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·基础研究·

Preparation and Characterization of Micellar Tryptanthrin*

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ABSTRACT Objective: The study was aimed to increase the solubility of tryptanthrin (TRYP) by preparing micellar tryptanthrin, improve its bioavailability further. **Methods:** Pegylated tryptanthrin was synthesized via acid-sensitive hydrazone bond and further prepared as micelles by employing the dialysis method. The particle size distribution of the micelles was evaluated by dynamic light scattering (DLS) and the morphology was observed using a transmission electron microscope. The critical micelle concentration (CMC) of micelles was estimated by fluorescence probe method. The in vitro drug-release profile was determined using a dynamic dialysis method at pH 5.5 and pH 7.4. The fracture behavior of hydrazone bond was studied by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). In vitro cytotoxicity of TRYP and PEGylated tryptanthrin micelles (PTMs) against MCF-7 cells was studied using the CCK-8 assay. **Results:** The solubility of PTMs was increased by 1493 fold compared with TRYP. The PTMs were prepared with a particle size of 228.8 nm and PDI of 0.1, and the micelles were spherical. The CMC of PTMs was 3.5×10^{-7} mol/L and the low CMC indicated the high stability of prepared micelles and facilitated further use. The fracture behavior of hydrazone bond at acidic pH was verified and 95% of TRYP was released from PTMs at lower pH 5.5 in 12 h, while the drug release was very slow at normal physiological pH 7.4. The cytotoxicity of PTMs decreased under normal physiological conditions compared with TRYP, indicating that the cytotoxicity of PTMs was reduced compared with that of TRYP, which may be due to the stability of TRYP when prepared as micelles. Whereas at pH 5.5, the PTMs showed comparable cytotoxicity, indicating successful drug release from the carrier in response to tumor intracellular pH. **Conclusions:** The pegylated tryptanthrin improved water solubility of tryptanthrin effectively, which would benefit its further improvement in its bioavailability. Besides, it could be a promising tumor targeting prodrug.

Key words: Nanomicelle; Polyethylene glycol; Tryptanthrin; Water soluble

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Introduction

Tryptanthrin (TRYP), an indole quinazoline alkaloid, is a major active constituent extracted from several Chinese herbal plants such as Strobilanthes cusia, Polygonum tinctorium lour and Isatis tinctorial [1-3]. It is reported that TRYP has a broad spectrum of biological activities including anticancer, anti-inflammatory, antiprotozoal, antiallergic, and antimicrobial activities [4-6]. Our previous study illustrated that TRYP has proliferation-attenuating and apoptosis-inducing effects on a leukemia cell line in vitro and might be a new candidate to treat leukemia[7]. However, the poor water solubility (1.339 μg/mL in water [8]) of TRYP limits its absorption in the body, resulting in its extremely low bioavailability.

Liposomes and micelles [9-11] can increase the solubility of poorly soluble drugs, and they have been extensively studied in recent years. The lipophilic drugs with the logarithms of oil/water partition coefficient (P) (lgP) higher than 4.5 could be prepared as lipidosomes. Hence, TRYP with a lgP of 2.37 [12] cannot form stable lipidosomes. Owing to the advantages of good solubilization effect and simple production process, polymer micelles have garnered considerable attention [13,14]. Polymer micelles consist of hydrophobic cores and hydrophilic shells, and polymer-drug-conjugated micelles (PDCM) are effective drug carriers that can be loaded with hydrophobic drugs to increase solubility and bioavailability of the parent drug[15,16]. Furthermore, micelles with nano-size can target to the tumor tissues passively based on the enhanced permeability and retention (EPR) effect and release loaded drugs in target sites [17,18]. Moreover, the preparation process of polymer drug conjugate micelles is relatively simple, and drugs can be linked to polymers by physical encapsulation or chemical binding [19,20], which greatly expands their range of applications.

Polyethylene glycol (PEG) is one of the polymers that commonly used in the preparation of polymer drugs. PEG is an ideal drug carrier material and the Food and Drug Administration (FDA)

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has approved PEG for human intravenous, oral, and dermal applications [21,22]. However, currently, most of the PEGylated drugs used in clinical practice are macromolecules and the key problem that restricts the application of PEGylated small-molecule drugs is the difficulty of separation of PEGylated drugs and free PEG. Because they have a similar molecular weight and polarity, the common column chromatography method is not applicable. Currently, the only small-molecule drug that is under clinical research is PEGylated irinotecan [23]. Molecular imprinting technology (MIT) has been widely applied in various fields to selectively separate target molecules because of its recognition specificity and application universality [24-26]. In our work, the PEG molecularly imprinted polymer was prepared using PEG as a template molecule and applied for the separation of PEGylated TRYP and mPEG-COOH of similar molecular weight.

In order to improve the solubility of TRYP, TRYP-loaded micelles were prepared in this study. The method was to connect TRYP and PEG through hydrazone bonds. The amphiphilic block copolymer, TRYP-PEG, was used to form micelles by employing the dialysis method (Fig. 1).

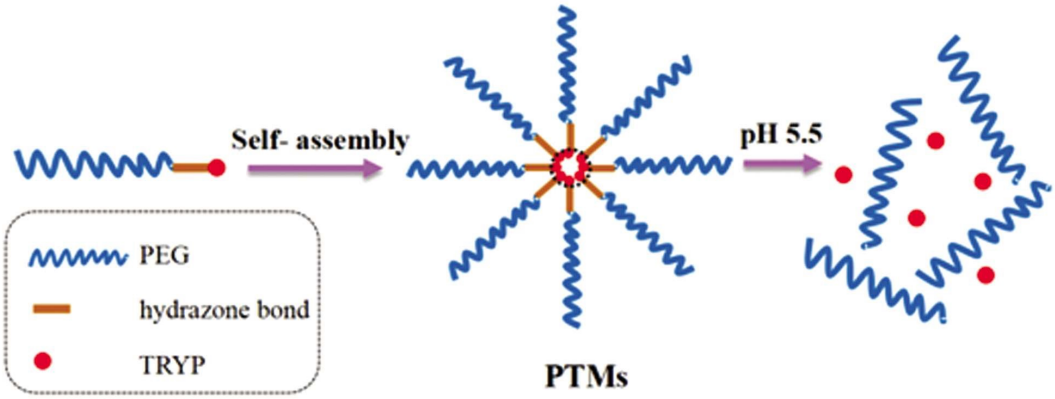

图 1 胶束化色胺酮制备示意图

Fig.1 Preparation of micellar tryptanthrin

1 Materials and Methods

1.1 Materials

Unless stated otherwise, all reagents were obtained from commercial sources and were analytical grade. TRYP was a gift from department of Natural medicine, the Air Force Medical University. Methoxy polyethylene glycol (molecular mass 2000 Da, purity :>95%) was purchased from Nanocs (America). Distilled deionized water was produced using the Millpak Reagent Water System (Millipore, USA). Pyrene, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), acrylamide, ammonium persulfate, 2-hydrazinoethanol, and N,N-dimethylacrylamide were purchased from Sigma Aldrich (USA). DMEM medium and CCK-8 were purchased from KeHao BioEngineering Inc.

1.2 Synthesis of TRYP−PEG

Tryptanthrin (248.0 mg) was dissolved in 25 mL of tetrahydrofuran (THF) and 2-hydroxyethyl hydrazine (76.0 mg) was dissolved in 3 mL of ethanol. The mixture was stirred under nitrogen atmosphere for 12 h. The reaction process was monitored by thin layer chromatography (TLC) on F254 silica gel pre-coated sheets (Qing Dao, China) and the products were purified by column chromatography.

mPEG2000-COOH (40.0 mg), TRYP-$NNHCH_2CH_2OH$ (9.2 mg), 4-dimethylaminopyridine (DMAP, 22.3 mg), and dicyclohexylcarbodiimide (DCC, 26.5 mg) were dissolved in 15 mL of dry dichloromethane, and then the solution was stirred in an ice bath for 24 h. The impurities were removed and the crude product was acquired by suction filtration. The unreacted mPEG-COOH was removed using PEG-imprinted polymer that was prepared as mentioned below.

1.3 Preparation and selectivity of molecularly imprinted polymer

mPEG-COOH (100.0 mg), acrylamide (3.6 mg), and N, N-dimethylacrylamide (39.6 mg) were dissolved in 30 mL of distilled water. The solution was then stirred at room temperature for 30 min. Thereafter, ammonium persulfate (100.0 mg) was added and the solution was placed in an oil bath at 85 ℃ under vigorous stirring with reflux condensation for 12 h. Ten microliters of anhydrous ethanol was added to the product. The sample was centrifuged at 12000 rpm for 10 min, and then the supernatant was discarded and the precipitate was re-suspended in ethanol. Centrifugation was repeated under the same condition three times. The supernatants were collected; the absorbance of the supernatant was detected using a UV spectrophotometer (MAPADA, Shanghai, China). When mPEG-COOH could not be monitored, washing was stopped. The collected precipitates were just contained imprinted polymers, with mPEG-COOH as the template molecule (PEG molecularly imprinted polymers, PMIPs). To measure the adsorption performance of PMIPs, 4 mL of 0.2 mg/mL mPEG-COOH solution was added to 11 mg of PMIPs. The sample

was placed in a constant temperature (25 ℃) shaker at 100 rpm, and then subjected to full wavelength scanning by ultraviolet spectrophotometry at 200-300 nm after 30 min. The process was repeated, except that the mPEG-COOH solution was replaced by TRYP-PEG solution. The changes in absorbance were observed and the adsorption rate of PMIPs to PEG or TRYP-PEG was calculated using the following equation.

$$\text{Adsorption rate (mg/g)}=\frac{(\text{total mass of material-mass of unabsorbed material})}{(\text{total mass of PMIPs})}$$

1.4 Characterization of TRYP-PEG

The synthesized polymers TRYP-PEG were confirmed by 1H-NMR. The NMR spectrum was obtained using Varian 400MHz (Bruker, Germany), with tetramethylsilane (TMS) as the internal standard. Chemical shifts are expressed as parts per million (ppm). TRYP-PEG was dissolved in 0.5 mL of D_2O. Fourier transformed infrared spectroscopy (FTIR) was also used to confirm the structure of TRYP-PEG. The spectra were obtained using the Nicolet 5DXC IR spectrometer (Nicolet, Madison, USA) at a resolution of 2 cm^{-1} and a spectral range of 4000-400 cm^{-1}.

1.5 Preparation of PEGylated TRYP micelles

TRYP-PEG (4.3 mg) was dissolved in 2 mL of DMSO. The solution was stirred rapidly for 2 h, and then 10 mL of deionized water was added dropwise into the solution. The solution was stirred for 12 h, and then transferred into a pretreated dialysis bag (MWCO: 1 kDa) and dialyzed against deionized water. The outer phase was replaced with fresh deionized water every 3 h. After 12 h, the PEGylated TRYP micelles (PTMs) were obtained and stored at 4 ℃ for further use.

To measure the drug loading content, PDMs (10.0 mg) was dissolved in 10 mL of HCl (1 mol/L), the solution was stirred rapidly for 2 h at 60 ℃. Then, 2 mL of the above solution was taken and determined by UV-vis at 251 nm. The content of TRYP was calculated using a standard curve. Drug loading could be calculated using the following equations:

$$\text{Drug loading content}(\%)=\frac{(\text{weight of TRYP in micelles})}{(\text{total mass of micelles})}\times 100.$$

1.6 Drug-release study

The in vitro drug-release profile was determined using a dynamic dialysis method. Briefly, PTMs was incubated with phosphate-buffered saline (PBS) solution of different pH (pH 5.5 and 7.4). Typically, 5 mL of PTMs solution (2 mg/mL) was dialyzed (MWCO: 1 kDa) against 25 mL of PBS solution at 100 rpm in a constant temperature shaker at 37 ℃. At predetermined intervals (0.5, 1, 2, 3, 6, 9, and 12 h), 1 mL of external buffer was removed and an equal volume of PBS solution was refilled. The concentration of TRYP released from polymeric micelles was quantified by UV at 251 nm.

As PEG is a large molecule and unfavorable for the chromatography, to investigate fracture behavior of hydrazone bond and drug release of TRYP-PEG, the structural change of TRYP-$NNHCH_2CH_2OH$ under acidic conditions instead was studied.

In brief, 0.5 mg of TRYP-$NNHCH_2CH_2OH$ was dissolved in 4 mL of acetic acid solution (pH 5.5) for 30 min. The samples, TRYP, TRYP-$NNHCH_2CH_2OH$, and TRYP-$NNHCH_2CH_2OH$ in acetum, were detected by thin layer chromatography (TLC), and the developing solvent used was ethyl acetate-petroleum ether (50: 50, v/v).

High performance liquid chromatography (HPLC) was used to detect the three samples further. Chromatographic separation was carried out on the Agilent 1260 Infinity LC system (Agilent, Germany). Assay was conducted using a C18 column (200 mm × 4.5 mm, 5 μm particle size) under isocratic elution with acetonitrile-water (47:53, v/v). The flow rate was 0.8 mL/min and the injection volume was 10 μL. The detection wavelength was 251 nm and the column temperature was maintained at 25 ℃.

1.7 Determination of solubility

The solubility of PTMs was measured according to the method of general rules of Chinese Pharmacopoeia (2015). The PTMs was freeze dried before the experiment. To 1.0 g of PTMs, an appropriate amount of PBS (pH 7.4) was added at 25 ℃ and shaken for 30 s. Then, PBS was added dropwise until no insoluble substance could be observed, and then the volume of PBS was measured and solubility was calculated.

1.8 Particle size of PTMs

The particle size distribution of the micelles was evaluated by dynamic light scattering (DLS) using the Delsa™ Nano C Particle analyzer (BECKMAN Coulter Instruments, USA). The morphology of polymeric micelles was observed using a transmission electron microscope (TEM, JEM-1230, Japan).

1.9 Critical micellar concentration determination

The critical micelle concentration (CMC) was estimated by fluorescence probe method[27]. Pyrene (10.0 mg) was dissolved in 25 mL of acetone (1×10^{-5} mol/L). The micelle samples were dispersed in PBS (pH 7.4) at a concentration range of 1-10 μg/mL. Then, 20 μL of pyrene solution was evaporated in dark to remove the solvent, and 2 mL of polymer solutions of different concentrations was added. The mixed solutions were ultrasonically shaken for 1 min. After 12 h, the fluorescence values were measured. The excitation wavelengths were set at 335 nm. The fluorescent intensity at 373 and 384 nm were measured and the ratio of them was calculated. The CMC of micelles was determined from the inflection points in the fluorescent intensity ratios versu micelles concentration curves.

1.10 Tumor cell line and cell culture

Human breast tumor cell line MCF-7 was cultivated in DMEM supplemented with 10% fetal calf serum and 1% antibiotics in a humidified 5% CO_2/95% air atmosphere at 37 ℃.

1.11 In vitro cytotoxicity test

The in vitro cytotoxicity of TRYP and PTMs was investigated using the Cell Counting Kit-8 (CCK-8) assay. The cells were seeded into 96-well culture plates at a density of 5×10^4 cells per well and incubated for 24 h. Then, the corresponding culture medium was replaced by 200 μL of DMEM medium containing TRYP and PTMs at different concentrations under different pH (7.4 and 5.5). After incubating for another 48 h, 10 μL of CCK-8 solution was added to each well of the plate and the cells were incubated for 2 h. Cell viability was determined by scanning using the Bio-Rad 680 microplate reader at 450 nm.

1.12 Statistical analysis

The results are presented as mean ± standard deviation of at least three repetitive experiments for all the treatment groups. Statistical analysis was conducted using the one-way ANOVA with Student's t-test using a SPSS 23.0 program; $P<0.05$ indicated significant difference.

2 Results

2.1 Characterization of TRYP−PEG

The synthetic route of TRYP-PEG is illustrated in Fig. 2A. Hydrophilic flexible chain (polyethylene glycol) was introduced into TRYP by pH-sensitive hydrazone bond. Pegylated TRYP was obtained by a two-step reaction. Firstly, TRYP was modified by 2-hydroxyethyl hydrazine and the pH-sensitive hydrazone bond was introduced at C6 of TRYP. Therefore, the intermediate product TRYP-$NNHCH_2CH_2OH$ was obtained. Then, TRYP-PEG was obtained by esterification between the carboxyl groups of PEG and hydroxy groups of TRYP-$NNHCH_2CH_2OH$. The excess mPEG was removed by molecular imprinting.

The chemical structure of TRYP-PEG was characterized by 1H-NMR and FTIR. As shown in Fig. 2B, the characteristic peak of both TRYP (δ 7.4-8.4 ppm) and PEG (δ 3.6 ppm) was observed, which proved that PEG was conjugated to TRYP successfully [28]. As shown in Fig. 2C, the IR spectrum of TRYP-PEG presented new absorption peaks at 1095 cm^{-1} (C-O-C stretching vibration) and 1778 cm^{-1} (C=O stretching vibration). The appearance of characteristic peaks of PEG and ester group verified the successful synthesis of TRYP-PEG.

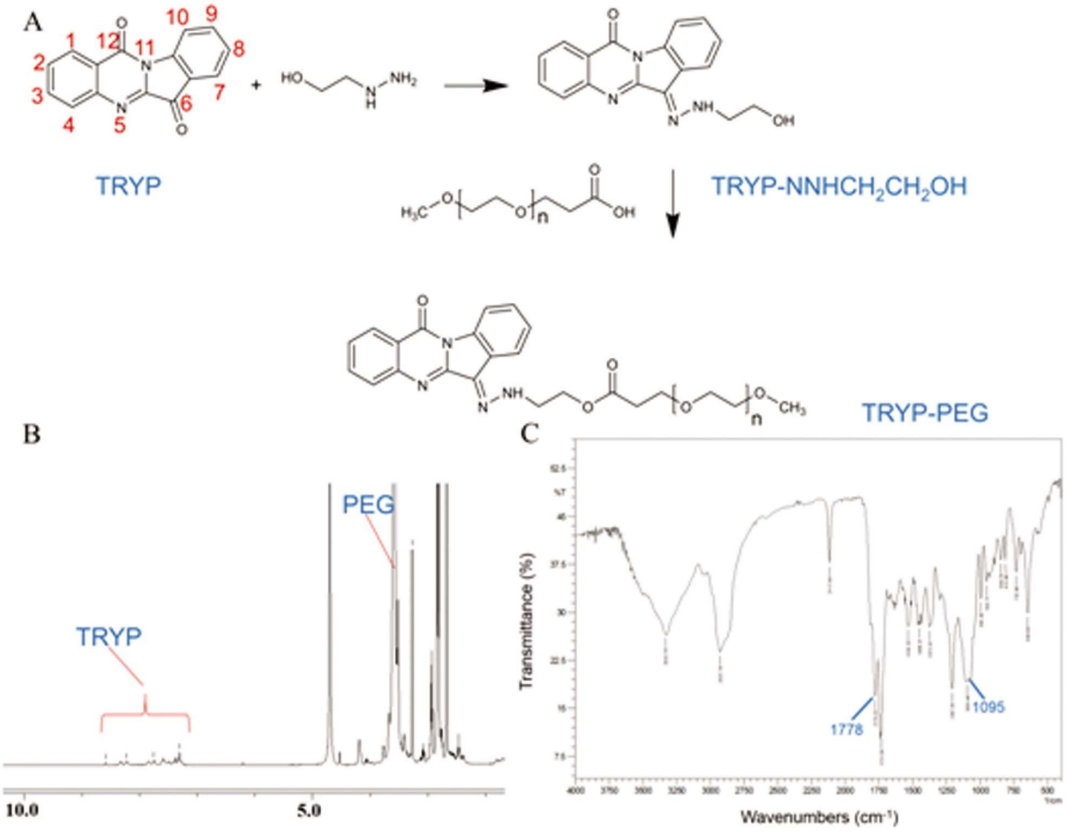

图 2 TRYP-PEG 的合成路线(A),核磁共振氢谱(B)和红外光谱(C)

Fig. 2 Synthetic route (A), 1H-NMR (B, 400 MHz, D2O) and FTIR (C) of TRYP-PEG

2.2 Selectivity of imprinted polymer

The adsorption capacity of PMIPs to mPEG-COOH and TRYP-PEG was evaluated by UV-vis spectrophotometry. The adsorption rates of PMIPs to mPEG-COOH and TRYP-PEG were 520.2 and 7.3 mg/g, respectively. The adsorbability of PMIPs to mPEG-COOH was 71 times higher than that of TRYP-PEG, showing that through molecular imprinting, mPEG-COOH could be absorbed by PMIPs efficiently, which indicated that relatively pure

TRYP-PEG could be obtained.

2.3 Drug-release study

The release of TRYP from PTMs was measured to confirm the pH sensitivity of drug release. The in vitro TRYP-release amount was investigated at different pH (pH 7.4 and 5.5). As shown in Fig. 3A, the release of TRYP was negligible at pH 7.4 with an accumulative release profile of 25% in 12 h, indicating that the hydrazone bond was relatively stable under physiological conditions. However, as the pH decreased from 7.4 to 5.5, there was an increase in the release of TRYP and approximately 95% of TRYP was released in 12 h. The pH of lysosome is below 5.5 and the value is lower in tumor sites [29]. It was obvious that the release of TRYP was pH dependent, and the drug was released rapidly and completely under acidic conditions, which will enable the application of the drug against tumor.

TRYP-$NNHCH_2CH_2OH$ was stable in PBS (pH 7.4). However, in acidic solution, hydrazone bond was cracked and TRYP was released. As shown in Fig. 3B, after incubation with acetum for 30 min, the spot of TRYP-$NNHCH_2CH_2OH$ disappeared, whereas a new spot was observed in the TLC plate, and its retardation factor (Rf=0.684) was the same as that of TRYP.

HPLC was used to further analyze the substance. Peaks of TRYP (tR=11.393 min) and TRYP-$NNHCH_2CH_2OH$ (tR=15.859 min) were observed in the spectra presented in Fig. 3C and 3D, respectively. As shown in Fig. 3E, after incubation with acetum for 30 min, a peak of TRYP (tR=11.344 min) appeared, which indicated the disruption of hydrazone bond and release of TRYP.

Drug loading content of PTMs was 11.32± 1.43%.

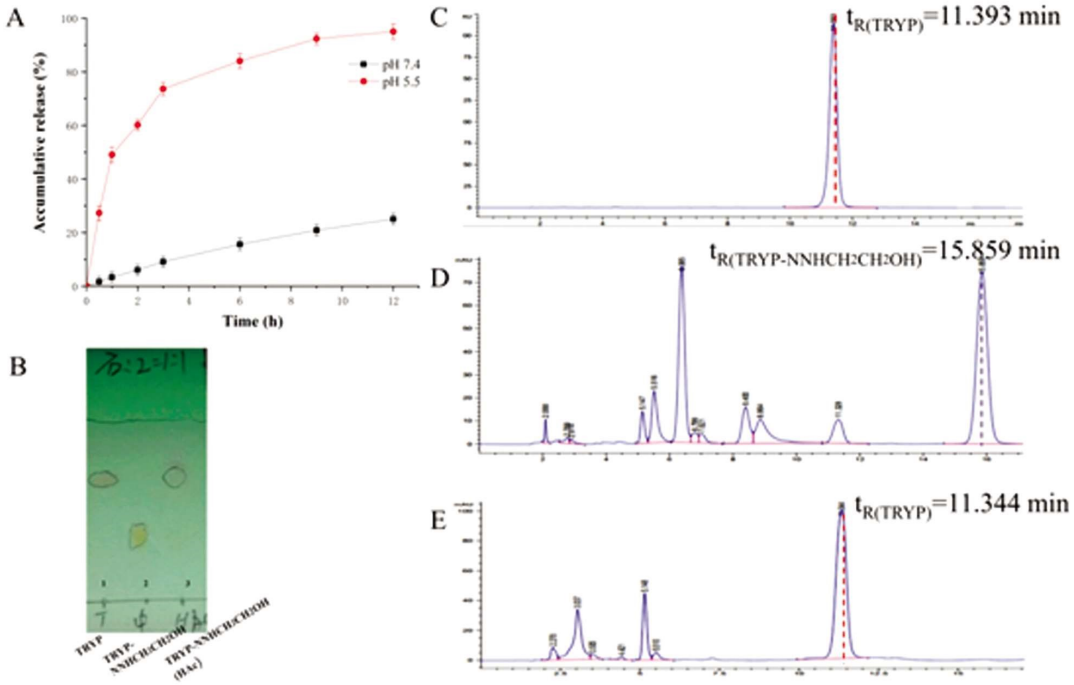

图 3 PTMs 在不同 pH 下色胺酮的累计释放率(n=3)(A),TRYP, TRYP-$NNHCH_2CH_2OH$ 和 TRYP-$NNHCH_2CH_2OH$ 在醋酸中作用 30 min 的解离产物的薄层色谱(B),TRYP (C),TRYP-$NNHCH_2CH_2OH$ (D), 以及 TRYP-$NNHCH_2CH_2OH$ 在醋酸中作用 30 min 的解离产物(E)的高效液相色谱

Fig. 3 Accumulative release profiles of TRYP from PTMs in different pH (n=3) (A). TLC of TRYP, TRYP-$NNHCH_2CH_2OH$, and TRYP-$NNHCH_2CH_2OH$ in acetum (B). HPLC of TRYP (C), TRYP-$NNHCH_2CH_2OH$ (D), and separated product of TRYP-$NNHCH_2CH_2OH$ in acetum for 30 min (E).

2.4 Solubility in aqueous solution

The solubility of TRYP in PTMs was 8.065 mmol/L, which was around 1493-fold higher than that of TRYP (5.4 μmol/mL in aqueous solution), indicating the strong solubilizing ability of PEG. The application of TRYP has been studied more and more, however, there is only little research on improving its solubility [30]. Our study provided a relatively simple but effective way to improve the solubility of TRYP.

2.5 Particle size and CMC of PTMs

The PTMs were prepared using the dialysis method, with a particle size of 228.8 nm and PDI of 0.1 (Fig. 4A). The morphology of PTMs was examined by TEM. As shown in Fig. 4B, the micelles were spherical with an average diameter of 50 nm, which was considerably smaller than the particle size measured by DLS. It was probably because the DLS analysis provided dynamic diameter in aqueous solution, which was larger than the size of dried particles. The CMC of PTMs was 3.5×10^{-7} mol/L (Fig. 4C). The low CMC indicated the high stability of prepared micelles and facilitated further use.

2.6 Cytotoxicity test

In vitro cytotoxicity of TRYP and PTMs against MCF-7 cells was studied using the CCK-8 assay. Fig. 5 shows the viability of MCF-7 cells incubated with different concentrations of TRYP or PTMs for 48 h. The pH of 7.4 and 5.5 was selected to simulate the

normal physiological condition and tumor site acidic condition, respectively.

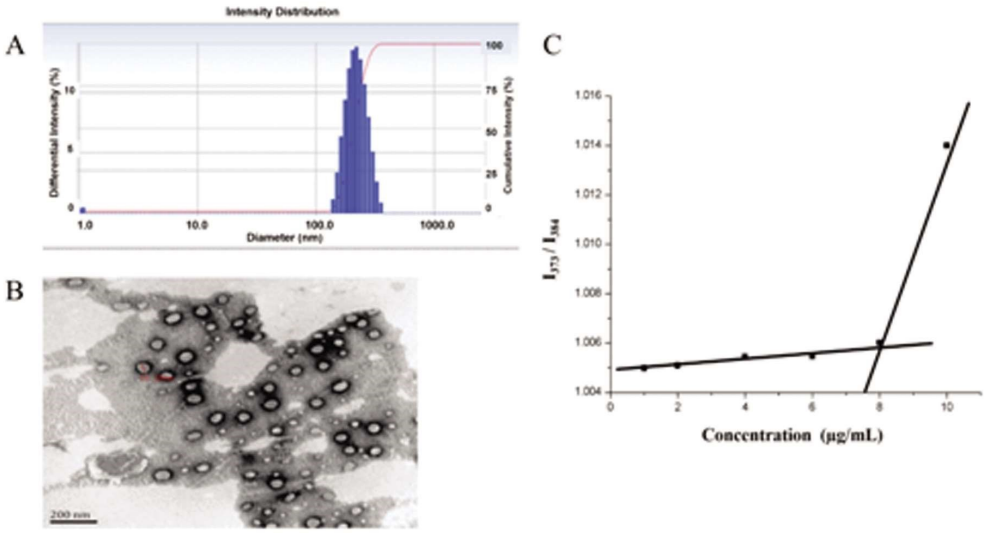

图 4 PTMs 的粒径及粒径分布(A)，透射电镜图(B)和临界胶束浓度(C)

Fig. 4 Particle size and distribution of PTMs (A), TEM of PTMs (B) and CMC of PTMs (C)

As shown, both drug and PTMs showed concentration-dependent toxicity. The viability of MCF-7 cells decreased with the increase in the concentration of TRYP or PTMs. The viability of PTM-treated cells was higher than that of TRYP-treated cells at pH 7.4 (P<0.05), which indicated that the cytotoxicity of PTMs was reduced compared with that of TRYP under normal physiological conditions. This may be due to the stability of TRYP when prepared as micelles. The PTMs exhibited the same cytotoxicity at pH 5.5, which indicated that PTMs had activity comparable to that of TRYP under acidic conditions.

Combined with previous study results, that is, the TRYP-release rate from micelles was higher under acidic conditions and the pH of tumor microenvironment is significantly higher than that of normal tissues, the PTMs have potential use as an antitumor agent.

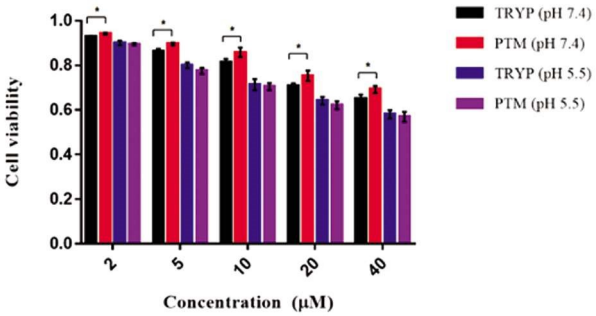

图 5 TRYP 和 PTMs 对 MCF-7 细胞的细胞毒性(*P<0.05)

Fig. 5 Cytotoxicity of TRYP and PTMs on MCF-7 cells (*P<0.05)

Herein, micellar tryptanthrin was prepared. The solubility of TRYP in PTMs was increased by 1493 fold compared with that of TRYP, respectively, making it possible for effective use. Besides, the micelles had relatively low CMC, which indicated the high stability of prepared micelles and was beneficial to the application of TRYP in vivo. The drug release from PTMs was pH dependent. The complex exhibited stability under physiological conditions and the drug-release rate was extremely low; however, TRYP was almost completely released within 12 h at lower pH 5.5, which was propitious for drug release, and exerted the pharmacological action under acidic condition. Moreover, compared with the parent drug TRYP, the cytotoxicity of PTMs decreased at pH 7.4, which indicated that the cytotoxicity of PTMs was reduced under normal physiological conditions. However, the PTMs showed comparable cytotoxicity due to the dissociated TRYP at tumor intracellular pH 5.5. It indicated that pegylated tryptanthrin not only improved water solubility of TRYP effectively but also could be a promising tumor targeting prodrug.

So, the preparation of micellar TRYP as nanomicelles improved the solubility of TRYP, which lays a foundation for further improvement in its bioavailability and application of anti-tumor.

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胶束化色胺酮的制备及表征 *

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摘要 目的：通过制备胶束化色胺酮，增加色胺酮的溶解度，并进一步提高其生物利用度。**方法：**以酸敏感的腙键连接聚乙二醇和色胺酮，并通过透析法，将聚乙二醇化色胺酮进一步制备成胶束。用动态光散射法测定胶束的粒径分布，用透射电镜观察胶束的形貌。通过芘荧光探针法测定胶束的临界胶束浓度。测定胶束在不同 pH 下的药物释放情况（pH5.5 和 7.4）。采用薄层色谱法和高效液相色谱法研究腙键的断裂行为。通过 CCK-8 法比较生理 pH 和酸性 pH 下，色胺酮和聚乙二醇化色胺酮胶束 (PTMs)对 MCF-7 细胞的体外细胞毒性。**结果：**与色氨酸相比，PTMs 的溶解度提高了 1493 倍。制备的胶束粒径为 228.8 nm，PDI 为 0.1，形貌为球形。PTMs 的临界胶束浓度为 3.5×10^{-7} mol/L，较低的 CMC 值表明制备的胶束稳定性高，便于进一步使用。腙键可在酸性条件下发生断裂，且在 pH 5.5 下，12 h 内 95%的色胺酮从胶束中释放，而在生理 pH 下（pH 7.4），药物释放缓慢。在生理条件下，胶束的细胞毒性低于色胺酮，说明胶束化色胺酮可降低药物毒性及胶束在生理条件下有一定的稳定性。而在 pH 5.5 时，色胺酮胶束与色胺酮的细胞毒性相近，表明胶束可响应肿瘤细胞内的低 pH 值，成功实现药物释放。**结论：**胶束化色胺酮不仅能有效改善色胺酮的溶解度，有利于进一步提高其生物利用度，而且是一种很有应用前景的肿瘤靶向前药。

关键词：胶束；聚乙二醇；色胺酮；水溶性

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