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Surface-Eroding Poly(ortho ester amides) for Highly Efficient Oral Chemotherapy

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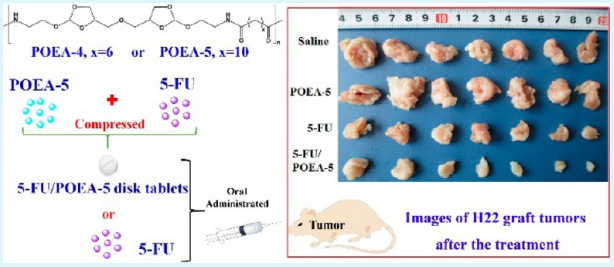
ABSTRACT: Two new poly(ortho ester amide) copolymers (POEA-4 and POEA-5) were synthesized via polycondensation of a new ortho ester diamine monomer with active esters of different aliphatic diacids. The kinetics of POEA mass loss and release of 5-FU were both nearly zero-order, suggesting predominantly surface-restricted polymer erosion and drug release. In vitro cytotoxicity tests demonstrated that both copolymers have excellent biocompatibility. In vivo acute toxicity tests suggested that oral administration of POEA-4 and POEA-5 did not cause any adverse effects on mice even at a very high dose (2000 mg/kg). In vivo antitumor efficacy against H22 transplanted tumors of 5-FU-loaded POEA tablets were fully examined. We envision that, with further optimization, POEA-based materials could have great potential as drug carriers for oral chemotherapy.



KEYWORDS: *poly(ortho ester), oral chemotherapy, controlled release, surface erosion*

1. INTRODUCTION

Over the past decades, oral chemotherapy has attracted increasing attention because of greater safety, convenience, and better patient compliance, compared to other drug delivery methods such as intravenous, intramuscular, implantable, subcutaneous, transdermal routes.[1−8] Despite obvious advantages, it is extremely difficult to achieve efficient oral delivery of anticancer agents, especially those with high potency, such as paclitaxel (PTX), doxorubicin (DOX), 5-fluorouracil (5-FU), because the human gastrointestinal (GI) tract presents significant barriers to drug delivery.[9−15]

Microspheres and nanoparticles made from biodegradable polymers have been used as oral drug carriers to improve drug solubilization, protect drugs against physicochemical and enzymatic degradation, and enhance drug absorption through size-dependent uptake of particulates by the intestinal epithelium.[16−21] Some micro- and nanoparticles have been proposed for delivering anticancer drugs orally, but evaluation of these material systems is often focused on in vitro cell studies with limited evidence to establish in vivo efficacy after oral administration.[22−25] Furthermore, biocompatible polyesters based on copolymers of lactic and glycolic acid (PLGA) have been the dominant type of material used in oral drug delivery because of excellent safety records in humans.[26−30] Despite much success, PLGA-based micro-/nanoparticles often exhibit multiphasic drug release profile with initial bursts, which is difficult to control accurately and could result in dose-related side effects.[21,31,32] Bulk erosion of PLGA is generally considered to be responsible for the burst and nonlinear drug release.

Poly(ortho esters) (POE) is a family of biodegradable and biocompatible materials with potential biomedical applications.[33,34] The ideal design of POE for drug delivery is a material that is both highly hydrolytically labile and hydrophobically stable, so that erosion of the polymer is limited only to the surface in contact with the aqueous environment. Such material design enables POE to have the much desired, surface-dominated erosion property and achieve near-zero-order release of encapsulated drugs without burst.[2,10,33,34] Although POE was considered in the past for use in orthopedic surgery and as implantable or injectable sustained drug delivery system, it has not been evaluated for oral drug delivery. Here we propose that POE may be suitable for oral delivery of anticancer drugs given its biocompatibility, surface eroding property, and the potential of achieving sustained drug release with minimal burst release.

Since the early 1970s, four generations of POE have been synthesized either by transesterification between an ortho ester and a diol, or through addition polymerization between a diol and a diketeneacetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro-[5,5]undecane (DETOSU).[33−36] These methods have serious weaknesses that include spontaneous isomerization and moisture sensitivity of DETOSU, or rigorous reaction conditions such as high vacuum, high temperature, and poor reproducibility. To overcome these weaknesses, our groups successfully developed a new pathway of syntheses to prepare a new family of poly(ortho ester amides) (POEA) by facile solution polycondensation between an acid-labile diamine monomer (4-aminomethyl-2-aminopentyloxy-2-methyl-[1,3]-dioxolan) with a built-in ortho ester bond and fatty diacid esters.[37] Three POEA 1−3 hydrogels

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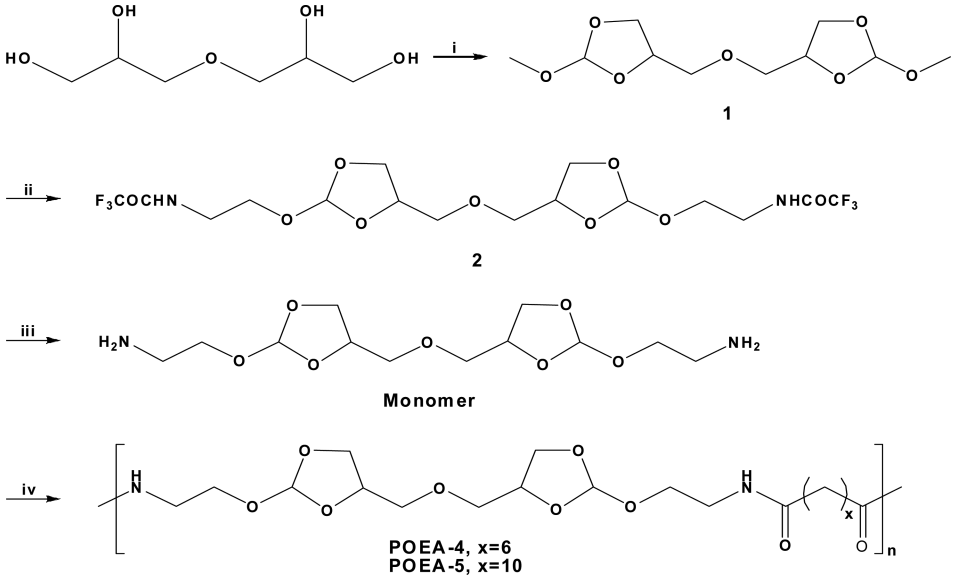

Figure 1. Synthetical route of POEA-4 and POEA-5. Reaction conditions: (i) trimethylorthformate, *p*-toluene sulfonic acid (*p*-TSA), acetonitrile; (ii) N-(2-hydroxyethyl)trifluoroacetamide, pyridinium-*p*-TSA; (iii) $NaOH/H_2O/THF$; (iv) disuccinimidylsuberate or disuccinimidyldodecanoate, triethylamine, DMF.

exhibited predominantly surface-restricted matrix erosion with nearly zero-order kinetics of drug release in response to biologically acidic environments. We hypothesize that the POEA polymers would be ideal materials for oral delivery of anticancer drugs, but improvement must be made to simplify the synthesis and purification of the crucially important ortho ester diamine monomer.[37] To establish proof-of-principle, we report a simple method of synthesizing POE-type polymer with surface-erosion property, in vitro characterization of drug release, and in vivo evaluation of antitumor activity in mice bearing malignant ascites using 5-FU as a model drug.

2. EXPERIMENTAL SECTION

2.1. Materials. Suberic acid bis(*N*-hydroxysuccinimide ester) and dodecanedioic acid bis(*N*-hydroxysuccinimide ester) were purchased from Thermo Fisher Scientific Inc. (MA, USA). Diglycerol, trimethyl orthoformate and N-(2-hydroxyethyl)trifluoroacetamide were obtained from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (MTT), Hoechst 33258 and p-tertiary-Octylphenoxy polyethyl alcohol (Triton X-100) were purchased from Sigma-Aldrich (St. Louis, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Gibco (Tulsa, OK, USA). Acetonitrile, tetrahydrofuran, dichloromethane, *N*,*N*-dimethylformamide, *p*-toluenesulfonic acid monohydrate and other reagent were purchased commercially and used after purification. Mouse fibroblast (NIH3T3) and murine hepatoma cell line (H22) were obtained from Cell Band of Shanghai Institutes for Biological Science cell resource center (Shanghai, China). Female ICR mice (18−22 g), supplied by the Department of Experimental Animals, Yangzhou University (Yangzhou, China), All animal experiments were done in accordance with the guidelines of the Use of Laboratory Animals and with the approval of Institutional Authority for Laboratory Animal Care of Anhui University.

*2.1.1. Synthesis of 4,4′-(Oxybis(methylene)) Bis(2-methoxy-1,3-dioxolane) (**1**).* In a nitrogen atmosphere, to mixture of diglycerol (25.0 g, 0.15 mol), trimethyl orthoformate (127.7 g, 1.20 mol), and acetonitrile (150 mL) was added p-toluene sulfonic acid (*p*-TSA; a trace amount). The mixture was reacted overnight at room temperature and was concentrated afford the crude product. The product was dissolved in ethyl acetate (250 mL), washed with saturated Na_2CO_3. The organic layer was dried over $MgSO_4$, and concentrated to yield 4,4′-(oxybis(methylene)) bis(2-methoxy-1,3-dioxolane) as oil (33.2 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.31−3.32 (m, 6H, O−CH_3), 3.56−3.73 (m, 4H, CH_2−O−CH_2), 3.78−4.15 (m, 4H, CH−O−CH_2−CH), 4.30−4.36 (m, 2H, CH_2−CH-CH_2),5.73−5.75 (d, 2H, CH-$(O)_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 51.8, 65.94, 65.96, 69.26, 69.37, 71.80, 71.84, 72.78, 116.54, 116.17. ESI-MS: ($C_{10}H_{18}O_7$), 250.3; found *m*/*z*, 251.3 ($M+H^+$).

*2.1.2. Synthesis of N,N′-(((4,4′-(Oxybis(methylene)) Bis(1,3-dioxolane-4,2-diyl)) Bis(oxy)) Bis(ethane-2,1-diyl)) Bis(2,2,2-trifluoroacetamide) (**2**).* A mixture of compound 1 (8.5 g, 34.0 mmol), N-(2-hydroxyethyl)trifluoroacetamide (11.68 g, 74.3 mmol), and pyridinium p-toluene sulfonate (174 mg, 0.693 mmol) was heated at 130 °C for 8 h. After cooling to room temperature, the residue was dissolved in ethyl acetate (250 mL), washed with an aqueous $NaHCO_3$ solution twice, dried over $MgSO_4$, and concentrated to yield N,N′-(((4,4′-(oxybis (methylene)) bis(1,3-dioxolane-4,2-diyl)) bis (oxy)) bis(ethane-2,1-diyl)) bis(2,2,2-trifluoroacetamide) as oil (13.88 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.67−3.72 (m, 4H, NH−CH_2), 3.48−3.60 (m, 8H, NH−CH_2−CH_2,CH_2−O−CH_2), 3.78−4.15 (m, 4H, CH−O−CH_2), 4.43−4.45 (m, 2H, CH_2−CH−CH_2), 7.16−7.74 (m, 2H, N*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 42.09, 60.46, 60.54, 61.98, 62.20, 65.55, 65.94, 74.54, 74.59, 75.28, 114.45, 115.80, 117.31, 157.16, 157.49, 157.86. ESI-MS: ($C_{16}H_{22}N_2F_6O_9$), 500.3; found *m*/*z*, 523.3 ($M+Na^+$).

2.1.3. Synthesis of 2,2′-((4,4′-(Oxybis(methylene)) Bis(1,3-dioxolane-4,2-diyl)) Bis(Oxy)) Diethanamine (Monomer). The compound 2 (13.88 g, 27.7 mmol) was dissolved in THF (80 mL), and sodium hydroxide (3 M, 83 mL) was added. The mixture was vigorously stirred overnight, extracted with diethyl ether, dried over $MgSO_4$, and concentrated to yield 2,2′-((4,4′-(oxybis (methylene)) bis(1,3-dioxolane-4,2-diyl)) bis(Oxy)) diethanamine as oil (6.12 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.61 (s, 4H, NH_2), 2.82−2.89 (d, 4H, CH_2-NH_2), 3.51−3.74 (m, 8H, NH−CH_2−CH_2, CH_2−O−CH_2), 3.77−4.19 (m, 4H, CH−O−CH_2), 4.29−4.50 (m, 2H, CH_2−CH−CH_2), 5.85−5.87 (d, 2H, CH−$(O)_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 41.69, 65.82, 66.13, 66.18, 67.02, 71.69, 72.55, 74.15, 75.08, 75.13, 115.62, 116.02. ESI-MS: ($C_{12}H_{24}N_2O_7$), 308.3; found *m*/*z*, 331.2 ($M+Na^+$). Anal. Calcd for $C_{12}H_{24}N_2O_7$: C, 46.75; H, 7.85; N, 9.09. Found: C, 46.67; H, 7.78; N, 9.15.

2.1.4. Synthesis of Poly(ortho ester diamide) (POEA). The two copolymers (POEA-4 and POEA-5) were synthesized according to the same procedure of polycondensation, and with the synthesis of POEA-4 as an example: disuccinimidylsuberate (1.207 g, 3.28 mmol) was added

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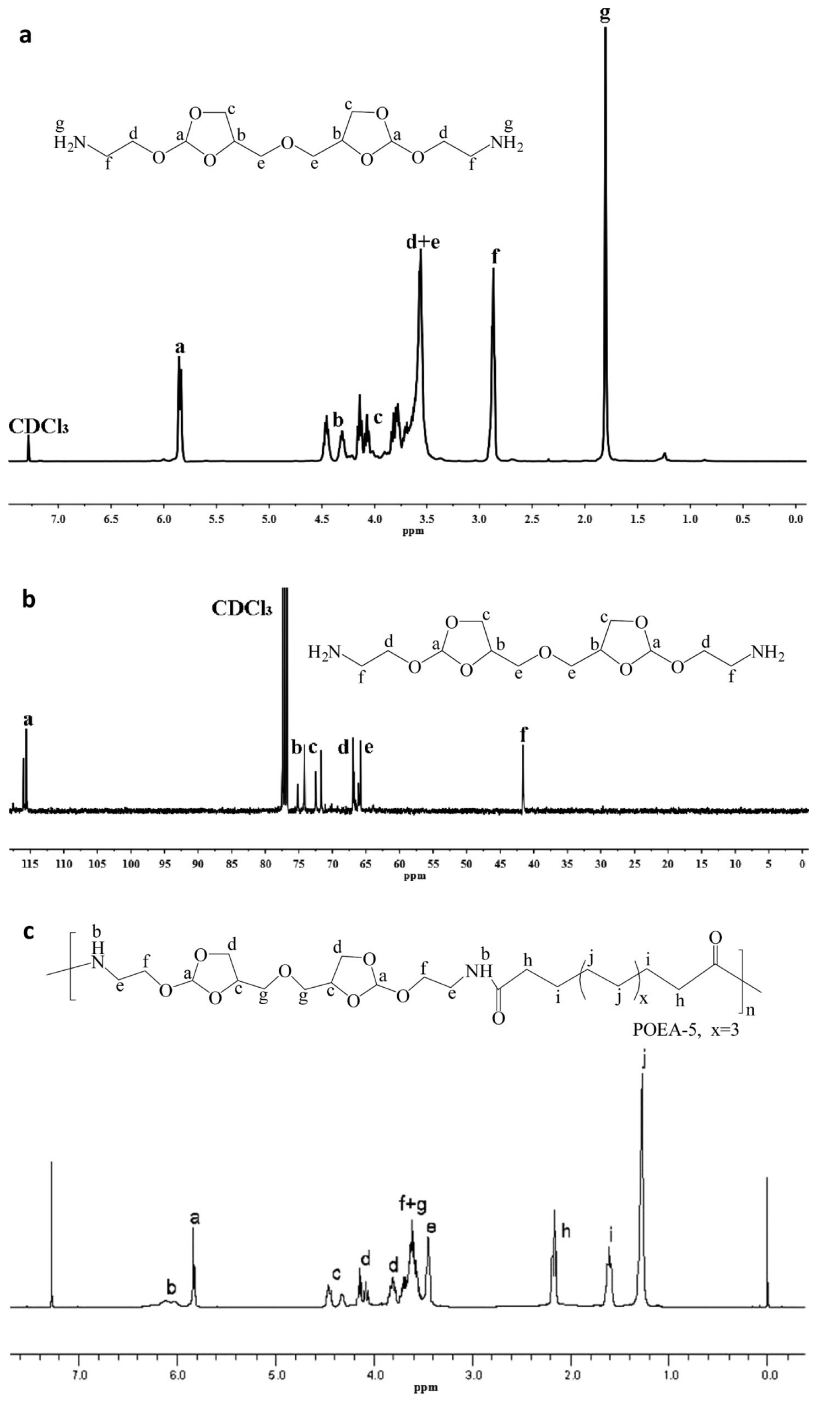

Figure 2. (a) ^{1}H NMR spectra of the monomer in $CDCl_3$; (b) ^{13}C NMR spectra of the monomer in $CDCl_3$; (c) ^{1}H NMR spectra of POEA-5 in $CDCl_3$.

to a stirred mixture of monomer (1.01 g, 3.28 mmol), TEA (1.40 mL, 10.05 mmol), and dimethylformamide (DMF; 5.0 mL) under nitrogen, reacted 5 days at room temperature, and added dropwise to stirred ethyl acetate. After pouring off the upper solution, the viscous oil in the bottom was collected, washed with ethyl acetate repeatedly, and dried under a high vacuum to yield POEA-4 as a semisolid (1.12 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.24−1.33 (m, 4H, CH_2−$(CH_2)_4$−CH_2), 1.62 (s, 4H, CH_2−$(CH_2)_4$−CH_2), 2.16−2.19 (m, 4H, CO−CH_2−CH_2), 3.44 (s, 4H, CH_2−CH_2−N), 3.50−3.75 (m, 8H, CH_2−CH_2−O), 3.56−4.19 (m, 4H, CH−CH_2−O), 4.33−4.46 (d, 2H, $(CH_2)_2$−CH−O), 5.83−5.84 (d, 2H, CH−$(O)_3$), 6.28 (s, 2H, CH_2−NH−CO).

2.2. Characterizations. The ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker Advance 400 NMR spectrometer, respectively. GPC analysis for copolymers was carried out with a Waters HPLC system. DMF was used as the mobile phase with a flow rate of 1 mL/min. All sample solutions were filtered through a 0.45-μm filter before injecting into the GPC system. A calibration curve was constructed using a series of polystyrene standards, from which number-average molecular weights, weight-average molecular weight and polydispersity indices were calculated. Mass Spectroscopy (ESI-MS) measurements were performed on a Micromass Platform LCZ system (Waters) equipped with an electro spray interface (ESI). Elemental analysis was conducted on a ElementarVario EL III analyzer. Thermo gravimetric analysis (TGA) was conducted on a SDT-2960 thermo gravimetric analyzer with a heating rate of 10 °C/min under a nitrogen atmosphere.

2.3. Determination of Cytotoxicity of POEA. The cytotoxicity of the POEA-4 and POEA-5 was assessed by the MTT assay. NIH3T3 cells

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were cultured in Dubecco's Eagle Medium with the addition of 10% fetal bovine serum, 100 U/mL penicillin/streptomycin, and incubated at incubators maintained at 37 °C under a humidified atmosphere containing 5% CO_2. Cells were seeded in 96-well plates with a density of 10 000 cells/well, and allowed to adhere for 24 h before assay, then treated with different concentrations of POEA-4 or POEA-5 (dose diluted by complete medium, 0−5000 μg/mL) for another 24 h. Afterward, the medium was removed, 180 μL of DMEM and 20 μL of the MTT solution (5 mg/L in PBS) were added to each well. After incubated for 4 h, the precipitate was dissolved in DMSO (100 μL/well) after the supernatant was removed. The absorbance was measured at 570 nm using a Microplate reader. The relative cell viability was calculated by the following equation: Relative cell viability % = $(OD_{POEA}/OD_{control}) \times 100$, where OD_{POEA} was obtained in the presence of POEA and $OD_{control}$ was obtained in the absence of POEA.

2.4. Determination of Cytotoxicity of POEA Degradation Products. 140 μL hydrochloric acid (1 M) was added to POEA powder (5 mg) to complete dispersion and incubated at 37 °C overnight. To eliminate cytotoxic effects due to changes in osmolarity and pH of the degradation products, more cell culture media and NaOH (1 M) were taken to adjust with physiological condition of pH 7.4. After NIH3T3 cells were adhered to 96-well, culture medium was replaced by the solution of polymer degradation products in a series of dilutions. Cells were incubated for 24 h and MTT assay was performed to evaluate cytotoxicity of degradation products as previously mentioned.

2.5. Cell Morphology by Direct Contact. POEA-4 and POEA-5 films were prepared by adding 100 μL solution of polymers in ethanol (10 mg/mL) to a 96-well plate, and allowing the ethanol to evaporate overnight. NIH3T3 cells were seeded over polymer film at 5000 cells per well and incubated for 24 h at 37 °C in 5% CO_2. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C and incubated with 5 μg/mL of Hoechst 33258 for 30 min at 37 °C in the dark. Cell morphology was examined under a fluorescent microscope (BX51, Olympus, Japan). Cells cultured on plates without exposure to polymer were used for comparison.

2.6. In Vivo Acute Toxicity. For assessment of acute toxicity, thirty-fivefemale mice were divided into seven groups randomly and individually administered with 0.2 mL of saline, POEA-4 or POEA-5 at a dose of 50, 300, and 2000 mg/kg by gavages. The mice were weighed and observed for adverse reactions for 14 days. Anticoagulant whole blood (heparin sodium) which collected via the ocular vein (about 1 mL each mouse) were centrifuged twice at 3000 rpm for 10 min in order to separate serum. The separated serum was tested biochemistry by kit (Nanjing Jiancheng Bioengineering Institute), including total protein (TP), urea nitrogen (BUN), creatinine (CREA), glutamate oxaloacetic transaminase (GOT), and lactic dehydrogenase (LDH). Mice were thereafter sacrificed for a thorough autopsy examination. Major organs (heart, liver, spleen, lung, kidney, stomach, and intestine) were quickly excised, washed with cold PBS (pH 7.4), and fixed in 4% neutral buffered paraformaldehyde.Tissues were paraffin embedded and sectioned, stained with hematoxylin and eosin (H&E) and pathological observed by a digital microscope.

2.7. Prepared Free POEA-5 and 5-FU+POEA-5 Disk Tablets. Free POEA-5, and POEA-5 loaded with 10 wt % of 5-FU were dissolved in ethanol, dried under vacuum, and compressed under a pressure of 25 MPa for 15 min to produce POEA-5 and 5-FU+POEA-5 disk tablets with a thickness of 1 mm. Afterward, the tablets were removed from the mold and cut into 2 × 2 mm, or 1 × 1 mm fragments.

2.8. In Vitro Erosion and 5-FU Release. 5-FU/POEA-5 disk tablets fragments with 2 × 2 mm were weighed and placed into test tubes with screw-top caps and immersed into 30 mL of 50 mM buffer solutins (pH 7.4, 5.0, or 1.0). The test tubes were transferred to a 37 °C incubator and gentle agitation in the dark. At various time points, in triplicate, tubes were removed from the incubator and buffer solutions

Table 1. Characterization of Polymers by GPC and TGA

polymer	M_n[a] (× 10^{-4})	M_w[a] (× 10^{-4})	PDI	TGA[b] (°C)
POEA-4	1.24	1.97	1.59	198.5
POEA-5	1.81	3.50	1.93	202.5

[a]Calculated based on the data from GPC. [b]Temperatureat 5% weight loss under nitrogen.

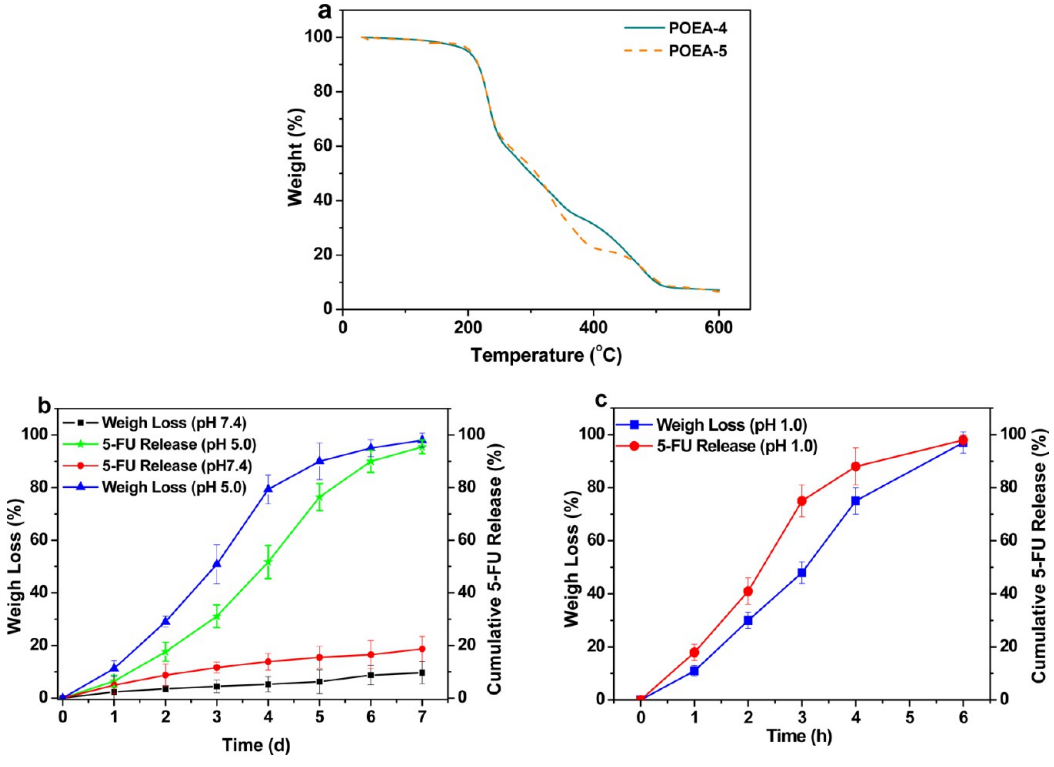

Figure 3. (a) TGA thermograms of POEA; (b) kinetics of POEA-5 erosion loss and release of 5-FU encapsulated into POEA-5 matrix at pH 7.4 and 5; (c) kinetics of POEA-5 erosion loss and release of 5-FU encapsulated into POEA-5 matrix at pH 1.

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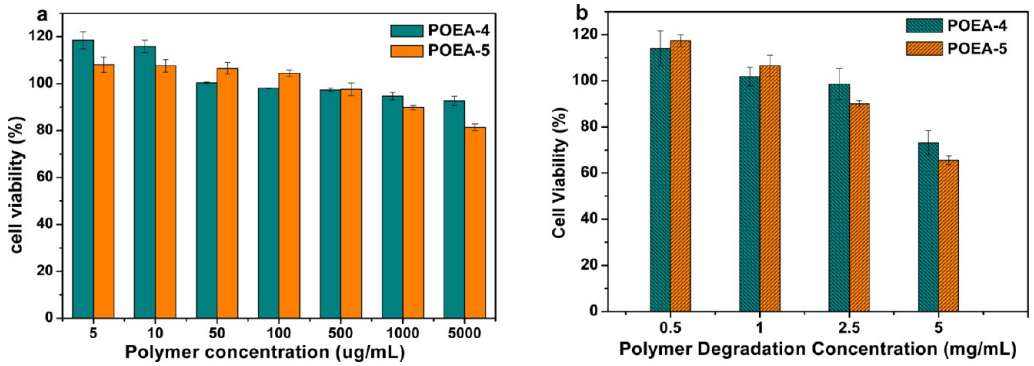

Figure 4. (a) Viability of NIH3T3 fibroblasts exposed to POEA degradation for 24 h; (b) viability of NIH3T3 fibroblasts exposed to POEA for 24 h tested by MTT assay.

were decanted, and released 5-FU content were measured by UV absorbance at 265 nm. The remaining tablet fragments were transfer to a 40 °C oven, dried overnight, and weighed.

2.9. In Vivo Antitumor Efficacy and Safety Evaluation of 5-FU+POEA-5 Disk Tablets. In vivo antitumor activity against subcutaneous tumor was evaluated in ICR mice. H22 murine hepatoma cells (2×10^6 cells, 0.2 mL) were implanted subcutaneously into the right axilla of the mice. Mice were divided into four groups (7 mice in each group) randomly, administered in gavages everyday for 7 days. Four groups as follows: normal control group (0.9% NaCl), negative control group (POEA-5 tablet fragments), positive control group (5-FU, 60 mg/kg), and group orally administered with 5-FU+POEA-5 tablet fragements (i.g. 5-FU, 60 mg/kg). Body weights of each mouse were measured every day. After the final administration, the mice were weighed, and sacrificed by cervical dislocation, and the tumor mass was harvested, photographed, and then fixed with paraformaldehyde and paraffin embedded, stained with hematoxylin and eosin (H&E), and pathologically observed by a digital microscope.

2.10. Statistical Analysis. Data were presented as mean ± SD. Significance between the mean values was calculated using Student's *t* test analysis with SPSS 12.0. A value of $P < 0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Preparation of Poly(ortho ester amide). Here we synthesized a new ortho ester diamine monomer (2,2′-((4,4′-(oxybis (methylene)) bis(1,3-dioxolane-4,2-diyl)) bis(Oxy)) diethanamine) with balanced hydrophobility and hydrophilicity using diglycerol as starting material. As shown in Figure 1, the new monomers were facilely synthesized in three steps and easily purified by extraction because of increased hydrophobility, which was confirmed by mass spectroscopy, ^{1}H NMR, and ^{13}C NMR (Figure 2, 2b). Two poly(ortho ester amide) polymers (POEA-4 and POEA-5) were successfully synthesized via polycondensation of the new ortho ester diamine monomer with active esters of different aliphatic diacid, whose structure was confirmed by ^{1}H NMR (Figure 2c). The average molecular weight of POEA-4 and POEA-5 were determined by GPC to be 1.24×10^4 and 1.81×10^4 with polydispersity index (PDI) of 1.59 and 1.93, respectively (Table 1). The temperatures of 5% weight loss of POEA-4 and POEA-5 were measured to be 198.5 and 202.5 °C by TGA, which revealed that the copolymers had relatively high thermal stability (Figure 3a, Table 1).

3.2. Preparation of 5-FU Loading POEA Tablets and in Vitro Release. Considering POEA-4 is a semisolid and not suitable for tableting, POEA-5 was chosen for further characterization as an oral 5-FU carrier. 5-FU was selected as a model drug, because it has been widely used to treat malignancy tumors such as brain, liver and breast cancer by intravenous route.[38]

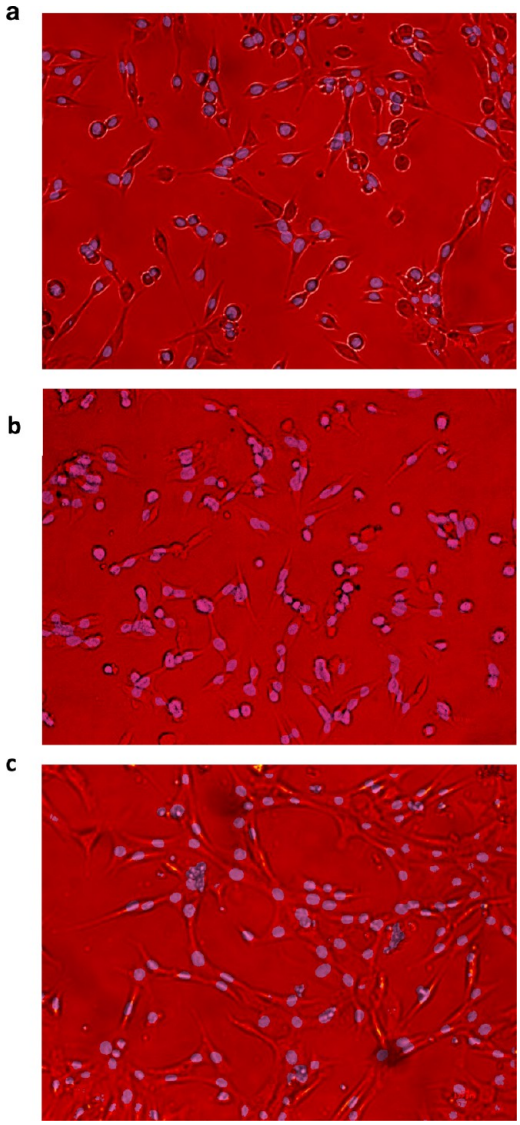

Figure 5. Cell morphology after direct contact with POEA film for 24 h. (a) Control, NIH3T3 fibroblasts cultured on a tissue culture platewithout exposure to POEA; (b) POEA-4; (c) POEA-5. Shown are merged white light and fluorescence images. Nuclei of cells were stained with Hoechst 33258. Magnification: 10× objective.

5-FU and POEA-5 were mixed at a mass ratio of 1:9 and compressed for 15 min to give 5-FU+POEA-5 disk tablets. These tablets were incubated at 37 °C in aqueous buffers of

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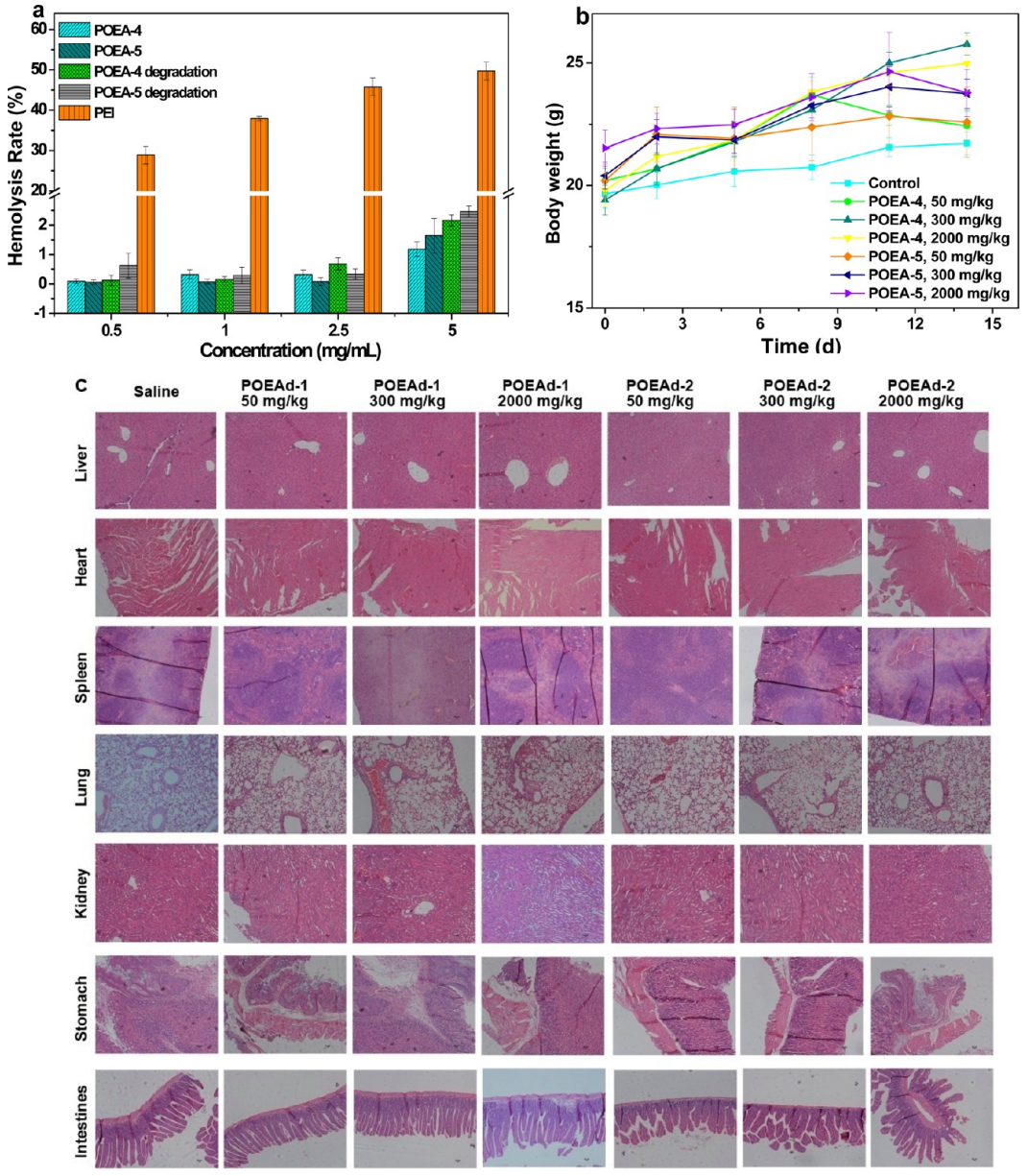

Figure 6. In vitro and acute toxicity evaluation of POEA using a mouse model. (a) Hemolytic activity of POEA, POEA degradation, and PEI against red blood cells; (b) body weights of the mice as a function of days after oral administration; (c) histological sections of organs including liver, heart, spleen, lung, kidney, stomach, and intestines after 14 days of gavages.

pH 7.4, 5.0, and 1.0. At different time points, the mass loss of tablets and the amount of 5-FU released into the buffers were determined. The disk tablets lost approximately 15% of its mass after 7 days at physiological pH 7.4, but eroded completely by day 7 at mildly acidic pH 5.0 (Figure 3b). Accordingly, only 20% of 5-FU was released after 7 days at pH 7.4, whereas full release was achieved by day 7 at pH 5. At strongly acidic pH of 1 the erosion rate of tablets and 5-FU release were much accelerated, and the disk tablets eroded completely after 6 h with a full release of incorporated 5-FU (Figure 3c). Interestingly, the process of POEA mass loss was nearly zero order and matched the profile of release kinetics very well at both pHs with no initial bursts, indicating that the release of 5-FU was predominantly driven by surface-restricted matrix erosion. These results demonstrated that POEA could undergo highly pH-sensitive surface erosion resulted from hydrolysis of pH-sensitive ortho ester bond in POEA main chains, thus enabling precise control over drug release.

3.3. Biocompatibility of Poly(ortho ester amide). To verify the biocompatibility of the POEA copolymers, we conducted in vitro cytotoxicity tests against mouse embryonic fibroblast cell line, NIH3T3 cells. NIH3T3 cells were treated with different concentrations of POEA-4 and POEA-5 (0−5000 μg/mL) for 24 h, and cell viability was assessed by MTT assay. As shown in Figure 4a, POEA-4 and POEA-5 did not show obvious cytotoxicity with relative cell viability of more than 80% even at the highest polymer concentration of 5000 μg/mL, suggesting both copolymers are cytocompatible. NIH3T3 cells were also exposed to degradation products of POEA-4 and POEA-5 for 24 h, and the relative cell viability was measured by MTT assay, as shown in Figure 4b. The relative cell viability treated with POEA-4 and POEA-5 degradation products was slightly decreased, but still more than 90% even at a high polymer concentration of 2.5 mg/mL. In addition, NIH3T3 cells were seeded on the surface of POEA-4 and POEA-5 films and cocultured for 24 h. The morphology of NIH3T3 cells on POEA-4

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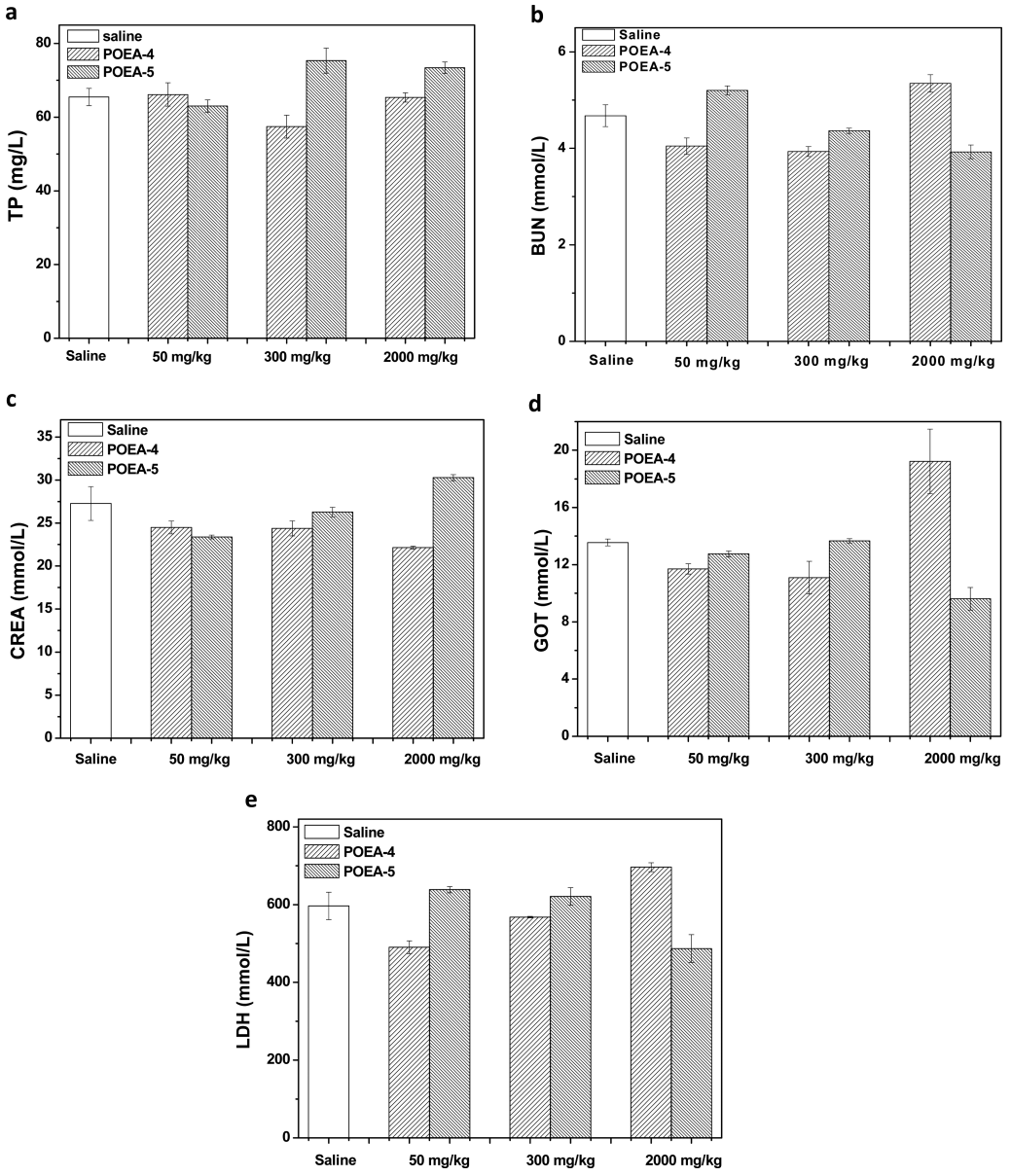

Figure 7. Serum chemistry analysis of acute toxicity study. (a) Total Protein (TP); (b) blood urea nitrogen (BUN); (c) creatinine (CREA); (d) glutamate oxaloacetic transaminase (GOT); (e) lactic dehydrogenase (LDH).

and POEA-5 films was observed under fluorescent microscope. As shown in Figure 5, NIH3T3 cells were able to adhere to the surface of the POEA films and showed spindle-shaped morphology, suggesting that POEA did not inhibit cell proliferation.

Blood compatibility of POEA-4, POEA-5, and their degradation products was evaluated in rabbit blood by hemolytic test, as shown in Figure 6a. PEI, a hemolytic material, was chosen as a positive control. Hemolysis increased significantly from 29% to 50% with increasing concentration of PEI. In contrast, all the POEA polymers and their degradation products did not show any hemolytic activity. Hemolysis was lower than 1% in the presence of 2.5 mg/mL of these materials. Even at the highest polymer concentration of 5 mg/mL, hemolysis due to POEA-4, POEA-5, and their degradation products was still less than 3%, indicating the excellent blood compatibility of POEA.

3.4. In Vivo Acute Toxicity. Figure 6b shows the body weight change of mice receiving different treatments during a 15 day period. It is found that all the mice grew normally and the body weight slightly increased. Moreover, no symptom of any abnormalities such as anxiety, convulsion, vomiting, and breathing difficulties was observed in any animals during the test period, suggesting that oral administration of POEA-4 and POEA-5 did not cause any adverse effects on mice even at a very high dose of 2000 mg/kg. After 15 days, thin sections of mouse organs including liver, heart, spleen, lung, kidney, stomach, and intestines were stained histologically and examined by optical microscopy. As shown in Figure 6c, none of the polymer treatments caused any significant lesion to the organs, suggesting minimal toxicity of POEA-4 and POEA-5 even at a high dose of 2000 mg/kg in a 2 week uptake period. The structure of liver lobules is clear and complete as well as myocardial cells were dense and neatly organized. No damage was found in the cells of the spleen, which is the largest lymphatic organ and part of the immune system. Glomerulus and renal tubular structure was normal without inflammatory cell infiltration. The structure of air tube, bronchus, and alveolar in the lung was clear and integrated.

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ACS Appl. Mater. Interfaces 2015, 7, 10436−10445

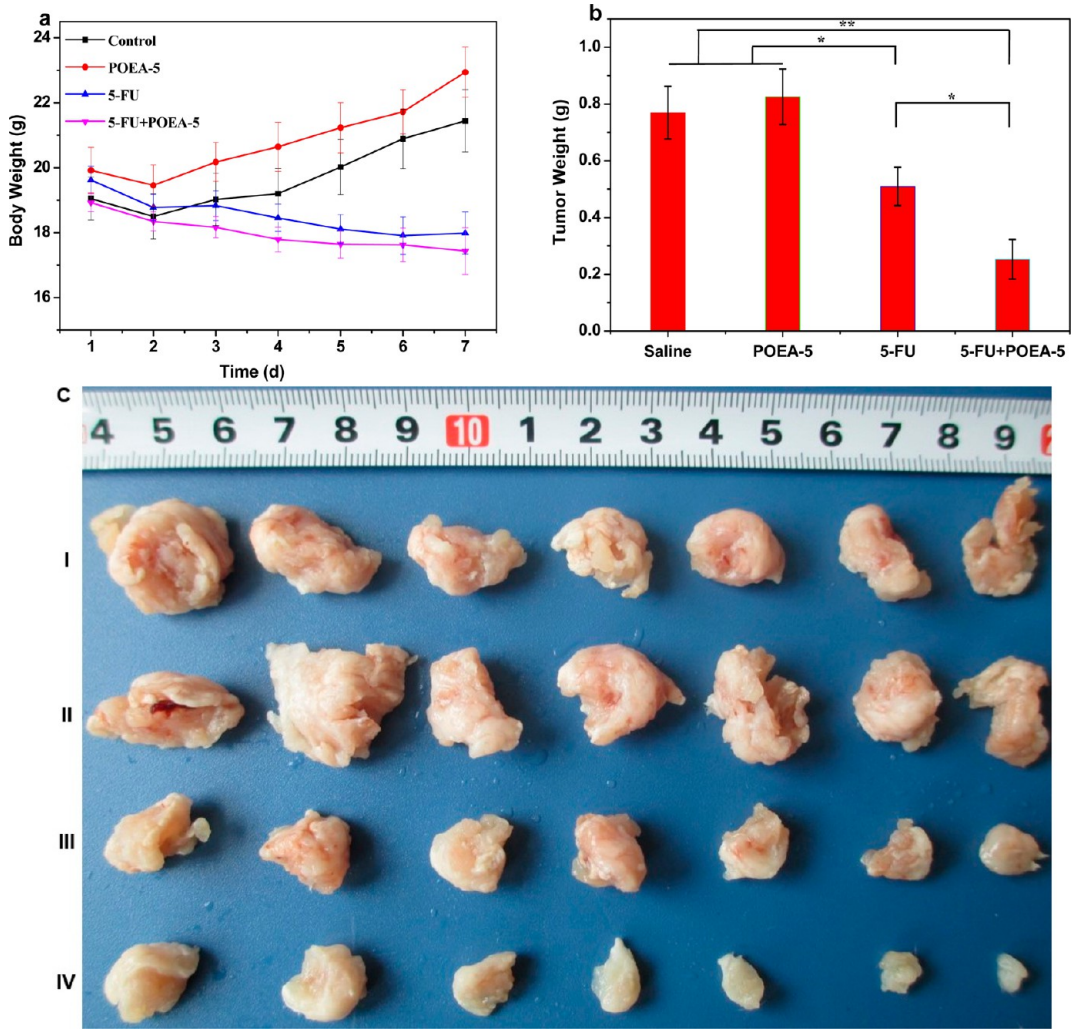

Figure 8. In vivo anticancer acitivity. (a) Weights of H22 graft tumors after daily administration for 7 days; (b) weights of H22 grafted tumors after daily administration for 7 days. * Represents $P < 0.05$ and ** represents $P < 0.01$; (c) images of H22 graft tumors at the end time point of the treatment; (I) saline; (II) POEA-5; (III) 5-FU; (IV) 5-FU+POEA-5.

For the intestine tissue, there were many vertical plica visible on cavity surface and many small villi distributed on mucosal surface.

Furthermore, blood samples were collected from the ocular vein and serum was collected. Blood biochemical parameters, including total protein (TP), blood urea nitrogen (BUN), creatinine (CREA), glutamic oxaloacetic transaminase (GOT), and lactate dehydrogenase (LDH) were measured, as shown in Figure 7. TP is the main plasma solid composition, which was composed of globulin and albumin. Gavages of POEA-4 or POEA-5 did not cause noticeable change in plasma protein content. BUN and CREA are the main terminal metabolism products and are indicators of renal function. BUN and CREA increase usually indicate renal disease, such as glomerulonephritis, acute or chronic renal failure. Compared to that of control, there is no significant statistical difference in the BUN and CREA levels after treatment. GOT mainly exists in the cytoplasm and mitochondria of liver cells and LDH that is found in the cytoplasm of cells in many organs, elevated serum GOT or LDH level often indicates liver and heart diseases. No significant change in GOT and serum LDH in any animals receiving high doses of POEA, demonstrating that the polymers did not cause liver and heart damage. All these experiments demonstrated that both POEA-4 and POEA-5 are biocompatible.

3.5. In Vivo Antitumor Activity. To investigate in vivo antitumor activity, we established a subcutaneous liver cancer mouse model using the mouse hepatocellular cancer cell line H22, and selected 5-FU as the model drug, which is widely used to treat malignant tumors such as brain, liver, and breast cancer by intravenous routes.[39] Free 5-FU and 5-FU+POEA-5 tablets were administrated via oral gavage every day for 7 days. Mice treated with saline and empty POEA-5 tablets were used as negative controls.

The average body weight of saline and empty POEA-5 treated mouse groups showed significant increases during the treatment, from 19 to 21.5 g and 19.5 to 23 g, because of the continuous growth of tumors (Figure 8a). In contrast, the body weight of 5-FU and 5-FU+POEA-5 tablets treated mice almost remained unchanged, indicating that 5-FU+POEA-5 tablets could effectively inhibit the tumor growth. At the end of treatment, mice were sacrificed and tumor tissues were excised, imaged, and weighed. Figure 8b showed the average weight of tumor from each mouse group. Mice treated with saline, POEA-5 tablets and free 5-FU had much heavier tumors on average (about 0.77, 0.83, and 0.51 g, respectively) than mice treated with 5-FU+POEA-5 tablets (only 0.28 g). The tumor growth inhibition of 5-FU+POEA-5 tablets treated mice reached 63.7%, which was

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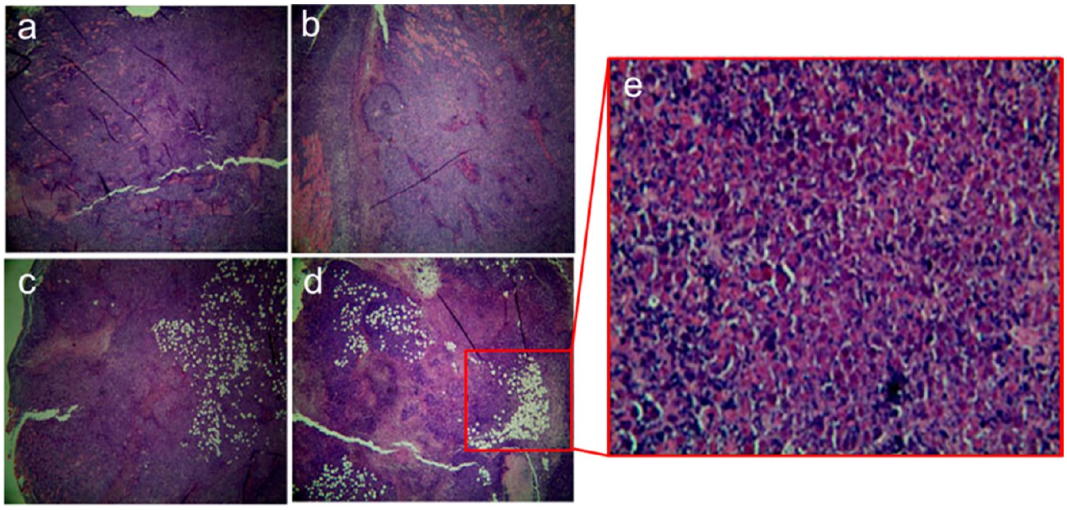

Figure 9. Images of the histological sections of H22 grafted tumors ((a−d) 40×; (e) 400×); (a) saline; (b) POEA-5; (c) 5-FU; (d) 5-FU+POEA-5; (e) 5-FU+POEA-5 (high magnification).

almost 2-fold as that of free 5-FU treated mice (about 33.7%) indicating the statistically significant difference of antitumor efficiency between the free 5-FU and 5-FU+POEA-5 tablets treated mice ($P < 0.05$). As depicted in Figure 8c, neither saline nor empty POEA-5 powder treatment was effective for tumor inhibition. The size of tumors in both groups increased rapidly during the 7-day treatment, reaching more than 2.0 cm on day 7. In contrast, mice treated with 5-FU+POEA-5 tablets had the smallest tumors (less than 1.0 cm in diameter), demonstrating effective suppression of tumor growth, which was superior than treatment with 5-FU alone.

To further examine the efficacy of different treatments, we examined hematoxylin and eosin (H&E) stained sections of tumors by optical microscopy. As show, in Figure 9a, b, a large amount of living cells are able to be seen in the tumors from the mice treated with saline and empty POEA-5 tablets, and the tumor cells were densely arranged and nuclei hyperchromatic. In free 5-FU-treated mice, a small area of tissue necrosis cytoplasm of tumor cells is visible where nuclei are stained pink and light (Figure 9c). On the other hand, the existence of a large area of necrotic region in the tumors from the mice treated with 5-FU+POEA-5 tablets (Figure 9d) and high magnification (Figure 9e) suggested the much higher necrotic rate in contrast to that of other groups. All these results fully demonstrated the notably enhanced anticancer efficacy of 5-FU+POEA-5 tablets, which establishes that POEA can be an effective anticancer drug carrier in oral administration.

4. CONCLUSIONS

In summary, we synthesized a new ortho ester diamine monomer (2,2′-((4,4′-(oxybis (methylene)) bis(1,3-dioxolane-4,2-diyl)) bis(Oxy)) diethanamine) with balanced hydrophobility and hydrophilicity using diglycerol as starting material. After then, two poly(ortho ester amide) copolymers (POEA-4 and POEA-5) were prepared via polycondensation of an ortho ester diamine monomer with active esters of different aliphatic diacids. In vitro cytotoxicity test demonstrated that both POEA-4 and POEA-5 have excellent biocompatibility and in vivo acute toxicity test suggested that oral administration of both polymers did not cause any adverse effects on mice even at a very high dose (2000 mg/kg). 5-FU, a widely used anticancer drug, was mixed and compressed with POEA-5 to give oral administrated disk tablets. The kinetics of POEA mass loss and release of 5-FU were nearly zero-order, suggesting predominantly surface-restricted polymer erosion and drug release. In vivo antitumor efficacy against H22 grafted tumors of 5-FU-loaded POEA tablets were fully examined, and the 5-FU+POEA-5 tablet-treated mice showed the best inhibition in tumor growth compared to free 5-FU-treated mice. We envision that, with further optimization, POEA-based drug carriers could have great potential applications in oral chemotherapy.

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Notes

The authors declare no competing financial interest.

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