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Chemically crosslinkable thermosensitive polyphosphazene gels as injectable materials for biomedical applications

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ABSTRACT

Chemically crosslinkable and thermosensitive poly(organophosphazenes) containing multiple thiol (–SH) groups along with hydrophobic isoleucine ethyl ester and hydrophilic α-amino-ω-methoxy-poly(ethylene glycol) of the molecular weight 550 have been synthesized and characterized as an injectable biomaterial. The aqueous solutions of these polymers were transformed into hydrogel with desired gel strength at body temperature via hydrophobic interactions, and the gel strength was further improved by the cross-linking of thiol groups with crosslinkers, divinyl sulfone (VS) and PEG divinyl sulfone (PEGVS) under physiological conditions. The kinetics of cross-linking behavior of polymer thiol groups with crosslinkers was studied in both *in vitro* and *in vivo* conditions. Field Emission-Scanning Electron Microscopy (FE-SEM), swelling experiments, and rheology study of present polymers revealed that the inner three-dimensional hydrogel networks depended on the degree of thiol units in the polymer network. From the *in vivo* (*in mice*) degradation studies, the dual cross-linked gels showed to have a controlled degradation. These results demonstrate that the inner network of the hydrogels can be tuned, gel strength and degradation rate can be controlled, and the chemically crosslinkable and thermosensitive poly(organophosphazenes) hold promises for uses as injectable systems for biomedical applications including tissue engineering and protein delivery.

1. Introduction

Injectable and in situ-forming hydrogels have potential for biomedical applications. Easy administration, tunable three-dimensional network and minimally invasive procedures [1,2] used for their localization into the body have made them more attractive in the field of drug delivery and tissue engineering [3]. Additionally, the in-situ forming biomaterials at gentle chemical and physical conditions are ideal for the delivery of protein or DNA, as well as living cells.

Injectable hydrogels can be obtained by different methods and they can be classified into two categories; physical cross-linking and chemical cross-linking. Physically cross-linked gels can be obtained due to hydrophobic interactions [2,4], ionic interactions [5] or by stereocomplexation [6,7]. Thermosensitive gels obtained by a physical cross-linking through hydrophobic interactions in polymer chains have been found for wide applications in the design of tissue engineered grafts and delivery of bioactive compounds due to their convenient administration and gelation under physiological conditions. However, the physical nature of thermal gelation limits their potency in a number of tissue engineering and drug delivery applications due to the limited control of gelation kinetics and material properties [8]. Weak mechanical gel strength of these polymers results in faster dissolution after gelation in a bioenvironment and the disruption of the hydrogel network may give rise to changes in the external environment such as temperature, pH, and ionic strength.

In contrast to the physical cross-linking, chemically cross-linked hydrogels obtained through a photocross-linking [9,10], Michael-type reactions [11,12], and enzymatic cross-linking [13,14] have been showed desired mechanical strength, stability and better control over the gelation kinetics. These properties subsequently impact polymer diffusivity and permeability, degradation rate, equilibrium of water content, elasticity, and modulus [15], providing materials that can be tailored to more closely mimic the mechanical properties of native tissues. However, the photocross-linking often requires a photoinitiator. Prolonged irradiation time, incomplete double bond conversion and heat production have limited for their uses [16]. Cross-linked gels can be obtained through Michael-type addition reactions between the thiols and acrylates or vinylsulfones. These gels may have application potentials for protein delivery and tissue engineering because of their gel strength and formation under mild conditions. However, their gelation reaction leading to

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a three-dimensional network is rather slow and limits their practical uses as injectable systems [17].

Recently research colleagues have focused on the development of injectable hydrogels based on physical and chemical methods to overcome the limitations associated with the individual methods. The hydrogels made by dual methods will play a major role as drug carriers in local delivery, and be controlled over the gelation kinetics, and the pore architecture of the gel network makes them attractive in tissue engineering applications. Until now, only a few thermosensitive and chemically crosslinkable polymers have been reported including crosslinkable PEO–PPO–PEO [18,19], Poly(NIPAAm-*co*-cysteamine) [20], Poly(NIPAAm-*co*-HEMA-acrylate) [21] and modified Pluronics [22]. Though those systems address some of the listed problems above, they still have the limitations of their own polymers, including the use of high concentration of the polymer to obtain thermogelation, non-degradability and/or cytotoxicity associated with the NIPAAm. If the thermogelation is able to be achieved by lowering polymer concentration, there will be several possible advantages. One is that it will require less amount of the crosslinker to chemically crosslink with the polymer system. The other is that the small amount is better than high amount. Even though it is relatively small decrease, it may be remarkable for the biomedical application.

In our previous work, we have developed various biodegradable and thermosensitive polyphosphazene derivatives [23–26] by balanced substitutions of hydrophilic, hydrophobic and other functional substituents onto the polymer backbone. The polyphosphazenes with well balanced side groups have many inherent advantages in the biomedical field, which include good biocompatibility and formation of non-toxic degradation products upon degradation. The degradation products are mainly phosphate, ammonia, and their side groups [27,28].

Most of the synthesized poly(organophosphazene) derivatives exhibited a temperature dependant sol–gel transition in aqueous solutions [23–26]. However, we observed that the gel strength exhibited by these thermosensitive gels was not enough in controlling the sustained release of loaded drugs in some cases [29]. Development of an in situ-forming biodegradable hydrogel with desired mechanical strength containing three-dimensional networks with low polymer concentration under mild conditions is necessary to overcome the above problems.

In this study we have focused on the design and the development of thiol-based thermosensitive, crosslinkable, and biodegradable polymers to address the limitations associated with the specified systems above. The physical properties of hydrogels were studied in terms of their viscosity, swelling ratio, and FE-SEM. The increase in the mechanical strength of the hydrogel network after chemical cross-linking was interpreted and discussed by the degree of swelling and storage modulus of gels. The gel degradation behavior under *in vivo* conditions was monitored by FE-SEM, and with fluoresceinamine tagged polymer.

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene was acquired from Aldrich (Milwaukee, WI, USA) and purified by sublimation at 55 °C under vacuum (about 0.1 mmHg). Poly(dichlorophosphazene) was prepared as described previously [30]. L-isoleucine ethyl ester (IleOEt) was prepared by the literature method [31]. α-Amino-ω-methoxy-poly(ethylene glycol) with molecular weight of 550 (AMPEG550) was prepared by the published method [32]. 2-cystamine dihydrochloride was obtained from Lancaster. Vinyl sulfone (VS) and sodium hydride (NaH) was obtained from Aldrich. 5,5′-Dithiobis (2-nitro-benzoicacid) was obtained from Sigma. Dithiothreitol was obtained from Duchefa Biochemie (Netherland). Polyethylene glycol of molecular weight 3350 was acquired from SigmaUltra. Tetrahydrofuran (THF) was dried by reflux over sodium metal and distilled under nitrogen atmosphere. All animal studies were conducted according to the ethical guidelines of the Institutional Animal Care and Use Committee of the Korea Institute of Science and Technology (KIST).

2.2. Preparation of cystamine free base

Cystamine dihydrochloride (30 g, 0.14 mol) was dissolved in distilled water (50 ml), 150 ml of chloroform was added. The mixture was cooled to −5 °C using ice bath (ice and NaCl). An ice cooled 40% aqueous NaOH solution (40 g, 1 mM) was slowly added to the above mixture drop by drop not exceeding the temperature 0 °C under continuous stirring and the mixture was allowed to stir for 15 min at same temperature. The organic layer was separated; the residue was additionally extracted with a 100 ml of chloroform. The combined organic layers were passed through $MgSO_4$ bed, evaporated to dryness at 30 °C and used immediately after preparation. The product slowly decomposes at room temperature. Characterization data: ^{1}H NMR ($CDCl_3$): d = 1.24(br, 4H), 2.69(m, 4H), 2.94(m, 4H).

2.3. Synthesis of PEG vinyl sulfone (PEGVS)

PEGVS was synthesized by coupling PEG–OH with an excess of divinyl sulfone by a published method [33]. Briefly, PEG–OH (MW 3350, 5 g) was dissolved in distilled tetrahydrofuran (80 ml) under inert atmosphere and refluxed in a Soxhlet apparatus filled with molecular sieves for 4–5 h. The solution was allowed to cool to 25 °C and sodium hydride (0.35 g), at 5-fold molar excess over OH groups, was added followed by the addition of divinyl sulfone (10.57 g), a 30-fold molar excess over OH groups. The reaction mixture was stirred for 3 days under argon atmosphere at room temperature. Afterwards, the reaction solution was neutralized with acetic acid, filtered, concentrated and precipitated in ice-cold diethyl ether. Precipitation was repeated three times to remove unreacted divinyl sulfone. The final product was dried under vacuum and stored under argon at −20 °C. Derivativeness was confirmed with ^{1}H NMR ($CDCl_3$): 3.6 ppm (PEG backbone), 6.1 ppm (d, 1H, $=CH_2$), 6.4 ppm (d, 1H, $=CH_2$), 6.8 ppm (dd, 1H, $-SO_2CH=$), 3.24 ppm (t-$SO_2CH_2CH_2$–O–) and 3.90 ppm (t-$SO_2CH_2CH_2$–O–). The degree of end group conversion was found to be 98% as calculated by proton NMR. Gel permeation chromatography was used to confirm that the starting material (PEG–OH) and the end-functionalized PEGVS had identical molecular weight distributions.

2.4. Synthesis of cystamine terminated polyphosphazenes

All reactions were carried out under an atmosphere of dry nitrogen using the standard Schlenk-line techniques. Synthesis of $[NP(IleOEt)_{0.87}(AMPEG550)_{0.56}(Cystamine)_{0.57}]_n$ (**A**). Polymer **A** (**IV**) was synthesized as elucidated below. L-isoleucine ethyl ester hydrochloride (6.00 g, 30.6 mM) suspended in dry THF (100 ml) containing triethylamine (12.93 ml, 92 mM) was added slowly to poly (dichlorophosphazene) (4.0 ml, 34.48 mM) dissolved in dry THF (100 ml). The reaction mixture was stirred for 8 h at −60 °C and then for 48 h at room temperature. AMPEG550 (7.96 g, 14.48 mM) dissolved in dry THF (50 ml) containing triethylamine (6.10 ml, 44 mM) was added to the above reaction mixture, stirred for 1 day at room temperature, 1 day at 45 °C. The above reaction mixture was added to freshly prepared cystamine (10.85 ml, 71.37 mM an excess of 3 equivalents) dissolved in dry chloroform containing triethylamine (10.85 ml, 77.23 mM) at 0 °C. The reaction mixture was stirred for 1 day at room temperature, 1 day at 45 °C and finally the reaction mixture was filtered. The filtrate was concentrated and poured into n-hexane to obtain a precipitate, which was reprecipitated twice in the same solvent system. The polymer product was further purified by dialysis in methanol for 4 days and then in distilled water at 4 °C for 4 days. The dialyzed solution was freeze-dried to obtain polymer **A** (**IV**). Yield: 81%. The other listed polymers were prepared in similar way as shown in Fig. 1(**I–IV**) by using different feed ratios of substituents.

2.4.1. $[NP(IleOEt)_{0.87}(AMPEG550)_{0.56}(Cystamine)_{0.57}]$ (**A**)(**IV**)

^{31}P NMR ($CDCl_3$), δ (ppm): 19.8. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.1 (s, 6H, $-CH_3$ and CH_2CH_3 of IleOEt), 1.2–1.4 (b, 3H, $-OCH_2CH_3$ of IleOEt), 1.4–1.6 (b, 2H, $-CH_2CH_3$ of IleOEt), 1.6–1.9 (b, 1H, $-CH(CH_3)CH_2CH_3$ of IleOEt), 2.8–2.9 (b, 4H, $-(SCH_2CH_2)_2$– of cystamine), 2.9–3.3 (b, 6H, $-(SCH_2CH_2)_2$– of cystamine and $-NHCH_2CH_2O$– of AMPEG), 3.4 (s, 3H, $-CH_3$ of AMPEG), 3.5–4.0 (b, 44H, $-(CH_2CH_2O)_{11}$– of AMPEG), 4.0–4.2 (b, 1H, –NHCH– of IleOEt), 4.2–4.4 (b,2H, $-OCH_2CH_3$ of IleOEt).

2.4.2. $[NP(IleOEt)_{0.82}(AMPEG550)_{0.57}(Cystamine)_{61}]$ (**B**) (**IV**)

Poly(dichlorophosphazene) (4 g, 34.48 mM), IleOEt (5.73 g, 29.31 mM), AMPEG550 (7.77 g, 14.13 mM), cystamine (11.63 g, 76.5 mM). Yield: 77%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.6. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.1 (s, 6H), 1.2–1.4 (b, 3H), 1.4–1.6 (b, 2H), 1.6–1.9 (b, 1H), 2.8–2.9 (b, 4H), 2.9–3.3 (b, 6H), 3.4 (s, 3H), 3.5–4.0 (b, 44H), 4.0–4.2 (b, 1H), 4.2–4.3 (b, 2H).

2.4.3. $[NP(IleOEt)_{0.76}(AMPEG550)_{0.53}(Cystamine)_{0.71}]$ (**C**) (**IV**)

Poly(dichlorophosphazene) (4 g, 34.48 mM), IleOEt (5.39 g, 27.58 mM), AMPEG550 (7.68 g, 13.96 mM), cystamine (12.5 g, 82.2 mM). Yield: 79%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.9. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.1 (s, 6H), 1.2–1.4 (b, 3H), 1.4–1.6 (b, 2H), 1.6–1.9 (b, 1H), 2.8–2.9 (b, 4H), 2.9–3.3 (b, 6H), 3.4 (s, 3H), 3.5–4.0 (b, 44H), 4.0–4.2 (b, 1H), 4.2–4.4(b, 2H).