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Injectable and Cross-linkable Polyphosphazene Hydrogel for Space-Filling Scaffolds†

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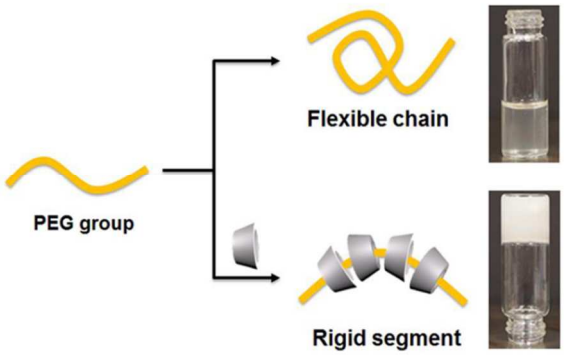
Low mechanical strength is a major disadvantage of injectable hydrogels. Although mechanical property of hydrogels can be improved by the introducing of robust materials, the use of conventional reinforcing agents limits the medical application of injectable hydrogels due to their non-biodegradable property. We synthesized and self-assembled an injectable hydrogel from biodegradable mixed-substituted polyphosphazene and natural organic molecule α-cyclodextrin (αCD) by host-guest inclusion. Photo-crosslinking was then employed for the hydrogel to transform into solid state with desired shape. By the approach, the mechanical property and anti-water solubility of polyphosphazene hydrogel can meet the requirement of potential application. In addition, the surface of the cured hydrogel can be readily tuned from cell-philic to cell-phobic by changing in the ratio of two side chains of substituted polyphosphazene.

1. Introduction

Developments in medical techniques have led to a large demand for a variety of injectable space-filling scaffolds, as they are easy to manipulate and their use effectively avoids trauma.[1-3] In this regard, hydrogels are ideal materials because they are not only injectable but they also solidify under specific conditions.[4,5] Injectable hydrogels have been regarded as new and emerging biomedical materials,[6,7] and prepared by use of physical and/or chemical cross-linking,[8,9] such as chitosan,[10,11] hyaluronic acid,[12,13] gelatin,[14] peptide hydrogels,[15] and polyphosphazene hydrogels.[16-18] Polyphosphazene polymers are inorganic–organic hybrid polymers that consist of an inorganic –N=P– backbone and two organic units attached to each phosphorus atom. These polymers are very designable and exhibit various characteristics arising from their substituents, which greatly influence their properties.[19] Their largest advantage in medical applications is their excellent biocompatibility and tunable biodegradability, especially amino acid ester substituted polyphosphazenes.[20-27] Furthermore, their degradation products are nontoxic.[28-31] Therefore, the use of polyphosphazene hydrogels in biomedical materials has received increasing attention in the last decade.[32-34] For example, Lee et al. reported a thermosensitive poly(organophosphazene) with functional groups that forms a hydrogel via phase transition at a temperature above the lower critical solution temperature of the aqueous polymer solution.[35,36] Tian et al. employed noncovalent interactions between polyethylene and α-cyclodextrin (αCD), adamantine, or β-cyclodextrin to prepare polyphosphazene hydrogels.[37,38] However, the poor anti-water solubility and poor mechanical properties of these hydrogels limit their practical application. Potta et al. attempted to introduce chemical cross-linking groups into a polyphosphazene hydrogel to prevent it from dissolving in water,[39-41] but the mechanical performance of the hydrogel still did not allow it use in biomaterial applications. In order to enhance their mechanical strength, nanoparticles,[42] carbon fibers,[43] graphene,[44,45] and montmorillonite[46] are usually added as cross-linked sites to hydrogels. Nevertheless, these reinforcing agents are not biodegradable thus limiting the application of their hydrogels in medical scaffolds. We observed that inclusion of polyethylene glycol (PEG) and αCD into hydrogels could improve the mechanical strength of the hydrogels by increasing the steric hindrance of polymer chains (**Scheme 1**). This strategy may have potential use in developing space-filling hydrogels. Another problem with hydrogels is their poor resistance to water, resulting in their dissolution in water after a certain period of contact with the solvent. Therefore, cross-linking of injectable hydrogels under specific condition in real applications must also be taken into account.



Scheme 1 The mechanism of host-guest inclusion and its rigid enhancing process.

Herein, we synthesized a series of polyphosphazene with two kinds of mixed substituents, glycine ethyl ester group (GlyEE) and monoacrylic-terminated PEG group (PEGac). The former was used to impart good cell affinity and biocompatibility, and the latter was used to enable self-assembly in the presence of αCD and to endow with anti-biological adhesion. The pendant polyphosphazene could self-assemble in the presence of αCD in water to form an injectable supermolecular hydrogel cream. After irradiation with ultraviolet (UV) light, the cream could form a cross-linked polyphosphazene hydrogel with good mechanical strength and high stability in water. Effects of the ratio of GlyEE to PEGac on the mechanical strength of the hydrogel and on cell adhesion were investigated.

2. Experimental

2.1. Materials

Hexachlorocyclotriphosphazene (HCCP) was purchased from Adamas-beta (Shanghai, China) and then purified twice by heating it at 50 °C under vacuum (about –0.1 MPa). Glycine ethyl ester hydrochloride, αCD, NaH (60% in paraffin), PEG with average molecular weight (M_n) of 600–1000 g/mol were obtained from Adamas-beta, acrylic anhydride was purchased from Xiya Reagent, and triethylamine (TEA) was purchased from Sigma-Aldrich. These were used without further purification. Tetrahydrofuran (THF; Shanghai Chemical Reagent Corporation) was dried by refluxing over sodium metal and then distilled under an argon atmosphere. All glasswares were dried overnight under vacuum at 110 °C before use. Bovine serum albumin (66 kDa, >98% purity), fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco BRL. HeLa cells were obtained from the Cell Resource Center of Shanghai Biological Sciences Institutes. Water used in the experiments was purified to a resistivity higher than 18.2 MΩ·cm by using a Hitech system.

2.2. Equipments

^{1}H NMR and ^{31}P NMR spectra were recorded on a Varian Mercury-400 spectrometer, ^{1}H NMR spectra were referenced to solvent signals, and ^{31}P NMR spectra were referenced to signals obtained with 85% phosphoric acid (external reference). Fourier transform infrared spectra (FTIR) were scanned on a Paragon 1000 (Perkin-Elmer) spectrometer. Molecular weight and polydispersity were estimated by using a Perkin-Elmer Series 200 gel permeation chromatograph (GPC) at 30 °C equipped with two linear mixed-B columns (Polymer Lab Corporation; pore size: 10 μm, column size: 300 × 7.5 mm) and a refractive index detector. DMF (0.01 mol/L LiBr) and polystyrene were used as the eluent (elution rate: 1.0 mL/min) and calibration standard, respectively. Wide-angle X-ray diffraction (WAXD) patterns of powdered samples were obtained on a Shimadzu XRD-6000 X-ray diffractometer with a Cu Kα radiation source (1.54 Å wavelength), and the supplied voltage and current were set to 40 kV and 30 mA, respectively. Diffraction patterns were collected from 0.5° to 40° at a speed of 5°/min. Transmission electron microscopy (TEM) images were taken by using a JEM-2010/INCA Oxford TEM (JEOL/Oxford) operated at 200 kV accelerating voltage. Samples were prepared on the surface of 300-mesh Formvar–carbon film-coated copper grids. Visual images were captured by a Canon IXUS 800IS digital camera (Canon, Japan). Rheology behaviors of the hydrogel samples at room temperature were determined with a TA-ARG2 rheometer using a 40 mm parallel-plate geometry. Compressive stress–train tests were conducted on water-swollen gels by using a tensile-compressive tester (Zwick/Roell Z020). A cylindrical gel sample with 15 mm diameter and 10 mm thickness was set on the lower plate and then compressed with the upper plate at a strain rate of 10%/min. The strain under compression is defined as the change in the thickness relative to the freestanding thickness of the sample.

2.3. Synthesis of crosslinkable PEG precursor PEG600ac

Poly(ethylene glycol) 600 (PEG600; 6.0 g, 10 mmol) was dissolved in 300 mL of anhydrous THF under an argon atmosphere. Subsequently, 1.514 g of acrylic anhydride (12.0 mmol) was added dropwise to this solution at 0 °C. The resulting mixture was stirred for 2 h and then heated at 55 °C overnight. The reaction mixture was precipitated in diethyl ether, filtered, washed with a large amount of diethyl ether, and then dried under vacuum to obtain PEG600ac. Yield: 73%. ^{1}H NMR (D_2O): 6.45 (d, 1H, –CH=CH_2), 6.24 (t, 1H, –CH=CH_2), 6.03 (s, 1H, –CH=CH_2), 4.36 (d, 2H, –OCH_2CH_2–), 3.88–3.53 (br, 50H, PEG600ac, –OCH_2CH_2–) (**Figure S1**).

2.4. Synthesis of crosslinkable PEG precursor PEG1000ac

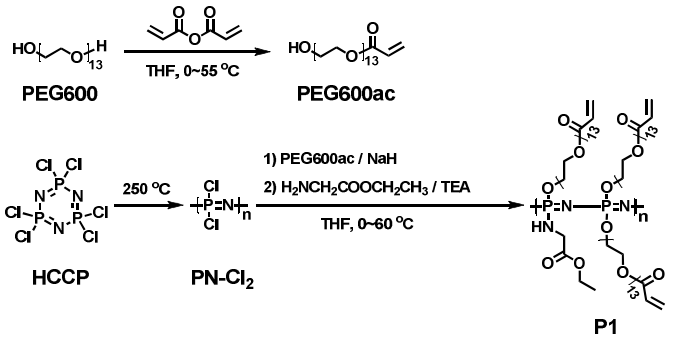
Preparation of monoacrylic polyethylene glycol 1000 was similar to that of PEG600ac. We utilized 10.0 g (10 mmol) of polyethylene glycol 1000 (PEG1000) and 1.640 g of acrylic anhydride (13.0 mmol). Yield: 81%. ^{1}H NMR (D_2O): 6.45 (d, 1H, –CH=CH_2), 6.24 (t, 1H, –CH=CH_2), 6.03 (s, 1H, –CH=CH_2), 4.36 (d, 2H, –OCH_2CH_2–), 3.88–3.53 (br, 86H, PEG1000ac, –OCH_2CH_2–) (**Figure S2**).

2.5. Synthesis of guest polyphosphazene polymer

Synthetic route of cross-linkable polyphosphazene polymer was shown in **Scheme 2**. Poly(dichlorophosphazene) (PN-Cl_2) was firstly prepared by thermal ring-opening polymerization of HCCP at 250 °C in evacuated Pyrex tubes.[47]

PN-PEG600ac-70%gly (P1). PEG600ac (3.38 g, 5.17 mmol) and NaH (0.207 g, 5.17 mmol) were mixed in anhydrous THF (60 mL) at 0 °C to form a clear yellowish suspension, which was afterward added dropwise to poly(dichlorophosphazene) (1 g, 8.62 mmol) dissolved in anhydrous THF (50 mL) at 0 °C for 1 hour, then kept at 40 °C for 1 day to give a partially substituted polymer. Meanwhile, in another separate flask, glycine ethyl ester hydrochloride (3.36 g, 24.0 mmol) was added to anhydrous THF (20 mL) containing triethylamine (4.85 g, 48.0 mmol), the suspension was stirred at 60 °C for 1 day and then filtered. The obtained aqueous solution was subsequently added to the polymer solution dropwise at 0 °C for 1 hour then at 60 °C to complete the substitution reaction overnight. Finally, the participation of the reaction system was removed by centrifugation, and the solution was concentrated and precipitated twice in n-hexane. The crude product was further dialyzed versus methanol, methanol/water (90/10), and again methanol, each for 1 day at room temperature. The final product was dried under vacuum to obtain a gel-like polymer. Yield: 63%. ^{31}P NMR ($CDCl_3$): δ 1.88 (br), δ −2.81 (br), δ −5.35 (br). ^{1}H NMR ($CDCl_3$): δ 6.23 (d, 1H, PEG600ac, –CH=CH_2), δ 5.97 (s, 1H, PEG600ac, –CH=CH_2), δ 5.71 (s, 1H, PEG600ac, –CH=CH_2), δ 4.44 (br, 2H, PEG600ac, –CH_2COO–), 4.20 (s, 2H, GlyEE, –OCH_2CH_3), δ 4.03 (d, 2H, –OCH_2CH_2–), δ 3.81 (s, 2H, GlyEE, –CH_2COO–), δ 3.64–3.49 (br, 44~52H, PEG600ac, –OCH_2CH_2–), δ 1.97 (br, H, GlyEE, –NH–), δ 1.23 (s, 3H, GlyEE, –CH_2CH_3).

PN-PEG600ac-30%gly (P2). PEG600ac (3.96 g, 6.05 mmol), NaH (0.242 g, 6.05 mmol), poly(dichlorophosphazene) (0.5 g, 4.31 mmol), glycine ethyl ester hydrochloride (0.72 g, 5.15 mmol), containing triethylamine (1.04 g, 10.3 mmol). ^{31}P NMR ($CDCl_3$): δ 1.88 (br), δ −2.81 (br), δ −5.35 (br). ^{1}H NMR ($CDCl_3$): δ 6.22 (s, 1H, PEG600ac, –CH=CH_2), δ 5.97 (s, 1H, PEG600ac, –CH=CH_2), δ 5.68



Scheme 2 Synthesis route of crosslinkable guest polyphosphazene

(s, 1H, PEG600ac, –CH=CH_2), δ 4.44 (br, 2H, PEG600ac, –CH_2COO–), 4.20 (s, 2H, GlyEE, –OCH_2CH_3), δ 4.03 (d, 2H, –OCH_2CH_2–), δ 3.81 (s, 2H, GlyEE, –CH_2COO–), δ 3.64–3.49 (br, 44~52H, PEG600ac, –OCH_2CH_2–), δ 1.97 (br, H, GlyEE, –NH–), δ 1.23 (s, 3H, GlyEE, –CH_2CH_3).

PN-PEG1000ac-30%gly (P3). PEG1000ac (6.38 g, 6.05 mmol), NaH (0.242 g, 6.05 mmol), poly(dichlorophosphazene) (0.5 g, 4.31 mmol), glycine ethyl ester hydrochloride (0.72 g, 5.15 mmol), containing triethylamine (1.04 g, 10.3 mmol). ^{31}P NMR ($CDCl_3$): δ 1.88 (br), δ −2.81 (br), δ −5.35 (br). ^{1}H NMR ($CDCl_3$): δ 6.22 (s, 1H, PEG1000ac, –CH=CH_2), δ 5.97 (s, 1H, PEG1000ac, –CH=CH_2), δ 5.68 (s, 1H, PEG1000ac, –CH=CH_2), δ 4.44 (br, 2H, PEG1000ac, –CH_2COO–), 4.20 (s, 2H, GlyEE, –OCH_2CH_3), δ 4.03 (d, 2H, –OCH_2CH_2–), δ 3.81 (s, 2H, GlyEE, –CH_2COO–), δ 3.64–3.49 (br, 80~88H, PEG1000ac, –OCH_2CH_2–), δ 1.97 (br, H, GlyEE, –NH–), δ 1.23 (s, 3H, GlyEE, –CH_2CH_3).

2.6. Supermolecular inclusion hydrogel

A representative procedure for preparing the supermolecular inclusion hydrogel is described in detail as follows. Two kinds of precursors, P2 (9 wt %) and αCD (9 wt %) water solutions were mixed at room temperature. The clear mixture turned to a white and opaque gel in several minutes under magnetic stirring.

2.7. Rheological measurements

A TA-ARG2 rheometer was used to investigate the gelation kinetics, and time sweep tests were performed at 5% strain at a constant oscillatory frequency of 1 rad/s. The samples were loaded on parallel plates with 25 mm diameter. The gap distance between the two plates was fixed at 0.3 mm.

2.8. Anti-dissolving ability of the hydrogel in water

The anti-dissolving ability of the hydrogel cream before and after UV cross-linking was compared by using a 10 mm Petri dish filled with water. The dissolution process was recorded by a digital camera.

2.9. Load-bearing test

Samples tested were cross-linked P3 (marked as P3U), P3 included in αCD (P3C), UV irradiated P3 included in αCD (P3CU), and P1C, P1U, P1CU and P2C, P2U and P2CU (the assembly systems of P1 and P2 were marked according to the same principle to P3). The first three were analyzed to determine the contribution of inclusion and cross-linking to the mechanical strength, and the latter two were analyzed to investigate the influence of PEG length on hydrogel strength.

2.10. Cell adhesion study

The hydrogel samples, P1CU, P2CU, P3CU, cut into 8 mm× 8 mm square were selected for cell adhesion study. The samples were sterilized and then added to a 24-well plate seeded with HeLa cells with a density of 1×10^5 cells/well. The cells were then cultured for 24 h in a medium consisting of DMEM, 10% FBS, and 1% penicillin/streptomycin solution at 37 °C and 5% CO_2. All samples were visualized under a Nikon-C1 laser scanning confocal microscope.

3. Results and discussion

3.1. Characterization of Mixed-Substituent Polyphosphazene

In order to avoid chain entanglement and ensure smooth progress of the succedent substitution reaction of GlyEE, we used PEG600 and PEG1000, which have relatively low molecular weight. This low-molecular-weight PEG also facilitates elimination by kidney *in vivo* after degradation of the polyphosphazene hydrogel, thus enabling practical clinical applications of the hydrogel.

Herein, polyphosphazene with various molar ratios of monoacrylic PEG groups to GlyEE groups were prepared. The ^{31}P NMR spectrum of P1 (**Figure S3**) shows signals at 1.88, −2.81, and −5.35 ppm, which are due to vibrations of [N–P–N], [N–P–O], and [O–P–O], respectively. The signal at −17.05 ppm corresponding to [Cl–P–Cl] disappeared, indicating that complete substitution had taken place. The amounts of PEG600ac and GlyEE were calculated according to the integral ratio of the peaks at 6.22 and 1.23 ppm in the ^{1}H NMR spectrum (**Figure S4**). The percentage of PEG600ac obtained by calculation was very close to the feed ratio (**Table 1**), proving the synthesis of the mixed-substituent polyphosphazene.

Table 1. Number-average Molecular weight (M_n), and Weight-average Molecular weights (M_w) and Molecular weight distribution (M_w/M_n) of mixed-substituent polyphosphazene and the proportions of each substitute in the corresponding polymers.

Polymer	PEGac	GlyEE	M_n	M_w	M_w/M_n
P1 (PEG600)	29.7%	70.3%	14,907	46,763	3.137
P2 (PEG600)	68.9%	31.1%	35,196	82,640	2.348
P3 (PEG1000)	71.4%	28.6%	46,814	122,465	2.616

3.2. Preparation of the Supermolecular Inclusion Hydrogel Cream

The supermolecular inclusion hydrogel creams were prepared by mixing an aqueous solution of the polymer and αCD (eq. concentration). The gelation time was dependent on the concentrations of the two precursors, that is, higher concentrations resulted in shorter gelation time. For example, when aqueous solutions of P2 (9 wt%) and αCD (9 wt%) were mixed at 25 °C, an opaque white gel was formed within 30 s, whereas 1.5 min was needed for gelation with 5 wt% P2 and αCD (**Figure 1a**). This difference is due to the more opportunities to form inclusion at higher concentrations of polymer and αCD. In addition, T_{gel} was closely related to the fraction and length of side-chain PEGac groups of polyphosphazene. As shown in **Figure 1b**, increasing the molecular weight and the amount of PEG segments resulted in higher T_{gel}, as more self-assembly sites formed. It has been proved that αCDs can form nanocrystallites with PEG.[50-54] TEM observations revealed the presence of nanocrystallite domains and the formation of nanocrystallites at higher concentrations of polyphosphazene and αCD (**Figure 1c** and **1d**). Abundant hydrogen bonds outside the threaded αCDs provide the physical cohesion force that induces αCD crystallization.

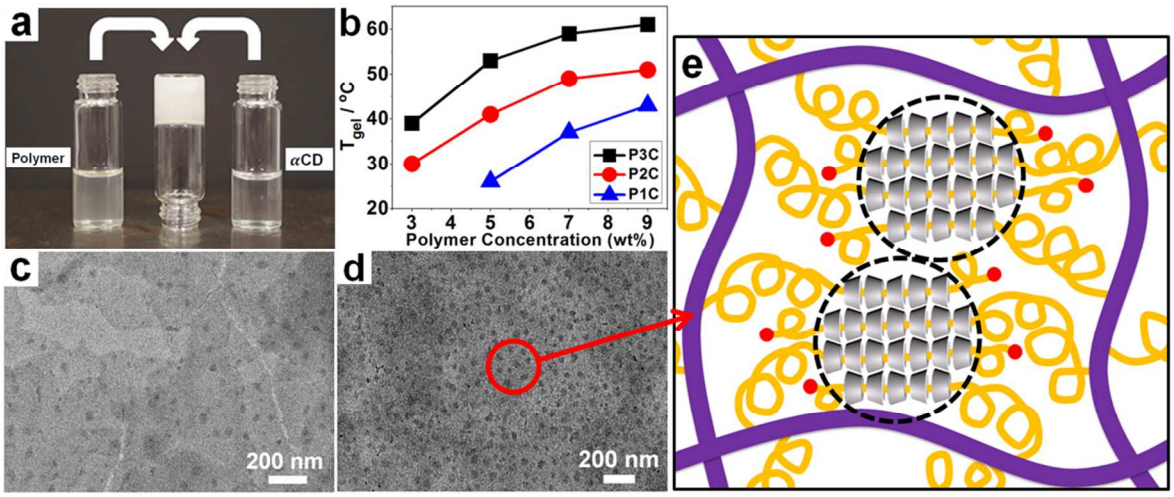


Figure 1 Supermolecular hydrogel consisting of P3C inclusions. (a) Formation of the hydrogel; (b) the relationship between gelation temperature (T_{gel}) and polymer concentration; (c) & (d) TEM images of P2C with 3 wt% and 9 wt% P2, respectively. (e) Illustration of the self-assembly structure.

A plausible structure of the assembly between αCD and the polyphosphazene with PEGac groups is illustrated in **Figure 1e**.

XRD is an effective tool for determining the inclusion structure of the host–guest inclusion hydrogels. Herein, XRD was employed to confirm whether the inclusion structure remained in the hydrogel after irradiation. Diffraction peaks at 7.4°, 12.9°, 19.7°, and 22.3° (**Figure 2**) imply the formation of the supermolecular inclusion structure.[55-65] After curing of the hydrogel under UV light, the intensity of the diffraction peaks remained the same, indicating retention of the inclusion structure.

3.3. Light Curing of the Supermolecular Inclusion Hydrogel and Its Anti-water solubility

The anti-water solubility of the supermolecular inclusion hydrogel cream before and after UV irradiation is compared in **Figure 3**. The cross-linked hydrogel showed high anti-water-solubility, whereas that without cross-linking partially dissolved in water within 4 h. Light curing induces cross-linking of acryloyl groups at the terminal of PEGac, producing abundant chemical cross-linking bonds and preventing the dethreading of αCD from the PEG segment.

3.4. Mechanical Performance of the Cross-linked Supermolecular Hydrogels

3.4.1. Rheological study

By definition, cream cannot return to its initial shape upon deformation. According to **Figure 4a**, sample P2C was a typical cream, as its storage modulus (220 Pa) was lower than its loss modulus (~1100 Pa). UV curing could induce the cream to turn into a hydrogel (P2CU) whose storage modulus is higher than its loss modulus. In addition, the storage modulus of P2CU (threaded with αCD) was approximately eight times higher than that of P2U without αCD, proving that the host–guest inclusion approach effectively enhanced the mechanical strength of the hydrogels. When a longer PEG segment was used, the rheological performance was enhanced (**Figure 4b**). For instance, the storage modulus of P3CU reached 130 kPa. This enhancement is due to the greater number of rigid inclusion structures in the hydrogel cream, which increases the mechanical strength. Even the storage modulus of the non-cross-linked P3C, was higher than its loss modulus. However, its storage modulus is still too low (<1000 kPa); it was a white viscous liquid that could easily be extruded from a narrow needle (diameter: d = 0.9 mm). This property, however, allows its application as an injectable hydrogel.

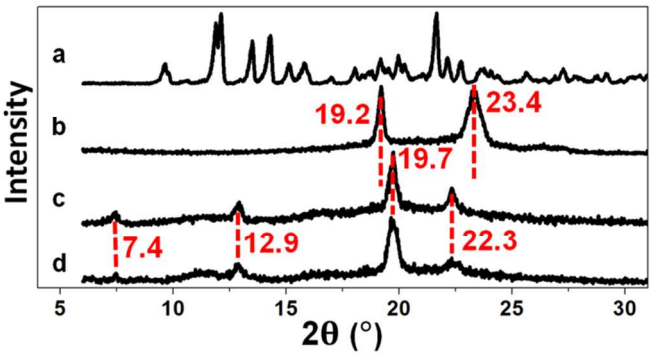


Figure 2 XRD spectra of (a) αCD, (b) P2, (c) P2C, and (d) P2CU.

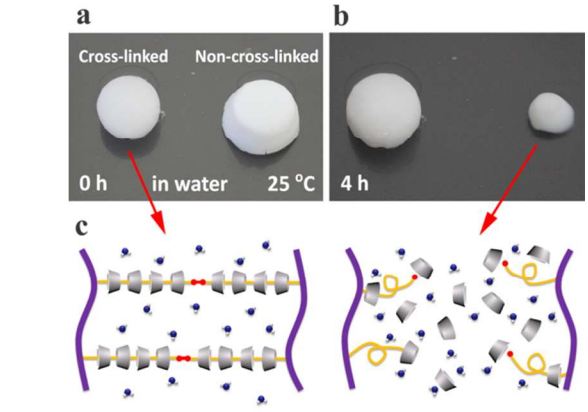


Figure 3 Anti-water solubility test of the P2C hydrogel cream before (a) and after (b) UV irradiation, and the proposed mechanisms of dissolution (c).

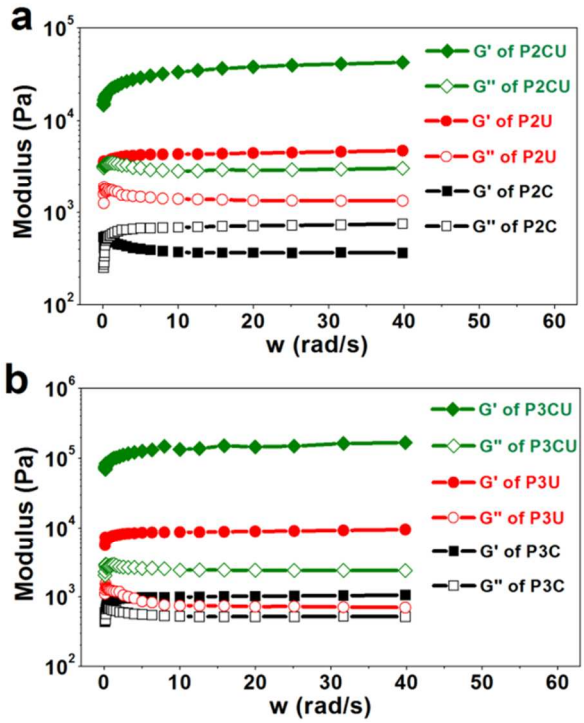


Figure 4 Rheology curves of the supermolecular inclusion hydrogel cream with (a) P2 and (b) P3.

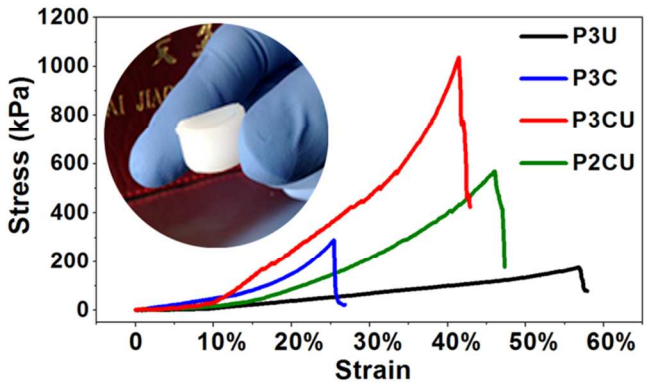


Figure 5 Load-bearing test of the supermolecular inclusion hydrogel cream P3C, and of the hydrogels P3U, P3CU, and P2CU.

3.4.2. Load-bearing study

A space-filling hydrogel must not only be anti-water-soluble and injectable, but also have good load-bearing property. Rigid structure formed via the host–guest inclusion process improves the mechanical strength of the hydrogel from 178 kPa (P3U) to 1036 kPa (P3CU), as shown in **Figure 5**. In addition, P3CU could bear more weight than did P2CU (566 kPa), consistent with the result of the rheological study.

3.5. Cell Adhesion Study

In general, a space-filling hydrogel requires a specific cell adhesion property. It may have a cell-philic surface or cell-phobic (antifouling) surface, depending on the surgical purpose.[66] Herein we tried to tune the cell adhesion property of the hydrogel surface by adjusting the proportion of GlyEE to PEGac on the polyphosphazene backbone.

Figure 6 shows the cell adhesion behavior on the cross-linked polyphosphazene hydrogels. P1CU provided a cell-philic surface as it contained a high proportion of GlyEE pendants (**Figure 6a**), whereas P3CU, which had abundant PEG pendants, exhibited cell-phobic property (**Figure 6c**). The hydrophilic PEG segments and the outer layer of the threaded αCD formed a hydration layer on the surface of P3CU, thereby preventing cells from attaching to the surface (**Figure 6d**). We also could shorten the PEG segment to obtain a surface for moderate cell adhesion (**Figure 6b**). Evidently, the cell adhesion property of the polyphosphazene hydrogel could be tuned by changing the ratio of PEG to GlyEE. Both cell-philic and cell-phobic (antifouling) surfaces are important to the surface design of biological materials.

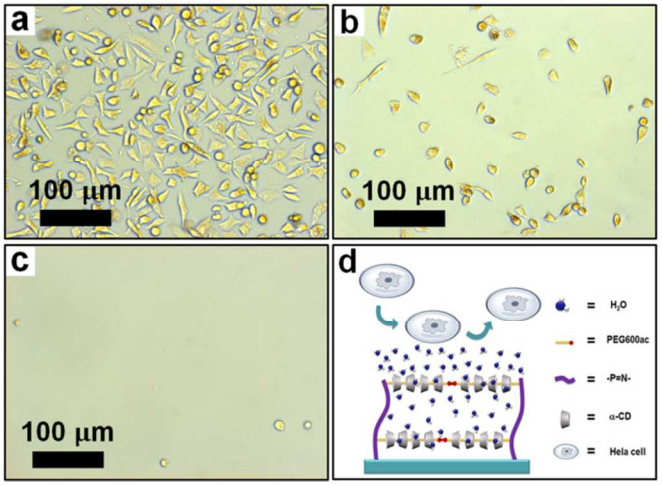


Figure 6 Cellular adhesion properties of (a) P1CU, (b) P2CU, and (c) P3CU. (d) An illustration of the cell-phobic mechanism.

4. Conclusions

A series of polyphosphazene with various ratios of GlyEE and PEGac were prepared. Host–guest inclusion using αCD and photo-cross-linking could markedly enhance the mechanical strength (up to seven times) and anti-water-solubility of their hydrogels. Through this approach, we prepared scaffold material from polyphosphazene hydrogel. In particular, the high-strength solid, P3CU, which was obtained by curing the injectable hydrogel cream P3C under UV light, was found to satisfy the requirements for medical space-filling scaffolds. Furthermore, the surface of the hydrogel could be converted from cell-philic to cell-phobic by tuning the ratio of GlyEE to PEGac. Our synthetic procedure provides a new approach for surface design of biomedical scaffold materials.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Detailed information of preparation method, water contact angle, protein adsorption, anticoagulant property of the membranes, platelet adhesion, cell culture, cell morphology on the membranes]. See DOI: 10.1039/b000000x/

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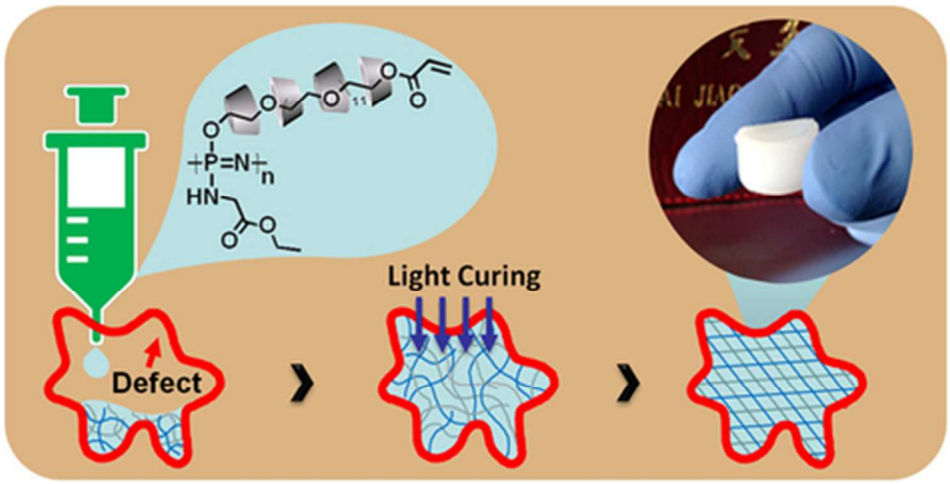

Injectable and Cross-linkable Polyphosphazene Hydrogel for Space-Filling Scaffolds
39x20mm (300 x 300 DPI)