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Peptide Modified Polymeric Micelles Specific for Breast Cancer Cells

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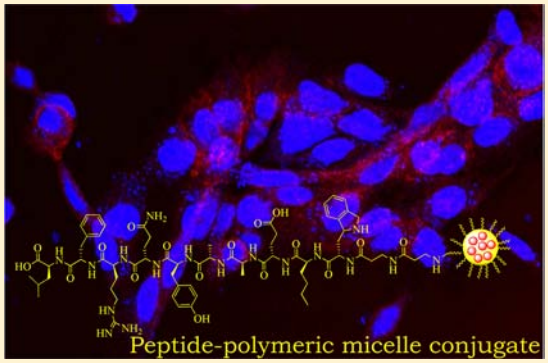
ABSTRACT: The specific targeting ability of novel breast cancer targeting peptides as ligands coupled to polymeric micelles was evaluated in the present study. In this context, engineered breast cancer cell targeting peptides, denoted as peptide **11** (RGDPAYQGRFL) and peptide **18** (WXEAAYQRFL), were compared with the lead 12-mer p160 peptide and cyclic RGDfK peptide. All four peptides were conjugated individually to poly(ethylene oxide)-*b*-poly(caprolactone) (PEO-*b*-PCL) diblock polymeric micelles to obtain targeted carrier systems PM11, PM18, PM 160, and PM c-RGD. Physical blending of the peptides **11** and **18** with PEO-*b*-PCL was also done to yield combination micelles, comPM11 and comPM18. The structural confirmation of polymer was carried out using ^{1}H NMR and MALDI-TOF, and the size distribution and zeta potential of the micelles were determined using dynamic light scattering. Lipophilic cyanine fluorescent probe DiI was physically incorporated in the polymeric micelles to imitate the hydrophobic drug loaded in the micellar core. The cellular uptake of DiI-loaded peptide-containing polymeric micelles by MDA-MB-435, MDA-MB-231, and MCF7 breast cancer cell lines, as well as HUVEC and MCF10A noncancerous cells, were analyzed using flow cytometry and confocal microscopy techniques. Modification of polymeric micelles with peptide **11** or **18** led to an increase in micellar uptake specifically in breast cancer cells compared to p160, c-RGD modified, or naked micelles. The peptide−micelle combinations (comPM11 and comPM18) displayed better uptake by the cells compared to the covalently conjugated PM11 and PM18 micelles; however, the combinations were less selective toward cancer cells. The results point to a potential for peptides 11- and 18-micelle conjugates as attractive platforms for improved performance of a wide range of chemotherapeutic drugs and/or imaging agents in cancer therapy and diagnosis.



INTRODUCTION

Success in cancer treatment using chemotherapy is constantly challenged by poor selectivity of anticancer drugs for the diseased cells and/or their limited access to the cancer cells.[1] Targeted drug delivery methods have been explored to improve the performance of chemotherapeutics through redirecting the anticancer drug away from healthy cells and toward cancerous tissues.[2−4] An increase in the selective accumulation and interaction of chemotherapeutics with cancer cells is expected to enhance the therapeutic index of anticancer agents not only by lowering drug side effects but also through enhancing drug efficacy. To date, many strategies have been explored to enhance the selectivity of anticancer drugs for cancer cells. Some of these strategies rely on the natural differences between cancer and healthy cells.[5−8] For example, overexpression of surface receptors on cancer cells that are absent or minimally expressed on healthy cells has been utilized to increase the selectivity of anticancer drugs to cancer cells.[9] Different ligands interacting with such receptors have been explored to target chemotherapeutic agents to cancer cells. This includes the use of engineered antibodies,[10,11] tumor-homing peptides,[12−17] and aptamers.[18,19] Among these approaches the use of antibodies has received a lot of attention for a number of years. While the use of antibodies is an interesting and viable option for targeting cancer cells, their level of success in cancer targeting has been limited because of their large size, possible immunogenicity, production cost, low physicochemical stability, and short *in vivo* half-life.[20] Peptides could overcome some of these limitations. Peptides are smaller, have excellent tissue penetration properties, and can easily be chemically conjugated with drugs and oligonucleotides. Besides, peptides are not taken up by the reticuloendothelial system (RES) like antibodies, so they are expected to cause minimal side effects to bone marrow, liver, or spleen.[21]

A number of peptide sequences have been identified by peptide phage display as targeting agents for different tumors and cell types.[22−24] Among these, tumor-homing peptides containing RGD and NGR sequences have received particular attention.[25,26] These peptides target the $\alpha v\beta 3$ integrin and aminopeptidase N receptors, respectively, that are overexpressed on the tumor cells and tumor vasculature.[27−30] Another 12-mer peptide, p160, with a so far unidentified receptor, has been shown to bind MDA-MB-435, MCF7, and WAC-2 human cancer cells strongly and specifically.[31−33] Further, *in vivo* biodistribution experiments in tumor-bearing mice showed greater uptake and retention of p160 peptide after organ

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perfusion by tumors compared to normal organs.[31] Relative to the RGD-4C peptide, p160 showed high accumulation in tumor versus normal organs.

Using p160 as a lead peptide, we previously designed and synthesized a library of 70 peptides on a cellulose membrane.[34] From this work we identified peptides **11** (RGDPAYQGRFL) and **18** (WXEAAYQRFL) with better targeting affinity than previously reported cancer targeting peptides p160 (VPWXE-PAYQRFL) and cyclic RGDfK.[34] Peptides **11** and **18** displayed up to 3-fold higher binding affinity for MDA-MB-435 and MCF7 cancer cell lines than for the p160 peptide, with insignificant affinity for noncancerous human umbilical vein endothelial cells (HUVECs). The apparent dissociation constant of peptide **18** for recognizing MDA-MB-435 cells was found to be in the low micromolar range (K_d 42 μM).[34]

These targeting peptides can be either linked directly to the chemotherapeutic agent or used to decorate the surface of nanocarriers such as polymeric micelles. Recent studies show that polymeric micelles have the potential to act as nanoscopic carriers unrecognizable by the phagocytic cells of RES, resulting in prolonged blood circulation. Polymeric micelles have therefore emerged as a promising tool for delivering various drugs with remarkable *in vitro* and *in vivo* success.[35−37] Studies suggest that attachment of cell-specific peptides on the surface of nanocarriers such as polymeric micelles can be used as an efficient strategy to enhance cellular internalization of nanocarriers loaded with chemotherapeutic agents in the desired tissue.[38,39] Previously we showed that p160-conjugated micelles display enhanced selective toxicity of encapsulated anticancer drug paclitaxel against MDA-MB-435 cancer cells over normal HUVEC and MCF10A cells.[40]

The aim of this study was to evaluate the binding of micelles modified with engineered derivatives of p160 (peptides **11** and **18**) with breast cancer cells. Peptides were either covalently conjugated to the surface of poly(ethylene oxide)-*b*-poly(caprolactone) (PEO-*b*-PCL) micelles (Figure 1) or physically mixed with the micellar solution. Physically encapsulated fluorescent probe (DiI) in PEO-b-PCL was used to label the polymeric micelles. PEO-*b*-PCL was chosen due to its biocompatibility, biodegradability, and sufficient *in vivo* stability, as well as great promise in tumor-targeted drug delivery through EPR effects.[40,41] Both the peptide−micelle conjugates and combinations were evaluated for specific uptake by the cancer and noncancerous cells using flow cytometry and fluorescence imaging techniques. Our results show that peptide−micelle conjugates with peptides **11** and **18** are excellent formulations for targeted delivery to breast cancer cells.

EXPERIMENTAL SECTION

Materials. Wang resin (1.1 mmol/g), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt), and the Fmoc-amino acids were purchased from NovaBiochem (San Diego, CA), while piperidine was obtained from Caledon. *N,N′*-Diisopropylcarbodiimide (DIC), *N,N*-dimethylformamide (DMF), *N*-methyl morpholine (NMM), trifluoroacetic acid (TFA), tetrahydrofuran (THF), ethylene oxide (EO), 3,3 diethoxy-1-propanol (DEP), and potassium naphthalene all were purchased from Sigma-Aldrich (St. Louis, MO, USA). ε-Caprolactone was purchased from Lancaster Synthesis (MA, USA). Fluorescent probe [1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide] (DiI), DAPI-antifade, and

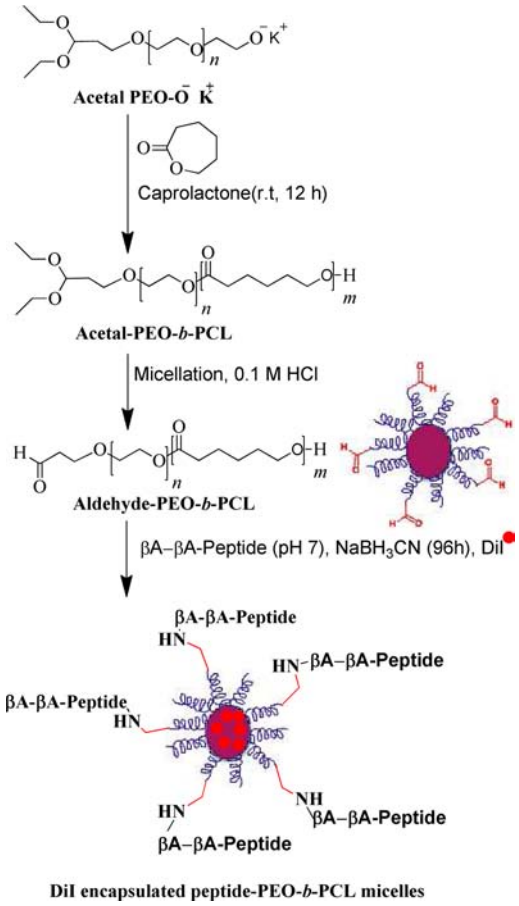

Figure 1. Synthetic scheme for the preparation of DiI encapsulated peptide-PEO-*b*-PCL micelles.

fluorescein 5-isothiocyanate (5-FITC) were purchased from Molecular Probes (USA). All amino acids including Fmoc-Lys(Dde)-OH were purchased from Novabiochem. All other reagents for synthesis were obtained from Sigma-Aldrich and used as received without further purification.

All cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and additives were from Invitrogen (Karlsruhe, Germany). Human breast cancer cell line MDA-MB-435 was cultured in RPMI-1640 medium, MCF-7 and MDA-MB-231 were cultured in DMEM, human mammary epithelial cell line (MCF-10A) was cultured in minimal essential growth medium MEGM (Lonza, Cedarlane) with the addition of 100 ng/mL cholera toxin (Sigma); and all were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 IU/mL streptomycin. Human umbilical vein endothelial cells (HUVEC) were a kind gift from the laboratory of Sandra Davidge, University of Alberta, Canada, and were cultivated using endothelial cell growth medium EGM (Lonza, Cederlane) containing 20% FBS, 2 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 ng/mL basic fibroblast growth factor (Roche Diagnostics, Mannheim, Germany). All cell lines were cultivated at 37 °C in a 5% CO_2/95% O_2 incubator.

Synthesis and Characterization of Peptide−Micelle Conjugates. Preparation of peptide−polymeric micelle (PM) conjugates was done in three steps. The first step involved the synthesis of aldehyde-PEO-*b*-PCL adopting a previously reported method (Figure 1).[38] Briefly, acetal-PEO-*b*-PCL was

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first synthesized through one-pot anionic ring-opening polymerization of ethylene oxide using initiator 3,3 diethoxy1-propanol (DEP) and potassium naphthalene solution in THF at room temperature under argon, followed by ε-caprolactone addition. Second, acetal-PEO-*b*-PCL (20 mg/mL) was allowed to self-assemble into micelles using a water/acetone mixture, after which the acetone was evaporated. The acetal groups on the border of the micelles were converted to aldehyde through the dropwise addition of HCl (0.5 mol/L) at room temperature, adjusting the pH of the medium to 2. After being stirred for 2 h, the mixture was neutralized with NaOH (0.5 mol/L) to stop the reaction. The resulting micellar solution was concentrated by ultracentrifugation with a MILLIPORE Centrifugal Filter Device (M_w cutoff 100 000 Da) followed by purification using a Sephadex G50 column using phosphate-buffered saline (PBS, pH 7.0) as an eluent. Finally, the micellar solution was extensively dialyzed (Spectrapor, MWCO 3500 Da) and freeze−dried for further use. The structural confirmation of the polymer aldehyde-PEO-*b*-PCL was done in $CDCl_3$ using ^1H NMR (Bruker AM-300) and MALDI-TOF using a Voyager spectrometer (Applied Biosystems). The size of the polymeric micelles was measured using a Malvern Zetasizer 3000 at polymer concentration of 4 mg/mL.

The second step of the synthesis of peptide−PM conjugates involved the synthesis of peptides by solid-phase peptide synthesis using Fmoc coupling protocols.[34] Linear peptides **11**, **18**, and p**160** were synthesized with two β-alanine residues at the N-terminal as a linker using Wang resin as a solid support. The prepared peptides were cleaved from the resin using TFA/TIPS/H_2O (95:2.5:2.5, 7.5 mL) for 2 h. The cyclized RGDfK (c-RGD) peptide was synthesized using similar procedures on chlorotrityl resin (0.1 mmol, 70 mg). First, N-α-Fmoc-Asp-OAll was coupled to the resin using a side chain carboxyl group followed by other amino acids. Finally, after addition of N-α-Fmoc-D-Phe-OH amino acid, the C-terminal allyl ester was removed by treatment with $Pd(PPh_3)_4$ (0.16 equiv) and $PhSiH_3$ (16 equiv) in DCM/DMF for 2 h. The N-terminal Fmoc group was then removed with piperidine/DMF (1:4) before addition of BOP (1.95 equiv, 86 mg), HOBt (27 mg, 2 equiv), and NMM (50 mL, 4.5 equiv) in DMF for 2 h to allow on-resin cyclization and give the cRGDfK peptide. Removal of the (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting group from the amino side chain of lysine was achieved by hydrazine monohydrate−DMF (2:98) for 3 min. The two Fmoc-β-alanine residues were linked to the NH_2 of lysine side chain and the final Fmoc group was removed using 20% piperidine. The peptide was treated with TFA/CH_2Cl_2 (1:1, 8 mL) for 2 h.[42]

The crude product was purified with a Varian Prostar HPLC system (Walkersville, MD, USA) using a Vydac C18 semi-preparative column (1 × 25 cm, 5 μm). The peptides were characterized using analytical HPLC and MALDI-TOF mass spectrometry. The Fmoc-peptides βAla-βAla-**11**, βAla-βAla-**18**, βAla-βAla-**160**, and βAla-Aβla-**c-RGD** eluted at 24, 14, 40, and 12 min, respectively, using an analytical C18 Vydac column (0.46 × 25 cm, 5 μm) with a flow rate of 1.2 mL/min using methanol/water. The $[M+H]^{+1}$ for the peptides was found to be 1643.36 (calcd. 1643.78), 1661.36 (calcd. 1660.80), 1883.50 (calcd. 1882.94), and 968.30 (calcd. 968.46), respectively. The third step involved the conjugation of the peptides to the aldehyde-PEO-*b*-PCL micelles. Peptides were conjugated to the aldehydic group on the micellar surface as described previously.[38] Briefly, peptides were added and stirred with the polymeric micelles (4 mg/mL) in sodium phosphate buffer (pH 7 ionic strength 0.1 M) solution at a 1:1 molar ratio (peptide:CHO-PEO-*b*-PCL) at room temperature. After 2 h, $NaBH_3CN$ (10 equiv) was added to the reaction mixture to reduce the Schiff's base. After 4 days of reaction, the micellar solution was purified using dialysis against water (MWCO, 3000 Da). The conjugation was monitored using reversed-phase (RP) HPLC. The resulting peptide−micelle conjugates (PM11, PM18, PM160, and PM c-RGD) were freeze−dried and stored at −20 °C until use. The conjugation was confirmed using mass spectrometry. The amount of conjugated peptide was calculated by subtracting the amount of unreacted peptide relative to the initial added peptide, as well as using the Micro bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA). The peptide-conjugated polymeric micelles were fluorescently labeled with the hydrophobic dye DiI using physical encapsulation.[38] The level of encapsulation efficiency was determined by measuring the fluorescence at excitation/emission 550/565 nm using Varian Cary Eclipse fluorescence spectrophotometer (Victoria, Australia).

Table 1. Different Peptide−Micelle Formulations Used in This Study

peptide	peptide−micelle conjugate[a]	peptide−micelle combination[b]
11	PM11 (PM-βA-βA-RGDPAYQGRFL)	comPM11 (acetal-PEO-*b*-PCL + FITC-11)
18	PM18 (PM-βA-βA-WXEAAYQRFL)	comPM18 (acetal-PEO-*b*-PCL + FITC-18)
p160	PM 160 (PM-βA-βA-VPWXEPAYQRFL)	-
cRGDfK	PM c-RGD (PM-βA-βA-cRGDfK)[b]	-

[a]Conjugates were prepared by the reaction of PM (CHO-PEO-*b*-PCL) with the βA-βA-peptide. [b]cRGDfK was N to C-terminal cyclized and β-ala was linked to the Lys side chain.

Preparation and Characterization of DiI Loaded Micelles. DiI loaded micelles were prepared by dissolving DiI (30 μg) and block copolymer (acetal or PM11) (3 mg) in acetone. The solution was added to double distilled water (3 mL) in a dropwise manner and stirred for 4 h, and the organic solvent was removed under vacuum. The obtained micellar solution was then centrifuged at 11,600 g to remove the free unencapsulated DiI. An aliquot of the micellar solution was diluted with equal volume of dimethyl sulfoxide (DMSO) to quantify the level of encapsulated DiI by fluorescence spectrophotometry (BioTek Synergy2, USA) at $\lambda_{ex/em}$ 550/565 nm.[40]

To determine the release of DiI from micelles, 0.5 mL DiI loaded micellar solution was diluted with FBS so that the final concentration of DiI was 0.5 μg/mL. The mixture was then placed is a dialysis bag (MWCO 3500 Da) and dialyzed against PBS (50 mL, pH 7.4) containing 10% FBS at 37 °C with moderate shaking. Aliquots were then removed from the dialysis media at regular intervals, followed by dilution with an equal volume of DMSO and the level of DiI in the solution was evaluated fluorometrically.[43]

Synthesis and Characterization of Peptide−Micelle Combinations. Peptide−micelle combinations (comPM) were prepared by physically mixing FITC-labeled peptides with the micellar solution. Peptides **11** and **18** were labeled with FITC on the N-terminus via a β-Ala-linker following a

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previously reported method to yield FITC-β-Ala-11 (or FITC-11) and FITC-β-Ala-18 (or FITC-18).[34] The peptides were purified using RP-HPLC and characterized using MALDI-TOF mass spectrometry. Purified peptides FITC-βAla-**11** and FITC-βAla-**18** eluted at 19 and 9 min, respectively, using an analytical C18 Vydac column (0.46 × 25 cm, 5 μm) with isocratic 32% IPA/water at a flow rate of 1 mL/min. The $[M+H]^{+1}$ for the peptides was found to be 1740.39 (calcd. 1740.07) and 1755.80 (calcd. 1755.00), respectively. Stock solutions of the peptides were prepared in 10% aqueous (sterile water) acetonitrile and kept at −20 °C. Peptide concentrations were determined by measuring fluorescence at 465 nm (excitation) and 535 nm (emission). FITC-peptides were then physically mixed with acetal-PEO-*b*-PCL micelles (4 mg/mL) in sodium phosphate buffer (pH 7 ionic strength 0.1 M) solution at a 1:2 molar ratio (peptide/acetal-PEO-PCL). The mixture was stirred for 4 h at room temperature. Thereafter, the micellar solution was purified using dialysis against water (MWCO 3000 Da). The resulting comPMs (comPM11 and comPM18) were freeze−dried and stored at −20 °C until use. The combination was confirmed through MALDI-TOF mass spectrometry and the amount of peptide in each comPM was determined using BCA protein assay. Physical loading of DiI dye was done as described for the peptide−micelle conjugates.

The quantification of peptides on the micelle surface in chemically conjugated as well as physically loaded PMs was done using the BCA protein assay. For the BCA assay, a calibration curve was made using known concentrations of BSA, and the unknown concentration was extrapolated from the calibration curve. According to the calibration curve, the peptide concentration in PM11, PM18, PM160, and PMcRGD was 11.5, 12, 16, and 10.5 μg/mL of polymer solution, respectively. The percentage of molar conjugation of peptides 11, 18, p160, and c-RGD to the polymer was found to be 12%, 12%, 16%, and 11%, respectively, which converts to approximately 1−2 peptides per 10 polymer chain. The amount of peptide present in peptide−micelle combinations (comPM) was determined similarly using the BCA assay, and was found to be approximately 12 μg/mL.

Measurement of Cellular Uptake by Flow Cytometry. The cellular uptake of DiI loaded PM11, PM18, PM160, and PMcRGD polymeric micelle conjugates was evaluated against three human breast cancer cell lines (MDA-MB-435, MCF-7, and MDA-MB-231) and two noncancerous cell lines (HUVEC and MCF-10A) using flow cytometry. Cells were grown in T-75 culture flasks containing media supplemented with FBS and antibiotics until they reached 80% confluence. After being washed twice with PBS, the cells were detached from the surface through incubation with trypsin solution at 37 °C. The cells were centrifuged at 500 *g* for 5 min and resuspended in media. Subsequently, the cells were placed into 6-well plates at a density of 10^6 for cancer cells and 3×10^5 for noncancerous cells in 3 mL of culture medium at 37 °C for 24 h. The next day, cells were washed using PBS and incubated for 45 min in serum-free media containing PM conjugates at a concentration of 10^{-5} mol/L according to the peptide concentration. The final DiI and polymer concentration was 10 μg DiI/10 mg polymer/mL. The media was then removed and the cells were washed three times with ice-cold PBS to remove the unbound PM conjugates. The cells were then scraped from the wells with a manual scrapper, transferred to the centrifuge tubes, and centrifuged at 1000 rpm for 7 min. The pellet was suspended in FACS buffer (PBS with 5% FBS, and 0.09% sodium azide), rewashed, and then resuspended in FACS buffer. Untreated cells as well as cells treated with PM only were subjected to the same steps. Finally, the samples were subjected to FACS analysis (using Becton-Dickinson Facsort) to acquire data. The data were analyzed using DakoCytomation Summit software. Measurement of cellular uptake of the peptide−micelle combinations comPM11 and comPM18 was done following the same procedure as described above.

Measurement of Cellular Uptake by Confocal Microscopy. MDA-MB-435, MDA-MB-231, and MCF10A cells (50 000) were cultured on a coverslip at 37 °C for 24 h. The medium was removed and replaced with 1 mL of fresh serum-free medium containing peptide−micelle conjugates at a concentration of 10^{-5} mol/L. The cells were incubated with the conjugates for 60 min at 37 or 4 °C. After incubation, the medium was removed and the cells were washed three times with 2 mL of serum-free media. The cells were fixed on ice with 2% formaldehyde for 20 min. The formaldehyde was removed through washing with medium three times. The coverslips were put on slides containing one drop of DAPI-Antifade to stain the nucleus. The cells were imaged under confocal microscope. Confocal laser scanning microscopy (Zeiss 510 LSMNLO, Jena, Germany) was performed using a 100× oil immersion lens. Confocal stacks were processed using Carl Zeiss LSM 5 Image software, which also controls the confocal microscope. A similar procedure was used to study the uptake of the peptide−micelle combination comPM18 by the MDA-MB-435 and MCF10A.

RESULTS AND DISCUSSION

Peptide−Micelle Conjugates and Peptide−Micelle Combinations. Polymers and peptides were synthesized and characterized for the preparation of two types of surface-modified micelles, peptide−micelle conjugates and combinations.

Polymers acetal-PEO-*b*-PCL and aldehyde-PEO-*b*-PCL (Figure 1) were synthesized and characterized using our previously established method.[44] The structure was confirmed using ^{1}H NMR spectra which showed all of the characteristic peaks for PEO and PCL segments. By comparison of the intensity of methylene proton peaks for PEO ($\underline{CH_2}\underline{CH_2}O$) at δ = 3.65 ppm to the intensity of peak of methyl protons for the acetal group ($\underline{CH_3}CH_2O$) at δ = 1.2 ppm, the average molecular weight of the PEO was calculated to be 3658 g/mol. The average molecular weight of the PCL block of the synthesized copolymer was estimated based on the peak intensity of the protons from acetal-PEO ($-CH_2CH_2O-$) at δ = 3.65 ppm compared to that of PCL methylene protons ($COCH_2CH_2CH_2CH_2\underline{CH_2}O$)-H at δ = 4.10 ppm, and was found to be 5040 g/mol. The conversion of the acetal group on the surface of the micelle to an aldehyde was confirmed by ^{1}H NMR, with the disappearance of the acetal group methyl protons ($\underline{CH_3}CH_2O)_2$ around δ = 1.2 ppm and the appearance of a CHO peak around δ = 9.8 ppm, as well as a shift in the methylene protons $OCH\underline{CH_2}$ from 1.9 ppm to appear further downfield with the methylene protons of PCL ($CO\underline{CH_2}CH_2CH_2CH_2CH_2O$)-H at 2.35 ppm. The acetal-PEO-*b*-PCL was also characterized using MALDI-TOF mass spectrometry. The mass spectra of the acetal-PEO-*b*-PCL revealed spacing of 44 and 114 Da of PEO and PCL repeat units, respectively. Polymer was converted into micelles using a cosolvent evaporation method where acetone was used as an organic solvent. Micelle formation was confirmed by studying

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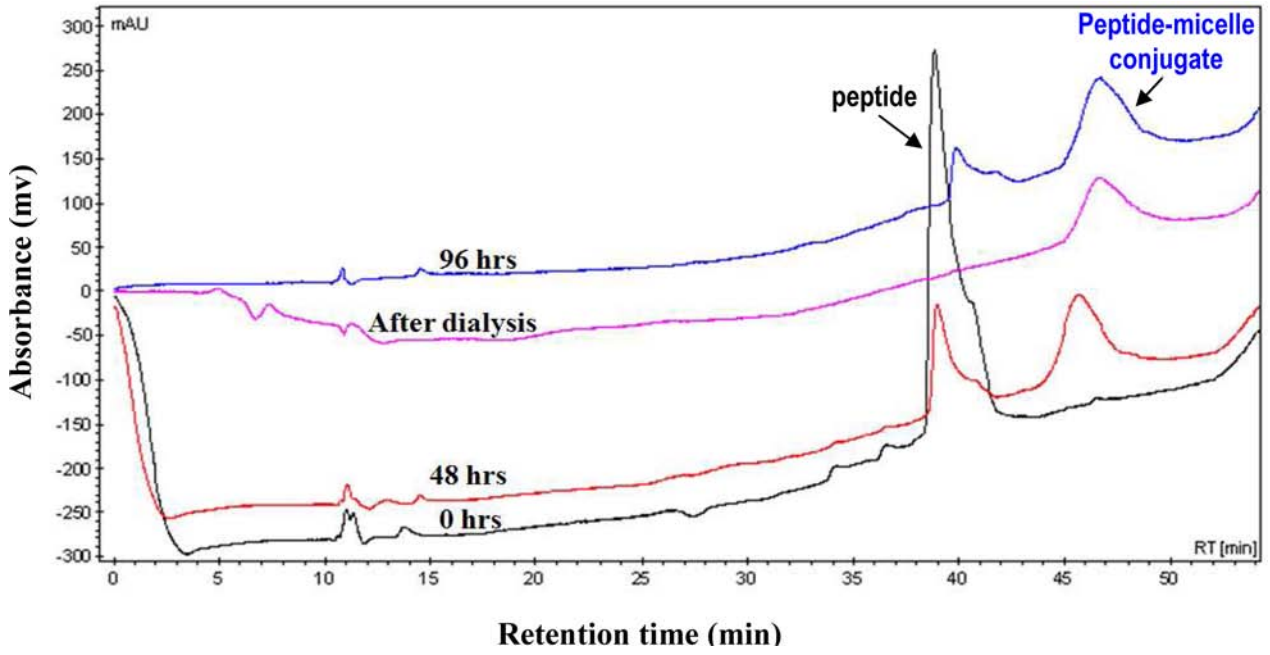

Figure 2. Progress of the peptide (p160) and polymer (aldehyde-PEO-*b*-PCL) conjugation reaction. Aliquots were removed from the reaction mixture at different time intervals (0, 48, 96 h) and conjugation was analyzed using reversed-phase HPLC with methanol/water as a mobile phase. At the end of the reaction (96 h), the sample was dialyzed before HPLC analysis.

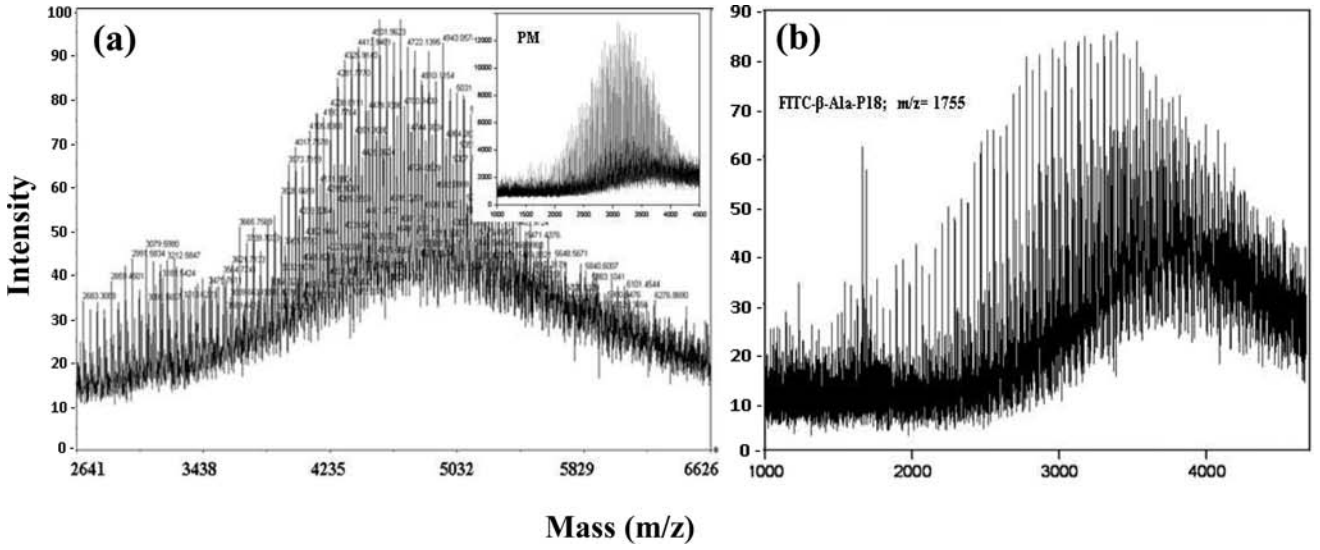

Figure 3. MALDI-TOF mass spectrometry characterization of the (a) PM18 conjugate (4200−5600) and PM (2700−3400; inset) and (b) comPM18. Mass spectrometry analysis of comPM18 shows mass of both PM (3000−4000) and the peptide FITC-18 (1755).

the size distribution using dynamic light scattering (DLS) measurements. On the basis of DLS measurements, the average diameter and polydispersity index of prepared polymeric micelles (PM) were 75 nm and 0.02, respectively.

For the preparation of peptide−micelle conjugates, peptides were chemically conjugated to the aldehydic groups on the surface of the polymeric micelles through their N-terminal groups (Figure 1). Peptides were synthesized using solid phase peptide synthesis. [34] Two β-alanine residues were added to the N-terminal of the peptides as a spacer to reduce the steric hindrance when reacting with the micelle surface. The use of the aldehyde group to covalently conjugate various ligands, such as monosaccharide derivatives and peptides, to the PEO-*b*-poly(lactide) micelles is a synthetic strategy developed and widely used by Nagasaki and co-workers.[44] In this strategy, the Schiff's base that is formed is later reduced using an excess of sodium cyanoborohydride. Using this method, four peptide−micelle conjugates (PM11, PM18, PM 160, and PM c-RGD) were obtained. PM 160 and PM c-RGD were used as positive controls. The progress of the reaction for each peptide was monitored using RP-HPLC. The reaction mixture was injected at time zero and then the reaction was monitored at 24, 48, and 96 h. The reaction progress revealed almost 100% conjugation at 96 h. Figure 2 shows the RP-HPLC trace of βAla-βAla-p160 conjugation to CHO-PEO-*b*-PCL micelles. The same pattern was observed for the conjugation reactions of all the other peptides. Further, the HPLC peaks corresponding to the

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Table 2. Characterization of Peptide−Micelle Conjugates

micelles	micellar size[b] (nm)	polydispersity[b] (PDI)	zeta potential (mV)[c]	DiI encapsulation efficiency[d] (%)	DiI release after 48 h[d] (%)
Acetal micelle[a]	110.5 ± 1.2	0.40 ± 0.08	−2.3 ± 0.6	81.6 ± 1.3	8.7 ± 0.1
PM11	107.6 ± 1.6	0.23 ± 0.01	−2.7 ± 0.3	91.6 ± 1.1	10.4 ± 0.3
PM18	122.7 ± 1.4	0.57 ± 0.02	−3.9 ± 0.3	nd	nd
PM 160	127.6 ± 1.7	0.25 ± 0.01	−4.2 ± 0.5	nd	nd

[a]Acetal micelle is the nontargeted acetal PEO-*b*-PCL micelle. [b]Determined by dynamic light scattering measurement. [c]Determined by electrophoretic light scattering measurement at pH 7.4. [d]nd stands for not determined.

conjugates were collected and analyzed using MALDI-TOF mass spectrometry.

All mass spectra showed shifts in the molecular weight of the polymer confirming the addition of the peptide to the polymer. Figure 3**a** shows an example of the PM18 micelle (mass 4300−5100 m/z) and the insert shows the mass spectrum of the unconjugated micelle (2700−3400 m/z) without the peptide **18** (1661 m/z).

Peptide−micelle combinations were prepared using acetal-PEO-*b*-PCL polymer where the absence of an aldehyde group rules out the possibility of any chemical interaction. Peptide−micelle combinations comPM11 and comPM18 were prepared by mixing peptide and polymer solutions at a 1:2 molar ratio. An excess of polymer was used to make the peptide concentrations in both chemically and physically conjugated micelles similar so that the comparisons are significant. For the peptide−micelle combinations, FITC-labeled peptides were used to allow fluorescence monitoring during the cell uptake studies. MALDI-TOF mass analysis of the combinations showed the presence of peptide and polymer confirming the existence of two different compounds physically blended together (Figure 3b).

The quantification of peptides on the micelle surface in chemically conjugated PMs revealed that the peptide concentration was about 10−16 μg/mL of the polymer which correlates to approximately 1−2 peptides per 10 polymer chains. Similarly, the amount of peptide present in peptide−micelle combinations was found to be around 12 μg/mL. For both of the micellar preparations (conjugates and combinations) the micelles were fluorescently labeled using physically encapsulated DiI hydrophobic dye,[40] and the level of encapsulation efficiency was found to be greater than 81%.

Table 2 shows further characterization of the peptide−micelle conjugates. The mean diameter of nontargeted (acetal) micelles and targeted peptide−micelle conjugates was around 107−127 nm with a narrow polydispersity index ranging from 0.23 to 0.57 nm. The zeta potential values show that the acetal micelles were negatively charged (−2.3). Peptide conjugated micelles either maintained the same charge such as PM11 (−2.7) or slightly larger negative values, e.g., PM18 (−3.9) and PM160 (−4.2). A slightly negative surface charge on acetal micelles has been previously reported with acetal-PEG-PDDL micelles.[45] The nonsignificant change in the zeta potential value of PM11 is due to the amphoteric (net charge zero) nature of the p11 peptide on the micelles. On the other hand, excess carboxylic groups on the p18 or p160 peptide residues (net charge −1) contribute to the absolute negative zeta potential values of PM18 and PM160. These changes in the surface charge of peptide-modified polymeric micelles further confirm successful conjugation of the peptide to the micellar surface. Next, the DiI encapsulation efficiency and release was evaluated for the PM11 micelles. Both the nontargeted (acetal) and targeted (PM11) micelles displayed more than 80% DiI encapsulation efficiency, most likely due to the lipophilic nature of the fluorescent dye. The *in vitro* release of DiI at 37 °C revealed similar release kinetics for the nontargeted and targeted micelles (Figure 4). During the first 5 h there was only

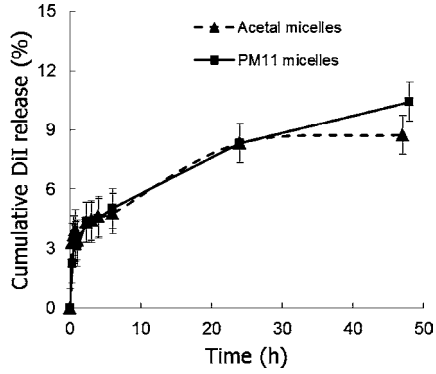

Figure 4. *In vitro* release of DiI from nontargeted (acetal) and targeted (PM11) micelles in PBS containing 10% FBS at 37 °C.

5% release of DiI, followed by release of another 4−5% by 48 h. After 48 h of incubation at 37 °C, the DiI remaining in acetal and PM11 micelles was 89.6% and 91.3%, respectively. This suggests that most of the encapsulated DiI will be retained in the micellar carrier during the time frame of the cellular uptake experiments.

Peptide−micelle Conjugate PM18 Shows Enhanced and Specific Uptake by Breast Cancer Cells. To study the specific uptake of the peptide−micelle conjugates by breast cancer cells, we investigated the binding of the conjugates to breast cancer cell lines MDA-MB-435, MCF-7, and MDA-MB-231; noncancerous human breast MCF 10A cells; and human endothelial HUVECs. There is some controversy about the origin of MDA-MB-435 cells. However, several recent studies report favorably on the use of MDA-MB-435 cells as a model breast cancer cell line.[46−48] Two additional breast cancer cell lines (MCF7 and MDA-MB-231) were used along with negative control cell lines MCF10A and HUVEC. MCF10A is derived from the same breast tissue as MCF7 but is noncancerous. HUVECs were selected as these are fast-multiplying endothelial cells which may be more prone to uptake by nonspecific cancer drugs. The three cancer cell lines used here belong to different breast cancer subtypes. Our previous studies suggest no difference in internalization capacity of unmodified polymeric micelles (without peptide) by these cells; however, different subtypes can have different clinical outcomes, different response to adjuvant therapy, and different pattern of metastasis.

Fluorescence-based experiments (flow cytometry and confocal microscopy) were used to monitor the uptake of peptide−micelle conjugates during in vitro cell uptake experiments. Based on the flow cytometry results (Figure 5), we found that

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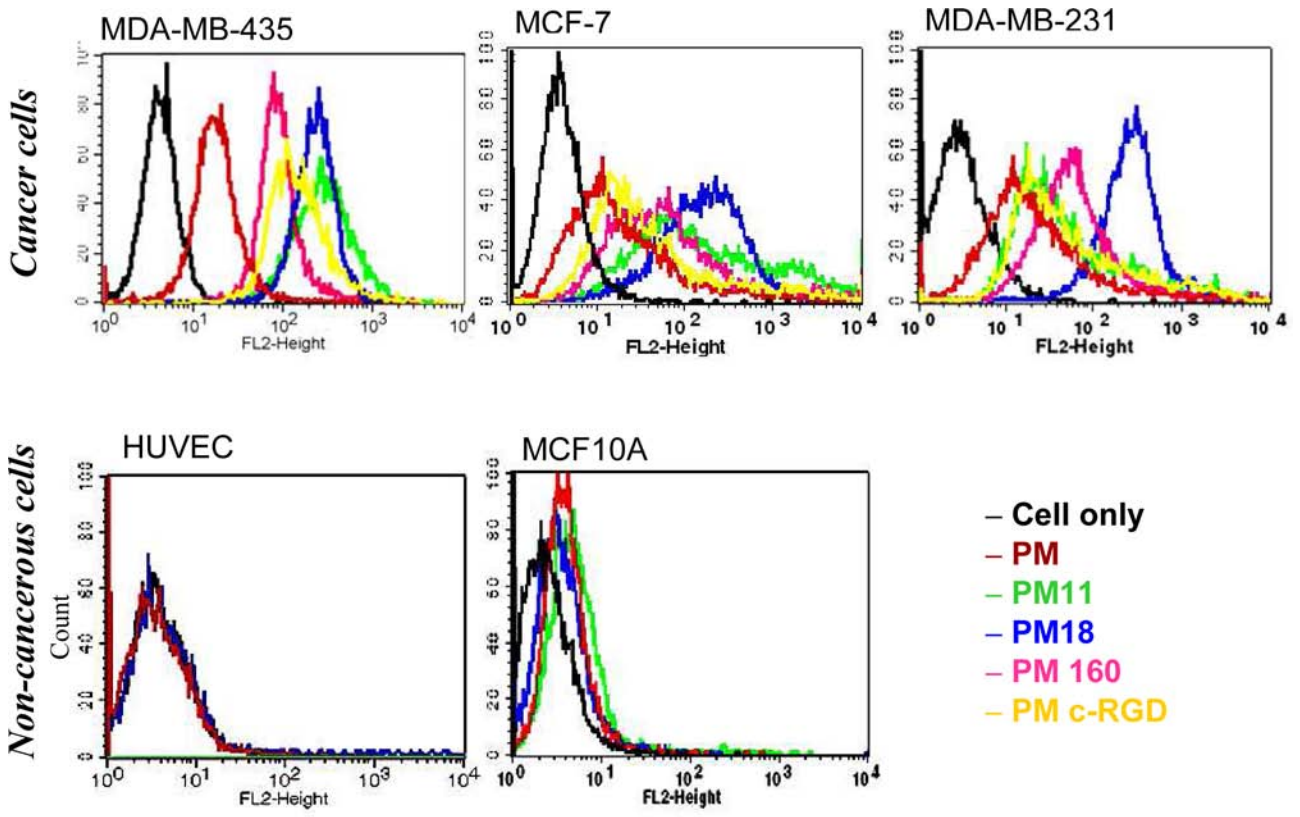

Figure 5. Cellular uptake of PM (brown), PM11 (green), PM18 (blue), PM 160 (pink), and PM c-RGD (yellow) peptide–micelle conjugates by different cancer cell lines (top row) or noncancerous cells (bottom row) by flow cytometry. Cancer cells MDA-MB-435, MDA-MB-231, MCF-7, or noncancerous HUVEC and MCF-10A were incubated with PM conjugates (10^{-5} mol/L) for 45 min at 37 °C, followed by measurement of the fluorescence (excitation/emission, 550/565 nm) or the uptake (n = 1–3). Autofluorescence of the cells is shown in black.

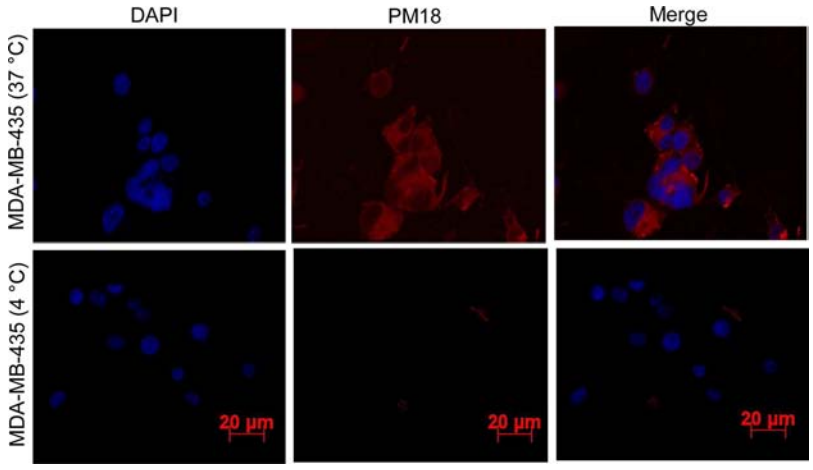

Figure 6. Confocal fluorescence microscopy images after incubation of PM18 (10^{-5} mol/L) with MDA-MB-435 cells at 37 °C (top row) and 4 °C (bottom row) for 60 min. Cell nuclei were stained blue with DAPI. Scale bar: 20 μm.

peptide–micelle conjugates greatly enhanced cellular uptake by the breast cancer cell lines compared to that of unconjugated polymeric micelles. After 1 h of incubation of the cancer cells with the peptide–micelle conjugates, PM11 and PM18 with peptides **11** and **18**, respectively, as a surface targeting ligand showed higher uptake compared to the PM 160 and PMcRGD peptide–micelle conjugates. In general, PM18 showed highest mean fluorescence intensity (average MFI 235 ± 18) for the three cancer cells lines. In comparison, the average MFI for PM 160 was 71 ± 16 and for PM (or acetal-PEO-*b*-PCL) was 31 ± 10. These results support our earlier findings where fluorescently labeled peptides **11** (FITC-11) and **18** (FITC-18) displayed superior targeting properties for breast cancer cell lines MDA-MB-435 and MCF-7 compared to the other cancer targeting peptides, such as p160.[34] It was also observed that the conjugates have low uptake by the HUVECs and MCF10A cells.

Based on the above findings, PM18 was selected for studying cellular uptake using fluorescence microscopy. In this experiment, MDA-MB-435 cells were incubated with the PM18

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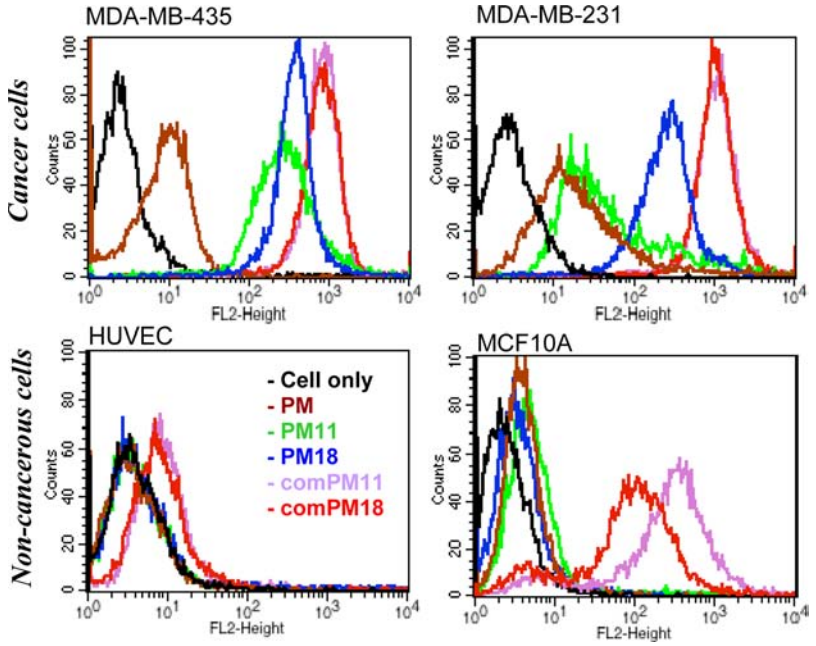

Figure 7. Comparison of DiI cell uptake between PM−peptide conjugate (PM11 or PM18) and PM−peptide combination (comPM11 or comPM18) by cancer and noncancerous cells. Cancer cells (MDA-MB-435 and MDA-MB-231) and noncancerous (HUVEC and MCF-10A) were incubated with polymeric micelles (10^{-5} mol/L) for 45 min at 37 °C, followed by measurement of the fluorescence (DiI) or the uptake (n = 1−3). Autofluorescence of the cells is shown in black.

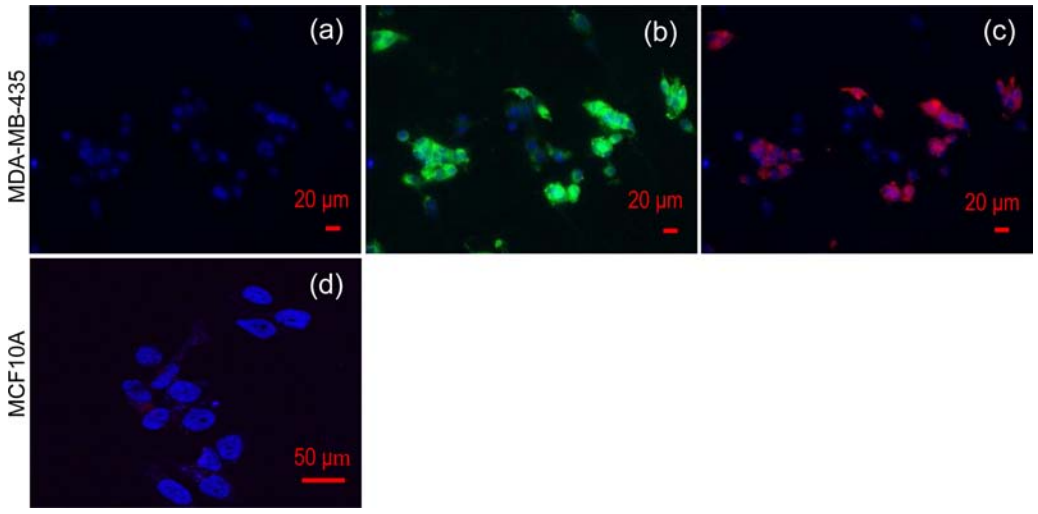

Figure 8. Confocal fluorescence microscopy images of comPM18 (10^{-5} mol/L) after incubation with MDA-MB-435 (a−c) or MCF10A cells (d) for 60 min at 37 °C. Peptide−micelle combination, comPM18, consists of FITC-labeled peptide (green) and DiI (red) containing micelle. (a) shows cell nuclei stained blue with DAPI, (b) is overlay of blue and green channels, and (c) is overlay of blue and red channels for MDA-MB-435 cells. (d) Overlay of blue, green, and red channels for MCF10A cells displaying small uptake (red fluorescence) of comPM18.

peptide−micelle conjugate for 60 min at 37 °C and the distribution of PM18 was examined using confocal microscopy. PM18 bound strongly to the cells as observed by the uniform distribution of DiI dye inside the cells (Figure 6). In addition, in order to prove that cellular uptake occurs through an active mechanism the experiment was repeated for MDA-MB-435 cells at 4 °C. A substantial decrease in the DiI fluorescence at 4 °C clearly points out that an energy dependent mechanism and perhaps receptor-mediated uptake is involved in the internalization of PM18 peptide−micelle conjugate. In this regard, we have previously shown that the cellular uptake of p160-modified polymeric micelles has been significantly reduced upon pretreatment of cells with free p160.[40]

Peptide−Micelle Combinations Show Better Uptake by Cancer Cells Compared to the Peptide−Micelle Conjugates. The second objective of this study was to compare the uptake as well as binding specificity of peptide−micelle conjugates with the peptide−micelle combinations. Peptides **11** and **18** were selected for this study. Peptide−micelle conjugates (PM11 and PM18), combinations (comPM11 and comPM18), and PMs alone were incubated with the MDA-MB-435 and MDA-MB-231 cells, and the uptake was determined based on the DiI fluorescence using flow cytometry. As shown in Figure 7, in cancer cells peptide−micelle combinations displayed higher uptake compared to peptide−micelle conjugates, followed by PMs alone. Based on

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the mean fluorescence intensity (MFI) for the MDA-MB-435 cells, the cell uptake for both comPM11 and comPM18 was about 35 times better than that of PM alone. PM11 and PM18 were 12 and 9 times better than PM, respectively. Similarly, comPM11 and comPM18 showed higher uptake for the MDA-MB-231 cells and the MFI for both was about 33 times higher than the PM alone. Clearly, the peptide−micelle combination where the peptide is physically mixed with the polymeric micelle showed better uptake by cancer cells. However, the noncancerous cell line MCF10A also showed uptake for the peptide−micelle combinations with comPM11 and comPM18 showing 16 and 6 times higher MFI compared to PM, respectively. The uptake of all the micellar preparations under study by the HUVECs was minimal.

Next, the uptake of comPM18 by the cancer cells MDA-MB-435 and noncancerous MCF10A was explored using confocal microscopy. Figure 8a−c illustrates enhanced cellular uptake of comPM18 by the MDA-MB-435 cells. A strong green and red fluorescence is observed due to the presence of FITC-peptide and the DiI dye inside the cells. The presence of the peptide and DiI inside the cells confirms that both are located within the cytoplasm of the cells and suggests that the uptake is most likely by a peptide-mediated mechanism. In contrast, the MCF10A cells derived from normal mammary cells show minimal uptake as shown by the minimal DiI fluorescence (Figure 8d). This further confirms our flow cytometry results (Figure 7) where a small uptake of comPM18 was observed for noncancerous MCF10A cells (MFI 102) compared to the cancer cells, MDA-MB-435 cells (MFI 733), and MDA-MB-231 (MFI 943).

The above results show that peptide−micelle combinations facilitate targeted uptake by breast cancer cells. However, peptide−micelle combinations seem to be less selective than peptide−micelle conjugates, as combination formulations display slightly enhanced uptake by the noncancerous HUVEC and MCF10A cells as well (Figure 7). The enhanced uptake of the peptide−micelle combinations by different cell types compared to peptide−micelle conjugates could be due to the synergistic cosolubilization and stabilization of the peptide−micelle mixture. Physical mixing of surfactants enhances cosolubilization and stabilization of emulsions. For instance, ubiquinone increased the stabilization and solubilization of omega-3 fatty acid esters by functioning as a kosmotropic agent in the micellar system.[49] In this regard, Sugahara et al. showed that coadministration of a tumor-penetrating peptide iRGD with the anticancer drug doxorubicin, nanoparticles carrying drug, or trastuzumab (a monoclonal antibody) improved the therapeutic index of drug by selectively delivering the combinations to the cancer site.[17]

The safety and cytotoxicity of the empty micelles and free peptides against the MDA-MB-435 cells at concentrations similar to those used in this study have been previously reported, [40] showing almost no potential toxicities from the empty carrier or the free peptide. We have also reported on the development of proteolytically stable analogues of peptide **18**, e.g., peptide **18−4**, that are stable in the presence of human serum and liver homogenate from mice and display up to 3.5-fold enhanced binding to cancer cells compared to peptide **18**.[50] Finally, a liposomal formulation of doxorubicin surface coated with peptide **18−4** has shown better cytotoxicity against MDA-MB-35 compared to nontargeted liposomes both in vitro and in vivo.[51,52]

CONCLUSIONS

Targeted polymeric micelles show enhanced cancer-specific uptake of the encapsulated lipophilic cyanine fluorescent dye DiI that mimics a hydrophobic drug in micellar core. The presence of multiple conjugated peptides on the micelle surface was responsible for the enhanced uptake, compared to the naked micelles which showed minimal uptake, by breast cancer cells. Peptide **11** and **18** conjugated micelles seem most promising for the targeted delivery of drugs or diagnostic moieties to the breast cancer site and such formulations can be further improved by conjugating proteolytically stable peptide analogues on the micelle surface, [50] or by employing multiple peptide combinations targeting different cancer cell receptors. These micellar formulations are evaluated for targeting breast cancer cells and their specificity toward other cancer types remains to be elucidated. Better homing of the drug into cancer cells and away from normal tissues using a peptide modified drug carrier can lead to a higher in vivo therapeutic index for the conventional chemotherapeutic agents.

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Notes

The authors declare no competing financial interest.

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