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Construction of anti-EGFR immunoliposomes via folate–folate binding protein affinity

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Abstract

A novel method for synthesis of anti-EGFR immunoliposomes using folate–folate binding protein (FBP) affinity is described. An anti-EGFR antibody (cetuximab or C225) was covalently linked to FBP via a thioether bond. Liposomes incorporating a lipophilic folate derivative (folate-PEG-cholesterol) were prepared by polycarbonate membrane extrusion. Anti-EGFR immunoliposomes were then obtained by combining FBP-C225 and folate-liposomes and evaluated for uptake and cytotoxicity in EGFR-overexpressing U87 human glioblastoma cells. Anti-EGFR immunoliposomes constructed via folate–FBP affinity exhibited excellent stability under physiological pH, and quickly released the bound FBP-C225 upon low pH (pH 3.5) treatment. Flow cytometry and fluorescence microscopy showed similar receptor-specific binding and internalization for both folate–FBP affinity-coupled and covalently coupled C225-immunoliposomes, but not for the non-targeted IgG-immunoliposomes. C225-immunoliposomes loaded with anticancer drug doxorubicin were more cytotoxic than non-targeted immunoliposomes in EGFR-overexpressing U87 glioma cells. Folate–FBP affinity is a potential method for construction of immunoliposomes and may have applications in synthesis of targeted drug carriers in general.
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Keywords: Immunoliposomes; Folate binding protein; Drug targeting; Folate; EGF receptor

1. Introduction

Liposomes, spherical vesicles formed by lipid bilayers, have been widely investigated as carriers of anticancer drugs (Allen et al., 1991; Papahadjopoulos et al., 1991). Conjugation of liposomes to a targeting ligand can potentially improve their selectivity for tumor cells. The ligands evaluated for liposome targeting include monoclonal antibodies (MAbs) (Kirpotin et al., 1997; Leserman et al., 1981), folate (Lee and Low, 1994), and transferrin (Singh, 1999). Antibody-conjugated liposomes (immunoliposomes) are particularly promising given increasing availability of a wide variety of MAbs. Covalent conjugation of MAbs to liposomes, however, may result in loss of MAbs activity due to variability in conjugation condition and often random sites of attachment on the MAb molecules. Antibodies can be conjugated to pre-synthesized liposomes containing a functionalized lipid (Bendas et al., 1999; Hansen et al., 1995; Torchilin et al., 2001; Zalipsky, 1993), or incorporated by incubating liposomes with micelles of lipid-derivatized MAbs, otherwise known as “post-insertion” (Iden and Allen, 2001). The later method, however, may suffer from low MAb incorporation efficiency and MAb denaturation due to the high temperature required for ligand insertion. Besides covalent conjugation of MAbs to liposomes, non-covalent conjugation, such as via biotin–avidin affinity, has also been evaluated in the preparation of immunoliposomes (Hansen et al., 1995; Schnyder et al., 2004). However, immunogenicity of avidin may impede potential clinical application of these liposomes. Another non-covalent conjugation strategy was based on affinity between nickel and polyhistidine, which has been frequently

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used in purification of recombinant proteins (Dietrich et al., 1996; Nielsen et al., 2006). However, introduction of nickel ions into liposomes introduces potential toxicity concern and the linkage may be unstable under in vivo conditions.

Folate receptor targeted liposomes have been reported to selectively deliver drugs into folate receptor expressing cells (Lee and Low, 1994). Folate binding protein (FBP), an endogenous protein, exhibits very high affinity for folate, with K_d in the nanomolar range (Henderson, 1990; Pan and Lee, 2004). In this study, a novel non-covalent conjugation system is evaluated that is based on the high affinity interaction between folate and FBP.

The human EGF receptor (EGFR) belongs to a family of receptors involved in cellular proliferation and differentiation (Cohen et al., 1981). EGFR is an attractive therapeutic target because its expression is amplified in many types of human cancers, including those of lung, breast, prostate, colon, ovary, and head and neck (Barth et al., 2002; Mendelsohn and Baselga, 2003; Nicholson et al., 2001; Schwechheimer et al., 1995). Cetuximab (Erbitux^{TM} or C225), was the first FDA approved EGFR-specific MAb for treatment of patients with EGFR-expressing metastatic colorectal and head/neck cancers (Baselga et al., 2000; Giaccone, 2005). Anti-EGFR immunoliposomes have been produced previously by a covalent method (Mamot et al., 2003). In this report, we present a non-covalent method for synthesis of anti-EGFR immunoliposomes based on the folate–FBP affinity. The liposomes were characterized for cellular uptake and cytotoxicity in glioma cells.

2. Material and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and 1,2-disteaoryl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy-polyethylene glycol$_{2000}$ (mPEG$_{2000}$-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Maleimido-PEG$_{3350}$-cholestrol (Mal-PEG$_{3350}$-Chol) was synthesized in our lab, as described elsewhere (Pan and Lee, 2004). cholesterol (Chol), cholesteryl chloroformate, folic acid, bovine milk folate binding protein (FBP), folate-agarose gel, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 8-hydroxypyrenetrisulfonic acid trisodium salt (HPTS), sheep IgG, 2-iminothiolane (Traut's Reagent), *N*-hydroxysuccinimide (NHS), PEG$_{3350}$-bis-amine, Sepharose CL-4B gels, and triethylamine (TEA) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Life Technologies (Grand Island, NY). PD-10 desalting column was purchased from GE Healthcare Life Sciences (Piscataway, NY). Bicinchoninic acid (BCA) protein assay kit and *m*-maleimidobenzoyl-*N*-sulfosuccinmide ester (Sulfo-MBS) were purchased from Pierce Chemical Co. (Rockford, IL). Polycarbonate membranes and Lipex^{TM} Extruder were obtained from Northern Lipids (Vancouver, Canada). C225 was generously provided by ImClone Systems (New York, NY) by way of Dr. Rolf Barth. All other chemicals were of reagent grade.

2.2. Cell culture

U87 human glioblastoma cells, which overexpresses EGFR, was kindly provided by Dr. Rolf F. Barth (The Ohio State University, Department of Pathology, Columbus, OH). Cells were cultured as a monolayer in DMEM media supplemented with 100 units/mL penicillin, 100 μM/mL streptomycin, 200 μg/mL of G418 and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Preparation of FBP-C225 conjugate

FBP was affinity purified from crude bovine milk FBP. Briefly, bovine milk FBP was mixed with folate-agarose in PBS (pH 7.4) and gently shaken at 4 °C overnight. After transferring the slurry into a 1 mL syringe stopped with glass wool, the folate-agarose was washed twice with 200 μL PBS (pH 7.4). The FBP was batch eluted with 500 μL of 0.2 M acetic acid, and the pH of the eluted FBP solution was adjusted to 7.4 with 0.5 M NaOH.

C225 was activated with 5× molar excess of Sulfo-MBS in PBS (pH 7.4) for 1 h at room temperature. Unreacted Sulfo-MBS was removed and the solution pH adjusted by passing through a PD-10 desalting column eluted with PBS (pH 6.5). The maleimide modified C225 was stored at 4 °C and used within 1 h. In parallel with MAb activation, FBP (0.4 mg) was thiolated with 5× molar excess of Traut's reagent in PBS (pH 8.0) for 1 h at room temperature. The reaction mixture was passed through a PD-10 column eluted with PBS (pH 6.5), and with UV detection at 280 nm. The thiolated FBP was then conjugated to maleimide modified C225 at a 1:1 molar ratio by coincubation at 4 °C for 4 h with gentle stirring. The product was purified by Sephacryl S-300 gel filtration column, which was pre-equilibrated and eluted with PBS (pH 7.4). The fractions containing FBP-C225 were collected and concentrated by Microcon (MWCO, 100 kDa) by centrifugation at 1000 × *g* for 3 min. Protein concentration was determined by BCA protein assay (Stoscheck, 1990). All elution buffers used above were degassed by N_2 to deplete oxygen.

2.4. Synthesis of folate-PEG-Chol

Synthesis of folate-PEG-Chol has been reported previously by our lab (Guo et al., 2000). Here we describe a modified synthetic route for folate-PEG-Chol (Fig. 1). First, Chol-PEG-NH_2 (**1**) was synthesized, as described elsewhere (Pan and Lee, 2004). Then, 1.2 M excess of folate-NHS (**3**) (34 mg, 63 nmol), synthesized as previously reported (Guo et al., 2000), was added to Chol-PEG-NH_2 (200 mg, 52 nmol) in $CHCl_3$ (20 mL), and the reaction mixture was stirred under N_2 in darkness at room temperature overnight. The reaction mixture was then loaded onto Sephadex LH20 column, and eluted with $CHCl_3$/MeOH (1:1, v/v). The fractions containing product folate-PEG-Chol (**4**) were pooled and evaporated to dryness under vacuum. The final product was analyzed by MALDI MS and by ^1H NMR.

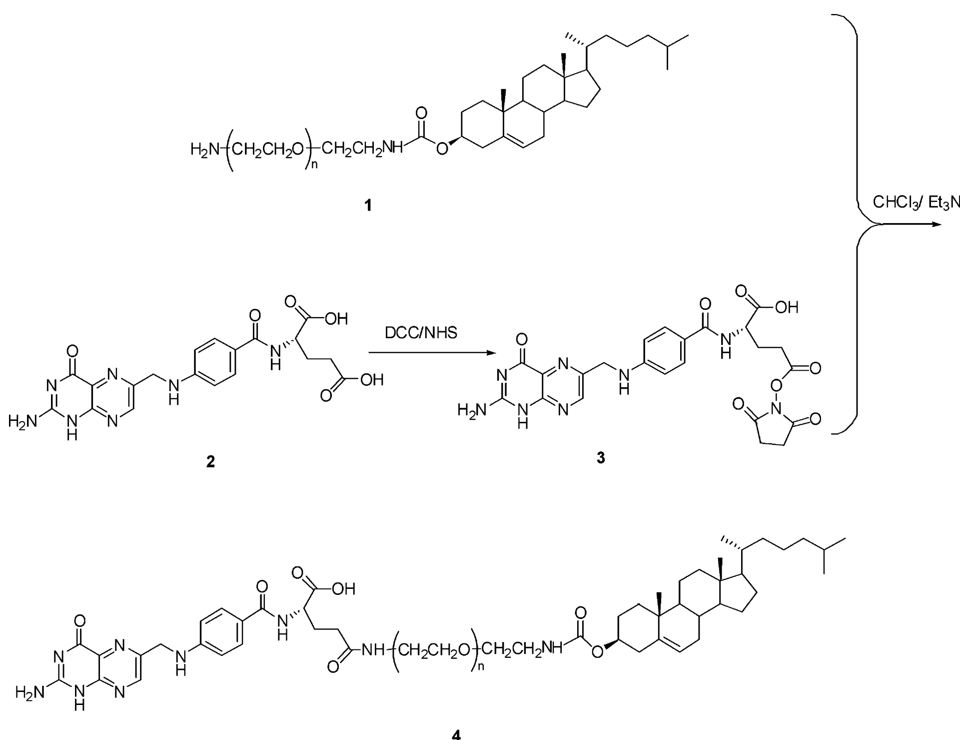

Fig. 1. Scheme for synthesis of folate-PEG_{3350}-cholesterol.

2.5. Preparation of immunoliposomes via folate–FBP affinity

Unilamellar folate-liposomes composed of HSPC/Chol/PEG_{2000}-DSPE/folate-PEG_{3350}-Chol (60:35:5:0.5, mol/mol) were prepared by polycarbonate membrane extrusion method (Olson et al., 1979). Briefly, a chloroform solution of lipids (50 mg) was dried into a thin film in a round-bottom flask on a rotary evaporator, and then further dried for 2 h under vacuum. The lipid mixture was hydrated in 1 mL of fluorescent dye HPTS (25 mM HPTS, in PBS, pH 7.4). The resulting suspension was subjected to 5 cycles of freezing and thawing, and extruded five times through polycarbonate membrane with pore size of 100 nm using Lipex™ Extruder driven by pressurized nitrogen at 200 psi at 65 °C. Unentrapped HPTS was separated from liposomes by gel filtration on a 10 mL PD-10 column equilibrated in PBS (pH 7.4). Non-covalently coupled C225-liposomes were obtained by incubating FBP-C225 and folate-liposomes at a ratio of 25 μg FBP-C225/μmol lipids at room temperature for 1 h, followed by passing through a CL-4B Sepharose column to remove any unbound C225-FBP conjugate. Liposome size was determined by dynamic light scattering (DLS) using a Nicomp Particle Sizer Model 370 (PSS, Santa Barbara, CA).

Meanwhile, covalently coupled C225-liposomes and IgG-liposomes loaded with fluorescence dye HPTS were also prepared by post-insertion method as positive and negative controls for targeted liposomes, respectively. Briefly, MAb (C225 or IgG) was thiolated by Traut's reagent, and thiolated MAb was conjugated to Mal-PEG-Chol, as described elsewhere (Pan and Lee, 2004). The resulting MAb-PEG-Chol micelles were incubated with liposomes consisting of HSPC/Chol/PEG_{2000}-DSPE (60:34:2, mole/mole) at 60 °C for 1 h at ratio of 35 μg MAb/μmol lipid. An insertion rate of ∼60% was observed. After purification by Sepharose CL-4B gel filtration, C225-liposomes or Ig-G liposomes were stored at 4 °C for further study.

For cytotoxicity study, liposomes were loaded with doxorubicin by ammonium sulfate generated pH-gradient driven remote-loading method, as described previously (Haran et al., 1993; Lasic et al., 1992). Briefly, dry lipids film was rehydrated in 250 mM ammonium sulfate, followed by extrusion as described above. The external ammonium sulfate buffer was then exchanged with 10% sucrose by tangential flow diafiltration. Liposomes were then incubated with doxorubicin at lipid-to-drug weight ratio of 25 for 30 min at 60 °C. Unencapsulated drug was removed by Sepharose CL-4B column. For synthesis of folate-liposomes encapsulating doxorubicin, above formulation was supplemented with an additional 0.5 mol% of folate-PEG_{3350}-Chol. C225-FBP was incubated with doxorubicin loaded folate-liposomes to form immunoliposomes, as described above. Doxorubicin containing covalently linked C225-liposomes for the targeted control group were prepared by post-insertion of C225 into liposomal doxorubicin, as described above.

2.6. Stability at different temperature

Folate-liposomes with FBP or C225-FBP were incubated at 4 and 37 °C for 1, 4 and 24 h in RPMI 1640 medium. Then, the liposomes were passed through a Sepharose CL-4B column equilibrated in PBS (pH 7.4) to determine the stability of FBP or C225-FBP associated liposomes. Change in particle sizes was monitored by DLS.

2.7. Cellular uptake study

Uptake of C225-FBP-liposomes was evaluated in U87 human glioblastoma cells. U87 cells cultured as a monolayer in T75 flask were harvested by treatment with trypsin/EDTA solution. Liposomes at approximately 500 μg/mL (lipid concentration) encapsulating HPTS were then incubated with U87 cells (5×10^5 cells) for 2 h at 37 °C. After extensively washing with PBS, the cells were kept on ice for flow cytometric assay. For fluorescence microscopy studies, U87 cells (5×10^5 cells) grown on chamber slides were treated with liposomes and incubated for 2 h at 37 °C. After extensively PBS washing, cells were fixed with paraformaldehyde (4% in PBS), and mounted on slides in glycerol, and visualized on a Zeiss LSM confocal microscopy.

2.8. Cytotoxicity determination

Immunoliposomes encapsulating doxorubicin were evaluated in U87 cells in 96-well plates. Cells were incubated in quadruplicates with serial dilutions of liposomes for 2 h at 37 °C, followed by extensive washing with PBS (pH 7.4). The cells was allowed to grow in fresh DMEM medium containing 10% FBS for an additional 48 h days, and then were analyzed by MTT assay.

3. Results

In the present study, novel non-covalently coupled immunoliposomes were developed based on the high affinity between folate and FBP. The mass spectra of the folate-PEG-Chol (**4**) produced a series of 44 Da-spaced lines, which is the molecular mass of one ethylene oxide unit, and the peaks were centered at 4154 Da. ^{1}H NMR ($CDCl_3$, 300 MHz)—δ: 7.71 (s, 1H), 7.61 (d, 2H), 6.74 (d, 2H), 5.38 (s, 1H), 3.80–3.50 (br, m, PEG protons, ~300H), 2.00–1.03 (m, Chol protons, ~26H), 1.00 (s, 3H), 0.91 (d, 3H), 0.87 (d, 6H), 0.67 (s, 3H).

The long ~3350 Da spacer of folate-PEG-Chol should minimize steric hindrance from PEG_{2000}-DSPE on the liposome surface and enable folate motifs on the liposome surface to access the FBP on the cell surface. Fluorescent HPTS loaded folate-liposomes were prepared by high-pressure extrusion through polycarbonate membrane with 100 nm pore size, and yielded a mean diameter of 115 ± 32 nm, as determined by DLS.

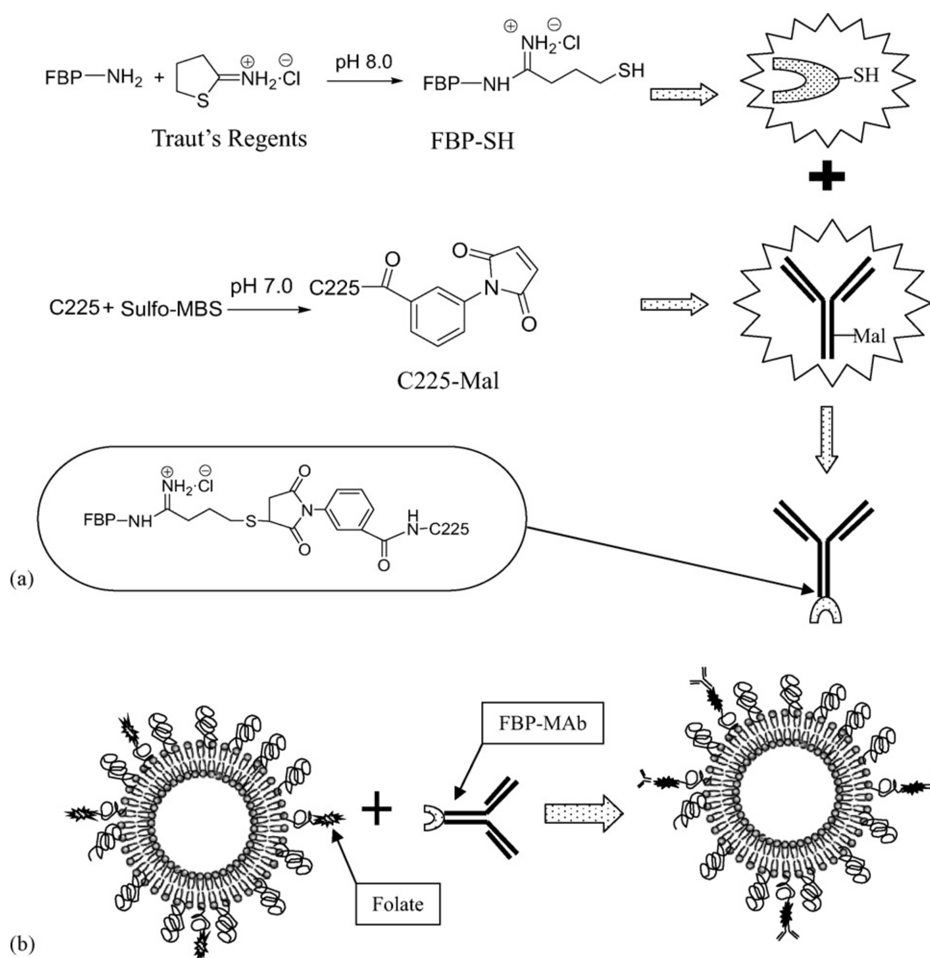

Fig. 2. Synthesis of FBP-C225 and anti-EGFR immunoliposomes. (a) Schematic depiction of synthesis of FBP-C225 conjugates. (b) Construction of non-covalently coupled anti-EGFR C225-liposomes by incubating FBP-C225 with folate-liposomes under a mild condition (neutral pH, room temperature).

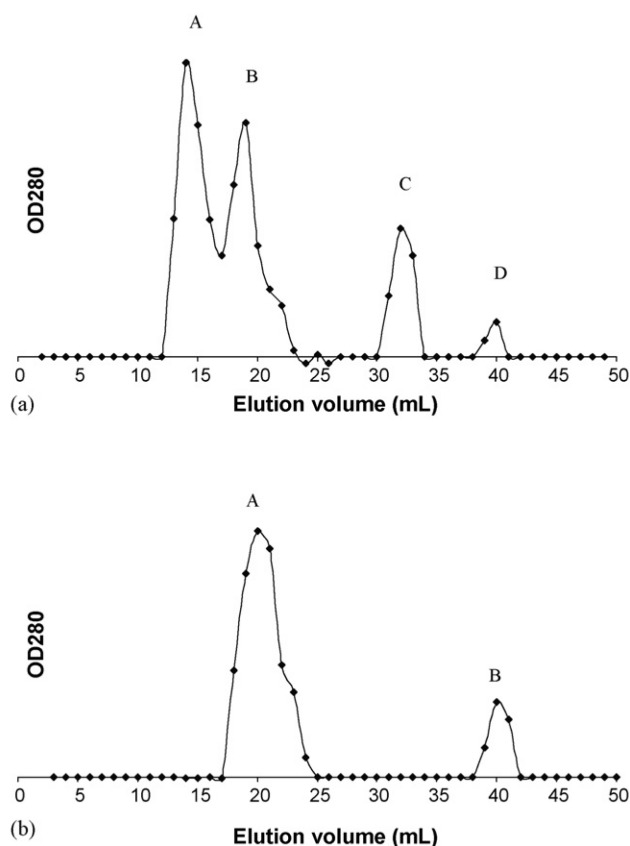

Fig. 3. Purification of C225-FBP conjugate by gel filtration chromatography. (a) Elution profile of C225-FBP conjugates. The reaction mixtures were loaded on to a Sephacryl S-300 column, and eluted with PBS (pH 7.4). Peaks: (A) C225-FBP, (B) unconjugated C225, (C) crosslinked FBP, and (D) unconjugated FBP. (b) Elution profile of free C225 (A) and FBP (B) on Sephacryl S-300 column.

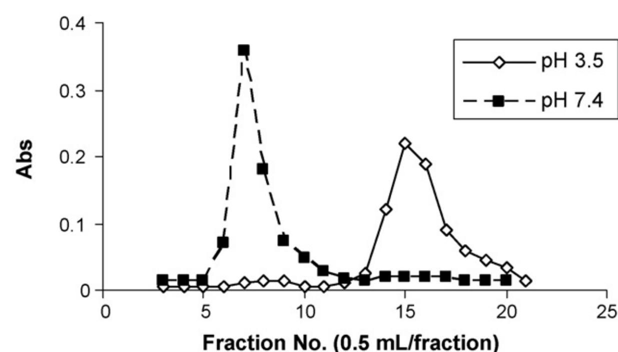

Fig. 4. Effect of pH on the affinity of C225-FBP to folate-liposomes. Non-covalently coupled C225-FBP/folate-liposomes were eluted at either pH 7.4 or 3.5 on Sepharose CL-4B gel filtration column. *Y*-axis, absorption at 280 nm.

About 300 folates were located on liposome surfaces, based on an average area per phospholipid molecule of 75 Å^2 (Kirpotin et al., 1997).

The scheme for construction of immunoliposomes by using non-covalent folate–FBP affinity is illustrated in Fig. 2. Maleimide groups were introduced onto C225 by sulfo-MBS, and thiol group on FBP by Traut's reagents. FBP was then conjugated to C225 via a covalent thioether bond formed between thiol and maleimide groups. The resulting C225-FBP conjugate was then purified by Sephacryl S-300 gel filtration column (Fig. 3). The conjugates were identified by their distinctive molecular weights. The yield for C225-FBP conjugation was approximately 40% based on BCA protein assay. Non-covalently coupled immunoliposomes were obtained by incubating folate-liposomes with purified C225-FBP at neutral pH 7.4, followed by purification by a Sepharose CL-4B column. The protein assay showed that over 90% of C225-FBP conjugate was bound to folate-liposomes after passing through the column (data not shown). The immunoliposomes had a mean diameter of 135 ± 41 nm as determined by DLS.

The binding of C225-FBP to folate-liposome was evaluated by gel filtration at neutral (PBS, pH 7.4) and acidic pH (PBS, pH 3.5), since FBP has high affinity for folate at neutral pH but loses affinity at acidic pH (Salter et al., 1972, 1981). In Fig. 4, C225-FBP conjugates were coeluted with folate-liposomes in the void volume at neutral pH. In contrast, they were eluted separately with folate-liposomes at acidic pH. Therefore, the FBPs retained their folate binding affinity-pH profile after conjugation to C225.

The stability of FBP and C225-FBP after binding to folate-liposomes was studied at 4 and 37 °C. FBP is reported to be highly stable over a wide range of temperature (Salter et al., 1972). FBP bound to folate-liposomes showed a high degree of stability with minimum dissociation. C225-FBP was also tightly bound to folate-liposomes over prolonged incubation at 4 or 37 °C (Table 1). The particle sizes of FBP or C225-FBP bound liposomes remained essentially unchanged after overnight incubation at 4 °C, and had only a slight increase after 37 °C overnight incubation.

Table 1
Retention of FBP and C225-FBP on folate-liposomes at different temperature and time

Temperature	Time					
	1 h		4 h		24 h	
	Bound %	Size (nm)	Bound %	Size (nm)	Bound %	Size (nm)
FBP						
4 °C	100	125 ± 29	100	127 ± 26	100	119 ± 33
37 °C	100	131 ± 32	~99	142 ± 45	~99	165 ± 52
C225-FBP						
4 °C	100	136 ± 29	~95	129 ± 41	~91	142 ± 46
37 °C	~96	145 ± 53	~90	168 ± 57	~82	219 ± 77

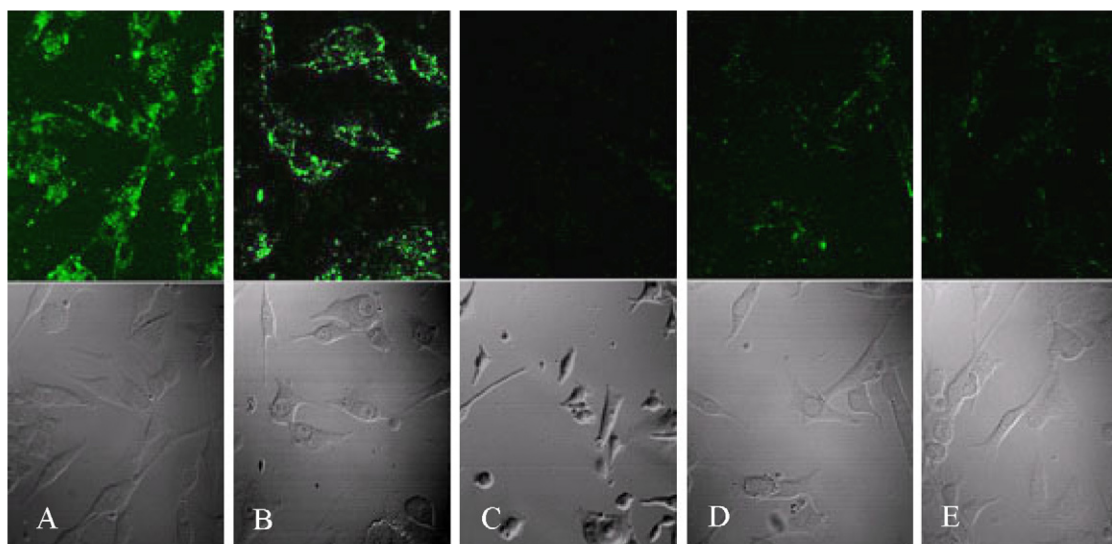

Fig. 5. Internalization of liposomes in EGFR-overexpressing U87 human glioblastoma cells. Phase contract (bright field images in the bottom panels) and fluorescence images (top panels). (A) C225-liposomes constructed by covalent coupling, (B) C225-FBP/folate-liposomes constructed by non-covalent coupling, (C) folate-liposomes, (D) IgG-liposomes constructed by covalent coupling, and (E) folate-liposomes bound to FBP.

Uptake studies in U87 human glioblastoma cells showed that non-covalently coupled C225-liposomes synthesized using folate–FBP affinity could efficiently bind to U87 cells (Fig. 5). Both non-covalently coupled and covalently coupled C225-liposomes demonstrated high intracellular accumulation in U87 cells. However, there was little uptake of non-C225 conjugated liposomes, including folate-liposomes, IgG-liposomes and FBP-folate-liposomes (folate-liposomes bound to FBP). Results of flow cytometric assay were consistent with the observation by fluorescent microscopy (Fig. 6). The fluorescence intensity of cells treated with non-covalently coupled C225-liposomes was only slightly reduced compared to directly coupled C225-liposomes. These data suggest that both C225 and FBP could retain their binding affinity after conjugation, and the resulting non-covalently coupled C225-liposomes were able to be internalized by cells via receptor-mediated endocytosis.

Doxorubicin, one of the most commonly used anticancer drugs, was loaded into the liposomes by pH-gradient remote-loading method. The loading efficiency was greater than 95%. In EGFR-overexpressing U87 human glioblastoma cells, cytotoxicity of both directly coupled and the non-covalently coupled C225-liposomes loaded with doxorubicin showed significant receptor-specific cytotoxicity ($IC_{50} = 0.72 \pm 0.11$ and 1.44 ± 0.34 μM, respectively), which approached that of free doxorubicin ($IC_{50} = 0.38 \pm 0.08$ μM). In contrast, doxorubicin loaded folate-liposomes and FBP-folate-liposomes showed almost 20-fold lower cytotoxicity compared to free doxorubicin. Summary of the cytotoxicity results was given in Table 2. The C225 immunoliposomes are expected to be much more cytotoxic to EGRF expressing cells in vivo since they have much longer circulation time than free DOX and are expected to accumulate in tumors based on increased vascular permeability of the tumor

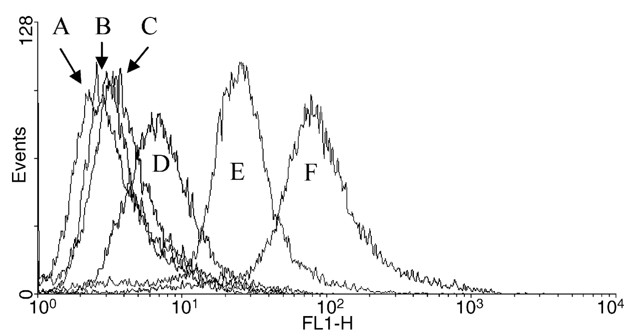

Fig. 6. Uptake of liposomes in EGFR-overexpressing U87 human glioblastoma cells evaluated by flow cytometry: (A) untreated U87 cells, (B) cells treated with folate-liposomes, (C) cells treated with folate-liposomes bound to FBP, (D) cells treated with IgG-liposomes prepared by covalent coupling, (E) cells treated with C225-FBP/folate-liposomes constructed by non-covalent coupling, and (F) cells treated with C225-liposomes constructed by covalent coupling.

Table 2
Cytotoxicity of liposomal doxorubicin in EGFR-overexpressing U87 human glioblastoma cells, $n = 3$

	Formulation	IC_{50} (μM)
Doxorubicin (DOX)	Free drug	0.38 ± 0.08
Folate-L-DOX	HSPC/Chol/mPEG2000-DSPE/folate-PEG3350-Chol at 60:34:5:0.5 molar ratio	18.6 ± 0.85
FBP/folate-L-DOX	HSPC/Chol/mPEG2000-DSPE/folate-PEG3350-Chol at 60:34:5:0.5 molar ratio. ~10 μg FBP/μmol lipid	15.2 ± 1.98
C225-L-DOX	HSPC/Chol/mPEG2000-DSPE at 60:34:2 molar ratio. ~20 μg C225/μmol lipid	0.72 ± 0.11
C225-FBP/folate-L-DOX	HSPC/Chol/mPEG2000-DSPE/folate-PEG3350-Chol at 60:34:5:0.5 molar ratio. ~25 μg FBP-C225/μmol lipid	1.44 ± 0.34

L-DOX: liposomal doxorubicin.

endothelium. In fact, non-targeted liposomal DOX has shown superior antitumor activity in murine models compared to free DOX.

4. Discussion

In this study, we presented a novel method for synthesis of non-covalently coupled immunoliposomes, where FBP conjugated anti-EGFR MAb C225 was tethered to folate-liposomes via folate–FBP affinity.

In previous studies, immunoliposomes have been constructed by combining streptavidin-conjugated OX26 (rat anti-transferrin MAb) and biotinylated liposomes (Schnyder et al., 2004). The resulting immunoliposomes showed binding and uptake by transferrin receptor expressing L16 cells. However, avidin (or streptavidin) has four biotin binding sites, which could cause crosslinking among biotinylated liposomes and result in aggregation. Additionally, avidin (or streptavidin), an exogenous protein, may trigger an antibody response after repeated injections. In another non-covalent approach, immunoliposomes were synthesized by mixing with Ni-NTA-lipids containing liposomes with $(His)_6$-tagged MAbs or fragments (Nielsen et al., 2006). This approach has been developed as a rapid and sensitive assay for the identification and characterization of tumor-specific antibodies with non-covalent conjugation to liposomes. However, this approach is still plagued by the potential toxicity of Ni and lack of sufficient stability in vivo.

FBP has several advantages as a linker protein for bioconjugation chemistry. First, FBP (M_W ∼40 kDa) has relatively low M_W and a single binding site, which precludes liposome crosslinking. In comparison, avidin (M_W 52 kDa), which has four binding sites. Secondly, as an endogenous protein, FBP is likely to be less immunogenic. Furthermore, the immunoliposomes may disassemble following internalization by endocytosis upon exposure to the low pH environment of the endosomal compartment, due to reduced affinity between folate and FBP under low pH. This internalization-triggered uncoupling may be beneficial to delivery of liposomal drug at the cellular level by allowing the sorting of the internalized liposomes to the appropriate subcellular compartment and/or facilitating intracellular release of drug molecules from the liposomes. The bovine milk FBP in the current study shared more than 80% sequence homology with the high affinity human FBPs (Henderson, 1990). In our study, FBP-C225 conjugation procedure was relatively simple, although the yield of 40% was somewhat low. This problem can possibly be solved in the future by generating a antibody–FBP fusion protein using recombinant methods. An important advantage of this non-covalently coupling method is elimination of the need to perform chemical reaction on or high temperature treatment of liposomes, which have limited stability.

Covalently coupled immunoliposomes containing 2–5% (molar percent) of PEG_{2000}-DSPE have shown prolonged systemic circulation (Mamot et al., 2005; Zalipsky et al., 1996). FBP level in human serum is negligible and its expression is absent in almost all normal tissues. We therefore expect the non-covalent coupled immunoliposomes to have good stability in the circulation. This will be further evaluated in future in vivo experiment.

In conclusion, we have developed novel non-covalently coupled anti-EGFR C225-liposomes by using folate–FBP affinity. The non-covalently coupled C225-liposomes can be efficiently transported into cells by means of receptor-mediated endocytosis, which was validated by flow cytometric assay and confocal microscopy. This novel method of conjugation can be similarly applied to the synthesis of other immunoconjugates and is potentially superior to the currently widely used biotin–avidin affinity system.

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References

Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., 1991. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta 1066, 29–36.

Barth, R.F., Yang, W., Adams, D.M., Rotaru, J.H., Shukla, S., Sekido, M., Tjarks, W., Fenstermaker, R.A., Ciesielski, M., Nawrocky, M.M., Coderre, J.A., 2002. Molecular targeting of the epidermal growth factor receptor for neutron capture therapy of gliomas. Cancer Res. 62, 3159–3166.

Baselga, J., Pfister, D., Cooper, M.R., Cohen, R., Burtness, B., Bos, M., D'Andrea, G., Seidman, A., Norton, L., Gunnett, K., Falcey, J., Anderson, V., Waksal, H., Mendelsohn, J., 2000. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. J. Clin. Oncol. 18, 904–914.

Bendas, G., Krause, A., Bakowsky, U., Vogel, J., Rothe, U., 1999. Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. Int. J. Pharm. 181, 79–93.

Cohen, S., Carpenter, G., King Jr., L., 1981. Epidermal growth factor-receptor-protein kinase interactions. Prog. Clin. Biol. Res. 66 (Pt A), 557–567.

Dietrich, C., Boscheinen, O., Scharf, K.D., Schmitt, L., Tampe, R., 1996. Functional immobilization of a DNA-binding protein at a membrane interface via histidine tag and synthetic chelator lipids. Biochemistry 35, 1100–1105.

Giaccone, G., 2005. HER1/EGFR-targeted agents: predicting the future for patients with unpredictable outcomes to therapy. Ann. Oncol. 16, 538–548.

Guo, W.J., Lee, T., Sudimack, J., Lee, R.J., 2000. Receptor-specific delivery of liposomes via folate-PEG-Chol. J. Liposome Res. 10, 179–195.

Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S., Allen, T.M., 1995. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. Biochim. Biophys. Acta 1239, 133–144.

Haran, G., Cohen, R., Bar, L.K., Barenholz, Y., 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta 1151, 201–215.

Henderson, G.B., 1990. Folate-binding proteins. Annu. Rev. Nutr. 10, 319–335.

Iden, D.L., Allen, T.M., 2001. In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. Biochim. Biophys. Acta 1513, 207–216.

Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., Papahadjopoulos, D., 1997. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. Biochemistry 36, 66–75.

Lasic, D.D., Frederik, P.M., Stuart, M.C., Barenholz, Y., McIntosh, T.J., 1992. Gelation of liposome interior. A novel method for drug encapsulation. FEBS Lett. 312, 255–258.

Lee, R.J., Low, P.S., 1994. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. J. Biol. Chem. 269, 3198–3204.

Leserman, L.D., Machy, P., Barbet, J., 1981. Cell-specific drug transfer from liposomes bearing monoclonal-antibodies. Nature 293, 226–228.

Mamot, C., Drummond, D.C., Greiser, U., Hong, K., Kirpotin, D.B., Marks, J.D., Park, J.W., 2003. Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells. Cancer Res. 63, 3154–3161.

Mamot, C., Drummond, D.C., Noble, C.O., Kallab, V., Guo, Z., Hong, K., Kirpotin, D.B., Park, J.W., 2005. Epidermal growth factor receptor-targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs in vivo. Cancer Res. 65, 11631–11638.

Mendelsohn, J., Baselga, J., 2003. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J. Clin. Oncol. 21, 2787–2799.

Nicholson, R.I., Gee, J.M., Harper, M.E., 2001. EGFR and cancer prognosis. Eur. J. Cancer 37 (Suppl. 4), S9–S15.

Nielsen, U.B., Kirpotin, D.B., Pickering, E.M., Drummond, D.C., Marks, J.D., 2006. A novel assay for monitoring internalization of nanocarrier coupled antibodies. BMC Immunol. 7, 24.

Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., Papahadjopoulos, D., 1979. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta 557, 9–23.

Pan, X., Lee, R.J., 2004. Tumour-selective drug delivery via folate receptor-targeted liposomes. Expert Opin. Drug Deliv. 1, 7–17.

Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C., et al., 1991. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc. Natl. Acad. Sci. U.S.A. 88, 11460–11464.

Salter, D.N., Ford, J.E., Scott, K.J., Andrews, P., 1972. Isolation of the folate-binding protein from cow's milk by the use of affinity chromatography. FEBS Lett. 20, 302–306.

Salter, D.N., Scott, K.J., Slade, H., Andrews, P., 1981. The preparation and properties of folate-binding protein from cow's milk. Biochem. J. 193, 469–476.

Schnyder, A., Krahenbuhl, S., Torok, M., Drewe, J., Huwyler, J., 2004. Targeting of skeletal muscle in vitro using biotinylated immunoliposomes. Biochem. J. 377, 61–67.

Schwechheimer, K., Huang, S., Cavenee, W.K., 1995. EGFR gene amplification—rearrangement in human glioblastomas. Int. J. Cancer 62, 145–148.

Singh, M., 1999. Transferrin as a targeting ligand for liposomes and anticancer drugs. Curr. Pharm. Des. 5, 443–451.

Stoscheck, C.M., 1990. Quantitation of protein. Methods Enzymol. 182, 50–68.

Torchilin, V.P., Levchenko, T.S., Lukyanov, A.N., Khaw, B.A., Klibanov, A.L., Rammohan, R., Samokhin, G.P., Whiteman, K.R., 2001. *p*-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via *p*-nitrophenylcarbonyl groups. Biochim. Biophys. Acta 1511, 397–411.

Zalipsky, S., 1993. Synthesis of an end-group functionalized polyethylene glycol–lipid conjugate for preparation of polymer-grafted liposomes. Bioconjug. Chem. 4, 296–299.

Zalipsky, S., Hansen, C.B., Lopes de Menezes, D.E., Allen, T.M., 1996. Longcirculating, polyethylene glycol-grafted immunoliposomes. J. Control Release 39, 153–161.