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Preparation and Characterization of Two Types of Tryptanthrin-Loaded Nanomicelles

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Abstract: Tryptanthrin has not been widely applied in clinical practice due to its poor solubility and low bioavailability in spite of possessing several biological and pharmacological activities. Here, to improve the solubility of tryptanthrin, two types of novel tryptanthrin-loaded micelles were prepared. One was tryptanthrin physically encapsulated by distearoyl phosphatidylethanolamine polyethylene glycol (DSPE-PEG) and the other was pegylated tryptanthrin synthesized by acid-sensitive hydrazone bond and further prepared as micelles. Molecular imprinting technology was used to separate pegylated tryptanthrin and free mPEG-COOH in the preparation of PEGylated tryptanthrin micelles with considerably high separation efficiency. The solubility of tryptanthrin-loaded DSPE-PEG micelles (TDMs) and PEGylated tryptanthrin micelles (PTMs) was increased by 300 and 1493 fold compared with that of tryptanthrin, respectively. The PTMs increased the solubility of tryptanthrin more effectively and 95% of tryptanthrin was released from PTMs at pH 5.5 in 12 h. The cytotoxicity of PTMs decreased under physiological conditions compared with that of tryptanthrin, whereas at pH 5.5, the PTMs showed comparable cytotoxicity with that of tryptanthrin, indicating successful drug release from the carrier in response to tumor cell pH. Overall, we elucidated an efficient method to improve water solubility of tryptanthrin and indicated pegylated tryptanthrin is a promising prodrug.

Keywords: nanomicelle; polyethylene glycol; tryptanthrin; water soluble

1. 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 [1,2]. It is reported that TRYP has a broad spectrum of biological activities including anticancer, anti-inflammatory, antiprotozoal, antiallergic, and antimicrobial activities [3,4]. 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 [5]. However, the poor water solubility (1.339 μg/mL in water [6]) of TRYP limits its absorption in the body, resulting in its extremely low bioavailability.

Liposomes and micelles [7-9] 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 [10] cannot form stable lipidosomes. Owing to the advantages of good solubilization effect and simple production process, polymer micelles have garnered considerable attention [11,12]. 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 [13]. Besides, the preparation process of polymer drug conjugate micelles is relatively simple, and drugs can be linked to polymers by physical encapsulation or chemical binding [14], which greatly expands their range of applications.

One of the polymers that is most commonly used in the preparation of polymer drugs is polyethylene glycol (PEG). Polyethylene glycol is an ideal drug carrier material and the Food and Drug Administration has approved PEG for human intravenous, oral, and dermal applications [15,16]. 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 [17].

Molecular imprinting technology (MIT) has been widely applied in various fields to selectively separate target molecules because of its recognition specificity and application universality [18,19]. MIT was innovatively applied for the separation of PEGylated TRYP and mPEG-COOH of similar molecular weight in our work.

In order to improve the solubility of TRYP, two types of TRYP-loaded micelles were prepared in this study. The first method was encapsulating TRYP with DSPE-PEG. Amphiphilic DSPE-PEG was used to form micelles by employing the solvent evaporation method and hydrophobic TRYP was encapsulated in it (Figure 1a). The second 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 (Figure 1b).

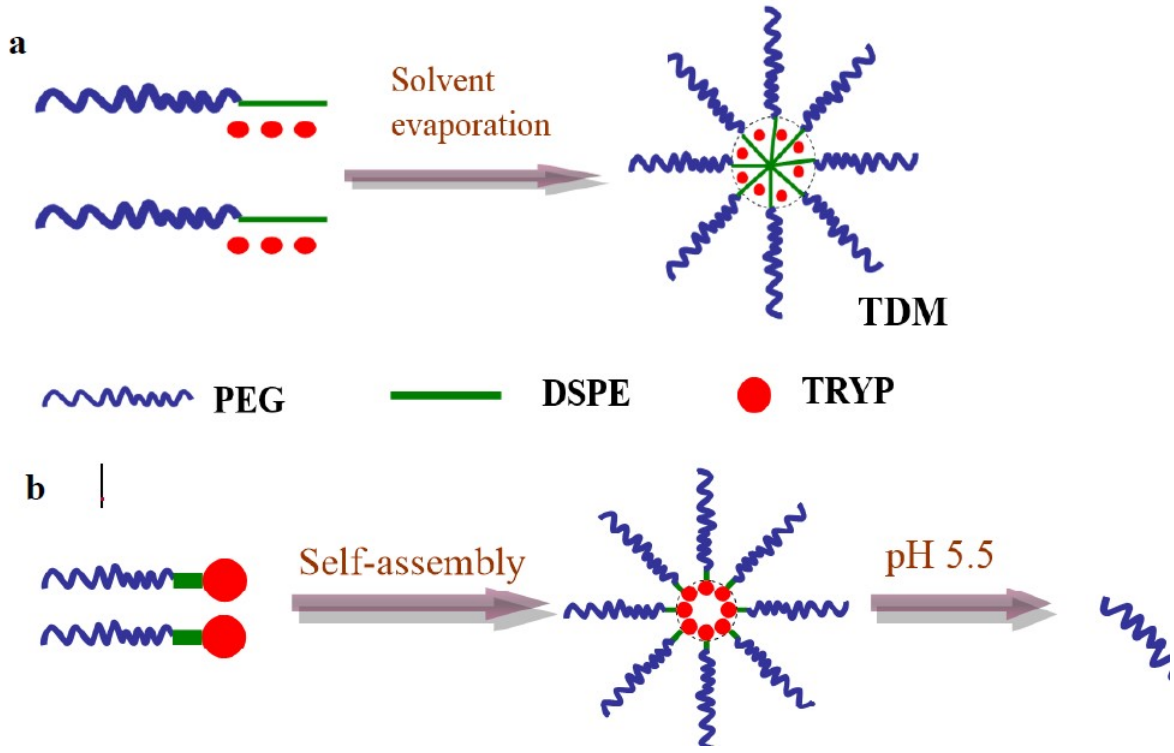

Figure 1. Schematic diagram of TDMs (a) and PTMs (b).

2. Materials and Methods

2.1. 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 Fourth Military Medical University. Distearoyl phosphatidylethanolamine -[methoxy (polyethylene glycol)-2000] (DSPE-PEG$_{2000}$) was purchased from Ruixi BioEngineering Inc. Methoxy polyethylene glycol (molecular mass 2000 Da, purity :>95%) was purchased from Nanocs(America). Acetonitrile (ACN)

was purified through 0.22 μm membrane. 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.

2.2. Preparation and verification of TRYP-loaded DSPE-PEG micelles

DSPE-PEG_{2000} (44.29 mg) and TRYP (100 mg) were dissolved in 30 mL of trichloromethane. After the removal of solvent by rotary evaporation and desiccation, a thin film was acquired. It was dissolved in phosphate buffer solution and passed through hydrophilic membrane filters (0.45 μm). The filtrate contained only TRYP-loaded DSPE-PEG micelles (TDMs).

The encapsulation of TRYP was verified by proton nuclear magnetic resonance (^{1}H NMR). The NMR spectra were obtained using Varian 400MHz (Bruker, Germany), with tetramethylsilane (TMS) as the internal standard. Chemical shifts are expressed as parts per million (ppm). TRYP and DSPE-PEG_{2000} (10 mg) were dissolved in 0.6 mL of $CDCl_3$. Thereafter, two equal parts of TDMs (10 mg) were dissolved in D_2O and $CDCl_3$, respectively.

2.3 Synthesis of TRYP-PEG

Tryptanthrin (248 mg, 1 mmol) was dissolved in 25 mL of tetrahydrofuran (THF) and 2-hydroxyethyl hydrazine (76 mg, 1 mmol) was dissolved in 3 mL of ethanol. The mixture was stirred at room temperature 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.

mPEG$_{2000}$-COOH (40 mg, 0.02 mmol), TRYP-$NNHCH_2CH_2OH$ (6.12 mg, 0.02 mmol), 4-dimethylaminopyridine (DMAP, 22.3 mg, 0.183 mmol), and dicyclohexylcarbodiimide (DCC, 26.5 mg, 0.136 mmol) 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.

2.4. Preparation and adsorption of imprinted polymer

mPEG-COOH (100 mg, 0.05 mmol), acrylamide (3.55 mg, 0.05 mmol), and N,N-dimethylacrylamide (39.6 mg, 0.4 mmol) were dissolved in 30 mL of distilled water. The solution was then stirred at room temperature for 30 min. Thereafter, ammonium persulfate (100 mg, 0.44 mmol) was added and the solution was placed in an oil bath at 85°C under vigorous stirring with reflux condensation for 12 h. Ten microliters of anhydrous ethanol was added to the product and the mixture was transferred to centrifuge tubes. The sample was centrifuged at 12000 rpm for 10 min, and then the supernatant was discarded and the precipitate was resuspended in ethanol. Centrifugation was repeated under the same condition three times or more. 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 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°C) 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 of the same concentration. The changes in absorbance were observed and the adsorption rate of PMIPs to PEG or TRYP–PEG was calculated using the following equation.

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

2.5. Characterization of TRYP–PEG

The synthesized polymers TRYP–PEG were confirmed by ^{1}H NMR. 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}. The sample was ground with KBr into a fine powder.

2.6 Drug-release study

The in vitro drug-release profile was determined using a dynamic dialysis method. Briefly, TRYP–PEG was incubated with phosphate-buffered saline (PBS) solution of different pH (pH 5.5 and 7.4). Typically, 5 mL of TRYP–PEG 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°C. At predetermined intervals (0.5, 1, 2, 3, 6, 9, and 12 h), 1 mL of external buffer was removed and a equal volume of PBS solution was refilled. The concentration of TRYP released from polymeric micelles was quantified by ultraviolet spectrophotometry at 251 nm.

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

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, and the developing solvent used was ethyl acetate-petroleum ether (50:50, v/v).

High performance liquid chromatography (HPLC) was used to detecte 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°C.

2.7 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 slowly 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°C for further use.

2.8 Determination of solubility

The solubility of TDMs and PTMs was measured according to the method of general rules of Chinese Pharmacopoeia (2015). The TDMs and PTMs were freeze dried before the experiment. To 1 g of TDMs and PTMs, an appropriate amount of PBS (pH 7.4) was added at 25°C and shaken for 30 s. Then, PBS was added dropwise until there were no insoluble substance, and then the volume of PBS was measured and solubility was calculated.

2.9 Particle size distribution of TDMs and 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).

2.10 Critical micellar concentration determination

The critical micelle concentration (CMC) was estimated by fluorescent spectroscopy [20]. Pyrene (10 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.

2.11 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 (containing penicillin and streptomycin) in a humidified 5% CO_2/95% air atmosphere at 37°C.

2.12In vitro cytotoxicity test

The in vitro cytotoxicity of TRYP-$NNHCH_2CH_2OH$ and TRYP–PEG 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 at 37°C 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 at 37°C. Cell viability was determined by scanning using the Bio-Rad 680 microplate reader at 450 nm.

2.13 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.

3. Results and Discussion

3.1. Preparation and verification of TDMs

As reported, the characteristic peaks of tryptamine were at δ 8.42 ppm (d), δ 7.66 ppm (t), δ 7.84 ppm (t), δ 8.02 ppm (d), δ 7.90 ppm (d), δ 7.42 ppm (t), δ 7.78 ppm (t), and δ 8.62 ppm (d), and PEG compounds presented large peaks around δ 3.6–3.7 ppm, which can be ascribed to repeated -CH_2 groups of the PEG chains [21]. The shell of TDMs was hydrophilic, and therefore, the micelle was stable in aqueous solution and tryptamine was encapsulated in the core of the micelle. Therefore, the characteristic peaks of tryptamine did not appear in the spectrum presented in Figure 2c. On the contrary, the "core-shell" structure of micelle was broken by trichlormethane and TRYP was exposed as shown in Figure 2d. The results of ^1H NMR verified the encapsulation of TRYP.

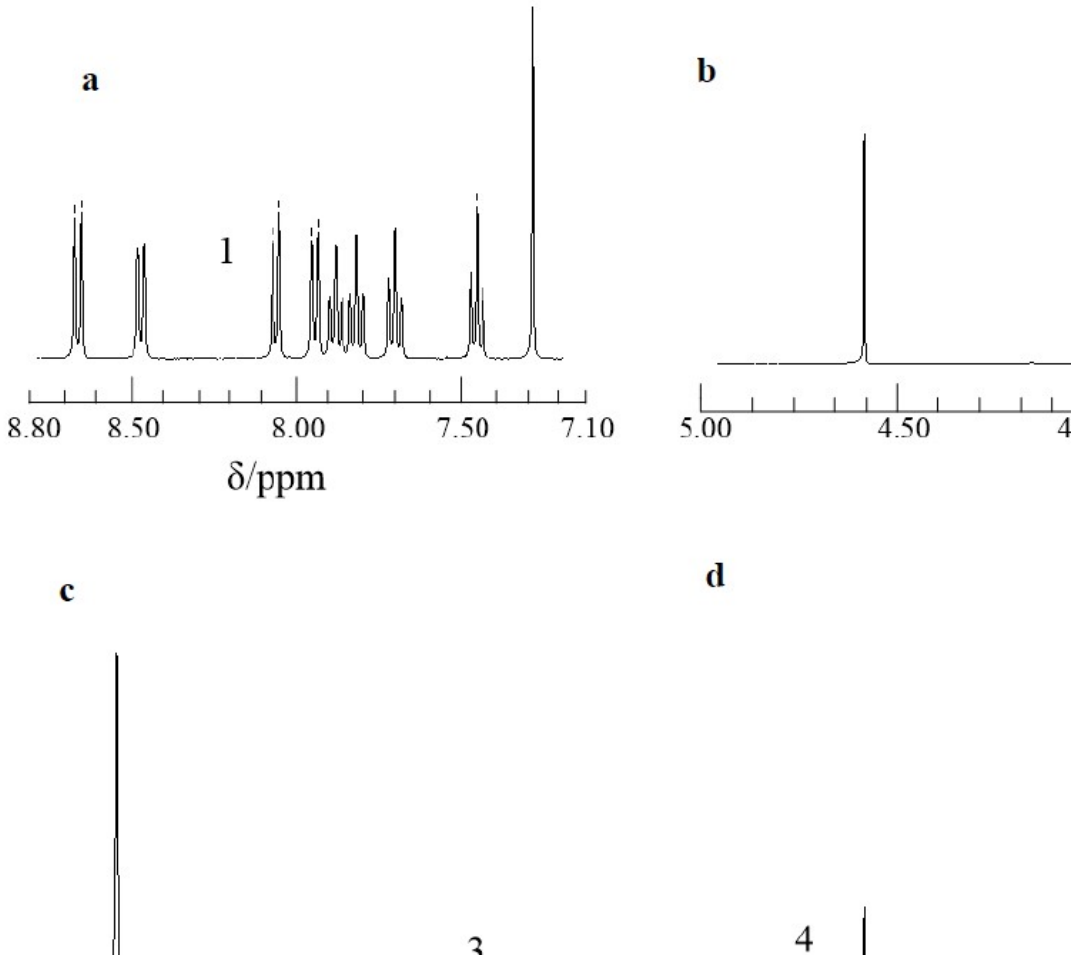

Figure 2. ^{1}H-NMR spectra of TRYP (a), DSPE-PEG$_{2000}$ (b), TDMs dissolved in D_2O (c), and TDMs dissolved in $CDCl_3$ (d).

3.2. Preparation and characterization of TRYP-PEG

The synthetic route of TRYP-PEG is illustrated in Figure 3a. 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$_2$CH$_2$OH was obtained. The reaction conditions were mild and the products were purified by column chromatography. Then, TRYP–PEG was obtained by esterification between the carboxyl groups of PEG and hydroxy groups of TRYP-NNHCH$_2$CH$_2$OH. The excess mPEG was removed by molecular imprinting and a pure product was obtained.

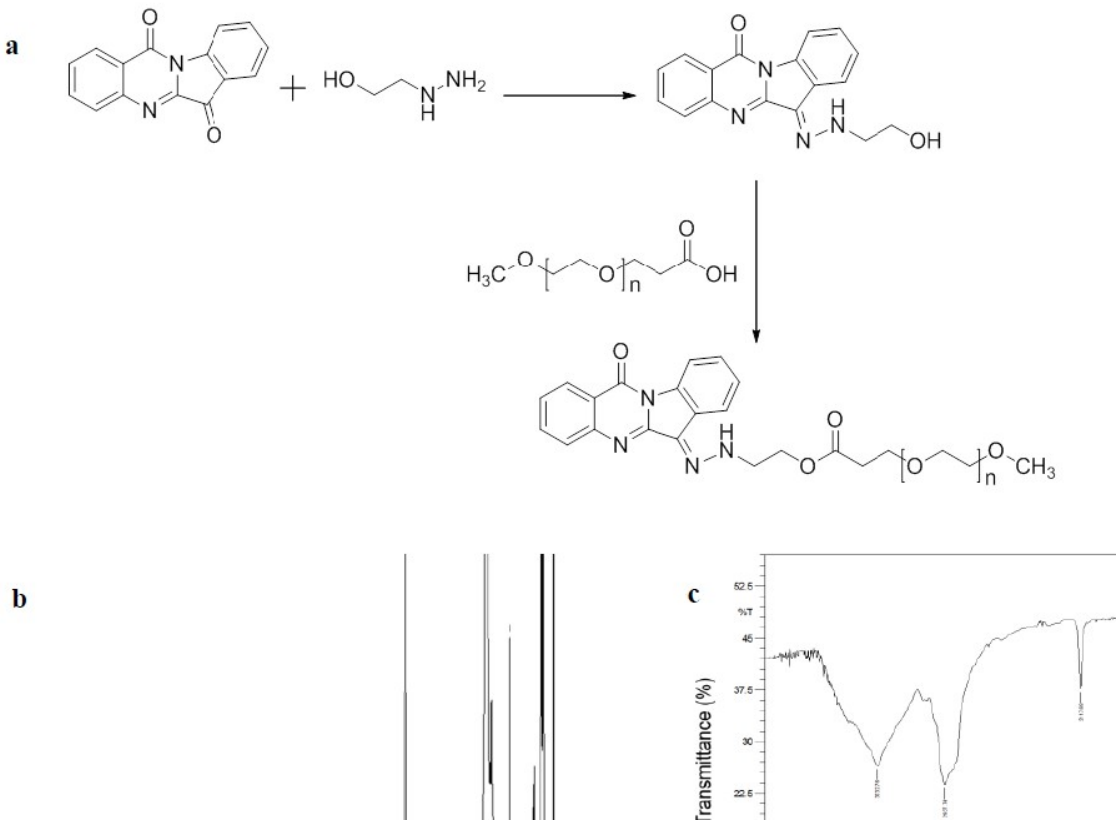

Figure 3. Synthetic route (a), ^{1}H-NMR spectra (b), and FTIR spectra (c) of TRYP–PEG.

The chemical structure of TRYP–PEG was characterized by ^{1}HNMR and FTIR. As shown in Figure 3b, the characteristic peak of both TRYP (δ 7.4–8.4 ppm) and PEG (δ 3.6ppm) was observed, which proved that PEG was conjugated to TRYP successfully. As shown in Figure 3c, the spectra 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.

3.3. Adsorption experiments 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 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 excess mPEG-COOH could be separated from TRYP–PEG effectively and relatively pure TRYP–PEG could be obtained.

3.4. Drug-release study

The release of TRYP from TRYP–PEG 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 Figure 4a, 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. 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.

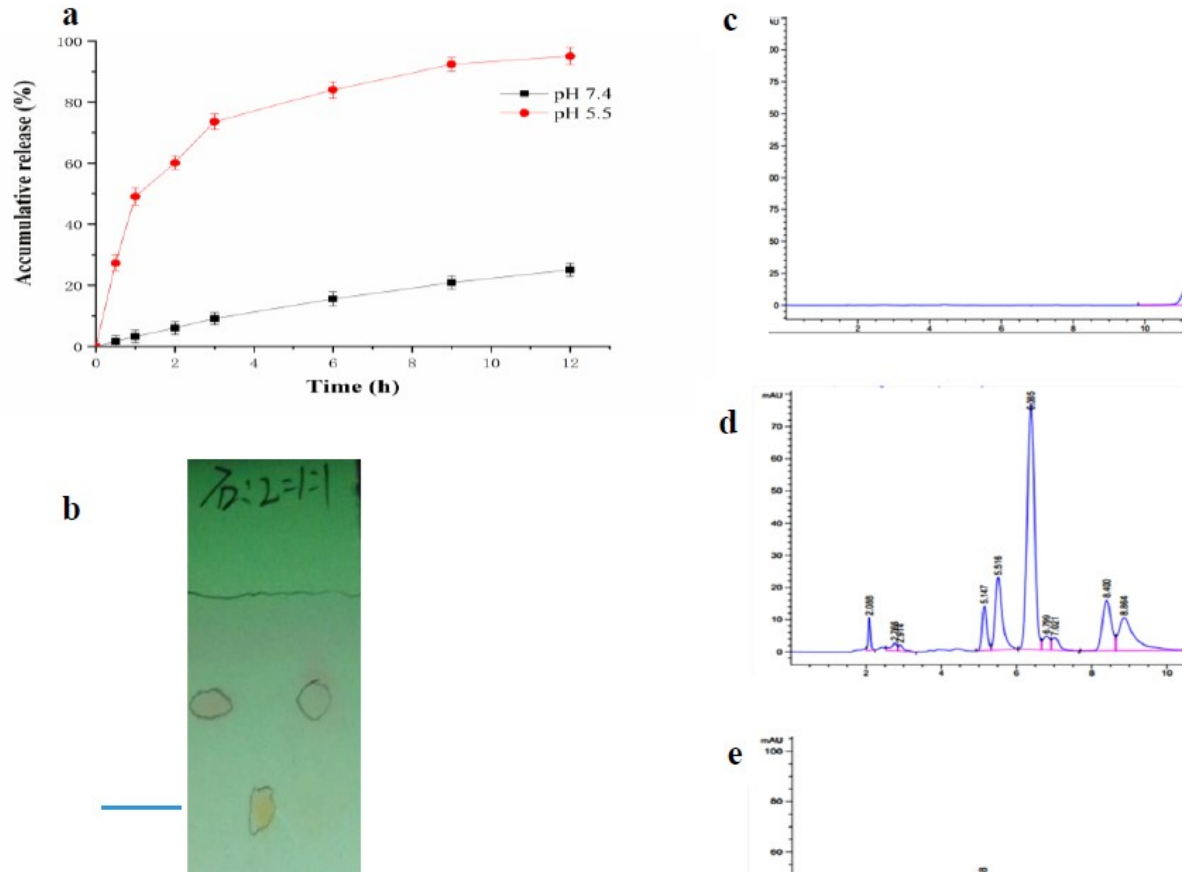

Figure 4. Accumulative release profiles of TRYP from TRYP–PEG under different pH (n = 3) (a). TLC chromatogram of TRYP, TRYP-NNHCH$_2$CH$_2$OH, and TRYP-NNHCH$_2$CH$_2$OH in acetum (b). HPLC of TRYP (c), TRYP-NNHCH$_2$CH$_2$OH (d), and separated product of TRYP-NNHCH$_2$CH$_2$OH in acetum for 30 min (e).

TRYP-NNHCH$_2$CH$_2$OH was stable in PBS (PH 7.4). However, in acidic solution, hydrazone bond was cracked and TRYP was released. As shown in Figure 4b, after incubation with acetum for 30 min, the spot of TRYP- NNHCH$_2$CH$_2$OH disappeared, whereas a new spot was observed in the TLC plate, and its retardation factor (R_f) was the same as that of TRYP.

High performance liquid chromatography was used to further analyze the substance. Peaks of TRYP (t_R = 11.39min) and TRYP- NNHCH$_2$CH$_2$OH (t_R = 15.85 min) were observed in the spectra presented in Figure 4c and 4d, respectively. As shown in Figure 4e, after incubation with acetum for 30 min, a peak of TRYP (t_R = 11.39min) appeared, which indicated the disruption of hydrazone bond and release of TRYP.

3.5. Solubility in aqueous solution

The solubility of TRYP in TDMs and PTMs was 1.625 and 8.065 mmol/L, respectively, which was around 300- and 1493-fold higher than that of TRYP (1.339 μg/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 [22]. Our study provided a relatively simple but effective way to improve the solubility of TRYP.

Furthermore, it is apparent that the method of preparing micelles by chemical bonding could increase the solubility of TRYP more effectively than physical encapsulation, therefore the PTMs were chosen for further analyses.

3.6. Particle size distribution of TDMs and PTMs

The TDMs were prepared using the solvent evaporation method, with a particle size of 112.5 nm and PDI of 0.15 (Figure 5a). The PTMs were prepared using the dialysis method, with a particle size of 228.8 nm and PDI of 0.1 (Figure 5b). The morphology of PTMs was examined by TEM. As shown in Figure 5c, 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.

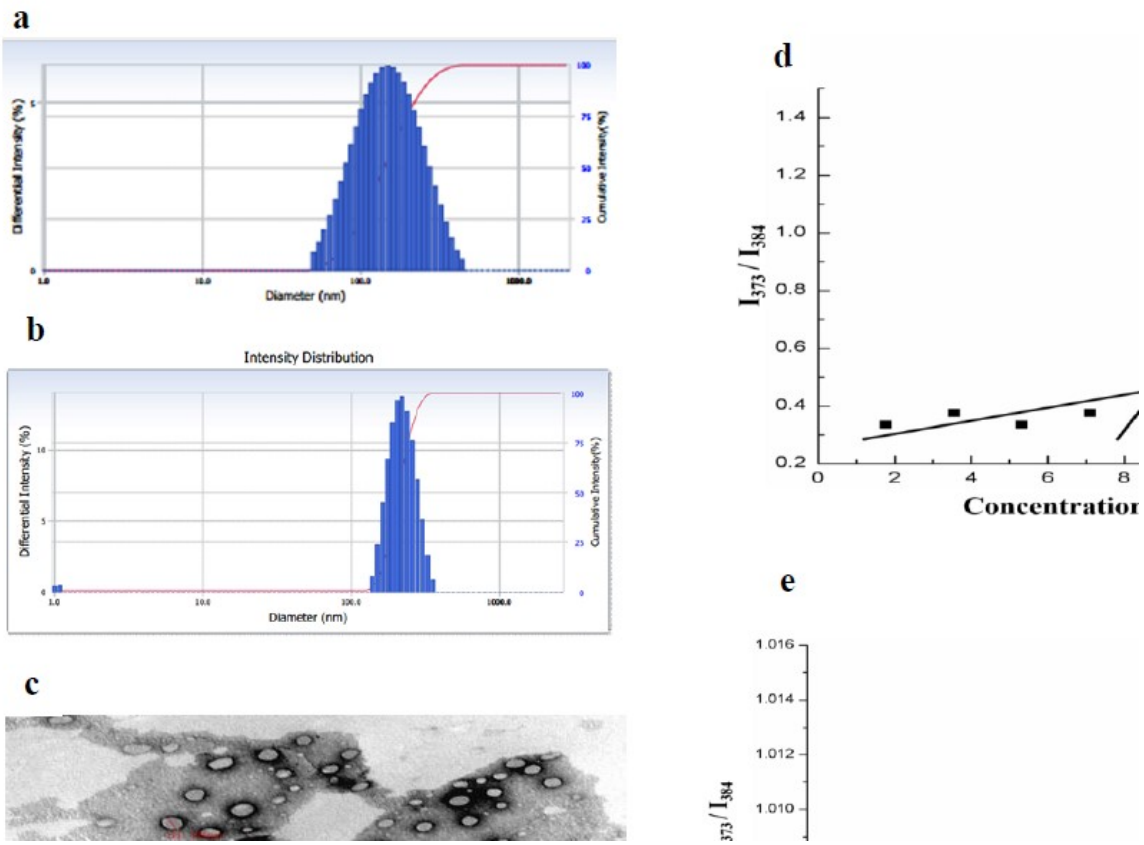

Figure 5. Particle size and distribution of TDMs (a) and PTMs (b). TEM of PTMs (c). CMC of TDMs (d) and PTMs (e).

The CMC of TDMs and PTMs was 8.93×10^{-6} and 3.5×10^{-7} mol/L, respectively (Figure 5d and 5e). The low CMC indicated the high stability of prepared micelles and facilitated further use; both of them were stable.

3.7. Cytotoxicity test

In vitro cytotoxicity of TRYP and PTMs against MCF-7 cells was studied using the CCK-8 assay. Figure 6 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.

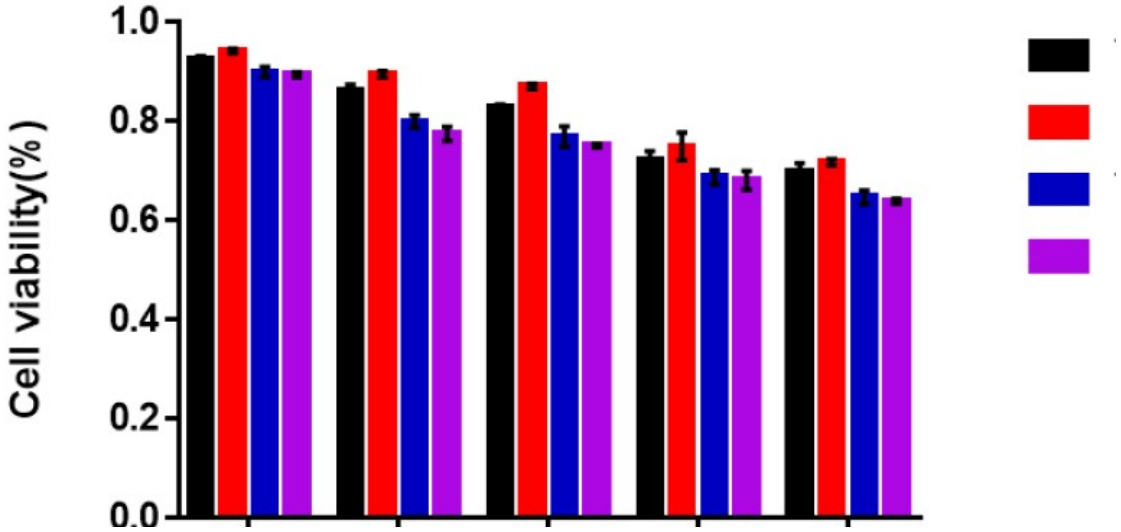

Figure 6. Cytotoxicity of TRYP and PTMs on MCF-7 cells at different pH (7.4 and 5.5).

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.

5. Conclusions

There have been more and more application studies of TRYP, however, only little research on increasing its solubility. Herein, we report two novel strategies to improve the solubility of TRYP. The solubility of TRYP in TDMs and PTMs was increased by 300 and 1493 fold compared with that of TRYP, respectively, making it possible for it to be used effectively. Besides, both these micelles had relatively low CMC, which indicated the high stability of prepared micelles and facilitated their further use.

The PTMs could improve the solubility of TRYP more obviously; therefore, these micelles were chosen for further research. 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 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. Whereas at pH 5.5, the PTMs showed comparable cytotoxicity with TRYP. The results indicated that these nanomicelles could be applied as an anti-tumor agent.

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

Author Contributions: H. W. conceived and designed the experiments; X. H., Q. Z. and Y.Q. performed the experiments; X. H. and Q. Z. analyzed the experimental data; Z.Y. made the graphics; X. H. wrote the paper; H. W. and T. Y. reviewed and edited the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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