

Degradation behavior and biosafety studies of the mPEG-PLGA-PLL copolymer

Zelai He ^{a, b}, Ying Sun ^a, Jun Cao ^c, Yourong Duan ^{a, *}

^a State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 20003, China

^b The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, 109 Xueyuan Western Road, Wenzhou 325027, China

^c Dahua Hospital, Xuhui District, Shanghai 200237, China

EXPERIMENTAL SECTION

1. Materials

PEG with a molecular weight (Mw) of 2000, tricaine, phenol red, 1-phenyl-2-thiourea (PTU) was obtained from Sigma-Aldrich (China) Co., Ltd. (Shanghai, China). L-lactide and glycolide were purchased from Yuanshengrong Co. (Beijing, China). N ϵ -carbobenzyloxy-L-lysine N-carboxyanhydride (N ϵ -CBZ-L-lysine NCA) was obtained from Shanghai Yuaniu Biotechnology Co. (Shanghai, China). Pluronic 188 (F68) was purchased from BASF (Ludwigshafen, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Fluorescein isothiocyanate (FITC) and inulin were obtained from Beyotime Institute of Biotechnology. Prothrombin time (PT) kit, activated partial thromboplastin time (APTT) kit and complement C3 kit were purchased from Jiancheng Bioengineering institute (Nanjing, China). Reactive

oxygen species (ROS) kit and acridine orange (AO) were obtained from Qcbio Science & Technologies Co., Ltd. Lactic acid, lysine, polystyrene standard and tetramethylsilane were purchased from National Institute of Metrology, China. All cell culture media and reagents were obtained from Gibco (Grand Island, NY, USA) unless otherwise specified. Glutaraldehyde solution, methylene chloride, acetone, ethyl acetate, et al.; other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

L929 (murine fibroblast cell) and Huh7 (human hepatic carcinoma cell) cell lines were grown in a high-glucose DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C under 5% CO₂.

Zebrafishs (AB strain of *Danio rerio*) were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS) and tested in a strict compliance with the “Guide for the Care and Use of Laboratory Animals”.

2. Synthesis of PEAL/FITC-PEAL copolymers

PEAL (Mw 12000, mPEG 10%) was prepared according to our previous protocol with minor modifications (Scheme 1) [1, 2, 3]. In brief, the PEAL was prepared as follows: (1) the mPEG-PLGA-OH copolymer was prepared using zinc lactate as a catalyst; (2) its end-group –OH was changed into –NH₂. The synthesis of mPEG-PLGA-poly(Nε-(Z)-L-lysine) was performed by ring-opening polymerization of the initiated NCA with the amino-terminated mPEG-PLGA; (3) the PEAL

copolymer was obtained in an HBr/CH₃COOH solution by removing the N ϵ -(carbonylbenzoxy) end-group of the mPEG-PLGA-poly(N ϵ -(Z)-L-lysine) copolymer.

PEAL, FITC and Triethylamine were mixed in DMSO in a molar ratio of 1: 1: 2. The solution was kept at room temperature overnight until completely reacted. The mixture was then transferred into a dialysis bag and stirred overnight to get rid of the FITC with a small molecular weight. During the dialysis, water was replaced with freshly distilled water every 4 h. After the material in the dialysis bag was frozen and dried, PEAL-FITC was collected. The whole experiments were performed in the dark to avoid any possible light influence.

3. Platelet adhesion test.

Fresh blood from healthy human volunteers was collected in siliconized tubes at a ratio of 9:1 between blood and sodium citrate (3.8 wt %). Platelet-rich plasma (PRP) was obtained by centrifugation at 1 200 rpm for 10 min [4]. PEAL polymer films and the control PLGA films were cut into 1 \times 1 cm with a thickness of about 0.2 mm, immersed in PBS solution (pH 7.4, 0.01 mol/L), sufficiently swelling equilibrium, and then immersed in PRP for 2 h at 37 $^{\circ}$ C. The films were gently washed for three times with PBS solution and fixed by using 2.5% glutaraldehyde solution for 12 h to fix the blood components on the films. Finally, they were dehydrated by successive immersion in ethanol at several different concentrations (50, 75, 90, 100% v/v) for 15-20 min and dried at room temperature [5, 6, 7]. A thin layer of Au film was sputtered on the platelet-attached surface to facilitate the scan electron microscopy (SEM)

observation [8].

4. Water contact angle measurements and water uptake test.

The polymer films (1×1 cm²) were immersed in deionized water at 37 °C. They were then taken out at predetermined time. Surplus water was removed by using filter paper [9, 10].

Contact angle measurements. After films co-cubated with deionized water for 12 h, the films were dried under vacuum and 10 μL of deionized water was gently released on the film using an Acura pipette. The contact angle measurements were done with a high performance photo camera connected to a computer for image acquisitions. The images were treated and analyzed using the Image J software to obtain the curvature angles of the water drops.

The water uptake test. The water uptake was calculated according to the following equation:

$$\text{Water absorption (\%)} = (W_t - W_0)/W_0 \times 100\%.$$

Where W_0 is the weight of the dry films and W_t is the weight of the wet films at time t.

5. In vitro degradation behavior.

The weight loss, gel permeation chromatograph and proton nuclear magnetic resonance (¹H-NMR). Polymer films (50-70 mg, 1×1 cm²) were placed in 5 mL of 0.1 M PBS solutions with pH values of 5.0, 7.4 and 9.1, respectively, at 37 °C and vibrated at 72 rpm. The PBS solution was replaced frequently to maintain a constant

pH value. At the predetermined time, the films were taken out and rinsed gently two times with distilled water to clear up the adhered salt, and then dried with vacuum over at 37 °C before testing.

The weight loss. The degree of degradation was calculated by the following equation:

$$\text{Weight loss (\%)} = (W_0 - W_t)/W_0 \times 100.$$

Where W_0 represents the initial weight of the films before degradation and W_t represents the dry weight of the films after t days of degradation.

Gel permeation chromatograph. The molecular weight and the distribution of the left polymers films were measured by Gel permeation chromatograph (GPC) performed on a Polymer PL-GPC 50 GPC system with an RI detector (Polymer laboratories, UK) under 240 nm wavelength. Chloroform was used as the mobile phase at a flow rate of 1.0 mL/min. 20 μ L of polymer solutions were pumped for each analysis. Polystyrene (molecular weight range from 500 to 480000) was used as the standard.

¹H-NMR. The composition of the left polymer films were determined by ¹H-NMR using a Bruker Avance 400 NMR spectrometer with CDCl₃ as a solvent. Tetramethylsilane was used as the internal reference to determine chemical shifts (δ) in ppm [11, 12].

The detection of pH value, lactic acid and lysine in degradation medium.

Preparation of samples and the detection of pH value: Polymer films (50 mg, 1 \times 1 cm²) were placed in 5 mL of 0.1 M PBS solution (containing 1% penicillin) with

pH values of 5.0, 7.4 and 9.1, respectively, at 37 °C and vibrated at 72 rpm. At the predetermined time, the pH value was measured using an FE20 pH meter (FiveEasy Plus, Shanghai, China). 100 µL of the degradation medium was collected, and diluted by 10 times before centrifugation to analyze the content of lactic acid and lysine. Meantime, 100 µL of original PBS solution was replenished.

The detection of lactic acid and lysine in the degradation medium: The amount of lactic acid and lysine was determined by an Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 µm) at 25 °C.

For lactic acid: The flow rate of mobile phase (methanol: 0.2% phosphoric acid = 5: 95, v/v) was 1 mL/min, and the UV detector wavelength was 210 nm. Twenty microliter supernatant was injected for each analysis. Lactic acid (500 µg/mL) was used as the standard.

For lysine: The flow rate of mobile phase (20 mM KH₂PO₄ solution: acetonitrile = 99:1, v/v) was 1.0 mL/min, and the wavelength displayed by the UV detector was 203 nm. Twenty microliters of supernatant was injected for each analysis. Lysine (500 µg/mL) was used as the standard.

6. MTT assay (degradation products).

The cytotoxicity of the PEAL degradation products was investigated by exposing the degraded compositions to cells. PEAL was accelerated degraded under high pH conditions by hydrolyzation. In brief, the copolymer (16 µmol) was placed in 2.0 mL of NaOH solution (1.0 mol/L) and incubated at 70 °C to ensure a complete

degradation. The pH value was adjusted to 7.4 using HCl solutions (1.0 mol/L). The degraded medium was filtered through a 0.22 µm filter membrane for sterilization. Then, it was diluted into 2000, 1000, 500, 250, 125, 62.5 µM using DMEM medium. Sterile PBS solution was similarly diluted with the medium as a control. These solutions were added to the cells in the 96-wells plates (200 µL/well), which were then incubated under a 5% CO₂ environment at 37 °C for 48 and 96 h. At the pre-determined time, the medium was aspirated, rinsed gently with PBS solution and then added to 200 µL of MTT solution (0.5 mg/mL, with serum-free DMEM culture medium preparation, and filtered) to each well. The incubation time was 2-4 h before the MTT solution was aspirated. 200 µL of DMSO was added into each well. 96-wells plates were shaken for 15 min at room temperature to solubilize the formazan product [13, 14]. The absorbance (OD) was measured with a microplate photometer at a wavelength of 450 nm. The relative growth rate (RGR) for cells was evaluated as follows:

$$\text{RGR} = (\text{OD}_t - \text{OD}_0) / (\text{OD}_{\text{nc}} - \text{OD}_0) \times 100\%$$

Where OD_t represents the absorbance of the testing sample, OD₀ and OD_{nc} represent the absorbance of the background and the negative control, respectively.

The half maximal inhibitory concentration (IC₅₀) values represent the concentration that the vitality of 50% of the total cells is inhibited, and IC₅₀ values were determined using SPSS 20.0 software [2].

7. Preparation of NPs.

The polymer (2000 nM) was first dissolved in 250 μ L of ethyl acetate, and then introduced into 1 mL of 1% (w/w) F68 solution. In consequence, the mixture was homogenized and emulsified by ultrasonic (400W, 25 times \times 2 s) (JY 92-II ultrasonic processor; Ningbo Scientz Biotechnology Co, Ltd, China). The resulting emulsion was rotated and evaporated in a rotary evaporator (SHZ-DIII, Shanghai Qiangqiang Industrial Development Co. Ltd, China) under 37 °C and a pressure of 0.05 MPa to evaporate the organic solvent. After 1 h evaporation, centrifugation at 15000 rpm for 45 min was conducted to obtain the NPs after freeze dry.

8. Characterization of NPs.

The diameter and zeta potential of the NPs were characterized using a ZetaSizer Nano ZS (Malvern Instruments Ltd., UK). The average diameter and the size distribution of the NPs were determined by dynamic light scattering. The Zeta potential (ζ) was determined under a He-Ne laser beam at a wavelength of 633.8 nm at room temperature. All samples were analyzed in triplicate batches for accuracy.

The morphology of the NPs was characterized using a JEM-1400 transmission electron microscope (TEM) from JEOL Ltd (Akishima, Japan). The samples were prepared by depositing 10 μ L suspensions of NPs on a 200 mesh, copper grid with formvar film, and then air-dried at room temperature [15, 16].

9. PT and APTT experiments

The exposure of NPs to blood can induce coagulation cascade. So coagulation

assay that containing PT and APTT measurements was carried out [17, 18]. Briefly, venous blood from healthy donors was collected in siliconized tubes containing 3.8 wt% sodium citrate with a blood: citrate ratio of 9:1. Platelet-poor plasma (PPP) was obtained by centrifugation at 3000 g for 15 min. PEAL NPs were added to PPP to form solutions with a final concentration of 0, 10, 100, 500 and 1000 μM . The samples were stirred for 30 min at 37 °C. Then, 0.5 mL of PT Hemostasis reagent (Labtest[®]) or APTT partial thromboplastin reagent (Labtest[®]) along with 0.5 mL of calcium chloride (0.025 mol/L) was added to the plasma samples (0.5 mL). The PT and APTT were determined by the appearance of a macroscopic clot as an indicator of the termination of the reaction. All experiments were performed at 37 °C within 2 h for 5 time for accuracy.

10. Complement activation

Human blood serum was collected from healthy donors' blood and put into glass vacutainers without anticoagulants. Blood was allowed to coagulate for 1 hour at 37 °C. The blood was then centrifuged at 1000 g for 20 min to get the serum. The PEAL NPs was incubated with 1000 μL of serum for 1 h at 37 °C. Then the turbidimetric method as kit instruction was followed for assessing the concentration of complement protein C3 that is left in the serum [19, 20].

11. Zebrafish maintenance and experimental set-up

Adult zebrafish were reared in fish water (FW) without chlorine gas under

standard laboratory conditions of 28.5 °C at 14 hours light/10 hours dark photoperiod. The zebrafish were spawned (female: male = 1: 2) within 15 min after fertilization. Their embryos were collected, selected, and transferred to Petri dishes filled with fresh E3 embryos medium (NaCl 0.875 g/L, KCl 0.0375 g/L, MgSO₄ 0.12 g/L, KH₂PO₄ 0.0205 g/L, Na₂HPO₄ 0.006 g/L, CaCl₂ 0.145 g/L, NaHCO₃ 0.06 g/L, penicillin 0.12 g/L, streptomycin 0.2 g/L) under a dissecting microscope for microinjection at 1-cell stage experiments and cardiovascular loading at 72 hour post fertilization (hpf) [21].

12. Loading of PEAL/PEAL-FITC NPs into zebrafish embryos at 1-cell stage by microinjection

The PEAL/PEAL-FITC NPs were loaded into zebrafish embryos by microinjection at 1-cell stage. Fine microinjection capillary tubes without filament (Warner Instruments, 1×0.53 mm outer/inner diameter) were pulled and separated into two microinjection pipettes with micropipette puller (Sutter Instruments). The pipettes were then sharpened with Narishige microforge (MF-900) before use. The microinjection pipette was connected to the microinjector (Narishige M300 Microinjector) for injection. The microinjection pipette was calibrated every time before microinjection.

Embryos were further selected and transferred into the microinjection plate filled with E3 medium. 3 nL of PEAL NPs (10, 100, 500, 1000 μM) or PEAL-FITC NPs (1000 μM) were then injected into the embryonic cell or yolk of the embryos with a

nitrogen-driven micro-injector (Narishige) within 45 min after fertilization. 3 nL of PBS solution (pH 7.4) was injected into the embryo as a negative control. After microinjection, the embryos were incubated at 28.5 °C for further development [21].

13. Effect of PEAL NPs treatment on heartbeat of larvae

After microinjection at 1-cell stage, the heartbeat rate per minute in the larvae after exposing to PEAL NPs for 24, 48, 72 and 96 hpf were evaluated under a dissecting microscope.

14. ROS measurement

After micro-injecting the PEAL NPs into the embryos at 1-cell stage, the generation of ROS in the larvae for 24, 48, 72 and 96 hpf were measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, 10 larvae were washed twice with PBS (pH 7.4) and then homogenized in a cold buffer solution (0.32 mM sucrose, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM phenyl methyl sulfonyl fluoride, pH 7.4). The homogenate was centrifuged at 15000 rpm for 20 min at 4 °C to collect the supernatant. 20 µL of the supernatant was added to a 96-well plate and incubated at room temperature for 5 min. Then, 100 µL of PBS solutions (pH 7.4) and 8.3 µL of DCFH-DA stock solutions (dissolved in DMSO, 10 mg/mL) were added to each well. The plate was incubated at 37 °C for 30 min in the dark. The fluorescence intensity at 530 nm was measured using a microplate reader (Synergy H4 Hybrid Microplate Reader, BioTek Instruments, Inc., USA) under an excitation wavelength of 485 nm

[22, 23].

15. Survival rate and morphologic analysis at 96 hpf after loading of PEAL NPs into the embryos at 1-cell stage

After PEAL NPs were injected into the embryos at 1-cell stage, the survival rate and the malformation rate at 96 hpf were evaluated under an IX51 microscope (OLYMPUS Corporation, Tokyo, Japan). 3 nL of PBS solutions (pH 7.4) or acetone ($3.40 \times 10^5 \mu\text{M}$) were used as the negative and positive control, respectively. 14 morphologies were observed at 96 hpf: yolk sac edema, bent body axis, eye, snout, jaw, otolith pericardial edema, brain, somite, caudal fin, pectoral fin, heart, pigmentation, trunk length and swim bladder.

16. AO staining assays

After the PEAL NPs (1000 μM) were microinjected into the embryos at 1-cell stage, these embryos were analyzed for cellular apoptosis and death at 24, 48, 72 and 96 hpf. Embryos were rinsed with FW and then incubated in 100 μL of 5 $\mu\text{g}/\text{mL}$ AO solutions for 1 h in the dark at 28 °C. Then, embryos were rinsed again with FW and fixed overnight in 4% paraformaldehyde. In consequence, they were stored in PBS solution at 4 °C. At last, they were analyzed and imaged using an IX51 fluorescence microscope (exposure time: 1/28 s) [23, 24].

17. Distribution of FITC-PEAL NPs after 0-12 h loading into the embryos at

1-cell stage

After FITC-PEAL NPs were microinjected into the embryos at 1-cell stage for 30 min, 3 h, 5 h and 12 h, the distribution of the NPs in the embryos were analyzed under an Olympus IX51 fluorescence microscope (exposure time: 1/30 s).

18. Distribution in the larvae after loading of FITC-PEAL NPs into the blood system

The microangiography method was used to load FITC-PEAL NPs into the circulation system of zebrafish embryos [21]. At 60-72 hpf, normal embryos were transferred to Petri dishes filled with E3 medium containing 0.04% tricaine to be anaesthetized for 3 min. Then they were mounted laterally in 0.3% agarose covered with E3 medium. 60 nL of FITC-PEAL NP solutions (1000 μ M, containing 5% phenol red) were injected into the tail venous with an nitrogen pulse provided by a Bel-Art Products at a pressure of 250 hPa and a time of 0.2 s. Free-FITC was used as a control. After injection, the embryos were transferred to the 96-well plate with E3 medium (containing 0.03% PTU) and incubated at 28.5 °C. During incubation, FITC-PEAL NPs would circulate throughout the whole vascular network, lighting up each blood vessel with active blood flow from the heart. These embryos were examined at 4 h, 24 h, 48 h and 72 h after microinjection under the Olympus IX51 fluorescence microscope.

19. Statistical analysis

All analyses were compiled using SPSS 20.0 software. The standard deviation (SD) for different samples was based on at least three independent experiments and the results were expressed as the mean \pm SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. In the zebrafish embryos tests, each exposure group consisted of at least 30 exposed embryos, and each group was repeated three times. The curves were fitted using Microsoft Excel software. All tests were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

References

1. He Z, Wang Q, Sun Y, Shen M, Zhu M, Gu M, Wang Y, Duan Y. The biocompatibility evaluation of mPEG-PLGA-PLL copolymer and different LA/GA ratio effects for biocompatibility. *J Biomater Sci Polym Ed*, 2014, 25(9): 943 – 964.
2. He Z, Shi Z, Sun W, Ma J, Xia J, Zhang X, Chen W, Huang J. Hemocompatibility of folic-acid-conjugated amphiphilic PEG-PLGA copolymer nanoparticles for co-delivery of cisplatin and paclitaxel: treatment effects for non-small-cell lung cancer. *Tumour Biol*, 2015, DOI: 10.1007/s13277-015-4634-1.
3. He Z, Sun Y, Wang Q, Shen M, Zhu M, Li F, Duan Y. Degradation and Bio-Safety Evaluation of mPEG-PLGA-PLL Copolymer-Prepared Nanoparticles.

The Journal of Physical Chemistry C, 2015, 119: 3348 – 3362.

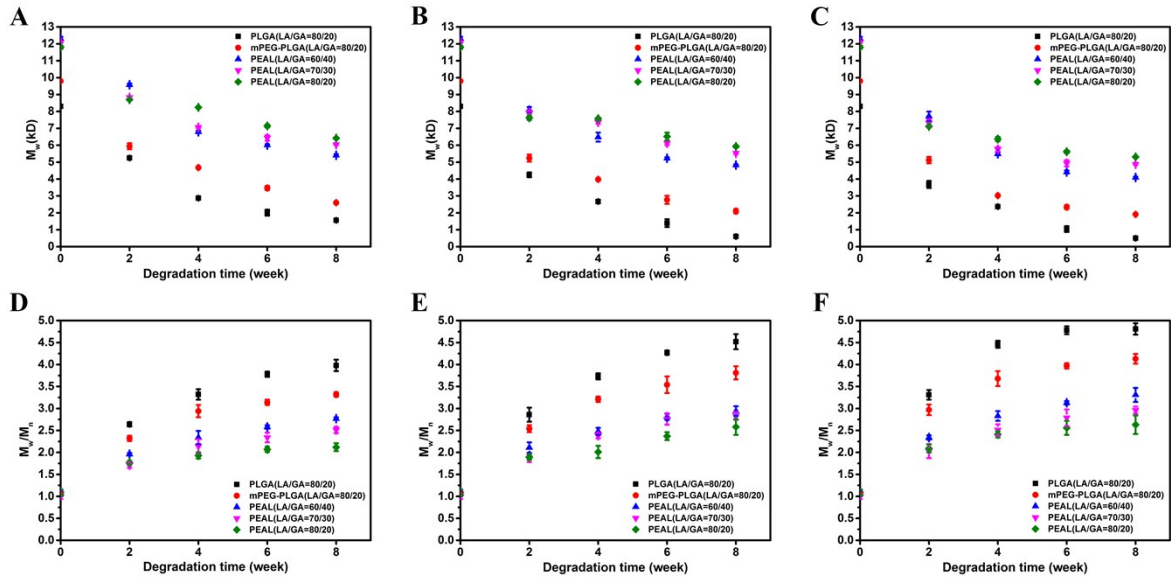
4. Pan CJ, Wang J, Huang N. Preparation, characterization and in vitro anticoagulation of emodin-eluting controlled biodegradable stent coatings. *Colloids and Surfaces B: Biointerfaces*, 2010, 77(2): 155 – 160.
5. Kim YH, Jeon J, Hong SH, Rhim WK, Lee YS, Youn H, Chung JK, Lee MC, Lee DS, Kang KW, Nam JM. Tumor targeting and imaging using cyclic RGD-PEGylated gold nanoparticle probes with directly conjugated iodine-125. *Small*, 2011, 7(14): 2052 – 2060.
6. Pan CJ, Shao ZY, Tang JJ, Wang J, Huang N. In vitro studies of platelet adhesion, activation, and protein adsorption on curcumin-eluting biodegradable stent materials. *J Biomed Mater Res Part A*, 2007, 82(3):740 – 746.
7. Koh LB, Rodriguez I, Venkatraman SS. The effect of topography of polymer surfaces on platelet adhesion. *Biomaterials*, 2010, 31(7): 1533 – 1545.
8. Jadidi-Niaragh F, Atyabi F, Rastegari A, Mollarazi E, Kiani M, Razavi A, Yousefi M, Kheshtchin N, Hassannia H, Hadjati J, Shokri F. Downregulation of CD73 in 4T1 breast cancer cells through siRNA-loaded chitosan-lactate nanoparticles. *Tumour Biol*, 2016, doi: 10.1007/s13277-015-4732-0.
9. Cardenas L, MacLeod J, Lipton-Duffin J, Seifu DG, Popescu F, Siaj M, Mantovani D, Rosei F. Reduced graphene oxide growth on 316L stainless

- steel for medical applications. *Nanoscale*. 2014, 6(15): 8664 – 8670.
10. Rivolo P, Severino SM, Ricciardi S, Frascella F, Geobaldo F. Protein immobilization on nanoporous silicon functionalized by RF activated plasma polymerization of Acrylic Acid. *Journal of Colloid and Interface Science*, 2014, 416: 73 – 80.
 11. Nickel O, Laurencin D, Bonhomme C, Sroga GE, Besdo S, Lorenz A, Vashishth D. Solid state NMR investigation of intact human bone quality: balancing issues and insight into the structure at the organic-mineral interface. *The Journal of Physical Chemistry C*, 2012, 116(10): 6320 – 6331.
 12. Mathew R, Gunawidjaja PN, Izquierdo-Barba I, Jansson K, García A, Arcos D, Vallet-Regí M, Edén M. Solid-State P and H NMR Investigations of Amorphous and Crystalline Calcium Phosphates Grown Biomimetically From a Mesoporous Bioactive Glass. *The Journal of Physical Chemistry C*, 2011, 115(42): 20572 – 20582.
 13. Ding M, Li J, Fu X, Zhou J, Tan H, Gu Q, Fu Q. Synthesis, Degradation, and Cytotoxicity of Multiblock Poly(ϵ -caprolactone urethane)s Containing Gemini Quaternary Ammonium Cationic Groups. *Biomacromolecules*, 2009, 10(10): 2857 – 2865.
 14. Bull-Hansen B, Berstad MB, Berg K, Cao Y, Skarpen E, Fremstedal AS, Rosenblum MG, Peng Q, Weyergang A. Photochemical activation of MH3-B1/rGel: a HER2-targeted treatment approach for ovarian cancer.

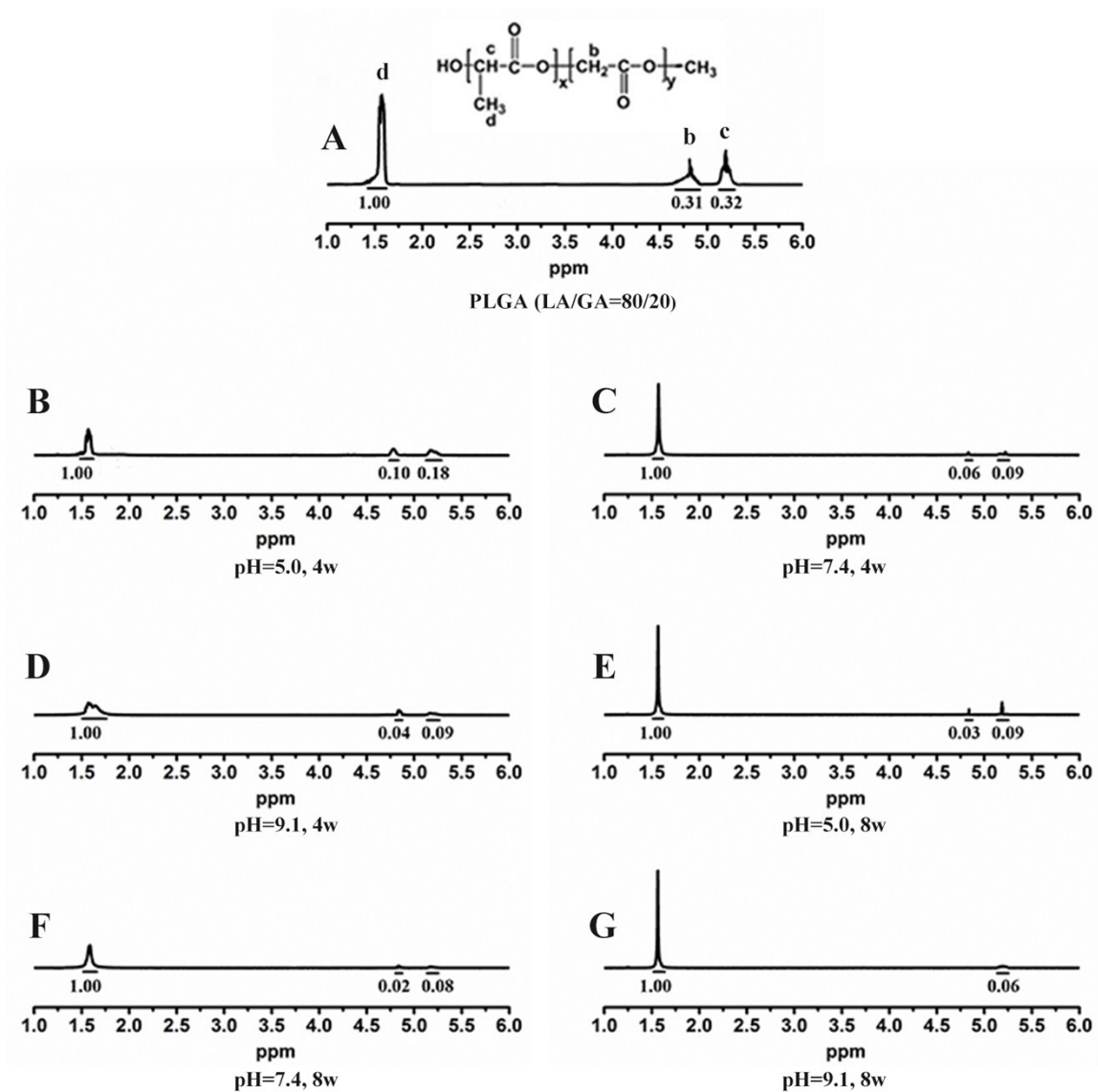
- Oncotarget. 2015, 6(14): 12436 – 12451.
15. Yang C, Xiong F, Dou J, Xue J, Zhan X, Shi F, Li M, Wu S, Luo S, Zhang T. Target therapy of multiple myeloma by PTX-NPs and ABCG2 antibody in a mouse xenograft model. *Oncotarget*, 2015, 6(29): 27714 – 27724.
 16. Wang H, Yin H, Yan F, Sun M, Du L, Peng W, Li Q, Feng Y, Zhou Y. Folate-mediated mitochondrial targeting with doxorubicin-polyrotaxane nanoparticles overcomes multidrugresistance. *Oncotarget*. 2015, 6(5): 2827 – 2842.
 17. Anitha A, Deepa N, Chennazhi KP, Lakshmanan VK, Jayakumar R. Combinatorial Anticancer Effects of Curcumin and 5-fluorouracil Loaded Thiolated Chitosan Nanoparticles Towards Colon Cancer Treatment. *Biochim Biophys Acta*, 2014, 1840: 2730 – 2743.
 18. Cenni E, Granchi D, Avnet S, Fotia C, Salerno M, Micieli D, Sarpietro MG, Pignatello R, Castelli F, Baldini N. Biocompatibility of Poly(D,L -lactide- co - glycolide) Nanoparticles Conjugated with Alendronate. *Biomaterials*, 2008, 29: 1400 – 1411.
 19. Durgadas CV, Sreenivasan K, Sharma CP. Bright Blue Emitting CuSe/ZnS/Silica Core/Shell/Shell Quantum Dots and Their Biocompatibility. *Biomaterials*, 2012, 33: 6420 – 6429.
 20. Wu CC, Yang YC, Hsu YT, Wu TC, Hung CF, Huang JT, Chang CL. Nanoparticle-induced intraperitoneal hyperthermia and targeted photoablation in treating ovarian cancer. *Oncotarget*, 2015, 6(29): 26861 -

26875.

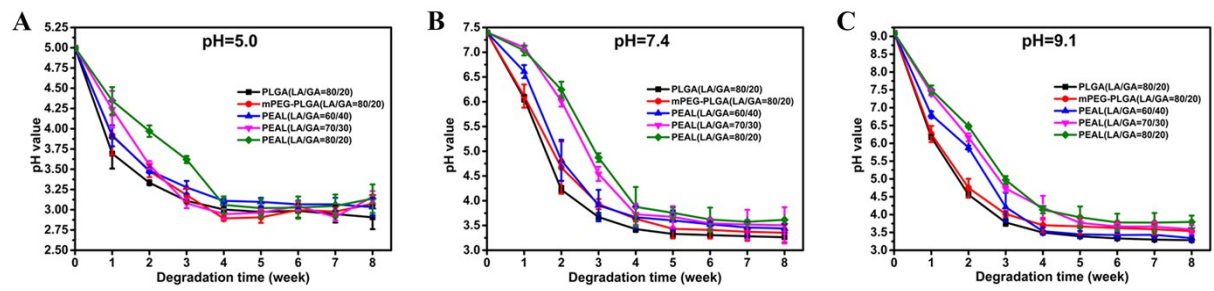
21. Cheng J, Chan CM, Veca LM, Poon WL, Chan PK, Qu L, Sun YP, Cheng SH. Acute and Long-term Effects after Single Loading of Functionalized Multi-walled Carbon Nanotubes into Zebrafish (*Danio rerio*). *Toxicol Appl Pharmacol*, 2009, 235: 216 – 225.
22. Zhao X, Wang S, Wu Y, You H, Lv L. Acute ZnO Nanoparticles Exposure Induces Developmental Toxicity, Oxidative Stress and DNA Damage in Embryo-larval Zebrafish. *Aquat Toxicol*, 2013, 136: 49 – 59.
23. Si J, Zhang H, Wang Z, Wu Z, Lu J, Di C, Zhou X, Wang X. Effects of $^{12}\text{C}^{6+}$ Ion Radiation and Ferulic Acid on the Zebrafish (*Danio rerio*) Embryonic Oxidative Stress Response and Gene Expression. *Mutat Res*, 2013, 745: 26 – 33.
24. Usenko CY, Harper SL, Tanguay RL. In vivo Evaluation of Carbon Fullerene Toxicity Using Embryonic Zebrafish. *Carbon N Y*, 2007, 45: 1891 – 1898.



Supplementary Figure S1. The M_w decrease is due to degradation of polymer in (A) pH 5.0, (B) pH 7.4 and (C) pH 9.1 medium with prolonged degradation time; PI (M_w/M_n) increase is due to the degradation of polymer in (D) pH 5.0, (E) pH 7.4 and (F) pH 9.1 medium with prolonged degradation time.



Supplementary Figure S2. ^1H -NMR spectra of PLGA (LA/GA = 80/20) in CDCl_3 .



Supplementary Figure S3. The pH decreased in the (A) pH 5.0, (B) pH 7.4 and (C) pH 9.1 media when degradation time was prolonged.