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A single partitioning step in aqueous polymer two-phase systems reduces hypotonized rat erythrocyte heterogeneity

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Abstract

Rat carrier erythrocytes prepared by hypotonic dialysis (80 mOsm/kg) are a heterogeneous cell population that can be fractionated into two well-defined cell subpopulations by a single partition step, in charge-sensitive dextran–poly(ethylene glycol) aqueous two-phase systems. One subpopulation (65% of total cells) has a decreased cell surface charge and is partitioned at the interface in a single step and then fractionated by counter-current distribution as a low-*G* subpopulation. The other subpopulation (35% of total cells) has charge surface properties more like those of the untreated control rat erythrocytes. These last cells are partitioned in the top phase in a single step and then fractionated by counter-current distribution as a high-*G* subpopulation. Partitioning is more effective in reducing cell heterogeneity in hypotonized rat erythrocyte populations than is density separation in Ficoll-paque which only separates a small less dense cell subpopulation (5% of total cells), with the most fragile cells, from a larger and more dense cell subpopulation (95% of total cells), with a mixture of fragile and normal cells. This simple cell separation procedure quickly reduces carrier erythrocyte heterogeneity in a single partitioning step so it can be used to prepare cells for in vivo studies.

Keywords: Aqueous two-phase systems; Partitioning; Erythrocytes

1. Introduction

Carrier erythrocytes for drugs, enzymes and other pharmacological agents are prepared by a hypotonic dialysis process which can temporarily open transitory membrane pores, for cell loading, and later reseal them with an isotonic solution [1–4]. After hypotonic dialysis, both human and rat erythrocytes are more heterogeneous than untreated control cells [4–10]. Some membrane modifications and changes in the intracellular composition that occur during this process might impair cell survival capacity [10,11].

Counter-current distribution (CCD) in aqueous dextran–poly(ethylene glycol) (D–PEG) two-phase systems makes it possible to perform multiple partioning steps that separate closely related cell populations [12,13]. CCD has been successfully used in our group to fractionate human and rat erythrocytes as a function of cell surface changes during cellular ageing [14–17]. More recently, this technique has been used to fractionate hypotonically [^{125}I]carbonic anhydrase-loaded rat erythrocytes into two well-defined low and high partition ratio subpopulations, the former containing the most fragile and superficially altered cells, that incorporate the most enzyme, and the later contains cells that incorporate less enzyme and whose surface properties are like those of the controls [10].

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Two particulates, such as cell types, can be separated from one another in a single extraction step when their partition ratio, P, differs significantly. One material (high P) will be recovered in the top phase and the other (low P) at the interface or bottom phase [13]. In this way, the low- and high-G subpopulations obtained after CCD of hypotonized rat erythrocytes, which have very different surface properties, would be good candidates for a single-step separation. This possibility, which would suppose a simple methodology to reduce carrier erythrocyte heterogeneity before use in in vivo studies, is analyzed in this paper. The cell separation was checked by a CCD procedure which confirmed that separation had taken place.

Differences in cell density after the hypotonic dialysis process have been described for human erythrocytes [9]. On this basis, the capacity of density centrifugation in Ficoll-paque to separate different cell subpopulations from the whole hypotonized rat erythrocyte population is analyzed and compared with subfractionation obtained by partitioning in D–PEG aqueous two-phase systems.

2. Experimental

2.1. Isolation of erythrocytes

Blood from male Wistar rats (150–200 g) was collected into heparinized (10 U/ml) tubes and filtered through α-cellulose-cellulose type 50 (mean size 50 μm) filters (Sigma, St. Louis, MO, USA) [18]. Two washings with phosphate buffered saline (PBS) (10 mM phosphate buffer, 3.5 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 145 mM NaCl, pH 8.0) at 400 g for 10 min at 4°C and once more at 2500 g for 5 min at 4°C, were carried out to obtain packed erythrocytes. PBS, pH 8, was used to prevent the rat hemoglobin gelation into erythrocytes [5,6].

2.2. Hypotonic-isotonic dialysis procedure

Dialyzed rat erythrocytes were prepared under hypotonic (80 mOsm/kg) conditions as previously described [6]. Briefly, 0.5 ml of packed erythrocytes (70% hematocrit) were placed in a dialysis bag (Medicell, molecular mass cutoff, 12–14 kDa) and dialyzed against 40 volumes of hypotonic PBS, under rotation at 4°C for 50 min. Dialysis bags were then incubated in isotonic PBS for 10 min at 37°C (annealing). Finally, erythrocytes were resealed by addition of 0.1 vol of hypertonic PIGPAC solution (5 mM adenine, 100 mM sodium pyruvate, 100 mM inosine, 100 mM NaH_2PO_4, 100 mM glucose, 12% (w/v) NaCl) to restore isotonicity and then incubated for 30 min at 37°C. Resealed erythrocytes were washed three times at 160 g, for 5 min at 4°C, in isotonic Hanks solution (6 mM glucose in PBS).

2.3. Preparation of phase systems

A 5.6% (w/w) D–4.6% (w/w) PEG, 0.03 M sodium chloride and 0.09 M sodium phosphate buffer charge-sensitive two-phase system was prepared by weight from the following stock solutions: 20% (w/w) dextran T-500 (D) (Pharmacia, Uppsala, Sweden) standardized by polarimetry, 40% (w/w) poly(ethylene glycol) 6000 (Serva, Heidelberg, Germany), 0.2 M sodium phosphate buffer (pH 6.8) and 1 M sodium chloride [12,13].

2.4. Single partition experiments

A 100-μl volume of erythrocytes (20% hematocrit) was added to a two-phase system formed using 2 g of bottom phase and 2 g of top phase from the above equilibrated phases. The system was mixed by 60 inversions and allowed to settle for 20 min, in a vertical position (to enhance cell separation), at 4°C [19–22]. The partition ratio, P (quantity of cells in top phase, at time of sampling, as a percentage of the total number of cells added) was calculated [13]. Top phase and interface were carefully collected separately and the corresponding cells were washed three times with PBS, pH 8, at 4°C to obtain packed erythrocytes.

2.5. Counter-current distribution (multiple partition)

A thin-layer CCD apparatus (Bioshef TLCCD, MK 3) with two thin layer units (60 concentric

cavities) was used for CCD cell fractionation [12,13]. The bottom capacity volume of the units was 0.85 ml (unit 1) and 0.81 ml (unit 2). Three adjacent cavities (0–2 and 30–32) each received a mixture of 0.46 ml (unit 1) or 0.42 ml (unit 2) of D-rich bottom phase plus 0.1 ml of the erythrocyte suspension, whereas the remaining cavities each received 0.56 ml (unit 1) or 0.52 ml (unit 2) of the D-rich bottom phase. Thus, bottom cavities were filled to about 64% of their total capacity). A 0.77-ml (unit 1) or 0.72-ml (unit 2) volume of the PEG-rich top phase was added to the 60 cavities. The top/bottom-phase volume ratio of both units was $L=1.4$. A partition step consisted of a 20-s shaking period followed by a 6-min settling period and a transfer of the top phase. With each transfer, the cells in the top phase are carried forward and re-extracted with fresh bottom phase whereas the cells partitioned at the interface are left behind to be re-extracted with fresh top phase. Thirty partition steps were performed at 4°C. Then, 1 ml of PBS was added to each cavity in order to transform the two-phase system into a single phase, and the content of each cavity was collected separately. The cell distribution profile is given by the hemoglobin absorbance values at 540 nm against the cavity number. Cells with an affinity for the top phase [high-partition ratio, $G=r_{max}/(n-r_{max})$ where r_{max} is the cavity number of the peak of a CCD curve and n is the number of transfers] were distributed as fast-moving cells in the cavities with the highest number. Cells with an affinity for the interface (lower-G values) tended to remain in the fractions with the lowest number as slow-moving cells [12,13]. CCD experiments were repeated at least four times using different erythrocytes.

2.6. Cell separation in Ficoll-paque

A 4-ml volume of hypotonized erythrocytes (20% hematocrit) was carefully placed over 3 ml of Ficoll-paque (1.077 g/ml, Pharmacia). After centrifugation at 400 *g* for 30 min at room temperature, two fractions were obtained; one from the bottom containing the more dense cells (F_1) and the other from the top containing the less dense cells (F_2). Both fractions were separated and washed three times with PBS at 4°C to remove Ficoll-paque.

2.7. Osmotic resistance curves

A 20-μl volume of erythrocytes (20% hematocrit) was suspended in 2 ml of hypotonic PBS of increasing osmolality up to 300 mOsm/kg, at room temperature for 5 min. The absorbance of released hemoglobin was determined at 540 nm in the supernatant. 100% hemolysis was taken as the absorbance of hemoglobin released in distilled water.

3. Results and discussion

Partitioning in the D–PEG aqueous two-phase system is a highly sensitive method for the separation and fractionation of cells on the basis of surface properties [12,13]. The sensitivity stems from the exponential relation between parameters involved in partitioning and the partition ratio, P, of cells. The phases are mild and the partitioned cells are viable and retain their biological function [13]. This method allows erythrocyte charge and non-charge (hydrophobicity) surface properties to be detected by the appropriate selection of polymer and salt concentrations (maintaining physiological isotonicity and pH). Ions of some salts (e.g. phosphate ions) have affinity for the D-rich bottom phase, which results in a Donnan potential between the two phases (PEG-rich top phase positive), and produces a charge-sensitive two-phase system. Other salts (e.g. NaCl) have ions with essentially equal affinities for the two phases, and there is no potential difference between the phases and this produces a non-charge-sensitive system [12,13].

Partitioning behaviour in both charge- and non-charge-sensitive two-phase systems of hypotonically dialyzed (80 mOsm/kg) rat erythrocytes in comparison with the controls has been studied previously by our group [10]. A 5.6% D/4.6% PEG polymer concentration seems to be the most appropriate for partitioning hypotonized erythrocytes as a function of cell surface charge properties. This is the lowest polymer concentration at which a relatively high quantity (35%, Table 1) of cells are in the top phase when the system contains high phosphate concentrations (0.09 M, i.e. charge-sensitive systems); less than 10% of cells are in the top phase in the absence of this ion, i.e. when phosphate was substituted by

Table 1
Partition ratio, P, in a 5.6% D–4.6% PEG charge-sensitive two-phase system of control and hypotonically dialyzed (D-80) rat erythrocytes as well as of the more dense (F_1) and less dense (F_2) fractions separated with Ficoll-paque from hypotonized erythrocytes

	Control	D-80	F_1	F_2
Top phase	67.86 ± 1.30	34.44 ± 1.55	42.97 ± 1.90	29.00 ± 1.00
Interface	32.14	65.56	57.03	71.00

Cells adsorbed at the interface are obtained by subtracting the percentage of cells in the top phase from 100.
Values represent the mean ± S.E. (n=5).

NaCl (0.15 M, non-charge-sensitive system). Thus, the higher number of top-phase cells in charge-sensitive systems than in non-charge sensitive systems is mainly due to the cell surface charge properties [12,13].

Hypotonically dialyzed rat erythrocytes are fractionated by CCD (30 partition steps) in the charge-sensitive system into two well-defined cell subpopulations with a low- (cavities 1 to 8) or a high- (cavities 9 to 24) partition ratio, G, (Fig. 1, bottom). The high-G subpopulation is found in cavities equivalent to the controls (Fig. 1, top) and, therefore, this subpopulation should contain cells with similar charge surface properties to the controls, whereas cells in the low-G subpopulation have apparently lower charge surface properties. Since the charge surface properties of these low- and high-G subpopulations seem to be significantly different, their separation in a single partition step was assayed.

Hypotonized erythrocytes partitioned into the top phase (35% of the total cells) and those absorbed at the interface (the remaining 65%) (Table 1), were carefully separated and the cell separation resolution analyzed by CCD. To obtain enough cells for CCD fractionation, several top-phases or interfaces from different experiments were pooled. The CCD profile for the top phase cells shows a homogeneous curve, located between cavities 9 and 25 (Fig. 2), i.e. similar to the profile for the controls (Fig. 1, top) and to the above-mentioned high-G hypotonized subpopulation (Fig. 1, bottom). Cells extracted from the interface fractionate in the first 1 to 10 cavities (Fig. 3) and showed a profile like the one for the low-G hypotonized cell subpopulation (Fig. 1, bottom). We conclude that a single partition step gives good

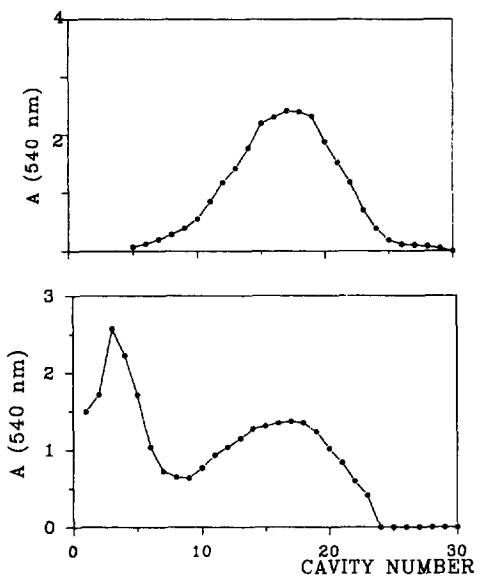

Fig. 1. Representative CCD (30 partition steps) fractionation profile (hemoglobin absorbance measured at 540 nm against cavity number), in a 5.6% D–4.6% PEG charge-sensitive (0.09 M phosphate buffer, pH 6.8 and 0.03 M NaCl) two-phase system of the control (top) and hypotonized (80 mOsm/kg) (bottom) erythrocytes. (From Ref. [10], with permission).

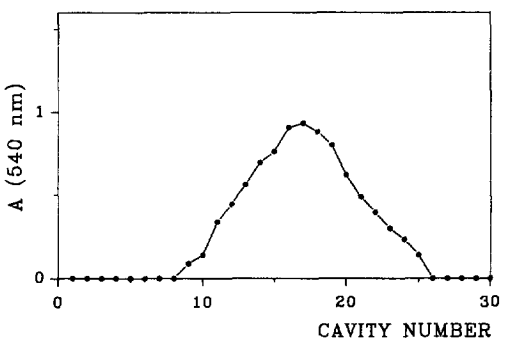

Fig. 2. Representative CCD (30 partition steps) fractionation profile (hemoglobin absorbance measured at 540 nm against cavity number), in a 5.6% D–4.6% PEG charge-sensitive (0.09 M phosphate buffer, pH 6.8 and 0.03 M NaCl) two-phase system of the hypotonized (80 mOsm/kg) erythrocytes partitioned in the top phase in a single partition step.

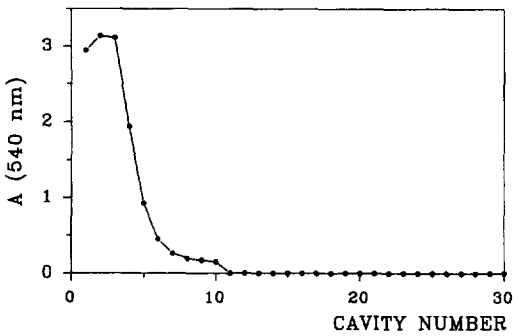

Fig. 3. Representative CCD (30 partition steps) fractionation profile (hemoglobin absorbance measured at 540 nm against cavity number), in a 5.6% D–4.6% PEG charge-sensitive (0.09 *M* phosphate buffer, pH 6.8 and 0.03 *M* NaCl) two-phase system of the hypotonized (80 mOsm/kg) erythrocytes absorbed at the interface in a single partition step.

resolution in the separation between cells with altered surface properties and the normal cells present in heterogeneous cell populations, as is the case of rat erythrocytes subjected to hypotonic dialysis.

Reduction of cell heterogeneity in the hypotonized erythrocyte populations was also undertaken according to cell density by centrifugation in Ficoll-paque. Two different cell fractions of high (F_1, >1.077 g/ml) and low (F_2, <1.077 g/ml) density were separated. F_1 contains most of the cells (95% of total) while F_2 contains only 5% of the total number of cells. Moreover, F_2 contains the cells with the highest osmotic fragility whilst F_1 contains cells with an osmotic fragility that is intermediate between the control and the whole hypotonized population (Fig. 4A), suggesting that the less dense fraction (F_2) removes the most altered cells during the hypotonic treatment. This agrees with earlier reports of density fractionations in human hypotonized erythrocytes [9].

The partitioning behaviour of F_1 and F_2 fractions was studied in 5.6% D–4.6% PEG charge-sensitive two-phase systems (Table 1). Charge surface properties of cells in F_2 were decreased (P=20%) while those of cells in F_1 were intermediate (P=50%) to the control and to the whole hypotonized population charge surface properties (Table 1), again suggesting that the most altered cells were located in the fraction F_2. Cell heterogeneity after Ficoll-paque separation was then analyzed by CCD only in the

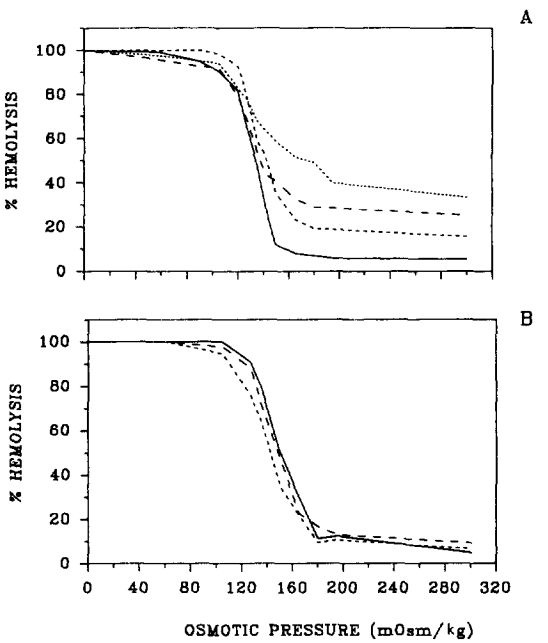

Fig. 4. Osmotic resistance curves of control (———) and hypotonically dialyzed (– – –) erythrocytes as well as of the more dense (- - -) and less dense (· · ·) fractions separated with Ficoll-paque from hypotonized erythrocytes, before (top) and after (bottom) CCD fractionation.

pooled F_1 fractions, since F_2 had too few cells to be fractionated.

F_1 was found to be a heterogeneous cell population when fractionated by CCD. Two well-defined subpopulations of low-*G* (cavities 1 to 10) and high-*G* (cavities 10 to 27), were repeatedly observed (results not shown). This CCD behaviour was similar to that obtained in the whole hypotonized cell population (Fig. 1, bottom). Therefore, F_1 contains a mixture of cells with charge surface properties and osmotic resistance (Fig. 4B) like the controls, i.e. it contains the cells least affected by the hypotonic dialysis process, as well as cells with decreased surface charge and increased osmotic fragility (some cell debris has also been observed by optical microscopy, results not shown). So, although some altered cells are removed in fraction F_2, this separation method is not as efficient in satisfactorily reducing the heterogeneity of hypotonized populations as single partition in two-phase systems.

In summary, the rat erythrocyte hypotonic dialysis process seems to be accompanied by membrane and

cell density alterations which result in heterogeneous erythrocyte populations. A single partition step in charge-sensitive D-PEG aqueous two-phase systems makes it possible to reduce such cell heterogeneity and to obtain two separate and well-defined cell subpopulations: cells with decreased surface charge, with affinity for the interface, that fractionate by CCD as a low-*G* subpopulation and other cells like the controls, with affinity for the top-phase, which fractionate by CCD as a high-*G* subpopulation.

It has been reported that the in vivo survival behaviour of carrier erythrocytes is characterized by a rapid loss of some cells during the first 24 h after reinjection, followed by a normal elimination of the remaining cells afterwards [11]. Surface charge altered cells are likely to be the first removed by the mononuclear phagocytic system, whereas cells with surface charge properties more similar to the controls might remain in blood. In this way, the low- and high-*G* subpopulations could have different potential pharmacological applications to direct carrier erythrocytes towards different organs thus performing a selective therapeutic action. Further in vivo studies with both separated cell subpopulations should improve our understanding about the effect of the hypotonic dialysis process on the survival of carrier erythrocytes.

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