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Immobilization of human red cells in gel particles for chromatographic activity studies of the glucose transporter Glut1

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Abstract

Chromatography on a novel stationary phase, human red cells immobilized in a gel bed, was introduced for analysis of activities of the glucose transporter Glut1 in the cell membrane. A gel containing positively charged ligands was synthesized from derivatized acrylamide monomers. Red cells were immobilized in gel particles which were packed into a column tube for chromatographic analyses over periods of 10–15 days. D-Glucose was separated from L-glucose on a 1.1-ml bed with a retention volume difference of 0.23 ml, approximately equal to the total inner volume of immobilized intact cells and of ghosts probably formed from lysed cells during the immobilization. The separation was suppressed by the glucose-transport inhibitor cytochalasin B. The interactions between D-glucose, the transport inhibitor forskolin and Glut1 were analyzed by quantitative frontal affinity chromatography. The dissociation constants at room temperature were 6.8 mM for D-glucose binding and 1.8 μM for glucose-displaceable binding of forskolin, in good agreement with published values. The results suggest that chromatography on immobilized cells is a potentially useful tool for studies on cellular membrane functions.

Keywords: Affinity chromatography; D-Glucose; Forskolin; Glucose transporter; Cell immobilization; Transport retention chromatography; (Human red cell)

1. Introduction

The glucose transporter Glut1 is abundant in the membrane of human red cells and is one of the most intensively studied transport proteins [1,2]. Glut1 selectively transports D-glucose across the cell membrane by facilitated diffusion and can be competitively inhibited by cytochalasin B (CB) [3], forskolin [4], phloretin [5] and ubiquinone Q_0 [6]. Research on Glut1 includes isolation and purification of the protein from the membrane [7–9], reconstitution of the protein into liposomes [5,10], specific photoaffinity labelling [11,12] and intrinsic fluorescence studies [13], as well as immunological [14] and molecular biological studies [15].

To study the glucose-binding component of red-cell membranes (now denoted Glut1), a method called retardation chromatography was introduced by Bobinski and Stein [16]. Related methods have been developed in our laboratory for chromatographic analysis of interactions of Glut1 with glucose and transport inhibitors [17–19]. In these studies, Glut1 was reconstituted and the proteoliposomes were im-

Abbreviations: ADMA, *N*-allyldimethylamine; CB, cytochalasin B; Glut1, human red cell glucose transporter

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mobilized sterically in chromatographic gel beads. Recently, quantitative frontal analysis of interactions between Glut1, D-glucose and CB were done on both immobilized proteoliposomes and vesicles of human red cell membranes that had been depleted of peripheral proteins [20,21]. The Glut1 affinity for glucose was found to be higher for Glut1 in the membrane vesicles than for Glut1 in proteoliposomes, which indicates that the native lipids, or other membrane components in the vesicles, favored the native structure of Glut1 and thus promoted high affinity.

Immobilization of whole cells [22] has attracted much attention in the recent years. Human red cells have, for example, been immobilized on various supports, e.g., polylysine-coated polyacrylamide [23], silylated silica beads [24], proteinated polymer films [25] and glucose-carrying polystyrene [26]. Although vesicles prepared from plant cells have been used as a chromatographic stationary phase [27], the present study is, to our knowledge, the first one to use immobilized cells for chromatographic characterization of a cell component, in our case the membrane protein Glut1. For this purpose, human red cells (and probably ghosts that were formed) were immobilized in a gel synthesized from derivatized acrylamide monomers and built up of positively charged polymer aggregates with big channels [28]. The binding constants for interaction of D-glucose and forskolin with Glut1 as well as the difference in retention volume of D-glucose and L-glucose were determined in this novel chromatographic system.

2. Materials and methods

2.1. Chemicals

Piperazine diacrylamide, *N,N,N′,N′*-tetramethylethylenediamine and ammonium persulfate were bought from Bio-Rad, Richmond, CA; methacrylamide and *N*-allyldimethylamine (ADMA) from Fluka, Buchs, Switzerland; D-[U-^{14}C]glucose (3.7 MBq/ml), L-[1(N)-^{3}H]glucose (37 MBq/ml) and [12-^{3}H]forskolin (37 MBq/ml) from NEN Life Science Products, Boston, MA; ATP, forskolin and CB from Sigma, St. Louis, MO; D-glucose (AnalaR) from BDH Laboratory Supplies, Poole, UK; and scintillation liquid (Flo-Scint V) from Packard Instruments, Groningen, The Netherlands. All other chemicals were of analytical grade.

2.2. Isotonic solutions

Isotonic phosphate buffer was prepared by adjusting 107 mM disodium hydrogen phosphate to pH 7.4 with 160 mM sodium dihydrogen phosphate. Phosphate-buffered saline was prepared by mixing 20 volumes of the isotonic phosphate buffer with 80 volumes of isotonic saline (160 mM NaCl). Solution A was prepared by diluting the phosphate-buffered saline to keep the osmolarity at 320 mosM upon supplementing the solution with KCl (5 mM) and ATP (1 mM). Solutions B containing different concentration of D-glucose were prepared by mixing solution A with isotonic D-glucose solution (320 mM). Solution B containing 5 mM D-glucose was denoted solution B-5.

2.3. Gel preparation

Gel derivatized with amine ligand or gel without this ligand was prepared as follows. Piperazine diacrylamide (0.125 g), methacrylamide (0.100 g), $(NH_4)_2SO_4$ (0.090 g) and ADMA (0–50 μl) were dissolved in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.0). The pH was then adjusted to 7.0 with 6 M HCl. Following de-aeration for 2 min, 15 μl of 10% (w/v) ammonium persulfate and 15 μl of 5% (v/v) *N,N,N′,N′*-tetramethylethylenediamine aqueous solutions were added and the mixture was allowed to polymerize for at least 24 h in closed test tubes at room temperature. The gel was dispersed mechanically to form irregular particles of sizes in the range of approximately 40–100 μm and was washed three times with 10 mM sodium phosphate buffer (pH 7.4) and three times with isotonic saline by suspension and centrifugation at $2500 \times g$ for 4 min. The sedimented gel, about 1.5 ml, was suspended in 2 ml of solution B-5.

The amine-ligand density obtained with different ADMA contents was determined essentially as described by Peterson and Sober [29] by suspending about 3 ml of sedimented gel in 15 ml of 0.5 M NaCl prepared from boiled water, adding a small excess of 0.1 M HCl and titrating back with 0.1 M NaOH. The titration was monitored with a pH meter.

Unless otherwise stated, the gels used in the experiments were made from a 1.5-ml monomer mixture with 10 μl ADMA (6.7 μl/ml) (see Section 3.1).

2.4. Preparation of red cells

Fresh human blood (type A^+) was collected at the Blood Bank of the University Hospital (Uppsala, Sweden) from healthy adult donors by venipuncture. EDTA was used as anticoagulant. After centrifugation at $950 \times g$ for 4 min at 5°C the plasma and the buffy coat were aspirated and discarded. The red cells were washed four times with isotonic saline by alternating suspension and centrifugation and were immobilized as described below within 4 h.

2.5. Immobilization of red cells, packing of a gel bed and determination of the number of immobilized cells

An 0.4-ml aliquot of the washed red cells (on the average $4.05 \cdot 10^9$ cells) was suspended in 0.4 ml of solution B-5 and mixed with 3.5 ml of the gel suspension. The mixture was incubated at room temperature for 60 min with occasional gentle agitation. The gel with immobilized red cells was packed in a plastic column tube (10×1.0 cm i.d.) at 4°C with solution B-5 at a flow-rate of 0.1 ml/min. Some cells lysed and the packing was continued until no more hemoglobin was released. The final gel bed height was 1.4 cm. Between chromatographic runs the bed was kept running with non-degassed solution B-5 at a flow-rate of 0.025 ml/min at 4°C.

The number of immobilized intact red cells was calculated on the basis of cyanmethemoglobin determinations [30] after cell lysis with 10 ml of 20 mM sodium phosphate buffer (pH 7.4) applied at a flow-rate of 0.1 ml/min. For calibration, washed red cells were diluted with isotonic saline and counted under a microscope; the non-diluted red cells were lysed by addition of the above buffer and the amount of released hemoglobin was determined.

2.6. Transport retention chromatography of D-glucose and L-glucose

The chromatographic system (illustrated in Fig. 1 in Ref. [21]) consisted of a dual-piston HPLC pump

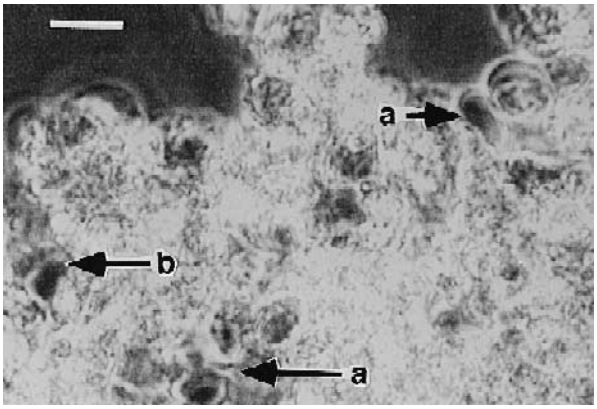

Fig. 1. Phase contrast micrograph of a gel particle with immobilized red cells. The sample was taken from a freshly packed bed and suspended in solution B-5. Arrows (a) show cells that were immobilized on the gel bed surface, and arrow (b) indicates cells trapped in a channel. The gel is shown in light grey; the dark areas at the top correspond to the buffer. Scale bar, 10 μm.

(Model 2248, Pharmacia Biotech, Uppsala, Sweden), an injector (Pharmacia V-7) with a 25-μl loop, a flow-cell scintillation detector with a 500-μl Teflon tubing detection cell (Radiomatic FLO-ONE 525TR, Packard Instruments, Meriden, CT), and a column with immobilized red cells, which was run at 23°C and at a flow-rate of 0.1 ml/min, unless otherwise stated. The scintillation liquid flow-rate was 1.0 ml/min. The energy windows were set at 20–160 keV for ^{14}C detection and 0–12 keV for ^{3}H detection [18].

The column was equilibrated with at least 5 ml of the mobile phase and a 25-μl aliquot was applied of a sample mixture of 30 μl of D-[^{14}C]glucose and 3 μl of L-[^{3}H]glucose diluted to 300 μl with solution A. A series of runs was done with solution A or solutions B, containing 0–200 mM D-glucose, as the mobile phase to determine the effect of D-glucose on transport retention of D-glucose and L-glucose. The elution profiles were smoothed and were used without cross-over correction.

2.7. Frontal analysis of the interactions of Glut1 with D-glucose and forskolin

The analyses were done essentially as described for D-glucose and CB in Ref. [20]. A 10-ml Super-

loop was used for supplying the sample (5–10 ml). The flow rate was 0.1 ml/min and the temperature 23°C. Two series of forskolin runs were performed to determine the binding constants of D-glucose and forskolin and the glucose-displaceable binding of forskolin, (a) with 0–20 mM D-glucose in the eluent, and (b) with forskolin concentrations in the sample of 0–10 μM (see Fig. 5 below). The chromatograms were smoothed and the elution volumes of the front were calculated at half the plateau heights.

3. Results

3.1. Immobilization of red cells in the positively charged gel bed

Red cells adhere easily to positively charged gel beads due to their negative surface charges [23]. The support used in the present study was a polymer gel with amine ligands which were positively charged at pH 7.4. With rough surfaces, large channels and the positively charged groups, this gel had a high capacity for immobilization of red cells. Cells adhered to the surfaces of the gel and were trapped in the channels (Fig. 1). An increase in the ADMA content of the monomer mixture increased the density of the charged amine ligand (Fig. 2a). Basically, this was beneficial for the immobilization of red cells. However, the number of intact cells immobilized reached a maximum when the content of ADMA was 6.7 μl/ml in the monomer mixture and decreased at higher contents (Fig. 2b), since the structure of the gel, particularly the size of the polymer aggregates and the channels, was altered by higher contents of ADMA. Cells could also be immobilized to a certain extent in the gel bed without amine ligands because of the channel structure and non-specific interactions with the gel.

An amount of $1.02–1.26 \cdot 10^9$ red cells per milliliter packed bed was immobilized; the exact number of cells was dependent on the blood sample. The preparation of the immobilized red cell column was reproducible if the cells came from a single blood sample, as shown by the number of cells immobilized, the difference in elution volume between D-glucose and L-glucose, and the elution volume for forskolin upon frontal analysis. The relative standard errors were 6%, 8% and 7%, respectively ($n = 3$).

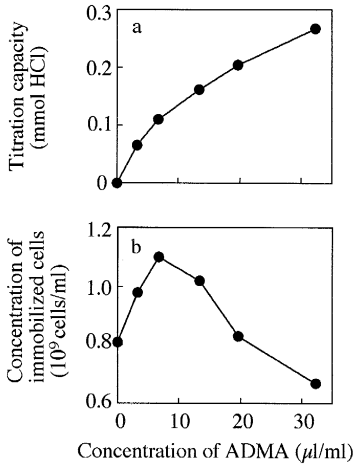

Fig. 2. (a) Titration of a gel bed derivatized with ADMA. Gel synthesized without ADMA was taken as blank. (b) The relationship between concentration of ADMA in the monomer mixture and concentration of red cells immobilized in the gel bed. The cells were prepared from a single blood sample.

3.2. The immobilized red cell column

A large proportion of the red cells immobilized in the beds retained the hemoglobin after being packed into the column. However, the immobilized cells were highly sensitive to column back-pressure and the composition of the mobile phase. To avoid too high flow resistance, a short bed height, a low ratio of height to diameter of the bed and a low flow-rate (0.1 ml/min) were chosen. More than 40% of the immobilized cells were released or lysed if the flow-rate was increased to 0.2 ml/min. Stopping the flow of mobile phase through the column caused the hemoglobin of the immobilized cells to blacken, although this process was reversible.

The mobile phase used contained several substances for maintaining the cells in a physiologically normal state. The presence of ATP greatly extended the time period available for chromatographic analysis of the cells by preserving the membrane deformability of red cells [31]. The 'life span' of the immobilized red cell columns was in the range of 10–15 days. The cells were relatively stable during the first 5 days, whereafter some hemoglobin was lost (Fig. 4a, below).

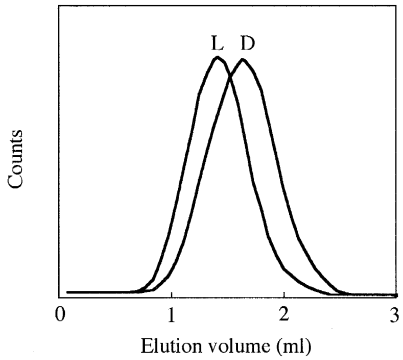

Fig. 3. Elution profiles of D-[^{14}C]glucose (D) and L-[^{3}H]glucose (L) on an immobilized red cell column upon transport retention chromatography in solution A. The height of the peaks were adjusted for easy comparison.

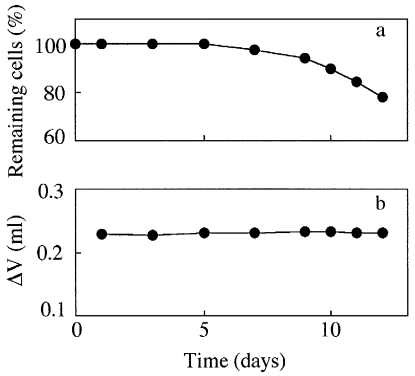

Fig. 4. (a) The percentage of immobilized red cells remaining in a gel bed over a period of 12 days, during which several runs were done. The data were obtained from determinations of hemoglobin in the eluate. (b) Separation of D-glucose and L-glucose (as illustrated in Fig. 3) versus gel bed age. The runs were done on the same immobilized red cell column and during the same time period as in (a).

3.3. Transport retention chromatography of D-glucose and L-glucose on the immobilized red cell column

Separation of D-glucose and L-glucose was achieved on the immobilized red cell columns, as illustrated in Fig. 3. The difference between the retention volumes of D-glucose and L-glucose (ΔV) was 0.23 ml on a 1.1-ml gel bed in which $1.38 \cdot 10^9$ cells were immobilized. D-Glucose in the eluent had no effect on the ΔV values at concentrations below 100 mM, whereas a 10% decrease in the ΔV values was observed at 200 mM D-glucose (not shown). The separation was nearly completely inhibited by including 2 μM CB in the mobile phase; the elution volume of L-glucose was unaffected. No separation occurred after hypotonic lysis of the cells or on a gel bed without cells.

Even though some cell lysis occurred (Fig. 4a), the ΔV value was essentially constant over a period of 12 days (Fig. 4b). Probably some of the lysed cells formed ghosts which still could transport D-glucose (see Section 4).

3.4. Frontal analysis of the interactions between Glut1, D-glucose and the inhibitor forskolin — an example of quantitative affinity chromatography on the immobilized red cell column

Chromatographic frontal analysis of D-glucose and forskolin affinities to Glut1 in a gel bed with immobilized red cells (and ghosts) is illustrated in Fig. 5.

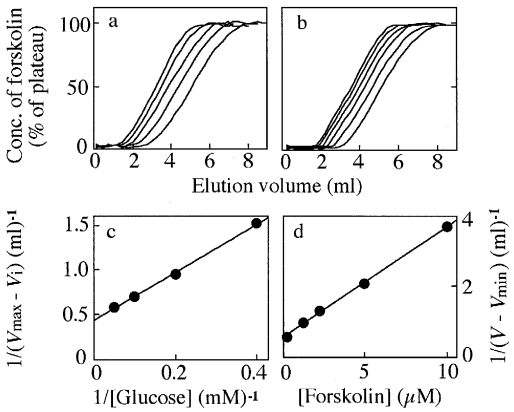

Fig. 5. Frontal analysis of interactions of Glut1 with forskolin and D-glucose on an immobilized red cell gel bed. (a) The elution profiles of 2 nM forskolin in solutions B with 0, 2.5, 5, 10 and 20 mM D-glucose (from right to left) (glucose series). (b) The elution profiles of forskolin at concentrations of 0.002, 0.2, 1.2, 2.2, 5 and 10 μM (from right to left) in solution A (forskolin series). (c) Eq. (1) in Ref. [20] was applied to data from the series of runs in panel (a). V_{max} is the maximal elution volume of forskolin (obtained at 2 nM forskolin in the absence of D-glucose; $V = V_{max}$ when [Forskolin] = 2 nM $\ll K_d$ forskolin) and V_i, the elution volume of forskolin in the presence of D-glucose at a given concentration. (d) Eq. (2) in Ref. [20] was applied to the series of runs with 0.2–10 μM forskolin in panel (b). V is the elution volume of forskolin at a given forskolin concentration in the absence of D-glucose; and V_{min}, the elution volume of forskolin when the specific interaction was suppressed completely. K_d (glucose), K_d (forskolin) and the concentration of forskolin binding sites were calculated from the slopes and intercepts. For details, see Ref. [20].

Table 1
Dissociation constants (K_d) for the interactions of human red cell Glut1 with D-glucose and forskolin (pH 7.4, 23°C, $I = 0.15–0.17$)

Object	K_d (glucose) (mM)	K_d (forskolin) (μM)
Present study:		
Red cells	6.8 [a]	1.8 [a]
Other studies:		
Red cells	7 [b]	2.6 [c]
Red cell membranes depleted of peripheral proteins	17 [d]	1.7 [e]
Glut1 proteoliposomes	46 [f]	1.8 [e]

[a] Single determination.
[b] Value determined by centrifugation [32] ($I = 0.21$) and osmotic swelling [33] (38°C, pH and I not given).
[c] Value determined by centrifugation [4] (pH 8.0).
[d] Value determined by frontal affinity chromatography (Ref. [21] and unpublished determination by L. Lu et al.).
[e] Frontal affinity chromatography (L. Lu et al., unpublished value).
[f] Value determined as in footnote [d]. Glut1 was prepared in octylglucoside solution.

The dissociation constants obtained and some values of other origin are listed in Table 1. Our value for D-glucose (6.8 mM) agreed well with values reported for the transporter in human red cells. D-Glucose showed stronger affinity to Glut1 in human red cells (and ghosts) than to Glut1 in red cell membranes depleted of peripheral proteins or in proteoliposomes. The affinity of forskolin to Glut1 was about the same with red cells, cell membranes and proteoliposomes.

4. Discussion

The transport retention separation of D-glucose and L-glucose is based on the accessibility of the intracellular aqueous volume to D-glucose via the glucose transporter [17,18]. However, the ΔV value (0.23 ml) obtained on an immobilized red cell column (Fig. 3) was much larger than the cell water volume (0.044 ml) of the intact immobilized cells ($1.38 \cdot 10^9$ cells; $3.1 \cdot 10^{13}$ cells per liter cell water [34]). This can be explained by the formation of sealed ghosts from cells lysed during the packing procedure. Spherical ghosts have a larger inner water volume than have disc-shaped red cells (1.9 times the cell water volume, according to data from Ref. [35]). The estimated total inner volume of ghosts in the packed beds was 0.163 ml, provided that all of the lysed cells ($2.67 \cdot 10^9$) formed immobilized ghosts. The total inner volume of intact cells and sealed ghosts thus was 0.21 ml, very close to the ΔV value obtained. When all the cells were lysed with hypotonic phosphate buffer, which leads to formation of a hole in the membrane [36,37], no separation occurred (retention volumes for both enantiomers similar to that previously obtained for D-glucose). An isotonic environment was necessary for the formation of the sealed ghosts which performed as intact cells in the transport of glucose [38].

Binding of D-glucose to Glut1 did not contribute to the D-glucose retention, since the affinity is too weak. The estimated affinity-based retardation was less than 1 μl, according to Eq. (9) in Ref. [39].

Differences in the retention volumes of D-glucose and L-glucose have also been obtained on gel beds with immobilized proteoliposomes in which Glut1 had been reconstituted [17,18]. Although the amount of Glut1 and the total internal liposome volume were similar to the corresponding amount and volume in the gel beds in the present work, the previous ΔV values were much lower, indicating that Glut1 in the natural red cell membrane has a higher D-glucose transport efficiency than Glut1 in artificial model membranes, and/or that the natural membrane shows lower glucose leakage.

The fact that D-glucose in the eluent did not decrease the ΔV value at concentrations below 100 mM seems reasonable. Since there may be about $5.3 \cdot 10^5$ Glut1 polypeptides per cell [40], and since a turnover number of up to about 10^3 molecules per binding site per second has been suggested [41,42], the cells and ghosts immobilized in the column may have a transport rate for D-glucose approaching 4 μmol/s. Under the conditions of 100 mM D-glucose in the eluent and a flow-rate of 0.1 ml/min, the rate of D-glucose influx into the column (0.17 μmol/s) was much lower than the transport rate of red cells. Therefore, the D-glucose in the eluent had little effect on the separation of D-glucose and L-glucose.

The hemoglobin determinations showed that $1.38 \cdot 10^9$ red cells had been immobilized in the gel bed. These cells contained $1.21 \cdot 10^{-9}$ mol Glut1 monomers on the basis of the above approximate value $5.3 \cdot 10^5$ Glut1 polypeptides per cell, whereas

the amount of operative forskolin binding sites (P) obtained from the frontal analysis was higher, about $2.07 \cdot 10^{-9}$ mol. However, $4.05 \cdot 10^{9}$ cells were added to the gel for immobilization. As also indicated by the transport retention chromatographic results, those cells that were not immobilized in intact form probably formed ghosts and became immobilized as such to play the same role as intact cells in the binding of forskolin and glucose. The total amount of Glut1 monomers in $4.05 \cdot 10^{9}$ cells and ghosts was estimated as above to be $3.55 \cdot 10^{-9}$ mol, corresponding to about 0.58 forskolin-binding sites per Glut1 monomer. This is higher than the values 0.30 derived from 400 pmol forskolin-binding sites per mg membrane protein [4], 0.66 pg membrane protein per ghost [43] and the above number of Glut1 polypeptides per cell.

The fact that the ΔV value was independent on the age of the column, even though some hemoglobin was lost (Fig. 4b), also indicates the formation of sealed ghosts after lysis of the immobilized cells, although direct evidence is lacking. Further studies may lead to improvement of the immobilized red cell column.

The results of our experiments with immobilized red cells show that the novel cell immobilization system can be used for studying the interactions between components of intact red cell membranes and their binding ligands. Cells of other origin can probably also be studied with this or similar systems. Compared with conventional methods for analysis of cells in suspension, such as equilibrium dialysis, centrifugation or fluorescent quenching, the chromatographic system based on immobilized cells has the following advantages: (1) this system allows convenient addition and removal of various substances to a single batch of cells to mimic in vivo situations, (2) on-line detection is used for analysis of the eluates which avoids the cumbersome detecting steps used with other methods, and (3) the immobilized cells can be used repeatedly for many runs, which makes the analysis inexpensive and affords the basis for a routine analytical technique.

Proteoliposomes have been extensively used in previous studies of interactions of membrane proteins with their binding ligands. Immobilization of the intact cell in a gel bed, rather than using a single component or a portion of the membrane, can keep the membrane protein in a state of high activity in its natural environment for studies of membrane functions.

Acknowledgements

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