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# Short Communication

# Behaviour of polyhydroxyethyl methacrylate sorbent with dextran-filled macropores in dye-affinity chromatography of proteins

Danica Mislovičová*

*Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava (Slovak Republic)*

Miroslav Petro

*Department of Analytical Chemistry, Faculty of Sciences, Comenius University, Mlynská dolina CH-2, 842 15 Bratislava (Slovak Republic) and Polymer Institute, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava (Slovak Republic)*

Dušan Berek

*Polymer Institute, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava (Slovak Republic)*

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ABSTRACT

A series of composite sorbents based on the rigid macroporous polyhydroxyethyl methacrylate (HEMA)-based support filled with soft dextran gel was prepared. The affinity dyes Cibacron Blue and Remazol Brilliant Blue were then immobilized on both the original HEMA and the composite (HEMA-D) sorbents. The properties of HEMA and HEMA-D sorbents, such as hydrophobic interactions with proteins and recovery of chromatographed enzymes (lactate dehydrogenase and malate dehydrogenase), in both original and dyed forms were studied. Important advantages of using HEMA-D supports in dye-affinity chromatography as compared with only HEMA supports were found.

## INTRODUCTION

The Separon polyhydroethyl methacrylate (HEMA) sorbents are based on a rigid, macroporous polymer matrix prepared by the suspension copolymerization of 2-hydroxyethylmethacrylate with ethylenedimethacrylate [1]. Column packings based on HEMA seem to be very suitable for biopolymer separations in various LC modes including high-performance affinity chromatography (HPAC) [2,3]. However, the hydrophobic interactions of unmodified HEMA sorbents with proteins often prevent their direct application in affinity-based separations. A suitable sorbent for affinity ligand immobilization can be prepared by glycosylation of the HEMA surface [4] or by modifying the HEMA support with polysaccharides [5].

This paper describes the preparation and ap-

* Corresponding author.

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plication of the HEMA sorbent with pores filled with dextran gels of various cross-linking density. The permeation and sorption properties of the supports prepared were examined. After subsequent derivatization with the reactive dyes Cibacron Blue 3G-A and Remazol Brilliant Blue R, the materials were applied in dye-affinity chromatography of the enzymes lactate dehydrogenase (LDH) and malate dehydrogenase (MDH).

## EXPERIMENTAL

### *Materials*

Macroporous Separon HEMA S 10 000, 25–40 $\mu$m (Tessek Prague, Czech Republic), was used as support. The exclusion limit of this material for dextrans is about $M_r$ $10^7$. Dextran D-40, $M_r$ $4 \cdot 10^4$ was obtained from Biotika (Slovenská L'upča, Slovak Republic). The cross-linking reagent 1,4-butanediol diglycidyl ether (BDGE) was from Aldrich (Milwaukee, WI, USA). The dye Cibacron Blue 3G-A (CB) was purchased from Ciba-Geigy (Basle, Switzerland), Remazol Brilliant Blue R (RBB) was from Hoechst (Frankfurt, Germany) and Coomassie Blue G-250 from Serva (Heidelberg, Germany). LDH (E.C. 1.1.1.27) from beef flank muscle was prepared as crude lyophilizate [6]. Other enzymes, such as LDH from rabbit muscle (Fluka, Buchs, Switzerland) and MDH (E.C. 1.1.1.37) from pig heart, were purchased from Boehringer Mannheim (Mannheim, Germany). NADH was from Reanal (Budapest, Hungary). CB–dextran T 10 was prepared according to the procedure previously described [7]. Dextran standards were from Pharmacosmos (Viby, Denmark). Other chemicals were purchased from commercial sources and were of analytical grade.

### *Apparatus*

Spekol 11 and Specord 40 (both from Carl Zeiss Jena, Germany) were used for spectrophotometric determinations of dyes, enzymes and proteins. Gradient HPAC equipment consisted of a GP3 gradient programmer, an LCI 30 manual loop injector, an LCD 2040 UV detector, an FCC 61 fraction collector and a TZ 4620 line recorder (all from Laboratory Instruments, Prague, Czech Republic) and of a VCR 40 high-pressure pump (from Workshops of the Czechoslovak Academy of Sciences, Prague, Czech Republic). Gel permeation chromatographic (GPC) equipment consisted of a Waters 510 HPLC pump (Waters, Division of Millipore, Milford, MA, USA), a Rheodyne 7120 injector (Rheodyne, Berkeley, CA, USA), an RIDK 101 differential refractometric detector and a TZ 4620 line recorder (both from Laboratory Instruments). A Baseline 810 chromatography workstation, (Dynamic Solutions, Division of Millipore, Ventura, CA, USA) was used for data collection and processing.

### *Methods*

The activities of LDH and MDH were determined spectrophotometrically [8] and the protein content was determined according to the method of Bradford [9].

### *Synthesis of HEMA S 10 000 with dextran-filled pores*

HEMA S 10 000 dried overnight at 120°C was treated with an alkaline solution of dextran (4.67 g of D-40 dissolved in 10 ml of 0.5 *M* aqueous NaOH) containing the appropriate amount of cross-linking agent. The mass ratio of BDGE *vs.* dextran was $0.74 \cdot 10^{-3}$ mol/g for HEMA-D1, $0.56 \cdot 10^{-3}$ mol/g for HEMA-D2 and $0.15 \cdot 10^{-3}$ mol/g for HEMA-D3. The amount of dextran incorporated into HEMA pores was 0.56 g/g, 0.33 g/g or 0.20 g/g for HEMA-D1, HEMA-D2 or HEMA-D3, respectively. The cross-linking reaction was stopped by neutralizing the system with 2% (v/v) nitric acid after 48 h. Afterwards the HEMA–dextran composite sorbent was allowed to swell in water for 5 h at 40°C and was then washed with deionized water.

### *Preparation of dyed HEMA sorbents*

The technique used originally for the attachment of dyes to cellulose beads [7] was applied to the preparation of the dyed derivatives of HEMA sorbents: 1 g of HEMA sorbent was suspended in 25 ml of water and then the

appropriate amounts of dye (25–80 mg) and sodium chloride (0.3 g) were added. After dissolution of the latter compounds, alkaline conditions in the reaction mixture were adjusted with 5 ml of 6% $Na_2CO_3$ (CB) or 5 ml of 8% NaOH (RBB). The reaction time was 2 h at 80°C (CB) or at ambient temperature (RBB). The reaction was stopped by neutralization and the unbound dye was washed out with distilled water until the filtrate was colourless. The degree of substitution was determined spectrophotometrically at 610 nm (CB) and at 595 nm (RBB) from the difference between the initial amount of the dye in the reaction mixture and the residual amount of dye in the washing solution.

*Packing the columns*

Glass minicolumns for elution and loading experiments were packed with the sorbents swollen in water. Stainless-steel columns for GPC and HPAC experiments were packed with the sorbents from water slurry under the maximum pressure of 20 MPa. The fines generated during preparation of composite HEMA-D sorbents were removed by repeatedly decanting them in water and methanol.

*Chromatographic experiments*

*GPC characterization.* GPC characterization of HEMA–dextran composite sorbents was performed with degassed distilled water as mobile phase. Columns of 25 × 0.8 cm I.D. were used. The flow-rate was 3 ml/min. A 20-μl aliquot of sample solution containing dextran standards, saccharose, glucose and deuterium oxide (1 mg/ml total amount) was injected.

*Elution experiments.* These were performed using glass minicolumns (1 × 1.1 cm I.D.) filled with 0.2 g of dyed sorbents and equilibrated with 50 m*M* phosphate buffer, pH 7. The enzyme solution (50 μl; *ca.* 10 U) was loaded. The unbound proteins were washed out with the equilibration buffer followed by elution with 2 *M* potassium chloride in equilibration buffer and finally with 1 m*M* NADH in equilibration buffer. The flow-rate was 0.2 ml/min and both the total enzyme activity and the protein content (in the case of crude preparation) were determined in 1-ml fractions. The non-specific interactions of HEMA sorbents with LDH were determined using a column (2.5 × 1.1 cm I.D.) containing 0.5 g of undyed support after loading about 10 U (1.8 mg of proteins) of crude LDH enzyme while the elution was performed only with the equilibration buffer.

*Loading experiments.* The same dyed support columns as for elution experiments were used. After equilibration with 50 m*M* phosphate buffer, the columns were loaded with the solution of crude LDH (3 mg/ml) in the equilibration buffer at a flow-rate of 0.2 ml/min. The activity of LDH in effluent was determined as above. After the column had been saturated, the excess enzyme was washed out with the equilibration buffer and the bound LDH was eluted with 50 μ*M* CB–dextran T 10 [10].

*HPAC-experiments.* The steel column (10 × 0.6 cm I.D.) filled with 2.8 ml of CB–HEMA-D2 containing 8.5 μmol dye per g of sorbent was used. A 20-μl aliquot of enzyme solution (about 5 U) was applied on the equilibrated column with 50 m*M* phosphate buffer, pH 7. The enzyme was eluted with equilibration buffer and then with a concentration gradient of KCl (0–3 *M*) or NADH (0–0.5 m*M*). The experiment was performed at a flow-rate of 0.2 ml/min, pressure 2 MPa and at ambient temperature. The activities of both enzyme and proteins were determined in 1-ml effluent fractions.

## RESULTS AND DISCUSSION

The pore structure of HEMA-D composite sorbents and bare HEMA support was characterized by inverse GPC. Fig. 1 shows the GPC calibration curves of dextran standards observed for HEMA sorbents with and without dextran gel filling. The permeation properties of prepared sorbents correlates well with the conditions of dextran cross-linking, mainly with the molar ratio of cross-linking agent to dextran. HEMA-D1 represents material with an exclusion limit of $M_r \approx 4 \cdot 10^3$ for dextran standards. Therefore the proteins are excluded from its pores. In the case of HEMA-D2 and HEMA-D3, with exclusion limits of $M_r$ $2 \cdot 10^5$ and $4 \cdot 10^5$, respectively, the proteins that are small enough to penetrate the dextran gel can interact with the

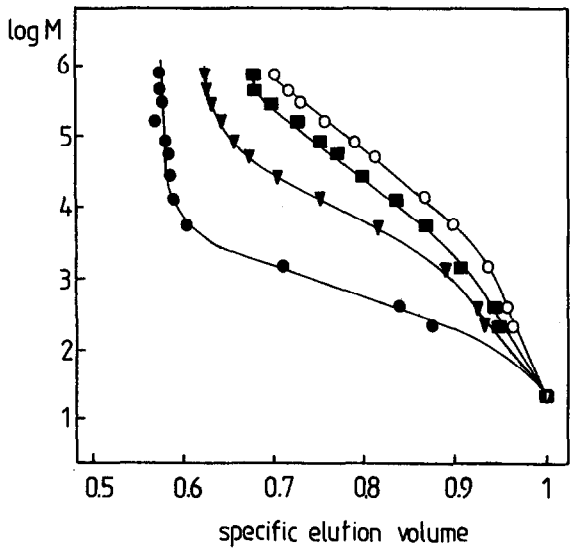


Fig. 1. GPC calibration curves of HEMA and HEMA-D sorbents. Columns, 25 × 0.8 cm, were packed with HEMA-D1 (●), HEMA-D2 (▼), HEMA-D3 (■) and bare HEMA (○). A 20-μl aliquot of calibration standard solution (dextrans, saccharose, glucose and deuterium oxide; 1 mg/ml) was injected onto the column with distilled water as eluent at a flow-rate of 3 ml/min . The specific elution volume was determined as elution volume of injected standard relative to the elution volume of deuterium oxide. M is (weight average) molar mass of injected standard.

HEMA surface. The highest reproducibility was achieved in the case of HEMA-D1 synthesis because the exclusion properties of the resulting sorbent were less sensitive to variation in the reaction conditions. The synthesis of HEMA-D2 and HEMA-D3 sorbents needed more precisely controlled reaction conditions [5].

The activities of LDH eluted from applied HEMA and HEMA-D sorbents were close to 100% of loaded enzyme activities, and no important differences in enzyme recoveries among the sorbents were revealed. Higher non-specific interactions were observed in the case of accompanying proteins. About 25–31% of loaded proteins was adsorbed on bare HEMA and on HEMA with pores filled with slightly cross-linked dextran (HEMA-D2 and HEMA-D3). In the case of composite containing the most densely cross-linked dextran network (HEMA-D1) the recovery of proteins reached 90% because there was no contact between proteins and the inside surface of the HEMA-matrix.

All described sorbents were derivatized with two kinds of dyes: CB and RBB. The concentration of immobilized RBB necessary was more than ten times higher than the concentration of CB required to bind the same amount of enzyme. As described in our previous papers [7,11], the interaction of LDH with CB is stronger than with RBB because of its different nature (CB is pseudobiospecific, RBB is mainly hydrophobic). The higher hydrophobicity of LDH–RBB interaction was expressed as difficulty in eluting bound enzyme with KCl solution as the elution agent. Thus it is preferable to use an affinity sorbent containing the immobilized CB dye.

Table I shows the results of affinity chromatography experiments with the crude preparation of LDH on the dyed sorbents. The loaded enzymes elute more easily from filled than from non-filled HEMA containing the same amount of a particular dye. The existence of hydrophobic interactions between solute and substrate is evident from the lack of elution of bound LDH with KCl solution. It seems that these non-specific enzyme–sorbent interactions play a major role in the case of dyed non-filled HEMA sorbent and they are partially reduced in the case of dyed dextran-filled HEMA. The presence of very small pores in HEMA-D1 causes only a negligible amount of enzyme to be bound on both CB- and RBB-dyed HEMA-D1. From the point of view of desired sorbent affinity to the LDH the dyed HEMA-D2 sorbents have the best properties for our purposes.

Sorbent CB–HEMA-D2 (8.5 μmol of CB per g) was used for the HPAC of LDH (beef muscle) with a concentration gradient of KCl and NADH. The same experiments were done with pure commercial MDH (pig heart) and LDH (rabbit muscle). It is demonstrated in Fig. 2 that enzymes can be selectively separated from accompaning proteins on HEMA–dextran with immobilized dyes. The optimum concentrations of eluting agent (KCl and NADH) as determined from HPAC gradient chromatograms are similar for all three enzymes (1.3 *M* KCl and 0.2 m*M* NADH). The decrease in the concentration of KCl in eluent down to the optimum in isocratic experiments caused an increase in the

TABLE I

ELUTION OF BOVINE LDH FROM RBB-DYED AND CB-DYED SORBENTS

| Support | Dye content (μmol/g) | LDH activity (%) | | |
|---|---|---|---|---|
| | | Elution with | | |
| | | Equilibr. buffer | 2 *M* KCl | 1 m*M* NADH |
| RBB–HEMA | 48.0 | 35.4 | – | 22.5 |
| RBB–HEMA-D1 | 51.4 | 94.2 | – | – |
| RBB–HEMA-D2 | 50.8 | 91.7 | 3.0 | 0.8 |
| | 73.3 | 38.8 | 32.6 | 25.5 |
| RBB–HEMA-D3 | 50.9 | 88.8 | – | 11.1 |
| | 71.9 | 13.0 | – | 85.3 |
| CB-HEMA | 8.3 | – | – | 93.2 |
| CB-HEMA-D1 | 7.6 | 78.6 | 20.2 | 5.0 |
| CB-HEMA-D2 | 7.8 | 6.7 | 66.7 | 22.0 |
| CB-HEMA-D3 | 9.0 | 6.8 | 33.7 | 59.4 |
| CB-HEMA-D3 | 30.1 | – | – | 100.0 |

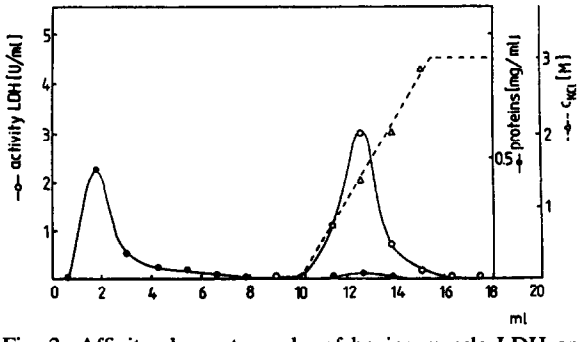


Fig. 2. Affinity chromatography of bovine muscle LDH on CB-dyed HEMA–dextran sorbent. Column: CB–HEMA-D2 (10 × 0.6 cm I.D., 8.5 μmol of CB per g). Mobile phase: 50 m*M* phosphate buffer, pH 7. Concentration gradient of KCl ($c_{KCl}$): 0–3 *M*. *A* 20-μl aliquot of lyophilizate solution, 11 U of LDH, 1.6 mg of protein, was injected. Flow-rate: 0.2 ml/min. Pressure: 2 MPa.

amount of bound LDH eluted from 66.7% (with 2 *M* KCl) to 78.6% (with 1.3 *M* KCl).

The loading capacities (activity of bound LDH/mass of sorbent) of CB-dyed HEMA–dextran composite sorbent and CB-dyed HEMA with about the same content of immobilized affinity ligands are not significantly different (Table II). However, the recovery of LDH from CB–HEMA-D2 obtained at maximum loading of LDH is several times higher than from CB–HEMA. In the case of CB–HEMA the small distance between affinity ligands bonded on the HEMA matrix surface probably results in the irreversible binding of LDH (multivalent interaction). On the other hand, the affinity ligands in CB–HEMA-D2 can be distributed throughout

TABLE II

THE RECOVERIES OF LDH OBTAINED ON CB–HEMA AND CB–HEMA-D2 SORBENTS

| Sorbent | Dye content (μmol/g) | LDH activity (U per g of sorbent) | | Recovery (%) |
|---|---|---|---|---|
| | | Loaded | Eluted | |
| CB-HEMA | 8.3 | 190.85 | 47.5 | 24.9 |
| CB-HEMA-D2 | 8.5 | 224.05 | 184.00 | 82.1 |

the whole dextran network incorporated into HEMA pores. This results in better accessibility of affinity ligands to LDH molecules. Also, for this reason, the enzymes can be easily eluted from dyed HEMA–dextran composites with aqueous KCl with the same purification effect as with NADH.

## CONCLUSIONS

New packing materials for liquid chromatography based on solid macroporous HEMA matrix filled with dextran gels were prepared. The incorporated dextran network acts as a suitable medium for immobilization of CB and RBB dyes. HEMA–dextran composites with immobilized affinity dyes show suppressed non-specific interactions with applied enzymes and improved recovery of specifically bound enzyme in comparison with dyed non-filled HEMA.

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