



## Short communication

## Water-soluble hypocrellin A nanoparticles as a photodynamic therapy delivery system

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## ABSTRACT

A novel delivery system for the hydrophobic photosensitizer, hypocrellin A, has been developed that uses hypocrellin A nanoparticles prepared by a reprecipitation method. In comparison to the unmodified photosensitizer, the hypocrellin A nanoparticles not only possess superior water solubility, higher water and light stability but also display high singlet oxygen production and DNA photocleavage capability.

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## 1. Introduction

Hypocrellins such as hypocrellin A (HA) (Fig. 1) and hypocrellin B (HB), a class of natural photosensitizers (PSs), have been studied intensively in the field of photodynamic therapy (PDT) owing to their very high singlet oxygen generation ability [1]. Originally, hypocrellins were utilized as potent therapeutic agents for vascular–capillary diseases or skin diseases such as white lesions of the vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis [2,3]. Recently, it was reported that these natural perylenequinonoid compounds could display light-induced antitumor and antiviral activities, most notably against human immunodeficiency virus (HIV) [4–6]. These significant biological activities have renewed attempts to study the potential of hypocrellins in the clinical application of PDT.

However, investigations have shown that, due to its hydrophobicity, i.e., poor-water solubility, the preparation of pharmaceutical formulations of hypocrellins was difficult. To solve this solubility problem, approaches such as structural modifications

and complexations with metal ions have been attempted. However, the resulting water-soluble derivatives show very poor photodynamic activity in vitro due to their reduced cellular uptake [7–12]. Alternatively, carriers such as surfactants, liposome, amylase, butter oil and ceramic nanoparticles have been used to deliver hypocrellins [13,14]. Upon systemic administration, such drug-doped carriers are preferentially taken up by tumor tissues. But unfortunately, the preparation of the above delivery systems is not only complicated but also can lead to side-effects, for example, using surfactants in the preparation of surfactant-based drug delivery system, tends to increase the systemic toxicity of the drug formulation.

Therefore, the development of novel drug formulation and delivery methods, without the addition of any external agents such as surfactants or other carrier vehicles, has received intensive attention. One method proposed for dispersion of hydrophobic compounds in water is the “reprecipitation method” [15–19]. This method is very simple, and the system has no external agents.

In this paper, it is reported that hypocrellin A nanoparticles (HANPs), which have been successfully prepared through the reprecipitation method, can improve the water-solubility, stability, and singlet oxygen quantum yield and photocleavage capability to DNA effectively. To our best knowledge, this is the first report to use this simple method with hypocrellin A.

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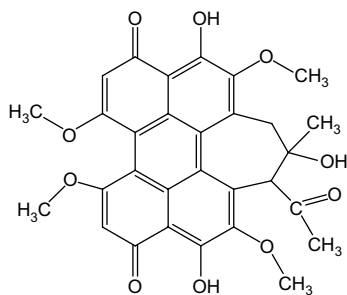


Fig. 1. Molecular structure of hypocrellin A (HA).

## 2. Materials and methods

### 2.1. Reagents

9,10-Anthracendipropionic acid, dimethyl sulfoxide (DMSO), ethidium bromide (EB) and calf thymus deoxyribonucleic acid (CT DNA) were all purchased from Sigma–Aldrich Corporation. Hypocrellin A was isolated from the fungus sacs of *Hypocrella bambusae*, and recrystallized twice from acetone before use. The purity of the hypocrellin A sample obtained was greater than 98% (determined by HPLC).

### 2.2. Preparation and characterization of HANP

HANP was prepared using the approach similar to that reported by the Nakanishi group and the Prasad group [20,21]. A solution of hypocrellin A in DMSO (200  $\mu$ L, 3 mmol) was injected into 10 mL of phosphate buffer (pH = 7.4) at room temperature with controlled stirring. After the formation of hypocrellin A nanoparticles, DMSO was removed completely by dialyzing the solution against phosphate buffer in a 12–14 kDa cutoff cellulose membrane for 12 h. In order to verify that the DMSO had been completely removed the energy dispersion spectrum (EDS) was used to detect the sulfur content of the samples before and after dialysis. The results indicated that the sample contains sulfur before dialysis, but after dialysis, the sulfur cannot be detected. Therefore, it indicates that the DMSO can be removed completely through the dialyzing method. Transmission electron microscopy (TEM) was employed to determine the morphology and size of the aqueous dispersion of nanoparticles, using a JEOL JEM 2020 electron microscope, operating at an accelerating voltage of 200 kV.

### 2.3. Light stability

The air-saturated solution of HANP or hypocrellin A was illuminated in a 1 cm cuvette by a high pressure mercury lamp (500 W) and its UV–vis spectra from 350 nm to 800 nm were recorded every minute.

### 2.4. Singlet oxygen detection

Singlet oxygen detection measurements were carried out in a plastic cuvette using the disodium salt of 9,10-anthracendipropionic acid (ADPA) as a singlet oxygen sensor [22]. In a typical experiment, an aqueous solution of ADPA (150  $\mu$ L, 5.5 mmol) was mixed with HANP (3 mL). The control experiment used ADPA mixed with aqueous solution of HA, which has been prepared by adding small amounts of concentrated DMSO solutions of HA to phosphate buffer. These solutions were irradiated with a 500 W high-voltage mercury lamp with 470 nm cutoff filter. The optical densities at

378 nm (characteristic absorption peak of ADPA) were recorded every 30 s using VARIAN Cary 5000 UV–vis spectrophotometer.

### 2.5. Photodegradation of CT DNA analysis

To study the PDT properties of HANP, the air-saturated buffer solution of CT DNA (8  $\mu$ mol/L) (10 mmol/L ammonium acetate, 100 mmol/L sodium chloride, pH = 7.0) containing ethidium bromide (16  $\mu$ mol/L) was used as phototherapeutic target, after adding HASN (HA) into the above solution, the mixture was irradiated with light above 470 nm, and the fluorescence spectrum of the mixture was recorded every 1 min [23–25].

## 3. Results and discussions

### 3.1. TEM imaging of HANP

A TEM image of HANP prepared by reprecipitation method is shown in Fig. 2. The particles are nearly cubical, having uniform size distribution, with an average diameter of 100 nm.

### 3.2. Optical spectroscopy and stability

Between 350 nm and 800 nm, free HA has three characteristic absorbance bands, located at 476 nm, 550 nm and 594 nm, respectively (Fig. 3). After HA was converted into HANP, all the three characteristic absorbance bands were slightly red-shifted to 483 nm, 552 nm and 596 nm, respectively. The intensity of the latter two absorbance bands increased slightly. This phenomenon indicated that the surroundings of HA molecules has been changed, i.e., the HA molecules on the surface of nanoparticles can prevent the inner HA molecules from water molecules, and the inner HA molecules exist in the hydrophobic surroundings [26].

Furthermore, the absorption spectrum of two aqueous solutions of HA and HANP has shown a remarkable difference after lifting to stand for three days (Fig. 4). The absorption intensity of three characteristic absorbance bands of HANPs has no obvious change but that of the HA aqueous solution decreased in intensity. Thus, comparing the changes in the absorption spectrum, it can be

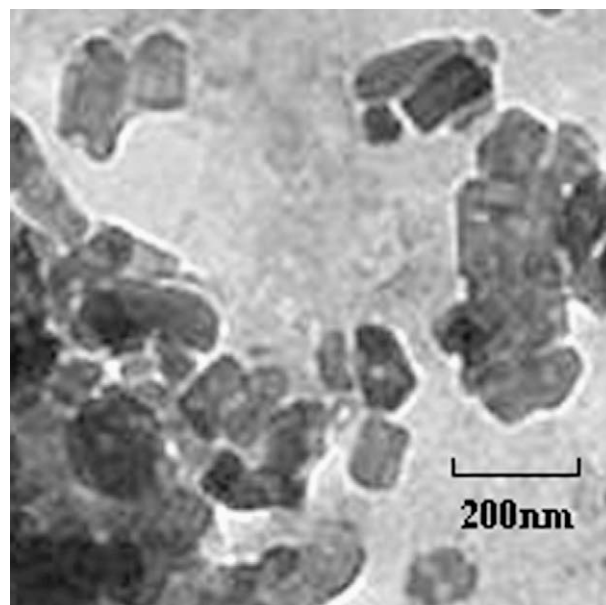


Fig. 2. TEM image of HANP.

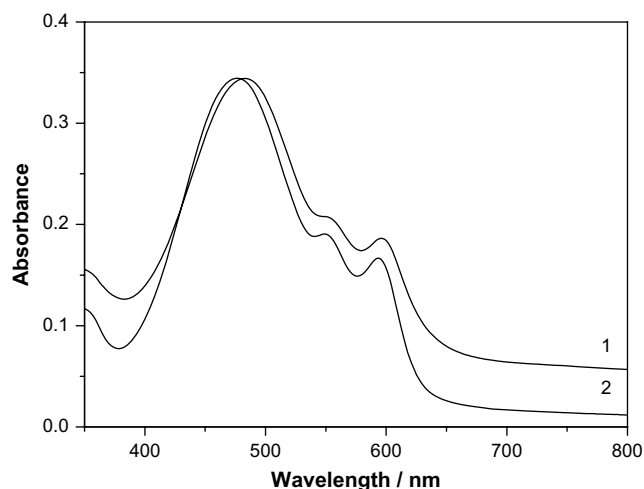


Fig. 3. Absorption spectra of HANP (1) and HA (2) in the aqueous solution.

determined that HANP has excellent stability in the aqueous solution. This could be due to negative surface charges on HANP, which is presumably derived from the deprotonation of the phenol hydroxyls [18].

### 3.3. Light stability

Most photosensitizers, including HA, will degrade during the process of PDT by photobleaching. In this process, absorption strength or fluorescence intensity will reduce [27]. Thus, the medicine that is used in PDT must not only have a strong lethal effect on cancer cell but must also have a slow photobleaching speed [28].

In order to study the photostability of the HANP, photobleaching experiments were carried out. When the air-saturated solution of HANP (or HA) was irradiated by a high pressure mercury lamp, the intensity of the absorption peaks decreased with the irradiation time (Fig. 5: inset panel).

Under the same experimental conditions, photobleaching efficiencies of HANP and free HA are 15.95% and 22.58%, respectively (Fig. 5), which illustrate that HANP has better photostability than HA due to the protection of the exterior HA molecules, and as a consequence HANP is more suitable to be studied in PDT than HA.

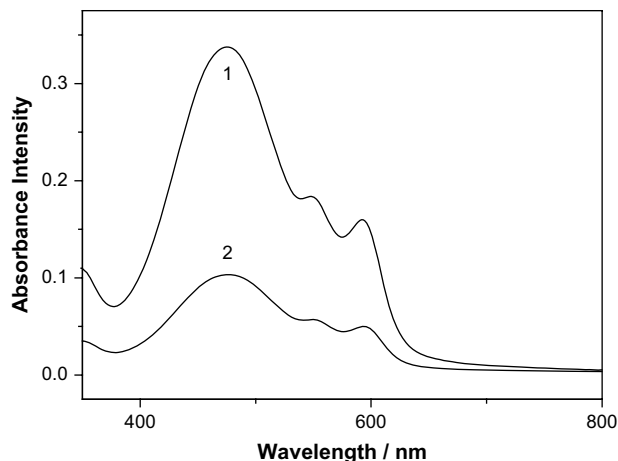


Fig. 4. Absorption spectra of aqueous solutions of HA (1) and of HA left to stand for 3 days (2).

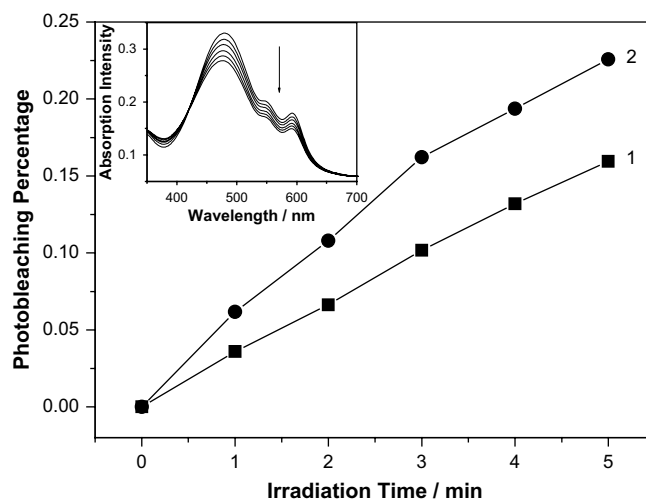


Fig. 5. Photobleaching percentages of HANP (1) and HA (2) with the irradiation time. (inset picture: absorption spectra of HANP after irradiation with visible light for 0, 1, 2, 3, 4, 5 min).

### 3.4. Detection of singlet oxygen

Singlet oxygen is believed to be one of the reactive intermediates in PDT. Consequently, the quantum yield of  $^1\text{O}_2$  is an important parameter to evaluate the application potential of the photosensitizer in PDT. Thus, the ADPA bleaching experiment was carried out to detect the  $^1\text{O}_2$  generation quantum yield of HANP, taking HA as a reference. If the absorption intensity of ADPA continuously decreased as the irradiated time increase, the generation of singlet oxygen is confirmed (Fig. 6 inset panel). Because  $^1\text{O}_2$  can react irreversibly with ADPA to produce the endoperoxide derivative of ADPA (Scheme 1), and the  $^1\text{O}_2$  generation quantum yield can be calculated through comparing the decrease of the absorption intensity of ADPA [29].

ADPA bleaching experiments indicated that the  $^1\text{O}_2$  generation efficiency of HANP is 1.08 (taking free HA as a reference) (Fig. 6). After allowing HANP and HA to left to stand for 3 days, ADPA

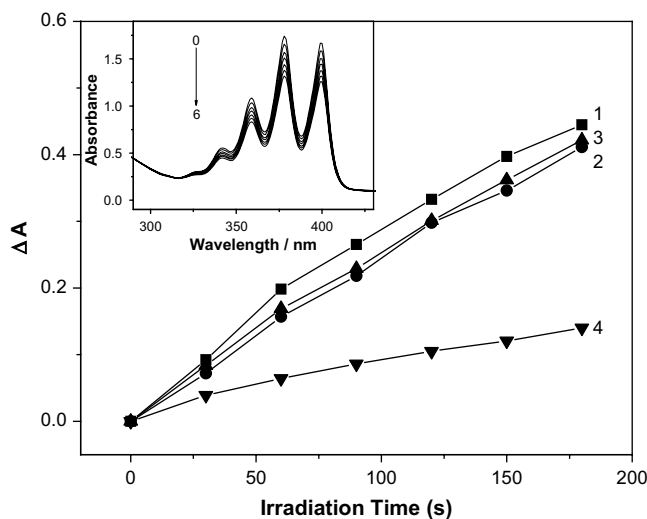
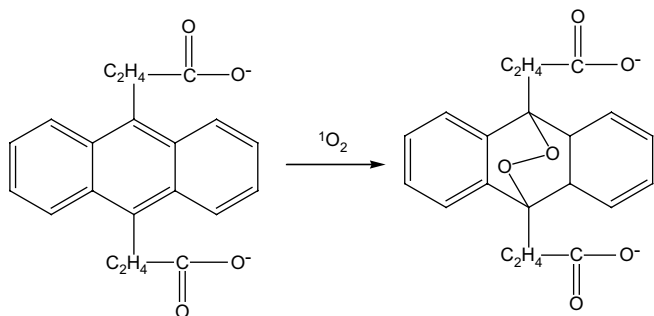


Fig. 6. Photosensitized ADPA bleaching by measuring absorbance decrease ( $\Delta A$ ) at 378 nm as a function of irradiation time. (1: HANP; 2: HA; 3: HANP was left to stand for 3 days; 4: HA was left to stand for 3 days) (inset picture: 0–6: absorption spectra of ADPA in HANP system were irradiated for 0, 30, 60, 90, 120, 150, 180 and 210 s).



Scheme 1. Reaction of ADPA with  $^1\text{O}_2$  to form the endoperoxide.

bleaching of HANP and HA were detected again, and the calculation results indicated that the  $^1\text{O}_2$  generation quantum yields of HANP and HA were 1.03 and 0.34, respectively. These values indicate that nanoparticulation can improve the  $^1\text{O}_2$  generation ability of HA effectively. This greater ability to generate singlet oxygen and improved stability suggests that HANP is a promising candidate for use in PDT.

### 3.5. Photodegradation of CT DNA analysis

Hypocrellins were investigated as a potential DNA cleavage agent. According to previous references, HA can abstract one electron from DNA upon irradiation, and thus induce damage and cleavage of DNA. One electron transfer from DNA to triplet HA will result in the formation of two reduced forms of HA,  $\text{HA}^{\cdot-}$  and  $\text{HAH}_2$ , which are the precursors of superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) and hydroxyl radical ( $\cdot\text{OH}$ ). On the other hand, singlet oxygen ( $^1\text{O}_2$ ) is formed via the energy transfer from triplet HA to molecular oxygen. These reactive oxygen species promote the cleavage of DNA [30].

When irradiation was carried out in the HANP-EB-CTDNA buffer aerobic solution with light above 470 nm, 36.88% binding sites can be destroyed during 6 min, while only 25.74% binding sites were damaged when free HA was used as the photosensitizer. These results suggest that the photocleavage ability of HANP is superior to free HA (Fig. 7), which is consistent with the  $^1\text{O}_2$  generation quantum yield results.

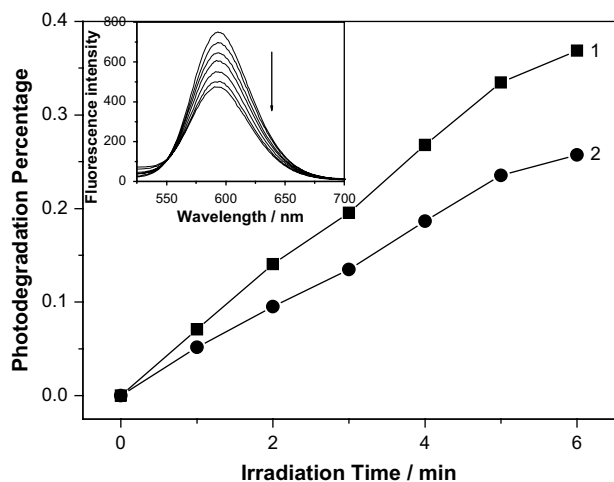


Fig. 7. Photocleavage percent of CT DNA by HANP (1) or HA (2) detected by photo-degradation percentage of ethidium bromide to the damaged CT DNA (inset picture: photodegradation of CT DNA by HANP by irradiated for 0, 1, 2, 3, 4, 5 and 6 min).

## 4. Conclusions

In conclusion, HANP can be prepared through the reprecipitation method greatly improving the water solubility, stability and ability of singlet oxygen generation. Furthermore, the high  $^1\text{O}_2$  generation quantum yield of HANP results in a higher capability to photocleave DNA than free HA. The properties of HANP make it a likely prospect for application in the clinical therapy of PDT.

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## References

- [1] Miller GG, Brown K, Ballangrud AM, Barajas O, Xiao Z, Tulip J, et al. Preclinical assessment of hypocrellin B and hypocrellin B derivatives as sensitizer for photodynamic therapy of cancer: progress update. *Photochemistry Photobiology* 1997;65:714–22.
- [2] Yang HL, Huang FR. Quantum chemical and statistical study of hypocrellin dyes with phototoxicity against tumor cells. *Dyes and Pigments* 2007;74: 416–23.
- [3] Zhao YW, Xie J, Zhao JQ. The photosensitization activity of a water-soluble carboxylate of hypocrellin B. *Dyes and Pigments* 2005;66:61–8.
- [4] Diwu ZJ, Lown JW. Hypocrellins and their uses in photosensitization. *Photochemistry Photobiology* 1990;52:609–16.
- [5] Petrich JW. Excited-state intramolecular H-atom transfer in nearly symmetrical perylene quinones: hypericin, hypocrellin, and their analogs. *International Reviews in Physical Chemistry* 2000;19:479–500.
- [6] Hudson JB, Zhou J, Chen J, Harris L, Yip L, Towers GH. Hypocrellin, from *Hypocrella bambuase*, is phototoxic to human immunodeficiency virus. *Photochemistry Photobiology* 1997;60:253–5.
- [7] He YY, Jiang LJ. Synthesis and EPR investigations of new aminate hypocrellin derivatives. *Free Radical Biology and Medicine* 2000;28:1642–51.
- [8] Zhou JH, Xia SQ, Chen JR, Wang XS. The photodynamic property improvement of hypocrellin a by chelation with lanthanum ions. *Chemical Communications* 2003;12:1372–3.
- [9] Xia SQ, Zhou JH, Chen JR, Wang XS, Zhang BW. A tyrosine-modified hypocrellin B with Affinity for and photodamaging ability towards calf thymus DNA. *Chemical Communications* 2003;23:2900–1.
- [10] Zhou JH, Liu JH, Feng YY, Wei SH. Synthesis and characterization of the monomer ruthenium complex of hypocrellin B. *Bioorganic and Medicinal Chemistry Letters* 2005;15:3067–70.
- [11] Zeng ZH, Qiao R, Zhou JH, Xia SQ, Zhang Y. Photodynamic properties of dipeptide-modified hypocrellin B derivatives: the role of tyrosine and tryptophan groups. *Journal of Physical Chemistry B* 2007;111:3472–9.
- [12] Qiao R, Zeng ZH, Xia SQ, Zhou JH, Liu YY. Using electrostatic interactions to increase the photodamaging ability of hypocrellin B: synthesis and study of 2-(dimethylamino) ethanethiol-modified HB. *New Journal of Chemistry* 2007;3: 196–8.
- [13] Zhao BZ, Xie J, Zhao JQ. A novel water-soluble nanocrystals of hypocrellin B and their interaction with a model protein-C-phycoerythrin. *Biochimica et Biophysica Acta* 2004;1670:113–20.
- [14] Zhou JH, Zhou L, Dong C, Feng YY, Wei SH, Shen J, et al. Preparation and photodynamic properties of water-soluble hypocrellin A-silica nanospheres. *Materials Letters* 2008;62:2910–3.
- [15] Ji XH, Fu HB, Xie RM, Xiao DB, Yao JN. Perylene nanocrystals prepared by reprecipitation method. *Chinese Journal of Chemistry* 2002;20:123–6.
- [16] Fu HB, Wang YQ, Yao JN. Characterization of the optical size-dependence of pyrazolines nanocrystals. *Chemical Physics Letters* 2000;322:327–32.
- [17] Kasai H, Nalwa HS, Okada S, Oikawa H, Nakanishi H. Fabrication and spectroscopic characterization of organic nanocrystals. *Nanostructure Materials* 2002;5:433–73.
- [18] Ji XH, Fu HB, Cao YA, Xie RM, Zhang XT, Yao JN. Preparation and characteristics of perylene nanocrystals. *Chemical Journal of Chinese University* 2001;22:1394–6.
- [19] Baba K, Kasai H, Okada S, Oikawa H, Nakanishi H. Fabrication of organic nanocrystals using microwave irradiation and their optical properties. *Optical Materials* 2003;21:591–4.
- [20] Kasai H, Nalwa HS, Oikawa H, Okada S, Matsuda H, Minami N, et al. A novel preparation method of organic microcrystals. *Japanese Journal of Applied Physics Part 2 Letters* 1992;31:1132–4.
- [21] Koichi B, Haridas EP, Indrajit R. New method for delivering a hydrophobic drug for photodynamic therapy using pure nanocrystal form of the drug. *Molecular Pharmaceutics* 2006;6:289–97.
- [22] Lindig BA, Rodgers MAJ, Schaap AP. Determination of the lifetime of singlet oxygen in D2O using 9,10-anthracenedipropionic acid, a water-soluble probe. *Journal of the American Chemical Society* 1980;102:5590–3.

- [23] Pruetz WA. Inhibition of DNA–ethidium bromide intercalation due to free radical attack upon DNA. (I) Comparison of the effects of various radical. *Radiation and Environmental Biophysics* 1984;23:1–6.
- [24] Pruetz WA. Inhibition of DNA–ethidium bromide intercalation due to free radical attack upon DNA. II. Copper (II)-catalysed DNA damage by  $O_2^-$ . *Radiation and Environmental Biophysics* 1984;23:7–18.
- [25] Wei SH, Zhou JH, Huang DY, Wang XS. Synthesis and type I/type II photosensitizing properties of a novel amphiphilic zinc phthalocyanine. *Dyes and Pigments* 2006;71:61–7.
- [26] Zou W, An JY, Jiang LJ. A study of spectral properties and the binding ability of hypocrellin A in liposomes. *Acta Biochemica et Biophysica Sinica* 1995;6: 685–9.
- [27] Yu KC, Cheng H, Jin L. Progress of synthesizing phthalocyanines used for photodynamic therapy. *Photographic Science and Photochemistry* 2003;21: 138–46.
- [28] Xu DY. Photosensitizers in photodynamic therapy – history, present status and future perspectives. *Chinese Journal of Laser Medicine and Surgery* 2001;10:44–7.
- [29] Tang W, Xu H, Kopelman R, Philbert MA. Photodynamic characterization and in vitro application of methylene blue-containing nanoparticle platforms. *Photochemistry and Photobiology* 2005;81:242–9.
- [30] He YY, Jiang LJ. Photosensitized damage to calf thymus DNA by a hypocrellinderivative: mechanisms under aerobic and anaerobic conditions. *Biochimica et Biophysica Acta* 2000;1523:29–36.