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# TPGS-g-PLGA/Pluronic F68 mixed micelles for tanshinone IIA delivery in cancer therapy

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ABSTRACT

Tanshinone IIA (TAN) has few clinical applications for anti-cancer therapy mainly due to its high lipophicity, low cellular uptake, and poor bioavailability. To improve the anti-cancer effect and bioavailability of TAN, we developed a mixed micelle system constituted with D-α-tocopheryl polyethylene glycol succinate-graft-poly(D,L-lactide-co-glycolide) (TPGS-g-PLGA) copolymer and Pluronic F68. TAN was encapsulated in the TPGS-g-PLGA/Pluronic F68 mixed micelles by using the thin film hydration technology optimized by the central composite design/response surface method (CCD/RSM). TAN-loaded mixed micelles were highly stable in the presence or absence of bovine serum albumin (BSA) and achieved sustained drug release *in vitro*. Compared with free TAN, TAN mixed micelles had higher cytotoxicity and pro-apoptotic effects against human hepatocellular carcinoma HepG2 cells. The significant enhancement on pro-apoptosis by TAN micelles was evidenced by increased chromosome condensation, mitochondria membrane potential loss, cell apoptosis, and cleavages of caspase-3 and PARP. Furthermore, pharmacokinetic studies revealed that TAN mixed micelles significantly prolonged the circulation time and improved bioavailability of TAN in rats. These results demonstrated that TAN-loaded TPGS-g-PLGA/F68 mixed micelles are an effective strategy to deliver TAN for cancer therapy.


## 1. Introduction

Tanshinone IIA (TAN), a lipophilic compound isolated from Radix *Salviae miltiorrhizae* (Danshen), has been approved for treating cardiovascular disease by U.S. Food and Drug Administration (FDA) (Tian and Wu, 2013; Xu and Liu, 2013). TAN has also received many attentions as an anti-cancer agent because it induces cell apoptosis in several types of cancers, including hepatocellular carcinoma (HCC) (Chen et al., 2013; Chiu et al., 2013; Hu et al., 2013; Huang et al., 2013; Xu et al., 2013; Yun et al., 2012; Zhou et al., 2012). However, the clinical application of TAN for anti-cancer therapy is hindered by its low water-solubility, poor cellular uptake (Chen et al., 2007), short half-life, and first pass metabolism (Zhang et al., 2012b; Yu et al., 2007). Many colloidal carriers including polymeric micelles (Wang et al., 2014), emulsions (Chu et al., 2012), micro-emulsions (Ma et al., 2013), and nanoparticles (Li et al., 2008) have been used to improve the solubility of TAN. However, the cytotoxicity and bioavailability of TAN still need to be improved.

Recently, polymeric micelles with a core–shell structure formed by the self-assembly of amphiphilic copolymers have been increasingly used to deliver lipophilic drugs (Kataoka et al., 2001). These micelles significantly improve drug solubility, increase drug-loading capacity, and has enhanced permeation and retention (Drbohlavova et al., 2013; Lian et al., 2013). Pluronics, also named Poloxamers, are a type of micelles amphipathic copolymerswith a basic ethylene oxide $(EO)_x$–propylene oxide $(PO)_y$–ethylene oxide $(EO)_x$ structure. Pluronics are approved by U.S. FDA and are one of the most attractive biomaterials for drug delivery due to their excellent biocompatibility and low toxicity (Batrakova and Kabanov, 2008; Kabanov et al., 2002). Use of Pluronics for drug delivery prolongs drug circulating in the blood (Zhang et al., 2011) and sensitizes cancer cells (Batrakova et al., 2010). Therefore, Pluronics can be used to overcome the aforementioned shortcomings of TAN. However, Pluronics have

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insufficient drug encapsulation capacity and high critical micelle concentration (CMC), leading to dissociation of micelles after intravenous injection (Kulthe et al., 2011). Thus, Pluronics are often used with other micellescopolymers to increase drug loading capacity improve its deficiency(Oh et al., 2004).

D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS), a derivative of vitamin E with an amphiphilic structure, has attracted increasing interests for use in food and drugs (Zhang et al., 2012c). But independent application of TPGS for drug delivery is limited because of its low hydrophobicity and insufficient drug loading capacity. TPGS has been utilized to form mixed micelles with other copolymers such as Pluronics (Gao et al., 2008) and poly(ethylene glycol)-phosphatidylethanolamine (Mu et al., 2005) to improve drug stability and bioavailability. TPGS-based copolymers such as TPGS-PLA (Mu et al., 2010) have been used widely for drug delivery.

Mixed micelles, formed by a combination of two or more chemically diverse copolymers, have synergistic effects on drug delivery (Ebrahim Attia et al., 2011). In particular, the TPGS-graft-poly(D,L-lactide-co-glycolide) (TPGS-g-PLGA)/Pluronic F68 mixed micelle system has been demonstrated to have increased emulsification ability, micelle–cell interaction, and cellular internalizing (Kerleta et al., 2009). In the present study, mixed micelles with TPGS-g-PLGA copolymer and Pluronic F68 were developed to encapsulate our model drug TAN. We used the central composite design/response surface method (CCD/RSM) model to optimize the preparation conditions for TAN-loaded mixed micelles. Next, we examined the cytotoxicity and pro-apoptotic effects of TAN micelles on human HCC HepG2 cells. Furthermore, we investigated the cellular uptake of the mixed micelles loaded with a fluorescent probe Nile red (NR). Finally, we measured the pharmacokinetic behavior of TAN mixed micelles *in vivo*. We found that TAN mixed micelles have excellent drug loading capacity and stability as well as higher cellular uptake and bioavailability compared with free TAN.

## 2. Materials and methods

### 2.1. Materials

TPGS, 1,3-diisopropylcarbodiimide (DCC), and 4-(dimethylamino)pyridine (DMAP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pluronic F68 was purchased from BASF Wyandotte (Jamesburg, NJ, USA). PLGA-COOH (IV(dl/g) = 0.08) was purchased from Daigang Biomaterial (Jinan city, China). Tanshinone IIA (TAN) (CAS No. 578-72-9) was purchased from MUST Bio-Technology Co., Ltd. (Chengdu, China). Water was deionized and ultrafiltered by a Milli-Q apparatus (Millipore, Billerica, MA, USA). HepG2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). All chemicals were of analytical grade.

### 2.2. Characterization of TPGS-g-PLGA copolymer

$^1$H NMR spectra were recorded on a superconducting Fourier transform nuclear magnetic resonance spectrometry (Bruker Company, Billerica, MA) at 400 MHz using deuterated chloroform ($CDCl_3$) as a solvent. FT-IR spectra were obtained by an infra-red spectrometer (PerkinElmer, Massachusetts, USA). Mean size distribution and zeta potential were measured by dynamic light scattering (DLS) at 25 °C with a Zetasizer Nano ZSP system (Malvern Instruments, Worcestershire, UK). The morphology of micelles was observed under a transmission electron microscopy (TEM, Tecnai G20, FEI Company, Oregon, USA) at an accelerating voltage of 200 kV. Briefly, one drop of the mixed micelle suspension was added to a copper grid and negatively stained with a phosphotungstic acid solution (2%, w/v) for 30 s. The sample was dried in air and observed under the TEM.

### 2.3. Synthesis of TPGS-g-PLGA copolymer

The TPGS-g-PLGA copolymer was prepared by integrating the polymers with active terminal groups. TPGS (15 mmol), PLGA (10 mmol), DCC (30 mmol), DMAP (30 mmol), and 10 μL of triethanolamine (TEA) were all dissolved in 50 mL anhydrous dichloromethane (DCM) and stirred for 24 h at room temperature under nitrogen protection. The mixture was concentrated in vacuum, precipitated in cold diethylether, and collected by filtration to remove *N*,*N*′-dicyclohexylurea (DCU) by-products. To remove unreacted TPGS, the filter through was dialyzed against DCM in a dialysis tube with molecular weight cut-off at 3.5 kDa. The final product (TPGS-g-PLGA) was collected and vacuum-dried at 40 °C for 24 h.

### 2.4. Preparation of TAN-loaded mixed micelles

TAN-loaded mixed polymeric micelles were prepared using the thin film hydration method (Wei et al., 2009). Briefly, the mixture containing 10–30 mg copolymers (TPGS-g-PLGA and Pluronic F68 at different ratios) and 1 mg TAN was dissolved in acetone. The mixed solution was evaporated with a rotary vacuum until a thin film was formed. The film was freeze-dried overnight at 40 °C and rehydrated in 5 mL of phosphate buffer solution by stirring at a water bath (60 °C) for 30 min to form a micellar suspension. Unencapsulated TAN crystals were removed by filtration with a 0.45 μm filter membrane, and an opalescence suspension of TAN-loaded mixed micelles was obtained. Blank mixed micelles were prepared according to the aforementioned procedures without adding TAN.

### 2.5. Optimization of the preparation conditions for TAN-loaded mixed micelles

In order to optimize the preparation conditions, the CCD–RSM method was applied to develop a second order response model (Kollipara et al., 2010). This method saves time and resources with fewer experimental runs than industrial procedures. The key parameters, including water bath temperature, TPGS-g-PLGA/Pluronic F68 (T/F) ratio, and polymer/drug (P/D) ratio were optimized *via* a number of preliminary experiments. A central composite design was carried out on these three independent variables (variable $X_1$: water bath temperature ranging from 30 °C to 60 °C; variable $X_2$: T/F ratio ranging from 0.25 to 4; variable $X_3$: P/D ratio ranging from 10 to 30) at five experimental levels: −1.682, −1, 0, +1, +1.682. Drug entrapment efficiency (EE%) and drug loading percentage (DL%) were selected as evaluation indexes $y_1$ and $y_2$, respectively. The overall desirability (OD) value was obtained as a dependent variable by mathematical transformation using the following equations according to the Hassan's method.

$$d_i = \frac{y_i - y_{min}}{y_{max} - y_{min}} \quad (1)$$

$$OD_i = (d_1^i \times d_2^i)^{1/2} \quad (2)$$

### 2.6. Drug loading and encapsulation efficiency

The drug concentration of TAN-loaded micelles was determined by Waters e 2695 HPLC equipped with a reverse phase C18 column