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PEGylated polycyanoacrylate nanoparticles as salvicine carriers: synthesis, preparation, and *in vitro* characterization[1]

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KEY WORDS polyethylene glycols; poly (alkyl cyanoacrylate); nanoparticles; salvicine

ABSTRACT

AIM: To synthesized poly (methoxypolyethyleneglycol cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) (PEGylated PHDCA) with polyethylene glycol (PEG, $M_r = 5000$), prepare PEGylated PHDCA and poly (*n*-hexadecyl cyanoacrylate) (PHDCA) nanoparticles loading salvicine and determine their *in vitro* characterizations. **METHODS**: The structure of PEGylated PHDCA was determined with ^{1}H-NMR, ^{13}C-NMR and Fourier transform infrared spectrum (FTIR). Its molecular weight was determined by gel permeation chromatography (GPC). Nanoparticles were prepared by emulsion/solvent evaporation method. **RESULTS**: ^{1}H-NMR, ^{13}C-NMR, and FTIR were consistent with structure of PEGylated PHDCA, whose average molecular weight is 6680. Entrapment efficiency could be determined by high pressure liquid chromatography (HPLC) method without endogenous interference at the retention time of salvicine. The entrapment efficiency was 92.6 % for PEGylated PHDCA nanoparticles and 98.9 % for PHDCA nanoparticles. The nanoparticles size was about 250 nm. The values of the zeta potential were obviously influenced by the composition of the copolymer. Compared with PHDCA nanoparticles (−23.1 mV), PEGylated PHDCA nanoparticles showed a low surface potential (−9.6 mV). Salvicine release from nanoparticles showed an initial burst effect, then a plateau for an extended period, and finally sustained release phase. **CONCLUSION**: These results showed that the PEGylated PHDCA nanoparticles could be an effective carrier for salvicine delivery in the respect of anti-tumor potency.

INTRODUCTION

Encapsulation of active compounds into nanoparticles becomes a very promising way of preventing drug degradation, decreasing toxic effects, and controlling the rate of drug release. However, the potential of nanoparticles is compromised by their rapid elimination from the bloodstream and their accumulation in the tissues of the mononuclear phagocyte system (MPS), especially the liver[1]. In past few years, there was an increasing interest to develop stealth nanoparticles or long-circulating nanoparticles as drug carriers, whose striking advantages were a less uptake by MPS, extending half-life, and a better role in controlled delivering the pharmacological agent to its target[2,3]. The stealth nanoparticles could be prepared by modification of their surface with hydrophilic, flexible, and non-ionic polymers, such as poly (ethylene glycol) (PEG)[4-6]. More recently, a more rapidly biodegradable copolymer, poly (methoxypolyethyleneglycol cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) (PEGylated PHDCA) has been developed for the preparation of stealth nanoparticles[2,7-10]. However, there is no report on PEGylated PHDCA nanoparticles as a specific drug carriers, and PEGylated PHDCA was synthesized only using a small molecular weight PEG ($M_r = 2000$), which could not be an ideal carrier in extending half-life of the drug enveloped.

In this study, we synthesized PEGylated PHDCA copolymer using a bigger molecular weight PEG ($M_r = 5000$), which was used to increase the proportion of hydrophilic fraction in copolymer and steric hindrance from methoxypolyethyleneglycol (MePEG) on the surface of the nanoparticles. The structure of copolymer was confirmed with ^{1}H-NMR, ^{13}C-NMR and FTIR. Its molecular weight was determined by gel permeation chromatog-

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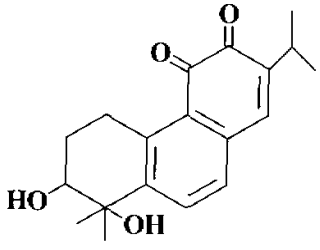
raphy (GPC). Salvicine, which is a novel anti-tumor compound obtained by structural modification of a natural product isolated from a Chinese medicinal plant *Salvia prionitis* Hance[11,12], was chosen as a model drug for encapsulation studies in the nanoparticles. The stealth nanoparticles were characterized in terms of size, zeta potential, and *in vitro* release of salvicine.



Salvicine

MATERIALS AND METHODS

Materials Cyanoacetic acid (purity >99 %), poly(vinylalcohol) (PVA) (M_r = 16 000, 98 % hydrolyzed) and monomethoxy polyethylene glycol (MePEG, M_r = 5000, purity >95 %) were obtained from Sigma Chemical Co (St Louis, MO). Salvicine presenting tangerine color crystalloid was kindly donated by Prof Zhang Jin-Sheng (Phytochemistry Department, Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Dichloromethane (DCM) of HPLC grade was used in this study. All other reagents and solvents were of analytical grade.

Synthesis of PEGylated PHDCA copolymer PEGylated PHDCA was synthesized by an appropriate modification for using MePEG (M_r = 5000)[7]. Briefly, MePEG cyanoacetate was obtained by esterification of the cyanoacetic acid (10 mmol) and MePEG (2.5 mmol) in DCM (50 mL) with 1, 3-dicyclohexylcarbodiimide (DCC, 10 mmol) and 4-(dimethylamino) pyridine (DMAP, 30 mg) as catalyst. The *n*-hexadecyl cyanoacetate was prepared according to the same procedure as MePEG cyanoacetate, except that hexane was added to the reaction system before filtration. PEGylated PHDCA was synthesized by polymerization of MePEG cyanoacetate and *n*-hexadecyl cyanoacetate (molar ratio; 1:5) in ethanol with formalin and dimethylamine and purified with GPC.

Measurement of copolymer The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker AM 400 MHz spectrometer. Recognition of methyl, methylene, methine, and quaternary carbon nuclei in ^{13}C-NMR spectra rests on the BB + DEPT-135.

Fourier transform infrared spectrum (FTIR spectrometer, Nicolet, Magna FTIR-750) was obtained from a neat film cast from the chloroform copolymer solution between KBr tablets.

GPC of PEGylated PHDCA was performed in tetrahydrofuran with a Waters Associaties Model ALC/GPC 244 apparatus. Molecular weight and molecular weight distribution of the copolymer were calculated by using polystyrene as the standard.

Preparation of nanoparticles Nanoparticles were prepared by emulsion/solvent evaporation procedure[8]. Briefly, PEGylated PHDCA or PHDCA (75 mg) and salvicine (50 mg) were dissolved in 5 mL DCM. The organic phase was poured into 50 mL PVA solution (0.4 %, w/v) and homogenized at 1×10^4 rpm for 2 min (Bailing Model DS-200, China). Thereafter, the emulsion (o/w) was diluted in 100 mL PVA solution (0.1 %, w/v) and the organic solvent was rapidly eliminated by evaporation under reduced pressure. Finally, the nanoparticles were collected by centrifugation at 25 000 × *g* (Beckman Model J2-21) for 20 min and washed three times with water.

Determination of the entrapment efficiency Salvicine was determined by HPLC method. A Shimadzu HPLC system was used with two LC-10AD pumps, one SPD-10A UV detector, and a C-R6A recorder. The column was Lichrosorb C_{18}, 5 μm, 5 mm × 150 mm. The mobile phase was a mixture of methanol and water (70 : 30, v/v), prepared and degassed daily. Chromatography assay was performed at 25 ℃ using a flow rate of 1 mL/min with a pressure of 490 Pa. Absorption was measured at 260 nm with 0.05 absorption units of full scale (AUFS).

The amount of salvicine encapsulated into nanoparticles was calculated by the difference between the total amount used to prepare the nanoparticles and the amount of non-entrapped salvicine. Each sample was assayed in triplicate.

Physicochemical characterization of nanoparticles The morphological examination of nanoparticles was performed using a transmission electron microscope (TEM, CM12 Philips, Netherlands) following negative staining with sodium phosphotungstate solution (0.2 %, w/v). The surface potential and size distribution of the nanoparticles were analyzed using a Zetasizer II (Malvern

Instruments, UK). The results were all normalized with respect to a polystyrene standard solution.

***In vitro* release experiment** Samples of 75 mg of nanoparticles with 27.8 mg and 29.6 mg of salvicine in PEGylated PHDCA nanoparticles and PHDCA nanoparticles, respectively, were suspended in 100 mL of phosphate buffer saline (PBS) pH 7.4 containing 0.02 % (w/v) Tween 80 and incubated at 37 ℃ under gentle magnetic stirring at 100 rpm. At predetermined time intervals, 2.5 mL samples were withdrawn and centrifuged at 25 000 × *g* for 20 min. The supernatant was assayed for salvicine release and replaced by 2.5 mL of fresh release medium. The amount of salvicine in the supernatant was determined by HPLC method as described above. Each nanoparticle batch was analyzed in triplicate.

RESULTS

Synthesis of PEGylated PHDCA copolymer

Esterification of the cyanoacetic acid with PEG or hexadecanol, and synthesis of PEGylated PHDCA copolymer was showed in Fig 1.

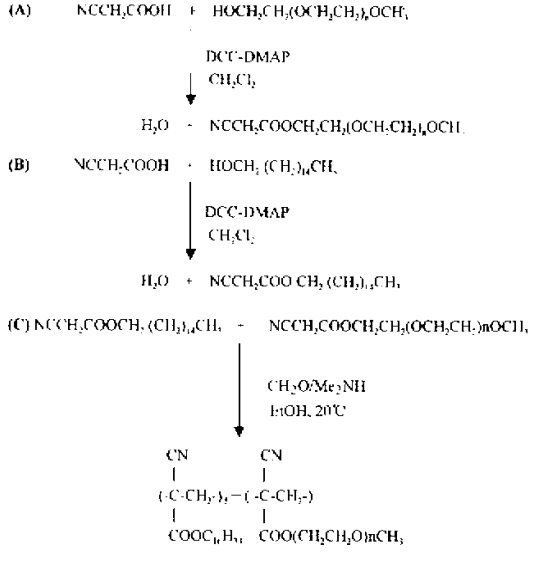


Fig 1. Esterification of the cyanoacetic acid with PEG (A) or hexadecanol (B), and polymerization of the cyanoacetates (C).

Measurement of copolymer The structure of PEGylated PHDCA was confirmed by ^{1}H-NMR (Fig 2).

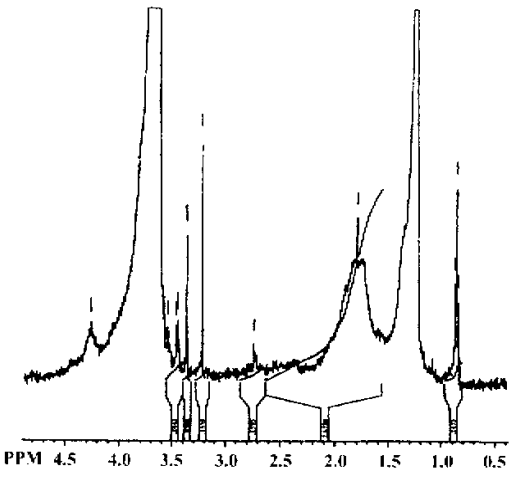


Fig 2. ^{1}H-NMR spectrum of the PEGylated PHDCA copolymer

One of the striking features was a large peak at 3.65 ppm, corresponding to the methylene groups of the MePEG. The peak at 4.2 ppm was attributed to the methylene in the α-position to the ester groups. The singlet at 3.36 ppm was attributed to the MeO terminal group of the PEG chain. Signals at 1.78, 1.10, and 0.88 ppm were assigned to the methylene and methyl protons of the hexadecyl chain.

The ^{13}C-NMR spectrum also is consistent with the proposed structure of the copolymer backbone (Fig 3). The hexadecyl chain exhibits several methylene groups between 33.3 and 21.9 ppm and a methyl group at 13.4 ppm. The signal at 38.5 ppm was assigned to the quaternary carbon atoms of the poly(cyanoacrylate) chain, whereas the methylene carbons bearing the oxygen atoms of the side chains appear at 67.5 ppm. The terminal MeO group of the MePEG chain shows a peak at 58.3 ppm, whereas the methylene presented a large signal at 69.8 ppm.

The FTIR spectrum is consistent with the structure of the expected copolymer (Fig 4). It showed absorption bands related to the C-N stretching vibration at 2167.6 cm^{-1} and the ester carbonyl at 1749.1 cm^{-1}. The C-O stretching of the PEG appeared at 1112.7 cm^{-1}.

Molecular weights and molecular weight distribution of the obtained copolymer were determined by means of GPC (Fig 5). The average molecular weight is 6680. The unimodal mass distribution excluded the presence of poly(MePEGacrylate) or poly(hexadecylacrylate).

Determination of the entrapment efficiency

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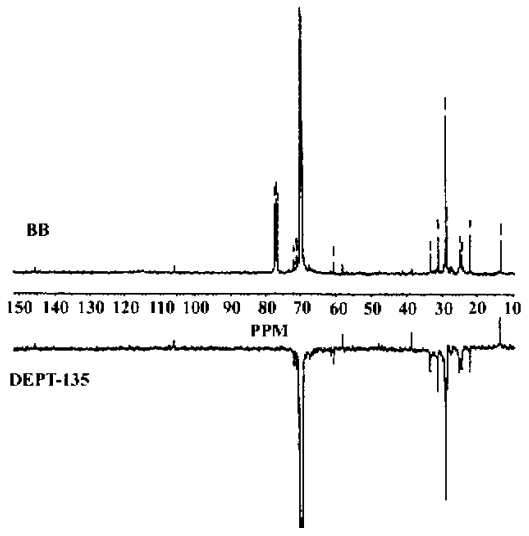

Fig 3. ^{13}C-NMR spectrum of the PEGylated PHDCA copolymer.

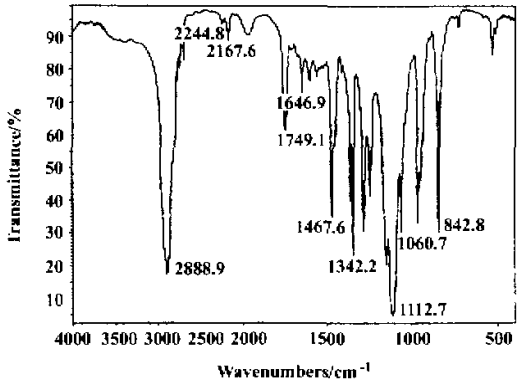

Fig 4. FTIR spectrum of the PEGylated PHDCA copolymer.

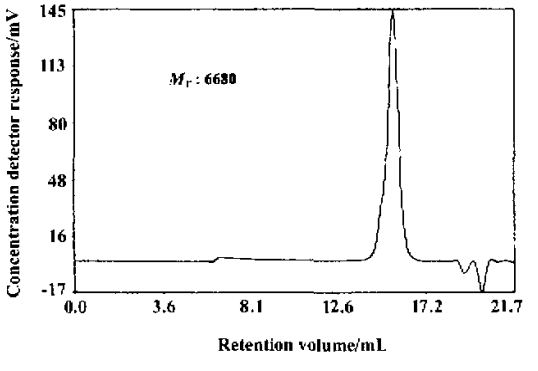

Fig 5. GPC chromatogram of the PEGylated PHDCA copolymer.

Salvicine was determined by HPLC method. There was no endogenous interference at the retention time of salvicine (7.1 min). The minimal detection concentration of salvicine was 5 μg/L (S/N≥3). The calibration for salvicine was linear over the range of 10 – 1000 μg/L. Linear equation: $A = 996.39\ C + 4.35$ ($r = 0.9999$). The average recovery of salvicine was 99.89 %. Relative standard variations (RSD) of both intra- and inter-day were 0.21 % and 0.35 %, respectively. The entrapment efficiency values achieved for salvicine were slightly influenced by the presence of PEG in the PHDCA chain (Tab 1). Compared with PHDCA nanoparticles (98.9 %), PEGylated PHDCA nanoparticles showed a little low encapsulation efficiency (92.6 %).

Tab 1. Physicochemical properties and entrapment efficiency of salvicine PEGylated PHDCA and PHDCA nanoparticles. $n = 3$. $x \pm s$.

Polymer	Size/nm	Zeta-potential /mV	Entrapment efficiency/%
PHDCA	273 ± 9	−23.1 ± 1.4	98.9 ± 1.4
PEGylated PHDCA	259 ± 10	−9.6 ± 1.6	92.6 ± 2.3

Physicochemical characterization of nanoparticles From micrographs, both nanoparticles containing salvicine mainly were spherical in shape (Fig 6). The nanoparticles size was about 250 nm. The values of the Zeta potential were obviously influenced by the composition of the copolymer. Compared with PHDCA nanoparticles (−23.1 mV), PEGylated PHDCA nanoparticles showed a lower surface potential (−9.6 mV) (Tab 1).

***In vitro* release of salvicine** The *in vitro* release profiles of salvicine were obtained by representing the percentage of salvicine release with respect to the amount of salvicine encapsulated (Fig 7). For two nanoparticles, salvicine release both occurred in three phases: (1) an initial burst release, a significant amount of drug was release within 12 h, 13.7 % for PEGylated PHDCA nanoparticles and 21.1 % for PHDCA nanoparticles; (2) after 12 h, salvicine release profiles displayed a plateau for an extended period; (3) a sustained release of the drug over 28 d. The amount of cumulated salvicine release over 28 d were 29.8 % for PEGylated PHDCA nanoparticles and 41.8 % for PHDCA nanoparticles, respectively.

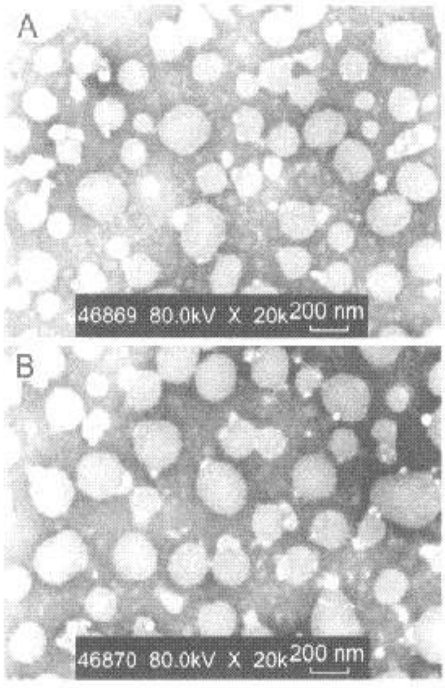

Fig 6. Transmission election microscope photographs of PEGylated PHDCA nanoparticles (A) and PHDCA nanoparticles (B).

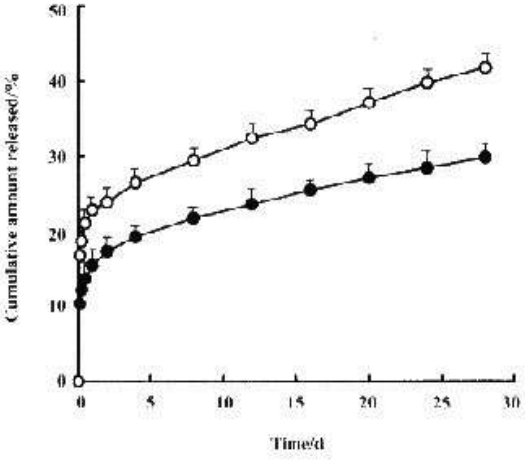

Fig 7. Release profiles of salvicine from PHDCA (○) and PEGylated PHDCA (●) nanoparticles. $n = 3$. $\bar{x} \pm s$.

DISCUSSION

More recently, a more rapidly biodegradable PEG-coated nanoparticles has been prepared by emulsion/polymerization of alkyl cyanoacrylate monomer in an aqueous medium in the presence of PEG, which initiated the polymerization of the cyanoacrylate monomer and chemically coupled to the growing PIBCA[3]. However, it is not suitable to hydrophobic compounds and these drugs that are not unstable in a low pH medium (< 1.5) to obtain nanoparticles with size under 300 nm. Therefore, a PEG-polycyanoacrylate copolymer for the formation of PEG-coated PACA nanoparticles is desirable. In this paper, we synthesized another MePEGcyanoacetate with MePEG ($M_r = 5000$) to increase the proportion of the hydrophilic part in the final copolymer and steric hindrance caused by MePEG on the surface of the nanoparticles to extend half-life of nanoparticles in the systemic circulation. Our results showed that ^{1}H-NMR, ^{13}C-NMR and FTIR were consistent with structure of PEGylated PHDCA, and the unimodal mass distribution of GPC also excluded the presence of poly(MePEGacrylate) or poly(hexadecylacrylate).

Salvicine showed a specific absorption at 260 nm, but its intensity of absorption is weak. HPLC method of determining salvicine was set up in this paper. The results showed no endogenous interference at the retention time of salvicine, and its determination by HPLC was accurate, sensitive, and easy.

The encapsulation efficiency achieved for salvicine was a little difference between PEGylated PHDCA nanoparticles and PHDCA nanoparticles. The difference could come from the presence of PEG in the PHDCA chain, but its mechanism is indistinct. Compared with PHDCA nanoparticles, PEGylated PHDCA nanoparticles showed a marked decrease in the surface charge. This could be related to a shift of the hydrodynamic phase of shear to greater distances from the nanoparticles surface. The same observations have been reported for PLGA-PLA; PEG coated nanoparticles[13]. In fact, these results also are an indication of the presence of the PEG at the surface of the nanoparticles.

For two nanoparticles, salvicine release all showed an initial burst effect. This fast release could be related part salvicine adsorbed onto the wall of the nanoparticles that would be immediately released during the initial stage. After the initial burst, salvicine release profiles displayed a plateau for an extended period, which could result from the only diffusion of the drug dispersed into the polymermatrix. Finally, a sustained release of the drug over 28 d should result from the diffusion of the salvicine through the polymer wall and the erosion of the polymers. However, the amount of cumulated salvicine

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release from two nanoparticles over 28 d was not more than 45 %, this result could be related partly the low solubility of salvicine (~0.15 g/L, 37 ℃). In this paper, a higher and faster salvicine release was observed for PHDCA nanoparticles than those of PEGylated PHDCA nanoparticles within 12 h. The difference should be relation to the presence of PEG in the PHDCA chain. These results showed that the PEGylated PLGA nanoparticles could be an effective carrier for salvicine delivery in the respect of anti-tumor potency. The pharmacokinetics and anti-tumor effect of the stealth nanoparticles is presently under investigation.

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聚乙二醇化聚氰酯毫微粒作为 salvicine 载体：合成、制备和体外特性[1]

李亚平[2,3,4]，周朝辉[3]，裴元英[4]，张贤英[3]，顾宙辉[3]，袁伟芳[3]（[3]中国科学院上海生命科学研究院药物研究所，上海 200031；[4]复旦大学药学院药剂教研室，上海 200032，中国）

关键词 聚乙烯二醇类；聚氰酯；毫微粒；salvicine

目的：合成和鉴定聚乙二醇化聚氰酯共聚物，制备聚乙二醇化聚氰酯共聚物和聚氰酯聚合物的毫微粒，测定二种毫微粒的体外特性。**方法**：用 ^{1}H-NMR，^{13}C-NMR 和 FTIR 测定聚乙二醇化聚氰酯共聚物的结构，用凝胶渗透色谱法测定共聚物的分子量，用乳化/蒸发法制备毫微粒。**结果**：^{1}H-NMR，^{13}C-NMR 和 FTIR 光谱与聚乙二醇化聚氰酯共聚物的结构相符，合成共聚物的分子量是 6680，用 HPLC 测定毫微粒的包封效率时，共聚物对 salvicine 的测定无干扰，聚乙二醇化聚氰酯共聚物毫微粒的包封率是 92.6 %。聚氰酯聚合物的包封效率是 98.9 %。二种毫微粒的粒径均为 250 nm 左右。Zeta 电位值受聚合物结构的影响，与聚氰酯毫微粒比较（-23.1 mV），聚乙二醇化聚氰酯毫微粒显示低的 Zeta 电位值（-9.6 mV）。毫微粒的体外释放显示一个开始的突释效应，然后缓慢释放达 28 天。**结论**：聚乙二醇化聚氰酯毫微粒可能是 salvicine 体内抗肿瘤作用的一个有效载体。

（责任编辑 吕 静）