molecular pharmaceutics

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Multifunctional Block Copolymer Micelles for the Delivery of ^{111}In to EGFR-Positive Breast Cancer Cells for Targeted Auger Electron Radiotherapy

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Received August 15, 2009; Revised Manuscript Received November 2, 2009; Accepted November 5, 2009

Abstract: Block copolymer micelles (BCMs) can improve the payload delivery of therapeutic agents to tumors. Our aim was to construct hEGF-modified BCMs for the delivery of ^{111}In to tumor cells for Auger electron-emission radiotherapy of EGFR-positive breast cancer (BC). Multifunctional nanosized BCMs were prepared from MePEG$_{2500}$-*b*-PCL$_{1200}$ and ^{111}In-DTPA-PEG$_{3000}$-*b*-PCL$_{1600}$ with or without hEGF-PEG$_{2900}$-*b*-PCL$_{1400}$ (^{111}In-hEGF-BCMs or ^{111}In-BCMs). The resulting BCMs were analyzed by dynamic light scattering and transmission electron microscopy. Cellular uptake and nuclear importation were assessed in MDA-MB-468, MDA-MB-231 and MCF-7 BC cells with decreasing EGFR density. *In vitro* antiproliferative effects were evaluated using the WST-1 assay after 48 h with ^{111}In-hEGF-BCMs, and the clonogenic assay was used to determine the survival fraction (SF) after a 21 h exposure. Results were compared with ^{111}In-DTPA-hEGF, an established Auger electron-emitting radiotherapeutic that is currently in clinical development. Cell uptake and nuclear importation of ^{111}In-hEGF-BCMs decreased in the following order: MDA-MB-468 > MDA-MB-231 > MCF-7. Cellular uptake of ^{111}In-hEGF-BCMs was less than ^{111}In-DTPA-hEGF ($P < 0.05$) but was 4-fold higher than for ^{111}In-BCMs ($P < 0.001$). There was a significant growth inhibition of MDA-MB-468 cells by ^{111}In-hEGF-BCMs (6-fold inhibition, $P < 0.05$) while the growth of MDA-MB-231 and MCF-7 were not significantly inhibited. The SF of MDA-MB-468 cells was decreased to 2.6% while that for MCF-7 cells was 132.7%. ^{111}In-DTPA-hEGF reduced the SF of MDA-MB-468 cells to 0.4%. Nontargeted ^{111}In-BCMs had minimal effect on the SF of BC cells. Therefore, the ^{111}In-hEGF-BCMs were bound, internalized and transported to the nuclei of EGFR-positive BC cells, where the Auger electron emissions were lethal. The ^{111}In-hEGF-BCMs are a promising delivery system for targeted radiotherapy of BC.

Keywords: Epidermal growth factor receptor; Auger electron radiotherapy; breast cancer; block copolymer micelle; ^{111}In

Introduction

Epidermal growth factor receptors (EGFR) are a subfamily of the receptor tyrosine kinases (RTK) that regulate cell growth and survival as well as adhesion, migration, differentiation and other cellular processes.[1,2] EGFR/ErbB1 is a 170 kDa transmembrane glycoprotein that is overexpressed in a number of human cancers including head and neck, bladder, pancreas and breast.[3,4] Potent ligands of EGFR/ErbB1 include epidermal growth factor (EGF), amphiregulin (AR) and transforming growth factor α (TGFα). The development of therapies directed toward specific molecular targets has revolutionized the treatment of breast cancer (BC).

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10.1021/mp900201v
Published on Web 11/05/2009

The earliest of these was tamoxifen, which is indicated for the treatment of estrogen receptor (ER)-positive BC. High response rates (>80%) have been reported with the use of antihormonal therapies directed toward ER-positive BC.[5] In some human epidermal growth factor-2-positive (EGFR-2/HER-2) BC patients, anti-HER-2 humanized monoclonal antibody (mAb) trastuzumab (Herceptin) was found to be effective.[6] However, molecular investigations have shown that sensitivity to antihormonal therapy correlates inversely with levels of EGFR, indicative of poor prognosis.[7,8] In addition, acquisition of resistance (and therefore disease relapse) to most of the currently available cytostatic and cytotoxic agents has been common in clinical settings. However, lower relapse rates have been observed when radiotherapy is used in combination with these agents for early stage disease.[5]

Targeted radiotherapy relies on the selective delivery of radionuclides to tumor cells using antibodies or peptides that recognize tumor-associated antigens or receptors. Radioimmunotherapy employing mAbs radiolabeled with β-emitting (^{131}I, ^{90}Y) radioisotopes has achieved some success in oncology especially in the treatment of hematological malignancies such as non-Hodgkin's B-cell lymphoma.[9] These promising results unfortunately have been difficult to reproduce for solid tumors in which response rates are very low.[9,10] A major limitation for treatment of solid tumors is the dose limiting, nonspecific irradiation and killing of bone marrow stem cells associated with the long-range (2−12 mm; 200−1200 cell diameters) and moderate-high energy of the β-particles (E_β 0.6−2.2 MeV). In contrast, Auger electrons emitted by radionuclides such as ^{111}In are unique because although their energy is low (<25 keV), it is deposited over nanometer to at most micrometer distances in tissues, and thus their linear energy transfer (LET) approaches that of α-emitters.[11] The magnitude of DNA damage in a cancer cell is correlated with LET; thus, α-emitters and Auger electron-emitters theoretically have the potential to be the most toxic to cancer cells.[11] In addition, the subcellular range of the Auger electron-emitters restricts their toxicity to cells that internalize these radionuclides. Reilly et al. previously discovered that ^{111}In-labeled human EGF (^{111}In-DTPA-hEGF) was cytotoxic to human BC cells that overexpress EGFR. The survival fraction (SF) of MDA-MB-468 BC cells (1×10^6 EGFR/cell) treated *in vitro* with ^{111}In-DTPA-hEGF for 24 h was reduced to 3% while the SF for MCF-7 cells with a 100-fold lower receptor density was almost 100%.[12,13] However, in EGFR-overexpressing mouse xenograft models the amount of radioactivity delivered to the tumor was generally low, typically 2% of the injected dose per gram (% ID/g), due to the short circulation half-life of the radiopharmaceutical.[14,15] In order to improve efficacy, strategies are needed to increase the amount of radioactivity and consequentially, the radionuclide dose delivered to the tumor *in vivo*. As well, novel approaches that deliver multiple radionuclides per ligand-receptor recognition event have the potential to improve on the success already achieved with ^{111}In-DTPA-hEGF.

Block copolymer micelles (BCMs) are nanosized supramolecular assemblies of amphiphilic copolymers that have

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shown great promise as drug delivery vehicles[16−21] and similarly have potential for the delivery of radionuclides.[22−24] In aqueous media, the micelles consist of a hydrophobic core surrounded by a hydrophilic corona. The corona can be functionalized to include various targeting ligands, metal chelators for radionuclides and/or other imaging probes (e.g., ^{111}In, or ^{64}Cu or Gd) while the core can be loaded with drugs notably anticancer agents. BCMs can improve the amount of drug reaching the tumor by passive targeting via exploitation of the enhanced permeation and retention (EPR) effect.[25−27] The EPR effect refers to the accumulation of macromolecules or nanoparticles at tumors due to the leaky vasculature and poor lymphatic drainage at these sites.[21,28]

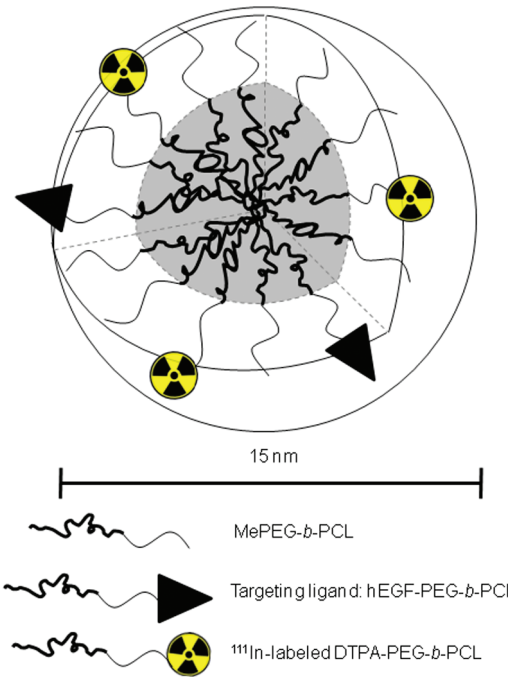

Figure 1. Schematic depicting ^{111}In-labeled-hEGF PEG-*b*-PCL copolymer micelles (^{111}In-hEGF-BCMs).

The macromolecules or nanoparticles must have prolonged residence times in the bloodstream in order to fully exploit the EPR effect.[21,25−27,29] Active targeting of BCMs to tumors can also be achieved by conjugation of moieties that recognize specific receptors or epitopes on the diseased tissues or organs.[30,31] Our group is currently exploring the use of functionalized BCMs for the treatment and diagnosis of EGFR overexpressing cancers.[30,32] In our recent study, it was shown that the incorporation of ^{111}In onto nontargeted BCMs (^{111}In-BCM) resulted in a ∼15-fold increase in the circulation half-life of the radionuclide, in comparison to ^{111}In-benzyl-DTPA and a consequent increase in tumor accumulation.[23] As well, *in vitro* studies revealed that hEGF-BCMs (hEGF > 0.05 nM) led to cell death in cells that overexpress EGFR.[23,30] For the first time in this report, we describe the potential of the nanosized hEGF-conjugated BCMs functionalized with DTPA and complexed with ^{111}In (Figure 1) for targeted Auger electron-emission radiotherapy of EGFR-positive BC cells. In this *in vitro* study we aim to

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evaluate cellular binding, internalization and cytotoxicity of multifunctional BCMs directed toward EGFR positive BC.

Materials and Methods

Materials. Methoxy polyethylene glycol (MePEG M_n = 2500, polydispersity index = 1.06) obtained from Sigma-Aldrich (St. Louis, MO) and H_2N-PEG (M_n = 3000, polydispersity index = 1.07) obtained from JenKem Technology (Allen, TX) were dried twice by azeodistillation in toluene. ε-Caprolactone (CL, 99%) was obtained from Sigma-Aldrich and was dried by *n*-butyllithium (at −78 °C), calcium hydride and molecular sieves, prior to use. Tetrahydrofuran (THF, 99.9%) and toluene (99.9%) were obtained from Sigma-Aldrich and were refluxed over a sodium−benzophenone complex and distilled under nitrogen. HCl (1.0 M in diethyl ether, HCl ether), sodium (99.5%) and diethylenetriaminetetraacetic acid dianhydride (DTPA dianhydride) were obtained from Sigma-Aldrich. hEGF was obtained from Upstate Biotechnology (Lake Placid, NY). $^{111}InCl_3$ was purchased from MDS Nordion (Kanata, ON, Canada). Carboxy-terminated poly(ethylene glycol)-*b*-poly(ε-caprolactone) (HOOC-PEG-*b*-PCL), methoxy-terminated PEG-*b*-PCL (MePEG-*b*-PCL) and amino-terminated PEG-*b*-PCL (H_2N-PEG-*b*-PCL) copolymers were prepared as previously reported.[32,33] *N*-Hydroxysuccinimidyl PEG-*b*-PCL activated ester (NHS-PEG-*b*-PCL) and hEGF-PEG-*b*-PCL were synthesized as previously described.[32] The conjugation efficiency (and the amount of hEGF conjugated to the micelle) was determined by the μBCA assay (Pierce Scientific) following the manufacturer's guidelines.

Synthesis of DTPA-PEG-*b*-PCL. To a stirred suspension of DTPA dianhydride (32 mg, 8.9 μmol) in dry DMF (2 mL), triethylamine (1.4 mg, 10 μmol) and N_2H-PEG-*b*-PCL (20 mg, 4.4 μmol) were added and the mixture was stirred at room temperature for 15 h. The reaction mixture was purified by flash column chromatography with an RP-C_8 stationary phase mini-prep column (Sigma, St. Louis, MO) using an isocratic (water) followed by a water/acetonitrile gradient. Unreacted DTPA was eluted with a gradient of water to water/acetonitrile 80/20 v/v, and pure DTPA-PEG_{3000}-PCL_{1600} was eluted from the column with water/acetonitrile 30/70 v/v. Acetonitrile was evaporated under N_2, and the DTPA-PEG-*b*-PCL residue was lyophilized prior to storage at 4 °C.

Characterization of Block Copolymers. ^1H NMR spectra were acquired with a Gemini 200 spectrometer (200 MHz for ^1H) in $CDCl_3$. The molecular weights of polymers were determined by analyzing the ratio of the proton intensities in the ^1H NMR spectra.[23] For example, the relative degree of polymerization of the polycaprolactone (PCL) block was calculated by ^1H NMR analysis (spectra not shown) by comparing the integrals of characteristic methylene peaks of the PCL block at 2.31 ppm and PEG block at 3.65 ppm in the ^1H NMR spectrum (Table 1). Gel permeation chromatography (GPC) measurements to determine the molecular weight and polydispersity index of the polymers were carried out at 40 °C using a Waters 2695 liquid chromatography system equipped with three Waters Styragel HR 4E columns (10 μm, 7.8 × 300 mm) connected in series (Waters Inc.; Mississauga, ON, Canada). The column effluent was connected to a refractive index detector (Waters Inc., Mississuaga, ON, Canada) which was previously calibrated using polystyrene standards (Polysciences Inc., Warrington, PA). THF with 1% triethylamine was used as mobile phase at a flow rate of 1.0 mL/min at 40 °C. The data was analyzed using Empower software (Waters Inc., Mississauga, ON, Canada). The critical micelle concentration (CMC) of the copolymer was determined using an established method with 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe.[34]

Table 1. Characteristics of Block Copolymers[a]

	MW-PEG	MW-PCL	PDI	CMC, mg/L
MePEG-*b*-PCL	2500	1200	1.12	102
H_2N-PEG-*b*-PCL	3000	1600	1.16	nd[b]
HOOC-PEG-*b*-PCL	2900	1400	1.13	nd

[a] PCL: Polycaprolactone. PEG: Polyethyleneglycol. PDI: Polydispersity index. CMC: Critical micelle concentration. MW: Molecular weights calculated from NMR spectra. [b] Not determined.

Preparation and ^{111}In-Labeling of Block Copolymer Micelles. MePEG-*b*-PCL micelles were prepared by the "dry-down" method as previously described.[35] Briefly, 50 mg of MePEG-*b*-PCL copolymer was dissolved in 1 mL of DMF and was stirred for 4 h, after which it was dried under an atmosphere of N_2 and reduced pressure in a vacuum overnight. The resulting dry film was then hydrated with 1 mL of filtered Milli-Q water at 60 °C with vortexing. The hydrated micelle solution was then stirred at room temperature for 48 h followed by 1 h sonication at room temperature.

DTPA-PEG-*b*-PCL (1−5 μg) was radiolabeled with ^{111}In-acetate ($^{111}InCl_3$/sodium acetate, 40−250 MBq) by incubation at pH 6.0 for 30 min. Purity of the complex was determined on Whatman ITLC-SG strips (Pall Life sciences, Mississuaga, ON, Canada) developed in 100 mM sodium citrate pH 5.0. ^{111}In-DTPA-PEG-*b*-PCL remains at the origin (R_f = 0) while free ^{111}In-DTPA/^{111}In-acetate migrates with the solvent front (R_f = 1).[23] ^{111}In-DTPA-PEG-*b*-PCL was obtained with high specific activity (40−50 MBq/μg) and radiochemical purity (96−98%). ^{111}In-hEGF-BCMs were prepared by adding different concentrations of hEGF-PEG-*b*-PCL and specific activities of ^{111}In-DTPA-PEG-*b*-PCL to the preformed MePEG-*b*-PCL BCMs. The mixture was stirred at 60 °C for 1 h and then at room temperature

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overnight. ^{111}In-BCMs were prepared in a similar manner but without hEGF-PEG-*b*-PCL. Radioactivity was measured using an automated gamma counter (Wallac, 1480 Wizard 3″, Perkin-Elmer, Turku, Finland).

Physico-Chemical Characterization of Block Copolymer Micelles. The hydrodynamic diameters of BCMs were determined by dynamic light scattering (DLS) at an angle of 90° at room temperature (90Plus Particle Size Analyzer, Brookhaven Instruments Corp., Holtsville, NY). Sample solutions of BCMs (1−20 mg/mL) were prepared in filtered Milli-Q water and analyzed by DLS. Micelle morphology was evaluated by transmission electron microscopy (TEM) with a Hitachi 7000 microscope operating at an acceleration voltage of 75 kV (Schaumburg, IL). 1 mg/mL samples of hEGF-BCMs and BCMs in water were diluted 1/1 v/v with a 2% solution of uranyl acetate and allowed to dry on negatively charged copper grids that had been precoated with carbon (Ted Pella Inc., Redding, CA). The copper grids were then blotted and left to stand to allow solvent evaporation. The average diameters of the copolymer aggregates in the TEM images were obtained using SigmaScan Pro software (Jandel Scientific).

Cell Culture. Human BC cells MDA-MB-468 (1×10^6 EGFR/cell), MDA-MB-231 (2×10^5 EGFR/cell) and MCF-7 (1×10^4 EGFR/cell) were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, for MDA-MB-231 and MCF-7) and RPMI 1640 medium (MDA-MB-468) containing 1% penicillin−streptomycin solution (1000 U/mL, Invitrogen, Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY). Subconfluent cells were harvested by trypsinization with trysin-EDTA (Sigma Aldrich, Mississauga, ON, Canada).

Receptor Binding Assay. ^{111}In-DTPA-hEGF was prepared by derivatization of hEGF with DTPA and labeled with ^{111}In (specific activity 8−10 MBq/μg) as previously described.[36] The binding of ^{111}In-hEGF-BCMs and ^{111}In-DTPA-hEGF to EGFR on MDA-MB-468 human BC cells was measured by a direct receptor binding assay. Briefly, ^{111}In-hEGF-BCMs (1, 2, or 4 nM, hEGF equiv) or ^{111}In-DTPA-hEGF (1, 2, or 4 nM, hEGF equiv) in 1 mL of 150 mM saline was added to microtubes containing 1×10^6 MDA-MB-468 cells. After incubation at 4 °C for 1 h, the tubes were centrifuged to separate the total bound radioactivity (TB) (cell pellet) from free radioactivity (supernatant). The cells were rinsed three times with 150 mM saline, and the rinses were added to the previously removed supernatant. Nonspecific binding (NSB) was determined by repeating the assay in the presence of 100 nmol/L hEGF. Specific binding (SB) was obtained by subtracting NSB from TB.

Intracellular Distribution of ^{111}In-hEGF-BCMs and ^{111}In-BCMs. The intracelluar distribution (cytoplasmic and nuclear radioactivity) of ^{111}In-hEGF-BCMs was determined by first seeding 2×10^6 MDA-MB-468, MDA-MB-231 or MCF-7 BC cells per well in 6-well plates. The cells were cultured for 24 h and then incubated with 1−1.5 MBq ^{111}In-hEGF-BCMs (equivalent to a concentration of 150 nM, hEGF), ^{111}In-BCMs, or ^{111}In-DTPA-hEGF (150 nM, hEGF) for 1 h at 37 °C. Unbound radioactivity was removed followed by rinsing three times with PBS. Cell surface-bound ^{111}In-hEGF-BCMs, ^{111}In BCMs, or ^{111}In-DTPA-hEGF was removed and collected by rinsing the cells with 200 mM sodium acetate, pH 2.5. Cytoplasmic and nuclear radioactivity were isolated using a nuclei isolation kit (Nuclei EZ Prep Nuclei Isolation Kit, NUC-101; Sigma-Aldrich, Mississauga, ON, Canada) according to the manufacturer's directions. The surface-bound, cytoplasmic, and nuclear fractions were measured using a gamma counter. This procedure for isolation of nuclei has been previously confirmed by our group to be of high quality.[37]

Cell Proliferation Assay. The effect of exposure to ^{111}In-hEGF-BCMs on the proliferation of BC cells was determined using the WST-1 assay (Roche Diagnostics Gmbh, Mannheim, Germany). The assay is a colorimetric assay that measures cell viability by determination of the rate of conversion of WST-1 reagent (light red) to formazan (dark red) by mitochondria enzyme succinic dehydrogenase. The resulting shift in absorbance is measured by a UV−vis spectrophotometer at 440 nm. Approximately 2×10^3 MDA-MB-468, MDA-MB-231 or MCF-7 BC cells per well were seeded into 96-well plates. Cells were treated with ^{111}In-hEGF-BCMs, ^{111}In-BCMs, ^{111}In-DTPA-hEGF, ^{111}In-acetate, DTPA-hEGF, or media alone (total volume of 1 mL) for 48 h. The following treatment groups and proportions of hEGF/^{111}In were used for studies of the ^{111}In-hEGF-BCMs: hEGF/^{111}In was 75 nM/50 kBq, 150 nM/50 kBq, 300 nM/50 kBq, 150 nM/100 kBq or 150 nM/200 kBq. ^{111}In-BCMs were studied at similar radioactivity amounts but without hEGF in the micelles. The concentration of MePEG-*b*-PCL was 125 μM. The ^{111}In-DTPA-hEGF treatment group consisted of hEGF/^{111}In in 75 nM/50 kBq, 150 nM/100 kBq or 300 nM/200 kBq ratios. The DTPA-hEGF group had similar concentrations of unlabeled hEGF as that for ^{111}In-DTPA-hEGF. Control groups consisted of ^{111}In-acetate, unlabeled BCMs, or media alone. Ten microliters of the cell proliferation reagent WST-1 was added to the wells followed by a 2 h incubation at 37 °C under 5% CO_2. The plates were read with a BioTek microplate reader (Winooski, VT) at 440 nm. The proportion of viable cells was calculated as follows: $(A_{\text{treated}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})$ where A is absorbance at 440 nM. The control group consisted of untreated cells.

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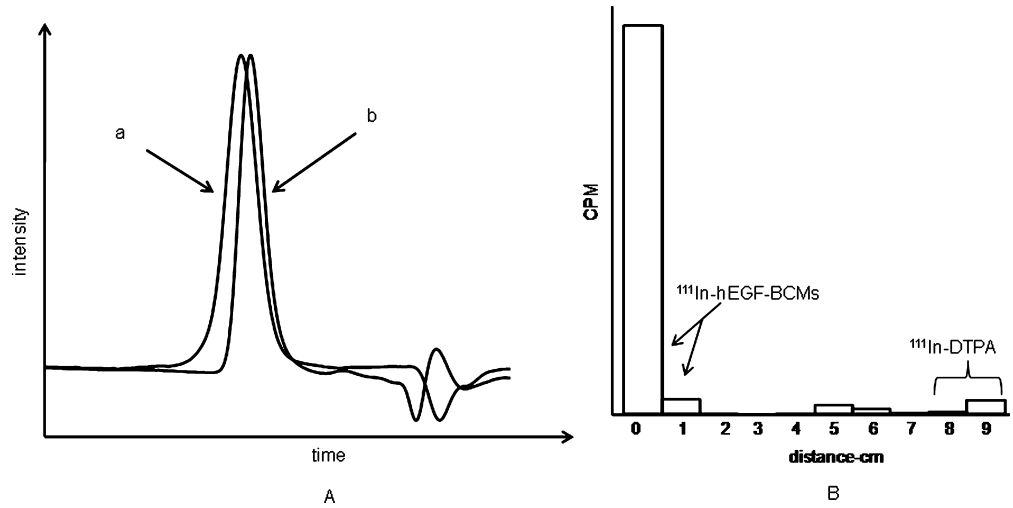

Figure 2. (A) Gel permeation chromatograms (GPC) of HOOC-PEG_{2900}-*b*-PCL_{1400} (a) and HOOC-PEG_{2900} (b). (B) Radiometric trace (ITLC chromatogram) of the crude labeled mixture of ^{111}In-DTPA-PEG-*b*-PCL.

Clonogenic Survival Assay. The clonogenic assay (or cell survival assay) measures the ability of a single cell to grow into a colony (>50 cells). The SF is a measure of the fraction of cells that are plated and grow (those unable to form colonies eventually die) to form colonies after treatment with cytotoxic agents.[38] The SF of BC cells exposed *in vitro* to ^{111}In-BCMs was determined by treating 1×10^6 MDA-MB-468, MDA-MB-231 or MCF-7 cells per well in 6-well plates for 21 h at 37 °C and 5% CO_2. The treatment groups were similar to those analyzed using the WST-1 assay (see above), however, the concentration of ^{111}In in each well was 10 times greater. After the 21 h treatment period, unbound radioactivity was removed followed by rinsing three times with PBS, pH 7.5. Approximately 1×10^3 to 1×10^4 cells were then seeded in T25 flasks, and the cells were cultured at 37 °C under 5% CO_2 in the appropriate growth medium supplemented with 10% FBS for 7−10 days, during which they developed colonies (>50 cells). The plating efficiency was determined by dividing the number of colonies formed in the control wells by the number of cells seeded. The SF was determined by dividing the number of colonies formed in the treated wells by the number of cells seeded and multiplying by the plating efficiency.

Statistical Analysis. All data was expressed as the mean ± standard deviation (SD) of at least 3 independent experiments. Statistical significance was assessed using a two-tailed Student's *t* test (receptor binding assay and intracellular distribution studies) and one-way ANOVA with Bonferroni's correction (*in vitro* WST-1 cytotoxicity studies) with $P < 0.05$ considered to be significant. All graphs were generated using GraphPad Prism (version 4.03; GraphPad, La Jolla, CA).

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Results

Synthesis and Characterization of Block Copolymer Micelles. The characteristics of the copolymers used in these studies are summarized in Table 1. DTPA was conjugated to H_2N-PEG_{3000}-*b*-PCL_{1600} with a yield of ~77%. Figure 2A shows the GPC chromatograms for HOOC-PEG_{2900}-*b*-PCL_{1400} (a) and HOOC-PEG_{2900} (b). Conjugation of hEGF was afforded via a lysine in hEGF using the *N*-hydroxysuccinimidyl activated ester of carboxyl PEG_{2900}-*b*-PCL_{1400} (NHS-PEG-*b*-PCL) at pH 8.5. A conjugation efficiency of 30−50% was obtained. A radiolabeling yield ≥98% was obtained for ^{111}In-DTPA-PEG_{3000}-*b*-PCL_{1600}. Figure 2B shows the radiometric trace of an ITLC of the labeling mixture.

DLS analyses revealed mean diameters of 15 ± 0.8 nm and 13 ± 1 nm with monomodal size distributions for the BCMs and hEGF-BCMs, respectively. BCMs were found

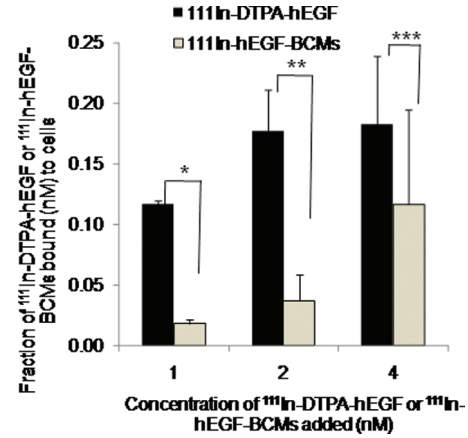

Figure 3. Plot of specific binding (total binding − nonspecific binding) of radioactivity for binding of ^{111}In-DTPA-hEGF and ^{111}In-hEGF-BCMs to MDA-MB-468 cells. The fraction of ^{111}In-hEGF-BCMs bound was less than ^{111}In-DTPA-hEGF at all concentrations ($P < 0.05$ for *, ** and ***).

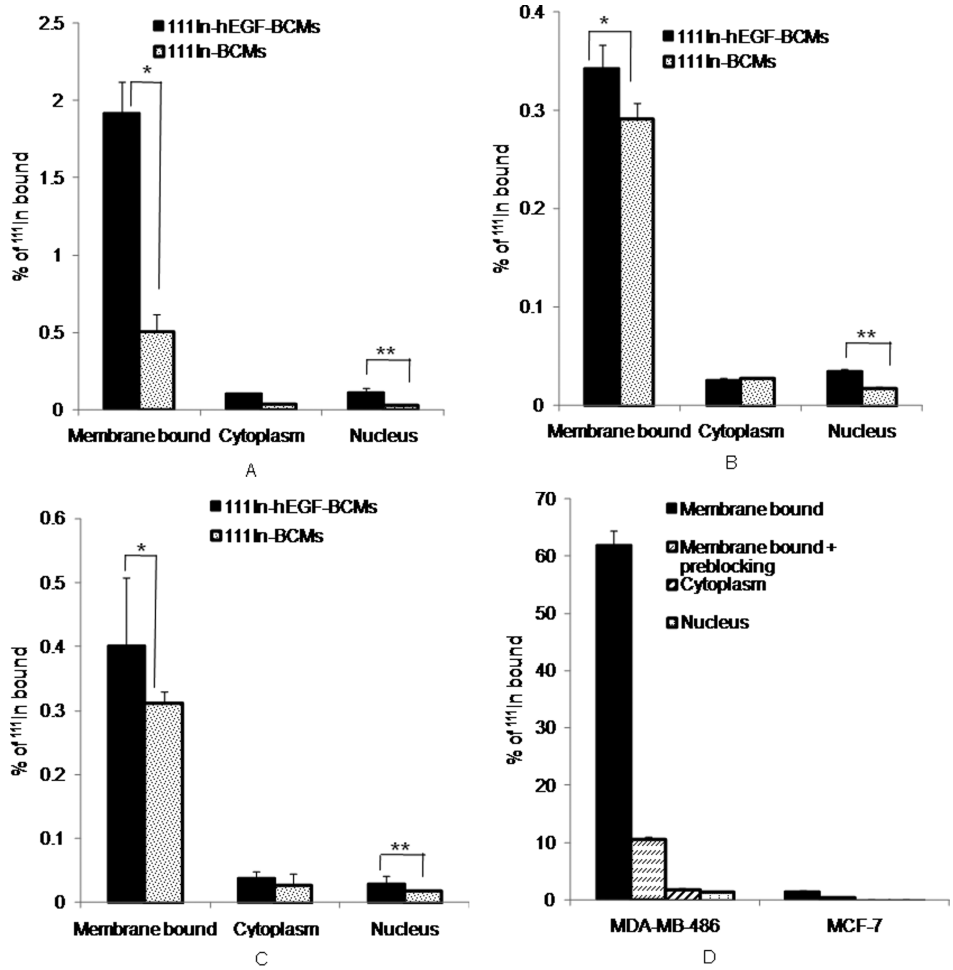

Figure 4. Cellular distribution of ^{111}In-hEGF-BCMs and ^{111}In-BCMs after 1 h incubation with (A) MDA-MB-468 cells (cell associated and nuclei imported ^{111}In-hEGF-BCMs were greater than that obtained for ^{111}In-BCMs with $*P = 0.0003$ and $**P = 0.008$, respectively); (B) MDA-MB-231 cells ($*P = 0.041$ and $**P = 0.006$); and (C) MCF-7 cells ($*P$ and $**P > 0.05$). (D) Cellular distribution of ^{111}In-DTPA-hEGF in MDA-MB-468 and MCF-7 cells.

to retain their size following incubation in plasma. TEM analysis of the BCMs and hEGF-BCMs showed mainly spherically shaped micelles with a few rodlike structures (results not shown).

Receptor Binding and Intracellular Distribution of ^{111}In-hEGF-BCMs. Figure 3 shows a comparison of the specific binding of ^{111}In-DTPA-hEGF and ^{111}In-hEGF-BCMs on MDA-MB-468 cells. The fraction of bound ^{111}In-DTPA-hEGF to MDA-MB-468 cells was higher than that for ^{111}In-hEGF-BCMs at all concentrations ($P < 0.05$).

As shown in Figure 4A, the amount of ^{111}In-hEGF-BCMs associated with MDA-MB-468 cells (1×10^6 EGFR/cell) following a 1 h incubation at 37 °C was 1.9 ± 0.2% (percentage of total ^{111}In-hEGF-BCMs incubated with the cells), which was almost 4-fold higher than that for ^{111}In-BCMs (0.37 ± 0.11%) ($P = 0.0003$). Nuclear importation was 5-fold higher for ^{111}In-hEGF-BCMs (0.11 ± 0.03%) than for ^{111}In-BCMs (0.02 ± 0.005%) ($P = 0.008$).

In the case of MDA-MB-231 cells (1×10^5 EGFR/cell), the ratio of cell-associated radioactivity for ^{111}In-hEGF-BCMs to ^{111}In-BCMs was 1.2 (Figure 4B) ($P = 0.041$) after 1 h incubation. Nuclear importation was 2-fold greater for the ^{111}In-hEGF-BCMs (0.035 ± 0.003%, of the total ^{111}In-hEGF-BCMs incubated with the cells) compared to ^{111}In-BCMs (0.017 ± 0.002%, of the total ^{111}In-BCMs incubated with the cells) ($P = 0.006$).

There were no significant differences in cell-associated ^{111}In-hEGF-BCMs (0.41 ± 0.1% of total ^{111}In-hEGF-BCMs incubated with the cells) compared to ^{111}In-BCMs (0.31 ± 0.02%) after 1 h incubation (Figure 4C) in MCF-7 cells (1×10^4 EGFR/cell) ($P = 0.231$). There was no significant difference ($P = 0.110$) in nuclear importation between ^{111}In-hEGF-BCMs and ^{111}In-BCMs in MCF-7 cells. In MDA-MB-468 cells 62 ± 2.4% of ^{111}In-DTPA-hEGF was cell-associated compared to 1.3 ± 0.24% in MCF-7 cells (Figure 4D), indicating the importance of cell surface EGFR expression on the binding of EGF-associated molecules. Also, the amount of ^{111}In-DTPA-hEGF that was internalized and imported into the nuclei of MDA-MB-468 cells was 20-fold higher than that for MCF-7 cells. In general, receptor-mediated internalization was found to be much more efficient when MDA-MB-468 cells were treated with ^{111}In-DTPA-hEGF (Figure 4D) in comparison to ^{111}In-hEGF-BCMs (Figure 4A).

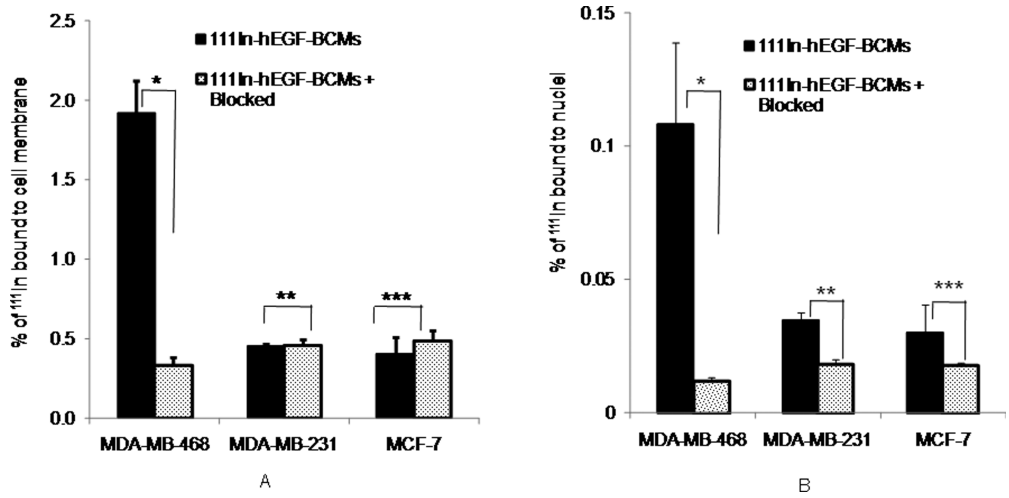

Figure 5. Effect of preblocking with hEGF (100 nM) on (A) cell uptake (*$P = 0.002$, **$P = 0.401$ and ***$P = 0.312$) and (B) nuclear importation of ^{111}In-hEGF-BCMs in MDA-MB-468, MDA-MB-231 and MCF-7 cells (*$P = 0.005$, **$P = 0.001$ and ***$P = 0.12$).

To further assess the targeting specificity of the ^{111}In-hEGF-BCMs, cells were preblocked with 100 nM of hEGF for 30 min prior to radiopharmaceutical incubation; the amount of cell-associated (Figure 5A) radioactivity and radioactivity imported into the nuclei (Figure 5B) were assessed. As shown in Figure 5A there was a significant decrease in cell-associated radioactivity for MDA-MB-468 ($P = 0.002$) while there was no significant difference in the case of MDA-MB-231 ($P = 0.401$) and MCF-7 ($P = 0.310$) cells when preblocked with hEGF. As can be seen in Figure 5B, MDA-MB-468 cells that were preblocked with hEGF had the least amount of radioactivity imported into the nucleus. Nuclear importation of ^{111}In-hEGF-BCMs in MDA-MB-468 and MDA-MB-231 cells decreased by 9- ($P = 0.005$) and 2-fold ($P = 0.001$), respectively, when the cells were preblocked compared to unblocked. There was no significant decrease in nuclear importation when MCF-7 cells were preblocked ($P = 0.12$) compared to unblocked.

Cell Proliferation Assay. After a 48 h treatment period, growth inhibition was found to be significant for all treatment groups compared to nontreated in MDA-MB-468 cells ($P < 0.001$). MDA-MB-231 and MCF-7 cells on the other hand were not significantly inhibited compared to the nontreated group ($P > 0.05$). As shown in Figure 6, ^{111}In-hEGF-BCMs and ^{111}In-DTPA-hEGF inhibited the growth of MDA-MB-468 cells by 6- and 3.5-fold compared to nontreated cells ($P < 0.001$), respectively (the difference between ^{111}In-hEGF-BCMs and ^{111}In-DTPA-hEGF was not statistically significant). ^{111}In-hEGF-BCMs and ^{111}In-DTPA-hEGF did not significantly inhibit the growth of MDA-MB-231 cells ($P > 0.05$). Compared to ^{111}In-hEGF-BCMs, growth inhibition of ^{111}In-BCMs on MDA-MB-468 was less profound (1.9-fold, $P < 0.05$).

Clonogenic Survival Assay. Figure 7 shows the SF of MDA-MB-468 and MCF-7 cells following various treatments. In general, the SF was lower for MDA-MB-468 cells than for MCF-7 cells for most treatment groups. The SF of MDA-MB-468 cells treated with increasing amounts (1 MBq, 2 MBq or 4 MBq) of ^{111}In-DTPA-hEGF (specific activity of 83 MBq/μmol) was <1% for the three different treatment groups while these treatments had no effect on the MCF-7 cells (for simplicity treatment groups with 2 MBq or 4 MBq are omitted in Figures 6 and 7).

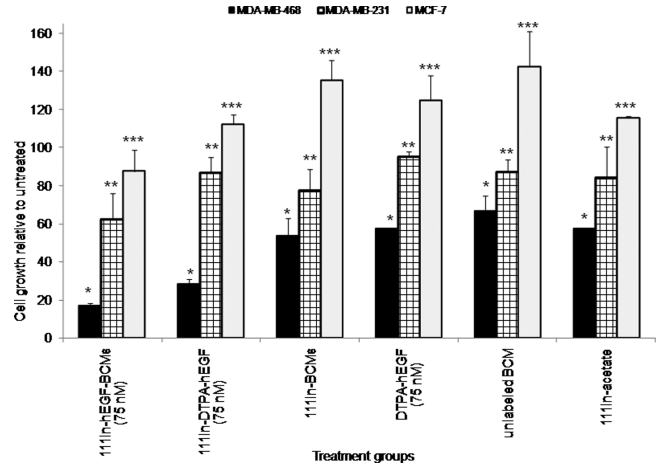

Figure 6. Proliferation assay (WST-1) for MDA-MB-468, MDA-MB-231 and MCF-7 cells after 48 h treatment (see text for description of treatment groups). *$P < 0.05$ for all treatment groups compared to untreated (media alone) for MDA-MB-468 cells and **$P > 0.05$ for all treatment groups compared to untreated for MDA-MB-231 and ***$P > 0.05$ for MCF-7 cells.

As shown in Figure 7 the cytotoxicity of ^{111}In-hEGF-BCMs ($0.4 \pm 0.01\%$) on MDA-MB-468 cells was less than for ^{111}In-DTPA-hEGF. The SF of MDA-MB-468 cells treated with ^{111}In-hEGF-BCMs (75 nM hEGF equivalent, 1 MBq) or (300 nM hEGF equivalent, 1 MBq) were $9.1 \pm 3.2\%$ or $2.6 \pm 2.0\%$, respectively. Both ^{111}In-hEGF-BCMs and ^{111}In-DTPA-hEGF were not cytotoxic to MCF-7 cells. As well the nontargeted ^{111}In-BCMs were not cytotoxic to MDA-MB-468 and MCF-7 cells.

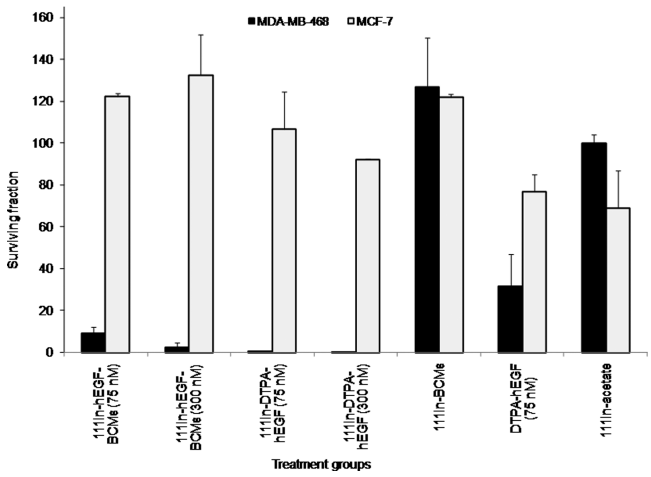

Figure 7. Survival fraction of MDA-MB-468 and MCF-7 cells after 21 h treatment (see text for description of treatment groups).

Discussion

This study represents the first to evaluate BCMs for delivery of therapeutic radionuclides to EGFR-positive BC cells. BCMs have several properties that make them very attractive as delivery systems. First, BCMs of different sizes and morphologies can be easily prepared to suit a particular application.[39] As well the BCMs can be engineered to have long circulation half-lives *in vivo* and thus accumulate at solid tumors via the EPR effect. In addition, BCMs can easily be functionalized to include different targeting moieties on their surface that recognize specific epitopes/receptors on diseased tissues, including tumors.[40] Recently, Mamot et al. demonstrated an enhanced antitumor effect of doxorubicin loaded anti-HER2 mAb targeted immunoliposomes compared to free doxorubicin or nontargeted doxorubicin loaded liposomes (mean size 86 nm) in a HER-2 overexpressing mouse xenograft model.[41] Although the tumor accumulations of the anti-HER2 immunoliposomes and nontargeted immunoliposomes were comparable, the anti-HER2 immunoliposomes enabled a 6-fold increase in cellular uptake at the tumor site compared to nontargeted liposomes 24 h following iv administration. One of the advantages of BCMs over many other colloidal carriers such as liposomes is that they can be made to have much smaller hydrodynamic diameters which may result in improved penetration in solid tumors.[42] In the present study, the hEGF-PEG-*b*-PCL BCMs had a spherical morphology with an average diameter of 15 nm and may therefore allow for enhanced tumor penetration *in vivo*.[43]

Many authors have shown that transport of BCMs across cell membranes proceeds via an endocytotic mechanism that is pH, temperature, time and energy dependent.[44,45] Transport of hEGF (and therefore ^{111}In-DTPA-hEGF) occurs via receptor mediated endocytosis.[1] Also, using flow cytometry it has been shown that anti-EGFR mAb immunoliposomes internalize into EGFR-positive MDA-MB-468 cells by receptor mediated endocytosis. As shown in Figures 4D and 5, preblocking cell surface EGFR with excess hEGF in MDA-MB-468 cells revealed that uptake of ^{111}In-DTPA-hEGF and ^{111}In-hEGF-BCMs was significantly reduced, thereby confirming that uptake and internalization of ^{111}In-hEGF-BCMs also occurs by receptor-mediated endocytosis.

To be most radiotoxic ^{111}In-hEGF-BCMs (as well as ^{111}In-DTPA-hEGF) have to be internalized into cancer cells and localize in close proximity to the chromosomal DNA due to the submicrometer range of the Auger electron-emitters.[47] Dosimetry estimates show that the lethal effect can be up to 35-fold greater when the ^{111}In is located in the nucleus compared to when it is located on the cell surface.[48] ^{111}In-hEGF-BCMs were evaluated in comparison to ^{111}In-DTPA-hEGF for delivery of ^{111}In to EGFR-positive BC cells and the nucleus of these cells *in vitro*. It was found that 1.4% of total ^{111}In-DTPA-hEGF incubated with cells was imported into the nuclei of MDA-MB-468 cells and this was diminished 20-fold in MCF-7 cells. On the other hand, the amount of ^{111}In-hEGF-BCMs imported into the nuclei (0.11% of total ^{111}In-hEGF-BCMs incubated) of MDA-MB-468 cells diminished 4-fold in MCF-7 cells. In the current study, nuclear importation of ^{111}In-hEGF-BCMs in the EGFR-positive cells decreased in the following order: MDA-MB-468 > MDA-

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MB-231 > MCF-7 (Figure 4A−C). Nuclear importation of ^{111}In-hEGF-BCMs was lower than ^{111}In-DTPA-hEGF at 1 h postincubation in MDA-MB-468 cells. The most significant difference between ^{111}In-DTPA-hEGF and ^{111}In-hEGF-BCMs is the cell-associated uptake of the radiopharmaceuticals after 1 h incubation. Cell-associated ^{111}In-DTPA-hEGF in MDA-MB-468 cells was 30-fold higher than for ^{111}In-hEGF-BCMs (Figures 4A and 4D). A plausible explanation for the decreased cell-association of ^{111}In-hEGF-BCMs is the steric hindrance that may arise due to the conjugation of hEGF to the surface of the micelles. In addition, cell uptake and internalization of BCMs and other nanoparticles generally occurs slowly.[46,49] Uptake of ^{111}In-DTPA-hEGF peaks after 1 h incubation while uptake of (nontargeted) BCMs is a slow process and peaks at 24 h post incubation for some BCMs.[49] However, receptor-mediated internalization of targeted nanoparticles such as anti-EGFR mAb-targeted immunoliposomes can be much more efficient and has been found to plateau as soon as 4 h.[46] Due to these limitations fewer ^{111}In were delivered to the nuclei of EGFR-positive BC cells *in vitro* by ^{111}In-hEGF-BCMs in comparison to ^{111}In-DTPA-hEGF. However, as has been observed for other nanoparticles, BCMs can increase delivery of radionuclide/drug to tumors *in vivo* via exploitation of the EPR effect.[21,27,29,42] Recently, we showed that incorporation of ^{111}In onto nontargeted BCMs led to a 15-fold increase in the circulation half-life of the radionuclide and improved tumor accumulation in mice bearing MDA-MB-231 tumor xenografts.[23] *In vivo* studies will need to be conducted to determine if the increase in tumor accumulation of ^{111}In that may be provided using hEGF-BCMs as a delivery system translates into improved efficacy, in comparison to ^{111}In-DTPA-hEGF.

The WST-1 assay measures the activity of mitochondrial succinate dehydrogenase for which decreased activity is one of the hallmarks of apoptosis (intrinsic apoptosis pathway). Therefore other triggers of apoptosis (e.g., extrinsic apoptosis pathway) and other forms of cell death cannot be measured by WST-1. In this way, the clonogenic assay provides a better measure of cell survival and death. Some major differences in growth inhibition were observed for the different treatment groups. Use of the WST-1 assay to analyze cells 48 h (Figure 6) after treatment showed ^{111}In-hEGF-BCMs were growth inhibitory toward MDA-MB-468 cells. This is in agreement with a previous study where we showed by MTT assay that unlabeled hEGF-BCMs (0.1−10 nM hEGF) resulted in profound death of MDA-MB-468 cells after 72 h treatment while 24 h treatment did not result in a significant cell death.[30] Unlabeled EGF paradoxically has growth-inhibitory effects on MDA-MB-468 cells with high levels of EGFR, but is much less toxic than ^{111}In-hEGF.[50] Some nonspecific uptake of ^{111}In-BCMs (significantly lower than ^{111}In-hEGF-BCMs in MDA-MB-468 and MDA-MB-231 cells) was observed in all three cell lines. Nonspecific binding of ^{111}In-BCMs inhibited the growth of MDA-MB-468 cells but not MDA-MB-231 and MCF-7 cells. However, this (low) nonspecific uptake did not affect the SF of the cells. The SF assay suggests that the higher the concentration of hEGF-PEG-*b*-PCL copolymer in the BCMs, the higher the amount of ^{111}In imported into the nuclei and, consequently, the lower the SF of MDA-MB-468 cells (Figure 7). The SF of MDA-MB-468 cells treated with ^{111}In-DTPA-hEGF (1 MBq, 83 μmol/MBq) was 0.4%, which is in agreement with another recent observation by our group.[13] Therefore, ^{111}In-DTPA-hEGF was more cytotoxic than ^{111}In-hEGF-BCMs.

Conclusion

We have shown that ^{111}In-hEGF-BCMs in comparison to ^{111}In-DTPA-hEGF have a lower affinity for EGFR *in vitro*. Despite their reduced affinity, ^{111}In-hEGF-BCMs were effective at targeting and killing BC cells that express high levels of EGFR. Nontargeted ^{111}In-BCMs inhibited the growth of MDA-MB-468 cells but did not affect their SF. Targeting and killing of BC cells were enhanced by the incorporation of EGF as targeting ligand, an effect that could potentially be further enhanced *in vivo* via exploitation of the EPR effect that is amenable to BCMs. The retention of cellular binding, internalization and cytotoxicity of ^{111}In-hEGF-BCMs in EGFR positive BC cells encourages further evaluation of this micelle formulation *in vivo*. Future studies will evaluate the pharmacokinetics, biodistribution and efficacy of the ^{111}In-hEGF-BCMs in comparison to ^{111}In-DTPA-hEGF in animal xenograft models of EGFR-positive BC.

Acknowledgment. This study was supported by a grant from the Canadian Institutes of Health Research and Canadian Breast Cancer Research to C.A. and RMR The authors also thank Dr. Conrad Chan for technical assistance.

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