Supporting Information

Angiopep-2 Functionalized Nanoformulation Enhances Brain Accumulation of Tanshinone IIA and Exerts Neuroprotective Effects Against Ischemic Stroke

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MATERIAL AND METHODS

1. Polymer Synthesis

1.1 Synthesis of PLGA

PLGA polymer was synthesized by solid phase polycondensation reaction with some modifications using D, L-lactide (LA) and glycolide (GA) as monomers and stannous octoate as the catalyst. Briefly, a pre-calculated amount of GA and LA were added to the reaction vessel at a 50: 50 molar ratio, and dehydrated for 2h at 70℃ under vacuum. The temperature was then gradually raised to 160℃, and 0.5% w/w stannous chloride was added as a catalyst. The reaction proceeded under magnetic stirring for 10 hours. The products were repeatedly washed with methanol, and dried under vacuum 12 h to obtain white crystalline solid PLGA polymers.

To obtain PLGA with carboxylate end groups (PLGA-COOH), PLGA (1.145 g, 0.05 mmol), succinic anhydride (0.036 g, 0.36 mmol), and 4-dimethylaminopyridine (DMAP) (0.037 g, 0.3 mmol) was dissolved in 15 ml of anhydrous 1, 4-dioxane, and reacted under nitrogen protection for 24 h. After reaction, 1, 4-dioxane was removed under reduced pressure at 40°C, and the reaction mixture was re-dissolved in 3 ml of methylene chloride. This solution was precipitated dropwise into 15 ml of ice-cold anhydrous ether to obtain a white viscous material which was further dissolved in 30 mL of dichloromethane, and washed consecutively with 10% hydrochloric acid (v/v, 30 ml × 3) and saturated NaCl solution (30 ml × 3). The organic phase was dried with anhydrous magnesium sulfate, filtered, and precipitated in ice-cold anhydrous ether.

1.2 Synthesis of MAL-PEG-PLGA copolymer

The MAL-PEG-PLGA copolymer was synthesized by the amidation reaction between PLGA-COOH and MAL-PEG_{3400}-NH_2. Briefly, 250 mg PLGA-COOH was dissolved in 2 ml dichloromethane in a dry three-necked glass flask. Next, 10.78 mg 4-Dimethylaminopyridine (DMAP), 10.15 mg N-hydroxy-succinimide (NHS) and 18.2 mg *N*, *N*'-Dicyclohexylcarbodiimide (DCC) were loaded into the reactor and stirred under nitrogen at room temperature. After 24 h of reaction, dicyclohexylurea (DCU) was removed by filtration, and the filtrate was slowly precipitated in 10 ml of ice-cold anhydrous ether to obtain PLGA-NHS. This product was washed three times with a mixture of ethyl ether and methanol, and dried under vacuum at 60℃ for 4 h.

To obtain Mal-PEG-PLGA, PLGA-NHS was dissolved in 2 ml of dichloromethane and then 33.2 mg of MAL-PEG_{3400}-NH_2 (with PLGA at a molar ratio of 1.3: 1) and 3 drops of N, N -diisopropylethylamine (DPIEA) was added into the reactor. The reaction went on for 12 h under nitrogen protection and magnetic stirring, and the products were precipitated in 10ml ice-cold anhydrous diethyl ether. The resulting white flocculent precipitate was washed with cold methanol and dried under vacuum for 4 h.

2. Polymer Characterization

2.1 Characterization of PLGA Polymers

PLGA polymers were characterized by Fourier transform-infrared (FT-IR) spectroscopy, ^{1}H Nuclear Magnetic Resonance (^{1}H-NMR), and Gel-permeation chromatography (GPC).

2.1.1. Fourier Transform Infrared (FT-IR) Spectroscopy of PLGA

In FT-IR spectra (**Figure S2**), the stretching vibration at 3517 cm^{-1} that confirmed the presence of the terminal hydroxyl group (-OH) attributed to PLGA. The peaks at 2958 cm^{-1} and 3004 cm^{-1} were the stretching vibration of saturated bonds -CH_3 and -CH_2 belonging to PLGA, respectively. A typical strong peak at 1749.5 cm^{-1}displayed the presence of C=O double bond, and the absorption signals at 1098 cm^{-1}～1171 cm^{-1} were assigned to C-O stretching vibration. All the obtained peaks in FT-IR spectra confirmed the successful synthesis of PLGA.

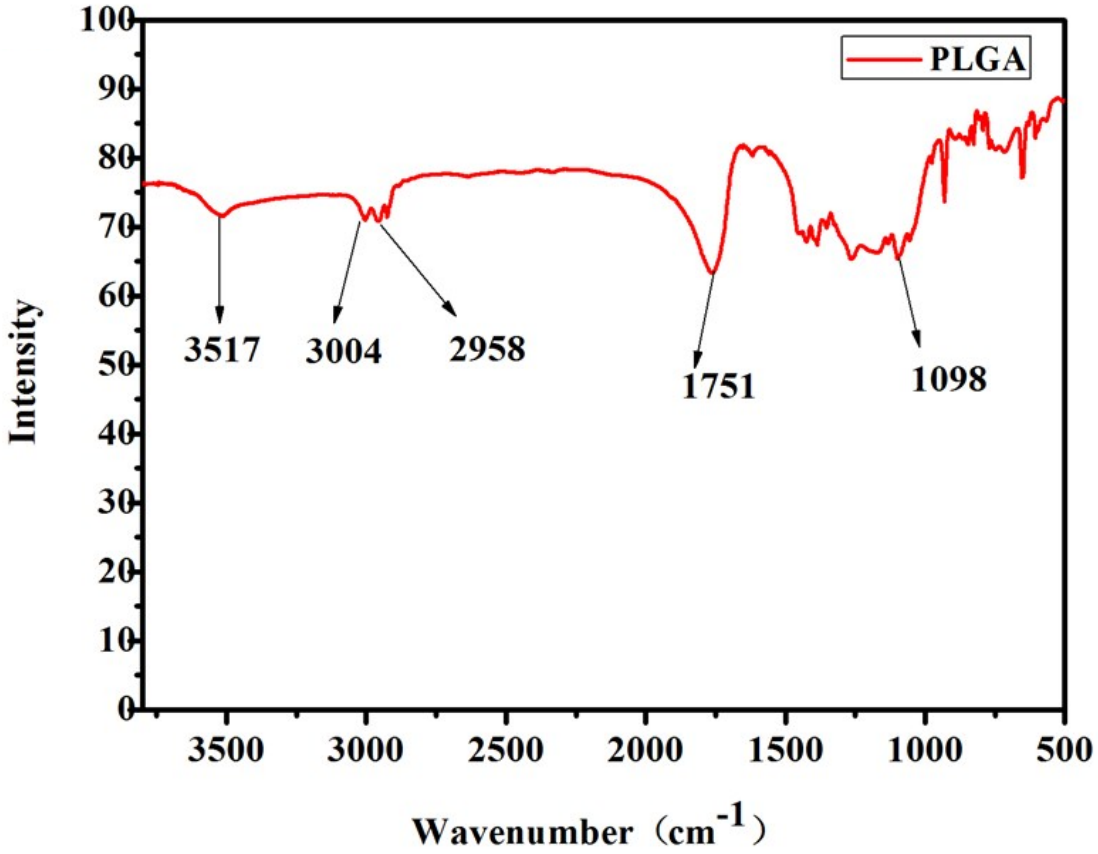

Figure S2 FT-IR spectra of synthesized PLGA polymers

2.1.2 ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) of PLGA

Similar to FT-IR spectra, the chemical structure of PLGA polymers was further confirmed through proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. As shown in ^{1}H NMR spectrum (**Figure S3**), PLGA block represented the characteristic shifts at 1.581 ppm, 4.787 ppm, and 5.232 ppm which confirmed the presence of methyl (m, -CH_3) proton peak of D, L-lactic acid (D, L-LA), methylene (m, -CH_2) proton peak of glycolide (GA) and methine (m, -CH) proton peak of D, L-LA in PLGA, respectively. The ^{1}H NMR results proved that the reaction successfully introduced the segments of D, L-LA and GA into the PLGA polymer. From the peak crack analysis of ^{1}H-NMR spectrum, we can find that except the absorption peak of -CH_3 is a typical double peak, the other peaks of -CH and -CH2 are multi-peak. The result also proved the direct melt polymerization reaction of lactide (LA) and glycolide (GA). The resulted PLGA is an amorphous polymer.

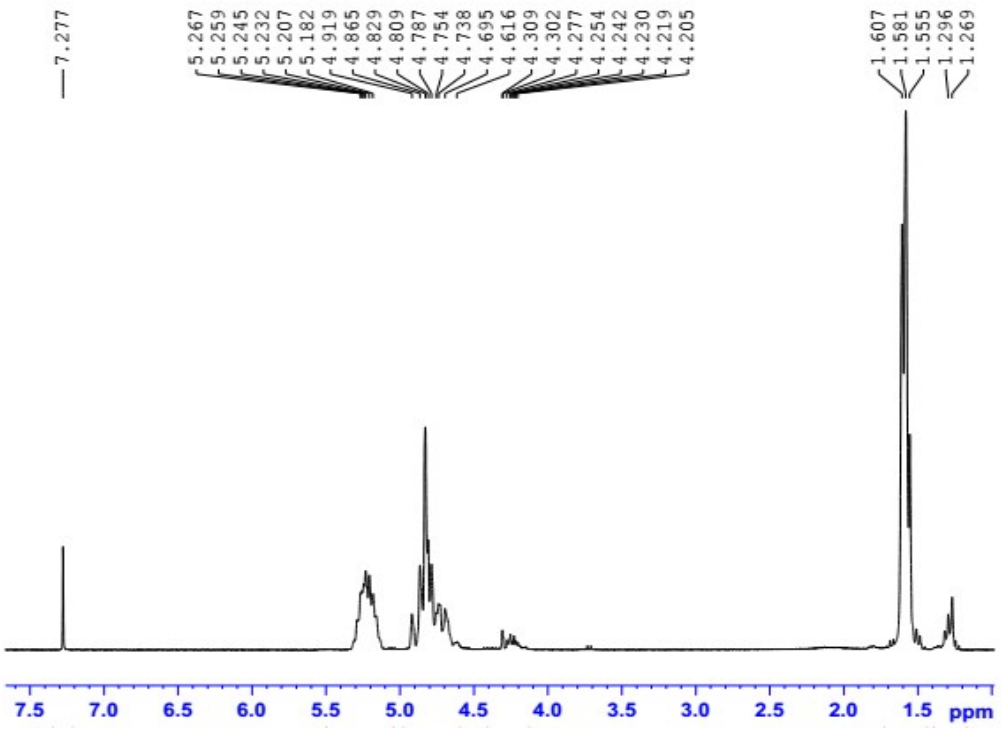

Figure S3 ^{1}H-NMR spectra of synthesized PLGA polymers

2.1.3 Thermal analysis of PLGA

Thermal transition characteristics of PLGA were determined using DSC (CDR-37P, Beijing, China). The sample size was approximately 5 mg, and the sample was subjected to thermal cycles from 0 to 90℃ at a rate of 10℃/min.

Thermal analysis of PLGA polymers was conducted by DSC as presented in **Figure S4**. The DSC thermogram of PLGA polymer exhibited a typical sharp endothermic band at 50.8℃ indicative of the relaxation peak following the glass transition temperature (Tg) of this kind of polymers.

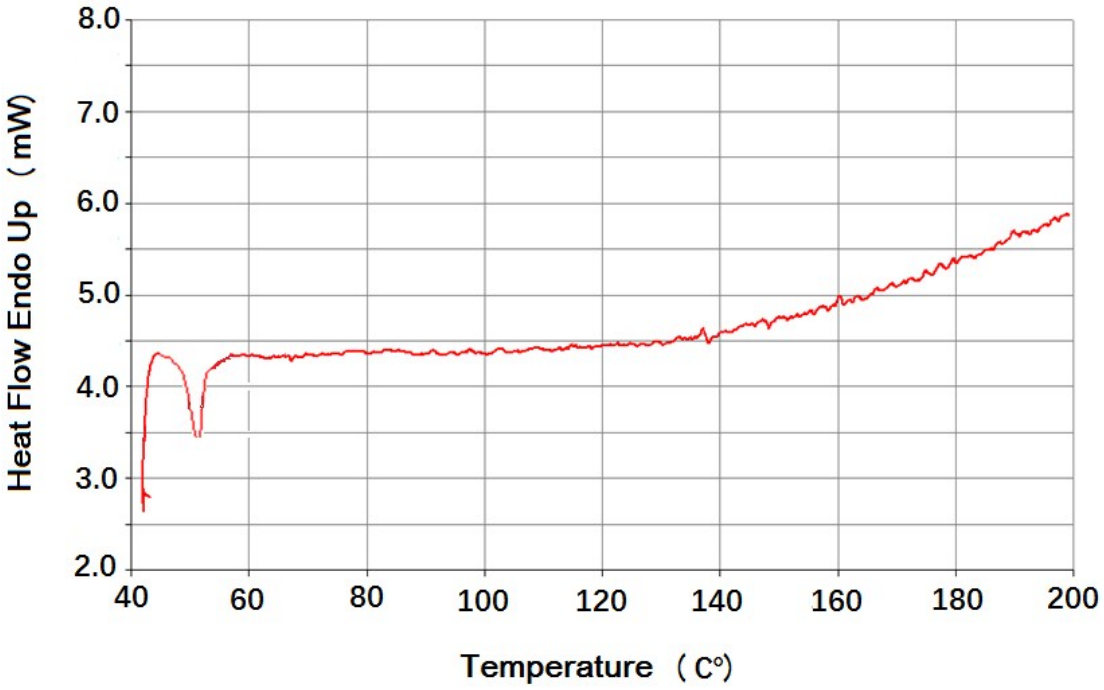

Figure S4 Differential scanning calorimetry (DSC) curve of the synthesized PLGA polymers

2.1.4 X-ray diffraction (XRD) analysis of PLGA

X-ray pattern analysis was carried out to assess the crystalline structure of the synthesized PLGA polymers. Dried powder of synthesized PLGA analyzed by X-ray diffractometer (X'Pert Pro PANALYTICAL, Netherlands) operated at 45 kV and 40 mA current at 25℃ temperature and diffraction pattern was recorded by CuKα1 radiation with λ of 1.54Å, step size 0.017 in the region of 2θ from 20 to 100.

As shown in X-ray diffraction (XRD) pattern of synthesized PLGA (**Figure S5**), a wide diffuse crystal diffraction peak appeared near 2θ of 18℃～20℃, which exhibited the characteristics of amorphous polymer. Therefore, it is speculated that the synthesized PLGA polymers have an "amorphous" microstructure.

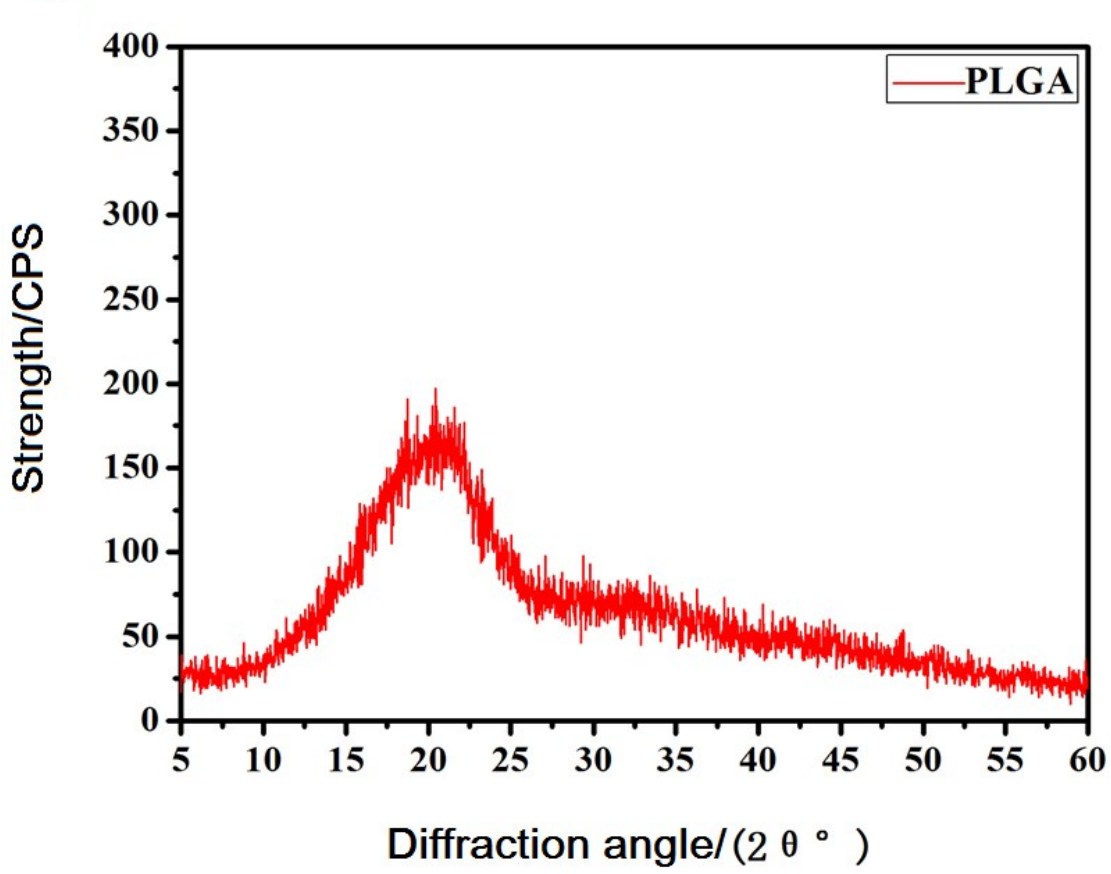

Figure S5 X-ray diffraction curve of synthesized PLGA polymers

2.2 Characterization of PEG-PLGA Diblock Copolymers

2.2.1 FT-IR Spectroscopy of PEG-PLGA

The structure of diblock PEG-PLGA was confirmed by Fourier transform infrared (FT-IR) spectroscopy. The copolymer samples were dissolved in chloroform and were cast on KBr plates before FT-IR analysis.

The PEG-PLGA copolymer was synthesized following a two-step synthesis and further characterized by FT-IR spectroscopy. The typical strong peak at 1749.5 cm^{-1} in FT-IR spectroscopy (**Figure S6**) of PLGA displayed the presence of C=O double bond. When the amino group (-NH_2) in MAL-PEG-NH_2 reacts with the carboxyl group (-COOH) in PLGA, the peak of C=O double bond at 1749.5 in MAL-PEG-PLGA is obviously weakened, indicating that MAL-PEG-PLGA was successfully synthesized. The signals at 1130 cm^{-1} and 952 cm^{-1} were the characteristic peaks of MAL-PEG-NH_2. The stretching vibration at 3458 cm^{-1} confirmed the presence of terminal hydroxyl group (-OH) of MAL-PEG-PLGA. The peaks at 3002 cm^{-1} and

2958 cm^{-1} attributed to the stretching vibrations of the saturated bonds of -CH_3 and -CH_2 in MAL-PEG-PLGA, respectively. The absorption peaks at 1170 cm^{-1} and 1282 cm^{-1} were assigned to C-O-C stretching vibrations of MAL-PEG-PLGA. All the obtained peaks in FT-IR spectra confirmed about the conjugation of PEG to PLGA.

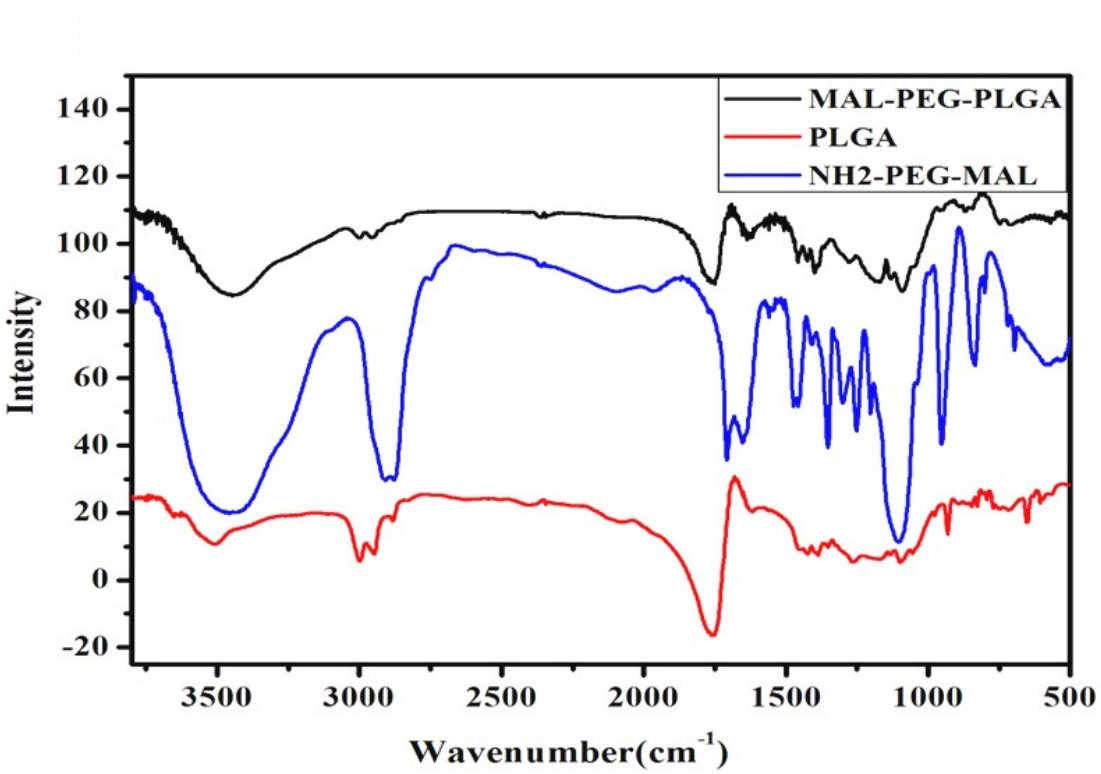

Figure S6 FT-IR spectra of synthesized MAL-PEG-PLGA compared with PLGA and MAL-PEG-NH_2

2.2.2. ^{1}H NMR of PEG-PLGA

^{1}H NMR spectra of the PEG-PLGA copolymers were recorded in $CDCl_3$ using Bruker AVANCE 500 MHz NMR spectrometer (Switzerland) to verify PEG conjugation to PLGA. The number average molecular weight (Mn) of the PEG-PLGA copolymers and the LA/GA ratio were determined by integration of the signals pertaining to each monomer, such as the peaks from the CH and CH_3 groups of lactide and from the CH_2 groups of ethylene glycol and glycolide.

The obtained PLGA polymers and PEG-PLGA copolymers were characterized by ^{1}H-NMR. In ^{1}H-NMR of PLGA (**Figure S7A** (a)), the integrated signals, around 1.573 ppm, were attributed to the methyl (m, -CH_3) protons of the D, L-lactic acid units. The corresponding peak, 1.573 ppm (**Figure S7B** (a)) also exists in ^{1}H-NMR

spectrum of PEG-PLGA. In ^{1}H NMR spectra of PEG-PLGA (**Figure S7B**), the larger peak at 3.636 ppm (m, -CH_2 of PEG) was mainly due to the methylene protons of PEG, while D, L-lactic acid -CH protons were assigned at 5.214 ppm (c) and the peaks of the glycolic acid -CH_2 protons were at 4.828 ppm (**Figure S7B** (b)). The aforementioned is indicative of the successful synthesis of PEG-PLGA diblock copolymers.

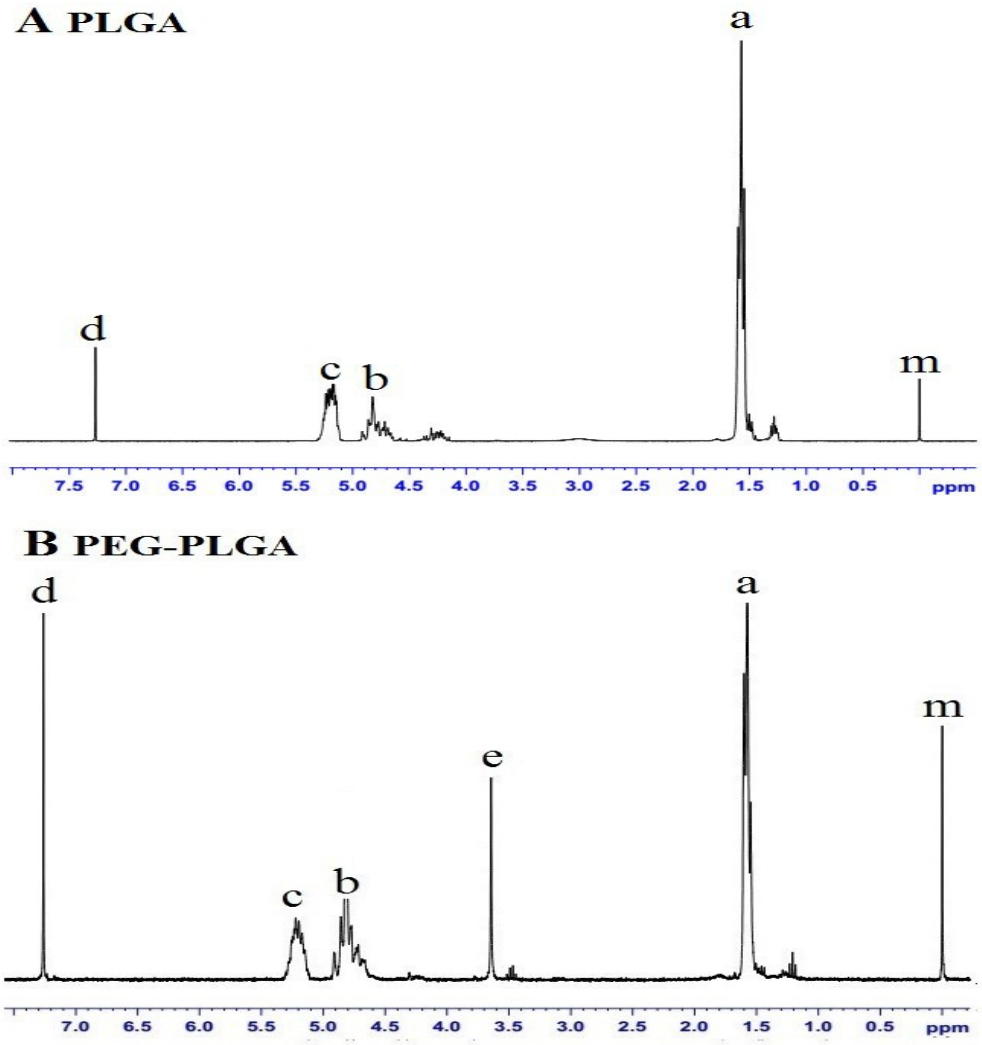

Figure S7 ^{1}H-NMR spectra of synthesized PLGA polymers (A) and synthesized PEG-PLGA diblock copolymers (B)

2.2.3. Gel Permeation Chromatography (GPC)

The molecular weights of the PEG-PLGA copolymers and their size distributions were determined using an Agilent 1100 GPC Addon apparatus and a RID-A refractive index signal detector coupled to Plgel® columns. Tetrahydrofuran was used as the

eluent solution at a flow rate of 1 ml/min. Polystyrene standard was used for calibration.

From the GPC diagram (**Figure S8**), it is clear that elution time of PEG-PLGA diblock copolymers is relatively shorter than that of PLGA polymers, indicating that the molecular weight of PEG-PLGA is greater than PLGA. The GPC measurements show a unimodal distribution with a number average molecular weight (Mn) (calculated by using the Polystyrene calibration) is 1.7868×10^4, and the polydispersity (Mw/Mn) of PLGA polymers was found to be approximately 1.33.

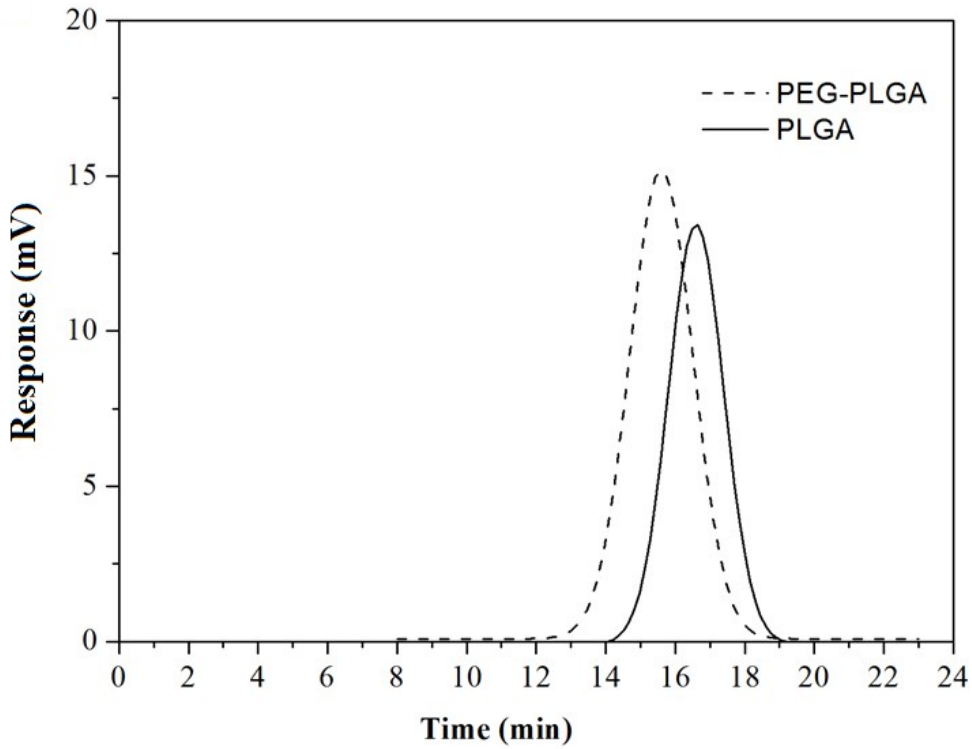

Figure S8 Gel-permeation chromatograph (GPC) diagram of PEG-PLGA diblock copolymers and PLGA polymers

Figure S9 shows a typical GPC chromatogram of MAL-PEG-PLGA, which is a nearly symmetric peak. The unimodal GPC trace with the low polydispersity (Mw/Mn = 1.42) of the diblock copolymers suggested that the reaction product is indeed a PEG-PLGA diblock copolymer with a narrow molecular weight distribution and its purity was sufficient to study the physical properties of the copolymers.

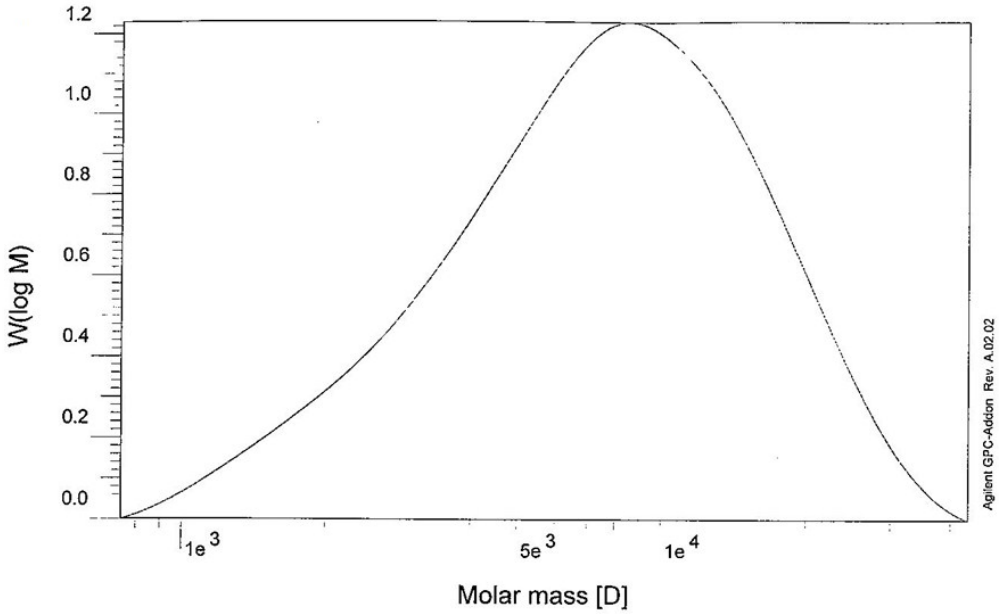

Figure S9 Gel-permeation chromatograph (GPC) of synthesized PEG-PLGA diblock copolymers

2.2.4. Elemental Analysis

The elemental composition of the PEG-PLGA copolymers was studied by energy-dispersive X-ray (EDX) spectroscopy. The dried PEG-PLGA samples were applied to the carbon-coated copper grid for measurement of elemental composition profile of the sample using EDX equipped with SEM (SUPRA 55 SAPPHIRE, ZEISS, Germany).

The EDX analysis was utilized to ascertain the presence of elemental signals as well as purity of the synthesized PEG-PLGA diblock copolymers. The EDX spectrum of the synthesized PEG-PLGA (**Figure S10B**) disclosed the occurrence of N element. In addition, the other elemental signals such as carbon and oxygen were also noticed both in PLGA (**Figure S10A**) and in PEG-PLGA (**Figure S10B**). In the present study, the synthesized PLGA polymers and PEG-PLGA diblock copolymers exhibited strong absorption spectra in the range between 2.0 to 2.5 keV. The results suggested that the NH_2-PEG-MAL has been conjugated on the surface of PLGA.

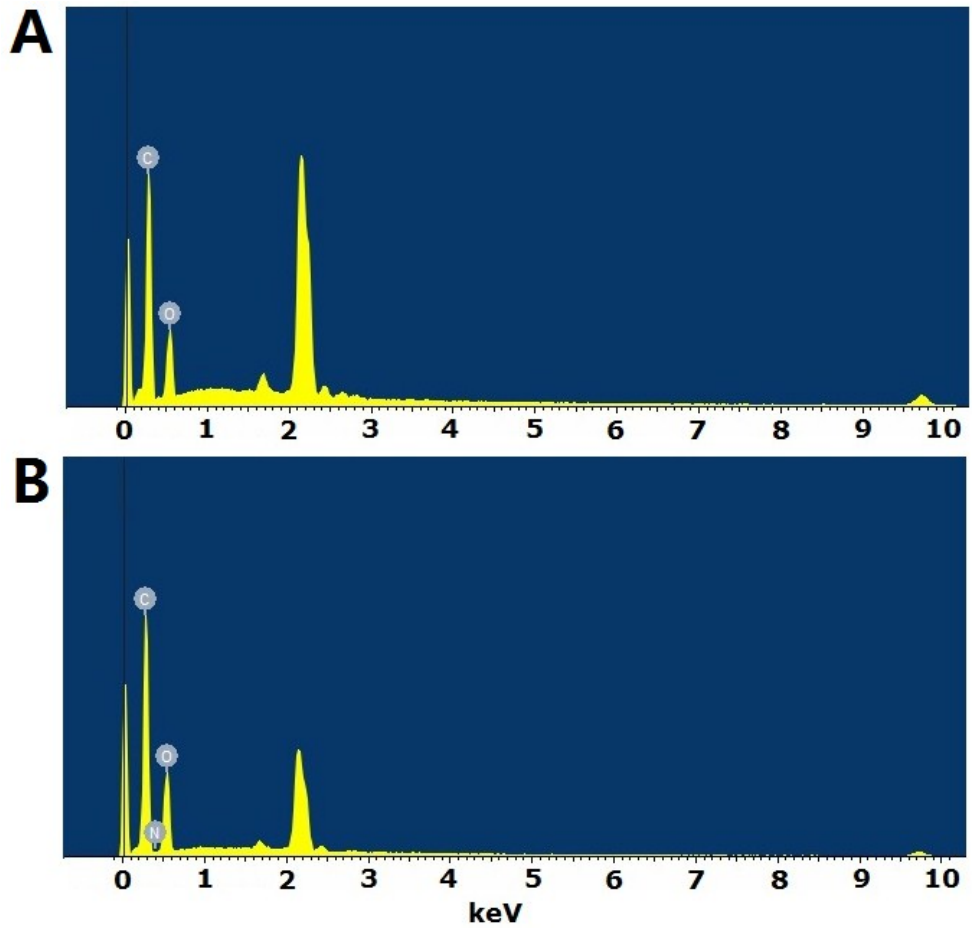

Figure S10 Energy Dispersive X-Ray (EDX) spectra analysis of synthesized PLGA polymers (A) and synthesized PEG-PLGA diblock copolymers (B)

2.3 Preparation and Characterization of Angiopep-2-TSⅡA-PEG-PLGA NPs

The tanshinone ⅡA-loaded unmodified PEG-PLGA nanoparticles (TSⅡA- PEG-PLGA NPs) were prepared via the optimized emulsion/solvent evaporation method according to our previous reports [1, 2]. Briefly, 30 mg MAL-PEG-PLGA and 2 mg TSⅡA were dissolved in 3 ml dichloromethane, and then added drop-wise with 1 ml of 0.4% polyvinyl alcohol-124 (PVA-124) aqueous solution. The mixture was then emulsified by sonication (200 w) under the conditions of ultrasound 10 s, intermittent 10 s with Digital Control Ultrasonic Instrument (KQ5200-DB, Kunshan Ultrasonic Instrument Co. Ltd., China) for 10 min in ice water bath. The emulsion was immediately poured into a 30 ml of stirring 0.5% PVA-124 solution for 20 min under magnetic stirring and dichloromethane was eliminated by evaporation under magnetic

stirring for 4 h. The resulted TSⅡA-PEG-PLGA NPs suspension was purified by centrifugation (15,000 rpm, 10 min) using AvantiJXN-30 centrifuge (Beckman Counter, USA) at 4℃. Thereafter, the TSAⅡ-PEG-PLGA NPs were resuspended and washed with Ultra-pure water to remove excess PVA-124.

For preparing TSⅡA-encapsulated Angiopep-2 modified PEG-PLGA nanoparticles (Angiopep-2-TSⅡA-PEG-PLGA NPs), the TSⅡA-PEG-PLGA NPs were dispersed in 0.01 M PBS buffer (pH 7.0), followed by the addition of Angiopep-2 in the same solvent at the molar ratio of 1:1 (maleimide: Angiopep-2) under stirring for 8 h. The obtained Angiopep-2 conjugated TSⅡA-loaded PEG-PLGA NPs were centrifuged under 12,000 rpm for 10 min. The resulted Angiopep-2 modified TSⅡA-encapsulated PEG-PLGA NPs were then eluted with 0.01 M PBS buffer (pH 7.0) through a 1.5×20 cm Sephadex G-25 column (PD-10, GE Healthcare, USA) to remove the unconjugated Angiopep-2 peptide.

To confirm the modification of Angiopep-2 peptide to the surface of TSⅡA-encapsulated PEG-PLGA NPs, ^{1}H NMR spectra (in D_2O) were recorded on Bruker AVANCE 500 MHz NMR spectrometer (Switzerland). The morphology of Angiopep-2-TSⅡA-PEG-PLGA NPs was observed by scanning electron microscope (SEM, SUPRA 55 SAPPHIRE, ZEISS, Germany). The particle size, size distribution and zeta potential of Angiopep-2 modified TSⅡA-encapsulated PEG-PLGA NPs were measured using a Nano ZS90 Zetasizer (Malvern Zetasizer, UK) based on the dynamic light scattering (DLS) technique. Drug encapsulation efficiency (EE%) and loading capacity (LC%) of Angiopep-2 modified TSⅡA-encapsulated PEG-PLGA NPs were also investigated by UltiMate® 3000 DGLC dual three element liquid chromatographic system (Thermo Scientific, USA) with a reverse-phase Waters C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was consisted of methanol and water (75: 25, v/v) and the flow rate was set at 1.0 ml/min and the detection

wavelength was 270 nm. The column temperature was maintained at 25℃.

The TSⅡA concentration was determined by HPLC. Drug loading efficiency (LE) and entrapment efficiency (EE) were calculated by:

$$LE\% = \frac{M_1 - M_2}{M_3} * 100\% \quad (1)$$

$$EE\% = \frac{M_1 - M_2}{M_4} * 100\% \quad (2)$$

Where M_1 is the total amount of TSⅡA added into Nano drug delivery system; M_2 is the free unloaded TSⅡA in Angiopep-2-TSⅡA-PEG-PLGA suspension; M_3 is total amount of Angiopep-2-TSⅡA -PEG-PLGA NPs; M_4 is total amount of free TSⅡA.

The exact chemical characteristics of Angiopep-2-PEG-PLGA NPs were analyzed by ^{1}H NMR. The morphology and particle size of Angiopep-2-TSⅡA-PEG-PLGA NPs dynamic light scattering zeta potential/particle size system. The coupling efficiency of Angiopep-2 to PLGA NPs was determined as described previously[3].

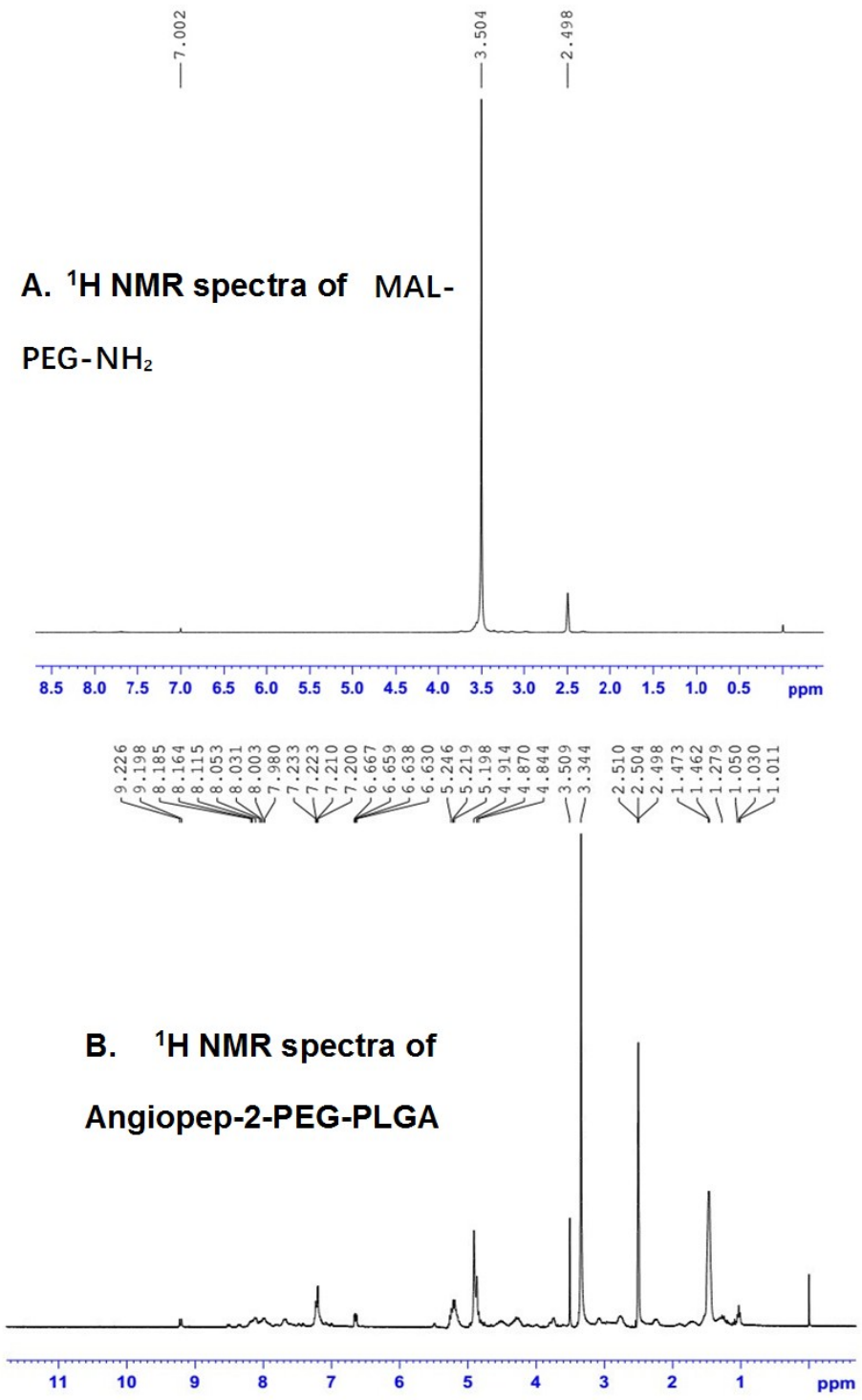

Figure S11 ^{1}H NMR spectra of MAL-PEG-NH_2 (A) and ^{1}H NMR spectra of Angiopep-2-PEG-PLGA NPs (B)

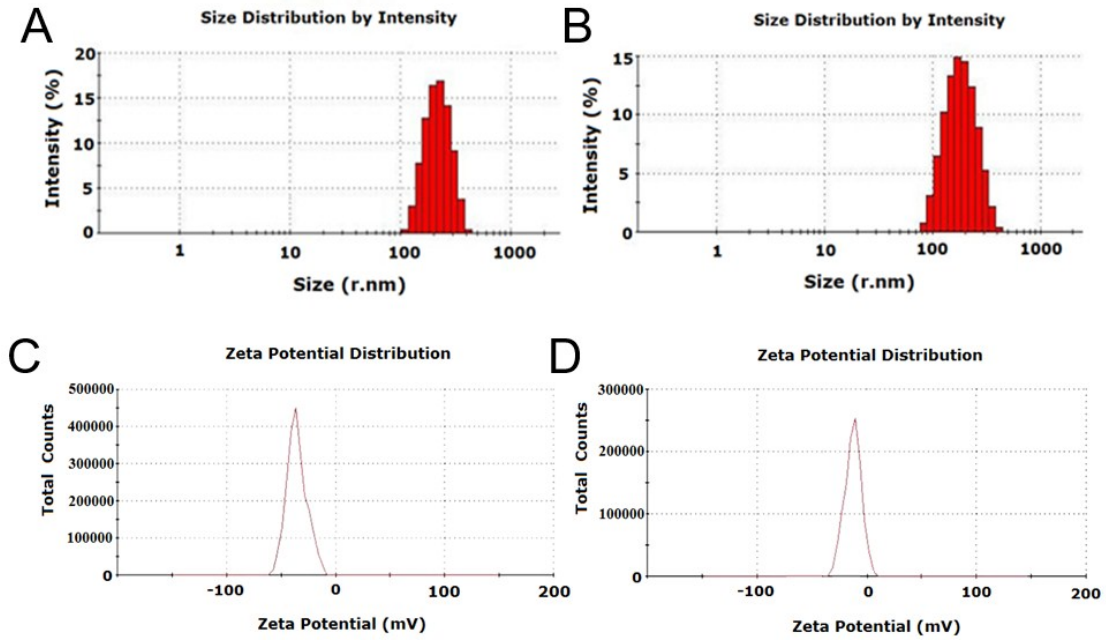

Figure S12 The particle size distribution of blank PLGA NPs (**A**) and Angiopep-2-TSⅡA-PEG-PLGA NPs (**B**), Potential distribution of blank PLGA NPs (**C**) and Angiopep-2-TSⅡA-PEG-PLGA NPs (**D**)

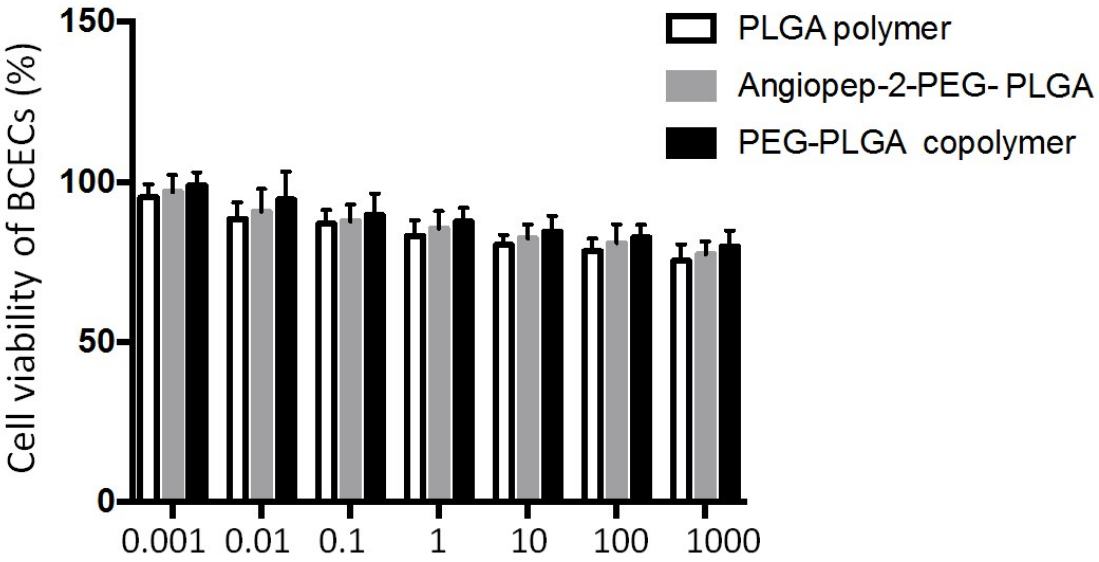

Figure S13 The cytotoxicity results of MTS proliferation assay performed on Angiopep-2-PEG-PLGA NPs, PEG-PLGA NPs and PLGA NPs after 48 h incubation at 37℃ with BCECs cell line

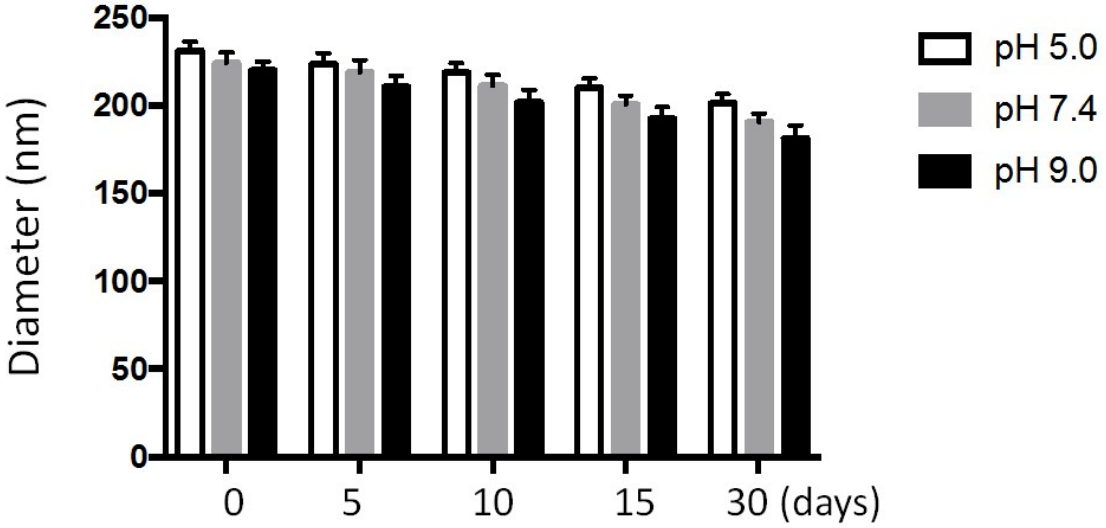

Figure S14 The stability of Angiopep-2-TSⅡA-PEG-PLGA NPs in solutions with varying pH as judged by their diameter

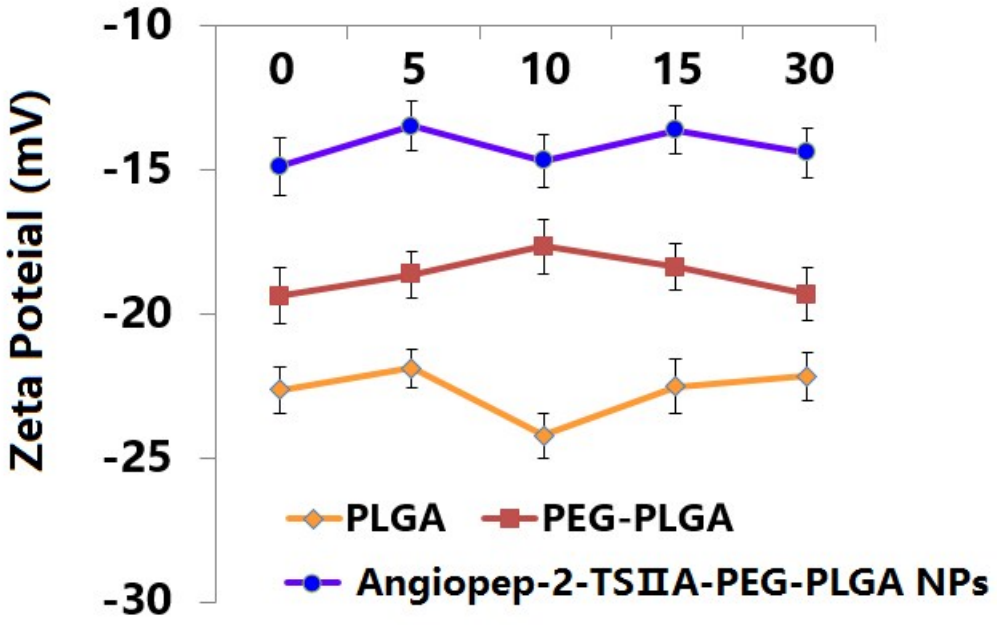

Figure S15 The stability of PLGA NPs, PEG-PLGA NPs and Angiopep-2-TSⅡA-PEG-PLGA NPs in solutions as judged by their zeta potential

2.4 *In Vitro* Cytotoxicity Assay of Nanoparticles by MTS

The cytotoxicity of Angiopep-2-PEG-PLGA NPs on brain capillary endothelial cells (BCECs) was evaluated by MTS assay using the NPs suspensions of unmodified PLGA polymer and PEGylated PLGA copolymer as control groups. To this end, an MTS test using CellTiter 96® AQueous One Solution kit (Cell Proliferation Assay,

Promega, USA) was performed. The test was executed on BCECs cell line. Cells were seeded in a 96-well plate, 3×10^3～5×10^3 cells/well. An equal volume of NPs suspensions, at increasing concentrations from 0.01 μg/ml to 1000 μg/ml, was added to the cell culture media (DMEM, 10% FBS) and incubated for 48 h at 37℃, 5% CO_2. The results of cell survival profile were expressed as a percentage of vital BCECs cells with respect to control (cells incubated without NPs).

2.5 Stability and Drug Release

To identify the stability of Angiopep-2-TSⅡA-PEG-PLGA NPs, the phosphate-buffered saline (PBS) solutions (0.1 M, pH 5.0, 7.4 or 9.0) were prepared, respectively. A total of 10 mg of Angiopep-2 modified TSⅡA-encapsulated PEG-PLGA NPs were suspended in 15 mL of PBS with continuous stirring under 120 rpm at 37℃. The sizes and zeta potential of Angiopep-2-TSⅡA-PEG-PLGA NPs were detected at 0, 1, 2, 3, and 5 days in order to determine the stability of Angiopep-2-TSⅡA-PEG-PLGA NPs by dynamic light scattering (DLS) [4].

Cumulative tanshinone IIA release from Angiopep-2-TSIIA-PEG-PLGA NPs was determined by dispersing 1 mg/ml of the NPs in 0.01 M phosphate buffer (pH 7.4), and incubating it at 37℃. Aliquots of the dispersion were withdrawn at 0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h and 72 h, and centrifuged at 15,000 rpm for 25 min. Volume of the dispersion withdrawn was replaced with fresh phosphate buffer. The supernatant was collected and TSIIA concentrations were quantitated by HPLC. The experiments were performed in triplicates.

2.6 Pharmacokinetics and Biodistribution

Pharmacokinetic characteristics of Angiopep-2-TSIIA-PEG-PLGA NPs were conducted in male SD rats with free TSⅡA suspension as control. Twenty SD rats were divided into 2 groups, and the same dose of TSIIA (10 mg/kg) in two different formulations was intravenously administrated through the rat tail vein, respectively. At each time point, 0.5 mL blood sample was collected and centrifuged (3000 rpm, 10

min) to obtain the plasma. TSIIA in the plasma were then extracted by addition of 1ml acetonitrile. The mixture was vortex-mixed for 1 min followed by centrifugation at 12,000 rpm for 10 min. The supernatant was evaporated under protection of nitrogen and then re-dissolved in 200 μl mobile phase before HPLC-MS assay. The pharmacokinetic parameters were calculated from the plasma concentrations of TSIIA using DAS 2.1 software system. The pharmacokinetic parameters (**Table S1**) we calculated include maximum plasma concentration (C_{max}), half-life ($t_{1/2}$), the area under the plasma concentration-time curve (AUC), and the mean residence time (MRT).

Table S1 The main pharmacokinetic parameters of tanshinone IIA following intravenous administration of free drug and Angiopep-2-TSⅡA-PEG-PLGA NPs.

Pharmacokinetic parameters	free TSⅡA	Angiopep-2-TSⅡA-PEG-PLGA NPs
$t_{1/2\beta}$ (h)	0.87 ± 0.21	8.21 ± 0.72
MRT (h)	1.54 ± 0.39	11.42 ± 0.56
AUC $_{0\sim\infty}$ (μg/l/h)	0.174 ± 0.062	1.078 ± 0.126
CLs [L/(h kg)]	1.75 ± 0.26	0.37 ± 0.05

Next, we investigated the biodistribution of Angiopep-2-TSIIA-PEG-PLGA NPs in SD rats to further evaluate its potential as a drug delivery system. SD rats were injected with Angiopep-2-TSIIA-PEG-PLGA NPs, TSIIA-encapsulated PEG-PLGA NPs or TSIIA solution via tail vein, respectively. Twelve hours post-injection, blood was collected retro-orbitally from all subjects. Six rats from each group were euthanized, the blood was removed prior to sacrificing animals, and their organs (hearts, livers, spleen, lungs, kidney and brain) were harvested. Before weighting, the organs were washed with saline, and were dried with filter paper. TSIIA concentration in the tissue samples of each group was measured with a HPLC method described previously [1, 2].

2.7 Stroke Modelling and Evaluation of Therapeutic Outcomes

Ischemic stroke in rats was modelled by middle cerebral artery occlusion (MCAO)

as described previously [1, 2, 5], and the treatment protocol is illustrated in **Figure S16**. Briefly, Rats were anesthetized with chloral hydrate (300 mg/kg, ip), and then the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated via a ventral midline incision. Branches of ECA were cauterized. The ECA was ligated and cut off at the distance of 5 mm from crotch of CCA. A nylon monofilament (φ 0.22～0.24 mm) with a rounded tip was inserted into right ICA about 18～20 mm through the open end of ECA, until laser Doppler flowmetry shows an abrupt 80%～90% reduction of cerebral blood flow, indicating the occlusion of the origin of middle cerebral artery. Sham-operated control rats received the same procedure except filament insertion. After 2 h of ischemia, the nylon suture was withdrawn to establish reperfusion.

SD Rats were randomly divided into 5 groups (n = 10). Group 1: sham-operated group injected with normal saline; group 2: model group, MCAO rats treated with normal saline; group 3: vehicle control group: MCAO rats treated with blank Angiopep-2-PEG-PLGA NPs; group 4: TSⅡA treatment group, MCAO rats treated with free TSⅡA (10 mg/kg); group 5: unmodified TSⅡA-PEG-PLGA NPs treatment group, MCAO rats treated with TSⅡA-encapsulated PEG-PLGA NPs (every day at a dose equivalent to 10 mg/kg of TSⅡA for a total of 3 days); group 6: Angiopep-2-TSⅡA-PEG-PLGA NPs treatment group, MCAO rats treated with Angiopep-2-TSⅡA-PEG-PLGA NPs (every day at a dose equivalent to 10 mg/kg of TSⅡA for a total of 3 days). All formulations were administered intravenously at the time of reperfusion following 2 h of ischemia, respectively.

The neurological function assessment was carried out at 24 h after MCAO. The neurological deficit score was assessed using a modified five point scoring system that developed from our previous studies [1, 2]. After the neurological assessment, rats were sacrificed and brains were harvested. Infarct volume was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining (n = 6). Briefly, the brains were immersed in ice-cold saline for 5 min, and then sectioned into 2 mm-thick coronal

slices, stained with a 2% TTC solution at 37℃ for 30 min, followed by fixation with 4% paraformaldehyde [1]. TTC-stained sections were photographed and the digital images were analyzed (Image-Pro Plus 5.1, USA) to calculate the infarct area percentage. For histology, brain coronal sections were fixed in 4% paraformaldehyde, then dehydrated, embedded in paraffin, and finally sectioned in 6 μm thick slices. Brain coronal sections were stained with Hematoxylin-Eosin (HE) in accordance with the standard procedures and examined with a microscopy.

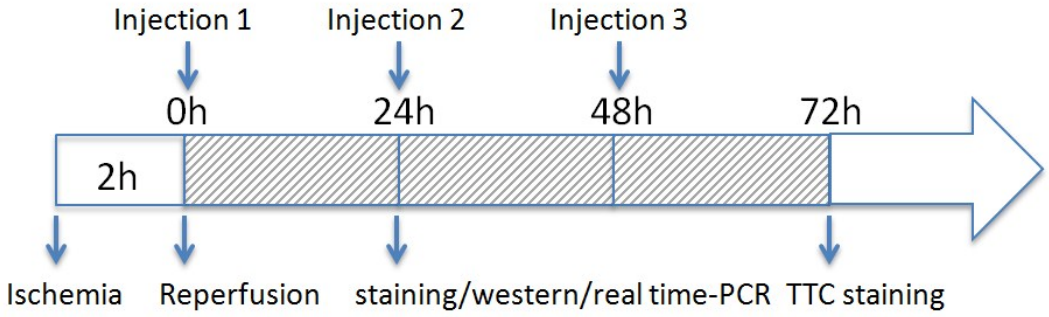

Figure S16 Scheme of surgery and drug administration schedule for therapeutic effect testing.

Table S2 Sequences of the amplification primers used in the real-time RT-PCR.

mRNA Species		**Oligonucleotides (5′→3′)**
HMGB1	forward	ATGGGCAAAGGAGATCCTA
	reverse	ATTCTCATCATCTCTTCT
NF-*κ*Bp65	forward	CGATCTGTTTCCCCTCATCT
	reverse	ATTGGGTGCGTCTTAGTGGT
IκB*α*	forward	TGGAGCCGACCTCAATAAACC
	reverse	TGCGACTGTGAACCACGATG
IKKβ	forward	GGAGTT TGGCATCACATCG
	reverse	GCCTCACCACCTCCT CTACT
TRAF6	forward	CGAAGAGGTCATGGATGCTAA
	reverse	TCAAAGCGGGTAGAGACTTCA
TLR2	forward	GAAAGATGCGCTTCCTGAAC
	reverse	CGCCTAAGAGCAGGATCAAC
TLR4	forward	GTTGGATGGAAAAGCCTTGA
	reverse	CCTGTGAGGTCGTTGAGGTT
TLR5	forward	CAGTTGCGAACCATAAGGACG
	reverse	GAGGTCACCGAGACAAAGCAC

MyD88	forward	ATTGGGGCAGTAGCAGATGAAG
	reverse	CAACCAGCAGAAACAGGAGTCT
IRAK-4	forward	CTGCTGCCAGATGCTGTTCC
	reverse	ACGGTTGTCCTGTTCTTTTCC
TRAM	forward	ATAAGTGCCCCCTTTCTTGG
	reverse	CCTCGTCGGTGTCATCTTCT
TRIF	forward	CAAGCCGTGCCCACCTACT
	reverse	TGTTCCGATGATGATTCCAG
GAPDH	forward	CAAGGTCATCCATGACAACTTTG
	reverse	GTCCACCACCCTGTTGCTGTAG

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