

# Photosensitive multifunctional poly(vinyl alcohol) micelles for enhanced antitumor effect

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## ABSTRACT

Polyvinyl alcohol (PVA) micelles were firstly synthesized by using hemin molecules as novel crosslinked bridges (PVA-H crosslinked micelles). On one hand, the crosslinked micelles can allow high stability against extensive dilution (1800-fold) to reduce side-effects; On the other hand, the bridges not only can destruct by means of laser irradiation (405 nm, 200 mw), but also can induce the production of singlet oxygen ( $^1O_2$ ) and the release of 5-fluorouracil (5FU) (i.e. 46% release in 360 min). But above all, that 5FU-loaded PVA-H crosslinked micelles irradiating by laser is more efficient than PVA-H crosslinked micelles and free 5FU in killing tumor cells, suggest the effective synergistic antitumor effect about chemotherapy and photodynamic therapy (PDT).

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## 1. Introduction

Various delivery systems loaded hydrophilic or lipophilic drugs had been engineered to improve the treatment outcomes of anti-cancer agents including targeted delivery [1–6], particle-based delivery [7–10], controlled delivery [11–13], etc. Among the hydrophilic drugs-loaded delivery systems, photosensitive polymeric micelles have presented many merits such as given time and space [14]. For instance, the 5-fluorouracil bonded to nanocarrier by photosensitive linker showed controlled release by photo-triggered response [15]. However, the treatment efficiency of them is not optimistic, not only because of the single therapeutic methods, but also low stability in vivo [16].

To improve the outcome of cancer treatment, combined therapy is attracting increasing attention. For example, Králová et al. [17] synthesized porphyrin-cyclodextrin conjugates for multifunctional drug delivery and therapy. Deng et al. [18] also reported a multimodal drug delivery system.

In order to restrain premature drug release in vivo, cross-linking micelles had been evolved [19]. Over the last few years, different crosslinking methods, for example cinnamate- or disulfide-crosslinking have been adopted in improving micelles stability [20]. However, overly

stable crosslinking micelles prohibit release of drugs even though they reach the target sites. Thus, the degradable linkages, stimulating by pH, redox or temperature, will be widely used as crosslinking bridges. Nevertheless, it is difficult to prepare cross-linking micelles with hotodegradation and multimodal cancer therapy in one single system.

As a drug delivery system, poly(vinyl alcohol) (PVA) possesses many advantages, such as nonimmunogenicity, biocompatibility and a large possibility of functionalization [21]. PVA has also been used as a surfactant in developing novel antisense oligonucleotide-formulated microspheres [22].

The objective of this study was to achieve crosslinking PVA micelles decorated with hemin molecules for the photo-triggered release of 5-fluorouracil (5-FU) as well as singlet oxygen ( $^1O_2$ ), which may be used as potential medicines applied in the synergetic therapy for cancer.

## 2. Experimental

### 2.1. Materials

P-toluenesulfonic acid (PTSA) and polyvinyl alcohol (PVA,  $M_w = 22$  KDa) (Sangon Company Limited (Shanghai, China)) were dried with anhydrous toluene azeotropic distillation. According to previous reports [23], the 4-(dimethylamino) pyridine-p-toluenesulfonate (DPTS) was synthesized. 5-fluorouracil (5FU), 2,2,6,6-Tetramethylpiperidine (TEMP), Hoechst 33,342, propidium iodide (PI), 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT),

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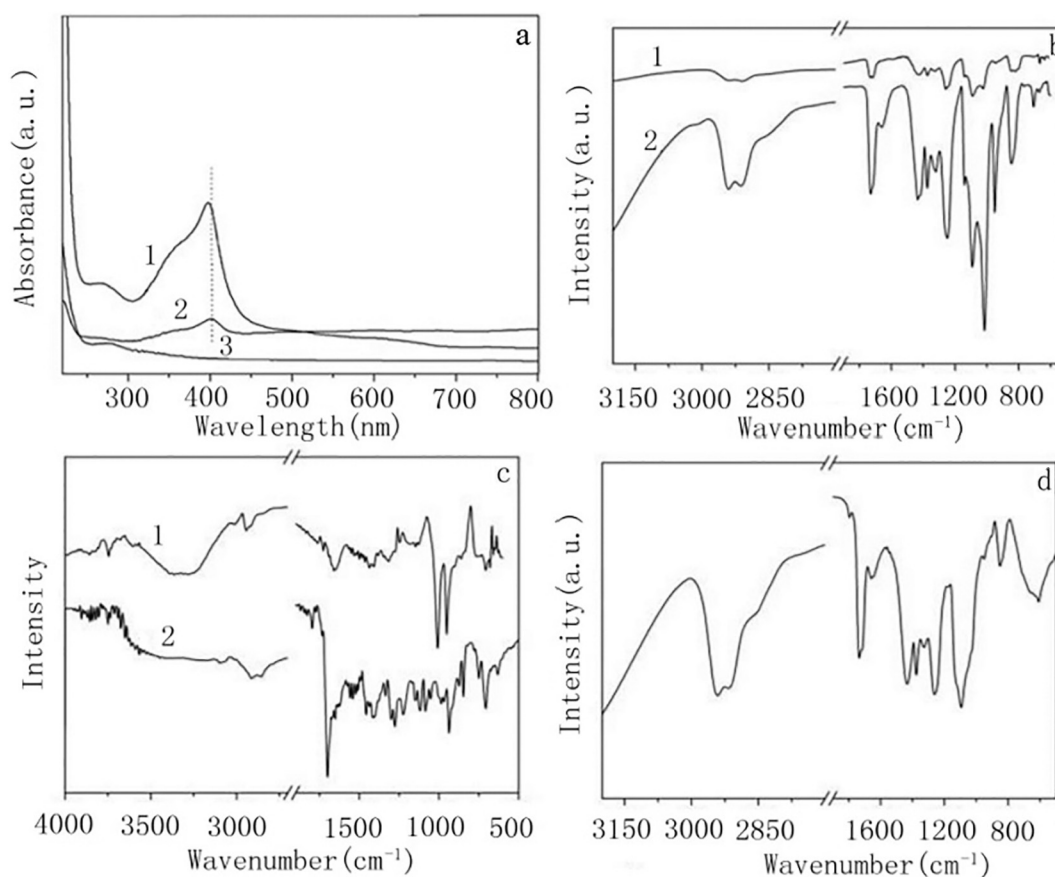


**Fig. 1.** (a) UV-vis spectra of (1) PVA-H, (2) hemin and (3) PVA; (b) The IR absorption spectra of (1) PVA and (2) PVA-H; (c) The IR spectra of (1) the difference spectrum (2-1) in Fig. 1 (b) and (2) hemin; (d) The IR absorption spectrum of crosslinking micelles.

4-dimethylaminopyridine (DMAP), *n*-dodecyl- $\beta$ -D-maltoside ( $\beta$ -DM), trimethylglycine (Tricine), sodium hydroxide (NaOH), hemin, dithiothreitol (DTT), 1, 3-Diphenylisobenzofuran (DPBF) and *N,N*-dicyclohexylcarbodiimide (DCC) were obtained from Sangon Company Limited (Shanghai, China).

## 2.2. Synthesis of PVA-H

1.0 g PVA was dissolved in 24 mL dimethyl sulfoxide (DMSO). Then, 0.012 g *N,N*-dicyclohexylcarbodiimide and 0.006 g hemin were added and stirred for 30 min. Under the protection of nitrogen, 0.0017 g DPTS in 10 mL DMSO solution was added, stirred for 48 h and precipitated with ethanol (cold). The obtained derivative was purified by the solution in DMSO, afterwards, precipitating with ethanol (cold). After reused for 3 times, the PVA-H was placed in vacuum oven at room temperature for 24 h.

## 2.3. Fabrication of micelles, crosslinking and de-crosslinking of PVA-H micelles

PVA-H micelles were formed using solvent exchange method. In short, 0.2 wt/v% (1 mL) PVA-H solution in DMSO, was dropwise added in 5 mL of distilled water under stirring at room temperature. The resulted PVA-H micelles were obtained through dialysis. The hydrophobic pyrene was used as the probe [24] to determine the critical aggregation concentration of micelles.

The crosslinking were formed after the adding of 0.0027 g DTT in the PVA-H micelle. Then stirring for 6 h (25 °C), the micelle solution also was dialyzed for one day. The de-crosslinking of PVA-H crosslinking micelles were executed by 405 nm laser irradiation (200 mw).

## 2.4. Drug loading

The loading of 5FU was carried out by adding water to 5FU and PVA-H solution in DMSO. After stirring, the drug-loaded micelles were dialyzed. The weight concentration of the polymer was 0.2 mg mL<sup>-1</sup>.

After the protection of nitrogen for 10 min in the above micelle, DTT was added and stirred for 6 h. The mixture was purified by dialysis for 1 day.

The substance in the dialysis bags was preserved by freezing and drying in a vacuum. To calculate drug loading content (DLC), 5FU loaded micelles solution in DMSO was analyzed with UV spectroscopy. From Eq. (1), the DLC was calculated.

$$\text{DLC (wt\%)} = \frac{\text{weight of loaded drug}}{\text{total weight of loaded drug and polymer}} \times 100\% \quad (1)$$

## 2.5. Drug delivery

The 5FU release from PVA-H crosslinking micelles was investigated using a dialysis bag at 37 °C in different conditions, i.e. laser irradiation or non-irradiation. For comparison, the 5FU release from non-crosslinking micelles was also studied. The 5FU release quantity was measured by UV-vis spectrometer (266 nm).

## 2.6. Cell viability evaluation and fluorescence images

The cell viability was evaluated by MTT assay. We choose the polymer concentration of 0.1–1.0 mg mL<sup>-1</sup> in vitro cytotoxicity of crosslinked micelles against HeLa cells based on the polymer concentration (0.2 mg mL<sup>-1</sup>) in the drug-loaded micelles. Fluorescence images were measured according to previous reports [25].

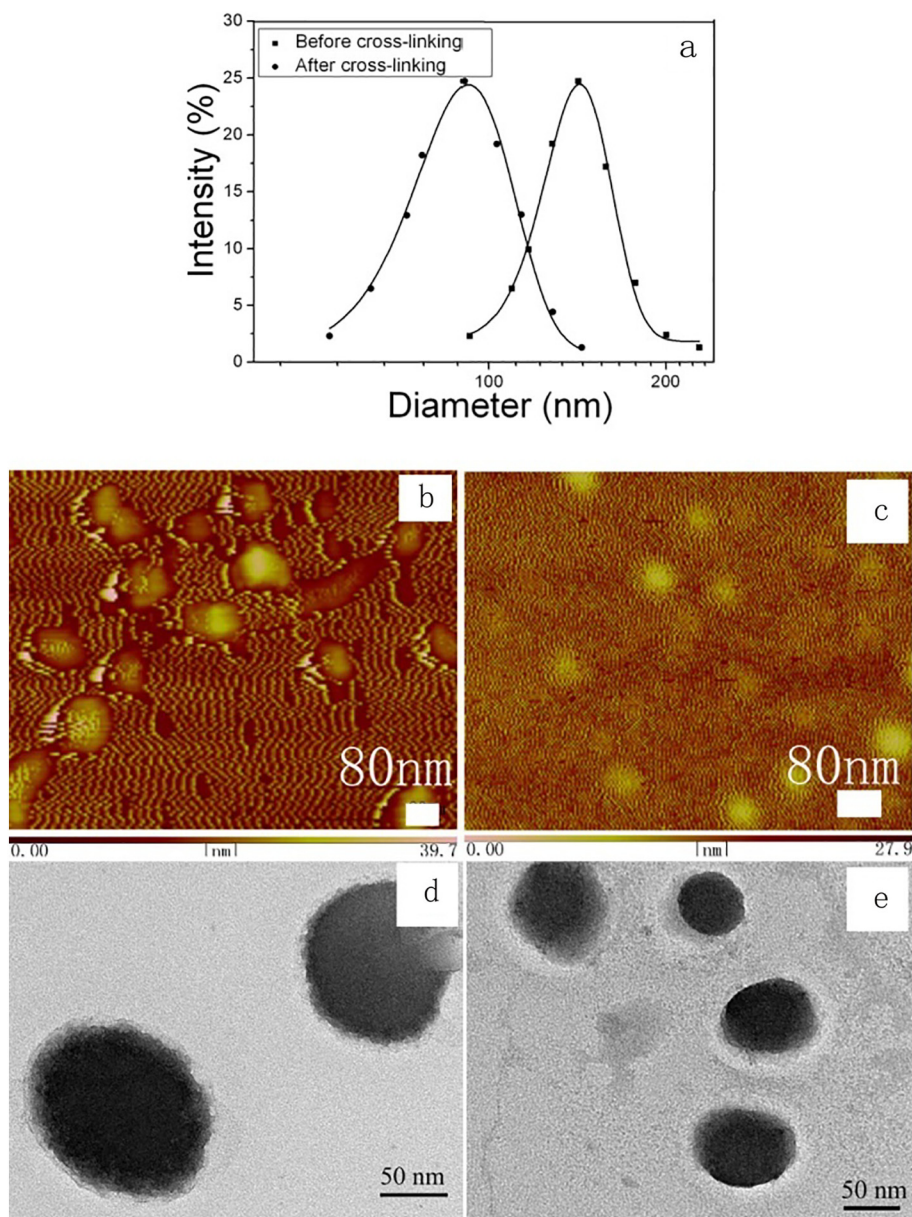


**Fig. 2.** (a) Size distribution of cross-linked micelles and micelles; AFM and TEM images of (b, d) non-crosslinked micelles and (c, e) crosslinked micelles.

### 3. Results and discussion

The PVA-H can be readily fabricated by the reaction between PVA and hemin in dimethyl sulfoxide. Fig. 1a exhibits the UV-vis spectrum of PVA-H, hemin and PVA. The spectrum shows a strong peak at 397 nm and a weak peak at the range of 250–300 nm (Curve 1), which can be assigned to the characteristic peaks of hemin and PVA, respectively. However, the former shifts toward shorter wavelength (from 401 to 397 nm) compared with alone hemin (curve 2 in Fig. 1a) and PVA (curve 3 in Fig. 1a).

Fig. 1b shows IR absorption spectra of the samples. The characteristic peaks of PVA in curve 1 can also be seen in curve 2. However, the relative intensity of the peaks at  $1010\text{ cm}^{-1}$  and  $1093\text{ cm}^{-1}$ , relating to the carboxyl stretching band (C—O) and the stretching vibrations of C—OH bonds in PVA, have changed, indicating the formation of the C—OH bonds for the esterification reaction of hemin and PVA. Moreover, two new bands at  $3005\text{ cm}^{-1}$  and  $950\text{ cm}^{-1}$ , attributing to stretching

vibration of C = C—H in hemin, can also be observed in curve 2 (Fig. 1b). These suggest that PVA-H was obtained. To further clarify the grafting, the difference spectrum is obtained in curve 1 (Fig. 1c), subtracting the original PVA contributing from the spectrum of the PVA-H. The spectrum shows the characteristic peaks of hemin (curve 2 in Fig. 1c), indicating the coupling hemin to PVA. It should be noted that a new peak ( $1010\text{ cm}^{-1}$ ) is observed in the difference spectrum comparing with curve 2 in Fig. 1c. However, the two peaks at  $3005\text{ cm}^{-1}$  and  $950\text{ cm}^{-1}$  have disappeared (Fig. 1d), which related to C=C—H group in hemin, suggesting a Michael addition reaction between vinyl and thiol [26]. So, hemin grafted on PVA can be used as bridges, and improve stability of micelles against premature drug release in vivo.

The PVA-H is easy to form micelles in water, and the critical aggregation concentration is  $0.288\text{ mg L}^{-1}$ . The hydrodynamic sizes of the non-crosslinking micelles and the crosslinking micelles are 130 nm and 90 nm (Fig. 2a), respectively. The formation of both micelles was further

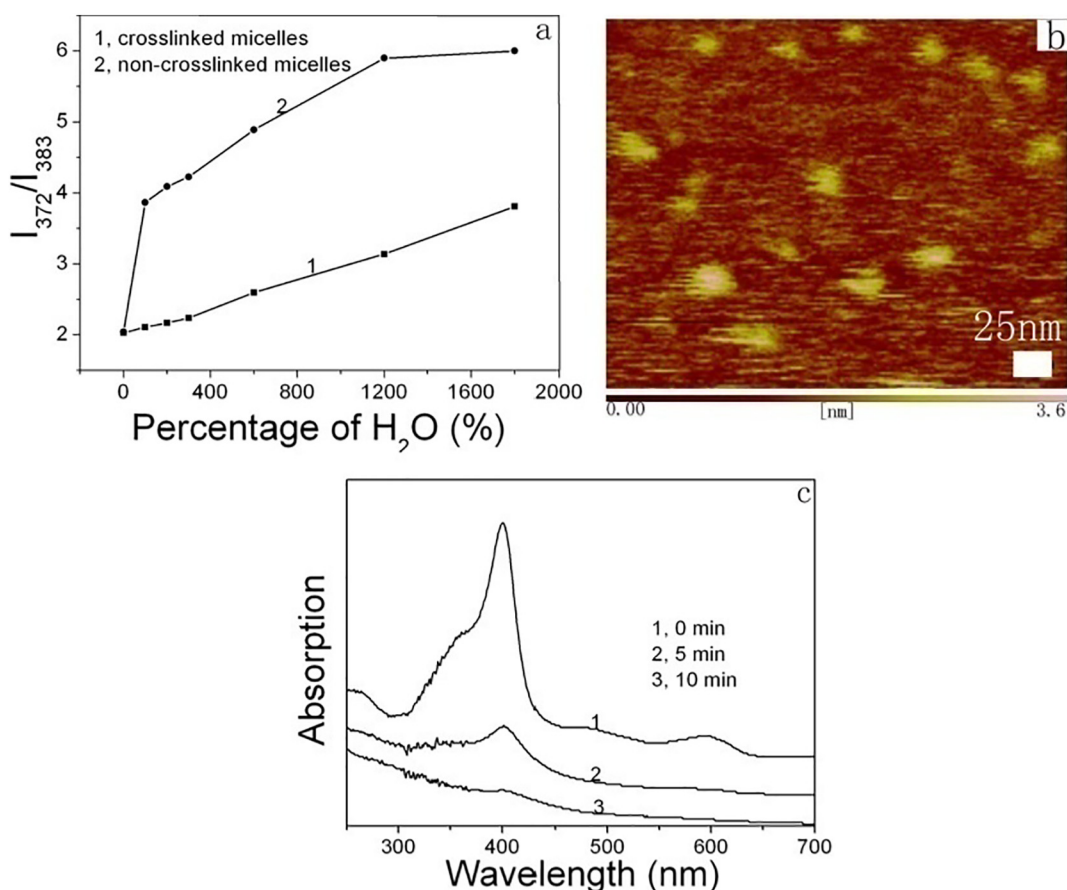


**Fig. 3.** (a) Change of  $I_{372}/I_{383}$  value of micelles upon addition of  $H_2O$ ; AFM images of (b) the crosslinking micelles after irradiation 30 min and (c) UV–vis spectra of hemin.

confirmed by AFM and TEM images (Fig. 2b, c, d, e). It is also found that the particle size is smaller after cross-linking by comparing Fig. 1b, d with Fig. 1c, e. In truth, the decrease in particle size is favorable in enhancing tissue penetration [14].

As shown in Fig. 3a, the relative peak intensity ( $I_{372}/I_{383}$ ) (3.81) in fluorescence spectrum of crosslinked micelles with 1800-fold dilution by addition of  $H_2O$  is smaller than that (3.87) of non-crosslinked ones with 100-fold dilution. So the cross-linking in PVA-H micelles can develop the stability of the micelles. Which actually not only prevent anticancer drugs losing in vivo transport, but also minimize the drugs destruction to the normal cells [27].

It was interesting to find another phenomenon that the size of crosslinked micelles reduced to about 25 nm after 30 min laser irradiation (Fig. 3b) compared with Fig. 2c. This is due to re-assembly behavior of polymer micelles as a result of the structural changes of hemin under laser irradiation. With the increase in the irradiation time, hemin (401 nm) peak intensity gradually decreased (Fig. 3c), suggesting potential application in phototriggered carriers for drug sentinel release.

The typical image of 5FU-loaded crosslinked micelles is presented in Fig. 4a, which shows a shape change, ellipsoid-like morphology with the length of about 1300 nm and a diameter of 700 nm comparing with the initial crosslinked micelles (Fig. 2c). The result demonstrates that 5FU-loaded micelles can be prepared by solvent exchange method [28], and introduction of 5FU causes the morphological change of crosslinked micelles. This phenomenon is similar to previous reports, i.e., doxorubicin hydrochloride loaded micelles are larger than those of the parent ones [29,30]. Upon adding a drug, transition from sphere to cylinder of poly(ethylene oxide)-b-poly[2-(diisopropylamino)-ethyl methacrylate] micelles had been observed in reference [31]. As we

know, each 5FU molecule has two aldehyde groups, which can form hydrogen-bond with hydroxyl groups on hemin in the micelles. The peak change of aldehyde groups from 265 nm to 275 nm (Fig. 4b) confirms the hydrogen bond formation, which is the driving force for drug loading and morphology changes of micelle. The 5FU loading is 1.9 wt% calculated by formula (1).

We then investigated the effect of the crosslinked and laser irradiation on drug release from micelles. The result reveals smallest release of 5-FU (ca. 11%) from the crosslinked micelles within 360 min (curve 3 in Fig. 4c) because of their cross-linking structure. In contrast, the corresponding non-crosslinked micelles release 42% 5FU under the same conditions (curve 2 in Fig. 4c). It is worth noting that the release behavior of the crosslinking micelles (curve 1 in Fig. 4c) under laser irradiation is similar to non-crosslinking micelles, possibly due to destroying the porphyrin ring structure. The results imply that the photo-triggered de-crosslinked is completed and rapid. Hence, the crosslinked micelles has resolved this issue of localized and controlled drug release by introduced hemin in micelles.

In addition, hemin can be used as a photosensitizer for potential application in PDT. To verify the generation of singlet oxygen, 1,3-Diphenylisobenzofuran (DPBF) is also used as a chemical trapping agent. In Fig. 4d, the absorption intensity of DPBF gradually decreased in the presence of hemin under laser irradiation, indicating an increase amount of singlet oxygen produced with reaction time [32], and hemin can be used in PDT.

The biocompatibility and antitumor effects of 5FU-loaded crosslinking micelles, PVA-H crosslinking micelles, and free 5FU, are estimated by fluorescence microscopy and MTT tests. From Fig. 5a, the cell survival rate is 35% after 24 hour incubation with free 5FU. For 4 hour

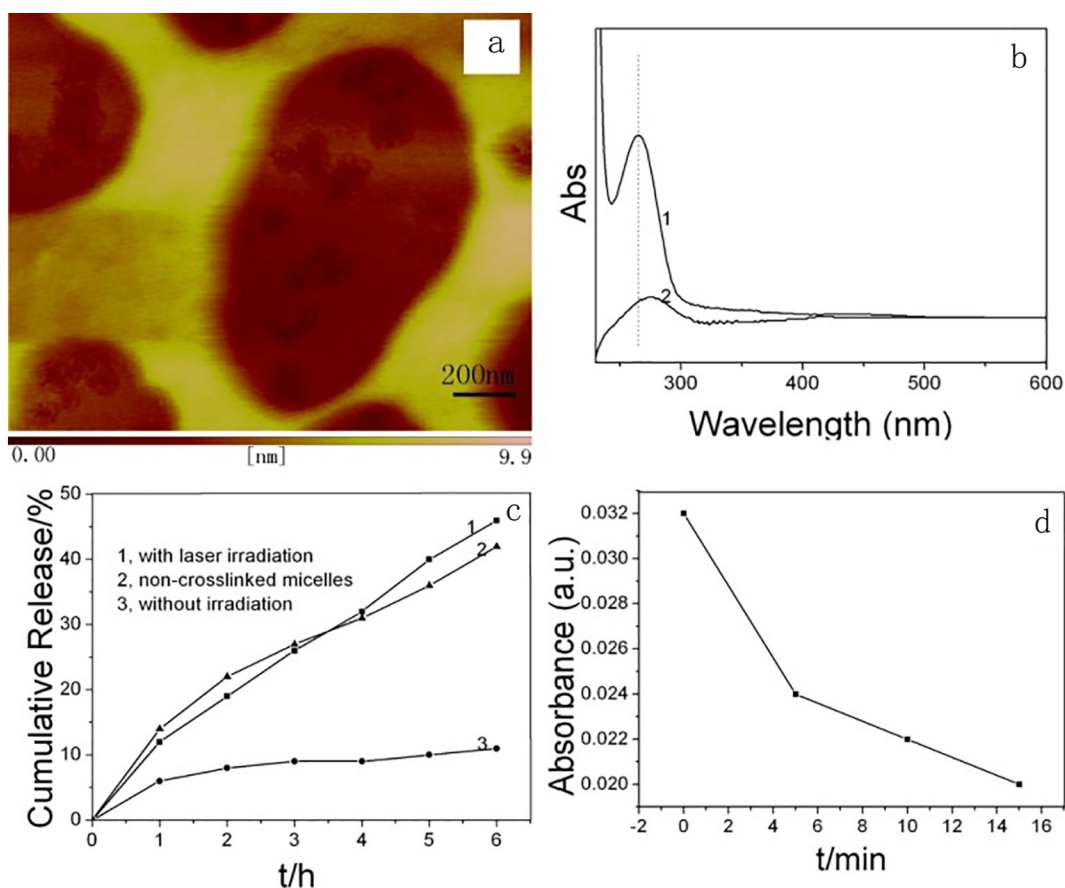


**Fig. 4.** (a) drug-loaded micelles; (b) UV-visible spectra of (1) 5FU and (2) 5FU-loaded crosslinked micelles; (c) 5-FU release profiles from crosslinked micelles (1) with and (3) without irradiation, and (2) non-crosslinked micelles without irradiation; (d) Photodegradation of DPBF by singlet oxygen produced with hemin.

incubation with the PVA-H crosslinking micelles with following 1 min irradiation, the cell viability is 29%, owing to the generation of  $^1\text{O}_2$ . It should be noted that the viability of cells hatching with 5FU-loaded crosslinking micelles under the same irradiation is only 16% due to excellent synergistic cancer therapy. It is noteworthy that the PVA-H crosslinking micelles show lower cytotoxicity to HeLa cells up to  $1.0 \text{ mg mL}^{-1}$  concentration testing (Fig. 5b).

It is consistent with the MTT results that the anticancer activity of different systems studied by using fluorescence microscopy (Fig. 6). The cell preincubation with 5FU-loaded crosslinking micelles for 1 min irradiation led to the maximum cells death indicated by the nuclei turning red (Fig. 6 h compared with Fig. 6 b, e). It is a reasonable

assumption for the hemin-crosslinked micelle carrier, not only possessing the advantages of localized drug release, favorable biocompatibility, convenient preparation, and degradability, but also improving therapeutic effects for tumor.

#### 4. Conclusion

In summary, we have developed a novel PVA-H micelle system for drug delivery by using hemin as a crosslinking bridge, which shows high stability, light-controlled drug release and multimodal cancer therapies. The combined treatment of PDT and chemotherapy is superior to

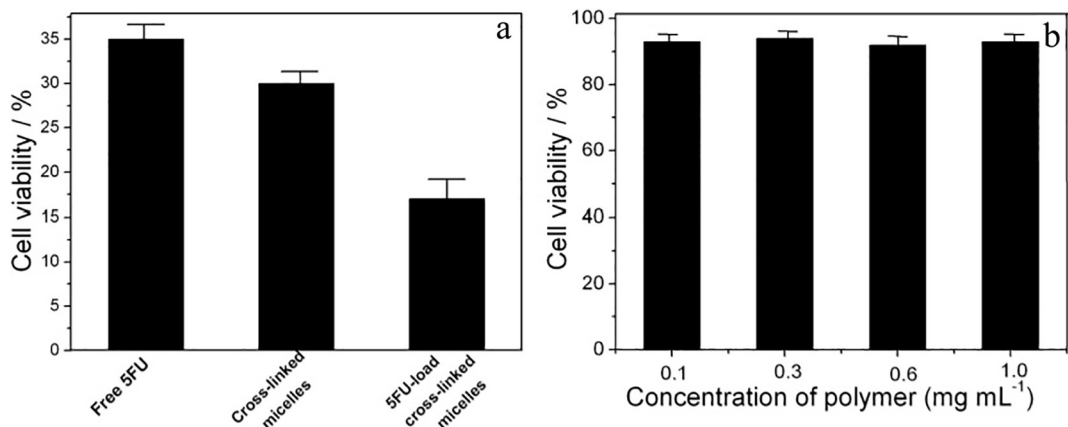


**Fig. 5.** (a) Viability of HeLa cells with 5FU-loaded crosslinking micelles, crosslinking micelles and free 5FU for 24 h; (b) The cytotoxicity of crosslinked micelles.

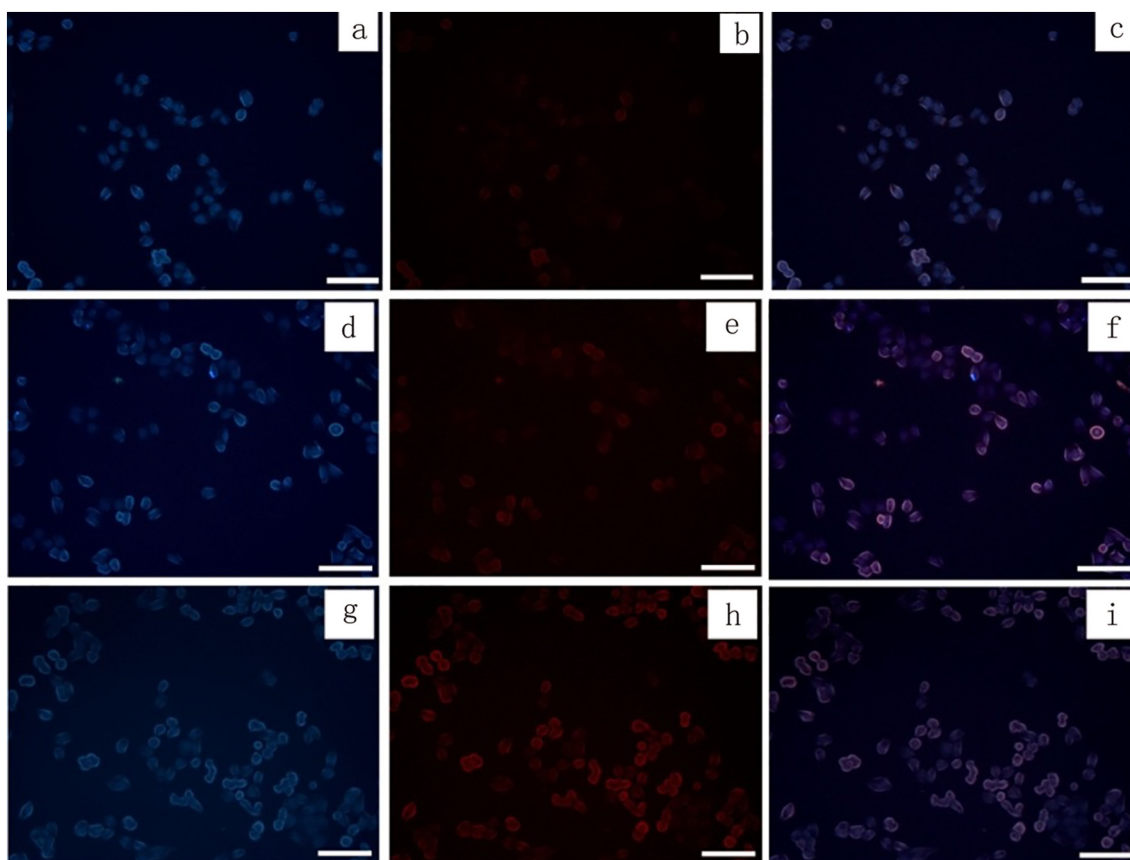


**Fig. 6.** Fluorescent images of cells incubating 3 h with (a–c) free 5FU, (d–f) PVA-H crosslinking micelles and (g–i) 5FU-loaded crosslinking micelles; The scale bars correspond to 80  $\mu\text{m}$  in all the images.

single in vitro studies. We believe this novel micellar delivery system can have huge potential impact on cancer treatment.

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