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Biochimica et Biophysica Acta 1285 (1996) 246–254

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Encapsulation of hemoglobin in a bicontinuous cubic phase lipid

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Received 26 March 1996; accepted 25 July 1996

Abstract

The effects of encapsulating bovine hemoglobin (BHb) in the bicontinuous cubic phase formed by monooleoylglycerol and water was investigated with Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction. Cubic phase was formed in the presence of 1–10 wt% BHb. Studies using X-ray diffraction reveal that at 0.5–2.5 wt% BHb, the cubic phase structure is characterized by the double diamond lattice (*Pn3m*). At 2.5–5 wt% BHb, coexistence of two cubic phase structures, *Pn3m* and the gyroid lattice (*Ia3d*), was observed while at BHb concentrations higher than 5 wt% the gyroid structure persists. FTIR shows there is an increase in intensity of the free $\nu C=O$ (1745 cm^{-1}) and a corresponding decrease in the intensity of the hydrogen bonded $\nu C=O$ (1720 cm^{-1}), as the BHb concentration is increased. The νC-O-CO peak shifts from 1183 cm^{-1} to 1181 cm^{-1} as the concentration of BHb is raised from 2.5 to 10 wt% indicating BHb may induce subtle changes in the interfacial region of cubic phase monoolein. The bandwidth of the $\nu_a CH_2$ stretch (2926 cm^{-1}) increased in the presence of 5 wt% BHb compared to samples with 2.5 or 10 wt% BHb. The increase in frequency of the $\nu_s CH_2$ stretch (2854 cm^{-1}) induced by increasing temperature 20 to 60°C was dampened when BHb was present compared to samples heated in isotonic buffer. Analysis of the amide I band at 1650 cm^{-1} showed that the secondary structure of BHb is not affected by encapsulation in monoolein. In vitro release studies showed that 45% of the entrapped BHb was released after 144 h at 37°C. The porous nature of bulk cubic phase was further demonstrated by diffusion of $K_2Fe(CN)_6$ and conversion of 73% of the oxyhemoglobin to methemoglobin after 1 h. These results suggest that the cubic phase may be useful for encapsulation of Hb as a red cell substitute and for the encapsulation and delivery of other bioactive agents.

Keywords: Cubic phase; Hemoglobin; Encapsulation; Drug delivery; Monoolein; Lipid

1. Introduction

Lipid based self-assembled microstructures have been developed extensively as vehicles for the delivery of a number of biologically active agents. Liposomes have been shown to extend the circulation persistence and reduce the toxicity of a number of biologically active compounds including antifungals,

Abbreviations: BHb, bovine hemoglobin; DAF, diaminofluorine; FTIR, Fourier transform infrared spectroscopy; GSH, glutathione; Hb, hemoglobin; H_{II}, reversed hexagonal phase; I_{II}, cubic phase; LEH, liposome encapsulated hemoglobin; MO, monoolein; PBS, phosphate-buffered saline

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PII S0005-2736(96)00169-1

anti-cancer drugs, and antibiotics (for a recent review see [1]). Liposomes and other lipid phases have also been used for cellular delivery of genetic constructs and topical delivery of drugs [2–4]. One application which has been extensively investigated is the encapsulation of hemoglobin in liposomes as a red blood cell substitute (for review see [5]). Liposome encapsulated hemoglobin (LEH) has been shown to be safe and efficacious in animal hemorrhagic shock models and to transit through the reticuloendothelial system over the course of 4–7 days [6,7]. The drawback to the use of liposomes for this and other applications is their limited encapsulation efficiency. The processing of liposome encapsulated hemoglobin by a number of standard liposome preparation methods typically incorporates only 25–30% of the protein in the encapsulation process [5]. This results in higher costs and increased processing time to obtain high encapsulation yields. Therefore, we have investigated the encapsulation of hemoglobin in the cubic phase of glycerol monooleate (monoolein) as a first step in developing an oxygen carrying resuscitative fluid and further developing this unique lipid phase for controlled release applications.

At physiological temperatures and low hydration (< 5 wt.%), monoolein forms a fluid isotropic phase (L_2) that with increasing hydration progresses through the lamellar liquid crystal phase (L_α), and finally enters the cubic phase (I_{II}) as the hydration reaches 20 wt% water [8]. Depending on the hydration and temperature, two cubic lattice structures of bicontinuous cubic phase exist, *Pn3m* [9] and *Ia3d* [10]. The *Pn3m*, or double diamond structure, formed at higher hydration levels exists in equilibrium with excess water. Both cubic phase lattices comprise a bulk three-dimensional network of channels that end in pores, the diameter of which is determined by the lattice parameters (for monoolein, 40–50 Å; Fig. 1).

Previous studies have investigated the use of cubic phase to encapsulate hydrophilic or hydrophobic solutes [11–13] or proteins [14] including lidocaine, gramicidin, insulin, and bovine serum albumin without altering the cubic phase structure. Very large molecular weight drugs such as vitamin E can be incorporated into cubic phase to concentrations as high as 200 mg/ml [13], further indicating the versatility of cubic phase as an encapsulation medium. Release of these solutes from cubic phase has been

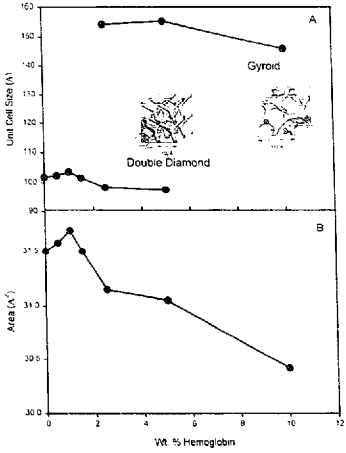

Fig. 1. Effect of bovine hemoglobin concentration on (A) unit cell size of cubic phase monoolein as determined by x-ray diffraction and (B) calculated effective lipid volume. The lipid concentration was maintained at 50 wt% in all samples. Cartoons of the structure of both double diamond and gyroid lattice are also shown

shown to be linear over time, even in the presence of simulated physiological fluids [13]. In addition to encapsulation, cubic phase has been used as a three-dimensional template for chemical reactions within the channels of the bicontinuous phase. One study utilized the porous structure of the cubic phase to diffuse Ca^{2+} into the channels of the bulk phase to gel sodium alginate trapped within the aqueous compartment of the cubic phase [15]. The release properties of albumin from the hydrogel/cubic phase composite was determined and described by a phase separation of monoolein/alginate. Although the majority of work has focused on the bulk cubic phase, the bulk phase has been dispersed by both chemical (mild detergent) and physical methods (sonication) to create particles (cubisomes) suitable for injection [11]. The goal of the present study is to utilize the high trapping efficiency of the bulk cubic phase and fabri-

cate cubisomes as a delivery vehicle for hemoglobin. The characterization of the phase properties of bulk cubic phase with encapsulated hemoglobin and its release kinetics is a first step toward achieving this goal.

2. Materials and methods

2.1. Materials

Monoolein was obtained from NuChek Prep (Elysian, MN) with a purity greater than 99% and used without further purification. Native tetrameric bovine hemoglobin (BHb) was purchased from Biopure (Boston, MA) and mixed with 30 mM glutathione (GSH; Calbiochem, LaJolla, CA) to give a final hemoglobin concentration of 20 g/dl. The BHb was stored at 4°C until use, at which time a sample was removed and allowed to warm to room temperature. The percent methemoglobin was assayed using commercial instrumentation (CO-Oximeter, Instrumentation Laboratories, Lexington, MA) before use, and samples with more than 10 percent methemoglobin were not used.

2.2. Lipid hydration

To assure a correct dry weight for the lipid, approx. 500 mg monoolein was dissolved in chloroform, dried under nitrogen, and lyophilized. The dry lipid was removed from the lyophilizer, weighed, and placed in a water bath at 40°C. Phosphate buffered saline (PBS; Sigma, St. Louis, MO) was added to the liquid monoolein to give 40 wt% water. Upon contact with the PBS the monoolein formed a viscous gel which was stirred with a spatula to give a uniform distribution and centrifuged for 45 min at 2000 × g and 30°C to remove air bubbles. Following centrifugation the samples were incubated for 24 h at room temperature to allow equilibration. Following incubation the samples were optically isotropic, indicating the formation of cubic phase. Samples hydrated with BHb were treated in the same manner except that they were hydrated to 40 wt% with 20 g/dl Bhb instead of PBS, resulting in a final lipid/water/protein ratio of 50:40:10.

2.3. X-ray studies

Structural phase information in the presence of varying concentrations of BHb was determined using monochromatic (0.15498 nm) focussed x-rays (National Synchrotron Source, Brookhaven, NY). The samples were sealed in vacuum-tight holders between two mica windows and the diffraction patterns were recorded on an image plate kept at a distance of 1 m from the sample. Sample temperature was maintained at 25°C using a constant temperature bath. Measured diffraction peaks were indexed to gyroid or double diamond cubic lattice. The gyroid phase is characterized by a set of reflections in the ratio 6:8:14:16:20:22 and the double diamond phase is characterized by reflections in the ratio 2:3:4:6:8:9:10 [16]. In our measurements the two phases showed at least three and five peaks respectively.

2.4. FTIR studies

Fourier transform infrared spectroscopy (FTIR) was used to characterize the phases formed from BHb/monoolein mixtures. Spectra were collected on a Nicolet Impact 400D FTIR (Nicolet Instrument, Madison, WI) equipped with a DTGS KBr detector and controlled by a personal computer. The optical bench and the sample chamber were continually flushed with dry nitrogen during spectral collection. Monoolein was hydrated as described above to 60:40 wt% lipid/PBS or 50:40:10, 50:45:5, or 50:47.5:2.5 wt% lipid/PBS/BHb and incubated at room temperature for 24 h. Following incubation the samples were sandwiched between CaF_2 windows separated by a 15 μm Teflon spacer. To prevent sample dehydration during scanning, the windows were sealed at their edges with parafilm. The sample was then loaded into a temperature controlled holder (Paige Instruments, Woodland, CA) and placed in the sample chamber of the FTIR. The chamber was flushed for 15 min before spectra were collected and samples were allowed to equilibrate for 2 min at each temperature before being scanned. 64 scans at 2 cm^{-1} resolution were averaged and Fourier transformed at each temperature.

Changes in BHb structure in the presence of monoolein were investigated by monitoring the amide

I band at 1650 cm^{-1} as described by Dong and Caughey [17]. Briefly, monoolein prepared as described above was incubated at 25°C for 2 or 24 h. Following incubation, samples were sandwiched between CaF_2 windows separated by a 6 μm Teflon spacer, sealed with parafilm, loaded into the temperature controlled holder, and placed in the sample chamber of the FTIR. The chamber was flushed with dry nitrogen for 30 min before scans were collected and total of 512 scans at 2 cm^{-1} resolution were collected and Fourier transformed to produce each spectra. Sample temperature was maintained at 37°C throughout scanning.

Because of the presence of the O-H stretching vibration from water in the region of the amide I band, it was necessary to subtract absorbance due to water from the sample spectra. A water spectra was collected as described above for the BHb samples and subtracted from the BHb/monoolein spectra. The subtraction was monitored by following the disappearance of the water absorbance band at ~ 2100 cm^{-1}. Following the subtractions, the second derivative of the spectra was determined. The resulting second derivative spectra was compared to a second derivative spectra of free BHb to calculate a correlation coefficient (r) using the equation developed by Prestrelski et al. [18]:

$$r = \frac{\sum^{N} x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}}$$

where x_i and y_i are absorbance values of the reference and sample spectra, respectively, at the ith frequency in the amide I region. A coefficient of 1 is consistent with structural similarity and deviations from 1 (i.e., reductions in the value of the coefficient) indicate conformational changes in the protein's secondary structure. The larger the conformational change in secondary structure, the smaller the value of r.

2.5. Release of entrapped BHb

In order to measure the release of BHb from MO/BHb, cylinders of MO/BHb were produced by loading the BHb containing MO (50:40:10, MO/PBS/BHb) into a plastic syringe following the centrifugation and allowing the sample to incubate for 24 h at room temperature. Samples of the MO/BHb cubic phase were then extruded for 2 cm and cut with a razor blade. This yielded a cylinder of MO/BHb 2 cm long with a diameter of 2 mm. The release of BHb from cubic phase MO was investigated by suspending the cylinders in PBS and monitoring the amount of BHb in the suspending PBS after various incubation times at 25 or 37°C. The amount of BHb released into the PBS was measured spectrophotometrically using a diaminofluorene (DAF) assay as described by Gebran et al. [19]. A total of three replicates were conducted.

2.6. BHb oxidation and accessibility

The stability of entrapped BHb was monitored by following the rate of conversion to methemoglobin. MO-BHb cubic cylinders, prepared as described above, were suspended in PBS in Eppendorf tubes and kept at 25 or 37°C. At various times samples were placed at 4°C for 24 h, resulting in the conversion of the MO into the lamellar phase and release of the entrapped BHb. Samples were centrifuged at 14000 × g for 10 min at 4°C to remove the lipid in the form of a solid white disk, and the percent methemoglobin in the supernatant was determined using the method of Tomita et al. [20]. Free BHb stored at 25 or 37°C for the same times served as controls, and a total of four replicates were performed.

Cubic phase containing BHb was exposed to CN^- to determine the accessibility of the entrapped BHb to ions in the media. Cubic cylinders containing BHb were placed in PBS containing 300mM NaCN and 1 M K_3FeCN_6. After various incubation times at 25 or 37°C the solution was decanted, the cylinders were rinsed with 2 volumes 'clean' PBS, suspended in PBS, and placed at 4°C for 24 h to release the BHb. The amount of methemoglobin formed as a result of exposure to the CN was determined as described above. Encapsulated BHb stored in PBS without exposure to the CN was used as a control.

2.7. Statistical analysis

The data were analyzed using a t-test on the SigmaStat program from Jandell Scientific Software (San Rafael, CA) with a $P < 0.05$ being significant.

3. Results and discussion

Structural characterization of MO/BHb mixtures. X-ray diffraction analysis shows that the cubic phase of MO is observed at concentrations of BHb between 1–10 percent of the total weight, corresponding to 2–20 g/dl Hb. The cubic lattice structure formed, however, is dependent upon the amount of BHb present (Fig. 1). Below 2.5 wt% BHb/MO, the double diamond lattice is observed, while between 2.5 and 5 wt% BHb the lattice structure is heterogeneous, with regions of coexistence of double diamond and gyroid lattice. If the BHb concentration is increased to 10 wt%, the gyroid cubic phase is observed. Investigation of the entire lipid/water/Hb phase diagram by x-ray analysis reveals that there is no other significant change in the phase diagram of monoolein in the presence of BHb (data not shown). However, the decrease in the unit cell size with increasing Hb concentration appears to suggest a shift in the excess water phase boundary to higher lipid concentration. Both these results, the change in cubic phase lattice and decrease in unit cell size in the presence of high concentrations of BHb (Fig. 1), may occur as a result of direct interaction of Hb with the lipid interface or may be related to changes in the hydration state of the lipid at high protein concentrations. The small decrease in the effective headgroup area, calculated form the measured unit cell size using the method described in Puvvada et al [16], suggests dehydration of the lipid at higher protein concentrations. This is further supported by the IR data discussed below.

Previously, investigators have shown that FTIR is useful in following structural changes in MO as a function of water content [21,22]. Specifically, the $\upsilon C=O$, $\upsilon_{as}C\text{-}O\text{-}OC$, $\upsilon C\text{-}OH_{sn\text{-}2}$, $\upsilon C\text{-}OH_{sn\text{-}3}$, and $\upsilon_{as}H_2O$ groups have been monitored to follow changes in the interfacial region of lipids and $\upsilon_s CH_2$, $\upsilon_{as}CH_2$, and the CH wagging groups to follow changes in the acyl chain order. The $\upsilon C=O$ band is composed of two peaks at 1740 cm^{-1} and 1722 cm^{-1} attributable to free and H-bonded C = O groups, respectively [23]. These groups, along with the $\upsilon C\text{-}OH$ and $\upsilon_{as}H_2O$ groups provide good insight into the structure of the MO interfacial region. Entrapment of BHb had no effect on the frequency of the C = O peaks although there was a decrease in the bandwidth

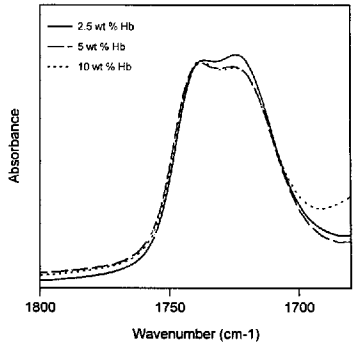

Fig. 2. Carbonyl bands of cubic phase monoolein in the presence of 2.5, 5.0, and 10.0% native tetrameric bovine hemoglobin at 37°C. Legends are given in the figure.

of the hydrogen bonded C = O and an increase in the bandwidth of the free C = O bands with increasing BHb concentration (Fig. 2). The increased bandwidth of the free C = O seen in the samples with higher BHb concentrations indicates that water penetration into the lipid is less, and hydrogen bonding is increased when BHb is present. The increased hydrogen bonding with increasing BHb is further indicated by an increase in the frequency of the water association band from 2092 cm^{-1} in samples with no BHb to 2130 cm^{-1} when BHb was present. The finding that there is no change in the frequency of the $\upsilon C=O$ spectra of MO in the presence of BHb is not inconsistent with the observation of Hb induced lattice change from double diamond to gyroid as little change in the $\upsilon C=O$ frequency or bandwidth was previously observed as a function of cubic phase structural changes [22]. Nilsson et al. [22] showed changes in cubic lattice have little effect on the $\upsilon C=O$ as measured by FTIR; however, the $\upsilon C\text{-}O\text{-}OC$ bandwidth is sensitive to lattice with considerable narrowing in the *Ia3d* lattice compared to the *Pn3m* lattice. We observe no change in this band in the presence of BHb, perhaps because of the mixed *Ia3d* and *Pn3m* lattice at the lower BHb concentrations.

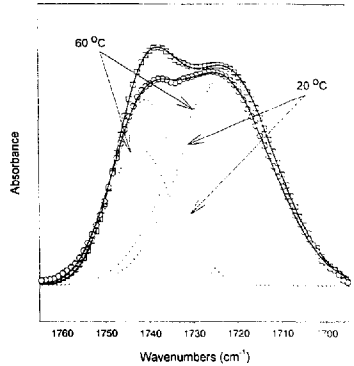

Fig. 3. The free and hydrogen bonded carbonyl bands in 50:40:10 wt% MO/PBS/BHb at 20°C (□) and 60°C (○).

Increasing the temperature to 60°C had a similar effect on both the PBS/MO and the BHb/MO C = O spectra, with an increase in the frequency of about 2 cm^{-1} for both the free and bound C = O and an increase in the bandwidth of the hydrogen bonded C = O at 1724 cm^{-1} (Fig. 3). These changes with increasing temperature are similar to those previously reported for monoolein/water mixtures and indicate weaker hydrogen bonding and greater water penetration into the lipid at higher temperatures [22].

The encapsulation of BHb resulted in a decrease in the order of the acyl chains at lower temperatures compared to PBS/MO (Fig. 4). This was evident as the vibrational frequency of the $\nu_s CH_2$ peak in the cubic phase of the BHb/MO mixtures was consistently higher than observed in buffer/monoolein cubic phase, indicating a slightly increased acyl chain disorder in the presence of the BHb. The BHb/MO samples were not substantially affected by increasing the temperature, while the PBS/MO showed a slight increase in lipid disorder with increasing temperature (Fig. 4) similar to what has been reported for H_2O/MO [22]. Unlike the results reported for H_2O/MO [22] we observe no change in the bandwidth with increasing temperature from 2°C to 60°C

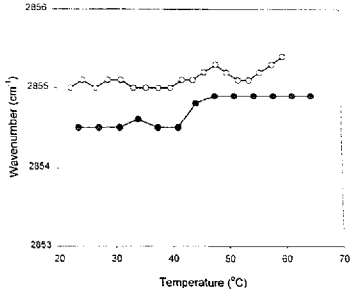

Fig. 4. Vibrational frequency of the symmetric CH_2 stretch of cubic phase monoolein hydrated to 60:40 wt% MO/PBS (●) or 50:40:10 wt% MO/PBS/BHb (○) as a function of temperature.

in either the BHb/MO or the PBS/MO samples (data not shown). Changing the amount of BHb did result in a change in the bandwidth of the asymmetric CH_2 peak, with the greatest bandwidth seen in the sample with 5 wt% BHb (Fig. 5). The increased

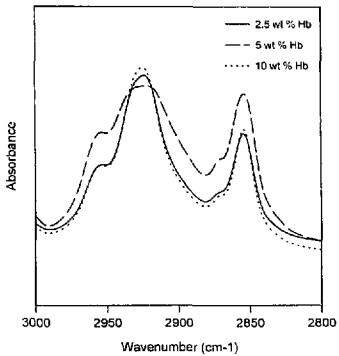

Fig. 5. Symmetric and asymmetric stretching bands of cubic phase monoolein in the presence of 2.5, 5.0, and 10.0% native tetrameric bovine hemoglobin at 37°C. Legends are given in the figure.

Table 1
Correlation coefficients of the amide I band from spectra of native tetrameric bovine hemoglobin entrapped in cubic phase monoolein

Incubation temperature (°C)	Incubation time (h)		
	2	24	Heat denatured [a]
25	0.95	0.92	-
80	-	-	0.37

Values are an average from three samples.
[a] Samples were incubated for 4 h at 80°C.

disorder of the acyl chains in the presence of 5% BHb may be a result of the mixed lattice seen at this Hb concentration (Fig. 1). Nilsson, et al. [22] report no change in CH_2 bandwidths with changes in lattice in H_2O/MO systems; however, they did not probe the region of lattice coexistence of the GMO phase diagram.

3.1. BHb structure

It is possible to gain information about the secondary structure of proteins by monitoring changes in the amide I band at ~1650 cm^{-1} [24]. Although the amount of specific secondary structures present in a sample can be quantified by determination of relative band areas [25], an overall comparison of protein secondary structure provides good insight into secondary structural changes [26]. Comparing the correlation coefficient from cubic phase encapsulated BHb to free BHb reveals that there is not a significant alteration in the secondary structure of the encapsulated BHb in the cubic phase (Table 1). The correlation coefficient of heat denatured BHb is presented to demonstrate changes in the coefficient as a result of significant changes in BHb structure.

Confirmation of oxyhemoglobin structure was made by examining the amount of methemoglobin formation in the cubic phase (Fig. 6). Although methemoglobin formation at 37°C in the cubic phase over 2 h followed a similar pattern to the methemoglobin formation in isotonic buffer, significantly more methemoglobin was formed in the presence of the MO. Additionally, oxygen carrying capacity measurements have been made on BHb encapsulated in the cubic phase and they are not substantially altered from values for native BHb (P_{50} of 27.9 and 29.5, respectively). The addition of $K_3Fe(CN)_6$ to the BHb encapsulated in the cubic phase or BHb in isotonic buffer resulted in a significant increase in methemoglobin formation (70%) over 2 h (Fig. 7). The rapid increase in methemoglobin formation in the cubic phase induced by $K_3FE(CN)_6$ indicates that these ions are able to diffuse through the pores of the cubic phase and oxidize the hemoglobin.

We have also investigated the release of BHb from the bulk cubic phase in vitro. The observed release kinetics show 12% of the measurable hemoglobin released from the cubic phase at 37°C after 2 h (Fig. 8). The release is significantly lower for the samples held at 25°C, with only 3% released after 2 h. After 24 h, however, the situation is reversed, with 41% and 52% of the total Hb released after 144 h at 37 and 25°C respectively. This indicates that the pore diameter of the cubic phase (45 Å) does allow for hemoglobin release although some of the released hemoglobin may desorb from the outer surface of the cubic phase. The greater release seen at 25°C after 25 h is surprising and we are unsure how to explain it. It may result from the high amount of methemoglobin

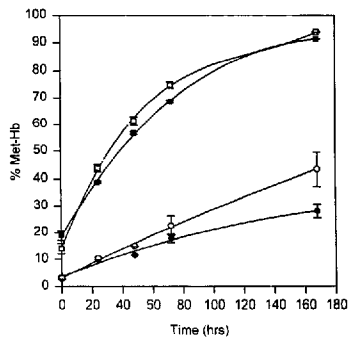

Fig. 6. Amount of methemoglobin formation for bovine hemoglobin entrapped in monoolein at 25 (○) and 37°C (□). Free hemoglobin incubated for the same time at the same temperature serves as a control (●, ■). Significantly more methemoglobin was formed in the presence of monoolein at 37°C; however, at 25°C the presence of monoolein did not significantly affect the amount of methemoglobin formed ($P < 0.05$; $n = 4$).

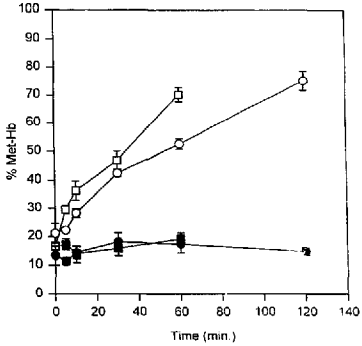

Fig. 7. Conversion of cubic entrapped bovine hemoglobin from oxy to methemoglobin at 25 (○) and 37°C (□) in the presence of $K_3FE(CN)_6$. Controls (●,■) are cubic entrapped bovine hemoglobin not exposed to $K_3FE(CN)_6$. The amount of methemoglobin formed in the presence of the $K_3FE(CN)_6$ is significantly higher than formed in the controls at both 25 and 37°C. ($P < 0.05$; $n = 4$).

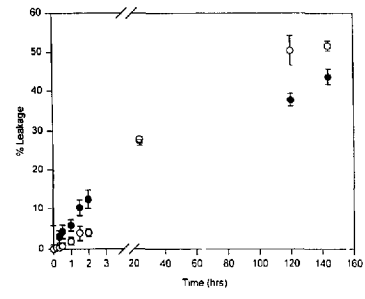

Fig. 8. Leakage of bovine hemoglobin from cubic phase monoolein over time at 25 (○) and 37°C (●). At incubations less than 24 h the amount of hemoglobin released from samples at 37°C is significantly higher than the amount released from samples at 25°C. At incubation times greater than 24 h the trend is reversed ($P < 0.05$; $n = 3$).

formed after long exposure at 37°C (Fig. 6), but this remains to be determined. The retention of Hb in the cubic phase is an important feature as there are a number of untoward physiological effects of free Hb such as hypertension and reticulo endothelial interaction (for review see Ref. [27]). It is possible to alter the release profile of cubic phase MO by the addition of a variety of amphophiles such as egg yolk lecithin and polyethalineglycol phosphatidylcholine (PEG-PE) to the MO before the formation of the cubic phase [12,28], and we are currently investigating the effects of these additives on the BHb-MO cubic phase.

These results indicate that the self-assembled cubic phase of monoolein is suitable for encapsulation of hemoglobin as a red cell substitute. The high encapsulation efficiency, routinely better than 95 percent of the available Hb is encapsulated, and the ease of making cubic encapsulated Hb demonstrate the potential of this system. We are currently conducting studies to determine the best methods of dispersing the cubic/Hb phase to make 'cubisomes' and are further investigating the functionality of dispersed cubisome encapsulated hemoglobin.

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