Journal of Chromatography, 432 (1988) 127–135
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4342

COLUMN-BASED SEPARATION OF ERYTHROCYTES USING AQUEOUS POLYMERIC TWO-PHASE SYSTEMS

DAVID R. SKUSE and DONALD E. BROOKS*

*Departments of Chemistry and *Pathology, University of British Columbia, 2211 Wesbrook Mall, Vancouver, B.C. V6T 1W5 (Canada)*

(First received April 11th, 1988; revised manuscript received June 7th, 1988)

SUMMARY

Cell separation using aqueous polymeric two-phase systems is well established. For separations of cells having similar partition coefficients a multi-step counter-current distribution procedure has been used. As an alternative strategy, we have developed a column-based technique in which the dextran-rich phase of a dextran–polyethylene glycol (PEG) phase system is immobilized onto derivatized chromatography beads. The PEG-rich phase is used as the eluent. A separation of erythrocytes from different species utilizing an affinity ligand was performed. The following behaviour was observed: (i) no elution occurs below a critical affinity ligand concentration; (ii) cell recovery is proportional to affinity ligand concentration; (iii) elution volume is independent of affinity ligand concentration. The above suggest that no multi-step partitioning occurs. However, the separation achieved is considerably better than in a corresponding single-tube separation. A 1:1 mixture of dog and human erythrocytes was separated to a purity of >98% using the column-based technique. The corresponding optimized single-tube separation gave a maximum purity of 84%.

INTRODUCTION

Aqueous polymeric phase systems are formed when two water-soluble but incompatible polymers are mixed in concentrations above a set of critical values [1,2]. Such solutions separate into two phases, each enriched in one of the polymers. These phases may be buffered and made compatible with a wide range of biological materials. When cells are added to such a system they often distribute unevenly between one of the phases and the liquid–liquid interface between the phases [2]. The distribution is a function of the interaction between the cell surface and the phases and is extremely sensitive to differences in cell surface properties. Moreover, there are a number of experimental variables which control the distribution. Consequently, the aqueous partitioning technique has been used to perform many cell separations and purifications [1,2].

0378-4347/88/$03.50

When it is necessary to separate cell types having similar partition coefficients, the usual single-tube separation technique does not give a separation of adequate resolution. In these cases it is necessary to use a multi-step procedure to increase the resolution. This has most usually been performed using a counter-current distribution (CCD) technique, in which a large number of extraction steps are carried out in sequence in a specially designed apparatus [2]. As an alternative strategy, for the separation of macromolecules, Müller [3] has developed a column-based technique in which the dextran phase of a dextran–polyethylene glycol (PEG) phase system is immobilized onto chromatography beads derivatized with polyacrylamide. The PEG-rich phase is used as the eluent. This technique gave a considerable increase in resolution and saving in time over CCD for macromolecular separations.

We have adapted Müller's technique [3] and investigated its applicability to the separation of cells. As a model we have investigated the column-based separation of dog and human erythrocytes, this being a well documented [4] and easily performed single-tube separation. The separation is based on the differing binding affinities of erythrocytes for a ligand consisting of a PEG head group esterified to a fatty acid. The species-dependent ability of PEG-fatty acids to bind to erythrocytes has been attributed to hydrophobic interactions between the fatty acid of the affinity ligand and membrane lipid, modified by interactions with sphingomyelin and phosphatidylcholine head groups [4]. Dog erythrocytes have high affinity for the ligands and human erythrocytes low. In phase systems formed by mixing dextran T40 and PEG 8000 and suitable buffers, in the absence of affinity ligand both dog and human erythrocytes accumulate at the interface between the phases [5]. The addition of PEG-oleate, which partitions strongly into the upper PEG-rich phase, causes the erythrocytes to repartition into the upper PEG-rich phase. By controlling the concentration of affinity ligand it is possible to separate dog and human erythrocytes between the upper phase and the interface.

In a previous communication [5], we have shown that the column-based partitioning behavior of fresh dog and human erythrocytes resembles that observed in conventional single-tube partitioning experiments. A mixture of fresh dog and human erythrocytes was separated on the basis of their differential interaction with PEG-oleate using a column-based technique. Columns consisted of polyacrylamide-derivatized agarose cell chromatography beads (diameter $\simeq 250\ \mu m$) with a bound layer of dextran-rich phase. Mixtures containing equal numbers of dog and human erythrocytes were loaded onto the column in PEG-rich phase and incubated with no flow to allow partition to take place. Dog erythrocytes were eluted by including PEG-oleate in the eluent. Human erythrocytes were then recovered by washing the beads with phosphate-buffered saline (PBS). The recovery of the dog erythrocytes loaded was 52% recovered at a purity $> 75\%$ and the recovery of the human cells was 30% at a purity $> 70\%$. Total cell recoveries were 80–90% of the cells loaded.

In the present paper we report on a more detailed investigation into this model separation. A simplified experimental procedure involving no incubation period was facilitated by the use of smaller chromatography beads. The use of glutaral-

dehyde fixed instead of fresh erythrocytes considerably simplified the sample preparation procedure. Columns consisted of polyacrylamide-derivatized silicic chromatography beads (diameter $\simeq 65$ μm) with bound dextran-rich phase of the same dextran T40–PEG 8000 system used in ref. 5. Fixed dog and human erythrocytes were pre-equilibrated with the appropriate concentration of PEG-oleate in PEG-rich phase, loaded onto the column and eluted isocratically with PEG-rich phase–PEG-oleate as the eluent. Elution profiles for dog and human erythrocytes were obtained independently as a function of PEG-oleate concentration. This information was then used to effect separations from a dog–human erythrocyte mixture. A parallel series of single-tube separation experiments were performed and the separations compared with those obtained using the column-based technique.

EXPERIMENTAL

Preparation of two-phase systems

A phase system containing 3% dextran T40 (Pharmacia, Dorval, Canada), 7.7% PEG 8000 (Union Carbide, Century Grade, Piscataway, NJ, U.S.A.), 0.079 *M* dibasic sodium phosphate, 0.025 *M* monobasic sodium phosphate and 0.006 *M* sodium azide (pH 7.2) was used. The constituents were mixed thoroughly and allowed to settle for 72 h before the phases were separated. The system was prepared and all experiments carried out at 22°C.

Preparation of chromatography beads

Fractogel TSK HW-65F (BDH, Vancouver, Canada) silicic chromatography beads were used. The beads were prepared for reaction by the following wash cycle: three washes with 50-ml aliquots of distilled water; one wash with a 50-ml aliquot of a solution containing 0.2 *M* sodium acetate and 0.001 *M* trisodium ethylenediamine tetraacetic acid monohydrate (EDTA); four washes with 50-ml aliquots of distilled water.

The reaction vessel was a 500-ml three-neck round-bottom flask. Neck 1 held a nitrogen inlet, neck 2 a reflux condenser topped with a nitrogen outlet via a paraffin bubbler and neck 3 a stoppered separating funnel with a pressure equalizing arm.

Washed beads (50 ml) and an acrylamide solution (40 g in 200 g distilled water) were placed in the reaction vessel and 0.2 *M* cerium(IV) ammonium nitrate [$Ce(IV)(NH_4)_2(NO_3)_6$] in 1 *M* nitric acid (11.6 ml) was placed in the separating funnel. The apparatus was degassed by bubbling nitrogen with magnetic stirring for 1 h. The reaction was initiated by adding the cerium(IV) solution to the acrylamide and beads, and allowed to proceed with magnetic stirring under nitrogen flow. After 3 h, the reaction was stopped by exposing the reaction mixture to air. The beads were washed with twenty 50-ml aliquots of distilled water and stored at 4°C in 0.006 *M* sodium azide.

Preparation of erythrocytes

Fresh blood was collected into citrate or heparin from human and dog donors, respectively. The erythrocytes were washed three times in 10 volumes of PBS.

Packed dog erythrocytes (approximately 0.2 ml) were incubated with 0.1 ml of $Na_2{}^{51}CrO_4$ ($3.7 \cdot 10^7$ Bq ml^{-1}) (Amersham, Montreal, Canada) and PBS (0.2 ml) at 37°C for 1 h. The cells were washed with five 1-ml aliquots of 1% glutaraldehyde in PBS and fixed by rotating end-over-end at 30 rpm for 20 h in 1% glutaraldehyde in PBS (5 ml).

Human erythrocytes were labelled in a similar fashion: packed erythrocytes (approximately 0.2 ml) were rotated end-over-end at 10 rpm for 1 h with 0.001 ml of $K^{125}I$ ($3.7 \cdot 10^9$ Bq ml^{-1}) (Amersham), four Iodobeads (Pierce, Rockford, IL, U.S.A.) and PBS (0.5 ml). The cells were washed with five 1-ml aliquots of 1% glutaraldehyde in PBS and fixed by rotating end-over-end at 30 rpm for 20 h in 1% glutaraldehyde in PBS (5 ml).

Fixed but not radiolabelled dog and human erythrocytes were also prepared: packed cells (1 ml) in 1% glutaraldehyde in PBS (10 ml) were rotated end-over-end at 30 rpm for 20 h.

Radiolabelled and unlabelled cells were blended to yield stock cell suspensions, containing $1 \cdot 10^8$ cells per ml and $2 \cdot 10^5$ Bq ml^{-1} in 1% glutaraldehyde in PBS.

Cell concentrations were determined by impedance cell counting (Electrozone Celloscope, Particle Data Incorporated, Elmhurst, IL. U.S.A.). Activities were measured using a gamma counter (1282 Compugamma, LKB, Bromma, Sweden).

Load mixes (suspensions of cells in PEG-rich phase) for both single-tube and column-based experiments were prepared in similar fashion. Fixed, labelled dog and/or human erythrocytes from the above stocks were washed with three 1.0-ml aliquots of glutaraldehyde-free PBS and resuspended in PEG-rich phase, such that the final cell concentration was $1 \cdot 10^8$ cells per ml ($5 \cdot 10^7$ ml^{-1} in each cell type for separation experiments). PEG-oleate was included in the PEG-rich phase as required for column-based experiments.

Single-tube experiments

The single-tube partition behaviour of dog, human and dog–human erythrocyte mixtures with variation in PEG-oleate concentration was determined as follows.

Aliquots of PEG-rich phase (2.0 ml) were added to equal volumes of dextran-rich phase in 1.0-cm^2 glass tubes. Varying concentrations of PEG-oleate in PEG-rich phase were added in volumes never exceeding 0.1 ml, such that the final PEG-oleate concentrations covered the range $0–3.5 \cdot 10^{-6} M$. Aliquots of load mix (0.1 ml) containing $1 \cdot 10^7$ of either dog or human erythrocytes or a 1:1 dog–human erythrocyte mixture were added. The systems were mixed well by inverting the tubes twenty times and then allowed to settle for 15 min. The PEG-rich phase was then sampled. The percentage of the loaded cells present in the PEG-rich phase was determined by comparing the isotopic activity of the PEG-rich phase sample with that of the load mix.

Preparation of columns

Derivatized beads (20–25 ml) were washed with three 50-ml aliquots of a solution containing 0.079 *M* dibasic sodium phosphate, 0.025 *M* monobasic sodium phosphate and 0.006 *M* sodium azide (pH 7.2) and one 25-ml aliquot of dextran-

rich phase. The beads were then resuspended in dextran-rich phase (50 ml) and rotated end-over-end at 30 rpm for 48 h. Columns of the resultant dextran-rich phase covered beads were packed at flow-rates of 6 ml h^{-1} to give beds of 1.0 cm $\times$ 1.0 cm^2. Each column was used only once.

Chromatographic procedure

Aliquots of load mix (0.1 ml), containing $1 \cdot 10^7$ of either dog or human erythrocytes or a 1:1 dog–human erythrocyte mixture in PEG-rich phase containing the requisite amount of PEG-oleate, were loaded onto the column and eluted isocratically with PEG-rich phase–PEG-oleate as the eluent at 6 ml h^{-1} (the optimum flow-rate for maximum cell recovery) for 2 h. The eluent was collected in 1.2-ml (12 min) aliquots using an automatic fraction collector (LKB). Erythrocytes remaining on the column at the end of this period were removed by washing with PBS. Erythrocyte concentrations were determined isotopically. The percentage of the loaded cells present in each aliquot was determined by comparing the isotopic activity of the aliquot with that of the load mix.

RESULTS AND DISCUSSION

Single-tube partition

The single-tube partition behaviours of glutaraldehyde fixed dog and human erythrocytes as a function of PEG-oleate concentration were investigated independently (Fig. 1). In the absence of PEG-oleate both types of erythrocyte accumulate almost totally at the interface between the phases. Addition of $< 1 \cdot 10^{-6}$ *M* PEG-oleate causes $>90\%$ of the dog erythrocytes but $<30\%$ of the human erythrocytes to partition into the PEG-rich phase. Further increase in PEG-oleate concentration does not appreciably increase the dog erythrocyte partition. However, human erythrocyte partition increases with increase in PEG-oleate concentration to $>90\%$ into the PEG-rich phase at PEG-oleate concentration $> 3.0 \cdot 10^{-6}$ *M*. These data indicate that fixed dog and human erythrocytes interact with PEG-oleate in a manner similar to fresh erythrocytes. Dog erythrocytes show a high affinity interaction and human erythrocytes a lower affinity interaction. Consequently, PEG-oleate concentrations in the range $0.5 \cdot 10^{-6}$–$3.5 \cdot 10^{-6}$ *M* were used to effect single-tube separations of 1:1 dog–human erythrocyte mixtures. The results obtained are summarized in Table I and Fig. 2. The purest samples of dog

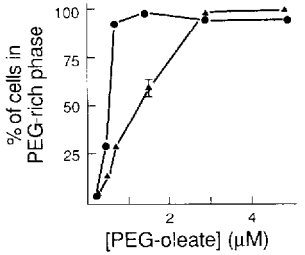

Fig. 1. Single-tube aqueous liquid–liquid partitioning behaviour of dog (●) and human (▲) erythrocytes obtained independently at various PEG-oleate concentrations.

erythrocytes (expressed as the number of dog erythrocytes present in the upper PEG-rich phase divided by the total number of erythrocytes in the PEG-rich phase) were obtained at PEG-oleate concentrations of $0.5 \cdot 10^{-6}$ and $1.2 \cdot 10^{-6}$ *M*, where 21 ± 2 and $99 \pm 2\%$, respectively, of the dog erythrocytes present partitioned into the PEG-rich phase at purities of 84 ± 3 and $81 \pm 3\%$. Further increase in PEG-oleate concentration led to increased numbers of human erythrocytes present in the upper phase and hence reduced purities.

Comparison of Figs.1 and 2 indicate differences in the partition behaviour of human erythrocytes in the two experiments. Human erythrocyte partition into the PEG-rich phase is lower when dog erythrocytes are present in the system than when the human erythrocytes are partitioned independently. Overall cell concentrations were $2.5 \cdot 10^6$ cells per ml in each set of experiments, thus in the separation experiments only $1.2 \cdot 10^6$ human cells per ml were present. However, it is thought unlikely that the observed difference in behaviour is a human erythrocyte concentration effect because previous unpublished observations in our lab-

TABLE I

PURIFICATION OF DOG ERYTHROCYTES FROM 1:1 DOG–HUMAN ERYTHROCYTE MIXTURES USING SINGLE-TUBE AND COLUMN-BASED AQUEOUS LIQUID–LIQUID PARTITIONING TECHNIQUES

PEG-oleate concentration ($\times 10^6$ *M*)	Single tube		Column			
	Percentage of cells in PEG-rich phase[a]	Purity[b] (%)	Overall		Best fraction	
			Recovery[c] (%)	Purity[d] (%)	Recovery[c] (%)	Purity[d] (%)
0.5	21	84	13	97	13	97
1.2	99	81	91	93	49	98
2.5	102	74	83	83	57	97
3.5	100	62	86	61	62	83

[a]Uncertainty of $< \pm 2\%$.
[b]Uncertainty of $< \pm 4\%$.
[c]Uncertainty of $< \pm 1\%$.
[d]Uncertainty of $< \pm 2\%$.

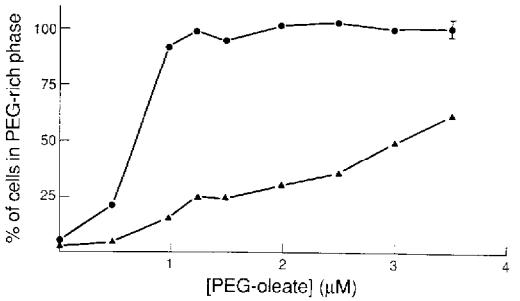

Fig. 2. Single-tube aqueous liquid–liquid partitioning behaviour of 1:1 dog (●)–human (▲) erythrocyte mixtures at various PEG-oleate concentrations.

oratory have shown human erythrocyte partition to be approximately independent of cell concentration over the range 10^4–10^8 cells per ml. Dog erythrocytes have a high affinity for PEG-oleate, hence their presence leads to a significant depletion of the PEG-oleate available to partition human erythrocytes into the PEG-rich phase, producing the observed difference in human erythrocyte partition behaviour.

Column-based partition

Elution profiles for dog and human erythrocytes were obtained independently at different PEG-oleate concentrations and are shown in Figs. 3 and 4. There are minimum critical PEG-oleate concentrations below which no erythrocyte elution occurred. These concentrations are $0.3 \cdot 10^{-6}$ *M* for dog erythrocytes and $1.2 \cdot 10^{-6}$ *M* for human erythrocytes. Above these critical concentrations erythrocyte recovery (taken as the percentage of the cells loaded eluted in the first six fractions) increased with increase in PEG-oleate concentration to $\simeq$ 90% for dog and $\simeq$ 80% for human erythrocytes. Elution volume was not proportional to PEG-oleate concentration (except at concentrations which gave very low recoveries). The above data suggest that the partition of erythrocytes from the PEG-rich phase to the interface is a one-step process and once erythrocytes have partitioned to the interface repartition does not occur. In the absence of PEG-oleate all the erythrocytes adhere to the beads and are not eluted. At PEG-oleate concentrations above the critical values, the erythrocytes presumably distribute between the PEG-rich phase and the interface, those in the PEG-rich phase being eluted. Further re-adsorption of the erythrocytes down the column does not occur. Hence the dependence of recovery on PEG-oleate concentration likely reflects the initial distribution of erythrocytes between the upper phase and the interface and there is no change in elution volume with change in PEG-oleate concentration. Thus column-based partition experiments performed in this manner appear to be analogous to conventional single-tube partition experiments and do not display the hoped for correlation with multi-step classical partition chromatography.

Fig. 3. Elution profile of dog erythrocytes from an aqueous liquid–liquid phase partitioning column at various PEG-oleate concentrations. (▲) $2.5 \cdot 10^{-6}$ *M*; (■) $1.2 \cdot 10^{-6}$ *M*; (○) $0.6 \cdot 10^{-6}$ *M*; (□) $0.3 \cdot 10^{-6}$ *M*.

Fig. 4. Elution profile of human erythrocytes from an aqueous liquid–liquid phase partitioning column at various PEG-oleate concentrations. (●) $5 \cdot 10^{-6}$ *M*; (▲) $2.5 \cdot 10^{-6}$ *M*; (■) $1.2 \cdot 10^{-6}$ *M*.

PEG-oleate concentrations in the range $0.5 \cdot 10^{-6}$–$3.5 \cdot 10^{-6}$ *M* were used to effect column-based separations of 1:1 dog–human erythrocyte mixtures. Elution profiles are shown in Fig. 5 and the results obtained are compared with corresponding single-tube separations in Table I. At a PEG-oleate concentration of $0.5 \cdot 10^{-6}$ *M* (Fig. 5a), although considerable purification of dog erythrocytes occurred, recoveries were impractically low. However, at a PEG-oleate concentration of $1.2 \cdot 10^{-6}$ *M* (Fig 5b) $91 \pm 1\%$ of the dog erythrocytes were eluted with only $6 \pm 1\%$ of the human erythrocytes, giving a dog erythrocyte purity of $93 \pm 2\%$. Furthermore, dog erythrocytes were obtained at even higher purities in the initial stages of elution. The highest purification occurred in the second fraction where $49 \pm 1\%$ of the dog erythrocytes loaded were present at a purity of $98 \pm 2\%$. The corresponding single-tube separation experiment yielded $99 \pm 2\%$ of the dog erythrocytes loaded at a purity of $81 \pm 3\%$. At higher PEG-oleate concentrations (i.e. $2.5 \cdot 10^{-6}$ and $3.5 \cdot 10^{-6}$ *M*) the dog erythrocyte purities obtained by collecting the whole dog erythrocyte elution peak were comparable to the purification obtained in the corresponding single-tube separation. However, the purity of the purest fraction remained much higher than in corresponding single-tube separations, e.g. at $2.5 \cdot 10^{-6}$ *M* PEG-oleate concentration, $57 \pm 1\%$ of the dog erythrocytes loaded in a column-based experiment were obtained at a purity of $97 \pm 2\%$. The corresponding single-tube separation yielded $102 \pm 2\%$ of the dog erythrocytes loaded at a purity of $73 \pm 3\%$.

Thus it seems that the column-based separation technique offers a considerable increase in resolution over conventional singe-tube procedures. Furthermore it should be noted that it is possible to recover non-eluted erythrocytes by diluting the phase system to one phase with buffer and then washing the beads. In this way it was usually possible to recover >95% of the erythrocytes loaded onto the column.

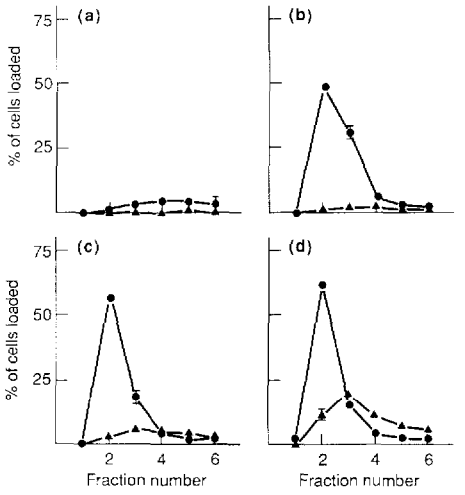

Fig. 5. Elution profiles of 1:1 dog (●)-human (▲) erythrocyte mixtures from aqueous liquid–liquid phase partitioning columns at PEG-oleate concentrations of $0.5 \cdot 10^{-6}$ *M* (a), $1.2 \cdot 10^{-6}$ *M* (b), $2.5 \cdot 10^{-6}$ *M* (c) and $3.5 \cdot 10^{-6}$ *M* (d).

Brooks [6] has shown that the maximum resolution obtained during conventional single-tube cell separation experiments does not approach that predicted by theory if thermodynamic equilibrium is assumed to hold throughout the separation process. It is hypothesized that the difference is due to the mechanical forces present during the phase demixing process which follows introduction of the cells and mixing of the phase system. It is thought that the enhanced resolution obtained in column-based experiments may occur because the fluid mechanical forces tending to remove cells from the phase boundary present during a column separation experiment are probably steadier and weaker than those present in a single-tube experiment. Thus the observed separation behaviour more closely resembles that predicted by theory.

Careful comparison of Figs. 3 and 4 with Fig. 5 indicates that the variation in human erythrocyte partition behaviour with inclusion of dog erythrocytes into the phase system observed for single-tube separations is also present in column-based experiments.

CONCLUSIONS

Column-based partitioning techniques can be used to separate fixed human and dog erythrocytes from a mixture on the basis of their differential interactions with PEG-oleate. The expected analogy with multi-step classical partition chromatography is not observed. Instead, column-based partition is a single-step process. However, the maximum resolution obtained in this process is considerably higher than in a corresponding single-tube separation. This difference may be ascribed to differences in the fluid mechanical environment during the separation processes.

ACKNOWLEDGEMENT

The financial support of the Medical Research Council of Canada through Grant MT-5759 to D.E.B. is gratefully acknowledged.

REFERENCES

1 P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley Interscience, New York, 1971.
2 H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous Two-Phase Systems, Academic Press, Orlando, FL, 1986.
3 W. Müller, Eur. J. Biochem., 155 (1986) 213.
4 E. Ericksson, P.-Å. Albertsson and G. Johansson, Mol. Cell. Biochem., 10 (1976) 123.
5 D.R. Skuse, W. Müller and D.E. Brooks, Anal. Biochem., in press.
6 D.E. Brooks, in S.K. Sikdar, M. Bier and P. Todd (Editors), Frontiers in Bioprocessing, CRS Press, Boca Raton, FL, in press.