FRET-trackable biodegradable HPMA copolymer-epirubicin conjugates for ovarian carcinoma therapy

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Abstract

To develop a biodegradable polymeric drug delivery system for the treatment of ovarian cancer with the capacity for non-invasive fate monitoring, we designed and synthesized *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-epirubicin (EPI) conjugates. The polymer backbone was labeled with acceptor fluorophore Cy5, while donor fluorophores (Cy3 or EPI) were attached to HPMA copolymer side chains via an enzyme-cleavable GFLG linker. This design allows elucidating separately the fate of the drug and of the polymer backbone using fluorescence resonance energy transfer (FRET). The degradable diblock conjugate (2P-EPI) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization using a bifunctional chain transfer agent (Peptide2CTA). The pharmacokinetics (PK) and therapeutic effect of 2P-EPI (Mw~100kDa) were determined in mice bearing human ovarian carcinoma A2780 xenografts. Compared to 1st generation conjugate (P-EPI, Mw<50kDa), 2P-EPI demonstrated remarkably improved PK such as four-fold terminal half-life (33.22 ± 3.18 h for 2P-EPI vs. 7.55 ± 3.18 h for P-EPI), which is primarily attributed to the increased molecular weight of the polymer carrier. Notably, complete tumor remission and long-term inhibition of tumorigenesis (100 days) were achieved in mice (n=5) treated with 2P-EPI. Moreover, *in vitro* cell uptake and intracellular drug release were determined via FRET intensity changes. The results establish a solid foundation for future *in vivo* tracking of drug delivery and chain scission of polymeric conjugates by FRET imaging.

Key words: *N*-(2-Hydroxypropyl)methacrylamide (HPMA), Epirubicin, Ovarian carcinoma, FRET

1. Introduction

Polymer-drug conjugates have a long history. The concept of polymer-drug conjugates was developed to address sub-optimal bioactivity and non-specificity of low molecular weight drugs in the human body [1-4]. Loading drugs on soluble macromolecules can improve pharmacokinetics and accumulation of drugs in solid tumors, resulting in enhanced therapeutic efficacy and reduced adverse side effects [5-7]. As the first example entering clinical trials for cancer treatment, (*N*-(2-hydroxypropyl)methacrylamide) (HPMA) copolymer-doxorubicin (DOX) conjugate demonstrated significant reduction of nonspecific toxicity. Maximum tolerated dose (MTD) of HPMA copolymer-DOX conjugate in humans was 320 mg/m² of DOX equivalent, whereas MTD of free (unbound) DOX in humans is 60-80 mg/m². The enhanced MTD of the polymer-bound DOX is primarily attributed to low uptake in heart tissue [8]. The use of polymeric drug delivery systems has become an established approach for improvement of cancer chemotherapy [9,10]. To gain more insight into the relationship between structure of polymer carrier and antitumor activity of polymer-conjugates, various fluorescent dyes have been exploited to investigate cellular uptake and drug release [11,12].

Recently we designed 2nd generation backbone-degradable HPMA copolymer carriers [13-15]. The combination therapy of A2780 human ovarian carcinoma xenografts with long-circulating HPMA copolymer-paclitaxel/gemcitabine conjugates showed distinct advantages over 1st generation conjugates [16]. Similarly, the backbone degradable HPMA copolymers possessed enhanced efficacy (when compared to 1st generation conjugates) in ovarian carcinoma xenografts in mice [17,18] and in a rat osteoporosis model [19].

Epirubicin (EPI), the 4'-epimer of the anthracycline DOX, is an antineoplastic agent that inhibits DNA replication, transcription and repair by binding to nucleic acids [20]. Epirubicin has been regarded as one of the most active drugs for the patients with cancer, particularly with metastatic disease [21]. It has shown equivalent cytotoxic effects to DOX in human ovarian cancer cells, but decreased cardiotoxicity and myelotoxicity than DOX at equimolar doses [22]. Thus, epirubicin is thought to have a better therapeutic index than DOX. Recently, EPI **has been** bound to various polymer carriers to improve its properties and delivery. For example, dextran [23], polyethylene glycol (PEG) [24], dendritic PEG [25], human monoclonal antibodies [26], **polyHPMA** [27,28] and polysialic acid [28] **were** used as (targetable) carriers.

Taking advantage of the inherent fluorescence of EPI, we synthesized 2nd generation HPMA copolymer-EPI conjugates aiming to develop a biodegradable polymeric drug delivery system with the capacity for non-invasive fate monitoring. Fluorescence resonance energy transfer (FRET) was used as a tool to track chain scission of the conjugates and to elucidate separately the fate of the polymer backbone and the drug. The in vitro cytotoxicity, pharmacokinetics (PK), and in vivo antitumor activity of the conjugates were first evaluated on human ovarian carcinoma xenografts. The backbone of FRET polymers was labeled with Cy5, whereas model drug Cy3 or EPI was attached to HPMA copolymer backbone via an enzyme-cleavable GFLG linker. The cell uptake and drug release were analyzed by changes in FRET intensity.

2. Materials and methods

2.1. Materials

Common solvents methanol, acetonitrile, **dimethyl**formamide (DMF), dichloromethane (DCM) were from Fisher Scientific (Pittsburgh, PA) as HPLC grade and used directly. Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), papain (EC 3.4.22.2, from papaya latex) and cathepsin B (EC 3.4.22.1, from bovine spleen) were from Sigma-Aldrich (St. Louis, MO). Epirubicin (EPI) was a kind gift from Prof. Kui Luo (Sichuan University, China). HATU was from AAPPTEC (Louisville, KY). **2,2-azobis**[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-

044) and **2,2-azobis**(2,4-dimethyl valeronitrile) (V-65) were obtained from Wako Chemicals (Richmond, VA). ¹²⁵Iodine was from Perkin-Elmer (Waltham, MA). Cy3-/Cy5-NHS ester and Cy5-amine were purchased from Lumiprobe (Hallandale Beach, FL). Various monomers including *N*-(2-hydroxypropyl)methacrylamide (HPMA) [29], *N*-methacryloylglycylphenylalanylleucylglycine (MA-GFLG-OH) [30], 3-(*N*-methacryloylglycylphenylalanylleucylglycyl) thiazolidine-2-thione (MA-GFLG-TT) [31], *N*-methacryloyltyrosinamide (MA-Tyr-NH₂) [32], 2-(*N*-methacryloylglycylphenylalanylleucylglycine)-*N*'-Boc-ethylenediamine (MA-GFLG-NH-Boc) [16], and RAFT agents, 4-cyanopentanoic acid dithiobenzoate (CPA) [33] and peptide2CTA (*N*^α,*N*^ε-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl)lysine) [15], were synthesized as previously described.

2.2. Cell culture

A2780 human ovarian cancer cells (ATCC) were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium (Gibco) supplemented with 10% FBS and a mixture of antibiotics (100 units/mL penicillin, 0.1 mg/mL streptomycin).

2.3. Synthesis and characterization of HPMA copolymer conjugates

2.3.1. Synthesis of polymerizable derivative of epirubicin (MA-GFLG-EPI)

N-(methacryloylglycylphenylalanylleucylglycyl) epirubicin (MA-GFLG-EPI) was synthesized by the reaction of MA-GFLG-OH with EPI in DMF using HATU/DIPEA as coupling agent. In brief, EPI (54 mg, 0.1 mmol) was first dissolved in 0.2 mL DMF. MA-GFLG-OH (50 mg, 0.11 mmol) was dissolved in 0.5 mL DMF, followed by addition of HATU (38 mg, 0.1 mmol) and DIPEA (45 μL, 0.25 mmol). After activation at room temperature for 2 min, MA-GFLG-OH/HATU/DIPEA solution was added to the vial containing EPI solution. The system was kept stirring in dark overnight. The reaction solution (10 μL, diluted with methanol) was then loaded onto an analytical column (Zorbax C18, 4.6×250 mm) and checked by HPLC. The peak of free EPI (15.34 min) disappeared, whereas a new peak showed up (20.14 min) indicating the reaction had finished. The solvent was removed by rotary evaporator under vacuum. The crude product was purified by column chromatography (silica gel 60 Å, 200-400 mesh) with elution 6:1 dichloromethane/methanol. A dark red powder was obtained after removal of the solvents with a yield of 70 mg (70%). The structure of the monomer (MA-GFLG-EPI) was confirmed by MALDI ToF MS (LTQ-FT, ThermoElectron) ([M+Na]⁺ 1024.43), and the purity was verified by HPLC (Agilent 1100 series).

2.3.2. Synthesis of HPMA copolymer-epirubicin conjugates (P-EPI/2P-EPI and P-Tyr-EPI/2P-Tyr-EPI)

HPMA copolymer-epirubicin conjugates (P-EPI and 2P-EPI) were synthesized by the copolymerization of HPMA with MA-GFLG-EPI using VA044 as initiator and 4-cyanopentanoic acid dithiobenzoate (CTA) or Peptide2CTA as chain transfer agent, respectively. As an example, HPMA (138 mg, 0.965 mmol) and MA-GFLG-EPI (35 mg, 0.035 mmol) were dissolved in 0.3 mL methanol under N₂ atmosphere. Peptide2CTA (60 μL with conc. 8.5 mg/mL in methanol, [M]/[CTA]=1400) and VA044 at a molar ratio of 3:1 were added using a syringe. The ampoule was bubbled with N₂ in ice bath for 5 min then sealed and polymerization was carried out at 40°C for 24 h. The copolymer was precipitated in acetone. The resultant orange-color copolymer was re-dissolved in methanol and re-precipitated in acetone to remove unreacted monomers. The dithiobenzoate end group was replaced by radical-induced end-modification

using excess of V-65 in methanol at 55 °C for 2 h. The final product (2P-EPI) was isolated by precipitation and dried under vacuum at room temperature with yield of 80 mg (50%). Similarly, P-EPI was obtained when CTA was used as RAFT agent with $[M]/[CTA]=550$ (Scheme 1).

The molecular weight and molecular weight distribution of the conjugates were determined by size-exclusion chromatography (SEC) on an ÄKTA FPLC system (GE healthcare) equipped with miniDAWN and OptilabEX detectors (Wyatt) with acetate/30% acetonitrile (pH 6.5) as mobile phase. Superose 6 HR10/30 column was used. The drug content in conjugates was determined by enzyme cleavage of free drug from polymer side chain GFLG linker using papain according to the procedure described previously [15].

To synthesize a radioisotope ^{125}I labeled conjugate, comonomer MA-Tyr-NH₂ (1.5% molar ratio in feed) was added and the same procedure shown above was used to produce P-Tyr-EPI/2P-Tyr-EPI.

Scheme 1. Synthesis of HEMA copolymer-epirubicin conjugates

2.3.3. Synthesis of polymer conjugates containing fluorophore Cy3 and/or Cy5

To synthesize polymer **conjugates** suitable for FRET evaluation, **P-Cy3-Cy5/2P-Cy3-Cy5**, the polymer precursor containing side chain thiazolidine-2-thione (TT) and GFLG linker terminated with protected amino group was first synthesized by RAFT copolymerization of the amino-protected monomer (MA-GFLG-NH-Boc [16]), HPMA and MA-GG-TT (Scheme 2A). After chain end-modification, the content of TT groups in the copolymer was determined by UV ($\epsilon_{305}=10,900 \text{ M}^{-1} \text{ cm}^{-1}$ in methanol) [31]. Cy5-NH₂ was dissolved in DMSO and reacted with polymer precursor. Unbound dye was removed using PD10 column. To incorporate the second dye, Cy3, the polymer was dissolved in water followed by addition of trifluoroacetic acid. The sample was kept stirring in ice-bath for 30 min, then condensed under reduced pressure and precipitated in precooled ether/acetone. The side-chain amino content in the deprotected polymer was analyzed by ninhydrin assay. Cy3-NHS was used to attach Cy3 to the polymer backbone via GFLG enzyme-cleavable linker. The content of Cy3 and Cy5 in the polymer chain was determined via UV-vis spectroscopy.

As control, HPMA copolymers containing fluorophore Cy3 or Cy5 (P-Cy3 or P-Cy5) were synthesized by polymer analogous reaction of HPMA polymer precursor containing pendant amino groups with Cy3-/Cy5-NHS ester (Scheme 2B). Free dye was removed using PD10

column (Amersham Biosciences). The content of Cy3/Cy5 in polymer conjugates was determined via UV-vis spectroscopy (Varian Cary 400 Bio UV-visible spectrophotometer).

Scheme 2. Synthesis of polymer conjugates containing fluorophores

2.3.4. Synthesis of HPMA copolymer-epirubicin conjugate containing fluorophore Cy5

FRET polymer P-EPI-Cy5 was synthesized in two steps (Scheme 3): First, RAFT copolymerization of HPMA (134 mg, 0.94 mmol), MA-GFLG-EPI (33 mg, 0.035 mmol) and MA-GG-TT (7.5 mg, 0.025 mmol) was conducted in methanol at 40 °C as described above. The dithiobenzoate end group was removed by addition of 40x V65 to the polymer solution at 55 °C for 2 h. The TT group was then aminolyzed by Cy5-NH₂. The free dye was removed using PD10 column. The final contents of EPI and Cy5 were determined by UV-vis spectroscopy.

Scheme 3. Synthesis of polymer conjugates containing epirubicin and Cy5 (P-EPI-Cy5)

2.4. FRET measurements

The concentration of fluorophore-labeled conjugate solution was first determined by UV-vis spectroscopy. Typically, 1.5 mg/mL conjugate in methanol was scanned within range of 400-800 nm. The solution was further diluted into ~100 g/mL, and the fluorescence intensity of each

sample was measured in duplicates using Infinite M1000 PRO (TECAN) with excitation wavelengths of 445 nm for EPI (P-EPI), 548 nm for Cy3 (P-Cy3) and 646 nm for Cy5 (P-Cy5), respectively. For FRET measurements, the donor was excited at 520 nm for Cy3 [34] and 445 nm for EPI; the emission spectra of the donor-acceptor were recorded at the range of 400 to 800 nm.

To compare the fluorescence intensity changes of the conjugate P-Cy3-Cy5 before and after cleavage of the donor Cy3, the conjugate was incubated in McIlvaine's buffer (50 mM citrate/0.1 M phosphate, pH 6) in the presence of papain (5 mg/mL, preactivated with 10 mM glutathione) at 37 °C for 1 h. The cleaved conjugate was diluted into methanol and determined with Ex 520 nm.

2.5 FRET change measurement in cancer and normal cells

To determine the cathepsin B dependence of drug release capability in our conjugates, FRET model conjugates, P-Cy3-Cy5 and 2P-Cy3-Cy5, were incubated with A2780 ovarian cancer cells (cathepsin B over-expressing) and NIH3T3 normal cells (cathepsin B low expression). The cells were first incubated with the conjugate (P-Cy3-Cy5 or 2P-Cy3-Cy5) at 37°C for 4 h, then cells were washed with fresh medium and subsequently cultured in fresh medium without conjugate for another 8 or 20 h. Cell lysates at different time intervals were measured by fluorescence spectrometer. FRET ratio = I_{Cy3} / I_{FRET} , where I_{Cy3} and I_{FRET} are the fluorescence intensity at 562 nm and 664 nm, respectively (excitation 520 nm). The data were presented as mean $\pm$ standard deviation (n=3).

2.6 Confocal microscopy of FRET changes in A2780 cancer cells

To visualize the FRET changes in cancer cells, A2780 cells were first incubated with conjugate P-Cy3-Cy5 at 37°C. After 4 h incubation, the cells were washed and fresh medium was added. Then a portion of treated cells was fixed with 4% paraformaldehyde immediately while the other cells were incubated for another 20 h and then were fixed. The fixed cells were observed under confocal microscope using the standard acceptor Cy5 photobleaching FRET method (the cells were exposed to high excitation intensity at an excitation wavelength of 646 nm for a 20 min period. In this experiment, both pre-bleach and post-bleach images were collected).

2.7. EPI release inside cultured cells

The release of drug EPI from Cy5-labeled polymeric conjugate was determined using FRET, with EPI serving as the donor fluorophore, and Cy5 as the acceptor. The conjugate P-EPI-Cy5 was incubated with A2780 cells at 37°C for 4 h. Then the treated cells were cultured in fresh medium for another 20 h. The cell lysate was measured by fluorescence spectrometer, before and after the 20 h additional culture. The "relative" FRET efficiency, also known as the FRET ratio, was calculated with the following equation FRET ratio = I_{EPI} / I_{FRET} , where I_{EPI} and I_{FRET} are the fluorescence intensity at 590 nm and 664 nm, respectively (excitation 490 nm).

2.8. Cell uptake study

Cellular uptake of the EPI and its conjugates (P-EPI and 2P-EPI) was analyzed using flow cytometry. A2780 human ovarian cancer cells (2×10^5) were seeded in 6-well plates. After 24 h culture, the cells were treated with free EPI, P-EPI, or 2P-EPI (EPI concentration: 100 nM) for 6 or 24 h. Untreated cells served as a negative control for background fluorescence. Thereafter, the cells were harvested and washed. An average of 1×10^4 cells was determined using flow

cytometry (BD Biosciences) and FlowJo software (Tree star). EPI uptake was analyzed based on the EPI fluorescence intensity (n=3).

2.9. In vitro cytotoxicity study

The cytotoxicity of free drug EPI and its polymeric conjugates (P-EPI, 2P-EPI) against A2780 human ovarian cancer cells was measured by CCK-8 assay (Dojindo). The cells were seeded in 96-well plates at the density of 10,000 cells/well in RMPI-1640 media containing 10% FBS. The cells were washed after 24 h, then incubated with media containing the drug EPI or its polymeric conjugates (P-EPI, 2P-EPI) at a series of drug concentrations. After 48 h of incubation, the number of viable cells was estimated using CCK-8 kit according to manufacturer's protocol. In brief, medium was discarded and replaced with 100 μ L fresh growth medium in each well, followed by the addition of 50 μ L 5 $\times$ diluted CCK-8 solution. Dehydrogenase activities in live cells converted the water-soluble tetrazolium salt WST-8 into a soluble yellow-color formazan dye. After the incubation of cells at 37°C, 5% CO₂ for 2 h, the absorbance was measured using a microplate reader at 450 nm (630 nm as reference). Untreated control cells were set as 100% viable.

2.10. Radiolabeling and pharmacokinetics study

HPMA copolymer-EPI conjugates containing tyrosinamide were reacted with Na¹²⁵I (Perkin Elmer) at room temperature in 0.01 M phosphate buffer containing chloramine-T for 30 min and then purified with PD-10 columns (GE Healthcare). The specific activity of the hot samples was in the range 40-60 μ Ci/mg. The ¹²⁵I labeling of polymer conjugates was conducted immediately before use. After radiolabeling, 6- to 8-week-old healthy female nude mice (22-25 g; Charles River Laboratories) were intravenously injected 0.5 mg (20 μ Ci/mouse) ¹²⁵I-labeled HPMA copolymer-drug conjugates (P-EPI and 2P-EPI, five mice per group). At predetermined intervals, blood samples (10 μ L) were taken from the tail vein, and the radioactivity of each sample was measured with Gamma Counter (Packard). The blood pharmacokinetic parameters for the radiotracer were analyzed using a two-compartmental model with WinNonlin 5.0.1 software (Pharsight).

2.11. Tumor model

All animal studies were carried out in accordance with the University of Utah IACUC guidelines under approved protocols. A2780 human ovarian cancer cells (5×10^6) in 100 μ L of phosphate buffered saline were subcutaneously inoculated in right flank of 6- to 8-week-old syngeneic female nude mice (22–25 g, Charles River Laboratories). When tumor reached approximately 4-5 mm in diameter (average 3 weeks after inoculation) treatment started.

2.12. In vivo antitumor activity

Female nude mice bearing subcutaneous A2780 ovarian tumors were randomly assigned to four groups (n=5 for each group). P-EPI and 2P-EPI were administered via tail vein with dose 5 mg/kg EPI equivalent on day 0, 4, and 8. Free drug EPI was also used for comparison. The mice in the control group were treated with saline. The day that mice received EPI or its conjugates treatment was set as day 0. The tumor size was measured to monitor the tumor growth. The tumor volume at day 0 was normalized to 100%. All subsequent tumor volumes and body weight were then expressed as the percentage relative to those at day 0. Mice were sacrificed at signs of sickness such as body weight loss >20%. Otherwise mice were sacrificed at 100 days.

2.13. Statistical analysis

The Student's t test was used to test differences in therapeutic efficiency, cell uptakes, pharmacokinetic parameters, and toxicity among different conjugates. Comparison among groups was performed using one-way ANOVA. The significance level was set at 0.05.

3. Results and discussion

3.1. Synthesis of HPMA copolymer-epirubicin conjugates

As an isomer of DOX, EPI has been reported to have different metabolic degradation and faster clearance from plasma after i.v. injection. As a result, EPI has less side effects compared with DOX at doses producing equivalent antitumor effects [35]. EPI therefore has been nanosized to various formulations such as micelles [36], liposomes [37], nanoparticles [38,39], and, most interesting for us, water-soluble conjugates to improve its therapeutic index. In general, polymer-EPI conjugates were produced via polymeranalogous reaction in which EPI is attached to polymer through active ester [27] or in the presence of coupling agents [25,28]. Here we report a different way to synthesize the conjugates - RAFT copolymerization. The polymerizable EPI derivative was synthesized first, and then copolymerized with HPMA with option of using MA-Tyr-NH₂ for isotope labeling. The major advantage of this approach is reproducibility with predetermined molecular weight and narrow polydispersity. In addition, the polymer is relatively pure without detectable free drug (Figure S1). Previously we have reported synthesis of long-circulating 2P-PTX and 2P-GEM in one step using a two-arm enzyme-cleavable chain transfer agent [16]. To assess this universal approach, we synthesized the 1st generation and 2nd generation HPMA copolymer-EPI conjugates; their molecular weight (including molecular weight distribution) and drug content are listed in Table 1.

Table 1. Characterization of HPMA copolymer-epirubicin conjugates

	Mn (kDa)	Mw (kDa)	Mw/Mn	EPI % (wt)
P-EPI	39	43	1.10	7.4
P-Tyr-EPI	26	28	1.08	6.5
2P-EPI	76	106	1.39	5.9
2P-Tyr-EPI	61	84	1.38	6.4

3.2. Cell uptake and in vitro cytotoxicity

The cell uptake of the conjugates (P-EPI and 2P-EPI) was analyzed using flow cytometry with EPI fluorescence signal. The free drug EPI was used as a control. A2780 human ovarian cancer cells were incubated with different EPI formulations, respectively. Free EPI showed a higher cell uptake than the two conjugates. This difference is likely due to their distinct entry pathways - diffusion (free drugs) vs. endocytosis (conjugates). In addition, the cytotoxicity of free EPI and its HPMA copolymer conjugates (P-EPI and 2P-EPI) against A2780 human ovarian cancer cells was determined. Figure 1A shows the representative cell-growth inhibition curves. Overall, free drug EPI and its conjugates (P-EPI, 2P-EPI) showed a dose-dependent cytotoxicity against A2780 cells. On the basis of the IC₅₀ values (Figure 1B), both HPMA copolymer-EPI

conjugates had less *in vitro* cytotoxicity than free EPI, due to the different mechanism of cell uptake [4,7].

Figure 1. Cell uptake and cytotoxicity of free drug EPI and its conjugates (P-EPI, 2P-EPI) in A2780 human ovarian cancer cells. (A) Flow cytometry analysis of cell uptake in the A2780 cells incubated with medium alone (non-treated), free EPI, or its conjugates (P-EPI, 2P-EPI) at 37 °C for 6 and 24 h. MFI, mean fluorescence intensity. (B) *In vitro* cytotoxicity of free drug EPI and its HPMA conjugates (P-EPI, 2P-EPI) toward A2780 human ovarian carcinoma cells. The data are presented as mean ± standard deviation (n=3-4).

3.3. Pharmacokinetic study of ^{125}I -labeled conjugates

It has been reported that conjugation of free drug to HPMA polymer carrier markedly **slows** its blood clearance [40]. For example, the fast initial clearance of DOX ($t_{1/2}$) is 4 min, however, there was still 55% polymer-bound drug in circulation **1 h after** injection of P-DOX (Mw 25 kDa) [40]. In this study, we compared pharmacokinetic profiles of 1st generation conjugate (P-(Tyr)-EPI, Mw 28 kDa) and 2nd generation conjugate (2P-(Tyr)-EPI, Mw 84 kDa) to highlight the effect of molecular weight on plasma concentration and circulation time. Tyrosine moiety was inserted into the conjugates for radiolabeling (^{125}I) in order to enhance accuracy and sensitivity of the analysis. The blood radioactivity-time profiles were determined and illustrated in Figure 2. The pharmacokinetic parameters of the two conjugates in mice are listed in Table 2, and the previously reported half-lives of 2P-PTX/P-PTX and 2P-GEM/P-GEM are cited here for comparison [16]. Higher Mw 2P-EPI conjugate showed a longer terminal half-life (33.22 h) than low Mw conjugate P-EPI (7.55 h). 2P-EPI (AUC=1060.48 %ID/mL) had a 10-fold higher systemic exposure than P-EPI (110.10 %ID/mL) ($p<0.001$). The increased exposure of 2P-EPI is mainly attributed to its significantly slower systemic clearance (CL) (2P-EPI: 0.09 mL/h vs. P-EPI: 0.91 mL/h) ($p<0.001$). Taken all together, the 2nd generation conjugate 2P-EPI having an increased Mw possesses an improved pharmacokinetic profile.

Figure 2. Pharmacokinetic profiles of ^{125}I -labeled conjugates P-EPI and 2P-EPI in mice. The data represent the mean radioactivity expressed as a percentage of the injected dose per gram of blood (n=5).

Table 2. Comparison of pharmacokinetic parameters for ¹²⁵I-labeled conjugates in mice

	P-EPI	P-PTX ^a	P-GEM ^a	2P-EPI	2P-PTX ^a	2P-GEM ^a
T _{1/2,α} (h)	0.33±0.07	0.88±0.11	0.26±0.02	0.18±0.06	1.13±0.13	1.45±0.36
T _{1/2,β} (h)	7.55±1.55	13.30±1.28	6.36±0.66	33.22±3.18	37.90±3.55	32.07±2.50
AUC (%ID h/mL blood)	110.10±14.06	420.95±26.05	108.66±6.74	1060.48±88.83	1206.42±85.97	1481.23±83.06
CL (mL/h)	0.91±0.12	0.24±0.01	0.92±0.06	0.09±0.01	0.08±0.01	0.07±0.004
MRT (h)	10.34±2.09	18.25±1.71	8.49±0.88	47.79±4.57	52.86±4.95	45.39±3.43
V _{ss} (mL)	9.39±0.82	4.34±0.16	7.82±0.38	4.51±0.11	4.38±0.16	3.06±0.10

Although individual drugs (EPI, PTX, and GEM) have different metabolism and clearance time [41], the new generation conjugate 2P-EPI showed significant improved pharmacokinetics with parameters similar to 2P-PTX and 2P-GEM, such as terminal half life, total body clearance, and steady-state volume of distribution (Table 2), which indicates conjugation of drug to polymer carrier can improve its stability in plasma, and the elimination rate of the conjugates is primarily determined by the polymer carrier.

3.4. In vivo anti-tumor activity

The therapeutic potential of the backbone degradable long-circulating HPMA copolymer-EPI conjugate (2P-EPI) was evaluated in female nude mice bearing A2780 human ovarian carcinoma xenografts. The mice were intravenously injected with three doses of 5 mg/kg EPI equivalent on days 0, 4, and 8. Free drug EPI and the 1st generation conjugate P-EPI were also administered for comparison. Tumor growth was closely monitored during and after treatment. At day 20, complete tumor regression was achieved in the five mice treated with conjugate 2P-EPI (Figure 3); the tumors treated with P-EPI shrank to 80±37% of the initial size. In contrast, free drug EPI at equivalent doses only slightly delayed tumor growth when compared with saline (control), and mice had to be sacrificed on day 20 as the tumor had reached 1772±840% of the baseline. However, there was no significant difference between treatment with 2P-EPI and P-EPI until day 35 when tumor started regrowth in P-EPI group, and four of the tumors grew back to ~1200% at day 80 (p<0.01) (Figure 3). On the contrary, no observable tumor was detected in the mice treated with 2P-EPI at day 100. These results demonstrate the importance of long-term experiments for evaluation of tumor growth inhibitory effect. The results also indicated that 2P-EPI is highly superior to both P-EPI and free EPI. The complete tumor regression and long-term inhibition of tumorigenesis by 2P-EPI treatment are attributed to long circulation time and sufficient extravasation of the conjugates at the tumor site by the enhanced permeability and retention (EPR) effect. In addition, this result also suggests that 2P-EPI conjugate may be able to arrest both tumor progenitor cells and differentiated cells as we observed in another scenarios [42].

For safety concern, body weight of the mice was closely recorded during and after treatment (Figure S2). The body weights of the mice temporarily decreased (less than 10%) when P-EPI and 2P-EPI were administered as multiple dosages but recovered gradually and remained stable after withdrawal, which suggests the doses used were tolerable.

Figure 3. Comparison of *in vivo* anti-tumor activity on female nude mice bearing A2780 human ovarian carcinoma xenografts. (A) The mice were intravenously injected with 3 doses of EPI or HPMA copolymer-EPI conjugates (P-EPI and 2P-EPI). Free drug and untreated groups were stopped on day 20 due to large size of tumors. (B) Long-term monitoring of tumor growth in conjugate treatments. (C) The tumor size on day 80 of the polymer conjugates, and (D) End point photographs of tumor-bearing mice from variable treatments.

3.5. Potential to track chain degradation and intracellular drug release via FRET

Similar to DOX, the fluorescence signal from EPI has been used for cellular uptake studies [39,43]. However, the polymer conjugation can affect the fluorescence emission [28]. Consequently, the data only from drug fluorescence may be misinterpreted.

Herein we propose to use FRET imaging as a tool to monitor drug release from HPMA copolymer conjugates. Initially, we designed and prepared a HPMA copolymer conjugate containing a popular FRET pair Cy3/Cy5. The donor fluorophore Cy3 was attached to HPMA polymer backbone as model drug via a cleavable (by lysosomal proteases) tetrapeptide linker Gly-Phe-Leu-Gly (GFLG), while the acceptor Cy5 was directly labeled on the HPMA backbone as a tag (Figure 4A, Scheme 2). To characterize its FRET property, the conjugate was determined using fluorescence spectrometry before and after exposure to papain, a thiol proteinase with specificity similar to lysosomal cathepsin B. Ex 520 nm was selected to minimize direct emission of Cy5. As shown in Figure 4B, detectable FRET occurred in the original non-treated conjugate, but not in the same conjugate incubated with papain. Loss of FRET was due to the cleavage of the linker GFLG by enzyme, and consequently, the release of Cy3 from backbone.

The FRET property of conjugates (P-Cy3-Cy5 and 2P-Cy3-Cy5) was further elucidated in living cells. Human ovarian cancer A2780 cells that overexpress cathepsin B were incubated with the FRET conjugate, and NIH3T3 mouse fibroblast cells (low cathepsin B expression) were used as control. The release of model drug Cy3 will result in decrease of FRET intensity that can be investigated via cell lysis using fluorescence spectrometry. According to the previous reports [44-46], the ratio, I_{Cy3}/I_{FRET} , was calculated to quantify the FRET change, where I_{Cy3} and I_{FRET} are the fluorescence intensity at 562 nm and 664 nm, respectively (excitation 520 nm). For the conjugate P-Cy3-Cy5, the ratio in medium alone was 2.35, and in initial stock was 1.97.

When the conjugate was incubated with A2780 cancer cells, the ratio increased to 2.71 at 4 h, then gradually increased to 3.99 at 12 h and 5.25 at 24 h, whereas in NIH3T3 cells, the ratio only increased to 2.73 at 24 h (Figure 4C). A similar FRET change in the 2nd generation conjugate (2P-Cy3-Cy5) was also observed (Figure S3). There was significant difference in the release efficacy between cancer and normal cells. This observation suggests an effective release of Cy3 from the conjugate, which is highly dependent on the cathepsin B level. Compared to normal cells, cathepsin B level is much higher in malignant tumors, such as ovarian cancer, breast cancer, melanoma, etc. It acts as an important proteinase of matrix materials to degrade surrounding proteins and other tissue components so that cancer cells can invade and metastasize [47]. Therefore, high expression of cathepsin B in tumor cells can induce a fast release of drugs from conjugates and thereby mediates a relatively high concentration of active free drug inside the tumor cells.

Figure 4. (A) Cartoon illustration of FRET principle of dual-labeled enzyme-cleavable polymer conjugates containing Cy3 (donor) and Cy5 (acceptor). (B) Fluorescence spectra of conjugate P-Cy3-Cy5 before and after cleavage by papain. The mixture of conjugates P-Cy5/P-Cy3 was measured as control (excitation 520 nm using methanol as solvent). (C) FRET ratios of P-Cy3-Cy5 in NIH3T3 mouse fibroblast cells (low cathepsin B expression) and A2780 ovarian cancer cells (high cathepsin B expression) at different time intervals. The cells were incubated with P-Cy3-Cy5 at 37°C for 4 h and then cultured in fresh medium for another 0, 8, or 20 h. Then cell lysis was measured by fluorescence spectroscopy. **FRET ratio = I_{Cy3}/I_{FRET}** , was calculated to quantify the FRET change, where I_{Cy3} and I_{FRET} are the fluorescence intensity at 562 nm and 664 nm, respectively (excitation 520 nm). Increase of the ratio revealed effective payload release following enzyme exposure.

The conjugate P-Cy3-Cy5 was also evaluated using FRET confocal microscopy, which is a more straightforward approach for performing FRET. In this experiment, pre-bleach and post-bleach images were collected. To do the photobleaching, the cells were exposed to high excitation intensity at an excitation wavelength of 646 nm (Ex of Cy5) for a 20 min period. After high-energy laser treatment, there was a dramatic reduction of Cy5 intensity in the bleached regions (Figure 5). In the cells immediately following 4 h incubation, it was found that bleaching the acceptor Cy5 resulted in FRET intensity significantly decreased, and donor Cy3 fluorescence substantial increase, because the acceptor can no longer accept energy from the donor. However, those intensity changes did not occur in the same batch of cells after additional 20 h culture (Figure 5). It indicated that the model drug Cy3 had been released from the backbone after cellular internalization and lysosomal cleavage.

Following the same principle, we synthesized the conjugate P-EPI-Cy5 (Scheme 3). The conjugate was characterized using FRET spectra. The cell uptake and the drug EPI release were determined by FRET intensity changes. In A2780 cancer cells, the ratio decreased from 0.89 (initial stock) to 0.84 at 4 h and gradually decreased to 0.77 at 24 h (Figure 6). The reduction of FRET ratio indicates that EPI molecules could be released from the conjugate inside A2780 cancer cells over time. These results showed potential to use FRET as a tool for

future in vivo real-time monitoring drug delivery, tracking chain scission of HPMA copolymer-drug conjugates, and for improved cancer diagnostics and therapy.

Figure 5. Visualization of payload Cy3 release from conjugate P-Cy3-Cy5 in cathepsin B over-expressing A2780 human ovarian cancer cells by FRET. The cells were first incubated with P-Cy3-Cy5 at 37°C for 4 h and then were washed. Half of the cells were fixed immediately, while the other half were incubated with fresh medium at 37°C for another 20 h and fixed. The fixed cells were observed under confocal microscope using the standard acceptor Cy5 photobleaching method. Bleached areas are indicated by red boxes. Representative images of pre- and post-bleaching are shown.

Figure 6. (A) Fluorescence spectra of FRET conjugate P-EPI-Cy5 compared with P-EPI, P-Cy5 and their mixture P-Cy5+P-EPI. (B) The change in FRET ratio of P-EPI-Cy5 conjugate revealed effective EPI release in A2780 ovarian cancer cells. The cathepsin B over-expressing A2780

cells were incubated with P-EPI-Cy5 at 37°C for 4 h and then were cultured in fresh medium for another 20 h. Then cell lysates at different time intervals were measured by fluorescence spectrometer. $\text{FRET ratio} = I_{\text{EPI}} / I_{\text{FRET}}$, where I_{EPI} and I_{FRET} are the fluorescence intensity at 590 nm and 664 nm, respectively (excitation 490 nm). The data are presented as mean $\pm$ standard deviation (n=3). *, p<0.01.

4. Conclusions

We have developed a biodegradable polymeric drug delivery system with the capacity for non-invasive fate monitoring using FRET-based methodology. Epirubicin served as both fluorescence donor and antineoplastic agent. The degradable diblock HPMA copolymer-EPI conjugate (2P-EPI) produced complete tumor remission and long-term inhibition of tumorigenesis (100 days) when treating mice bearing human ovarian carcinoma A2780 xenografts. This and PK data provide strong evidence that 2nd generation backbone degradable HPMA copolymer-drug conjugates remarkably enhance circulation time and treatment efficacy. Moreover, in vitro cell uptake and intracellular drug release determined via FRET intensity changes clearly demonstrated cathepsin B levels are decisive and responsible for drug anti-tumor activity. This aspect will be further investigated ex vivo and in vivo using near-infrared FRET pairs to ensure deeper tissue penetration and better imaging quality.

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Table, Scheme and Figure captions

- Table 1.** Characterization of HPMA copolymer-epirubicin conjugates
- Table 2.** Comparison of pharmacokinetic parameters for ^{125}I -labeled conjugates in mice
- Scheme 1.** Synthesis of HPMA copolymer-epirubicin conjugates
- Scheme 2.** Synthesis of polymer conjugates containing fluorophores
- Scheme 3.** Synthesis of polymer conjugates containing epirubicin and Cy5 (P-EPI-Cy5)
- Figure 1.** Cell uptake and cytotoxicity of free drug EPI and its conjugates (P-EPI, 2P-EPI) in A2780 human ovarian cancer cells. (A) Flow cytometry analysis of cell uptake in the A2780 cells incubated with medium alone (non-treated), free EPI, or its conjugates (P-EPI, 2P-EPI) at 37 °C for 6 and 24 h. MFI, mean fluorescence intensity. (B) *In vitro* cytotoxicity of free drug EPI and its HPMA conjugates (P-EPI, 2P-EPI) toward A2780 human ovarian carcinoma cells. The data are presented as mean $\pm$ standard deviation (n=3-4).
- Figure 2.** Pharmacokinetic profiles of ^{125}I -labeled conjugates P-EPI and 2P-EPI in mice. The data represent the mean radioactivity expressed as a percentage of the injected dose per gram of blood (n=5).
- Figure 3.** Comparison of *in vivo* anti-tumor activity on female nude mice bearing A2780 human ovarian carcinoma xenografts. (A) The mice were intravenously injected with 3 doses of EPI or HPMA copolymer-EPI conjugates (P-EPI and 2P-EPI). Free drug and untreated groups were stopped on day 20 due to large size of tumors. (B) Long-term monitoring of tumor growth in conjugate treatments. (C) The tumor size on day 80 of the polymer conjugates, and (D) End point photographs of tumor-bearing mice from variable treatments.
- Figure 4.** (A) Cartoon illustration of FRET principle of dual-labeled enzyme-cleavable polymer conjugates containing Cy3 (donor) and Cy5 (acceptor). (B) Fluorescence spectra of conjugate P-Cy3-Cy5 before and after cleavage by papain. The mixture of conjugates P-Cy5/P-Cy3 was measured as control (excitation 520 nm using methanol as solvent). (C) FRET ratios of P-Cy3-Cy5 in NIH3T3 mouse fibroblast cells (low cathepsin B expression) and A2780 ovarian cancer cells (high cathepsin B expression) at different time intervals. The cells were incubated with P-Cy3-Cy5 at 37°C for 4 h and then cultured in fresh medium for another 0, 8, or 20 h. Then cell lysis was measured by fluorescence spectroscopy. $\text{FRET ratio} = I_{\text{Cy3}}/I_{\text{FRET}}$, was calculated to quantify the FRET change, where I_{Cy3} and I_{FRET} are the fluorescence intensity at 562 nm and 664 nm, respectively (excitation 520 nm). Increase of the ratio revealed effective payload release following enzyme exposure.

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2P-EPI