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Research paper

Injectable poly(organophosphazene)–camptothecin conjugate hydrogels: Synthesis, characterization, and antitumor activities

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ABSTRACT

The objective of this study is to develop an effective polymer therapeutics involving camptothecin (CPT) with enhanced efficacy and lessened systemic side-toxicity for cancer treatment. Polymer–CPT conjugates (PCCs), which consisted of CPT–20-glycinate and poly(organophosphazene) bearing carboxylic acid, were synthesized, characterized for physicochemical properties, *in vitro* degradation and CPT release behaviors from the PCC, and evaluated their anticancer activity. The aqueous solutions of all these PCCs showed a thermo-responsive sol–gel transition behavior for injectable application near room temperature. The CPT incorporated into the hydrogel was proven to be stable *in vitro* over 15 days. The *in vitro* cytotoxicity of the PCC was verified to be effective against four kinds of human cancer cell lines. The *in vivo* anticancer activity study with HT-29 colon cancer cell xenografted mice showed that the intratumorally injected PCC hydrogel inhibited the tumor growth more effectively relative to CPT alone (−29% vs. 130% in tumor size).

1. Introduction

Polymer therapeutics is a comprehensive term to describe a curative means using polymers such as polymeric drugs or sequestrants, polymer–protein conjugates, polyplexes (polyelectrolyte complex) consisting of a polymer–DNA complex, polymer–drug conjugates (PDCs), and polymeric micelles containing a drug [1]. Since the first notable paper about a PDC was reported by Ringsdorf in 1975 [2], many research groups around the world have studied and developed this novel technique in the biomedical area for clinical and commercial purposes [1,3–5]. Especially, many efforts have been concentrated on polymer therapeutics for cancer therapy due to its unique merits including improved pharmacokinetics, enhanced efficacy, and decreased toxicity relative to a small-molecule drug [6]. Among the polymer therapeutics, PDCs have been researched focusing on four polymers such as HPMA copolymer, poly-L-glutamic acid, PEG, and dextran [6], because these polymers were known to be bio-inert or biocompatible and water-soluble. The advantages of PDC, such as improved drug solubility, extended circulation time depending on the polymer carrier, and drug targeting (passive or active), have made it an attractive polymer therapeutics [3].

During the past 5 years, our group has developed the locally injectable and biodegradable polyphosphazene–anticancer drug conjugates for the purpose of the intratumoral injection [7,8]. The aqueous PDC solutions maintained their sol-states at ambient temperature making them injectable, while they could be transformed to the gel-states at body temperature leading them to be useful as drug-depots. After they exhausted the involved drugs, the formulations were disappeared thus not requiring removal by surgical treatment. It was reported that the injected poly(organophosphazene)–doxorubicin (DOX) conjugate hydrogel showed a better inhibition effect against SNU-601 xenografted gastric cancer compared to a DOX solution during the experimental period without a noticeable toxicity [8]. Additionally, poly(organophosphazene)-paclitaxel (PTX) conjugate hydrogel exhibited much longer and more effective *in vivo* antitumor activity than a PTX solution alone against HSC-45M2 xenografted human gastric cancer over a month [7]. The significantly improved antitumor activities of these injectable hydrogels were due to the localization into the tumor tissue with little systemic circulation, sustained release without initial burst effect, and non-toxic degradation products, which were proved by release test and imaging systems [7,8]. Thus, it would be expected that the drug formulation can be locally injectable to lessen systemic circulation,

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need not be removed after its use, be targeted via a passive targeting (the enhanced permeation and retention effect), and circumvent multidrug resistance as well as the previously mentioned merits [3] by using the thermosensitive biodegradable polyphosphazene hydrogels as drug carriers or polymer backbones in the PDC.

Camptothecin (CPT), a pentacyclic quinolone alkaloid, is an antitumor antibiotic extracted from a tree called *Camptotheca acuminate* in 1958 [9], and it is one of the four drugs (doxorubicin, camptothecin, paclitaxel, and platinate) that have been used frequently to develop new polymer therapeutics [6]. Its anticancer mechanism can be explained as a type I DNA topoisomerase (topo) inhibitor. The positive results of CPT in preclinical testing have led to ongoing clinical trials [6]. However, the two main reported obstacles: poor water solubility causing low bioavailability and an unstable active lactone form that could be transformed to inactive hydrolyzed carboxylate form under basic conditions making it toxic, have limited the clinical usage of CPT [6,9,10]. The *in vivo* antitumor effect of several formulations for the CPTs has been extensively reviewed by Venditto recently [10]. The reviewed formulations for which the development objectives have been to overcome the problems of CPT ranged from CPT modification to macromolecular therapeutics [10].

The purpose of this study is to develop the locally injectable and biodegradable polyphosphazene–CPT conjugate hydrogel for cancer therapy. To do this, we synthesized several sets of the polymer–CPT conjugates and characterized their physicochemical properties. We introduced glycine as a linker between CPT and polyphosphazene to preserve the stability of CPT [11] and to endow the system with pH-dependent drug release behavior. We checked the viscosity of the conjugate solution as a function of temperature to see its local injectability. *In vitro* release tests of CPT conjugates were accomplished at physiological pH (7.4) and 37 °C. To verify the efficacy of the developed therapeutics, *in vitro* and *in vivo* antitumor effects of the conjugate hydrogel were studied using a sulforhodamine B (SRB) assay and HT-29 xenografted animal model, respectively.

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene obtained from Aldrich (Milwaukee, WI, USA) was purified by sublimation at 55 °C under reduced pressure (about 0.1 mm Hg). Poly(dichlorophosphazene), L-isoleucine ethyl ester hydrochloride (IleOEt·HCl), glycylglycine allyl ester trifluoroacetic acid salt (GlyGlyOAll·TFA), and α-amino-ω-methoxy-poly(ethylene glycol)s (AMPEG)s having a number average molecular weight of 550 (M_n = 550) were prepared according to the literature, respectively [12–15]. N,N′-Dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIPC), 4-dimethylaminopyridine (DMAP), anhydrous methylene chloride (MC), and isobutyl chloroformate (IBCF) were obtained from Aldrich. +(−)Camptothecin (98%) was purchased from Acros (New Jersey, USA) and used without further purification. Tetrahydrofuran (THF) and triethylamine (TEA) were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan) and purified under a dry nitrogen atmosphere by distilling and refluxing over sodium metal (Aldrich)/benzophenone (Acros) and barium oxide (Acros), respectively. A549 (human lung carcinoma), DLD-1 (human colon adenocarcinoma), HCT 116 (human colon carcinoma), and HT-29 (human colon adenocarcinoma) cells were purchased from the Korean Cell Line Bank (KCLB) and cultured in RPMI 1640 (90%) with L-glutamine (300 mg/L), 25 mM HEPES, 25 mM $NaHCO_3$ and heat inactivated fetal bovine serum (FBS, 10%) at 37 °C under a humidified atmosphere containing 5% CO_2.

2.2. Synthesis of poly(organophosphazene) containing acid group (acid polymer) (I)

An acid polymer was synthesized following our previous work [7,8,12]. In brief, the prepared substitutes, IleOEt·HCl, GlyGlyO-All·TFA, and AMPEG 550, were stepwise added into the poly(dichlorophosphazene) dissolved in THF through the nucleophilic substitution process. The acid polymer was prepared by the allyl ester cleavage reaction of the obtained polymer using 0.20–0.25 equivalent of tetrakis (triphenylphosphine)palladium(0) ($(Ph_3P)_4Pd(0)$) and 20 equivalent of morpholine. The detailed ^{1}H NMR analysis of Acid Polymer 1 and 2 can be found in the Supporting Information.

2.3. Synthesis of camptothecin–20-glycinate TFA salt (Gly–CPT·TFA) (II)

Gly–CPT·TFA salt was prepared in two steps as reported by Greenwald and its structure was confirmed by ^{1}H NMR [16]. Briefly, the calculated amount of DIPC (3 equiv.), DMAP (2 equiv.), and CPT (1 equiv.) was added into *t*-Boc-glycine (3 equiv.) dissolved in anhydrous MC at 0 °C and warmed to room temperature with stirring overnight. After washing the reactant solution with 1.0 N HCl aqueous solution, MC was removed under a vacuum. The product, CPT–20-ester of *t*-Boc-glycine, was obtained by recrystallization from methanol (MeOH). Gly–CPT·TFA was prepared by reaction between TFA and CPT–20-ester of *t*-Boc–glycine in MC at room temperature for 1 h. ^{1}H NMR (DMSO-d_6), δ (ppm): 1.0 (t), 1.6 (d), 2.2 (m), 4.4 (m), 5.4 (s), 5.6 (s), 7.2 (s), 7.7–8.8 (m).

2.4. Preparation of poly(organophosphazene)-CPT conjugates (III) (Scheme 1)

Before synthesis, the obtained acid polymer (0.4 g) and Gly–CPT·TFA were dried *in vacuo* at 40–50 °C for 2 days. Into the reaction flask containing the acid polymer, anhydrous THF (10 mL) was added and stirred until dissolving the polymer at room temperature. Dried TEA (29 μL) was added into the polymer solution. To activate the acid groups of the polymer, the calculated amount of IBCF (13 μL) was injected into the reaction solution and the reactants mixture was incubated with stirring for 30 min at 0 °C. And then, THF solution (3 mL) containing Gly–CPT·TFA (43 mg) after desalting with TEA (0.1 mL) was added into the activated polymer solution drop by drop. The reaction was progressed at 0–4 °C for 30 min and at room temperature for 5 h. The product was precipitated into the reaction solution by adding KF solution (2.0 M). The obtained precipitate was dissolved in MeOH and dialyzed against both MeOH and distilled water using dialysis membrane (MWCO: 12–14 kDa) for 4 days. The solution was filtrated through a 0.45 μm syringe filter and lyophilized. The final product was stored at −20 °C before characterization and experiment. The detailed ^{1}H NMR analysis of poly(organophosphazene)-CPT conjugates can be found in the Supporting Information.

2.5. Characterization of substituents and polymer–CPT conjugates (PCCs)

^{1}H NMR was measured with a Varian Gemini-300 spectrometer operating at 300 MHz in the Fourier transform mode using $CDCl_3$ or DMSO-d_6 and tetramethylsilane (TMS) as solvents and an internal reference, respectively. Proton-decoupled ^{31}P NMR spectra were obtained with the same spectrometer operating at 121.4 MHz using triphenyl phosphate as an external standard. A high performance liquid chromatography system (HPLC, Agilent 1200 series, USA) was used to indirectly prove chemical conjugation of PCCs and