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Settling-time dependence of rat bone marrow cell partition and counter-current distribution in charge-sensitive aqueous two-phase systems

Relationship with the cell partitioning mechanism

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SUMMARY

Differences in the settling-time dependence of single and multiple cell partitions have been found between heterogeneous (bone marrow cells) and homogeneous (erythrocytes) populations when using charge-sensitive dextran–poly(ethylene glycol) aqueous two-phase systems. The cell populations were partitioned using both single test-tube experiments and multiple thin-layer counter-current distribution. Lengthening the settling time, to favour phase separation, and decreasing the upper phase volume are more effective in fractionation by the counter-current distribution of heterogeneous cell populations than increasing the interfacial tension, although all three were employed to speed phase settling. On the basis of these results, the original cell partitioning mechanism proposed for non-charge-sensitive systems has been extended to charge-sensitive systems.

INTRODUCTION

Aqueous two-phase systems are formed when buffered and isotonic mixtures of dextran (D) and poly(ethylene glycol) (PEG) are allowed to separate over time. Mixtures of D and PEG near the critical point (low interfacial tension systems) need a relatively long settling time for phase separation. The higher the interfacial tension (far away from the critical point), the faster is the speed of phase separation. A partition ratio, P, between the total amount of cells in the PEG-rich upper phase and the cells adsorbed at the interface is obtained in single-tube experiments. Cell partitioning, which is based on differences in cell surface properties and their interaction with the phases, can be altered in various ways. Ions of some salts affect the physical

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properties of the systems. The affinity of phosphate for the D-rich bottom phase results in charge-sensitive systems, with a positive upper phase[1–4].

To increase the resolution of cells with close P values, multiple partitions can be carried out using a thin-layer counter-current distribution (CCD) procedure[1,4]. To produce a shorter time of phase separation, and thus increase the speed of phase settling, the height of the phase column of the CCD unit was reduced by Albertsson[1] in the thin-layer rotor. Other conditions of the CCD procedure can be varied to facilitate phase separation. A direct correlation between length of settling time and efficiency of fractionation was observed in charge-sensitive two-phase systems for heterogeneous cell populations from rat and human bone marrow[5–7] and from developing chicken erythrocytes[8–11]. As erythrocytes with very close P values are present in the blood of adult animals (rats and chickens), the settling time does not affect cell age fractionation in these homogeneous erythrocyte populations. This paper confirms that settling time is a determinant parameter for partitioning in single-tube experiments and for bone marrow cell fractionation by CCD in charge-sensitive two-phase systems.

Increasing system interfacial tension is an additional condition that can determine a shorter time of phase separation and thus affect the dependence of P on time[4,12]. Increasing polymer concentrations were then used in charge-sensitive systems to increase their interfacial tension. However, a higher interfacial tension did not affect the improved CCD fractionation observed with the lengthened settling time in the heterogeneous cells.

It has been reported recently that the decrease in time of phase separation, which can be produced by reducing the height of the phase column in the thin-layer CCD rotor, may result in a lower cell fractionation efficiency[13]. In our experience, shortening the height of the phase column (by reducing the volume of the upper phase in the cavities of the CCD rotor) and simultaneous lengthening of settling time are required for more efficient fractionation of heterogeneous cells[6], even when systems with higher interfacial tension were used, as shown here.

Finally, the results presented in this paper are interpreted in the light of the cell partitioning mechanism proposed for erythrocytes in non-charge-sensitive systems[12,14,15]. Taking into account the charge of the system and the lower surface charge of bone marrow cells with respect to erythrocytes[16], this mechanisms may serve to explain the higher efficiency of bone marrow cell fractionation by CCD with lengthened settling time[6].

EXPERIMENTAL

Preparation of cells

Male Wistar rats (150–250 g) were anaesthetized with dicthyl ether and killed by exsanguination. Bone marrow cell suspensions were prepared from the femur and tibia of at least five rats using a phosphate buffer (pH 6.8) saline solution (SSP) as previously described[17]. Bone marrow cells were resuspended in SSP, usually 1:1 (v/v). The total number of cells in the bone marrow cell suspensions was $9.48 \pm 3.61 \cdot 10^8$ cells/ml ($n = 36$). Blood was collected into heparinized tubes and, after centrifugation (400 g for 10 min), erythrocytes were washed three times with SSP.

Preparation of two-phase systems

Charge-sensitive two-phase systems, containing 0.03 mol/l sodium chloride and 0.09 mol/l sodium phosphate buffer (pH 6.8), were prepared and equilibrated as previously described[6,16]. The increasing polymer concentrations, expressed as % (w/w) dextran T-500 (D) (Pharmacia)–% (w/w) polyethylene glycol 6000 (PEG) (Serva), were 5.0–4.0, 5.2–4.2, 5.3–4.3 and 5.6–4.6.

Single partition experiments

Either 100 μl of bone marrow cell suspension or 5 μl of packed erythocytes were added to the phase systems formed by 2 g of bottom phase and 2 g of top phase from the above equilibrated phase systems. As equal volumes of both phases (phases density *ca.* 1)[4] are then being used, the top-to-bottom phase-volume ratio at equilibrium is $L = 1$, which is the normal phase-volume ratio used for cells in single partition experiments[3,12]. Systems were mixed by 60 inversions and allowed time to separate at 4°C. Duplicate aliquots were removed from the top phase at different times (10, 20, 40, 60 or 80 min). Haemoglobin[18] and protein[19] concentrations were measured and cells were counted in a Model ZBI Coulter counter. The partition ratio, P, is the amount of haemoglobin, protein or cells in the top phase given as a percentage of the total cells added to the system. P values were calculated by taking into account phase dilution from sample addition and top phase volume[3,4]

Counter-current distribution (multiple partitions)

An automatic thin-layer CCD apparatus (Bioshef TLCCD, MK3) with two 60-cavity circular rotors was used[4,20]. Each of five adjacent cavities of the rotor received a mixture of 0.6 ml of D-rich bottom phase and 0.1 ml of bone marrow cell suspension, while the other 55 cavities each received 0.7 ml of the bottom phase. Two different volumes (0.9 or 0.4 ml) of the top PEG-rich phase were then added to all sixty cavities to reach two different heights of phase columns. These are given by the top-to-bottom phase-volume ration, L, which was 1.3 (*i.e.*, 0.9/0.7 ml) or 0.6 (0.4/0.7 ml). The normal phase-volume ratio at phase equilibrium used for red cells on CCD is $L = 1.3$[3,21]. A partition step is formed by a 20-s shaking time, settling time (5 or 20 min) and a twist of the top rotor plate to transfer the top phase cavity to the next bottom phase cavity on the lower rotor plate. With each transfer the cells in the top phase are carried forward and re-extracted with the fresh bottom phase. Cavities of the lower (stator) plate contained 88% of their total capacity, thus allowing enough space for the cells to be adsorbed at the interface during each partition step. Fifty-five transfers were repeated at 4°C. More details are given in ref. 6.

The distribution of cells in the different cavities along the extraction train (*i.e.*, the CCD profile) is given in terms of absorbance at 410 nm (Uvikon 810 spectrophotometer; Kontron) and haemoglobin concentration[18]. Cells with affinity for the top phase (high P values) are distributed as fast-moving cells and migrate to the higher numbered fractions (*i.e.*, towards the right-hand side of the CCD profile). Cells with affinity for the interface (lower P values), and as slow moving cells, tend to remain in the fraction with a lower number (*i.e.*, towards the left-hand side of the CCD profile).

RESULTS AND DISCUSSION

Cell partition in single test-tube experiments

The partitioning of erythrocytes[3,12,16] and bone marrow cells[16], as a function of the increase in polymer concentration used to increase the interfacial tension, is mainly determined by hydrophobicity, in non-charge-sensitive systems, or charge, in charge-sensitive systems, of the cell surface. In all these studies a standard settling time (usually 20 min) was used. However, partitioning depends on the specific interaction between cells and microscopic D droplets suspended in the PEG-rich upper phase and the settling speed of cell–D droplet complexes to the bulk interface as phase separation proceeds[12]. This means that cellular P values are a function of the sampling time, *i.e.*, the phase settling time. Our objective was to study the dependence of P values on settling time (up to 80 min) with increase in interfacial tension, for both homogeneous (erythrocytes) and heterogeneous (bone marrow) cell populations in-charge-sensitive systems. Low, intermediate and high interfacial tension systems, formed by concentrations that were very close [5.0% (w/w) D–4.0% (w/w) PEG], close [5.3% (w/w) D–4.3% (w/w) PEG] and at some distance [5.6% (w/w) D–4.6% (w/w) PEG] from the critical point[4] were used here.

Erythrocyte partition

Low and intermediate interfacial tension systems. The dependence of P values on settling time with increase in interfacial tension has only been studied for erythrocytes in non-charge-sensitive systems[12,15]. The marked decrease with time of P values (60% at 80 min) observed in a low interfacial tension system [5.0% (w/w) D–4.0% (w/w) PEG] is a consequence of almost all the erythrocytes being associated with D droplets in the PEG-rich top phase and the fact that cell–D droplet complexes settle to the interface more rapidly than do free cells[12,14]. The interaction of cells with D droplets is reduced in charge-sensitive systems with a low interfacial tension[3,12] and, therefore, more free cells should be present in the upper phase. The high P values shown by erythrocytes in these systems[3,12,16,22] could therefore be due to slow sedimentation by the abundant free cells in the upper phase.

The decrease with time of P values in charge-sensitive systems with a low [5.0% (w/w) D–4.0% (w/w) PEG; Fig. 1A] or intermediate [5.3% (w/w) D–4.3% (w/w) PEG; Fig. 1B] interfacial tension seems not to be very pronounced (*ca.* 10% decrease at 20 min and 20–25% decrease at 80 min in both instances). It may be that erythrocytes (negatively charged at pH 6.8) tend to remain in the positive PEG-rich upper phase as free cells. The upper phase is positive with respect to the D-rich bottom phase, because phosphate partition towards this phase is asymmetric. Another contribution to the increase in the number of free cells in the upper phase may be electrostatic repulsion between cells and the phosphate-rich negative D droplets in the upper phase. As a consequence, interaction between cells and D droplets would be weaker in charge-sensitive systems than in non-charge-sensitive systems and a larger number of free cells would be present in the upper phase of charge-sensitive systems[12]. As free cells settle more slowly than cell–D droplet complexes, a smaller number of total cells are adsorbed at the interface at any given settling time and the decrease over time of the high P values is slow at both interfacial tensions (Fig. 1A and B).

The fact that erythrocytes are mainly free in the upper phase was supported by

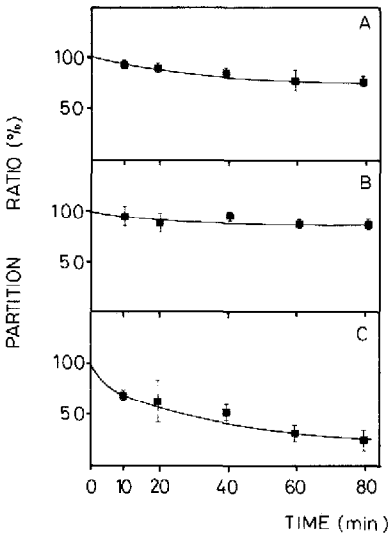

Fig. 1. Partitioning behaviour of erythrocytes with increase in phase settling time and interfacial tension in charge-sensitive systems. Partition values, P, in (A) 5.0% (w/w) D–4.0% (w/w) PEG, (B) 5.3% (w/w) D–4.3% (w/w) PEG and (C) 5.6% (w/w) D–4.6% (w/w) PEG two-phase systems are given in terms of haemoglobin content in the top phase as a percentage of that present in the total cells added. Points represent the means (± S.D.) at least of four separate partition experiments, each samples and measured in duplicate.

the results of additional cell partitioning experiments. A mixture of erythrocytes and the upper phase of a charge-sensitive system was carefully added to an equal volume of bottom phase from a similar system (thus forming a D droplet-free two-phase system). The proportion of cells remaining unsedimented after an 80-min settling time (higher than 80%; results not shown) is similar to P values obtained by mixing the two-phase system (Fig. 1A).

High interfacial tension systems. Increasing interfacial tension leaves a higher proportion of cell–D droplet complexes in the upper phase of non-charge-sensitive systems and results in a more pronounced decrease in P values with time than that found in low interfacial tension systems[12] The partition of erythrocytes in charge-sensitive systems of 5.6% (w/w) D–4.6% (w/w) PEG (far away from the critical point) also shows a marked decrease with time (50% at 20 min; 75% at 80 min) (Fig. 1C). This means that, in comparison with the data in Fig. 1A and B, a smaller number of free cells (*i.e.*, a larger proportion of cell–D droplet complexes) are present in the upper phase at any given settling time. The larger proportion of cell–D droplet complexes and their faster settling at the interface can explain the marked decrease in partition values over time (Fig. 1C). The effect of the large potential difference between the phases of charge-sensitive systems is then overcome by the effect of the high interfacial tension. This agrees with the decrease in sensitivity by charge-sensitive systems to the surface charge of erythrocytes with increase in polymer concentration[16].

Bone marrow cell partition

Low and intermediate interfacial tension systems. Variations with time of P values are shown in Fig. 2 for low [5.0% (w/w) D–4.0% (w/w) PEG; Fig. 2A] and intermediate [5.3% (w/w) D–4.3% (w/w) PEG; Fig. 2B] interfacial tension systems. Because of bone marrow cell heterogeneity, P values are expressed in terms of cell number, protein content and haemoglobin levels (as an index of erythroid cells). No significant differences were found between these parameters.

A small decrease in P values with time (*ca.* 20–25% at 20 min and 30–40% at 80 min) is observed in charge-sensitive systems with low interfacial tension (Fig. 2A). This decrease is slightly higher than that shown by erythrocytes in a similar system (10% at 20 min and 20–25% at 80 min; Fig. 1A). The surface-charge dependence of P values on the electrical potential difference between the phases is lower for bone marrow cells (as a reflection of their lower surface charge at pH 6.8) than for erythrocytes[16]. Interaction with the positive PEG-rich upper phase will then be weaker for bone marrow cells, implying that a smaller number of free cells (and a larger amount of cell–D droplet complexes) are present in the upper phase at any settling time. The larger proportion and the higher mobility of cell–D droplet complexes at the interface serve to explain the small decrease in P values over time in Fig. 2A.

The fact that a smaller number of free bone marrow cells are present in the upper phase is also supported by additional experiments carried out as for erythrocytes. A mixture of bone marrow cells and upper phase was carefully added to the bottom phases. The proportion of unsedimented cells after 80 min (*ca.* 80%; results not shown) is higher than P values obtained by mixing the two-phase system (60–70%; Fig. 1A).

The decrease with time of P values in intermediate interfacial tension systems is clearly higher for bone marrow cells (45–50% at 20 min and 75–80% at 80 min; Fig. 2B) than for erythrocytes (10% at 20 min and 20–25% at 80 min; Fig. 1B). Both the low negative surface charge of the bone marrow cells[16] and the increase in interfacial

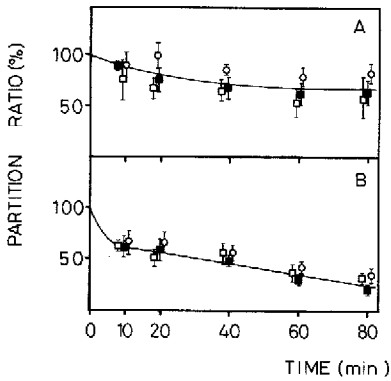

Fig. 2. Partitioning behaviour of bone marrow cells with increase in phase settling time and interfacial tension in charge-sensitive systems. Partition values, P, in (A) 5.0% (w/w) D–4.0% (w/w) PEG and (B) 5.3% (w/w) D–4.3% (w/w) PEG two-phase systems are given in terms of (■) haemoglobin, (□) protein content and (○) cell number in the top phase as a percentage of that present in the total cells added. Points represents the means (± S.D.) of at least four separate partition experiments, each sampled and measured in duplicate.

tension favour a decrease in P values over time. The proportion of rapidly moving cell–D droplet complexes is higher in the upper phase. Therefore, the tendency for bone marrow cells to remain as free cells in the upper phase can be assumed to be lower.

High interfacial tension systems. Experiments were also carried out at a 5.6% (w/w) D–6.6% (w/w) PEG polymer concentration (results not shown). The decrease in P values over time was nearly total (75% at 20 min and >90% at 80 min), indicating that this cell population moves quickly (in the form of cell–D droplet complexes) to the interface. The effect of this high interfacial tension again overcomes the effect of the potential difference on the partitioning of bone marrow cells. The number of cell–D droplet complexes formed increases progressively from low to high interfacial tension values. The increase in interfacial tension then favours the adsorption of cell–D droplet complexes at the interface more in bone marrow cells than in erythrocytes.

Bone marrow cell fractionation by counter-current distribution

Improved fractionation of heterogeneous cell populations has been observed previously when the settling times are longer (20 min) than those usually employed (5–7 min) during thin-layer CCD in low-interfacial charge-sensitive systems. CCD profiles with at least two or three peaks (due to the presence of different cell subpopulations) were always obtained. The increase in settling time did not improve the fractionation of homogenous erythrocyte populations where single-peak profiles were observed[5,6,8–11].

These observations were studied in this work using higher interfacial tension-systems, which facilitate phase separation and therefore sedimentation of the cell–D droplet complexes. As P values for bone marrow cells decrease with increase in interfacial tension (Fig. 2A and ref. 16), the possibility exists of employing a shorter settling time during the fractionation and so improve cell resolution.

Another factor determining the speed of phase settling is the height of the phase column. The more rapid settling of phases produced by reducing the height of the phase column in the thin-layer CCD rotor seems to decrease the interaction of cells with D droplets, thereby interfering with the cell partitioning mechanism, which, in turn, may result in a lower efficiency of cell fractionation[13]. As weak interaction between cells and D droplets in charge-sensitive systems has already been shown (preceding section), reducing the height of the phase column in the CCD rotor should not affect the cell partitioning mechanism in these systems significantly. In fact, fractionation of heterogeneous bone marrow cells is not improved by just shortening the height of the phase column (for instance, reducing the volume of the upper phase) in the cavities of the CCD rotor. A longer settling time (20 min) than that usually employed (5–7 min) was simultaneously required to improve cell fractionation[6]. Therefore, experiments were carried out at both a short (5 min; Fig. 3) and a long (20 min; Fig. 4) settling time, using three systems with a range of low and intermediate interfacial tension values [5.0% (w/w) D 4.0% (w/w) PEG; 5.2% (w/w) D–4.2% (w/w) PEG; 5.3% (w/w) D–4.3% (w/w) PEG]. Bone marrow cells are totally adsorbed at the interface of systems with higher interfacial tension (preceding section), and therefore they cannot be used.

Fractionation at short settling times. Representative CCD profiles for the fractionation of bone marrow cells, at a short settling time (5 min), are shown in charge-sensitive systems with low (Fig. 3A) and intermediate (Fig. 3B and C) interfacial tensions. A high (L = 0.9/0.7 = 1.3) and a low (L = 0.4/0.7 = 0.6) phase column were employed. Results are given for a high phase column (L = 1.3; Fig. 3) as similar single-peak profiles, located in the earlier cavities of the rotor, were also obtained for a low-phase column (L = 0.6; results not shown).

Assuming that a high proportion of free bone marrow cells is present in the upper phase of low interfacial tension systems (high P values; see discussion for Fig. 2A) and that free cells settle slowly towards the interface[12], a displacement of free cells in the top phase of each cavity towards the next one may take place during the CCD procedure. A low fractionation efficiency (single-peak profile) is obtained as a consequence of free cell displacement towards the high-numbered cavities of the CCD rotor (Fig. 3A and ref. 6). In contrast, the fractionation of bone marrow cells in non-charge-sensitive systems, at both a similar interfacial tension value and a short settling time, gave rise to two broad subpopulations[23,24], as a result of the small proportion of free bone marrow cells in the upper phase of low interfacial tension systems (low p values; ref. 16) and the fact that cells bound to D droplets settle rapidly towards the interface[12].

Owing to the rising affinity of bone marrow cells for D droplets with increase in interfacial tension, the P values decrease (Fig. 2 and ref. 16). However, improved fractionation was not achieved by increasing the interfacial tension of the systems. As shown, a single-peak profile was also obtained in intermediate interfacial tension

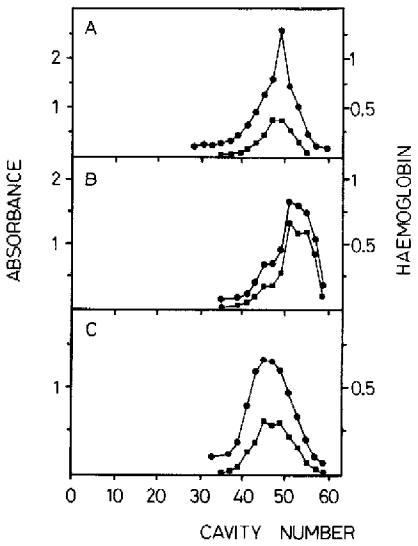

Fig. 3. Influence of interfacial tension on the fractionation by thin-layer CCD of rat bone marrow cells, at a short settling time (5 min). Two-phase systems contained (A) 5.0% (w/w) D–4.0% (w/w) PEG, (B) 5.2% (w/w) D–4.2% (w/w) PEG and (C) 5.3% (w/w) D–4.3% (w/w) PEG polymer concentrations. Distribution of cells is given in terms of (●) absorbance (410 nm) and (■) haemoglobin (mg/ml) present in each cavity of the distribution rotor. See text for details.

systems [5.2% (w/w) D–4.2% (w/w) PEG, Fig. 3B, 5.3% (w/w) D–4.3% (w/w) PEG, Fig. 3C]. In summary, a short settling time (5 min) was not long enough to improve the fractionation of bone marrow cells at any suitable polymer concentration.

Fractionation at long settling times. Representative CCD profiles for bone marrow cell fractionations at a long (20 min) settling time, in charge-sensitive systems with low and intermediate interfacial tension values, are shown in Fig. 4A–C. When using a low phase column (upper phase 0.4 ml; $L = 0.6$), fractionation into two broad subpopulations at any polymer concentration is observed (Fig. 4). However, when using a higher phase column (upper phase 0.9 ml; $L = 1.3$) only single-peak profiles were obtained (ref. 6 and results not shown). This means that settling time combined with the decrease in the height of the phase column affects the fractionation of heterogeneous bone marrow cells more significantly than does the increase in polymer concentration. Similar conclusions have been reached in experiments with heterogeneous avian red cell populations during animal development. CCD fractionation in two subpopulations was obtained, in this instance, by using a slightly higher volume of upper phase (0.7 ml) and a 20-min settling time[9–11].

A comment must be made in relation to the improvement in fractionation using a low phase column, *i.e.*, those made using a lower volume (0.4 ml) of upper phase. As free cells predominate in the upper phase of charge-sensitive systems, the more rapid settling of phases produced by reducing the height of the phase column should not, by itself, affect the partitioning behaviour of cells during CCD fractionation. However, the shorter distance between the cells ad the interface, and the longer settling time, are both parameters that favour free cell sedimentation and therefore influence CCD separation. It can then be suggested that most of the free cells (those with a high surface charge in the bone marrow cell population) would be those located in the high-numbered cavities of the CCD profile (around cavities 30–50, Fig. 4), whereas

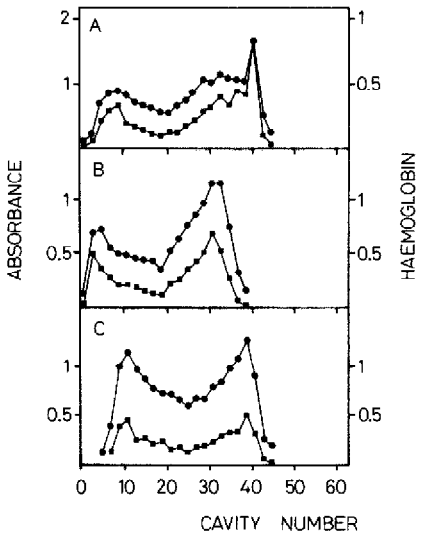

Fig. 4. Influence of interfacial tension on the fractionation by thin-layer CCD of rat bone marrow cells, at a long settling time (20 min). Two-phase systems and symbols as in Fig. 3. See text for details.

cell–D droplet complexes, because of their faster sedimentation, would be located in the low-numbered cavities (between 5 and 20, Fig. 4).

In summary, more efficient CCD fractionation of bone marrow cells is achieved when a long settling time (20 min) and a short phase column ($L = 0.6$) are used. Changes in interfacial tension then seem not to be as important as variations in settling time and phase column height to improve the efficiency of bone marrow fractionation. The above results support the necessity of taking these two parameters into account when attempting the CCD fractionation of heterogeneous cell populations in charge-sensitive systems.

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