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Alteration of Properties of Natural Pigments by Conjugation with Fibroin or Polyethylene Glycol

Asako Ishii, Makoto Furukawa, Ayako Matsushima, Yoh Kodera, Akira Yamada, Hirokazu Kanai & Yuji Inada*

Toin Human Science and Technology Center,
Department of Materials Science and Technology, Toin University of Yokohama,
Kurogane-cho, Aoba-ku, Yokohama, 225 Japan

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ABSTRACT

Almost all natural pigments exhibit defects when the pigments are extracted from living bodies; they are much less stable to light than synthetic dyes, and this accounts for why these pigments have not been widely utilized industrially. Another reason is often the poor solubility of natural pigments both in aqueous media and in organic solvents. To overcome these drawbacks, chlorophyll a, *β-carotene and astaxanthin were adsorbed on the silk protein, fibroin, to form pigment–fibroin conjugates. These conjugates exhibit high stability to visible and UV light. Similarly, melanin, an insoluble dermal pigment, was coupled with polyethylene glycol (PEG). The PEG–melanin conjugate is soluble not only in aqueous media but also in organic solvents, and exhibits a photoprotective effect to UV radiation on the growth of* Escherichia coli *cells.*

INTRODUCTION

We have a longstanding interest in the synthesis of bioconjugate materials, including the modification of proteins and enzymes by polyethylene glycol (PEG) derivatives to yield products with nontoxic, amphipathic and non-immunogenic properties.[1] The therapeutic effectiveness of protein drugs is much enhanced by such modification;[2] moreover, PEG-proteins lose their immunoreactivity to corresponding antisera[3] and have prolonged

* To whom correspondence should be addressed.

blood circulation times.[4] In addition, PEG-hydrolases are soluble in organic solvents such benzene, toluene and chlorinated hydrocarbons and catalyze the reverse reactions of hydrolysis; ester synthesis and ester exchange reactions proceeded effectively with PEG-lipase in organic solvents.[5,6] This concept was further expanded to prepare bioactive substances conjugated with inorganic compounds or with PEGs. Chlorophyll–bentonite conjugates[7] exhibit high photostability against the irradiation of light; the absorption maximum, 678 nm, is in good agreement with that of chlorophylls observed in an intact leaf. During the course of these investigations, we became interested in natural pigments such as chlorophyll (green), carotenoids (yellow or red) and melanin (black). Many of these natural pigments are quite unstable to light when they are extracted or isolated from living bodies, and melanin presents even further problems in that it is insoluble in both aqueous media and organic solvents. To overcome these drawbacks, chlorophyll *a* and carotenoids were adsorbed onto silk fibroin, and melanin was 'coupled' with PEG.

EXPERIMENTAL

Chlorophyll *a* from *Spirulina*, and β-carotene from carrots, were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and Sigma Chemical Co. (St Louis, MO). Astaxanthin and silk powder were donated by KI Chemical Industry Co. Ltd (Shizuoka, Japan) and Ikeda Corp. (Tokyo, Japan), respectively. Activated PEG_2, 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine, was synthesized as reported previously.[8] Melanin was obtained from black human hair by treating it with concentrated hydrochloric acid and sodium hydroxide.[9] The molecular weight of melanin was estimated to be 14 000 by GPC (data not shown).

Preparation of pigment–fibroin conjugates

Silk fibroin was dissolved in 26% (w/w) ethanol containing 32% $CaCl_2$, and the fibroin solution was dialyzed against water to form a fibroin suspension. Natural pigments dissolved in organic solvents, i.e. chlorophyll *a* in ethanol, β-carotene and astaxanthin in acetone, were added to the suspension, and each mixture was stirred for 20 min in the dark. The pigment–fibroin conjugates were collected by centrifuging and washed with water to remove solvents prior to being freeze-dried. The amounts of pigment conjugated with 1 g of fibroin were 19 mg for chlorophyll *a*, 9·6 mg for β-carotene and 9·5 mg for astaxanthin.

These conjugates were irradiated with visible light (60 W incandescent

lamp, from a distance of 10 cm) and with UV light at 254 nm (Spectronics XL-1000 UV crosslinker, Westbury, NY; 8 W × 5, from a distance of 15 cm) after adding a trace amount of water. At a given time, the absorbance at absorption maximum of each pigment–fibroin conjugate was measured by the opal glass method.[10]

Preparation of PEG–melanin conjugate

To 2 ml of 0·2 M sodium borate buffer (pH 10·0) containing 3·6 μmol of melanin was added activated PEG_2 (molar ratio of PEG_2/melanin = 0–10) and the reaction mixture was stirred for 12 h at 37°C to obtain PEG–melanin conjugates. After the reaction was completed, the degree of modification of amino groups in the melanin molecule was determined with trinitrobenzenesulfonate.[11] Then 20 ml of chloroform and 2 g of sodium chloride were added to the reaction mixture and the PEG–melanin conjugate was extracted from the reaction mixture into the chloroform layer. On addition of 40 ml of hexane to the chloroform solution, PEG–melanin conjugate was obtained as a dark brown precipitate.

Photoprotection with PEG–melanin

Escherichia coli (XL1-Blue) cells ($1·25 \times 10^6$ cells ml^{-1}) suspended in Luria-Bertani (LB) medium were added to a sterilized quartz cuvette (light path 1 cm). A PEG–melanin solution in another quartz cuvette was placed adjacent to the cuvette containing the bacterial cells. UV light (20 W m^{-2}) at 254 nm (Manasulu-Light, Manasulu Chem. Ind. Co., Tokyo, Japan) was irradiated directly onto the cell-culture cuvette through the PEG–melanin cuvette. After irradiating for 10 min, the cuvette containing bacterial cells was incubated at 37°C while shaking. At a given time, the cell number was estimated by measuring the optical density of the cell suspension at 600 nm.

RESULTS AND DISCUSSION

Natural pigments, chlorophyll *a*, β-carotene and astaxanthin, dissolved in organic solvents, were adsorbed onto silk fibroin to form pigment–fibroin conjugates. The absorption spectrum of the chlorophyll *a*–fibroin conjugate, as measured by the opal glass method, showed two peaks at 669 nm and 740 nm. β-Carotene and astaxanthin adsorbed onto fibroin, and their conjugates had absorption maxima at 450 nm and 500 nm, respectively. The absorption maxima of β-carotene in hexane, and of astaxanthin in ethanol, were 450 nm and 476 nm, respectively.

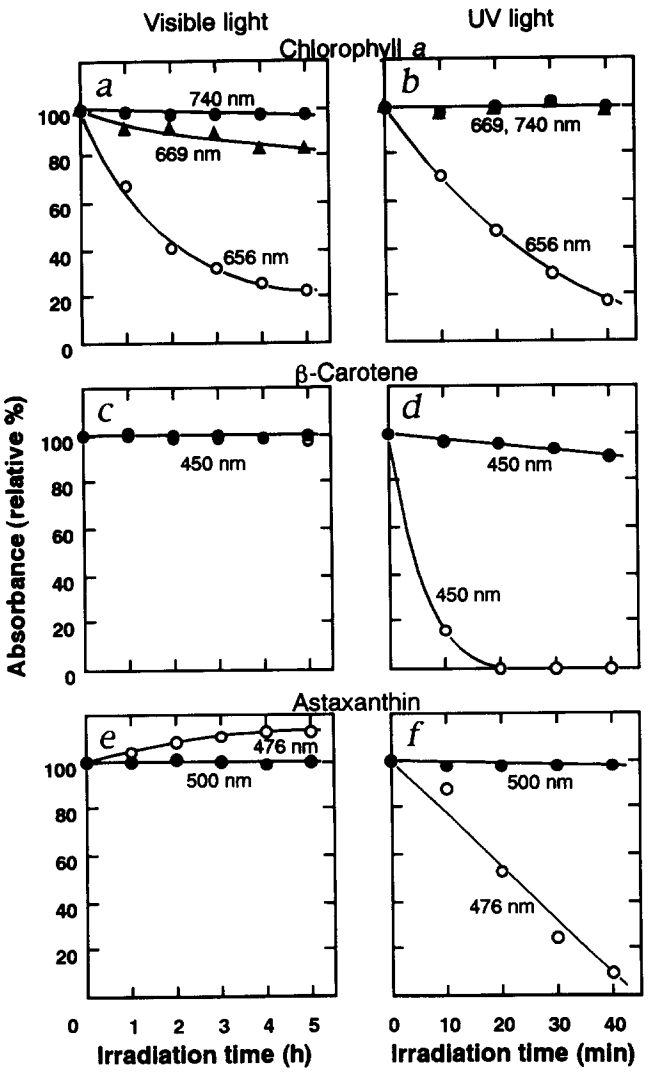

Fig. 1. Photostability of natural pigments to visible (left) and UV (right) light. Chlorophyll *a* (a and b), β-carotene (c and d) and astaxanthin (e and f). Closed and open symbols: pigment–fibroin conjugates and free pigments, respectively.

Figure 1 shows the stability of the pigment–fibroin conjugates, together with the free pigments, as a function of irradiation by visible and UV light. In the case of the chlorophyll *a*–fibroin conjugate, the absorption maxima at 740 nm and 669 nm were minimally lowered by either type of irradiation (Figs 1(a) and (b)). On the other hand, free chlorophyll *a* dissolved in ethanol was rapidly decolorized on irradiation by both visible and UV light. Similarly, the β-carotene–fibroin conjugate (Figs 1(c) and (d))

and the astaxanthin–fibroin conjugate (Figs 1(e) and (f)) were also stable to visible and UV irradiation. On the other hand, fibroin-free pigments were rapidly decolorized by irradiation with UV light (Figs 1(d) and (f)), although they were not decolorized by visible light (Figs 1(c) and (e)). The absorbance of free astaxanthin was slightly increased (Fig. 1(e)) but the reason for this is unclear. The effective photostabilization of chlorophyll *a*–fibroin conjugates depends on the presence of a trace amount of water. Shibata *et al.*[12] reported that water-soluble chlorophyll *a* bound to bovine serum albumin has an absorption maximum at 740 nm. The peak at 740 nm may reflect the aggregation of chlorophyll *a* molecules coordinated with two molecules of water $(\text{Chl}a \cdot 2H_2O)_n$.[13]

Melanin, a black pigment found in living organisms such as mammalian hair, skin and eyes, is known to have a photoprotective function for human beings.[14,15] Because of its insolubility in aqueous media or organic solvents, little is known about the details of its physiological and biological function. In order to effect its solubilization, melanin isolated from black human hair was coupled with activated PEG_2, an amphipathic macromolecule. Figure 2(a) shows the degree of modification of the amino groups in the melanin molecule and the amount of the conjugate extracted with chloroform. The solubility was markedly increased by increasing the molar ratio of activated PEG_2 to melanin. After coupling 36% of the total amino groups in the melanin molecule with activated PEG_2, the PEG–melanin conjugate reached a solubility in water of 12 g per 100 g, and in organic solvents such as benzene, ethanol and chloroform solubilities ranging from 6 g per 100 g to 11 g per 100 g (Fig. 2(b)). Thus, the PEG–melanin conjugate can now be useful in various circumstances, where before it was inert. To find whether PEG–melanin has photoprotective capability, dividing *E. coli* cells were exposed to UV light in the presence and absence of PEG–melanin (Fig. 2(c)). The number of bacteria increased logarithmically with time in the absence of UV irradiation. After 10 min irradiation with UV light cell growth virtually stopped, indicating that dividing *E. coli* cells are photosensitive. When PEG–melanin solutions were placed in the light path between the cells and the UV lamp, the growth of cells clearly depended upon the concentration of PEG–melanin. At 15 mg ml^{-1} of PEG–melanin, the growth curve was almost the same as that in the absence of irradiation. These results indicate that PEG–melanin conjugates can play an important role in the photoprotection of cell growth against UV radiation.

In summary, the stability of various natural pigments is markedly improved by adsorption onto silk fibroin, apparently because the fibroin substitutes for proteins that naturally stabilize these materials *in vivo*. Furthermore, melanin can be solubilized by conjugation to PEG, and the

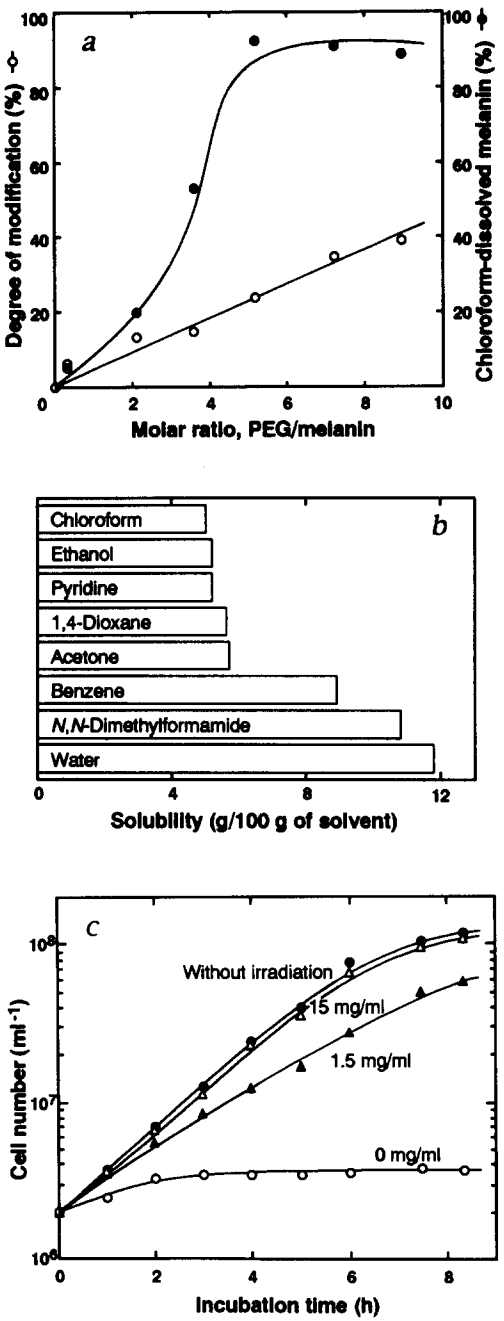

Fig. 2. Solubilization of melanin by conjugating with activated PEG_2 (a and b) and photoprotection of *E. coli* cell growth with PEG–melanin solution against UV irradiation (c). The concentration of PEG–melanin was spectrophotometrically determined assuming the extinction coefficient ($E^{1\%}$) at 460 nm to be 66·7.

product has been shown to be capable of photoprotection in the UV region. Experiments are now in progress to find whether PEG–melanin conjugates play a role in scavenging radicals formed *in vivo* and *in vitro*. These techniques can be extended to the manufacture of other bioconjugate materials which may open a new avenue to biomedical and biotechnical processes.[16–18]

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