PREPARATION OF UNIFORM SIZE BEADS WITH ENHANCED HYDROPHILICITY OF PORE SURFACE, BASED ON MACROPOROUS POLY(GLYCIDYL METHACRYLATE-*co*-ETHYLENE DIMETHACRYLATE)

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Dedicated to Professor Otto Wichterle on the occasion of his 80th birthday.

Relative hydrophilicity of poly(glyceryl methacrylate-*co*-ethylene dimethacrylate) beads before and after modification with epichlorohydrin followed by hydrolysis has been studied. The hydrophilicity was determined from the retention of human serum albumin in a column packed with the polymers using various mobile phases. Hydrophilization dramatically decreases the extent of human serum albumin adsorption and provides beads suitable for size-exclusion HPLC of hydrophobic proteins.

Developed in 1950's, hydrophilic porous beads based on polysaccharides such as cellulose, dextran or agarose revolutionized separation techniques in biochemistry and opened a new era of chromatography[1]. Synthetic porous polymers were first used for size exclusion chromatography[2] in 1964; however, the crosslinked polystyrene matrix was rather hydrophobic and could not be used for separation in aqueous media. Introduction of HPLC brought separation media in form of micrometer-sized porous beads based on silica and polystyrene[3]; their limited pH stability and high hydrophobicity, respectively, largely disqualified them for use in separation of biopolymers (peptides, enzymes, nucleic acids, etc.) due to the requirements necessary for preserving their biological activity. Search for hydrophilic separation media more suitable for aqueous HPLC continued over the decades and resulted in various packings with enhanced hydrophilicity. The achievement of Czech scientists in this area, based on the pioneering work of Wichterle with 2-hydroxyethyl methacrylate (HEMA)[4], was amazing. At the beginning of 1970's, Čoupek developed and commercialized an excellent beaded separation medium for HPLC in aqueous medium prepared by suspension polymerization of HEMA (ref.[5]).

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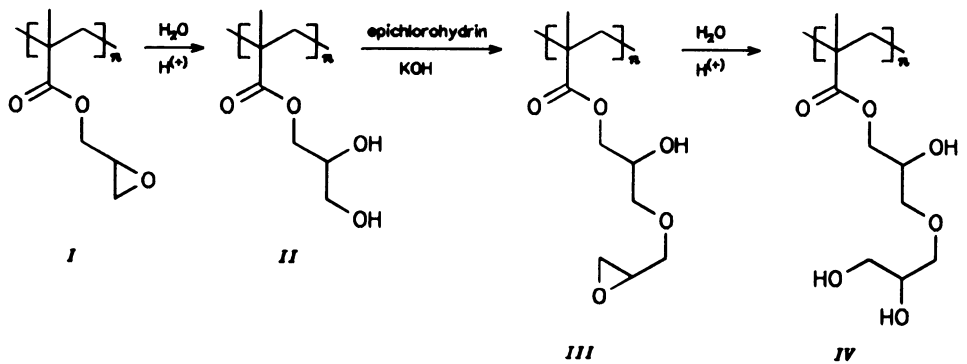
Similar separation media based on glycidyl methacrylate were also developed[6] in Prague in the mid-1970's.

Further development of the HPLC separation media for biopolymers was aimed at improvement of their performance and resulted in hydrophilized monodisperse polystyrene beads[7] commercialized in Sweden as FPLC™ by Pharmacia[1]. In order to avoid using hydrophobic polystyrene core of FPLC beads, we have recently developed a method producing uniformly sized beads with controlled pore size distribution based on more hydrophilic poly(glyceryl methacrylate-*co*-ethylene dimethacrylate)[8,9]. Though the copolymer is rather hydrophilic, the matrix is still not hydrophilic enough to completely avoid hydrophobic type interactions with the aliphatic polymer backbone exposed on the pore surface. Therefore, an additional treatment is desirable to shield the hydrophobic areas of the pore surface and thus further decrease the protein adsorption.

This short communication concerns the reaction of uniform-size porous hydrolyzed poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) beads with epichlorohydrin followed by an acid-catalyzed hydrolysis of the additionally attached epoxide groups (Scheme 1). The modification results in beads with dramatically decreased non-specific albumin sorption. These beads are better suited for size-exclusion high-performance liquid chromatography of proteins.



SCHEME 1

EXPERIMENTAL

Preparation of Polymer Beads

Uniform-size porous 10 μm poly(glyceryl methacrylate-*co*-ethylene dimethacrylate) (*II*) beads were prepared by swelling monodisperse polystyrene shape-template particles with glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) in various ratios and with porogenic solvents followed by a suspension polymerization[8,9]. Epoxide groups were then hydrolyzed keeping the poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (*I*) beads in aqueous 0.1 mol/l perchloric acid at room temperature for 120 h.

Hydrolyzed beads prepared from 60 vol.% GMA and 40 vol.% EDMA were used for reaction with epichlorohydrin. Dry beads (4 g) were dispersed in 40 ml water and after about 12 h the excessive water was removed by filtration. The beads were redispersed in 50 wt.% aqueous KOH (40 ml) and left to swell for 1 h. Suction-dried beads were transferred into a flask containing a solution of 20 ml epichlorohydrin in 20 ml water or dioxane. The reaction mixture was then heated to 60 °C for 3 or 8 h while stirred with an overhead stirrer.

Activated beads *III* containing epoxide groups were hydrolyzed to afford hydrophilic beads *IV* using the procedure described above.

The content of epoxide groups in both original and epichlorohydrin-modified beads and the chromatographic and porous properties were determined as described elsewhere[8].

Determination of Relative Hydrophilicity of Polymer Beads

Polymer beads were packed into a stainless steel column (80 × 8 mm) by the slurry method using constant pressure 11 MPa with water as the driving solvent. The measurements were carried out using a high-pressure pump model 64 (Knauer, Berlin, Germany) and a Rheodyna 7125 valve loop injector. Peaks were monitored by a UV detector (Knauer) at 280 nm. A constant flow rate 1 ml/min was used in all measurements.

Human serum albumin (HSA, Essentially Fatty Acid Free, Sigma, U.S.A.) solution (10 wt.%) in 0.1 mol/l phosphate buffer (pH 3.8) containing 0.15 mol/l NaCl was injected (20 ml) into the column and the peak of the non-adsorbed protein monitored. The mobile phase was changed to a 0.1 mol/l phosphate buffer (pH 7) containing 0.15 mol/l NaCl and the peak of the released protein monitored. Eventually, the mobile phase was changed to a 10 mol/l ethylene glycol solution in 0.1 mol/l phosphate buffer (pH 7) and the peak monitored. Released protein was collected and the total amount of recovered protein compared with the amount of injected HSA. Both data were typically equal within the range of experimental errors.

RESULTS AND DISCUSSION

Protein molecules are known to change their conformation upon changes of pH of the surrounding medium. Conformation changes result not only in a new shape of the protein molecule but also in different types of amino acid residues exposed on the surface of the protein. For example, HSA has hydrophobic character when dissolved in a buffer solution at pH less than 4.5 while at pH above 5, the exposed groups are more hydrophilic[10,11]. Therefore, HSA is an excellent probe for determination of relative hydrophilicity or hydrophobicity of chromatographic packings. In an ideal hydrophilic material, HSA must not be retained at any pH. Beads exhibiting hydrophilicity lower than that of the ideal packing will retain HSA according to the extent of hydrophobic areas, their character and accessibility. HSA method distinguishes the extent of hydrophilicity measured as a fraction of HSA unretained in a buffer of pH 3.8 and two different levels of hydrophobic interaction: First, weak interactions between the packing and HSA that cease after an increase of pH from 3.8 to 7 and, second, strong interactions that can be destroyed only with highly concentrated ethylene glycol solution.

Hydrolyzed poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (*I*) possessing many hydroxy groups is more hydrophilic than a typical hydrophobic poly(styrene-*co*-divinylbenzene) but still not enough to be an ideal hydrophilic material. The most important contributions to hydrophobicity of the glyceryl methacrylate-based copolymer (*II*) come from the aliphatic backbone and from the ethylene bridge of the ethylene dimethacrylate crosslinker. Figure 1 shows the effect of EDMA content in the porous copolymer on the hydrophilicity. Beads prepared solely from EDMA adsorb almost all the protein at any pH and release it only upon treatment with glycol. Also beads containing more than 60 vol.% EDMA do absorb all offered HSA at pH 3.8 but a part of the protein is bound weakly. As the content of the hydrophilic glyceryl methacrylate monomeric units increases, hydrophilicity also increases and a part of the protein passes the column without any retention. A tempting idea of improving the hydrophilicity would be to decrease the EDMA content below 20 vol.%. However, the beads with low crosslinking do not have porous properties required for chromatographic separation medium and extensively swell in water causing loss in mechanical stability.

Since the copolymer composition itself does not allow any further increase in hydrophilicity, the pore surface must be hydrophilized. Turková[12] reacted poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) beads with epichlorohydrin to render the polymer reactive for immobilization of affinants. This method was found to be sufficiently simple and reliable for attachment of 2,3-epoxypropyl groups to a surface covered with hydroxy groups.

Reactions with epichlorohydrin proceeded in both dioxane which is known to swell the polymethacrylate matrix and water which swells the beads only little. Table I collects the results of experiments. Two observations may be made. First, there is almost no difference between the epoxide content in beads modified in dioxane and water, and, second, the increase in reaction time does not increase the concentration of attached

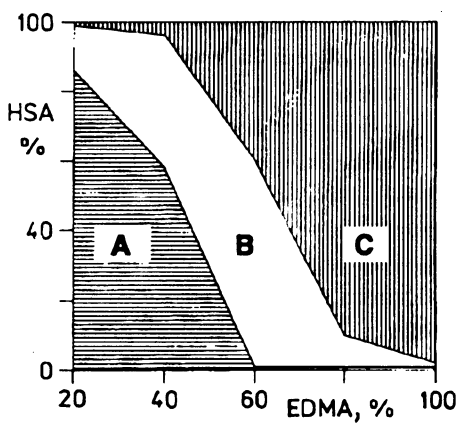


FIG. 1

Relative hydrophilicity of poly(glyceryl methacrylate-*co*-ethylene dimethacrylate) (*II*) beads containing various amounts of EDMA, expressed as a percentage of human serum albumin (HSA) released in buffer solution of pH 3.8 (area **A**), percentage of HSA released in buffer solution of pH 7 (area **B**) and percentage of HSA eluted with ethylene glycol solution (area **C**)

epoxide groups. Rather the opposite, the content of epoxide groups after 8 h reaction in water is lower than that after only 3 h. The results of additional measurements describing properties of the beads are summarized in Table I.

The surface modification reaction does not change porous properties measured in the dry state, such as specific surface area (nitrogen adsorption–desorption) and pore volume (Hg intrusion porosimetry). Chromatographic properties, on the other hand, depend primarily on the type of solvent used. Beads modified in water pack better and

TABLE I
Properties and chromatographic data of polymer beads based on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (*I*) (60 : 40 wt.%)

Polymer	Epoxide mmol/g	Solvent[a] Time[a], h	S m^2/g	V_p ml/g	$N \cdot 10^{-3}$ plates/m	$M_0 \cdot 10^{-4}$	D_{50} nm
I	2.9	–	–	–	–	–	–
II	0	–	69	1.17	21	35	21.4
IVa	0.63[b]	dioxane 3	78	1.22	23	59	24.8
IVb	0.68[b]	water 3	69	1.11	30	73	19.3
IVc	0.48[b]	water 8	68	1.07	29	70	20.9

S Specific surface area; V_p pore volume; *N* column efficiency; M_0 upper exclusion limit; D_{50} pore size at 50% of pore volume. [a] Refers to reaction *II* → *III*; [b] refers to the respective *III* precursor.

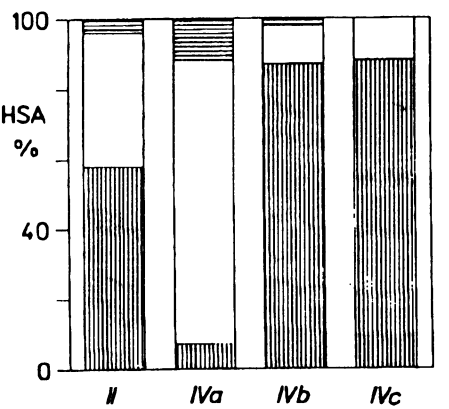


FIG. 2
Relative hydrophilicity of polymer beads: percentage of human serum albumin (HSA) unretained in buffer solution of pH 3.8 (vertical hatching); percentage of HSA released in buffer solution of pH 7 (void space); percentage of HSA eluted with ethylene glycol (horizontal hatching)

column efficiency is higher. Though the porous properties measured in the dry state do not change very much after the modification reaction, the upper exclusion limit determined by chromatographic size exclusion of dextran standards in water, is surprisingly twice as high as that of original beads. This suggests that the beads swell more in water or, in other words, they are more hydrophilic. Higher hydrophilicity should be reflected by lower adsorption of HSA. Indeed, the amount of HSA recovered at a pH of 3.8 is much higher for beads modified in water than for those modified in dioxane (Fig. 2). Moreover, the beads modified in dioxane actually exhibit higher adsorption of HSA compared to the original beads. The reason for this unexpected behaviour may be the amphiphilic nature of the beads. Dioxane is a relatively good solvent for polymethacrylates and does not prefer solvation of any part of the polymer. The epoxide groups attached in the reaction are better distributed in the polymer mass. In water, the reaction proceeds primarily on the pore surface as water swells the polymer beads less than dioxane. This effect is complex since, during the reaction in water, some epoxide groups are already hydrolyzed and become a source of hydroxy groups for further addition reaction. This also explains the fact that epoxide content in beads after 8 h reaction is lower than that after 3 h but the hydrophilicity of the latter beads is higher.

Figure 2 documents that beads prepared by 8 h modification with epichlorohydrin in water followed by hydrolysis of the epoxy groups are close to the ideal hydrophilic non-adsorbing separation medium. After 3 h reaction in water about 2% of HSA remain bound to the surface at pH 7, while the reaction product after 8 h in water no longer binds HSA at pH 7.

This short study of further hydrophilization of hydrophilic chromatographic separation medium shows that chemical modification may improve the already good hydrophilic surface properties. Thus, the modified poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) beads may become a separation medium of choice for HPLC size-exclusion chromatography of hydrophobic proteins.

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