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Apoptotic Epidermal Growth Factor (EGF)-Conjugated Block Copolymer Micelles as a Nanotechnology Platform for Targeted Combination Therapy

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Abstract: The overexpression of epidermal growth factor receptor (EGFR) in human epithelial cancers has been associated with aggressive disease, poor patient prognosis, and a high incidence of metastases. In the present study, block copolymer micelles are conjugated with epidermal growth factor (EGF), which acts as both a targeting ligand for the drug carrier and an apoptotic factor against EGFR-overexpressing cancers. Drug-free EGF-conjugated micelles are shown to result in cell-cycle arrest at the G_1 phase and subsequent induction of cell-type-specific apoptosis in EGFR-overexpressing breast cancer cells as demonstrated by flow cytometric analysis. EGF delivered as EGF-conjugated micelles was found to be 13-fold more potent than free EGF; the IC_{50} was decreased from 0.98 $\pm$ 0.1 nM for free EGF to 0.076 $\pm$ 0.01 nM for EGF micelles. The apoptotic micelles, however, are non-antiproliferative to cells expressing a low level of EGFR, suggesting that the apoptotic micelles have minimal or no toxicity against normal healthy tissues. Ellipticine, a chemotherapeutic agent, was loaded into the EGF–micelles after it had been shown, using the combination index–isobologram equation, to act synergistically with EGF. A 10-fold increase in EGF content in the ellipticine-loaded micelles lowered the IC_{50} of ellipticine in EGFR-overexpressing breast cancer cells by more than 18-fold. The EGF–micelles have the potential to be further pursued as a versatile nanotechnology platform for targeted delivery of a wide range of chemotherapeutic agents as a combination therapy for the treatment of EGFR-overexpressing cancers.

Keywords: Combination therapy; ellipticine; epidermal growth factor (EGF); block copolymer micelles; drug delivery; active targeting

Introduction

Epidermal growth factor receptor (EGFR) is overexpressed in at least 33–50% of common human epithelial cancers, including head and neck, lung, ovarian, and breast.[1–5] In particular, 45% of human breast cancers have been associated with EGFR overexpression, and these cases account for almost all of the poor prognosis forms of metastatic breast cancer.[6–9] For these reasons, EGFR has become recognized as a rational target for the development of cancer therapeutics.

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Most of the EGFR-targeted therapeutic agents, including those that are Food and Drug Administration (FDA)-approved (i.e., Iressa and Erbitux), are designed to result in the blockade of EGFR-mediated signal transduction pathways.[1,10] However, a few preclinical studies have focused on the development of EGFR-targeted delivery systems that aim to deliver other active agents to EGFR-positive cells.[11–14] Previously, our group reported on the synthesis and characterization of epidermal growth factor (EGF)-conjugated block copolymer micelles (EGF–micelles) for the delivery of hydrophobic drugs to EGFR-overexpressing cancers.[14] The EGF–micelles were found to enhance the intracellular uptake of micelle-encapsulated agents in EGFR-overexpressing human breast cancer cells. Furthermore, the empty EGF–micelles were found to have an antiproliferative effect, which was attributed to the apoptotic nature of EGF toward this cell line.[14]

The EGF–EGFR system is known to be involved in cell-growth regulation. Activation of EGFR via EGF binding can be mitogenic or apoptotic depending upon the level of EGFR expression and concentration of EGF.[15] Binding of EGF to EGFR results in growth stimulation in cells with a low expression of EGFR. Cell proliferation can also be triggered in cells expressing high levels of EGFR when exposed to EGF concentrations that are below or at physiological levels. It has also been well-documented that supraphysiological concentrations of EGF can induce apoptosis in cancer cells expressing a high level of EGFR.[15–17] It is postulated that EGF-induced apoptosis is a result of the upregulation of the cyclin-dependent kinase inhibitor $p21^{WAF-1/CIP-1}$ and subsequent cell-cycle arrest at the G_1 phase.[18] Although a few studies have reported on the use of EGF as a targeting moiety for nano-sized drug carriers,[11,12] the evaluation of these carriers as pro-apoptotic macromolecules against EGFR-overexpressing cancers has not been documented. To the best of our knowledge, the use of EGF as both a targeting moiety for drug delivery and an active component against EGFR-overexpressing cancers has not been explored.

In this study, we aimed to develop the EGF–micelles as a cell-type-specific apoptotic carrier, as shown in Scheme 1, for the delivery of synergistic drug combinations (i.e., EGF and a chemotherapeutic drug). It has been reported that the combined use of therapeutic agents that have distinct cytotoxic mechanisms can result in additive or synergistic effects.[19] Therefore, the EGF–micelles with an encapsulated chemotherapeutic agent may serve as a novel synergistic and potent treatment for EGFR-overexpressing cancers. In drug delivery, micelles assembled from amphiphilic block copolymers include a hydrophilic shell that is usually comprised of poly(ethylene glycol) (PEG) and a hydrophobic core.[20,21] In the current study, poly(ε-caprolactone) (PCL) was selected as the hydrophobic, core-forming block of the copolymer because of the established compatibility between this polymer

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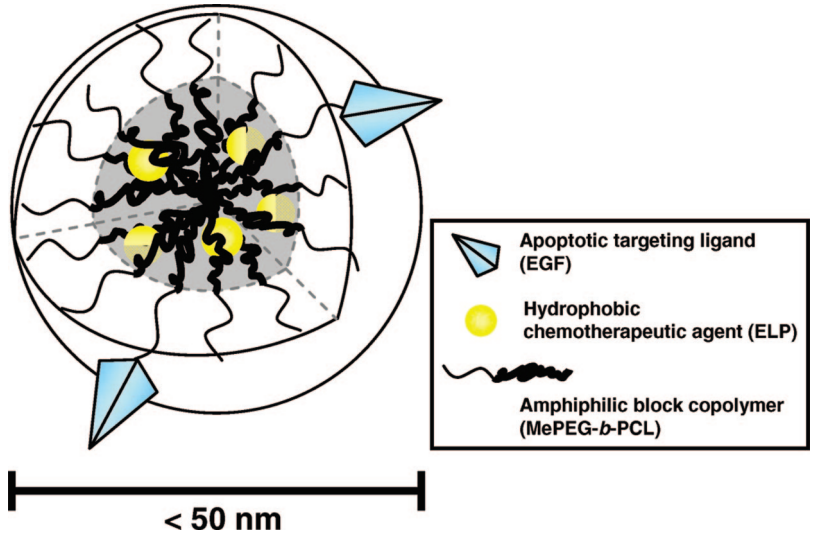
Scheme 1. Schematic of Block Copolymer Micelles Loaded with a Chemotherapeutic Agent in the Hydrophobic Core and the Apoptotic Targeting Ligand EGF Conjugated to the Hydrophilic Shell



and the drug of interest, ellipticine (ELP).[22] ELP is an antineoplastic agent, which has growth inhibitory effects against a wide range of cancers.[23] However, clinical development of this drug was halted because of its systemic toxicity and limited aqueous solubility (i.e., 0.15 μg/mL).[22,23] Our group has reported on the use of PCL-based copolymer micelles to increase the aqueous solubility and provide sustained delivery of ELP.[22,24] Here, the drug combination of EGF and ELP, delivered as free and micellar agents, was examined using the combination index (CI)–isobologram equation to validate the use of EGF–micelles as a combination therapy platform with ELP as the model drug.[19]

Experimental Section

Materials. Ethylene oxide (EO, 99.5%), ε-caprolactone (CL, 99%), 5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole (ELP), and 3-mercaptopropionic acid (MPA, 99%) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and were dried by *n*-butyl lithium (at −78 °C), calcium hydride, and molecular sieves, respectively, prior to use. Tetrahydrofuran (THF) and toluene were refluxed over the sodium–benzophenone complex and were distilled under nitrogen. HCl (1.0 M in diethyl ether, HCl ether), sodium (99.5%), potassium (99.95%), naphthalene (98%), human EGF, thiazolyl blue tetrazolium bromide (MTT), *N*,*N*-dimethylformamide (DMF), and all other chemicals purchased from Sigma-Aldrich, Inc. were used as received. Fluorescein-conjugated EGF, fluorescein isothiocyanate (FITC)-Annexin-V, propidium iodide, and 7-aminoactinomycin D (7-AAD) were purchased from Molecular Probes, Inc. (Eugene, OR).

Synthesis of Polymers. The α-carboxy-ω-hydroxy PEG (HOOC-PEG) was prepared using a previously established method.[25] Briefly, potassium–naphthalene (5.0 mL, 1.0 M, 5.0 mmol) was added dropwise (at 0 °C) to a flame-dried round-bottom flask containing the thiol anionic initiator MPA (0.26 g, 2.5 mmol) and distilled THF (50 mL). Purified EO (5.0 g, 5.7 mL, 114 mmol) was added to the reactor, which was then sealed and maintained for 48 h at 40 °C. The reaction was terminated by the addition of HCl (0.3 mL, 36%). The KCl precipitate was removed by filtration, and the polymer was recovered by precipitation in diethyl ether.

The carboxy-terminated poly(ethylene glycol)-*block*-poly(ε-caprolactone) (HOOC-PEG-*b*-PCL) copolymers were synthesized using a method outlined in detail elsewhere.[25] The HOOC-PEG (1 mmol) was added to a flame-dried flask and was azeo-distilled twice with dry toluene. Dichloromethane (10 mL), CL monomer (2.0 g, 17.5 mmol), and HCl in diethyl ether (1 M, 3 mmol) were then added to the flask. The reaction was maintained for 24 h at room temperature and was terminated by the addition of triethylamine. The final product was obtained by filtration and precipitation in diethyl ether. The molecular weight of the HOOC-PEG and the PCL blocks were found to be 2000 and 1920 g/mol, respectively. The molecular-weight distribution of the copolymer was 1.16. Methoxy-PEG-*b*-PCL (MePEG-*b*-PCL) was synthesized by a similar procedure using MePEG as the macroinitiator.[22,25]

N-Hydroxy-succinimide-PEG-*b*-PCL (NHS-PEG-*b*-PCL) copolymer was synthesized using a procedure reported

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elsewhere.[14] Briefly, 0.5 g of HOOC-PEG-*b*-PCL (M_n = 4000, 0.13 mmol), 0.040 g of *N*,*N*′-dicyclohexyl carbodiimide (1.5 × excess, 0.19 mmol), 0.022 g of NHS (1.5× excess, 0.19 mmol), and 5 mL of dichloromethane were added to a flame-dried round-bottom flask under nitrogen. The reaction was maintained for 24 h at room temperature. The NHS-PEG-*b*-PCL copolymer was collected by precipitation in cold diethyl ether.

All polymers were characterized using gel-permeation chromatography (GPC) and ^{1}H nuclear magnetic resonance (NMR) analyses. The GPC traces and ^{1}H NMR spectra were published elsewhere.[14,22,25]

Conjugation of EGF to NHS-PEG-*b*-PCL Copolymers. A total of 2.5 mg of NHS-PEG-*b*-PCL was added to a 0.01 M phosphate-buffered saline (PBS) solution containing 200 μg of EGF and 12 μg of fluorescein–EGF (pH 8.0−8.5) and allowed to stir for 4 h at room temperature. An additional 2.5 mg of NHS-PEG-*b*-PCL was then added to the mixture, and the reaction was maintained overnight at room temperature. The mixture was dialyzed against water using a cellulose ester membrane with a molecular-weight cut-off of 8000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA) for 24 h to remove the uncoupled EGF. Following dialysis, the mixture was divided into separate vials and lyophilized (FreeZone 6L Free-Dry System; Labconco Corp., Kansas City, MO) prior to storage. The conjugation efficiency was determined by measuring the fluorescence intensity (Fluoro Max-3; Jobin Yvon, Inc., Edison, NJ) of the coupled fluorescein–EGF at λ_{ex} = 494 nm and λ_{em} = 518 nm.

Preparation of MePEG-*b*-PCL Copolymer Micelles. A total of 0.2 mg of ELP and 10 mg of MePEG-*b*-PCL copolymer were dissolved in DMF. The ELP–copolymer mixture was stirred for 4 h and dried under nitrogen to form a dry ELP–copolymer film. The film was hydrated with 1 mL of PBS (pH 7.4) at 60 °C to form ELP-loaded nontargeted micelles (without EGF). The ELP–micelles were filtered through a 0.2 μm filter for sterilization and removal of non-incorporated ELP aggregates prior to *in vitro* cytotoxicity studies. The final drug loading was measured using the fluorescence-based method described below. Targeted micelles (with EGF) were prepared according to a method reported elsewhere.[14] Briefly, lyophilized EGF–PEG-*b*-PCL copolymer dissolved in filtered water was added to the filtered nontargeted micelles and stirred for 1 h at 60 °C and overnight at room temperature to allow for uniform distribution of the EGF-conjugated copolymer in the preformed micelles. "EGF–ELP" refers to the free agent combinations, while "EGF–micelles" and "EGF–ELP–micelles" refer to drug-free and ELP-loaded micelles incorporated with EGF-conjugated copolymers. The prefixes high, medium, and low refer to free agent or micelle combinations that contain varying amounts of EGF at EGF/ELP molar ratios of 1:300, 1:1000, and 1:3000. Unless otherwise noted, experiments including cell-cycle analysis, apoptosis studies, and evaluation of the bystander effect were performed with the drug-free high EGF–micelles.

Characterization of Copolymer Micelles. The micelles were evaluated in terms of their physicochemical characteristics. The size and size distribution of the micelles were measured by dynamic light scattering (DLS) at an angle of 90° and a temperature of 25 °C (DynaPro-MS/X; Protein Solutions, Inc., Lakewood, NJ). The mean diameter and the size distribution of the micelles were obtained from the regularization algorithm (DYNAMICS V6 version 6.7.1, Wyatt Technology Corp.). The amount of ELP loaded in the micelles was measured using a fluorescence-based assay.[22] In brief, a linear calibration curve was obtained for ELP concentrations ranging from 0.04 to 0.6 μg/mL in DMF. The ELP–micelles were diluted in DMF to concentrations within the linear range of the calibration curve. The fluorescence intensity of the ELP–micelles was measured at λ_{ex} = 294 nm and λ_{ex} = 423 nm.

Cell Culture. The MCF-7 breast cancer cells (10^4 EGFR/cell) and EGFR-overexpressing breast cancer cells (MDA-MB-468, 10^6 EGFR/cell) were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). The DMEM was supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin–streptomycin (100 units/mL penicillin G and 100 μg/mL streptomycin). Cells were allowed to grow in a monolayer in a tissue culture flask incubated at 37 °C in a 5% CO_2 atmosphere and 90% relative humidity.

Cell-Cycle Analysis by Flow Cytometry. MCF-7 or MDA-MB-468 cells were added to a 6-well plate at a cell density of 500 000 cells/well and were incubated for 24 h to allow for growth recovery. The growth medium was then removed and replaced with 2 mL of fresh medium containing 10 nM free EGF, drug-free EGF–micelles (10 nM, EGF equivalent), and drug-free MePEG-*b*-PCL micelles. All treatments contained a total of 5% (v/v) PBS, including the untreated control. After a 24 h incubation period, cells were trypsinized and washed with PBS by centrifuging at 1200 rpm for 10 min under 10 °C. The cell pellet was then resuspended in 0.5 mL of PBS at room temperature, with the subsequent addition of absolute ethanol (2 mL, −20 °C). After fixation overnight at −20 °C, the cells were rehydrated with 5 mL of PBS for 15 min. Cell pellets were obtained via centrifugation; the cell pellets were resuspended with 1 mL of propidium iodide staining solution [0.1% (v/v) Triton X-100, 0.2 mg of DNase-free RNase, and 12 μL of 1 mg/mL of propidium iodide solution, in 1 mL of 0.01 M PBS] and incubated for 15 min. Cell samples were kept on ice until flow cytometry analysis (FACSan; BD Biosciences, San Jose, CA), and the data were acquired by CELLQuest. Data were fit using the cell-cycle analysis model available in the ModFit LT for Mac version 2.0 software.

Flow Cytometric Assay of Apoptosis. MCF-7 or MDA-MB-468 cells were added to a 6-well plate at a cell density of 500 000 cells/well and were incubated for 24 h to allow for growth recovery. The growth media was then removed and replaced with 2 mL of fresh medium containing 10 nM free EGF, drug-free EGF-micelles (10 nM, EGF equivalent),

and drug-free MePEG-*b*-PCL micelles. All treatments contained a total of 5% (v/v) PBS, including the untreated control. After a 36 h incubation period, nonadherent and adherent cells were collected and washed with PBS by centrifugation at 1200 rpm for 10 min at 10 °C. The cell pellets were resuspended in 200 μL of 1× Annexin-V binding buffer (Molecular Probes, Inc., Eugene, OR), with the addition of 7 μL of FITC–Annexin-V and 2 μL of 7-AAD, and were incubated for 30 min at room temperature. After incubation, 800 μL of 1× Annexin-V binding buffer was added to each sample. Cell samples were kept on ice until flow cytometry analysis (FACSan; BD Biosciences, San Jose, CA), and the data were acquired by CELLQuest.

Evaluation of the Bystander Effect. MCF-7 or MDA-MB-468 cells were added to a 6-well plate at a cell density of 500 000 cells/well and were incubated for 24 h to allow for growth recovery. The growth medium was then removed and replaced with 2 mL of fresh medium containing 10 nM free EGF, drug-free EGF-micelles (10 nM, EGF equivalent), and drug-free MePEG-*b*-PCL micelles. All treatments contained a total of 5% (v/v) PBS, including the untreated control. After 72 h of incubation, the incubation medium was collected and filtered to remove cell debris (0.2 μm sterile syringe filters). The filtered medium was added to MCF-7 cells that were grown on a 96-well plate at a cell density of 5000 cells/well. Cell viability was measured using the colorimetric MTT assay following a 72 h incubation period.[26]

Determination of Inhibitory Concentration 50. MCF-7 or MDA-MB-468 cells were added to a 96-well plate at a cell density of 5000 cells/well. The growth medium was removed following a 24 h incubation and replaced with 150 μL of fresh medium that contained various concentrations of free drug, free EGF, or the corresponding micelle formulations. The plates were allowed to incubate for 24 or 72 h. For cells treated with free drug or free EGF, the total amount of dimethylsulfoxide (DMSO) and PBS used were 0.5 and 2%, respectively. It was found that there was no significant difference between the IC_{50} of EGF determined in 0 and 0.5% DMSO ($p > 0.05$); hence, 0.5% DMSO was used to solubilize ELP in the EGF–ELP combination treatment. For treatment with the micelle formulations, the final concentration of PBS was kept constant at 5% (v/v) to ensure that the cell proliferation was not affected by the solution added. The cell viability was measured using the MTT assay as reported previously.[14] The cell viability was reported relative to the control groups, which were also treated with the same concentration of DMSO or PBS. The concentrations of each treatment that result in 50% cell viability (i.e., inhibitory concentration 50, IC_{50}) were estimated from the growth inhibition curves.

Determination of the Combination Index (CI). The CI for each treatment was determined to analyze the drug combination effect of EGF and ELP using a method developed by Chou and Talalay.[19,27] The growth inhibitory results are plotted using the median-effect plot [i.e., log(fraction of nonviable cells/fraction of viable cells) versus log(drug concentration)] to determine whether the treatment combinations were mutually nonexclusive (i.e., drugs acting independently or having different actions) or mutually exclusive. The CI is then calculated according to the following:

For mutually nonexclusive combination,

$$\mathrm{CI} = \frac{D_1}{(D_m)_1} + \frac{D_2}{(D_m)_2} + \frac{D_1 D_2}{(D_m)_1 (D_m)_2} \quad (1)$$

For mutually exclusive combination,

$$\mathrm{CI} = \frac{D_1}{(D_m)_1} + \frac{D_2}{(D_m)_2} \quad (2)$$

where $(D_m)_1$ and $(D_m)_2$ represent the IC_{50} of treatments 1 and 2 applied separately, while, D_1 and D_2 are IC_{50} of treatments 1 and 2 applied as a combination. CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively.[19,28]

Statistical Analysis. All results were obtained from data groups of $n \geq 3$ and are expressed as mean ± standard deviation (SD). Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 14.0). A two-sample *t* test was used to measure statistical significance between pairs of results, and $p < 0.05$ was considered to be significant. For statistical analyses among three or more groups, one-way analysis of variance (ANOVA) was used and subsequent multiple comparisons with Bonferroni correction was performed if any statistical significance was detected by the ANOVA *F* test.

Results

Characterization of the EGF–Micelles and the ELP-Loaded Micelles. ELP–micelles were prepared, by the dry down method,[22] to achieve a final drug concentration of 50 μg/mL, which increased the water solubility of ELP by more than 300-fold. The micelles were found to have a monomodal size distribution, with mean diameters of 25 ± 3 nm for both the ELP–micelles and EGF–ELP–micelles as determined by DLS, while the drug-free micelles were found to have mean diameters of 26 ± 4 and 27 ± 3 nm with (EGF–micelles) or without EGF (MePEG-*b*-PCL micelles) on the micelle surface, respectively.

Effect of Drug-Free EGF–Micelles on Cell-Cycle Distribution Profiles. To determine whether the EGF–micelles were capable of arresting EGFR-overexpressing cancer

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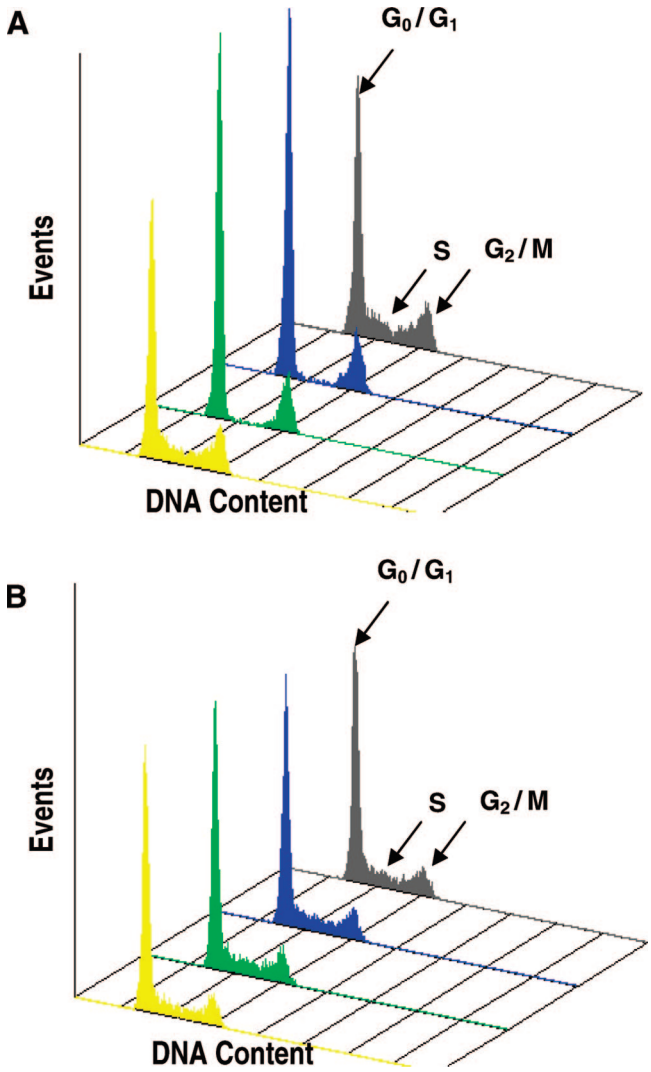

Figure 1. Effect of free EGF (green), drug-free EGF-micelles (blue), and drug-free MePEG-*b*-PCL micelles (gray) on the cell-cycle distribution in (a) MDA-MB-468 breast cancer cells (10^6 EGFR/cell) and (b) MCF-7 breast cancer cells (10^4 EGFR/cell). Cells were treated for 24 h prior to DNA content staining with propidium iodide for flow cytometric analysis. The yellow traces represent the cells that were treated with cell-culture media and 5% (v/v) PBS (untreated control).

cells at the G_1 phase, flow cytometry was used to evaluate the cell-cycle distribution of MDA-MB-468 cells (10^6 EGFR/cell) treated with drug-free EGF–micelles. As shown in Figure 1a, treatment of MDA-MB-468 cells with free EGF (10 nM) and EGF–micelles (10 nM, EGF equivalent) resulted in 75 ± 5 and 75 ± 4% of the cell population at the G_0/G_1 phase, respectively, versus 57 ± 1% for the untreated control and 53 ± 3% for cells treated with MePEG-*b*-PCL micelles. For breast cancer cells expressing a low level of EGFR (MCF-7 cells express 10^4 EGFR/cell) (Figure 1b), all treatments were found to have statistically insignificant effects on the cell-cycle distribution ($p = 0.157$). Specifically, 62 ± 3 and 60 ± 2% of MCF-7 cells were found to reside at the G_0/G_1 phase when treated with free EGF (10 nM) and

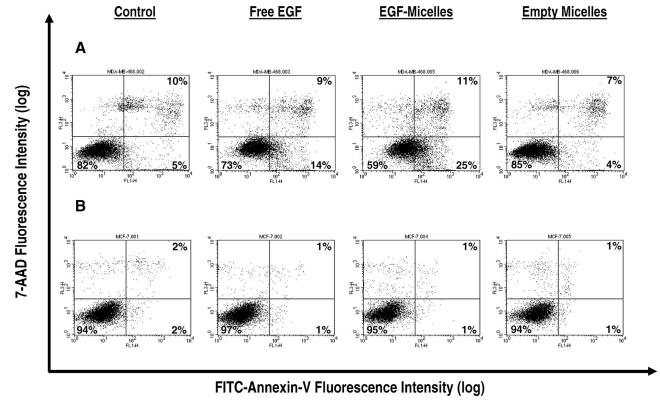

Figure 2. Apoptotic assessment of (a) MDA-MB-468 breast cancer cells (10^6 EGFR/cell) and (b) MCF-7 breast cancer cells (10^4 EGFR/cell) by flow cytometric analysis with FITC–Annexin-V and 7-AAD staining. Both the bottom-right quadrant (early apoptotic cells) and top-right quadrant (late apoptotic cells) include the populations of cells that are Annexin-V-stained. Cells were treated with cell-culture media with 5% (v/v) PBS (untreated control), 10 nM free EGF, drug-free EGF–micelles (10 nM, EGF equivalent), and empty MePEG-*b*-PCL micelles for 36 h prior to flow cytometric analysis.

EGF–micelles (10 nM, EGF equivalent), respectively, when compared to 64 ± 2 and 60 ± 3% for the controls (untreated and treated with MePEG-*b*-PCL micelles).

Cell-Type-Specific Apoptotic Nature and Growth Inhibitory Effect of Drug-Free EGF–Micelles. The extent to which EGF–micelles induce apoptosis was evaluated by measuring the externalization of phosphatidylserine (PS) on the cell membrane, which is one of the characteristics of apoptotic cells.[29] FITC–Annexin-V was used for labeling externalized PS, and 7-AAD was used to identify populations of dead cells. Parts a and b of Figure 2 include the results from flow cytometric analyses of MDA-MB-468 and MCF-7 cells, respectively, treated with 10 nM free EGF and EGF–micelles (10 nM, EGF equivalent) for 36 h. For MDA-MB-468 cells treated with free EGF and EGF–micelles, 14 and 25% of the cells were found to be in the early apoptotic stage (FITC–Annexin-V positive and 7-AAD negative), respectively. Conversely, free EGF and EGF–micelles failed to trigger apoptosis or cell death in MCF-7 cells (i.e., less than 2% of the MCF-7 cells were Annexin-V-positive).

The MTT colorimetric assay was also used to assess the growth inhibitory effect of the drug-free EGF–micelles. The growth inhibitory effects of free EGF and drug-free EGF–micelles on MDA-MB-468 and MCF-7 cells are presented in Figure 3. Treatment of MDA-MB-468 cells with free EGF for 24 and 72 h resulted in similar growth inhibitory effects

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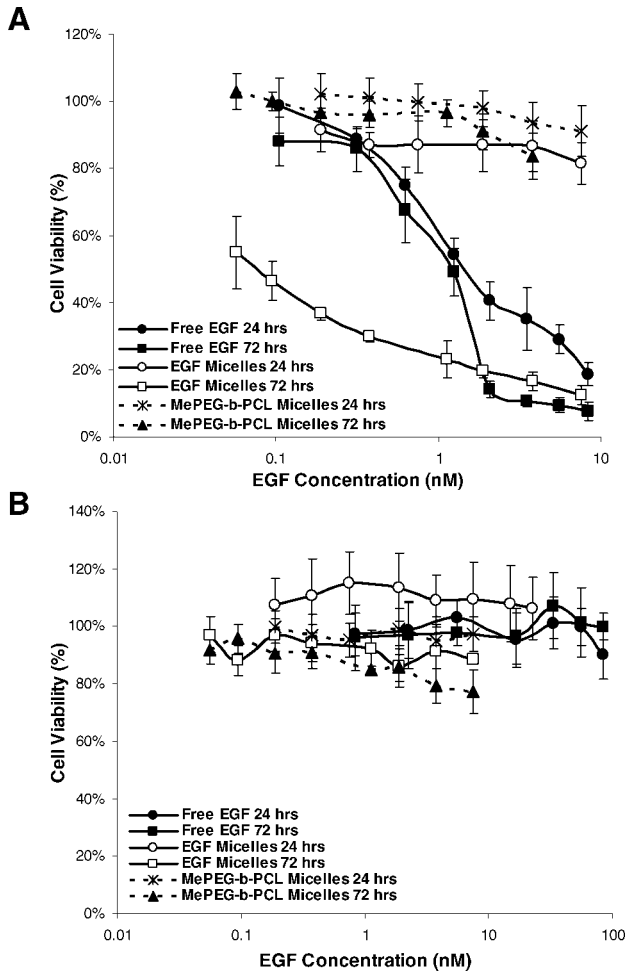

Figure 3. Growth inhibitory effects of free EGF and empty EGF–micelles in (a) MDA-MB-468 breast cancer cells (10^6 EGFR/cell) and (b) MCF-7 breast cancer cells (10^4 EGFR/cell). Cell viability was assessed by the colorimetric MTT assay following 24 or 72 h incubation periods. The copolymer concentrations used in treatments with drug-free MePEG-*b*-PCL micelles (nontargeted) is equivalent to the copolymer concentrations employed in treatments with EGF micelles. The amount of EGF molecules in the empty EGF–micelles was equivalent to that of the amount of EGF present on the micelle surface of the high EGF combination micelles (i.e., EGF/ELP molar ratio of 1:300).

(Figure 3a). In contrast, the growth inhibitory effect of EGF–micelles was highly dependent upon the incubation time. For instance, a 24 h treatment of the MDA-MB-468 cells with EGF–micelles was significantly less effective than treatment with free EGF. However, a 72 h treatment with EGF micelles ($IC_{50,EGF} = 0.076 \pm 0.01$ nM) inhibited the growth of MDA-MB-468 cells to a much greater extent than that of the treatment with free EGF ($IC_{50,EGF} = 0.98 \pm 0.1$ nM). In addition, the amount of EGF present on the EGF–micelles was found to affect the potency of the system. As illustrated in Table 1, the EGF–micelles with medium and low levels of EGF resulted in $IC_{50,EGF}$ values of 0.27 ± 0.06 and 0.46 ± 0.01 nM, respectively, in comparison with the EGF–micelles with the highest level of EGF, which had an $IC_{50,EGF}$ value of 0.076 ± 0.01 nM. In general, the EGF–micelles are much more effective at inhibiting the growth of MDA-MB-468 cells only following 72 h of incubation. In contrast, treatment of MCF-7 cells with free EGF or EGF–micelles seemed to result in a slight increase in cell viability (Figure 3b).

Drug Combination Effect of EGF and ELP Delivered as Free Agents. The combination effect of EGF and ELP was assessed by comparing three combinations that vary in terms of the mole ratio of EGF/ELP of 1:300 (high EGF–ELP), 1:1000 (medium EGF–ELP), and 1:3000 (low EGF–ELP). The median effect plot demonstrated that the EGF–ELP combination was mutually nonexclusive (i.e., drugs acting independently or having different actions) (data not shown).[19] Using eq 1, the combination effects of EGF and ELP were quantified and summarized in Table 1. All of the drug combinations delivered as free agents were found to exert synergistic or additive effects on MDA-MB-468 cells following a 72 h treatment period. The drug combinations also effectively lowered the IC_{50} of ELP ($IC_{50,ELP}$) for MDA-MB-468 cells. Note that no growth inhibitory effect was observed in MCF-7 cells treated with EGF; hence, the combination effect of EGF and ELP in MCF-7 cells could not be evaluated.[28]

Combined Growth Inhibitory Effect of ELP Encapsulated in EGF–Micelles. The growth inhibitory effect of the EGF–ELP–micelles in MDA-MB-468 and MCF-7 cells are illustrated in parts a and b of Figure 4, respectively. As shown in Figure 4a and Table 1, the cell viability of the MDA-MB-468 cells decreased, as the amount of EGF at the micelle surface was increased, while the concentration of ELP was held constant. Also, the EGF–ELP–micelles were more effective in inhibiting MDA-MB-468 cell growth when compared to the nontargeted ELP–micelles or the drug-free EGF–micelles. In the case of MCF-7 cells (Figure 4b), the inhibitory curves for the combinations were equivalent to the inhibitory curve for the ELP–micelles (nontargeted, without EGF). This indicates once again that EGF does not contribute to a growth inhibitory effect in cells that express low levels of EGFR.

The Bystander Effect Induced by Drug-Free EGF–Micelles. As mentioned above, treatment of cells expressing low levels of EGFR (e.g., MCF-7) with EGF–micelles were shown to be growth stimulatory or nongrowth inhibitory. For this reason, a phenomenon, termed the bystander effect, was evaluated as a preliminary assessment of the extent to which the EGF–micelles may be efficacious against tumors that include cells with heterogeneous levels of EGFR expression. Figure 5 illustrates the bystander effect that is induced in MCF-7 cells (nearby cells) by treatment of MDA-MB-468 cells (target cells) with EGF–micelles. MDA-MB-468 cells were treated with 10 nM free EGF or EGF–micelles (10 nM, EGF equivalent) for 72 h, the incubation media was then collected and filtered to remove cell debris. The filtered media was added to MCF-7 cells and incubated for an additional 72 h prior to assessment of cell viability. Incuba-

Table 1. Cytotoxicity and Combination Effects of Epidermal Growth Factor and Ellipticine Delivered as Free Agents or Micellar Agents after an Incubation Period of 72 h

		IC_{50}		
cell line[a]	treatment	ELP (μM)	EGF (nM)	combination effect[b](CI)
MDA-MB-468	free EGF		0.98 ± 0.1	
	free ELP	0.55 ± 0.05		
	high EGF–ELP [c]	0.14 ± 0.04	0.48 ± 0.09	slight synergism (0.87)
	medium EGF–ELP [c]	0.12 ± 0.01	0.12 ± 0.01	strong synergism (0.37)
	low EGF–ELP [c]	0.42 ± 0.1	0.14 ± 0.04	additive (1.0)
MCF-7	free EGF		nd[d]	
	free ELP	0.96 ± 0.3		
	high EGF–ELP	0.90 ± 0.04	3.0 ± 0.1	
	medium EGF–ELP	0.76 ± 0.02	0.76 ± 0.02	
	low EGF–ELP	0.73 ± 0.1	0.25 ± 0.04	
MDA-MB-468	high EGF–micelles		0.076 ± 0.01	
	medium EGF–micelles		0.27 ± 0.06	
	low EGF–micelles		0.46 ± 0.01	
	ELP–micelles	0.34 ± 0.01		
	high EGF–ELP–micelles	0.013 ± 0.006	0.043 ± 0.006	moderate synergism (0.63)
	medium EGF–ELP–micelles	0.11 ± 0.03	0.11 ± 0.03	slight synergism (0.86)
	low EGF–ELP–micelles	0.24 ± 0.03	0.08 ± 0.01	additive (1.0)
MCF-7	EGF–micelles		nd	
	ELP–micelles	0.73 ± 0.06		
	high EGF–ELP–micelles	0.66 ± 0.08	2.2 ± 0.2	
	medium EGF–ELP–micelles	0.70 ± 0.09	0.70 ± 0.09	
	low EGF–ELP–micelles	0.68 ± 0.04	0.23 ± 0.02	

[a] MDA-MB-468 and MCF-7 are breast cancer cells expressing 10^6 and 10^4 EGFR/cell, respectively.[17] [b] Drug combination effects are determined by calculating the combination index from the IC_{50} values and are defined as follows:[27] CI > 1.3, antagonism; CI = 1.1–1.3, moderate antagonism; CI = 0.9–1.1, additive; CI = 0.8–0.9, slight synergism; CI = 0.6–0.8, moderate synergism; CI = 0.4–0.6, synergism; CI = 0.2–0.4, strong synergism. [c] High, medium, and low EGF–ELP refer to EGF–ELP combination treatments that contain mole ratios of EGF/ELP of 1:300, 1:1000, and 1:3000, respectively. [d] nd = not detectable.

tion of MDA-MB-468 cells with EGF–micelles (10 nM, EGF equivalent) reduced the viability of MCF-7 cells to 30 ± 4%, while MDA-MB-468 cells treated with free EGF (10 nM) and empty MePEG-*b*-PCL micelles failed to induce the bystander effect in MCF-7 cells (viability of 96 ± 4 and 102 ± 4%, respectively).

Discussion

Clinical studies have shown that cancer patients diagnosed with EGFR-overexpressing tumors are often associated with a higher incidence of recurrence and metastases, as well as reduced survival.[7,30] Also, the aggressiveness of the disease has been attributed to an amplified growth stimulatory effect that results from overactivation of the EGFR signal transduction pathways via increased EGF–EGFR binding. However, many have overlooked that supraphysiological concentrations of EGF can trigger a cascade of cellular events in EGFR-overexpressing cancer cells that ultimately result in apoptotic cell death. Therefore, the goal of the current study was to explore the use of EGF–micelles as a pro-apoptotic drug carrier and investigate the combination effect that may be exerted on the EGFR-overexpressing cancer cells by EGF and an encapsulated drug.

From clinical studies, EGFR overexpression has been defined as values of EGFR/mg of membrane protein that are greater than 10 fmol. This value corresponds to approximately 7×10^5 EGFR/cell.[4,6,31–34] The levels of EGFR per cell in normal epithelial tissues, such as lung and liver, are reported to range from 2×10^4–10^5 EGFR/cell.[30,35] Two breast cancer cell lines, MDA-MB-468 (10^6 EGFR/cell) and MCF-7 (10^4 EGFR/cell), were selected for the investigation of the targeted apoptotic nature and growth inhibitory effect of the drug-free EGF–micelles. These cell lines have been

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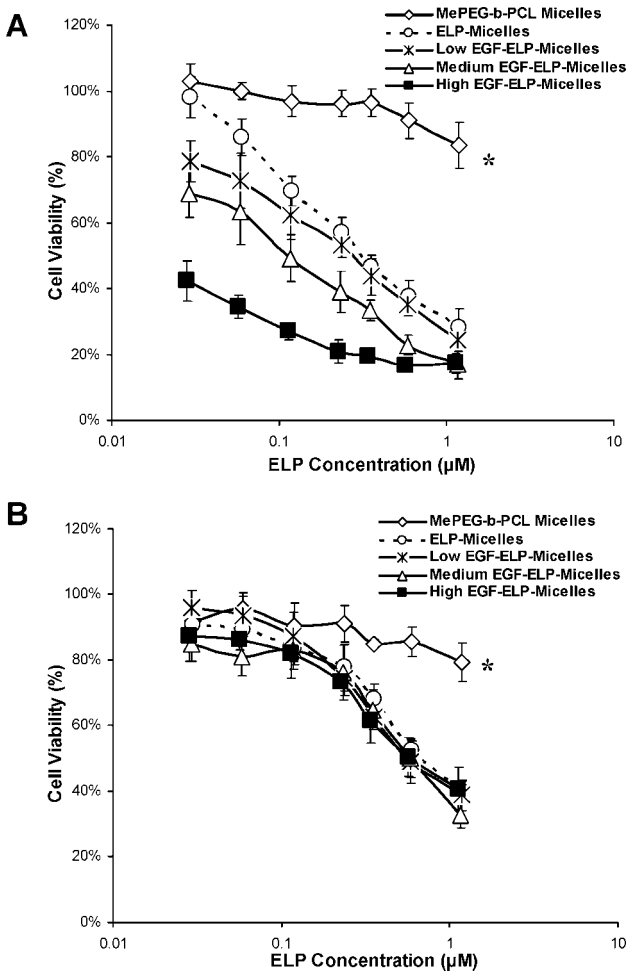

Figure 4. Inhibition of the growth of (a) MDA-MB-468 and (b) MCF-7 breast cancer cells treated with drug-free MePEG-*b*-PCL micelles (◊), ELP-incorporated micelles (○), or EGF-conjugated ELP–micelles, for 72 h. Drug combinations of high EGF–ELP–micelles (■), medium EGF–ELP–micelles (△), and low EGF–ELP–micelles (∗) correspond to EGF/ELP molar ratios of 1:300, 1:1000, and 1:3000, respectively. (∗) The copolymer concentrations used in treatments with drug-free MePEG-*b*-PCL micelles is equivalent to or greater than the copolymer concentrations employed in all other treatments at each corresponding ELP concentration.

used widely as *in vitro* models for the development of EGFR-targeted therapeutics.[18,36–40] It was expected that the EGF–micelles would be pro-apoptotic in the MDA-MB-468

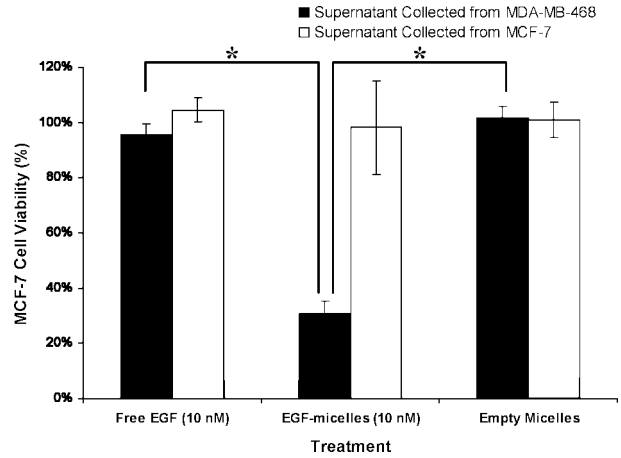

Figure 5. Bystander effect of free EGF, drug-free EGF–micelles, and empty MePEG-*b*-PCL micelles in MCF-7 breast cancer cells (10^4 EGFR/cell). MDA-MB-468 cells or MCF-7 cells were treated for 72 h; the supernatant was collected and filtered (0.22 μm pore size) to remove cell debris. The supernatant was then added to MCF-7 cells and treated for 72 h prior to cell viability assessment by the colorimetric MTT assay. (∗) $p < 0.01$.

breast cancer cells, while the MCF-7 cells would serve as a negative control.

Cell-Cycle Arrest and Apoptosis Induced by Drug-Free EGF–Micelles. The treatment of EGFR-over-expressing cancer cells with high concentrations of EGF has been shown to result in G_1 phase cell-cycle arrest and subsequent apoptotic cell death.[18,41,42] Several studies have documented the chronological phases of EGF-induced apoptosis.[41,43] As outlined, a 24 h treatment with EGF resulted in cell morphological changes, detachment from the

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substrate, and more than a 10% increase in the cell population residing at the G_1 phase. However, the majority of the cell population did not show apoptotic features at this point. Cells treated with EGF for 48 h were then found to show late apoptotic features, including massive DNA fragmentation and nuclear condensation.[43]

In the current study, similar observations were noted, such as MDA-MB-468 cells (10^6 EGFR/cell) treated with EGF–micelles for 24 h, resulting in only minimal FITC–Annexin-V staining, as well as cell morphological alterations and detachment (data not shown). Treatment of these cells with free EGF and drug-free EGF–micelles also resulted in a 20% increase in the number of cells residing at the G_0/G_1 phase (Figure 1a). The effect of EGF–micelles on the cell-cycle distribution of MDA-MB-468 cells can be attributed to the presence of EGF on the micelle surface because treatment of the cells with nontargeted MePEG-*b*-PCL micelles resulted in no significant effect on the cell-cycle distribution ($p > 0.05$). After a 36 h treatment of MDA-MB-468 cells with free EGF and EGF–micelles, early apoptotic cells were detected (Figure 2a). Finally, the cells entered the late apoptotic stage at 48 h post-treatment as an increase in the population of late apoptotic cells (FITC-Annexin-V and 7-AAD positive) was noted, accompanied with a reduction in the early apoptotic population (FITC–Annexin-V positive and 7-AAD negative) (data not shown). All of the above demonstrated that the biological function of EGF (i.e., activation of the EGFR signal transduction pathways) was retained following conjugation onto the PEG-*b*-PCL micelles.

Enhanced Toxicity of the Drug-Free EGF–Micelles. MDA-MB-468 cells treated with EGF–micelles resulted in a higher percentage of apoptotic cells (FITC–Annexin-V positive) in comparison to treatment with free EGF ($p < 0.05$) (Figure 2a). This was also confirmed by the growth inhibitory studies as illustrated in Figure 3a, in which prolonged treatment with EGF–micelles ($IC_{50,EGF} = 0.076 \pm 0.01$, 0.27 ± 0.06, and 0.46 ± 0.01 nM for high, medium, and low EGF–micelles, respectively) showed a much greater suppression in the growth of MDA-MB-468 cells than free EGF ($IC_{50,EGF} = 0.98 \pm 0.1$ nM). It should be noted that such an effect was only observed in cells treated for more than 24 h. Conversely, the growth inhibitory effect of free EGF on MDA-MB-468 cells showed no time dependence. These results suggest that the mode of delivery of EGF (i.e., free versus micelle-conjugated) plays a critical role in determining the kinetics and magnitude of its growth inhibitory effect.

The accessibility of micelle-conjugated EGF to cell-surface EGFR is limited because of steric effects, in comparison to free EGF. The treatment of cells with free EGF versus EGF–micelles may be likened to bolus versus sustained exposure of EGF to the cell-surface EGFR. In this way, the binding and internalization kinetics of EGF–micelles are likely slower when compared to that of free EGF. The more rapid internalization kinetics of the EGF–EGFR complexes in cells that are exposed to free EGF will likely result in more of an initial downregulation of EGFR[44,45] when compared to the EGF–micelles. It has been reported that the downregulation of cell-surface EGFR because of EGF exposure at concentrations greater than or equal to 10 nM stimulates apoptosis. Also, cell-surface EGFR expression has been inversely correlated with the extent of apoptosis induced.[15] It is likely for these reasons that treatment with free EGF was more effective at inhibiting the growth of MDA-MB-468 cells, in comparison to the EGF–micelles, following the 24 h incubation period. It has also been shown that, after internalization of the EGF–EGFR complexes, the EGF molecules are degraded following endosomal sorting.[46–48] Hence, prolonged incubation (i.e., 72 h) of free EGF with the cells may result in a more rapid depletion of EGF from the incubation medium, in comparison to EGF–micelles. The conjugation of EGF to the micelle surface provides a sustained exposure of EGF to the cells, because of the slower binding and internalization kinetics of this complex (i.e., EGFR–EGF–micelles), which may result in continual apoptotic signaling. Further studies are currently underway to confirm this hypothesis.

EGF–Micelles as a Targeted Combination Therapy Platform. To evaluate whether the apoptotic EGF–micelles can serve as a platform for combination therapy, ELP was encapsulated into the EGF–micelles. A trend was noted in which the growth inhibitory effects of the system were more profound as the amount of the EGF on the ELP–micelle surface was increased (Figure 4a). Also, the EGF–ELP–micelles (combination treatment) were proven to be more effective than the micelles without EGF (ELP–micelle) at the same ELP concentrations, indicating that less ELP is required to achieve the same *in vitro* response in the presence of EGF. This effect may be attributed to both the combination effect between EGF and ELP and the increased intracellular delivery of the drug because of active targeting.

For treatment of advanced or metastatic cancers, the combination of two or more therapeutic agents (e.g., EGF

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and a chemotherapeutic drug) may result in better toxicity profiles, as well as improved therapeutic efficacy if the drug combination exerts additive or synergistic effects.[19,27,49] Using the established CI–isobologram equation, originally put forth by Chou and Talalay,[19,27] it was found that all three combinations of free ELP and free EGF investigated exert additive or synergistic effects in MDA-MB-468 cells. In addition to the combination effect of EGF–micelles and the encapsulated ELP, it is believed that the enhanced growth inhibitory effect of the EGF–ELP–micelles may also be attributed to the active targeting effect.

Many studies have demonstrated that actively targeted drug carriers can increase drug transport into target cells and result in a decrease in the IC_{50}.[36,50] We have also demonstrated in our previous study that the EGF–micelles increased the uptake of the encapsulated agents in MDA-MB-468 cells when compared to the uptake of nontargeted micelles.[14] It is therefore likely that active targeting contributed to the enhanced cytotoxicity of the EGF–ELP–micelles in the EGFR-overexpressing cells. To distinguish between the active targeting effect and the combination effect of EGF–ELP, MDA-MB-468 cells were treated with drug-free EGF–micelles and ELP–micelles (nontargeted) at the identical ratio to that of the "high EGF–ELP–micelles" (i.e., EGF/ELP mol ratio of 1:300) for 72 h. The IC_{50} values were found to be 0.50 ± 0.09 nM and 0.15 ± 0.03 μM, with respect to EGF and ELP concentrations, respectively. These IC_{50} values were almost identical to that determined in MDA-MB-468 cells treated with high EGF–ELP free agent combination (i.e., 0.48 ± 0.09 nM and 0.14 ± 0.04 μM). The independent delivery of ELP and EGF in separate micelles resulted in a similar growth inhibitory effect as ELP and EGF as free agents. Because the IC_{50} of the high EGF–ELP–micelles is significantly lower (i.e., $IC_{50,EGF} = 0.043 \pm 0.006$ nM and $IC_{50,ELP} = 0.013 \pm 0.006$ nM) than that of the EGF–micelles and ELP–micelles applied as separate treatments, this indicates that the active targeting effect plays a significant role in enhancing the synergistic effect of the high EGF–ELP–micelles.

Because of the active targeting effect, it was expected that the medium and low EGF–ELP–micelles would also be associated with increased synergism and decreased IC_{50} values in comparison to the corresponding free agent combinations. However, these two EGF–ELP–micelles were found to have decreased and/or unchanged drug combination effects. For instance, the IC_{50} of the medium EGF–ELP–micelles (0.11 ± 0.03) was similar to the medium EGF–ELP free agent combination (0.12 ± 0.01). It was believed that the medium EGF–ELP–micelles did not have sufficient EGF to induce a significant active targeting effect because the EGF content of the medium EGF–ELP–micelles is 30 times less than the actively targeted EGF–micelles employed in our previous report.[14] Although the active targeting effect was not reflected in the growth inhibitory effects of the medium and low EGF–ELP–micelles, it was confirmed that an increase in the degree of EGF functionalization on the micelles is associated with an increase in the potency of the system (Table 1). However, the medium and low EGF–ELP–micelles were found to have statistically insignificant differences in their IC_{50} values with respect to the EGF concentrations ($IC_{50,EGF} = 0.11 \pm 0.03$ nM and $IC_{50,EGF} = 0.08 \pm 0.01$ nM; $p = 0.22$). On the other hand, the $IC_{50,ELP}$ of medium EGF–ELP–micelles was significantly lower than that of the low EGF–ELP–micelles ($p = 0.0036$) with respect to the ELP concentrations. These results suggest that the EGF–ELP combination effect over-rules that of the EGF growth inhibitory effects in these two systems when there is a lack of active targeting.

As a result of the enhanced potency of the EGF–micelles compared to free EGF, all EGF–ELP–micelles should have led to much stronger synergistic effects despite the insufficient active targeting effect. It was believed that the different modes of delivery of the free agents and the micellar agents altered the EGF–ELP combination effect (i.e., sequence dependent). When the drug combination is delivered as free agents, the cells were exposed to the EGF and ELP simultaneously. In the case of the EGF–ELP–micelles, the cells first encountered the EGF on the micelle surfaces; the drug is then subsequently released as previous studies demonstrated that PEG-*b*-PCL micelles provide sustained release of ELP.[22] It has been reported that the therapeutic response of drug combinations may be dependent upon the sequence in which the drug combinations are delivered.[28,51] In the current study, the distinct cell-cycle checkpoints of MDA-MB-468 cells in response to EGF and ELP treatments could be the sequence-dependent factor.[16,41,52,53] The anticancer activity of ELP has been associated with DNA intercalation, inhibition of topoimerase II, and cell-cycle arrest at the G_2/M phase,[52] while EGF induces apoptosis via G_1 phase cell-cycle arrest and upregulation of $p21^{WAF\text{-}1/CIP\text{-}1}$ expression. In the case of EGF–ELP–micelle treatment, a major portion of the MDA-MB-468 cells was arrested at the G_0/G_1 phase upon exposure to the EGF–micelles (Figure 1a); progression of the cells into the G_2/M phase is therefore largely inhibited,

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antagonizing the G_2/M phase arrest that may otherwise be imposed by the micelle-encapsulated ELP.

In general, it is difficult to translate the sequence- or ratio-dependent synergy observed by various drug combinations *in vitro* to meaningful results *in vivo*.[53,54] This is largely due to the unique physicochemical properties of each drug, which gives rise to their individual pharmacokinetics and biodistribution profiles *in vivo*. In this way, it is particularly challenging to achieve the desired ratio of two drugs at the target site simultaneously. Unlike conventional injectable formulations, drug carriers with long circulation half-lives and sustained drug-release profiles can control the pharmacokinetics of co-encapsulated drugs and simultaneously deliver the drug combination to the site of interest.[53] Therefore, along with their active targeting effect, the EGF–micelles may remain a valuable tool as a combination therapy platform for *in vivo* applications because the MePEG-*b*-PCL systems have been shown to have a prolonged circulation half-life ($t_{1/2,\beta}$ = 30 h) and provide sustained drug release.[22,55]

Targeted Toxicity of the EGF–Micelles and the Bystander Effect. As illustrated in the flow cytometry results (Figures 1b and 2b) and the growth inhibitory data (Figures 3b and 4b), the apoptotic effect of the EGF–micelles is only specific to cells having an overexpression of EGFR. Indeed, this is in accordance with a report by Filmus et al., which demonstrated that the number of cell-surface EGFR present had to reach a critical threshold for EGF-induced apoptosis to occur.[17] However, tumors are known to be heterogeneous in terms of the distribution and levels of receptor expression.[17,56,57] For instance, subclones of the MDA-MB-468 cell line, which express a low level of EGFR (i.e., approximately 10^4 EGFR/cell, similar level as MCF-7 cells), have been identified. Treatment of the isolated subclones with high concentrations of EGF were not found to be growth inhibitory.[58] Also, regions of a tumor can be semi- or avascular (e.g., necrotic region) and thus have limited exposure to the administered therapeutic agents.[59] A phenomenon known as the bystander effect may be exploited to resolve the specificity issues and binding barriers of the EGF–micelles that may be encountered *in vivo*.

The bystander effect, which has mostly been explored in the fields of gene and radiation therapy, refers to the decrease in the viability of neighboring cells as a result of targeting one type of cells within a mixed population.[60,61] The mechanisms underlying the bystander effect have been attributed to intercellular interactions, including gap junctional cell–cell communication, and/or the release of soluble factors, such as cytokines and reactive oxygen species, into the surrounding medium.[60] The bystander effect of the EGF–micelles observed in the current study was shown to be induced via the latter mechanism.

The rationale for choosing the MCF-7 cells as the "nearby" cells is based on the results obtained, which demonstrated that free EGF and EGF–micelles did not inhibit the growth of this cell line (Figures 2 and 3 and Table 1). A decrease in the cell viability of the MCF-7 cells treated with the conditioned medium collected from the target cells (i.e., MDA-MB-468 cells) is therefore truly indicative of the bystander effect. To date, the induction of the bystander effect *in vitro* via media transfer has only been reported for gene and radiation therapy.[37,60–62] To the best of our knowledge, the EGF–micelles were among one of the first drug carrier systems shown to be capable of inducing a potent bystander effect because of its intrinsic toxicity. Conditioned medium collected from EGF–micelles with treated MDA-MB-468 cells reduced the viability of MCF-7 cells by 70%; however, free EGF at an equivalent concentration had a negligible effect. It was believed that 10 nM free EGF was not potent enough to induce the bystander effect in MCF-7 cells, while MDA-MB-468 cells treated with 100 nM free EGF reduced the cell viability of MCF-7 cells to 53% (data not shown). Indeed, the superior potency of the bystander effect induced by EGF–micelles may have contributed to the increased growth inhibitory effect of the EGF–micelles in comparison to that of free EGF (Figures 2a and 3a).

Conclusion

In summary, an apoptotic EGF-conjugated micelle system was developed for targeted combination therapy against EGFR-overexpressing cancers. The drug-free EGF–micelles were capable of inducing the bystander effect as well as cell-type-specific apoptosis via mechanisms similar to that of free EGF. Also, EGF–micelles were found to be more potent than

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free EGF at inhibiting EGFR-overexpressing breast cancer cell growth. The combined delivery of EGF with the chemotherapeutic drug, ELP, using block copolymer micelles resulted in a synergistic growth inhibitory effect in EGFR-overexpressing breast cancer cells. Overall, the conjugation of an apoptotic factor to block copolymer micelles may serve as a targeted nanotechnology platform for improved cancer therapy.

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