PLLA-Grafted Gelatin Amphiphilic Copolymer and Its Self-Assembled Nano Carrier for Anticancer Drug Delivery

Beibei, Du; Tiantang, Fan; Jiafeng, Li; Qin, Zhang; Wuyou, Ye; Wang, Wenxin; Tai, Hongyun; Zhongyong, Fan

Macromolecular Chemistry and Physics

DOI:
10.1002/macp.201800528

Published: 05/03/2019

Peer reviewed version

PLLA grafted gelatin amphiphilic copolymers and self-assemble to form Nano carriers for anticancer drug delivery

Du Beibei[1], Fan Tiantang[1], Li Jiafeng[1], Gong Li[1], Zhang Qin[1], Ye Wuyou[1], Tai Hongyun[2], Wang wenxin[3], Fan Zhongyong[1*]

1Department of Materials Science, Fudan University, Shanghai, PR China

2School of Chemistry, Bangor University, Bangor, UK

3Charles Institute of Dermatology, School of Medicine, University College Dublin, Dublin 4, Ireland

* Corresponding author: Fan Zhongyong, Department of Materials Science, Fudan University, Shanghai, PR China, Tel: +86-21-65642395, Email: zyfan@fudan.edu.cn

Abstract

A series of poly(L-lactide)-grafted gelatin (Gel-g-PLLA) copolymers were synthesized by coupling the amino of gelatin and the carboxyl of carboxyl-terminated poly(L-lactic acid) (PLLA-COOH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). According to the fourier transform infrared (FT-IR) and nuclear magnetic resonance (^{1}H NMR) results, the successful bonding between gelatin and PLLA was confirmed. The effect of the gelatin length over the micelles was evaluated by the self-assembly behavior. The size and micellar morphology were determined by dynamic light scattering (DLS) as well as transmission electron microscopy (TEM). Meanwhile, the *in vitro* drug-release behavior revealed that the synthesized Gel-g-PLLA micelle showed excellent sustained-release properties at different lengths of gelatin when used as the carriers of the anticancer drug paclitaxel (PTX). PTX in the micelles exhibited a burst release about 70% in the first 24 h. All of the mentioned attractive features, together with the superior biocompatibility, make the present micelles a remarkable potential drug delivery system for cancer therapy.

Key words**:** *nanomaterials, gelatin, PLLA, self-assembly, drug release*

1.Introduction

Gelatin, a natural hydrophilic macromolecule obtained by partial hydrolysis of collagen extracted from skin, bones, cartilage, ligaments [1, 2], is composed of amino acids with peptide linkages. Thence, gelatin does not produce harmful byproducts during *in vivo* degradation [2]. Its inherent electrostatic binding properties, excellent biocompatibility, biodegradability, and non-toxicity [3] lead to its being widely used in pharmaceutical, cosmetic, tissue engineering and regenerative medicine [4, 5]. Another advantage of using gelatin to design biomaterials originates from its rich functional groups, such as hydroxyl, amine, and carboxyl groups [6, 7], because these groups can be used for further chemical grafting, inducing hydrophilicity, and providing protons [3].

Gelatin microsphere was largely used as a delivery vehicle of many drugs as it can form ionic complexes with charged hydrophilic therapeutic drugs such as proteins, nucleotides and polysaccharides [8, 9]. Although gelatin has wide applications, there are many drugs such as PTX, which are difficult to be embedded in gelatin microspheres. In previous studies, it has

been reported that amphiphilic polymer micelles used for drug delivery form a hydrophobic core, in which lipophilic drugs can be physically incorporated [10, 11]. Similar to gelatin microspheres, nano-micelles have many unique advantages as drug carriers such as sustained or controlled release, and targeting properties because of their nanoscale size [5, 12-15]. Therefore, great efforts have been made to explore the synthesis and self-assembly behavior of different types of amphiphilic polymers.

Poly(L-lactic acid) (PLLA), a synthetic polyester, has desirable biocompatibility, biodegradability, nontoxicity, and good mechanical properties [16-20]. Normally, PLLA is polymerized by L-lactic acid which is primarily derived from corn starch [21-23]. PLLA have been widely used as versatile biomaterials in many fields such as medical tissue engineering scaffolds [24, 25] and controlled drug release systems [26]. However, PLLA has the following disadvantages, which limit its application in the field of biomedical materials: 1) it contains a large amount of ester groups, resulting in its high hydrophobicity, lack of reactive groups on the surface, and poor adhesion to cells; 2) its degradation rate is difficult to control, the degradation products of it are acidic, and the affinity of the products to the cells is poor [22, 27-29]. In order to adjust its physicochemical properties and increase its hydrophilicity, PLLA can be copolymerized with hydrophilic polymers so that to create amphiphilic copolymers. At present, many such copolymers based on PLLA have been developed, and *in vivo* and *in vitro* properties of them have been studied [26-29]. PLLA commonly used as a hydrophobic segment to synthesize amphiphilic block copolymers with hydrophilic segments such as PNIPAAm and PEG. Recently, several PLLA-PEG [26, 27, 30, 31] and PLLA-PNIPAAm[28] block copolymers micelles have been reported. However, these hydrophilic segments are synthetic.

Given success applications of graft copolymer [32-34], we design a novel fully biodegradable and biocompatible micelles system based on the amphiphilic graft copolymers of PLLA and gelatin, Gel-g-PLLA, which takes advantages of the unique strengths of both PLLA and gelatin. Carboxyl-terminated PLLA (PLLA-COOH) was obtained by ringing opening polymerization of L-lactide (LLA). PLLA was grafted to gelatin by coupling agents EDC and NHS. EDC/NHS activation of carboxyl has been widely applied to the synthesis of various polymers [35]. We investigated the structure and self-assembly behavior of Gel-g-PLLA. We synthesized several Gel-g-PLLA samples using gelatin with different molecular weight and different ratio of

EDC/NHS. Then, we investigated the drug release behavior of drug-loaded Gel-g-PLLA micelles with the aim to prepare the micelles with desired release properties as clinical medicines.

2.Experimental section

2.1. Materials

L-lactide (LLA, SCRC, 98%) was purified by recrystallization in ethyl acetate for three times. After filtering, the solid was washing with diethyl ether prior to drying in vacuum at 40 °C for three days. Ethyl acetate (Greagent, 99.5%), dichloromethane (Greagent, 99.5%), diethyl ether (Greagent, 99.0%), EDC (Aladdin, 98.0%), NHS (Aladdin, 98.0%), dimethylsulfoxide (DMSO, Adamas, 99.9%) and phosphate-buffered saline (PBS, pH = 7.4) were used without further purification. Gelatin ($\overline{M}_n$ = 32247 g mol^{-1}, PDI = 2.79) was supplied by Raybo Biotech Company (Sichuan, China) and used for further process. PTX was obtained from Techwell Biopharmaceutical Ltd. (Shanghai, China). Dialysis membrane tube (molecular weight cut off, MWCO=3500, 7000 g mol^{-1}) were obtained from Spectrumlabs and used as received.

2.2. Characterization

Fourier Transform infrared spectroscopy (FTIR) spectra were recorded on a Bruker Tensor 27 spectrometer at frequencies ranging from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} at room temperature. The samples were obtained by mixing the powder with KBr crystals and pressing the powder into pellets.

Nuclear magnetic resonance (^{1}H NMR) spectroscopy were recorded on a Bruker DRX-400 spectrometer at room temperature. DMSO-d_6 (Adamas) was used as the solvent, and tetramethylsilane (THF) was used as the internal reference. The number-average molecular weight of tactic polymer can be calculated from the ^{1}H NMR spectra.

Gel permeation chromatography (GPC) analysis was performed using a Shimadzu apparatus equipped with a refractive index (RI) detector. 1) THF was used as the solvent at a flow rate of 1 mL min^{-1}. 50 μL of 1.0 w/v% solution were injected for each analysis. The system was calibrated with polystyrene (PS) standards with molecular weights of 500 to 4×10^6 g mol^{-1}. 2)H_2O was used as the solvent at a flow rate of 1 mL min^{-1}. 50 μL of 1.0 w/v% solution were injected for each analysis. The system was calibrated with polyethylene glycol (PEG) standards with molecular weights of 500 to 1×10^6 g mol^{-1}.

Dynamic light scattering (DLS) was examined using a Zetasizer Nano ZS (Malvern Instrument) equipped with a HeNe laser (λ = 633 nm) at room temperature with 90 °C scattering angle. Several aqueous solutions of copolymers with a concentration of 2.0 mg mL^{-1} were filtered through a 0.45 μm PES filter before measurements to remove impurities. The size distributions were obtained from the correlation functions, and the data were analyzed by the Malvern Zetasizer software.

Transmission electron microscopy (TEM) was performed on a Tecnai G2 20 TWIN, (FEI, America) instrument with 200 kV accelerating voltage. 5 μL of micellar solution at a concentration of 1.0 mg mL^{-1} were dropped onto a carbon coated copper grid, and air dried before measurements.

The critical micelle concentration (CMC) was determined on a Force Tensiometer-Kruss100, which is used for analyzing surfactants and solid surfaces. Aqueous solutions of the graft copolymers were prepared in deionized water with concentrations ranging from 0.001 to 1.0 mg mL^{-1}, and were incubated overnight. The Surface tension of the solutions was measured at 25 °C with the plate method of CMC measurement. The surface tension was recorded 3 times with each concentration. The CMC was taken as the low point of regression lines from the plots of the Surface tension against the graft copolymer concentration.

High Performance Liquid Chromatography (HPLC) was performed with a LC-10A apparatus (Shimadzu) equipped with a UV detection (SPD-10A, Shimadzu) and a 218MR54 column (4.6×250 mm, pore size 5 μm, C18, Vydac, USA). The detection wavelength was 227 nm. The sample solution of mobile phase (acetonitrile/water 55:45 v/v) was filtered through 0.22 μm filter before injection. The flow rate was 1.0 ml/min. The retention time was controlled at 11.8 min, and the calibration curve was linear in the range of 0.05-20 mg L^{-1} with a correlation coefficient of R2=0.999. The area of each eluted peak was integrated and used for PTX quantification.

2.3. Thermal degradation of low molecular weight (LMW) gelatin

Gelatin solution was prepared by swelling gelatin particles (40 g) in the deionized water (300 mL) and dissolving at 100 °C in a flask equipped with a magnetic stirrer and a thermometer. After several hours (2,4,6,8,10 and 12 h), 50 mL of the solution was collected and cooling, respectively. Then, the resulting solutions were lyophilized to yield gelatin in the form of solid.

2.4. Synthesis of PLLA-COOH

PLLA-COOH was synthesized by ROP of LLA, with zinc lactate as catalyst and H_2O as initiator. The monomers (9.94 g, 69 mmol), catalyst (0.01 g, 0.41 mmol) and initiator (0.06 g, 3.33 mmol) were loaded in the polymerization tube (10 mL). The polymerization was reacting at 140 °C for 4 h under vacuum. Finally, at preset action sampling time, the simple purification via the dissolution in dichloromethane and the precipitation in ethanol gave a white powder after being dried under vacuum at 37 °C to a constant weight.

2.5. Synthesis of Gel-g-PLLA copolymers

Firstly, 0.56g degraded gelatin was dissolved in 20 mL deionized water at 45 °C to form a transparent solution. Then, 0.2 g PLLA-COOH was dissolved in 20 mL DMSO at 30 °C in a flask equipped with a magnetic stirrer and a thermometer (pH=7). The carboxyl of PLLA-COOH was needed to be activated by reacting with EDC. Besides, the procedure required NHS for stabilization. Briefly, 48 mg EDC was added to the solution. After reacting for 10 minutes, 7.2 mg NHS was added and the solution was stirred for 2h at 30 °C. The degraded gelatin aqueous solution was then dropwise added into the above mixture with continuous stirring for 16 h. The resulting solution was purified by dialysis through the dialysis membrane tube (MWCO 3500 g mol^{-1}) in deionized water for 3 days. Finally, after filtering the mixture solution, the solid product was obtained through the vacuum freeze-drying equipment.

2.6. Preparation of PTX-loaded Gel-g-PLLA micelles

The PTX-loaded micelle was obtained by direct dissolution method to encapsulate PTX within Gel-g-PLLA micelles. Firstly, 1 mg PTX (2%) and 50 mg dry copolymer of Gel-g-PLLA were mixed in deionized water in a beaker at room temperature. Afterwards, the suspension was under continuous stirring for 24 h, yielding self-assembled micelles at room temperature. Unloaded PTX was separated by filtering through filter membrane (0.45μm) and then stored at 4 °C before use, followed by analysis using HPLC. The encapsulation efficiency (EE) and drug loading (DL) were calculated by the following equations (1, 2):

$$EE(\%) = \frac{W_{PTX}}{W_{total\ PTX}} \times 100\% \quad \text{Equation (1)}$$

$$DL(\%) = \left(\frac{W_{PTX}}{W_{PTX}+W_{NPs}}\right) \times 100\% \quad \text{Equation (2)}$$

where W_{PTX} is the amount of PTX incorporated in micelles, $W_{total\ PTX}$ is the initial amount of

PTX in the system and W_{NPs} is the amount of nanoparticles after freeze-drying.

2.7. *In vitro* PTX releasing experiment

The release of drug-loaded micelles *in vitro* was studied by the dialysis method and evaluated in 50 mL of phosphate-buffered saline (PBS, 0.1 M, pH = 7.4) against 1 mL of PTX-loaded micelle (10 mg mL^{-1}) solution that was loaded in a dialysis bag (MWCO 3500 g mol^{-1}) beforehand, and the dialysis bag was placed into 50 mL of PBS and the system was kept at constant temperature of 37 °C and 500 rpm stirring. At several regular time intervals, 1.0 mL of the release medium was taken out and replaced immediately by the same volume of fresh PBS medium, followed by analysis using HPLC. The cumulative PTX release was determinate by the following equation (3):

$$Cumulative\ \ PTX\ \ release(\%) = \frac{V_t \sum_1^{n-1} C_i + V_0 b C_n}{W_{PTX}} \times 100\% \qquad \text{Equation (3)}$$

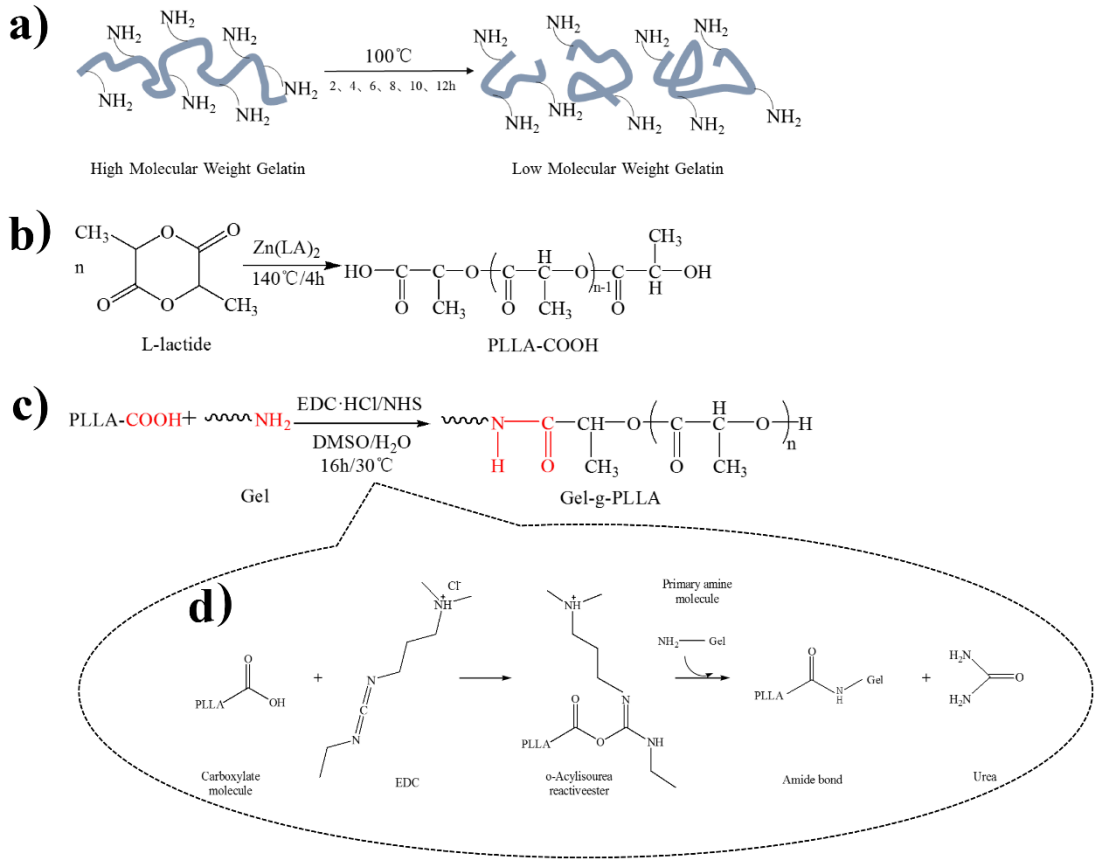
where V_0 equals to the total volume of release media, V_t is the volume of replaced media, C_n is the concentration of PTX in the sample.

3.Results and discussion

In order to optimize the properties of the grafted amphiphilic copolymers, we firstly prepared a large variety of Gel-g-PLLA amphiphilic copolymers using different molar ratio of EDC to NHS. Then we used the best molar ratio of EDC to NHS to prepare Gel-g-PLLA using PLLA with different molecular weight to investigate whether the hydrophobic chain length would affect the graft copolymer properties.

3.1. Synthesis and Characterization of Gel-g-PLLA Copolymers.

A series of Gel-g-PLLA were synthesized for this study using three steps (Scheme1): (a) thermal degradation of LMW gelatin, (b) synthesis of PLLA-COOH, and (c) synthesis of amphiphilic copolymer Gel-g-PLLA. Scheme1(d) shows the reaction mechanism of EDC/NHS conjugation chemistry between carboxyl and amine-containing molecules. EDC is a carboxyl-reactive cross linker. EDC reacts with a carboxyl group first and forms an amine-reactive O-acylisourea intermediate that quickly reacts with an amino group to form an amide bond and release of an isourea by-product. The intermediate is unstable in aqueous solutions, and therefore, two-step conjugation procedures require NHS for stabilization.



Scheme1. The synthetic route of Gel-g-PLLA. a) thermal degradation of LMW gelatin, b) synthesis of PLLA-COOH, c) synthesis of amphiphilic copolymer Gel-g-PLLA, and d) reaction mechanism of EDC reaction with carboxyl and amine-containing molecules.

Specifically, LMW gelatin was prepared from swelling long chain gelatin particles in the deionized water and dissolving at 100 °C for 2,4,6,8,10 and 10 h, respectively. The number average molecular weight of degraded gelatin was characterized by GPC. As we all known, the earlier the peak appears, the larger the molecular weight is. Apparently, With the degraded time getting longer, the peak appears late in Figure 1a. Figure1b demonstrates that the number average molecular weight of gelatin thermal degradation product gradually decreases with the prolongation of the time. At the beginning of the reaction (0-6 h), The number average molecular weight decreases rapidly, indicating that the reaction rate is fast. However, the curve declines slowly then (6-12h), which proves that gelatin thermal degradation reaction is a variable reaction process. Figure1c shows the ^{1}H NMR spectra of the LMW gelatin, the amide protons signal at 8.00 ppm exhibits weaker from 0 h to 12 h. It is apparently that there are less amide groups in LWM gelatin through thermal degradation.

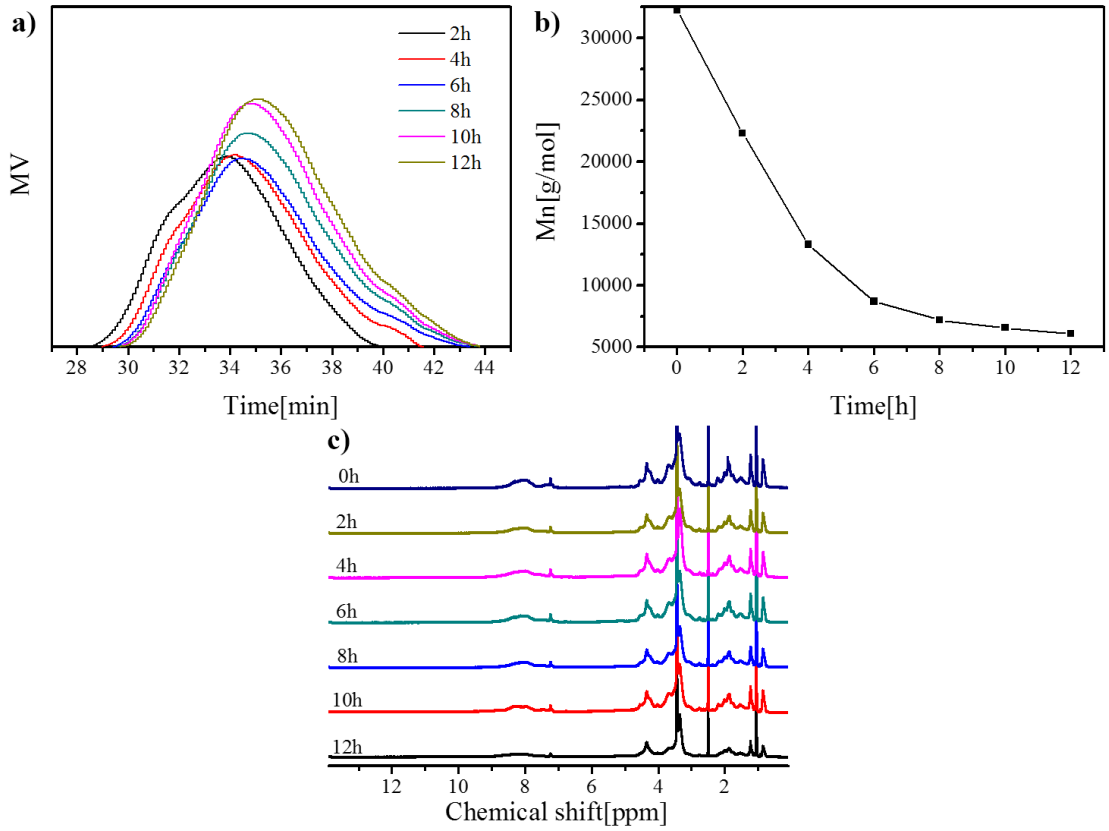


Figure 1. a) GPC curves of degraded gelatins after thermal degradation with different degradation time in H_2O, b) the number average molecular weight changes with degradation time, c) ^1H NMR spectra of gelatin with different degradation time in DMSO-d6.

PLLA-COOH was synthesized by ROP of LLA, with zinc lactate as catalyst and H_2O as initiator. The number average molecular weight of PLLA-COOH was 3144 g mol^{-1} ($\overline{M}_n$, GPC) and the polydispersity indices (PDI) was 1.08 using GPC analysis (Figure 2a). Besides, PLLA-COOH was characterized by ^1H NMR (Figure 2b). The characteristic signals at 1.45 (c) and 5.22 (b) ppm are assigned to the methyl and methine groups of PLLA-COOH, respectively. Besides, the signal at 4.27 (d) ppm belongs to the hydroxyl of its end units. The signal at 13.06 (a) ppm is assigned to the labile proton of terminal carboxyl group. The degree of polymerization of PLLA-COOH is determined by the ratio of the integrals of the macro-monomer backbone signal at 5.22 ppm (b) to the end-unit at 4.27 ppm (d). Through ^1H NMR analysis, the degree of polymerization obtained was 39.41 and the number average molecular weight of PLLA-COOH was around 2855 g mol^{-1} ($\overline{M}_n$, NMR) ,which is close to the data by GPC analysis. These findings confirmed the successful ROP of LLA oligomers.

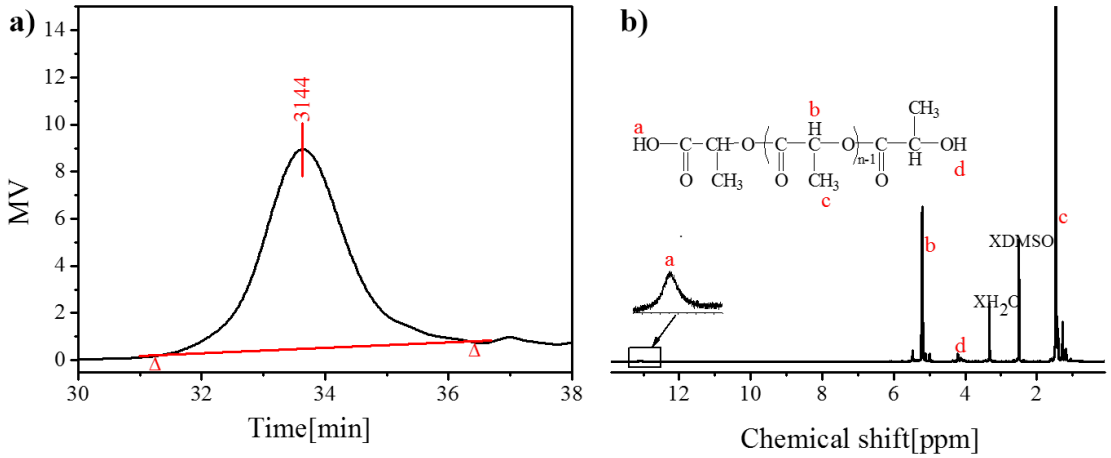


Figure 2. a) GPC curve of PLLA-COOH inTHF; b) ^{1}H NMR spectra of PLLA-COOH in DMSO-d6.

Samples were synthesized with the ratio of coupling agents (EDC/NHS=n, the molar ratio) as the variable (samples Gel-g-PLLA $_{n=1}$, Gel-g-PLLA $_{n=4}$, and Gel-g-PLLA $_{n=8}$, respectively). The appropriate molar ratio of EDC/NHS was chosen to go on further experiment. The other amphiphilic graft polymers used in the current work consisted of gelatin hydrophilic chain with $\overline{M}_n$ of 16000, 10000, and 7000 g mol^{-1} and PLLA hydrophobic chain with $\overline{M}_n$ of 3144 g mol^{-1} (samples Gel$_{16k}$-g-PLLA, Gel$_{10k}$-g-PLLA, and Gel$_{7k}$-g-PLLA, respectively). The experimental details of 6 samples prepared in this work were presented in Table 1. As summarized in Table 1, the molar ratio of EDC to NHS (n) was ranged from 1 to 8, and the degree of substitution of PLLA was calculated by ^{1}H NMR analysis.

Table 1. Experimental details and summary of the synthesized amphiphilic polymers.

Amphiphilic Copolymers	$\overline{M}_n$ of Gel (g mol^{-1},GPC)	n[a]	DS[b] (%)	Yield (%)	$\overline{M}_n$ (g mol^{-1},GPC)	PDI (GPC)
Gel$_{7k}$-g-PLLA $_{n=1}$	7000	1	6.79	23	9350	1.96
Gel$_{7k}$-g-PLLA $_{n=4}$	7000	4	7.86	47	9540	2.01
Gel$_{7k}$-g-PLLA $_{n=8}$	7000	8	7.14	36	8460	1.37
Gel$_{16k}$-g-PLLA $_{n=4}$	16000	4	12.83	44	17300	1.86
Gel$_{10k}$-g-PLLA $_{n=4}$	10000	4	8.13	46	13700	1.52
Gel$_{7k}$-g-PLLA $_{n=4}$	7000	4	7.47	49	8940	2.00

[a] The molar ratio of EDC to NHS.

[b] The degree of substitution (DS) of PLLA calculated using ^{1}H NMR analysis.

3.2. The molar ratio of EDC to NHS

EDC and NHS as the coupling agents were indispensable for the reaction between PLLA-COOH and gelatin. Three juxtaposed samples were synthesized with the different ratio of coupling agents (n=1, 4, 8, respectively) and investigated the influence of the ratio to graft copolymers. Different amount of the activator EDC, but the same amount of stabilizer NHS were added in the reaction. That is to say, the more amount of EDC added, the bigger "n" is. As shown in Table1, when the "n" equals 4, the degree of substitution of PLLA and the yield of reaction all reach highest point. Besides, the properties of micelles are presented in Table 2 and demonstrated in Figure 3. Apparently, the CMC value as well as the particle size decrease with the "n" increasing. Actually, the "n" value indirectly influences the chain length of the PLLA segment. Finally, the appropriate ratio (n=4) was chosen for further research.

Table 2. Characterization of the micelles.

Amphiphilic Copolymers	CMC[a] (g L^{-1})	D_h[b] (nm)	PDI[c]	EE(%)	DL(%)
Gel_{7k}-g-PLLA $_{n=1}$	0.4	171±20	0.871±0.03	-	-
Gel_{7k}-g-PLLA $_{n=4}$	0.1	55±5	0.374±0.01	-	-
Gel_{7k}-g-PLLA $_{n=8}$	0.1	23±2	0.293±0.01	-	-
Gel_{16k}-g-PLLA $_{n=4}$	0.9	152±15	0.610±0.02	50.5	1.00
Gel_{10k}-g-PLLA $_{n=4}$	0.2	58±5	0.422±0.03	53.4	1.06
Gel_{7k}-g-PLLA $_{n=4}$	0.1	45±3	0.643±0.01	57.7	1.14

[a] CMC determined by surface tension tester.

[b] The hydrodynamic diameter of Gel-g-PLLA micelles measured by DLS.

[c] The polydispersity index determined by GPC.

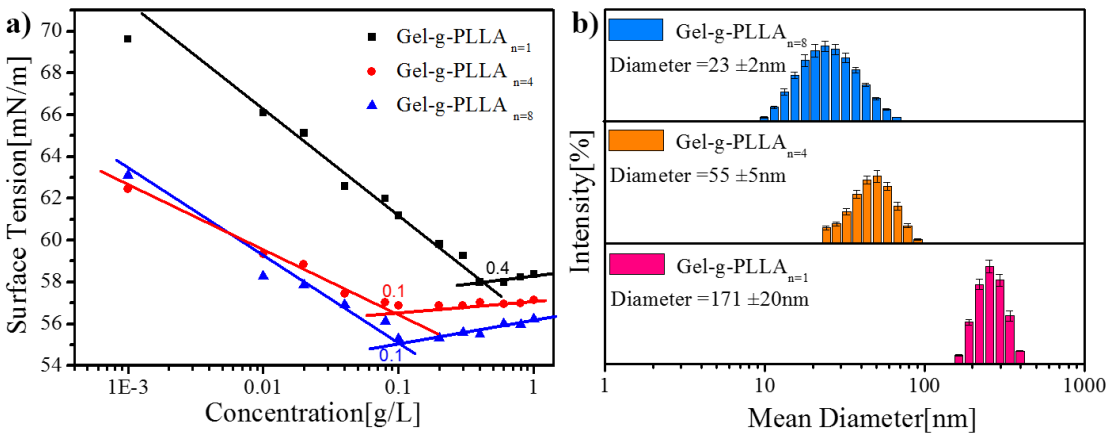


Figure 3. a) surface tension changes as a function of copolymer concentration, b) size distribution of micelles measured by DLS at room temperature.

3.3. Chain length of Gel

The functional groups in Gel-g-PLLA、PLLA-COOH and Gel were characterized by FTIR (Figure 4a) and the obtained data can be analyzed as follows. A peak at 3629 cm^{-1} (B) is attributed to the terminal carboxyl group of PLLA-COOH. The peaks at 1188, 1122 and 1023 cm^{-1} (B) belong to the C–O–C stretching vibrations. The peak at 3533 and 3263 cm^{-1} (A) are attributed to the stretching vibration of N–H of amide bond. The peak at 1685 cm^{-1} (C) is related to the stretching vibration of the C=O in the amide segment. However, the C=O bond in polyester segment is observed a characteristic peak at 1757 cm^{-1} (B). Apparently, the two peaks both apparent in the curve of Gel-g-PLLA (A). The peak around 1530 cm^{-1} (A、C) is assigned to the formation vibration of the N–H in the amide segment. Some peaks around 1446 cm^{-1} (A、C) and 1404 cm^{-1} (A、C) are assigned to the stretching vibration of the C–N in the amide group. The bending vibration of N–H in the amide group of the graft point presents a characteristic peak at 645 cm^{-1} (A). These results indicate that PLLA has been grafted to Gel.

Another method of confirming the chemical bond between PLLA-COOH with gelatin is by ^{1}H NMR measurements. The ^{1}H NMR spectrum of Gel-g-PLLA in DMSO-d_6 is shown in Figure 4b. The peaks at 1.56 (f) and 5.30 (h) ppm are assigned to the proton signal of methyl and methine groups of PLLA-COOH, respectively. The hydroxyl of PLLA-COOH end units is observed at 4.30 (g) ppm. Besides, the new bond of amide is found at 8.00 (e) ppm. The relative integral area of amide protons (8.00 ppm) in Gel-g-PLLA (e) is larger than that in gelatin (i), suggesting that PLLA has been successfully grafted to Gel. This result suggests that we have

obtained structure-defined Gel-g-PLLA.

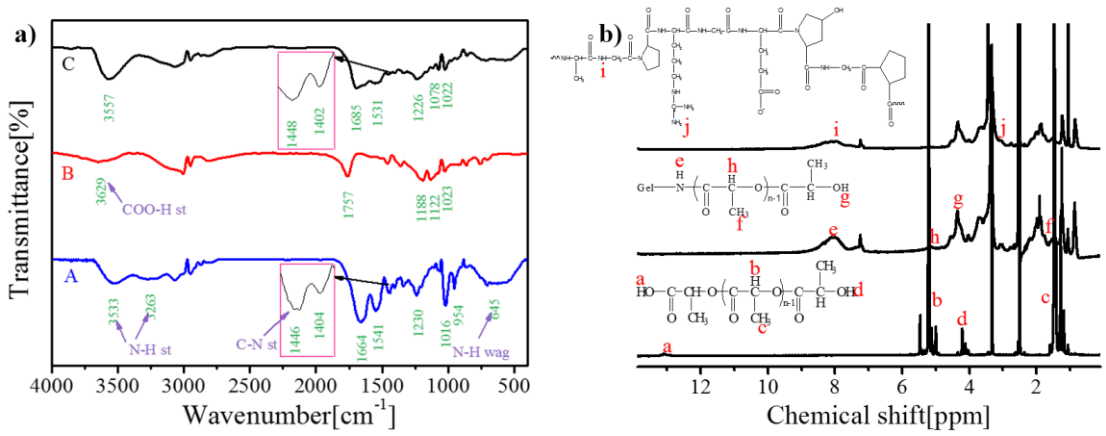


Figure 4. a) FTIR spectra of Gel-g-PLLA (A), PLLA-COOH (B) and Gel (C), b)^{1}H NMR spectra of PLLA-COOH, Gel and Gel-g-PLLA in DMSO-d6.

3.3.1. The degree of substitution

Through Figure 5b, the percentage of PLLA segments grafted to one Gel segment can be calculated. Several ^{1}H NMR spectra of Gel_{16k}-g-PLLA, Gel_{10k}-g-PLLA, Gel_{7k}-g-PLLA and their corresponding gelatin are shown as follows. The degree of substitution (DS) of PLLA-COOH ends with the amino groups of gelatin was determined by comparing the integrals of the signals derived from the amino protons (3.00ppm, e) with the amide protons (8.00ppm, d) in gelatin and Gel-g-PLLA, respectively, which is presented in Table 1, calculating from the ^{1}H NMR spectra. We can get the results through the following equation (4):

$$DS = \frac{d_2 - d_1}{d_1 + e_1} \qquad \text{Equation (4)}$$

where d_1, d_2 represent the amide protons in gelatin and Gel-g-PLLA, respectively, e_1 is the amino protons in gelatin.

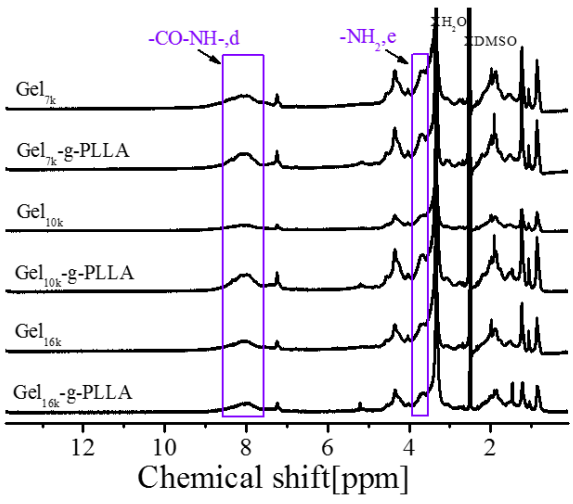


Figure 5. ^{1}H NMR spectra of Gel_{16k}-g-PLLA, Gel_{10k}-g-PLLA, Gel_{7k}-g-PLLA and the corresponding gelatin in DMSO-d6.

DS of all the copolymer samples are presented Table 1. Apparently, DS increases with the chain length of gelatin getting longer. Since the longer gelatin has more reactive functional groups. Besides, the yield of the reaction varies from 44 to 49%. This result indicates that the reaction yield needs to be improved with feasible methods.

3.3.2. The self-assembly behavior

The self-assembly behavior of Gel-g-PLLA in aqueous medium was also investigated by several experimental techniques. For the tests, the aqueous solutions of the copolymers were prepared as follows. Firstly, the copolymers were dissolved in water. Then, the homogeneous solutions were further sonicated at 25 °C for 5 min (sonicator: Kunshan Shumei KQ3200E) before to volume. It is clear that the copolymers are soluble in deionized water homogeneously without any specific process.

Based on the proverbial self-assembly mechanism of amphiphilic graft copolymers, the amphiphilic Gel-g-PLLA generated numerous spherical micelles in aqueous solution with a hydrophilic Gel corona and a hydrophobic PLLA core, which can be characterized by DLS and TEM (Figure 6). As shown in Figure 6, the mean diameter of the micelles determined by DLS are 45±3, 58±5 and 152±15 nm, which is a bit larger than that measured by TEM analysis. The data determined in this figure is summarized in Table 2. The large deviation between TEM and DLS measurements could be caused by different sample preparation methods, as the former is measured in the dry state, while the latter is measured in the aqueous solution. Therefore, the data measured by DLS is the particle size of the whole dispersion, which is statistically more significant than that measured by TEM.

Apparently, with the increase of gelatin chain length, the mean diameter of the micelles increases. Aggregates of amphiphilic copolymer tend to a lower surface energy state. As the hydrophilic chain decreases, the polymer assembly morphology changes smaller, thereby reducing the interface free energy, so that the energy of the system decreases to be more stable.

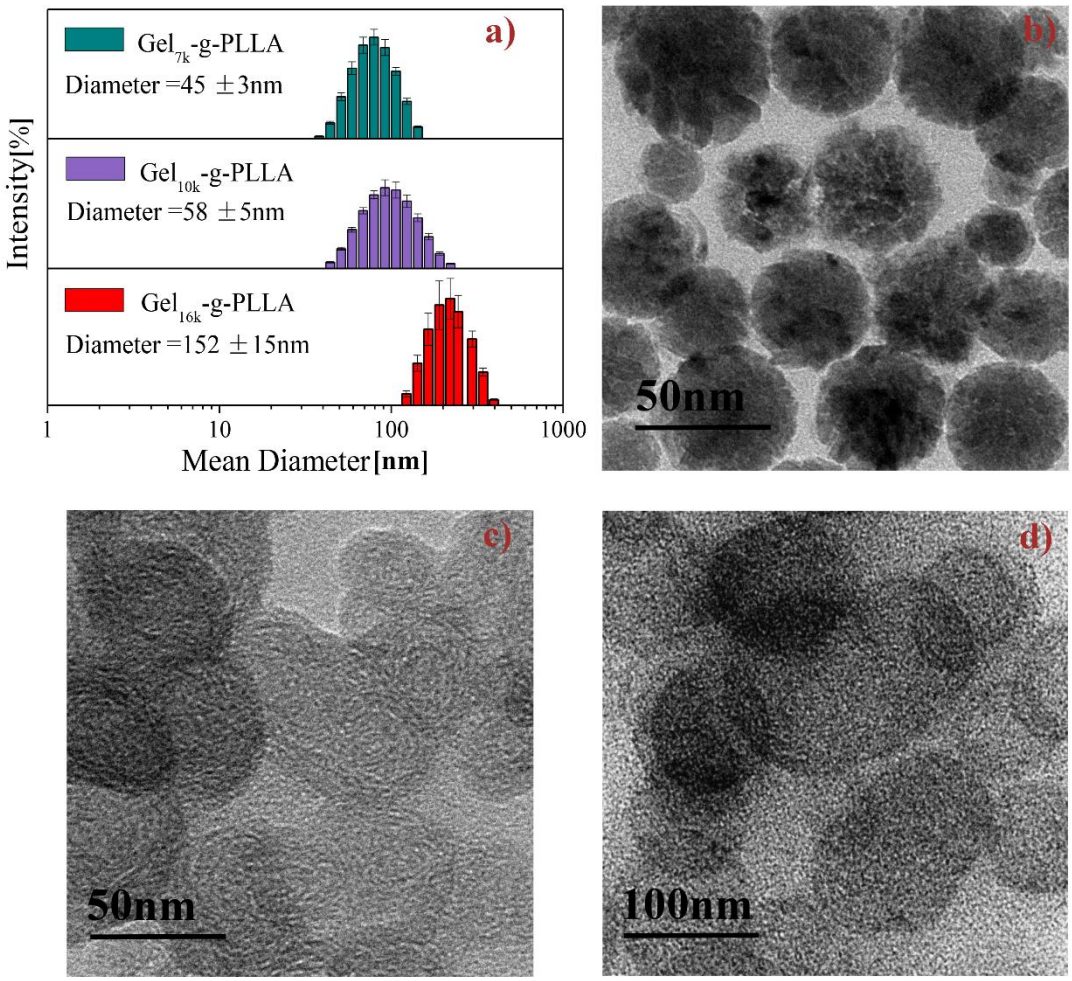


Figure 6. a) DLS data and TEM for micelles of b) Gel_{7k}-g-PLLA, c) Gel_{10k}-g-PLLA, d) Gel_{16k}-g-PLLA.

The amphiphilic nature of Gel-g-PLLA graft copolymers, including hydrophilic Gel and hydrophobic PLLA segments, can endow the copolymer with the ability to self-assemble in aqueous media so that the copolymers present a critical micelle concentration (CMC) to indicate the amphiphilicity. Upon introduction of amphiphilic Gel-g-PLLA into water, they will initially partition into the interface, reducing the system free energy by lowering the energy of the interface, and removing the hydrophobic parts (PLLA) of the Gel-g-PLLA from contacting with water. Subsequently, when the surface coverage of Gel-g-PLLA increases, the surface tension decreases and the copolymers start aggregating into micelles, thus again decreasing the system's free energy by decreasing the contact area of PLLA segment of the copolymer with water. Upon reaching CMC, any further addition of Gel-g-PLLA will just increase the number of micelles. Thus, the CMC value of several samples is presented in Table 2, characterized by Surface

Tension Tester.

The copolymers Gel_{7k}-g-PLLA, Gel_{10k}-g-PLLA and Gel_{16k}-g-PLLA aqueous solutions were prepared in deionized water with concentrations ranging from 0.001 to 1.0 mg mL^{-1}, and then measured by surface tension instrument. As demonstrated in Figure 7, the CMC value of samples ranges from 0.1 to 1 g L^{-1}. In deionized water, aggregation of neutral graft copolymers is primarily decided by the balance between the hydrophilic segments and the hydrophobic segments. These data suggest that CMC increases with the molecule gelatin chain prolongation. An increase in the proportion of hydrophilic segments weakens their mutual aggregation in the water, which results in amphiphilic copolymers forming micelles at higher concentrations. As the reduction of the hydrophilic segment, in other words, the addition of the hydrophobic segment, leads to a decrease in the stability of the copolymer in the water, so that aggregation tends to occur easily.

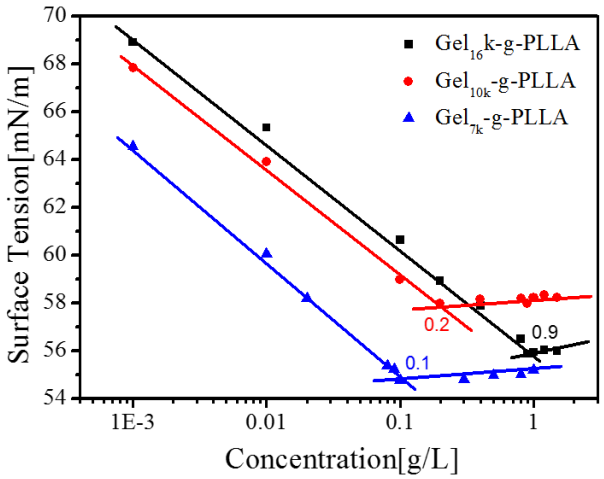
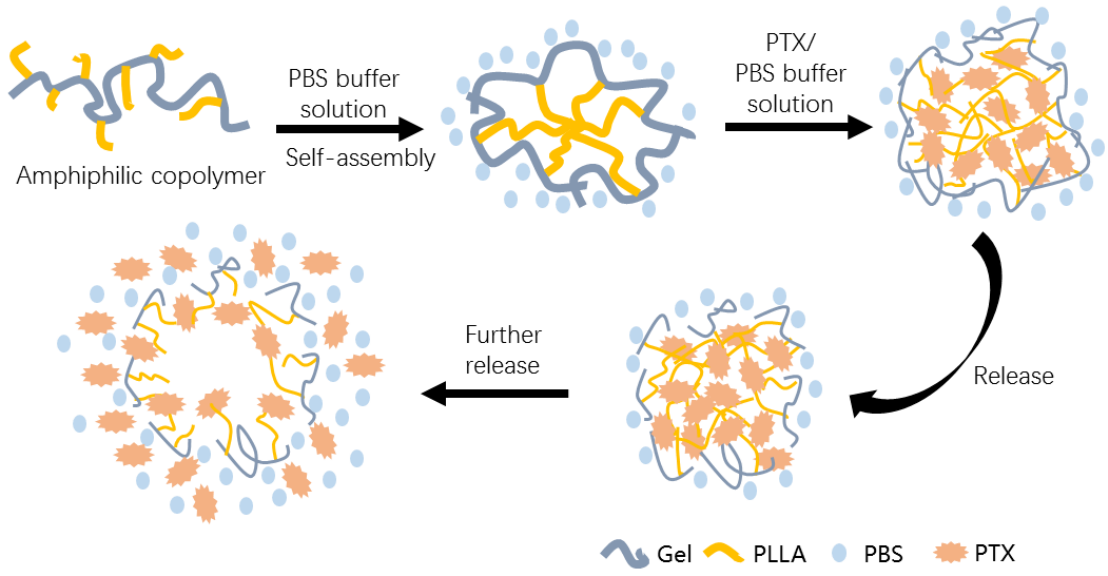


Figure 7. Surface tension changes as a function of copolymer concentration.

3.3.3. *In vitro* release behavior

PTX has been extensively used in clinical cancer treatments such as breast cancer, lung cancer, and ovarian cancer. The process of sustained release of PTX after embedding into micelles is drawing in the Scheme 2. Firstly, the amphiphilic copolymer was solved in PBS with the concentration above CMC. Then, it self-assembled into micelles. The PTX-loaded micelles were prepared using direct dissolution method, as mentioned in 2.6. Finally, the PTX in the micelles released *in vitro* after several hours and even days, like showing in Scheme 2.



Scheme 2. The release process of PTX-encapsulated Gel-g-PLLA micelles.

Table 2 presents that the encapsulation efficiency increases with increasing the length of gelatin chain, reaching a maximum value to 57.7%. Besides, the drug loading increases with increasing the length of gelatin chain, reaching a maximum value to 1.14%. In fact, the polymer chain length does not show significance both for the encapsulation efficiency and the drug loading using 1 mg PTX. For a copolymer with a fixed chain length of a hydrophilic segment, with the increasing length of the hydrophobic segment is increased, the drug loading will increase. As the increasing length of the hydrophobic segment can increase the partition coefficient of the drug between the micelle and medium, which leads to the integrating number of each micelle and core capacity increasing. As the chain length of gelatin increasing, in a sense, the chain length of PLLA is decreasing. For the release study, the three samples were all considered.

Many studies revealed prolonged release of all drugs in spite of an initial burst. Similarly, in the case of our PTX-loaded micelles, PTX exhibits a burst release in the first 24 h (Figure 8b), followed by a slower release (Figure 8a). Then, after 15 days, drug release proceeds gradually to reach 91%,87% and 82%, respectively. It is observed that the behavior after 24 h of release is quite different from the initial hours of analysis. The interpretation of this behavior may be related to the different interactions of PTX with the segments of the copolymer. Owing to hydrophobicity, the interaction of PTX with the hydrophobic segment of the copolymer (PLLA) is stronger, which results in a slow release. Thereby, the release of PTX is expected to occur as the polymer is hydrolytically degraded in this case. Considering the first several hours of the

release experiment, PTX released may be derived from weak interactions with gelatin.

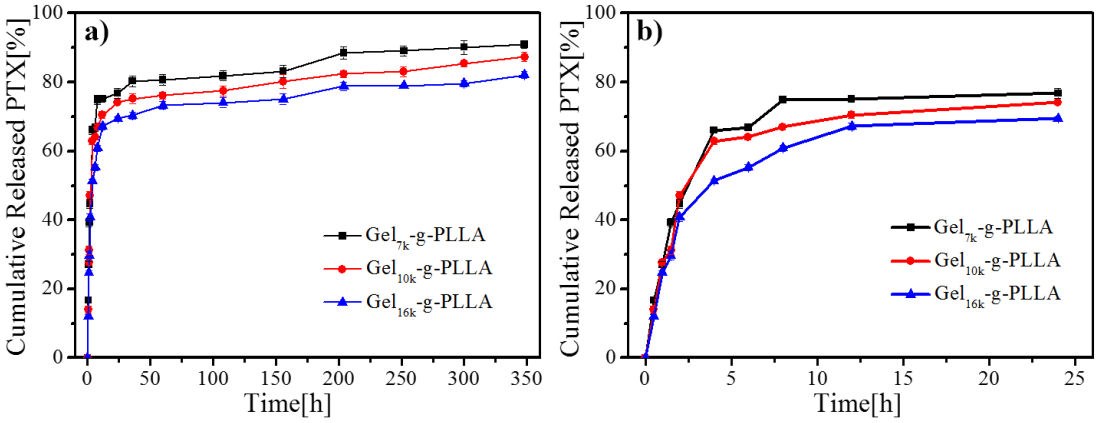


Figure 8. *In vitro* release of drugs from PTX-loaded micelles at 37°C and pH 7.4: a) during 350 h, b) or 25 h.

It is noteworthy that PTX is released faster in Gel_{7k}-g-PLLA micelles. In addition, with the chain length of gelatin increases, the release speed increases. This result confirms the release profile, for the slow release of PTX is related to the interaction of the hydrophobic segments of the nanoparticles, requiring relaxation and/or degradation of the nanoparticle chains to release PTX.

4.Conclusion

In this paper, a series of Gel-g-PLLA graft copolymers were successfully synthesized via a coupling agent route, which exhibited self-assembly and drug release behaviors in aqueous solutions. FT-IR and ^{1}H NMR studies confirmed that the reaction induced amide linkages between the polymers by using the inherent functional carboxyl groups of PLLA-COOH and amine groups of gelatin. The size and morphology of the prepared micelles are determined by TEM and DLS analysis showing the size of micelles from 20 to 200 nm with the increase in the gelatin content. Through CMC measurements, CMC varies from 0.1 to 0.9 g L^{-1} with the increase in gelatin chain length. A hydrophobic anticancer drug, PTX, was successfully encapsulated in micelles with an encapsulation efficiency of approximately 55%. PTX in all the micelles exhibits a burst release about 70% in the first 24 h. Apparently, Gel_{7k}-g-PLLA, prepared using gelatin with molecular weights of 1.7×10^{4} g mol^{-1} and the molar ratio of EDC to NHS of 4:1, has the best encapsulation efficiency, drug loading and release speed.

The above results highlight the importance of the hydrophilic segment on the self-assembly

behavior of Gel-g-PLLA copolymers. In addition, Gel-g-PLLA nanoparticles with adjustable micelle size, CMC and release time have a remarkable potential as carriers for hydrophobic drug delivery in cancer therapy.

Acknowledgements

The authors thank the National Natural Science Foundation of China (51373041 & 51673046). SICHUAN RAYBO BIOTECH company limited is appreciated.

References

[1] Wang W, Xiao J, Chen X, Luo M, Liu H, Shao P.: Fabrication and characterization of multilayered kafirin/gelatin film with one-way water barrier property. Food Hydrocolloids, **81**, 159-168 (2018).

Doi: 10.1016/j.foodhyd.2018.02.044

[2] Wang H, Hansen MB, Loewik DWPM, van Hest JCM, Li Y, Jansen JA, Leeuwenburgh SCG.: Oppositely Charged Gelatin Nanospheres as Building Blocks for Injectable and Biodegradable Gels. Advanced Materials, **23**, H119-H124 (2011).

Doi: 10.1002/adma.201003908

[3] Yoon S, Chen B.: Elastomeric and pH-responsive hydrogels based on direct crosslinking of the poly(glycerol sebacate) pre-polymer and gelatin. Polymer Chemistry, **9**, 3727-3740 (2018).

Doi: 10.1039/C8PY00544C

[4] Etxabide A, Uranga J, Guerrero P, de la Caba K.: Development of active gelatin films by means of valorisation of food processing waste: A review. Food Hydrocolloids, **68**, 192-198 (2017).

Doi: 10.1016/j.foodhyd.2016.08.021

[5] Nguyen AH, McKinney J, Miller T, Bongiorno T, McDevitt TC.: Gelatin methacrylate microspheres for controlled growth factor release. Acta Biomaterialia, **13**, 101-110 (2015).

Doi: 10.1016/j.actbio.2014.11.028

[6] Wang Y, Zhang X, Qiu D, Li Y, Yao L, Duan J.: Ultrasonic assisted microwave synthesis of poly (Chitosan-co-gelatin)/polyvinyl pyrrolidone IPN hydrogel. Ultrasonics Sonochemistry, **40**, 714-719 (2018).

Doi: 10.1016/j.ultsonch.2017.08.003

[7] Heidariyan Z, Ghanian MH, Ashjari M, Farzaneh Z, Najarasl M, Larijani MR, Piryaei A.: Efficient and cost-effective generation of hepatocyte-like cells through microparticle-mediated delivery of growth factors in a 3D culture of human pluripotent stem cells. Biomaterials, **159**, 174-188 (2018).
Doi: 10.1016/j.biomaterials.2018.01.005

[8] Young S, Wong M, Tabata Y, Mikos AG.: Gelatin as a delivery vehicle for the controlled release of bioactive molecules. Journal of Controlled Release, **109**, 256-274 (2005).
Doi: 10.1016/j.jconrel.2005.09.023

[9] Hayashi K, Tabata Y.: Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions. Acta Biomaterialia, **7**, 2797-2803 (2011).
Doi: 10.1016/j.actbio.2011.04.013

[10] Kim SW, Park K.: Polymer micelles for drug delivery, in Targeted Delivery of Small and Macromolecular Drugs: Problems Faced and Approaches Taken. CRC Press, Taylor and Francis Group, Boca Raton (2010).

[11] Zhao B, Brittain WJ.: Polymer brushes: surface-immobilized macromolecules. Progress in Polymer Science, **25**, 677-710 (2000).
Doi: 10.1016/S0079-6700(00)00012-5

[12] Xie Y, Ma X, Liu X, Long Q, Wang Y, Yao Y, Cai Q.: Carrier-Free Microspheres of an Anti-Cancer Drug Synthesized via a Sodium Catalyst for Controlled-Release Drug Delivery. Materials, **11**, 281 (2018).
Doi: 10.3390/ma11020281

[13] Wang G, Zhao D, Li N, Wang X, Ma Y.: Drug-loaded poly (epsilon-caprolactone)/Fe3O4 composite microspheres for magnetic resonance imaging and controlled drug delivery. Journal of Magnetism and Magnetic Materials, **456**, 316-323(2018).
Doi: 10.1016/j.jmmm.2018.02.053

[14] De Smet L, Ceelen W, Remon JP, Vervaet C.: Optimization of drug delivery systems for intraperitoneal therapy to extend the residence time of the chemotherapeutic agent. The Scientific World Journal, **2013**, 720858 (2013).
Doi: 10.1155/2013/720858

[15] De Clercq K, Schelfhout C, Bracke M, De Wever O, Van Bockstal M, Ceelen W, Remon

JP.: Genipin-crosslinked gelatin microspheres as a strategy to prevent postsurgical peritoneal adhesions: In vitro and in vivo characterization. Biomaterials, **96**, 33-46 (2016).
Doi: 10.1016/j.biomaterials.2016.04.012
[16]Zhong Y, Li T, Lin H, Zhang L, Xiong Z, Fang Q, Zhang G.: Meso-/macro-porous microspheres confining Au nanoparticles based on PDLA/PLLA stereo-complex membrane for continuous flowing catalysis and separation. Chemical Engineering Journal, **344**, 299-310 (2018).
Doi: 10.1016/j.cej.2018.03.080
[17]Moriya A, Maruyama T, Ohmukai Y, Sotani T, Matsuyama H.: Preparation of poly(lactic acid) hollow fiber membranes via phase separation methods. Journal of Membrane Science, **342**, 307-312 (2009).
Doi: 10.1016/j.memsci.2009.07.005
[18]Xiong Z, Liu F, Lin H, Li J, Wang Y.: Covalent Bonding of Heparin on the Crystallized Poly(lactic acid) (PLA) Membrane to Improve Hemocompability via Surface Cross Linking and Glycidyl Ether Reaction. ACS Biomaterials Science & Engineering, **2**, 2207-2216 (2016).
Doi: 10.1021/acsbiomaterials.6b00413
[19]Wang J, Caceres M, Li S, Deratani A.: Synthesis and Self-Assembly of Amphiphilic Block Copolymers from Biobased Hydroxypropyl Methyl Cellulose and Poly(L-lactide). Macromolecular Chemistry and Physics, **218**, 160055810, (2017).
Doi: 10.1002/macp.201600558
[20]Kelnar I, Kratochvil J, Fortelny I, Kapralkova L, Zhigunov A, Khunova V, Nevoralova M.: Effect of halloysite on structure and properties of melt-drawn PCL/PLA microfibrillar composites. Express Polymer Letters, **10**, 381-393 (2016).
Doi: 10.3144/expresspolymlett.2016.36
[21]Kim Y, Suhr J, Seo H, Sun H, Kim S, Park I, Kim S.: All Biomass and UV Protective Composite Composed of Compatibilized Lignin and Poly (Lactic-acid). Scientific Reports, **7**, 43596 (2017).
Doi: 10.1038/srep43596
[22]Zeng J, Li K, Du A.: Compatibilization strategies in poly(lactic acid)-based blends. RSC Advances, **5**, 32546-32565(2015).

Doi: 10.1039/c5ra01655j

[23]Hua S, Chen F, Liu Z, Yang W, Yang M.: Preparation of cellulose-graft-polylactic acid via melt copolycondensation for use in polylactic acid based composites: synthesis, characterization and properties. RSC Advances, **6**, 1973-1983 (2016).

Doi: 10.1039/c5ra23182e

[24]Wang J, Chen Q, Du B, Cao L, Lin H, Fan Z, Dong J.: Enhanced bone regeneration composite scaffolds of PLLA/β-TCP matrix grafted with gelatin and HAp. Materials Science and Engineering: C, **87**, 60-69 (2018).

Doi: 10.1016/j.msec.2018.02.011

[25]Hamad K, Kaseem M, Yang HW, Deri F, Ko YG.: Properties and medical applications of polylactic acid: A review. Express Polymer Letters, **9**, 435-455 (2015). Doi: 10.3144/expresspolymlett.2015.42

[26]Hu Y, Darcos V, Monge S, Li S.: Thermo-responsive drug release from self-assembled micelles of brush-like PLA/PEG analogues block copolymers. International Journal of Pharmaceutics, **491**, 152-161 (2015).

Doi: 10.1016/j.ijpharm.2015.06.020

[27]Jelonek K, Li S, Kaczmarczyk B, Marcinkowski A, Orchel A, Musial-Kulik M, Kasperczyk J.: Multidrug PLA-PEG filomicelles for concurrent delivery of anticancer drugs-The influence of drug-drug and drug-polymer interactions on drug loading and release properties. International Journal of Pharmaceutics, **510**, 365-374(2016).

Doi: 10.1016/j.ijpharm.2016.06.051

[28]Li P, Zhang Z, Su Z, Wei G.: Thermosensitive polymeric micelles based on the triblock copolymer poly(D,L-lactide)-b-poly(N-isopropyl acrylamide)-b-poly(D,L-lactide) for controllable drug delivery. Journal of Applied Polymer Science, 134, 45304 (2017).

Doi: 10.1002/app.45304

[29]Gatti S, Agostini A, Palmiero UC, Colombo C, Peviani M, Biffi A, Moscatelli D.: Hydrazone linked doxorubicin-PLA prodrug nanoparticles with high drug loading. Nanotechnology, **29**, 305602 (2018).

Doi: 10.1088/1361-6528/aac0d3

[30]Poudel AJ, He F, Huang L, Xiao L, Yang G.: Supramolecular hydrogels based on poly

(ethylene glycol)-poly (lactic acid) block copolymer micelles and alpha-cyclodextrin for potential injectable drug delivery system. Carbohydrate Polymers, **194**, 69-79 (2018).
Doi: 10.1016/j.carbpol.2018.04.035

[31]Shimizu T, Abu Lila AS, Fujita R, Awata M, Kawanishi M, Hashimoto Y, Okuhira K.: A hydroxyl PEG version of PEGylated liposomes and its impact on anti-PEG IgM induction and on the accelerated clearance of PEGylated liposomes. European Journal of Pharmaceutics and Biopharmaceutics, **127**, 142-149 (2018).
Doi: 10.1016/j.ejpb.2018.02.019

[32]Shao L, Cao Y, Li Z, Hu W, Li S, Lu L.: Dual responsive aerogel made from thermo/pH sensitive graft copolymer alginate-g-P(NIPAM-co-NHMAM) for drug controlled release. International journal of biological macromolecules, **114**, 1338-1344 (2018).
Doi: 10.1016/j.ijbiomac.2018.03.166

[33]Dang X, Yuan H, Shan Z.: An eco-friendly material based on graft copolymer of gelatin extracted from leather solid waste for potential application in chemical sand-fixation. Journal of Cleaner Production, **188**, 416-424 (2018).
Doi: 10.1016/j.jclepro.2018.04.007

[34]Wang J, Xu Y, Xu H, Zhang F, Qian Y, Zhu B.: Synthesis of an amphiphilic glucose-carrying graft copolymer and its use for membrane surface modification. Journal of Applied Polymer Science, **109**, 2914-2923(2008).
Doi: 10.1002/app.28400

[35]Guler Z, Sarac AS.: Electrochemical impedance and spectroscopy study of the EDC/NHS activation of the carboxyl groups on poly(ε-caprolactone)/poly(m-anthranilic acid) nanofibers. Express Polymer Letters, **10**, 96-110(2016).
Doi: 10.3144/expresspolymlett.2016.11