Encapsulation of hemoglobin in phospholipid vesicles

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Hemoglobin has been encapsulated in phospholipid vesicles by extrusion of hemoglobin/lipid mixtures through polycarbonate membranes. This technique avoids the use of organic solvents, sonication, and detergents which have proven deleterious to hemoglobin. The vesicles are homogeneous, with a mean size of 2400 Å as determined by photon correlation spectroscopy. The encapsulated hemoglobin binds oxygen reversibly and the vesicles are impermeable to ionic compounds. Hemoglobin encapsulated in egg phosphatidylcholine vesicles converts to methemoglobin within 2 days at 4°C. By contrast, when a mixture of dimyristoyl phosphatidylcholine, cholesterol and dicetyl phosphate is used there is no acceleration in methemoglobin formation, and the preparation is stable for at least 14 days at 4°C.

Hemoglobin *Phospholipid* *Vesicles* *Liposomes* *Encapsulation* *Blood*

1. INTRODUCTION

Situations exist in which adequate supplies of blood of suitable type are not available, consequently there is an acknowledged need [1] for a blood surrogate. Ideally, such a fluid would bind oxygen cooperatively, have no toxic or antigenic properties and be sufficiently stable to allow the patient to survive until whole blood could be administered. Here we report on our effort to prepare a blood surrogate based on encapsulation of hemoglobin in large unilamellar phospholipid vesicles. Our encapsulation method follows the extrusion procedure in [2] and yields stable vesicles narrowly distributed in size. Our method differs in several significant respects from that in [3,4].

2. MATERIALS AND METHODS

Egg phosphatidylcholine (EPC), dimyristoyl phosphatidylcholine (DMPC), and bovine brain phosphatidylserine (BPS) were purchased from Avanti Polar Lipids; horse heart cytochrome *c*, the sodium salt of diphosphoglyceric acid and tetramethylenephenylenediamine from Sigma; cholesterol and dicetylphosphate (DCP) from Supelco, Inc. Hemoglobin derived from outdated human blood at ~150 mg/ml was supplied by Fisher Diagnostics. All other chemicals were reagent grade and used without additional purification. Polycarbonate membranes were obtained from Nucleopore.

Oxygen dissociation curves were measured on an unmodified Aminco 'Hem-o-Scan'. Optical spectra were measured on a Cary 219C at 20°C. Lipid concentration was determined as in [5]. Hemoglobin was assayed as the CN-met derivative [6]. Cytochrome *c* was measured spectrophotometrically taking ϵ_{mM} = 11.9 at 555 nm for the reduced protein. The photon correlation spectroscopy apparatus consisted of a He–Ne laser, associated optics, a Malvern 48 channel correlator, and an Apple II microcomputer.

Hemoglobin is encapsulated as follows: The solvent is removed with a rotary evaporator from 75 μmol of a mixture of EPC, cholesterol and BPS 5:4:1 or of DMPC, cholesterol and DCP 5:4:1. Concentrated hemoglobin (3 ml) in buffer (100 mM NaCl, 20 mM HEPES, 1 mM EDTA, pH 7.4) is added to the lipid, which is dispersed by vortexing. The mixture is then placed at 20°C in an ultra-filtration cell (Amicon Model 12) and forced

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by argon pressure through polycarbonate membranes twice each through membranes with 6000 Å, 4000 Å and 2000 Å pores. The resulting mixture of vesicles and free hemoglobin is separated at 4°C on a column of Sepharose CL-6B (Pharmacia). The purified vesicles may then be concentrated on an M_r 100000 cut-off ultrafilter (Nucleopore), or by centrifugation at 5000 rev./min for 20 min.

Vesicle size is determined from diffusion coefficients derived from photon correlation spectroscopy on dilute suspensions of vesicles [7,8]. Measurements are done at a scattering angle of 90° for different sampling-time intervals. No laser heating effects were found. The hemoglobin molecules do not affect the time dependence of the correlation function for the range of sampling-time intervals used.

3. RESULTS AND DISCUSSION

Vesicles formed in hemoglobin solution from EPC, cholesterol and BPS elute near the void volume of the CL-6B gel, are quite turbid and pink in color. When hemoglobin alone is subjected to extrusion and gel filtration none elutes with the void volume. The absorption spectrum of the vesicle hemoglobin fraction (upper trace fig.1) is that of oxyhemoglobin superimposed upon a steep background resulting from light scattering by the vesicles. The background may be eliminated and the spectra rendered more readily interpretable by recording the data as the first derivative ($dA/d\lambda$). In this mode (lower trace fig.1), the α and β bands are depicted with maxima at 582 and 548 nm and minima at 570 and 528 nm. Photon correlation spectroscopy reveals that this fraction contains uniform-sized vesicles of 2315 Å ± 52 Å with a polydispersity of 0.066 ± 0.058. The vesicles are stable over 50 h at 4°C in that the average size changes by ~4% to 2407 Å ± 18 Å, and the polydispersity only increases to 0.23 ± 0.08.

Co-elution of hemoglobin with the void-volume vesicle fraction supports either protein encapsulation, hemoglobin binding to vesicles, or formation of large protein aggregates. To demonstrate explicitly that the hemoglobin is encapsulated, an aliquot of a modified Drabkin's reagent (200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KH_2PO_4, in 100 ml, pH 7.4) [6] was added to a solution of

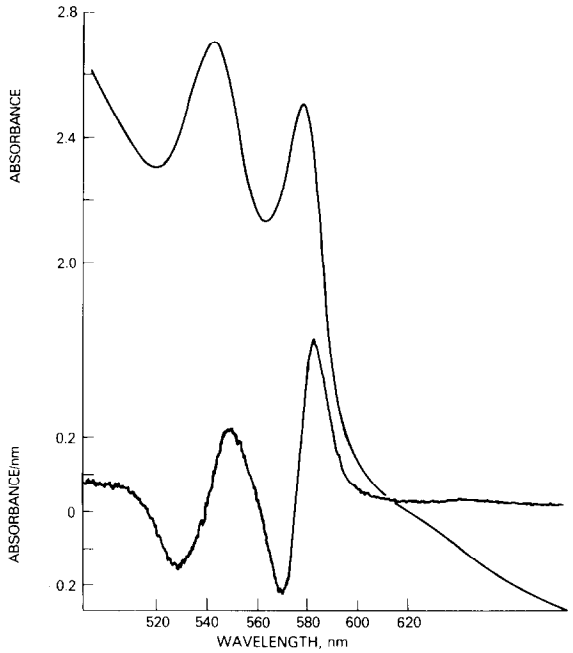

Fig.1. The absorption spectrum of a dilute suspension of vesicles containing hemoglobin, shown both as the absolute spectrum and in first derivative mode (as $dA/d\lambda$).

vesicles. Accessible hemoglobin would be converted to CN-methemoglobin, changing the absorption spectrum. Derivative spectra of vesicle-encapsulated hemoglobin in the absence and presence of Drabkin's reagent are compared in fig.2. The spectra are identical (the change in intensity in the presence of reagent is an effect of dilution). Adding the detergent Triton X-100 (0.1%) lyses the vesicles resulting in immediate conversion of released hemoglobin to CN-methemoglobin as shown by the broad derivative band with a crossover point near 530 nm. Two conclusions follow from this experiment:

(1) None of the hemoglobin is exposed on the vesicle surface or extends through the bilayer;
(2) The bilayer itself is impermeable to added CN^- and $Fe(CN)_6^{3-}$.

The EPC/cholesterol/BPS vesicles maintain their integrity against ion permeation for at least 24 h at room temperature. As expected, after 24 h there is spectral evidence of methemoglobin formation. However, the CN-methemoglobin derivative is not formed until the vesicles are lysed with detergent.

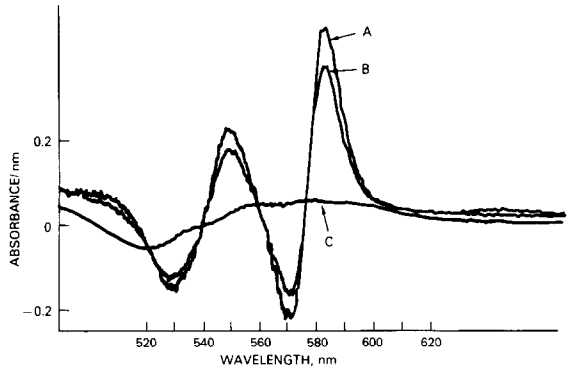

Fig.2. Three derivative traces of an encapsulating vesicle suspension of EPC/Chol/BPS: (A) pure suspension; (B) after addition of the modified Drabkin's reagent; (C) after addition of Triton X-100.

Vesicle integrity in the presence of hemoglobin is a function of the lipids used in the encapsulation. When hemoglobin is encapsulated using pure egg EPC the vesicles are leaky to Drabkin's reagent. That this is related to interaction of hemoglobin and lipid is demonstrated by the following experiments. Oxidized cytochrome *c* was encapsulated in EPC vesicles by the same procedure used for hemoglobin. Addition of sodium ascorbate (6 mM) to the outside of the vesicles did not produce a spectrum of reduced cytochrome *c*. Subsequent addition of the transmembrane electron transfer agent tetramethylenephenylenediamine resulted in rapid reduction of the cytochrome *c*, but no loss of light scattering due to the vesicles. Reduction was also accomplished upon treatment of the vesicles with Triton X-100 in the presence of external ascorbate. Thus EPC vesicles loaded with cytochrome *c* are impermeable to ascorbate. However, when an equimolar mixture of hemoglobin and cytochrome *c* was encapsulated and then ascorbate added, cytochrome *c* was immediately reduced. We conclude that pure EPC vesicles, which are intact when formed in the presence of cytochrome *c*, do not maintain their impermeability in the presence of hemoglobin. Addition of BPS and cholesterol restores structural integrity to the vesicular membrane in the presence of a protein which otherwise renders the bilayer leaky.

Oxygen dissociation measurements (fig.3) show very similar oxygen affinities for the precursor and the encapsulated hemoglobin (P_{50} of 9.1 and

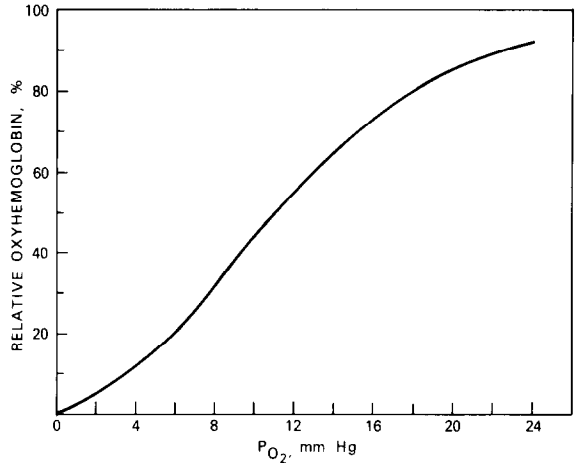

Fig.3. Oxygenation curve of hemoglobin encapsulated in vesicles of EPC/Chol/BPS as determined on the Hem-o-Scan: temp., 20°C; gas mixtures included carbon dioxide.

11 mm Hg, respectively). Cooperativity is maintained with a Hill coefficient of 3 for the precursor and 2.9 for the encapsulated hemoglobin.

It is possible to calculate the trapped volume per lipid and thence the trapping efficiency if the lipid concentration and the concentrations of the hemoglobin before and after encapsulation are known:

$$\frac{\text{Trapped volume}}{\text{Mole lipid}} = \frac{[\text{Hb}]_{\text{final}}/[\text{Hb}]_{\text{initial}}}{[\text{Lipid}]_{\text{final}}}$$

Using EPC/cholesterol/BPS, ~1.8 l of the initial hemoglobin solution are trapped/mol lipid. A theoretical trapped volume may be calculated assuming all vesicles are unilamellar, spherical and trap hemoglobin at its initial concentration. For a bilayer thickness of 40 Å (slightly thicker than the pure EPC bilayer because of ordering of the phospholipids by cholesterol) and an area/lipid molecule of 48 Å^2 [9], trapping volume is ~5 l/mol for vesicles of 2300 Å diameter. Thus, the trapping efficiency for this lipid mixture is in the range of 35%.

Although the vesicles described above are intact, oxygenate normally, and are well-characterized, the use of EPC presents one major problem in achieving the goal of a red cell surrogate – the stability of hemoglobin in the vesicles is diminished significantly compared to that in solution. The en-

capsulated protein converts to methemoglobin and further degradation products within 2 days at 4°C. Hemoglobin which is extruded in the absence of lipid or is in contact with an unextruded lipid dispersion retains its normal stability. Substituting DMPC and DCP for EPC and BPS, respectively, in the lipid mixture results in a profound improvement in hemoglobin stability. The vesicles themselves are unusually stable and resist lysis by Triton X-100 or other commonly available detergents. They may be concentrated by centrifugation and resuspended without loss of contents. While the size of these vesicles is identical to those formed with the unsaturated lipids, the trapping efficiency is up to 50% of the theoretical limit, and the hemoglobin remains stable against oxidation to methemoglobin for ≥ 14 days at 4°C.

Our preparation compares favorably to much less well-defined multilamellar liposomes in which hemoglobin has been entrapped [3,4]. The size of those liposomes was presumed to range from 0.02–1 μm. Although some cooperative O_2 binding by the hemoglobin was demonstrated, no estimation was given of the total amount of functional hemoglobin.

One significant advantage of the extrusion procedure over other methods of encapsulation is that it is gentle to the hemoglobin. Detergent-based methods, which can yield large unilamellar vesicles, have not been successful for hemoglobin encapsulation. For example, the deoxycholate-based method [10] works well for cytochrome *c*, but gave low and erratic trapping for hemoglobin. The octylglucoside dialysis procedure [11] resulted in aggregation and denaturation of hemoglobin upon dialysis of this detergent.

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