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Short communication

Improved lectin-mediated immobilization of human red blood cells in superporous agarose beads

Ingo Gottschalk[a,*], Per-Erik Gustavsson[b], Bo Ersson[c], Per Lundahl[a]

[a]*Department of Biochemistry, Biomedical Center, Uppsala University, P.O. Box 576, SE-751 23 Uppsala, Sweden*
[b]*Department of Pure and Applied Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*
[c]*Center for Surface Biotechnology, Biomedical Center, Uppsala University, P.O. Box 577, SE-751 23 Uppsala, Sweden*

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Abstract

A new type of agarose bead, superporous agarose, was used as a gel support for immobilization of human red blood cells (RBCs) mediated by wheat germ lectin. The number of immobilized cells was similar to that obtained with commercial wheat germ lectin–agarose but the cell stability appeared to be superior. This allowed improved frontal affinity chromatographic analyses of cytochalasin B (CB)-binding to the glucose transporter GLUT1 which established a ratio of one CB-binding site per GLUT1 dimer for both plain RBCs or those treated with different poly amino acids. The measured dissociation constants, 70±14 n*M* for CB and 12±3 m*M* for glucose binding to GLUT1, are similar to those reported earlier.

Keywords: Immobilization; Superporous agarose; Wheat germ agglutinin; Cytochalasin B; GLUT1

1. Introduction

Human red blood cells (RBCs) have previously been immobilized in small chromatographic columns ([1–3], also reviewed in Ref. [4]). The human RBC glucose transporter GLUT1 [4–6] in the membranes of the immobilized cells was studied using immobilized biomembrane affinity chromatography (IBAC) [7] in the frontal mode. This allowed the determination of both the ligand binding affinity and the number of sites. Our analyses on gel beds of commercial wheat germ lectin (WGA)-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) [2] indicated a ratio *r* of cytochalasin B (CB) binding sites to GLUT1 monomers of 0.5 (state 1 [3]), which seemed to shift to 1.0 (state 2 [3]) upon treatment of the immobilized cells with poly-Lys, although these values had large error limits.

The original purpose of the poly-Lys treatment was to improve the column stability [2]. In the present study we tried to reproduce the CB-binding shift by treatment of the immobilized RBCs with poly-Arg and in addition we used poly-Glu to indicate that the shift was due to a charge effect. Further aims were to improve the accuracy of the CB binding results by use of the recently developed superporous agarose gel [8] and to improve the RBC immobilization capacity and stability.

*Corresponding author. Tel.: +46-18-471-4599; fax: +46-18-552-139.
E-mail address: ingo.gottschalk@biokem.uu.se (I. Gottschalk).

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Superporous agarose beads [8] feature both the normal small diffusion pores and so-called flow- or superpores with diameters (e.g. ≈30 μm) large enough to allow RBCs access to the interior of the beads. The superporous agarose beads were filtered through graded metal screens and beads of a relatively narrow size range (180–224 μm) were used for this study. The beads have proved useful as a chromatographic support at high flow-rates for ion-exchange [8], affinity [9] and hydrophobic interaction chromatography [10].

WGA-mediated RBC adsorption as in Ref. [2] seemed to be the mildest of the reported methods for RBC-immobilization and was therefore also applied for the present study, accomplished by CNBr activation of the superporous gel beads [11].

2. Materials and methods

2.1. Materials

Superporous agarose beads of particle diameter 180–224 μm with an average superpore size of 30 μm were prepared as in Ref. [8] by a double emulsification procedure. Wheat germ agglutinin was purified from wheat germ as described in Ref. [12] and stored as freeze-dried material. Fresh human blood (A Rh+ or 0 Rh+ in citrate) was bought from the Blood Bank of the University Hospital, Uppsala; WGA-agarose (Sepharose 4B, [WGA]=1.96 mg/ml), HR 10/2 and HR 16/5 columns (I.D. 10 and 16 mm, respectively) and [4(*n*)-^{3}H]CB from Amersham Biosciences (Uppsala, Sweden); CB, poly-L-arginine (M_r 70 000–150 000) hydrochloride, poly-L-lysine (M_r 150 000–300 000) hydrobromide and *N*-acetyl-D-glucosamine from Sigma (St. Louis, MO, USA); and poly-L-glutamic acid (M_r 50 000–100 000) sodium salt from Fluka (Sigma–Aldrich Sweden, Stockholm, Sweden).

Buffer solution A, consisting of 139 m*M* NaCl, 5 m*M* KCl, 50 m*M* mannitol, 3 m*M* NaN_3 and 10 m*M* sodium phosphate buffer, pH 7.4, was used for RBC column preparation and running, unless otherwise stated. Buffer solution B was as buffer solution A with 10 m*M* Tris–HCl, pH 7.4, instead of the phosphate buffer. Buffer solution C was 0.5 *M* NaCl in 0.1 *M* Tris–HCl, pH 8.5, and buffer solution D 0.5 *M* NaCl in 0.1 *M* Na-acetate, pH 4.5. All solutions were filtered through cellulose acetate filters (0.2 μm pore size) and degassed before use.

2.2. WGA-coupling to superporous agarose

Superporous agarose was CNBr-activated using a modification of the in situ activation protocol from Ref. [11]. The following procedures were all performed at a flow-rate of 10 ml/min. Gel beds (1.4–8.5 ml) of superporous agarose beads were packed in water in a HR 16/5 glass column and washed with 25 ml 1 *M* K_3PO_4–HCl, pH 11. The gel was activated for 5 min by circulation of a solution of 0.5 g CNBr dissolved in 1 ml acetonitrile diluted in 15 ml K_3PO_4–HCl, pH 11. The activated bed was washed with 2×25 ml cold 0.1 *M* $NaHCO_3$, pH 8.2. WGA was circulated through the bed in the latter solution at 4 °C overnight at 0.3 ml/min (1.5 mg WGA/ml and 4–10 mg WGA/ml agarose). The gel was then blocked by circulating 1 *M* glycine (≈40 ml) through the column for 2 h and was stored at 4 °C in 1 *M* NaCl and 3 m*M* NaN_3. The concentration of coupled WGA per milliliter of superporous agarose was determined by automated amino acid analysis of an aliquot of the gel.

2.3. RBC column preparation

RBCs were immobilized on WGA-agarose (superporous beads or commercial homogenous beads) as described in Ref. [2]. In brief, gel beds of 0.6–1.4 ml were packed in buffer solution A at 2 ml/min. RBCs were washed three times by 5 min sedimentation at 950 *g* and the final pellet was suspended in 3 vols of buffer. This cell suspension was pumped through the gel for 5 min at 0.15 ml/min followed by incubation for 30 min. Cell loading and incubation were repeated another four times. Cells to be treated with poly amino acids were exposed to 0.025 mg/ml solution of the polymers in buffer solution A (poly-Glu or poly-Lys) or buffer solution B (poly-Arg) at a flow-rate of 0.15 ml/min for 2 h. The columns were washed overnight with buffer solution A at 0.1 ml/min before the start of the analyses.

2.4. Immobilized biomembrane affinity chromatography

The immobilized RBC membranes served as IBAC targets. The affinities of CB and D-glucose for GLUT1 in the membranes and the *r* values were determined as in Refs. [2,3], except that a flow-rate of 0.5 ml/min was used and that the columns were washed at 0.1 ml/min overnight. The experimental set-up was as drawn in Ref. [13]. The frontal elution volumes of [^{3}H]CB were determined by fitting the data from an on-line flow-scintillation detector to Eq. (1) in Ref. [3]. The glucose affinity and the non-specific CB retention volume were determined by linear regression analysis [14] of frontal runs of 2 n*M* [^{3}H]CB inhibited by 0–50 m*M* D-glucose. The CB-affinity and the number of binding sites were gained from frontal runs of 2–100 n*M* CB, fitting the data to Eq. (2) in Ref. [2].

For determination of the number of immobilized cells, the columns were washed with water and the amount of hemoglobin released was determined by automated amino acid analysis. The values of 32 ± 2 pg hemoglobin per cell [15] and 0.51×10^6 GLUT1 monomers per cell [2,16] allowed calculation of the number of immobilized GLUT1 molecules. The *r* values were obtained by dividing the number of immobilized CB-binding sites by this number of GLUT1 monomers.

3. Results and discussion

3.1. Immobilization capacity and stability

The yield of lectin coupling to the superporous agarose beads varied greatly and resulted in WGA concentrations of 0.08–4.3 mg/ml packed gel. Light-microscopic images of RBCs immobilized on this gel support are shown in Fig. 1. Panel A shows a bead colored dark red by adsorbed cells. In panel B, the focus is set at the interior of a bead to show superpores filled with intact cells. The density of immobilized RBCs per ml gel bed was found to be dependent on the concentration of coupled WGA (Fig. 2). A hyperbolic curve-fit for all data excluding the measuring point at 0.08 mg WGA/ml showed a cell-saturation level of $(1.2\pm0.2)\times10^9$ cells per ml gel bed with half-maximal binding at 1.2 mg WGA/ml gel bed (Fig. 2). Most of the cell density data were obtained after 2 days of IBAC analyses. At 1.2 mg WGA/ml gel bed, the immobilized cell density did not differ significantly (data not shown) for columns prior to or after IBAC, indicating good column stability during these experiments (see below). The corresponding immobilization capacity of the commercial WGA-agarose (Sepharose 4B) measured after IBAC analyses in Ref. [2] at 1.96 mg

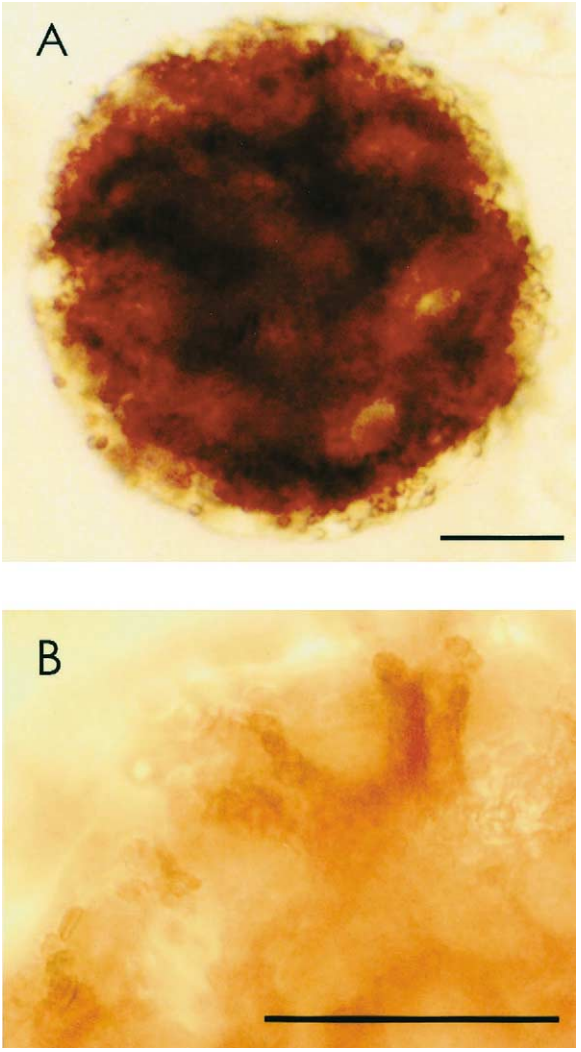

Fig. 1. RBCs adsorbed in WGA-superporous agarose beads. Panel A shows a whole bead, panel B an enlarged section of a bead. The scale bars in both panels have a length of 50 μm.

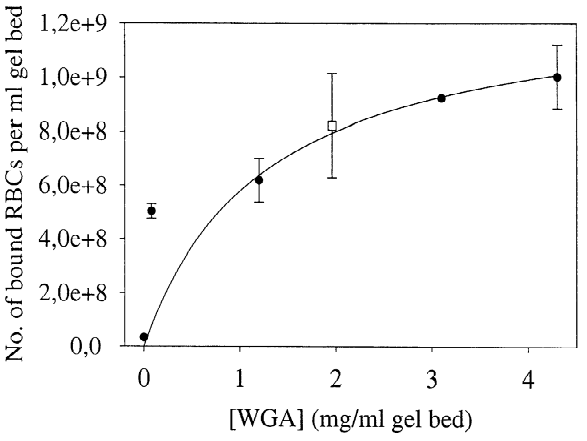

Fig. 2. The number of immobilized RBCs per ml gel bed of WGA-coupled superporous agarose (●) or WGA-Sepharose 4B (□) [2] plotted versus the WGA concentration in the gel. Error limits are SEM and the number of used columns was one, four, nine, one and three at 0, 0.08, 1.2, 3.1 and 4.3 mg WGA/ml superporous agarose, respectively, and three for WGA-Sepharose 4B. The hyperbolic curve fit (omitting the point for 0.08 mg WGA/ml) yielded the equation: $f([\mathrm{WGA}]) = \frac{(1.3\times10^{9}[\mathrm{WGA}])}{(1.3+[\mathrm{WGA}])}$ cells/ml gel, with [WGA] in mg/ml.

WGA/ml gel was $0.8\pm0.2\times10^{9}$ cells per ml gel, the same as for superporous agarose gel at the same lectin concentration, as depicted in Fig. 2. The estimated surface area available for RBCs in our superporous agarose batch (≈480 cm^2/ml) was slightly higher than that of Sepharose 4B (≈340 cm^2/ml gel, assuming an average bead size of 105 μm) but perhaps narrow sections of the superpores are blocked by RBCs. Smaller superporous agarose beads offer larger surface areas and may be able to accommodate higher densities of RBCs.

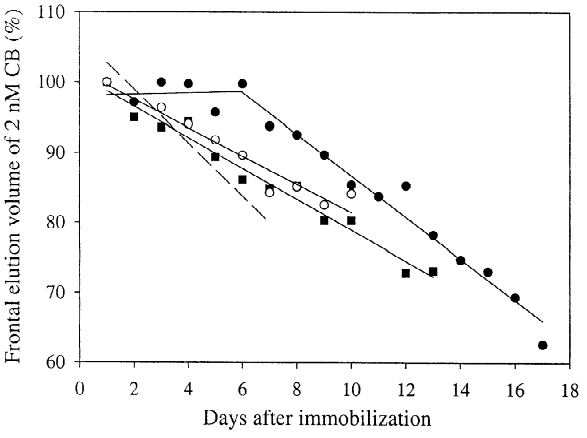

Fig. 3. The frontal elution volume of 2 n*M* CB versus the number of days after RBC immobilization for a superporous agarose column with 4.3 mg WGA/ml (●), a restored and reused column (see text) of WGA-superporous agarose (○), and a column with 1.2 mg WGA/ml superporous agarose (■). The dashed line (no symbols) represents data for RBCs on WGA-Sepharose 4B.

The elution volume in daily frontal runs of 2 n*M* [^{3}H]CB at a buffer flow-rate of 0.3 ml/min was used to monitor the CB-binding to the immobilized RBCs versus time. The results are illustrated in Fig. 3. The maximal number of days after which at least 90% stability could be observed (T_{90}) was chosen as a measure for column stability. A column containing 4.3 mg WGA/ml was essentially 100% stable for about 6 days. Then a decrease by 3% per day began, resulting in a T_{90} of 9 days. A corresponding series of data was measured on a column with 1.2 mg WGA/ml gel and on a column of previously used gel (4.3 mg WGA/ml) that had been restored by washing with water, buffer A supplemented with 0.1 *M* *N*-acetyl-D-glucosamine, and finally with buffer solutions C and D. The T_{90} of these columns decreased to 5 and 6 days, respectively, and there was no grace period as in the upper case. Apparently, the RBCs bound more weakly to columns containing less or inactivated lectin. Another feature of the superporous agarose support was that, regardless of the WGA concentration, most cells remained immobilized upon resuspension and washing of the gel beads, which was not the case for cells on the WGA-Sepharose 4B gel beads.

In similar stability tests (daily frontal runs of CB) on RBCs immobilized on WGA-Sepharose 4B, the elution volumes normally began to decrease after one to 3 days. A dashed line for a typical WGA-Sepharose 4B column is shown for comparison in Fig. 3 omitting the data symbols to retain a good survey.

Clearly, the higher WGA concentration in our superporous agarose support increased the column stability (Fig. 3). However, also superporous agarose with lower [WGA] than on WGA-Sepharose 4B gave better stability and IBAC results (Fig. 4), probably due to steric protection of the cells within the superpores of the new support.

3.2. CB-binding to GLUT1

The *r* values determined on adsorbed RBCs (plain

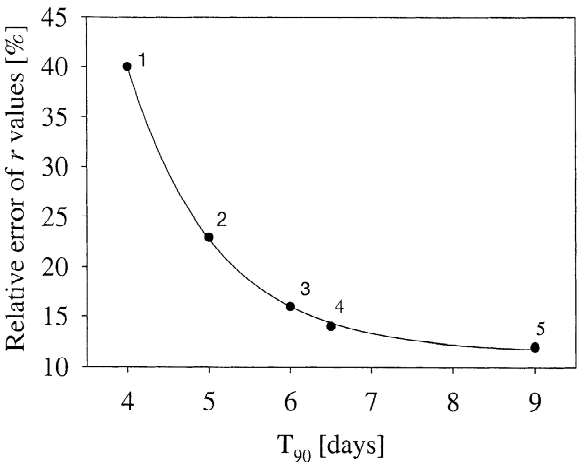

Fig. 4. Relative errors of r values versus the stability of the columns. The data points are for RBCs immobilized (1) on WGA-Sepharose 4B and treated with poly-Lys, (2) on WGA-Sepharose 4B, (3) on WGA-superporous agarose with [WGA]≤ 1.2 mg/ml, (4) by streptavidin–biotin interaction, and (5) on WGA-superporous agarose with [WGA]≥3.1 mg/ml. The data for the symbols 1, 2 and 4 originate from Refs. [2], [2] and [3], respectively.

cells or treated with different poly amino acids) by IBAC [7] are given in Table 1. It is obvious that all of the binding ratios are close to one CB molecule per GLUT1 dimer (the grand average is 0.54±0.09), which has been considered to be the protein's natural state [3,17]. Remarkably, even the values for poly-Lys- and poly-Arg-treated cells (0.40±0.05 and 0.59±0.02, respectively) were far lower than the value, 0.99±0.27 (n=3), reported in Ref. [2]. An attempt to reproduce this high binding ratio for cells immobilized on WGA-Sepharose 4B and treated with poly-Lys gave 0.56±0.24 (n=3) leading to a combined value of 0.77±0.33 (n=6). Obviously, this kind of measurement on WGA-Sepharose 4B led to unreasonably large relative errors. The reason was probably an irreproducible loss of hemoglobin upon poly-Lys treatment. The present data for superporous agarose provided all lower error limits and suggest that the CB binding state of GLUT1 is not altered by treatment with positively or negatively charged poly amino acids. The relative errors for r values for five different systems (see legend to Fig. 4) decreased exponentially with increasing estimated stability of the systems (Fig. 4). We assume that the higher stability offered by the WGA-superporous agarose system, that is, a decreased lysis or loss of cells from the columns during the experiments, was the reason for the improved results. A consequence of the present data for the conversions of GLUT1 that were postulated in Ref. [3] is that the protein in the RBC-membrane (see top section of Fig. 3 in Ref. [3]) probably always stayed in its ground state with an r value of 0.5. The high r value for GLUT1 in suspended membrane vesicles of 0.97±0.04 [3] is, however, valid and shows that the transporter dimers are capable of reversible dissociation from the functional point of view. A lower r value for membrane vesicles that was reported in Ref. [18] was in fact obtained with pH 10.5-washed vesicles that retained most of the cytoskeleton, leading to overestimation of the number of GLUT1 monomers [19]. The value in Ref. [3] is reliable, as it was derived from new experiments with cytoskeleton-free vesicles. The dissociation constants for CB- or D-glucose-binding to GLUT1 in Table 1 are in good agreement with earlier results [3] and the average values may apply for all of the materials.

Table 1
Number of CB-binding sites per GLUT1 monomer (r) and the dissociation constants for CB (K_{dCB}) and D-glucose (K_{dglc}) binding to GLUT1 measured on RBCs immobilized in superporous agarose beads

RBC treatment	r	K_{dCB} [nM]	K_{dglc} [mM]
None	0.61±0.11	71±7	16±3
Poly-Glu	0.54±0.04	85±21	12±1
Poly-Arg	0.59±0.02	62±11	12±0
Poly-Lys	0.40±0.05	57±11	7±2
Average (n=12)	0.54±0.09	70±14	12±3

Data are given for either plain cells or RBCs washed with poly amino acids (Glu, Arg or Lys). The given error limits are SEM, with n=3 except for plain RBCs (n=4) and poly-Arg (n=2).

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