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The effect of hydrophilic chain length and iRGD on drug delivery from poly(ε-caprolactone)-poly(N-vinylpyrrolidone) nanoparticles

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ABSTRACT

Poly(ε-caprolactone)-b-Poly(N-vinylpyrrolidone) (PCL-b-PVP) copolymers with different PVP block length were synthesized by xanthate-mediated reverse addition fragment transfer polymerization (RAFT) and the xanthate chain transfer agent on chain end was readily translated to hydroxy or aldehyde for conjugating various functional moieties, such as fluorescent dye, biotin hydrazine and tumor homing peptide iRGD. Thus, PCL-PVP nanoparticles were prepared by these functionalized PCL-b-PVP copolymers. Furthermore, paclitaxel-loaded PCL-PVP nanoparticles with satisfactory drug loading content (15%) and encapsulation efficiency (>90%) were obtained and used *in vitro* and *in vivo* antitumor examination. It was demonstrated that the length of PVP block had a significant influence on cytotoxicity, anti-BSA adsorption, circulation time, stealth behavior, biodistribution and antitumor activity for the nanoparticles. iRGD on PCL-PVP nanoparticle surface facilitated the nanoparticles to accumulate in tumor site and enhanced their penetration in tumor tissues, both of which improved the efficacy of paclitaxel-loaded nanoparticles in impeding tumor growth and prolonging the life time of H22 tumor-bearing mice.

1. Introduction

Recently, increasing interest and active effort have been devoted to nanoscale carriers for biomedical use, especially, delivering antitumor agents *in vivo* [1]. Due to the specific "leaky" structure of tumor vasculature, nanocarriers in circulatory system are passively trapped and retained in tumor sites, addressed as enhanced permeation and retention (EPR) effect [2]. To fully utilize EPR effect and diminish opsonization *in vivo*, stealth coating, in most case, poly(ethylene glycol) (PEG), a flexible, hydrophilic, uncharged biocompatible polymer is often used to decorate carriers' surface to gain prolonged plasma residence time for achieving better "passive targeting" [3,4]. Furthermore, well established anionic polymerization of PEG is capable to generate various functional end groups, such as amino, acetal, sugar, azide and alkyne [5–8]. The predominance of functionality makes PEG very suitable to conjugate tumor targeting ligand for further "active targeting". However, with in-depth study and feedback from clinical applications, the defects of PEG have also gradually being found. For example, Hoang et al. revealed that PEG coated nanocarriers preferential accumulate in spleen [9]. Ishida et al. found that plasma concentration and residue time of repeatedly injected PEGylated micelles dramatically reduced once administrating the formulation with several days interval, known as accelerated blood clearance (ABC) phenomenon [10,11]. These may eventually influence injected dose, treatment index, or even lead to side effect and toxicity of patients [12].

An alternative to PEG as surface modifier and stabilizer is the use of water-soluble poly(N-vinylpyrrolidone) (PVP). PVP is a well-known hydrophilic biocompatible polymer and used in many drug delivery systems, such as polymer conjugates and liposomes [13,14]. Leroux et al. pioneeringly investigated poly(N-vinylpyrrolidone)-poly(-D,L-lactide) (PVP-PLA) nanoparticles (usually size > 200 nm), including paclitaxel (PTX) loading, formulation characterization, antitumor effect and plasma protein adsorption ability and found that PTX-loaded nanoparticles showed greater *in vivo* antitumor activity than free drug [15–17]. Torchilin et al. found that PVP modification made liposome long-circulating *in vivo* [13]. More recently, Ishihara et al. reported that PVP-coated nanoparticles did not trigger ABC phenomenon and had reproducible pharmacokinetics and pharmacodynamics profiles [10].

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Our previous work revealed that PVP-coated nanoparticles with 100 nm size had a limited accumulation both in liver and spleen [18].

Although extensively studied, most of PVP polymers are prepared via free radical polymerization and barely bear reactive end groups at both ends (i.e. on α and ω terminals). Thus, it is difficult to label active ligands (folate, peptide, antibody etc.) or radioactive isotopes (^{99m}Tc, ^{131}I, ^{111}In etc.) on the surface of nanoparticles such as PCL-PVP for tumor targeting studies. Recently, the establishment of xanthate-mediated reverse addition fragment transfer (RAFT) method made molecular weight control in PVP synthesis possible [19,20]. Furthermore, xanthate end group on synthesized PVP could be readily modified to thiol, hydroxyl and aldehyde functional groups [14], providing a nice opportunity to functionalize the surface of PVP-based nanocarriers and exploit their *in vivo* behaviors.

It is worth to note that N-end cysteine peptide tumor-homing peptide (iRGD, CRGDK/EGPD/EC) recently have been identified as a highly efficient, deep penetrating peptide [21,22]. It can make the drug deep into extravascular tumor tissue when iRGD is chemically conjugated to or co-administrated with a drug. iRGD also homes to tumors in a tumor-specific and neuropilin-1–dependent manner. Thus, when the polymer nanoparticles are modified or co-administrated with iRGD, the tissues penetrating and targeting abilities of drug-loaded nanoparticles may be improved.

In the present work, PCL-b-PVP block copolymers with different length of PVP blocks were synthesized from PCL bearing xanthate chain transfer agent (CTA) motif based on RAFT process. Then the xanthate end was used to generate hydroxyl or aldehyde end for labeling fluorescent dye, tumor targeting peptide (iRGD) and biotin hydrazine, respectively. These copolymers were next employed to produce PCL-PVP nanoparticles. The propose of this study is to evaluate the influence of hydrophilic PVP chain length and iRGD targeting moiety from PCL-PVP nanoparticles on cytotoxicity, cellular uptake, protein resistance, *in vivo* biodistribution and circulation time. Moreover, *in vivo* antitumor activity and tumor penetration of PTX-loaded PCL-PVP nanoparticles were examined.

2. Materials and methods

2.1. Materials

N-vinypyrrolidone (VP), potassium O-ethyl xanthate, D,L-dithiothreitol (DTT), biotin hydrazine, isobutyl alcohol, sodium cyanoborohydride ($NaCNBH_3$) were obtained from Acros. ε-Caprolactone (ε-CL), NIR-797 isothiocyanate, Rhodamine B isothiocyanate and 2-bromopropionyl bromide were purchased from Sigma–Aldrich. Linear iRGD (CRGDRCPDC) was customized from GL Biochem Ltd. (Shanghai, China). Rat anti-mouse CD31 (BD Pharmingen™). trypblue, Alex-488 streptavidin, donkey anti-rat Alex-594 were obtained from Invitrogen. Paclitaxel was purchased from Meilian Pharm Co., Ltd. (Chongqing, China). Taxol® was purchased from Bristol-Myers Squibb (Princeton, NJ, USA). Murine hepatic H22 cell line was obtained from Shanghai Institute of Cell Biology (Shanghai, China). Male ICR mice (6–8 weeks old and weighing 18–22 g) were purchased from Animal Center of Drum Tower Hospital (Nanjing, China).

2.2. Synthesis and functionalize PCL-b-PVP

As shown in Scheme S1 (See Support information), monohydroxyl poly(ε-caprolactone) (PCL-OH) was first prepared by stannous octotate (0.05% w/w) mediated ring opening polymerization of ε-CL (15 g, 0.13 moL) in the presence of isobutyl alcohol (0.27 mL, 3 mmoL) using a well established method described elsewhere [23]. ^{1}H NMR and GPC equipped static light scattering detector (DMF with 0.03 M LiBr as eluent) were used to characterize the molecular weight of obtained PCL_{5K}–OH (13.8 g, Mn_{NMR} = 4900 Da, Mn_{GPC} = 5120 Da, Mw = 6240 Da, polydispesity (PDI) = 1.22, yield 92%). Thereafter, xanthate chain transfer agent (CTA) was grafted to hydroxyl end group via two steps [20]. Initially, PCL_{5K}–OH (2 g, 0.40 mmoL) was placed in an ice-cold three-neck flask containing 120 μL pyridine and 10 mL anhydrous dichloromethane, 2-bromopropionyl bromide was then added slowly. The mixture was stirred overnight at room temperature (r.t.). After filtered off white precipitates, concentrated residual solvent, precipitated the solution into excess diethyl ether thrice, dried in vacuum overnight, PCL_{5K}–Br (1.8 g, Purity > 95% by ^{1}H NMR, yield 90%) was obtained. Next, PCL_{5K}–Br (2 g, 0.40 mmoL) was dissolved in 15 mL anhydrous dichloromethane and 1 mL pyridine in a three-neck flask. Potassium O-ethyl xanthate (0.20 g, 1.25×10^{-3} moL) was added portion wise. After 16 h stirring at r.t. and separated with the same process as PCL_{5K}–Br mentioned above, PCL_{5K}–CTA was successfully produced (1.84 g, purity 70%, yield 92%). (See Support information Figure S1). Unreacted potassium O-exthyl xanthate was removed via a chromatography of silica gel using chloroform as eluent.

PCL_{5K}–CTA (500 mg, 0.1 mmoL), AIBN (1 mg, 6×10^{-3} mmoL) and fresh distilled VP (8 mL, 75 mmoL) monomer were placed in three Schlenk flasks and degassed via three freeze-pump-thaw cycles. These three flasks were immersed in a 60 °C oil batch and stopped at 22 h, 36 h and 60 h, respectively, precipitated via aforementioned method. Three batches of PCL_{5K}-b-PVP copolymers, noted as PCL_{5K}-b-PVP_{3K} (Mn_{PVP} = 3 k Da), PCL_{5K}-b-PVP_{6K} (Mn_{PVP} = 6 k Da) and PCL_{5K}-b-PVP_{12K} (Mn_{PVP} = 12 k Da), were prepared according to the integral ratio of ^{1}H NMR (DPX 300M, Bruker, Germany) assignments of VP ring (-CH_2–CO–, 3.1–3.2 ppm, b) and CL segment (–$COOCH_2$–, 4.1 ppm, t). In addition, successful chain extension of PVP from PCL_{5K}-CTA was confirmed through ^{1}H NMR diffusion order spectroscopy (DOSY) (see Support Information Figure S2). Continuously, O-ethyl xanthate moiety on PCL_{5K}-b-PVP-CTA quantitatively transformed to hydroxyl (PCL_{5K}-PVP-OH) and aldehyde (PCL_{5K}-PVP-CHO) groups, respectively, according to previous work [14]. Then, PCL_{5K}-b-PVP_{3K}–OH (50 mg, 6.25×10^{-3} mmoL) was reacted with NIR-797 isothiocyanate (1.4 mg, 1.59×10^{-3} mmoL) in anhydrous DMF containing several drops of triethylamine. The reaction proceeded for 24 h. The produced mixture was precipitated into diethyl ether and the precipitate was dried for use later. About 20% of copolymers were labeled with NIR-797 (PCL_{5K}-b-PVP_{3K}-NIR797). Similarly, the PCL-b-PVP copolymer was labeled with rhodamine-B isothiocyanate in same manner (PCL_{5K}-b-PVP_{3K}-Rhod).

2.3. Preparation of PCL-b-PVP nanoparticles

PCL-PVP nanoparticles were prepared by established method described in our previous work with minor modification [18]. Briefly, 20 mg of copolymer was dissolved in 0.4 mL of mixed solvent and then dropwise dispersed the homogeneous solution in distilled water to generate nanoparticles. The ratio of acetone to ethanol in mixed solvent was 2:1, 1:1 and 2:3 corresponding to PCL_{5K}-b-PVP_{3K}, PCL_{5K}-b-PVP_{6K} and PCL_{5K}-b-PVP_{12K}, respectively, leading to roughly comparable particle size from 40 nm to 54 nm, and the nanoparticle was named as NP3K, NP6K, NP12K, respectively. The purification of prepared nanoparticles included vacuum evaporated acetone and ethanol under reduced pressure, dialyzed and passing through a 100 nm single-pore membrane (Whatman, UK) to separate the aggregates and bacteria in the solution using a syringe pump (Longer Precision Pump, Hebei, China) at speed of 0.1 mL/h.

NIR797- and rhodamine B-labeled nanoparticles were prepared by the process described above using mixed PCL_{5K}-b-PVP-NIR797 and PCL_{5K}-b-PVP, and mixed PCL_{5K}-b-PVP-Rhod and PCL_{5K}-b-PVP, respectively.

Biotin-conjugated nanoparticles were obtained by following: For example, PCL_{5K}-PVP_{3K} nanoparticles (8 mg/mL) were first prepared by PCL_{5K}-b-PVP_{3K}-CHO (80 mg, 0.01 mmoL) copolymer following aforementioned procedure in PBS (pH 7.4). Then, the nanoparticle solution were used to conjugate biotin hydrazine (1 mg, 3.87×10^{-3} mmoL) with stirring at r.t.. 1 h later, 0.3 mL of 0.13 mM aqueous solution of $NaCNBH_3$ was added to reduce the Schiff base generated, and the solution kept stirring for 140 h. Finally, the reacted nanoparticles were purified by dialyzed against distilled water extensively, freeze-dried. The obtained product was marked as NP3K-Biotin.

iRGD-conjugated nanoparticles were prepared though a thiazolidine ring [22]. Briefly, PCL-PVP nanoparticles (80 mg, 0.010 mmoL, 8 mg/mL) prepared by PCL_{5K}-b-PVP_{3K}-CHO were dispersed in PBS, DTT (3.3 mg, 0.021 mmoL) and linear iRGD (CRGDRCPDC) (9.5 mg, 0.010 mmoL) were added, stirred at r.t. for 5 days. Then reacted nanoparticles were purified by dialyzed sequentially against PBS (pH 7.4) and distilled water, and freeze-dried. The obtain product was denoted as NP3K-iRGD.

To reduce the labeled ligands' influence on the basic properties of nanoparticles, each conjugated moiety (NIR797, iRGD, Rhodamine B and Biotin) would be around 10% of surface chain ends.

2.4. Preparation and characterization of PTX-loaded PCL-b-PVP nanoparticles

PTX-loaded PCL-PVP nanoparticles were prepared using well performed method developed by our group [18]. Briefly, 20 mg of copolymer and 5 mg of PTX were dissolved in 0.4 mL of acetone and ethanol co-solvent to form a homogeneous solution. Then, the solution was dropwise dispersed in distilled water to form the drug-loaded nanoparticles. The remaining organic solvents were removed by rotary vacuum evaporation. The obtained PTX-loaded PCL-PVP nanoparticles were filtrated through 0.22 μm sized cellulose acetate filter membrane to remove non-incorporated drug crystals as well as copolymer aggregates. Finally, the drug-loaded nanoparticles were lyophilized for use later. The drug-loaded nanoparticles had a satisfied drug loading content (D.L.) (about 15%) and encapsulation efficiency (E.E.) (more than 90%) (See Support Information Table S1).

The purified nanoparticles were used to as the samples for both dynamic light scattering (DLS) and static light scattering (SLS) measurements with a Brookheaven