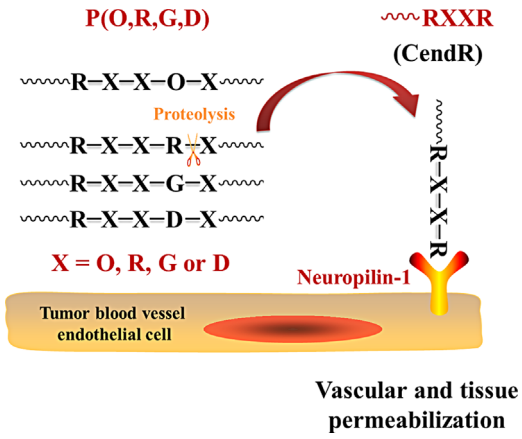
Poly(ornithine-co-arginine-co-glycine-co-aspartic Acid): Preparation via NCA Polymerization and its Potential as a Polymeric Tumor-Penetrating Agent[a]

Haiyang Yu, Zhaohui Tang,* Dawei Zhang, Wantong Song, Taicheng Duan, Jingkai Gu, Xuesi Chen

A novel random copolypeptide of ornithine, arginine, glycine, and aspartic acid [Poly(ornithine-co-arginine-co-glycine-co-aspartic acid), Poly(O,R,G,D)] has been prepared through ring-opening polymerization of *N*-δ-carbobenzoxy-l-ornithine *N*-carboxyanhydride [Orn(Cbz)-NCA)], l-glycine *N*-carboxyanhydride (Gly-NCA) and β-benzyl l-aspartate *N*-carboxyanhydride [Asp(Bn)-NCA], following by subsequent deprotection and guanidization. The structure of Poly(O,R,G,D) was confirmed by nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC). Low cytotoxicity of Poly(O,R,G,D) was confirmed from MTT assay. The Poly(O,R,G,D) contain some internal sequences of RXXR (X = O, R, G, or D) that could be proteolytically cleaved to expose the cryptic CendR element and bind to Neuropilin-1. This would lead to vascular and tissue permeabilization. Therefore trypsin-cleaved Poly(O,R,G,D) increase the vascular leakage of Evans blue from dermal microvessels at the injection site *in vivo* skin permeability assay. The intratumoral injection of the Poly(O,R,G,D) significantly enhanced the concentration of cisplatin-loaded nanoparticles in MCF-7 solid tumors. These results show that Poly(O,R,G,D) could increase the vascular leakage and tissue penetration of nanoparticles in a solid tumor and can be used as a potential polymeric tumor-penetrating agent.



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1. Introduction

The therapeutic efficacy of nanomedicines to many solid tumors is limited by their poor penetration into tumor tissue.[1,2] Yuan et al. reported that most stealth liposomes (90 nm) were located within a distance of 30 μm from the tumor vessel wall in about 1 week post injection.[3] Lee's study revealed that the traveling distance of block copolymer micelles in a solid tumor was extremely limited (less than 42 μm in 2 d post injection) even if the size of the micelles was as small as 25 nm.[4] Huo et al. reported that most of gold-coated nanoparticles (100 nm) were localized in the periphery of the tumor spheroid and around blood vessels, hindering deep penetration into tumors.[5] Therefore, nanomedicines actually had a low probability to reach a majority of target cells within a treated solid tumor, which was one of the main reasons that nanomedicines generally

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failed to provide superior efficacy to free drug systems in clinical trials.[6–11] To improve the efficacy of nanomedicines in terms of tumor permeability an agent with enhanced permeability of tumor tissue is highly desired.

Recently Ruoslahti et al. found that CendR peptide, where R/KXXR/K motif was exposed at the C-terminal of the peptide, could interact with the b1 domain of Neuropilin-1 (NRP-1) and cause cellular internalization and vascular leakage.[12–14] In particular, a tumor-penetrating peptide with a sequence of CRGDK/RGPD/EC (iRGD) was identified.[15–21] The iRGD showed a tumor-specific tissue penetration activity. When administered via the tail vein, the iRGD targeted to the tumor vascular because of the RGD motif affinity with integrins. The internal RGDK/R could be exposed and become an activated CendR peptide by a cleavage at the presence of a protease. The resultant RGDK/R interact with NRP-1, enhancing the tumor permeability and the tumor accumulation of coadministrated drugs such as nab-paclitaxel (ABX), doxorubicin (DOX), DOX liposomes, and trastuzumab.[16]

Polypeptides have found wide uses in biomedical application such as drug/gene delivery,[22–26] tissue engineering,[27,28] diagnostics,[29] and biosensors.[30] Polypeptides can be synthesized by the ring-opening polymerization (ROP) of *N*-carboxy anhydrides (NCA) of α-amino acids, which has become the most common technique used for large scale preparation of polypeptides with high molecular weight.[31–39] The NCA polymerization is well controlled without detectable side reactions for degree of polymerization close to 100.[40] When ammonium salts with non-nucleophilic tetrafluoroborate anions are used as initiators, multigram scale polyglutamate with defined molecular weight (up to 800 units) and low polydispersity (<1.2) can be synthesized by the ROP of NCAs.[41] Many copolypeptides have been reported to be prepared by the NCA polymerization.[42–45] Especially, as a typical copolypeptide, Glatiramer Acetate (copolymer-1; Cop-1; Copaxone®) has been approved as an effective treatment in relapsing multiple sclerosis (MS). [46] These indicate the great potential of copolypeptide in biomedical applications.

Herein we present the preparation of a random copolymer of ornithine, arginine, glycine, and aspartic acid [Poly(O,R,G,D)] through the NCAs polymerization followed by deprotection and guanidization. As a random copolymer, the Poly(O,R,G,D) should contain some internal sequences of RXXR, herein X represents O, R, G, or D. The internal sequences of RXXR can be proteolytically cleaved to expose the cryptic CendR element that then mediates binding to NRP-1. This will cause vascular and tissue permeabilization (Figure 1). Therefore Poly(O,R,G,D) after proteolysis should have the properties of CendR peptides. We therefore, for the first time prepared Poly(O,R,G,D) as a potential polymeric tumor-penetrating agent and evaluate its *in vivo* study.

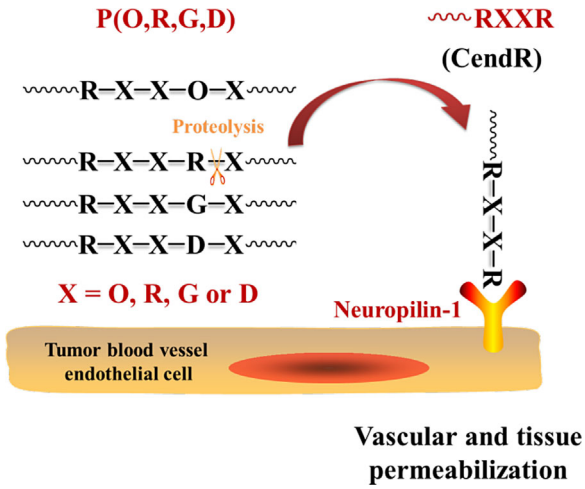

Figure 1. Schematic representation of the Poly(O,R,G,D) that is composed of –RXXOX–, –RXXRX–, –RXXGX–, and –RXXDX–. The –RXXRX– is proteolytically cleaved to expose the cryptic CendR element, –RXXXR, at the C-terminus. The activated CendR element then mediates binding to NRP-1, which causes vascular and tissue permeabilization.

2. Experimental Section

2.1. Materials

N-δ-Carbobenzoxy-l-ornithine, l-glycine and β-benzyl-l-aspartate were purchased from Shanghai Yeexin Biochem&Tech Co., Ltd., China. *N*-δ-Carbobenzoxy-l-ornithine *N*-carboxyanhydride [Orn(Cbz)-NCA)], l-glycine *N*-carboxyanhydride (Gly-NCA) and β-benzyl l-aspartate *N*-carboxyanhydride [Asp(Bn)-NCA] were prepared according to literature reported methods with slight modification.[47,48] Orn(Cbz)-NCA, Gly-NCA and Asp(Bn)-NCA were purified by recrystallization from ethyl acetate and n-hexane, then dried in vacuo at room temperature before use. Poly(l-glutamic acid) (PLG) and cisplatin-loaded nanoparticles (Cisplatin-NPs) were similarly prepared to our previous works.[49,50] n-Hexylamine, ether, 33 wt.-% solution of HBr in acetic acid and trifluoroacetic acid (TFA) were bought from Aladdin Industrial Corporation. Hyperbranched polyethylenimine (PEI, 25 kDa, PEI25K) was purchased from Aldrich. Peptide RPARPAR were custom-made by ChinaPeptides Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), branched polyethylenimine (average Mw ~25 000, PEI25K) and trypsin inhibitor from Glycine max (soybean) were purchased from Sigma–Aldrich. *N*, *N*′-dimetylformamide (DMF) was dried with CaH_2 for 3 d and distilled over CaH_2 under reduced pressure. 3,5-Dimethyl-1-pyrazolylformamidinium nitrate was purchased from J&K Scientific Ltd., China. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd., China and used as received.

2.2. Characterizations

^{1}H NMR and ^{13}C NMR spectra were recorded on an AV-300 or AV-400 NMR spectrometer (Bruker) at room temperature in trifluoroacetic acid-*d* (TFA-*d*) or D_2O/NaOD solution.

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Quantitative ^{13}C NMR were recorded at 300 K on a Bruker AVANCE III HD 500 MHz spectrometer with a 5 mm BBFO probe at 300 K for sample solution in D2O. The spectral widows were set to 31 KHz (250 ppm). The 90-degree high power pulse was 10.48 μs for ^{13}C. The inverted gated decoupling pulse sequence were generated and integrated in the Shape Tool of Topspin software version 3.2 pl6. A total of 3 000 scans were recorded with 6 s recycle delay for each sample. All ^{13}C chemical shifts are referenced to the resonances of DSS standard ($d = 0.00$). Gel permeation chromatography (GPC) measurement of Poly[O(Cbz), G,D(Bn)] was conducted on a Waters GPC system (Waters Styragel HT6E column, 1515 HPLC pump with OPTILAB DSP interferometric refractometer as the detector). The eluent was DMF containing 0.01 M lithium bromide (LiBr) at a flow rate of 1.0 mL min^{-1} at 40 °C. PMMA standards were used for calibration. GPC measurements of the Poly(O,G,D) and Poly(O,R,G,D) were performed on a Waters GPC system (Waters Ultrahydrogel Linear column, 1515 HPLC pump with 2414 Refractive Index detector). The eluent was phosphate buffer (PB, 0.2 M, pH 7.4) at flow rate of 1.0 mL min^{-1} at 25 °C. Monodispersed poly(ethylene glycol) standards were used to generate a calibration curve. Inductively coupled plasma mass spectrometry (ICP-MS, Xseries II, Thermoscientific, USA) was used for the quantitative determination of levels of platinum.

2.3. Synthesis of Poly[O(Cbz),G,D(Bn)]

Orn(Cbz)-NCA (0.400 g, 1.45 mmol), Gly-NCA (0.146 g, 1.45 mmol) and Asp(Bn)-NCA (0.361 g, 1.45 mmol) were dissolved in anhydrous DMF (20 mL) under an argon atmosphere. Then n-hexylamine (9.03 mg, 0.0893 mmol) in anhydrous DMF solution (1.0 mL) was added. The polymerization was performed under stirring at 25 °C. After 3 d, the solution was precipitated into excessive ether. Poly[O(Cbz),G,D(Bn)] was obtained by drying under vacuum at room temperature for 24 h. ^1H NMR (300M, TFA-*d*, 298K) of Poly[O(Cbz),G,D(Bn)]: δ 7.17 ppm (br, 10H, $-CH_2C_6\boldsymbol{H}_5$), 5.03 ppm (br, 4H, $-C\boldsymbol{H}_2C_6H_5$), 4.95 ppm (br, 1H, –C(O)C***H***< from Orn residues), 4.47 ppm (br, 1H, –C(O)C***H***< from Asp residues), 4.08 ppm (br, 2H, –C(O)C$\boldsymbol{H}_2$– from Gly residues), 3.11 ppm (br, 2H, $-C\boldsymbol{H}_2$COO– from Asp residues), 2.98 ppm (br, 2H, $-CH_2C\boldsymbol{H}_2$NH– from Orn residues), 1.84 ppm (br, 2H, $-C\boldsymbol{H}_2$–CH< from Orn residues), and 1.57 ppm(br, 2H, $-CH_2C\boldsymbol{H}_2CH_2$– from Orn residues). ^{13}C NMR (100 MHz, TFA-*d*, 298 K) of Poly[O(Cbz),G,D(Bn)]: δ 175–172 (br), 134.9, 130.0, 129.7, 129.4, 129.2, 128.8, 128.5, 71.7, 69.6, 55.0, 54.1, 51.3, 43.6, 35.8, 41.1, 36.3, 29.2, and 24.0 ppm. M_n determined by GPC: 25.8×10^3 g mol^{-1}. M_w/M_n determined by GPC: 1.08.

2.4. Synthesis of Poly(O,G,D)

The obtained Poly[O(Cbz), G,D(Bn)] (0.65 g) was dissolved in 6.5 mL of trifluoroacetic acid. The reactor was placed in a ice-water bath, and then 2 mL of HBr/acetic acid (33 wt.-%) was slowly added in. The solution was stirred at 25 °C for 2 h before precipitated into excess ether. After drying under vacuum, the precipitate was dialyzed with distilled water and freeze-dried to give the Poly(O,G,D). ^1H NMR (400 M, NaOD/D_2O, 298 K): δ 4.65 ppm (br, 1H, –C(O)C***H***< from Orn residues), 4.36 ppm (br, 1H, –C(O)C***H***< from Asp residues), 3.99 ppm (br, 2H, –C(O)C$\boldsymbol{H}_2$– from Gly residues), 3.03 ppm (br, 2H, $-C\boldsymbol{H}_2$COO– from Asp residues), 2.72 ppm (br, 2H, $-CH_2C\boldsymbol{H}_2$NH– from Orn residues), 1.75 ppm (br, 4H, >CHC$\boldsymbol{H}_2C\boldsymbol{H}_2$– from Orn residues). ^{13}C NMR (100 MHz, NaOD/D_2O, 298 K) of the Poly(O,G,D): δ 177.4, 174.1, 173.3, 172.2, 171.4, 53.3, 51.5, 42.5, 42.8, 38.8, 38.3, 27.7, 23.2 ppm. M_n determined by GPC: 3.5×10^3 g mol^{-1}. M_w/M_n determined by GPC: 1.27.

2.5. Preparation of Poly(O,R,G,D)

Poly(l-ornithine, l-arginine, l-Glycine, l-aspartate) [Poly(O,R,G,D)] was prepared by the guanidization of the obtained Poly(O,G,D) with 3,5-dimethyl-1-pyrazolylformamidinium nitrate. The Poly(O,G,D) (0.20 g) and 3,5-dimethyl-1-pyrazolylformamidinium nitrate (0.152 g) were dissolved in 15 mL of distilled water, then the pH of the solution was adjusted to 9.3 with 1 mol L^{-1} sodium hydroxide. The reaction mixture was kept under stirring at 37 °C for 48 h, and then dialyzed against distilled water for 48 h. The solution was freeze-dried to give the Poly(O,R,G,D) product in white powders. ^1H NMR (300 M, NaOD/D_2O, 298 K) of Poly(O,R,G,D): δ 4.69 ppm (br, 1H, –C(O)C***H***< from Orn and Arg residues), 4.36 ppm (br, 1H, –C(O)C***H***< from Asp residues), 4.00 ppm (br, 2H, –C(O)C$\boldsymbol{H}_2$– from Gly residues), 3.05 ppm (br, 2H, $-C\boldsymbol{H}_2$COOH), 2.78 ppm (br, 2H, $-C\boldsymbol{H}_2$NH–), 1.77 ppm (br, 4H, >CHC$\boldsymbol{H}_2C\boldsymbol{H}_2$– from Orn and Arg residues). ^{13}C NMR (100 MHz, NaOD/D_2O, 298 K) of Poly(O,R,G,D): δ 177.3, 174.1, 173.4, 172.1, 171.4, 156.7, 53.5, 51.4, 42.5, 40.5, 38.9, 38.2, 27.9, 24.4, and 23.3 ppm. M_n determined by GPC: 5.0×10^3 g mol^{-1}. M_w/M_n determined by GPC: 1.19.

2.6. Cell Culture and Tumor Model

MCF-7 (Human breast adenocarcinoma cell line) and PC-3 cells (Human prostate cancer cell line) were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U mL^{-1}) and streptomycin (50 U mL^{-1}).

All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Animal Care and Use Committee of Jilin University. MCF-7 xenografts were created by injecting female Balb/C nude mice (6 weeks old, 20 g body weight, Beijing HFK Bioscience Co. Ltd., China) with MCF-7 cells (0.15 mL, 1.5×10^6 cells) orthotopically into the mammary fat pad of each mouse.

2.7. Cytotoxicity Assay

Two kinds of cells, MCF-7 and PC-3 were used to test the *in vitro* cytotoxicity of the Poly(O,R,G,D). MCF-7 and PC3 cells were seeded in 96-well culture plates at a density of 10^4 cells per well in 100 uL DMEM and allowed to attach for 24 h. Then cells were reseeded with the Poly(O,R,G,D), PEI25K or PLG at different concentrations and incubated for another 48 h. The cell viability was analyzed by using MTT and measured in a Bio-Rad 680 microplate reader at a wavelength of 490 nm.

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2.8. *In vivo* Skin Permeability Assay

Firstly, the obtained Poly(O,R,G,D) was dissolved in phosphate-buffered saline (PBS, pH 7.4, 0.2 M) to produce solutions with Poly(O,R,G,D) concentration of 0.0658, 0.329, or 1.646 mg mL^{-1}. The solutions were treated with crystalline trypsin (0.625 μg mL^{-1}) at 37 °C for 30 min. After that, the proteolytic reaction was terminated with soybean trypsin inhibitor (5 mg mL^{-1}). The trypsin-cleaved Poly(O,R,G,D) solutions were obtained and used without further purification.

A modified Miles vascular leakage assay was applied to evaluate the influence of trypsin-cleaved Poly(O,R,G,D) on tissue penetration.[14] Balb/c nude mice were injected intravenously with a tracer Evans Blue (0.24 mg) in 200 μL of PBS. Ten minutes later the mice were injected intradermally on the ventral skin with PBS (40 μL), trypsin (0.625 μg mL^{-1}) + soybean trypsin inhibitor (5 mg mL^{-1}), polyglutamic acid, RPARPAR, Poly(O,R,G,D) solutions (0.0658, 0.329, or 1.646 mg mL^{-1} in 40 μL of PBS) or trypsin-cleaved Poly(O,R,G,D) solutions (0.0658, 0.329, or 1.646 mg mL^{-1} in 40 μL of PBS). Thirty minutes later the mice were killed by cervical dislocation, and the skin in the injection area was removed. Samples of skin (Square: ~0.5 cm^2) were cut out from the injection sites and photographed.

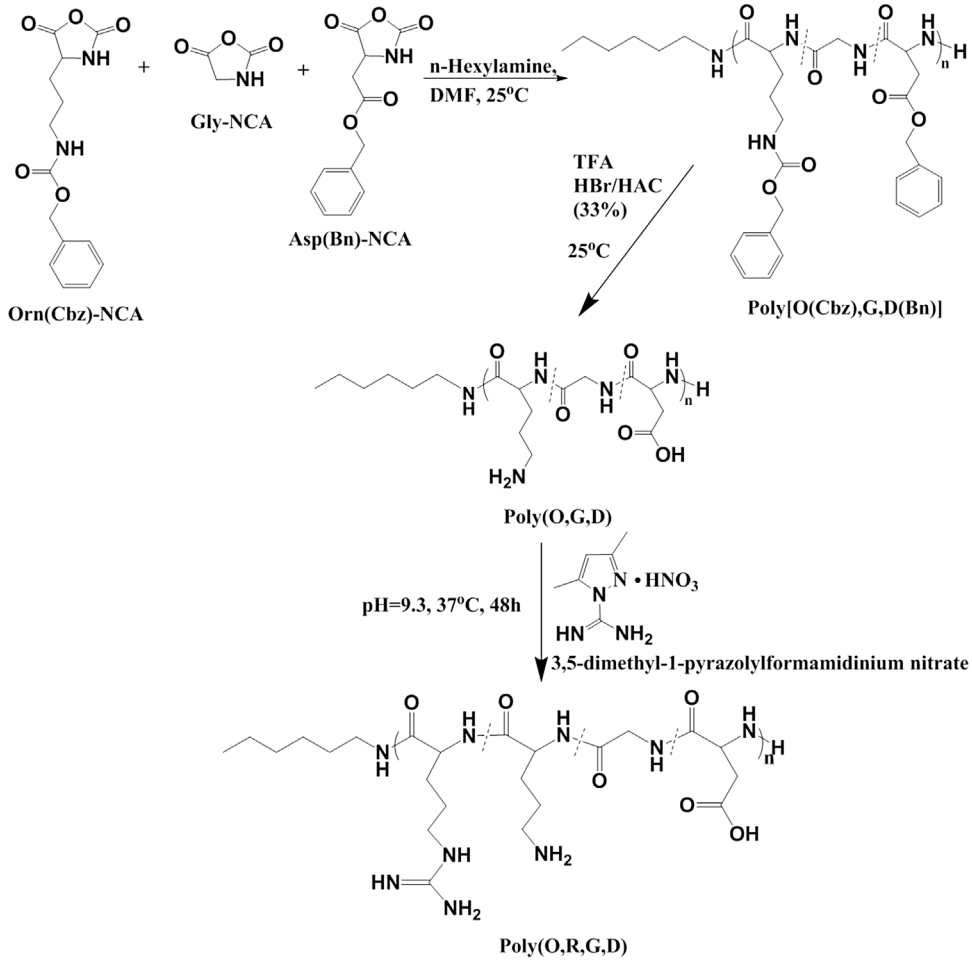
2.9. Tumor Concentration of Cisplatin-NPs With or Without Poly(O,R,G,D)

When MCF-7 tumor volume was approximately 250 mm^3, the tumor-bearing mice were divided into three groups [Control, Poly(O,R,G,D) and trypsin-cleaved Poly(O,R,G,D)]. Each group was composed of 5 mice. Poly(O,R,G,D) (20 mg kg^{-1}) and trypsin-cleaved Poly(O,R,G,D) [20 mg kg^{-1} on a Poly(O,R,G,D) basis] were intratumorally injected into the MCF-7 tumors, respectively. After 30 min, Cisplatin-NPs were administered to each mouse via tail vein injection at a dose of 12 mg kg^{-1} on a cisplatin basis. The mice were sacrificed after 4 h of the Cisplatin-NPs treatment. The tumors were excised and decomposed on heating in nitric acid. The platinum concentration in the solution was measured by ICP-MS.

3. Results and Discussion

3.1. Synthesis and Characterization of Poly(O,R,G,D)

The Poly(O,R,G,D) was prepared in three steps. As shown in Scheme 1, Poly[O(Cbz), G,D(Bn)] was firstly synthesized



Scheme 1. Synthesis of Poly(O,R,G,D).

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by the random copolymerization of Orn(Cbz)-NCA, Gly-NCA, and Asp(Bn)-NCA using n-hexylamine as initiator. Poly(O,G,D) was then generated from the deprotection of β-benzyl groups and *N*-δ-carbobenzoxy groups of the Poly[O(Cbz), G,D(Bn)] in HBr/acetic acid. Finally Poly(O,R,G,D) was prepared by the guanidization of the Poly(O,G,D) with 3,5-dimethyl-1-pyrazolylformamidinium nitrate in aqueous solution (pH = 9.3). The ^{1}H NMR spectra of the Poly[O(Cbz), G,D(Bn)], Poly(O,G,D), and Poly(O,R,G,D) were shown in Figure 2. For the Poly[O(Cbz), G,D(Bn)], the signals at δ 4.95, 2.98, 1.84, and 1.57 ppm were assigned to the protons of methine (a), –CH_2CH_2NH– (d), –CH_2–CH< (b) and –$CH_2CH_2CH_2$– (c) groups of Orn units, respectively. The signals at δ 4.08 ppm was attributed to the protons of methylene (e) groups of Gly units. The resonances at δ 4.47 and 3.11 ppm were assigned to the protons of the methine (f) and –CH_2COOH of Asp units, respectively. The signals at δ 7.17 ppm (–$CH_2C_6H_5$) and 5.03 ppm (–$CH_2C_6H_5$) in the spectrum of the Poly[O(Cbz), G,D(Bn)] disappeared in the spectrum of the Poly(O,G,D), indicating the complete removal of the β-benzyl groups and *N*-δ-carbobenzoxy groups. The ^{13}C NMR spectra of the Poly[O(Cbz), G,D(Bn)], Poly(O,G,D) and Poly(O,R,G,D) were shown in Figure 3. The signals of benzyl groups in the spectrum of the Poly[O(Cbz), G,D(Bn)] completely disappeared in the spectrum of the Poly(O,G,D), further confirming the successful removal of the β-benzyl groups and N-δ-carbobenzoxy groups. Compared with the ^{13}C NMR spectrum of the Poly(O,G,D), three new peaks at δ 156.7 ppm [HN=$C(NH_2)$–], 40.5 ppm [–CH_2NHC-(NH)NH_2] and 24.3 ppm [–$CH_2CH_2CH_2$–] that were attributed to arginine residues, appeared in the spectrum of the obtained Poly(O,R,G,D). This phenomenon indicates that the guanidization of the Poly(O,G,D) was successful. In the ^{13}C NMR spectrum of the Poly(O,G,D) (Figure 3(b)), the carbonyl groups at the backbone of the copolymer did not show three narrow peaks but four broad signals at δ 174.1, 173.3, 172.2, and 171.4 ppm, the methine and methylene groups at the backbone of the copolymer displayed three slightly broad signals at δ 53.3, 51.5, and 42.5 ppm, suggesting that the Poly(O,G,D) was not a block copolymer but a random copolymer. The Poly(O,R,G,D) was also a random copolymer because it was prepared from the Poly(O,G,D). The molar ratio of O(Cbz)/G/D(Bn) of the obtained Poly[O(Cbz), G,D(Bn)] was 0.96/1.1/1.0, determined from the integration ratio of resonances at δ 2.2–1.3 ppm [>CHCH_2CH_2–, b + c (Orn)], 4.08 ppm [–C(O)CH_2–, e (Gly)] and 3.11 ppm [–CH_2COOH, g (Asp)] as shown in Figure 2(a). Using a similar method, the molar ratio of O/G/D (1.0/1.2/1.0) of the obtained Poly(O,G,D) was determined from the Figure 2(b). The molar ratio of (O + R)/G/D in the obtained Poly(O,R,G,D) was 1.0/1.0/1.0, which was calculated based on the intensities ratio of signals at δ 4.00 [–C(O)CH_2–, e (Gly)], 3.05 [–CH_2COOH, g

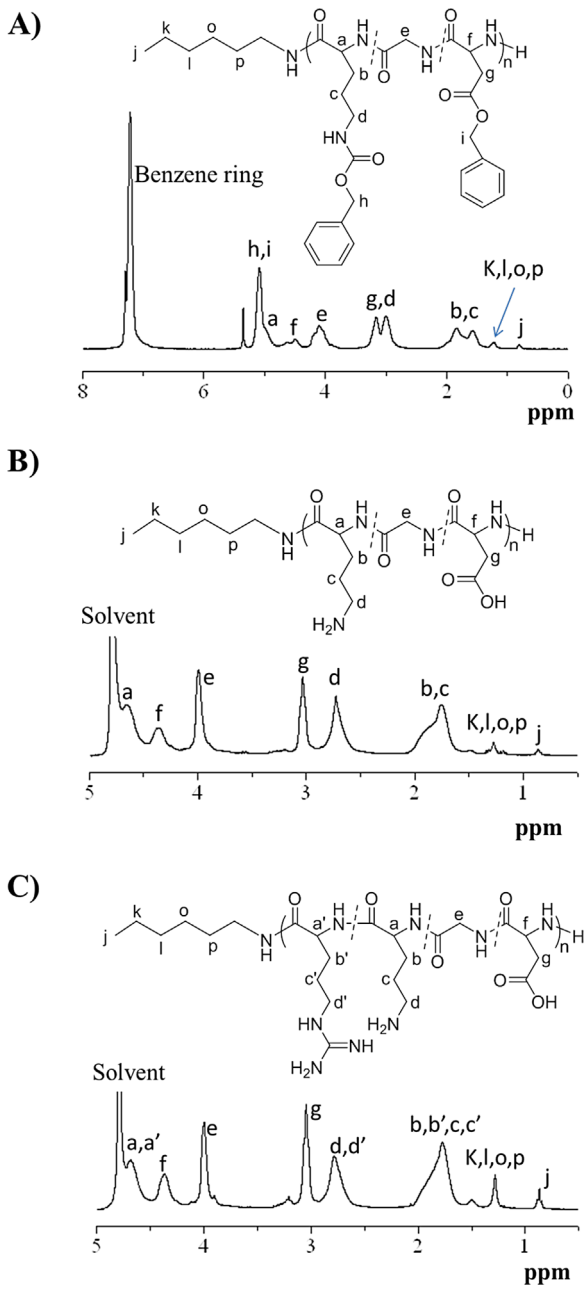

Figure 2. ^{1}H NMR spectra of Poly[O(Cbz),G,D(Bn)] in trifluoroaceticacid-*d* (a), Poly(O,G,D) (b) and Poly(O,R,G,D) (c) in NaOD/D_2O.

(Asp)] and 1.77 ppm [>CHCH_2CH_2–, bb′cc′ (Arg + Orn)] in the ^{1}H NMR spectrum of the Poly(O,R,G,D). This suggest that the resultant molar ratio of (O + R)/G/D of the Poly(O,R,G,D) was close to the feed molar ratio of Orn(Cbz)-NCA/Gly-NCA/Asp(Bn)-NCA (Table 1). The molar ratio of O/R was 0.28/0.72, calculated according to the intensity ratio of signals at δ 24.4 (–$CH_2CH_2CH_2$– from Arg residues) and 23.3 ppm (–$CH_2CH_2CH_2$– from Orn residues) in the quantitative ^{13}C NMR spectrum of the Poly(O,R,G,D)

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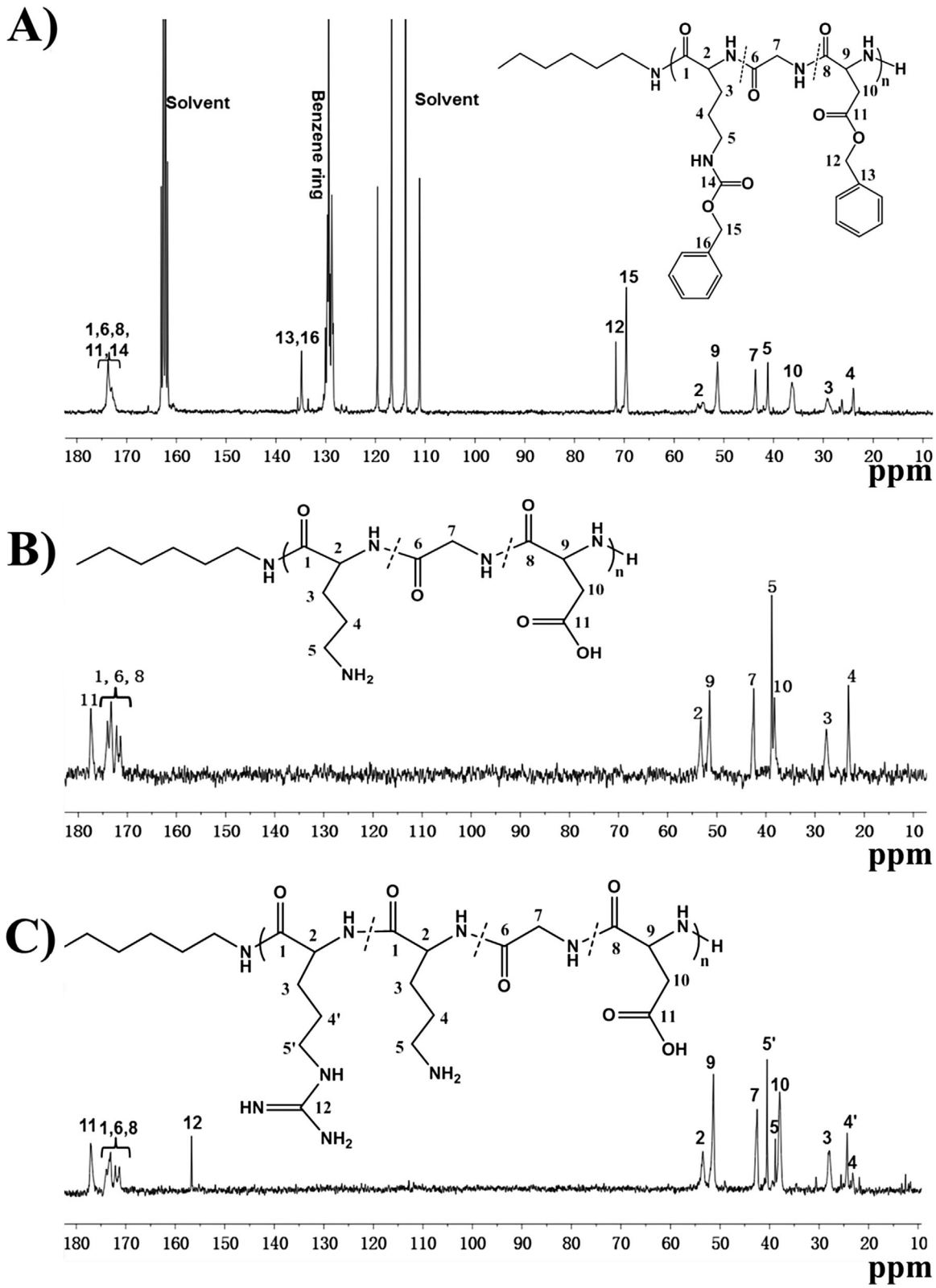

Figure 3. ^{13}C NMR spectra of Poly[O(Cbz),G,D(Bn)] in trifluoroaceticacid-*d* (a), Poly(O,G,D) (b) and Poly(O,R,G,D) (c) in NaOD/D_2O.

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Table 1. Characterization of polypeptides.

	Feed molar ratio[a]	Resultant molar ratio	X_n^{NMR} [e]				$M_n^{NMR} \times 10^{-3}$ [g mol^{-1}][f]	$M_n^{GPC} \times 10^{-3}$ [g mol^{-1}][g]	D[j]
			O	R	G	D			
Poly[O(Cbz), G,D(Bn)]	1.0/1.0/1.0	0.96/1.1/1.0[b]	16	–	18	16	8.4	25.8[h]	1.08[h]
Poly(O,G,D)	–	1.0/1.2/1.0[c]	15	–	18	15	4.6	3.5[i]	1.27[i]
Poly(O,R,G,D)	–	0.28/0.72/1.0/1.0[d]	4	11	15	15	4.9	5.0[i]	1.19[i]

[a]Feed molar ratio of Orn(Cbz)-NCA/Gly-NCA/Asp(Bn)-NCA; [b]Resultant molar ratio of O(Cbz)/G/D(Bn) determined by ^{1}H NMR; [c]Resultant molar ratio of O/G/D determined by ^{1}H NMR; [d]Resultant molar ratio of O/R/G/D determined by ^{1}H NMR and quantitative ^{13}C NMR; [e]Degree of polymerization determined by NMR; [f]Number-average molecular weight determined by NMR; [g]Number-average molecular weight determined by GPC; [h]Eluent: DMF containing 0.01 M LiBr, PMMA standards; [i]Eluent: PB (0.2M, pH 7.4), PEG standards. [j]Dispersity determined by GPC.

(Figure 4). As can be seen in Figure 5(a), the GPC analysis of the Poly[O(Cbz), G,D(Bn)] revealed a narrow monomodal molecular weight distribution ($M_w/M_n = 1.08$), indicate well controlled polymerization of the NCA. However, low molecular weight tailing was observed in the GPC profiles of the Poly(O,G,D) and Poly(O,R,G,D) (Figure 5(b,c)), which might be due to slight breakage of the backbone of the copolymers in the deprotection process or the coexistence of polypeptides in different superstructures.[40] The average molecular weight of the Poly(O,R,G,D) was higher than that of Poly(O,G,D) (Table 1) confirmed the successful preparation of Poly-(O,R,G,D).

3.2. *In vitro* Cytotoxicity

The relative cytotoxicity of the Poly(O,R,G,D) was evaluated with MTT assays with PEI25K as positive control and PLG as negative control. Two cell lines, MCF-7 and PC-3 were applied. As shown in Figure 6, the viabilities of MCF-7 and PC-3 cells treated with the Poly(O,R,G,D) were around 100% at all tested concentrations in 48 h even at 500 μg mL^{-1}. In contrast, the PEI25K showed significant inhibition to the proliferation of MCF-7 and PC-3 cells when the concentration of Poly(O,R,G,D) was above 15.625 μg mL^{-1}. These results proved the low cytotoxicity of Poly(O,R,G,D).

3.3. Poly(O,R,G,D) Causes Vascular Leakage and Tissue Penetration

CendR peptide is known to be able to regulate vascular permeability and cause vascular leakage via CendR peptide–NRP-1 interaction.[14] As a random copolypeptide, the Poly(O,R,G,D) should possess some internal RXXR sequences (X = O, R, G, or D) that can be activated to CendR peptides by proteolysis. To test this, we treat Poly(O,R,G,D)

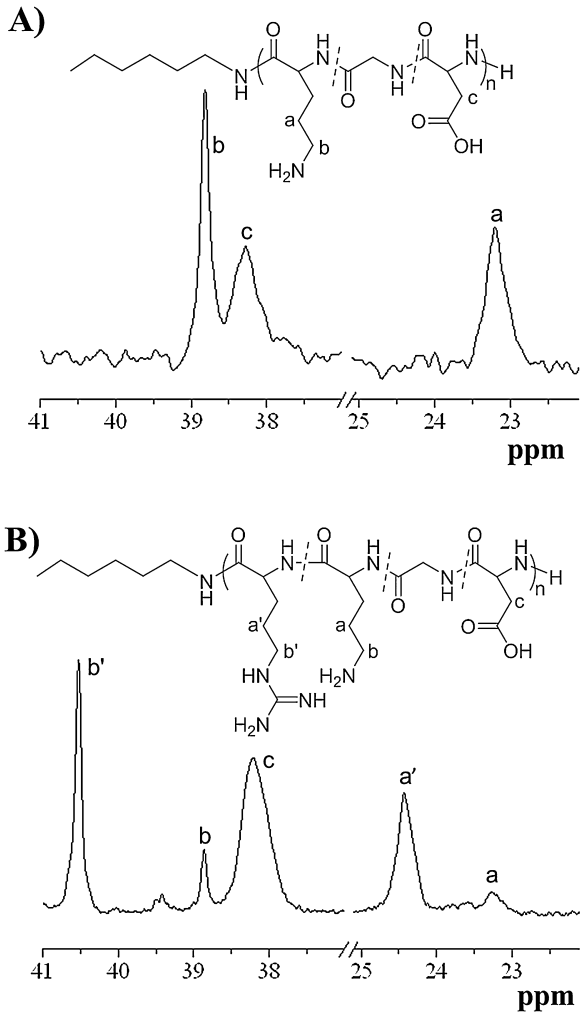

Figure 4. Selected regions of quantitative ^{13}C NMR spectra of Poly(O,G,D) (a) and Poly(O,R,G,D) (b) in NaOD/D_2O.

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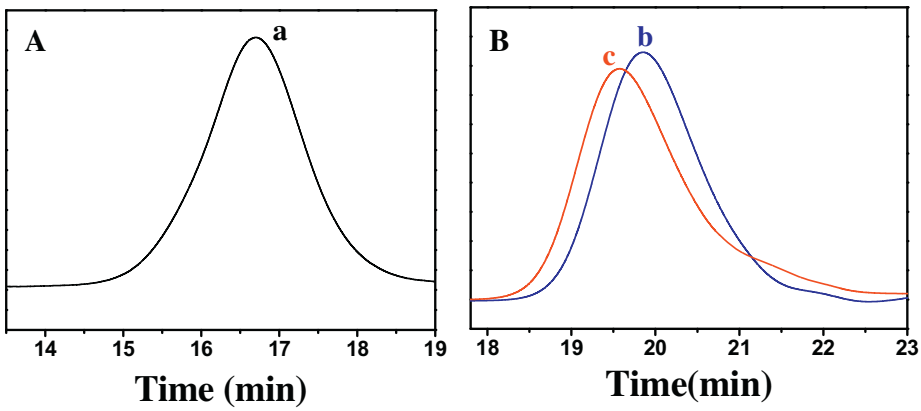

Figure 5. GPC curves of Poly[O(Cbz), G,D(Bn)], Poly(O,G,D) and Poly(O,R,G,D). (a) *a* = Poly[O(Cbz), G,D(Bn)] using DMF containing 0.01 M LiBr as eluent; (b) *b* = Poly(O,G,D), *c* = Poly(O,R,G,D) using PB (pH = 7.4 0.2 M) as eluent.

with trypsin, which cleaves peptide chains mainly at the carboxyl side of the arginines in the random copolypeptide.[51] A modified Miles vascular leakage assay was used to investigate the influence of trypsin-cleaved Poly(O,R,G,D) on vascular leakage and tissue permeability of skin. RPARPAR, a typical CendR peptide, was used as positive control.[14] Polyglutamic acid was used as negative control. Evans blue administered via intravenous injection was used as a trace. As shown in Figure 7, both the trypsin-cleaved Poly(O,R,G,D) and RPARPAR peptide increased the vascular leakage of the tracer from dermal microvessels at the injection site in a concentration-dependant manner. In contrast, the injection of PLG, uncleaved Poly(O,R,G,D), trypsin solution (0.625 $\mu g\,mL^{-1}$ trypsin + 5 $mg\,mL^{-1}$ soybean trypsin inhibitor) or PBS did not enhance the vascular leakage of Evans blue at the injection site. This indicates that trypsin-cleaved Poly(O,R,G,D) was a potential agent that could cause vascular leakage and tissue penetration.

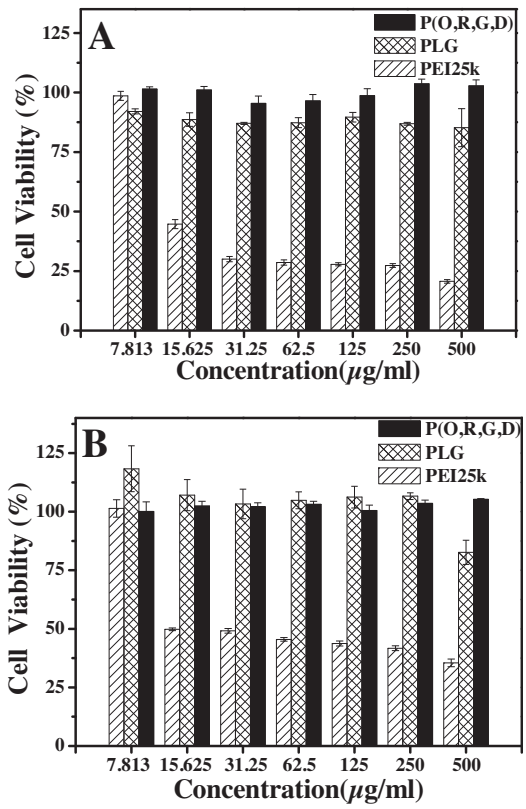

Figure 6. In vitro cytotoxicities of Poly(O,R,G,D), PLG and PEI25K to MCF-7 cells (a) and PC-3 cells (b) in 48 h. Data are presented as the mean ± STD (*n* = 3).

In order to study the ability of the Poly(O,R,G,D) to enhance the vascular leakage and permeability of solid tumor tissue, the influence of Poly(O,R,G,D) and trypsin-cleaved Poly(O,R,G,D) on the concentration of systemically administered Cisplatin-NPs in a solid tumor was investigated. MCF-7 xenografted BALB/c nude mouse model was used because NRP-1 was expressed in MCF-7 cells.[52] Poly(O,R,G,D) and trypsin-cleaved Poly(O,R,G,D) were directly injected into the MCF-7 tumors, respectively. As shown in Figure 8, tumors injected with the Poly(O,R,G,D) or trypsin-cleaved Poly(O,R,G,D) exhibited significant higher platinum concentration than that of control group while Poly(O,R,G,D) group showed similar platinum concentration to the trypsin-cleaved Poly(O,R,G,D) group. These phenomena suggested that the Poly(O,R,G,D) could increase the vascular leakage and tissue penetration in a solid tumor. The intratumor injection of Poly(O,R,G,D) increase the concentration of Cisplatin-NPs in MCF-7 tumors. This could be explained on the basis of in situ proteolysis of the Poly(O,R,G,D) in the treated tumor which generates various C-terminal RXXR sequence (CendR peptide) capable of binding to NRP-1 and induces vascular and tissue permeabilization. A cryptic CendR element could be exposed through the

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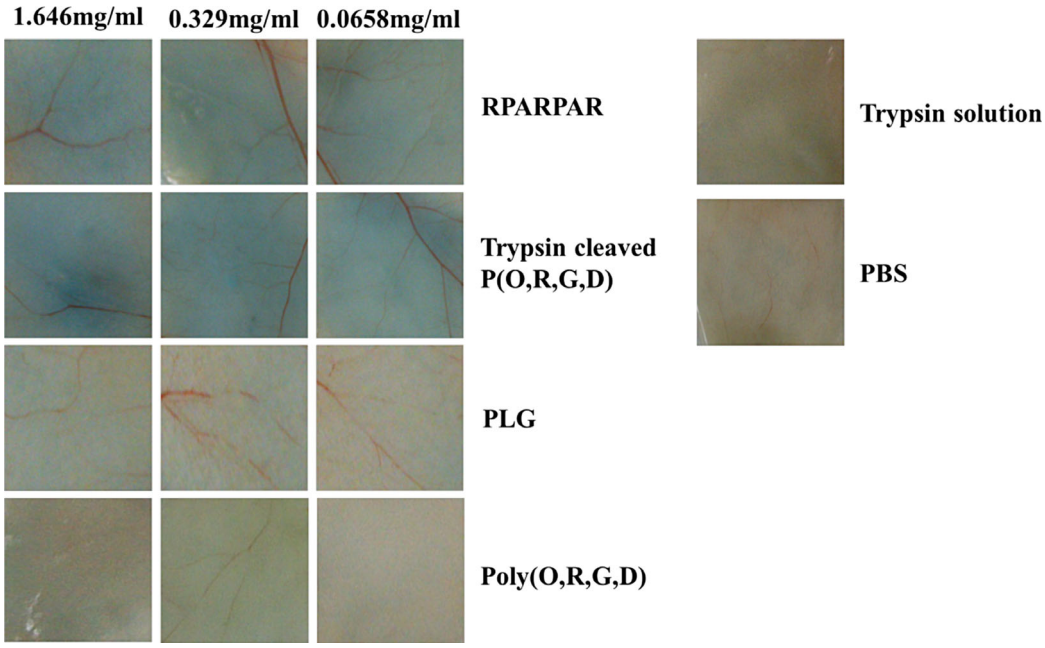

Figure 7. Miles vascular leakage assay: Macroscopic appearance of Evans blue leakage in skin samples of mice preinjected intradermally with RPARPAR peptide, trypsin-cleaved Poly(O,R,G,D), PLG, Poly(O,R,G,D), trypsin solution in 40 μL of PBS or PBS only (40 μL). Trypsin-cleaved Poly(O,R,G,D) was a cocktail of copolymer–copolymer fragments-trypsin–soybean trypsin inhibitor with [trypsin] = 0.625 μg mL^{-1} and [soybean trypsin inhibitor] = 5 mg mL^{-1}. Trypsin solution: 0.625 μg mL^{-1} trypsin + 5 mg mL^{-1} soybean trypsin inhibitor.

proteolytical process in a solid tumor, which was consistent with literature report.[16] The *in vivo* expression of trypsin by vascular endothelial cells may be connected with proteolytical process of the Poly(O,R,G,D). [53]

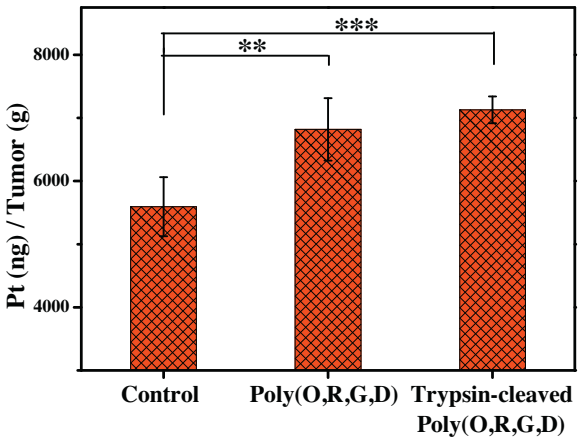

Figure 8. Platinum concentration in the tumor at 4 h after a single administration of Cisplatin-NPs (12 mg kg^{-1} on a cisplatin basis) in MCF-7 bearing mice *via* tail vein injection. Control: blank; 30 min before the administration of Cisplatin-NPs, trypsin-cleaved Poly(O,R,G,D) and Poly(O,R,G,D) groups were administered via intratumoral injection with trypsin-cleaved Poly(O,R,G,D) and Poly(O,R,G,D), respectively. Data are presented as the mean ± STD ($n = 5$) and analyzed for statistical significance using Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to control.

4. Conclusion

A novel polymeric tumor-penetrating agent Poly(O,R,G,D) has been presented. The random copolypeptide Poly(O,R,G,D) was successfully prepared by random polymerization of Orn(Cbz)-NCA, Gly-NCA, and Asp(Bn)-NCA followed by deprotection and guanidization. The Poly(O,R,G,D) showed low toxicity and good cell compatibility. Trypsin-cleaved Poly(O,R,G,D) exhibit an ability of increasing vascular leakage of Evans blue from dermal microvessels at the injection site. The intratumoral injection of Poly(O,R,G,D) significantly enhanced the concentration of cisplatin-loaded nanoparticles in MCF-7 solid tumors. These phenomena suggest that Poly(O,R,G,D) could increase the vascular leakage and tissue penetration in a solid tumor. Therefore, Poly(O,R,G,D) is a potential polymeric tumor-penetrating agent that can enhance tumor concentration and efficacy of nanomedicines in the treatment of solid tumors.

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