

Magnetic Filtration of Vesicles Containing Iron-Dextran Particles

Colin P.S. Tilcock, Eric F. Sommerman, Robert S. Molday, and Pieter R. Cullis Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia, Canada

Liposomes containing dextran-coated magnetite can be filtered from dispersion by a magnetic filtration procedure. The utility of the technique is demonstrated for the separation of liposomes from plasma components.

INTRODUCTION

We present here a novel method for the separation of lipid vesicles, based on high-gradient magnetic filtration (1-3) in which superparamagnetic dextran-coated magnetite (Fe-Dex) particles are encapsulated within the vesicles. The vesicles with entrapped magnetite may be removed from suspension by passage over a column containing a steel filter placed in the fringe field of a magnet pole gap, whereas vesicles without magnetite pass unhindered through the column.

The pharmacodynamics of liposomally encapsulated drugs is known to be influenced by several factors including the chemical composition of the

Address reprint requests to: Colin P.S. Tilcock, Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.

vesicles (4,5), vesicle size (5,6), dosage (6), and route of administration (7). Within the context of vesicle-serum interactions, it is clearly important to establish to what extent the nature of the protein absorbed onto the vesicle surface from the plasma influences the lifetime of the vesicles in the circulation. We demonstrate the principle and utility of the magnetic separation method, by using vesicles with encapsulated Fe-Dex to determine the binding of plasma proteins to the surface of the vesicles. Advantages, limitations, and other applications of the technique are discussed.

METHODS

Preparation of Iron-Dextran (Fe-Dex) Particles

Dextran-stabilized magnetite particles were prepared using a modification of the procedure of Molday and Molday (3). Typically, 0.75 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.4 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were dissolved in 5 ml of distilled water to which was added 5 ml of a 50% w/w aqueous solution of dextran T 40 (Pharmacia), followed by the bulk addition of 10 ml of 14% v/v NH_4OH with vigorous stirring. The mixture was stirred for 4 hr at room temperature, centrifuged ($\times 17,300$ g, 30 min) to remove large fragments, then dialyzed exhaustively against 0.1 M NaCl until the pH of the dialysis medium was less than 8. Unassociated dextran was removed by chromatography over Sephacryl S-300 (Pharmacia) with 0.1 M NaCl as eluant. The particles obtained were stored at 4°C until required. Fe-Dex particles were sized using a Nicomp 270 Laser Particle Sizer (Nicomp Instrument, Goleta, CA) operating at 632.8 nm and 5 mW. A multicomponent distribution analysis indicated that the Fe-Dex particles had a diameter of 50 ± 10 nm, with no evidence of larger aggregates after storage for several months. In some instances particles were prepared using a 50% dextran T 40 solution labeled with 0.05 mCi of [^{14}C]carboxyl dextran (NEN, Canada). Attempts to prepare smaller Fe-Dex particles with a lower molecular weight dextran (T 10, Pharmacia) were unsuccessful, giving rise to particles that aggregated with time.

Preparation of Lipid Vesicles

Twenty to 40 μmoles total of an equimolar mixture of egg phosphatidylcholine (EPC) and cholesterol (Chol) containing 5-10 μCi of either [^{14}C]cholesterol oleate or [^3H]DPPC (NEN, Canada) was dispersed by vortex mixing with 0.5-1 ml of the final Fe-Dex preparation to yield multilamellar vesicles (MLVs). Untrapped Fe-Dex particles were removed by

washing eight times in 10 volumes of 0.1 M NaCl followed by centrifugation ($\times 4,000$ g, 20 min). One preparation containing ^3H -labeled lipid and ^{14}C -labeled Fe-Dex was used to determine that six to eight washes were sufficient to obtain a pellet with a constant $^3\text{H}/^{14}\text{C}$ ratio.

Vesicles produced by extrusion techniques (VETS) were prepared according to the method of Hope et al. (8). MLVs (unwashed) were transferred to cryovials (Simport, Canada), subjected to five freeze-thaw cycles in liquid nitrogen, then extruded through two stacked 200 or 400 nm filters at 900 psi. The filters were exchanged for two fresh filters with the same pore size and the suspension extruded another 10 times at 500 psi. Untrapped Fe-Dex particles were removed by chromatography on Sepharose 4B at 4°C using a 23 X 2 cm column, 0.5 ml load, 0.1 M NaCl as eluant, and a flow rate of 0.1 ml/min. Only fractions up to and including the peak in the lipid count elution profile were used in further experiments.

Background binding of Fe-Dex particles was determined by preparing ^3H -labeled EPC/Chol 1:1 200 nm diameter VETS, VET_{200} (20 $\mu\text{mol}/0.5$ ml, 0.1 M NaCl), and incubating with 0.5 ml of ^{14}C -labeled Fe-Dex overnight at 4°C , followed by passage over Sepharose 4B as described above. Less than 2% of the total Fe-Dex counts associated with VET_{200} s prepared from MLVs is due to background binding of Fe-Dex particles to the surface of the LUVs. Quasielastic light scattering (QEL) showed no evidence of significant aggregation of VET_{200} s on incubation with externally added Fe-Dex particles. Vesicles were stored up to 5 days at 4°C prior to use and showed no evidence of precipitation.

Preparation of Plasma

Outdated human plasma was obtained from the Red Cross Unit (Vancouver). Plasma was centrifuged (16000 \times g, 30 min) to remove residual red blood cells and stored at 4°C until used.

Magnetic Separation

A custom-built water-jacketed Pyrex column (internal diameter 2.5 mm) was placed between the pole faces of a custom-built electromagnet operating at approximately 0.3 Tesla in the pole gap. The region inside the column between the pole gap was filled with 100 mg of 25 μm diameter stainless steel wire (Type 302, Goodfellow Metals, Cambridge, UK). The column was flushed with 0.1 M NaCl and connected to a fraction collector via a peristaltic pump. The column fluid was run down to the top of the

wire, then with the field applied and with (ambient temperature) water flowing through the column water jacket, 2-5 μmol of lipid (in 0.1 M NaCl or mixed with plasma for 30 min at room temperature) was gently loaded via hypodermic needle onto the fluid surface. Movement of the (brown) Fe-Dex-loaded vesicles to the surface of the wire occurred within 30 sec; then the column was filled with 0.1 M NaCl and eluted (10 ml at 0.2 ml/min). The field was then removed, the column removed from the vicinity of the magnet due to residual hysteresis of the iron cores, and the column eluted for a further 10-15 ml at 0.5 ml/min. Fractions were then sampled for counts. To avoid potential problems with magnetic remnance, the stainless steel filter was changed after every third run.

Gel Electrophoresis

Delipidation of vesicle-associated plasma proteins was performed by the method of Folch (9). The vesicle-associated plasma proteins from MLVs, VET₂₀₀s, and VET₄₀₀s as well as plasma protein standard were analyzed by SDS-PAGE electrophoresis using 7.5% acrylamide gels according to the method of Laemmli (10) and stained with Coomassie Blue according to the method of Anderson (11).

RESULTS

Figure 1 shows the separation of a mixture of two populations of EPC/**Chol** 1:1 MLVs, one of which was prepared in the absence of Fe-Dex particles. It is evident that the Fe-Dex-loaded vesicles are retained (>90%) on the wire matrix in the presence of an applied field, whereas a large percentage (~50%) of the nonloaded vesicles are eluted. The resolution of the two populations is clearly influenced by nonspecific associations between the lipid vesicles, such as macroscopic entrapment of nonloaded MLVs within microaggregates of magnetite-loaded MLVs.

Figures 2, 3, and 4 show the retention characteristics of VET₂₀₀ systems, VET₄₀₀, and MLVs, respectively, all loaded in 0.1 M NaCl. In all instances, if no field was applied, the vesicles eluted from the column in the first six to eight fractions (data not shown), dependent upon the amount of lipid applied. This, together with the result shown in Figure 1, demonstrates that the stainless steel filter was not a macroscopic barrier to passage of the vesicles. Figures 2-4 show that in the presence of an applied field, greater than 90% retention of the vesicles was observed. The recovery of added counts was greater than 98%, indicating no hold up in the filter.

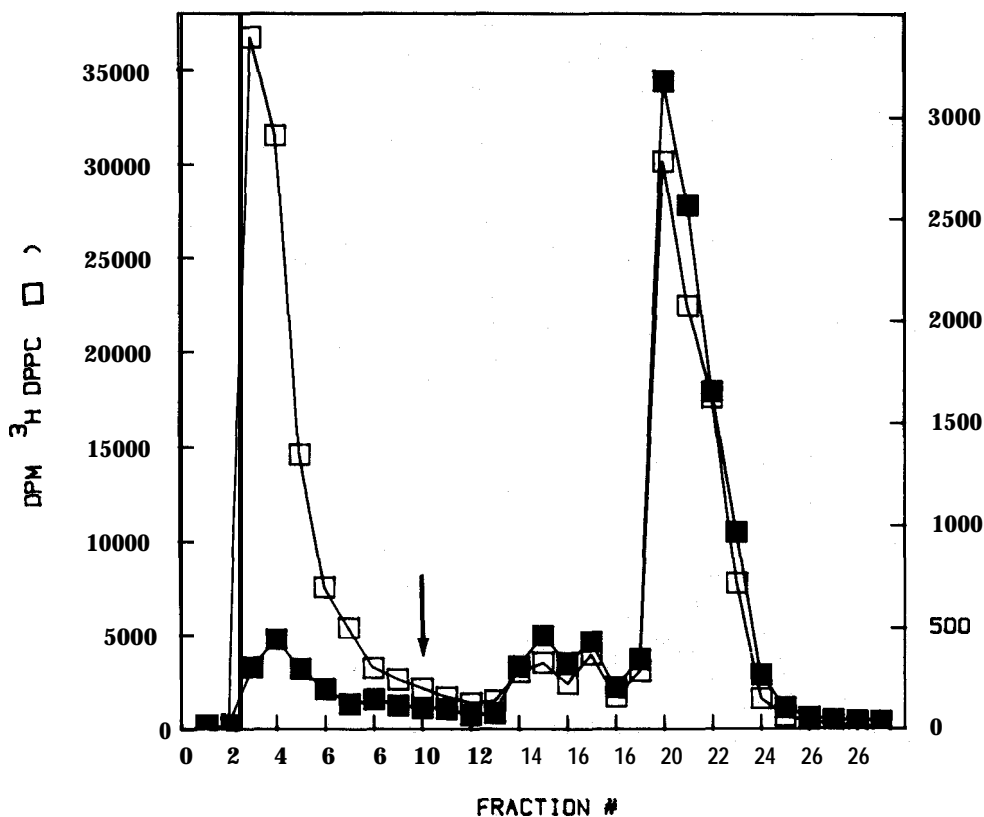


Figure 1. Resolution of a mixture of two populations of EPC/Chol 1:1 MLVs. ³H-Labeled MLVs (3.3 μ moles lipid), without entrapped Fe-Dex particles and ¹⁴C-labeled MLVs (3.3 μ moles lipid) containing Fe-Dex particles were loaded in a total volume of 150 μ l 0.1 M NaCl. The arrow indicates the fraction at which the magnetic field was cut.

Because the entrapment of **Fe-Dex** particles within the lipid vesicles is random upon dispersal (and also extrusion in the case of **VET**₂₀₀ and **VET**₄₀₀ systems), it is possible that a subpopulation of the vesicles does not contain **Fe-Dex** particles. This may partly explain the observation that for **VET**₂₀₀s (Fig. 2), approximately 10% of the total counts passed through the filter even with the field on. This interpretation is supported by the observation (not shown) that similar VETS prepared by extrusion through 100 nm filters exhibited much poorer (40-60%) retention characteristics.

After incubation with plasma, for both the **VET**₂₀₀ and **VET**₄₀₀ systems a greater percentage of the lipid-associated counts (>15%) eluted from the

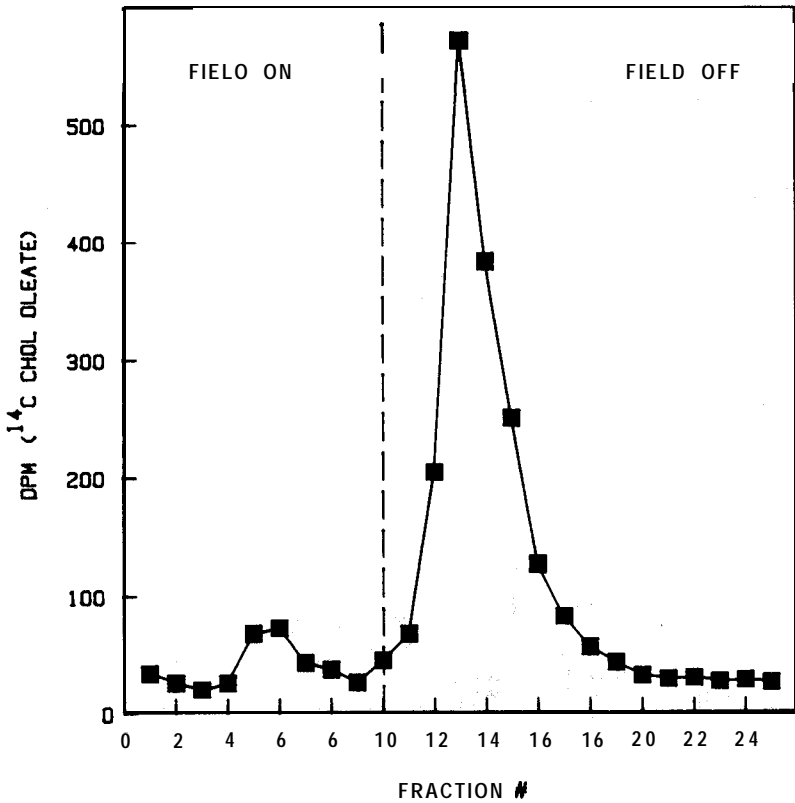


Figure 2. Retention of Fe-Dex-loaded EPC/Chol 1:1 VET,,. Load was 2.3 μ mole lipid in 200 μ l 0.1 M NaCl.

column with the field applied (Figs. 5,6). There are several possible explanations for this observation. As before, there may be a subpopulation of vesicles, not loaded with Fe-Dex particles. Alternatively, this may reflect the aggregation of vesicles by serum proteins or simply the binding of protein to the vesicle surface. In either event this will result in larger particles with greater effective Stokes radius, which will consequently be subject to greater viscous drag forces resulting in reduced retention by the wire filter (2). The counts eluted in the presence of the applied field could also represent lipid transferred to serum lipoproteins via exchange proteins (12). It was noted that the MLV systems did not exhibit decreased retention when mixed with plasma (Fig. 7) and were also quite stable in the presence of serum, as indicated by the small amount of entrapped aqueous matter, inulin, released over the course of the experiment.

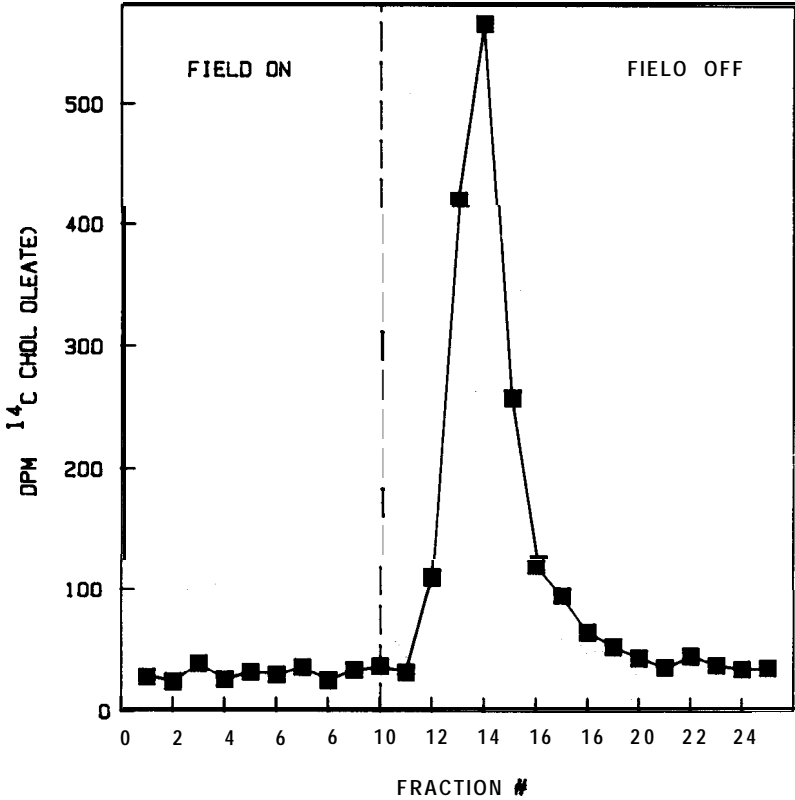


Figure 3. Retention of Fe-Dex-loaded EPC/Chol 1:1 VET₄₀₀. Load was 3.2 μ moles lipid in 200 μ l 0.1 M NaCl.

Finally, Figure 8 shows the results of SDS-PAGE electrophoresis of the associated protein with VET₂₀₀, VET₄₀₀, and MLVs. While the size of the vesicles seems to make no appreciable difference to the binding of the plasma proteins, the pattern of the associated proteins was significantly different from the total plasma proteins, in that many of the minor bands were attenuated.

DISCUSSION

Techniques that have been used for the separation of lipid vesicles from serum include gel filtration (13), density gradient centrifugation (14), and selective precipitation (15). Chromatographic procedures lack general applicability because of the coelution of the larger lipoprotein species, low-

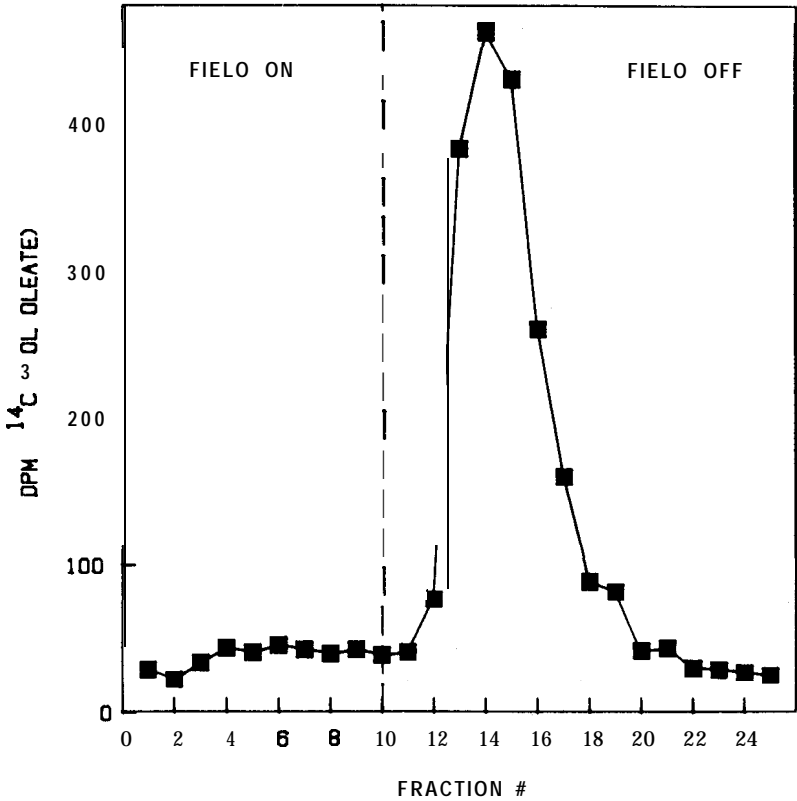


Figure 4. Retention of Fe-Dex-loaded EPC/Chol 1:1 MLVs. Load was 5.5 μmoles lipid in 250 μl 0.1 M NaCl.

density and very-low-density lipoproteins, with small lipid vesicles (16). Incomplete resolution of lipid vesicles and lipoproteins also restricts the utility of the centrifugation method, particularly with regard to the quantitation of protein binding. Dialysis procedures will only remove low-molecular-weight contaminants and selective precipitation requires the use of high salt concentrations, which may be expected to affect any electrostatic associations between plasma proteins and the vesicle surface.

We have shown that magnetic filtration of magnetite-loaded vesicles permits the recovery of lipid vesicles from serum by a method that does not involve tedious fractionations based on minor differences in size or density. The advantages of the method are that it is applicable to vesicles of any lipid composition and with diameter greater than approximately 200 nm. More importantly, the technique involves no perturbation of the mem-

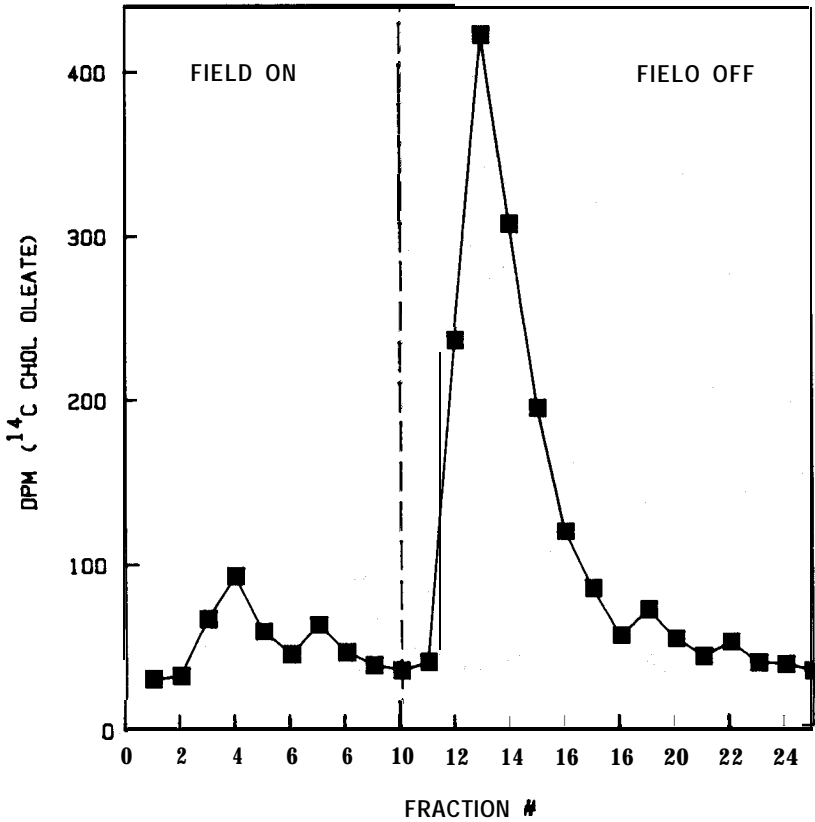


Figure 5. Retention of Fe-Dex-loaded EPC/Chol 1:1 VET, from plasma. Two μ moles of lipid in 200 μ l 0.1 M NaCl was mixed with 200 μ l of plasma for 30 min at room temperature. The combined vesicles in plasma were applied to the column.

brane surface. The principal disadvantage of the technique is that it is inapplicable to vesicles whose diameter is of the same order or less than the diameter of the Fe-Dex particle to be entrapped. We are currently investigating the in situ formation of magnetite inside lipid vesicles in response to a pH gradient (17) as a way of overcoming this limitation.

The magnetic attractive force experienced by the vesicle may be expressed in terms of the total volume magnetic susceptibility (1,18), which in our system must be considered for the vesicle as a whole and not just the entrapped Fe-Dex particle. Since the entrapment of Fe-Dex particles is stochastic, the total number of particles entrapped will vary to a first approximation as r^3 , where r is the vesicle radius. On this basis an MLV will

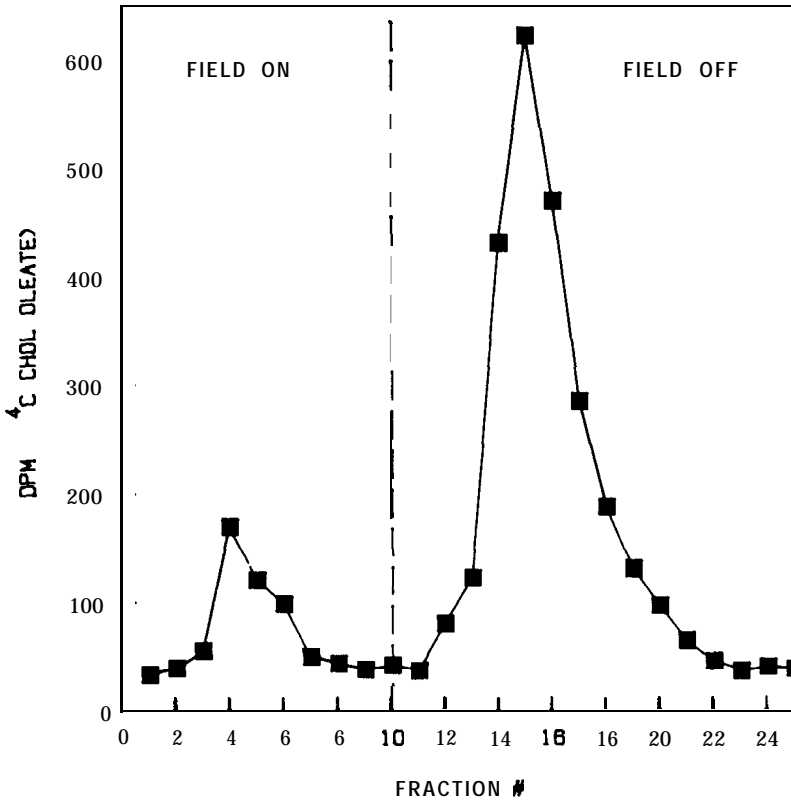


Figure 6. Retention of Fe-Dex-loaded EPC/Chol 1:1 VET, from plasma. 2.8 μ moles of lipid in 200 μ l of 0.2 M NaCl was mixed with 200 μ l of plasma for 30 min at room temperature before being applied to the column.

have a greater volume magnetic susceptibility than a unilamellar vesicle and will behave as if it were a stronger magnet. Conversely, viscous drag forces, at low Reynolds number, will vary as r . Thus the observation that the MLV system (Fig. 7) did not exhibit decreased retentions when mixed with plasma may be a simple consequence of the balance of (opposing) hydrodynamic shear forces and attractive magnetic forces.

Our experiments indicated that the magnetic separation technique is sensitive to the amount of lipid applied for a given weight of filter wire, and particularly to the flow rate. In our hands, for a filter of 100 mg of wire, application of more than 10 μ moles of lipid led to irreproducible and low retentions upon the filter. Flow rates in excess of 0.2 ml/min also gave low retentions. To some extent these constraints may be imposed by the

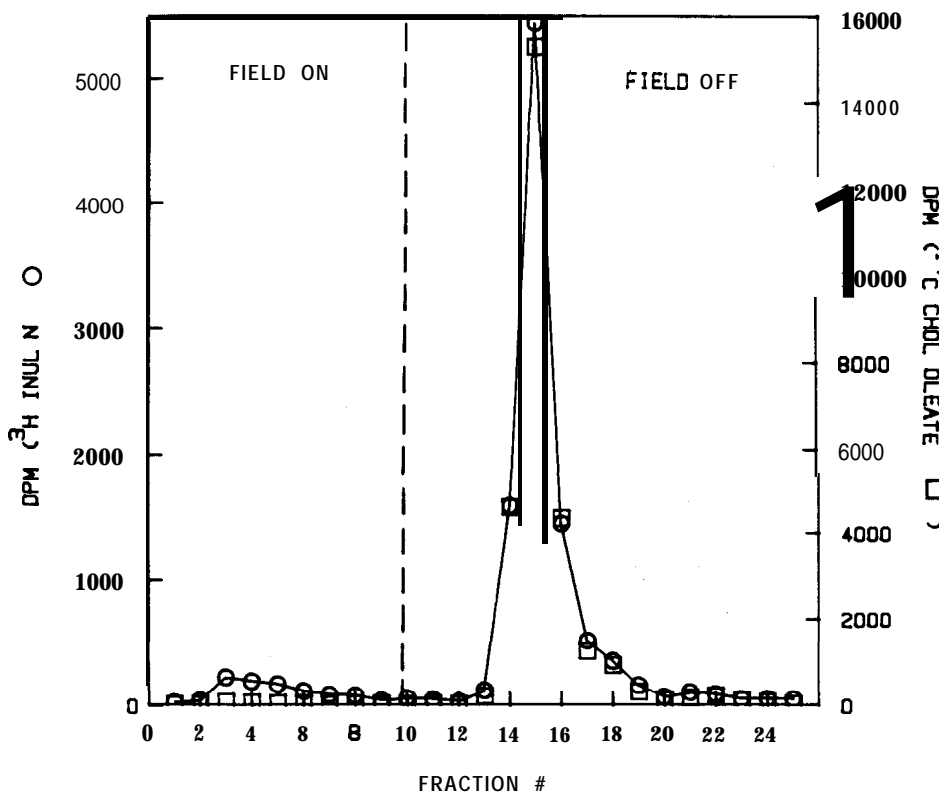


Figure 7. Retention of Fe-Dex-loaded EPC/Chol 1:1 MLVs from plasma. Twelve μmoles of lipid in $50 \mu\text{l}$ 0.1 M NaCl was mixed with $150 \mu\text{l}$ plasma for 30 min at room temperature before being loaded onto the column.

geometry and limitations of the equipment used. Wider-bore columns combined with lower flow rates may reduce shear forces that will tend to sweep vesicles off the wire filter (2). It should be noted that due to the limitations of the equipment available to us we were operating at field strengths well below the saturation magnetization of magnetite (approximately 2.5 Tesla [19]). The use of higher field strengths may be expected to yield improved retentions at higher flow rates. We noted that the physical manner in which the stainless steel filter was packed into the column did not influence the retention characteristics. For a given preparation of vesicles, duplicate runs under identical conditions of loading and flow rate, but with a different filter, gave similar elution profiles.

We envisage that the magnetic separation technique may be of considerable value in other areas of research beyond the protein binding described

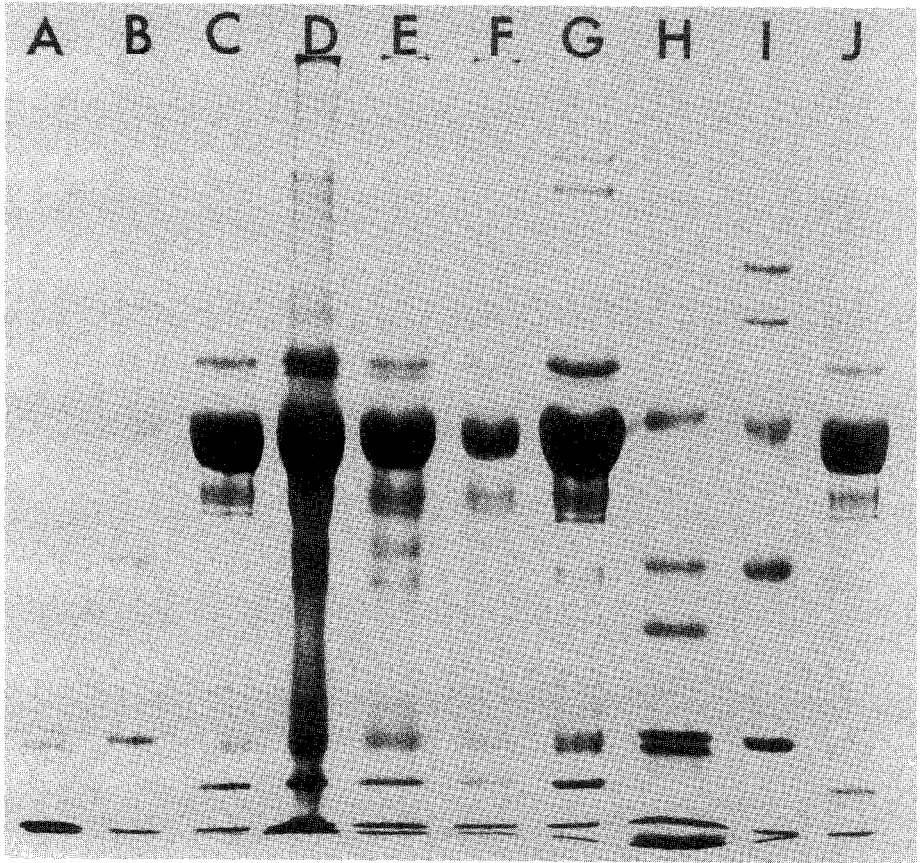


Figure 8. SDS-PAGE electrophoresis analysis of plasma proteins and vesicle-associated protein. Lanes A,B,H,I are molecular weight standards; C,G,J are of total plasma protein; lane D shows the plasma proteins associated with MLVs; lane E, those proteins associated with VET_{400S}; and lane F, the proteins associated with VET_{200S}.

here. Examples include studies on the function of lipid exchange proteins or monitoring the exchange of lipids and other surface markers during vesicle aggregation or fusion. Additionally, the vesicles with entrapped magnetite are themselves of interest as contrast agents for magnetic resonance imaging (20), as potential drug-delivery vehicles-targeted to tumor sites by utilizing external magnetic fields in a manner similar to that described for magnetic albumin microspheres (21)-or as agents for decreasing phase separation times in two-phase aqueous polymer systems (22).

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and the British Columbia Health Care Foundation.

REFERENCES

1. Watson, J.H.P. (1973). Magnetic filtration. *J. Appl. Phys.* 44:4209.
2. Lawson, W.F., Simons, W.H., and Treat, R.P. (1977). The dynamics of a particle attracted by a magnetized wire. *J. Appl. Phys.* 48:3213.
3. Molday, R.S., and Molday, L.L. (1984). Separation of cells labeled with immunospecific iron-dextran microspheres using high gradient magnetic chromatography. *FEBS Lett.* 170:232.
4. Abraham, I., Goundalkar, A., and Mezei, M. (1984). Effect of liposomal surface charge on the pharmacokinetics of an encapsulated model compound. *Biopharm. Drug Dispos.* 5:387.
5. Juliano, R.L., and Stamp, D. (1975). The effect of particle size and charge on the clearance rates of liposomes and liposome-encapsulated drugs. *Biochem. Biophys. Res. Commun.* 63:651.
6. Abra, R.M., and Hunt, C.A. (1981). Liposome disposition *in vivo* III. Dose and vesicle-size effects. *Biochim. Biophys. Acta* 666:493.
7. Poste, G., Kirsh, R., and Koestler, T. (1984). The challenge of liposome targeting *in vivo*. In: Gregoriadis, E. (ed.) *Liposome Technology*, Boca Raton, CRC Press, vol. 3, p. 1.
8. Hope, M.J., Bally, M.B., Webb, G., and Cullis, P.R. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* 812:55.
9. Folch, J., Lees, M., and Sloane-Stanley, G.A. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497.
10. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
11. Anderson, L., and Anderson, N.G. (1977). High resolution two-dimensional electrophoresis of human plasma proteins. *Proc. Natl. Acad. Sci.* 74:5421.
12. Bonté, F., and Juliano, R.L. (1986). Interactions of liposomes with serum proteins. *Chem. Phys. Lipids* 40:359.
13. Damen, J., Dijkstra, J., Regts, J., and Scherphof, G. (1980). Effect of lipoprotein-free plasma on the interaction of human plasma high density lipoprotein with egg yolk phosphatidylcholine liposomes. *Biochim. Biophys. Acta* 620:90.
14. Tall, A.R., Hogan, V., Askinazi, L., and Small, D.M. (1978). Interaction of plasma high density lipoproteins with dimyristoyllecithin multilamellar liposomes. *Biochemistry* 17:322.

15. Warnick, G.R., and Albers, J.J. (1979). A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *J. Lipid Res.* 19:65.
16. Scherphof, G.L., Damen, J., and Wilshut, J. (1984). Interactions of liposomes with plasma proteins. In: Gregoriadis, E. (ed.) *Liposome Technology*, Boca Raton, CRC Press, vol. 3, p.205.
17. Mann, S., Hannington, J.P., and Williams, R.J.P. (1986). Phospholipid vesicles as a model system for biomineralization. *Nature* 324:565.
18. Owen, C.S. (1982). High gradient magnetic capture of red blood cells. *J. Appl. Phys.* 53:3884.
19. Scholten, P.C. (1978). Colloid chemistry of magnetic fluids. In: Berkovsky, B. (ed.) *Thermomechanics of Magnetic Fluids*, Washington, Hemisphere, p.1.
20. Runge, V.M., Clanton, J.A., Lukehart, C.M., Partain, C.L., and James, A.E. (1983). Paramagnetic agents for contrast-enhanced NMR imaging: a review. *Am. J. Radiol.* 141:1209.
21. Widder, K.J., Morris, R.M., Poore, G.A., Howard, D.P., and Senyei, A.E. (1983). Selective targeting of magnetic albumin microspheres to the Yoshida sarcoma: ultrastructural evaluation of microsphere disposition. *Eur. J. Cancer Clin. Oncol.* 19:141.
22. Wikström, P. (1987). Magnetically enhanced aqueous two-phase separation. Abstract, 5th International Conference on Phase Partitioning, Oxford, UK.