FERRITIN AS A LABEL FOR HIGH-GRADIENT MAGNETIC SEPARATION

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ABSTRACT In three model systems, particles the size of cells or smaller have been surface labeled with ferritin to make them slightly paramagnetic, by virtue of the iron in the ferritin. In each case it was possible to show that labeled particles could be magnetically removed from a flowing suspension by the high-gradient magnetic separation (HGMS) technique. The first system of particles consisted of small (1 μm) carboxylate-modified latex spheres to which ferritin was covalently bound to create stable paramagnetic particles analogous to a ferritin-labeled subcellular membrane preparation. In the second system polyacrylamide beads that more closely approximated whole cells in size (5–50 μm) were labeled with immunoferritin. The third system was a biomembrane preparation: erythrocyte ghosts labeled with a ferritin-lectin conjugate. A field of 7 T (tesla) (70 kG) was used in each case, along with buffer flow rates through the HGMS column in the range 0.1–1.0 ml/min.

INTRODUCTION

Magnetic methods for separating subpopulations of cells from a suspension have been considered by a number of authors. In addition to the widespread use of magnets to remove phagocytes that have ingested iron particles, there are also magnetic iron oxide particles that can be bound to surface proteins or glycoproteins on cells of interest (1–6). In general, however, reproducible synthesis of these ferromagnetic particles from one laboratory to another has proven difficult, and in many cases the particles have a strong tendency to clump together due to their permanent magnetic moments. To address this problem, labeling materials that are not permanently magnetized (i.e., paramagnetic, as opposed to ferromagnetic) can be used, if stronger magnetic fields and field gradients are available to generate forces of the required strength on the labeled cells and effect the desired separation.

High-gradient magnetic separation (HGMS) has been applied to the retention of biological cells from a stream of flowing buffer in several experimental systems where the cells to be captured were erythrocytes that contained paramagnetic heme iron (7–14). Cell suspensions were passed through columns that were loosely packed with magnetic stainless steel wire and placed in a magnetic field. The fine wire served to concentrate the magnetic field lines, which produced large field gradients near the wire surfaces and resulted in strong attractive forces on magnetizable cells. As long as buffer flow rates were slow enough that the viscous forces were less than the magnetic forces, erythrocytes in each case could be retained on the wire matrix of the column while diamagnetic leukocytes would flow through essentially unhindered. Captured cells could be recovered by simply turning off the magnetic field or removing the column from the magnet.

The HGMS technique originated with particle physicists (15) and was developed by electrical engineers in the mining industry (16). It has been applied to the enrichment of paramagnetic erythrocytes containing deoxygenated hemoglobin (7–9), methemoglobin (9–11), and hemoglobin breakdown products in the malaria-infected cell (17). In addition to the separation of intrinsically paramagnetic cells, HGMS has been applied to the enrichment of leukocyte subpopulations that bore receptors for a surface determinant on a xenogeneic erythrocyte (12–14). The red cells were made paramagnetic by oxidation of the cell hemoglobin, and the white cells of interest became paramagnetically labeled when they bound to the red blood cells and formed small clusters (rosettes). Rosettes, and red blood cells, were retained in the HGMS column and thus separated from unlabeled leukocytes.

One of the most interesting possibilities for biological applications of HGMS to the enrichment of surface-labeled cells or subcellular particles, however, has been the question of whether conventional ferritin-labeling methods could confer enough paramagnetism upon particles to result in their capture in an HGMS column. The experiments that are described in this report have demonstrated HGMS capture of ferritin-labeled particles using a superconducting magnet and three model systems of essentially uniform particles. Initially, latex beads of 1 μm diam with ferritin covalently attached were used because this relatively stable preparation allowed good particle recovery and quantitation of magnetic capture. Magnetic retention based on surface labeling by ferritin-antibody conjugates

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was demonstrated with polyacrylamide beads that had diameters (5–50 μm) more comparable to those of typical cells that might be sorted in eventual applications. Finally, in a completely biological system, ferritin-concanavalin A conjugates were used to label erythrocyte membranes (ghosts). In each case, the use of homogeneous samples eliminated complications that would have arisen if both labeled and unlabeled subpopulations had been present in the original sample. It allowed the partial capture of particles at a particular flow rate to be interpreted in terms of the probability of capture of each particle upon encountering the magnetized steel wire.

MATERIALS AND METHODS

Magnet and Column Systems

Glass columns having an inside diameter of 0.5–1 cm were loosely packed to a height of 10 cm with 25-μm diam Type 430 stainless steel wire (Brunswick Corp., Deland, FL). The packing fraction f, (volume occupied by the wire itself divided by the total bed volume) was ~0.04. The magnet was a 7 T (tesla) superconducting solenoid of height 10 cm and inside diameter 5 cm (Thor Cryogenics Ltd, Oxford, England). It was mounted in a superinsulated Dewar flask (Superconducting Technology Inc., Mountain View, CA) that had an overall height of 75 cm and a room-temperature "warm bore" of diameter 3.2 cm, in which the column was suspended.

Samples and Assays

Carboxylate-modified latex spheres (1 μm diam, Dow Diagnostics, Dow Chemical Co., Indianapolis, IN) were coupled to ferritin by the *N*-hydroxysuccinimide "activated ester" method. Latex spheres (50 μl packed particle volume) were incubated 3 h at 4°C in 0.5 ml water plus 23 mg *N*-hydroxysuccinimide and 37 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The mixture was diluted with phosphate-buffered saline pH 7.5 and centrifuged at 10^4 *g*. The particles were resuspended in 1.0 ml phosphate-saline, 0.6 ml of ferritin solution (100 mg/ml, Calbiochem-Behring Corp., San Diego, CA) was added and the mixture stirred overnight at 4°C. Finally, 200 mg glycine was added to the mixture and allowed to react 30 min, and the particles were washed three times by centrifugation and resuspended in 10 ml of phosphate buffered saline. Examination under the phase microscope (100x magnification) revealed no large clumps of particles, although about half the sample consisted of small aggregates of 2–5 particles. The originally white particles were visibly tan in color by virtue of the bound ferritin.

Relative numbers of ferritin-conjugated spheres in the "passed" and "held" fractions and an aliquot of the initial suspension that was placed on the column were assayed by light scattering. Each was spun down, if necessary, resuspended in 2.0 ml phosphate-saline, and then further dilutions were made until all gave the same measured absorbance at 500 nm (Spectronic 20, Bausch & Lomb Inc., Rochester, NY). The volume of each sample was then proportional to the total number of particles, and dimensionless ratios could be calculated from these numbers, as described below.

The hydrophilic polyacrylamide beads for the immunoferritin experiments were 5–50 μm in diameter, and were conjugated with goat antibodies against rabbit IgG (Bio-beads, Biorad Laboratories, Richmond, CA). A sandwich technique was used to introduce the paramagnetic label onto the particles. Approximately 2×10^6 beads in 0.5 ml phosphate saline buffer were incubated with rabbit peroxidase–antiperoxidase (PAP) complexes (Cappel Laboratories Inc., Cochranville, PA) overnight at 4°C to coat the particles with rabbit IgG. They were washed by centrifugation and labeled in a 1 h incubation with a ferritin conjugate of goat antibody (IgG fraction) against rabbit IgG (Cappel Laboratories). Samples were washed two times before being passed onto the HGMS column. The numbers of beads in various fractions were determined by counting under the phase microscope in a hemocytometer chamber.

Erythrocyte sealed ghosts wee prepared from freshly drawn human blood by the method of Steck and Kant (18). Preparations were assayed for protein content by the method of Lowry (19). Ghost samples typically containing 0.1–0.3 mg protein were incubated for 1 h at 4°C in a volume of 0.5 ml phosphate-buffered saline containing 0.25 mg of ferritin–Con A conjugate (Miles Laboratories Inc., Elkhart, IN). After the incubation the sample was washed by centrifugation (11,000 *g*, 30 min) and resuspended in 1.1 ml phosphate-buffered saline for the HGMS fractionation.

The amount of membrane material in different fractions was assayed by the enhancement of fluorescence emission of the hydrophobic probe diphenylhexatriene, according to the method of Shinitsky and Inbar (20). Material in each fraction that came off the column was concentrated by centrifugation (20,000 *g*, 30 min) before being assayed.

Fractionation Procedure

Samples in each case were suspended in 1.1 ml phosphate saline, of which 0.1 ml was saved to assay for the amount of material in the 1.0 ml sample that was introduced into the HGMS column. The sample was pumped through the system vertically downward by a syringe pump to minimize pulsations in the flow. The running buffer was phosphate-buffered saline to which 0.5% bovine serum albumin had been added to minimize nonmagnetic capture of sample by the wire matrix in the column. Liquid was collected below the magnet until the eluate was essentially free of particles. Typically, a total passed volume was collected that was 5–10 times the bed volume of the column. The column was then removed gently from the magnet and the particles that were held in the magnetic field were eluted using a volume of buffer 3–8 times the bed volume of the column. In some experiments the first portion of this elution was carried out at the same speed as the run in the magnet. With or without this step, however, a final rapid flush of the column was accomplished by forcing buffer from a syringe through by hand to dislodge as many captured particles as possible. In the experiments with 1-μm ferritin-conjugated spheres, an additional extraction was effected by disassembling the column and mechanically agitating the wire in the upper half of the matrix while rapidly forcing 10 ml of buffer through it.

The portions collected while the column was in the magnet were pooled and assayed for particle or membrane content, and this was termed the passed fraction, P. Similarly, the material recovered after the column was removed from the field was assayed to yield a value for the held fraction, H, and the 0.1 ml that was saved from the original sample was assayed and multiplied by 10 to give the value for the initial sample, I. From these numbers were calculated the fraction of the initial sample that was recovered, R, and the fraction of the recovered material that was passed (transmitted) by the HGMS column, T:

$$R = (P + H)/I \tag{1}$$

$$T = P/(P + H). \tag{2}$$

RESULTS

Ferritin-based HGMS was initially demonstrated with latex spheres to which the protein was covalently bound. The efficiency of retention of these particles by the HGMS column was measured as a function of buffer flow rate. The retention of particles by the column ranged from 13 to 90% of the total number of particles recovered (the sum of the passed and held fractions), at flow rates between 13 cm/min and 1.3 cm/min (9.4 and 0.9 ml/min, respec-

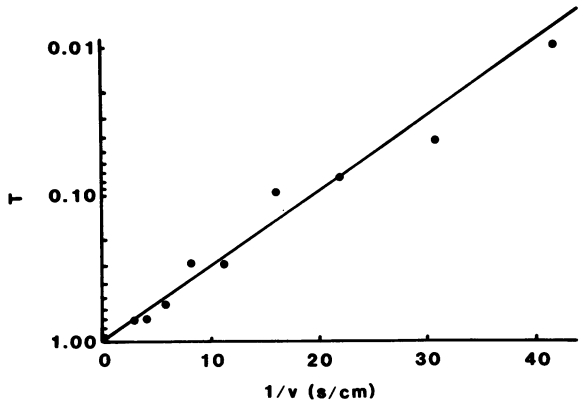

FIGURE 1 Velocity dependent magnetic capture of ferritin-conjugated 1-μm diam latex spheres in a 7 T (70 kG) field. The fraction of recovered particles that was passed by the HGMS filter, the transmittance T, is shown on an inverted logarithmic scale vs. the reciprocal of the buffer flow velocity, for purposes of comparison with theory (see text). The column had a cross-sectional area of 0.64 cm^2 and flow rates from 0.9–9.3 ml/min were used. The wire matrix occupied 4% of the bed volume.

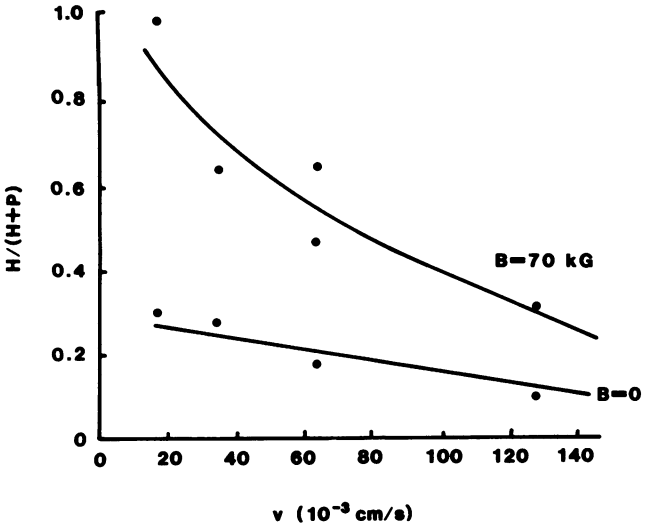

FIGURE 2 Velocity-dependent magnetic capture of immunoferritin-labeled hydrophilic beads, 5–50 μm diam. The column had a cross-sectional area of 0.29 cm^2 and contained 1.3 g of 25-μm wire that occupied 4% of the bed volume.

tively, in this column). In Fig. 1 the results have been graphed in a format in which theoretical models of the HGMS process (11, 16, 21) predict that observed data points should lie on a straight line passing through the origin. A good linear dependence can be seen in the log-reciprocal plot of Fig. 1 over the 10-fold range of flow velocities used.

To confirm more directly that magnetic forces were responsible for the observed particle retention, a run at 7.3 cm/min was repeated with the magnet turned off. Whereas 71% of the particles recovered from the first run were in the held fraction, this value dropped to 0.4% with the magnet off.

Beads for immunoferritin labeling were first examined by fluorescence spectroscopy, and enhanced uptake of labeled antibody was observed when a double-labeling sandwich technique was used. A fluorescein-conjugated rabbit IgG against horse ferritin (Cappel Laboratories) was allowed to bind, as an antigen, to washed beads (Bio-Rad). Alternatively, it was allowed to bind ferritin on beads that had previously been incubated with PAP and washed, followed by an incubation with ferritin-conjugated goat IgG against rabbit IgG. The latter procedure resulted in at least a threefold greater uptake of fluorescence by the beads. On the basis of these and similar experiments, it was decided to carry out the ferritin-HGMS study using samples of beads that were surface labeled with PAP, followed by ferritin-conjugated goat anti–rabbit IgG.

Labeled immunobeads were passed through an HGMS column and capture was determined as a function of buffer flow velocity. The results have been graphed on conventional axes in Fig. 2. The retention ranged from 30 to 98% and was greater at slower flow rates, as expected. At each speed it was approximately three times as great as for the corresponding control run in the absence of the magnetic field. The recovery, R, ranged from 69 to 100% in these experiments.

In one case the beads that had been separated into passed and held fractions by HGMS were used as samples for subsequent fractionations. As should be expected for

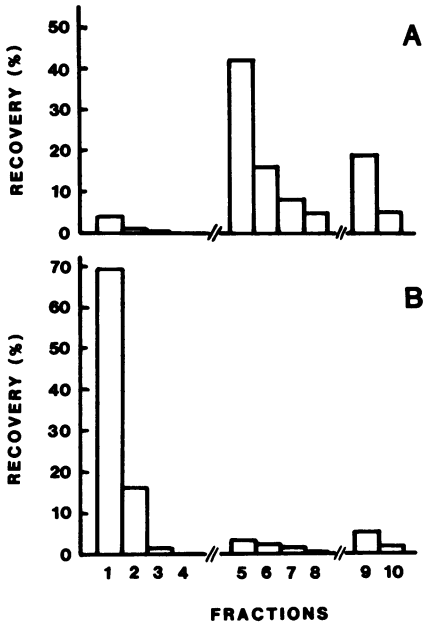

FIGURE 3 Magnetic retention of red blood cell ghosts. Membranes were labeled with ferritin–Con A in the absence or the presence of competing alpha-methyl-D-glucoside for curves A and B, respectively. Fractions 1–4 were collected while the column was in the magnet, 5–8 at the same 0.3 ml/min flow speed with the column outside the magnet and 9 and 10 were rapid elutions to dislodge remaining material. The column had a cross-sectional area of 0.95 cm^2 and contained 25 μm wire filling 3% of the 10 ml bed volume. The height of each bar gives the percentage of the total recovered material which was observed to be in that fraction.

uniformly labeled samples, particles in the two fractions showed exactly the same probability of magnetic capture when rerun through the magnet as they had in the first passage. The transmittance, T, was equal to 0.36 in the initial fractionation and the recovery, R, was 78%. The passed and held material from that run then yielded values for T of 0.28 and 0.33, respectively, when rerun under the same conditions.

Ferritin HGMS was demonstrated in a completely biological system by using a conjugate of ferritin and concanavalin A to label glycoproteins on erythrocyte plasma membranes (red blood cell ghosts). Sample material going onto the column or recovered in the eluted fractions was assayed by fluorescence enhancement of diphenylhexatriene (DPH) dye (20). The assay was tested for linearity using various dilutions of red blood cell membrane material (18) containing 0–20 μg protein. When fluorescence intensity at 420 nm was graphed vs. the concentration of ghost material (curve not shown), the assay was linear over the range 0–15 μg ghost protein/ml with a correlation coefficient $r = 0.999$.

Histograms presenting the elution patterns for ghost membranes recovered from the HGMS column are shown in Fig. 3. Four fractions were collected with the magnetic field applied to the column, four with the column removed from the magnet and two of buffer forced rapidly through to dislodge remaining particles. The upper histogram, in Fig. 3 *A*, shows results for ferritin–Con A labeling, and the lower (3 *B*) shows the effect of the inclusion of alpha-methyl-D-glucoside in the initial incubation. In the first case only 5% of the recovered ghosts passed through the magnetized column and were recovered in fractions 1–4. The corresponding value for the control in which lectin binding was inhibited was 87%. It is also clear from the figure that the material that was retained on the column was firmly held as long as the magnet was on. Labeled ghosts were not being slowly eluted and no increase in the "passed" fraction would have been observed if a larger volume had been collected.

The effect of varying the buffer flow rate can be seen in Fig. 4. In this graph the data point at 5×10^{-3} cm/s was obtained under the same conditions as the run shown in Fig. 3 *A* and the observed transmittance agreed with the earlier value (0.06 and 0.05, respectively). For comparison, the experiment of Fig. 3 *B* has been included (○), in which alpha-methyl-D-glucoside inhibited lectin binding and the transmittance increased to 0.87. The data have been plotted on conventional axes in Fig. 4 *A* and on log-reciprocal axes in Fig. 4 *B*.

The total recovery of ghost material was 60–90% for these experiments. Losses of sample membrane material, which tended to lower the observed values for R, were not only due to the HGMS column but also occurred during the centrifugation of ghosts from the relatively dilute passed and held samples, which was done to concentrate them for the fluorescence assay.

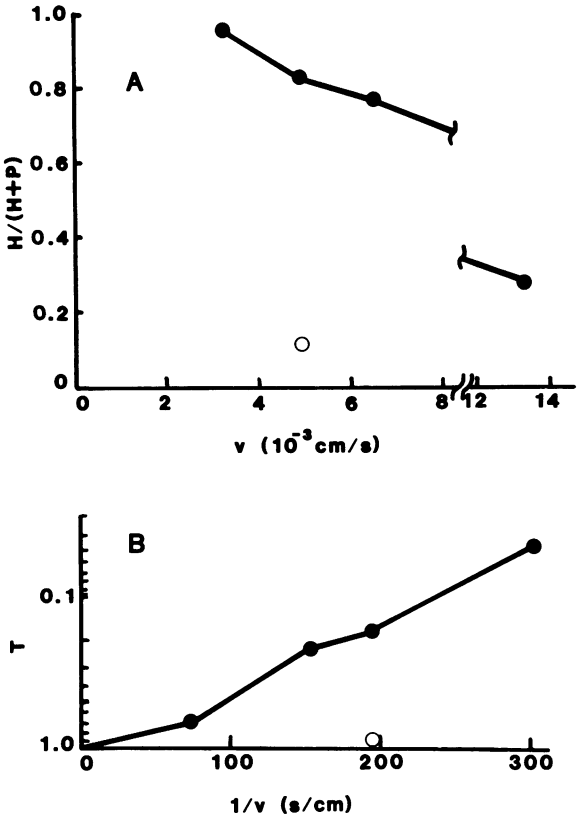

FIGURE 4 Dependence on flow rate for magnetic retention of red blood cell ghosts labeled with ferritin-Con A. The data have been plotted on conventional axes in *A* and in the log-reciprocal format in *B*. The data from the alpha-methyl-D-glucoside control experiment of Fig. 3*B* have been replotted in Fig. 4 as the open circle (○) in each graph.

DISCUSSION

For some applications, HGMS holds promise of significant advantages over other techniques for fractionating cell subpopulations. For example, it shares with fluorescence-activated cell sorting (FACS) the feature that cells are labeled in a procedure that is distinct from the process used to effect the enrichment. This means that the efficiency of the separation can readily be assessed by simply counting the number of labeled cells in different fractions. On the other hand, an advantage that HGMS shares with cell affinity chromatography is that it is based on passing samples through separation columns. This means that the method is capable of a much greater throughput than the cell-by-cell FACS method. In addition, because the forces binding the captured cells onto the HGMS column are mainly magnetic, cell recovery has usually been excellent when the field has been turned off and the column flushed.

The use of soluble materials such as ferritin conjugates to label cell or organelle subpopulations greatly enhances the capacity of the HGMS column in comparison with the use of paramagnetic red blood cells as labels (i.e., rosette enrichment). In the former case, unbound paramagnetic

material can be eliminated by washing cells before placing them on the column. In the rosette case, unbound red blood cells must go onto the column along with the rosettes and free leukocytes. For example, a column that can hold 10^9 cells could accommodate 10^9 ferritin-labeled cells, which might be included in an initial sample of anywhere from 2×10^9 to 100×10^9 cells. On the other hand, to use the same column to purify rosettes would mean that only 10^9 red blood cells could be used to form rosettes, which would imply an overall sample size including only 10^8 white cells in each sample administered to the column.

Soluble labeling reagents also have an important advantage in that they are stable and do not have to be as frequently prepared as red blood cell conjugates. Another important advantage may be the compatibility with the FACS technique. If the immunoferritin reagent to be used were also fluorescent, a single labeling step could prepare a sample both for magnetic presorting and for further enrichment on the FACS. The HGMS first stage would have the capacity to handle relatively large samples, which is important when one is attempting to purify cells that are present in the initial sample in very low amounts.

This study has shown in three model systems that ferritin-based HGMS can be observed in a strong magnetic field. The greatest magnetic retention was observed with 1-μm latex spheres to which ferritin was covalently bound. The fit of the data points of Fig. 1 to a straight line over a full 10-fold range of flow rates is in agreement with theoretical predictions for the HGMS process (16, 21).

The principal drawback of ferritin as a paramagnetic label is clearly that it binds relatively little iron to target cells and they are only weakly paramagnetic as a result. This implies the need for strong magnetic fields and slow buffer flow rates in the HGMS system. For purposes of estimating the order of magnitude of this effect, one can assume that a typical cell of interest might bind on the order of 7,000 molecules of ferritin, containing a total of roughly 10^7 atoms of iron. In comparison, the red blood cell contains approximately 10^9 heme iron groups.

The theory of HGMS (16, 21) predicts that the probability of capture of any paramagnetic cell will be proportional to $\chi B/v$, where B is the applied field, v the buffer flow velocity, and χ the paramagnetic susceptibility of the labeled cells. Our observations are consistent with this prediction and a susceptibility based on the above estimate. The value of B/v for the red cell containing methemoglobin is on the order of 10^5 G s/cm for 90% cell capture by the same wire used in this study (11), and B/v from Fig. 4 is ~200-fold greater for ferritin-labeled ghosts. This is the differential that would be expected if the magnetic susceptibility contributed by ferritin is approximately two orders of magnitude less than that due to high-spin heme iron in the intact red cell.

The relatively slow flow rates that are required to achieve retention of ferritin-labeled ghosts lead to increased amounts of nonmagnetic retention, as well. This limits the enrichment that can be expected if the system is used to extract labeled cells from a mixed initial sample. Drawing on the red cell ghost data of Fig. 4, there was a 12% retention of unlabeled ghosts (alpha-methyl-D-glucoside control) while labeled material was 95% held. This means that an initial sample that consisted of 50% labeled and 50% unlabeled cells would be expected to produce an enrichment up to the level of 90% labeled material in the magnetically retained fraction. By the same token, one can calculate that labeled cells that were present at an extremely low frequency in an initial mixed sample would be enriched eightfold by each passage through the HGMS system.

In principle, one could improve the rate of magnetic capture of cells by using more wire in the HGMS column. This would theoretically allow one to speed up the buffer flow velocity somewhat, since the resulting decrease in the probability of capture each time a cell encounters a segment of wire should be compensated for by the increased number of cell-wire collisions. In practice, however, experiments in this laboratory with methemoglobin red blood cell samples have indicated that wire packing densities above 5% by volume appear to give rise to increased ratio of nonmagnetic trapping of cells. It was assumed that a similar onset of nonmagnetic capture would occur for ferritin-labeled cells, and the density of wire in the columns was kept low, accordingly. An additional practical consideration was that 25-μm diam wire was used because of its availability, even though theory (22, 23) and one experimental demonstration (24) have suggested that the optimum diameter would probably be 12–15 μm if such wire were available in an alloy that was at least as magnetizable as the larger wire.

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