in an extracorporeal circuit (so called "hemoperfusion") [4]. Enzymes, vita-

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mins, hormones, heparin, albumin, antigens and antibodies, DNA, protein A, and many other biologically active molecules have been incorporated with a variety of carrier systems and have been used for specific hemoperfusion therapy [5–7].

Systemic lupus erythematosus (SLE) is considered as an autoimmune disease [8]. Autoantibodies against DNA are of primary importance for the diagnosis and pathogenesis of SLE. The level of anti-DNA antibodies correlates well with the disease activity and organ involvements, such as nephritis and cerabritis. In such cases the removal of anti-DNA antibodies from plasma may lead to a clinical improvement. For this reason application of DNA immobilized sorbents has been considered as a novel therapeutic method.

The first ex-vivo application of DNA-attached sorbents was achieved by Terman et al. [9]. They removed anti-DNA antibodies from the plasma of positively immunized rabbits by circulating their blood through an extracorporeal shunt containing immobilized DNA. The first clinical trial of immunoadsorption was also done by Terman et al. in 1976, in which they used DNA immobilized sorbents to treat a female patient suffering from SLE [10]. Nikolaev et al. have successfully applied DNA-activated carbon hemoperfusion to the treatment of patients with psoriasis [11].

The present paper describes our recent efforts in the preparation of a bioaffinity sorbent containing DNA for selective removal of human IgG and anti-DNA antibodies from human plasma. We have selected polyhydroxyethylmethacrylate (PHEMA) based microbeads as the basic carrier, by considering possible applications of these sorbents in direct hemoperfusion extracorporeal therapy [12–14]. It should be noted that PHEMA is one of the most widely used hydrophilic polymers in medicine, and has found a wide variety of biomedical applications due to its high biocompatibility [15–17]. PHEMA in different forms have also been used for enzyme immobilization [18], for controlled release of drugs [19–21], immobilization of cells [22], in immunochemical studies [23] and as sorbents in chromatography [24–26].

2. Experimental

2.1. Preparation of PHEMA microbeads

The basic monomer, 2-hydroxyethylmethacrylate (HEMA), was purchased from Sigma (St. Louis, MO, USA), and was purified by vacuum distillation under a nitrogen atmosphere. The comonomer ethyleneglycoldimethacrylate (EGDMA, Merck, Darmstadt, Germany) was used as the crosslinking agent. The polymerization initiator was 2,2′-azobisisobutyronitrile (AIBN) (BDH, Dorset, UK). The dispersion medium was a saturated aqueous solution of magnesium oxide (MgO) (Sigma).

PHEMA microbeads were prepared by a suspension polymerization technique [12]. Polymerizations were carried out in an aqueous dispersion medium containing magnesium oxide which was used to decrease the solubility of the monomer, HEMA, in the medium. The monomer-phase containing HEMA, EGDMA and AIBN was added to the dispersion medium within a laboratory type reactor (i.e. a two-neck flask with a volume of 500 ml) provided with a blade-type stirrer. In order to produce polymeric microbeads of about 200 μm in diameter and with a narrow size distribution, the HEMA/EGDMA ratio, the monomer-phase/dispersion-phase ratio, the amounts of EGDMA and AIBN, and the agitation speed were 1:3 (v:v), 1:10 (v:v), 0.33 (mol EGDMA/mol HEMA), 0.0015 (mol AIBN/mol HEMA), 30 g, respectively. Polymerization was carried out at 70°C for 3 h and then at 90°C for 1 h. After cooling, the polymeric microbeads were separated from the polymerization medium and residual MgO was removed by washing with a dilute HCl solution. The microbeads were also washed with water and ethanol, and then dried in a vacuum desiccator at room temperature. Fig. 1 shows a scanning electron photograph of PHEMA microbeads used in the studies described in this paper.

2.2. CNBr activation

Prior to the activation process, the PHEMA microbeads were kept in distilled water for about

Fig. 1. Scanning electron photograph of PHEMA microbeads.

24 h and washed on a glass filter with 0.5 *M* NaCl solution and water in order to remove impurities. Cyanogen bromide (CNBr, Sigma) aqueous solutions (100 ml) with different initial concentrations (5–50 mg CNBr/ml distilled water) were prepared. The pH of this solution was quickly adjusted to 11.5 with 2 *M* NaOH while it was magnetically stirred. One gram of PHEMA microbeads was then added to this solution and the activation procedure was continued for 60 min at a constant pH of 11.5. After the activation reaction, in order to remove the excess activation agent, the PHEMA microbeads were washed with 0.1 *M* $NaHCO_3$ and any remaining active groups (e.g. isourea) on the surfaces were blocked by treatment with ethanolamine (pH 9, 1 *M*) and $FeCl_3$ solution for 1 h. Then, the activated PHEMA microbeads were washed four times with distilled water containing 0.5 *M* NaCl.

2.3. DNA immobilization

DNA (from herring testes) was purchased from Sigma. One gram of the freshly CNBr-activated PHEMA microbeads was magnetically stirred at a constant temperature of 20°C for about 4 h (i.e. equilibrium time) with 50 ml of a DNA solution. In order to observe effects of pH and the initial CNBr concentration on the covalent coupling of DNA, pH and the initial CNBr concentration were changed between 3.0 and 9.0, and 10 and 50 mg/ml, respectively. The initial concentration of DNA in the medium was 2 mg/ml. To measure the effects of DNA concentration on coupling, the initial concentration of DNA was changed between 0.02 and 2 mg/ml with the pH of the solution (containing 0.1 *M* $NaHCO_3$ + 0.5 *M* NaCl) being 5.0.

In each experiment, after coupling, the DNA-immobilized PHEMA microbeads were washed with 0.1 *M* CH_3COONa (pH 4.5) + 0.15 *M* NaCl, and finally with 0.1 *M* $NaHCO_3$ (pH 9.5) + 0.5 *M* NaCl. The amount of DNA immobilized on the CNBr-activated microbeads was determined by measuring the decrease of the DNA concentration and also by considering the DNA molecules adsorbed non-specifically (not through the active sites), by spectrophotometry [12].

2.4. HIgG adsorption from aqueous solutions

Adsorption of human IgG (HIgG, Sigma, Cat. No: 160101) on the DNA-immobilized PHEMA microbeads (the DNA-PHEMA microbeads) was studied in batch experiments. One gram of DNA-PHEMA microbeads containing 2.75 mg DNA per gram of PHEMA was incubated with 50 ml of HIgG solution at 20°C for 4 h (i.e. equilibrium time). In order to study the effects of the pH on adsorption of HIgG, the pH of the adsorption medium was varied between 6.0 and 9.0. The initial concentration of HIgG in the adsorption medium was 0.20 mg/ml.

In order to study the effects of the amount of immobilized DNA on the adsorption of HIgG, DNA-PHEMA microbeads containing between 0.45 and 2.75 mg DNA/g PHEMA were incubated with the adsorption medium containing 0.20 mg HIgG/ml at pH 7.0.

Adsorption of HIgG from the media containing different amounts of HIgG (between 0.05 and 0.40 mg/ml) at pH 7.0 was also studied. DNA-PHEMA microbeads containing 2.0 mg DNA/g PHEMA were used.

In order to remove the non-specifically adsorbed HIgG molecules, after the HIgG adsorption, in each adsorption experiment mentioned above, the PHEMA microbeads were washed with 0.0625 *M* borate buffer (pH 8.8) + 0.15 *M* NaCl, with 2 *M* urea + 0.15 *M* NaCl, and finally with 0.1 *M* $NaHCO_3$ (pH 9.5) + 0.5 *M* NaCl. The amount of HIgG adsorbed through DNA on

the PHEMA microbeads was determined by the Lowry method by measuring the decrease in the HIgG concentration and also by considering the HIgG molecules non-specifically adsorbed (not through the active sites).

2.5. HIgG and anti-DNA antibody adsorption from human plasma

Blood samples taken from a healthy donor and a patient with systemic lupus erythematosus were used in these studies. Blood samples were centrifuged at 500 *g* for 30 min at room temperature to separate the plasma. A 0.2-g amount of DNA-PHEMA microbeads containing different amounts of DNA (between 0.7 and 2.75 mg DNA/g PHEMA) was incubated at 20°C for 20 min with 2 ml of the plasma obtained either from the healthy donor or the patient.

The original plasma of the healthy donor contained 10.5 mg HIgG/ml, as determined by a solid-phase enzyme-linked immunosorbent assay method (ELISA). The amount of HIgG adsorbed after the incubation period was obtained by measuring the decrease in the HIgG concentration in the plasma by the same assay.

The amount of anti-DNA antibody adsorbed on the DNA-PHEMA microbeads was determined by RIA by measuring the decrease in the anti-DNA antibody concentration in the plasma of the patient.

3. Results and discussion

3.1. DNA immobilization

In the first part of this study, effects of the medium pH, initial concentrations of CNBr and DNA on the immobilization of DNA onto the PHEMA microbeads were investigated in batch experiments. Effects of each parameter are presented separately in the following sections.

Effect of pH

This group of experiments were performed at pH values between 3.0 and 9.0 with the PHEMA microbeads activated by using solutions containing 10 mg CNBr/ml. The initial concentration of DNA in the incubation medium was 2 mg/ml. As shown in Fig. 2 the effect of pH on DNA immobilization is important. The saturation plateau of DNA (2.75 mg DNA/g PHEMA) was observed at a pH of 5.0. Note that both the three dimensional structure and the ionization degree of several groups on the DNA molecules can change with pH. At pH 5.0 DNA molecules probably have the most suitable structural properties for binding through CNBr active sites on the surface of PHEMA microbeads. Significantly lower immobilization capacities were obtained below and above pH 5.0.

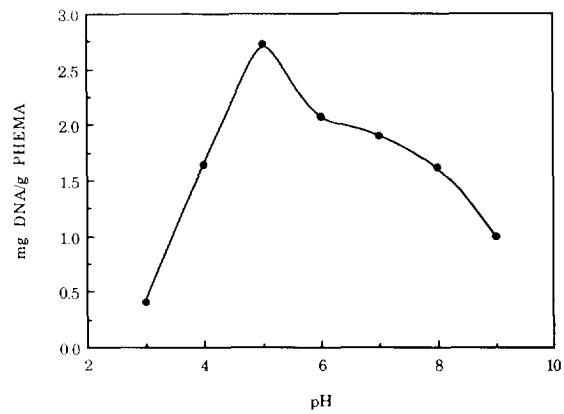

Fig. 2. Effect of incubation medium pH on DNA immobilization onto PHEMA microbeads.

Effect of initial CNBr concentration

The initial CNBr concentration in the activation medium was changed between 5 and 50 mg/ml in order to change the number of active sites on the PHEMA microbeads, and to immobilize different amounts of DNA on their surfaces. The initial concentration of DNA in the incubation medium was 2 mg/ml and the medium pH was 5.0.

Fig. 3 shows the effects of the initial CNBr concentration on DNA immobilization. The amount of DNA immobilized on the carrier first increased, then reached a saturation value of 2.75 mg DNA/g PHEMA at an initial concentration of 10 mg CNBr/ml. Note that a higher initial CNBr concentration corresponds to a

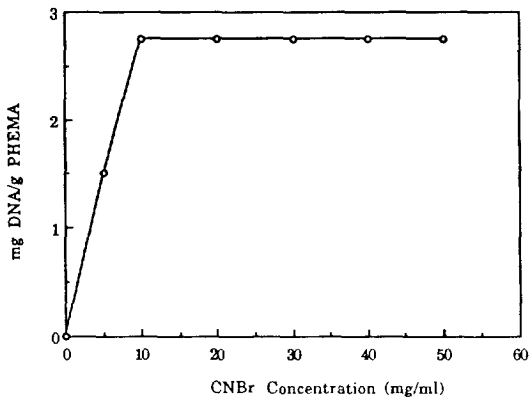

Fig. 3. Effect of CNBr concentration on DNA immobilization.

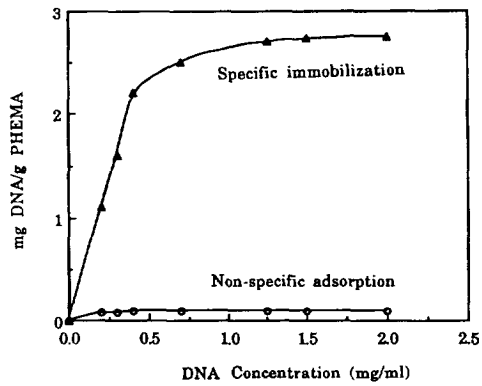

Fig. 4. Effect of DNA concentration on DNA immobilization onto PHEMA microbeads.

higher number of activated sites on the carrier. Therefore, it was expected that the amount of DNA would increase with an increase in the initial CNBr concentration. However, due to the size of the giant DNA molecules (molecular mass is approximately $1 \cdot 10^7$), it was not possible to immobilize more than 2.75 mg DNA/g PHEMA. It should be noted that the non-specific adsorption (i.e. adsorption on the non-activated PHEMA microbeads) was very low, only 0.1 mg DNA/g PHEMA, which may be considered as one of the advantages of using PHEMA microbeads as the carrier

Effect of initial DNA concentration

In this group of experiments, DNA adsorption onto the PHEMA microbeads was performed from aqueous solutions containing different amounts of DNA (0.2–2.0 mg/ml) at a pH of 5.0. Note that the PHEMA microbeads were activated in a medium containing 10 mg CNBr/ml.

Fig. 4 shows both non-specific adsorption (i.e. adsorption on the non-activated PHEMA microbeads) and specific immobilization (i.e. immobilization through active sites) onto the PHEMA microbeads. The non-specific adsorption was low, about 0.1 mg DNA/g PHEMA, while specific immobilization of DNA molecules first increased and then reached a saturation value (at 2.75 mg DNA/g PHEMA) at an initial DNA concentration of 2.0 mg/ml, as expected.

Optimal conditions for DNA immobilization

In the present study, effects of medium pH, and the initial CNBr and DNA concentrations on the immobilization of DNA onto the PHEMA microbeads were investigated. The optimal conditions for DNA immobilization are as follows: pH, 5.0; temperature, 20°C; CNBr concentration, 10 mg/ml; and DNA concentration, 2 mg/ml. The specific sorbents prepared at these conditions contain 2.75 mg DNA/g PHEMA.

3.2. HIgG adsorption from aqueous solutions

In this study, human IgG adsorption onto the DNA-PHEMA microbeads containing different amounts of immobilized DNA, from aqueous solutions containing different amounts of HIgG and at different pH values was studied. Effects of three different parameters on adsorption are discussed separately in the following sections.

Effect of pH

Adsorption experiments were performed at different pH values (6.0–9.0). The specific sorbents prepared under optimal conditions contained 2.75 mg DNA/g PHEMA. The initial concentration of HIgG in the adsorption medium was 0.2 mg/ml.

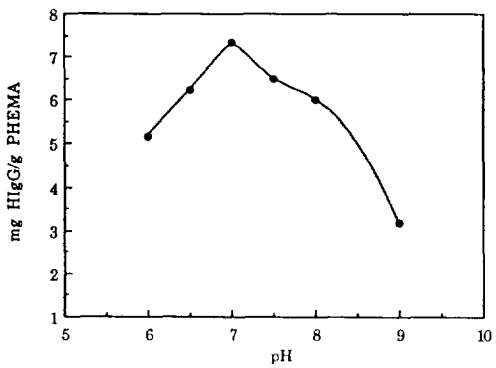

Fig. 5. Effect of pH on HIgG adsorption onto DNA-PHEMA microbeads.

Fig. 5 illustrates the effect of pH. The maximum adsorption of HIgG was observed at pH 7.0 (i.e. 7.35 mg HIgG/g DNA-PHEMA). Significantly lower immobilization capacities were obtained below and above this pH. Most probably, at pH 7.0, the structures of both the immobilized ligand (DNA) and the biomolecule to be removed (HIgG) had the most suitable conformations for coupling.

Effect of DNA surface concentration

In this group of experiments, HIgG adsorption onto the DNA-PHEMA microbeads carrying different amounts of DNA on their surfaces (0.45–2.75 mg DNA/g PHEMA) was investigated. Note that the initial concentration of HIgG in the adsorption medium and the medium pH were 0.2 mg/ml and 7.0, respectively.

Table 1
Effect of DNA surface concentration on HIgG adsorption

Amount of DNA immobilized (mg/g)	Amount of HIgG adsorbed (mg/g)
0.45 ± 0.05	1.28 ± 0.21
0.70 ± 0.07	2.70 ± 0.34
1.05 ± 0.04	3.60 ± 0.27
1.60 ± 0.08	4.50 ± 0.19
1.90 ± 0.04	5.20 ± 0.30
2.00 ± 0.01	7.00 ± 0.20
2.75 ± 0.01	7.35 ± 0.10

Table 1 shows the effect of DNA surface concentration on HIgG adsorption onto PHEMA microbeads. When the surface concentration of DNA increased the amount of HIgG coupled to the carrier also increased, as expected. The maximum amount of HIgG adsorbed was 7.35 mg/g DNA-PHEMA. There were no significant differences in adsorption capacity above the DNA surface concentration of 2.0 mg DNA/g PHEMA, due to the possible steric hindrance at a high ligand surface concentration.

Effect of initial HIgG concentration

In this experiment, HIgG adsorption onto the plain PHEMA beads and PHEMA microbeads containing 2.0 mg DNA/g PHEMA from aqueous solutions containing different amounts of HIgG (0.05–0.4 mg/ml) was studied at a constant pH of 7.0. Fig. 6 shows the non-specific and specific adsorption on the PHEMA microbeads. There was no pronounced adsorption (i.e. non-specific adsorption) on the plain PHEMA microbeads (0.1 mg HIgG/g PHEMA). However, significantly higher adsorption (i.e. specific adsorption) on DNA-PHEMA microbeads (up to 7.0 mg HIgG/g PHEMA) was observed. Note that the amount of HIgG coupled to the DNA-PHEMA microbeads via DNA molecules reached almost a plateau value at 0.2 mg/ml, possibly due to steric hindrance.

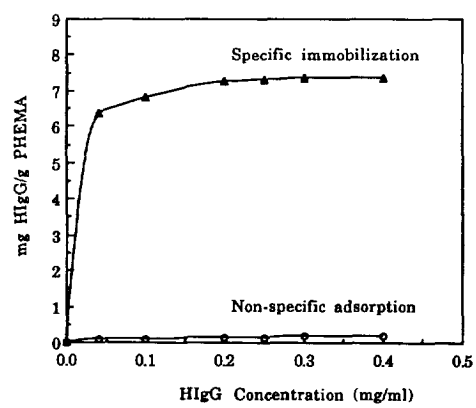

Fig. 6. Effect of HIgG concentration on HIgG adsorption onto PHEMA microbeads.

3.3. HIgG and anti-DNA antibody removal from human blood plasma

Blood samples taken from a healthy donor and a patient with systemic lupus erythematosus were used in this group of studies. A 2.0-ml volume of the plasma separated from the blood samples was incubated with 0.2 g of DNA-PHEMA microbeads containing different amounts of DNA (between 0.7–2.75 mg DNA/g PHEMA), at 20°C for 20 min.

HIgG removal

Table 2 shows the adsorption data for the HIgG removal from plasma obtained from the healthy donor. There was a very low non-specific adsorption of HIgG (0.22 mg HIgG/g PHEMA) on the plain PHEMA microbeads, while much higher adsorption values (up to 23 mg HIgG/g DNA-PHEMA) were observed when the DNA immobilized PHEMA microbeads were used. Note that adsorption of HIgG onto the PHEMA microbeads containing 2.75 mg DNA/g PHEMA from human plasma was approximately 3.5-fold higher (23.46 mg HIgG/g PHEMA) than those obtained in the studies in which aqueous solutions were used. This may be due to the conformation of the HIgG molecules within their native medium (i.e. plasma, pH 7.4) being much more suitable for interaction with the DNA molecules on the PHEMA microbeads. The high concentration of HIgG in the plasma (10.5 mg/ml) may contribute to this high absorption value.

Anti-DNA antibody removal

Table 3 shows the amount of anti-DNA antibodies adsorbed onto the PHEMA microbeads. There was a very low non-specific adsorption of anti-DNA antibodies (0.19 mg anti-DNA antibody/g PHEMA) onto the plain PHEMA microbeads, while much higher adsorption values (up to 40 mg anti-DNA antibody/g DNA-PHEMA) were observed when DNA immobilized PHEMA microbeads were used. Adsorption increased with increasing surface concentration of DNA. However, there was no significant change in the adsorption capacity above a certain DNA surface concentration (at 2.0 mg/g). Note that anti-DNA antibody adsorption from the patient plasma was approximately 7-fold higher than HIgG adsorption from the healthy donor plasma. There are two reasons for the difference in the adsorption values of HIgG and anti-DNA antibodies: (i) as expected DNA molecules exhibit much higher affinity for anti-DNA antibodies than HIgG, (ii) anti-DNA antibody concentration (10.5 mg/ml) was much higher than HIgG concentration (0.20 mg/ml), which determines the extent of the adsorption.

Table 2
HIgG removal from the plasma of a healthy donor

Amount of DNA immobilized (mg/g)	Amount of HIgG adsorbed (mg/g)
0.00	0.22 ± 0.04
0.70 ± 0.07	4.75 ± 0.17
1.60 ± 0.08	9.17 ± 0.54
2.00 ± 0.01	18.43 ± 0.27
2.75 ± 0.01	23.46 ± 1.17

Table 3
Anti-DNA antibody removal from the plasma of a patient with SLE

Amount of DNA immobilized (mg/g)	Amount of anti-DNA antibody adsorbed (mg/g)
0.00	0.19 ± 0.22
0.70 ± 0.07	7.99 ± 0.37
1.60 ± 0.08	25.83 ± 1.14
2.00 ± 0.01	39.71 ± 0.40
2.75 ± 0.01	40.08 ± 0.48

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References

[1] M.P. Deutscher (Editor), *Guide to Protein Purification, Methods in Enzymology, Vol. 182*, 1990.

[2] P.V. Sundaram and F. Eckstein, *Theory and Practice in Affinity Techniques*, Academic Press, New York, NY, 1978.

[3] J.K. Inman, in I.M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, Orlando, FL, 1983.

[4] S. Sideman and T.M.S. Chang, *Hemoperfusion: Kidney and Liver Support and Detoxification*, Hemisphere, Washington D.C., 1980.

[5] H. Klinkman, D. Falkenhagen and J.M. Courtney, *Int. J. Artif. Organs*, 2 (1979) 296.

[6] D.S. Terman, T. Travel, D. Petty, M.R. Racic and G. Buffaloe, *Clin. Exp. Immunol.*, 28 (1977) 180.

[7] P.K. Ray, *Plasma Therap. Transfusion Technol.*, 4 (1983) 289.

[8] E.M. Tan, P.H. Shur, R.I. Carr and H.G. Kunkel, *J. Clin. Invest.*, 45 (1966) 1732.

[9] D.S. Terman, H. Harbeck, A. Hoffman, I. Steward, J. Robinetto and R. Carr, *Fed. Proc.*, 34 (1975) 976.

[10] D.S. Terman, I. Steward, J. Robinette, R. Carr and R. Harbeck, *Clin. Exp. Immunol.*, 24 (1976) 231.

[11] V.G. Nicolaev, V.V. Sarnatskaya, E.V. Eretskaya, E.A. Snezhkova and N.V. Belitser, *Clin. Mat.*, 11 (1992) 125.

[12] A. Denizli, Ph.D. Thesis, Hacettepe University, Ankara, Turkey, 1992.

[13] A. Denizli and E. Pişkin, *Clin. Mat.*, 11 (1992) 129.

[14] A. Denizli, A.Y. Rad and E. Pişkin, *J. Chromatogr. B*, 1994 (accepted to publication).

[15] N.A. Peppas (Editor), *Hydrogels in Medicine and Pharmacy, Vol. 1*, CRC Press, Boca Raton, FL, 1987.

[16] J.D. Andrade (Editor), *Hydrogels for Medical and Related Applications, American Chemical Society, Washington DC, ACS Symp. Ser., No. 31*, 1976.

[17] B.J. Tighe, in N.A. Peppas (Editor), *Hydrogels in Medicine and Pharmacy, Vol. 2*, CRC Press, Boca Raton, FLorida, 1987, p. 53.

[18] M.Y. Arıca and V. Hasırcı, *Biomaterials*, 14 (1993) 803.

[19] A. Denizli, M. Kiremitçi and E. Pişkin, *Biomaterials*, 9 (1988) 257.

[20] A. Denizli, M. Kiremitçi and E. Pişkin, *Biomaterials*, 9 (1988) 363.

[21] M. Kiremitçi, A. Denizli, A. Öncü, E. Ertürk and E. Pişkin, *Life Support Systems*, 4 (1986) 405.

[22] V. Dixit, A. Denizli, M. Arthur, G. Gitnick and E. Pişkin, *Cell Transpl.*, 1 (1992) 391.

[23] A. Rembaum, S.P.S. Yen and R.S. Molday, *J. Macromol. Sci. Chem.*, A13 (1979) 603.

[24] A. Denizli, M. Olcay, M. Kiremitçi and E. Pişkin, *Artif. Organs*, 14 (1990) 138.

[25] A. Jayakrishnan, M.C. Sunny and B.C. Thanoo, *Polymer*, 31 (1990) 1339.

[26] A. Denizli, M. Kiremitçi and E. Pişkin, *Biomat. Artif. Cells Immobil. Biotechnol.*, 21 (1993) 183.