PURIFICATION AND CHARACTERIZATION OF CARBOXYMETHYLCHITIN-COATED LIPOSOMES ENCAPSULATING HEMOGLOBIN AS POTENTIAL BLOOD SUBSTITUTES

by

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ABSTRACT

In view of the desirability to increase the survival time of the liposome-based artificial red blood cells in vivo, bovine hemoglobin-loaded liposomes (LEHb) are coated with a polyanionic polymer, a highly substituted type of carboxymethylchitin. The purpose is to simulate the presence of a protective negative charge on the surface of living cells arising from the carboxylic extremities of sialoglycoproteins. In order to predict the in vivo response, the necessary experiments for the in vitro system characterization have been carried out.

The liposomes are prepared by a modified Reverse Phase Evaporation technique and then purified using a Sepharose 4B column. The purified LEHbs display a unimodal size distribution in the submicron range. Analysis of the lipid/Hb content of the liposomes reveals that the variations in the Hb encapsulation efficiency \( E_{Hb} \) as a function of the initial Hb concentration \( [Hb]_0 \) are insignificant compared to the net augmentation of \( E_{Hb} \) as a function of the increasing initial lipid concentration. Meanwhile high \( [Hb]_0 \)'s are necessary for the preservation of oxyhemoglobin. A comparative study between experimental techniques for the determination of adsorption efficiency suggests that FT-IR spectroscopy gives a more accurate quantitative adsorption index while the chitinase-based enzymatic assay should be used as a qualitative detection tool.
RÉSUMÉ

Afin d'augmenter la période de survie des liposomes encapsulant de l'hémoglobine d'origine bovine (LEHb) dans la circulation, il s'agit de recouvrir la surface de ces "globules rouges artificiels" par un polymère anionic, un type de carboxymethylchitine hautement substituée. Les cellules vivantes sont recouvertes d'une charge négative protectrice provenant des extrémités carboxyliques des sialoglycoprotéines. L'objectif est de simuler la protection physiologique. Avant de prévoir la performance de ces substituts sanguins \textit{in vivo}, l'analyse du système a été effectuée \textit{in vitro}. Les liposomes sont préparées par une modification de la méthode "Reverse Phase Evaporation" et ensuite purifiées par la chromatographie à base de gel Sépharose 4B. Les LEHbs purifiées possèdent une distribution de taille unimodale se situant dans la région sub-micrométrique. L'analyse du contenu des liposomes en lipides et Hb indiquent que les variations au niveau d'efficacité d'encapsulation d'Hb (E_{Hb}) en fonction de la concentration initiale d'Hb ([Hb]_{0}) sont négligeables, comparées à la hausse progressive d'E_{Hb} observée lors de l'augmentation de la concentration initiale de lipides. Par contre une haute [Hb]_{0} est une condition nécessaire pour la sauvegarde de l'oxyhémoglobine. L'évaluation des techniques expérimentales dans le cadre d'une étude comparée pour déterminer l'efficacité d'adsorption suggère que la spectroscopie FT-IR est une méthode plus précise pour les analyses quantitatives tandis que la méthode enzymatique à base de chitinase est utile lors des analyses qualitatives de détection.
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**NOMENCLATURE**

**RBC**  Red Blood Cell  
**Hb**  Hemoglobin  
**MetHb**  Methemoglobin  
**SFHb**  Stroma-free Hemoglobin  
**CMC**  Carboxymethylchitin  
**LEHb**  Liposome Encapsulating Hemoglobin  
**CMC-LEHb**  Carboxymethylchitin-coated Liposomes  

**[Hb]₀**  Initial Total Hb Concentration present in SFHb Solution  
**Met[Hb]₀**  Initial Methemoglobin Concentration present in SFHb Solution  
**S[Hb]₀**  Initial Sulfhemoglobin Concentration present in SFHb Solution  

**THb**  Concentration of SFHb inside the LEHbs  
**HbO₂**  Oxyhemoglobin in the LEHbs  
**MHb**  Methemoglobin in the LEHbs  
**SHb**  Sulfhemoglobin in the LEHbs  

**[L]₀**  Initial Phospholipid and Cholesterol Concentration equivalent to the Initial Lipid to Hemoglobin Loading  
**REV**  Reverse Phase Evaporation
I. INTRODUCTION

The shortage of blood donors, the transmission of hepatitis (B, C, non-A, non-B) and AIDS, requirements for cross-matching, and short durations of storage are some of the problems associated with the use of donor blood in transfusion. There are also special cases related to rare blood groups, religion and special forms of surgery.

Liposomes encapsulating hemoglobin (LEHb) represent one approach to provide a universal red blood cell (RBC) substitute. Liposomes are obtained by dispersing phospholipids in solvents so that they orientate their molecules to form one or more membranes around an aqueous phase, in this case hemoglobin. Oxygen diffuses freely through the bilayers due to the very high permeability of lipids to $O_2$. The preserved biophysical parameters of encapsulated Hb, as well as the physiological compatibility of the phospholipid bilayer(s) are essential requirements for therapeutic blood transfusions. So far LEHbs have not been used for clinical trials because studies on animal models have shown that the liposomes are subject to rapid chemical degradation in the host's circulation. In order to overcome this drawback several modifications have been attempted: Increasing rigidity of layers through changes in their compositions (34,62), the \textit{in vivo} $t_{1/2}$ is 4-20 hrs depending on rodents (9); using lipids which can interact to polymerize (51); embedding glycoproteins (72) and glycolipids (79) into the lipid bilayer, the \textit{in vitro} $t_{1/2}$ is 36 hours for the glycolipids. It takes 4-6 days for newly synthesized
RBCs to mature, hence at the present stage the LEHbs cannot be even used as short term blood substitutes.

Another approach consists of modifying surface properties simulating those of living cells which have negative surface charges arising from the presence of sialoglycoproteins, N-Acetyl neuraminic acid (NANA). Carboxymethylchitin (CMC) is a polyanionic polymer used to coat the surface of the LEHbs. Its negative charge protects the liposomes from lysis by the complement factors mimicking NANA, without compromising their oxygen carrying capacity (53,54).

The purpose of this project is to study the adsorption of CMC on liposomes with regard to the desirability to increase their $t_{1/2}$ in vivo. So far, the in vitro system characterization has been achieved; the CMC-coated LEHbs have been: i) synthesized by a modification of the Reverse Phase Evaporation (REV) method, ii) purified by gel chromatography, iii) characterized for their size and lipid/Hb content, iv) analyzed for adsorption efficiency. In addition the variables influencing optimum Hb capture and preservation were also investigated. All experiments were conducted in vivo.
II. BACKGROUND

A. Hemoglobin and its Functions

Whole blood which is slightly alkaline (pH=7.4), can be divided into about 55 vol% plasma, and 45 vol% cells. The cells consist of 95% by number red blood cells (RBC)s or erythrocytes. The prime function of RBCs are the transport of O₂ and CO₂ throughout the body and buffering the blood so as to regulate pH. Dimensions of a typical RBC are shown in Figure 1: The average RBC is 8.4 μm in diameter, 2.4 μm thick at the periphery and 1μm thick at its narrowest portion in its native state (32). The cell has the shape of a biconcave disc which can deform, however, into a bullet-shaped entity during passage through small capillaries. Hemoglobin which is present to the extent of more than 33 wt% inside the RBC, consists of 4 long protein chains to which are bound 4 "heme" groups (a ferroprotoporphyrin, a chelating structure composed, in turn, of 4 pyrrole rings) and has a molecular weight of 68,000. Figure 2 shows the structures of Hb ferrihemoglobin and ferrohemoglobin the deoxygenated and the oxygenated conformations respectively. The heme portion binds to O₂ and CO₂ while the globin portion is important as a buffering agent.

Hb must be maintained in the reduced state in order to deliver oxygen adequately. The metabolism of the RBC is regulated by various enzymatic systems geared to this task. Methemoglobin the non-functional Hb derivative is formed when the iron of the Hb molecule is oxidized from the ferrous (Fe²⁺) to ferric (Fe³⁺)
Figure 1: Dimensions of a Typical RBC

Figure 2: Structures of Ferri & Ferro Hemoglobin

Chemistry of hemoglobin. The hemoglobin molecule is made up of four of the units shown on the left. The abbreviations M, V, and P stand for the groups shown on the molecule on the left. (From Ganong, W. F., Review of Medical Physiology, 5th ed., Lange Med. Publ., Los Altos, California, 1971, p. 379.)
form, which does not combine further with $O_2$, hence Hb is no longer fulfilling its primary function of delivering $O_2$ to the tissues. Individuals with high levels of Methemoglobin called methemoglobinemia exhibit a bluish grey skin appearance and their blood has a chocolate brown color. Hereditary methemoglobinemia can be caused by enzymatic deficiency(ies) (e.g. acatalasemia) or abnormal Hb (e.g. hemoglobin M); acquired hemoglobinemia occurs when certain aromatic amino and nitro compounds are absorbed through the skin, absorbed, or ingested. MetHb is continuously being formed in the RBC under normal in vivo conditions but it is continuously reduced to its functional state by various mechanisms (Fig. 3a) namely the NAD and NADP methemoglobin reductase system which in turn is dependent on a series of cascade reactions initiated from $H_2O_2$ breakdown by catalase (11,57) (Fig. 3b).

The production of oxygen species such as hydroxyl radicals in the body ([a]) is often mediated by iron. $H_2O_2$ and $O_2^-$ are capable of attacking cellular lipids, proteins, carbohydrates and nucleic acids causing a wide variety of pathologic manifestations.

In the RBC, Hb tends to auto-oxidize into MetHb generating for each molecule of MetHb one molecule of superoxide radical (18). Enzymatic $O_2^-$, $H_2O_2$ scavengers such as catalase and superoxide dismutase (SOD) convert these compounds to non-toxic species in erythrocytes ([b]&(c)).

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH \quad [a]$$

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad [b]$$

sod
Figure 3a: Mechanisms of Methemoglobin Regulation

(HEMOGLOBIN) (ferrous protoporphyrin) 2Hgb++ 2Hgb+++ (METHEMOGLOBIN) (ferrirpro*oporphyrin 9)

REACTIONS OF EMBDEN-MEYERHOF PATHWAY
G-3-P 1,3-PGA
NAD → NADH

REACTIONS OF PENTOSE PHOSPHATE PATHWAY
6-P-gluconolactone G-6-P

GSH

NADPH → NADP

NADPH

NON-ENZYMATIC

dehydroascorbate GSSG

pentose-PO4 6-P-G

Figure 3b: Role of Catalase in Methemoglobin Regulation

1 catalase
2 glutathione peroxidase
3 glutathione reductase
4 glucose 6-phosphate dehydrogenase
CATALASE

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad [c] \]

The average normal [MetHb] in humans determined by spectrophotometric methods is 0.76% of the total hemoglobin and increases with the age of RBC in circulation (57).

B. Blood Substitutes based on Hemoglobin

B.1 Stroma-Free Hemoglobin

Over twenty years ago Rabiner et al. (87) proved that the removal of RBC membranes called stromas is effective in sustaining life at near zero hematocrit. This approach constituted an active field of research until the early 80’s when the following issues were raised:

1. Inside the RBC hemoglobin is a tetramer. It consists of 2 α-units and 2 β-units. The half-life of stroma-free hemoglobin (SFHb) in vivo is only 1 hr because of dissociation of the hemoglobin tetramer into dimers which are excreted by the kidneys (26).

2. When Hb is inside the RBCs, there is 2,3-DPG (diphosphoglycerate) attached available (Fig. 4a). The binding of 2,3-DPG to Hb results in low oxygen affinity, insuring adequate oxygen release to the tissues. When Hb is outside the RBC 2,3-DPG is not available (Fig. 4b). In this form Hb has a high oxygen affinity not effective in releasing oxygen.

3. Inside the RBCs, Hb does not contribute to the plasma oncotic (protein) pressure. The concentration of Hb in the whole blood is about 14 g/dl. If the total Hb in the blood is present
Figure 4a: Hb with 2,3-DPG inside RBC

0-0 cytoskeletal membrane protein

phospholipid-cholesterol bilayer of the RBC membrane

Figure 4b: Hb without 2,3-DPG outside RBC
in free solution it will exert an oncotic pressure twice that of iso-osmotic pressure. The Hb solution for infusion can only contain 7 g/dl of Hb.

B.2 Microencapsulation of Hemoglobin

B.2.1 Purpose

In 1957 Chang proposed the encapsulation of Hb, in ultrathin synthetic membranes substituting for the natural RBC membrane (stroma). Hb in the artificial cells is no longer exposed to the extracellular environment. The synthetic membranes are much more stable than the biological RBC membranes. There are no blood group antigens, hence the artificial cells do not react with blood group antibodies. In addition these materials are impermeable to 2,3-DPG.

He showed that red cell hemolysate enveloped in collodion membrane artificial cells prepared by interfacial precipitation retains its ability to combine reversibly to oxygen (22). Nylon and Silastic rubber followed as membrane materials for Hb microencapsulation by interfacial polymerization, the mean diameter of the blood substitutes being as low as 5 μm or less (22,23,25). The major problem was that they stayed in the circulation for a very short time after intravenous injections compared to the average t\(_{1/2}\) of an RBC which is 120 days. Attempts to improve the survival time included (i) Decreasing the diameter of the artificial cells: They can have a membrane thickness comparable to that of RBCs and diameters as small as 1 μm or less; (ii) Adding a strong negative surface charge to the membrane by the use of
sulfonated groups and (iii) Changes in the other chemical properties of the artificial cells such as membrane composition: Membrane types expanded to cross-linked protein membranes and other materials (23,24,25). In 1968, Mueller and Rudin combined their technique of bimolecular membrane formation with the technique of preparing artificial cells described by Chang in 1964 (23), to form "cellules" of about 90 μm diameter (25). Each of these consisted of RBC hemolysate enveloped in a spherical bilayer lipid membrane which was 60 to 100 Å thick. However these artificial cells were not stable and further studies were carried out by Chang in 1969 to incorporate lipid into polymer or cross-linked protein membranes (25).

More recently lipid membranes were used to prepare artificial cells of submicron size called liposomes.

B.2.2. Liposomes

a. General Characteristics

The logic behind the use of lipids is to confer properties such as fluidity, flexibility, electrical resistance, impermeability to highly polar compounds associated with the physiological cell membrane due to the presence of phospholipids and cholesterol which compose more than 70% of the bilayer (Fig. 5). Liposomes are obtained by dispersing phospholipids in solvents so that they orientate their molecules to form one or more membranes around an aqueous compartment (Fig. 6). Oxygen diffuses freely through the membranes due to the high permeability of oxygen through lipid materials.
Figure 5: Singer & Nicholson Fluid Mosaic Model of the Cell Membrane

Figure 6: Detailed Structure of the Liposome Bilayer(s)
<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Structure</th>
<th>Function</th>
</tr>
</thead>
</table>
| DPPC       | \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\] | hydrophobic tail |
| Phosphatidyl ethanolamine | \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\] | polar or hydrophilic head |
| Phosphatidyl serine | \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\] | |
| Phosphatidic acid (to impart + charge) | \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\] | |
| Stearylamine (to impart + charge) | \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\] | |
| Cholesterol (to modify thermotropic phase transition) | \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\] | |

Vitamin forms of \[\begin{align*} \text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_n\text{CH}_3 \\
\text{CH}_2\text{O} &-\text{C}-(\text{CH}_2)_m\text{CH}_3 \end{align*}\]
Some typical phospholipids can impart positive, negative or neutral charge depending on the components used. Having a hydrophilic-lipophilic balance (TABLE A), the phospholipid molecules orientate in solution to form a bilayer that will tend to adsorb available substances. At a particular temperature range, the fatty acyl side chains of the lipids become more loosely packed (Fig. 7). This phase transition occurs over a narrow range of temperature with homogeneous phospholipids and causes the bilayer(s) to become much more permeable. Depending on the means used to prepare liposomes, multilamellar vesicles (MLV), small unilamellar vesicles (SUV), or large unilamellar vesicles (LUV) are obtained. The diameters of the MLVs, LUVs and SUVs are in the order of 0.4-10 μm, 0.06-10 μm and 0.02-0.064 μm respectively (36). Hydrophilic drugs and biologically active materials such as hemoglobin can dissolve in the aqueous internal compartment(s) of the liposomes. Being colloidal in dimensions and composed of biocompatible materials, liposomes can be injected intravenously. The survival of the liposomes in the circulation is governed by the Reticulo-Endothelial System (RES). Liposomes may interact with cells via endocytosis, fusion and lipid exchange. The extent of the interaction of the liposomes with the host's defense mechanism depends on their physical properties such as charge, size, and bilayer fluidity. Extensive non-specific uptake of liposomes by the mononuclear phagocytes of the RES has been studied in vivo using radioactive labels for the aqueous and lipid phases (63). Results presented in TABLE B lead to the following conclusions:
Charge: Macrophage uptake of negatively charged liposomes is substantially higher than that of positively charged liposomes, and the uptake of the latter is in turn higher than that of the neutral liposomes. Through electrostatic interactions with the positively charged heme, saturated negatively charged phospholipids elicit Hb denaturation, while unsaturated (−) charged ones prevent Hb-induced lipid peroxidation (92). A combination of several lipids naturally occurring or synthetic (51), glycolipids (79), glycoproteins (72) and cholesterol (37) is used to obtain the desired membrane charge.

Size: The circulation time of the liposomes in the body varies with size. SUVs remain in circulation longer than LUVs which in turn are less easily uptaken by the MLVs. The average size and size distribution depend on the method of preparation.

Fluidity: The rigidity of the bilayer is a function of its composition. Studies show that a liposome with low fluidity has a higher circulation time in the body independent of size than, a liposome with high fluidity. Phospholipids with higher phase transition temperatures ($T_c$) stay longer in circulation because their permeability is not affected at 37°C (body temperature). $T_c$ decreases with the number of double bonds and increases with the number of carbon atoms depending on the type of phospholipid used. Cholesterol is often incorporated in the membrane to alter chain fluidity and so to diminish the effect of phase transition on bilayer permeability.
Figure 7: Effects of Phase Transition at $T_c$

Table B: Results of RES Uptake

<table>
<thead>
<tr>
<th>Size</th>
<th>large &gt; small</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Charge</td>
<td>- &gt; + &gt; neutral</td>
</tr>
<tr>
<td>$T_c$ or Fluidity</td>
<td>low* &gt; high**</td>
</tr>
</tbody>
</table>

* accumulation in the spleen  
** accumulation in the liver
The protective actions of cholesterol against Hb-induced lipid peroxidation in case of unsaturated (−) phospholipids and Hb denaturation by saturated (−) fatty acids are well documented (37, 92).

The extent of lysis of liposomes by serum components mainly lipoproteins also increases with bilayer fluidity. The distribution of liposomes to organs is also a function of bilayer fluidity. Low fluidity liposomes mainly accumulate in the spleen and cause splenomegaly. On the other hand the liposomes with high fluidity are mainly accumulated in the liver (42).

b. Preparation Methods

Liposomes can be prepared by several methods at laboratory and pilot scales.

At the laboratory scale the Film Method based on Sonication (79), the Detergent Dialysis Method (52), Extrusion through Polycarbonate Membranes (6, 46, 81) and Reverse Phase Evaporation (REV) (93) are used.

At the pilot scale the Bulk Method with Ultrasound (42), the Parr Bomb batch process (42), the Microfluidization, with Continuous Extrusion process (66-68) and the Homogenization, with Extrusion continuous process (40, 99), have capacities in the order of (dL)s, (L)s, (dL/hr) and (L/hr) respectively. Sonication is associated with possible Hb degradation (40), Detergent Dialysis is a slow process with limited clinical applications.

The Extrusion Process requires in average 3.5 times more lipid to
achieve the same Hb capture rate than the REV technique (67,99). Both methods give the same particle average diameter along with a uniform size distribution (13,99). The preservation of oxygen carrying capacity of the encapsulated Hb is an essential requirement for selecting the adequate encapsulation technique. Since neither of the procedures is associated with Hb degradation, the cost efficient REV method is chosen.

Despite efforts to minimize the uptake by RES of the liposomes by operating at optimal charge, size, and fluidity independent of the method of preparation, the longest in vivo circulation time of liposomes is 15-20 hrs in mice (9). In case of blood loss, it takes 4-6 days for newly synthesized RBCs to mature in the body (44), hence at the present stage LEHbs cannot be administered as short term blood substitutes.

B.3 Surface Modification of Liposomes

All living cells have negative surface charges arising from the presence of sialoglycoproteins, N-Acetyl neuraminic acid (NANA) or sialic acid. Studies conducted by Chang (24,26) have proven that the elimination of sialic acid from RBCs with sialidase causes an exponential decrease in the half-life of the treated cells, therefore suggesting that the survival of erythrocytes is a function of their surface charge. These findings led to the preparation of artificial cell membranes namely "sulfonated-nylon" with strong negative charge groups conferred by sulfonated groups; polysaccharides with strong sulfonated groups have also been incorporated in the membranes (25). The use of these negatively
charged membranes resulted in an increase of the \textit{in vivo} \( t_{1/2} \) of the artificial cells (24,25). These results are confirmed by more recent studies showing that cells subjected to sialidase are being accumulated in spleen, liver and phagocytosed (35,48). However, the addition of sialic acid (NANA) to the surface decreases the extent of lysis of liposomes by Complement Factors, (82).

\textbf{B.3.1 Objectives}

The purpose is to modify the surface properties of the liposomes by coating the particles with a biocompatible compound which confers:

(i) a negative charge to the membrane simulating the protective function of sialoglycoproteins embedded in the stroma of the RBC (Figs. 8a&8b). The surface potential of the RBC membrane is \(-18\) mV.

(ii) rigidity to the bilayer mimicking the viscoelastic properties essential to RBC movement in the microcirculation, preventing leakage in the microenvironment due to lack of mechanical stability.

\textbf{B.3.2. Chitin and its Derivatives as Biomaterials}

The structure of chitin is similar to that of NANA due to the \textit{N}-acetyl groups and the ring structure (refer to Figure 8c). Chitin occurs as a major skeletal component in all arthropods, it confers rigidity to the exoskeleton. It is extremely abundant in nature with an estimated annual production of 150,000 metric tons (69). It is a polysaccharide of high molecular weight and consists of unbranched chains (1-4) \(\beta\)-linked 2-acetamido-2 deoxy-D-glucose or \textit{N}-Acetyl D-Glucosamine (NAG) residues, the unit being the biose. Three crystalline forms (\(\alpha,\beta,\gamma\)) of chitin are known, and these differ in the arrangements of the chains and the presence of water-
Figure 8a: Structure of N-Acetyl Neuraminic Acid (NANA)

![Structure of N-Acetyl Neuraminic Acid](image)

Figure 8b: Structure of N-Acetyl Glucosamine

![Structure of N-Acetyl Glucosamine](image)

Figure 8c: Structure of Chitin

![Structure of Chitin](image)

carboxymethylchitin: \[ R_1 = -\text{NHCOCH}_3 \quad R_2 = -\text{CH}_2\text{OCH}_2\text{COOH} \]

chitosan: \[ R_1 = -\text{NH}_2 \quad R_2 = -\text{CH}_2\text{OH} \]

Figure 8d: Structures of Carboxymethylchitin & Chitosan

![Structures of Carboxymethylchitin & Chitosan](image)
bound molecules. Only α-chitin has been reported from arthropods, the type which is commercially available. Although chitin has been extensively studied, no definitive work has been done on its molecular weight due to its intractable nature. Chitin is polydisperse, insoluble in general organic solvent as a result of its previously described rigid crystalline structure supported by hydrogen bonds between hydroxyl groups and acetamido groups, dissolves with degradation in concentrated mineral acids, but can be dispersed in hot, concentrated, aqueous solutions of certain mineral salts. From viscosity data for solutions in nitric acid, Meyer and Wehrli (71) concluded that the molecular weights of cellulose and chitin were the same magnitude. Light Scattering was used to determine the molecular weight and degree of polymerization (d.p.) of chitin and carboxymethylchitin (CMC) for the commercially available α-chitin, extracted from crab shells (43). Results are summarized in TABLE C.

Chitin is considered biocompatible and is used in the surgical fields as sutures (74) and artificial skins (58). This biocompatibility would be due in part to the presence of N-acetyl groups in the structures, since it is reported that partially deacetylated chitin e.g. carboxymethylchitin has a potent activity to activate peritoneal macrophages at a certain dosage in vivo, while intact chitin has no such activity (78). Chitin derivatives namely chitosan and carboxymethylchitin (Fig. 8d) are used instead of chitin in the biomedical field because (i) depending on the chemical modification they can confer a (-) or (+) charge to the
### TABLE C: Molecular Weights of Chitin and Carboxymethylchitin
Determined by Light Scattering

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mol. Wt</th>
<th>Mol. Wt of the unit</th>
<th>Wt. average (Av.(M_w)*10^6)</th>
<th>d.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxymethylchitin</td>
<td>252.7</td>
<td>1.896</td>
<td>7503</td>
<td></td>
</tr>
<tr>
<td>(Na salt in 0.5M NaCl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxymethylchitin</td>
<td>252.7</td>
<td>1.338</td>
<td>5295</td>
<td></td>
</tr>
<tr>
<td>(Na salt in 2.5M NaCl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitin* in 5.55M LiSCN</td>
<td>199.0</td>
<td>1.036</td>
<td>5206</td>
<td></td>
</tr>
</tbody>
</table>

*α-Chitin from Crab shells
surface while chitin itself is neutral, (ii) highly substituted derivatives are soluble at the physiological pH of 7.4. The charge of chitin derivatives depends on their degree of substitution (d.s.): d.s. is the chemical shift due to the modified carbon at C$_3$ or C$_6$ of the (NAG) residue as the result of acetylation; it is used to define the extent of carboxymethylation of chitin derivatives. The feasibility of production of these derivatives at industrial scale is under investigation (84).

Chitosan has been applied to the microencapsulation of microbial cells (69). In the membrane forming process, chitosan, a natural polycationic polymer, is complexed with sodium alginate. The polysaccharide has been also used for heavy metal chelation, wound healing and cell immobilization (60,73,80). Chitosan having structural characteristics similar to glycosaminoglycans responsible in the body for regenerating blood/tissue interfaces seems to mimic their functional behaviour and is used in the development of hemodialysis membranes, artificial skin and drug targeting (21).

Carboxymethylchitin, is a polyanionic polymer used to coat the surface of liposomes (Fig. 9a). Its negative charge protects the liposomes from lysis by Complement Factors mimicking NANA, while increasing the T$_c$ of the bilayer (2). Freeze fracture images of the adsorbed liposomes indicate that CMC does not form a continuous additional layer on the entire cell surface but rather sits in patches on the surface presumably forming a mesh-like structure (Fig. 9b).
Figure 9a: Schematic Representation of a CMC-LEHb

Figure 9b: Freeze Fracture Section of a CMC-LEHb
B.3.3 CMC-Coated Liposomes as Blood Substitutes

It has been reported that liposomes coated with chitin derivatives are fairly stable to lysis and their oxygen carrying capacity is comparable to that of RBCs (53,54).

In contrast to the uncoated LEHbs which undergo disintegration to a great extent by the action of fibrinogen, and are affected insignificantly by either albumin and globulin (90), the CMC-LEHbs were found quite resistant to fibrinogen and globulin while they were slightly broken down by albumin (53). More recent in vitro studies show that the $t_{1/2}$ of leakage of $[^3]$H sucrose (entrapped in the aqueous phase) in buffer and in 75% plasma were estimated to be 94 and 8.5 hrs for uncoated LEHbs and 96 and 36.5 hrs for CMC-coated liposomes (80). The phagocytosis of coated liposomes is governed by a balance between electrostatic interactions which are a function of d.s. and the presence of N-Acetyl groups; liposomes coated with (CMC)$_1$ (d.s. 83%) activate the macrophages more than (CMC)$_2$ (d.s. 50%) even though the surface potential of (CMC)$_1$, $\xi_1$ (-21.24 mV) is more negative than $\xi_2$ (-18.79 mV): A higher extent of N-deacetylation may have occured during the preparation of (CMC)$_1$ since severer conditions are necessary to achieve a higher degree of carboxymethylation (45,95,96).

These carboxymethylchitin-coated liposomes containing stroma-free hemoglobin (CMC-LEHb)s meet the following specifications:

(i) The microcapsule membrane is biodegradable and its physiological compatibility can be adjusted by the d.s. of CMC.

(ii) The liposomes are allowed to pass unrestrictedly through normal capillaries. The mean diameter reported is 0.35 $\mu$m (53,54),
approximately 1/3 of the minimum thickness of RBCs.

(iii) The modified Reverse Phase Evaporation Technique does not diminish Hb's biological activity.

Presently, the use of CMC-LEHbs as blood substitutes constitutes a very active field of research. Acute toxicity studies have been performed on male mice (BALB/c) and the LD₅₀ value for 2 ml of intravenous injection was evaluated to be 13.8 ml/kg in terms of the total particle volume of the injected coated liposomes per kilogram of body weight of the animal (54). Further investigations are necessary to determine the t₁/₂ of the CMC-LEHbs in vivo.

B.4 Cross-linked Hemoglobin

Cross-linked Hb is in turn divided into three categories: pyridoxylated polyhemoglobin, intra-molecular cross-linked Hb and conjugated hemoglobin.

B.4.1 Pyridoxylated Polyhemoglobin

In 1964 Chang used bifunctional reagents namely sebacoyl chloride and later gluteraldehyde, to cross-link Hb into polyhemoglobin (polyHb) (25). Hb cross-linked into polyHb remains as a polymer of the tetramers, has a good t₁/₂ and at 14 g/dl is iso-oncotic. Intramolecular cross-linking of each individual Hb is a further extension, however as a single molecule only 7 g/dl is possible. The major problem is that in the absence of 2,3-DPG the oxygen affinity of Hb is very high. In 1975, Benesch's group linked pyridoxal phosphate to the 2 β-units of the Hb inside the RBC (10). This solves the high oxygen affinity previously
encountered but the conversion of pyridoxylated Hb into dimers results in rapid removal proceeding infusion (28). By cross-linking pyridoxylated polyHb, the tetramer structure is maintained, thus a 7 hr \textit{in vivo} $t_{1/2}$ achieved (27). This allows the use of Hb at 14 g/dl and adequate release of oxygen to organs is obtained.

\textbf{4.2 Intra-molecular Cross-linked Hemoglobin}

Cross-linked Hb also results in intramolecular cross-linking by adjusting the reaction. The use of other 2,3-DPG analogues instead of pyridoxal phosphate for $\beta-\beta$ linkage is currently under investigation namely NFPLP (2-nor-2-formylpyridoxal-5'-phosphate)-Hb and ATP-Hb (29). Crosslinking between $\alpha$ chains has also been performed (98).

\textbf{4.3 Conjugated Hemoglobin}

Hemoglobin can be cross-linked to polymers like polyamide. This forms a solid microsphere of conjugated Hb (23,25). Instead of cross-linking to molecules like nylon, Hb can also be cross-linked to soluble macromolecules namely dextran and ethylene glycol. These soluble conjugated Hbs have a much longer $t_{1/2}$ than particulate conjugated Hb. However they have high $O_2$ affinity. By pyridoxylating the Hb before conjugating it to soluble polymers the above problem is solved but only at a [Hb] of 7 g/dl (28).
C. Blood Substitutes based on Synthetic Oxygen Carriers

In view of the problems associated with unmodified Hb solutions, the main approaches towards RBC substitutes are presented.

C.1 Fluorocarbons

Artificial fluorocarbons namely Fluosol-DA are the first type of artificial blood substitutes to be ready for clinical trials (26). The major problems associated with their use are (33):

(i) Their short survival time in the circulation.

(ii) The accumulation of fluorocarbons in the body causes the saturation of the RES.

(iii) They only have 15% of the oxygen carrying capacity of the blood, hence they have a poor release of oxygen to the organs.

C.2 Oxygen-Carrying Chelates

Efforts in the synthesis of oxygen-carrying chelates have concentrated on the design of artificial compounds which mimic the reversible oxygenation of Hb.

C.2.1 Synthetic Porphyrins

Various types of porphyrins are most likely to have the desired characteristics. The problems encountered by the proponents of free porphyrins in solution are numerous ranging from unsuitable oxygen binding characteristic at room temperature to rapid removal in circulation due to the low molecular weight (<2000 daltons)(4). Baldwin et al. have eliminated the above-mentioned drawbacks by developing the "strapped-capped" porphyrin. But this heme derivative is only functional under an oxygen atmosphere which
induces RES toxicity (5).

C.2.2. Lipid/Heme

The lipid/heme synthesized by Tsuchida and Nishide (97) utilizes the hydrophobic region of the lipid membrane as the carrier of heme, instead of the globin which is the case for natural Hb. Despite desirable oxygen carrying capacity and long shelf life (one year) at room temperature, their use represents the following drawbacks:

(i) The hydrophobic heme derivatives are susceptible to irreversible oxidation in aqueous media (31).

(ii) Sterilization against viral contamination and pyrogenicity requires heating to 200 °C which denatures the heme at 45 °C and the lipid at 60 °C. The cost associated with the maintenance of sterile conditions and pyrogen-free raw materials contributes to the unit price of the blood substitute.

Terms Used

1 (LUV)s Commonly referred to as (REV) Liposomes
2 RES Major immunological defense system for the removal of foreign matter from the body
3 Endocytosis Processes used by the cell for the internalization of macromolecules involving cell surface interactions
4 Mononuclear Phagocytes White blood cells of the immune system which engulf and degrade foreign matter
5 Microfluidization Based on (REV) Lab scale Procedure
6 Homogenization Extrusion through Polycarbonate Membranes Lab scale Procedure
7,8,9 Fibrinogen, albumin, globulin Plasma proteins
III. OBJECTIVES

I. To select a liposome preparation technique satisfying the following criteria:

1. No Hb degradation during encapsulation
2. Narrow size distribution of particles, submicron in dimension
3. High encapsulation efficiency ($E_{Hb}$)
4. Fast preparation process
5. Cost effective
6. Suitable for surface modifications of liposomes

II. To purify and characterize the LEHb population in terms of composition, dimension, size distribution and particle count.

III. To determine the optimum operating conditions for reproducible high Hb capture and HbO$_2$ preservation by studying the effects of:

1. $[L]_0$, the initial lipid/cholesterol concentration
2. $[Hb]_0$, the initial hemoglobin concentration

IV. To synthesize and characterize a highly substituted, water-soluble type of carboxymethylchitin.

V. To prepare and purify CMC-coated liposomes.

VI. To select a method to quantify surface adsorption in order to determine adsorption efficiency ($E_{Ad}$).
IV. MATERIALS and EXPERIMENTAL METHODS
MATERIALS

The following are purchased from Sigma Chemicals:

1. HSPC: Hydrogenated Soybean Phosphatidylcholine dissolved in chloroform 100 mg/ml (P-6263), is used instead of PC from animal sources in order to reduce cost.

2. DMPC: Dimyristoyl phosphatidylglycerol powder (P-9274).

3. Cholesterol powder (C-3292).

4. α-Tocopherol or vitamin E (T-3251) dissolved in chloroform 5 mg/ml.

5. Chitin powder (C-3387) practical grade from crab shells.

6. Chitinase from Streptomyces Griseus (EC 3.2.1.14, C-1525).


Bovine Hemoglobin is purchased from the McIntyre Animal Centre. Its use instead of human blood is justified by the following factors:

i. Its biophysical properties are very close to that of human hemoglobin (40).

ii. It is cost-efficient and available upon request.

iii. No risk of Hepatitis or AIDS contraction.
EXPERIMENTAL METHODS
A. Preparation of Stroma-Free Hemoglobin (SFHb)

SFHb is prepared by the method of Chang & Keipert (56). The purpose of this procedure is to remove the membrane (stroma) of the RBCs.

1. Separation of RBC from other blood components

Bovine blood is centrifuged at 6,000 rpm for 20 min at 4°C using the IEC Centrifuge Model B-20A. The plasma and buffy coat containing the leukocytes and platelets are removed by aspiration and discarded.

2. Continuous washing of RBCs

The sedimented RBCs that remain are washed 4 times by suspension in 3 vol. ice-cold, sterile isotonic saline. After each wash the cells are resedimented by centrifugation.

3. Lysing RBCs

The cells are lysed by adding 2 vol. of hypotonic saline phosphate buffer (PBS, 0.154 ionic strength, pH=7.4) while swirling gently the vessel.

4. First toluene extraction

After standing 20-30 min, the lysed cells are poured into a large separator and 0.5 vol. cold toluene is added. The mixture is shaken vigorously in order to create an emulsion, and then left for 3 hrs at 4°C. The extraction mixture separated in 3 layers: an upper layer of stroma and lipid dissolved in toluene, a thin middle layer of cellular debris; and a lower layer of aqueous Hb solution. The two upper layers are removed by vacuum aspiration. The solution is clarified by high speed refrigerated centrifugation.

5. Second toluene extraction

A second toluene extraction with 0.3 vol. cold toluene is performed overnight at 4 °C. The lower layer of dark red aqueous SFHb is separated and centrifuged at 14,500 rpm for 1 hr.

6. Dialysis

The SFHb is dialyzed for 3 days against the buffer of choice using a standard hollow fiber dialyzer (Dialaid, A = 1 m²) in order to remove excess intracellular potassium and balance electrolytes and pH. The process yield is 20% on a volumetric basis.

The choice of buffer is critical in order to preserve the oxygen carrying capacity of Hb. The rate of MetHb formation is a very sensitive function of pH and has been extensively investigated (39,99). The conversion to Methemoglobin is minimized at pH=7.4.
B. Liposome Preparation

Liposomes are prepared by Reverse Phase Evaporation (REV), a technique which satisfies the criteria of selection dictated by objective I.

Liposomes are prepared by the technique of Szoka et al. (93). HSPC, Cholesterol, DMPG are mixed initially in the following molar ratio 10:9:1. α-tocopherol is added to the mixture (1% of total lipid) to prevent the oxidation of phospholipids (81). 100 mg of the above mixture dissolved in chloroform at 66 μmol/ml is subjected to solvent evaporation using a rotary vacuum pump (Model 75, Precision Scientific Company). Two mls of methylene chloride are added to the dry lipid. The lipid solution is then hydrated with 2 ml of SFHb solution. Vigorous shaking of the emulsion in a vortex mixer follows for 30 s. Twenty mls of phosphate buffer saline (PBS, Ionic strength 0.154; pH=7.4) are added to create a W/O/W emulsion followed by 3 hrs of stirring at 4°C.

Once entrapped in the bilayer HSPC, DMPG are respectively slightly (+) and (-) charged while cholesterol and α-tocopherol are neutral resulting in an electrically neutral bilayer. A schematic diagram of the procedure is presented in Figure 10.

Since the objective is to achieve high Hb capture the effect of the following factors on the encapsulation efficiency will be examined:

i. Vary the initial lipid/cholesterol concentration ([L]₀) from 66-180 μmol/ml.

ii. The initial (Hb) concentration ([Hb]₀) from 4.95-15.1 g/dl.

The effect of the buffer's ionic strength is well documented in literature (93). Hb capture increases with decreasing ionic strength until it levels off at 0.154 M under which the encapsulation is carried out.

Following their preparation the REV liposomes are centrifuged at 10,000 rpm for 30 min. A significant portion of the non-encapsulated lipid and SFHb sediment at the bottom of the tube while the liposomes remain suspended in the supernatant. The liposomes are ready for further purification.
step 1: Lipids suspended in chloroform are subjected to vacuum evaporation.

step 2: Suspend lipids in methylene chloride.

step 3: Addition of the hemoglobin (aqueous phase).

step 4: Vortex for 30s, inverted micelle formation.

steps 5, 6 & 7: Removal of methylene chloride by evaporation (5). As the solvent is removed the gel state collapses (6) releasing their encapsulated material (Hb); the remaining lipid contributes to the formation of the additional layers (7).
C. Liposome Purification and Characterization

Since spectroscopy based upon the phenomena of Absorption and Light Scattering is extensively used for quantitative and qualitative characterization, a brief theoretical background from the following consulted references (12,76,89) is presented in APPENDIX A. IR frequencies functional groups under investigation are also presented in APPENDIX A.

C.1 Detection and Purification

C.1.1 Column Chromatography

Gel chromatography is used to separate compounds by molecular weight (MW) or size in solution (61). The small molecules diffuse through the beads ($V_t-V_o$) while the big molecules travel down in the void volume ($V_o$) as represented in Figure 11. In the absence of all other possible gel/eluent interactions (electrostatic, chemical reaction) the smaller the particle size the higher the elution volume associated. The diffusion coefficient is related to the particle size by Stokes-Einstein's Law (eq. 1):

$$D = \frac{kT}{6\pi\eta a}$$  \hspace{1cm} (1)

where

- $a$: particle radius
- $T$: absolute temperature
- $\eta$: kinematic viscosity
- $k$: Boltzmann's constant

The XK 16/40 Pharmacia column is used to separate the liposomes from the non-encapsulated (lipid, Hb) (94) and non-adsorbed CMC. compounds. The gel used is Sepharose 4B (Pharmacia 17-0120-01) with the following specifications:

<table>
<thead>
<tr>
<th>Working pH range</th>
<th>Approximate wet bead diameter</th>
<th>Useful M.W. fractionation range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-11</td>
<td>45-165</td>
<td>$3 \times 10^4 - 5 \times 10^6$</td>
</tr>
</tbody>
</table>

The gel is insensitive to the composition and charge of the eluent at the operating pH of 7.4 (Fig. 12). The upper limit of the dextran fractionation range is useful for MW characterization of CMC but as reported chitin and its derivatives are high MW compounds, polydisperse molecules hence their characterization requires more elaborate techniques such as HPLC (45), while the lower limit of the protein fractionation range is not sensitive enough to distinguish between the 2 possible forms of SFHb dimer (MW 34,000) and tetramer (MW 68,000). This separation involves a combination of gel filtration and molecular sieve. In the case of the non-purified LEHb suspension, the non-encapsulated aqueous Hb will diffuse through the pores of the beads. The liposomes will
flow in the void volume because they are larger than the largest pores in the beads.

A simplified schematic diagram of the process is presented in Figure 13.

2.5 ml of the supernatant containing liposomes are injected to the column using a Gilson peristaltic pump operating at 9 ml/hr. An automatic fraction collector (Gilson Model FC-80K) is used for sample collection. The dimensions of the column from Pharmacia are as follows:

\[ H = 35 \text{ cm} \quad d = 1.5 \text{ cm} \]

The total height of packing or bed height \( H_p \) is 30 cm. The maximum operating flowrate for \( H_p \) of 30 cm packed with Sepharose 4B gel is \( 11.5 \text{ ml cm}^{-2} \text{ h}^{-1} \) which is equivalent to \( 20.3 \text{ ml h}^{-1} \) for a 1.5 cm wide column (41). Since the objective of this preliminary study is adequate separation and not recovery time optimization the pump flowrate is set at 9 ml hr\(^{-1}\).

For analytical purposes a sample volume of 1–5% bed volume \( (V_t) \) is recommended where

\[ V_t = H_p \times \pi d^2/4 = 53 \text{ cm}^3 \quad (2) \]

The sample volume (2.5 ml) represents 4.7% of \( V_t \).

Before starting any experiment the homogeneity of the packed bed was tested by running through blue dextran at [2 mg/ml] operating under designed experimental conditions. The quality of the packing was checked by monitoring the progress of the color zone spectrophotometrically at 254 nm.

C.1.2 Particle Intensity using Light Scattering

The IL MULTISTAT III PLUS F/LS Centrifugal Analyzer operating at the Light Scattering mode is used to determine the particle intensity. The reference used is latex beads (mean diameter 288 nm). The particles scatter light at an emission wavelength of 405 nm. The ratio of the reading at 405 nm over the control gives a qualitative index representing the particle intensity. The higher the reading the higher the number of particles present. Each fraction collected is subjected to this test. The liposomes are concentrated in the fractions corresponding to the highest reading.

* The interactions between CM-Chitin and Sepharose 4B gel have not been studied.
Figure 11: Diagrammatic Representation of $V_t$ & $V_0$

Figure 12: Structure of Sepharose
Figure 13: Schematic Diagram of the Purification Process

1: reservoir of liposomes/buffer
2: peristaltic pump
3: sepharose 4B column
4: fraction collector
C.1.3 Phospholipid Determination

As previously shown in TABLE A (Section II.B.2.2.a), phosphoglycerides, a principal class of phospholipids contain 2 fatty acids side chains that are esterified to 2 hydroxyl groups of a glycerol. The third hydroxyl group of the glycerol is esterified to phosphoric acid which in turn is esterified to a hydrophilic compound such as serine, ethanalamine and choline. Since most identification methods monitor the extent of lysis of the ester bonds by the release of glycerol, it can be safely assumed that for analytical purposes phosphoglyceride degradation can be simulated by that of triglycerides.

The criteria of selection for a quantitative procedure are as follows:

i. Accuracy
ii. Ease of operation
iii. Time economy (80 samples/experiment)

For the determination of triglycerides chemical methods involving end group analysis using dyes as indicator (83) or titration techniques are available. These procedures involve many chemical steps and are slow processes. After purification a small amount of sample is collected for the analytical tests. In order to obtain accurate results larger volumes than the ones available are required.

Gas Chromatography (64) is an accurate method of quantitative analysis but it is a long procedure given the number of samples. Hence the above techniques are not suitable for the purpose of this analysis and other alternatives are considered.

a. Enzymatic/Spectrophotometric Method

The IL Test Triglycerides Kit (Cat. No. 35190) based on an enzymatic assay is investigated (15). The MULTISTAT III PLUS F/LS Centrifugal Analyzer operating at the Absorbance mode at a wavelength of 340 nm. The enzymatic reaction sequence employed in this assay of triglyceride is as follows:

\[
\begin{align*}
\text{triglyceride} & \xrightarrow{\text{lipase}} \text{glycerol + ADP} \\
\text{glycerol + ATP} & \xrightarrow{\text{kinase}} \text{glycerol-1-phosphate + ADP} \\
\text{phosphoenol pyruvate + ADP} & \xrightarrow{\text{kinase}} \text{pyruvate + ATP} \\
\text{pyruvate + NADH + H}^+ & \xrightarrow{\text{dehydrogenase}} \text{lactate + NAD}^+
\end{align*}
\]

The decrease in absorbance due to the oxidation of NADH to NAD is directly proportional to the triglyceride present in a 30 µl sample. The reaction takes place at T= 37°C C with an estimated t, (reaction time) of 5 minutes. 17 samples are analyzed per run.
The Precinorm L Special Lipid Control Serum (Boehringer Mannheim # 781827) is lyophilized human serum and serves as control. The error associated with this assay is 2-4%.

b. IR Spectroscopy

This procedure is not used for detecting the liposomes. It will complement the enzymatic analysis; only the fractions containing the liposomes are tested by IR.

IR spectroscopy is used in this case because of the lack of spectral interference in the IR region as compared to the visible range in which lipids and hemoglobin peaks frequently overlap (38,50). Infrared studies on the structure of lipid bilayers on CaF₂ crystals have been performed (19,20,88,65,100). In this case Fourier Transform IR Spectroscopy (FT-IR) is used because it allows for better signal/noise ratios, increased resolution with shorter acquisition times due to multiplexing, i.e., simultaneous detection of all wavelengths (89).

50 µl of pure phosphatidylcholine at various concentration dissolved in chloroform is deposited on a 13x2 mm CaF₂ crystal (International Crystal Labs, # 0002D-403) and the sample is placed in an oven at 37°C until the chloroform evaporates. Infrared spectra at 4 cm⁻¹ resolution (Δσ) for a scan# of 32 are recorded from 1000-1800 cm⁻¹ using the Bomem FT-IR spectrometer. The samples are referenced against dried chloroform. A characteristic peak is registered at 1734 ± 5 cm⁻¹ (Fig. I) within the expected frequency range for saturated acyclic esters (1735-1750 cm⁻¹, TABLE A1), and its intensity which is directly proportional (r = 0.997) to the triglyceride content is used for a calibration curve (APPENDIX B). The lipid content of the liposome can be determined by the above methodology using FT-IR.
Figure 1: IR Spectrum of Phosphatidylcholine
C.1.4 Cholesterol Determination

The criteria of selection for cholesterol analysis are the same as above (Section IV.C.1.3). Cholesterol is a steroid. Gas chromatography is usually used for the separation and quantitative determination of steroids (75). This method does not satisfy the time economy criteria, therefore the following alternative was considered.

a. Enzymatic/Spectrophotometric Method

The Boehringer Cholesterol C-System Kit (Cat No 290319) based on an enzymatic colorometric method is investigated (55). The MULTISTAT III PL/5 F/LS Centrifugal Analyzer is used at the absorbance mode at a wavelength of 500 nm. The enzymatic reaction sequence employed in this assay of cholesterol is as follows:

\[
\text{cholesterol esterase} \\
\text{cholesterol ester} + \text{H}_2\text{O} \rightarrow \text{cholesterol} + \text{RCOOH} \\
\text{cholesterol oxidase} \\
\text{cholesterol} + \text{O}_2 \rightarrow \lambda^4-\text{cholestenone} + \text{H}_2\text{O}_2 \\
\text{peroxidase} \\
2\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + \text{phenol} \rightarrow 4-(\rho\text{-benzoquinone-mono-imino})\text{-phenazone} + 4\text{H}_2\text{O}
\]

The change in absorbance due to the oxidation of phenol bound to phenazone dye is directly proportional to the cholesterol concentration in the 25 \(\mu\)l sample. The reaction is carried out at 37 \(^\circ\)C and the colorimetric development takes 10 minutes. 17 samples are analyzed per run. Ethanol concentrations ranging from 10-200 mg/dl are used as control. The error associated with this assay is 0-5%.
C.1.5 Hemoglobin Determination

a. Spectrophotometric Method

When a scan of (Hb) is performed from 400 to 600 nm, 3 characteristic absorption bands are detected (Fig. II). The position of these bands and their molar absorptivity at these wavelength are well documented (38). Nevertheless, a scan of various solutions of SFHb at different concentrations are run using the PERKIN - ELMER Lambda 4B Spectrophotometer to obtain a calibration curve for the following reasons:

1. There might be an extinction factor associated with the change in molar absorptivity (Δε) from to the removal of the stroma (14).

2. To improve the accuracy of the results because the amounts encapsulated are very small; hence the actual ε could deviate from the theoretical one.

The absorbance at 540 ±2 nm is used for the calibration curve which fits a 2nd order linear model (r = 0.998) (APPENDIX B).

b. IR Spectroscopy Method

Infrared Spectroscopy can be used to study the behavior of globular proteins such as Hb in solution, but the water background prevents this method from being a useful quantitative tool (59). SFHb at different concentrations is deposited on CaF₂ crystals. The sample is placed in an oven operating at 37 °C until the water evaporates. An IR spectrum from 1400-1800 cm⁻¹ (Δσ = 4 cm⁻¹, scan# 32) is recorded using the Bomem FT-IR Spectrometer. The intensity of the Amide I band registered at 1653 cm⁻¹ which falls within the expected frequency range (1650± 5 cm⁻¹, TABLE A1), is directly proportional to the [SFHb] is used for the calibration curve (Fig. III) which fits a 2nd order linear model (r = 0.996) (APPENDIX B).

This procedure can be used to determine the amount of Hb encapsulated to complement the spectrophotometric analysis. Only the fractions containing the liposomes are tested.
Figure II: Scan of SFHb in the Visible Region

Figure III: IR Spectrum of SFHb
C.1.6 Electron Microscopy

After all the fractions are analyzed, they are subjected to electron microscopy in order to obtain a visual confirmation of the detection and purification techniques. Negative stain electron micrographs are prepared by the following technique (81): Carbon coated grids (300 mesh) are covered with a 0.1 mg/ml of Bacitracin and blotted dry. Liposomes that have been dialyzed against 0.15 M ammonium acetate/0.5 mM EDTA pH 7.0, are applied onto the grid at concentrations of 0.6 μm/mol and drawn off by filter paper. A drop of ammonium molybdate (2% w/v) is applied to the grid, drawn off with a piece of filter paper and allowed to dry for 3 hrs. A Philip 300 electron microscope at 80 KV is used at 49,440 magnification.
C.2 Average Size and Size Distribution of Liposomes

The diameter as well as the size distribution of the liposomes is determined using a NICOMP Size Analyzer Model 370 which operates by Light Scattering. The instrument operating range is from 5-5000 nm and is mainly used for the analysis of ceramic particles and thin spherical or vesicles in emulsion such as liposomes. For a schematic diagram of the analyzer followed by a simplified theoretical background refer to APPENDIX A.

A summary of the sequence of operation follows:

\[ I_s \rightarrow D \rightarrow a \]

The scattered intensity per unit incident intensity \( I_s \) at a distance \( r \) from the particle radius \( a \) is given by equation (3) where \( m \) is the refractive index of the particles relative to the medium, \( \lambda \) is the wavelength of the beam (8).

\[
I_s = \frac{16\pi^4 a^6}{r^2 \lambda^4} \left[ \frac{1}{(m^2 - 1)} \right] \left[ \frac{1}{(m^2 + 2)} \right] \tag{3}
\]

The scattered intensity per unit incident intensity \( I_s \) is a function of the particles' diffusion coefficient given by the Stokes-Einstein (eq. 1).

Three types of size distributions can be obtained namely intensity, volume, number. From the displayed intensity weighted distribution, the volume weighted and the number weighted diameters are obtained.

Each diameter \( d_i \) of the intensity weighted distribution should be weighted by the factor \( N_i(v_i)^2 \) where \( N_i \) represents the number of particles having diameter \( d_i \), and \( v_i \) their individual volume, which is proportional to \( d_i^3 \) according to equation (3). To obtain the volume weighted average diameter \( d_v \), the intensity weighted factors \( I_{s_i} \) are divided by \( v_i \) or \( d_i^3 \) (eqs. 4&5); similarly for the number weighted average diameter \( d_n \) the intensity weighted factors are divided by \( v_i^2 \) in order to obtain a final weighing factor of \( N_i \) (eqs. 6&7).

\[
I_{s_i}/v_i = F_{v_i} = \frac{N_i(d_i)^6}{(d_i)^3} = \frac{N_i(d_i)^3}{N_i} = N_i v_i \tag{4}
\]

\[
d_v = \Sigma F_{v_i} d_i \tag{5}
\]

\[
I_{s_i}/v_i^2 = F_{N_i} = \frac{N_i(d_i)^6}{(d_i)^6} = N_i \tag{6}
\]

\[
d_n = \Sigma F_{N_i} d_i \tag{7}
\]

The control was a sample of commercially available latex beads characterized as monodisperse, with a mean diameter of 288 nm. The volume weighted peak of 260 nm is shifted by almost 10% below the nominal value, while the number weighted diameter is reduced even more to 185 nm.
D. Methemoglobin Determination

For the determination of ferrihemoglobin in the presence of other hemoglobin derivatives, gasometric and optical methods have been used (17,39,57,91). It is desirable to use a method requiring:

1. Quantitation in the visible spectrum which is available on the usual laboratory spectrophotometer.
2. A reasonably simple and rapid technique
3. A calculation which compensates for differences in total hemoglobin concentration, to give the accuracy needed. The error associated with the procedure should not exceed 5% for MetHb determination.

The photoelectric method of Evelyn & Malloy (39) satisfies the above requirements. The procedure is the following:

1. 100 µl of sample is suspended in 10 ml of M/60 phosphate buffer (PB; pH=6.6) and a reading $L_1$ (vs PB) is made at 635 nm on the Beckman Model 4B Spectrophotometer. After the addition of 4 µl of neutralized 10% NaCN another reading $L_2$ is taken. The difference ($L_1 - L_2$) is proportional to [MetHb].

2. To the solution is added 4 µl of NH₄OH and a reading $L_3$ is made at 620 nm (vs water). $L_3$ is related to [SHb].

3. 2 ml of the above solution are suspended in 8 ml of PB (M/15, pH=6.6) and 4 µl of 20% KCN are added. This allows the conversion of all HbO₂ to MHB; then 4 µl of 10% NaCN are added to convert the MHB to MHB-CN. A reading $L_4$ is taken at 540 nm (vs PB M/15, 4 µl of KCN & NaCN) which is proportional to the total hemoglobin concentration.

The concentrations of MHB, SHb, HbO₂ and THb in g/dl are computed using equations (8, 9, 10, 11) (98).

\[
THb = \frac{(100 \times L_4)}{2.38} \quad (8)
\]

\[
MHB = \frac{[100 \times (L_1 - L_2)]}{2.77} \quad (9)
\]

\[
SHb = \frac{[1000 \times L_3 - (8.5 \times MHB + 4.4 \times THb)]}{100} \quad (10)
\]

\[
HbO_2 = THb - (MHB + SHb) \quad (11)
\]

The liposome sample has to be preconditioned before methemoglobin analysis. Each 24 hrs a 3 ml sample of the supernatant containing the liposomes is centrifuged at 19,000 rpm. In this fashion the liposomes are disintegrated and the hemoglobin content is released in the supernatant from which 100 µl is withdrawn daily for analysis.

SFHb stored at 4°C has to be prepared every 3 weeks because of high methemoglobin levels.
E. CM-Chitin Synthesis and Characterization

E.1 Synthesis

Carboxymethylchitin is not commercially available. The carboxymethylation of chitin was carried out using two different methods. The overall reaction steps of the synthesis are summarized in Figure IV.

E.1.1 Method I

a. Proposed Procedure

This is the procedure proposed by Tokura et al. (96), the reported yield is 89% on a dry basis.

α) Preparation of alkali-chitin

20 g of Chitin powder are suspended in 80 ml of a 40% NaOH solution containing 0.2% sodium dodecylsulfate (SDS) at 4 °C. The slurry is made to stand at −20 °C overnight after aging for 1 hr at 4 °C. During freezing (SDS) detergent facilitates the penetration of the alkali into chitin micelles.

β) Acetylation

Frozen alkali-chitin is suspended in 200 ml of 2-propanol at room temperature and monochloroacetic acid is added stepwise for 4 hrs until the mixture is neutralized with mechanical stirring.

γ) Washing

The product is collected by filtration and washed with ethanol. The residue is extracted at room temperature with 2 L of water using mechanical stirring.

δ) Extraction and Drying

The water extract is slowly added to 5 L of acetone to precipitate the CM-Chitin. The product is washed with acetone in order to wash-out the NaCl three times and then placed in the oven at 37°C overnight for the acetone to evaporate.

b. Variation of [base]

The [NaOH] is varied from 40 to 60% (95, 77).

c. Change of acetylation agent

Monochloroacetic acid is replaced by trichloroacetic acid.

d. 2nd reaction pass

The obtained CM-Chitin is a reactant under the original conditions.
Figure IV: Schematic Diagram of CMC Synthesis Steps

**Crustacean Shells**

Deproteinization

Decalcification

![Diagram of CMC synthesis steps](image)

**Carboxymethylation** of

-CH₂OH

-CH₂O⁻ Na⁺

[C₈H₁₇COOH]

N-Decacylation

of

-NHCOCH₃

Na⁺ OH⁻

[-CH₂O⁻ Na⁺]

[C₈H₁₇COOH]

-CH₂O⁻ Na⁺

+C₈H₁₇COOH

+ NaCl

-CH₂OCH₂COOH
E.1.2 Method II

a. Proposed Procedure

This procedure proposed by Hirano (45) is a modification of Method I with a reported yield of 85%.

α) 1st Acetylation

The alkali chitin prepared as above (94) is added to 1 L of 2-propanol containing 47.5 g of chloroacetic acid in an ice bath over 30 min. The reaction is exothermic and a ΔT of 30 °C is observed.

β) Filtration and Washing

The mixture is mechanically stirred over room temperature for one hour, the product is collected by filtration through a glass filter paper, washed with ethanol to give a powdered material.

γ) 2nd Acetylation

The material is suspended in 1,500 ml of water by stirring at room temperature. The viscous solution is recooled in an ice bath where 8 ml of acetic anhydride are added to reduce the extent of the N-deacetylation reaction.

δ) 1st Dialysis and Extraction

The mixture is dialyzed against water for 3 days. The dialysate is centrifuged at 5,000 rpm for 20 min, in order to remove insoluble material, and 3 vol. acetone are added to the supernatant. After standing overnight the precipitate is collected by centrifugation (5,000 rpm for 20 min) and washed with acetone.

ε) Washing and Filtration

The product is resuspended in ethanol (150 ml) collected by filtration and dried to give Na-CM-Chitin salt.

ζ) 2nd Dialysis and Freeze Drying

The salt is redissolved in 600 ml of water and acidified by addition of HCl. The resulting solution is dialyzed against distilled water for 1 day and lyophilized to give salt-free Carboxymethylchitin.

b. Variation of [base]

The [NaOH] is varied from 40 to 60% as previously.

* Conversion of amide to amine
E.2 Characterization

E.2.1 Water-Solubility of CM-Chitin

Chitin is insoluble in water. The degree of carboxymethylation of chitin, contributing to its water-solubility is regulated by [NaOH] during the freezing process. The water-solubility becomes apparent when the degree of substitution is over 0.6 (95). Hence the water-solubility could serve as a qualitative index to characterize the product.

E.2.2 Kinetics of Chitinase as a Function of D.S.

Chitinase is specific for linear polymers of NAG; chitosan, glycochitin and carboxymethylchitin in addition to chitin (49). The degree of enzymatic hydrolysis of CM-Chitin is evaluated by measuring the increase in reducing power of CM-Chitin according to the modified Schales procedure (47).

Stock solutions of chitinase [100 µg/ml] (54) from Streptomyces Griseus and CM-Chitin are prepared by dissolving them in a phosphate buffer solution (PBS ionic strength 0.154, pH =7.4). All assays for enzymatic activity are performed at 37 °C and the reaction is started by adding enzyme solution to substrate. The physical conditions and the buffer are chosen to simulate physiological conditions. After incubation, the reaction mixture is allowed to stand in a water bath at 100 °C to stop the reaction.

Samples are analyzed spectrophotometrically at different time intervals as follows: 1.5 ml of sample is mixed with 2 ml of coloring reagent KFe(CN)$_3$ are incubated in boiling water for 15 min in a test tube stoppered with an aluminum foil. After cooling the mixture for 30 min, the optical density at 420 nm is read versus water. The change in optical density is directly proportional to the NAG concentration and hence a standard curve for [CMC] is obtained (Figs. V a&b) & (APPENDIX B).
Figure Va: Absorbance vs. $[\text{CMC}]_0$ at Various Reaction Times

$[\text{CMC}]_0$ (mg/ml)

Figure Vb: Absorbance vs. $[\text{CMC}]_0$ at $t = 120$ min

$[\text{CMC}]_0$ (mg/ml)
E.2.3 IR Spectroscopy

IR Spectroscopy is extensively used to determine the configuration and molecular structure of polysaccharides having the advantages over the UV-visible range spectrophotometers of i) not requiring electronically polarizable derivatives such as KFe(CN)₃ to bind the for the detection weak electronic chromophores namely (COO⁻, acetamido groups) ii) capacity to handle solid samples.

CM-Chitin is an amide as well as an ester. The degree of substitution (d.s.) can be determined by IR spectroscopy. The ratio of C=O stretching at 1735 cm⁻¹ Ester band and Amide I band at 1655 cm⁻¹ can be used for the calibration curve because the absorption at 1655 cm⁻¹ is not changed by the acetylation reaction (78).

Since the CM-Chitin products synthesized differ in morphology two different techniques were used. In both cases an infrared spectrum is recorded from 1400 to 1800 cm⁻¹ (Δσ = 4 cm⁻¹, scan# 32) and the absorbance is evaluated by adjusting the baseline at 1800 cm⁻¹. The background d.s. associated with a sample of chitin run as control is 6.4% (Fig. VIa).

a. KBr Pellet

This procedure is used to characterize the products obtained by Method I. About 3 mg of CM-Chitin powder crushed using the Siebtechnik crusher (220 V, f=50 Hz) to pass through a 200-mesh sieve is mechanically blended with 400 mg of KBr powder to prepare a KBr disk (45).

b. CaF₂ Crystal

This procedure is used to characterize the products obtained by Method II. This procedure is not documented in literature. CaF₂ crystals previously recommended for lipid analysis (Section IV.C.1.3.b) are used following the same methodology. A 2 mg/ml solution or suspension depending on the d.s. is prepared. 50 µl of the sample is deposited on a CaF₂ IR crystal. The choice of material for the crystal is dictated by the range of frequencies it absorbs through (1100 - 4000 cm⁻¹) and its affordable price.

By superimposing absorbance spectra of CMC using the spectra subraction technique, 1st order linear calibration curves at the monitored frequencies are obtained (APPENDIX B). This technique consists of comparing the intensities of the IR absorbance of the Amide I (1655±5 cm⁻¹) and Ester bands (1735±5 cm⁻¹) for i) Various types of CMC at a given concentration (Fig. VIb), ii) Various [CMC]s for a given type of carboxymethylchitin (Fig. VII).
Figure VIa: IR Spectrum of Chitin
Figure VII: Quantitative Characterization of CMC by IR

Figure VII: Quantitative Characterization of CMC by IR
F. Analysis of CMC-Coated Liposomes

F.1 Adsorption

CM-Chitin* is dissolved in (PBS)(pH= 7.4, ionic strength 0.154) at [2 mg/ml]. The REV liposomes are prepared using standard procedure but the emulsion obtained is added to 20 ml of the CMC solution instead of buffer to yield a W/O/W complex emulsion. After 10 min of stirring another 20 ml of CMC solution are added. Stirring is continued for 3 hrs until all the methylene chloride has evaporated. The adsorption is carried out at 4°C in order to minimize methemoglobin formation even though in the original procedure adsorption takes place at 20 °C (53,54).

The same initial composition of the lipid/cholesterol is used. The presence of the adsorbed CMC confers a negative charge to the otherwise electrically neutral bilayer.

F.2 Purification and Detection

The suspension is then centrifuged at 2,500 rpm for 10 min and 10,000 rpm for 30 min. 3 ml of supernatant are injected to a Sepharose 4-B column as previously. The samples are analyzed, the liposomes are detected and characterized as previously. In addition the fractions collected are analyzed for their CMC content enzymatically. There is an absorbance associated with phospholipids and Hb around the 400 nm region (50).

In order to minimize lipid and Hb interference with the enzymatic assay, the samples are processed as follows: After the reaction is stopped each collected fraction is centrifuged at 5,000 rpm for 30 min to remove the precipitate (denatured Hb). The recovered supernatant is filtered 3 times (polycarbonate membrane, pore size = 0.2 μm) through an extruder and used as sample for spectrophotometric analysis.

In order to check the validity of this method a 3 ml sample of [2 mg/ml] pure CM-Chitin is injected through the column as control and fractions are analyzed enzymatically for CMC content. Results show that (CMC) does not interact with the gel at the column's operating conditions (Fig.C1, APPENDIX C). Hence gel chromatography is a suitable method for purifying CMC-coated liposomes.

* The most highly substituted derivative is used
F.3 Determination of Adsorption Efficiency

For the determination of adsorption efficiency, the preparation of samples is modified to increase purity. Purified REV liposomes collected in fractions are mixed with a [2 mg/ml] CMC solution. Adsorption is carried out for 3 hrs at 4°C. The CMC concentration chosen is the solubility of the compound at the operating conditions. 3 ml of the sample are injected through the column and the fractions are analyzed and characterized individually as described previously (Section IV.C.1-5) and the purified CMC-coated liposomes are collected to measure the adsorption efficiency.

F.3.1 Enzymatic Method

Calibration curves used for quantifying pure CMC content (Section IV.E.2.2) are used to evaluate the amount of CMC adsorbed provided that precautions are taken to minimize the lipid and Hb background (Section IV.F.2).

F.3.2 IR Spectroscopy

Infrared Spectra can be used directly for quantitative measurements of polysaccharides, the usual requirements being a solubility of at least 1% in the solvent (1). The solubility of CMC in PBS buffer is 0.2% therefore a liquid sample cannot be used for this type of analysis.

The CMC-LEHbs are analyzed by FT-IR using CaF$_2$ crystal by the procedure described (Section IV.F.3.2). 50 μl samples from the tubes containing CMC-coated liposomes are collected.

The Amide I peak at 1655 cm$^{-1}$ is used to quantify the amount of CM-Chitin adsorbed. The interference at that peak caused by Hb is taken into account by subtracting its contribution (Fig. VIIIb) from the overall intensity registered at that peak. The cholesterol and the other lipids do not interfere given their concentrations at the monitored frequency (Fig. VIIa). The Ester band in the vicinity of 1735 cm$^{-1}$ cannot be used because of the strong absorbance of the ester bonds of phosphatidylcholine (Fig. I) which interferes with the ester bond of the CM-Chitin (Fig. VIa).
Figure VIIIa: IR Spectrum of Lipid/Cholesterol Mixture

Figure VIIIb: IR Spectrum of LiHbs

#Peaks = 3

#Peaks = 12

66
V. RESULTS
A. Liposome Purification and Characterization

A.1 Detection and Purification

Plots of particle intensity, heme absorbance, [cholesterol], [triglyceride] vs. the collected fractions are presented in Figures X-XIII respectively. The data is summarized in APPENDIX C.

Two main regions are observed in all plots.

The first region extends from fraction # 13 to 20 is an area of overlap. Electron Microscopy (Fig. IX) confirms that the REV liposomes are eluted from the column in this region.

The second region can be analyzed as follows:

Figure X: fraction # 35-43 represent non-encapsulated particles scattering light, since the lipid/cholesterol content is hardly detected, one can conclude that these are Hb particles scattering light.

Figure XI: fraction # 35-46 represent free hemoglobin. SFHb contains some cholesterol and triglycerides, the contribution of this content can be observed in Figures XII & XIII in the same region.

Figure XII: fraction # 54-69 represent free cholesterol particles.

Figure XIII: fraction # 47-59 represent free triglyceride particles.
Figure IX: Electron Microscopy Picture of REV Liposomes

Magnification 49,440
Figure X: Scattering Intensity vs. Collected Fractions

![Scattering Intensity vs. Collected Fractions](image)

Figure XI: Heme Absorbance vs. Collected Fractions

![Heme Absorbance vs. Collected Fractions](image)
Figure XII: [Cholesterol] vs. Collected Fractions

Figure XIII: [Triglyceride] vs. Collected Fractions
A.2 Encapsulation Efficiency and Lipid Capture

The encapsulation efficiency ($E_{Hb}$) and lipid capture can be computed once the hemoglobin and lipid contents of the purified liposomes are determined (Section IV.C.1.3-5), using the following equation:

$$E(\%) = \left(\frac{A_n}{A_i}\right) \times 100$$ (12)

$A_n$: Amount in liposome fraction n (mg)
$A_i$: Amount initially available for encapsulation (mg)

TABLE I summarizes the effects $[Hb]_o$ and $[L]_o$ on encapsulation efficiency:

1. $E_{Hb}$ increases as a function of $[L]_o$ (Fig. XIV).
2. $E_{Hb}$ increases as a function of $(Hb/L)^{**}$ (Fig. XV).
3. $[Hb]_o$ has no effect on $E_{Hb}$ (Fig. XVI).

* For the determination of $E_{Hb}$ pure PC liposomes were used
** $(Hb/L)_e$ The ratio of hemoglobin encapsulated & lipid entrapped (mg/mg)
Table I: Effects of \([\text{Hb}]_0\) and \([\text{L}]_0\) on Hb Capture

<table>
<thead>
<tr>
<th>([\text{L}]_0) ((\mu\text{mol/ml}))</th>
<th>([\text{Hb}]_0) (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>66 120 180</td>
<td>4.95 8.1 15.1</td>
</tr>
</tbody>
</table>

\([\text{Hb}]_0\) (g/dl): 8.1  \([\text{L}]_0\) (\(\mu\text{mol/ml}\)) : 66

<table>
<thead>
<tr>
<th>(\text{THb}) (g/dl)</th>
<th>0.051 0.122 0.178</th>
<th>0.036 0.051 0.115</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{E}_{\text{Hb}}) (%)</td>
<td>6.3 15 22</td>
<td>7.2 6.3 7.6</td>
</tr>
<tr>
<td>(Hb/L) (_t) (mg/mg)</td>
<td>0.453 0.520 0.635</td>
<td>0.478 0.453 0.490</td>
</tr>
</tbody>
</table>

* Results determined by IR Spectroscopy
Figure XIV: $E_{ab}$ vs. $[L]_o$

![Graph](image1)

Figure XV: $(Hb/L)_r$ vs. $[L]_o$

![Graph](image2)
Figure XVI: $E_{\text{Hb}}$ vs. $[\text{Hb}]_o$
A.3 Size Distribution and Particle count

A.3.1 Average Size and Size Distribution

The particle size distribution determined by Gaussian Analysis is presented in Figure XVIIa. The data fits a log normal distribution function where $D_g$ is the geometric mean diameter and $\sigma_g$ the geometric standard deviation (eqs. 13&14).

$$dP/d(log D) = [100/\log \sigma_g 2\pi] \cdot \exp\left[-(log D - log D_g)^2 / (2 \log^2 \sigma_g)\right]$$ (13)

$$\sigma_g = \frac{\sum (log D - log D_g)^2}{\sum d(log D)}$$ (14)

A unimodal distribution was obtained for all samples.

A broader size distribution is observed in the case of the supernatant. The volume averaged diameter of the REV liposomes is .115 µm while that of non-purified liposomes from the supernatant is .356 µm. Purification shifts the distribution curve to the left (Fig. XVIIb).

The diameters varies significantly with the choice of weighing. The value of the volume weighted $d_v$ (.115 µm) of the purified liposomes is twice the value of the number weighted $d_n$ (.048 µm)(Fig. XVIIc).

Diffusion coefficients are also given by the size analyzer. Results show that the smaller the particle the higher its diffusion coefficient (TABLE II).

A.3.2 Particle Count

From the size distribution a weighted average volume per liposome as well as the total liposome count per ml of suspension can be calculated, assuming that the particles are spherical can be obtained.

$$v_{ave} = \pi/6 \cdot \sum x_i (d_{vi})^3$$ (15)

where

$v_{ave}$ : weighted average volume/liposome
$d_{vi}$ : particle diameter
$x_i$ : fraction of particles with $d_i$

$N_p = 1 \text{ ml (liposome suspension)}/ v_{ave}$ (16)

Computed values of $v_{ave}(\text{ml})$ and $N_p$ are $3.045 \times 10^{-15}$ and $3.285 \times 10^{14}$ respectively.
Figure XVIIa: Log Normal Particle Size Distribution of Liposomes
Figure XVIIb: Effect of Purification on Size Distribution

Figure XVIIc: Size Distributions based on $d_v$ and $d_n$
TABLE II: $d_v$ and Average Diffusivity ($D_{ave}$) of Eluted Particles

<table>
<thead>
<tr>
<th></th>
<th>$d_v$</th>
<th>$D_{ave}$</th>
<th>fraction #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µm)</td>
<td>(cm²/s)</td>
<td></td>
</tr>
<tr>
<td>LEHb Purified</td>
<td>0.115</td>
<td>$4.5 \times 10^{-8}$</td>
<td>13 - 20</td>
</tr>
<tr>
<td>LEHb Supernatant</td>
<td>0.356</td>
<td>$1.31 \times 10^{-8}$</td>
<td>----</td>
</tr>
<tr>
<td>Latex Beads.</td>
<td>0.260</td>
<td>$1.66 \times 10^{-8}$</td>
<td>23 - 25</td>
</tr>
</tbody>
</table>

* nominal $d_{ave} = 0.288$ µm
B. Methemoglobin Determination

SFHb is encapsulated at 3 different concentrations. The concentrations of methemoglobin measured under different [L]₀ and [Hb]₀ are presented in TABLE III. Data analysis reveals that:

1. The lower the [Hb]₀ the higher the Met[Hb]₀ and the encapsulated [MHb] (Fig. XVIII).

2. The Met[Hb]₀ is higher in solution than the [MHb] encapsulated under all encapsulation conditions.

Figure XVIII: Effect of [Hb]₀ on Methemoglobin Formation
Table III: Stability of LEHb Solutions as a Function of Storage Time and [Hb]₀, at [L]₀ = 66 (μmol/ml) and 4 °C

<table>
<thead>
<tr>
<th>SOLUTIONS</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb]₀ (g/dl)</td>
<td>4.95</td>
<td>8.1</td>
<td>15.1</td>
</tr>
<tr>
<td>Met[Hb]₀ (g/dl)</td>
<td>0.543</td>
<td>0.329</td>
<td>0.13</td>
</tr>
<tr>
<td>S[Hb]₀ (g/dl)</td>
<td>0.026</td>
<td>0.167</td>
<td>0.375</td>
</tr>
</tbody>
</table>

0 hr

<table>
<thead>
<tr>
<th>HbO₂%</th>
<th>99.6</th>
<th>99.9</th>
<th>99.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHB%</td>
<td>0.4</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>SHB%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

24 hrs

<table>
<thead>
<tr>
<th>HbO₂%</th>
<th>91.7</th>
<th>96.8</th>
<th>98.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHB%</td>
<td>7.1</td>
<td>3.24</td>
<td>1.7</td>
</tr>
<tr>
<td>SHB%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

48 hrs

<table>
<thead>
<tr>
<th>HbO₂%</th>
<th>90.9</th>
<th>95.7</th>
<th>97.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHB%</td>
<td>9.1</td>
<td>4.3</td>
<td>2.16</td>
</tr>
<tr>
<td>SHB%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

72 hrs

<table>
<thead>
<tr>
<th>HbO₂%</th>
<th>88.3</th>
<th>94</th>
<th>95.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHB%</td>
<td>11.7</td>
<td>6</td>
<td>4.5</td>
</tr>
<tr>
<td>SHB%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
C. Carboxymethylchitin Synthesis and Characterization

The results are summarized in TABLE IV.

C.1 Synthesis

Na-type CM-Chitin is obtained as an air-dried powder.

C.1.1 Method I

a. The product obtained from the original procedure has a very low d.s. and is insoluble in water.
b. The d.s. of CMC increases with [NaOH], hence improving the water-solubility of the product. At [NaOH] = 60% the CMC-H₂O mixture is a colloidal suspension.
c. Recycling the CMC for a second reaction pass increases the d.s. by 23.9 %.
d. The substitution of monochloroacetic acid by trichloroacetic acid improves the d.s. by 20.9%.

Despite the d.s. increase water-solubility is still not achieved.

C.1.2 Method II

The morphology of the white fibrous material obtained is analogous to the texture of styrofoam.

a. At a low base concentration ([NaOH]₀ = 40%) the degree of substitution is comparable to the one obtained by Method I.
b. At [NaOH]₀ = 60% water solubility is achieved as well as a high d.s. of 80.4.
Table IV: CMC Synthesis Methods and Characterization

<table>
<thead>
<tr>
<th>Methods of Synthesis/Analysis</th>
<th>Nishi &amp; Tokura</th>
<th>KBr pellet</th>
<th>d.s. (%) by FT-IR Analysis</th>
<th>H₂O¹ Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NaOH]₀ : (wt%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>80</td>
<td>21.6</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>63</td>
<td>30.0</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>72</td>
<td>33.9</td>
<td>suspension</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>45.5</td>
<td>suspension</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>42</td>
<td>42.3</td>
<td>suspension</td>
<td></td>
</tr>
<tr>
<td>Hirano</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[NaOH]₀ : (wt%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>17.5</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>19</td>
<td>80.4</td>
<td>good</td>
<td></td>
</tr>
</tbody>
</table>

* Solubility in PBS buffer pH=7.4 determined by visual inspection

83
C.2 Characterization

C.2.1 Kinetics of Carboxymethylchitin

The samples used are the ones with the highest d.s. from each method of synthesis.

Figure Va represents absorbance as function of \([\text{CMC}]\) at various reaction times for CMC d.s. 80.4. Data analysis shows that at 2 hrs the reaction is completed. Therefore values of absorbance at 120 min (Fig. Vb) will be used for the calibration curve (APPENDIX B).

Figures XIX a&b: Kinetics of CM-Chitin hydrolysis can be simulated by a Michaelis Menten Model (eq. 17). A Lineweaver-Burke plot (eq. 18) confirms the adequate fit \((r = 0.94)\).

\[
\frac{V}{V_{max}} = \frac{[S]}{(K_a + [S])} \quad (17)
\]

\[
\frac{1}{V} = \frac{K_a}{V_{max}[S]} + \frac{1}{V_{max}} \quad (18)
\]

where

- \(V\) : CMC hydrolysis rate \((\text{mg.ml}^{-1}.\text{hr}^{-1})\)
- \(V_{max}\) : Maximum hydrolysis rate \((\text{mg.ml}^{-1}.\text{hr}^{-1})\)
- \([S]\) : Substrate concentration \((\text{mg/ml})\)
- \(K_a\) : Michaelis Menten kinetic constant \((\text{mg/ml})\)

The Kinetic data is summarized in TABLE V. Results indicate that the \(K_a\) varies as a function of the degree of substitution.

C.2.2 IR Spectroscopy

IR spectroscopy is used as a qualitative tool. Figure Vlb illustrates the above statement.

As described previously in Section IV.E.2.3 the absorbance ratio of the Ester \((1735\pm5 \text{ cm}^{-1})\) to Amide I peak \((1655\pm5 \text{ cm}^{-1})\) gives the degree of substitution.
Figure XIXa: Michaelis Menten Plot of CMC Hydrolysis

Figure XIXb: Lineweaver Burk Plot of CMC Hydrolysis
TABLE V: Chitinase Kinetics as a Function of D.S. at [E]₀ = 0.1 mg/ml

<table>
<thead>
<tr>
<th>Substrate</th>
<th>D.S. (%)</th>
<th>Chitinase Source</th>
<th>(K_{\text{mav}}) (mg/ml)</th>
<th>(V_{\text{max}}) (mg/ml/hr⁻¹)</th>
<th>pH, T(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin</td>
<td>0</td>
<td>Streptomyces</td>
<td>0.1(47)</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antibiotics</td>
<td>5.1, 37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td>45.5</td>
<td>Streptomyces</td>
<td>0.971*</td>
<td>1.091</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Griesus</td>
<td>7.4, 37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td>80.4</td>
<td>Streptomyces</td>
<td>0.571**</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Griesus</td>
<td>7.4, 37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The kinetic constants \(K_{\text{mav}}\) are averaged values obtained for:

* Method I, 3 independent experiments at 10 values of \([\text{CMC}]_0\)

** Method II, 2 independent experiments at 17 values of \([\text{CMC}]_0\)
D. Carboxymethylchitin-Coated Liposomes

D.1 Detection and Purification

Plots of particle intensity, [triglyceride], [cholesterol] and CMC absorbance .vs. the collected fractions are presented in Figures XX-XXIII respectively. The data is summarized in APPENDIX C.

One main region of overlap fraction # 10 to 20 is observed in all plots. The other peaks consist of free non-encapsulated or non-adsorbed particles.

Figures XXI & XXIII: Fractions # 10-12 contain non-adsorbed liposomes while fractions # 13-19 consist of CMC coated liposomes.

D.2 Assumptions

1. The Hb and lipid/cholesterol capture of the CMC-LEHbs liposomes are not affected by the adsorption process since the encapsulation takes place before the addition of the CMC-PBS buffer solution.

2. The average size and size distribution of the CMC liposomes is the same as the non-coated liposomes since there has been no shift in the elution pattern of the liposomes. The fractions under investigation are from # 13-19. The reported $d_{av}$ of CMC non-purified coated liposomes is 0.35 $\mu$m (2,3) which is the same as the average size of the particles of the non-purified LEHb supernatant.
Figure XX: Scattering Intensity of CMC-LEHbs

![Graph showing scattering intensity of CMC-LEHbs](image)

Figure XXI: Triglyceride Concentration Profile of CMC-LEHbs

![Graph showing triglyceride concentration profile of CMC-LEHbs](image)
Figure XXII: Cholesterol Concentration Profile of CMC-LEHbs

Figure XXIII: CMC Absorbance Profile of CMC-LEHbs
D.3 Adsorption Efficiency

The adsorption efficiency \( E_{Ad} \) is defined in equation (19):

\[
E_{Ad}(\%) = \left( \frac{(CMC)_{Ad}}{(CMC)_i} \right) \times 100
\]

\((CMC)_{Ad} : \) Amount of CM-Chitin adsorbed on the liposomes (mg)
\((CMC)_i : \) Amount initially available for adsorption (mg)

Before quantifying surface adsorption, it is important to consider the efficiency of the adsorption procedure itself: Triglycerides/cholesterol enzymatic analysis reveals that the ratio of the non-adsorbed to adsorbed liposomes is 1:9 (mg/mg).

D.3.1 Enzymatic Method

All eluted fractions are tested for CMC content (Fig. XXIII). \( E_{Ad} \) computed from the analysis of fraction # 13-19 is 27.65% while the free CM-Chitin represent 72.35% of the total amount available for adsorption.

D.3.2 IR Spectroscopy

IR scans of fraction # 13-19 adsorbed on CaF\(_2\) crystals (Section IV.F.3.2) reveal that \( E_{Ad} \) is 17.42 %. Figure XXIV is a sample IR spectrum of CMC-LEHbs contained in fractions # 17 & 18.
Figure XXIV: IR Spectrum of CMC-coated Liposomes
VI. DISCUSSION
Cell Structure: Figure IX shows a negative staining micrograph of liposomes. A mixture of unilamellar and multilamellar structures with a liquid phase interior Hb is observed. The staining technique requires blotting the cells dry therefore the liposomes do not appear spherical as expected in theory. Meanwhile, scanning electron micrographs taken of REV liposomes show that the cells are spherical in shape (53).

Encapsulation Efficiency: The Hb capture rates of the REV liposomes are similar to the ones reported by Mayhew et al. for the same size range and lipid/cholesterol composition (66-68). As previously shown by Figure XIV $E_{Hb}$ increases with $[L]_o$.

IR Spectroscopy shows that at $[L]_o = 66 \mu$mol/ml the ratio of Hb encapsulated to lipid entrapped is 1 (mg/mg) (TALE I). As $[L]_o$ is increased, relatively more lipid is available to entrap the Hb solution during encapsulation. The same trend has been shown by Vidal-Naquet & Beissinger (99) using high pressure extrusion and homogenization techniques but for the same initial lipid to Hb loading $[L]_o$, the reported $E_{Hb}$ is 3.5 less than the computed efficiency of the REV liposomes.

The encapsulation efficiency increases as a function of $(Hb/L)_o$ (Fig. XV) at different $[L]_o$s and $[Hb]_o$s. This improvement is caused by the presence of extra lipid available for Hb encapsulation because $E_{Hb}$ is independent of $[Hb]_o$ (Fig. XVI).
Average Size and Size Distribution: There is some evidence that physical processes leading to emulsion formation should be expected to produce log-normally distributed components having a well defined mean diameter and half width (8). The width of the unimodal distribution is a function of the stoichiometry of the starting compounds, the duration and detailed nature of the preparation technique employed, such as in the case of the REV liposomes the duration of vortexing. A literature survey confirms that the REV preparation technique results in a reproducible unimodal size distribution (66-68).

The discrepancy between the values of $d_n$ and $d_v$ can be understood qualitatively by reviewing the relationships between the scattering intensity and weighing factors for number weighing $F_{n_i}$ and volume weighing $F_{v_i}$ (eqs. 6&4): Number weighing involves one additional division of $I_{S_i}$ by the particle volume. The experimental $d_n$ of 0.048 $\mu$m matches that of Mayhew et.al (67) while $d_v$ of 0.115 $\mu$m lies within the size range reported for REV liposomes (93). Manual scale-adjusted measurements of liposome performed on pictures obtained by electron microscopy (Fig. IX) reveal that $d_v$ is a more realistic representation of the average particle size. In addition $d_v$ for the control sample of latex beads was closest to the actual nominal value. Hence, the volume averaged diameter $d_v$ will serve as a basis of comparison for the rest of the discussion.
Particle Count: Pidgeon and Hunt have calculated the number and surface area of liposomes in suspensions (85). For \( d_v \) of 0.115 \( \mu \)m the interpolated number of particles \( N_p \) is \( 6.80 \times 10^{14} \). The \( N_p \) obtained 3.28 \( \times 10^{14} \) from equation (15) for the REV liposomes is in the same order of magnitude.

Purification: The liposomes were separated from the non-encapsulated materials (lipid/cholesterol/Hb) and in the case of CMC-LEHbs the non-adsorbed CMC was recovered using a Sepharose 4B column. The feasibility of this separation can be explained by electrostatic repulsions between the Sepharose and CMC (Figs. 12 & 8d) because at pH = 7.4 both are negatively charged. As previously described for particles in suspension travelling in the void volume, the concepts of gel chromatography do not apply and the column serves as a sieve. The following hypothesis can be postulated: Ideally in the absence of any electrostatic interaction between gel/liposome or gel/lipid, the particle’s radius is the limiting factor in diffusion; the smaller ones are contained in the first eluted fractions. This is illustrated by the data in TABLE II which obey Stokes-Einstein’s law (eq. 1): As the average size decreases the diffusion coefficient increases. Meanwhile, purification of the liposomes shifts the size distribution to the left, it disposes of the larger non-encapsulated lipid/cholesterol fragments.

Methemoglobin: The rate of methemoglobin formation is higher at low \([\text{Hb}]_o\) (Fig. XVIII). As a protein hemoglobin’s stability is a function of its concentration. At low \([\text{Hb}]_o\) the heme is more
susceptible to oxidation from ferrous to ferric state. As [Hb]$_0$ is increased the heme stabilizes and a decrease in ferrihemoglobin concentration is observed. The value of bovine $M_{Hb}$ is 4% of the total Hb concentration (35) and compares to the experimental value obtained for $M_{Hb}$ of 4.5% after 72 hrs of encapsulation for a $[Hb]$ of 15.1 g/dl (TABLE III). Therefore the REV technique does not degrade, if not preserve the oxygen carrying capacity of SFHb.

The Met$[Hb]_0$ is higher than $M_{Hb}$ encapsulated. This can be contributed to two factors related to peroxide regulation.

1) $H_2O_2$ can readily cross the lipid bilayer (5), while the ability of $O_2^-$ to cross the membrane is still debated (10). The extent of permeability to these species does not rule out the hypothesis that $H_2O_2$ and $O_2^-$ can peroxidize the liposome bilayer containing DMPG, an unsaturated fatty acid. The incorporation of fat soluble $\alpha$-Tocopherol or vitamin E into the bilayer which is a free radical trap, reduces the penetration of peroxides in the liposomes.

2) The activity of liposome entrapped catalase is higher than that of free (ncn-encapsulated) enzyme in the SFHb solution. Although this hypothesis remains to be tested, encapsulation concentrates the enzyme which results in enhanced stability due to the increased protein-protein interactions; this has been proven for a variety of enzymatic systems (30).

The combination of the increased catalase activity and decreased amounts of $H_2O_2$ substrate could result in the reduced concentrations of ferrihemoglobin.

**Carboxymethylchitin:** Apart from extra purification steps in Method II namely freeze-drying, the major difference between the procedures during chemical synthesis is the cooling and the addition of acetic anhydride in order to minimize the probability of the N-deacetylation of the acetamido group (Fig. IV): After the 1st acetylation and wash not all the base has been removed therefore
the excess NaOH can attack the acetamido group. N-deacetylation can occur at room temperature (20 °C) and low base concentrations (84). Reducing the temperature favors the more exothermic reaction which uses acetic anhydride as acetylation agent in combination with the excess base to push the reaction towards the carboxymethylation of the remainder of the unreacted hydroxyl groups, and hence reduce the extent of N-deacetylation.

In Method I the substitution of mono- by tr.-chloroacetic acid increases the probability of the nucleophilic substitution reaction (Fig. IV). The additional chlorine atoms make the acetylation agent more reactive for the attack of alkali-chitin in order to yield CMC-NaCl salt.

Recycling CMC-Chitin as a substrate for a second reaction pass allows the unreacted chitin residues to be carboxymethylated. The d.s. is comparable to the product obtained by the previous modification.

Nevertheless the initial base concentration [NaOH], remains an important factor in CMC synthesis independent of the procedure used. At [NaOH], = 60% Method II is chosen as the carboxymethylation procedure leading to the desired highly substituted and water-soluble chitin derivative.

It has been shown that the kinetics of Chitin degradation by chitinase from *Streptomyces Antibioticus* follow a Michaelis Menten model characterized by a $K_m$ (mg.ml$^{-1}$) value of 0.1 in citrate buffer at pH = 5.1 and $T = 37 ^\circ$C (49). The experimental values of $K_{nave}$ (mg.ml$^{-1}$) for the hydrolysis of CMC by chitinase from *Streptomyces Griseus*,

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are in the same order of magnitude but higher than that of chitin regardless of d.s.: 0.971 and 0.571 (mg.m\(^{-1}\)) for d.s. of 45.5 and 80.4 respectively. Chitin is not water-soluble but its solubility/degradation (hydrolysis) is achieved by decreasing the pH (71). Since the kinetic experiments are not run under the same pH (physiological restrictions, pH= 7.4) it is not possible to conclude that the enzyme has a lower affinity for the carboxymethylated chitin derivative. Nevertheless, assuming that the extent of initial substrate solubilization-induced hydrolysis caused by the acidification of chitin is taken into account for \(K_m\) determination, a balance between solubility and charge could contribute to this decrease in affinity:

1. At pH\textasciitilde5.1 the acetamido group of chitin is slightly positively charged while at pH \textasciitilde7.4 the polymer is neutral. At pH\textasciitilde7.4, the higher the degree of carboxymethylation the more negatively charged the CMC becomes, due to the substitution in the C\textsubscript{3} or C\textsubscript{6} of the polar but uncharged hydroxyl group (-CH\textsubscript{2}OH) by the negatively charged carboxymethyl side chain (-CH\textsubscript{2}OCH\textsubscript{2}COOH). The negative charge exerts an inhibitory effect on the enzyme compared to (+) charged chitin. But this does not explain why the highly substituted carboxymethylchitin has a lower \(K_m\) than the low d.s. CMC.

2. The majority of the C\textsubscript{3} or C\textsubscript{6} in the highly substituted derivative are water-soluble while for the CMC of d.s. 51.4 only half its side chains are H\textsubscript{2}O soluble, hence the enzyme has better access to the more soluble compound.

**CMC-coated Liposomes**: Qualitative proof of adsorption such as variations in surface potential of liposomes calculated from electrophoretic mobility, change in \(T_c\) of the lipid/cholesterol bilayer upon the fixation of CMC on the surface exist (53,54). Attempts have been made to quantify the amount adsorbed enzymatically using chitinase (3), however the supernatant was not
purified and the method measures the adsorbed as well as the free carboxymethylchitin in solution. The adsorption efficiencies measured by IR spectroscopy and chitinase assay are 17.42% and 27.65% respectively. The discrepancy arises from the source of error associated with the enzymatic method. The background reading associated with the phospholipids at 420 nm can be minimized but not completely eliminated. Therefore the $E_{Ad}$ computed from IR spectroscopy is a more accurate quantitative adsorption index while the enzymatic method is a qualitative detection tool.

**RBCs vs. CMC-LEHbs:** TABLE VI presents a comparison between 1 ml of blood (hematocrit of 45%) and 1 ml of a prepared liposome suspension ($E_{Hb}$ = 22%). Since the average diameter ($d_v$) of a LEHb is about 73 times smaller than that of an erythrocyte the ratio surface area to volume ($S/V$) available for gaseous exchanges is 28 times larger. Even though the cell count for the LEHbs is close to 64,300 times that of RBCs 1Hb is 85 times smaller per ml of suspension.

The bilayer composition is 87.4% lipids (phospholipids, cholesterol) and 12.6% CMC for the CMC-LEHbs. The biological rat erythrocyte membrane is comprised of 87.8% lipid and lipid-bound components (phospholipids, cholesterol, lipoproteins, glycolipids) while the other 12.2% is constituted by carbohydrates and sugar-bound compounds such as glycoproteins, oligosaccharides and sialic acid (35).

---

1. So far, there is no evidence proving that the enzymatic systems in the RBC are removed during the SFHb preparation
Table VI: Comparative Summary of RBCs and CMC-LEHb

<table>
<thead>
<tr>
<th></th>
<th>RBC</th>
<th>CMC-LEHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{av}$ ($\mu m$)</td>
<td>8.4</td>
<td>0.115</td>
</tr>
<tr>
<td>$(S/V)_p$</td>
<td>1.87</td>
<td>52.6</td>
</tr>
<tr>
<td>Particle Count</td>
<td>$5.1 \times 10^9$</td>
<td>$3.28 \times 10^{14}$</td>
</tr>
<tr>
<td>(per ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$THb$ (g/dl)</td>
<td>15.1</td>
<td>0.178..</td>
</tr>
<tr>
<td>Membrane Composition (wt %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipids</td>
<td>87.8</td>
<td>87.4</td>
</tr>
<tr>
<td>carbohydrates</td>
<td>12.2</td>
<td>12.6</td>
</tr>
</tbody>
</table>

* Spherical particles are assumed
** Improvements required refer to Section VIII Recommendations
*** Classification includes all lipid-bound and protein-bound components
VII. CONCLUSION

Blood substitutes based on liposomes encapsulating bovine stroma-free hemoglobin have been prepared by the Reverse Phase Evaporation technique. Purification follows synthesis by passing these LEHbs through a Sepharose 4B column. The average size ($d_v$) of the liposomes is 0.115 $\mu$m while the particle count is $3.28 \times 10^{14}$/ml. Studies examining the effects $[\text{Hb}]_o$ and $[\text{L}]_o$ on $E_{\text{Hb}}$, $\langle Hb \rangle$, the encapsulated Hb to lipid loading ($\text{Hb}/\text{L}$), and $\text{HbO}_2$ preservation show that: i) $E_{\text{Hb}}$ and $\langle \text{Hb}/\text{L} \rangle$, increase with $[\text{L}]_o$ while $E_{\text{Hb}}$ is independent of $[\text{Hb}]_o$. The optimum $E_{\text{Hb}}$ of 22%, is achieved at $[\text{L}]_o = 180 \mu$mol/ml with $[\langle \text{Hb} \rangle] = 0.178$ g/dl. ii) The higher the $[\text{Hb}]_o$ the lower the $M_{\text{Hb}}$. The lowest $M_{\text{Hb}}$ recorded was 4.5%, 72 hrs after encapsulation for $[\text{Hb}]_o = 15.1$ g/dl.

The LEHbs are adsorbed with a highly substituted (d.s. 80.4) water-soluble type of carboxymethylchitin. Characterization follows purification by gel chromatography as previously. The coated liposomes are analyzed qualitatively by a chitinase assay simulating the kinetics of hydrolysis of the adsorbed CMC by a Michaelis Menten Model. The adsorption efficiency ($E_{\text{ad}}\%$) of 17.4 measured by FT-IR spectroscopy coupled to lipid/cholesterol analysis reveal the following: i) The weight ratio of adsorbed to non-adsorbed liposomes is 9:1 (mg/mg), ii) The overall composition of the bilayer(s) is close to that of the natural RBC membrane in terms of total lipids and carbohydrates at 87.8% (phospholipids & cholesterol) and 12.2% (CMC) respectively.
VIII. RECOMMENDATIONS

I. Hb capture rates ($E_{Hb}$) and the $\frac{(Hb/L)}{t}$ increase with $[L]_o$. Another way to improve the $\frac{(Hb/L)}{t}$ ratio is to extrude sequentially the purified REV liposomes through membranes with pore sizes of 1 to 0.1 μm ($d_v = 0.115 \, \text{μm}$) in order to reduce the percentage of multilamellar liposomes and hence reduce the lipid content. This additional step will inevitably narrow the liposomes' size distribution.

II. The adsorption efficiency of 17.42 % can be improved by increasing the $[\text{CMC}]_0$. The water-solubility of carboxymethylchitin is not only a function of d.s. but also its size or molecular weight. Reducing the size of the macromolecule will increase water-solubility. This has been achieved in the case of chitosan (69). Solutions of molecular weight-reduced chitosan of up to 1% (wt) can be prepared, while the limiting solubility of the highest substituted CMC remains at 0.2% (wt).

III. More experiments with suspension of known average size and size distribution have to be conducted to study the behavior of the Sepharose 4B column as a sieve.

IV. An extra step can be added in the preparation of SFHb in order to prevent dimerization of Hb during the removal of stroma by the addition of pyridoxal phosphate. Pyridoxylation also decreases the Hb's oxygen affinity, insuring adequate oxygen delivery (76). This process concentrates the Hb, stabilizes the heme, therefore Hb is less susceptible to oxidation. The addition of ascorbic acid (vitamin C) and reduced glutathione which are physiological non-enzymatic regulators of [MetHb] in the RBC (Fig. 3a) to the SFHb solution also reduces the rate of methemoglobin formation (16). As indicated by the data the above-mentioned modifications are not necessary for in vitro experiments, but are recommended for in vivo trials.
IX. REFERENCES


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X. APPENDICES
A. Absorption Spectroscopy

Absorption spectroscopy consists of placing the substance under investigation between an energy source that provides electromagnetic radiation in the frequency range being studied. The wavelength (λ) is inversely proportional to the adsorbed energy (E) (eq. 20), while the wavenumber σ is the inverse of λ.

\[ E = h\nu = \frac{hc}{\lambda} \]  

where

- λ: wavelength
- h: Planck constant
- c: velocity of light (10^8 m.s^-1)
- E: Energy of the source
- ν: frequency of radiation

The wavelength regions for detecting functional groups of interest are 200-400 nm (UV), 400-800 nm (visible), 2-16 μm (IR).

Transmittance (T) of the solution is the fraction of the incident radiation transmitted by the solution (eq. 21). The absorbance (A) is defined by equation (22).

\[ T = \frac{E}{E_0} \]  
\[ A = -\log_{10}(T) \]

Beer's Law states that at a given wavelength the absorbance directly proportional to the path length (b) through the solution and the concentration of the absorbing specie [c] (eq. 23), assuming that all particles behave independently.

\[ A = \varepsilon b[c] \]

where

- \( \varepsilon \): Molar absorptivity (L.cm^-1.mol^-1)
- b: path length (cm)
- [c]: molarity (mol.L^-1)

Deviations from Beer's Law are observed for

1) Solutions close to the solubility limit.
2) Protein solutions whose stability is dependent on intermolecular interactions.
A.1 Detection in the UV and Visible Ranges

The absorption of ultraviolet or visible radiation by species results in the excitation of bonding electrons; as a consequence the wavelengths of absorption peaks can be correlated with the types of bonds that exist in the species under study. At a certain wavelength the magnitude of $\varepsilon$, molar absorptivity depends upon the capture cross section of the species and the probability for an energy absorbing transition to occur. The relationship between $\varepsilon$ and these parameters has been shown to be:

$$\varepsilon = 8.7 \times 10^{19} \text{ PA}$$  \hspace{1cm} (24)

where

$P$ : Transition probability  
$A$ : cross section target area

Typical spectrophotometers contain the following components:

1. source of radiant energy (heated solid, lamp)  
2. sample compartment  
3. wavelength or scan region selector  
4. photoelectric detector or transducer of radiant energy  
5. Signal Processor (computer, chart recorder)

A.2 Detection in the IR Range

Electronic transitions require energies in the UV and visible regions; absorption of IR is confined to molecular species for which small energy differences exist between various vibrational and rotational states. In order to absorb IR radiation the molecule must undergo a net change in dipole moment as a consequence of its vibrational and rotational motion. The frequency of radiation that will bring this change is identical to the vibration frequency of a harmonic oscillator consisting of 2 masses connected by a spring (bond). That is,

$$E_{\text{rad}} = h\nu = \Delta E = h\nu_m = [h/2\pi](k/\mu)^{1/2}$$  \hspace{1cm} (25)

or

$$\nu = \nu_m = [1/2\pi](k/\mu)^{1/2}$$  \hspace{1cm} (26)

where

$\Delta E$: change in energy between vibrational states 
$\nu_m$: vibrational frequency of the mechanical model (cm$^{-1}$) 
$\nu$: radiation frequency in wavenumbers (cm$^{-1}$) 
k : force constant for the bond (dynes.cm$^{-1}$) 
$\mu$ : reduced mass of the 2 point masses
The principle of interferometry states that light at a given wavelength is considered as a time dependent wave whose period is related to the frequency of the light energy. For a continuous source the interferogram can be represented as a sum of an infinite number of cosine terms. That is,

\[ P(\delta) = \int_{-\infty}^{\infty} B(\sigma) \cos(2\pi \sigma \delta_n) \, d\sigma \]  

where

- \( B(\sigma) \): Incident power of the IR beam (frequency domain)
- \( P(\delta) \): Amplitude of the interferogram signal (time domain)

The Fourier Transform of this integral is

\[ P(\sigma) = \int_{-\infty}^{\infty} B(\delta) \cos(2\pi \sigma \delta_n) \, d\delta \]  

FT-IR consists of recording \( P(\delta) \) as a function of \( \delta \) (eq. 27) and then mathematically transforming this relation to one that gives \( P(\sigma) \) as a function of \( \sigma \) (the frequency spectrum) (eq. 28). The resolution of a FT-IR spectrometer can be described in terms of the difference in wavenumber between two lines that can be just separated by the instrument. That is,

\[ \Delta \sigma = \sigma_1 - \sigma_2 \]  

where \( \sigma_1 \) and \( \sigma_2 \) are wavenumbers for a pair of barely resolvable IR lines.

Since the spectrum is the summation of time dependent signals, its quality improves with the acquisition time which in turn increases with the number of scans performed.

Fourier Transform Infrared spectrometers consist of a continuum source, an interferometer, a sample department and a detector (Fig. A1). The source energy from a He-Ne laser passes through the interferometer to produce time dependent signal which then passes through the sample and to the detector. The computer then transforms the time dependent waveform at discrete time intervals into the frequency domain with incorporated FT-IR algorithms.
B. Light Scattering

Light scattering is based upon the principle that illuminated particles scatter the incident source of radiation in all directions. A schematic diagram of the instrument is presented in Figure A2.

Light from a HeNe laser (5 mW) is focussed at an angle of $\theta=90^\circ$ into a cuvette containing a dilute suspension of particles diffusing by Brownian motion, held at constant temperature. The incident light wave consists of rapidly oscillating (f=10 $10^{15}$) electric field of amplitude $E_0$. The arrival of this alternating field causes the polarizable electrons in the vicinity of the particles, to radiate in all directions the scattered light wave, creating a new oscillating electrical field $E_s$ related to $I_s$ (eq.30).

$$I_s = (E_s)^2 \tag{30}$$

The fluctuations of $I_s$ in time are a function of particle size. Small particles move in solution very fast resulting in a rapidly fluctuating intensity signal; by contrast larger ones diffuse more slowly with negligible variations in $I_s(t)$. For particles diameters of 150 nm or smaller (within the predicted experimental range) and $a<\lambda$ where $a$ is the particle radius and $\lambda$ is the wavelength of the light beam (632.8 nm) the Raleigh theory for small Isotropic Particles applies: The scattered intensity per unit incident intensity $(I_s)$ at a distance $r$ from the particle radius $a$ is given by equation (3) where $m$ is the refractive index of the particles relative to the medium, $\lambda$ is the wavelength of the beam.

$$I_s = \frac{16\pi^4 a^6}{r^2 \lambda^4} \left[ \frac{(m^2-1)}{(m^2+2)} \right] \tag{3}$$

For particle size with $a>150$ nm (Mie region) correction factors are added to equation (3).

The variations of $I_s(t)$ captured by the PMT detector can be mathematically expressed by a weighted sum ($f_i$ weighing coefficient) of exponentially decaying functions $C(t')$ (eq.31), each of which corresponds to a different particle diameter with a different decay time constants $\tau_i$, corresponding to a particular particle diffusivity $D_i$ in the solvent. $D_i$ is related to $\tau$ by equation (32) where $K$ is called the "scattering wavevector". It is a constant which depends on the laser wavelength $\lambda$, $\theta$ the scattering angle, $n$ the refractive index of the solvent (eq. 33). For each $D_i$ a particle radius $a_i$ is calculated using the Stokes - Einstein's law (eq. 1):

$$C(t') = A \left\{ \sum f_i \exp \left(-0.5 \frac{t'}{\tau_i} \right) \right\}^2 + B \tag{31}$$

$$D_i = \frac{1}{(2K^2)(1/\tau_i)} \tag{32}$$

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\begin{align*}
K &= \left(\frac{4\pi n}{\lambda}\right) \sin \left(\frac{\Theta}{2}\right) \\
D &= \frac{kT}{6\pi \eta a}
\end{align*}

where

- $a$: particle radius
- $T$: absolute temperature
- $\eta$: kinematic viscosity
- $k$: Boltzmann's constant

A summary of the analyzer's sequence of operation follows:

1. $E_o \rightarrow E_s \rightarrow I_s \rightarrow D \rightarrow a$

The microcomputer processes the time dependent scattering intensity fluctuations resulting in visual display of size distribution(s).

* Values are specific to the Nicomp 370 Model

** The optical and physical properties of PBS buffer (i.e., refractive index, viscosity) are assumed as those of water
### TABLE A1: CHARACTERISTIC INFRARED ABSORPTIONS OF FUNCTIONAL GROUPS

<table>
<thead>
<tr>
<th>Group</th>
<th>Intensity Range (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ESTER STRETCHING</strong></td>
<td></td>
</tr>
<tr>
<td>saturated, acyclic</td>
<td>1735-1750</td>
</tr>
<tr>
<td><strong>AMIDES</strong></td>
<td></td>
</tr>
<tr>
<td>Carbonyl Stretching</td>
<td></td>
</tr>
<tr>
<td>or AMIDE I</td>
<td></td>
</tr>
<tr>
<td>Primary, solid and concentrated solution</td>
<td>1650 ± 5</td>
</tr>
<tr>
<td>Primary, dilute solution</td>
<td>1690 ± 5</td>
</tr>
<tr>
<td>N-H Bending</td>
<td></td>
</tr>
<tr>
<td>or AMIDE II</td>
<td></td>
</tr>
<tr>
<td>Primary amides, dilute solution</td>
<td>1590-1620</td>
</tr>
<tr>
<td>Secondary amides, dilute solution</td>
<td>1550-1510</td>
</tr>
</tbody>
</table>
Figure A1: Schematic Representation of a Fourier Transform Infrared Spectrometer

Figure A2: Block Diagram of the NICOMP Size Analyzer
A. Determination of [SFHb]

A.1 Spectrophotometric Method

The calibration curve (35) at 540 nm fits a 2\textsuperscript{nd} order linear model with a correlation coefficient of $r = 0.998$

$$\text{Abs}_{540} = 0.033 + 10.09 \text{[SFHb]} - 16.22 \text{[SFHb]}^2 \quad (35)$$

This equation is valid for [SFHb] from 0 to 1.75 mg/ml.

A.2 FT-IR Analysis

The calibration curve (36) at 1653 cm\textsuperscript{-1} also fits a 2\textsuperscript{nd} order linear model with a correlation coefficient of $r = 0.996$

$$\text{Abs}_{1653} = 0.0005 + 2.079 \text{[SFHb]} - 10.37 \text{[SFHb]}^2 \quad (36)$$

The equation is valid within the following range of concentrations: 0 - 1.5 mg/ml.

B. Determination of [CMC]

B.1 Enzymatic Method

The calibration curve (37) at 420 nm fits a 4\textsuperscript{th} order linear model ($r = 0.999$).

$$\text{Abs}_{420} = -0.003 + 1.071 \text{[CMC]} + 0.278 \text{[CMC]}^2 - 0.7 \text{[CMC]}^3 + 0.203 \text{[CMC]}^4 \quad (37)$$

This model is valid from [CMC]s ranging from 0-2 mg/ml.

B.2 FT-IR Analysis

The calibration curves (38,39) at 1655 and 1734 cm\textsuperscript{-1} both fit 1\textsuperscript{st} order linear model with $r$ values of 0.995 and 0.990 respectively.

$$\text{Abs}_{1655} = 0.002 + 0.037 \text{[CMC]} \quad (38)$$

$$\text{Abs}_{1734} = 0.001 + 0.009 \text{[CMC]} \quad (39)$$

These models are valid for [CMC]s ranging from 0-2 mg/ml.
C. Determination of [Phosphatidylcholine]

C.1 FT-IR Analysis

The calibration curve (40) for [PC] at 1734 cm$^{-1}$ fits a 1st order linear model ($r = 0.997$).

$$\text{Abs}_{1734} = 0.0468 + 0.333 \times [\text{PC}] \quad (40)$$

This equation is valid for [PC] ranging from 0-100 mg/ml.
<table>
<thead>
<tr>
<th>Column 1: fraction #</th>
<th>Column 2: [cholesterol] (mg/dl)</th>
<th>Column 3: [triglyceride] (mg/dl)</th>
<th>Column 4: scattering intensity at 405 nm</th>
<th>Column 5: heme absorbance at 540 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.00</td>
<td>0.00</td>
<td>44.60</td>
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<td>2.00</td>
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<td>38.05</td>
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<td>3.00</td>
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<td>6.00</td>
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**TABLE C1: LEHBs PURIFICATION and CHARACTERIZATION**

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Column 3: [triglyceride] (mg/dl)  
Column 4: scattering intensity at 405 nm  
Column 5: heme absorbance at 540 nm

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Column 3: [triglyceride] (mg/dl)
Column 4: [cholesterol] (mg/dl)
Column 5: CMC absorbance at 420 nm

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### TABLE C2: CMC-LEHbs PURIFICATION and CHARACTERIZATION

<table>
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<tr>
<th>Column 1: fraction #</th>
<th>Column 2: scattering intensity at 405 nm</th>
<th>Column 3: [triglyceride] (mg/dl)</th>
<th>Column 4: [cholesterol] (mg/dl)</th>
<th>Column 5: CMC absorbance at 420 nm</th>
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Figure C1: Elution Profile of Pure Carboxymethylchitin at [2 mg/ml]