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# Injectable, dual cross-linkable polyphosphazene blend hydrogels

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## ABSTRACT

A new class of injectable, self cross-linkable, and thermosensitive polyphosphazene-based blending systems of functional thiolated and acrylated polymers was designed and synthesized to develop an ideal injectable carrier, and to overcome many barriers associated with developing the injectable carriers, such as the uses of monomeric crosslinkers, catalysts, oxidants, pH adjustments, initiators, UV light, heat production and organic solvent. The aqueous solutions of the polymer blends were exhibited a solution state at low temperature, and transformed into a hydrogel state with desired mechanical strength at body temperature via thermosensitive hydrophobic interactions. The mechanical strength was further improved by the cross-linking of thiol groups with acrylate groups in the polymer network under physiological conditions. The thermoresponsive hydrophobic interactions in the polymer network accelerated the chemical cross-linking to improve the mechanical property. The mechanical strength, inner three-dimensional network, and degradation rate can be tuned through the degree of cross-linking between the thermosensitive and functional blended polymers. The results suggest that the self cross-linkable thermosensitive polyphosphazene blend systems have great potentials to play a crucial role as an injectable carrier because of their improved suitable mechanical properties for application potentials, in addition to their inherent advantages such as injectable, biodegradable and thermosensitive properties.

## 1. Introduction

Hydrogels are exceptional materials with a variety of biomedical applications [1–4]. In particular, injectable hydrogels have attracted much attention recently in the field of injectable carriers because of easy administration, minimally invasive procedures [5] used for their site specific introduction and patient convenience. Especially, injectable thermosensitive gels obtained by hydrophobic interactions are extensively studied in the fields of biomedical applications because of their three-dimensional (3D) network formation under mild conditions [6–10]. However, one of the main limitations using those gels to engineer tissue like networking systems *in vivo* is their weak mechanical properties [11,12]. The scientists are focusing on the development of injectable hydrogel with desired mechanical properties under physiological conditions by utilizing the self assembled dual cross-linking systems such as physical and chemical cross-linking systems.

Until now, only a few dual cross-linking systems have been come into a view [13–15]. These systems are based on the cross-linking of the thermosensitive and chemically reactive gels, which are functional polymers and monomers. Though those systems are suitable candidates as an injectable carrier material, the use of the monomers (hydrophilic or hydrophobic) to develop the chemical cross-linking system may disturb the thermosensitive property of the entire system after the typical mixing. This result may lead to the collapse of the thermosensitivity of the thermoresponsive and chemically cross-linkable system, and result in leaching out of the monomer cross-linker before getting cross-linking with the polymer network.

On the other hand to the thermosensitive and chemically cross-linkable gels, the dual cross-linking systems based on the thermosensitive and photopolymerizable gels have been reported recently [16]. Though these dual cross-linkable systems can give the desired mechanical property to the hydrogels, the uses of many barriers such as monomeric crosslinkers [17], catalysts [18], oxidants [19], pH adjustments [20], initiators [21], UV light [22], heat production [23] and organic solvent, limit their potentials in clinical applications.

In the previous work, we have developed physically and chemically cross-linked polyphosphazene derivatives [24] for improving the mechanical properties of the hydrogels. The reported system utilizes monomeric crosslinkers to develop the dual cross-linking in the polymer network. Though the system

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addresses some of the limitations in improving mechanical properties of the hydrogels, the use of the monomeric crosslinkers limits their potentials in various clinical applications. The use of the monomeric crosslinkers may disturb the thermosensitivity of the polymeric systems. Furthermore, the molecular weight of the crosslinkers may also affect the intended applications. The use of the crosslinkers with low molecular weight may cause leaching problems if it is not properly cross-linked with the polymeric system on time. The use of the crosslinkers with high molecular weight may cause a viscous nature and leads to the difficulty in delivery through a needle.

Herein, we report a new strategy for the *in situ* preparation of injectable hydrogels with tunable physical and chemical properties under physiological conditions by blending of two chemically cross-linkable and thermoresponsive polymeric systems (acrylated and thiolated). The bioactive components can be mixed with the polymer blend around room temperature (25 °C), and then can be injected into the target site. The instantaneous thermogelation property of the blend makes the loaded bioactive components holding in the target site, and then further chemical cross-linking in the blend system improves the mechanical properties, which may play a vital role in maintaining the hydrogel integrity in the *in vivo* environment.

In this study, self cross-linkable, biodegradable, injectable, and thermosensitive polymer blending systems were designed and synthesized to overcome many barriers such as the use of monomeric crosslinkers, initiators, UV light, heat production, and organic solvent. We have tried to create ideal injectable carriers that hold a promise in many biomedical applications. The physical properties of the hydrogels before and after blending were evaluated by viscosity, swelling ratio, FE-SEM and storage modulus. *In vivo* gel degradation behavior was monitored by FE-SEM and fluoresceinamine tagged polymer. An *in vivo* mass loss study of gel was carried out as well. The effect of thermogelation property on the chemical cross-linking process between the polymers in the polymer blend was investigated both *in vitro* and *in vivo*.

## 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 [25]. L-isoleucine ethyl ester (IleOEt) was prepared by the literature method [26]. α-Amino-ω-methoxy-poly (ethylene glycol) with molecular weight of 550 (AMPEG550) was prepared by the published method [27]. 2-cystamine dihydrochloride and 4-(Dimethylamino) pyridine (DMAP) were obtained from Lancaster (Lancashire, UK). 5, 5′-Dithiobis (2-nitro-benzoicacid) was obtained from Sigma (Steinheim, Germany) and acrylic acid was obtained from Dae Jung reagents and chemicals (Gyonggi-do, Korea). Dithiothreitol was obtained from Duchefa Biochemie (Haarlem, Netherland). N,N′-Dicyclohexylcarbodiimide (DCC) was obtained from Acros (New Jersey, USA). Fluorescein *O*-acrylate was obtained from Aldrich (Wisconsin, USA). 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. Synthesis of aminoethanol functionalized polyphosphazenes

All reactions were carried out under an atmosphere of dry nitrogen using the standard Schlenk-line techniques. Synthesis of $[NP(IleOEt)_{1.09}(AMPEG550)_{0.45}(AEOH)_{0.46}]_n$ (**C**). Polymer **C** (**IV**) (precursor of polymer **3**) was synthesized as elucidated below. L-isoleucine ethyl ester hydrochloride (6.41 g, 32.75 mmol) suspended in dry THF (100 ml) containing triethylamine (9.94 g, 98.27 mmol) was added slowly to poly (dichlorophosphazene) (4.0 g, 34.48 mmol) dissolved in dry THF (100 ml). The reaction mixture was stirred at −60 °C for 8 h and then at room temperature for 48 h. AMPEG550 (7.58 g, 13.79 mmol) dissolved in dry THF (50 ml) containing triethylamine (4.18 g, 41.37 mmol) was added to the above reaction mixture, stirred at room temperature for 1 day and at 45 °C 1 day. The above reaction mixture was added to aminoethanol (4.10 g, 67.24 mmol an excess of 3 equivalents) dissolved in dry THF containing triethylamine (6.8 g, 67.24 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 1 day, at 45 °C 1 day 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 **C** (**IV**). Yield: 76%. The other listed polymers (**1**&**2**) precursors (**A**&**B**) were prepared in similar way as shown in Fig. 1(**I**–**IV**) by using different feed ratios of the substituents.

#### 2.2.1. $[NP(IleOEt)_{0.91}(AMPEG550)_{0.66}(AEOH)_{0.43}]_n$ (A) IV

$^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.9. $^{1}$H -NMR ($CDCl_3$), δ (ppm): 0.91–1.05 (s, 6H), 1.05–1.40 (b, 3H), 1.40–1.62 (b, 2H), 1.62–1.85 (b, 1H), 2.80–3.18 (b, 4H), 3.38 (s, 3H), 3.50–3.91 (b, 46H), 3.91–4.00 (b, 1H), 4.11–4.40 (b, 4H).

#### 2.2.2. $[NP(IleOEt)_{1.22}(AMPEG550)_{0.48}(AEOH)_{0.30}]_n$ (B) IV

$^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.8. $^{1}$ H -NMR ($CDCl_3$), δ (ppm): 0.91–1.05 (s, 6H), 1.05–1.40 (b, 3H), 1.40–1.62 (b, 2H), 1.62–1.85 (b, 1H), 2.80–3.18 (b, 4H), 3.38 (s, 3H), 3.50–3.91 (b, 46H), 3.91–4.00 (b, 1H), 4.11–4.40 (b, 4H).

#### 2.2.3. $[NP(IleOEt)_{1.09}(AMPEG550)_{0.45}(AEOH)_{0.46}]_n$ (C) IV

$^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.9. $^{1}$H -NMR ($CDCl_3$), δ (ppm): 0.91–1.05 (s, 6H,$-CH_3$ and $CH_2CH_3$ of IleOEt), 1.05–1.40 (b, 3H, $-OCH_2CH_3$ of IleOEt), 1.40–1.62 (b, 2H, $-CH_2CH_3$ of IleOEt), 1.62–1.85 (b, 1H, $-CH(CH_3)CH_2CH_3$ of IleOEt), 2.80–3.18 (b, 4H $-NHCH_2CH_2O-$ of AMPEG and$-NH-CH_2-CH_2-$of AEOH)), 3.38 (s, 3H,$-CH_3$ of AMPEG), 3.50–3.91 (b, 46H,$-(CH_2CH_2O)_{11}-$of AMPEG and $NH-CH2-CH_2-$of AEOH), 3.91–4.00 (b, 1H,$-NHCH-$of IleOEt), 4.11–4.40 (b, 2H,$-OCH_2CH_3$ of IleOEt.

### 2.3. Synthesis of acrylated polyphosphazenes

Acrylated polyphosphazenes were prepared as shown in Fig. 1 (**IV**–**V**). In a representative example, polymer **C** (**IV**) (2.0 g, 1.6 mmol) was dissolved in dry THF (50 ml) containing triethylamine (0.33 g, 3.2 mmol) and stirred at 0 °C for 15 min. N,N′-Dicyclohexylcarbodiimide (1.04 g, 5.0 mmol), 4-(Dimethylamino) pyridine (0.19 g, 1.5 mmol) and acrylic acid (0.36 g 5.0 mmol) dissolved separately in dry THF (each 10 ml) were added one after another to the ice cold polymer solution. The reaction mixture was stirred at room temperature for 48 h under argon atmosphere. The reaction mixture was filtered and filtrate was concentrated. A mixture of chloroform and diethyl ether (50–50) was added and a white precipitate (N,N′-Dicyclohexyl urea) was removed by filtration. The filtrate was evaporated and the 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 **3** (**V**). Yield: 72%. The other listed polymers were prepared in a similar way.

#### 2.3.1. $[NP (IleOEt)_{0.91}(AMPEG550)_{0.66}(AEAC)_{0.43}]$ (1) (V)

Polymer **3** (**V**) (2.05 g, 1.4 mmol), acrylic acid (0.31 g, 4.3 mmol) were used. Yield: 74%. $^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.7. $^{1}$H -NMR ($CDCl_3$), δ (ppm): 0.91–1.05 (s, 6H), 1.05–1.40 (b, 3H), 1.40–1.62 (b, 2H), 1.62–1.85 (b, 1H), 2.80–3.18 (b, 4H), 3.38 (s, 3H), 3.50–3.91 (b, 44H), 3.91–4.00 (b, 1H), 4.11–4.40 (b, 4H) 5.82 (s, 1H) 6.2 (t, 1H), and 6.42 (d, 1H).

#### 2.3.2. $[NP (IleOEt)_{1.22}(AMPEG550)_{0.48}(AEAC)_{0.30}]$ (2) (V)

Polymer **2** (**V**) (2.01 g, 1.1 mmol), acrylic acid (0.23 g, 3.19 mmol) were used. Yield: 68%. $^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.9. $^{1}$H -NMR ($CDCl_3$), δ (ppm): 0.91–1.05 (s, 6H), 1.05–1.40 (b, 3H), 1.40–1.62 (b, 2H), 1.62–1.85 (b, 1H), 2.80–3.18 (b, 4H), 3.38 (s, 3H), 3.50–3.91 (b, 44H), 3.91–4.00 (b, 1H), 4.11–4.40 (b, 4H) 5.82 (s, 1H) 6.2 (t, 1H), and 6.42 (d, 1H).

#### 2.3.3. $[NP(IleOEt)_{1.09}(AMPEG550)_{0.45}(AEAC)_{0.46}]_n$ (3)(V)

$^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.9. $^{1}$H -NMR ($CDCl_3$), δ (ppm): 0.91–1.05 (s, 6H, $-CH_3$ and $CH_2CH_3$ of IleOEt), 1.05–1.40 (b, 3H, $-OCH_2CH_3$ of IleOEt), 1.40–1.62 (b, 2H, $-CH_2CH_3$ of IleOEt), 1.62–1.85 (b, 1H, $-CH(CH_3)CH_2CH_3$ of IleOEt), 2.80–3.18 (b, 4H $-NHCH_2CH_2O-$ of AMPEG and $-NH-CH_2-CH_2-$ of AEAC), 3.38 (s, 3H, $-CH_3$ of AMPEG), 3.50–3.91 (b, 44H, $-(CH_2CH_2O)_{11}-$ of AMPEG), 3.91–4.00 (b, 1H, $-NHCH-$ of IleOEt), 4.11–4.40 (b, 4H, $-OCH_2CH_3$ of IleOEt and $NH-CH2-CH_2-$ of AEAC) 5.82 (s, 1H $CH_2{=}CH-$ of AEAC) 6.2 (t, 1H $CH_2{=}CH)-$ of AEAC), and 6.42 (d, 1H $CH_2{=}CH)-$ of AEAC).

### 2.4. Synthesis of thiolated polyphosphazenes

Thiolated polyphosphazenes were prepared as described previously [24].

#### 2.4.1. $[NP(IleOEt)_{0.88}(AMPEG550)_{0.54}(Cysteamine)_{0.58}]$ (4)

$^{31}$P -NMR ($CDCl_3$), δ (ppm): 19.9. $^{1}$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, 3H, $-CH_2CH_3$ of IleOEt and $-CH_2CH_2SH$ of cysteamine), 1.6–1.9 (b, 1H, $-CH(CH_3)CH_2CH_3$ of IleOEt), 2.6–2.8 (b, 2H, $-CH_2CH_2SH$ of cysteamine), 2.9–3.3 (b, 4H, $-CH_2CH_2SH-$ of cysteamine 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), 4.1–4.4 (b,2H, $-OCH_2CH_3$ of IleOEt).