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Preparation and characterization of mPEG-g-α-zein biohybrid micelles as a nano-carrier

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ABSTRACT: A new kind of block copolymer micelles methoxy polyethylene glycol (mPEG) grafted α-zein protein (mPEG-*g*-α-zein) was synthesized. The chemical composition of mPEG-*g*-α-zein was identified with the help of FT-IR and ^{1}H-NMR. The biohybrid polymer can self-assemble into spherical core–shell nanoparticles in aqueous solution. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to investigate the self-assembled morphology of mPEG-*g*-α-zein. Dynamic light scattering (DLS) results showed that the particle size of mPEG-*g*-α-zein was about 90 nm. Moreover, the nanoparticles had a very low critical micelle concentration value with only 0.02 mg/mL. Then, the anticancer drug curcumin (CUR) was encapsulated into the biohybrid polymer micelles. The *in vitro* drug release profile showed a zero-order release of CUR up to 12 h at 37°C. Cell viability studies revealed that the mPEG-*g*-α-zein polymer exhibited low cytotoxicity for HepG2 cells (human hepatoma cells). Consequently, the mPEG-*g*-α-zein micelles can be used as a potential nano-carrier to encapsulate hydrophobic drugs and nutrients. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42555.

KEYWORDS: biomaterials; biomedical applications; degradation; nanoparticles; nanowires and nanocrystals

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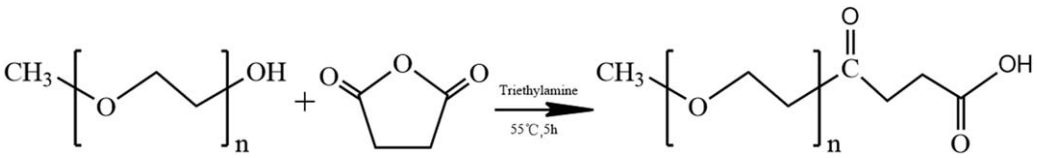
INTRODUCTION

Amphiphilic polymer self-assembled nano-carriers have attracted worldwide attention during the last few decades in biomedicine field.[1–3] Numerous synthetic polymers have been used to formulate smart drug delivery systems to maximize drug action and minimize side effects. However, most of these materials are synthetic polymers and are seldom generally recognized as safe (GRAS).[4,5] These synthetic materials may provoke several adverse effects. For examples, synthetic biodegradable materials, such as PCL, PLA and etc., could cause tissue acidity rise or damage.[6,7] Recently, reports of food-derived biological materials gradually increased, such as natural polysaccharides, polypeptides and proteins. Protein-based biopolymers are especially popular for the design of colloidal drug delivery systems, due to their high nutritional values, abundant renewable sources, drug binding capacity, and significant uptake into targeted cells.[8]

Zeins, natural storage proteins derived from foods, are the ideal materials for delivery of nutrients and drugs.[9–11] In the U.S., zein has been classified as Generally Accepted as Safe (GRAS) food ingredient by the FDA. Zeins are characterized by their high hydrophobicity and are classified as α, β, γ and δ-zein, depending on their solubility, molecular weight, and structure. Among these four groups, α-zein is the most abundant fraction and comprise 75–85% of total zeins. Recent years, people used small angle X-ray scattering (SAXS), two-dimensional high-resolution nuclear magnetic resonance, and computer simulation methods to determine the acid sequence and molecular conformations of α-zein.[12] α-zein has a typical helix secondary structure and an asymmetric globular folding contour. α-Zein shows highly hydrophobic property and conserved chemical composition, which makes it an excellent starting material for encapsulating hydrophobic molecules.[13,14] However, to the best of our knowledge, the preparation of copolymers with purified α-Zein has not been carried out.[15]

Hererin, we chose α-Zein as starting material to graft with mPEG hydrophilic segments. When the hydrophobic α-Zein grafts with mPEG, it exhibits the amphiphilic properties. The



Scheme 1. Schematic synthetic reactions for mPEG-COOH.

α-Zein-based copolymers can self-assemble into narrow size distribution nanoparticles in water, using for encapsulating hydrophobic drugs (e.g., Curcumin,[11] a naturally occurring compound from Curcuma longa, has been widely studied for its chemopreventive and chemotherapeutic effects against various types of human cancers), controlling release of nutrients and improving bioavailability. The objective of this work was using α-Zein-based materials to reach the goal of increasing bioavailability of hydrophobic drugs and nutrients by encapsulating it in mPEG-*g*-α-Zein micelles.

EXPERIMENTAL

Materials and Equipment

Zein (Z8010, Batch #057k0156), CUR and mPEG (M_z: 5 kDa), TEMED (Sigma). Aacrylamide, methylene bisacrylamide, mercaptoethanol, tris, glycine, and low-molecular weight standard proteins were purchased from Solabio company. N-methyl pyrrolidone (NMP), ammonium persulfate (AP), Coomassie Brilliant Blue and TEMED (Sigma) were analytical grade and purchased from Sinopharm chemical Reagent Co. Methylene chloride, hexane, and ethanol were purchased from Beijing Chemical Plant.

UV–vis spectra were recorded on a Tu-1901 double-beam UV–Vis spectrometer (Spectral Analysis of General Instrument, Beijing). Dynamic light scattering (DLS) particle size were recorded on a W3058 analyzer (American Microtrac company). A JY600C electrophoresis system and JY-SCZ8 vertical electrophoresis tank were used for the electrophoretic separation of proteins (Beijing Junyi Dong fang Electrophoresis Equipment Co., Ltd.). Morphology and composition of micelle were characterized on JEM-1200EX scanning electron microscope (SEM) and Bruker AV400 type 400 MHz NMR spectrometer. DLS were measured by DSY5000X fluorescence microscope. Cytotoxicity of micelles were employed HepG2 cell line and evaluated by DNM-9602 fluorescence plate reader (Beijing new Technology Co.).

Purification and Characterization of α-Zein

Zein was dissolved in NMP (0.5 g/mL), and extracted five times in dichloromethane. After centrifugation (8000 r/min, 3 min), we can obtain the purified zein without fat. The product was dissolved in 95% ethanol, centrifuged and the supernatant was diluted with water to a concentration of 20%, then lyophilized to gain a purified α-Zein powder.

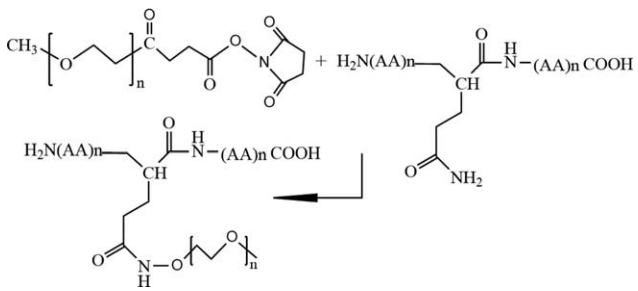
SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool for resolving the molecular weight of macromolecules and its detection limit can reach the picomole range. A SDS-PAGE assay was performed as follows: 1 mg α-Zein was dissolved in 50% ethanol. Then, 125 m*M* Tris-HCl, 2 wt % SDS, 10 wt % glycerol, 5 wt % BME and 0.05 wt % Bruce blue were added under the condition of pH = 6.8. After boiling, 5 μL of the supernatant was added to 4% stacking gel and 10% separating gel. It was kept under 300 V for 3 h. Gel was stained into 0.1% Coomassie blue for 1–2 h. SDS-PAGE photographs were taken after bleaching in bleaching solution 1–2 h. The standard protein molecular weight range from 14.4 to 97.4 kDa.

MALDI-TOF was performed as follows: 40 m*M* of HABA (matrix: 2-hydroxyl-4-amino butyric acid) was dissolved in 65% ACN (acetonitrile) and 0.3% TFA (9.69 mg/mL). The mass spectrometer was operating in the linear mode with 425 ns delay, nitrogen laser 337 nm,a 25 kV accelerating voltage. The ion flight path is 1.3 m, partial search mode using the center, spectra were averaged over 250 laser shots using two laser shots per position.

Synthesis and Characterization of mPEG-*g*-α-Zein

The PEGlation of α-Zein was performed by two steps. The first step was the esterification of mPEG as shown in Scheme 1. mPEG and succinic anhydride (20 times excess) were dissolved in CH_2Cl_2. The reaction mixture stired at 55°C for 5 h. After cooling to room temperature, the crude product was precipitated into diethyl ether for three times. Then the crude product was dialyzed (MWCO 3.5 kDa) against water for 2 days and lyophilized. The mPEG—COOH was obtained with yields of 64%.

The second step was the amidation to gain the mPEG-*g*-α-zein as shown in Scheme 2. mPEG—COOH, EDC and NHS (molar ratio was 1 : 1.2 : 1.2) were dissolved in NMP. The carboxyl group on mPEG-COOH was activated by stirring for 3 h. Then the mixture was added dropwise to α-Zein solution (DMSO as solvent, 10 mg/mL) and stirred for 24 h at room temperature. The crude product was precipitated in diethyl ether for three times and then was dialysed (MWCO 3.5 kDa) against water for 3 days and lyophilized to gain the final product mPEG-*g*-α-zein.



Scheme 2. Schematic synthetic reactions for mPEG-*g*-α-zein.

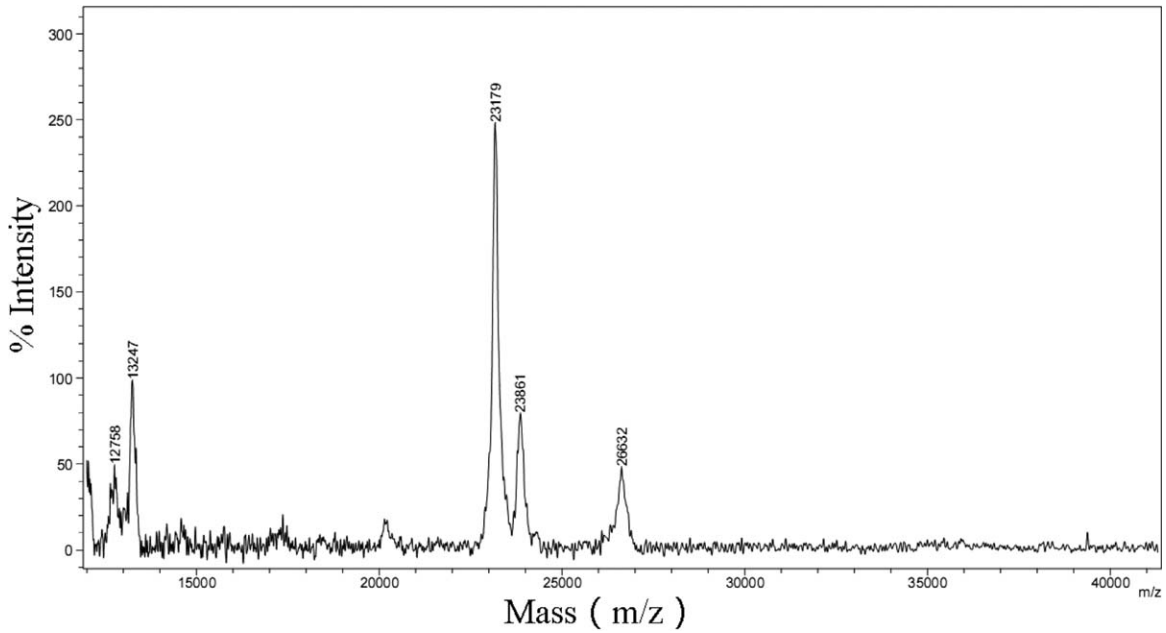


Figure 1. The MALDI-TOF characterization of purified α-zein component.

The obtained mPEG-*g*-α-zein was characterized by FT-IR and ^{1}H NMR. The critical micelle concentration (CMC) was determined by using pyrene as a fluorescence probe. Steady state fluorescence spectra were obtained by a Shimadzu RF5301 luminescence spectrometer. The excitation wavelength is set as 339 nm, the excitation and emission slit widths were set to 3 nm and 3 nm, the emission intensity of the recording from 350 to 400 nm.

Preparation and Characterization of mPEG-*g*-α-Zein Drug-Loaded Micelles

Curcumin (2 mg) and mPEG-*g*-α-zein (100 mg) was dissolved in 2 mL 90% ethanol at 37°C. The mixture was stirred at 500 rpm overnight. 10 mL of water was injected into the mixture using micro injection pump at a rate of 0.5 mL/min. The mixture was dialyzed (MWCO 10 kDa) against deionized water to remove excess alcohol and lyophilized to gain the CUR loaded mPEG-*g*-α-zein micelles.

The detection wavelength was 480 nm. Standard curve of CUR (0.031 to 1 μg/mL) in 90% ethanol was generated for the determination of CUR amount. Free CUR in the supernatant was subtracted from CUR in the micelles. The encapsulation efficiency was calculated as percent mg of drug loaded per mg of the mPEG-zein relative to the theoretical loading of CUR. Encapsulation efficiency was expressed as a mean of three experiments (±SD).

The encapsulation efficiency and loading content of CUR were calculated as follows:

$$\text{Loading content} = (W_t - W_f)/W_{np} \times 100\%$$

where W_t is the total weight of CUR fed, W_f is the weight of non-encapsulated free CUR, and W_{np} is the weight of nanoparticles.

$$\text{Encapsulation efficiency} = (W_t - W_f)/W_t \times 100\%$$

where W_t is the total weight of CUR fed, and W_f is the weight of non-encapsulated free CUR.

The measurements of the average size and ς potential of CUR loaded mPEG-*g*-α-zein micelles were performed on a W3058 analyzer (American Microtrac company) at 25°C, employing a semiconductor laser source ($\lambda = 633$ nm) at an scattering angle of 173°. Micells were gold coated (300 A) with an Emi-tech K575 sputter coater (Ashford, UK) to improve the electrical conductivity of sample surfaces. SEM images were observed by a XL-30 ESEM FEG SEM (FEI). AFM image of micells surface topography were observed by Japan Seiko SPI3800N scanning probe workstation, used the Olympus OMCL-AC240TS probe under tapping mode (Tap Mode).

In Vitro Curcumin Release Study

The *in vitro* drug release of CUR was under simulated physiological conditions. The CUR loaded micelles were vortex mixed and stand for 30 min. 5 mL of the above drug-loaded gel was added to 3.5 kDa MWCO dialysis bag and placed in 10 mL of phosphate buffer solution at 37°C under oscillation. At selected time intervals, 1 mL of the buffer solution outside the dialysis bag was removed for UV–vis analysis and replaced with an equal volume of fresh buffer solution. The release amount of CUR was calculated by UV–vis spectroscopy from the absorbance at 480 nm with the help of a calibration curve of CUR in the same buffer.

Cytotoxicity Measurements of Blank Micelles

Cytotoxicity of the zein-based biohybrids was evaluated using the CCK-8 assay in HepG2 cell line. The cells were cultured Dulbecco modified eagle medium (DMEM), supplemented with in 10% fetal bovine serum (FBS), under 5% CO_2 and 95% relative humidity atmosphere. The HepG2 cells were seeded in a 96-well plate at a density of about 10,000 cells/well in 100 L of growth medium and incubated for 24 h, after which time the

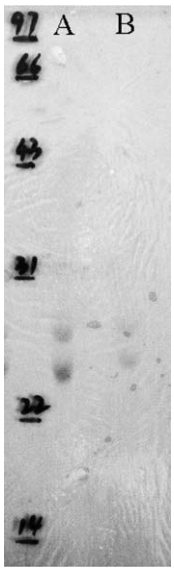


Figure 2. SDS-PAGE photograph of α-zein and mPEG-*g*-α-zein.

growth medium was replaced with DMEM medium containing different cocentrations of the zein-based biohybrids. Then, 10 μL of CCK-8 was added and the plate was incubated for 2 h. Finally, the PERLONG DNM-9602 microplate reader was used to measure the absorbance values at 450 nm wavelength.

RESULTS AND DISCUSSION

Purification of α-Zein

α-Zein was purified from the crude zein protein according to our previous work.[16] α-Zein could dissolve in 95% ethanol while the *β*, *γ*, and *δ*-zein could only dissolve in 60% ethanol.[17,18] The MALDI-TOF was used to characterize the purified α-zein. In Figure 1, the ion peaks at m/z 23179, 23861 and 26632 were attributable to the z19 and z22 fraction of α-zein respectively. In SDS-PAGE photograph (Figure 2), the left side was the standard molecular weight proteins marker, which used to estimate the molecular weight of the unknown protein (MW). Line A presented two strips of purified α-zein which was Z19 and Z22, respectively (molecular weight of 23 kDa, 26 kDa). Line B presented two strips of mPEG-α-zein. These results were in conformity, so we concluded that the product was purified α-zein.

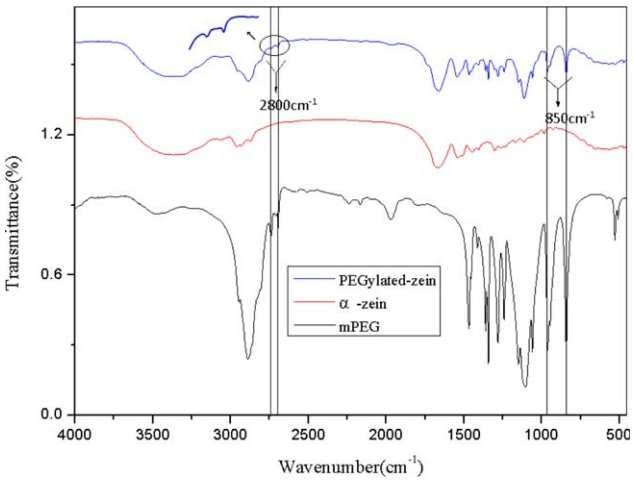


Figure 3. FT-IR spectra of α-zein, mPEG and mPEG-*g*-α-zein. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

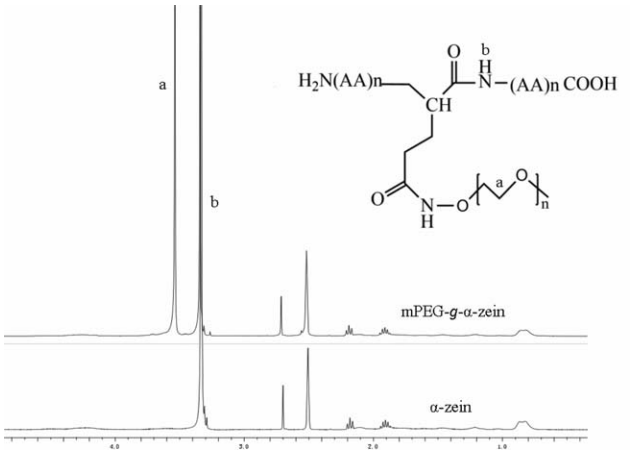


Figure 4. ^{1}H NMR spectrum of mPEG-*g*-α-zein and α-zein (DMSO-*d6*; 25°C).

Table 1. The Particle Size and the *ξ* Potential Changes Before and After Drug Loading

Micelles	Particles size (nm)	*ξ* Potential (mv)
Blank	78 ± 5	+39
Curcumin	97 ± 5	+43

Preparation and Characterization of mPEG-*g*-α-Zein

α-Zein is composed of about 21% of asparagine and glutamic acid,[19] where its glutamine has a N-terminal. The m-PEG-N-hydroxy succinimidyl ester (5 kDa) was used to form an amide bond with the terminal amino group in zein. So we can prepare mPEG-*g*-α-zein under mild conditions using EDC and NHS. The

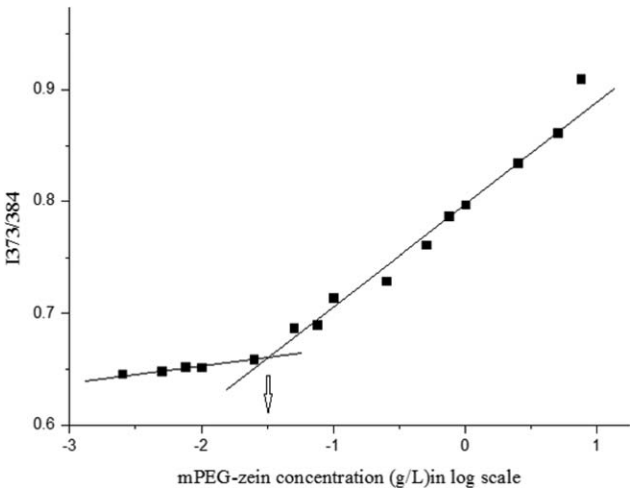


Figure 5. Critical micelle concentration of mPEG-*g*-α-zein.

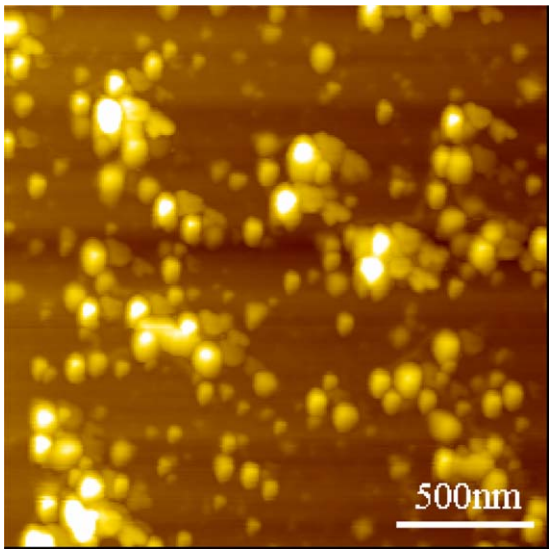


Figure 6. Images of mPEG-*g*-α-zein micelles by atomic force microscopy. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

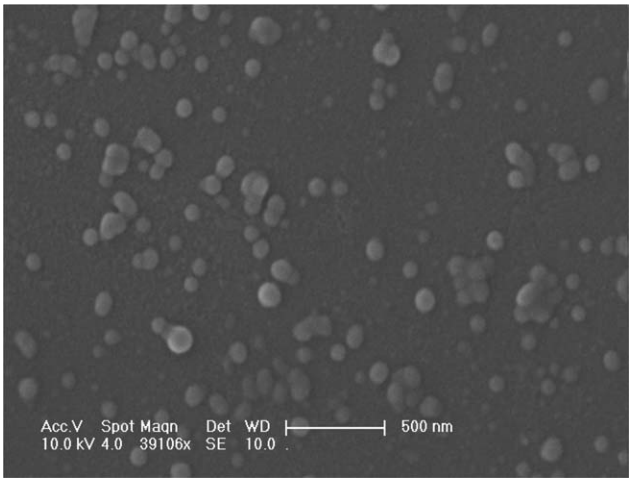


Figure 7. Image of mPEG-*g*-α-zein micelles by scanning electron microscopy.

infrared spectra of α-zein, mPEG, and mPEG-*g*-α-zein were shown in Figure 3. We can see from the FT-IR spectrum for the underivatized and mPEG. The stretching vibration of carbonyl in CH_2CH_2O groups appeared at 850 cm^{-1} in the FT-IR of mPEGylated-zein, but not in that of its precursor, Moreover, the band at 2800 cm^{-1} corresponding to methyl group appeared in the spectrum of α-zein grafted, which indicates that the mPEG groups were successfully introduced onto α-zein chains. The chart of mPEG-*g*-α-zein ^1H NMR was was further confirmed by ^1H NMR (Figure 4) spectrum, the ethylene proton signal of mPEG was seen at 3.6 ppm, which peak was not observed in α-zein. In SDS-PAGE photograph (Figure 2), line A represented α-zein, line B represented molecular weight of α-zein increased after the grafting of PEG. All these proved that grafting was succeed.

Characterization of mPEG-*g*-α-Zein Micelles

mPEG-*g*-α-zein micelles had a typical core–shell structure. Its biologically inert surface could prevent its self-aggregation and proteins absorption in bloodstream and inside the cell, due to PEG chains and their nanoparticle size so that it could avoid the recognition and phagocytosis of reticulo endothelial system (RES) and easy to implement long cycle.[20,21] As a poorly soluble drug reservoir, hydrophobic core had a high load capacity of drug.[22,23] Table 1 showed the particle size and the ξ potential changes before and after drug loading. The CUR was buried in the hydrophobic core of α-zein by the driving force in the hydrophobic. Particle diameter of micelles increased from 80 nm to 100 nm seen from DLS. The ξ potential of mPEG-*g*-α-zein micelles before and after drug loading were (39 ± 2 mv) and (43 ± 1 mv) respectively, and showed little change.

We measured the CMC value of mPEG-α-zein in the aqueous solution. CMC of mPEG-*g*-α-zein was determined by using pyrene as a fluorescence probe. Steady state fluorescence spectra were obtained by a Shimadzu RF5301 luminescence spectrometer. When the amphiphilic molecules exceeds the CMC, amphiphilic molecules self-assemble into micelles in solution, the solubility increases, the value of I_{373}/I_{384} will be changed, which can determine CMC of the amphiphilic molecules. The ratio of absorbance of pyrene 373 and 384 nm is plotted against

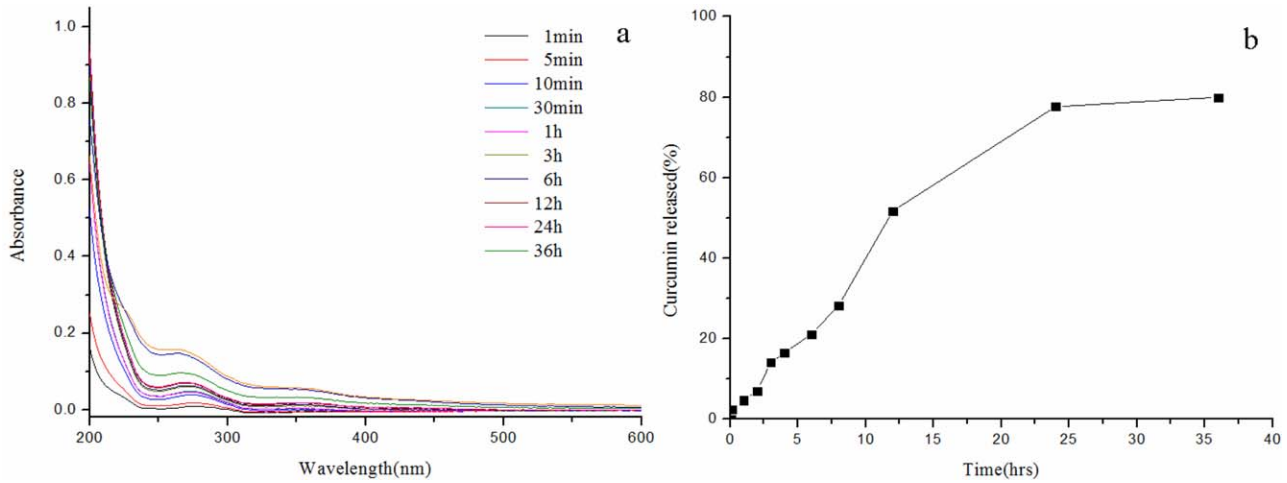


Figure 8. The release profile of micelles in a PBS solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

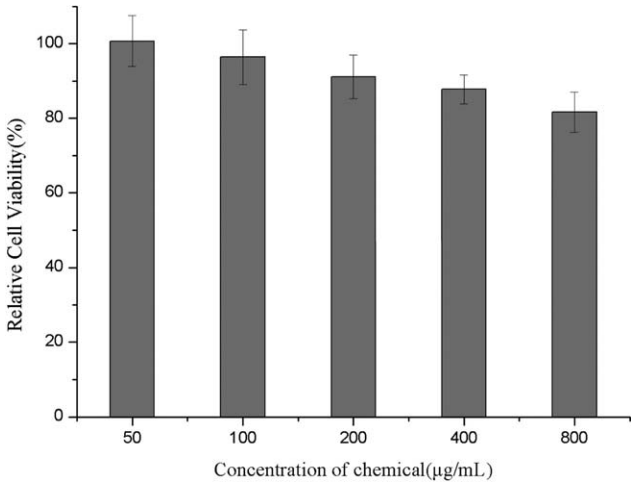


Figure 9. *In vitro* cytotoxicity of mPEG-*g*-α-zein in HepG2 cells, mean ± standard derivation ($n = 4$).

logarithmic concentration (g/L) of mPEG-*g*-*a*-zein. CMC was about 0.02 mg/mL shown in Figure 5.

By SEM and atomic force microscopy, the self-assembled morphologies of mPEG-*g*-α-zein micelles were observed (Figures 6 and 7). mPEG-*g*-α-zein aggregates exhibited regular spherical morphologies, and the size was about 90 nm. The results were agreement with our measurement from DLS results (Table 1).

In Vitro Curcumin Release Study

The *in vitro* release of CUR from mPEG-*g*-α-zein micelles was studied. The drug loading efficiency (DLE) was about 96%. Figure 8(a) showed the UV absorbent curves of micelles in a PBS solution. 420 nm was the absorption peaks of CUR. As can be seen from the release profile [Figure 8(b)], α-zein microspheres wrapped CUR has been in a steady release phase with no significant burst release *in vitro*. The degradation of the surface of nanospheres made the drug gradually released in 0–24 h, so it had the effect of sustained release.

In Vitro Cytotoxicity of the mPEG-*g*-α-Zein Micelles

Cytotoxicity of mPEG-*g*-α-zein at different concentrations was examined by CCK-8 assay. Cell viability treated by mPEG-*g*-α-zein was about 100% under the concentration of 50 μg/mL shown in Figure 9. When the concentration of the polymer increased to 400 μg/mL, the cell viability is 90% with no significant increase in cytotoxicity. When the polymer concentration further increased to 800 μg/mL, cell viability decreases, but still more than 80%. Based on the data above, it can be concluded that the amphiphilic mPEG-*g*-α-zein has excellent biocompatibility.

CONCLUSIONS

We prepared the novel polymer-biohybrids using methoxy polyethylene glycol (mPEG) grafted with α-zein protein (mPEG-*g*-α-zein). It can self-assemble into micelles in aqueous solution as a drug delivery vehicle. Hydrophobic core can extent the capacity of CUR and other hydrophobic drugs to improve their bioavailability. CMC of nanospheres was low. When the concentration of the grafted polymer increases, the increase of cell toxicity was not obvious, indicating that the grafted polymer had a lower cytotoxicity. These results showed that the self-assembly amphiphilic mPEG-*g*-α-zein had high utility as a drug carrier, surface modifiers, and in the biomedical field.

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