# The Synthesis of a Reconstituted $C_{60}$-Modified Protein

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We designed and synthesized reconstituted $C_{60}$-modified myoglobin ($C_{60}$-Mb) and found that fundamental characteristics, except autoxidation rate constants, of $C_{60}$-Mb and native Mb were almost identical.

We report here the first synthesis of a reconstituted $C_{60}$-modified protein. Fullerenes possess wide ranging biological reactivity including antiviral activity againt HIV[1,2], selective DNA cleavage[2], and promotion of chondrogenesis[3]. Chen and coworkers[4] recently described the preparation of antibodies specific for fullerenes and discussed protein-fullerene interactions. Fullerenes are also strong photosensitizing reagents and the photophysics, photochemistry and photobiochemistry of fullerenes are of exciting areas.[5] Our objective is to introduce a fullerene moiety into proteins to create novel proteins with specific function. The construction of such systems using proteins may open new fullerene fields in chemistry and biochemistry. There are two reports related to this area.[6,7] Methano[$C_{60}$] fullerene dicarboxylic acid was covalently attached, by Son and coworkers[6], to the amino-residues of native and denatured bovine serum albumin and the modified proteins were purified and characterized. Hill and coworkers[7] recently described the preparation of a fullerene-modified redox protein, azurin mutant S118C, prepared by site-directed mutagenesis methods followed by chemical modification and reported the electrochemical behavior of the protein.

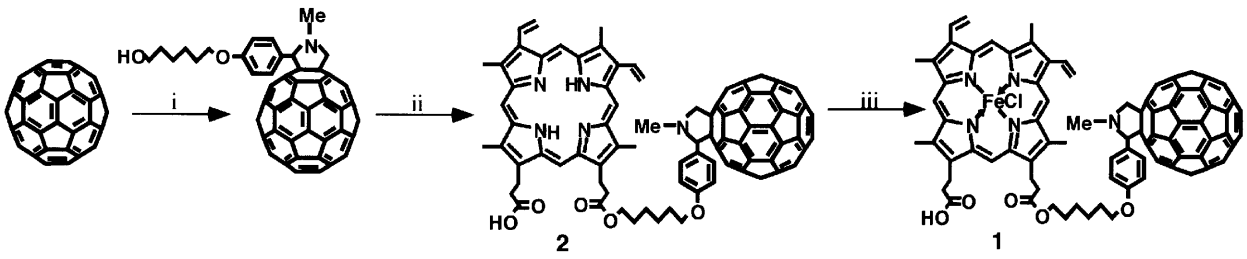
Our strategy for preparing a fullerene-modified protein is to use the heme protein reconstitution method[8]. By substitution of the heme moiety of a protein with hemin derivatives, reconstituted artificial proteins can be prepared. We describe here the preparation of a reconstituted $C_{60}$-modified myoglobin ($C_{60}$-Mb), in which the prosthetic group consists of a hemin-fullerene conjugate. This modified protein is of interest as the functional component of artificial photosynthetic systems. The construction of biodevices based on fullerene-protein conjugated novel materials has possible biological, biochemical, pharmaceutical and medical applications.

A protoporphyrin IX-$C_{60}$ conjugate, **2**, and a hemin-$C_{60}$ conjugate, **1**, were synthesized according to Scheme 1. The reaction of $C_{60}$ with *N*-methylglycine and 4-((6-hydroxyhexyl)oxy)benzaldehyde in chlorobenzene produced *N*-methyl-2-((6-hydroxyhexyl)oxyphenyl)-3,4-fulleropyrrolidine, which was condensed with protoporphyrin IX (acid chloride form) in chloroform to give compound **2**. Iron insertion to **2**[9] produced compound **1**[10].

Apo-Mb from met-Mb (horse heart, Sigma) was prepared according to the literature.[11,12] The purity of apo-Mb was 99% as determined by spectrophotometry.[11-12] Preparation of $C_{60}$-Mb was conducted as follows. A pyridine solution (100 μL) of **1** (0.5 mg, $3.3 \times 10^{-7}$ mol) was added drop-wise to an apo-Mb (1.3 mL, $3.3 \times 10^{-7}$ mol) aqueous phosphate buffer (10 mmol $dm^{-3}$, pH 7.0) at 4 °C. Twenty μL of the buffer solution was added each for one-drop addition of the pyridine solution. In this procedure, the total pyridine content in the buffer was varied between 5-15 vol% and 10 vol% resulted in highest yield of $C_{60}$-Mb. The precipitate was separated by centrifugation (6000 g), followed by through column-chromatography purification (Sephadex G-25, eluent: 50 mmol $dm^{-3}$ phosphate buffer, pH 7.5) to produce $C_{60}$-Mb.

The molecular extinction coefficient the Soret-band of $C_{60}$-Mb (met form) was determined to be 189000,[13] which is very close to that of native Mb (188000)[14]. Figure 1 shows UV-vis absorption spectra of $C_{60}$-Mb alone and the presence of potassium fluoride or sodium azide. The absorption maxima of the Soret- and Q-bands of $C_{60}$-Mb (met-form) appeared at 408 nm and 504 and 630 nm, respectively, which are very close to those of native Mb(met-form, Soret-band: 408 nm, Q-bands: 502 and 630 nm, respectively)[14]. The Soret- and Q-bands of $C_{60}$-Mb in the presence of potassium fluoride were 407 and 602 nm, which are close to those of corresponding native Mb (Soret-band: 406 nm, Q-bands: 604 nm) [14] in the presence of potassium fluoride. The Soret- and Q-bands of $C_{60}$-Mb in the presence of sodium azide appeared at 419 nm and 538 and 571

**Scheme 1.**




i) N-methylglycine, 4-((6-hydroxyhexyl)oxy)benzaldehyde, chlorobenzene, reflux, 43 %; ii) protpporphyrin IX (diacid chloride form), $Et_3N$, THF, rt, 25 %; iii) $FeCl_2$, DMF, 65 °C, 13 %.

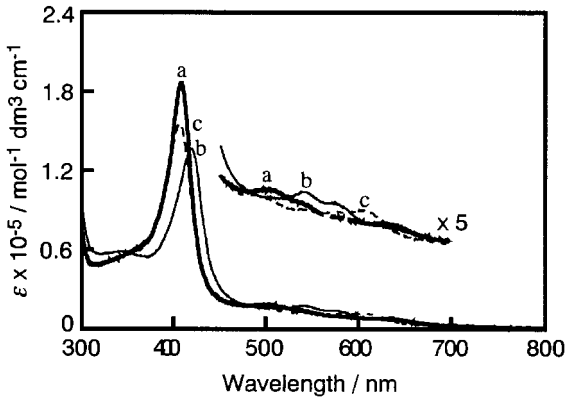



**Figure 1.** UV-vis absorption spectra of $C_{60}$-Mb alone (a) and in the presence of sodium azide (b) or potassium fluoride (c) in phosphate buffer solution (50 mmol $dm^{-3}$,pH 7.5).

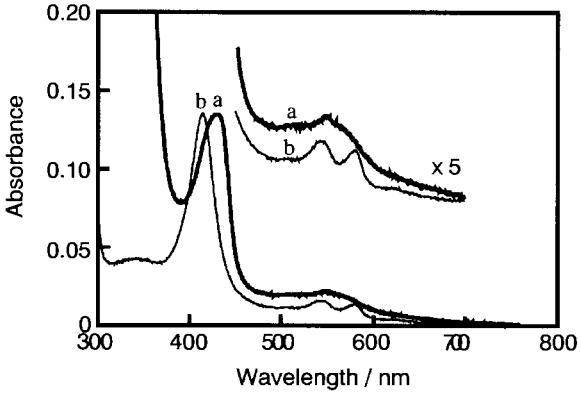



**Figure 2.** UV-vis absorption spectra of $C_{60}$-Mb in phosphate buffer solution (50 mmol $dm^{-3}$, pH 7.5) after the addition of sodium dithionate (a), followed by dioxygen bubbling.

nm, respectively, which are also close to those of corresponding native Mb (Soret-band: 418 nm, Q-bands: 540 and 572 nm) [14]. These results indicate that the heme moieties in both proteins are located in similar microenvironments.

Mb is known to act as an oxygen carrier in mammalian cells. The oxygen binding properties of $C_{60}$-Mb and native Mb were compared. The addition of sodium dithionate to the buffer solution of $C_{60}$-Mb (met-form) produced $C_{60}$-Mb($Fe^{II}$)[15] , and then dioxygen was introduced. Reduced $C_{60}$-Mb was found to form the oxygen complex (Figure 2), whose Soret- and Q-bands appeared at 415 nm and 543 and 578 nm, respectively, which are very close to those of the oxygen complex of native Mb (Soret-band: 418 nm, Q-bands: 541 and 578 nm) [14]. The stability of the oxygen complex of $C_{60}$-Mb was monitored by the time course in absorbance of Q-band at 578 nm. The oxy-form of $C_{60}$-Mb($Fe^{II}$) gradually autoxidized to the met-form with the first-order rate constant of 0.2 $h^{-1}$, which is ca. 6-times faster than that of native Mb measured under the same experimental condition. Introduction of the bulky fullerene moiety to the protein probably contributes to this faster autoxidation.

In conclusion, we have succeeded in the synthesis of reconstituted $C_{60}$-modified myoglobin and found that fundamental characteristics, except the autoxidation rate constants, of $C_{60}$-Mb and native Mb were almost identical. Intense effort is currently underway in our laboratory to define fully the structural characteristics and physicochemical and biological properties and function of $C_{60}$-Mb. We are also preparing zinc-substituted $C_{60}$-Mb as well as fullerene-modified proteins using another proteins instead of Mb.

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9 IR (KBr): 1728 (C=O, ester), and 1710 (C=O, carboxylic acid) $cm^{-1}$. $^1H$ NMR (400 MHz, $CDCl_3$, TMS): δ 1.12-1.84 (m, 8H, $(CH_2)_4$), 2.55 (s, 3H, $NCH_3$), 3.25 (t, 4H, $13^2$, $17^2$, $CH_2$), 3.58 – 3.74 (m, 12H, 2,7,12,18,$CH_3$), 3.85, 4.72 (d, *J* = 9.3 Hz, 1H each , $NCH_2C_{60}$), 3.98 (t, 2H, $COOCH_2$), 4.35 (s, 1H, $NCH(Ar)C_{60}$), 4.40 (m, 6H, $13^1$, $17^1$, $CH_2$, $ArOCH_2$), 6.19, 6.36 (d each, *J* = 11.0 Hz, *J* = 16.9 Hz, 2H each, $=CH_2$), 6.54, 7.55, 7.72 (d, *J* = 7.33 Hz, m, m, 2H, 1H, 1H, ArH), 8.26 (m, 2H, CH=), 10.05 - 10.20 (m, 4H, *meso*-H). Anal. Calcd for $C_{109}H_{55}N_5O_5$ + $8H_2O$: C, 78.93; H, 4.31; N, 4.22 %. Found: C, 78.57; H, 4.42; N, 4.62 %. The absorption maxima of Soret- and Q-bands of **2** in $CHCl_3/CH_3OH$ = 10/1 (v/v) appeared at 407.0 nm and 506.0, 541.5, 575.5 and 688.5 nm, respectively.
10 The absorption maxima of Soret- and Q-bands of **1** in $CHCl_3/CH_3OH$ = 10/1 (v/v) appeared at 402.5 nm and 489.0 and 589 nm, respectively.
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15 Soret- and Q-bands appeared at 431 and 557 nm, respectively, which are close to those of corresponding native Mb($Fe^{II}$) (Soret-band: 435 nm, Q-band: 556 nm).