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Separation and characterization of red blood cells with different membrane deformability using steric field-flow fractionation

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Abstract

Human red blood cells were treated in different ways to alter their membrane deformability, and the hydrodynamic behavior of these altered cells was studied using the steric field-flow fractionation (FFF) technique. The relationships between cell retention in the FFF channel, flow-rate of the carrier fluid and the applied field strength were studied for normal and glutaraldehyde-fixed human red cells, and separation conditions were optimized. The effect of flow-induced hydrodynamic lift forces on red cell retention in the steric FFF channel was studied, and the results suggest that the membrane deformability of the red cell is an important factor contributing to the lift force, besides other previously described effects due to density and flow velocity. Using steric FFF, a mixture of normal and glutaraldehyde-fixed human red cells was completely separated with a resolution twice that found in published data from gel permeation, another hydrodynamic separation technique. Partial loss of membrane deformability, induced by different degrees of glutaraldehyde-fixation, by diamide, or by a thermal treatment, has also been studied. Steric FFF is thus shown to have potential for rapid separation and differentiation of red cells with different density and membrane deformability, conditions known to be associated with, e.g., cell senescence and certain hematological diseases.

1. Introduction

The deformability of erythrocytes is an important factor in blood circulation, allowing red blood cells to undergo the shape deformations required for them to pass through capillaries with diameters even smaller than that of the relaxed discoid cells. It is further responsible for reducing the bulk viscosity of blood flow in large vessels, thereby causing a non-Newtonian viscoelastic behavior of the fluid [1]. The ability of red cells to undergo shear deformation is gradually lost as the cell ages, and viscoelastic studies clearly show the deformation under shear, expressed as the ratio of length to width, as being significantly higher for young than for old cells [1].

The intrinsic deformability of the cell is determined by three major factors [1]: the viscoelastic properties of the membrane; the viscosity of the intracellular hemoglobin-rich fluid (internal fluid viscosity); the geometry of the cell, including the ratio of surface area to cell volume.

Various methods have been used to analyze red cell deformability by characterizing its stress–

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strain (and stress–rate of strain) relations [1,2]. These analyses include measurements of the average deformability of a large cell population, using procedures such as Millipore or paper filtration, rheoscopy, cinephotomicrography, cone-plate and cone-in-cone viscometry, ektacytometry, and centrifugation, or, by measurements of deformability of single cells, by e.g. the micropipette technique [1,3]. These measurements do not allow the fractionations and further characterizations of cell sub-populations.

In the present work, the sedimentation-based steric field-flow fractionation (FFF) technique has been used to separate and isolate red cells with different densities and deformabilities. FFF is a family of analytical separation techniques applicable to macromolecules and particles over a 10^{15}-fold particle mass range, corresponding to a 10^{5}-fold particle diameter range [4]. The technique is based on elution from a narrow flow channel, much like in chromatography, but the separation takes place in a single phase and is induced and controlled by a field or gradient applied in a direction perpendicular to the flow. Steric FFF, a relatively new form of FFF, provides rapid separation of particles in the 1–100 μm diameter range; the boundaries of this range are somewhat flexible and depend on the choice of experimental parameters. The field responsible for accomplishing this separation can be electric, sedimentation, a cross-flow, a thermal gradient or other [4]. Although steric behavior is present in all forms of FFF, the bulk of present applications derive from the use of sedimentation fields (i.e., either gravity or a field generated in a centrifuge) [4]. In steric FFF, separation occurs because particles transported through the separation channel are held in close proximity to the wall by their interaction with the field. Under these circumstances, larger particles protrude further into the flow stream than smaller ones, and are as a result carried downstream at a faster rate. Previous work has involved the separation and characterization of fresh red blood cells from different species [4–6], and the separation of viable HeLa cells from fixed red cells [4]. These examples have clearly demonstrated that human and animal cells are suitable samples for the steric FFF technique.

It has been shown [4,7] that particle migration in the steric FFF channel depends on size, shape and density, as well as on the viscosity (unpublished) and density of the medium. In addition, the relative migration rate of the particles is also highly influenced by hydrodynamic lift forces [4,7], which are induced by the flow of carrier fluid. Due to a loss of membrane deformability upon aging, senescent and young red blood cells are known to experience different hydrodynamic behavior under high shear in laminar flow [1]. Since it is reasonable to expect that red cells with different membrane deformability will also respond differently to the laminar flow in the thin FFF channels, we have undertaken the present study on red cell behavior under different conditions of field and flow in sedimentation steric FFF. Specifically, we wish to examine the effects of changes in membrane deformability on cell migration in this system. If differences in migration are significant, it is our hypothesis that the steric FFF technique may offer a convenient way of separating cells based on differences in membrane rigidity. In addition, the separation patterns may have potential use as diagnostics of the senescence or pathological state in a red blood cell population.

2. Experimental

2.1. Blood

Freshly drawn human blood was collected in EDTA-containing tubes (Becton Dickinson, Rutherford, NJ, USA); the red cells were sedimented and washed three times in isotonic phosphate buffered saline (PBS) solution (0.01 *M* phosphate, 0.137 *M* NaCl, 0.0027 *M* KCl, final pH was adjusted to 7.4 with 1 *M* NaOH) to remove the blood plasma, white cells and platelets. The final cell concentration in the PBS suspension was 5% to 10% by volume.

2.2. Chemical fixation

Human red cells were fixed with glutaraldehyde (J.T. Baker, Phillipsburg, NJ, USA) to different degrees. After being washed with iso-

tonic PBS, a certain volume of the supernatant of the red cell suspension (10% cells by volume) was replaced by an equal volume of glutaraldehyde solution buffered by PBS. The suspensions were then incubated at room temperature for 2 h. After fixation the cells were washed with PBS to remove all unreacted materials.

A mixture of normal and glutaraldehyde-fixed red cells was obtained by washing freshly drawn human blood with PBS buffer, and taking one part of the washed RBCs for glutaraldehyde-fixation. After fixation, fresh and glutaraldehyde-fixed cells were mixed to form the injection plug.

Chemical alteration of the red cell membrane was also induced by diamide. Human blood was collected as described before, and washed 3 times in isotonic 0.05 m*M* Tris-buffered saline (0.05 *M* Tris-HCl, 0.15 *M* NaCl, final pH was adjusted to 7.4 with 1 *M* NaOH). The washed cells (10% by volume) were incubated with different concentrations (0.2–0.8 m*M*) of diamide (Sigma, St. Louis, MO, USA) in Tris-buffered saline for 90 min at 370°C, and then transferred to ambient conditions and injected into the steric FFF channel immediately.

2.3. Thermal treatment

Washed human red cells were heated in a water bath where the temperature was held constant at 49°C ± 0.05°C for 15 min. The cell sample was injected into the steric FFF channel immediately after heating.

2.4. Cell density measurement

The density of various red cell fractions was determined by density gradient fractionation. Different density media were prepared by mixing Nycodenz 1.077 g/ml and Nycodenz 1.150 g/ml (NycoPrep, reagents for preparation of density gradients, GIBCO Laboratories, Grand Land, NY, USA) at different volume ratios. Washed cells were diluted three times in TBS (0.1 *M* NaCl, 10 m*M* EDTA, and 25 m*M* Tris, pH 8.0), layered carefully on top of a Nycodenz solution with known density, and centrifuged at 600 *g* for 20 min at room temperature. If the density of a cell is higher than that of the Nycodenz solution, it will settle to the bottom of the centrifuge tube; if it is lower than that of the Nycodenz solution it will stay on the top; if it is equal to the Nycodenz density the cell population should distribute evenly in the solution. By centrifuging cells with a series of Nycodenz solutions with different densities, the red cell density can thus be determined with an accuracy of ±0.001 g/ml.

2.5. The steric FFF system

The set-up of the steric FFF system was the same as that used for sedimentation FFF, and has been described in detail elsewhere [4,8]. The walls of the channel were made of a corrosion-resistant alloy (Hastelloy C) which was polished to a smooth finish. Unless otherwise specified, the channel dimensions were 0.0254 cm × 2.0 cm × 94.5 cm, yielding a measured void volume, V^0, of 4.7 ml [4]. The rotor radius was 15.5 cm in both cases, and the sedimentation field G (units of gravity, 1 $g = 980$ cm/s^2) can be calculated from the rotational speed rpm and the rotor radius r: $G = (\text{rpm} \cdot 2\pi/60)^2 \cdot r$.

The carrier flow was fed to the channel by a peristaltic pump (Rainin Instruments, Woburn, MA, USA). Different sedimentation fields were achieved by changing the spin rate of the centrifuge. Both flow-rate and spin rate were under computer control (ZEOS 386SX PC). The effluent from the channel was monitored with a Linear UV-106 (Linear Instruments, Reno, NV, USA) detector, which was operating with a 254-nm light source.

Sample injection was made by insertion of a syringe needle through the septum injection port which was welded onto the inner wall of the channel at the apex of its V-shaped inlet [4]. Small volumes of cell suspensions (4–8 μl) with cell concentrations around 5–10% by volume, were injected into the channel and exposed to a sedimentation field under a constant channel flow. Following injection, the flow was stopped for a short period to allow the sample to sediment to the channel wall (relaxation). The appropriate relaxation time varied with the sample and the applied external field. At the resumption of flow the volume of effluent was being measured,

and the appearance of the cell peak in the effluent was indicated by the detector.

During the separation, a sedimentation force F_s, is affecting the cells. The magnitude of this force is given by:

$$F_s = V\Delta\rho G \quad (1)$$

where V is the particle volume, $\Delta\rho$ is the density difference between particle and carrier fluid and G is the applied sedimentation field.

As the laminar channel flow is initiated, a hydrodynamic lift force is immediately developed which tends to move the cells towards the channel center. The exact nature of this lift force is unknown, but experimental studies on rigid spheres of polystyrene and silica have shown that it depends on particle size, velocity and lateral position in the flow profile [4,7,9]. An equilibrium between the two opposing forces is rapidly reached, and the cells will migrate through the separation channel at velocities characteristic of their degrees of levitation.

In practice, the observed retention ratio R, computed as the ratio of void volume V° to elution volume V_e, reflects the average migration velocity of the particle population in the steric FFF channel, so that R values for less retained particles are higher than those for more retained particles. The retention ratios for fresh and glutaraldehyde-fixed red cells were studied both by increasing the sedimentation field while keeping the flow-rate constant, and by increasing flow-rate under constant sedimentation field.

3. Results and discussion

In previous steric FFF work, blood cells and other types of cells were successfully separated according to differences in either size or density [4,8]. It was noticed that besides these two effects, a velocity-induced lift force played an important role in governing cell migration through the FFF channel. Other studies of flow effects on cells near walls [16] have shown that an increase of shear stress caused an increased hydrodynamic lift force directed away from the wall, and a tentative interpretation of this phenomenon was that cell membrane deformability increases with increasing shear stress. Since changes in deformability are of clinical importance, it is the purpose of the present study to explore the effect of deformability on retention in steric FFF. The lateral migration of red cells in laminar flow is illustrated by the early findings [16] that cells are not evenly distributed in a blood vessel through which blood is flowing, but rather tend to migrate away from the vessel walls, leaving behind a cell-depleted layer (also referred to as the skimming layer or plasma layer). This inward migration is thought to be due to the effect of hydrodynamic lift forces, and to be highly influenced by cell deformability [16]. If so, the normal and glutaraldehyde-fixed cells should have significantly different behavior also in a sedimentation steric FFF system, even after migration effects due to size and density have been taken into consideration.

3.1. Effect of glutaraldehyde fixation

The glutaraldehyde fixation of red cells has long been known to induce a cross-linking of the cellular proteins, including those in the cell membrane. A highly fixed cell can be considered as a discoid-shaped non-deformable rigid body, and as an extreme in terms of membrane rigidity. Examples of the typical FFF elution behavior of normal and glutaraldehyde-fixed cells are shown in Fig. 1. Here, both samples are analyzed under optimal conditions (for optimization, see below), with a flow-rate of 3 ml/min and field strength of 4 g (wall shear rate 230 s^{-1}). As seen, the normal red cells eluted from the channel at a significantly faster rate than their glutaraldehyde-fixed counterparts. A mixture of two cell populations can be successfully separated, as seen from the fractogram in Fig. 2, in which the peak positions of the normal and fixed cells are consistent with those observed in Fig. 1. The quantities of cells of each type in the mixture were randomly chosen, which explains the difference in peak height between fresh and fixed cells.

The fixed cells in Figs. 1 and 2 were obtained through cross-linking with rather substantial

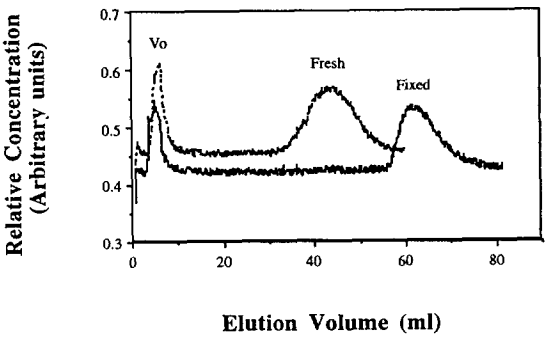

Fig. 1. Fractograms of fresh and 1% glutaraldehyde-fixed red cells. The system was operated under optimal conditions: sedimentation field strength was kept constant at 4 *g* and the flow-rate was kept constant at 3 ml/min.

amounts (1% w/v) of glutaraldehyde. It was therefore of interest to examine the retention behavior of cells exposed to fixation under less severe conditions. For this purpose, different degrees of red cell fixation were induced by changing glutaraldehyde concentration in the cross-linking solution, from 0.001%, 0.005%, 0.01%, 0.015%, 0.1%, up to 0.3%. The resulting FFF migration rates (represented by retention ratio *R*) of the cells were found to decrease with the increase in glutaraldehyde concentration, as shown in Fig. 3. In particular, very large changes in cell behavior are seen to take place in the interval of 0–0.025% glutaraldehyde, while the hydrodynamic behavior is seen to reach a plateau above 0.1% glutaraldehyde.

The exposure of the red cells to glutaraldehyde

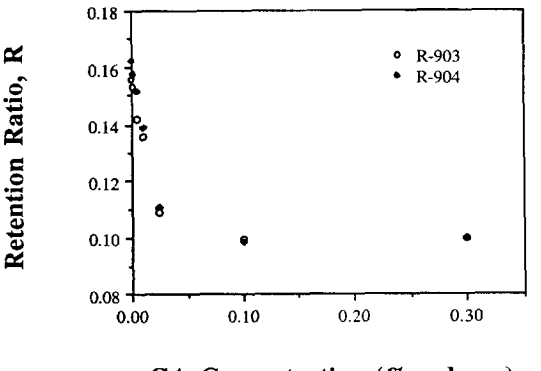

Fig. 2. Fractogram of a mixture of fresh and 1% glutaraldehyde-fixed red cells at optimal conditions.

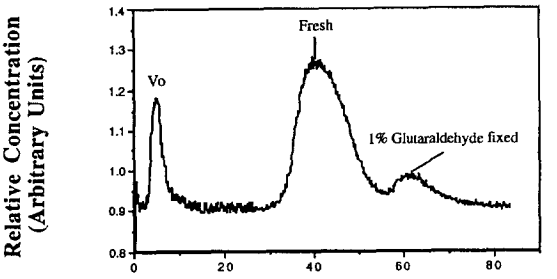

Fig. 3. The effect of partial fixation of red cells with increasing glutaraldehyde concentration on the retention ratio *R*. The samples were collected from two individuals: R-903 (individual A) and R-904 (individual B) (performed in a similar steric FFF system with channel dimensions of 0.0254 cm × 2.0 cm × 89.5 cm, and void volume of 4.9 ml).

changes their physical properties in several ways: deformability decreases, density increases (see below), and cell volumes might be reduced. All factors are likely to influence retention. In order to evaluate the effects of cross-linking on cell volume, the normal and glutaraldehyde-fixed cell preparations were both examined by microscopy, and were found to show no appreciable difference in size, as shown in Table 1. In addition, two other FFF studies [6,11] have suggested that no direct correlation exists between red cell retention and cell volume. This may be due to the fact that red cells are highly deformable, and change their shapes in response to changes in their environment.

The density effect is somewhat more difficult

Table 1
Measured diameters of fresh and fixed red cells

Glutaraldehyde (% w/v)	Diameter (μm)	S.D. (μm)
1	9.28	0.57
0.5	9.34	0.46
0.1	9.24	0.34
0[a]	9.32	0.21

Red cells are fixed in different glutaraldehyde concentrations as shown.
[a] Fresh red blood cells, without glutaraldehyde treatment.

to assess. As mentioned before, the hydrodynamic lift forces are balanced by the settling force, which is proportional to the density difference $\Delta\rho$ between the cells and the carrier fluid (see Eq. 1). Therefore, the retention time is likely to increase with $\Delta\rho$. Since we did observe a density increase after glutaraldehyde-fixation, and since this would generate a higher settling force for fixed than for normal cells under the same field strength G, it was important to examine whether the increased retention upon glutaraldehyde-fixation was primarily an effect of this increased density, or whether it derived from other factors such as reduced membrane deformability. A particular red cell sample with 0.015% glutaraldehyde fixation was selected to demonstrate the existence of retention effects other than those due to increases in density difference between the cells and their carrying medium. The densities of the cross-linked cell population as well as of its normal counterparts were carefully determined by the differential centrifugation technique. A carrier solution was then prepared to exactly compensate for the increase in cell density upon fixation, so that the density difference between the treated cells and the adjusted carrier was the same as that between the fresh cells and the normal carrier. The fractograms of (1) normal cells in normal carrier, (2) 0.015% glutaraldehyde-fixed cells in normal carrier, and (3) 0.015% glutaraldehyde-fixed cells in density-compensated carrier are shown in Fig. 4. It is clear that despite density compensation, the treated cells still elute at a slower rate than do the fresh cells, although their migration velocity is somewhat faster than for the treated cells in the lower-density carrier, as expected. This important fact suggests that factors other than volume and density, with deformability as a likely candidate, do indeed play a role in governing red cell behavior under laminar flow.

3.2. System optimization

As mentioned before, the lift forces acting on cells and other particles under flow in narrow channels and ducts are highly influenced by the average flow velocity of the carrier fluid [7]. To

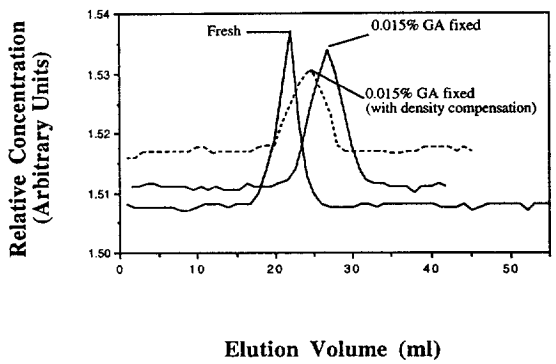

Fig. 4. The fractograms of fresh red cells with normal PBS carrier; 0.015% glutaraldehyde-treated red cells with normal PBS carrier; and 0.015% glutaraldehyde-treated red cells with density-compensated PBS carrier (performed in a similar steric FFF system with channel dimensions of 0.0254 cm × 2.0 cm × 89.5 cm, and void volume of 4.9 ml).

examine the effect of flow-induced lift on red cell retention in the steric FFF channel, the retention behavior of normal and glutaraldehyde-fixed cells was studied under various conditions of field strength and flow-rate. Similar studies were performed early on using normal fresh red cells [4], but these observations did not involve glutaraldehyde-fixed human red cells. The relationships between retention and flow-rate for normal and glutaraldehyde-fixed cells in the metallic channel used here are illustrated in Fig. 5; for the former there is good agreement with the earlier observations [4]. It is interesting to note that when the

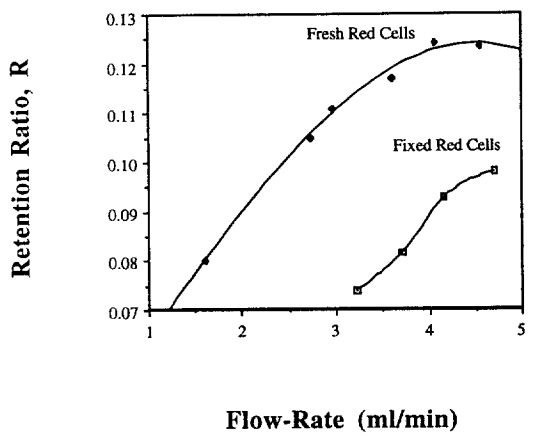

Fig. 5. Relationships between retention ratio R and flow-rate of carrier fluid for normal and glutaraldehyde-fixed cells. The sedimentation field was kept constant at 4 g (150 rpm).

flow-rate decreased beyond a threshold value (3 ml/min at field strength = 4 *g*), most of the glutaraldehyde-fixed cells were retained in the channel until the field was removed, while the normal cells were able to elute from the channel even at flow-rates as low as 1.5 ml/min under the same field strength. This suggests that the hydrodynamic lift force for fresh deformable and less dense red cells is greater than that of the glutaraldehyde-fixed cells under the same field and flow conditions. When the flow-rate was raised beyond the optimal level, the difference in retention between the normal and glutaraldehyde-fixed cells decreased, making the separation process less selective.

To optimize the steric FFF separation, the relationships between retention and sedimentation field for normal and glutaraldehyde-fixed cells were also studied. High sedimentation forces and potentially strong wall interactions will offset the lift forces and increase retention to the point where the particles are forced to adsorb to the channel wall. This point is reached at much lower *g*-force for the fixed than for the fresh cells, as seen in Fig. 6. It was observed that, under a constant flow-rate of 3.1 ml/min (wall shear rate of 230 s^{-1}), the sedimentation fields could be increased up to a threshold value of around 7 *g*, beyond which no measurable amount of glutaraldehyde-fixed cells was able to elute from the channel. The corresponding threshold

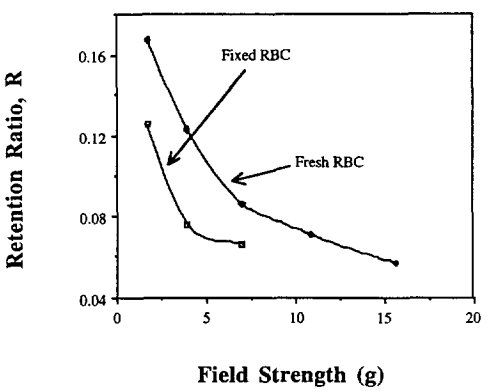

Fig. 6. Relationship between retention ratio *R* and sedimentation field strength for normal fresh and glutaraldehyde-fixed cells. The flow-rate for the carrier fluid was kept constant at 3.1 ml/min (wall shear rate = 230 s^{-1}).

for normal fresh cells was found to be much higher (around 16 *g*). This observation agreed with the above study of the relationship between retention and flow-rate, which suggested that lift forces for normal red cells are significantly greater than for glutaraldehyde-fixed cells.

Combining the results from the observed relationships between retention and flow-rate and between retention and sedimentation field strength, the optimal separation condition in our system under operation with a PBS carrier was found to be a flow-rate of 3.0 ml/min at a field of 4 *g*.

3.3. Other induced changes of membrane deformability

A partial loss of red cell membrane deformability was also induced by diamide, $(CH_3)_2NCON=NCON(CH_3)_2$, a thiol-oxidizing agent which is known to cause membrane polypeptide aggregation, decreased micro-pipette deformation and other changes in the red cell membrane similar to those caused by G-6-PD deficiency in patients with certain chronic hemolytic pathologies [12,13]. Fig. 7A shows the fractograms of fresh red cells and those incubated in 0.4 m*M* of diamide. Indeed, a treatment of fresh cells with diamide caused a measurable shift in retention. The magnitude of this shift was related to the diamide concentration in the incubation mixture, and appeared to reach saturation when the diamide concentration was above 0.4 m*M*, as seen in Fig. 7B. This shift, although not as great as that caused by extensive glutaraldehyde-fixation, gives an indication that retention measurements by the steric FFF method may be of clinical relevance.

Similar to the effects of diamide, the effect of thermal treatment on red cell membrane deformability has been previously studied [14]. It was found that a thermal treatment of red cells will increase their membrane rigidity due to a partial denaturation of the membrane proteins. Other changes will also be induced by heating, such as increased osmotic and mechanical fragility, increased resistance to packing, formation of microspherocytes and budded forms, and in-

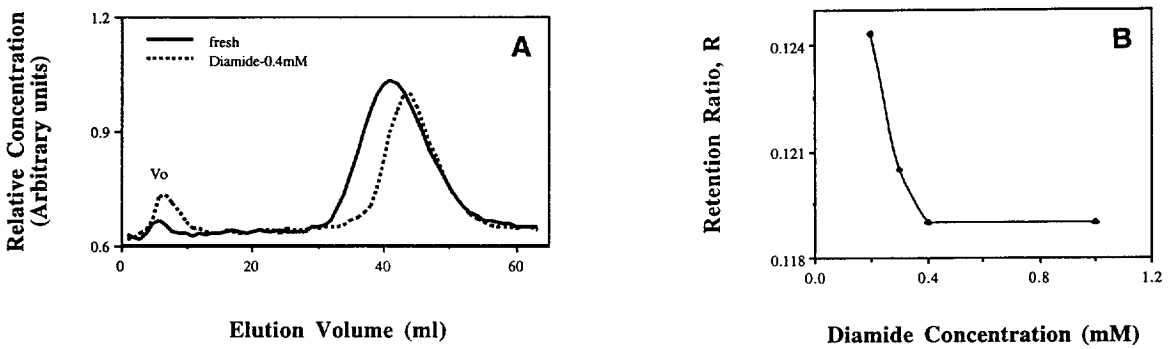

Fig. 7. (A) Fractograms of diamide-incubated red cells. A field condition of 4 *g* and a flow-rate of 3.0 ml/min were used for the separation. (B) Retention ratio (peak maximum) of diamide-incubated red cells vs. diamide concentration.

creased viscosity of the cell suspension [14]. The fractogram of red cells which had been under heat treatment at 49°C for 15 min is compared with that of normal red cells in Fig. 8. The substantially increased void peak in the fractogram of heated red cells reflects the products of thermal treatment, including released hemoglobin, which were not retained in the FFF channel. The results support findings by other characterization methods which have concluded that thermal treatment will increase the membrane rigidity of red cells.

3.4. System resolution

Recently, a different hydrodynamic separation method, gel permeation, was used to assess red cell deformability [15]. The effort offered some insights into how red cell membrane deformability can be visualized by other hydrodynamic characterization methods. Since both the steric FFF and the gel permeation belong to the family of size-based separation techniques, their separation performance can be compared by means of their respective resolution index R_s [5] for mixtures of fresh and fixed red cells. From Fig. 9, which represents a fractogram (adopted from Ref. [15]) of a 1:1 mixture of normal and glutaraldehyde-fixed red cells using gel permeation, we estimate the R_s-value to be 0.6. By comparison, the R_s value for the separation in Fig. 2 is estimated to be 1.2, well in excess of the $R_s = 1$ indicative of complete segregation of the two particle types. The difference in resolution provided by the two techniques indicates the

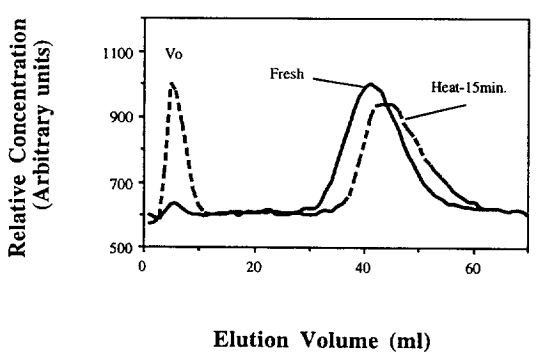

Fig. 8. Fractograms of thermally treated and normal red cells under optimal conditions.

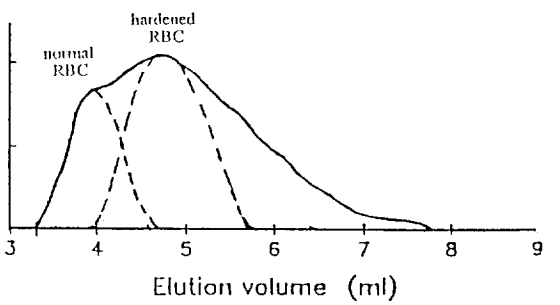

Fig. 9. Adopted from Fig. 4 of Ref. [9], gel permeation profile of a 1:1 mixture (solid line) of normal and glutaraldehyde-hardened red cells. Separation resolution is computed as 0.6 by fitting the profile with two gaussian peaks (dotted lines) representing normal and glutaraldehyde-hardened red cells, respectively.

separation performance of sedimentation steric FFF to be significantly better than that of gel permeation for samples of this general character.

4. Conclusions

Red cells with different membrane properties behave differently in laminar flow. By cross-linking red cells with glutaraldehyde in different concentrations one may produce increasingly larger retentions with higher degrees of fixation. Since the fixation reaction is known to affect both the cell density and rigidity, the density effect needs to be compensated for if one wants to draw conclusions regarding the relationship between cell membrane deformability and lateral migration in laminar flow. This compensation can be done by performing the analysis in a carrier whose density increase exactly matches the density increase of the cells. Even with this compensation, one sees a clear increase in retention that now can be ascribed to the increase in rigidity. Similar increases are seen after other treatments known to enhance the rigidity of the cell membrane, such as exposure to diamide or to elevated temperature. These observations indicate that in addition to the previously observed effects of flow velocity, settling field strength and cell density, there are cell specific properties of other kinds that influence the red cell behavior in laminar flow. By inference from other hydrodynamic studies regarding the effect of membrane deformability on red cell response to shear flow, it is suggested that deformability has a substantial impact on retention in steric FFF.

By means of this technique, normal red cells are seen to be completely resolved from a subpopulation of cells fixed with 1% glutaraldehyde. This resolution is higher than that reported for a similar sample analyzed by gel permeation. Such rapid and selective separation of red cells of different deformability suggests that the steric FFF has potential to serve as a diagnostic tool for detecting pathological shifts in red cell samples.

Acknowledgements

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