Microencapsulation IV: Cross-Linked Hemoglobin Microcapsules

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Abstract □ Hemoglobin microcapsules were prepared through cross-linking of hemoglobin itself with various acyldichlorides. Variations in the reticulation conditions were performed in order to ameliorate the oxygen dissociation curve, the mean diameter, and the possibility for the microcapsules to be lyophilized. With terephthaloylchloride, as the cross-linking agent, incorporation of inositol hexaphosphate and glucose, followed by stabilization through glutaraldehyde and using a high stirring speed, allowed preparation of stable hemoglobin microcapsules, 5 μm in diameter, which suffered rapid lysis by proteases. They were able to ensure oxygen transfer: the dissociation curve was sigmoidal with a p50 = 13 mm Hg. They retained these properties after lyophilization followed by rehydration.

Keyphrases □ Hemoglobin—preparation of cross-linked microcapsules □ Microencapsulation—preparation of cross-linked hemoglobin microcapsules □ Cross-linking—proteins, microencapsulation of hemoglobin

Human hemoglobin has been subjected to microencapsulation by several authors: Chang (1–4) was the first to prepare hemoglobin microcapsules through interfacial polymerization or coacervation processes. However, the membrane made of nylon, polystyrene, collodion, or silicon rubber (dimethicone membrane[1]) failed to be biodegradable.

More recently, Kondo (5–8) used polyphthaloyl-L-lysine as the wall-constituting polymer and the microcapsules could be digested by proteolytic enzymes.

Vigneron (9, 10) obtained similar results through interfacial polymerization between sebacoyl chloride and hexamethylene diamine with L-lysine admixed.

On the other hand, whereas cross-linking of hemoglobin by various reagents [dialdehydes (11, 12), carbodiimides (13), diepoxides, diisocyanates (14), and unsaturated sulfones (14, 15)] has been extensively studied, application of the procedure to clinical problems has been restricted to the preparation of hemoglobin, modified in order to increase its life-span when used as a blood replacement fluid.

In continuation of microencapsulation experiments through cross-linking of proteins (16–18), this paper deals with the application of this concept to hemoglobin.

EXPERIMENTAL

Reagents—Human hemoglobin was used. Red blood cells were collected from citrated human blood by centrifugation and washed with isotonic sodium chloride solution. After centrifugation, the red cells were subjected to hemolysis. Stromas were eliminated by successive filtration through membranes with porosities of 3 μm then 0.2 μm. The hemoglobin solution (6%, w/v) was added to glucose (2%, w/v) and then lyophilized.

Several buffers were prepared with pH's ranging from 9.8 (0.45 *M* Na_2CO_3; HCl to pH 9.8); [0.5 *M* bis(2-hydroxyethyl)amino-tris(hydroxymethyl) methane(I)[2]; HCl to pH 9.8] to 10.5 (0.45 *M* Na_2CO_3; HCl to pH 10.5); and 11.5 (0.45 *M* Na_2CO_3; HCl to pH 11.5), (2 *M* Na_2CO_3; HCl to pH 11.5).

The organic solvent was chloroform–cyclohexane (1:4, v/v). It was used to prepare solutions of terephthaloylchloride[3] (2.5 and 5 g%, w/v), sebacoyl chloride[3] (2.25 and 4.5 g%, w/v), and succinylchloride[4] (0.25, 0.50, and 1 g%, w/v).

An etheral solution of 12% glutaraldehyde (v/v) was prepared from a 25% aqueous solution[3] (v/v) through extraction with diethyl ether from a sodium chloride-saturated solution.

The surfactants were sorbitan trioleate[5] [2, 5, and 10% solutions (v/v) in the organic solvent] sorbitan monolaurate[5] [5 and 10% solutions (v/v) in the organic solvent], lecithin from soybean[6] [1, 2, 3, and 5% solutions (w/v) in the organic solvent]. Polysorbate[5] was used for the transfer of microcapsules, as a 1:3 mixture (v/v) with glycerin.

The other reagents were inositol hexaphosphate[2], sodium chloride solution 0.9 g% (w/v), and buffered sodium chloride solution, pH 7.3.

Macromolecular solutions (plasma substitutes) were used for resistance assays (dextran[7], modified gelatin[8,9]).

Materials—A stirrer[10], fitted with a 5-bladed screw (maximal speed: 1800 rpm) was first used and later replaced by an homogenizer[11], fitted with a metallic double blade (maximal speed: 23,000 rpm).

The oxygen dissociation curves were recorded with an analyzer[12].

Procedures—The hemoglobin lyophilisate (1.4 g, corresponding to 0.35 g of glucose and 1.05 g of hemoglobin) was dissolved in the buffer (3 ml) and emulsified with 15 ml of 2% surfactant solution (1200 rpm) at 0°.

The acylchloride solution (15 ml) was added and stirred for 3 min. The organic solvent (30 ml) was then mixed into the suspension. After centrifugation (350×*g*, 30 sec), the sediment was resuspended in the glycerin–surfactant mixture (5–10 ml) to which 50 ml of 0.9% aqueous NaCl was then added. The suspension was centrifuged (350×*g*, 5 min) and the sediment washed twice in 0.9% NaCl. Finally, the hemoglobin microcapsules were suspended in 30 ml of buffered sodium chloride solution.

After freezing at −30°, the suspension was easily lyophilized to yield a light brownish powder.

Modifications in the Reticulation Conditions—*Buffer*—Several 0.45 *M* Na_2CO_3 buffers (pH increasing from 9.8 to 11.5) were used during the polymerization step. Other attempts were made with a 2 *M* Na_2CO_3 buffer (pH 11.5) and with a I buffer[2], pH 9.8.

Acylating Agent—Several acyldichlorides were used as acylating agents at different concentrations: terephthaloylchloride, sebacoyl chloride, succinyldichloride, and a dialdehyde:glutaric dialdehyde.

Surfactant—Sorbitan trioleate, sorbitan monolaurate, and soybean lecithin were checked at different concentrations during the polymerization step.

Stirring—Variations of stirring conditions were made by replacement of the stirrer[10] by an homogenizer[11].

Temperature—Microcapsules were prepared at 20, 0, and −10°. Use of a nitrogen atmosphere was attempted to avoid oxidation of hemoglobin.

Further Treatment of the Microcapsules—Before lyophilization

[1] Silastic.

[2] Sigma.
[3] Aldrich.
[4] Fluka.
[5] Seppic Montanoir.
[6] Metabio.
[7] Rheomacrodex, Egic.
[8] Plasmagel, Roger Bellon.
[9] Haemaccel, Hoechst.
[10] Heidolph stirrer—stirring motor type RZRII—Adaptation system type RK6.
[11] Virtis 23 homogenizer, Bioblock.
[12] Hem-O-Scan analyzer, Aminco.

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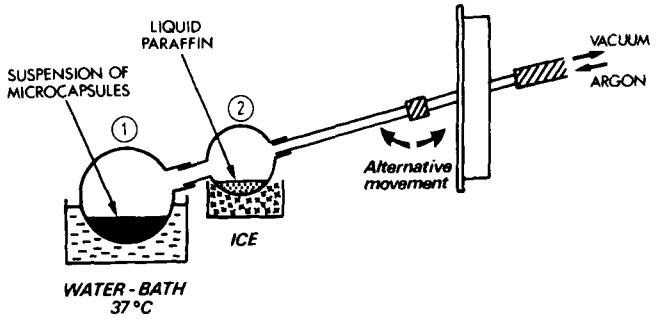

Figure 1—*Scheme of the apparatus used for deoxygenation of microencapsulated hemoglobin.*

glucose was added to the microcapsule suspension, as a hemoglobin protector (19).

Stabilization with Glutaraldehyde—To strengthen the cross-linked wall, microcapsules were prepared according to the following process: Microcapsules were obtained from hemoglobin and terephthaloylchloride at 0° in a pH 9.8 buffer with 5% sorbitan monolaurate (stirring speed: 1800 rpm). The capsules were centrifuged, resuspended in 60 ml of 0.9% NaCl solution, and 0.4 ml of a 25% aqueous glutaraldehyde solution was added. After a 3-min reaction, the capsules were isolated as before.

Incorporation of Inositol hexaphosphate—Inositol hexaphosphate is known to lower the oxygen affinity of hemoglobin (20, 21). Therefore, it was added to the hemoglobin microcapsules, either through incubation (20 hr, 4°) of the microcapsules in the phosphate solution (external phase) or during the preparation of the microcapsules (internal phase).

Assays—*Centrifugation*—Microcapsules were centrifuged for 60 min at 100,000×*g* (4°).

Resistance of Microcapsules to Ultrasound—Evaluation followed a 15-min treatment at 45 kHz.

Resistance to Freezing–Defreezing Cycles—Freezing at −30° followed by melting at 20° was repeated 4 times.

Aging—Suspensions of capsules in water or in 0.9 g% NaCl solution were stored at +4°.

Resistance to Macromolecular Solutions—Microcapsules were suspended in different macromolecular plasma substitutes at 37°.

Degradability Effect of Proteases—Microcapsules were incubated at 37° in different media containing proteases: pepsin (0.32 g %, w/v) in a hydrochloric acid solution (pH 2); pancreatin (1 g %, w/v) in a KH_2PO_4–NaOH buffer (pH 7.5); pronase 4×10^{-3} g % (w/v) in a $Na_2B_4O_7$–KH_2PO_4 buffer (pH 8) with $Ca(CH_3COO)_2$ (0.176 g %, w/v).

Oxygen Transfer Properties—Permeability toward oxygen was studied by using the apparatus depicted in Fig. 1.

A suspension of microcapsules of suitable concentration was prepared in a phosphate buffer (pH 7.5). Flask 1 contained the suspension and was heated to 37°. Flask 2 contained liquid petrolatum[13] and was cooled in an ice bath. The apparatus was continuously agitated and evacuated to 10^{-1}–10^{-2} T, after which argon was introduced. After several cycles, the apparatus was placed in an upright position so as to cover the deoxygenated suspension with liquid petrolatum. The microcapsule suspension was then transfered into the measurement cell of a spectrometer through a syringe containing liquid petrolatum so as to avoid any contact with air.

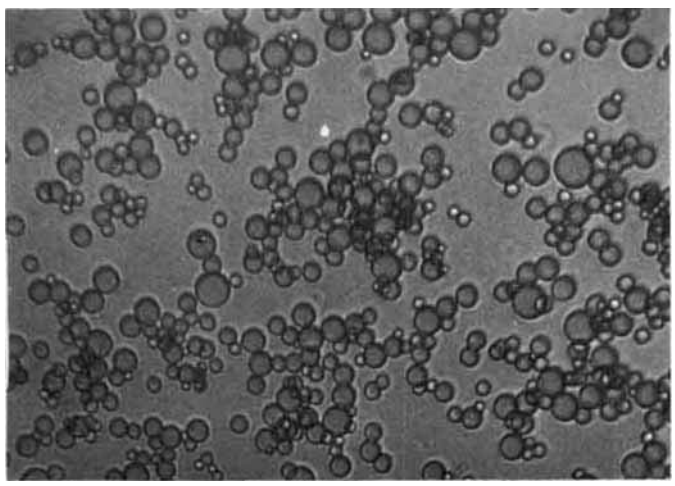

Figure 2—*Photomicrograph of hemoglobin microcapsules with cross-linked hemoglobin membrane (terephthaloylchloride).*

[13] Nujol.

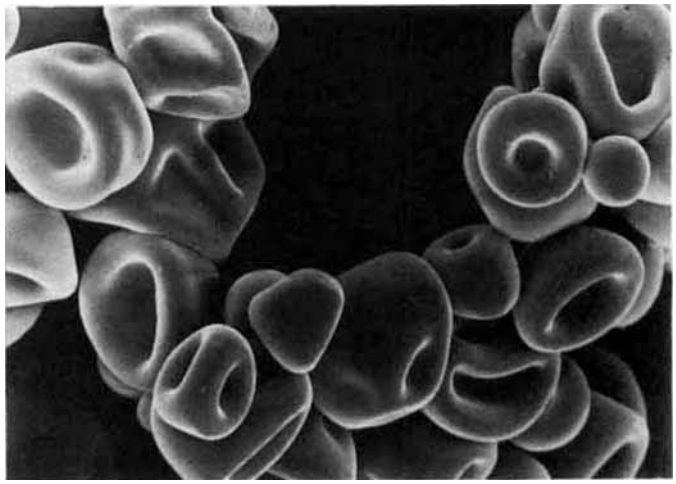

Figure 3—*Scanning electron micrograph of hemoglobin microcapsules with cross-linked hemoglobin membrane (terephthaloylchloride).*

After recording of the initial spectrum of the microcapsules, progressive reoxygenation was performed by bubbling air through the suspension with a syringe (0.5 ml in 15 sec) while the spectra were successively measured.

The saturation curve was recorded by plotting the percentage of oxyhemoglobin *versus* the partial pressure of oxygen.

RESULTS AND DISCUSSION

The initial typical procedure gave reddish spherical microcapsules. Their size was ~60 μm (σ = 23 μm) (Fig. 2).

A scanning electron micrograph of the dehydrated microcapsules is presented in Fig. 3.

Hemoglobin was chemically modified by terephthaloylchloride, which was indicated by examination of the film, isolated at the interface of an aqueous solution of hemoglobin and an organic solution of terephthaloylchloride. The IR spectrum of the film showed significant differences from that of hemoglobin in the fingerprint area (700–1500 nm).

After a 60-min centrifugation at 100,000×*g* (4°), the microcapsules were found mainly unaltered. However, the supernate was slightly colored, indicative of some hemoglobin leak. The microcapsules resisted a 15-min treatment by ultrasound at 45 kHz. Four freezing–defreezing cycles did not affect the microcapsules. Rehydration of the lyophilized microcapsules regenerated their spherical shape; however, a large amount of hemoglobin (20–25% of the total amount of hemoglobin used) was oxidized to methemoglobin during the process. Suspending the capsules in water resulted in turgescence, diffusion of hemoglobin, and, finally, lysis of the membrane. In 0.9% NaCl solution, hemoglobin also slowly diffused into the medium. After suspension in macromolecular solutions, optical microscopic observation showed the membrane to be collapsed, especially in the case of the larger capsules. The original spherical shape was regenerated through resuspension in an isotonic aqueous medium.

Table I—Hemoglobin Microcapsules with a Cross-Linked Hemoglobin Wall (Terephthaloylchloride): Influence of Nature and Concentration of the Lipophilic Surfactant [a]

Surfactant	Concentration, %	Mean diameter, μm	σ, μm
Sorbitan trioleate	2	40.8	18.9
	5	32.6	13.9
	10	23.4	10.8
Sorbitan monolaurate	5	24.9	8
	10	27.4	11.5
Soybean lecithin	1	27	10.2
	2	30.6	12.5
	3	30.2	12.5
	5	26.9	13

[a] Stirring speed: 1600 rpm.

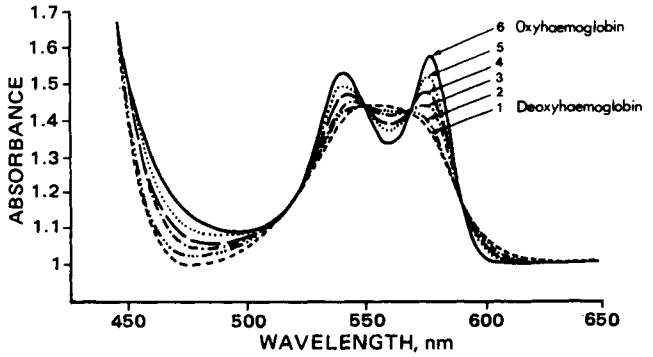

Figure 4a—*Visible absorption spectra of hemoglobin microcapsules: progressive reoxygenation of deoxyhemoglobin (curve 1) to oxyhemoglobin (curve 6).*

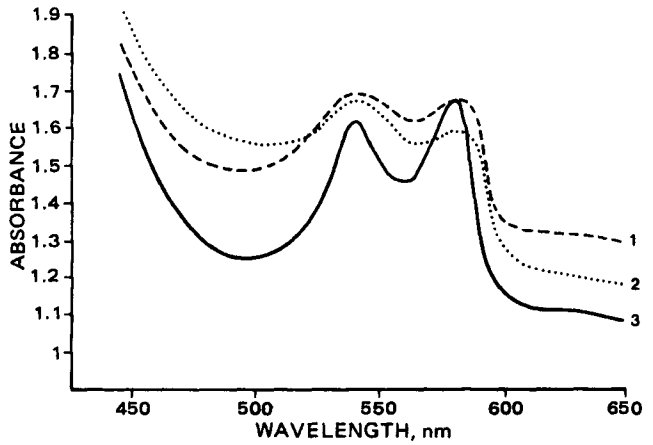

Figure 4b—*Absorption spectra of hemoglobin microcapsules, influence of the cross-linking reagent. Key: curve 1, terephthaloylchloride; curve 2, sebacoyl chloride; curve 3, glutaraldehyde.*

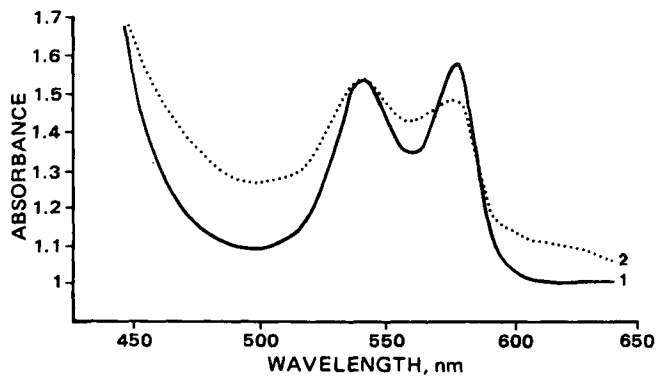

Figure 4c—*Absorption spectra of lyophilized hemoglobin microcapsules (terephthaloylchloride as cross-linking agent). Key: curve 1, glucose added prior to lyophilization; curve 2, without glucose.*

The microcapsules were destroyed after a 2-min incubation in pepsin or pronase, 1-min in pancreatin.

The electronic spectrum of the deoxygenated capsules (Fig. 4a) was characteristic of deoxyhemoglobin and gradually changed to the spectrum of oxyhemoglobin upon oxygenation.

The oxygen dissociation curve (curve 1, Fig. 5a) was not sigmoidal and differed from that of a hemoglobin solution (curve 3) or of normal human blood (curve 4). The p50 was only 6 mm Hg.

The initial procedure then allowed preparation of relatively stable hemoglobin microcapsules, the membranes of which were resistant, biodegradable, and permeable to oxygen.

However, the mean diameter was far too large, presence of methemoglobin had to be avoided, the lyophilization process needed improvements, and the oxygen affinity had to be reduced.

The following results were gained upon modifications of the procedure: Attempts to raise the pH of the 0.45 *M* Na_2CO_3 buffer from 9.8 to 11.5,

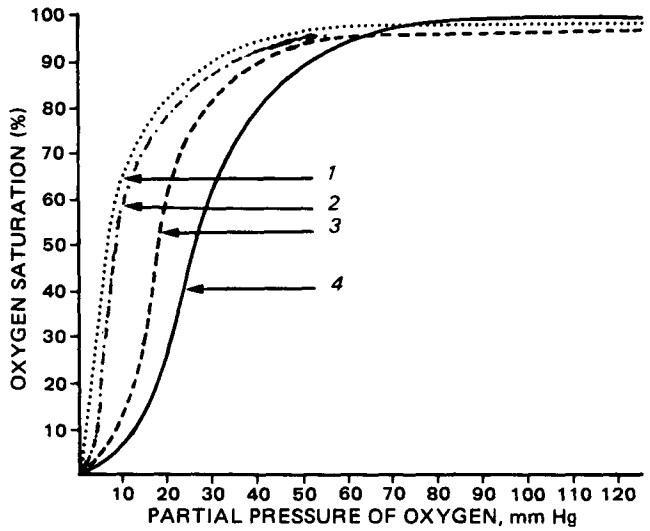

Figure 5a—*Oxygen dissociation curves. Key: curve 1, hemoglobin microcapsules with terephthaloylchloride as cross-linking agent; curve 2, hemoglobin microcapsules with glutaraldehyde as cross-linking agent; curve 3, hemoglobin solution; curve 4, normal human blood.*

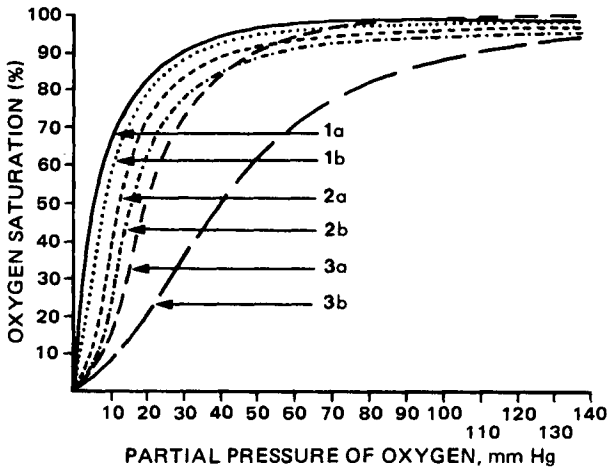

Figure 5b—*Oxygen dissociation curves of hemoglobin microcapsules without subsequent treatment and after stabilization with glutaraldehyde: effect of adding inositol hexaphosphate. Key: 1a, untreated microcapsules (p50 = 6 mm Hg); 1b, untreated microcapsules with inositol hexaphosphate added to the external phase (p50 = 8 mm Hg); 2a, stabilized microcapsules (p50 = 11 mm Hg); 2b, stabilized microcapsules with inositol hexaphosphate incorporated in the internal phase (p50 = 13 mm Hg); 3a, hemoglobin solution (p50 = 18 mm Hg); 3b, hemoglobin solution + inositol hexaphosphate (p50 = 39 mm Hg).*

as well as increasing the molarity to 2 *M* were not useful. However, replacing the sodium carbonate buffer by a 0.5 *M* I buffer, pH 9.8, gave good results. Increasing the concentration of terephthaloylchloride provided partial denaturation of hemoglobin. Sebacoyl chloride and succinylchloride gave poor results: the membranes were tiny and hemoglobin diffused into water, while the absorption spectrum was strongly altered (Fig. 4b, curves 1 and 2). On the contrary, glutaraldehyde yielded nice microcapsules which suffered practically no diffusion of hemoglobin. The absorption spectrum (Fig. 4b, curve 3) was unaltered and the p50 was 7 mm Hg. The oxygen dissociation curve was slightly sigmoidal (Fig. 5a). The microcapsules could be easily lyophilized. They were destroyed within 2 min in pepsin (pH 2) and only 2–3 hr in pancreatin (pH 7.5).

From the various surfactants checked (Table I), 5% sorbitan monolaurate seemed to give the best results regarding the average size and the distribution of diameters. Soybean lecithin also gave interesting results. Variation in the nature and concentration of surfactant did not affect the absorption spectrum nor the affinity curve.

The stirrer[10] used previously allowed only a maximum speed of 1800 rpm, but the use of the homogenizer[11] (metallic double blade) drastically improved the size of capsules (Table II). While graduation 100 of the

Table II—Hemoglobin Microcapsules with a Cross-Linked Hemoglobin Wall (Terephthaloylchloride): Influence of Stirring Conditions

Stirrer	Speed	Surfactant Compound	Surfactant Concentration, %	Mean diameter, μm	σ, μm
Stirrer[a]	1800 rpm	Sorbitan trioleate	10	18.7	8.6
		Soybean lecithin	1	20.6	7.7
		Sorbitan monolaurate	5	19.2	6.5
Homogenizer[b]	Graduation 10	Sorbitan monolaurate	5	9.8	4.9
	Graduation 40	Sorbitan monolaurate	5	4.9	1.2

[a] Heidolph stirrer-stirring motor type RZRII-Adaptation System type RK6. [b] Virtis 23 homogenizer, Bioblock.

apparatus corresponds to a maximum speed of 23,000 rpm, graduation 40 yielded capsules with a 4.9-μm diameter (σ = 1.2).

With microcapsules prepared at 20°, hemoglobin was altered. At −10°, hemoglobin rapidly diffused from the microcapsules. Again, the best results were gained at 0°. An attempt was made to prepare the microcapsules from deoxyhemoglobin under nitrogen. No improvement in the qualities of the capsules (p50 = 6 mm Hg) could be observed. Adding an amount of glucose equal to the weight of hemoglobin used prevented alteration of the hemoglobin during lyophilization (Fig. 4c).

When the capsules prepared from hemoglobin and terephthaloylchloride were further stabilized with glutaraldehyde they became very resistant. They resisted ultracentrifugation (100,000×g, 60 min, + 4°), freezing, melting cycles, and lyophilization. They were, however, rendered less deformable than previously and showed greater resistance to proteases (pepsin, 2 min; pancreatin, 30 min; pronase 2–3 hr). Diffusion of hemoglobin out of the wall was much slower. Interestingly, their p50 was now 11 mm Hg and the curve became slightly sigmoidal in shape (Fig. 5b).

Incorporation of inositol hexaphosphate induced modifications of the oxygen dissociation curves (Fig. 5b).

With standard microcapsules [not stabilized with glutaraldehyde (p50 = 6 mm Hg)], inositol hexaphosphate was only efficient when added in the external phase (p50 = 8 mm Hg). This result was probably due to inositol hexaphosphate being washed out during the isolation step of microcapsules.

On the contrary, with glutaraldehyde-stabilized capsules (p50 = 11 mm Hg), inositol hexaphosphate was not efficient when added to external phase (p50 unchanged = 11 mm Hg), but raised the p50 to 13 mm when in the internal phase. These last two results show that inositol hexaphosphate did not diffuse through glutaraldehyde-stabilized membranes.

The hemoglobin microcapsules prepared through the emulsion–reticulation method present interesting properties: one could actually obtain stable and biodegradable microcapsules with a 5-μm diameter, with a membrane permeable to oxygen but not to hemoglobin and inositol hexaphosphate, with a sigmoidal saturation curve (p50 = 13 mm Hg), and which could be lyophilized. Complementary work is necessary to evaluate their rheological and biological properties.

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