Department of Pharmaceutical Sciences[1], Shenyang Pharmaceutical University; School of Pharmacy[2], Zhengzhou University, Zhengzhou, China

Investigation of a new injectable thermosensitive hydrogel loading solid lipid nanoparticles

XINHONG GUO[1,2], FUDE CUI[1], YABING XING[2], QIAN MEI[2], ZHENZHONG ZHANG[2]

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Fude Cui, Department of Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Whenhua Road, Shenyang, 110016, China
gxh371@126.com

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For improving the effectiveness of cancer chemotherapy and avoiding rapid clearance of solid lipid nonoparticles (SLN) from the systemic circulation following systemic administration, 2-methoxyestradiol (2-ME) as model drug, PLGA-PEG-PLGA as hydrogel material, an injectable SLN loaded hydrogel was developed. Integrity of SLN within and released from the hydrogel was confirmed by direct visualization by a scanning electron microscope (SEM), particle size measurement by laser light scattering, and free drug concentration in the release medium by ultracentrifugation. Moreover, *in vitro* release, thermo-sensitive properties and rheological behavior were investigated. The results indicated that SLN were stable in the hydrogel. In the release medium, most 2-ME existed in the SLN and intact 2-ME SLN could be released from the hydrogel for a prolonged period over 46 days. Their concentration showed a significant effect on the release rate, in contrast to particle size and pH value of the release medium. In addition, the SLN loaded hydrogel could still exhibit reversible thermo-sensitive properties and better syringeability. These results suggested that the SLN loaded hydrogel could transport SLN to the target site and control prolonged release of SLN, which may increase the efficacy of cancer chemotherapy.

1. Introduction

Implantable depot devices such as copolymeric hydrogels delivery systems provide both physical targeting to the target body site and controlled prolonged release of the drug, resulting in high local concentrations at specific anatomical sites (Zentner et al. 2001; Yang et al. 2009). These properties are particularly useful for chemotherapy drugs where local delivery of cytotoxic agents directly to the tumor site may improve patient outcome and potentially overcome limitations associated with systemic administration. However cancer cells in solid tumors tend to be resistant to chemotherapy due to various drug permeation barriers or cellular mechanisms, which makes it difficult to achieve high intratumoral drug concentrations (Miglietta et al. 2000; Ozben et al. 2006).

Solid lipid nanoparticles (SLN) offer great promise to improve the therapeutic effectiveness of cancer chemotherapy. SLN formulations of anticancer agents have sometimes shown greater cytotoxicity to the cancer cells than the corresponding free drug and offer a promise to overcome susceptibility to induce drug resistance, SLN may therefore improve the effectiveness of chemotherapeutic treatment in cancers that are comparatively refractory to drug therapy (Gieseler et al. 2003; Nielsen et al. 1996). It has been shown by fluorescence microscopy that cell uptake of some anticancer drugs formulated in SLN can be much higher than that of the free drugs, and in cell viability experiments the former can be more effective than the latter (Miglietta et al. 2000). However, following systemic administration, SLN are rapidly cleared from the systemic circulation by the mononuclear phagocyte system (RES) so only lymph nodes, liver or spleen can be targeted efficiently. For other cancers, RES clearance is a major barrier to systemic cytotoxic drug delivery by SLN (Wong et al. 2007).

Based on those considerations, a 2-phase drug depot consisting of drug-loaded SLN entrapped in a PLGA-PEG-PLGA hydrogel was designed. A new delivery system is conceived from a combination of the hydrogel- and the lipid nanoparticles-based delivery systems and can thus integrate the advantages and avoid the drawbacks of the two systems (Lalloo et al. 2006; Paavola et al. 2000), which could transport drug-loaded SLN directly to target/tumor site and sustain prolonged release of drugs-loaded SLN from the hydrogel. For water-insoluble anticancer drugs, SLN could improve water solubility. As we know, the other two-phase systems including the entrapment of liposomes (Gariepy et al. 2002; Bochot et al. 1998; Feng et al. 2004) or microspheres in copolymer matrices (Stenekes et al. 2000) have been investigated to control drug release profiles. To our knowledge, few work was conducted to investigate SLN entrapped in hydrogels up to now. 2-Methoxyestradiol (2-ME), a metabolite of 17-ß-estradiol, is an effective and water-insoluble anticancer drug against a wide spectrum of solid tumors such as breast cancer and prostatic carcinoma by its anti-proliferation and anti-angiogenic properties (Guo et al. 2009). So in this paper, with 2-ME as a model anticancer drug, the two-phase delivery system of 2-ME SLN loaded hydrogel was investigated and was characterized for the stability of SLN, rheological behav-

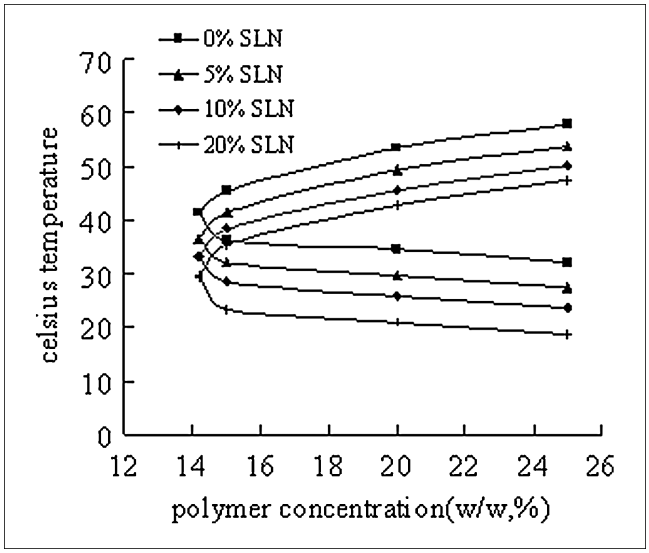

Fig. 1: Effect of concentration (w/w,%) of polymer and 2-ME SLN on sol-gel temperature (as shown in anadrome side of the curve) and gel-pre transition temperature (as shown in catadrome side of the curve)

ior, thermo-sensitive properties and *in vitro* release of 2-ME SLN.

2. Investigations, results and discussion

2.1. Preparation of 2-ME SLN loaded hydrogel

The characteristics of 2-ME SLN prepared are shown in the Table 1. The copolymer solution after incorporating SLN powder at about room temperature became milky and uniform without demixing and precipitation, which indicated that the copolymer solution was highly compatible with the SLN.

2.2. Thermo-sensitive properties

The copolymer solution after incoporating 2-ME SLN still exhibited reversible thermo-sensitive properties in the range of 15–60 °C. However, the concenration of 2-ME SLN significantly influenced the thermo-sensitive properties of the copolymer solution . After incorporating different concentration of SLN, sol–gel and gel-pre transition temperature of copolymer solution decreased accordingly and the higher concentration of SLN resulted in lower sol–gel and gel-pre transition temperatures of the copolymer solution (Fig. 1).

2.3. Rheological behavior

The rheological characteristics of the SLN-loaded copolymer solution systems before gelling was temperature-dependent and concentration-dependent as shown in Fig. 2. Generally, the viscosity of the 2-ME SLN-loaded copolymer solution increased with increase of temperature and SLN concentration. The viscosities of 2-ME loaded SLN copolymer solutions were relatively low and less than 1 Pas at around room temperature when the concentration of SLN was lower than 20%(w/w), so it could easily pass the syringe needle (Yang et al. 2009).

2.4. Integrity of 2-ME SLN in the hydrogel

The integrity of 2-ME SLN incorporated within, and released from the hydrogel was confirmed by four approaches, namely direct visualization by SEM, particle size measurement by laser light scattering, opalescence observation by eyes and free drug

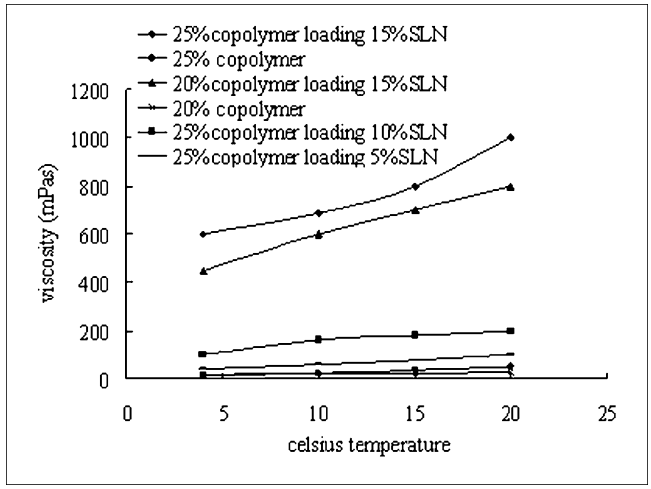

Fig. 2: Effect of concentration of copolymer and SLN and temperature on viscosity of the copolymer solution before forming gel (n = 3)

concentration determination in the release medium by ultracentrifugation.

Fig. 3 shows SEM images of the 2-ME SLN (Fig. 3A), and the 2-ME SLN loaded hydrogel (Fig. 3B). It could be seen from Fig. 3B that the spherical SLN kept intact after incorporating into the hydrogel. However, because of coating of the copolymer, their particle size was larger than the free 2-ME SLN (Fig. 3A).

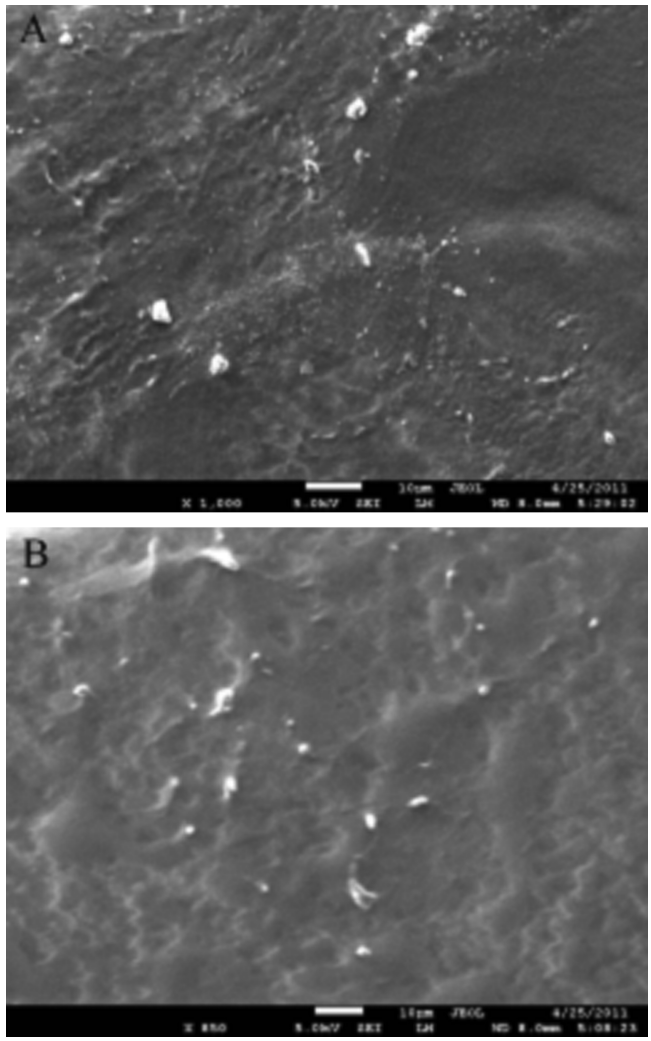

Fig. 3: SEM images of 2-ME SLN (A) and 2-ME SLN loaded hydrogel (B)

Table: Characteristics of 2-ME SLN after re-dissolving 2-ME SLN power in 5% glucose and particle size of 2-ME SLN released from the hydrogel (25%,w/w copolymer) (mean ± S.D., n = 3)

2-ME SLN				Particle size of released SLN (nm)	
Particle size(nm)	Zeta potential (mv)	EE(%)	DL(%)	after 1day	after46 days
151 ± 22	–40.01 ± 1.80	91.27 ± 2.45	2.23 ± 0.35	147 ± 25[a]	161 ± 28[a]
228 ± 17	–38.35 ± 1.62	89.64 ± 3.37	2.18 ± 0.29	241 ± 28 [b]	233 ± 23 [b]
94 ± 31	–35.26 ± 2.24	87.49 ± 2.85	2.05 ± 0.27	403 ± 32 [c]	411 ± 24

[a, b, c] No statistical significance with the particle size of corresponding 2-ME SLN ($p > 0.05$).

In the release process, it was observed that the release medium displayed a significant bluish opalescence. Moreover, the released SLN were of particle sizes close to those of the free SLN. These were clear evidences that supported the SEM observation (Table).

In each release medium sampled, the concentration of free 2-ME (about 0.5 μg/ml) was far lower than its solubility (about 40 μg/ml) and its concent was only 0%–4% (w/w), which indicated that 2-ME was mostly entrapped in SLN and intact 2-ME SLN could be released from the hydrogel. Moreover, this indicated that the release of drug from the SLN was very slow.

2.5. In vitro SLN release from the hydrogel

In the release process, it was found that the state of 2-ME SLN loaded hydrogel was maintained all along and gradually became smaller because of degradation of the copolymer. 2-ME SLN were released from the hydrogel for prolonged period over 46 days without significant burst release. So the hydrogel could control a prolonged release of 2-ME SLN.

The concentration of 2-ME SLN showed a significant effect on their release profile from the hydrogel (Fig. 4). The higher concentration resulted in a slower release rate. However, their particle size of 161 nm−394 nm and pH value of the release medium of 4.7–7.4 all showed no significant effect on the release rate of 2-ME loaded SLN (Figs. 5–6).

3. Discussion

In this study, a 2-ME SLN hydrogel was successfully prepared by a formerly mentioned method. Certainly, the 2-ME SLN loaded hydrogel could be prepared by another method. The copolymer was added in SLN suspension and then it was placed in ice-box at 4 °C until forming a milky solution with bluish opalescence. However, this method could not adjust the concentration of 2-ME SLN in the hydrogel. So in this study, the formerly mentioned method was employed to prepare the 2-ME SLN loaded hydrogel.

Thermoreversible gelation in polymeric systems is a well-known phenomenon (Qiao et al. 2005). At lower temperatures, hydrogen bonding between hydrophilic PEG segments of the copolymer chain and water molecules dominates in the aqueous solution, resulting in their dissolution in water. As the temperature increased, the hydrogen bonding became weaker, while hydrophobic forces among the hydrophobic PLGA segments strengthened leading to sol–gel transition. As the temperature continued to increase, excessive hydrophobicity destroyed the micellar framework and resulted in precipitation of copolymer. Addition of 2-ME SLN into the copolymer solution resulted in a decrease of its sol-gel and gel-pre transition temperature (Fig. 1), which could be related to that superficial hydrophilicity of 2-ME SLN weakened hydrogen bonding and strengthened hydrophobicity of the copolymer. Inversely, addition of a hydrophobic drug or delivery system such as microspheres showed no effect on the phase transition temperature (data no shown). It was desirable that a copolymer solution incorporating SLN should not gel at room temperature for easy injection, and could maintain the gel state at body temperature (37 °C) for sustained release of SLN. As we know, PEG molecular weight and molar ratio of LA and GA of the copolymer all significantly influenced the phase transition temperature (Qiao et al. 2005). So the transition temperature of 2-ME SLN loaded copolymer solution could be adjusted by selecting a suitable copolymer.

2-ME SLN could be stable in the hydrogel and intact 2-ME SLN could be released from the hydrogel. Moreover, the hydrogel could control the prolonged release of 2-ME SLN from

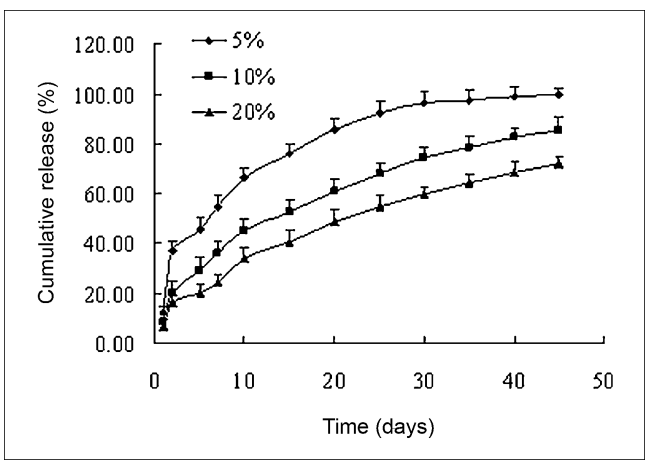

Fig. 4: Effect of SLN concentration on *in vitro* release of SLN (particle size of 280 ± 28 nm) from 0.2 ml hydrogel (20%,w/w, copolymer) at 37 °C (n = 3)

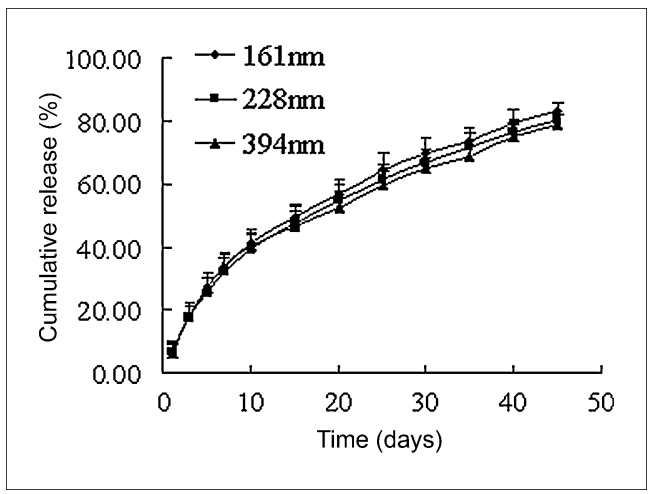

Fig. 5: Effect of particle size of 2-ME SLN on *in vitro* release of 2-ME SLN from 0.2 ml hydrogel (20%,w/w, copolymer) at 37 °C (n = 3)

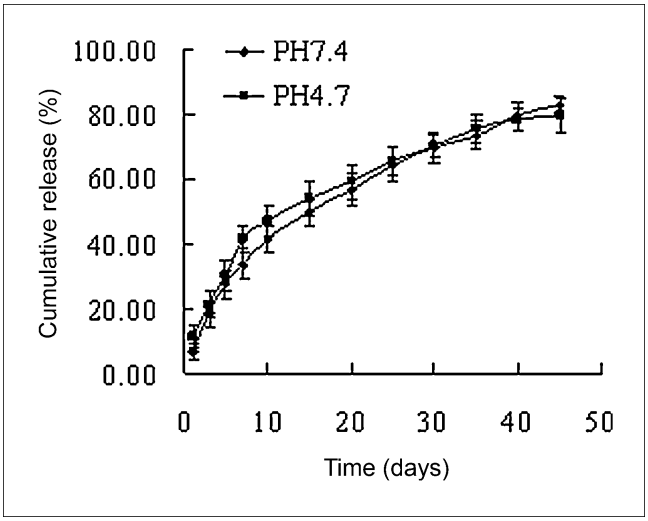

Fig. 6: Effect of pH value of the release medium on the release rate of 2-ME SLN from 0.2 ml hydrogel (mean ± S.D. n = 3)

the hydrogel over one-two months. In addition, pH value of the release medium did not significantly influence their release rate, which indicated that both intratumoral injection and peritumoral injection all were feasible administration method for the 2-ME SLN loaded hydrogel. The above results indicated that the hydrogel could transport 2-ME SLN to the target/tumor site and then 2-ME SLN educed role in efficient prolonged chemotherapy of cancer. As we know, the clinical success rate of chemotherapy is particularly low in the treatment of solid tumors (Tannock et al. 2002). The unique environment inside a solid tumor leads to a number of non-cellular drug resistance mechanisms. Irregularly developed intratumoral vasculature and the extracellular matrix of tumor cells both prevent efficient drug penetration into the tumor (Bhattacharya et al. 2004; Croix and Kerbel 1997). High interstitial pressures that can build up in solid tumors further exacerbate this problem (Jain 1987). In addition to cellular mechanisms, cancer cells in solid tumors tend to be more resistant to chemotherapy than non-aggregating cancer cells due to various drug permeation barriers, which makes it difficult to achieve high intratumoral drug concentrations (Gieseler et al. 2003; Baird and Kaye 2003). Local drug delivery systems and SLN offer the promise to overcome at least some of these obstacles. In this study, a 2-ME SLN loaded hydrogel was successfully developed and integrated the advantages of the two delivery systems. The SLN loaded hydrogel could transport SLN to the target site and control the prolonged release of SLN, which was highly important for anticancer drug to increase the efficacy of cancer chemotherapy.

4. Experimental

4.1. Materials

2-ME (99.5 % in purity) were home-made. Phosphatidylcholine (PC, injection grade) were purchased from Siwei (Zhengzhou, China). Poloxamer188 (P188) was supplied by Shenyang Jiqi Pharmaceutical Co., Ltd. (China). Compritol 888 (ATO888) was purchased from Gattefosé, France. Poly (DL-lactide-co-glycolide) -poly (ethyleneglycol)-poly (DL-lactide-co-glycolide) (PLGA-PEG-PLGA) (PEG1500, MW4000–6000) with DL-lactide/Glycolide (LA/GA) molecular ratio of 6/1 was purchased from (JiNan daigang Co., Ltd., China). All reagents for high performanc liquid chromatography (HPLC) analysis. Other chemicals used were of analytical grade.

4.2. Preparation and characterization of 2-ME SLN powder

SLN were prepared using hot homogenization-ultrasonication. Briefly, 20 mg 2-ME was added to 250 mg monostearin and 50 mg ATO888 previously melted at 80 °C. 300 mg PC and 200 mg F_{68} was dissolved in 10 ml double distilled water and heated up to 80 °C in a beaker. When a clear homogenous lipid phase was obtained, the hot aqueous surfactant solution was added to hot lipid phase and homogenization (Ultra Turrax IKA T25, Germany) was carried out at 10,000 rpm for 2 min at 80 °C. The obtained pre-emulsion was probe ultrasonied (LTD JY92-II, Scientz Biotechnology Co., China) at 100W for 3 min at 80 °C and then cooled down in an ice bath to form SLN suspension. 4% (w/v) trehalose and 6% (w/v) mannitol as cryo-protectant were added in the SLN suspension. The samples were frozen at −80 °C for 12 h followed by drying at −50 °C for 24 h under 20 Pa vacuum by a laboratory freeze drier (HERMLE CT/DW 110, Germany).

The volume average diameter and zeta potential of 2-ME SLN power lyophilized after re-dissolved in 5% glucose were determined with Zeta sizer-Nano-ZS90 (Malvern Instruments, Malvern, UK). The morphology of 2-ME SLN power lyophilized after re-dissolved in 5% glucose was observed by SEM (JSM7500F, JEOL, Japan). The samples were placed on polycarbonate substrate and left to dry at room temperature (25 °C). They were further dried under vacuum (DZF-6050, ShangHaiJingHong, China), and sputter coated with gold in a metallizer and examined under the SEM operating at an accelerating voltage of 20 kV.

Drug entrapment efficiency (EE) and drug loading (DL) was determined by ultracentrifugation (Venishetty et al. 2007). The EE and DL were calculated from the ratio of the drug amount incorporated into SLN to the total charged drug amount and lipid amount, respectively. Ultracentrifugation was carried out using Centrisart, consisting of a filter membrane (molecular weight cut off 10,000 Da) between the outer chamber and the sample recovery chamber. About 0.5 ml of SLN dispersion was placed in the outer chamber. The unit was centrifuged at 3000g for 15 min. SLN along with encapsulated drug remained in the outer chamber and dispersion medium including free drug moved to the sample recovery chamber through the filter membrane. The drug concentration in the dispersion medium was estimated by HPLC analysis (Agilent 1200 series, USA). The chromatographic conditions were as follows: C_{18} column (150 mm × 4.6 mm, 5 μm) (Agilent, USA), column temperature of 30 °C, injection volume of 20 μl, mobile phase consisting of methanol and water (65:35, v/v), flow rate of 1.0 ml/min, the excited wavelength and emission wavelength of 285 nm and 325 nm. The calibration curve for the quantification for 2-ME was linear over the range of standard concentration between 25 and 1250 ng ml^{-1} with a correlation coefficient of $R^2 = 0.9996$.

4.3. Preparation of thermosensitive 2-ME SLN-loaded hydrogels

The lyophilized 2-ME SLN power was incorporated directly into the copolymer solution. The copolymer was dispersed in water and then was placed in an ice-box at 4 °C until forming a transparent copolymer solution. 2-ME SLN power was suspended rapidly in copolymer solution at about room temperature before the experiment. Then copolymer solution incorporating 2-ME SLN power formed a hydrogel at 37 °C.

The 2-ME SLN loaded hydrogel was cooled and then reached a solution state. SEM was used to analyze the particle size and morphology of 2-ME SLN within the solution by the same protocol as that for the 2-ME SLN (Section 3.2).

4.4. Determination of phase transition temperature

Specific solution(sol)–gel and gel-precipitation (pre) transition temperatures were obtained through tube inversion experiments (Yang et al. 2009). The samples (1 ml) were sealed in small glass tubes (internal diameter about 10 mm) at 4 °C and heated in a temperature controllable water bath from 5 °C to 50 °C at a heating rate of 1 °C/min. When the liquid in the tube became immobile within 30 s, the temperature was recorded as the sol–gel temperature. When the gel in the tube was immobile and then transformed to precipitation within 30 s, the temperature was recorded as the gel–pre temperature.

4.5. Rheological behavior

The rheological behavior of the copolymer solution incorporating 2-ME SLN were investigated by studying their viscosity as functions of temperature (Yang et al. 2009) in a digital rotary viscometer (NDJ-8S, Dalong Instruments, China). After incubating at 4 °C for 24 h, the cold aqueous solutions (50 ml) were put into the glass container which was attached to a controllable circulation water bath with a temperature precision of ± 0.1 °C, respectively. Before each measurement, temperature was stabilized for 10 min, and the interval for measurement was 1 °C.

4.6. In vitro release of SLN from the hydrogel and stability of SLN in the hydrogel

The release experiment was performed in triplicate (Lalloo et al. 2006; Qiao et al. 2005). 0.2 ml copolymer solution incorporating 2-ME SLN

power in Eppendorf tubes solidified to gel without fluidity and were then incubated in 1 ml release medium (PBS buffer, 0.02% sodium azide, 1% Tween80, pH = 7.4 or 4.8) under agitation at 37 °C and 75 strokes/min (ZD-85, zhejiangjintan, China). At desirable time intervals, 1 ml supernatant was withdrawn and replaced with 1 ml fresh release medium. The particle size of the SLN and the SLN amount in the release medium were measured periodically in the release process. The SLN amount in the release medium was determined by measuring the drug amount entrapped into SLN. The total drug amount was determined as the above HPLC method by lysing the SLN with methanol to release the drug. The free drug amount in the release medium was determined by ultracentrifugation according to the above method of EE determination.

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