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Macroporous hydrogels based on 2-hydroxyethyl methacrylate

Part III *Hydrogels as carriers for immobilization of proteins*

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Four series of macroporous hydrogels based on crosslinked copolymers of 2-hydroxyethyl methacrylate (HEMA)—sodium methacrylate (MANa), copolymer HEMA—[2-(methacryloyloxy)ethyl]trimethylammonium chloride (MOETACl), terpolymer HEMA—MANa—MOETACl and on a polyelectrolyte complex were used as carriers for immobilization of proteins, chicken egg white albumin and avidin. The adsorption capacity of the hydrogels for the two proteins, kinetics and pH dependence of albumin adsorption and desorption were studied. The morphology of the hydrogels with and without immobilized albumin was studied by low-vacuum scanning electron microscopy.
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1. Introduction
Cell technologies have achieved exponential growth in the last ten years. The cultured cells including human cells are employed in biology for study of the function of tissue systems under defined conditions and in industry for assessment of biological safety of pharmaceutical and cosmetic products and for production of new therapeutic substances using biotechnological procedures. The use of cultured cells makes it possible to minimize the number of laboratory animals and human volunteers and enhances the safety of new medical technologies and products. New horizons for technologies have opened by extensive research on embryonic/organ stem cells. These cells need a well-defined niche, which includes also molecular microenvironment encoded by these elements as necessary for maintenance of the stem cell phenotype. Similarly, specific microenvironment is necessary for the differentiation of stem cells to desired cell lines [1]. The extensive use of cells in medical technologies including cell therapy will also increase the exploitation of cultured cells in medicine [2].

A majority of nontransformed animal and human cells can grow only after their adhesion to cultivation supports. Many authors extensively studied this process and the results were summarized by Smetana *et al.* [3, 4]. A mere support is not sufficient for cell adhesion and subsequent cultivation. The cells need the presence of biologically active pro-adhesive molecules (proteins, glycoproteins and polysaccharides) on the surface of polymer supports, which are recognized and decoded by cell surface receptors. These bioactive substances are usually adsorbed on the support surface from the cultivation medium. Only polymers exhibiting appropriate physical properties stimulate adsorption of biological substances in correct conformation, where the biologically active parts of these molecules are accessible to cell surface receptors.

Suitable bioactive materials can be prepared as polymer composites or, particularly, by immobilization of biologically active molecules or their parts on a polymer support appropriate for a specific application due to its mechanical properties, durability, non-toxicity, etc. Covalent attachment of biologically active molecules to reactive polymer groups is stable and the biological activity of ligands can be preserved [4, 5]. However, this process is rather complex and the spectrum of immobilized bioactive molecules cannot be changed for various applications. In contact with physiological fluids or cell culture media, immobilized small active molecules can be easily overlayered by large proteins with low

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biological activity (such as albumin) and hence their accessibility considerably reduced [6].

The main aim of this paper is a model study of protein adsorption on the surface of macroporous hydrogels. The used proteins were selected based on their different values of the isoelectric point. The second aim is to verify the posibility of the new bioactive cultivation carrier preparation by an inexpensive and simple immobilization of bioactive molecules on the surface of macroporous hydrogels prepared earlier [7, 8]. The preservation of biological activity of immobilized ligands and their accessibility to seeded cells is assumed to be a necessary feature of the technology. The system avidin-biotin could be a good candidate for the cultivation support preparation, because they form complexes, which have been used in histochemistry for many years, with excellent results and high selectivity [9].

2. Materials and methods

The macroporous hydrogels were prepared by the method described earlier [7, 8], using the fraction of sodium chloride below 30 μm, 30–50 and 50–90 μm. Four series of hydrogels were studied:

Series 1: copolymer 2-hydroxyethyl methacrylate (HEMA)—sodium methacrylate (MANa)
Series 2: copolymer HEMA—[2-(methacryloyloxy) ethyl]trimethylammonium chloride (MOETACl)
Series 3: terpolymer HEMA—MANa—MOETACl
Series 4: polyelectrolyte complex obtained by the reaction of Series 1 copolymers with linear poly(MOETACl)

The contents of ionogenic comonomers (MANa, MOETACl) in macroporous hydrogels were 1–17 mol%.

The immobilization of proteins (albumin and avidin, from chicken egg white, Sigma) was carried out in a physiological solution, or in the presence of 0.667 M phosphate buffers (pH 5–8). A sample of hydrogel (dry weight m_H) was put into a solution (volume V_0) with a known concentration of protein (c_0) and maintained there for 12 h. Then the concentration of protein in the solution was determined (c) by standard Bredford's spectrophotometric method [10] using a Helios Beta spectrometer (Thermo Spectronic, Great Britan). The capacity of hydrogel carrier for protein immobilization (S) was calculated as

$$S = (V_0 c_0 - V_0 c)/m_H, \quad c_0 = 1 \text{ g}/100 \text{ ml}$$
$$m_H = 0.02 \text{ g} \quad V_0 = 20 \text{ ml}$$

For the study of kinetics of adsorption, a hydrogel sample (cube 0.34 cm^3) was put into a protein solution and, at given time intervals, the protein amount immobilized on hydrogel (S_i) was calculated as

$$S_i = \frac{\left(V_0 c_0 - \sum_{i=1}^{i-1} V' c_i\right) - V_i c_i}{m_H},$$

where V' is the volume of the sample for spectroscopy measurement (0.1 ml), c_i concentration of protein in solution at the time t_i, V_i volume of solution at the time t_i.

Morphology of macroporous hydrogels was studied using a low-vacuum electron microscope AquaSEM (Tescan, Czech Republic) described earlier [7, 8].

3. Results and discussion

Fig. 1 shows the kinetics of albumin adsorption on macroporous hydrogels for the four series. It follows from the figure that a major part of albumin (90–95%) is immobilized during first 3–4 h and this time period does not depend on the structure of carrier. The amount of non-electrostatic adsorbtion of albumin on poly(HEMA) is 8.75 mg protein per 1 g of dry hydrogel.

However, the amount of immobilized albumin strongly depends on the composition of hydrogel carrier in the order Series 2 $\gg$ Series 3 $\sim$ Series 4 > Series 1 and increases with increasing content of charged groups on the carrier (Fig. 2) especially for hydrogels with quaternary ammonium groups (Series 2, 3). The hydrogel carrier based on a polyelectrolyte complex immobilizes albumin almost independently of the charged group content in polymer chain. In this complex all negative charges are saturated by a very strong interaction with positive charges of poly(MOETACl) [8]; this interaction is much stronger than the interaction of weakly charged parts of protein with the charges of the hydrogel carrier. Thus, the immobilization of albumin

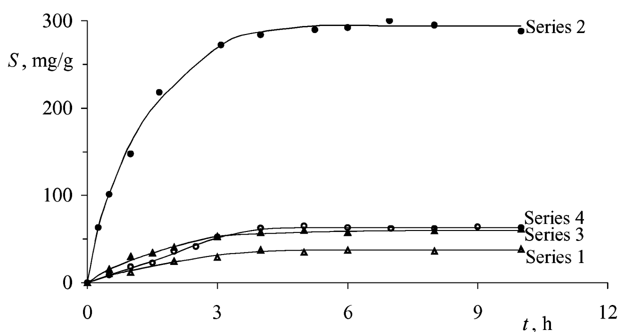


Figure 1 Time dependence of adsorption capacity of hydrogel carrier (S, mg of protein per g of dry hydrogel) for albumin. Ionogenic comonomer content 9.59 mol%, average pores size 40 μm, pH 7.

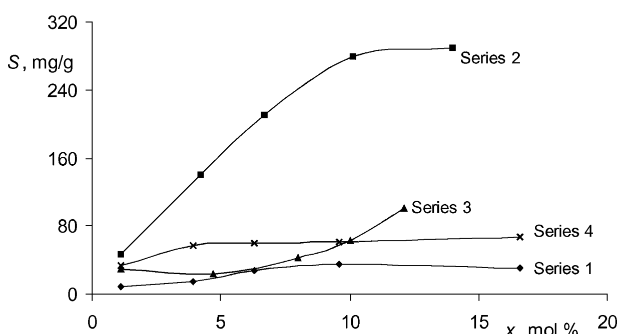


Figure 2 Dependence of adsorption capacity of hydrogel carriers for sorption of albumin on the ionogenic comonomer content. Average pore size 40 μm, pH 7.

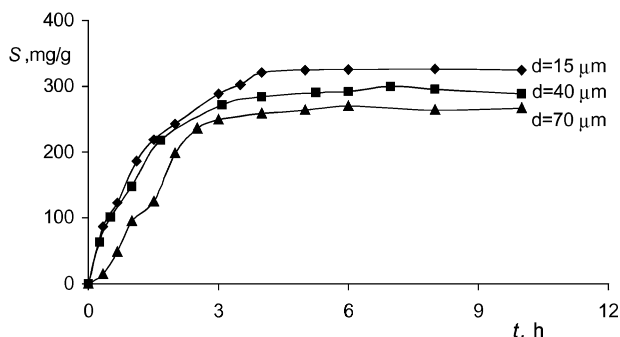


Figure 3 Dependence of adsorption capacity of Series 2 macroporous hydrogels with 9.59 mol% of MOETACl at three pore sizes, pH 7.

TABLE I Dependence of adsorption capacity of albumin on hydrogel carriers with 9.59 mol% of ionic comonomers on pH. Average pores size 40 μm

pH	Series 1	Series 2	Series 3	Series 4
5	34.6	307.5	67.6	63.3
6	35.3	301.7	61.9	64.3
7	37.6	295.4	63.3	65
8	40.2	288.3	67	65.5

by the Series 4 carrier is almost non-electrostatic, i.e. adsorption proceeds on the whole surface of hydrogel and the amount of immobilized protein is only little affected by chemical composition of the polyelectrolyte complex. Consequently, this amount of immobilized protein is higher than in a hydrogel without charges based on poly(HEMA) (8.75 mg/g mentioned above). The polyelectrolyte complex positively influences the adsorption.

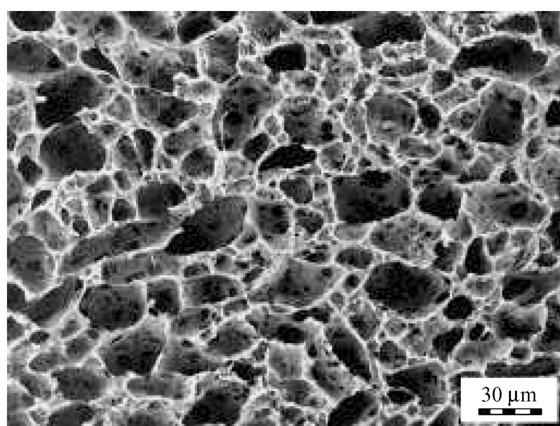
The fact that the gel with positive charges exhibits the strongest adsorption (Fig. 2) is not surprising, because albumin is a protein with a low isoelectric point (p*I* 4.9) [11] and the interaction of its acid groups with positive charges of the hydrogel carrier predominates over the interaction of basic groups with negative charges of the hydrogel. If the hydrogel contains both positive and negative charges (Series 3), its sorption capacity for albumin lies just between the values for hydrogels of Series 1 and 2.

Protein immobilization by the hydrogel carrier is strong, reversible desorption does not proceed. After immobilization, a hydrogel sample (capacity 295.4 mg of albumin per 1 g of dry hydrogel, Series 2 with 9.59 mol% of ionogenic comonomer) was put into fresh physiological solution and, after 48 h the concentration of protein in solution was measured. The solution contained 0.2 mg protein/100 ml solution. This amount issues probably from the non-immobilized protein in pores of hydrogel. After a third washing of the hydrogel with physiological solution, no protein in solution could be found.

Table I shows the pH dependence of albumin adsorption on hydrogel carriers with 9.59 mol% of ionic comonomers. Although the protein adsorption on neutral surface is the highest at the isoelectric point [12], in our case this applies to immobilization of albumin by the Series 2 hydrogel. In contrast, the adsorption of albumin on the Series 3 and 4 hydrogels is almost pH-independent and, for the negatively charged hydrogels (Series 1), the adsorption in the isoelectric point is even the lowest. With increasing pH, the dissociation of carboxyl groups of the hydrogel carrier rises and the interaction of carboxyls with albumin amino groups increases.

Kinetics of albumin adsorption on macroporous Series 2 hydrogels of three different porosities containing 9.59 mol% of MOETACl are shown in Fig. 3. Due to the fact that the overall pore surface decreases with their average size [8], the increase in capacity with decreasing pore dimensions, hence with increasing surface area of all pores could be expected. In reality, however, the capacity grows with growing size of pores. The fact can be ascribed to the advantage of the high-molecular-weight protein to occupy sufficient space inside the pore rather than to the high surface area of the hydrogel.

Fig. 4 shows the morphology of Series 2 hydrogel carriers containing 6.34 mol% of MOETACl without (a) and with adsorbed protein (b). As follows from almost identical morphologies, aggregation of protein does not occur as individual protein molecules cannot be observed on electron micrographs at given resolution.



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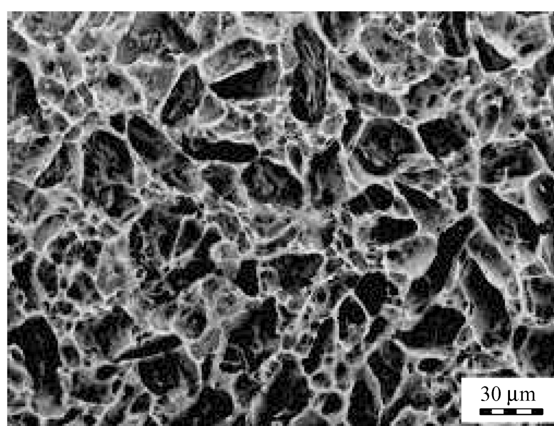

b

Figure 4 AquaSEM microphotographs (see refs. [7, 8] for experimental details) of a Series 2 hydrogel with 6.34 mol% of MOETACl. (a) hydrogel without albumin, (b) hydrogel with immobilized albumin.

TABLE II Capacity of hydrogels with 9.59 mol% of charged comonomers. Average pores size 40 μm, pH 7

Hydrogel	Capacity (mg/g)
Series 1	282.5
Series 2	77.5
Series 3	138.0
Series 4	104.0

Capacities of the measured hydrogels containing 9.59 mol% of ionogenic comonomer for adsorption of avidine are given in Table II. Due to the high isoelectric point of avidine (*pI* 10.5) [13], the carboxyl-containing hydrogel (Series 1) shows the highest and the hydrogel with quaternary ammonium groups (Series 2) the lowest immobilization capacity for avidine.

From a comparison of capacities for avidine (Table II) and albumin (Fig. 2) follows that avidine adsorbs much more on Series 3 and 4 hydrogel carriers. The presence of positive charges hence affects the avidine adsorption much less than the presence of negative charges does in albumin adsorption. At the same time, the non-electrostatic adsorption of avidine on hydrogel without charge (8.3 mg/g dry hydrogel) is almost identical with the albumin adsorption (8.75 mg/g). Hence, only specific adsorption on charged groups of the hydrogel carrier is responsible for enhanced adsorption of avidine.

4. Conclusion

The synthesized macroporous hydrogels are suitable for immobilization of both the investigated proteins: hydrogels with positive charges are most appropriate for albumin while those with carboxylic groups for avidine. The amount of immobilized protein increases with increasing amount of ionogenic comonomer in the hydrogel. After immobilization, the proteins do not aggregate and hence their biomedical applications are possible.

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