Supporting Information

Synthesis and evaluation of cytocompatible alkyne-containing poly(β-amino esters)-based hydrogels functionalized *via* click reaction

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Materials and method

Materials

Polyethylene glycol diacrylate (PEGDA, Mw 600), propynylamine, sodium ascorbate, ammonium persulphate (APS), copper sulfate, ibuprofen, *N*, *N′*-carbonyldiimidazole (CDI), dimethyl sulfoxide (DMSO), [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] (MTT), *N, N, N′, N′*-Tetramethylethylenediamine were purchased from Aladdin. 2-Chloro-trityl chloride resin, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate, fluorenylmethoxycarbonyl (Fmoc) protected amino acids were supplied by GL Biochem. All the other reagents were used as received.

Synthesis of Fmoc-L-Lys(N_3)-OH

Fmoc-L-Lys-OH (736 mg, 2 mmol), potassium carbonate (552 mg, 4 mmol), and imidazole-1-sulfonyl azide hydrochloride (461 mg, 2.2mmol) and copper sulfate pentahydrate (10 mg, 0.04mmol) and distilled water (1 mL) were dissolved in methanol (20 mL). The mixture was stirred for overnight at room temperature, and then the solvent was removed under reduced pressure. 20 mL of distilled water was added to mixture, and then acidified to pH 4.0 with hydrochloride. The mixture was extracted by ethyl acetate, washed with water, brine, and dried with anhydrous sodium sulphate. The solvent was removed under reduced pressure to obtain Fmoc-L-Lys(N_3)-OH (670 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.76 (2H, d J=7.6 Hz), 7.59 (2H, d, J=6.8 Hz), 7.40(2H, t, J=7.4 Hz), 7.31(2H, t, J=7.4 Hz), 5.32(1H, d, J=8.4 Hz), 4.42 (2H, t, J=6.4 Hz), 4.22 (1H, t, J=6.8 Hz), 3.28 (2H, t, J=6.6 Hz), 1.74-1.46(6H, m).

Synthesis of PAE containing alkynyl groups

Acrylate terminated PAE were synthesized by mixing PEGDA and propynylamine monomers in a feeding ratio of 1.05:1. The polymerization reaction was conducted under stirring at 90 ℃ for 24 h to obtain acrylated terminated PAE containing alkynyl groups.

Peptide synthesis and purification

K(N_3)RGD peptide was synthesized by solid phase peptide synthesis (SPPS) using 2-chloro-trityl chloride resin with Fmoc chemistry procedures[28]. The amino acid was coupled with HBTU and diisopropylethylamine (DIEA) in DMF as a coupling reagent, and Fmoc was deprotected by 20 % piperidine in DMF. The synthesized peptide was cleaved by mixing trifluoroacetic acid (TFA)/trisisopropylsilane (TIS)/water (95:2.5:2.5). The organic solvents were removed under reduced pressure, and then precipitated in ethyl ether. The crude peptide was dissolved in distilled water, filtered and purified by HPLC.

Preparation of RGD conjugated PAE

Acrylate terminated alkynyl PAE (66mg, 0.1mmol) and K(N_3)RGD (5 mg, 0.01 mmol) and copper sulfate pentahydrate (1.245mg, 0.005mmol) were dispersed in the distilled water (5 mL) under nitrogen. An aqueous solution (0.2 mL) of sodium ascorbate (1.9811mg, 0.01 mmol) was added dropwise to the mixture by a syringe. The resulting mixture was stirred for 4 h. The resulting mixture was dialyzed in a dialysis bag (Mw 500) against distilled water for 24 h.

Preparation of peptide RGD conjugated hydrogel

K(N_3)RGD conjugated/non-conjugated acrylated terminated PAE and PEGDA were dissolved in distilled water (1 mL). 10 % APS and 1 μL of TEMED was added to PAE/PEGDA solution, and then heated to 50 °C for 4 h to obtain peptide RGD conjugated hydrogel (the parameters of PAE/PEGDA hydrogels was shown in **Table S1**). Freshly prepared hydrogel was immersed in distilled water at 37 °C for 24 h to remove the unreacted small molecules or physically entrapped in the hydrogel.

Mechanical properties

To investigate the mechanical properties, the circular cylinders hydrogels were prepared to the same dimension (d=1 cm). The thickness of hydrogels was examined by a vernier caliper with a precision of 0.001 mm. Mechanical properties such as compressive strength, Young's modulus, were operated on an universal mechanical testing machine at a crosshead speed of 10 mm/min. The measurements were replicated five times, and the results were expressed by the average values and standard deviations.

Swelling ratio

The hydrogels were dehydrated by a graded ethanol series (50%, 70%, 90% and 100%), and dried in the oven. The weight of dried samples was recorded, and the hydrogels were immersed in phosphate buffer saline (PBS) for 12 h. After a predetermined period, the wet weight of hydrogels was measured. The swelling ratio and Higuchi model were calculated by the following equation:

$$\text{Swelling Ratio (SW)} = \frac{\text{Wet Weight of hydrogel} - \text{dried Weight of hydrogel}}{\text{Dried weight of hydrogel}} \times 100\%$$

$$\text{Higuchi equation} \qquad \frac{Mt}{Mi} = \kappa_H \times t^{1/2}$$

Characterization

Proton (^{1}H) NMR spectra were performed on a NMR instrument (Avance III HD 400M, Bruker) at room temperature, where D_2O, DMSO-*d6* were used as solvents. Chemical shift were recorded in ppm and referenced to residual solvents resonances. The molecular weight of PAE was conducted on a gel permeation chromatography (GPC, Waters1515) instrument

with PL aquagel-OH MIXED-M column connected to refractive index detector (RI2414), and the molecular weight was calculated according to standard PEG. Scanning Electron Microscopy (SEM) images were collected on a Hitachi SEM (S-3400N II, Japan). The surfaces and cross-sections of hydrogels were sprayed with a thin palladium layer. The coated palladium layer did not affect the morphology observation.

Hydrogel rheology

Rheological test was performed by a HAAKE MARS rheometer (Thermo Scientific). Amplitude time sweep rheology of samples was conducted to determine the storage modulus and the gelation points of hydrogels. Briefly, various solutions (1 mL) were added to a Thermo rheometer plate and a 35 mm parallel plate (P_{35}/Ti) to monitor in situ polymerization at 50 °C. The storage modulus and loss modulus were investigated, and the gelation points of hydrogels were analyzed.

Wettability

The wettability of hydrogels was investigated on a water contact angle measurement system (JY-PHb, Chengde Jinhe Instruments, China). A water droplet was dosed on surface of samples with weak inertia, and the contact angle was recorded on a horizontal microscope. Then, the contact angles of dehydrated hydrogels (gradient dehydration with ethanol, dried state) and swollen hydrogel (immersed in PBS, wet state) were conducted. The average contact angle was conducted for three times, and expressed by the mean values and their standard deviations.

Degradability

In vitro degradability behaviors of hydrogels were investigated by gravimetric method. Briefly, PAE/PEGDA hydrogels were incubated in a predetermined volume of PBS (pH 7.4), and the experiments were performed at 37 °C. After a predetermined period (24 h), fresh medium was replenished. The hydrogels were collected at the set time, dried and weighed. The mean degradation rate was determined by the following formula:

$$\text{Degradation rate (\%)} = \frac{\text{Original weight} - \text{residual weight}}{\text{Original weight}} \times 100\%$$

Cell viability

In vitro cytotoxicity of PAE/PEGDA hydrogel were conducted against L929 cells by MTT assay. The rat fibroblast L929 cells were grown in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 10 % FBS at a humidified atmosphere containing 5 % CO_2. L929 cells were planted (5000 cells/well), and then incubated with DMEM medium containing the extracts of KRGD conjugated PAE/PEGDA hydrogels for 24 h at 37 °C. After adding MTT (10 μL, 5 mg/mL), the culture medium was further incubated for 4 h. The culture

medium was removed, and then DMSO (100 μL) was added to dissolve the formazan. The absorbance was analyzed by a microplate reader (Bio-Rad).

Live/Dead staining

Primary Schwann cells were collected from the sciatic nerves of one-day-old Sprague-Dawley Rat according to previous method approved by Animal Care and Use Committee at the Nantong University. Schwann cells were grown in DMEM containing 10 % fetal bovine serum (FBS, Gibco) at 37 ℃ at a humidified atmosphere containing 5 % CO_2. After co-incubation on various KRGD conjugated PAE/PEGDA hydrogels, Schwann cells were stained by EthD-1/calcein AM (Introgen) for 40 min. Schwann cells on the surface were observed by a Leica fluorescence microscopy.

Hemo-compatibility of hydrogels

The erythrocytes was collected from fresh mouse blood by centrifuging (1000 rmp, 10 min), and then washed by saline for three times. After diluted with saline to a concentration of 5 % (v/v), the erythrocytes solution (50 μL) was incubated with hydrogels with gentle shaking at 37 ℃ for one hour. The mixture was centrifuged again (1000 rmp, 10 min), and the supernatant was collected. The absorbances of the suspensions were conducted on a micro-plate reader (Bio-Rad, USA). The lysis treated with 0.1% triton was taken as the positive control, and the sample incubated with PBS was considered as the negative control. The hemolysis percentage of samples was determined by the following formula:

$$\text{Hemolysis ratio (\%)}=\frac{\text{Absorbance of sample}-\text{Absorbance of Saline}}{\text{Absorbance of positive control}-\text{Absorbance of Saline}}\times 100\%$$

Statistical Analysis

All results were presented as mean ± standard deviation based on at least three replicated tests. Statistical significance is determined using Student's t-test. $^{*}P < 0.05$,** $P< 0.01$,*** $P< 0.001$.

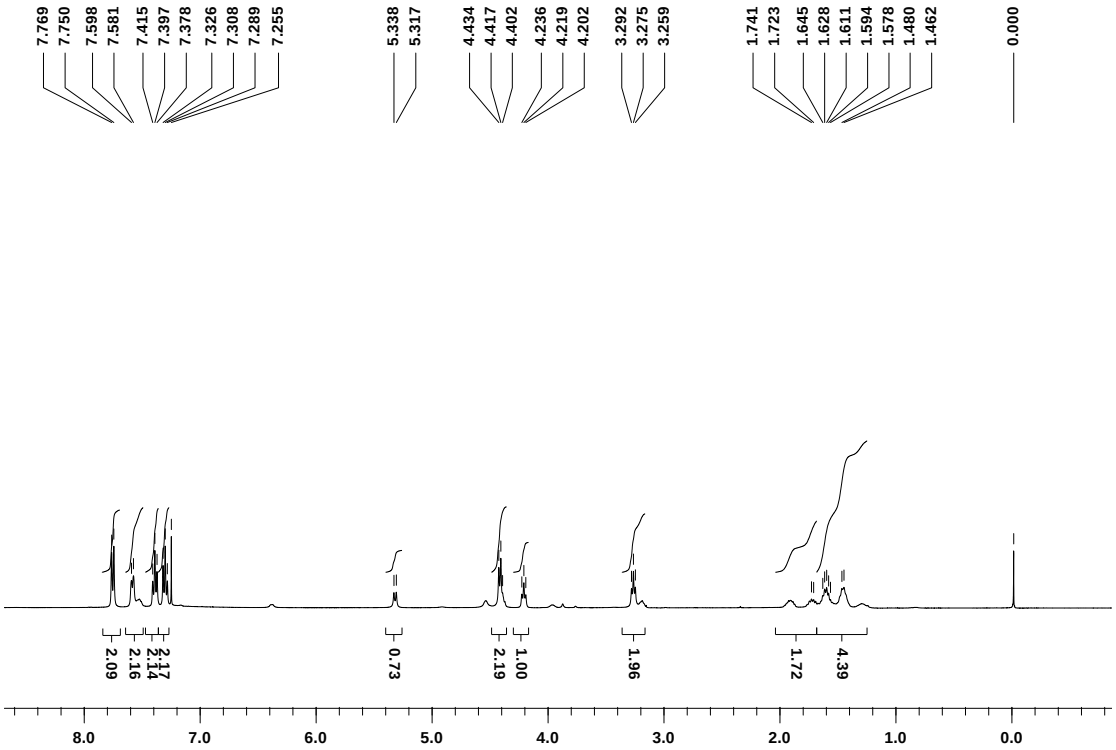


Figure S1 ^{1}H NMR of Fmoc-Lys-N_3 [K(N_3)].

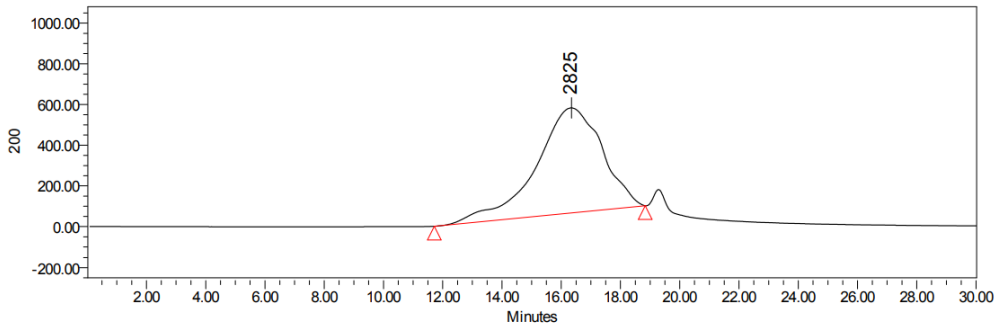
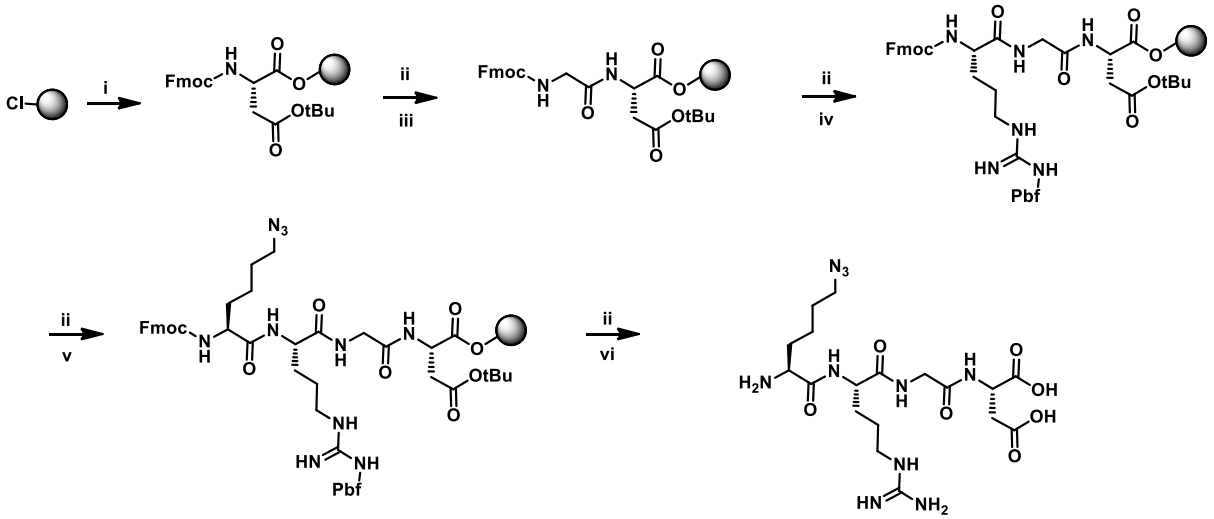


Figure S2 GPC of PAE containing alkynyl groups.



Scheme S1 Synthetic route of K(N_3)RGD peptide. (i) Fmoc-Asp(OtBu)-OH, DIEA, CH_2Cl_2; (ii) Piperidine, DMF; (iii) Fmoc-Gly-OH, DIEA, HBTU, DMF; (iv) Fmoc-Arg(Pbf)-OH, DIEA, HBTU, DMF; (v) Fmoc-Lys(N_3)-OH, DIEA, HBTU, DMF; (vi) TFA, TIPS, H_2O.

Table S1 Parameters for the three types of hydrogels investigated.

Hydrogel	**Gel-1**	**Gel-2**	**Gel-3**
RGD-PAE/g	0.05	0.075	0.125
PEGDA/g	0.1	0.15	0.25
10%APS/μL	10	15	25
TEMED/μL	1	1	1
Water/mL	1	1	1

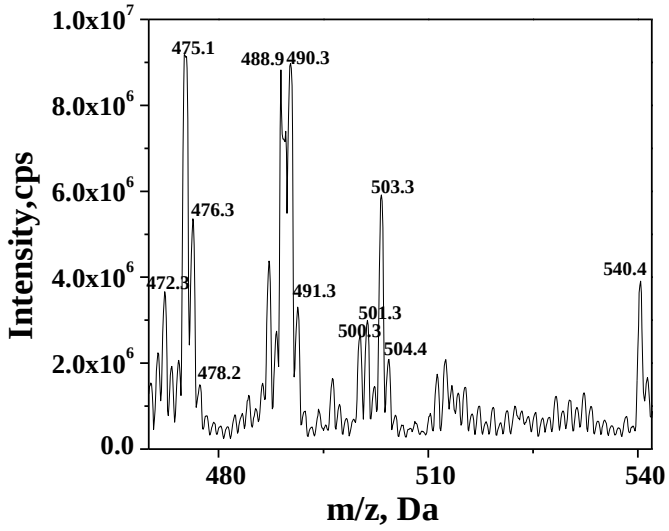


Figure S3 Mass spectrum of K(N_3)-RGD, the molecular weight calculated at 500.3, founded at 501.3 $[M+H]^+$.

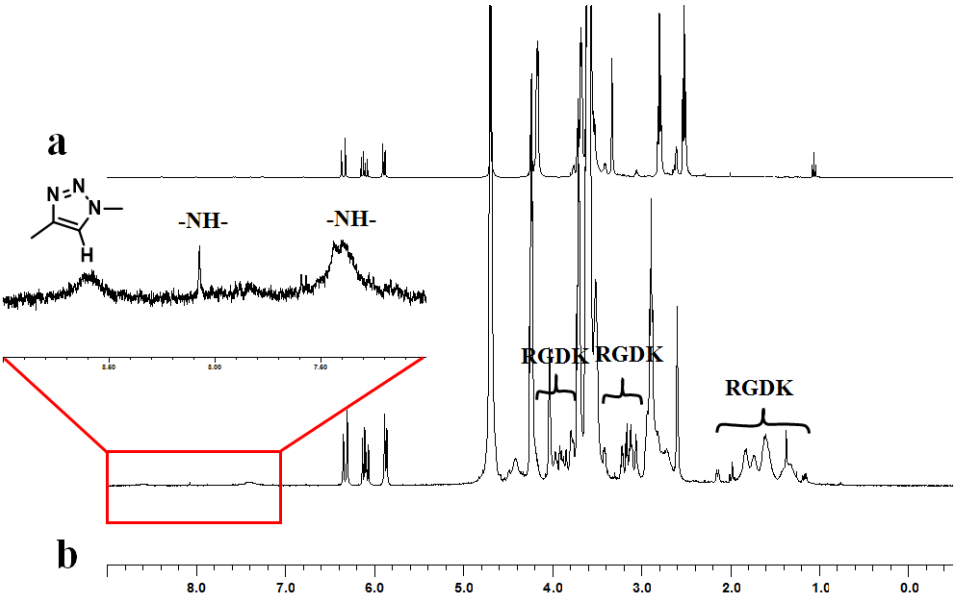


Figure S4 ^{1}H NMR spectra of PAE (a) and RGD conjugated PAE (b).

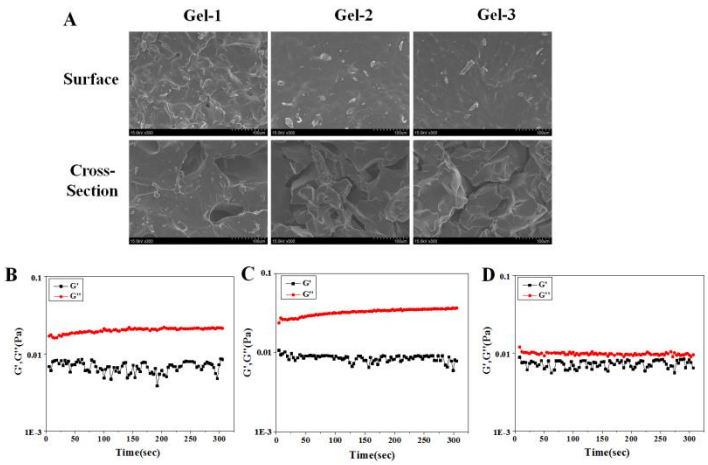


Figure S5 (A) Surface and cross-section SEM images of the lyophilized KRGD conjugated PAE/PEGDA hydrogels. Typical rheology analysis of KRGD conjugated PAE polymerization (B, 5 % PAE; C, 7.5 % PAE; D, 12.5 %);

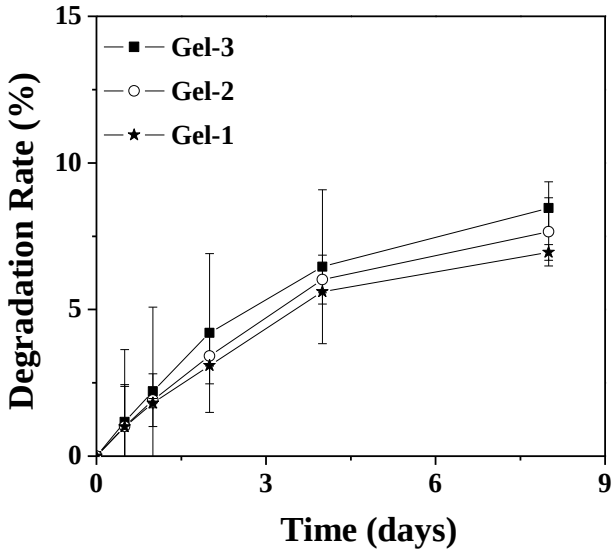


Figure S6 Degradation behavior of KRGD conjugated PAE/PEGDA (Gel-1, Gel-2 and Gel-3) hydrogels in PBS.

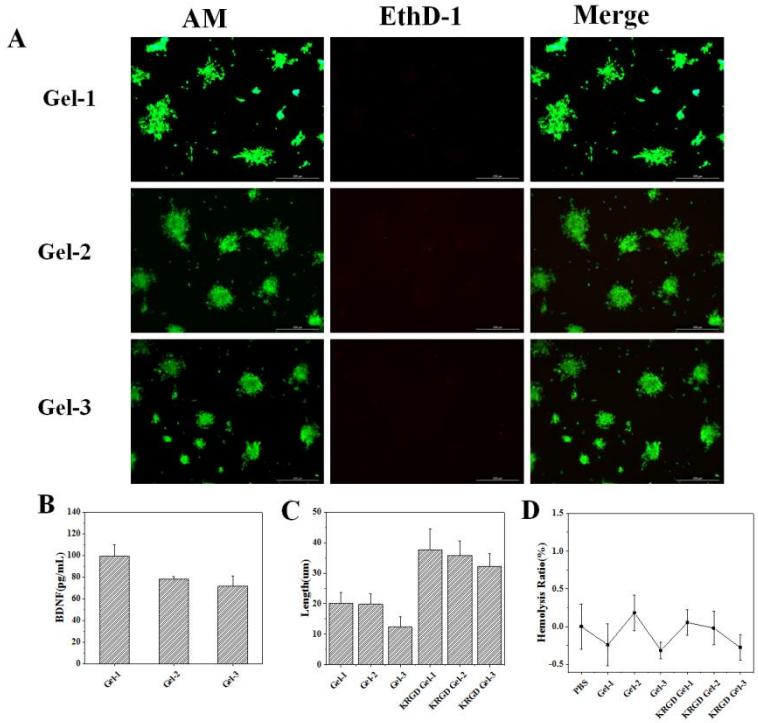


Figure S7 LIVE/DEAD viability assay (A), secreted BDNF (B), length (C), of Schwann cells and hemolysis ratio (D) of PAE/PEGDA hydrogels. Nonconjugated PAE/PEGDA hydrogels (Gel-1 and Gel-3), KRGD conjugated PAE/PEGDA hydrogels (KRGD Gel-1 and KRGD Gel-3), the scale bar represents 200 μm.