SEPARATION OF LANTHANIDE-BINDING CELLS

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ABSTRACT

A new method for magnetic separation of cells is described. Based on cellular concentration of complexed paramagnetic cations from a carrier solution, the method achieves highly repeatable separations, at lower magnetization fields, than existing HGMS cell-separation methods. Feasibility has been shown for erbium and dysprosium, with whole blood as the sample. Single-pass magnetic separation efficiencies significantly greater than those attainable with paramagnetic erythrocytic hemoglobin are readily available, e.g., 90% versus 37%, at 0.3 Tesla and 0.75ml/min flow rate. The method requires no cellular incubations, is not technically demanding, and does not visibly affect cellular integrity; it promises to provide a flexible and general technique for cell separation.

INTRODUCTION

For large products of field intensity and gradient, intrinsic hemoglobin permits erythrocytes to be magnetically separated from whole blood following its modification into paramagnetic deoxy [1,2], met [2,3] or catabolic [4] forms; and diamagnetic repulsion of erythrocytes containing physiologic oxyhemoglobin has been suggested as yet another separation mechanism [5]. Lacking an intrinsic component having suitable magnetic properties, certain leukocytes and other cellular classes have been magnetically separated at lower field strengths by first allowing them to phagocytize magnetic particles [6,7], bind magnetic microspheres [8-11], or rosette erythrocytes containing paramagnetic methemoglobin [12]. These methods all address separation of living cells made to exhibit a net paramagnetism from a carrier fluid that is essentially diamagnetic. Those involving erythrocytes require a large intensity-gradient product for practical separation efficiencies because of the low magnetic contrast of these cells; those for leukocytes result in improved magnetic contrast but require incubations during which metabolic processes may degrade cellular integrity. A separation method that is useful at low field levels, that uses a soluble agent needing no incubation period, and that potentially permits differential separation of a selected cell class from a mixed cellular suspension would offer important advantages over existing methods.

Lanthanide ions are widely used as calcium analogs in studies characterizing transport processes of biological membranes, an application in which they show high-affinity binding to cellular membrane components [13-15]. Some of the trivalent lanthanides have magnetic moments nearly twice those of paramagnetic hemoglobins: 9.5 and 10.5 BM for erbium (Er^{3+}) and dysprosium (Dy^{3+}), respectively, compared with 5.8 and 5.35 BM for high-spin met- and deoxy- hemoglobins, respectively. These high magnetic moments suggest that cells otherwise diamagnetic may be separable from a carrier fluid containing low levels of such paramagnetic ions, due to concentration of ions on the cellular membranes; such separation should occur at intensity-gradient products considerably less than those currently needed for erythrocytes and without requiring the incubations needed in present separative methods for leukocytes. The involvement of binding sites on the membrane suggests the further possibility of differentially separating a selected cell class, by preferentially inhibiting access of the paramagnetic ion to its binding sites. This paper examines the first possibility and describes experimental separations, at relatively low magnetizing fields, of cells from whole blood diluted with a solution containing centimolar levels of the lanthanides erbium or dysprosium, complexed [16-18] with the chelating agent ethylenediaminetetra-acetic acid (EDTA) to form, generically, $Ln(EDTA)^-$.

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MATERIALS AND METHODS

To prepare working solutions, the desired amount of solid erbium or dysprosium chloride hexahydrate (Aldrich Chemical Co.) was dissolved in a buffered solution containing disodium EDTA (Fisher Scientific Co.) in a molar ratio of 1.1:1 with the lanthanide, to ensure that all of the ion was complexed. The buffer was 1,4-piperazine-bis(ethane-sulfonic acid) (Sigma Chemical Co.), at a final concentration of 20mM. Mixing was done in a plastic container, and pH was adjusted to 7.0-7.1 by addition of sodium hydroxide. For lanthanide concentrations so permitting, solution osmolarity was then adjusted to physiologic levels with sodium chloride. Working solutions were prepared within two hours of each experiment, from stock solutions no more than one week old.

Venous blood samples were drawn from volunteers into evacuated tubes (6451 Vacutainer; Becton-Dickinson) containing 10.5mg disodium EDTA. The samples were verified for normalcy (S-Plus Coulter Counter; Coulter Electronics, Inc.) before use on the day of drawing. Manual 1:101 dilutions were made in polystyrene flasks and shaken gently to give homogeneous cell suspensions. Cellular morphology was observed in wet mounts made by adding a few microliters of suspension to the standard solution of RPMI-1640 (Gibco Laboratories) with 5% bovine albumin (Sigma Chemical Co.). Once it was known that essentially normal morphology could be retained, suspensions were monitored for abnormality via their volume distributions (ZB Coulter Counter with C-1000 Channelyzer). Spectrophotometry (Model 575; Coleman Instruments Div.) showed the characteristic oxyhemoglobin peaks.

For comparative purposes, an experiment was done in which sodium dithionite at 10mM final concentration was substituted for the lanthanide; EDTA was omitted, solution osmolarity being adjusted to that of 40mM erbium by addition of sodium chloride. In a second dithionite experiment, the previous protocol [2] was used. For both, deoxygenation was confirmed spectrophotometrically.

Separability of cells from the suspensions was examined using a High-Gradient Magnetic Separation (HGMS) system as previously described [2], except for substitution of a different filter circuit. The filter was a D-shaped plastic chamber 13mm long and $118mm^2$ in cross-section, filled with stainless steel wire as noted in the legends; packing was random, but with the major component parallel to flow. The filter was mounted between 50mm polepieces on a 10cm electromagnet (V-4005, with a V-2901 supply; Varian Associates) and subjected to magnetizing fields to 2 Tesla (T) as measured with a Hall effect gaussmeter (615, with HTB1-0608 probe; F.W. Bell, Inc.). Suspension flows upward through the filter were controlled by a variable-speed syringe pump, fitted with a 3ml syringe to contain the cell suspension.

Unless otherwise noted, the filter was flushed manually with 15ml of the experimental working solution before each separation determination, to clean the matrix and to clear the circuit of air; with the electromagnet still de-energized, the circuit was then overfilled rapidly with the corresponding cell suspension and the syringe pump activated. After 1.2ml of cell suspension had been delivered at the chosen flow rate, a one-milliliter aliquot (A1) of filter effluent was collected, for use in calculating mechanical filtration levels. With filter

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backflow prevented, the pump syringe was refilled, the magnet energized at the preselected field intensity, and the delivery and collection (aliquot A2) steps repeated. This sequence was then repeated for each selected combination of flow rate and field intensity. A 2ml control aliquot (A3) was taken for each suspension from the reservoir used to fill the filter and the syringe pump. The aliquots were analyzed for cell count and volume, count data (A1, A2, A3) being used to calculate filter characteristics as follows (for percentages, multiply by 100):

Mechanical filtration, $F = (A3 - A1)/A3$

Magnetic filtration, $M = (A1 - A2)/A3$

Magnetic efficiency, $E = (A1 - A2)/A1 = M/(1 - F)$

Total filtration, $T = (A3 - A2)/A3 = M + F$

RESULTS AND DISCUSSION

Table I summarizes experimental filtration data according to the concentration of $Er(EDTA)^-$ in the working solution; data for various field intensities are given for the higher ion concentrations, all at constant flows of 0.75ml/min. The data demonstrate that the diamagnetic blood cells do acquire useful paramagnetic contrast over the paramagnetic carrier solution, giving practical separations at 2 Tesla of cells from whole-blood suspensions containing $Er(EDTA)^-$ concentrations in the 5mM range. At identical HGMS conditions, 10mM erbium gave separations exceeding those for equimolar dithionite, a content well above that needed to produce complete deoxygenation of cellular hemoglobin [2]; even less-efficient separations occurred when 20mM sodium nitrite was used to oxidize intracellular hemoglobin to the met form (see also [2]).

Thus the tabular data emphasize an important advantage of the experimental mechanism: Although the resultant magnetic contrast is concentration-limited, the operative limit is not the concentration of an intrinsic cellular component as with the hemoglobin-conversion mechanisms, but rather the concentration of the extrinsic ion. Consequently (see also Fig. 1), magnetic separation efficiencies exceeding those available with these mechanisms are easily attainable, at $Ln(EDTA)^-$ concentrations

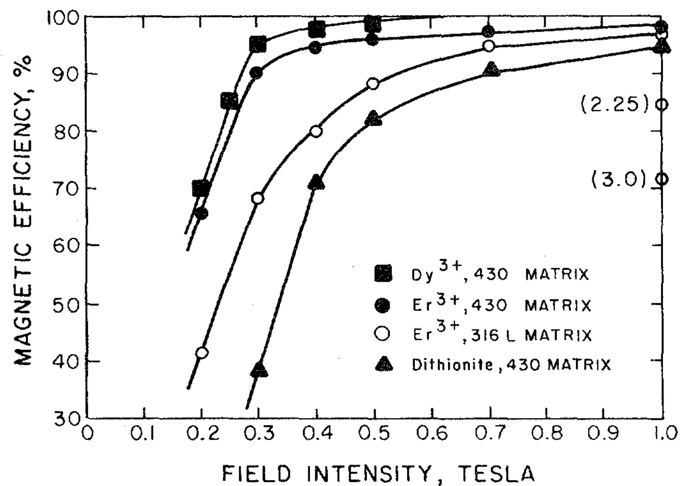

Fig. 1. Field-dependency of separation efficiency for blood cells suspended in 40 mM $Ln(EDTA)^-$, or in 10mM sodium dithionite according to [2], at 0.75ml/min. The 316L stainless matrix described in Table I was used to acquire data indicated by open circles, including the comparative flow data at 2.25 and 3.0 ml/min. Other data were acquired with a 430 stainless mesh woven of wire 50 micrometers in diameter, filling 15.5% of the filter.

giving physiologic osmolarities. Further, if the cellular sequelae of elevated osmolarities can be accepted, $Ln(EDTA)^-$ concentrations above 35mM can be used to realize single-pass separation efficiencies not readily obtainable with the conversion mechanisms and permitting use of considerably lower magnetizing fields as shown by Fig. 1. Higher flows can also be used, a flow-dependency study at 1.0T (see figure) giving separations of 94.8, 94.3, 83.5 and 71.3% at 0.75, 1.5, 2.25 and 3.0 ml/min, respectively, for 40mM $Er(EDTA)^-$.

Also illustrated in Table I is the consistency observed in separation efficiency, cf., the two sequences for 50mM erbium concentration. These were completed on consecutive days, using the same batch of working solution but blood from different donors. Similarly, identical efficiencies of 94.8% were obtained in the 40mM, 1.0 Tesla, flow- and concentration- dependency studies, although done on different days using different donors. Note too the consistency below saturation of separation efficiencies at constant products of ion concentration and field intensity, e.g., 5mM at 2.0T and 10mM at 1.0T; or 10mM at 2.0T, 20mM at 1.0T and 40mM at 0.48T. Separation efficiencies were more repeatable than those seen previously [2] with either dithionite deoxygenation or (particularly) nitrite oxidation, and $Ln(EDTA)^-$ solutions were definitely more stable than dithionite ones.

As noted in the Materials section, cellular hemoglobin remains in the oxygenated (and therefore the antagonistically diamagnetic) state throughout the separation process. Cell lysis was not detected, cellular morphology was not visibly altered (beyond reversible osmotic effects), and cell agglutination due to the heavy metal was prevented by EDTA. Separated cells mainly included erythrocytes, due to the thousand-fold superiority in natural occurrence, but other formed bodies also bind the paramagnetic ions and so are retained; specifically, magnetic separation efficiencies for leukocytes in resuspended buffy-coat preparations were comparable to those for whole blood. As predictable from geometrical considerations, platelets were retained, but at lesser efficiencies than either leukocytes or eythrocytes.

The data composing Table I and the open circles of Fig. 1 were obtained using unannealed 316L austenitic wire as the matrix; comparison of this curve with data for 40mM erbium in the table demonstrates a decrease of over 5% in separation efficiency at 0.3 and 0.5 Tesla.

Table I. Filtration data at 0.75ml/min for whole blood diluted 1:101 with working solution including erbium complex at the indicated concentrations. Matrix material was unannealed, 316L austenitic stainless wire 35 micrometers in diameter, randomly packed to 11.4% of the filter volume. (See text for meaning of symbols.)

$[Er^{3+}]$	Field, T	E,%	M,%	F,%	T,%
1mM	2.03	31.6	24.6	22.2	46.8
5mM	2.03	83.2	---	---	---
10mM	2.03	96.6	90.8	6.0	96.8
	1.00	83.2	---	---	---
20mM	1.00	94.2	---	---	---
40mM	1.00	94.8	93.1	1.7	94.8
	0.48	92.4	86.8	6.0	92.8
	0.28	71.7	67.4	6.0	73.4
50mM	1.00	98.7	92.9	5.9	98.8
	0.48	91.1	85.7	5.9	91.6
50mM*	1.00	98.4	94.1	4.3	98.4
	0.48	92.2	88.2	4.3	92.5
Sodium dithionite, substituted for erbium and EDTA.					
10mM	1.00	76.9	68.4	11.0	79.4
	0.30	13.0	11.6	11.0	22.6

* This working solution was aged one day.

This decline was traced to apparent magnetic annealing, due to the many operational cycles of the electromagnet, of the 316L stainless-steel's magnetic properties, originating in its work-hardening. Consequently, the filter was repacked, but with 430 ferritic stainless mesh, and the other curves of Fig. 1 were developed.

The pattern seen for the 316L matrix was again obtained, except that the differences favoring erbium were accentuated by the easier magnetization of the 430 alloy. With erbium, separations exceeding 90% could be achieved at fields of 0.3 Tesla, at which value a 37% efficiency was demonstrated for dithionite; at identical flows, dithionite required a field of 0.7 Tesla to provide an efficiency of 90%. At 1.0 Tesla with erbium, single-pass magnetic efficiencies greater than 99% were attained, at flows of 0.75ml/min; repeatable separations at such efficiencies are not practical with dithionite. As indicated in the figure, $Dy(EDTA)^-$ consistently gave magnetic separation efficiencies some 5% greater than $Er(EDTA)^-$, below saturation. From the separation perspective, the experimental mechanism is clearly superior to the conversion mechanisms.

Paramagnetic carrier fluids have been suggested as magnetically controlled density media for separation of inorganic, diamagnetic materials [19-24]; more recently, use of such carriers in HGMS systems has received attention [25-28]. Because of their magnetic moments, lanthanides have been considered for the magnetogravimetric systems [19-24], while manganese chloride has been used in the HGMS work [26-28]. These efforts depend on diamagnetic particles contrasting with paramagnetic fluid; the present work depends on a net cellular paramagnetism due to concentration of the lanthanide complex and so on an unrelated mechanism. Uncomplexed erbium (which lacks useful solubility characteristics at the physiologic pH and electrolyte levels required for undamaged cells) has isolated wear particles and tissue fragments in a ferrographic system [29,30].

No separations of individual mammalian cells comparable to those reported here are described in the accessible literature.

CONCLUSION

A new method has been described which permits magnetic separation of cells from whole blood, with repeatabilities and efficiencies here-to-fore unattainable. Based on use of a soluble paramagnetic material, the method requires no cellular incubations, does not degrade cellular integrity, and is not technically demanding. Feasibility has been shown with $Er(EDTA)^-$ and $Dy(EDTA)^-$; paramagnetic (lanthanide or other) complexes in conjunction with specific inhibitory agents may allow differential magnetic separation of blood cells. The technique should be generally extensible to other cellular species, as well as to particles.

The superior magnetic separation efficiencies seen with the new mechanism permit practical separations at lower fields than is possible with existing methods. As an illustration, 1" by 1" samarium-cobalt magnets ¼" in thickness were used with the ferritic matrix to provide separation efficiencies exceeding 50%, from a HGMS system occupying less than one cubic inch; as with the reported data, no attempt was made to optimize the matrix packing and wire size, so improvements can yet be made. It is this potential, of using small permanent-magnet sources in practical cell-separation systems, that is most promising.

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