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Delivering a hydrophobic anticancer drug for photodynamic therapy by amorphous formulation

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ABSTRACT

An amorphous formulation of hypocrellin A for photodynamic therapy is reported which can provide stable aqueous dispersion of such hydrophobic photosensitizers. In vitro studies have demonstrated the active uptake of amorphous formulation of hypocrellin A into the mitochondria of tumor cells. Compared with Tween-80 micelle embedded hypocrellin A, low dark-toxicity but similar light-toxicity of the amorphous one to drug impregnated tumor cells was observed. Thus, the potential of using amorphous formulation of hypocrellin A as drug delivery system for photodynamic therapy has been demonstrated.

Photodynamic therapy (PDT) has been investigated since the early 20th century, and is currently well established either as primary or as an adjunctive treatment for several types of cancer.[1] Its clinical use has been limited by its poor bioavailability because commonly used photosensitizers (PSs) are hydrophobic.[2] Some approaches to enhance water-solubility have been attempted such as structural modification, complexation with metal ions, and encapsulations of PSs by surfactants, liposomes, amylase, butter-oil, polyelectrolyte-multilayer, ceramic nanoparticles, etc. However, these synthetic processes are inherently complex and expensive, and some resulting water-soluble derivatives show poor photodynamic activity in vitro due to reduced cellular uptake.[3] Moreover, the use of surfactants or the byproducts produced during preparation of drug delivery systems tends to increase toxicity of the drug. Ideally safe and efficient PDT drug delivery must use a minimal number of additional ingredients apart from the PSs while effectively preserving the photodynamic capability of the PSs.[4]

In the past years, there has been high interest in the use of amorphous forms as active pharmaceutical ingredients because of their potential for improving oral bioavailability of poorly soluble drugs.[5] When prepared in amorphous forms, many insoluble drugs exhibit significantly higher solubility and faster dissolution than their crystalline form. It has been demonstrated that when amorphous compounds are appropriately prepared and delivered orally, their enhanced dissolution rate and solubility result in improved bioavailability if the gastrointestinal tract is the limiting factor for absorption.[6]

Even though the amorphous formation of hydrophobic compounds has been well studied in drugs taken orally, little studies have been carried out using this technique for PDT drug delivery through intravenous injection. To investigate this concept for PDT, we have prepared the amorphous formulation of hypocrellin A (AM-HA) based on our previous studies.[7,8] Hypocrellin A (Cambridge crystallographic data centre number 663042) is a photosensitive agent, that belongs to second-generation photosensitizers, including 5-aminolevulinic acid (ALA), phthalocyanins, temoporfin (mTHPC), etc. In clinical trials, HA has produced promising results in the treatment of various skin diseases such as white lesions of vulva and vitiligo.[9] Furthermore, HA can kill tumor cells efficiently and has been proposed as a potential photosensitizer for PDT because of their high quantum yields of singlet oxygen, availability in pure monomeric form and low dark-toxicity.[10] In this letter, amorphous formation of hypocrellin A was prepared using the reprecipitation procedure and the Tween-80/hypocrellin A (TW-HA, 2% Tween-80 v/v) micelle of the same drug concentration was used as a control delivery system.[11]

The X-ray diffraction patterns (XRD) (Fig. 1A) of the raw HA exhibits intense crystalline peaks between 3° and 40°.[12] This proves that the raw HA was highly crystalline. However, only one broad and diffuse maximum peak was detected in the pattern of the amorphous formulation of HA (the aqueous solution can be lyophilized to obtain the solid form of AM-HA). Furthermore, the differential scanning calorimetry (DSC) curve of HA shows a melting point at 220 °C. In contrast, the AM-HA showed glass

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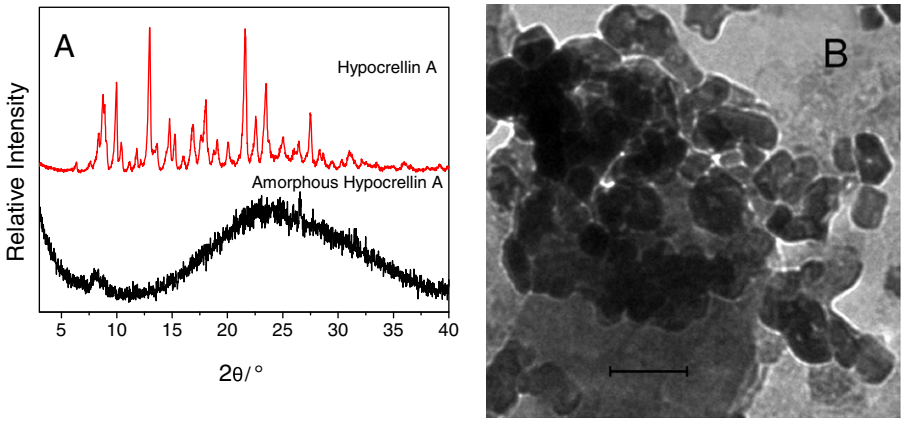

Figure 1. (A) XRD of raw HA and amorphous formulation of HA; the TEM image of AM-HA (Bar = 200 nm).

transitions without any crystallization or melting derived from confirming the formation of amorphous forms. In addition, sodium of 9,10-anthracenedipropionic acid (ADPA) bleaching experiments indicated that the $^1O_2$ generation efficiency of AM-HA is 1.08 (taking free HA as a reference) in aqueous solution using the protocol procedure.[8,13] This value indicated that AM-HA is not homogenously distributed in water and further morphology detection using transmission electron microscopy (TEM) verified this hypothesis. The TEM image (Fig. 1B) showed that AM-HA is nanoparticle formation because of the reprecipitation procedure.[8,14] The increased $^1O_2$ generation ability of AM-HA comparing with free HA was possibly because the nanoparticle formation protected the interior HA molecules from exposure to the aqueous environment, slowing down the $^1O_2$ quenching speed.

The absorption and fluorescence emission spectrum of TW-HA and AM-HA are similar with HA, indicating no changes in the HA chromophore upon preparing amorphous formulation. These results demonstrate that AM-HA formulation can be used both in photodynamic therapy and bioimaging because its absorbance and emission are not affected by the crystal form transition.

Mitochondria have been identified as a sensitive intracellular targets for PDT, and HA has been found to localize to the mitochondria.[7,15] The localization of AM-HA and TW-HA were studied by co-incubating photosensitizers (AM-HA or TW-HA) treated HeLa cells with Rhodamine 123, a mitochondria specific fluorescent probe.[16] From the results summarized in Figure 2, the fluorescence microscope images clearly indicated that the AM-HA and TW-HA localized in the same subcellular region as the mitochondrial marker, suggesting their affinity to mitochondria. Therefore, the crystal form transition did not affect the subcellular distribution of HA.

The cell nuclear morphology change is very important index of cell death and the Hoechst 33342 is sensitive to DNA and was used to assess changes in nuclear morphology by the protocol method.[17] Results of Hoechst 33342 staining assay showed that the fluorescence of normal cells chromatin stained dimly and occupied the majority of the cell (Fig. 3A). But after irradiation the AM-HA (Fig. 3B) or TW-HA (Fig. 3C) treated cells, nuclear morphology changes such as nuclear shrinkage, chromatin condensation or fragmentation were demonstrated and the fluorescence of chromatin was condensed, intensely stained, or shifted to the periphery of the cell.

In order to estimate the cell viability, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to examine the IC_{50} of HeLa cells after treatment with AM-HA and TW-HA with or without visible light.[18] Comparing with TW-HA, AM-HA have similar light-toxicity but lower dark-toxicity in vitro. The ratios (R_f) of dark-toxicity to light-toxicity for AM-HA and TW-HA are 7.65 and 3.64, respectively. These ratios indicate that compared to traditional delivery vehicles, the water-soluble HA, as an amorphous formulation can retain high light-toxicity and simultaneously reduce dark-toxicity because the surfactant-based drug dispersions, Tween-80 micelle, tend to increase the toxicity of the drug formulation.[4]

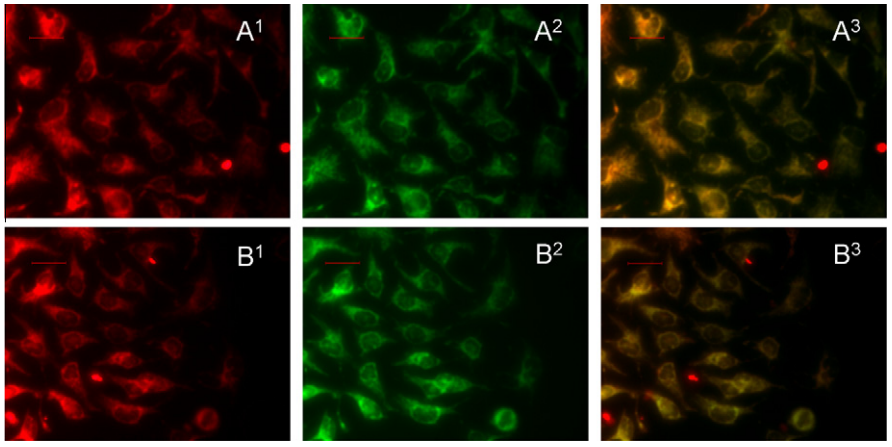

Figure 2. Fluorescence microscope images of the double-stained HeLa cells with AM-HA (or TW-HA) and Rhodamine 123 (each 5 μM incubated for 4 h). (A^1) AM-HA; (A^2) Rhodamine 123; (A^3) overlay of A^1 and A^2; (B^1) TW-HA; (B^2) Rhodamine 123; (B^3) overlay of B^1 and B^2. Bar = 100 μM.

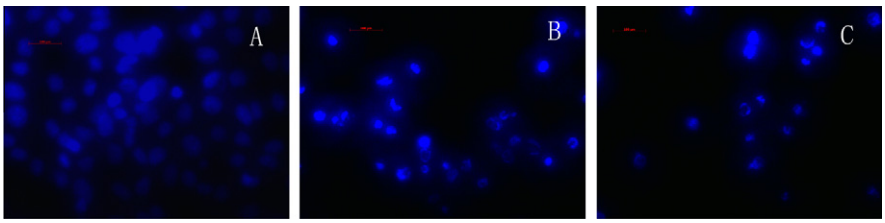

Figure 3. Fluorescence micrographs of HeLa cells stained with Hoechst 33342. The normal cells (A), the AM-HA (B) and TW-HA treated cells after irradiation by light. Bar = 100 μm.

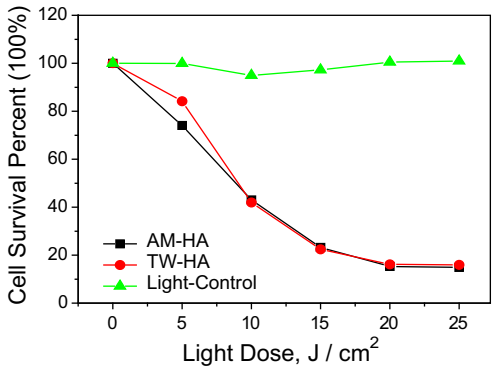

Figure 4. In vitro PDT photosensitivity of AM-HA (2 μM) versus TW-HA (2 μM). The cells were exposed to laser light at a dose of 5 J/(cm^2 min). Light control: cells were not incubated with photosensitizers, but were exposed to laser light.

As shown in Figure 4, the HA in the amorphous formulation manifested a similar light dose response compared to the conventional Tween-80 micelle formulation, demonstrating that the efficacy of the drug in vitro is not affected in this surfactant-free formulation.

In summary, we have reported the synthesis of the amorphous formulation of hypocrellin A. It exhibits much better water-solubility than free hypocrellin A, and higher ratios (R_f) of dark-toxicity to light-toxicity than the traditional delivery vehicle, Tween-80 micelle. Thus amorphous formulation of hypocrellin A may serve as an excellent candidate in the field of PDT and this method also provide a promising future to effectively improve water-solubility for other hydrophobic PS.

Acknowledgments

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References and notes

1. (a) Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. *J. Natl. Cancer Inst.* **1998**, *90*, 889; (b) Dolmans, D. E.; Fukumura, D.; Jain, R. K. *Nat. Rev. Cancer* **2003**, *3*, 380; (c) Bechet, D.; Couleaud, P.; Frochot, C.; Viriot, M. L.; Guillemin, F.; Barberi-Heyob, M. *Trends Biotechnol.* **2008**, *26*, 612.
2. (a) Wolinsky, J. B.; Grinstaff, M. W. *Adv. Drug Deliv. Rev.* **2008**, *60*, 1037; (b) Nikolaeva, I. A.; Misharin, A. Y.; Ponomarev, G. V.; Timofeev, V. P.; Tkachev, Y. V. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2872.
3. (a) Zhou, J. H.; Xia, S. Q.; Chen, J. R.; Wang, X. S.; Zhang, B. W. *Chem. Commun.* **2003**, *12*, 1372; (b) Zhou, J. H.; Liu, J. H.; Feng, Y. Y.; Wei, S. H.; Gu, X. T.; Wang, X. S.; Zhang, B. W. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3067; (c) Zhao, B. Z.; Xie, J.; Zhao, J. Q. *Biochim. Biophys. Acta, Gen. Subj.* **2004**, *1670*, 113; (d) Zhou, J. H.; Zhou, L.; Dong, C.; Feng, Y. Y.; Wei, S. H.; Shen *Mater. Lett.* **2008**, *62*, 2910; (e) Wang, K. W.; He, Q.; Yan, X. H.; Cui, Y. *J. Mater. Chem.* **2007**, *17*, 4018; (f) Roy, I.; Ohulchanskyy, T. Y.; Pudavar, H. E.; Bergey, E. J.; Oseroff, A. R.; Morgan, J.; Dougherty, T. J.; Prasad, P. N. *J. Am. Chem. Soc.* **2003**, *125*, 7860; (g) Zhou, L.; Liu, J. H.; Wei, S. H.; Feng, Y. Y.; Zhou, J. H.; Yu, B. Y. *Monatsh. Chem.* **2009**, *140*, 1167; (h) Zhou, L.; Dong, C.; Wei, S. H.; Feng, Y. Y.; Zhou, J. H.; Liu, J. H. *Mater. Lett.* **2009**, *63*, 1683.
4. Baba, K.; Pudavar, H. E.; Roy, I.; Ohulchanskyy, T. Y.; Chen, Y. H.; Pandey, R. K.; Prasad, P. N. *Mol. Pharmaceutics* **2007**, *4*, 289.
5. (a) Muller, R. H.; Jacobs, C.; Kayser, O. *Adv. Drug Deliv. Rev.* **2001**, *47*, 3; (b) Lindfors, L.; Forssn, S.; Skantze, P.; Skantze, U.; Zackrisson, A.; Olsson, U. *Langmuir* **2006**, *22*, 911; (c) DiNunzio, J. C.; Miller, D. A.; Yang, W.; McGinity, J. W.; Williams, R. O. *Mol. Pharmaceutics* **2008**, *5*, 968.
6. (a) Panchagnula, R.; Bhardwaj, V. *Drug Dev. Ind. Pharm.* **2008**, *34*, 642; (b) Zhao, Z. J.; Wang, Q.; Zhang, L. *J. Phys. Chem. B* **2007**, *111*, 13167.
7. Zhou, L.; Liu, J. H.; Zhang, J.; Wei, S. H.; Feng, Y. Y.; Zhou, J. H.; Yu, B. Y.; Shen, J. *Int. J. Pharm.* **2010**, *1*, 131.
8. Zhou, L.; Zhou, J. H.; Dong, C.; Ma, F.; Wei, S. H.; Shen, J. *Dyes Pigm.* **2009**, *82*, 90.
9. Hu, Y. Z.; An, J. Y.; Jiang, L. J.; Chen, D. W. *J. Photochem. Photobiol., A* **1995**, *89*, 45.
10. He, Y. Y.; An, J. Y.; Jiang, L. J. *Dyes Pigm.* **1999**, *41*, 93.
11. A solution of HA in dimethyl sulfoxide (DMSO) (200 μL, 3 mM) was injected into 10 mL of water at room temperature with controlled stirring for 30 min at 100 rpm/min. After the formation of AM-HA, DMSO was removed completely by dialyzing the solution against water in a 12–14 kDa cutoff cellulose membrane for 24 h.
12. XRD was performed to investigate the crystallinity of HA. The X-ray powder diffraction patterns were obtained with a *D*/max 2500VL/PC rotating anode X-ray powder diffractometer at 40 kV and 100 mA. Data were collected from 3° to 40° (2θ) in continuous scan mode increasing at a step size of 0.02°.
13. An aqueous solution of ADPA (150 mL, 5.5 mmol/L) was mixed with AM-HA (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.
14. Al-Kaysi, R. O.; Muller, A. M.; Ahn, T. S.; Lee, S.; Bardeen, C. J. *Langmuir* **2005**, *21*, 7990.
15. Chen, Y.; Gryshuk, A.; Achilefu, S.; Ohulchansky, T.; Potter, W.; Zhong, T.; Morgan, J.; Chance, B.; Prasad, P. N.; Henderson, B. W.; Oseroff, A.; Pandey, R. K. *Bioconjugate Chem.* **2005**, *16*, 1264.
16. (a) Lei, W. H.; Xie, J. F.; Hou, Y. J.; Jiang, G. Y.; Zhang, H. Y.; Wang, P. F.; Wang, X. S.; Zhang, B. W. *J. Photochem. Photobiol., B* **2010**, *98*, 167 (b) HeLa cells were seeded in a DMEM supplemented with 10% fetal bovine serum (FBS) at a concentration of around 1×10^5/mL in a 6-well cell culture plate. The plates were then placed overnight in an incubator at 37 °C with 5% CO_2. The next day, serum free medium was replaced on the plates, at this stage; the localization of AM-HA was investigated by co-incubating with mitochondria targeting probe rhodamine 123 for 30 min. The cells were then rinsed briefly with phosphate-buffered saline (PBS) and examined immediately by fluorescence microscopy.
17. (a) Cao, J.; Liu, Y.; Jia, L.; Zhou, H. M.; Kong, Y.; Yang, G.; Jiang, L. P.; Li, Q. J.; Zhong, L. F. *Free Radical Biol. Med.* **2007**, *43*, 968 (b) After treatment with AM-HA overnight and irradiated by light, HeLa cells were washed with PBS three times and treated with 25 μg/ml Hoechst 33342 at 37 °C with 5% CO_2 in the dark for 15 min. Nuclear morphology change were observed under a fluorescence microscope.
18. (a) Gao, D.; Xu, H.; Philbert, M. A.; Kopelman, R. *Nano Lett.* **2008**, *8*, 3320; (b) Zheng, Q. D.; Bonoiu, A.; Ohulchanskyy, T. Y.; He, G. S.; Prasad, P. N. *Mol. Pharmaceutics* **2008**, *5*, 389; (c) For studying cell viability, 96-well plates were inoculated with cells at 2×10^5/mL density overnight. The medium was removed and the wells were rinsed using sterile PBS and 100 μL serum free medium with AM-HA or TW-HA was replaced into each well. After incubated overnight, the cell were irradiated by light and the percentage of dead cells was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay.