Published in final edited form as:

Mol Pharm. 2017 September 05; 14(9): 3121–3133. doi:10.1021/acs.molpharmaceut.7b00355.

EGFR-Targeted Cationic Polymeric Mixed Micelles for Codelivery of Gemcitabine and miR-205 for Treating Advanced Pancreatic Cancer

Goutam Mondal, Saud Almawash, Amit Kumar Chaudhary, Ram I. Mahato*

Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska 68198, United States

Abstract

Gemcitabine (GEM), a first-line chemotherapy for pancreatic cancer undergoes rapid metabolism and develops chemoresistance after repeated administration. We previously demonstrated that the combination of GEM and miR-205 provides an effective therapeutic strategy to sensitize GEM-resistant pancreatic cancer cells. Since epidermal growth factor receptor (EGFR) is overexpressed in pancreatic cancer cells, in this study, we aimed to deliver mixed micelles containing GEM and miR-205 decorated with EGFR-targeting cetuximab (C225) monoclonal antibody for targeted therapy. Cetuximab C225 was conjugated to maleimido-poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylene carbonate-*graft* *t*-dodecanol (C225-PEG-PCD) to prepare mixed micelles with mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA for targeted codelivery of GEM and miR-205. This mixed micelle formulation showed a significant enhancement in EGFR-mediated cellular uptake in GEM-resistant MIA PaCa-2^R cells. Further, an enhanced tumor accumulation of C225-micelles conjugated with near-infrared fluorescent Cy7.5 dye and Dy677-labeled miR-205 in orthotopic pancreatic tumor bearing NSG mice was evident after systemic administration. In addition, inhibition of tumor growth was also observed with increased apoptosis and reduced EMT after treatment with C225-micelles containing GEM and miR-205. Therefore, we believe that the targeted delivery of GEM and miR-205 in combination could be a novel strategy for treating advanced pancreatic cancer.

Graphical Abstract

*Corresponding Author Tel: 402-559-5422; Fax: 402-559-9543; ram.mahato@unmc.edu.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.molpharmaceut.7b00355](https://doi.org/10.1021/acs.molpharmaceut.7b00355). NMR spectra, particle size distribution and Zeta potentials of micelles, complexation and dissociation of miRNA from micelles, cytotoxicity of micelles without GEM and miR-205 and EGFR expression in MIA PaCa-2R cells, cellular uptake of free BLOCK-iT fluorescent oligo in MIA PaCa-2R cells, transfection efficiency of fluorescent labeled oligonucleotide in MIA PaCa-2R cells, MTT assay of free GEM and miRNA complexed with lipofectamine RNAiMAX into MIA PaCa-2R cells, cytotoxicity effect of micelles containing GEM and miR-205 or scrambled miRNA in MIA PaCa-2R, and NIR fluorescence imaging of micelles containing conjugated Cy7.5 and DY677-miR-205 (PDF)

The authors declare no competing financial interest.

Keywords

pancreatic cancer; gemcitabine; miR-205; EGFR; targeted drug delivery; polymeric mixed micelles; C225

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related deaths in the United States, with a five-year survival rate of less than ~8%.¹ Pancreatic cancer treatment faces multiple challenges due to the presence of desmoplasia and emergence of chemoresistance resulting in recurrence and metastatic spread. Gemcitabine (Gemzar, GEM), a nucleoside analogue, is the first-line chemotherapy to treat pancreatic cancer, with a mere 6.8 month survival rate. However, GEM is rapidly deaminated by cytidine deaminase (CDA) to its inactive metabolite 2,2'-difluorodeoxyuridine (dFdU), resulting in its short in vivo half-life (8–17 min).^{2–4} Further, dense desmoplastic pancreatic tumor tissue impedes effective GEM delivery to the cancer cells. GEM was encapsulated into nanoparticles and liposomes to overcome these limitations, which exhibited greater anticancer activity than GEM alone. However, poor encapsulation efficiency, rapid leakage and reticuloendothelial system (RES) mediated uptake of these particles limit their effectiveness.^{3,5,6} Therefore, GEM was conjugated to stearic acid and formulated into PEG2000-hydrazine-C18 pH-sensitive micelles, which showed improved efficacy.⁷ Recently, Chitkara et al. conjugated GEM to poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylene carbonate) (PEG-PCC). This drug-polymer conjugate could self-assemble into micelles and show sustained drug release and negligible plasma metabolism of the drug.⁸ Intravenous administration of these micelles into tumor-bearing NSG mice showed significant inhibition of tumor growth.⁸ Moreover, Kattel et al. demonstrated that GEM and dodecanol (DC) conjugated mPEG-*b*-PCC-*g*-GEM-*g*-DC formed micelles increased GEM accumulation in the orthotopic pancreatic tumor by 2.5 fold compared to free GEM after systemic administration.⁹

Repeated administration of GEM at a high dose is known to result in chemoresistance and tumor relapse. A similar limitation is also evident for GEM derivatives/conjugates, and even in clinical setup. Further, several phase II trials have been conducted to improve the overall

survival of the patients using GEM in combination with cisplatin, 5-fluorouracil (5-FU), irinotecan, and oxaliplatin. Although phase II results were encouraging, phase III trials failed to improve the survival rate over GEM.^{10–14} A recent report showed a median survival of 8.5 months in the combination of albumin-bound (nab)-paclitaxel and GEM in patients with metastatic pancreatic cancer compared to 6.7 months for the patients treated with GEM alone and post-treatment one-year survival rate of 35% vs 22%.¹⁵ Therefore, different combination approaches are urgently needed to combat with advanced pancreatic cancer.

PDAC harbors a small population (1–2%) of self-renewing cancer stem cells (CSCs) which are responsible for tumor initiation, propagation, chemoresistance, and recurrence. These CSCs are regulated by small noncoding RNAs (17–25 nucleotides) known as miRNAs.^{16–18} Singh et al. identified aberrantly expressed miRNAs in CSCs isolated from GEM-resistant MIA PaCa-2^R cancer cells and in tumors of pancreatic cancer patients, whereby there was significant downregulation of miR-205, which controls several genes involved in cancer progression, invasion, and metastasis.¹⁹ Recently, Chaudhary et al. showed that miR-205 sensitized GEM resistance MIA PaCa-2^R cells and inhibited proliferation of pancreatic CSCs by stably overexpressing miR-205.²⁰ Despite its dominant role in cancer, the clinical potential of miR-205 has not been realized due to the challenges involved in its in vivo delivery.²¹ Thus, combination of drug with miR-205 could be viable strategy for enhancing the antitumor efficacy of small drug molecules in treating advanced pancreatic cancer. To coformulate GEM and miR-205 mimic into micelles, Mittal et al. synthesized GEM conjugated polymer, poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylene carbonate-*graft*-dodecanol-*graft*-tetraethylenepentamine) (PEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA), by attaching cationic pendant chains. The cationic polymer formed stable micelles with miR-205 and exhibited high transfection efficiency, with minimal cytotoxicity. The micelles carrying GEM and miR-205 were effective in chemosensitizing GEM-resistant MIA PaCa-2^R and significantly reduced tumor growth in pancreatic cancer xenograft model.²²

We previously used ectopic pancreatic tumor model to check combination effect of miR-205 mimic and GEM where tumors were grown under the skin, not in the pancreas. A major disadvantage of this tumor model is that after tumor formation, tumor cells do not develop actual tumor microenvironment. In contrast, in the orthotopic model, tumor cells are implanted in the pancreas where tumor cells experience similar tumor microenvironment.²³ Thus, systemic delivery of miR-205 mimic and GEM to orthotopic pancreatic tumor is an attractive approach for treating advanced pancreatic cancer.

Micelle-based targeted drug delivery could be the most efficacious approach for inhibiting tumor growth with reduced systemic toxicity.²⁴ Global efforts are being directed toward developing nanomedicine that can target overexpressed receptors to facilitate selective drug binding and internalization, resulting in enhanced therapeutic efficacy and reduced side effects.^{25–27} We selected epidermal growth factor receptor (EGFR) since it is overexpressed in almost 95% pancreatic cancer patients. Furthermore, EGFR overexpression is correlated with disease progression.^{28,29} In our previous studies, we found GE11 peptide (YHWYGYTPQNVI)-linked mixed micelles containing GEM showed MIA PaCa-2 cells derived orthotopic tumor growth inhibition by ~1.5 fold compared to unmodified micelles.³⁰

Recently, monoclonal antibody (mAb) C225 conjugated anti-EGFR interleukins (ILs) and mAb C225 conjugated gold nanoparticles showed excellent in vivo efficacy in breast cancer and advanced pancreatic cancer, respectively.^{31,32} Although full-length antibody has more mitogenic and pro-angiogenic capability than a peptide, we selected cetuximab (C225) for targeting EGFR to achieve excellent tumor growth inhibition in advanced pancreatic cancer. C225 was approved by the FDA for the treatment of EGFR-positive colorectal cancer and has now also been used for the treatment of head and neck, lung, and pancreatic cancers.

In this study, we conjugated C225 to maleimido-poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylene carbonate-*graft* *t*-dodecanol (C225-PEG-PCD). We also synthesized mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA and prepared mixed micelles using C225-PEG-PCD and PEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA for enhanced GEM and miR-205 delivery to EGFR receptor overexpressing pancreatic tumor cells. We showed that C225-micelles containing conjugated GEM and complexed miR-205 exhibited a better cytotoxic effect compared to nontargeted IgG-micelles and unmodified micelles. Systemic administration of GEM conjugated miR-205 complexed C225-micelles exhibited significant tumor growth inhibition in MIA PaCa-2^R cells derived orthotopic pancreatic tumor bearing mice via apoptosis of tumor cells.

EXPERIMENTAL SECTION

Materials.

Gemcitabine hydrochloride was procured from AK Scientific (Union City, CA). Immunoglobulin G (IgG), t etraethylene pentamine (TEPA), 1 -ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC), triethylamine (TEA), dodecanol (DC), hydroxybenzotriazole (HOBT), benzyl bromide, tin(II) 2-ethylhexanoate (Sn(Oct)₂), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), puromycin dihydrochloride, methoxy poly(ethylene glycol) (mPEG, Mn = 5000, PDI = 1.03), 2,2-bis(hydroxymethyl) propionic acid, were purchased from Sigma-Aldrich (St. Louis, MO). C225 was purchased from UNMC pharmacy. Maleimide PEG hydroxyl (MW 5000) was procured from JenKem Technology (Beijing, China). Matrigel matrix basement membrane was procured from Corning (Chicago, IL). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was procured from Biosynth Chemistry & Biology (Itasca, IL). Polybrene was purchased from Santa Cruz Biotechnology (Dallas, TX). Lentiviral particle encoding luciferase reporter gene (LP-hLUC-Lv201-0200) was procured from GeneCopoeia (Rockville, MD). mirVana miR-205 mimic (hsa-miR-205-5p) and mirVana miRNA mimic negative control #1 were purchased from Life Technologies (Carlsbad, CA). Custom miRIDIAN mimic hsa-miR-205-5p w/5' Dy677 was purchased from GE Healthcare Dharmacon, Inc. (Lafayette, CO). Cyanine7.5 amine was purchased from Lumiprobe Corporation (Hallandale Beach, FL). Dead End Fluorometric TUNEL System was procured from Promega Corporation (Madison, WI). Mouse IgG1 mAb (Phycoerythrin)-Isotype control and mouse mAb to EGFR (Phycoerythrin) were procured from Abchem (Cambridge, MA). All other reagents and chemicals were procured from Sigma-Aldrich (St. Louis, MO).

Synthesis of Polymers.

Synthesis of 2-Methyl-2-dodecane oxycarbonylpropylene Carbonate (MDC).—

2-Methyl-2-benzyloxycarbonyl-propylene carbonate (MBC) and 2-methyl-2-carboxylpropylene carbonate (MCC) were synthesized as reported by Li et al.³⁵ DC was conjugated to the carboxyl group of MCC using HOBT/EDC coupling reaction. Briefly, MTC-OH (240 mg), HOBT (253 mg), and EDC (360 mg) were dissolved in 20 mL of DMF followed by the addition of 245 μ L of TEA. After 1 h, DC (235 mg) was added to the reaction mixture and left for 18 h under N₂ atmosphere at RT. Then, 150 mL ethyl acetate was added to the reaction vessel, and distilled water was used to wash organic layer, and organic solvent dried over sodium sulfate. The solvent was evaporated by the rotary evaporator to yield 2-methyl-2-dodecane oxycarbonylpropylene carbonate (MDC), which was purified by column chromatography.

Synthesis of MAL-PEG-PCD.—MAL-PEG-PCD was synthesized as reported by Mondal et al.³⁰ Briefly, MDC (450 mg) and MAL-PEG-OH (300 mg) were dissolved in 15 mL anhydrous CH₂Cl₂ and kept under N₂ atmosphere at RT with continuous stirring. Then, 40 μ L DBU was diluted in 1 mL anhydrous CH₂Cl₂ and added to the reaction mixture and left for 3 h under stirring. At the end of the reaction, 250 mg benzoic acid was added to the reaction mixture, and solvent was removed. Then, the product was dissolved with CHCl₃ and we used diethyl ether and isopropyl alcohol to get the precipitate. Finally, the precipitate was dried under high vacuum at RT and got ~80% yield of purified MAL-PEG-PCD lipopolymer.

Conjugation of C225 and IgG to MAL-PEG-PCD.—EGFR targeting C225 (10 mg) was first thiolated by Traut's reagent (5-fold excess) in HEPES buffer (pH 8.0) for 1 h at RT. PD-10 column was used to remove unreacted Traut's reagent using degassed HEPES buffer (pH 6.5) as an elution buffer. MAL-PEG-PCD (25 mg) was dissolved in distilled water and stirred under N₂ at RT. Thiolated C225 (35 mg) and TECP (100 mM, 100 μ L) was added to the reaction and left for 24 h at RT under N₂ atmosphere with gentle stirring. Then, 1 mM MEA (50 μ L) was added to the reaction mixture and left for 30 min at RT to quench excess maleimide groups. Sepharose CL-4B gel filtration was used to remove unbound mAb and MEA. Bicinchoninic acid assay (BCA) was carried out to determine the coupling efficiency as reported previously.³⁶ Similarly, IgG-conjugated MAL-PEG-PCD polymer was synthesized.

Synthesis of mPEG-b-PCC-g-GEM-g-DC-g-TEPA.—mPEG-PCC and mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA were synthesized using our previously reported method.²² Briefly, mPEG-PBC was synthesized by ring-opening polymerization of mPEG and MBC using stannous-2-ethyl hexanoate as a catalyst at 100 °C for 18 h under reduced pressure. Then, the polymer was dissolved in chloroform, precipitated in cold hexane-diethyl ether mixture and dried under vacuum to get pure polymer with ~65% yield. Debenzylation of mPEG-PBC was carried out by hydrogenation reaction. Briefly, mPEG-PBC was tetrahydrofuran and methanol (1:1) mixture containing palladium on carbon (Pd/C), charged with hydrogen at a pressure of 40 psi and left for 18 h. Then, reaction mixture was filtered to remove Pd/C and mPEG-PCC was obtained by solvent evaporation using a rotary evaporator with ~95%

yield. To synthesize mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA, mPEG-PCC (100 mg), HOBt (54 mg) and EDC (77 mg) were added to reaction vessel containing 10 mL DMF and left for 2 h stirring at RT under N₂ atmosphere. GEM (60 mg), DC (37 mg) and TEPA (38 mg) were added to the reaction mixture and followed by the addition of 125 μ L DIPEA. Then, the reaction mixture was left for 72 h continuous stirring under N₂ atmosphere at RT. The crude product was precipitated using cold isopropyl alcohol and then dissolved in chloroform and precipitated in cold diethyl ether. Then, the precipitate was purified by dialysis against water using 2 kDa MWCO dialysis tubing after dissolving in acetone. Finally, purified dialysis polymer solution was lyophilized to yield mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA. Similarly, mPEG-*b*-PCC-*g*-Cy7.5-*g*-DC-TEPA was synthesized.

Formulation and Characterization.

C225-, IgG-, and unmodified-micelles containing GEM and miR-205, were prepared by film hydration as reported earlier with slight modification. Briefly, 10 mg of C225-PEG-PCD (10–30% weight ratio) and mPEG-*b*-PCC-*g*-GEM-*g*-DC-TEPA (70–90% weight ratio) were taken in a glass vial, dissolved in CHCl₃, and followed by evaporation under reduced pressure to form a thin film. Similarly, IgG- and unmodified-micelles were prepared using 10 mg of IgG-PEG-PCD (30% weight ratio) and mPEG-*b*-PCC-*g*-GEM-*g*-DC-TEPA (70% weight ratio) and 10 mg of mPEG-*b*-PCC-*g*-GEM-*g*-DC-TEPA, respectively. HEPES buffer (10 mM, pH 6.5, 1 mL) containing miR-205 was then added to hydrate the film and solution was vortexed for 5 min and allowed to stand for 30 min at 37 °C to enable miRNA complexation. It was then centrifuged at 5000 g for 5 min and filtered through 0.22 μ m filter (Millipore). The particle size distribution and zeta (ζ) potential of 1 mg/mL of micelles (C225-, IgG-, and unmodified-micelles containing GEM and miR-205) was determined by dynamic light scattering using a Malvern Zetasizer. GEM loading to the copolymer was determined by alkaline hydrolysis as described previously.⁸ Agarose gel retardation assay was carried out to determine the appropriate N/P ratio resulting in effective complex formation between miR-205 and TEPA of the polymer. miRNA dissociation from micelles was carried out by polyanion competition assay as reported earlier.²²

Cellular Uptake Studies.

MIA PaCa-2 cells were obtained from ATCC (Manassas, VA) and GEM resistant MIA PaCa-2^R cells were maintained in DMEM containing 10% FBS and 1% antibiotic in an incubator at 37 °C/5% CO₂. Cellular uptake study was carried out in MIA PaCa-2^R cells treated with free BLOCK-iT fluorescent Oligo and C225-, IgG-, and unmodified-micelles containing BLOCK-iT fluorescent Oligo. Briefly, 2 $\times$ 10⁵ cells were seeded per well in six-well plates for 24 h. Free BLOCK-iT fluorescent Oligo and C225-, IgG-, and unmodified-micelles containing BLOCK-iT fluorescent Oligo (50 nM/well) were added to each well. After 4 h, the cells were washed with PBS and observed under an epifluorescence microscope. Further, for quantitative analysis of BLOCK-iT fluorescent oligo, cells were washed with phosphate buffer saline, trypsinized, again washed with cold PBS, and centrifuged. Then, the cell pellet was resuspended in PBS containing 4% paraformaldehyde and kept for 10 min at RT. Cells were centrifuged, washed with PBS, resuspended in 1 mL of PBS, and analyzed by a flow cytometer (BD LSRFortessa X-20). For competition studies, an excess of C225 was diluted with medium and added to cells 1 h before the addition of

C225-micelles. After 4 h, the cells were washed with PBS and images were taken under an inverted microscope (Zeiss, Germany).

Cytotoxicity Studies.

For checking transfection efficiency of miRNA in pancreatic cancer cells, MIA PaCa-2^R cells were seeded into 6-well plate at a density of 2×10^5 cells/well in serum-free DMEM medium. 3'-Fluorescein labeled oligonucleotide (50 nM/well) was complexed with lipofectamine RNAiMAX and transfected according to manufacturer's instructions (Thermo Fisher Scientific, MA). Four hours post-transfection, cells were observed under a fluorescent microscope (Zeiss, Germany). Similarly, for cytotoxic effect of GEM and miR-205 in combination, 5000 MIA PaCa-2^R cells per well were used in 96-well plate and cell viability was assessed by MTT assay after 72 h post-treatment with 500 nM GEM and 50 nM miR-205 mimic. Cytotoxicity of C225-, IgG-, and unmodified-micelles containing GEM and miR-205 were evaluated by MTT assay by treating MIA PaCa-2^R cells with free GEM (abbreviated F-GEM), GEM conjugated polymer (mPEG-*b*-PCC-*g*-GEM-*g*-DC, abbreviated as P-GEM), C225-, IgG-, and unmodified-micelles containing 500 nM GEM and 50 nM miR-205.

Biodistribution Studies.

All in vivo experiments were carried out following the NIH guidelines and protocol was approved prior to in vivo study by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center, Omaha, NE. MIA PaCa-2^R cells were stably transfected with lentiviral vector encoding luciferase and green fluorescent protein (GFP) (hLUC-Lv201-0200) using polybrene as described previously.³⁰ The orthotopic pancreatic tumor model was generated by implantation of 2×10^6 MIA PaCa-2^R-GFP-luciferase cells suspending in PBS:matrigel (1:1 v/v) into the pancreas of 6–8 week-old (NOD.Cg-Prkdcscid Il2rgtm1wjl/SzJ) NSG mice ($n = 3$). C225-, IgG-, and unmodified-micelles containing Cy7.5 (2 mg/kg) and hsa-miR-205-5p w/5' Dy677 (2 mg/kg) were injected into mice via the tail vein. After 6 h and 24 h post injection of mixed micellar formulations, D-luciferin (150 mg/kg) was intraperitoneally administered to each animal via injection, and bioluminescent as well as fluorescent was measured by IVIS System (Caliper Life Sciences). After bioluminescent as well as fluorescent measurement, mice were sacrificed and all major organs and tumors were removed for ex-vivo imaging using the IVIS Imaging System (Caliper Life Sciences) to determine the organ distribution of Cy7.5 dye, and Dy677 labeled miR-205.

Efficacy of C225-Micelles Carrying GEM and miR-205 in Orthotopic Pancreatic Tumor Bearing Mice.

After 7 days post implantation of luciferase and GFP stably expressing 2×10^6 MIA PaCa-2^R into NSG mice, we started bioluminescent measurement using IVIS Imaging System (Caliper Life Sciences) by intraperitoneally injection of D-luciferin (150 mg/kg) into mice. When bioluminescent value of tumor load was $\sim 4 \times 10^7$ p/sec/cm²/sr, mice were then randomly sorted into five groups ($n = 6$): control, C225-, IgG-, unmodified-micelles containing GEM and miR-205, and C225-micelles containing GEM and scrambled miRNA. Treatment was started at that time via intravenous administration thrice a week for 2 weeks

at the dose of 40 mg/kg GEM and 2 mg/kg miR-205 mimic or scrambled miRNA. Bioluminescent was measured thrice a week for 19 days' post-treatment. Mice were sacrificed and tumors were removed after final bioluminescent measurement. Tumors were fixed with 10% formaldehyde in PBS, and paraffin-embedded sections were immunostained for Ki-67 (cell proliferation marker), cleaved caspase 3 for apoptosis and hematoxylin and eosin (H&E). To detect tumor cells apoptosis induced by GEM and miR-205 or scrambled miRNA, MIA PaCa-2^R cells derived orthotopic tumor bearing mice ($n = 3$) were intravenously administered with C225-, IgG-, unmodified-micelles containing GEM and miR-205, and C225-micelles containing GEM and scrambled miRNA thrice a week for 2 weeks after 12 days post tumor inoculation. Mice were sacrificed 72 h post last injection, tumors were removed. Frozen tumors were cryosectioned and fixed with 10% paraformaldehyde and immunostained with a TUNEL kit as stated in the manufacturer's protocols and counterstained with DAPI and imaged under an inverted microscope.

Statistical Analysis.

Results are represented as the means $\pm$ SEM. The statistical analysis of the results was done using two-tailed Student's test. * $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis and Characterization of Polymers.

MAL-PEG-PDC, mPEG-*b*-PCC-*g*-GEM-*g*-DC-TEPA, mPEG-*b*-PCC-*g*-Cy7.5-*g*-DC-*g*-TEPA, and MCC were characterized by ¹H NMR (500 MHz, DMSO-d₆). mPEG-PCC was characterized by ¹H NMR (400 MHz, DMSO-d₆) and MDC was characterized by ¹H NMR (500 MHz, chloroform-d). MAL-PEG-PDC copolymer was synthesized by ring opening polymerization using 2-methyl-2-dodecanoxycarbonylpropylene carbonate (MDC) and MAL-PEG₅₀₀₀-OH and characterized by ¹H NMR analysis with desired peaks of PEG ($-\text{CH}_2-\text{CH}_2-\text{O}-$) at δ 3.5, PCC ($-\text{CO}-\text{O}-\text{CH}_2-$) at δ 4.2, $\text{CH}_3-(\text{CH}_2)_9-$ at δ 0.9, $\text{CH}_3-(\text{CH}_2)_9-$ at δ 1.2–1.45, $\text{CH}_3-(\text{CH}_2)_9-\text{CH}_2-\text{CH}_2-$ at δ 1.5–1.8, $\text{CH}_3-(\text{CH}_2)_9-\text{CH}_2-\text{CH}_2-$ at δ 4.02, $-\text{NH}-\text{CO}-\text{CH}_2-\text{CH}_2-$ at δ 2.5–3.2, $-\text{NH}-\text{CO}-\text{CH}_2-\text{CH}_2-$ at δ 4.6, $-\text{CO}-\text{CH}-\text{CH}-\text{CO}-$ at δ 7.2–7.5, and $-\text{NH}-\text{CO}-\text{CH}_2-\text{CH}_2-$ at δ 8.4 as described before (Figure S1).³⁰ Since C225 targets EGFR, we next synthesized C225-PEG-PCD using thiolated C225, MAL-PEG-PCD, and tris(2-carboxyethyl) phosphine hydrochloride (TECP) as shown in Figure 1A. For effective combination therapy, GEM, DC, and TEPA were conjugated to pendant carboxyl groups of mPEG-PCC to get mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA (Figure 1B). mPEG-PCC polymer showed characteristic ¹H NMR peaks of PEG ($-\text{CH}_2-\text{CH}_2-\text{O}-$) at δ 3.5, PCC ($-\text{CO}-\text{O}-\text{CH}_2-$) at δ 4.2, and $-\text{COOH}$ at δ 12–14 as described before (Figure S2).⁸ By peak integration of mPEG and PCC proton, we found ~30–32 PCC units with average molecular weight of 9800 Da. Further, the final mPEG-*b*-PCC-*g*-GEM-*g*-DC-TEPA showed characteristic ¹H NMR peaks of PEG ($-\text{CH}_2-\text{CH}_2-\text{O}-$) at δ 3.5, PCC ($-\text{CO}-\text{O}-\text{CH}_2-$) at δ 4.2, $\text{CH}_3-(\text{CH}_2)_9-$ at δ 0.9, $\text{CH}_3-(\text{CH}_2)_9-$ at δ 1.2–1.45, $\text{CH}_3-(\text{CH}_2)_9-\text{CH}_2-\text{CH}_2-$ at δ 1.5–1.8, $\text{CH}_3-(\text{CH}_2)_9-\text{CH}_2-\text{CH}_2-$ at δ 4.02, GEM 5'- and 3'-OH groups at δ 6.18–6.39, $-\text{CO}-\text{NH}-$ at δ 8.26, $-\text{CO}-\text{N}-\text{CH}-\text{CH}-$ at δ 8.15, and $-\text{CO}-\text{N}-\text{CH}=\text{CH}-$ at δ 5.35, TEPA ($-\text{CO}-\text{NH}-$) at δ 7–8, $-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}_2$ at δ 2.7–3.0, $-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}_2$ at δ 2.5–2.7, and $-\text{NH}-\text{CH}_2-\text{CH}_2-$

NH-CH₂-CH₂-NH-CH₂-CH₂-NH-CH₂-CH₂-NH₂ at δ 3–3.2 (Figure S3). Similarly, mPEG-*b*-PCC-*g*-Cy7.5-*g*-DC-*g*-TEPA were synthesized (Figure 1C) and characterized by ¹H NMR (Figure S4). MCC was synthesized by hydrogenation of MBC and characterized by ¹H NMR peaks of –COOH at δ 13.35, –CO–O–CH₂– at δ 4.2, and –O–CH₂–C(CH₃)– at δ 1.5–1.8 (Figure S5). EDC/HOBT coupling method was used to conjugate DC with the carboxyl group of MCC. At the end, MDC was purified and characterized by ¹H NMR showing characteristic peaks of –CO–O–CH₂– at δ 4.2, –O–CH₂–C(CH₃)– at δ 1.5–1.8, CH₃–(CH₂)₉– at δ 0.9, CH₃–(CH₂)₉– at δ 1.2–1.45, CH₃–(CH₂)₉–CH₂–CH₂– at δ 1.5–1.8, CH₃–(CH₂)–CH–CH– at δ 4.02 as reported earlier (Figure S6).³⁵

Formulation Development and Evaluation.

The efficiency of C225 conjugation to Mal-PEG-PCD was determined to be ~60% by BCA using IgG as a standard. The prepared 10–30% C225-, IgG-, and unmodified-micelles containing GEM and miR-205 showed the mean particle size of 121.7 ± 7 , 122.3 ± 6 , 124.6 ± 4 nm, 127.7 ± 4 , and 76.6 ± 6 nm with ζ potentials of -1.05 ± 0.4 , -1.82 ± 0.7 , -2.36 ± 0.6 , -2.73 ± 0.65 , and 4.73 ± 1.65 mV, respectively (Figures S7 and S8). After preparation of C225-micelles using 10 mg of C225-PEG-PCD (10–30% w/w) and mPEG-*b*-PCC-*g*-GEM-*g*-TEPA-*g*-DC (70–90% w/w), we determined the antibody concentration on micelles surface by BCA as reported previously.³⁶ We found ~510 μ g/mL C225-micelles (C225-PEG-PCD, 30% w/w, total 10 mg polymer), ~340 μ g/mL C225 (C225-PEG-PCD, 20% w/w, total 10 mg polymer), and ~170 μ g/mL C225 (C225-PEG-PCD, 10% w/w, total 10 mg polymer). GEM loading of 10% w/w in mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA was achieved as determined by alkaline hydrolysis. The complex formation between miR-205 and C225-, IgG-, and unmodified-micelles was determined at N/P ratios ranging from 4:1 to 128:1. At N/P ratio of 32:1, C225-, IgG-, and unmodified-micelles formed complete complex with miR-205 without any interference from C225, IgG, and GEM (Figure S9A). Further, the dissociation of miR-205 from these micelle formulations was observed at miRNA:heparin weight ratio of 1:8 (Figure S9B).

EGFR Mediated Cellular Uptake of Micelles.

Before going into cellular uptake study, we first investigated the cytotoxicity nature of C225-, IgG-, and unmodified-micelles. In our observation, these micelle formulations carrying BLOCK-iT fluorescent oligo did not show any cytotoxicity on MIA PaCa-2^R cells (Figure S10A). Since our objective was to achieve targeted delivery of C225 conjugated micelles, cellular uptake was studied on MIA PaCa-2^R cells after confirming EGFR expression (Figure S10B). As a result, higher uptake of 30% w/w C225-micelles was observed compared to IgG-micelles and unmodified micelles, whereas there was no significant differences between 10 and 20% w/w C225-micelles, IgG-micelles, and unmodified micelles indicating C225 density is crucial for targeting EGFR (Figure 2). Significant decrease in cellular uptake of 30% w/w C225-micelles was observed when MIA PaCa-2^R cells were presaturated with C225 (Figure 2). Similar pattern of cellular uptake was also evident in flow cytometry analysis (Figure 3A,B). We observed negligible cellular uptake in control experiment with free BLOCK-iT fluorescent oligo treated MIA PaCa-2^R cells (Figure S11).

Restoration of Chemosensitivity in Resistant Pancreatic Cancer Cells.

To determine whether there is a correlation between miR-205 expression level and the degree of GEM resistance, MIA PaCa-2^R cells were subjected to cytotoxicity assays. In good agreement with our previous studies, combination of GEM and miR-205 suppressed the proliferation of MIA PaCa-2^R cells by 38.9% compared to monotherapy (Figure S12B).^{19,22} These results indicate that the combination of GEM and miR-205 was more effective in suppressing the growth and could be an appropriate strategy to treat GEM resistant PDAC. Since miR-205 plays an important role in sensitizing GEM resistant pancreatic cancer cells, we further checked the efficacy of C225-micelles containing 500 nM GEM and 50 nM miR-205. As a result, MIA PaCa-2^R cells treated with C225-micelles showed significant reversal of chemoresistance compared to IgG- and unmodified-micelles containing GEM and miR-205 and C225-micelles containing GEM and scrambled miRNA (Figure S12C).

In Vivo and ex Vivo Imaging.

For whole body imaging, mice were intravenously injected with different micelles. As a result, 30% w/w C225-micelles containing conjugated Cy7.5 dye and complexed Dy677 labeled miR-205 showed significantly higher level of fluorescent signal in whole body at 6 and 24 h compared to IgG- and unmodified-micelles (Figures 4A and S13A). An enhanced level of tumor accumulation of C225-micelles was observed compared to IgG- and unmodified-micelles confirming EGFR mediated uptake of micellar formulation (Figures 4B and S13B). The quantitative analysis of Cy7.5 dye intensity in tumor tissue at 6 and 24 h were nearly 1.5 and 2-fold higher in C225-micelles compared to IgG- and unmodified-micelles (Figure 4C). Similarly, fluorescence intensity of Dy677 labeled miR-205 in tumor tissue at 6 and 24 h were nearly 1.6 and 3-fold higher in C225-micelles compared to IgG- and unmodified-micelles (Figure 4C), indicating C225-micelles to form stable electrostatic complex with miR-205 and protecting it during in vivo delivery.

In Vivo Therapeutic Study.

The synergistic effect of GEM and miR-205 after intratumoral administration in subcutaneous tumor bearing mice in our earlier study prompted us to evaluate the effect of this combination in more realistic orthotopic pancreatic tumor model.²² To continue this endeavor, we evaluated comparative therapeutic potential between EGFR targeted C225- and unmodified-micelles containing GEM (40 mg/kg) and miR-205 (2 mg/kg) after systemic administration. A significantly low level of bioluminescence intensity and smaller tumor size was observed in mice treated with C225-micelles containing GEM and miR-205 compared to others (Figure 5A,B,C). In addition, mice treated with C225-micelles containing GEM and miR-205 showed smallest tumor volume and tumor weight compared to others (Figure 6A,B) without any changes in body weight (Figure 6C). H&E staining of all the treated mice showed no obvious pathological change in the major organs, such as heart, liver, spleen, and kidney (Figure 7). Immunohisto-chemical staining of the tumor tissue from mice treated with C225-micelles containing GEM and miR-205 showed the lowest Ki-67 and highest cleaved caspase-3 expression compared to all other treatment groups (Figure 8). In addition, mice treated with C225-micelles containing GEM and miR-205 showed more TUNEL positive cells compared to all other treatment groups (Figure 9). Immunohistochemical

analysis of tumor specimen of mice treated with C225-micelles containing GEM and miR-205 showed significantly enhanced E-cadherin and reduced ZEB1 expression compared to other treatment groups (Figure 10).

DISCUSSION

GEM based chemotherapy does not completely improve the outcome of pancreatic cancer. Most patients develop drug resistance after repeated administration of GEM at a high dose.^{22,37} Therefore, to overcome GEM resistance, global effort has been directed to uncover molecular mechanisms behind this phenomenon. Most of the study has observed decrease in chemoresistance in preclinical studies, but not in clinical trials.

miRNAs modulate several genes in the post-transcriptional level or target mRNA degradation. Since several oncogenic and tumor suppressor miRNAs are linked to drug resistance, miRNA replenishment therapy would be an attractive approach to overcome GEM resistance. However, very few miRNAs (miR-218, miR-1246, miR-15a, miR-214, miR-29a, and miR-200c) have been examined for their roles in GEM resistance in pancreatic cancer.^{38–42} We have previously demonstrated that miR-205 was highly downregulated in pancreatic cancer tissues and GEM-resistant MIA PaCA-2^R cells and transient expression of miR-205 mimic reversed GEM resistance in MIA PaCA-2^R cells. The role of miR-205 in many different cancers, including melanoma, lung cancer, prostate cancer, and breast cancer, have been reported.^{43–46} Thus, a combination of miR-205 mimic with GEM could be an advantageous therapeutic approach to reverse GEM resistance and inhibit advanced pancreatic tumor progression.

Effective GEM delivery is hindered due to its hydrophilicity leading to low drug loading and rapid release. For codelivery of GEM and miRNA to overcome GEM resistance needs efficient nanomedicines that can conjugate GEM to enhance its loading and protect from plasma metabolism as well as a complex with miRNA to protect it from rapid degradation in circulation and increase membrane penetration. Recently, therefore, Mittal et al. synthesized amphiphilic copolymer mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA that has PEG forming the hydrophilic segment and polypropylene carbonate forming the hydrophobic segment, and this segment was grafted with GEM, DC, and cationic TEPA chains, which efficiently complexed with miR-205.²² Polyethylenimine (PEI) and poly(L-lysine) (PLL) are widely used as a cationic block grafted to polyester end groups for gene delivery, but this cationic segment has high toxicity in various cell lines.^{47,48} Xiong et al. used polycaprolactone backbone in cationic poly(ethylene oxide)-*b*-poly(ϵ -caprolactone-*g*-poly-amine) for siRNA delivery, but we used polycarbonate hydrophobic backbone in mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA for miR-205 delivery which improves biocompatibility as its degradation products are CO₂ and H₂O instead of acids like polyesters.⁴⁹ GEM hydrophilicity creates a problem for its encapsulation in nanocarriers. Thus, chemical conjugation approach was used to get better GEM loading (~10–12% w/w) and slow release compared to previously reported PEGylated GEM prodrugs.^{50,51}

Passive targeting is based on the enhanced permeation and retention (EPR) effect, whereas active targeting is based on specific interactions of drug or drug carrier with the target cells,

known as ligand–receptor interactions.⁵² This ligand–receptor interaction for active targeting is dependent on many factors, such as the receptor overexpression in target cells compared to nontarget cells and receptor availability in the cell surface of the target cells.⁵² EGFR, a cell surface receptor, and its over-expression in tumor cells facilitates selective targeting, and preclinical and clinical reports, indicating that EGFR targeting for cancer therapy is a valid strategy.⁵³ Several approaches have been developed for EGFR targeting by antibody-based immunoconjugates, such as immunoliposomes for targeted drug delivery to EGFR overexpressing cancer cells. Recent studies demonstrated that mAb C225 conjugated anti-EGFR immunoliposomes exhibit efficient activity in an EGFR-overexpressing MDA-MB-468 breast cancer cells and U-87 glioblastoma cells compared to nontargeted liposomes.^{31,54} Patra et al. proved that C225 conjugated gold nanoparticles exhibit effective GEM delivery to EGFR expressing pancreatic cancer and inhibited orthotopic pancreatic tumor growth.³² In our previous studies, we confirmed EGFR expression in MIA PaCa-2 cells, and we assumed that this expression would not change by GEM resistance. Further, we confirmed EGFR expression in MIA PaCa-2^R cells by FACS analysis (Figure S10B). Therefore, we selected C225 as a targeting agent for codelivery of GEM and miR-205 to EGFR expressing MIA PaCa-2^R cells in vitro and MIA PaCa-2^R derived orthotopic pancreatic tumor.

Chemical conjugation is the best strategy for covalent conjugation of targeting agent with the polymer otherwise, there is a chance for impairment of functional activity of targeting agent or reduction of targeting capability.⁵⁴ We previously reported GEM conjugation to the polymer, mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA, but we failed to conjugate C225 to the polar part of this polymer. To solve this synthetic complexity, we synthesized C225–PEG-PCD and IgG–PEG-PCD using thiolated C225 or IgG and MAL–PEG-PCD. We prepared mixed micelles using mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA and C225–PEG-PCD for EGFR targeting. We also prepared nontargeted mixed micelles using mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA and IgG–PEG-PCD. In our previous study, we found that miR-205 did not affect GEM release in PBS at pH 5.5 and 7.4 from the micelles as GEM is conjugated to the polymer via an amide bond, and GEM release critically depends on the cleavage of amide bond that is not disturbed by electrostatic interaction between miR-205 and cationic TEPA chain. We assume similar release pattern of GEM would be achieved from C225-micelles and IgG-micelles as thiolated C225 and thiolated IgG have conjugated via thiol bond to MAL–PEG-PCD polymer, respectively and this thiol bond are stable at pH 5.5 and 7.4 as well as would not disturb GEM release.⁵⁵ Micelles should protect miR-205 from degradation in a biological environment. We found that our unmodified-micelles, C225-micelles, and IgG-micelles improved miR-205 stability compared to naked miR-205 up to 24 h in serum (data not shown). This improved stability was observed due to stable electrostatic complex formation between miR-205 and cationic TEPA chain and steric effect exerted by PEG corona reduces miR-205 interaction with the exogenous RNases that are present in serum.²²

As EGFR mediated cellular uptake depends on EGFR recognition and internalization of C225 on the surface of C225-micelles, we noticed highest uptake of BLOCK-iT of C225-micelles (C225–PEG-PCD, 30%) in MIA PaCa-2^R cells compared to unmodified-, C225- (C225–PEG-PCD, 10–20%), and IgG-micelles (Figures 2 and 3A,B). However, the cellular

uptake of C225-micelles significantly diminished when MIA PaCA-2^R cells were presaturated with C225 before transfection, indicating C225-micelles specificity toward EGFR (Figure 2A–C). Although all C225-micelles have the same C225-targeting moiety and ζ potential close to zero, the cellular uptake efficacy improved with increase in C225–PEG-PCD weight percentage that may be due to C225 density on the micelle surface. We measured the amount of C225 per mL micelles (10 mg total polymer) using BCA method and found ~510 μg antibody/mL micelle (10 mg total polymer) in C225-micelles (C225–PEG-PCD, 30% w/w) and ~170–340 μg antibody/mL micelle (10 mg total polymer) in C225-micelles (C225–PEG-PCD, 10–20% w/w), respectively. It seems that at density ~170–340 μg /mL micelle (10 mg total polymer), the C225 might fail to reach surface saturation and may be responsible for poor EGFR targeting.⁵⁶ Therefore, we assume that this phenomena could be due to the changes in valency of C225 that increases EGFR binding affinity which is consistent with earlier study.^{57,58} Previous report also showed that cellular internalization can be increased with an optimal density of targeting agents on the surface of micelles.³⁰ On the other hand, the higher uptake of C225-micelles was due to EGFR mediated strong uptake of BLOCK-iT compared to IgG-micelles.⁵⁹ Since the combination of GEM and miR-205 has shown significant inhibition of MIA PaCa-2^R cells (Figure S12B) compared to GEM and miR-205 alone, we concentrated on GEM and miR-205 in combination to further evaluate its targeted delivery using C225 as a targeting ligand. We observed a significant enhancement in cytotoxicity when MIA PaCa-2^R cells were treated with C225--micelles containing GEM and miR-205 compared to IgG- and unmodified-micelle formulation carrying GEM and miR-205 (Figure S12C). This observation further confirms EGFR mediated cellular uptake of C225-micelles indicating the capability of GEM and miR-205 in combination to reverse GEM resistance. As 30% of C225-micelles containing GEM and miR-205 showed better cellular uptake and cytotoxicity profile, we used this formulation for in vivo study.

Cy7.5 labeling of mPEG-*b*-PCC-*g*-Cy7.5-*g*-DC-*g*-TEPA and Dy-677 labeling of miR-205 allowed in vivo and ex vivo near-infrared (NIR) fluorescence imaging, as well as tracking of both micellar carrier and miR-205 in the tumor. Mice treated with C225-micelles containing Cy7.5 and Dy677-miR-205 showed enhanced tumor accumulation of both components at 6 h and 24 h compared to unmodified and IgG-micelles, indicating the effect of C225 conjugation (Figures 4A–C and S13A,B). Unlike chemically conjugated Cy7.5, Dy677-miR-205 might dissociate from micelles or degraded in circulation after intravenous administration. Interestingly, C225-micelles protected Dy677-miR-205 in circulation and delivered Dy677-miR-205 to the tumor as Dy677 fluorescence intensity in tumors increased at 24 h than 6 h (Figure 4C), indicating excellent potential of C225-micelles for miRNA and drug delivery. This enhanced tumor accumulation of C225-micelles containing Cy7.5 and Dy677-miR-205 compared to other formulations was due to the presence of C225 on micelle surface for EGFR targeting and this is in good agreement with the work of Xiong et al.⁴⁹

A recent report showed that various miRNAs like miR-21, miR-34a, miR-143/145 cluster, miR-219–1–3p, miR-34a, miR-217, miR-29a, 330–5p, and miR-29c can be administered at doses ranging from 0.5 to 4 mg/kg for preclinical pancreatic cancer therapy.⁶⁰ Although in our previous work, we used 1 mg/kg miR-205 dose for intratumoral injection, we selected 2 mg/kg miR-205 or scrambled miRNA dose for intravenous administration. Mice treated with

C225-micelles containing GEM and miR-205 showed significantly low photon value compared to IgG-micelles, and unmodified micelles containing GEM and miR-205 treated mice. More importantly, mice treated with C225-micelles containing GEM and miR-205 showed a pronounced effect compared to C225-micelles containing GEM and scrambled miRNA treated mice and control mice (Figure 5A–C). Our findings clearly indicate that EGFR targeted codelivery of GEM and miR-205 has the potential to inhibit advanced pancreatic tumor growth. Tumor bioluminescent measurement is not corresponding absolute tumor volume but gives an indirect idea about tumor load.⁶¹

Average tumor volume and weight of mice treated with C225-micelles containing GEM and miR-205 showed significantly low compared to IgG-micelles, and unmodified micelles containing GEM and miR-205, C225-micelles containing GEM, and scrambled miRNA treated mice as well as the control mice. This further confirmed the strong therapeutic potential of C225-micelles containing GEM and miR-205 (Figure 6A,B). There was no change in body weights of mice in all treatment groups, suggesting little systemic toxicity of these formulations (Figure 6C). H&E staining of the control mice showed loss of cellular architecture with hypercellularity, whereas mice treated with C225-micelles containing GEM and miR-205 showed significant pyknosis, compared to IgG-micelles, and unmodified micelles containing GEM and miR-205 treated mice. This was more drastic when we compared with C225-micelles containing GEM, and scrambled miRNA treated mice (Figure 8). Analysis of Ki-67 and cleaved caspase 3 expressions of mice treated with C225-micelles containing GEM and miR-205 showed significant reduction in proliferation of tumor cells and the highest level of apoptosis compared to the control mice as well as all other treatment groups (Figure 8). Also, we found that treatment with C225-micelles containing GEM and miR-205 led to significant increase in dead cells in pancreatic tumors compared to all other treatment groups that were confirmed by TUNEL assay (Figure 9).

Wang et al. showed that GEM-resistant pancreatic cancer cells were associated with EMT and high level expression of ZEB1 and mesenchymal markers.⁶² Arumugam et al. found an inverse correlation between E-cadherin and ZEB1 in GEM-resistant pancreatic cancer cell lines.⁶³ Our IHC of control tumor tissue which was developed orthotopically in NSG mice using GEM-resistant MIA PaCa-2^R cells showed high expression of ZEB1 and low expression of E-cadherin that is consistent with a previous report (Figure 10). A recent report showed that miR-205 expression positively associated with E-cadherin expression but negatively correlated with ZEB1 expression in breast cancer.⁶⁴ Gregory et al. showed that ectopic expression of miR-205 in MDCK-Pez cells, was generated by overexpressing PTP-Pez in Madin-Darby canine kidney (MDCK) epithelial cells, induced mesenchymal to epithelial transition (MET) with upregulating E-cadherin and downregulating ZEB1.⁶⁵ Another report showed that downregulation of miR-205 expression level in esophageal squamous cell carcinoma cells reduced E-cadherin.⁶⁶ We have previously shown that combination formulation of GEM and miR-205 enhanced E-cadherin and reduced ZEB1 expression in MIA PaCa-2^R cells in vitro. Immunohistochemical staining of C225-micelles containing GEM and miR-205 treated mouse tissue showed enhanced E-cadherin and reduced ZEB1 expression compared to IgG-micelles and unmodified micelles containing GEM and miR-205. More importantly, C225-micelles containing GEM and scrambled miRNA treated mice showed E-cadherin and ZEB1 expression that is comparable to the

control mice, which further confirmed miR-205 has an important role in regulating E-cadherin and ZEB1.

CONCLUSION

In conclusion, we have synthesized C225-PEG-PCD and mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA for preparing micelles containing GEM and miR-205 and decorated with C225 antibody, which targets EGFR overexpressed on pancreatic cancer cells. There was significant enhancement in cellular uptake by tumor cells and reversal of GEM resistance, leading to enhanced tumor accumulation and consequent tumor regression. Uncovering other targeting ligands more potent than C225 would be an additional advantage to enhance the therapeutic outcome of this combination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We acknowledge the financial support from the National Institutes of Health (1R01EB017853) and the Faculty Start-up fund from the University of Nebraska Medical Center.

REFERENCES

- (1). Siegel RL; Miller KD; Jemal A Cancer statistics, 2017. *Ca-Cancer J. Clin* 2017, 67 (1), 7–30. [PubMed: 28055103]
- (2). Hwang RF; Moore T; Arumugam T; et al. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res* 2008, 68 (3), 918–926. [PubMed: 18245495]
- (3). Graeser R; Bornmann C; Esser N; et al. Antimetastatic effects of liposomal gemcitabine and empty liposomes in an orthotopic mouse model of pancreatic cancer. *Pancreas* 2009, 38 (3), 330–337. [PubMed: 19142174]
- (4). Brusa P; Immordino ML; Rocco F; Cattel L Antitumor activity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs. *Anticancer Res* 2007, 27 (1A), 195–199. [PubMed: 17352232]
- (5). Maksimenko A; Mougin J; Mura S; et al. Polyisoprenoyl gemcitabine conjugates self assemble as nanoparticles, useful for cancer therapy. *Cancer Lett* 2013, 334 (2), 346–353. [PubMed: 22935679]
- (6). Rejiba S; Reddy LH; Bigand C; Parmentier C; Couvreur P; Hajri A Squalenoyl gemcitabine nanomedicine overcomes the low efficacy of gemcitabine therapy in pancreatic cancer. *Nanomedicine* 2011, 7 (6), 841–849. [PubMed: 21419876]
- (7). Zhu S; Lansakara-P DS; Li X; Cui Z Lysosomal delivery of a lipophilic gemcitabine prodrug using novel acid-sensitive micelles improved its antitumor activity. *Bioconjugate Chem* 2012, 23 (5), 966–980.
- (8). Chitkara D; Mittal A; Behrman SW; Kumar N; Mahato RI Self-assembling, amphiphilic polymer-gemcitabine conjugate shows enhanced antitumor efficacy against human pancreatic adenocarcinoma. *Bioconjugate Chem* 2013, 24 (7), 1161–1173.
- (9). Kattel K; Mondal G; Lin F; Kumar V; Mahato RI Biodistribution of self-assembling polymer-gemcitabine conjugate after systemic administration into orthotopic pancreatic tumor bearing mice. *Mol. Pharmaceutics* 2017, 14, 1365.
- (10). Choi M; Razzaque S; Kim R Systemic therapy of advanced pancreatic cancer: Has the landscape changed? *Clin Adv. Hematol Oncol* 2012, 10 (7), 442–451. [PubMed: 22895284]

- (11). Teague A; Lim KH; Wang-Gillam A Advanced pancreatic adenocarcinoma: A review of current treatment strategies and developing therapies. *Ther. Adv. Med. Oncol* 2015, 7 (2), 68–84. [PubMed: 25755680]
- (12). Rocha Lima CM; Savarese D; Bruckner H; et al. Irinotecan plus gemcitabine induces both radiographic and CA 19–9 tumor marker responses in patients with previously untreated advanced pancreatic cancer. *J. Clin. Oncol* 2002, 20 (5), 1182–1191. [PubMed: 11870159]
- (13). Flerov MA The intensity of cerebral mono-, di- and triglyceride metabolism in growing animals under normal conditions and in hypoxia. *Nerv Sist* 1975, 15, 112–115. [PubMed: 1214930]
- (14). Rocha Lima CM; Green MR; Rotche R; et al. Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. *J. Clin. Oncol* 2004, 22 (18), 3776–3783. [PubMed: 15365074]
- (15). Von Hoff DD; Ervin T; Arena FP; et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N. Engl. J. Med* 2013, 369 (18), 1691–1703. [PubMed: 24131140]
- (16). Ji Q; Hao X; Zhang M; et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PLoS One* 2009, 4 (8), e6816. [PubMed: 19714243]
- (17). Danquah M; Singh S; Behrman SW; Mahato RI Role of miRNA and cancer stem cells in chemoresistance and pancreatic cancer treatment. *Expert Opin. Drug Delivery* 2012, 9 (12), 1443–1447.
- (18). Wang Z; Li Y; Ahmad A; et al. Pancreatic cancer: Understanding and overcoming chemoresistance. *Nat. Rev. Gastro-enterol. Hepatol* 2011, 8 (1), 27–33.
- (19). Singh S; Chitkara D; Kumar V; Behrman SW; Mahato RI miRNA profiling in pancreatic cancer and restoration of chemo-sensitivity. *Cancer Lett* 2013, 334 (2), 211–220. [PubMed: 23073476]
- (20). Chaudhary AK; Mondal G; Kumar V; Kattel K; Mahato RI Chemosensitization and inhibition of pancreatic cancer stem cell proliferation by overexpression of microRNA-205. *Cancer Lett* 2017, 402, 1–8. [PubMed: 28536008]
- (21). Chitkara D; Mittal A; Mahato RI miRNAs in pancreatic cancer: Therapeutic potential, delivery challenges and strategies. *Adv. Drug Delivery Rev* 2015, 81, 34–52.
- (22). Mittal A; Chitkara D; Behrman SW; Mahato RI Efficacy of gemcitabine conjugated and miRNA-205 complexed micelles for treatment of advanced pancreatic cancer. *Biomaterials* 2014, 35 (25), 7077–7087. [PubMed: 24836307]
- (23). Khan JA; Kudgus RA; Szabolcs A; et al. Designing nanoconjugates to effectively target pancreatic cancer cells in vitro and in vivo. *PLoS One* 2011, 6 (6), e20347. [PubMed: 21738572]
- (24). Singh R; Lillard JW Jr. Nanoparticle-based targeted drug delivery. *Exp. Mol. Pathol* 2009, 86 (3), 215–223. [PubMed: 19186176]
- (25). Accardo A; Aloj L; Aurilio M; Morelli G; Tesaro D Receptor binding peptides for target-selective delivery of nanoparticles encapsulated drugs. *Int. J. Nanomed* 2014, 9, 1537–1557.
- (26). Peters C; Brown S Antibody-drug conjugates as novel anti-cancer chemotherapeutics. *Biosci. Rep* 2015, 35 (4), e00225. [PubMed: 26182432]
- (27). Vhora I; Patil S; Bhatt P; Gandhi R; Baradia D; Misra A Receptor-targeted drug delivery: Current perspective and challenges. *Ther. Delivery* 2014, 5 (9), 1007–1024.
- (28). Bloomston M; Bhardwaj A; Ellison EC; Frankel WL Epidermal growth factor receptor expression in pancreatic carcinoma using tissue microarray technique. *Dig. Surg* 2006, 23 (1–2), 74–79. [PubMed: 16717472]
- (29). Baselga J Why the epidermal growth factor receptor? the rationale for cancer therapy. *Oncologist* 2002, 7 (90004), 2–8. [PubMed: 12202782]
- (30). Mondal G; Kumar V; Shukla SK; Singh PK; Mahato RI EGFR-targeted polymeric mixed micelles carrying gemcitabine for treating pancreatic cancer. *Biomacromolecules* 2016, 17 (1), 301–313. [PubMed: 26626700]
- (31). Mamot C; Ritschard R; Wicki A; et al. Immunoliposomal delivery of doxorubicin can overcome multidrug resistance mechanisms in EGFR-overexpressing tumor cells. *J. Drug Target* 2012, 20 (5), 422–432. [PubMed: 22519893]

- (32). Patra CR; Bhattacharya R; Wang E; et al. Targeted delivery of gemcitabine to pancreatic adenocarcinoma using cetuximab as a targeting agent. *Cancer Res* 2008, 68 (6), 1970–1978. [PubMed: 18339879]
- (33). Rocha-Lima CM; Soares HP; Raez LE; Singal R EGFR targeting of solid tumors. *Cancer Control* 2007, 14 (3), 295–304. [PubMed: 17615536]
- (34). Owonikoko TK; Sun SY; Ramalingam SS The role of cetuximab in the management of non-small-cell lung cancer. *Clin. Lung Cancer* 2009, 10 (4), 230–238. [PubMed: 19632939]
- (35). Li F; Danquah M; Mahato RI Synthesis and characterization of amphiphilic lipopolymers for micellar drug delivery. *Biomacromolecules* 2010, 11 (10), 2610–2620. [PubMed: 20804201]
- (36). Pan X; Wu G; Yang W; Barth RF; Tjarks W; Lee RJ Synthesis of cetuximab-immunoliposomes via a cholesterol-based membrane anchor for targeting of EGFR. *Bioconjugate Chem* 2007, 18 (1), 101–108.
- (37). Oettle H; Neuhaus P; Hochhaus A; et al. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: The CONKO-001 randomized trial. *JAMA* 2013, 310 (14), 1473–1481. [PubMed: 24104372]
- (38). Liu Z; Du R; Long J; et al. microRNA-218 promotes gemcitabine sensitivity in human pancreatic cancer cells by regulating HMGB1 expression. *Chin J. Cancer Res* 2015, 27 (3), 267–278. [PubMed: 26157323]
- (39). Hasegawa S; Eguchi H; Nagano H; et al. MicroRNA-1246 expression associated with CCNG2-mediated chemoresistance and stemness in pancreatic cancer. *Br. J. Cancer* 2014, 111 (8), 1572–1580. [PubMed: 25117811]
- (40). Zhang XJ; Ye H; Zeng CW; He B; Zhang H; Chen YQ Dysregulation of miR-15a and miR-214 in human pancreatic cancer. *J. Hematol. Oncol* 2010, 3, 46–8722–3–46.
- (41). Nagano H; Tomimaru Y; Eguchi H; et al. MicroRNA-29a induces resistance to gemcitabine through the wnt/beta-catenin signaling pathway in pancreatic cancer cells. *Int. J. Oncol* 2013, 43 (4), 1066–1072. [PubMed: 23900458]
- (42). Ma C; Huang T; Ding YC; et al. MicroRNA-200c overexpression inhibits chemoresistance, invasion and colony formation of human pancreatic cancer stem cells. *Int. J. Clin Exp Pathol* 2015, 8 (6), 6533–6539. [PubMed: 26261532]
- (43). Dar AA; Majid S; de Semir D; Nosrati M; Bezrookove V; Kashani-Sabet M miRNA-205 suppresses melanoma cell proliferation and induces senescence via regulation of E2F1 protein. *J. Biol. Chem* 2011, 286 (19), 16606–16614. [PubMed: 21454583]
- (44). Tellez CS; Juri DE; Do K; et al. EMT and stem cell-like properties associated with miR-205 and miR-200 epigenetic silencing are early manifestations during carcinogen-induced transformation of human lung epithelial cells. *Cancer Res* 2011, 71 (8), 3087–3097. [PubMed: 21363915]
- (45). Bhatnagar N; Li X; Padi SK; Zhang Q; Tang MS; Guo B Downregulation of miR-205 and miR-31 confers resistance to chemotherapy-induced apoptosis in prostate cancer cells. *Cell Death Dis* 2010, 1, e105. [PubMed: 21368878]
- (46). Adachi R; Horiuchi S; Sakurazawa Y; Hasegawa T; Sato K; Sakamaki T Erbb2 down-regulates microRNA-205 in breast cancer. *Biochem. Biophys. Res. Commun* 2011, 411 (4), 804–808. [PubMed: 21787752]
- (47). Arote R; Kim TH; Kim YK; et al. A biodegradable poly(ester amine) based on polycaprolactone and polyethylenimine as a gene carrier. *Biomaterials* 2007, 28 (4), 735–744. [PubMed: 17034844]
- (48). Ogris M; Brunner S; Schuller S; Kircheis R; Wagner E PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* 1999, 6 (4), 595–605. [PubMed: 10476219]
- (49). Xiong XB; Lavasanifar A Traceable multifunctional micellar nanocarriers for cancer-targeted co-delivery of MDR-1 siRNA and doxorubicin. *ACS Nano* 2011, 5 (6), 5202–5213. [PubMed: 21627074]
- (50). Vandana M; Sahoo SK Long circulation and cytotoxicity of PEGylated gemcitabine and its potential for the treatment of pancreatic cancer. *Biomaterials* 2010, 31 (35), 9340–9356. [PubMed: 20851464]

- (51). Pasut G; Canal F; Dalla Via L; Arpicco S; Veronese FM; Schiavon O Antitumoral activity of PEG-gemcitabine prodrugs targeted by folic acid. *J. Controlled Release* 2008, 127 (3), 239–248.
- (52). Bae YH; Park K Targeted drug delivery to tumors: Myths, reality and possibility. *J. Controlled Release* 2011, 153 (3), 198–205.
- (53). Mamot C; Ritschard R; Kung W; Park JW; Herrmann R; Rochlitz CF EGFR-targeted immunoliposomes derived from the monoclonal antibody EMD72000 mediate specific and efficient drug delivery to a variety of colorectal cancer cells. *J. Drug Target* 2006, 14 (4), 215–223. [PubMed: 16777680]
- (54). Mamot C; Drummond DC; Noble CO; et al. Epidermal growth factor receptor-targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs in vivo. *Cancer Res* 2005, 65 (24), 11631–11638. [PubMed: 16357174]
- (55). Christie RJ; Fleming R; Bezabeh B; et al. Stabilization of cysteine-linked antibody drug conjugates with N-aryl maleimides. *J. Controlled Release* 2015, 220, 660–670.
- (56). Elias DR; Poloukhine A; Popik V; Tsourkas A Effect of ligand density, receptor density, and nanoparticle size on cell targeting. *Nanomedicine* 2013, 9 (2), 194–201. [PubMed: 22687896]
- (57). Crothers DM; Metzger H The influence of polyvalency on the binding properties of antibodies. *Immunochemistry* 1972, 9 (3), 341–357. [PubMed: 4113719]
- (58). Poon Z; Chen S; Engler AC; et al. Ligand-clustered “patchy” nanoparticles for modulated cellular uptake and in vivo tumor targeting. *Angew. Chem., Int. Ed* 2010, 49 (40), 7266–7270.
- (59). Raynal I; Prigent P; Peyramaure S; Najid A; Rebuzzi C; Corot C Macrophage endocytosis of superparamagnetic iron oxide nanoparticles: Mechanisms and comparison of ferumoxides and ferumoxtran-10. *Invest. Radiol* 2004, 39 (1), 56–63. [PubMed: 14701989]
- (60). Merhautova J; Demlova R; Slaby O MicroRNA-based therapy in animal models of selected gastrointestinal cancers. *Front. Pharmacol* 2016, 7, 329. [PubMed: 27729862]
- (61). Klerk CP; Overmeer RM; Niers TM; et al. Validity of bioluminescence measurements for noninvasive in vivo imaging of tumor load in small animals. *BioTechniques* 2007, 43, S7–S13 30..
- (62). Wang Z; Li Y; Kong D; et al. Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway. *Cancer Res* 2009, 69 (6), 2400–2407. [PubMed: 19276344]
- (63). Arumugam T; Ramachandran V; Fournier KF; et al. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. *Cancer Res* 2009, 69 (14), 5820–5828. [PubMed: 19584296]
- (64). Lee JY; Park MK; Park JH; et al. Loss of the polycomb protein mel-18 enhances the epithelial-mesenchymal transition by ZEB1 and ZEB2 expression through the downregulation of miR-205 in breast cancer. *Oncogene* 2014, 33 (10), 1325–1335. [PubMed: 23474752]
- (65). Gregory PA; Bert AG; Paterson EL; et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol* 2008, 10 (5), 593–601. [PubMed: 18376396]
- (66). Matsushima K; Isomoto H; Yamaguchi N; et al. MiRNA-205 modulates cellular invasion and migration via regulating zinc finger E-box binding homeobox 2 expression in esophageal squamous cell carcinoma cells. *J. Transl. Med* 2011, 9, 30–5876–9–30.

Figure 1.

Synthesis schemes of copolymers. (A) C225-PEG-PCD copolymer; (B) mPEG-*b*-PCC-*g*-GEM-*g*-DC-*g*-TEPA; (C) mPEG-*b*-PCC-*g*-Cy7.5-*g*-DC-*g*-TEPA. Reagents: (i) HEPES buffer (pH 8.0), 1 h, RT; PD-10 column, HEPES buffer (pH 6.5); (ii) KOH, DMF, benzyl bromide, 100 °C, 15 h; (iii) triphosgene, pyridine, CH₂Cl₂, -78 to 0 °C; (iv) 10% Pd/C, H₂, ethyl acetate, RT, 40 psi, 3 h; (v) EDC, HOBT, TEA, DMF, dodecanol, RT, 18 h; (vi) DBU, MAL-PEG₅₀₀₀-OH, 3 h, RT; (vii) distilled water, TECP (100 mM), RT, N₂, 24 h; 1 mM MEA, Sepharose CL-4B; (viii) 10% Pd/C, H₂, THF:MeOH (1:1), RT, 40 psi, 18 h; (ix) Sn(Oct)₂, mPEG₅₀₀₀-OH, 100 °C, 18 h; (x) EDC, HOBT, DIPEA, DMF, GEM, TEPA, DC, RT, N₂, 72 h; (xi) EDC, HOBT, DIPEA, DMF, Cy7.5-NH₂, TEPA, DC, RT, N₂, 72 h.

Figure 2.

EGFR mediated cellular uptake of micelles containing BLOCK-iT fluorescent oligo. Inverted microscopic images of MIA PaCa-2^R cells treated with C225-micelles (C225-PEG-PCD, 10–30% w/w), IgG-, and unmodified-micelles and preincubated with free C225 antibody prior treated with C225-micelles (C225-PEG-PCD, 30% w/w). Scale bar, 100 μ M.

Figure 3.

Flow cytometry quantification of EGFR mediated cellular uptake. Overlay histogram of red fluorescent protein (RFP) expression (A) and mean fluorescence intensity (B) of MIA PaCa-2^R cells treated with C225- micelles (10–30% C225–PEG-PCD), IgG- micelles, unmodified micelles containing BLOCK-iT Fluorescent Oligo (50 nM/well). To evaluate EGFR mediated cellular uptake, MIA PaCa-2R cells were pre-incubated with C225 and then treated with C225-micelles (30% C225–PEG-PCD). *** $p < 0.001$ compared to C225-micelles (30% C225-PEG-PCD); 1: C225-micelles +Block-IT (10% C225–PEG-PCD); 2: C225-micelles+Block-IT (20% C225–PEG-PCD), 3: C225-micelles+Block-IT (30% C225–PEG-PCD), 4: IgG-micelles+Block-IT, 5: Unmodified-micelles+Block-IT, 6: C225+C225-micelles+Block-IT (30% C225–PEG-PCD). Data presented as the mean $\pm$ SEM ($n = 3$).

Figure 4.

Near-infrared (NIR) fluorescence in vivo and ex vivo imaging of micelles containing conjugated Cy7.5 and DY677-miR-205. NSG mice bearing orthotopic MIA PaCa-2^R-GFP-LUC cells derived pancreatic tumors injected intravenously with unmodified, IgG-, and C225-micelles containing Cy7.5 (2 mg/kg) and DY677-miR-205 (2 mg/kg). Tumor and other vital organs were removed after 24 h post injections. The bioluminescence, fluorescence, and overlay images of NIRF probes in live mice were imaged at 24 h after injection (A), ex vivo NIR fluorescence images of Cy7.5 and Dy677 NIR fluorescent probes in dissected vital organs and tumor were taken (B), and quantitative analysis of Cy7.5 and DY677 probes intensity in tumor at 6 and 24 h (C). Lu: lung; H: heart; S: spleen; K: kidney; Li: liver; and T: tumor. Data presented as the mean SEM ($n = 3$). * $p < 0.05$ compared to C225-micelles containing Cy7.5 and DY677-miR-205 treated group.

Figure 5.

In vivo efficacy of C225-micelles containing GEM (40 mg/kg) and miR-205 (2 mg/kg) after intravenous administration in NSG mice bearing orthotopic MIA PaCa-2^R-GFP-LUC cells implanted pancreatic tumor. Representative bioluminescence images (BLI) of mice after treatment with unmodified, IgG-, and C225-micelles containing GEM and miR-205 and C225-micelles containing GEM and scrambled miRNA ($n = 6$) (A). BLI photon intensity measurement started when tumor load was $\sim 4 \times 10^7$ of all treated groups and control group and continued up to final measurement (B). Data represented as the mean $\pm$ SEM ** $p < 0.01$; * $p < 0.05$ compared to C225-micelles containing GEM and miR-205 treated group. After the end of in vivo experiment, tumors were dissected from all groups and representative tumor images were taken (C).

Figure 6.

Combination of GEM with miR-205 suppressed tumor volume and weight. At the end of experiment tumor from all groups were dissected, and tumor weight (A) and tumor volume (B) were measured. Data presented as the mean $\pm$ SEM ($n = 5$) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (A) and, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to C225-micelles containing GEM and miR-205 treated group (B). After first BLI measurement, body weights of all mice was measured up to end of the experiment to check unwanted toxicity due to the combination therapy of GEM with miR-205 (C).

Figure 7.
Hematoxylin and eosin (H&E) staining of vital organs (heart, liver, kidney, and spleen).
Scale bar, 100 μ M.

Figure 8.

Immunohistochemical staining of tumor sections for hematoxylin and eosin (H&E), cell proliferation marker (Ki-67) and apoptosis marker cleaved caspase 3. At the end of in vivo study, small piece of tumor tissue of all groups was fixed in formalin, paraffin embedded cryosectioned and immunostained for H&E (upper panel), Ki-67 (middle panel) and cleaved caspase 3 (lower panel). Images were taken at 40× magnification. Scale bar, 100 μm .

Figure 9.

TUNNEL assay of tumor specimens of orthotopic MIA PaCa-2^R cells derived pancreatic tumor bearing NSG mice treated with unmodified, IgG-, and C225-micelles containing GEM and miR-205 and C225-micelles containing GEM and scrambled miRNA. Tumor tissues were fixed, paraffin cryosectioned and immunostained for TUNEL to detect apoptosis of tumor cells (green, upper panel), nucleus stained with DAPI (blue, middle panel). Images were taken at 40× magnification. Scale bar, 100 μ m.

Figure 10.

Immunohistochemical staining of micelles containing GEM and miR-205 treated tumor tissue for zinc-finger E-box binding homeobox 1 (ZEB1) and E-cadherin. Intravenous administration of unmodified, IgG- and C225-micelles containing GEM and miR-205 and C225-micelles containing GEM and NC miRNA treated tumor tissue and control tumor tissue were fixed, paraffin embedded cryosectioned and immunostained for ZEB1 (upper panel) and E-cadherin (lower panel). Images were taken at 40× magnification. Scale bar, 100 µm.