Block Copolymer Micelles Conjugated with anti-EGFR Antibody for Targeted Delivery of Anticancer Drug

TAIHO NOH,1 YEON HEE KOOK,2 CHIYOUNG PARK,3 HYEWON YOUN,2 HANA KIM,1 EUN TAX OH,2 EUN KYUNG CHOI,3 HEON JOO PARK,2 CHULHEE KIM1

1Department of Polymer Science and Engineering, Inha University, Incheon 402-751, Korea
2Department of Microbiology, Center for Advanced Medical Education by BK21 Project, College of Medicine, Inha University, Incheon, 400-712, Korea
3Department of Radiation Oncology, College of Medicine, University of Ulsan, Seoul, 138-736, Korea

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ABSTRACT: Antiepidermal growth factor receptor antibody (anti-EGFR antibody) was conjugated with the block copolymer micelle based on poly(ethylene glycol) (PEG) and poly(ε-caprolactone) (PCL) for active targeting to EGFR overexpressing cancer cells. Doxorubicin (DOX) was encapsulated in the core of the block copolymer (MePEG-b-PCL) micelle (DOX-micelle). The mean diameters of the DOX-micelle and the anti-EGFR-PEG-b-PCL copolymer micelles loaded with DOX (DOX-anti-EGFR-micelle) were about 25 and 31 nm, respectively. The RKO human colorectal cancer cells expressing moderate degree of EGFR were incubated with free DOX, DOX-micelle, or DOX-anti-EGFR-micelle to study the distribution of DOX in the cells. When cells were incubated with free DOX, moderate degree of DOX fluorescence was observed in the nuclei. In the cells treated with DOX-micelle, the DOX fluorescence intensity in the cytoplasm was much greater than that in the nuclei. On the other hand, the nuclei of the cells treated with DOX-anti-EGFR-micelle exhibited DOX fluorescence intensity similar to that in the cytoplasm. The cytotoxicity of DOX-anti-EGFR-micelle to induce apoptosis in RKO cells was significantly greater than that of free DOX or DOX-micelle. These results demonstrated that the presence of anti-EGFR antibody on the DOX-micelle surface (DOX-anti-EGFR-micelle) increased the internalization of the DOX-micelle and nuclear accumulation of DOX, and enhanced the DOX-induced cell death. ©2008 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 46: 7321–7331, 2008

Keywords: anti-EGFR; block copolymers; doxorubicin; drug delivery systems; micelles

INTRODUCTION

In recent years, there has been great interest in the use of a variety of nanoscale colloidal particles such as liposomes, polymeric micelles, silica nanoparticles, and gold nanoparticles to improve the delivery of cytotoxic drug to cancer cells.1–8 Among these nanocarriers, polymeric micelles have a potential to bring several advantages to therapeutic systems because of the storage capability and chemically tailorable structure. For the use of polymeric micelle as a drug delivery carrier, there are requisite requirements such as
biocompatibility, high drug loading capacity, long circulation time, and controlled release profiles. For this purpose, water-soluble polymers such as poly(ethylene glycol) (PEG), poly(2-ethyl-2-oxazoline), and poly(ε-caprolactone) (PCL) have also been utilized as a hydrophilic block for drug encapsulation. In addition, biocompatible and biodegradable polyesters such poly(D,L-lactic-co-glycolic acid) and poly(ε-caprolactone) (PCL) have also been utilized as a hydrophobic block for drug encapsulation. One of the challenging topics has been the design of drug carriers with active targeting capability that would result in higher intracellular drug concentrations in a selective manner. In our studies, we have focused on the development of drug-loaded polymeric micelles conjugated with a targeting ligand as a target-selective delivery vehicle. Specifically, first, we selected PEG–PCL block copolymer micelle composed of PEG as a hydrophilic block and PCL as a hydrophobic block for drug encapsulation. Second, anti-EGFR antibody was conjugated with the PEG–PCL block copolymer micelle for active targeting to cancer cells overexpressing EGFR.

EGFR (ErbB-1; HER1 in humans) is a cell-surface receptor for the members of the epidermal growth factor family (EGF-family), and is overexpressed on the surface of a number of different human cancer cells including colorectal, breast, and lung cancer cells. In recent years, efforts have been made to develop drug delivery systems exploiting the overexpression of EGFR on the cell surface. For example, the possibility of using EGF-conjugated polymer micelle as a vehicle for targeting hydrophobic drugs to EGFR overexpressing cancers has been investigated. Significant progress has also been made in recent years developing new cancer treatment strategies using various EGFR inhibitors and some of them are in clinical trials. In this study, we have investigated the feasibility of using anti-EGFR antibody-conjugated polymer micelle as a vehicle for the delivery of anticancer drugs DOX to EGFR overexpressing cancer cells. It is known that the ligand–receptor complexes are internalized via receptor-mediated endocytosis and that the endocytosis of receptors to lysosomes leads to degradation of the receptors. Interestingly, on the other hand, there has been emerging evidence that ligand–EGFR complexes are translocated into nuclei after EGFR-mediated internalization. Although it is unclear how the translocation of the EGFR–ligand complexes into nuclei takes place, it may be expected that anti-EGFR antibody-micelle may also be translocated into nuclei following endocytosis, and thus the anti-EGFR antibody-micelle may be used to deliver anticancer drugs such as DOX to nuclei of EGFR overexpressing cancer cells.

The mechanism of the anticancer action of DOX is complex, although DNA replication has been known to be the major target of the drug. However, wider clinical use of DOX is limited because of its low water solubility and acute toxicity to normal tissue. To reduce the acute toxicity and improve the therapeutic efficacy of DOX, various delivery vehicles for DOX have been proposed.

This report describes the development of DOX-loaded PEG–PCL block copolymer micelle conjugated with anti-EGFR antibody (DOX-anti-EGFR-micelle). We elucidated the targeting capability of DOX-anti-EGFR-micelle and its cytotoxicity against RKO human colorectal cancer cells expressing in vitro.

EXPERIMENTAL

Materials and Equipments

Monomethoxy-poly(ethylene oxide) (MePEG, \(M_n = 2000\), Sigma-Aldrich) and \(\varepsilon\)-carboxy-\(\omega\)-hydroxy-PEG (COOH-PEG, \(M_n = 2000\), \(M_w/M_n = 1.16\), Polymer Sources) were purified by azeotropic distillation with toluene. \(\varepsilon\)-Caprolactone and triethylamine (Sigma-Aldrich, Inc., St. Louis, MO) were dried over calcium hydride and distilled under reduced pressure. Mouse monoclonal anti-EGFR antibody (mouse IgG1 isotype, MW 170 kDa, Polymer Sources) were distilled before use. The cellulose membrane (molecular weight cutoff \(= 12,000\), Sigma-Aldrich), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), stannous octoate [Sn(Oct)\(_2\)], and phosphate buffered saline (PBS) (pH = 7.4, 0.01 M) were used as received (Sigma-Aldrich). Doxorubicin (DOX) was purchased from Sigma-Aldrich and hydrophobic DOX was prepared following the literature procedure. Tetrahydrofuran (THF) and toluene were distilled before use. The cellulose membrane (molecular weight cutoff \(= 12,000\), Sigma-Aldrich, Inc.) was used for dialysis. The molecular weights and block compositions of the block copolymers were determined with the \(^1\)H-NMR spectra which was recorded on a Varian UNITY INOVA 400 at 400 MHz. The molecular weight distributions were determined using a GPC equipped with a Waters Associates 410 RI detector, 510 HPLC.
pump and μ-Styrigel columns with pore sizes of $10^2$, $500$, $10^3$, and $10^4$ Å. The eluent was THF and the molecular weights were calibrated with poly-styrene standards. The UV-visible spectra were recorded on HP-8453A diode array spectrophotometer.

Cell Lines and Culture Conditions

The RKO human colorectal cancer cells were used. The RKO cells are known to express moderate amount of EGFR. The cells were cultured in 25 cm$^2$ plastic tissue culture flasks with Dulbecco’s modified Eagle’s medium (DMEM, Hycclone Laboratories Inc., Logan, UT) supplemented with 10% fetal bovine serum (FBS, Hycclone Laboratories Inc.) and 1% penicillin/streptomycin (Hyclone Laboratories Inc.) in a humidified 5% CO$_2$/95% air incubator at 37 °C.

Synthesis of MePEG-b-PCL

Sn(Oct)$_2$ (12.51 µg, 30.9 µmol) was added into the toluene solution (100 mL) of MePEG (10.0 g) and ε-caprolactone (13.13 g, 115 mmol) at room temperature under nitrogen. After stirring for 72 h at 130 °C, the solvent was removed under reduced pressure. The product was obtained by precipitation from THF into n-hexane (yield 19.5 g, 87%).

$^1$H-NMR (400 MHz, CDCl$_3$) δ 1.4 (b, 50H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)), 1.6 (b, 95H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)), 2.25 (b, 50H, \(-\text{CO-CH}_2-\text{CH}_2-\text{O}--\)), 3.6 (m, 200H, \(-\text{CH}_2-\text{CH}_2-\text{O}--\)), 4.1 (b, 46H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)).

Synthesis of COOH-PEG-b-PCL

A toluene solution (30 mL) of ω-carboxy-ω-hydroxy-PEG (0.10 g) and ε-caprolactone (0.13 g, 11.2 mmol) was stirred at room temperature under nitrogen. After adding Sn(Oct)$_2$ (4.17 µg, 10 µmol), the reaction mixture was stirred for 72 h at 130 °C. The product was purified by a repeated precipitation from THF into n-hexane (yield 0.21 g, 87%).

$^1$H-NMR (400 MHz, CDCl$_3$) δ 1.4 (b, 1H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)), 1.6 (b, 2H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)), 2.25 (b, 1H, \(-\text{CO-CH}_2-\text{CH}_2-\text{O}--\)), 3.6 (m, 4H, \(-\text{CH}_2-\text{CH}_2-\text{O}--\)), 4.1 (b, 1H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)).

Synthesis of NHS-PEG-b-PCL

A THF solution (1 mL) of NHS (12.5 mg, 0.11 mmol) and TEA (0.015 mL, 0.11 mmol) was added to a THF solution (5 mL) of COOH-PEG-b-PCL (0.10 g, and DCC (0.022 g, 0.11 mmol). After stirring for 12 h at 45 °C, the precipitated urea was filtered off. The purified product was obtained by precipitation from THF into diethyl ether (yield 0.08 g, 80%).

$^1$H-NMR (400 MHz, CDCl$_3$) δ 1.4 (b, 50H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)), 1.6 (b, 95H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)), 2.25 (b, 50H, \(-\text{CO-CH}_2-\text{CH}_2-\text{O}--\)), 3.6 (m, 200H, \(-\text{CH}_2-\text{CH}_2-\text{O}--\)), 4.1 (b, 46H, \(-\text{CO-CH}_2-\text{CH}_2-\text{CH}_2-\text{O}--\)).

Synthesis of Anti-EGFR-Conjugated PEG-b-PCL (anti-EGFR-PEG-b-PCL)

Anti-EGFR antibody (12.4 mg, 72.9 nmol) was added to a PBS solution (0.4 mL, pH = 7.4) of NHS-PEG-b-PCL (1.7 mg) and stirred for 48 h at room temperature. The reaction mixture was then dialyzed against PBS solution for 48 h. Anti-EGFR-PEG-b-PCL was characterized by using BCA (bicinchoninic acid, Sigma-Aldrich) protein assay and dot blot assay.

Preparation of DOX-Micelle

A THF solution (1.0 mL) of DOX (4.0 mg) and MePEG-b-PCL (40.0 mg) was stirred for 1 h, and the mixture was then added dropwise to water (35 mL) with stirring. The mixture was then opened to air overnight, allowing slow evaporation of THF and formation of micelles. The residual THF was completely removed under reduced pressure. The solution was dialyzed against water for 72 h (molecular weight cutoff: 12,000 Da) and then lyophilized.

Preparation of DOX-Anti-EGFR-Micelle

A THF solution (1.0 mL) of DOX (4.0 mg) and MePEG-b-PCL (40.0 mg) was stirred for 1 h at room temperature, and then THF was removed under nitrogen. The mixture of DOX and MePEG-b-PCL was hydrated with PBS solution (pH = 7.4, 30 mL), and then anti-EGFR-PEG-b-PCL (10.1 mg) was added to the preformed DOX-micelle solution. After stirring at 60 °C for 1 h and additional 12 h at room temperature, the mixture was dialyzed against water.
Determination of DOX-Loading Content

The micelle loaded with DOX was dissolved in chloroform/DMSO (1:1, v/v) solution, and the DOX-loading content was determined by measuring the UV-vis absorbance at 485 nm.

Dynamic Light Scattering

Dynamic light scattering measurements were performed at 25 °C with a Brookhaven BI-200SM goniometer and BI-9000AT digital autocorrelator. The scattered light of a He-Ne laser (Research Electrooptics, 35 mW) operated at 632.8 nm was measured at an angle of 90° and collected on an autocorrelator. The sample solutions were purified by passing through a 0.20-μm Millipore filter. The hydrodynamic diameters (d) of micelles were calculated using the Stokes-Einstein equation $d = k_B T/3πηD$, where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $η$ is the solvent viscosity, and $D$ is the diffusion coefficient. The polydispersity factor of micelles, represented as $μ_2/μ_1^2$, where $μ_2$ is the second cumulant of the decay function and $μ_1$ is the average characteristic line width, was calculated from the cumulant method. CONTIN algorithms were used in the Laplace inversion of the autocorrelation function to obtain micelle size distribution.

Transmission Electron Microscopy (TEM)

TEM was performed with a Philips CM 200, operated at an acceleration voltage of 120 kV. For the preparation of dispersed samples in water, a drop of sample solution (1 g/L) was placed onto a 200-mesh copper grid coated with carbon. About 2 min after deposition, the grid was tapped with filter paper to remove surface water, followed by air drying. Negative staining was performed by using a droplet of a 5 wt % uranyl acetate solution. The samples were air dried before measurement.

Dot Blot Assay

Nitrocellulose membrane was labeled for protein elution fractions and placed on the top of wet Whatman paper for keeping moist. Samples (2 μL each) were loaded from each micelle fraction on the membrane, and allowed to dry. The membrane was incubated in blocking solution of 5% skim milk for 1 h. After washing twice with Tris-buffered saline supplemented with 0.05% Tween-20 (TBST), the membrane was immersed into the secondary antibody (antimouse immunoglobulin G; Amersham Pharmacia Biotech) solution, followed by a droplet of a 5 wt % uranyl acetate solution. The samples were air dried before measurement.

Fluorescence Microscopy Study for DOX Uptake

RKO cells were grown in eight-well chamber slides and treated with 10 μM of free DOX and DOX-micelle or DOX-anti-EGFR-micelle with equivalent amount of DOX, i.e., 10 μM, at 37 °C for 0–24 h. Cells were washed with PBS, fixed using 4% (v/v) paraformaldehyde (Sigma-Aldrich) at 4 °C for 30 min, and permeabilized with 0.25% (w/v) Triton X-100 (Sigma-Aldrich) at room temperature for 2 min. After washing several times with PBS, the nuclei were stained with DAPI solution containing ProLong Gold-antifade reagent (Invitrogen, Eugene, OR). The slides were covered with cover glasses, and the cells were examined with a fluorescence microscope (Nikon TE-2000E, Tokyo, Japan). To quantitate the DOX localized in the nuclei, DOX-derived fluorescence was captured and analyzed by ImageJ 1.41e image analysis software (NIH) by measuring the intensity of the fluorescence.
DOX-derived fluorescence in nucleus corresponding the area expressing DAPI-derived fluorescence.

RESULTS AND DISCUSSION

Preparation of DOX-Anti-EGFR-Micelle

As shown in Figure 1, the block copolymers, MePEG-b-PCL, and COOH-PEG-b-PCL, were prepared by ring-opening polymerization of ɛ-caprolactone using MePEG and COOH-PEG as macroinitiators, respectively. The number average molecular weight, $M_n$, of MePEG-b-PCL was estimated to be 4500 by comparing the peak integration of the methylene protons of the PCL block ($\text{CH}_2\text{CH}_2\text{O}, \delta = 3.6 \text{ ppm}$) to that of the PEG block ($\text{CH}_2\text{CH}_2\text{O}, \delta = 2.25 \text{ ppm}$) in the $^1\text{H}$-NMR spectrum. From the $^1\text{H}$-NMR analysis, $M_n$ of

<table>
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<th>Block Copolymers</th>
<th>Feed Ratio $^b$</th>
<th>$M_n$ $^c$</th>
<th>Composition Ratio $^d$</th>
<th>wt % of PCL $^e$</th>
<th>$M_w/M_n$ $^f$</th>
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<tr>
<td>MePEG-b-PCL</td>
<td>0.50</td>
<td>4500</td>
<td>0.46</td>
<td>56</td>
<td>1.15</td>
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<tr>
<td>COOH-PEG-b-PCL</td>
<td>0.50</td>
<td>4600</td>
<td>0.50</td>
<td>57</td>
<td>1.25</td>
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</tbody>
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$^a$ All the samples were prepared by using PEG with $M_n$ of 2000.
$^b$ Molar feed ratio of ɛ-caprolactone to the repeating unit of PEG.
$^c$ Estimated by $^1\text{H}$-NMR.
$^d$ Molar composition ratio of the repeating units of PCL to that of PEG by $^1\text{H}$-NMR analysis.
$^e$ Weight percentage of hydrophobic PCL block in the block copolymers.
$^f$ Estimated by GPC.
COOH-PEG-b-PCL was calculated to be 4600 as summarized in Table 1. The polydispersity, $M_w/M_n$, of the copolymers was in the range of 1.15–1.25 as determined by GPC analysis. The carboxyl end group of COOH-PEG-b-PCL was chemically activated by the reaction with NHS to yield NHS-PEG-b-PCL. Anti-EGFR antibody was conjugated to the block copolymer by allowing NHS-PEG-b-PCL to react with the anti-EGFR antibody in PBS to yield anti-EGFR-PEG-b-PCL.

The micelle formation of the block polymers in aqueous phase was confirmed by DLS and TEM analysis. As summarized in Table 2, the mean diameter of the micelle derived from MePEG-b-PCL-micelle (PEG-PCL-micelle) was 25 nm (polydispersity factor, $\mu_d/T^2 = 0.22$). PEG-PCL-micelle and DOX-micelle exhibited similar mean diameter (25 nm, $\mu_d/T^2 = 0.29$). The micelles visualized by TEM were spherical as shown in Figure 2, and the size of DOX-micelle was about 25 nm. DOX-anti-EGFR-micelle was prepared by adding anti-EGFR-PEG-b-PCL into a DOX-micelle solution. The mean diameter of DOX-anti-EGFR-micelle was 31 nm ($\mu_d/T^2 = 0.31$). As shown in Figure 2(c,d), the CONTIN analyses of the autocorrelation function for DOX-micelle and DOX-anti-EGFR-micelle respectively, showed monomodal distributions. The quantities of encapsulated DOX in DOX-micelle and DOX-anti-EGFR-micelle were 3.0 and 4.0 wt % of total weight, respectively.

The conjugation of anti-EGFR antibody with the block copolymer micelles was studied using the dot blot method. As shown in Figure 3, anti-EGFR antibody was detected only in DOX-anti-EGFR-micelles but not in the DOX-micelles or

<table>
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<th>Sample</th>
<th>Loading Content (wt %)</th>
<th>$d$ (nm)</th>
<th>$\mu_d/T^2$</th>
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<tr>
<td>PEG-PCL-micelle</td>
<td>–</td>
<td>25</td>
<td>0.22</td>
</tr>
<tr>
<td>DOX-micelle</td>
<td>3.0</td>
<td>25</td>
<td>0.29</td>
</tr>
<tr>
<td>DOX-anti-EGFR-micelle</td>
<td>4.0</td>
<td>31</td>
<td>0.31</td>
</tr>
</tbody>
</table>

*a* Mean diameters of micelles in distilled water at 25 °C.

*b* Polydispersity factor.

Figure 2. TEM images of (a) DOX-micelle and (b) DOX-anti-EGFR-micelle. Size distribution histograms of (c) DOX-micelle and (d) DOX-anti-EGFR-micelle at scattering angle of 90° (from CONTIN analysis of the autocorrelation function).
free DOX, indicating that anti-EGFR antibody was conjugated onto DOX-micelles. The number of anti-EGFR antibody conjugated to a micelle, quantified using the Micro BCA assay method (162 μg/mg), was 1.1 assuming that the aggregation number of the micelle is ~200.

**In vitro Release of DOX from Polymer Micelles**

Figure 4 shows the rate of release of DOX from DOX-micelle in phenol red-free DMEM. Approximately 10% of DOX was released from DOX-micelle in DMEM in 4 h of incubation, and no further release occurred thereafter.

**Cellular Uptake of DOX with Fluorescence Microscopy**

Figure 5(a) shows the DOX fluorescence distribution, nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI), and merging of the DOX fluorescence and DAPI staining in the RKO cells incubated with free DOX, DOX-micelle, and DOX-anti-EGFR-micelle for 1, 4, and 8 h. Note that the results of only 1–8 h uptake are shown since considerable fractions of cells died of apoptosis after 8 h treatment. Since DOX works as a topoisomerase inhibitor, nucleation of DOX is important for killing of the target cancer cells. In the cells incubated with free DOX for 1 h, weak DOX fluorescence could be observed in the nuclei as well as outside the nuclei. As the incubation time with free DOX was increased to 4 and 8 h, the intensity of DOX fluorescence in the nuclei slightly increased while that around the nuclei diminished. When the cells were incubated with DOX-micelle for 1 or 4 h, the nuclei exhibited little DOX fluorescence whereas rather strong DOX fluorescence intensity could be observed around the nuclei. Upon incubation with DOX-micelle for 8 h, the DOX fluorescence intensity in the nuclei slightly increased and that around the nuclei still remained strong. It appeared that DOX-micelle was trapped outside the nuclei, probably in the nuclear membrane, endosomes, or lysosomes, as reported by other investigators.23,46 In the cells incubated with DOX-anti-EGFR-micelle for 1 h, intense DOX fluorescence could be observed both inside and outside the nuclei. As the incubation time with DOX-anti-EGFR-micelle was increased to 4 and 8 h, the nuclear DOX fluorescence intensity further increased while that in the cytoplasm appeared diminished slightly. The relative intensity of DOX fluorescence in the nuclei of the cells treated with free DOX, DOX-micelle, and DOX-anti-EGFR-micelle for 1–8 h were quantitated and shown in Figure 5(b). The intensity of nuclear DOX fluorescence in the cells treated with free DOX and that of cells treated with DOX-micelle were similar, and it increased only slightly during the 8 h incubation. On the other hand, the nuclear DOX fluorescence intensity in the cells treated with DOX-anti-EGFR-micelle progressively increased during the 8 h incubation.
importantly, the DOX fluorescence intensity in the nuclei of the cells treated with DOX-anti-EGFR-micelle was about twofold greater ($p < 0.05$) than that in the cells treated with free DOX or DOX-micelle throughout the 8 h incubation period. It could be concluded that DOX was effectively delivered to nuclei when cells were treated with DOX-anti-EGFR-micelle. Pertinent to our conclusion, there are compelling experimental evidence indicating that EGFR and its ligands are

Figure 5. (a) Fluorescence microscopy images of RKO cells incubated with free DOX, DOX-micelle, and DOX-anti-EGFR-micelle for 1–8 h. The images show the DOX fluorescence (red), nuclear staining by DAPI (blue) and merged. (b) Relative intensity of DOX fluorescence in the nuclei of RKO cells incubated with free DOX, DOX-micelle, or DOX-anti-EGFR-micelle for 1–8 h. Means of more than 40 nuclei with 1 S.E. are shown.
accumulated in the nuclei of various human tissues and cancer cell lines.\textsuperscript{34,40} It is known that when ligands including EGF bind to cell surface EGFR, the ligand–receptor complexes are rapidly internalized through endocytosis to lysosomes and undergo proteolytic degradation. On the other hand, some of the ligand–EGFR complexes are translocated into nuclei possibly through the conventional nuclear importing system.\textsuperscript{34} Note that, in this study, we used an anti-EGFR antibody that binds to non-EGF-binding site, unlike other anti-EGFR antibody which is known to block ligand-binding sites and undergo degradation without internalization.\textsuperscript{35}

\textit{In vitro} Cytotoxicity Assay

Figure 6 shows the flow cytometrically determined apoptosis in RKO cells treated with free DOX, DOX-micelle, or DOX-anti-EGFR-micelle. About 30 and 50\% of cells died of apoptosis when cells were treated with free DOX or DOX-micelles for 24 h, respectively. The DOX-anti-EGFR-micelle was most effective among the three drug preparations causing apoptosis in about 70\% of the cells in 24 h. The differences in \% apoptosis among the three different groups were statistically significant at 16 h. However, after 24 h incubation, the \% apoptosis caused by DOX-anti-EGFR-micelle was statistically greater than that caused by free DOX, but not that caused by DOX-micelle. Note that encouraging results have been observed in recent clinical trials to test the effectiveness of anti-EGFR antibodies alone or in combination with other anticancer agents for the treatment of lung or breast cancer.\textsuperscript{47–50} We may attribute the significantly greater apoptosis by DOX-anti-EGFR-micelle relative to that by DOX alone or DOX-micelle to the increased nuclear accumulation of DOX and thus increased cell death by DOX.

In using cell surface molecules as a target for delivery of anticancer agents to the cells, important factors are specificity, stability, and availability of the target. Other factors that are also important for successful delivery of drugs to targets inside the cells are the rate of internalization and the extent and rate of the drugs to escape from the endosomes and/or lysosomes. As shown in Figure 5(a), considerable amount of DOX remained outside of nuclei when cells were treated with DOX-micelle or DOX-anti-EGFR-micelle possibly because the vehicles were trapped in the endosomes or lysosomes in the cytoplasm. Further work to improve the anticancer effect of DOX-anti-EGFR-micelle \textit{in vitro} as well as \textit{in vivo} using RKO xenografts grown in nude mice are in progress in our laboratory.

CONCLUSIONS

A cancer targeting ligand, anti-EGFR antibody, was introduced to the PEG-PCL block copolymer micelle which contained a hydrophobic anticancer drug, DOX, and the uptake of DOX and the drug-induced apoptosis were investigated using EGFR-positive RKO cells. The fluorescence microscopy study indicated that the cellular and nuclei accumulation of DOX was greater in the cells treated with DOX-anti-EGFR-micelles as compared with that in the cells treated with free DOX or DOX-micelles. Likewise, treating the cells with DOX-anti-EGFR-micelle was more effective to cause apoptosis than treating the cells with free DOX or DOX-micelles. Our results are in agreement with the reports by others that ligand–EGFR complexes are internalized and translocated into cell nuclei.\textsuperscript{34,39,40} It is concluded that the anti-EGFR antibody conjugated with copolymer micelles is an efficient vehicle for the delivery of cytotoxic drugs to cancer cells overexpressing EGFR.

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REFERENCES AND NOTES


