COMPARISON OF VOLUME AND SURFACE MECHANISMS FOR MAGNETIC FILTRATION OF BLOOD CELLS

M.D. Graham

Coulter Biomedical Research Corporation, Conoord, Massachusetts 01742, U.S.A.

Abstract - Erythrocytes containing diamagnetic hemoglobin were magnetically filtered from diluted whole blood via a new mechanism involving cellular magnetization through surface binding of soluble paramagnetic lanthanide chelates. Filtration efficiencies for diluents containing 40mM concentrations of erbium or dysprosium chelates are compared to those obtained by paramagnetization of internal erythrocytic hemoglobin and shown to be significantly greater, for equivalent non-saturated filtration conditions. As a consequence, useful filtration efficiencies can be reached at field strengths and gradients previously ineffective.

Erythrocytes are unique among mammalian cells in that 95% of their dry mass is a protein (hemoglobin, Hb) containing four Fe atoms per molecule. Although attempts were made to isolate erythrocytes through the effects of magnetic fields on Hb iron (1,2), practical separations had to await development of high-gradient magnetic filtration (HGMF). Separations have now been achieved through several mechanisms, all involving paramagnetization of the intracellular Hb (3-6). Because the concentration of cellular Hb is set by physiologic considerations and its total amount is limited by cellular volume, magnetic contrasts possible with these erythrocyte-specific mechanisms require intense magnetization fields for useful filtrations. Practically, magnetic filtrations of blood cells would be more attractive if mechanisms capable of greater cellular contrasts were available, so that smaller, less-expensive magnetic systems could be used.

Recently a promising new technique was described in which blood cells containing diamagnetic Hb were filtered from whole blood diluted with a solution of lanthanide chelates (7,8). In this non-specific approach, soluble paramagnetic ions are bound to the cellular surface in sufficient amounts to give the cell appreciable magnetic contrast over its paramagnetic suspending media. The technique causes minimal cellular insult (8), and its dependence on chelate concentration (8) and magnetizing field (7) have been described. Here, efficiencies of magnetic filtrations via surface binding of Er and Dy chelates from 40mM diluents are compared with those resulting via two volume mechanisms based on Hb paramagnetization. Calculations depending from the experimental chelate data predict essentially total filtrations at magnetizing fields of 0.3T, easily attainable with small magnetic circuits.

Résumé - Des érythrocytes contenant de l’hémoglobine diamagnétique ont été magnétiquement filtrés d’une solution sanguine entière en mettant en œuvre une approche nouvelle. Celle-ci est fondée sur un mécanisme de magnétisation cellulaire par fixation en surface de chelates lanthanide paramagnétique et soluble. L’efficacité d’une filtration contenant une dilution de 40mM en concentration chelatée d’erbium ou de dysprosium a été comparée avec celle d’une filtration obtenue par paramagnétisation d’hémoglobine érythrocytaire interne. Les résultats ont démontré une efficacité supérieure dans le premier cas, pour des conditions de filtrations non-saturées équivalentes. En conséquence, il apparaît maintenant possible d’obtenir des efficacités de filtrations utiles à des niveaux d’intensité et à des gradients de champ magnétique qui se sont dans le passé avérés inefficaces.
EXPERIMENTAL METHODS

Commercial isotonic saline (Isoton II; Coulter Diagnostics) was used as base for the Hb-paramagnetization diluents; as buffer, disodium hydrogen phosphate (Fisher Scientific Co) was added in ratios of 1.942g per liter for oxidizing solutions including 20mM sodium nitrite (Fisher Scientific Co) or 1.11g per gram of sodium dithionite (J.T. Baker Chemical Co) in the 10mM deoxygenating solutions (9). Mixed the day of use, paramagnetization diluents were vacuum-filtered through 0.45-micron filters and had their pH adjusted to 7.0-7.1 by addition of either NaOH or HCl. Owing to its high oxygen reactivity, dithionite was transferred and weighed in flowing nitrogen, and the buffered saline used in deoxygenating diluent was saturated with nitrogen before addition of the dithionite. Filtration, pH adjustment, and pre-use storage of deoxygenating diluent were done under nitrogen.

Because lanthanides precipitate at physiologic pH in solutions buffered by phosphate (or carbonate), the lanthanide diluents were mixed de novo from phosphate-free stock solutions (8) made within a week of use. Effectively, 40mM of Er or Dy chloride hexahydrate (Aldrich Chemical Co) was removed from a desiccator and dissolved in a 44mM solution of disodium ethylenediaminetetra-acetic acid (disodium EDTA; Fisher Scientific Co) buffered to pH = 7.0-7.1 with 20mM 1,4-piperazinebis(ethane-sulfonic acid) in free-acid form (PIPS; Sigma Chemical Co). Mixing was done in plastic glassware, and pH adjustment was with NaOH. Osmolarity of the diluents was approximately 310mOsm.

To keep diluent osmolarity differences from affecting filtration results through volume or shape effects, all diluents were adjusted to 310mOsm by addition of 3M NaCl or distilled water. Diluents were stored in capped polystyrene culture flasks until use.

Filtration data were acquired using diluted blood from 5 male volunteers. Bloods were drawn by venipuncture into evacuated tubes (6451 Vacutainer; Becton-Dickinson) containing 10.5mg disodium EDTA as anticoagulant. The samples were processed within six hours of drawing and were analyzed (Table 1) for normalcy (S-Plus Coulter Counter; Coulter Electronics, Inc) made on the influx and effluent of the filter; averaged results for the 5 donors are given in Fig. 1.}

Hemoglobin constituency of the suspended cells was determined by further diluting a sample of each suspension to a final ratio of 1:500 with the respective diluent (to minimize scattering effects) and measuring its absorbances at 560, 576, and 630nm against the cell-free diluent in a second 1.0cm cuvette as reference (Model 575; Coleman Instrument Division). Published extinction coefficients (10) were used to calculate the suspension's oxyHb, deoxyHb and methHb concentrations; the fraction of each component was taken as the ratio of its calculated concentration to the sum of concentrations of these three Hb species (9). As reference, cells suspended in the unmodified commercial diluent were equilibrated with oxygen by bubbling it through the suspension for 10 minutes prior to spectrophotometry.

Filtration of cells from the suspensions was accomplished with the HGMF system used previously (7,8). The magnetic filter, based on a design by Paul (6), was a D-shaped chamber 1.4ml in volume (13mm long in the flow direction and 117mm² cross-sectional area for 6.4mm of its length) machined in a 2.5cm x 2.5cm plastic block 1.25cm thick. The chamber was filled 15.5% by volume with a mesh woven of 430 stainless steel wire 50μm in diameter; insofar as possible the longer side of the mesh was arranged parallel to flow and perpendicular to the magnetizing field. The magnetic filter was mounted between 5cm polepieces on a 10cm electromagnet (V-4005, with V-2901 supply; Varian Associates) and subjected to magnetizing fields as measured by a Hall-effect gaussmeter (Model 615, with HT81-0608 probe; F.W. Bell, Inc). Suspension flows of 0.75ml/min upward through the filter were controlled by a motor-driven syringe pump fitted with a 3ml syringe to contain the suspension samples. Magnetic filtration efficiencies were calculated as has been described (7) from cell counts (ZB Coulter Counter with C-1000 Channelizer; Coulter Electronics, Inc) made on the influx and effluent of the filter; averaged results for the 5 donors are given in Fig. 1.
RESULTS AND DISCUSSION

Table 1. S-Plus data for the five male donors from whose blood suspensions were made for use in filtration studies. Multiple samples were taken from each donor, to allow compilation of the data in Fig. 1 over several experimental sessions. Entries are donor averages, while the Mean (Standard Deviation) values are for all samples.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cell Count $\times 10^6$/mm$^3$</th>
<th>Hemoglobin Hb, g/dl</th>
<th>Hematocrit Hct, %</th>
<th>Cell Volume MCV, $\mu$m$^3$</th>
<th>Cell Hb MCH, pg</th>
<th>Cell Hb MCHC, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.26</td>
<td>14.6</td>
<td>45.2</td>
<td>86.0</td>
<td>27.8</td>
<td>32.3</td>
</tr>
<tr>
<td>2</td>
<td>5.72</td>
<td>15.4</td>
<td>49.5</td>
<td>86.4</td>
<td>27.0</td>
<td>31.2</td>
</tr>
<tr>
<td>3</td>
<td>5.29</td>
<td>15.5</td>
<td>48.9</td>
<td>92.4</td>
<td>29.3</td>
<td>31.7</td>
</tr>
<tr>
<td>4</td>
<td>5.27</td>
<td>16.0</td>
<td>48.7</td>
<td>92.3</td>
<td>30.4</td>
<td>32.9</td>
</tr>
<tr>
<td>5</td>
<td>5.25</td>
<td>15.1</td>
<td>46.0</td>
<td>87.5</td>
<td>28.7</td>
<td>32.7</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>5.42(0.23)</td>
<td>15.3(0.5)</td>
<td>47.9(1.9)</td>
<td>88.4(3.0)</td>
<td>28.3(1.3)</td>
<td>31.9(0.7)</td>
</tr>
</tbody>
</table>

Spectrophotometry on cells suspended in oxygenated commercial diluent indicated that nearly all of the cellular Hb (0.98 to 0.99+) was in the oxygenated state, the rest being methHb. For cells in deoxygenating dithionite diluent, a similar division was seen, the major component being deoxyHb. However for cells suspended in oxidizing nitrite diluent the conversion was less complete, with 0.93 to 0.97 of the total Hb content being calculated as methHb; the remainder usually had as its major component oxyHb, a finding consistent with earlier work (9). In the lanthanide diluents, 0.90 to 0.97 of the total Hb was oxyHb, the rest calculated to be predominantly deoxyHb. When these suspensions were oxygenated by bubbling, as was done prior to their filtration, the calculated oxyHb content increased to 0.98+.

Filtration efficiencies for cells suspended in the four experimental diluents appear in Fig. 1. Data for the paramagnetizing diluents reflect the differences seen in Hb conversion and may be compared with earlier results obtained using the same filter at a flow rate of 1.0ml/min and 8% filling factor (9). Similarly, data for the lanthanide diluents demonstrate the advantage in magnetic moment Dy has over Er (10.5 vs. 9.5 Bohr magnetons, respectively), as well as the clear superiority both have over the paramagnetizing diluents.

Fig. 1. Magnetic filtration efficiencies for human erythrocytes suspended in two volume-active paramagnetizing diluents (dithionite and nitrite) and surface-active diluents containing 40mM Er or Dy. The same filter was used in all determinations, at 0.75ml/min flow rate.
A semi-quantitative comparison can be obtained through use of the filter equation developed by Watson (11), \((1 - E) = \exp(-Kx)\), where \(E\) is the magnetic filtration efficiency in percent divided by 100, \(x\) is the relative cellular magnetic susceptibility (or contrast), and \(K\) is defined by Eq. 1, below. The intent is to calculate \(K\) based on the experimental filtration data for the best-characterized suspension and then use the result to calculate \(x\) for the other diluents.

Mammalian erythrocytes consist of a cellular membrane several nanometers thick surrounding anuclear cytoplasm that is essentially Hb (12); their shape is a disk concave on both of its faces. In physiologic conditions, human erythrocytes are approximately \(7.7\mu m\) in diameter and \(2.8\mu m\) in maximal thickness, thinning to \(1.5\mu m\) in their central region (13). Mean cell volume (MCV) is \(87\mu m^3\) for males compared to \(90\mu m^3\) for females; per volume of blood, the cell count (C), Hb content (Hb) and hematocrit (volume fraction of packed erythrocytes, Hct) are greater in males, at \(5.4 \times 10^6/mm^3\), 16.0g/100ml and 47%, respectively. Both sexes have average cellular Hb content (MCH) of 29pg and Hb concentration (MCMC) of 35g/100ml. Thus, data in Table 1 compare well with reference values for males, except for MCHC which is slightly below the normal range of 32-37g/100ml. Because cell Hb concentration is an important parameter in the paramagnetization methods, all calculations have been done using the mean values listed in Table 1; molarity of cellular Hb is then \(4.95mM\), corresponding to \(2.64 \times 10^8\) Hb molecules per erythrocyte.

Erythrocytes contain approximately 95% of their dry mass as Hb (e.g., 14) so that the average dry mass here is 29.8pg/cell. Given an average erythrocytic density \(\rho = 1.096\ g/cc\), the total cell mass is 97pg/cell, leaving 67pg/cell as water (75.9% by volume). Assuming that Hb \((\rho = 1.335g/cc; 15)\) and the residual dry mass differ little in density, 28.3pg of Hb occupies about 22.9% by volume of each cell. The volume fractions can be combined with published susceptibility data to provide estimates of limiting cellular magnetic susceptibilities under the various experimental conditions. Hemimolar susceptibilities \(x_M\) are \(11.910 \times 10^{-6}\ cgs/mol\) (16) and \(2460 \times 10^{-6}\ cgs/mol\) (17), respectively for human Hb in the deoxygenated and oxygenated states; a similar value was not found for metHb, so \(x_M = 13.875 \times 10^{-6}\ cgs/mol\) for bovine metHb at \(pH = 7.05\) has been used (18). These values give expected cellular susceptibilities of \(-3.88\), \(-7.94\) and \(-3.57 \times 10^{-6}\ MKSu\) for cells containing deoxyHb, oxyHb and metHb, respectively, when the above volume fractions are used and it is assumed that all cellular Hb is in a single form and that diamagnetic moieties equal water in susceptibility.

Watson’s equation assumes spherical particles traversing at low flow rate a filter containing only a few percent by volume of matrix material. The erythrocytic geometry and the 15.5% filling factor used in these experiments both fail to meet assumed conditions; further, both paramagnetization diluents may fail to totally convert all cellular Hb or produce Hb degradation products that may not be paramagnetic. Diluent osmolarities were standardized to control erythrocytic volume (and shape), and their formulation was selected to give reliable Hb conversion without detectable Hb degradation (9). Under these conditions it seems permissible to write \(K' = xK\), where \(x\) is a dimensionless factor intended to account for the non-spherical shape of the cells and the higher filling factor of the filter matrix. Then, for

\[
F = \text{filling factor of filter} = 0.155
\]

\[
L = \text{effective filter length} = 1.11cm
\]

\[
s = \text{radius of matrix wire} = 25\mu m
\]

\[
M_S = \text{wire saturation magnetization} = 1.5 \times 10^5\ A/m
\]

\[
H_0 = \text{magnetizing field}
\]

\[
\nu_o = \text{permeability constant}
\]

\[
V = \text{cell volume, MCV} = 88.4\mu m^3
\]

\[
R = \text{cell radius} = 3.85\mu m
\]

\[
\chi = \text{cell magnetic contrast}
\]

\[
\eta = \text{viscosity of diluent}
\]

\[
V_o = \text{suspension velocity} (= 0.0149cm/sec)
\]

\[
X_c = \text{susceptibility of cell}
\]

\[
X_d = \text{susceptibility of diluent}
\]

\[
K = \frac{FLM_sB_p}{9\pi\nu_o R} V x = K' / x
\]

\[
(1 - E) = \exp(-Kx)
\]

\[
\chi = -\ln(1 - E) / K
\]

\[
\chi = -\ln(1 - E) / K' = x_c - x_d
\]
Of the parameters listed, four \((H_0, v_0, n, X)\) are functions of the protocol, the remaining one being defined by properties of either the filter or the cells. Fig. 1 was compiled by selecting \(H_0\) at a volume flow of 0.75ml/min for all determinations; for the filter cross-sectional area of 117mm\(^2\), this flow rate corresponds to a mean flow velocity through the filter of 0.0149cm/sec = \(v_0\). In Fig. 1, non-saturated comparisons can be made at a magnetizing field of 0.3T for diluents containing Dy, Er, and dithionite; the latter can be similarly compared with the nitrite oxidizing diluent at a field of 0.4T. Since the dithionite diluent is believed to be the most repeatable of the two paramagnetizing diluents (9), \(K'\) will be calculated using Eq. 3b, the experimental data, and the susceptibility for deoxygenated cells, \(-3.88 \times 10^{-6}\). At 0.3T, \(E = 0.374\), giving \(K' = 9.06 \times 10^4\); this corresponds to contrasts \((\text{MKSu})\) of 25.9 \(\times 10^{-6}\) for Er \((E = 0.904)\) and 34.5 \(\times 10^{-6}\) for Dy \((E = 0.956)\). At 0.4T, the deoxygenated and nitrite-treated cells gave filtration efficiencies of 0.716 and 0.495 respectively, leading to \(K' = 2.44 \times 10^5\) for the first and a contrast of 2.81 \(\times 10^{-6}\) for the latter. These values assume that the susceptibility of the paramagnetization diluents is essentially that of water, \(x_w = -9.05 \times 10^{-6}\), and that the viscosity of the diluents approximates that of isotonic saline, \(\eta = 1.019\text{cp}\). A rough check on all assumptions is provided by the cellular susceptibility of the metHb-containing cells, \(x_c = -6.24 \times 10^{-6}\) (cf. \(-3.57 \times 10^{-6}\) calculated assuming total Hb conversion).

From the CGS molar susceptibilities of ErCl\(_3\) and DyCl\(_3\), \(\chi_M = +0.0746\) and +0.0896 respectively, the MKS susceptibilities of the two 40mM diluents can be calculated as 0.0375 and 0.0450; these values are essentially the cellular susceptibilities, given the small percentages of them the cellular contrasts represent. Thus, it appears the chelates interact sufficiently with the cells to give the latter effective susceptibilities at least two orders greater than the relative paramagnetism attainable with the Hb conversion mechanisms (Table 2).

Unlike the paramagnetization diluents which achieve their effect by acting on the erythrocyte's volume content of Hb, the lanthanide diluents do not impact the cellular contents. It is thought that the chelates bind to cellular surfaces, possibly through further complexation of the lanthanide; the net cellular paramagnetic moment thus requires that any surface accretion of chelate exceed the volume amount of chelate excluded by the membrane. For normal human erythrocytes the surface area (13) is about 130\(\mu\)m\(^2\) and the average volume for the cells here is 88.4\(\mu\)m\(^3\). Thus, each cell excludes about 2.13 \(\times 10^9\) molecules of the 40mM chelate in these diluents and must therefore surface-bind slightly more, about 1.64 \(\times 10^7\) chelates/\(\mu\)m\(^2\) for uniform distribution.

Human erythrocytes have been shown to be macropolyanions possessing about \(10^7\) ionic groups per cell, of which more than 60% are the carboxyl group of N-acetylneuraminic acid, the remainder thought to include some \(\alpha\)-carboxyl groups on amino acids (19). If it is assumed that each anionic site binds a single chelate molecule, about 200 times the available sites are required to produce the calculated binding densities. This suggests that individual sites bind multiple chelates, that sites not identified by the usual marking methods become involved in chelate binding, or that sites normally covert are exposed by the experimental diluents. The binding mechanism is not yet understood, although it is thought to essentially saturate at concentrations of 10-15mM for either Er or Dy (7); for 10mM Er, calculations similar to the above predict that \(10^8\) binding sites should give filtration efficiencies comparable to dithionite, while in fact efficiencies considerably greater are observed (7). This result further supports involvement of more than the usual anionic membrane sites.

### Table 2. Summary of cellular contrasts, diluent susceptibilities, and cellular susceptibilities resulting from calculations based on Fig. 1. All values are in MKSu, \(x \times 10^6\).

<table>
<thead>
<tr>
<th>Agent</th>
<th>(x)</th>
<th>(x_d)</th>
<th>(x_c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>deoxyHb</td>
<td>5.17</td>
<td>-9.05</td>
<td>-3.88</td>
</tr>
<tr>
<td>metHb</td>
<td>2.81</td>
<td>-9.05</td>
<td>-6.24</td>
</tr>
<tr>
<td>Er</td>
<td>25.9</td>
<td>+37490</td>
<td>+37515</td>
</tr>
<tr>
<td>Dy</td>
<td>34.5</td>
<td>+45030</td>
<td>+45065</td>
</tr>
</tbody>
</table>
Finally, it should be noted that all corrections for diamagnetic moieties have been neglected in these calculations, including that for the oxygenated Hb contained in the chelate-binding cells. Further, stated molar values are likely to over-estimate actual diluent content of the lanthanides or dithionite, the first being hydroscopic and the second being both impure in the as-purchased state and liable to combine with oxygen (and so be rendered less effective) during its storage and handling.

CONCLUSION

Filtration efficiencies for erythrocytes diluted with two volume-active paramagnetization diluents have been compared with those for cells from the same donors subjected to experimental surface-active diluents containing lanthanide chelates. The results show a clear advantage for the latter. Calculations pivoting on the filtration system characteristic as determined for deoxygenated cells indicate that the lanthanides Er and Dy give cellular magnetic contrasts (MKSu, x 10^6) of 25.9 and 34.5, respectively, compared to 5.17 for the deoxygenated cells and 2.81 for cells containing metHb. These contrasts obtain despite the cells being suspended in a highly paramagnetic fluid, leading to effective cellular susceptibilities greater by at least two orders than for cells whose Hb has been converted by the paramagnetization mechanisms.

The improved filtration efficiencies attainable with the surface-active diluents permits useful filtration efficiencies at fields as low as 0.3T, field strengths incompatible with high-efficiency filtrations by the paramagnetization mechanisms. For example, if the flow rate were to be reduced to one-half the value used in these experiments, Eq. 3b predicts that either Dy or Er would permit filtration of at least 98% of the cells at 0.3T magnetizing field. It is this prospect, of achieving practical filtrations using field intensities available from compact magnet assemblies, that is greatly attractive.

REFERENCES