Synthesis of a Novel Polymeric Material Folate-Poly(2-ethyl-2-oxazoline)-Distearoyl Phosphatidyl Ethanolamine Tri-Block Polymer for Dual Receptor and pH-Sensitive Targeting Liposome

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The *in vivo* distribution of antitumor drugs is usually lack of selectivity, and thus, leading to a low efficacy of chemotherapy on cancers and high toxicity to normal cells. Receptor-mediated targeting liposome with pH-sensitivity as a dual drug delivery system is one of the efficient approaches to overcome the disadvantages. The study was to synthesize a novel smart polymeric material (folate-poly(2-ethyl-2-oxazoline)-distearoyl phosphatidyl ethanolamine, F-PEOz-DSPE), which can combine with the folate-receptor (FR) overexpressed on cancer cells and respond to pH changes in endosome–lysosome system in cancer cells to rapidly release drug simultaneously. The F-PEOz-DSPE was synthesized by the method of asymmetric synthesis of organic polymer and characterized by IR, ^{1}H-NMR, electrospray ionization (ESI)-MS and gel permeation chromatography (GPC). To investigate the properties of targeting and pH-sensitivity of F-PEOz-DSPE, blank liposomes, blank fluorescently labeled liposomes and doxorubicin (DOX)-loaded liposomes containing F-PEOz-DSPE or PEOz-DSPE or DSPE were prepared. The cytotoxicity, cellular uptake and drug cumulative release *in vitro* were investigated. Blank liposomes modified with PEOz block had little cytotoxicity *in vitro*. The liposomes containing F-PEOz-DSPE showed a higher affinity to human ovarian cancer cell SKOV3, a FR$^+$ cancer cells, than those with PEOz-DSPE. A higher drug cumulative release from DOX-loaded liposomes containing F-PEOz-DSPE or PEOz-DSPE *in vitro* was found in phosphate buffered saline at pH 5.0 medium than at pH 7.4. These results indicate that F-PEOz-DSPE exhibits selective targeting, pH-sensitivity and little cytotoxicity, and may be a promising polymeric material for dual receptor and pH-sensitive targeting liposome.

Key words synthesis; polymeric material; pH-sensitivity; folate-poly(2-ethyl-2-oxazoline)-distearoyl phosphatidyl ethanolamine; receptor-targeting; liposome

The *in vivo* distribution of antitumor drugs is usually lack of selectivity, and thus, leading to a low efficacy of chemotherapy on cancers and high toxicity to normal cells.[1] Receptor-mediated targeting liposome with pH-sensitivity as a dual drug delivery system is one of the efficient approaches to overcome the disadvantages. Because it does not only improve the selectivity of anticancer drugs to the tumor tissue, but also rapidly release drug in tumor cells simultaneously. The liposomes modified with intelligent polymer material have great potential to realize the high selectivity for antineoplastic in tumor tissue.

Folate receptors (FR) have rare expression or no expression in healthy tissues, however, overexpression on the cell membrane surface in malignant tumor tissue, and the expression level increases with the increase of the cancerous malignant. Overexpression of FR occurs in many human malignancies, especially when associated with aggressively growing cancers. Therefore, Folic acid (F), the natural ligand of FR, is a crucial molecule that can interact with FR on cancer cells and also can help selectively targeting antitumor drugs to tumor cells. Liposomes modified with folic acid have shown selective targeting toward many human tumors, including ovarian, renal, breast and endometrial tumors.[2–5]

It is reported that there are some significant differences between solid tumor and its surrounding normal tissues. The pH value of extracellular microenvironment in tumor tissues (6.5–7.2) is slightly lower than that of normal tissue or blood (7.4). In addition, the pH value in different cell organelles is also different. For example, the pH value in endosomes is 5.5–6.0, while it is 4.5–5.0 in lysosomes.[6–9] Some studies have reported that the polymer micelle prepared by poly(L-lactic acid)-*b*-poly(ethyleneglycol)-*b*-poly(L-histidine) (PLA-PEG-polyHis) tri-block copolymer or polyHis-*b*-PEG or PLA-PEG copolymer could greatly improve the targeting of the antitumor drugs in tumor tissue. Besides, the polymer micelle prepared by PLA-poly(2-ethyl-2-oxazoline)-PLA (ABA type) tri-block copolymer acted on intracellular endosome–lysosome system, which could improve the drug concentration in the cytoplasm and achieve the best curative effect.[9–12]

Poly(2-ethyl-2-oxazoline) (PEOz), synthesized by cationic ring-opening polymerization,[13] has water solubility, biocompatibility, non-toxicity and no-immunogenicity. In recent years, PEOz has been shown to be a pH-sensitive polymer with a favorable pK_a value (4–6).[14,15] This pH sensitivity is very beneficial for achieving rapid release of anticancer drugs in acid conditions, such as in tumor tissue and the endosome–lysosome system. The pH-sensitivity of PEOz had been confirmed in many studies utilizing PEOz as a block of the

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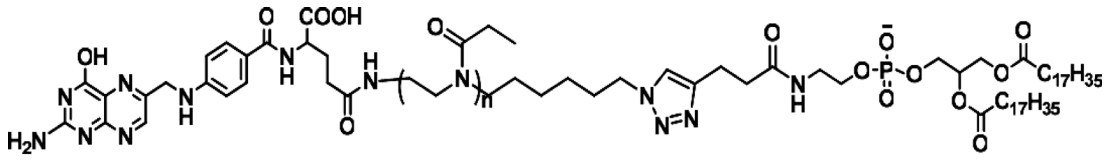

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Fig. 1. Structure of F-PEOz-DSPE (**7**) Tri-Block Polymer

micelle carrier. However, few studies have been reported about using PEOz or (folate-poly(2-ethyl-2-oxazoline)-distearoyl phosphatidyl ethanolamine (F-PEOz-DSPE, **7**) (Fig. 1) as a pH-sensitive modifier of liposomes to achieve its pH-sensitive property in endosome–lysosome system. So a novel smart polymeric material was designed to respond to the different pH microenvironment and facilitate the antitumor efficacy of the antineoplastic agents.

Based on these facts, F-PEOz-DSPE was designed. The aim of this study was to synthesize a novel smart polymeric material F-PEOz-DSPE tri-block polymer, which can combine with the FR over-expressed on cancer cells and respond to pH changes in endosome-lysosome system in cancer cells to release drug rapidly.

The F-PEOz-DSPE was synthesized by the method of asymmetric synthesis of organic polymer and characterized by IR, ^{1}H-NMR, electrospray ionization (ESI)-MS and gel permeation chromatography (GPC).[16–18] It has been reported that the polymer materials contained PEOz block mostly belong to AB or ABA type, which means there is only one functional group at a single end or two same groups at both ends of PEOz. One of the innovations of this study is to bind two different functional groups at both ends of PEOz molecule. After many attempts and explorations, a relatively reasonable and feasible synthetic route was designed. The "click" (click chemistry) reaction[16] was applied to the synthesis of liposome polymeric material (F-PEOz-DSPE tri-block polymer), which is a novel design, and similar reports have not been seen at home and abroad.

To investigate the targeting and pH-sensitivity of F-PEOz-DSPE tri-block polymer, blank liposomes, blank fluorescently labeled liposomes and doxorubicin (DOX)-loaded liposomes containing F-PEOz-DSPE or PEOz-DSPE or DSPE were prepared, respectively. The cytotoxicity, cellular uptake and drug cumulative release *in vitro* were investigated.[19,20] These results indicate that F-PEOz-DSPE exhibits selective targeting, pH-sensitivity and little cytotoxicity, and may be a promising polymeric material for dual receptor-mediated targeting and pH-sensitive liposomes.

Experimental

Figure 2 was shown the synthesis route of F-PEOz-DSPE tri-block polymer.

Synthesis of 6-Azidohexan-1-ol (1) 6-Chloro-1-hexanol (10 g, 73 mmol), NaN_3 (14.3 g, 220 mmol) and NaI (1.1 g, 7.3 mmol) were dispersed in 100 mL dried *N,N*-dimethyl formamide (DMF). The reaction was performed at 80°C for 18 h. Then the solvent was removed from a rotary evaporator. **1** was obtained as off-white powder in yield (95%). ^{1}H-NMR (300 MHz, $CDCl_3$, tetramethylsilane (TMS), ppm) δ: 1.3–1.7 (m, 8H), 3.28 (t, 2H), 3.65 (t, 2H).

Synthesis of 6-Azido-1-tosylate Hexane (2) **1** (10 g, 70 mmol), triethylamine (20 mL, 145 mmol) and trimethylamine hydrochloride salt (1.3 g, 13.5 mmol) were dissolved in 300 mL dried chloroform (solution A). Tosyl chloride (20 g, 105 mmol) was dissolved in 60 mL dried chloroform (solution B). Then solution B was added dropwise to solution A under ice bath. The reaction mixture was left to react at 0°C for 1 h and ambient temperature for 1 h, respectively. After removing the organic solvent, **2** was obtained as off-white powder in yield (50%). ^{1}H-NMR (300 MHz, $CDCl_3$, TMS, ppm) δ: 1.27 (m, 4H), 1.4–1.6 (m, 4H), 2.46 (s, 3H), 3.23 (t, 2H), 4.02 (t, 2H), 7.34 (d, 2H), 7.79 (d, 2H). FT-IR (KBr, cm^{-1}): 2097 (N_3).

Synthesis of PEOz-N_3 (3) ($PEOz_{2000}$-N_3, for Example) **3** was synthesized by cationic ring-opening polymerization of 2-ethyl-2-oxazoline (EOz). EOz (8.5 mL, 84 mmol) and acetonitrile (25 mL) was transferred to a Schlenk flask on Schlenk line. Then **2** (0.62 mL, 2.5 mmol) were added to the flask under nitrogen. The reactant was stirred at 70°C for 24 h. Then 23.6 mL of NH_3/acetonitrile solution was transferred into the flask on Schlenk line at ice bath and left to react for 24 h under ambient temperature to terminate the reaction species. After that, K_2CO_3 (25 g, 181 mmol) was added and the suspension was stirred under room temperature for 24 h to remove the *p*-toluenesulfonic acid. After filtering K_2CO_3, **3** was obtained by precipitation in ethyl ether and dried in vacuum. **3** was off-white powder in yield (76%). ^{1}H-NMR (300 MHz, $CDCl_3$, TMS, ppm) δ: 1.13 (m, $CH_2C\underline{H}_3$), 2.44 (m, $CH_3C\underline{H}_2CO$), 3.57 (m, $C\underline{H}_2C\underline{H}_2N$). FT-IR (KBr, cm^{-1}): 2099 cm^{-1} (N_3), 1639 cm^{-1} (O=CN). GPC was used for determining molecular weight of **3** [Waters Styragel HT3 (tetrahydrofuran (THF)) columns (7.8×300 mm), mobile phase (THF), flow rate of mobile phase (1.0 mL/min), oven temperature of column (30°C)].

(In order to obtain different molecular weights of PEOz-N_3, three ratios of EOz/initiator were used: 33/1, 66/1 and 133/1.)

Synthesis of DSPE-yne (4) 4-Pentynoic acid (0.8 g, 8.2 mmol) and *N,N'*-dicyclohexylcarbodiimide (DCC) (1.2 g, 5.8 mmol) were dissolved in 50 mL dried chloroform and left to react for 4 h under ambient temperature. DSPE (3 g, 4 mmol) and pyridine (200 μL) were added to the aforementioned solution. The reactant was stirred at 50°C for 12 h. The side product *N,N*-dicyclohexylurea (DCU) was filtered off. After removing the organic solvent and dried in vacuum, **4** was obtained as off-white powder in yield (75%). ESI-MS: *m/z* 826.6 $[M-H]^-$. FT-IR (KBr, cm^{-1}): 2300 cm^{-1} (–C≡CH).

Synthesis of PEOz-DSPE (5) **3** (500 mg, 0.25 mmol), **4** (400 mg, 0.48 mmol) and *N,N,N',N',N'*-five methyl dien (PMDETA) (110 μL, ρ=0.829 g/mL) were dissolved in 2 mL dried chloroform. The reaction mixture was degassed by three freeze-pump-thaw cycles and the flask was filled with nitrogen. CuBr (72 mg, 0.5 mmol) was added and the flask was sealed under vacuum. The reaction was left at 50°C for 72 h. Then the solution was diluted by chloroform and filtered over neutral alumina, **5** was harvested by precipitation in ethyl

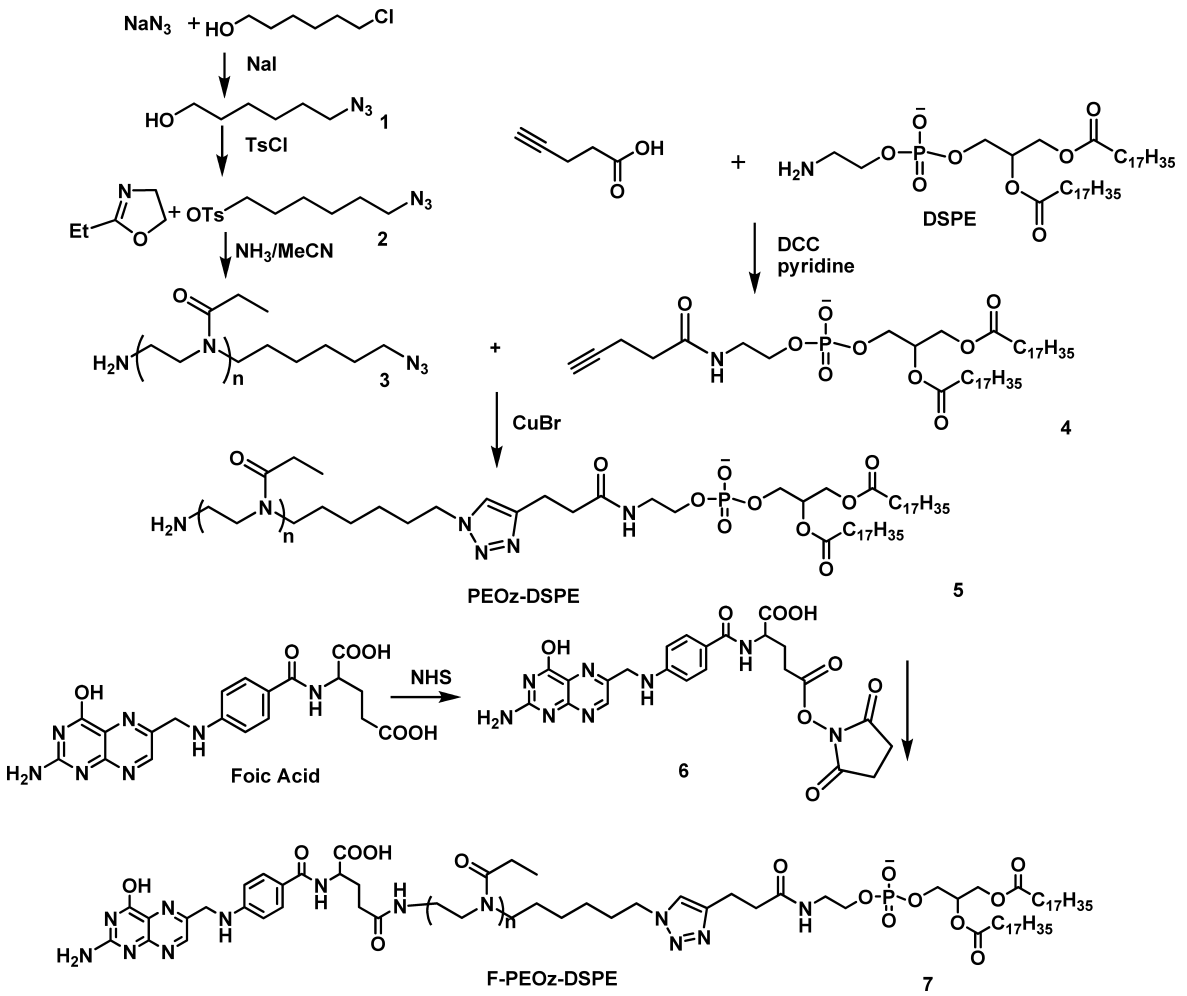


Fig. 2. Synthetic Route of F-PEOz-DSPE Tri-Block Polymer

ether and filtration as off-white powder in yield (63%).

Synthesis of F-*N*-Hydroxy Succinimide (NHS) (6) Folic acid (5.0 g, 11.3 mmol), NHS (1.3 g, 11.3 mmol) and 25 mL anhydrous triethylamine (TEA) were added into anhydrous dimethyl sulfoxide (DMSO) (40 mL). The mixture was stirred at ambient temperature under nitrogen atmosphere in dark until folic acid was completely dissolved. DCC (2.5 g, 12.1 mmol) were added to this solution, and the mixture was stirred for 24 h at ambient temperature under nitrogen atmosphere in dark. After filtration, the filtrate was poured into 400 mL of ethyl acetate. **6** was obtained as yellow powder in yield (74%). ^{1}H-NMR (300 MHz, D_2O/NaOD, TMS, ppm) δ: 8.16 (1H), 7.26 (2H), 6.18 (2H), 4.15 (2H), 4.05 (1H), 2.30 (4H), 2.10 (2H), 1.85, 1.80 (2H).

Synthesis of F-PEOz-DSPE (7) **5** (300 mg, 0.11 mmol), **6** (180 mg, 0.33 mmol) and 900 μL of anhydrous TEA were added into anhydrous DMSO (1.5 mL). The mixture was stirred at ambient temperature under nitrogen atmosphere in dark for 10 min and then was left at 50°C for 72 h. After that, **7** was harvested by precipitation in ethyl ether, filtration and dialysis. **7** was off-white powder in yield (55%). ^{1}H-NMR (300 MHz, DMSO, TMS, ppm) δ: 8.70 (1H), 8.00 (1H), 7.70 (2H), 6.71 (2H), 4.45 (2H), 4.25 (1H), 3.35 (m), 2.28 (m), 1.26 (m), 0.94 (m). GPC was also used for determining molecular weight of **7**, M_n=3907 [Waters Styragel HT3 (THF) columns (7.8×300 mm), mobile phase (THF), flow rate of mobile phase (1.0 mL/min), oven temperature of column (30°C)].

Preparation of Liposomes First, 3 types of blank liposomes were prepared, which were composed of (HSPC), cholesterol (Chol), DSPE or $PEOz_{2000}$-DSPE or F-$PEOz_{2000}$-DSPE (2 : 1 : 0.1 mol : mol : mol) by the method of thin-film evaporation and heat extrusion. Then, DOX was encapsulated into 3 types of blank liposomes by ammonium sulfate gradient method to gain 3 types of DOX-loaded liposomes. Unloaded DOX was separated and removed by gel filtration chromatography (Sephadex-G25 columns, Pharmacia & Upjohn, Sweden). The particle sizes of the 3 types of DOX-loaded liposomes were measured using a Particle Analyzer (Zetasizer Nano ZS, Malvern) at 25°C.

To investigate cell uptake, fluorescently labeled liposomes were prepared by integrated with 25-NBD-cholesterol into blank liposomes bilayer in a molar ratio of 5%. The cell uptake could be observed under the fluorescence microscopy at Ex/Em of 488 nm/505 nm when incubated with these fluorescently labeled liposomes. So the same operations were performed in accordance with the above, the 2 types of blank fluorescently-labeled liposomes were obtained. The one was blank fluorescently-labeled liposomes containing $PEOz_{2000}$-DSPE (P_{2000}), the other one was blank fluorescently-labeled liposomes containing F-$PEOz_{2000}$-DSPE (F-P_{2000}).

Cytotoxicity of Blank Liposomes *in Vitro* The cytotoxicity of three blank liposomes containing F-$PEOz_{2000}$-DSPE or

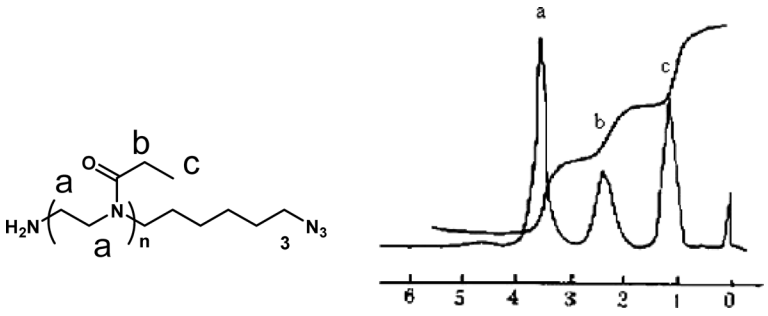


Fig. 3. ^{1}H-NMR of PEOz-N_3 (300 MHz)

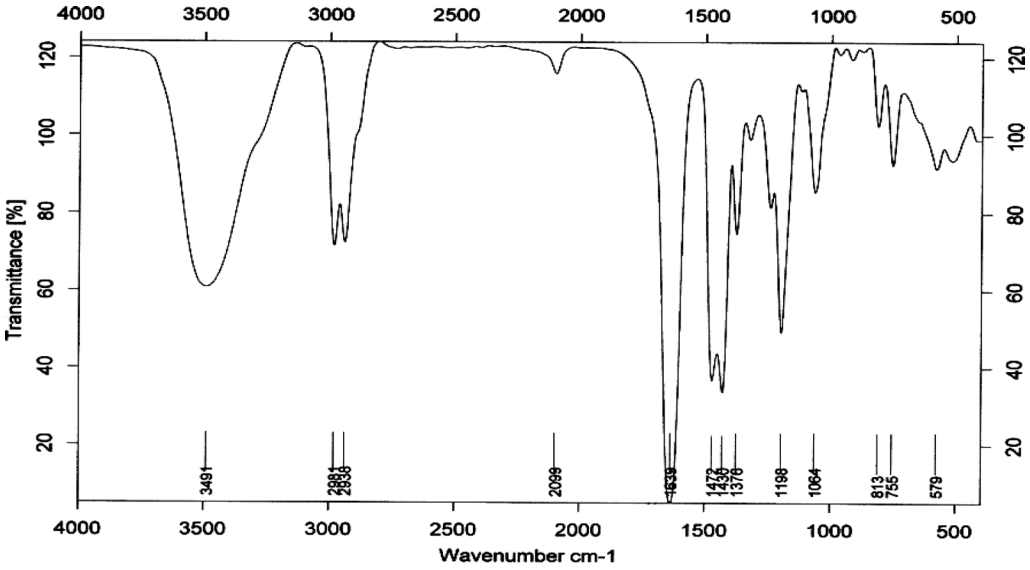


Fig. 4. IR Spectrum of PEOz-N_3

$PEOz_{2000}$-DSPE or DSPE, were assessed in human ovarian cancer cell line SKOV3 by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell proliferation assay. The cells were seeded in a 96-well plate at the concentration of 1×10^4 cell/well with triplicate and maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin overnight at 37°C with 5% CO_2 in a humidified atmosphere. Next day, culture medium was replaced by 100 μL of medium containing serial dilutions of three blank liposomes. Cell viability was tested by MTT assay after 48 h incubation. Briefly, 10 μL of MTT (5 mg/mL) was added into each well followed by 4 h incubation at 37°C in 5% CO_2 incubator. Subsequently, the medium was removed carefully and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve formazan. Finally, optical density, which is directly correlated with cell quantity, was read at 490 nm using ELx800 Universal Microplate Reader (Bio-TEK Instruments, Inc., U.S.A.). Cells cultured with medium alone were used as control and the cell viability was considered as 100%.

Qualitative Study of Cell Uptake of Blank Liposomes *in Vitro* Human ovarian cancer cells (SKOV3), which overexpress FR, were used to assess the cell uptake of liposomes. SKOV3 cells were grown using folate free RPMI 1640 media supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO_2. To observe cell uptake, SKOV3 cells were incubated with blank fluorescently-labeled liposomes P_{2000} and F-P_{2000} in same concentration at 37°C for 2 h, respectively. At different time duration, the sampled cells were rinsed gently by phosphate-buffered saline (PBS) for 3 times in order to remove the free liposomes. Then, cell uptake of liposomes could be visible under the fluorescence microscope.

Competition assay was conducted to further confirm folate receptor-targeting of F-P_{2000} liposomes. SKOV3 cells were cultured using folate free RPMI-1640 media supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO_2. Free folate (800 μM) as a competitor was added 10 min before incubated with blank fluorescently-labeled liposomes containing $PEOz_{2000}$-DSPE or F-P_{2000}-DSPE. Cell uptake of liposomes was measured by the same method as described above.[21)]

Drug Release from DOX-Loaded Liposome *in Vitro* Release studies *in vitro* were performed by the dialysis bag method. The dialysis bag (molecular weight cut off 1000) was soaked in distilled water for 24 h before use. DOX-loaded liposomes of F-$PEOz_{2000}$-DSPE or $PEOz_{2000}$-DSPE or DSPE were prepared with a DOX concentration of 1.0 mg/mL, respectively. Then, 1 mL of the 3 types of liposomes was placed in the dialysis bag, respectively, and the receptor compartment was filled with 100 mL PBS at pH 5.0 or 7.4 medium at 37°C with gentle agitation (40 rpm). The next, 1.0 mL of the dissolution

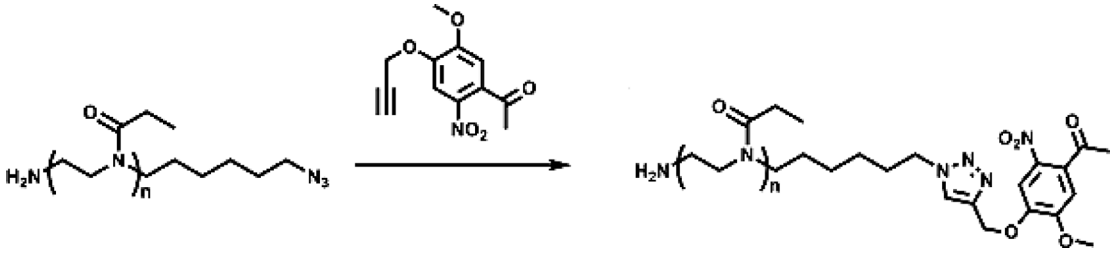


Fig. 5. "Click" Reaction of PEOz-N_3 and 1-Acetyl-2-nitro-4-calkynyl Oxygen Base-5-methoxy Benzene

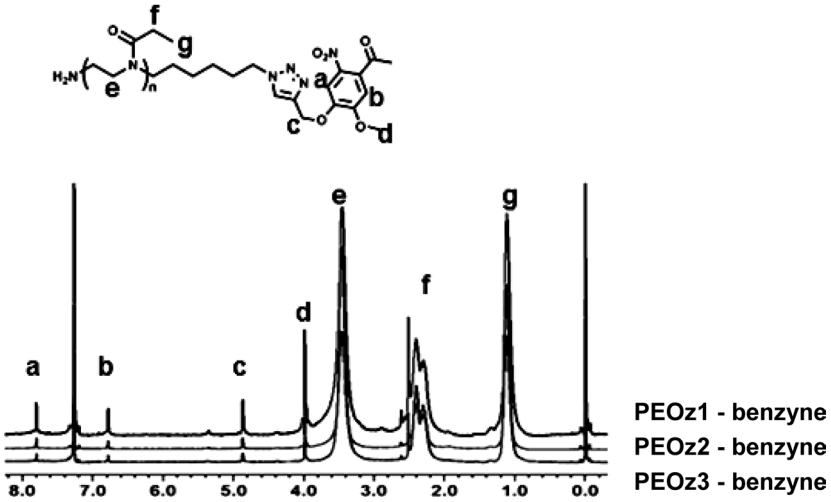


Fig. 6. ^{1}H-NMR Spectra of PEOz-Benzyne (300 MHz)

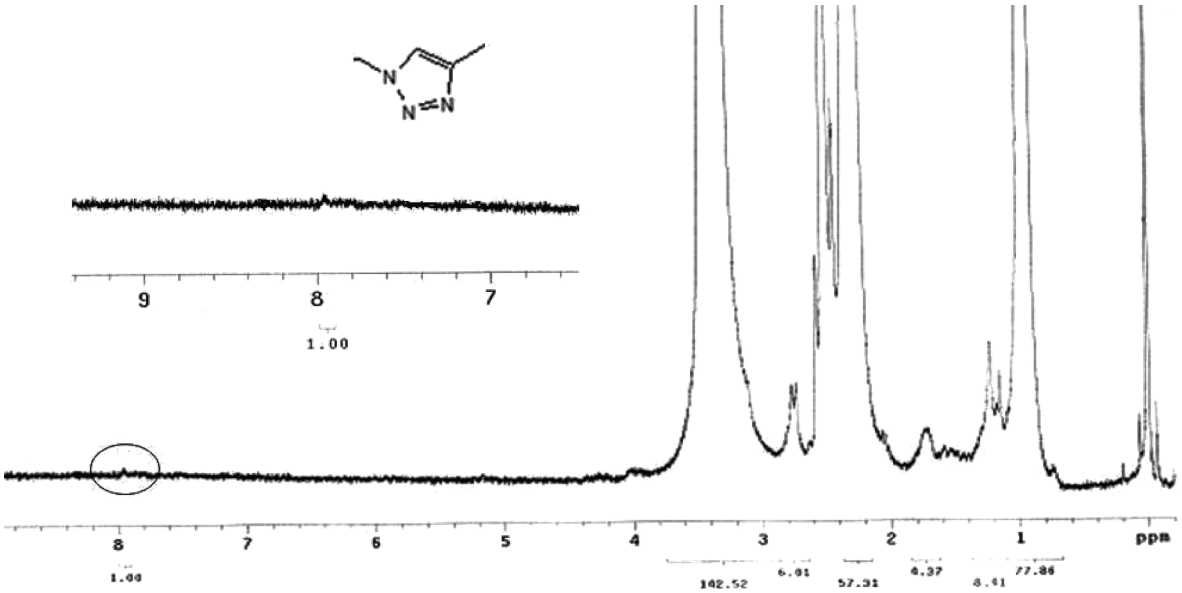


Fig. 7. ^{1}H-NMR Spectrum of PEOz-DSPE (300 MHz)

Table 1. Molecular Weights of PEOz-N_3 (**3**)

Sample No.	Molar ratio of 2-ethyl-2-oxazoline monomer and initiator (**2**)	Conversion rate of polymerization reaction (%)	Molecular weight (M_n)		
			GPC	From ^{1}H-NMR data	From conversion data
PEOz1-Benzyne	33 : 1	76	2247	1900	2500
PEOz2-Benzyne	66 : 1	61	3923	3300	4000
PEOz3-Benzyne	133 : 1	56	7139	6500	7500

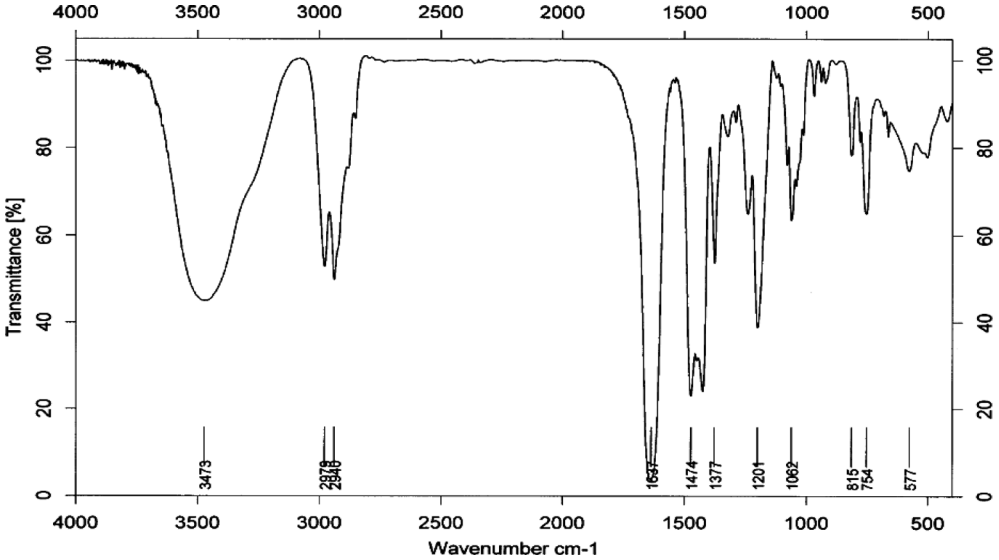


Fig. 8. IR Spectra of PEOz-DSPE

Table 2. Molecular Weights of PEOz-DSPE (**5**) and F-PEOz-DSPE (**7**)

Polymer code	M_n [a)]	M_n [b)]	M_w [b)]	PDI [b)]
PEOz-DSPE (**5**)	2900	3146	3476	1.10
F-PEOz-DSPE (**7**)	3400	3907	3990	1.02

a) Estimated by ^{1}H-NMR. *b*) Estimated by GPC.

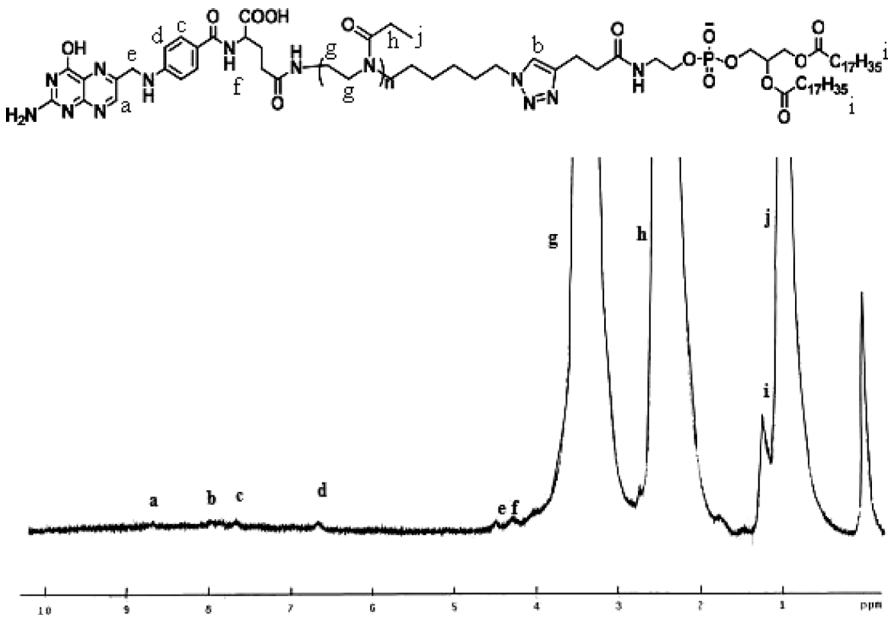


Fig. 9. ^{1}H-NMR Spectrum of F-PEOz-DSPE (300 MHz)

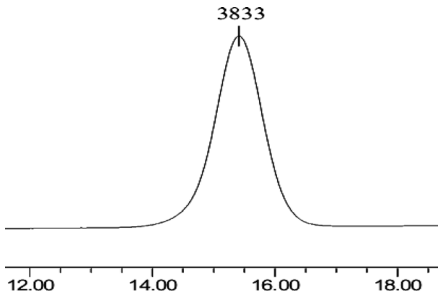
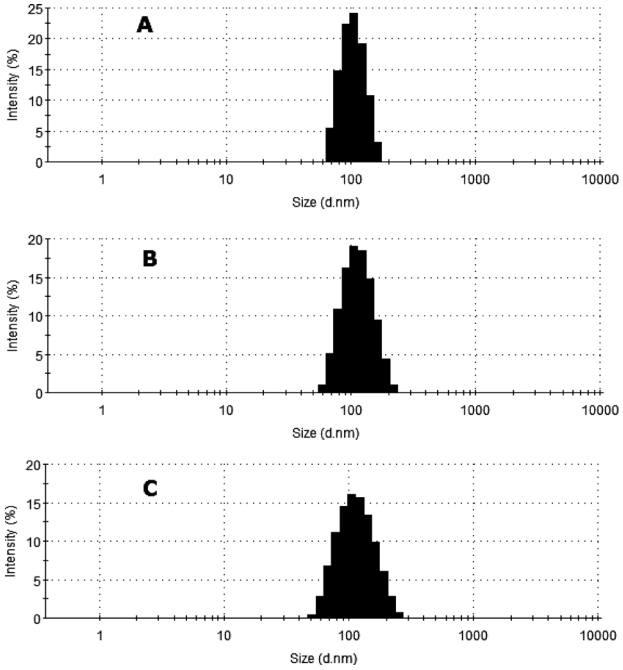


Fig. 10. GPC Chromatograph of F-PEOz-DSPE

Table 3. Characterization of DOX-Loaded Liposomes Containing DSPE, $PEOz_{2000}$-DSPE and F-$PEOz_{2000}$-DSPE

Sample	DOX-Loaded liposomes containing DSPE	DOX-Loaded liposomes containing $PEOz_{2000}$-DSPE	DOX-Loaded liposomes containing F-$PEOz_{2000}$-DSPE
Mean particle size/nm	102.8	108.2	122.4
PDI	0.098	0.106	0.163
Particle size distribution	(see A below)	(see B below)	(see C below)
Encapsulation efficiencies/%	96	91	94
DOX concentration/mg/mL	1.12	1.05	1.10



medium was withdrawn from the receptor compartment at intervals of 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h. And replace with the same volume of fresh dialysis medium. The DOX content of the samples was analyzed by HPLC at various time points during the dialysis process. All analyses were performed in triplicate to calculate the averages.[20]

Results and Discussion

Synthesis and Characterizations of F-PEOz-DSPE Tri-Block Polymer First, the PEOz-N_3 (**3**) was synthesized by cationic ring-opening polymerization using 6-azido-1-tosylate hexane (**2**) as an initiator. The chemical structure verified by ^{1}H-NMR (Fig. 3) and FT-IR (Fig. 4). To determine molecular weight of PEOz-N_3 (**3**), PEOz-N_3 and 1-acetyl-2-nitro-4-calkynyl oxygen base-5-methoxy benzene were performed by "click" reaction to obtain PEOz-benzyne (Fig. 5). Using integral ratio between the H of the benzene ring and the H of repeating units of PEOz by ^{1}H-NMR spectrum of PEOz-benzyne (Fig. 6), calculate molecular weight and conversion rate of PEOz-N_3. The polymerization degrees of 3 types of PEOz-N_3 were 19, 33 and 65, respectively. GPC was also used for determining molecular weight of PEOz-N_3. The molecular weight of PEOz1-N_3, PEOz2-N_3 and PEOz3-N_3 were 2247, 3923 and 7139 (Table 1). PEOz1-N_3 (molecular weight of PEOz chain is around 2000) was used to synthetize PEOz-DSPE (**5**) and F-PEOz-DSPE (**7**), label as $PEOz_{2000}$-DSPE and F-$PEOz_{2000}$-DSPE.

Second, PEOz-DSPE (**5**) was synthesized by "click" reaction between PEOz-N_3 (**3**) and DSPE-yne (**4**). A specific peak belonging to triazole (^{1}H-NMR, δ=7.96) was observed (Fig. 7) and in IR spectra, the specific peak belonging to azido (2099 cm^{-1}) disappeared, implying the consumption of azido group (Figs. 4, 8). Based on the above results, the PEOz and DSPE were linked with triazole after "click" reaction. The GPC and ^{1}H-NMR data was shown in Table 2.

Finally, F-PEOz-DSPE (**7**) tri-block polymer was synthesized successfully through an amide linkage between the carboxyl group of folate and the primary amine of PEOz-DSPE. The resultant F-PEOz-DSPE was analyzed by ^{1}H-NMR (300 MHz, DMSO) (Fig. 9). Chemical signal belonging to proton on folate (δ: 8.70 (1H), 7.70 (2H), 6.71 (2H), 4.45 (2H), 4.25 (1H)), proton on triazole (δ: 8.00 (1H)), proton on PEOz (δ: 3.35 (m), 2.28 (m), 0.94 (m)), and proton on alkane chain of DSPE (δ: 1.26 (m)) were observed clearly in ^{1}H-NMR. GPC was used for determining molecular weight of F-PEOz-DSPE (**7**). GPC trace of **7** was shown in Fig. 10 and the molecular weight determined by GPC and ^{1}H-NMR was shown in Table 2.

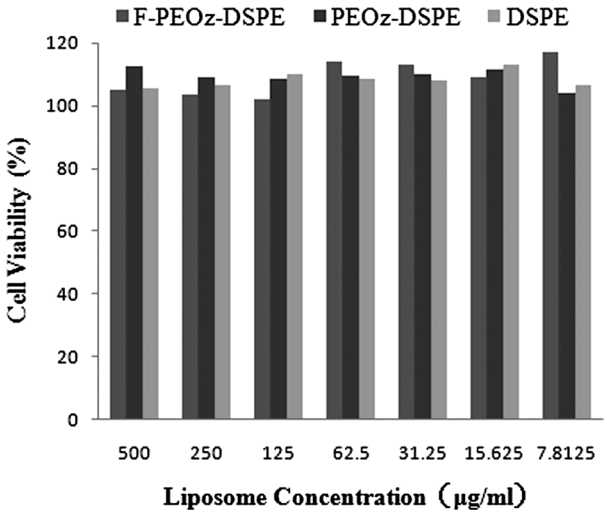


Fig. 11. Cytotoxicity of Blank Liposomes in SKOV3

F-PEOz-DSPE: Blank liposomes containing F-$PEOz_{2000}$-DSPE
PEOz-DSPE: Blank liposomes containing $PEOz_{2000}$-DSPE
DSPE: Blank liposomes containing DSPE

Characterization of Liposomes The mean particle sizes

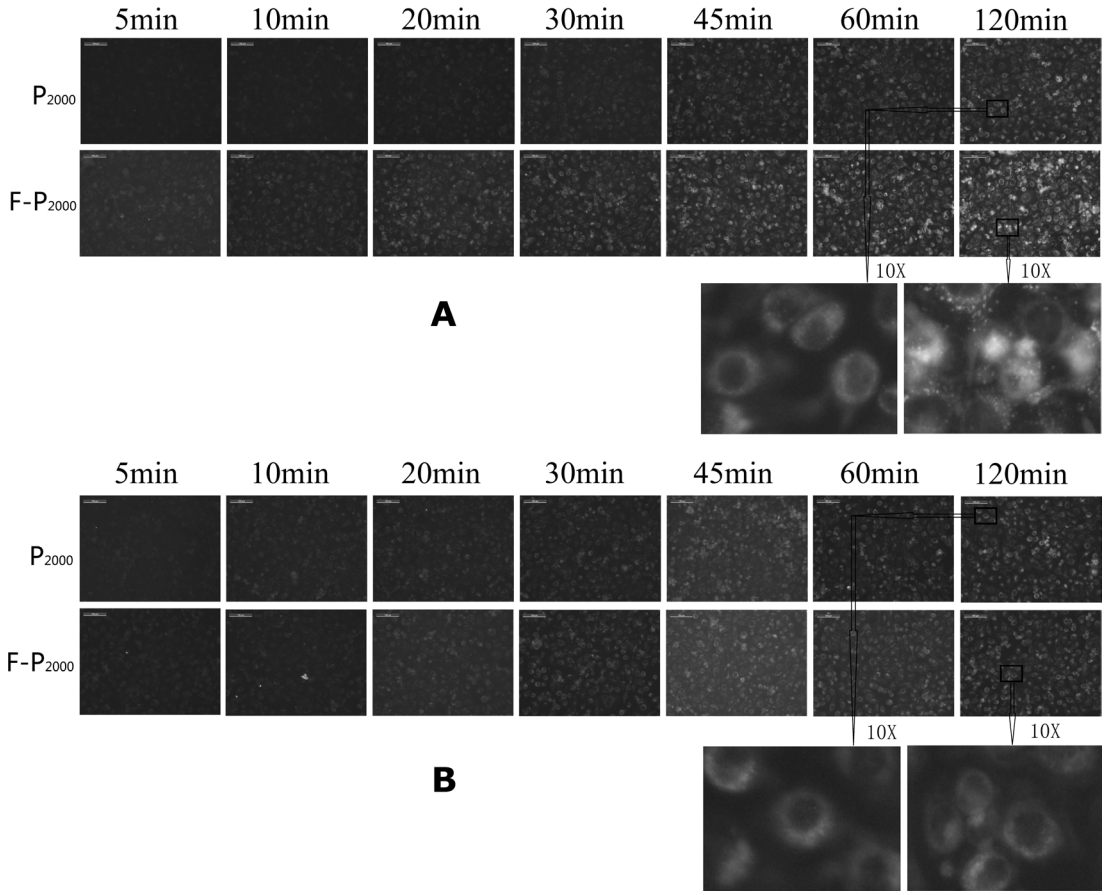


Fig. 12. Cell Uptake and Competition Assay of 2 Types of Liposomes by SKOV3

P_{2000}: Blank fluorescently-labeled liposomes containing $PEOz_{2000}$-DSPE; F-P_{2000}: Blank fluorescently-labeled liposomes containing F-$PEOz_{2000}$-DSPE. A: No blocking agent. B: Folate as a receptor blocking agent.

of 3 types of DOX-loaded liposomes containing DSPE or $PEOz_{2000}$-DSPE or F-$PEOz_{2000}$-DSPE ranged from 100 to 150 nm (PDI <0.200). Their encapsulation efficiencies were higher than 91.0%, DOX concentrations were 1.0 mg/mL or so (Table 3).

Evaluation for Cytotoxicity of Blank Liposomes *in Vitro* The effects of blank liposomes modified with PEOz block on cell viability were evaluated using SKOV3 cells by MTT assay. As shown in Fig. 11, the cell viability was expressed as percentage ratios of viable cell between groups treated with liposomes and cell control groups according to optical density. No cell growth inhibition could be observed even if the concentration of liposomes was increased to 500 μg/mL, indicating that the prepared blank liposomes modified with PEOz block had little cytotoxicity *in vitro*.

Evaluation of Targeting *in Vitro* For the assessment of specific cell uptake, SKOV3 cells were incubated with blank fluorescently-labeled liposomes containing $PEOz_{2000}$-DSPE (P_{2000}) or F-$PEOz_{2000}$-DSPE (F-P_{2000}) for different time duration, respectively. The results showed that the fluorescence intensity presented by SKOV3 cells in both groups was substantially enhanced with increasing incubation time. Besides, the cell uptake of liposomes in the F-P_{2000} treated groups was much higher than that in the P_{2000} treated group when the incubation time was over 5 min (Fig. 12A).

Competition assay was clearly showed that there was little difference in cell uptake efficacy from F-P_{2000} to P_{2000} liposomes using free folate as a receptor blocking agent, and the capability of F-P_{2000} liposomes binding to SKOV3 cells was greatly reduced when folate-receptor on SKOV3 cells was blocked (Fig. 12B). This indicated that the liposomes modified with folate could selectively bind to SKOV3, a FR^+ cancer cell, *via* a receptor-mediated mechanism. It was concluded that F-PEOz-DSPE has the ability to target the FR^+ cancer cells.

Evaluation of pH-Sensitivity *in Vitro* To evaluate the pH-sensitivity of PEOz-modified liposomes ($PEOz_{2000}$-DSPE and F-$PEOz_{2000}$-DSPE liposomes), the drug release rate and cumulative amount *in vitro* are important parameters. As shown in Figs. 13A–C. The drug releases of three types of DOX-loaded liposomes containing F-$PEOz_{2000}$-DSPE or $PEOz_{2000}$-DSPE or DSPE were studied in PBS at pH 5.0 and pH 7.4 medium. The cumulative release of DOX from liposomes containing F-$PEOz_{2000}$-DSPE and $PEOz_{2000}$-DSPE at 48 h was 40–45% at pH 7.4 medium and was 90% or so at pH 5.0 medium. The release rate of DOX from liposomes containing F-$PEOz_{2000}$-DSPE or $PEOz_{2000}$-DSPE in pH 5.0 PBS medium was faster than that in pH 7.4 medium at all times measured (from 15 min to 48 h). There were little differences of the release rates and cumulative amount of released DOX cargo between F-$PEOz_{2000}$-DSPE liposomes and $PEOz_{2000}$-DSPE liposomes under pH 5.0 conditions, and neither were under pH 7.4 conditions. Little differences of the release rates and cumulative amount of released DOX cargo could be observed in the DSPE liposomes between in pH 5.0 and 7.4 PBS medium. It was indicated that PEOz-modified liposomes ($PEOz_{2000}$-DSPE and F-$PEOz_{2000}$-DSPE liposomes) have the

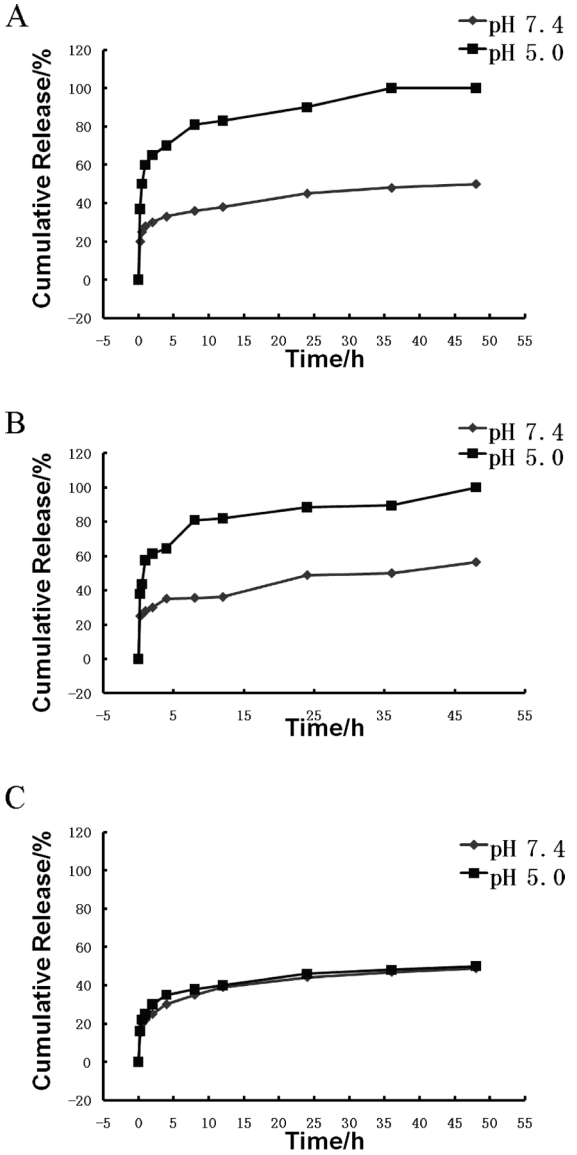


Fig. 13. The *in Vitro* Release Profile of DOX-Loaded Liposomes Containing F-PEOz$_{2000}$-DSPE (A), PEOz$_{2000}$-DSPE (B) and DSPE (C) in PBS at 5.0 and 7.4 pH

characteristic features of pH-sensitivity (Fig. 13).

Detailed mechanism of pH-sensitive behavior is still unclear at this stage, but the proposed mechanism might be destabilization of PEOz-modified liposomes, and then leading to the leakage of the encapsulated drug to achieve the goal of drug release, as discussed in previously reported literature.[14,15,22)]

Conclusion

A novel smart polymeric material F-PEOz-DSPE tri-block polymer was synthesized successfully. Making using of the materials, a bi-functional receptor-mediated targeting liposomes with pH-sensitivity was prepared, which could bind to the folate receptor over-expressed on cancer cells and respond to pH changes in the endosome-lysosome system in cancer cells. So it could not only improve the selectivity of anticancer drugs to the tumor tissue, but also rapidly release drug in tumor cells simultaneously. The liposomes modified with intelligent polymer material F-PEOz-DSPE have great potential to realize the high selectivity. The liposomes containing F-PEOz-DSPE had a higher affinity for SKOV3, a FR$^+$ cancer cells, than those with PEOz-DSPE. The *in vitro* release experiment demonstrated that the release rate of DOX-loaded liposomes containing F-PEOz-DSPE increased significantly in phosphate buffered saline (PBS) at pH 5.0 medium compared with at pH 7.4 medium. These results indicate that F-PEOz-DSPE exhibits selective targeting, pH-sensitivity and little cytotoxicity, and may be a promising polymeric material using for preparing targeted liposomes with pH-sensitivity.

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References

1) Jemal J. A., Siegel R., Xu J., Ward E., *CA Cancer J. Clin.*, **60**, 277–300 (2010).
2) Müller C., Schibli R., *J. Nucl. Med.*, **52**, 1–4 (2011).
3) Ledermann J. A., Raja F. A., *Gynecol. Oncol.*, **119**, 151–156 (2010).
4) Dainty L. A., Risinger J. I., Morrison C., Chandramouli G. V., Bidus M. A., Zahn C., Rose G. S., Fowler J., Berchuck A., Maxwell G. L., *Gynecol. Oncol.*, **105**, 563–570 (2007).
5) Low P. S., Henne W. A., Doorneweerd D. D., *Acc. Chem. Res.*, **41**, 120–129 (2008).
6) Stubbs M., McSheehy P. M., Griffiths J. R., Bashford C. L., *Mol. Med. Today*, **6**, 15–19 (2000).
7) Yahara T., Koga T., Yoshida S., Nakagawa S., Deguchi H., Shirouzu K., *Surg. Today*, **33**, 243–248 (2003).
8) Luo L., Tam J., Maysinger D., Eisenberg A., *Bioconjug. Chem.*, **13**, 1259–1265 (2002).
9) Lee E. S., Na K., Bae Y. H., *J. Controlled Release*, **91**, 103–113 (2003).
10) Lee E. S., Oh K. T., Kim D., Youn Y. S., Bae Y. H., *J. Controlled Release*, **123**, 19–26 (2007).
11) Wang C. H., Wang C. H., Hsiue G. H., *J. Controlled Release*, **108**, 140–149 (2005).
12) Kim D., Lee E. S., Oh K. T., Gao Z. G., Bae Y. H., *Small*, **4**, 2043–2050 (2008).
13) Mero A., Pasut G., Dalla Via L., Fijten M. W., Schubert U. S., Hoogenboom R., Veronese F. M., *J. Controlled Release*, **125**, 87–95 (2008).
14) Hsiue G. H., Wang C. H., Lo C. L., Wang C. H., Li J. P., Yang J. L., *Int. J. Pharm.*, **317**, 69–75 (2006).
15) Lee S. C., Lee H. J., *Langmuir*, **23**, 488–495 (2007).
16) Kolb H. C., Finm M. G., Sharpless K. B., *Angew. Chem. Int. Ed. Engl.*, **40**, 2004–2021 (2001).
17) Zhao X., Chen D., Gao P., Ding P., Li K., *Chem. Pharm. Bull.*, **53**, 1246–1250 (2005).
18) Hashizaki K., Taguchi H., Itoh C., Sakai H., Abe M., Saito Y., Ogawa N., *Chem. Pharm. Bull.*, **53**, 27–31 (2005).
19) Fathi-Azarbayjani A., Chan S. Y., *Chem. Pharm. Bull.*, **58**, 143–146 (2010).
20) Liu D., Hu H., Zhang J., Zhao X., Tang X., Chen D., *Chem. Pharm. Bull.*, **59**, 63–71 (2011).
21) Kim Y. K., Choi J. Y., Yoo M. K., Jiang H. L., Arote R., Je Y. H., Cho M. H., Cho C. S., *J. Biotechnol.*, **131**, 353–361 (2007).
22) Fattal E., Couvreur P., Dubernet C., *Adv. Drug Deliv. Rev.*, **56**, 931–946 (2004).