Targeting Doxorubicin to Epidermal Growth Factor Receptors by Site-Specific Conjugation of C225 to Poly(L-Glutamic Acid) through a Polyethylene Glycol Spacer

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Purpose. Targeted delivery of chemotherapeutic agents through antibody-polymer conjugates has met with limited success. One of the limiting factors is the loss of antibody's binding affinity upon conjugation with polymeric carriers because of lack of control over the number and site of attachment. This study aims to synthesize monovalent polymeric immunoconjugates through site-specific conjugation and to evaluate the *in vitro* binding activities of the resulting construct.

Methods. Antibody C225 against epidermal growth factor receptors was coupled to the terminus of a doxorubicin-bound block copolymer, poly(L-glutamic acid)-co-polyethylene glycol (PG-PEG). Western blot analysis, confocal fluorescent microscopy, and cytotoxicity assay were performed to confirm the specific binding of C225-PEG-PG-Dox to EGFR.

Results. C225 was conjugated to PEG-PG-doxorubicin conjugates by reacting sulfhydryl group introduced to C225 with vinylsulfone group introduced at the terminus of PEG-PG block copolymer. Polymeric immunoconjugate C225-PEG-PG-Dox, but not control (i.e., conjugate without antibody), selectively bound to human vulvar squamous carcinoma A431 cells that overexpress epidermal growth factor receptors. Receptor-mediated uptake of C225-PEG-PG-Dox occurred rapidly (within 5 min), whereas nonspecific uptake of PEG-PG-Dox required an extended period of time (24 h) to internalize. Binding of C225-PEG-PG-Dox to A431 cells could be blocked by pretreatment with C225 antibody. C225-PEG-PG-Dox was more potent than free doxrubicin in inhibiting the growth of A431 cells after a 6-h exposure period.

Conclusion. Site-specific conjugation of a monoclonal antibody to the terminus of a polymeric carrier enhances receptor-mediated delivery of anticancer agents.

KEY WORDS: targeting; epidermal growth factor receptors; doxorubicin; poly(L-glutamic acid).

INTRODUCTION

Because many anticancer agents are highly toxic not only to tumor cells but also to normal cells, drug-delivery systems that can spare the toxic effect of anticancer agents to normal tissue are highly desired. Delivery of drugs to target cells can theoretically allow the use of a reduced dose to achieve the same therapeutic response, with a consequent decrease in systemic toxicity. A number of methods have been used to selectively target tumors with therapeutic agents. For example, immunoconjugates using intermediate polymeric carriers, such as *N*-(2-hydroxypropyl)methacrylamide copolymer, dextran, and polyamino acids have been synthesized and evaluated (1–3). Compared with direct coupling of drug to antibodies, conjugation through polymer intermediates can substantially increase the drug payload without significantly impairing its antigen binding activity. Rowland *et al.* (4) were the first to propose the use of poly(L-glutamic acid) (PG) as an intermediate polymer carrier. PG polymer was loaded with *p*-phenylenediamine mustard, and the conjugate was then linked to an antibody through a "concentration-dilution" technique to minimize antibody crosslinking. About 66% of the antibody activity was retained in the conjugate (4). Generally speaking, conjugation of monoclonal antibodies (mAbs) to linear polymers through their side chain functional groups usually reduces receptor binding affinity because of folded configuration of polymers that imbed the targeting moiety in the random coiled structure.

One approach to overcome these limitations is to conjugate mAb to the termini of polymer-drug conjugates in a site-specific manner. Mann *et al.* (5) described the use of dextran with a uniquely reactive terminus for univalent attachment of proteins to avoid antibody crosslinking. Kato *et al.* (6) reported the conjugation of an anti-α-fetoprotein antibody to the end of PG conjugated with daunomycin. The resulting immunoconjugate retained most of the antigen-binding activity of the parent antibody and was more cytotoxic than the parent drug, underlining the validity of this approach.

Polyethyleneglycol (PEG)-modified proteins and liposomes have been shown to exhibit reduced liver uptake and increased blood circulation half-lives, resulting in improved biologic activity (7–9). We previously described the synthesis of a PEG-conjugated mAb with a radionuclide attached to one terminus of the PEG chain (10). The conjugate exhibited significantly reduced nonspecific interaction and improved nuclear imaging properties (11). In this study, we extended our previous work and introduced a doxorubicin-bound polymer instead of a radionuclide to one terminus of the PEG chain. Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. It is overexpressed on the cells of more than one third of all solid tumors, including bladder, breast, colon, ovarian, prostate, renal cell, and squamous cell (nonsmall cell lung and head and neck) carcinomas (12). MAb C225 specifically binds to the external domain of EGFR with an affinity comparable to that of the natural ligand (13). C225 appears to be a suitable targeting agent and was used as a model mAb here to construct polymeric immunoconjugate.

MATERIALS AND METHODS

Materials

Diisopropylcarbodiimide, dimethylformamide (DMF), PG (MW 31,000), triethylamine, ninhydrin, and pyridine were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Doxorubicin hydrochloride (Dox·HCl) was obtained from Sigma (St. Louis, MO, USA). Dox free amine was obtained

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by extracting an aqueous solution of Dox·HCl and triethylamin (molar ratio, 1:3) with chloroform. Vinylsulfonyl *N*-hydroxysuccinimidyl PEG (VS-PEG-NHS; MW, 3400) was obtained from Shearwater (Huntsville, AL). N-succinimidyl S-acetylthioacetate (SATA) and hydroxyamine were obtained from Pierce (Rockford, IL, USA). BODIPY hydrazide was obtained from Molecular Probe (Eugene, OR, USA). C225 was kindly provided by ImClone Systems Inc. (New York, NY, USA). Spectra/Pro 7 dialysis tubing (MWCO 10,000) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Silica gel plates for thin-layer chromatography (TLC) were purchased from EM Science (Gibbstown, NJ, USA). PD-10 disposable columns were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel was obtained from BioWhittaker Molecular Applications (Rockland, ME, USA).

Analytical Methods

UV measurements were recorded using a Beckman DU640 spectrophotometer (Fullerton, CA, USA). ^{1}H-NMR spectra were recorded at 300 MHz on a Brucker Avance 300 spectrometer (Billerica, MA, USA). The concentration of C225 in the immunoconjugate was estimated by UV at 650 nm using Bio-Rad Laboratories protein assay kits (Hercules, CA, USA). The measurements were made against PEG-PG-BODIPY background using known concentrations of C225 as a reference standard. The concentrations of BODIPY and Dox in the immunoconjugates were quantified by UV at 503 nm and 480 nm, respectively. Gel permeation chromatography (GPC) was performed with a Waters HPLC system (Waters Corporation, Milford, MA, USA) consisting of a 2410 refractive index detector and a 2487 dual λ UV detector. Samples were eluted with 0.1 M phosphate buffer (pH 7.4) containing 0.1% LiBr at a flow rate of 0.5 mL/min or 1 mL/min through a Superdex 200 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protein conjugates were purified using an AKTA fast-protein liquid chromatography system (Amersham Pharmacia Biotech) equipped with a Resource Q 1-mL anion exchange column. The samples were eluted with 20 mM Tris buffer (pH 7.5) and a linear gradient of 0–100% 20 mM Tris buffer containing 1.0 N NaCl in 20 mL at a flow rate of 3 mL/min. Each fraction collected was 0.5 mL. The products were detected by UV absorbance at 254 nm. Particle size was determined by laser light scattering with a Nicomp particle size analyzer (Santa Barbara, CA, USA).

Synthesis of Vinyl Sulfone-PEG-PG (VS-PEG-PG) and VS-PEG-PG-Dox Conjugates

To a solution of PG (500 mg, ~0.016 mmol) in 3 mL of 1.0 M phosphate-buffered saline (PBS; pH 8) was added VS-PEG-NHS (200 mg, 0.059 mmol) in small increments over a course of 2 h. The reaction mixture was stirred for an additional 5 h at room temperature. Ninhydrin spray was used to monitor the consumption of unreacted NH_2 at the terminal of the PG polymer. The reaction solution was acidified with 1 N HCl to pH 3.0, and the precipitate was recovered by centrifugation at 450 ×g for 10 min. The solid was washed twice with distilled water and lyophilized to yield 360 mg (75%) of the conjugate product in acid form. ^{1}H NMR (D_2O): δ3.72 ppm (s, 296H, -CH_2CH_2- in PEG), δ4.29-4.34 (m, 240H, α-CH in PG), δ2.19-2.34 ppm (m, 480H, γ-CH_2 in PG), and δ1.89-2.04 ppm (m, 480H, β-CH_2 in PG). The molar ratio of PEG to PG was approximately 0.96 based on integrals of NMR peaks at δ3.72 ppm and δ4.29-4.34 ppm corresponding to -CH_2CH_2O- in PEG and α-CH in PG, respectively.

To couple Dox to VS-PEG-PG polymer, Dox free amine (40 mg, 74 μmol), 30 μL of diisopropylcarbodiimide (24.3 mg, 192 μmol), and 100 μL of pyridine were added sequentially into a solution of 100 mg VS-PEG-PG in 5 mL of DMF. After the solvent was evaporated under vacuum, the residual solid was extracted with ether and dried under vacuum. The solid was dissolved in 0.1 N $NaHCO_3$, filtered through a 0.22-μm filter, and dialyzed against distilled water (molecular weight cut off, 10 K). For further purification, an aqueous solution of the polymer conjugate was acidified with 1.0 HCl. The precipitate was collected by centrifugation, washed with water, re-dissolved in 0.1 N $NaHCO_3$, and dialyzed again. One hundred and twenty milligrams of polymer conjugate was obtained (45% yield based on Dox·HCl; 90% based on VS-PEG-PG).

Conjugation of mAb C225 to VS-PEG-PG-Dox

Sulfhydryl group was first introduced to C225. An aliquot of SATA in DMF (190 μL, 8 mg/mL, 6.6 μmol) was added into C225 (50 mg, 0.33 μmol) in 5 mL of PBS (pH 7.2). The mixture was stirred for 1 h at room temperature, and then hydroxylamine in water (0.5 mL, 50 M) was added to the solution. The reaction mixture was stirred for additional 2 h and was concentrated to 1–2 mL by ultracentrifugation (MWCO, 10K; Millipore Corp., Bedford, MA, USA). After passing through a PD-10 column to remove small molecular weight contaminants, the resulting SH-containing C225 was mixed with 3 ml PBS solution of VS-PEG-PG-Dox (110 mg, 2.78 μmol) with a molar ratio of C225 to polymer of 1:8. The solution was stirred overnight at 4°C and then passed through a nickel affinity column (Freezyme conjugate purification kit, Pierce) to remove unreacted polymer. The column was washed with Tris buffer (25 mM, pH 7.2) containing 0.15 mM NaCl, and then subjected to elution with Tris buffer (25 mM, pH 7.2) containing 0.15 mM NaCl and EDTA (Pierce). The fractions containing C225-PEG-PG-Dox were collected, pooled, and further purified by anion-exchange chromatography to remove free mAb from polymer-bound mAb. C225 in the purified product was detected by the formation of blue precipitate when Bio-Rad protein assay reagents were added to the conjugate solution. Two milliliter of C225-PEG-PG-Dox with a Dox concentration of 0.085 mg/mL was recovered. The yield for C225 was 8.7% assuming a molar ratio of 1.0 between C225 and the polymer in C225-PEG-PG-Dox.

Immunoprecipitation and Western Blot Analysis

Human vulvar squamous carcinoma A431 cells were grown in Dulbecco's modified Eagle's medium -F12 medium containing 10% fetal bovine serum at 37°C. Cell pellets were treated with cold lysis buffer containing 1x protease inhibitor cocktails (Sigma, St Louis, MO, USA) on soft ice for 30 min, and then subjected to centrifugation to remove cell debris. Each test drug was added to 200 μL of supernatants in 0.5-mL microcentrifuge tubes. Two microliters of protein-A beads

(Sigma) were then added into each tube. The microcentrifuge tubes were incubated at 4°C for 1 h and centrifuged, and the beads were washed three times with 0.5 mL of lysis buffer. The beads were heated to 95°C in 20 μL of 1× SDS-PAGE laemmli sample buffer (Bio-Rad Laboratories) for 5 min and analyzed by 7% SDS-PAGE. Western blot analysis was performed by electronically transferring the samples into a nitrocellulose membrane and incubating the membrane for 1 h with an anti-EGFR antibody (Sigma). The receptor signals in the membrane were developed by the ECL chemoluminescence detection kit (Amersham Pharmacia Biotech).

Intracellular Localization by Confocal Laser Microscopy

A confocal fluorescent microscope was used to investigate the binding and subsequent internalization of C225-PEG-PG-Dox (excitation/emission: 480/540-nm) by EGFR-positive A431 cells (American Type Cell Culture, MD, USA) and EGFR-negative NIH3T3 Clone 4 (courtesy of Dr. Zhen Fan, M. D. Anderson Cancer Center, Houston, TX). Cells were grown on a Lab-Tek II Chamber Slide (Nalge Nunc International, Naperville, IL, USA) to 50% confluence and incubated with C225-PEG-PG-Dox or PEG-PG-Dox at an equivalent Dox concentration of 4.8 μg/mL at 37°C for various times. Free Dox was incubated with both cell lines for 30 min at a concentration of 5.8 μg/mL. Cells were washed three times with PBS, fixed in 95% ethanol, and then treated with 1 μM TO-PRO-3 iodide (excitation/emission: 642/661 nm; Molecular Probes, Eugene, OR, USA) for 15 min for nuclei staining. Fluorescent images of cells were analyzed using LMS-510 confocal microscopy with ×40 or ×60 object oil lens (Zeiss, Thornwood, NY, USA). The chamber slides were mounted on a microscope in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). The pinhole of the microscope was adjusted to be close to one.

Fluorescence Microscopic Studies

The selective uptake of C225-PEG-PG-Dox in A431 cells was also studied by competitive binding with C225 antibody using fluorescence microscope. Thus, A431 cells were seeded on cover slips in a 24-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and incubated in Dulbecco's modified Eagle medium/F12 culture medium (0.5 mL/well) overnight. C225-PEG-PG-Dox was added into each well to a final equivalent Dox concentration of 4.8 μg/mL. Alternatively, antibody C225 (final concentration, 240 μg/mL) was added to the culture medium 1 h prior to the addition of C225-PEG-PG-Dox. After an incubation period of 15 min at 37°C, cells were washed three times with PBS, fixed in 95% ethanol, and then treated with 1 μM TO-PRO-3 iodide for nuclei staining. Cells were washed twice again with PBS and the cover slips were mounted for microscopic examination using DMR microscope (Leica Microsystems Inc., Bannockburn, IL, USA). The microscope was equipped with 75W Xenon lamp, 640 nm/680 nm and 480 nm/535 nm filters (Chroma Technology Corp., Brattleboro, VT, USA), Hamamatsu B/W Chilled CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan), and Image-Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD, USA). In the images, TO-PRO-3 and Dox were pseudocolored red and green, respectively.

Cytotoxicity

One hundred microliters of growth medium suspending 2000 cells per well was plated out in 96-well plates and incubated for 2 days to allow the cells to attach. Cells were exposed to various concentrations of Dox or C225-PEG-PG-Dox for 6 h at 37°C, washed twice with fresh culture medium, and then incubated for an additional 72 h. Twenty microliters of MTT solution from a Promega Cell Proliferation Assay kit (Madison, WI, USA) were added to the wells. The microplates were then incubated for 1 h at 37°C. Absorbance was measured at 490 nm using a microplate reader (Molecular Devices Corp, Sunnyvale, CA, USA). The data reported represent the means of triplicate measurement; the standard errors of the mean were less than 15%.

RESULTS

Synthesis and Characterization

The synthetic scheme is outlined in Fig. 1. Block copolymer VS-PEG-PG containing functionalized VS terminal group was obtained by directly reacting heterofunctional polyethylene glycol, VS-PEG-NHS, with an amino group at one end of the PG polymer chains. To simplify the purification scheme, an excess of PEG was used to ensure complete conversion of PG to PEG-PG. Unreacted PEG molecules were efficiently removed by an acidification/polymer precipitation process. The structure of the conjugate was confirmed by ^{1}H NMR spectrum, which showed approximately 1:1 molar ratio of PEG to PG in PEG-PG conjugate. It is unlikely that the product was a physical mixture of PEG and PG because water-soluble PEG could not precipitate out from the aqueous solution when pH value was adjusted to 3. The nega-

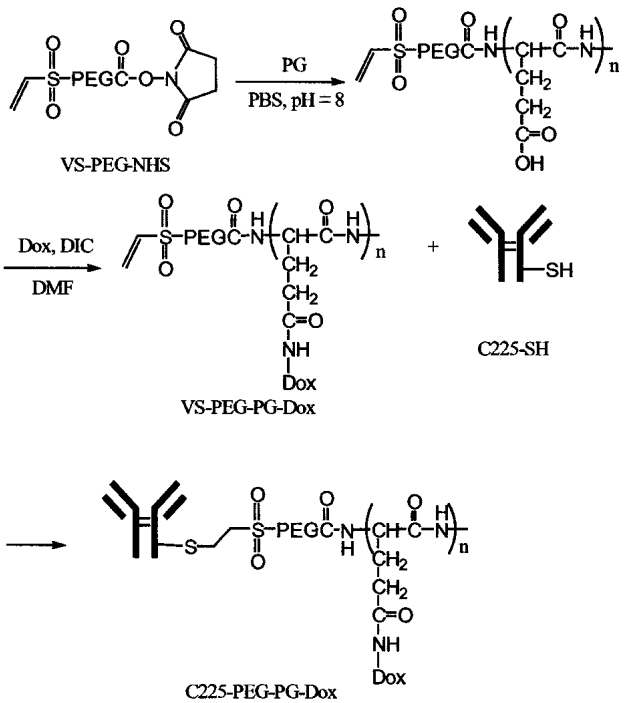

Fig. 1. Synthesis of polymeric immunoconjugate C225-PEG-PG-Dox from block copolymer VS-PEG-PG-Dox and C225.

tivity of ninhydrin test further suggests complete conversion of PG to PEG-PG.

Dox was conjugated to VS-PEG-PG via a carbodiimide-mediated coupling reaction (Fig. 1). Yokoyama *et al.* (14) conjugated Dox to PEG-poly(L-aspartic acid) in aqueous solution in the presence of triethylamine using a water-soluble carbodiimide as the coupling agent. Because of concern over possible hydrolysis of vinylsulfone in basic aqueous solution, anhydrous dimethylformamide was used as a solvent in our synthesis. Non-covalently associated Dox was removed by solvent extraction, followed by dialysis and polymer precipitation. No free Dox was detectable by silica gel TLC (volume ratio of n-butanol:acetic acid:water; 4:1:1) or by GPC. The amount of Dox in the polymer was 15% (w/w) as measured by UV at 480 nm. Each polymer chain contained approximately 11 Dox molecules.

To conjugate C225 to VS-PEG-PG-Dox, the mAb was pretreated with SATA/hydroxylamine to introduce sulfhydryl groups. Mixing C225-SH with VS-PEG-PG-Dox afforded the target compound, C225-PEG-PG-Dox (Fig. 1). A nickel chelate column was first used to remove unreacted PEG-PG-Dox. Free C225 was then readily separated from the immunoconjugate by anion-exchange chromatography (Fig. 2). Fractions 3-5 with a retention time of 4.4 min in the elution profile corresponded to free C225 and fractions 14-21 with a retention time of 7.6 min corresponded to C225-PEG-PG-Dox (Fig. 2). Fractions containing C225-PEG-PG-Dox were pooled, concentrated by ultracentrifugation, and stored at 4°C for future use. Figure 3 compares the GPC chromatogram of purified C225-PEG-PG-Dox with that of non-C225 modified PEG-PG-Dox and C225. While PEG-PG-Dox had a broad molecular weight distribution with a peak appearing at 15.81 min and a peak at 23.81 min, the chromatogram of C225 PEG-PG revealed only one narrowly distributed peak at 15.68 min. No free C225 was detected in the elution profile of C225-PEG-PG (Fig. 3).

We could not estimate the concentration of C225 in C225-PEG-PG-Dox because the conjugate precipitated when protein assay reagent was added. Therefore, an immunoconjugate C225-PEG-PG-BODIPY containing BODIPY dye molecules was synthesized and purified using the same procedures. The molar ratio of C225 to PEG-PG-BODIPY polymer in C225-PEG-PG-BODIPY was estimated to be 1.0 based on protein assay of C225 (measured at 650 nm) and quantification of BODIPY (measured at 503 nm). Assuming that the molar ratio between C225 and the polymer in C225-PEG-PG-Dox was also one, the yield of C225 was estimated to be 8.7%.

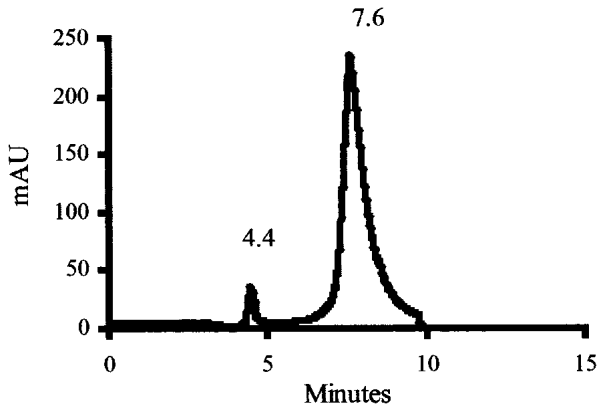

Fig. 2. Purification of C225-PEG-PG-Dox by FPLC using a Resource Q anion-exchange chromatography. The column was eluted with 20 mM Tris buffer (pH 7.5) and a linear gradient of 0–100% 20 mM Tris buffer containing 1.0 N NaCl at a flow rate of 3 ml/min. The peaks at 4.4 min and 7.6 min corresponded to free C225 and C225-PEG-PG-Dox, respectively.

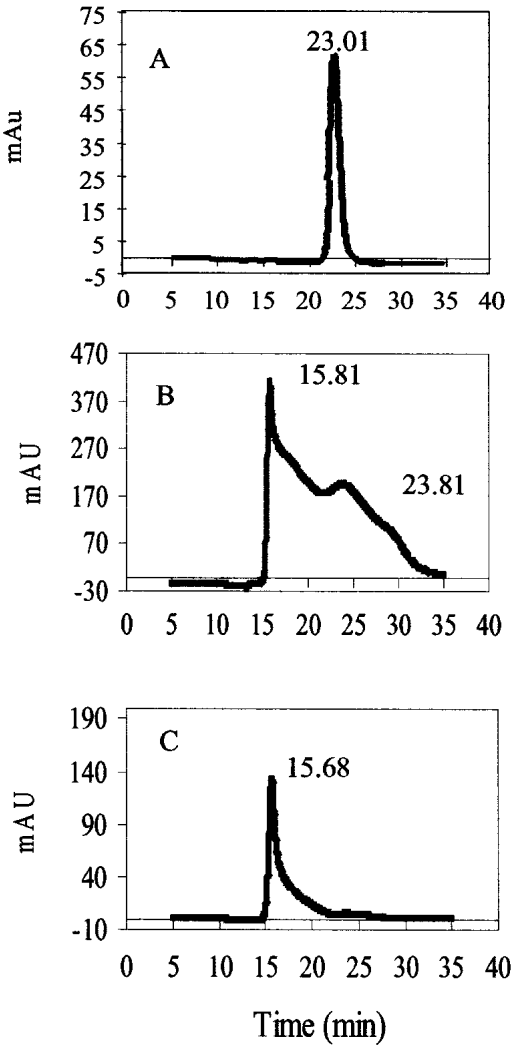

Fig. 3. Gel permeation chromatography of C225 (A), PEG-PG-Dox (B), and purified C225-PEG-PG-Dox conjugate (C) using a Superdex 200 column (1.0 × 30 cm). The compounds were monitored by measuring absorbance at 254 nm. Samples were eluted with 0.1 M phosphate-buffered saline (pH 7.4) containing 0.1% LiBr at a flow rate of 0.5 ml/min through a Superdex 200 column.

Light scattering analysis of the aqueous solutions of PEG-PG-Dox and C225-PEG-PG-Dox showed the presence of nano-sized particles at pH 7.4. The volume-average diameter of PEG-PG-Dox decreased from 207 nm to 16 nm upon conjugation with C225 (data not shown). On the other hand, no particles were detected when PEG-PG-Dox was dissolved in DMF or when the pH of the aqueous solution of PEG-PG-Dox was adjusted to 11.

Binding of C225-PEG-PG-Dox Immunoconjugate to EGFR

Expression of EGFR in A431 cells was confirmed by the strong signal in western blot analysis (Lane 5, Fig. 4). The

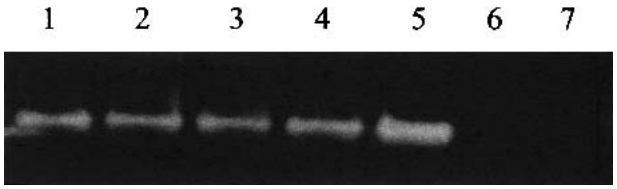

Fig. 4. Immunoprecipitation of EGF receptor from A431 cells by C225 and its polymer conjugate. Western blot analysis with anti-EGFR antibody revealed the binding of C225-PEG-PG-Dox molecules to EGF receptor in A431 cells. Lanes 1–3: 5, 2.5, and 1.0 μL of C225-PEG-PG-Dox (2.3 mg C225/ml), respectively. Lane 4: 10 μL of C225 (2.4 mg/mL). Lane 5: 5 μL of cell lysate without immunoprecipitation. Lane 6: 50 μL of PEG-PG-Dox (2.3 mg/mL). Lane 7: phosphate-buffered saline as negative control.

binding activity of C225-PEG-PG-Dox to EGFR on the surface of A431 cells was comparable to that of C225 (Lanes 1–4, Fig. 4). PEG-PG-Dox without covalently associated C225 did not have any EGFR signal (Lane 6, Fig. 4).

Internalization of C225-PEG-PG-Dox by A431 Cells

Figure 5 shows A431 cells visualized after 5-min to 60-min incubation with C225-PEG-PG-Dox (Fig. 5A-5D) or with PEG-PG-Dox (Fig. 5E–H). C225-PEG-PG-Dox, but not control conjugate (i.e., without C225), bound to A431 cells. Furthermore, C225-PEG-PG-Dox co-localized with cell nucleus, whereas PEG-PG-Dox did not (Fig. 5D vs. Fig. 5H).

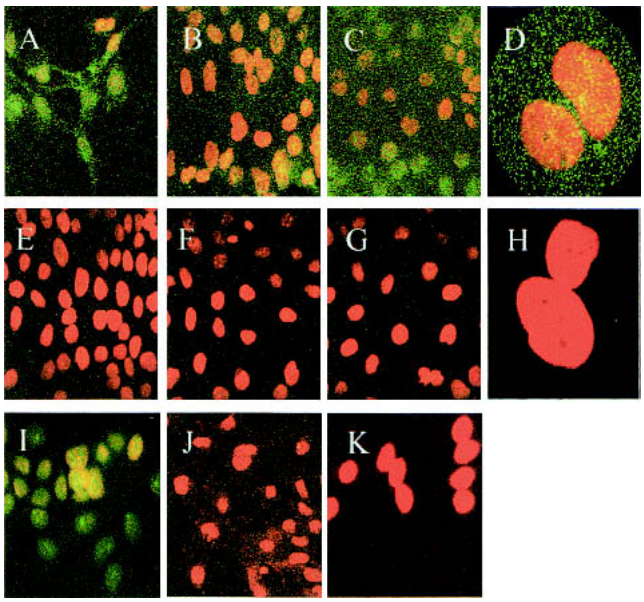

Fig. 5. Confocal fluorescent microscopy showing selective binding of C225-PEG-PG-Dox (4.8 μg eq. Dox/mL) to epidermal growth factor receptor-positive A431 cells (A–D). PEG-PG-Dox (4.8 μg eq. Dox/mL) was used as a non-C225-modified polymer control (E–H). Cells were incubated with polymeric conjugates at 37°C for 5 min (A, E), 15 min (B, D, F, H), and 60 min (C, G). Also shown are A431 cells after 24 h incubation with PEG-PG-Dox (I); A431 cells after 30 min incubation with free Dox at a dose of 5.8 μg/mL (J); and EGFR-negative NIH3T3 (clone 4) cells after 60 min incubation with C225-PEG-PG-Dox (k). Cells were fixed in 95% ethanol and treated with 1 μM TO-PRO-3 (red) for nuclei staining. Green: fluorescence of doxorubicin. Original magnification of all images (except D and H): ×40 objective; ×60 (D, H).

The process of cellular uptake of immunoconjugate into A431 cells was very rapid, with internalization of the conjugate observed as early as 5 min after drug exposure. In comparison, nonspecific uptake of PEG-PG-Dox through adsorptive endocytosis could be detected only after an extended incubation time (24 h; Fig. 5I). When A431 cells were incubated with free Dox, no detectable fluorescence was observed for a 30-min exposure (Fig. 5J), and weak fluorescence signal was detectable only after a 60-min incubation period (data not shown). Incubation of C225-PEG-PG-Dox with a cell line NIH3T3 (clone 4) that does not express EGFR also failed to show detectable Dox fluorescent signal (Fig. 5K). Figure 6 shows the uptake of C225-PEG-PG-Dox in A431 cells with or without the presence C225. Pretreatment of C225 completely blocked the binding of C225-PEG-PG-Dox to A431 cells (Fig. 6).

A431 Cells Response to Immunoconjugate

After a 6-h exposure period to A431 cells, free Dox was incapable of achieving 50% cell death even at the highest concentration (10 μM) used. In contrast, about 80% cells were dead at an equivalent Dox concentration of 10 μM when cells were exposed to C225-PEG-PG-Dox (Fig. 7). The IC_{50} of C225-PEG-PG-Dox after a 6-h exposure was estimated to be 1.7 μM.

DISCUSSION

The results of a recent theoretical and experimental study indicate that extended polymer chains such as PEG have a dramatic effect on molecular recognition (15). Deguchi *et al.* (16) found that a PEG linker, but not a short linker with 14 atoms, releases a steric hindrance between epider-

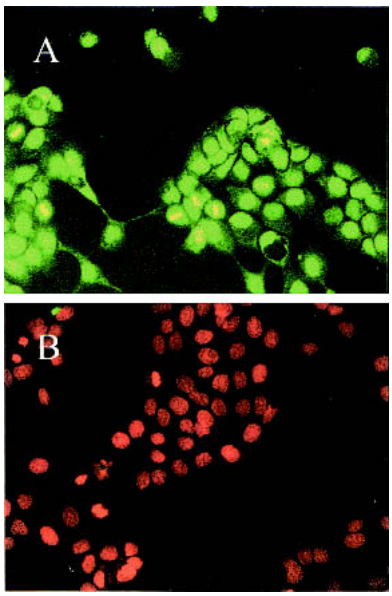

Fig. 6. Fluorescence microscopy showing the blockage of the binding of C225-PEG-PG-Dox to A431 cells by C225. Cells were either incubated with C225-PEG-PG-Dox (4.8 eq. μg Dox/mL) for 15 min (A) or with C225 (240 μg/mL) for 1 h followed by C225-PEG-PG-Dox for 15 min (B). Cells were fixed in 95% ethanol and treated with 1 μM TO-PRO-3 (red) for nuclei staining. Green: fluorescence of doxorubicin. Original magnification ×40.

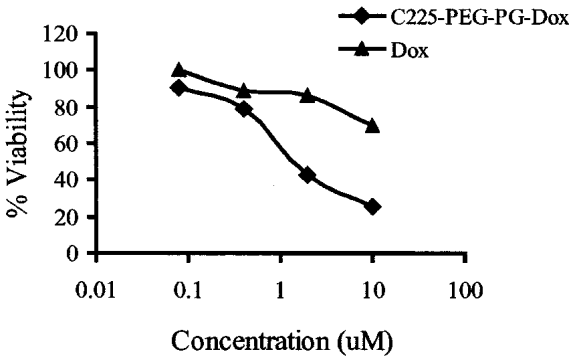

Fig. 7. Cytotoxicity of C225-PEG-PG-Dox against A431 cells by MTT assay. Cells were incubated for 6 h, washed, and incubated for additional 72 h. The x-axis represents the equivalent Dox concentrations in μM. Data are presented as means of triplicates. The standard errors are smaller than the symbols shown.

mal growth factor (EGF) and a mAb against transferrin receptor, allowing for retention of the bifunctionality of a EGF-mAb conjugate with binding to both EGF and transferrin receptors. These results underline the importance of a flexible chain between a homing ligand and the cargo it carries in targeted drug delivery. In this study, we have synthesized a polymeric immunoconjugate where the targeting device is site-specifically attached to the terminus of a block copolymer PEG-PG in such a way that PEG serves as a flexible spacer. PG is a biodegradable polymer that contains multiple side-chain functional groups for drug attachment. Conjugation between mAb C225 and the block copolymer PEG-PG was achieved through the selective Michael addition reaction between sulfhydryl group on mAb C225 and vinyl sulfone group introduced to the terminus of PEG-PG copolymer (Fig. 1). To facilitate interpretation of the *in vitro* binding data, it is important that the resulting immunoconjugate is free of unreacted precursor molecules. We used a purification scheme combining a nickel-affinity column to remove unreacted PEG-PG Dox polymer and an anion-exchange chromatography column to remove free C225. It was anticipated that the nickel chelate column would bind the immunoconjugate through Fc region of the associated antibody (17), allowing removal of unreacted block PEG-PG-Dox conjugate. This procedure was proven to be extremely inefficient, as substantial amount of C225-conjugated product was eluted together with PEG-PG-Dox in the washing step, suggesting that the chelating affinity of the mAb to nickel column was compromised once PG polymer chains were coupled to the antibody. Nevertheless, we were able to obtain pure C225-PEG-PG-Dox conjugate devoid of unreacted C225 and PEG-PG-Dox (Figs. 2 and 3). The immunoconjugate was shown to be free of noncovalently associated Dox by TLC and GPC analyses.

The GPC profiles of both VS-PEG-PG-Dox and C225-PEG-PG-Dox showed a peak with retention times at around the dead volume of the column (Fig. 3B), suggesting the formation of polymeric aggregates in aqueous solution. In light of a previous report that PEG-poly(L-aspartic acid) block copolymer formed micelles when Dox was coupled to poly(L-aspartic acid) (18), it is speculated that similar nanoparticles may be formed when PEG-PG-Dox was exposed to aqueous solution as a result of hydrophobic interaction of Dox. This notion is supported by the detection of particles with a volume-average diameter of 207 nm in the aqueous solution of PEG-PG-Dox. The particle size decreased to 16 nm upon conjugation with C225, possibly resulting from an increased contribution of hydrophilic segment. Furthermore, no particle was detected in DMF solution of PEG-PG-Dox. Increasing the pH level to more than 11 caused complete dissolution of the particles. These observations indicate that destruction of the hydrophobic core in PEG-PG-Dox copolymer destabilized and prevented the formation of nanoparticles. Thus, it is conceivable that C225-PEG-PG-Dox may have adapted a structural feature in which C225 molecules reside on the surface of polymeric nanoparticles (Fig. 8).

The selective binding of the polymeric immunoconjugate to EGFR was established by immunoprecipitation and western blot analysis (Fig. 4). The fact that C225-PEG-PG-Dox, but not PEG-PG-Dox, was taken up by A431 cells suggest that the internalization of C225-PEG-PG-Dox in A431 cells was mediated through EGFR (Fig. 5A-5H). The role of EGFR in this process is further confirmed by the facts that C225-PEG-PG-Dox could not bind to EGFR-negative NIH3T3 (Clone 4) cells (Fig. 5K), and that an anti-EGFR antibody, C225, completed blocked the uptake of C225-PEG-PG-Dox in A431 cells (Fig. 6). Interestingly, the fluorescence signal was observed in the nuclei of A431 cells as early as 5 min after incubation with C225-PEG-PG-Dox (Fig. 5A). This may be better visualized at higher magnification, in which yellow colored spots arisen from fused green color of Dox and the red color of cell nuclei were clearly visualized (Fig. 5D). The reason for the rapid uptake of polymeric immunoconjugate in the nuclei of A431 cells is not clear at present. Nevertheless, the selective cellular uptake of C225-PEG-PG-Dox and the ability to delivery Dox rapidly into the nuclei are likely responsible for the enhanced cytotoxic effect of C225-PEG-PG-Dox against A431 cells as compared to free Dox during a 6-h exposure (Fig. 7).

In summary, we have developed a synthetic approach toward univalent immunoconjugates in which the mAb is attached to one end of polymer chains through a PEG linker. Using mAb C225 as a model targeting device, we demonstrated rapid binding and internalization of the immunoconjugates to A431 cells overexpressing EGFR. Receptor-mediated drug targeting may facilitate selective delivery of polymeric drug to cancer cells, resulting in improved therapeutic window. Further studies are needed to confirm that the enhanced cellular uptake of targeted polymeric delivery system will lead to increased antitumor activity and improved therapeutic index in appropriate tumor models.

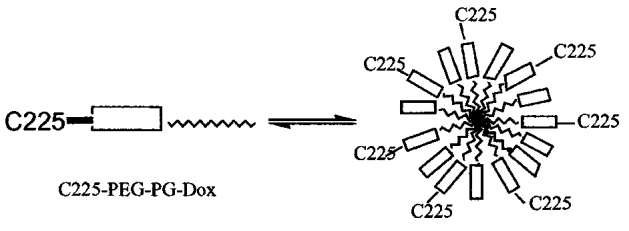

Fig. 8. Hypothetical structure of a targeted micellar delivery system. The targeting units C225 are attached to the surface of nanoggregates formed via hydrophobic interaction among Dox drug molecules and stabilized by hydrophilic PEG chains.

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