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Development of Worm-like Polymeric Drug Carriers with Multiple Ligands for Targeting Heterogeneous Breast Cancer Cells

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In this study, wormorm-like polymeric micelles were construted from poly(L-lactic acid)-*b*-poly(ethyelen glycol) (PLLA-*b*-PEG) block copolymers *via* worm-like (or cylindrical) self- assembly that consisted of a relatively long PLLA block (M_n 7K Daltons) at the core and a relatively short PEG block (M_n 2K Daltons) as the shell. Several cancer-targeting moieties (such as folate, cobalamin, and cyclic arginine-glycine-aspartic (RGD) peptide) were chemically coupled with the succinylated or maleimided PEG block of PLLA-*b*-PEG to act as a cancer cell-specific targeting ligand for breast cancer. The worm-like micelles with muplite cancer cell-specific ligands proved to be successful in recognizing different breast cancer cells at once. This has the potential to aid in cancer-specific drug delivery and to be used as an effective treatment for breast cancer.

Key Words: Worm-like micelle, Multiple ligands, Tumor targeting

Introduction

Solid cancers are frequently characterized by either specific antigens or overexpressed receptors on cell surfaces.[1-3] Antigens, or receptors, provide uptake routes for nutrients, as well as signals from the surrounding environment, which is essential for active growth of the cancer cells. Targeting antigens or receptors have been extensively utilized as an important delivery mode for macromolecular and/or nano-sized carriers to kill cancer cells or suppress aggressive cell growth.[4-9] However, drug carriers exploit these uptake pathways by presenting surface ligands or antibodies on the nanocarriers, and usually exhibit limited efficacy in clinical settings.[10,11] The heterogeneous antigen or receptor expression on cell surfaces and various cell populations in cancer tissues[4-15] have reduced the efficiency of these drug carriers in cancer targeting.

Breast cancers include various cell lines such as SKBR3, MDA-MB-231, MCF-7, BT-20, T-47D, HCC38, SKBR3, AU-565, ZR751, and HCC70, and, according to the individual cell line, have different biological characteristics.[14,15] For example, the well-known HER2 antigen (human epidermal growth factor receptor 2 protein) has been detected in 20 - 30% of human breast cancer cells and has been partially targeted by Herceptin®.[16] Only 20 - 50% of breast cancer cells express the folate receptor (FR),[13] which decreases the pharmaceutical efficacy of FR-targeting drug carrier systems for *in vivo* breast cancer.[17,18] This issue may imply the necessity for a novel drug delivery system that is able to overcome imperfect expression of specific antigens or receptors on breast cancer cells.

Non-spherical worm-like polymeric micelles, bio-inspired by filovirus that infects human cells, have been attracting great interest as a new class of drug delivery carriers.[19-21] The worm-like micelles, which are self-assembled and prepared in water from amphiphilic block copolymers, exhibit the potential to deliver a large quantity of drugs per carrier.[19-21] Furthermore, due to their flexible, long shapes, which reduce phagocytic clearance in body,[19] the worm-like micelles have the enhanced

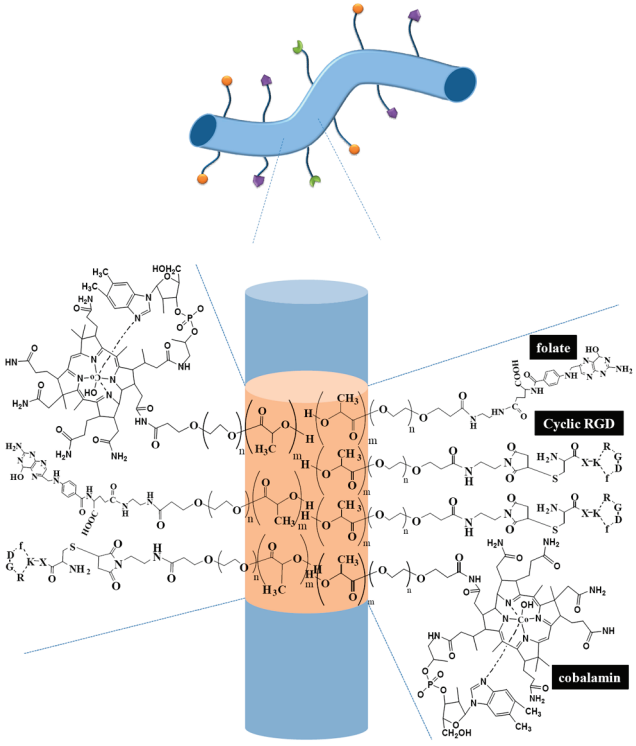

Figure 1. Schematic diagram depicting non-spherical polymeric micelles with multiple ligands. See the text for more details.

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blood circulation (with improved blood stability) for up to 1 week, which may be longer than any other synthetic nanoparticle.[19-21] In particular, the relative broad surface area of the worm-like micelles present positive features for surface functionality.

In this study, we first developed non-spherical worm-like polymeric micelles with multiple ligands (i.e., folate, cobalamin, cyclic RGD) (Figure 1). This system was designed to improve the cancer specificity of drug carriers and to effectively multi-target heterogeneous breast cancer cells. The folate or cobalamin, as vitamin B, was utilized for targeting FR-expressing or cobalamin receptor-expressing breast cancer cells.[16-18,22] The cyclic RGD was coupled with the micelles to specifically target breast cancer cells that overexpressed integrin$\alpha_v\beta_3$ (as a receptor for cyclic RGD).[23,24] Herein, we investigated the morphological properties of worm-like micelles with multiple ligands and evaluated their cancer specificity in an *in vitro* cell uptake test.

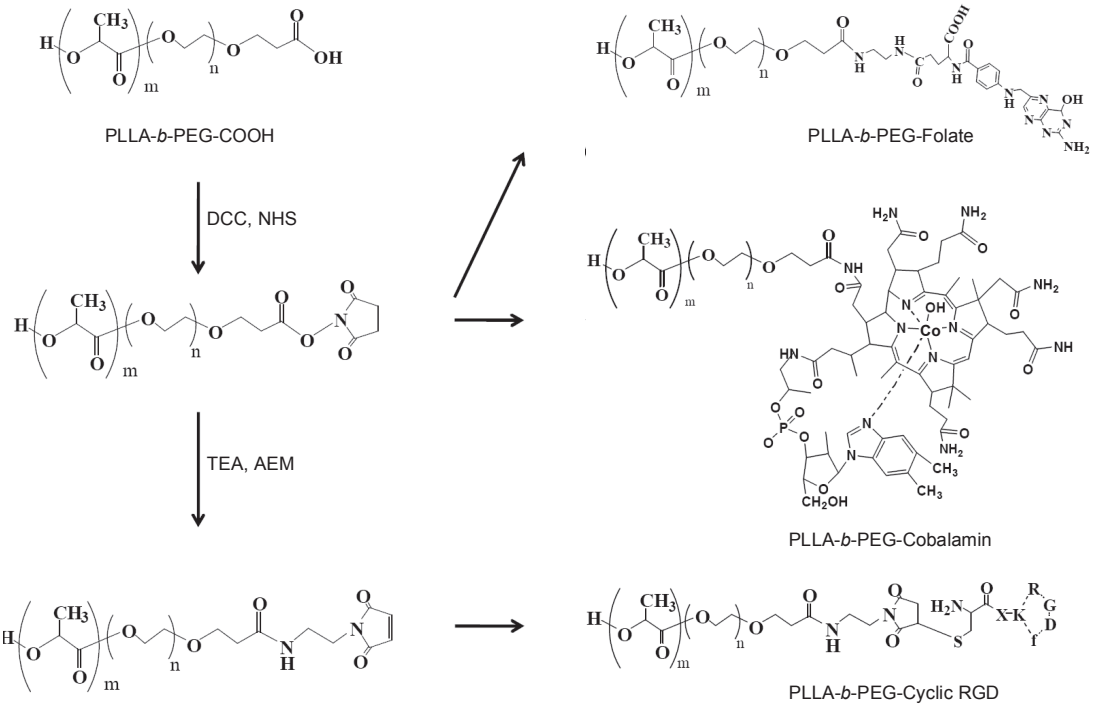
Experimental

Materials. *N*-Hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), triethylamine (TEA), *N*-(2-aminoethyl) maleimide (AEM), dimethylsulfoxide (DMSO), dichloromethane (DCM), fluorescence isothiocyanate (FITC), tetramethylrhodamine-succinyl ester (TMRHD), folate, cobalamin, ethylenediamine, *n*-propyl galate, glycerol, and doxorubicin·HCl (DOX·HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penicillin-streptomycin, Tris-HCl (pH 8.4), fetal bovine serum (FBS), 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution, and RPMI1640 medium were purchased from Gibco (Uxbridge, UK). Cyclic RGD (Cys-KRGDf: Cys-Lys-Arg-Gly-Asp-phe) was purchased from Peptron Inc. (Republic of Korea). Cy5.5® Bis NHS ester was provided by GE Healthcare (Piscataway, NJ, USA). PLLA (M_n 5012: 5K, 6982: 7K, 10413: 10K Daltons)-*b*-PEG (M_n 2K Daltons)-COOH[25,26] and folate-amine[27,28] were synthesized as described in detail in our previous studies.

PLLA-*b*-PEG-COOH with multiple ligands. Before coupling between the ligand and PLLA-*b*-PEG-COOH, the carboxyl group of PLLA-*b*-PEG-COOH (20 mmol) was pre-activated with NHS (24 mmol) and DCC (24 mmol) in DCM at room temperature for 4 hours (Scheme 1). After this reaction, the solution was filtered to remove 1,3-dicyclohexylurea (DCU) (as a byproduct), and was then mixed with excess diethyl ether to precipitate the product. The dried pre-activated PLLA-*b*-PEG-COOH (PLLA-*b*-PEG-NHS, 20 mmol) was further activated with AEM (40 mmol) in DCM with TEA (40 mmol) at room temperature for 8 hours (Scheme 1). The resulting PLLA-*b*-PEG-AEM was recrystallized from excess diethyl ether.

The ligand (cobalamin or folate-amine, 24 mmol) was conjugated with PLLA-*b*-PEG-NHS (20 mmol) in DMSO with TEA (30 mmol). Thiol group of cyclic RGD (1 mmol) was coupled with PLLA-*b*-PEG-AEM (1 mmol) in a DMSO/water mixture (80:20 vol %) (Scheme 1). After the reaction was completed, the solution was transferred to a pre-swollen dialysis membrane tube (Spectra/Por; MWCO 2K) and dialyzed against deionized water to remove unconjugated ligands for 2 days. The resulting solution was freeze-dried for 2 days.

Preparation of non-spherical micelles. PLLA-*b*-PEG without ligands or PLLA-*b*-PEG with ligands [PLLA-*b*-PEG -folate (10 wt %), PLLA-*b*-PEG-cobalamin (10 wt %), PLLA-*b*-PEG-cyclic RGD (6 wt %), PLLA-*b*-PEG (74 wt %)] were dissolved in DCM with or without DOX and transferred to a 50 mL round flask. DCM in the solution was eliminated using a rotary evapo-



Scheme 1. Synthesis of PLLA-*b*-PEG with different ligands such as folate, cobalamin, and cyclic RGD

rator. The polymers coated on the round flask were mixed with 20 mL of deionized water and then sonicated at a frequency of 120 kHz for 20 min to fabricate rod-like or worm-like polymeric micelles. Subsequently, the solution was stirred for 2 days at 20 - 90 ^{o}C.

Before loading the DOX into the micelles, DOX·HCl (1 mol) was stirred into a two-mole ratio of TEA in DMSO overnight in order to increase the hydrophobicity of DOX.[25,26] When a total of 9 mg of polymer with 1 mg of DOX was used to produce micelles, 0.95 mg of the DOX was encapsulated into the micelles. The amount of entrapped DOX was determined by measuring the UV absorbance at 481 nm of the drug-loaded polymeric micelles dissolved in DMSO (as previously described).[25,26]

Morphology observation. In order to confirm the morphology change of the non-spherical micelles, each sample was prepared by casting a dilute micelle solution (0.1 mg/mL) in phosphate buffer saline (PBS) pH 7.4 on a slide glass, which was then dried in vacuo. The morphology of the samples was imaged by Field Emission Scanning Electron Microscopy (FE-SEM, Hitachi s-4800).

In addition, the ligand distribution on the non-spherical worm-like micelles was investigated using a confocal microscope (Leica TCS NT, Leica, Germany). For this test, each ligand was labeled with fluorescent dyes such as FITC, TMRHD, and Cy5.5. Briefly, the residual carboxyl group of folate coupled with PLLA-*b*-PEG (1 mmol) was aminated with excess ethylenediamine in DMSO for 4 hours, and was then tagged with Cy5.5® Bis NHS ester (1.2 mmol) for 8 hours. Cobalamin (10 mmol), coupled with PLLA-*b*-PEG, was labeled with FITC (12 mmol) in DMSO for 8 hours. Each solution was dialyzed against deionized water for 2 days to remove unconjugated ligands, and then lyophilized. Cyclic RGD-TMRHD was purchased from Peptron Inc. and was used for the conjugation with PLLA-*b*-PEG-AEM. Next, the micelles were prepared from PLLA-*b*-PEG with fluorescent ligands. The obtained micelle solution was casted on a slide glass and dried prior to imaging with a confocal microscope. A coverslip was mounted on a glass microscope slide with a drop of anti-fade mounting medium (5% *N*-propyl galate, 47.5% glycerol and 47.5% Tris-HCl at pH 8.4). The specimens for fluorescence were examined under a confocal microscope (FITC: λ_{ex} 488 nm and λ_{em} 510 nm, TMRHD: λ_{ex} 570 nm and λ_{em} 595 nm, Cy5.5: λ_{ex} 670 nm and λ_{em} 700 nm).

Drug release test. A DOX-loaded polymeric micelle solution (0.5 mL, DOX content: 0.1 mg) was added to a dialysis membrane bag (Spectra/Por MWCO 15K). The dialysis membrane bag was sealed and then immersed in a vial containing fresh PBS (10 mL, ionic strength = 0.15) at 150 mM PBS pH 7.4 solution. The release test of DOX from the micelles was performed under mechanical shaking (100 *rev.*/min) at 37 ^{o}C. The outer phase of the dialysis membrane bag was extracted and replaced with a fresh buffer solution at predetermined time intervals in order to maintain a sink for DOX. The DOX concentration was measured using a UV/visible spectrophotometer.[25-28]

Fragmentation of micelles. In order to evaluate the physical scissoring of the worm-like micelles in PBS pH 7.4 (ionic strength = 0.15) under mechanical shaking (100 *rev.*/min) at 37 ^{o}C, the micelle solution was extracted at a given time and coated on a slide glass. The contour length of the dried micelles on the slide glass was analyzed by FE-SEM and was fit to a Gaussian distribution (for 100 worm micelles).[19-21]

Cell uptake test. Human breast carcinoma BT-20 and T-47D cells (from Korean Cell Line Bank) were maintained in RPMI-1640 medium with 2 mM L-glutamine, 5% penicillin-streptomycin, 10% fetal bovine serum in a humidified incubator at 37 ^{o}C and 5% CO_2 atmosphere. Before testing, cells (1×10^5 cells/mL) growing as a monolayer were harvested by trypsinization using a 0.25% (w/v) trypsin/0.03% (w/v) EDTA solution. Cells suspended in a RPMI-1640 medium (200 μL) were seeded into a 6-well plate and cultured for 24 hours prior to *in vitro* cell testing.

The uptake of the micelles with fluorescent ligands into BT-20 and T-47D cells was examined using a confocal microscope (FITC: λ_{ex} 488 nm and λ_{em} 510 nm, TMRHD: λ_{ex} 570 nm and λ_{em} 595 nm). First, the worm-like micelles were pretreated in PBS pH 7.4 (ionic strength = 0.15) under mechanical shaking (100 *rev.*/min) at 37 ^{o}C for 1 day. After 4 hours of incubating the cells (1×10^3 cells/ml) with the pretreated micelles (the micelles with multiple ligands (cobalamin-FITC, cyclic RGD-TMRHD) or the micelles with only cobalamin-FITC] (10 μg/mL for RPMI-1640 medium), the cells were washed three times with PBS (pH 7.4) and fixed with 1% formaldehyde in PBS for 10 min at room temperature. A cover slip was mounted on a microscope slide with a drop of anti-fade mounting media (5% *N*-propyl galate, 47.5% glycerol and 47.5% Tris-HCl, pH 8.4) to reduce fluorescence photo bleaching.[29]

Results and Discussion

Preparation of non-spherical micelles. Self-assembly of PLLA-*b*-PEG provides a fundamental driving force to build spherical or non-spherical nanostructures. It was verified that the ratio of hydrophilic or hydrophobic to total polymer mass reflects the ability of micelles to form several nanostructures, such as spherical micelles, cylindrical micelles, and polymersomes.[19-21] In this study, PLLA-*b*-PEG with different molecular weights of PLLA block were prepared and utilized to explore the optimal ratio of hydrophobic to total polymer mass for constructing cylindrical micelles. In a preliminary study, the micelles fabricated by the film-rehydration method[19-21] using PLLA (M_n 5K)-*b*-PEG (M_n 2K) led to mixed miceller structures with spherical and cylindrical shapes (data not shown). This is comparable with the PLLA (M_n 3K)-*b*-PEG (M_n 2K) that has been often used to produce nano-sized spherical micelles for drug delivery.[25-29]

Morphological analysis of micelles. Figure 2 shows the morphological changes in the non-spherical micelles prepared from PLLA (M_n 7K)-*b*-PEG (M_n 2K) or PLLA (M_n 10K)-*b*-PEG (M_n 2K), according to the temperature condition during micelle preparation. The morphology of micelles changed from spherical to worm-like and from worm-like to rod-like, as the temperature increased. It was assumed that dehydration of the hydrophilic PEG block upon heating led to an elevated hydrophobicity of PLLA-*b*-PEG (resulting in the formation of ordered worm-like micelle), followed by a decrease in the flexibility of the interfacial curvature (resulting in the formation of ordered rod-like micelles).[30-32] As shown in Figure 2(a), the transition to the

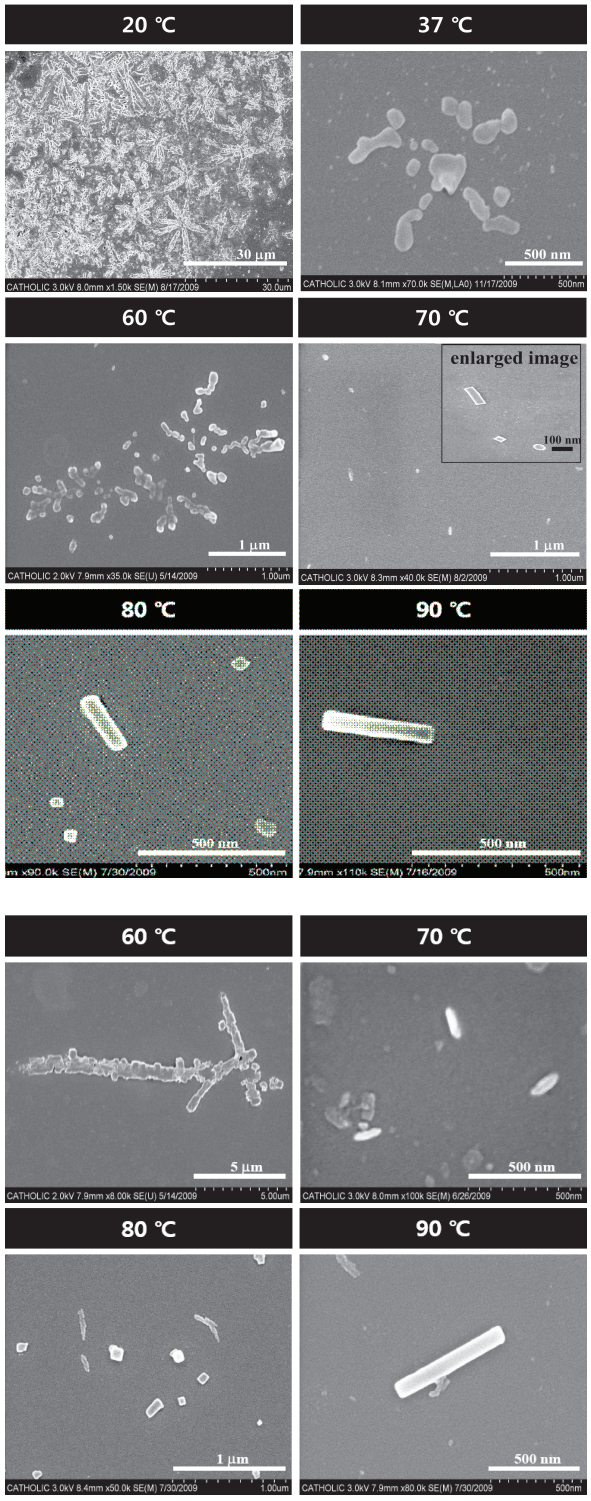


Figure 2. FE-SEM images of non-spherical micelles fabricated under different temperature conditions (20 - 90 °C). (a) PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles and (b) PLLA (M_n 10K)-*b*-PEG (M_n 2K) micelles.

worm-like micelles between 37 - 60 °C and to the rod-like micelles between 70 - 80 °C was significant. These results are consistent with the morphology changes of PLLA (M_n 10K)-*b*-PEG (M_n 2K) micelles at these temperature conditions (Figure 2(b)). However, unlike PLLA (M_n 7K)-*b*-PEG (M_n 2K), which formed

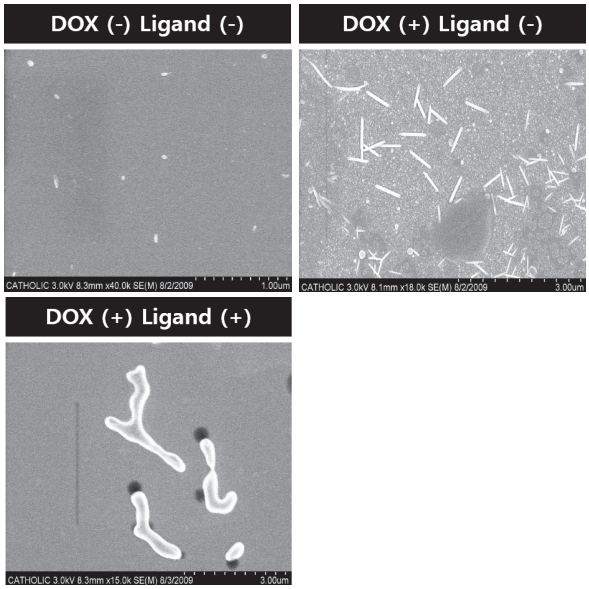


Figure 3. FE-SEM images of non-spherical PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles with (indicate '+') or without (indicate '-') ligands or DOX. Non-spherical micelles were fabricated at 70 °C.

worm-like micelles at 60 °C, PLLA (M_n 10K)-*b*-PEG (M_n 2K) micelles produced mixed morphologies of both worm-like and rod-like micelles at the sample temperature. This difference may be due to the relatively higher hydrophobicity of PLLA (M_n 10K)-*b*-PEG (M_n 2K) relative to PLLA (M_n 7K)-*b*-PEG (M_n 2K). In addition, it was observed that relatively low temperatures (i.e., 20 °C) were not sufficient to stabilize the worm-like or rod-like micelles, thereby triggering the formation of lamellar-type microaggregations.[30-32]

Figure 3 shows the morphology changes of micelles when an anticancer drug (DOX) was encapsulated into the micelles or multiple ligands were bounded to the micelles. DOX-loaded rod-like micelles grew longer, from an average of 0.15 to 1.5 μM, which may be related to the improved hydrophobicity at the worm core. Moreover, the conjugation of hydrophilic multiple ligands, such as cobalamin, folate, and cyclic RGD, seemed to provide flexibility at the interfacial curvature of the rod-like nanostructures, resulting in yielding flexible worm-like micelles (average 2.4 μm) (Figure 1). These results indicate that physical or chemical stimuli for non-spherical drug carriers may deform their morphologies, which will ultimately affect their pharmacokinetics or therapeutic efficacies[19,33,34]

Figure 4 shows the distribution of ligands existing on worm-like surfaces. PLLA-*b*-PEG bock copolymers with fluorescent ligands (i.e., PLLA-*b*-PEG-folate-Cy5.5, PLLA-*b*-PEG-cobalamin-FITC, and PLLA-*b*-PEG-cyclic RGD-TMRHD) were self-assembled to visualize the surface of the worm-like micelles using a confocal microscope. Folate tagged with Cy5.5 (blue color), cobalamin with FITC (green color), and cyclic RGD with TMRHD (red color) were randomly distributed along the surface of the worm-like micelles, suggesting a heterogeneous distribution of ligands.

Drug release with different morphologies of micelles. Figure 5 shows the sustained drug (DOX) release behavior of PLLA-

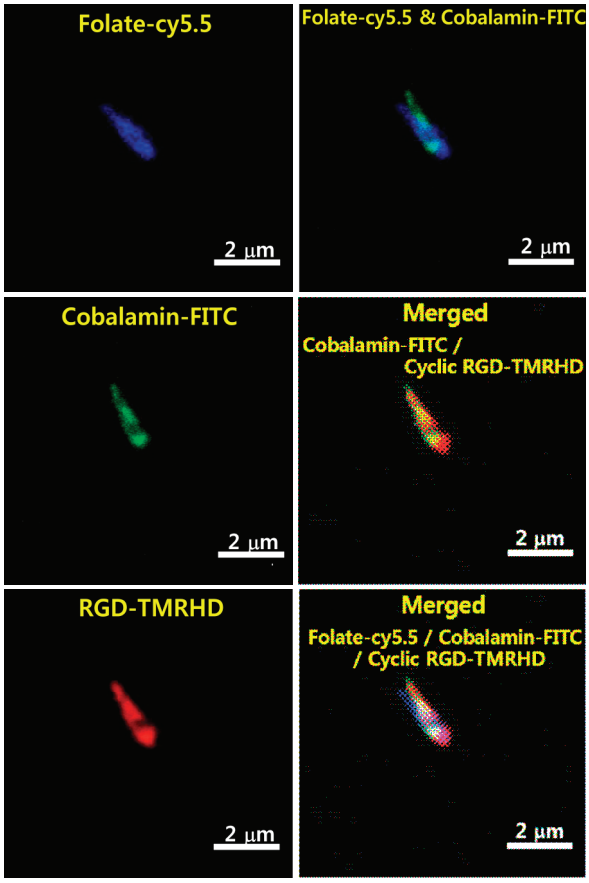


Figure 4. Confocal images of non-spherical PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles with multiple ligands (folate-Cy5.5, cobalamin-FITC, cyclic RGD-TMRHD). Worm-like micelles were fabricated at 37 °C.

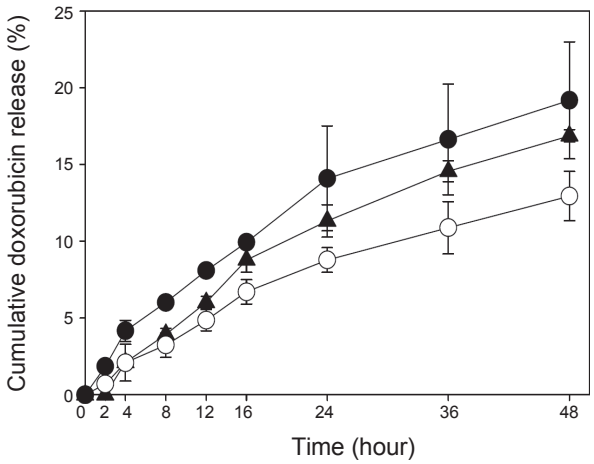


Figure 5. DOX release behavior of polymeric micelles with different morphologies: **(●)** spherical PLLA (M_n 2K)-*b*-PEG (M_n 2K) micelles, (▲) rod-like PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles with DOX, (○) worm-like PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles with DOX and multiple ligands. (n = 3). Worm-like micelles and rod-like micelle were fabricated as described in Figure 3.

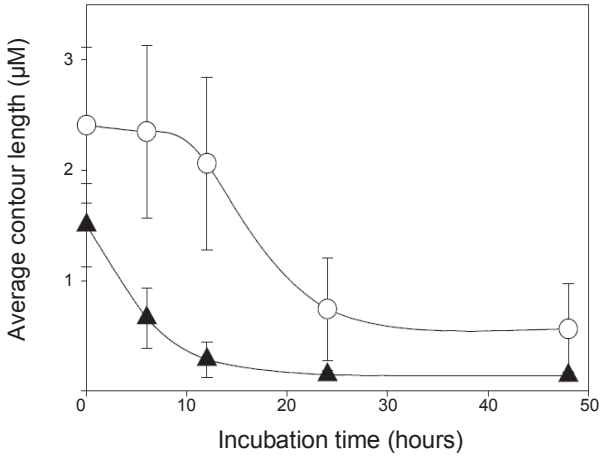


Figure 6. Time-dependent change in the contour length of worm-like PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles with DOX and multiple ligands (○) or rod-like PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles with DOX (▲) in PBS pH 7.4 (ionic strength = 0.15) under mechanical shaking (100 *rev.*/min) at 37 °C. (n = 3) Worm-like micelles and rod-like micelle were fabricated as described in Figure 3.

b-PEG micelles under mechanical shaking (100 *rev.*/min) at 37 °C. For this study, spherical micelles (average particle size: 96 nm) as a control group were prepared from PLLA (M_n 2K)-*b*-PEG (M_n 2K) at 70 °C. As expected, the worm-like micelles or rod-like micelles consisting of PLLA (M_n 7K)-*b*-PEG (M_n 2K) displayed slower DOX release behaviors compared to the spherical micelles, which was due to the relatively large hydrophobic PLLA block. However, despite consisting of the same PLLA block, the rod-like micelles exhibited comparatively higher drug release rates than the worm-like micelles. Unlike the flexible worm-like micelles, the rod-like (non-flexible) micelles have been reported to be poorly resistant to mechanical shear stress (100 *rev.*/min). This property of rod-like micelles is assumed to cause extensive fragmentation of micelles.[19-21, 30-32] It was observed that the rod-like micelles underwent rapid fragmentation during 6 - 24 hours of incubation (Figure 6). While, the length of worm-like micelles slowly decreased with time (Figure 6), suggesting a relatively high resistance of worm-like micelles to mechanical shear stress. It seemed that this fragmentation in the rod-like micelles also resulted in accelerated drug release at early stage (for the first 48 hours). Of course, the drug release patterns of worm-like or rod-like micelles for the next 1-2 weeks were not significantly changed (data not shown). It is known that the degradation pattern of PLLA-*b*-PEG significantly depends on the block length and affects the drug release behavior.[35] Because the polymer (PLLA-*b*-PEG) with the same block length was used for the preparation of worm-like or rod-like micelles in this study, it seemed that the drug release behaviors of the worm-like or rod-like micelles were similar at later stage (for the next 1-2 weeks) (data not shown).

Tumor targeting. Figure 7 shows the confocal images of BT-20 or T-47D breast cancer cells treated with the worm-like micelles. In this study, we used fragmented worm-like micelles. Considering that the worm-like micelles were gradually fragmented in PBS pH 7.4 (ionic strength = 0.15) under mechanical shaking (100 *rev.*/min) at 37 °C (Figure 6), the fragmentation of worm-like micelles in the blood stream may be accelerated. Therefore, to simulated *in vivo* conditions, we pretreated the

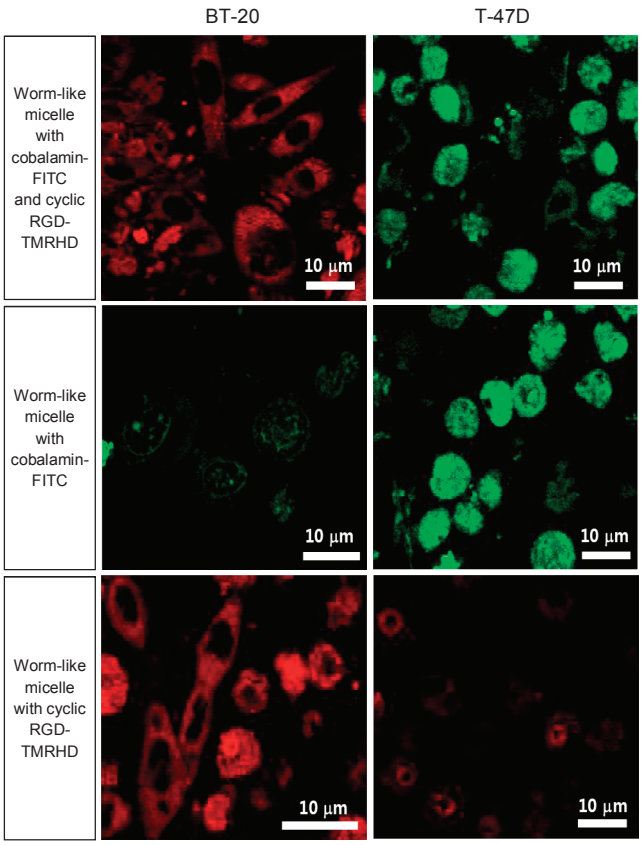


Figure 7. Confocal images of BT-20 and T-47D tumor cells treated with PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles (10 μg/mL) with multiple ligands (cobalamin-FITC and cyclic RGD-TMRHD) or worm-like PLLA (M_n 7K)-*b*-PEG (M_n 2K) micelles (10 μg/mL) with cobalamin-FITC (or cyclic RGD-TMRHD) for 4 hours. Worm-like micelles were fabricated at 37 °C and were pretreated in PBS pH 7.4 (ionic strength = 0.15) under mechanical shaking (100 *rev.*/min) at 37 °C for 1 day.

worm-like micelles in PBS pH 7.4 (ionic strength = 0.15) under mechanical shaking (100 *rev.*/min) at 37 °C for 1 day. The partially fragmented micelles were then mixed with RPMI-1640 medium and added to cancer cells grown on a 6-well plate. As shown in Figure 7, the uptake of worm-like micelles with multiple ligands (cobalamin-FITC: green color, cyclic RGD-TMRHD: red color) in BT-20 (expressing integrin$\alpha_v\beta_3$) and T-47D cells (expressing cobalamin receptor) was significant in both cells. However, cellular uptake of micelles containing only one ligand (cobalamin) by BT-20 cells was low. Similarly, cellular uptake of micelles containing only one ligand (cyclic RGD) by T-47D cells was also low.

Although it was reported that human lung-derived epithelial cells can uptake large worm-like micelles,[34] the endocytosis of large worm-like micelles remains an issue.[19-21] Nevertheless, the fragmented micelles may still be effective to extravasate into the cancer stroma and be internalized into cancer cells.[33]

In addition, the irregular fluorescence observed in the confocal images may be due to the presence of fragmented or non-fragmented micelles.

Overall, these results suggest that worm-like micelles with multiple ligands hold promise for improving cancer cell-specific interactions.

Conclusion

Non-spherical worm-like polymeric micelles with multiple ligands--such as folate, cobalamin, and cyclic RGD--were prepared to mediate multiple ligand/receptor pair interactions, which were devised to multi-target different breast cancer cell lines simultaneously. In this study, we investigated the morphological changes of worm-like micelles under various temperature conditions during micelle preparation, ligand conjugation, and drug encapsulation. We found that worm-like micelles coupled with multiple ligands and encapsulated with DOX exhibited flexible worm-like shapes and enhanced their cancer cell-specific recognition abilities for heterogeneous breast cancer cells. This may be advantageous for maximizing the therapeutic activity of drug carrier systems for *in vivo* breast cancer. Further *in vivo* investigations will be necessary to verify the potential efficacy of this system.

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