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Surface Modification of Polymeric Micelles by Strain-Promoted Alkyne-Azide Cycloadditions

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Abstract

Organomicelles modified by surface dibenzylcyclooctyne moieties can conveniently be functionalized by strain-promoted alkyne-azide cycloadditions. The ligation approach is highly efficient, does not require toxic reagents and is compatible with a wide variety of functional modules. Interactions of proteins with surface ligands of the micelles have been studied by AFM, which revealed that it leads to disassembly of the particles thereby providing a mechanism for triggered drug release.

Keywords

click chemistry; bioconjugation; nanocarrier; drug delivery; AFM

Introduction

Polymeric micelles, which are formed by the self-assembly of an amphiphilic block copolymer in aqueous environment, are emerging as attractive vehicles for drug delivery.[1] These nanocarriers can solubilize and increase longevity of lipophilic drugs in the blood stream, offer controlled release by environmental sensitive or external stimuli, and accumulate in solid tumors by enhanced permeability and retention effect.[2] The therapeutic efficiency of nanoparticle drug delivery systems can be further improved by surface functionalization by a tissue targeting ligand, a cell-penetrating molecule such as TAT or arginine rich peptide,[3] or by an appropriate signaling peptide for targeting an organelle such as the nucleus.[4]

Early efforts of surface functionalization of polymeric nanoparticles employed activated esters for condensation with amines,[5] maleamides for reaction with thiols,[6] aldehydes or ketones for reaction with amines[7] or biotin for binding to avidin.[8] More recently, copper (I)-catalyzed azide-alkyne 1,3-dipolar Huisgen cycloadditions (CuAAC)[9] of azides with alkynes to give stable triazoles have been employed for the functionalization of particles[10] and polymeric materials[11] including polymersomes.[12] This reaction employs chemical stable entities (azide and alkyne) that react fast at ambient temperature, and tolerates a wide range of functional groups and solvents including water. A major disadvantage of copper-

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catalyzed cycloaddition is, however, the cellular toxicity of the metal catalyst. This problem has been addressed by a strain-promoted alkyne-azide cycloadditions (SPAAC), which do not require a cytotoxic metal catalyst,[13] and has led to the application of cyclooctyne-azide cycloaddition to labeling of cell-surface glycans and proteins of living cells, proteomics and tissue reengineering.

We report here that SPAAC is an efficient tool for surface modification of organo-micelles. In particular, polymers composed of poly(ethylene oxide) (PEO) and poly(ε-carprolactone) (PCL) that are modified at the polar PEO terminus with a DIBO residue can be employed for the controlled preparation of organomicelles. The latter particles could readily be modified with a variety of azido-containing probes and a number of studies have shown that the probes of the resulting particles are exposed for biological recognition. Atomic force microscopy (AFM) and dynamic light scattering (DLS) have demonstrated that surface modification does not significantly affect size and morphology. Finally, it has been found that the interaction of proteins with surface ligands of the micelles leads to disassembly of the particles thereby providing a mechanism for triggered drug release.

Results and Discussion

We envisaged that micelles modified with 4-dibenzocyclooctynol (DIBO) would provide an attractive nanocarrier that can be modified by a variety of azido-containing probes. In this respect, we have already demonstrated that DIBO can easily be prepared and reacts fast with azido-containing saccharides and peptides in the absence of a metal catalyst and it can be employed for visualizing metabolically labeled glycans of living cells.[13b] A variety of probes can easily be modified by an azido group and hence DIBO modification of micelles and reaction with azido-containing probes was envisaged to offer an attractive technology for making materials with enhanced function.

Thus, we developed an efficient approach for the preparation of amphiphilic block co-polymers **7**, which is composed of poly(ethylene oxide) (PEO) and poly(ε-carprolactone) (PCL) and modified at the polar PEO terminus with a DIBO residue. Furthermore, block copolymer **4** was synthesized which has the polyethylene glycol moiety terminating as a methyl ether to control the density of DIBO at the micelle surface. Co-polymerization of commercially available methoxy polyethylene glycol (**1**, n ≈ 45) or azido polyethylene glycol (**2**) with caprolactone in the presence of tin(II) 2-ethylhexanoate (SnOct) at 130 °C for 24 h gave **4** and **5**, respectively[14] (m ≈ 26, M_n = 5000, PDI ≈ 1.5). The azido moiety of **4** was reduced by hydrogenation over palladium on activated carbon and the resulting amine **6** condensed with activated carbonate **3** to give the DIBO modified copolymer **7**.

DIBO-functionalized micelles **B** were prepared by addition of nanopure water to a mixture of block copolymer **4** and **7** (1/9, w/w) in THF followed by dialysis. In addition, micelles **A** were prepared that were only composed of unfunctionalized block polymer **4** (Table 1). Next, micelles **B** were exposed to azido-modified fluorescein probe **8** (Figure 1) for 18 h and then extensively dialyzed against water to give micelles **C**. To determine the degree of functionalization, micelles **C** were freeze-dried, dissolved in DMSO/$CHCl_3$ (1:1, v/v) and the fluorescence intensity measured, which indicated that the fluorescein probe had modified 70% of the DIBO residues of the micelles. As expected, no fluorescent intensity was measured when micelles **A** were treated by the same procedure, indicating that the fluorescein probe was covalently attached to DIBO of micelles **B**. Furthermore, TLC analysis of copolymers **4** and **7**, fluorescein probe **8**, the micelles **B** and **C**, respectively confirmed that the probe was covalently attached to the DIBO-PEO-PCL block polymer.

To examine the generality of the methodology, the DIBO modified micelles **B** were modified with mannoside **9**, RGD peptide **10**[15] and biotin probe **11** (Figure 1) to give micelles **D**, **E** and **F**, respectively. The mannoside, peptide and biotin incorporation was 76%, 65% and 58%, respectively as determined by quantitative carbohydrate, amino acid and biotin analysis. These results demonstrate that a wide variety of compounds can be attached to the micelles and that the majority of the DIBO functionalities are exposed for reaction with the probes.

The micelles **A**–**F** had similar diameters (32–42 nm) as shown by dynamic light scattering (DLS) highlighting that surface modification does not significantly affect size (see Table 1). The morphology of the particles in solution was examined by AFM by employing tips that were cleaned by UV exposure, covered with a magnetic film (35 nm) by ion-beam deposition and then coated on the backside by a thin Au layer (20 nm) to protect the magnetic coating, and to enhance reflectivity thereby achieving the highest possible resolutions and sensitivity. Images of the micelles in solution were obtained by MAC mode[16] AFM and the best results were achieved by using a MICA substrate surface that was exposed to 3-aminopropyltriethoxysilane (APTE) vapor. As can be seen in Figure 2, a substantial number of micelles **A**–**F** had been absorbed to the modified MICA surface. The micelles had spherical morphologies with approximate diameters of 24–44 nm, which compared well with the results obtained DLS. When gold was employed as a substrate surface, only a small number of micelles **A**–**E** adhered to the gold surface. In contrast, a large number of biotin-modified micelles **F** were absorbed, most likely due to a strong interaction between the sulfur of biotin and gold of the surface.

Next, we explored whether micelles **D**, which are modified by mannosides, can be selectively attached to a gold surface modified with the mannose binding lectin ConA. Thus, ConA was modified by thiols by treatment with the NHS active ester of lipoic acid.[17] The chemically modified lectin was dialyzed, exposed to a gold substrate surface, which was thoroughly washed to remove unbound ConA. Examination of the surface by AFM indicated dense packing of the proteins (see Figure S7). Next, a suspension of micelles **D** was exposed to the ConA modified surface, which was imaged by AFM. Interesting, a significant number of micelles were observed, which had similar morphologies as the micelles adhered to a mica surface (Figure 3a). The use of peptide-modified micelles **E** did not lead to significant absorption to the ConA modified surface highlighting that specific interactions were formed between micelles **D** and ConA. Although micelles **D** adhered to a ConA modified surface were stable for a significant period of time, the addition of a solution of unmodified ConA led to micelle disassembly (Figure 3b–d). Thus, a reduction in micelle diameter (20~25 nm) was observed after incubation for 15 min with soluble ConA followed by reexamination of the surface by AFM. An exposure time of 30 and 45 min led to only remnants of the micelles. No disassembly of the micelles was observed after exposure to BSA, highlighted that specific interactions between the mannosides of the micelles and ConA are responsible for disassembly. Probably, protein-ligand binding results in a significant change in the balance between hydrophilic-lipophilic forces resulting in the disassembly of the micelles. Recently, similar observations were made and for example extravadin treatment of self-assembled amphiphilic dendrimers modified with biotin and loaded with pyrene resulted in a decrease in emission intensity of pyrene.[18] This result was rationalized by release of pyrene due to protein-induced disassembly and release of the guest.

Next, the cytotoxicity and the loading capacity of the surface modified micelles were examined. For the latter, 7-ethyl camptothecin (7-Et-CPT) was employed, which is a lipophilic and cytotoxic drug that inhibits topoisomerase I and is being examined as an anticancer agent.[19] Thus, 7-Et-CPT was incorporated into unfunctionalized (**A**) and RGD-modified (**E**) micelles to give micelles **G** and **H**, respectively. The free 7-Et-CPT was

removed by dialysis against water and the amount of 7-Et-CPT was quantified by fluorescent measurement of freeze-dried samples that were redissolved in DMSO/$CHCl_3$ (1/1, v/v). It was found that both micelle preparations contained 1.0±0.2% 7-Et-CPT (w/w), showing that the surface peptides did not affect the loading capacity of the micelles.

MCF-7 cells, which is a human breast cancer cell line that expresses $\alpha_V\beta_3$-integrins[20], were exposed for one and three days to different concentrations of micelles **A, E**, **G** and **H**. The unloaded particles **A** and **E** did not influence cell viability demonstrating that the DIBO-derived conjugation linkage is non-cytotoxic (data not shown). Furthermore, micelles **G** and **H** loaded with 7-Et-CPT induced similar dose-dependent cytotoxicities (Figure 4). The RGD peptides of micelles **E** and **H** bind to $\alpha_V\beta_3$-integrins on tumor endothelial cells. [15c, 15d] Surface-modified micelles can attach to the cell membrane and act as an extracellular sustained-release drug depot or can be internalized by endocytosis and then release their load.[21] The observation that unmodified (**G**) and RGD-modified (**H**) micelles loaded with 7-Et-CPT exhibit similar cytotoxicities indicate that micelles **H** act as an extracellular release depot. In this respect, binding to $\alpha_V\beta_3$-integrins may induce a significant number of micelles to disassemble before they can be endocytosed. Alternatively, it may be possible that a more potent cyclic RGD peptide is required for proper targeting of integrins[22] thereby significantly increasing uptake. Future studies are underway to test these various hypotheses.

Conclusion

In conclusion, we have shown, for the first time, that soft materials such as organomicelles modified by a cyclooctyne can conveniently be functionalized with various biological relevant modules using strain-promoted alkyne-azide cycloadditions. The ligation approach is highly efficient, does not require toxic reagents, and is compatible with a wide variety of functional modules. Modification of the surface of organomicelles with a cyclooctyne for reaction with modules containing an azido moiety is in particular attractive because azides can easily be installed in a wide variety of compounds.[23] It is to be expected that the methodology reported here can be extended to other types of nano-size materials. The AFM technology described here made it possible, for the first time, to visualize protein-mediated disassembly of micelles and it is to be expected that it will contribute to understanding the mode of targeted drug delivery.

Experimental Section

The preparation and characterization of compounds and polymers are provided in the supporting information.

General procedure for the preparation of organomicelles without loading

A mixture of block copolymers (10 mg) in THF (0.8 mL) was slowly added to water (15 mL) under sonication. The final mixture was exposed to air overnight to allow evaporation of THF and formation of micelles. The resulting solution was then dialyzed against nanopure water (2.0 L) using a pre-swollen semi-permeable membrane (cutoff 12,000 – 14,000 Da) for 4 h, and the water was replaced every hour.

General procedure for the preparation of loaded organomicelles

7-Et-CPT (1 mg) was added to a solution of block copolymer (10 mg) in THF (0.8 mL). The mixture was slowly added to water (15 mL) under sonication. The resulting mixture was exposed to air overnight to allow evaporation of THF and formation of micelles. The resulting solution was then dialyzed against nanopure water (2.0 L) using a pre-swollen semi-permeable membrane (cutoff 12,000 – 14,000 Da) for 4 h, and the water was replaced

every hour. The micelle solution was passed through a syringe filter (pore size 0.45 μ m; Millipore, Billerica, MA) to remove drug aggregates.

General procedure for surface modification of DIBO containing organomicelles

A mixture of block copolymers (10 mg) with or without loading in THF (0.8 mL) was slowly added to water (15 mL) under sonication. The final mixture was opened to air overnight to allow slow evaporation of THF and formation of micelles. A solution of azido-functionalized ligand (2.0 eq. to DIBO) in aqueous solution was added to the micelle solution under shaking. After standing for another 18 h, the solution was dialyzed against nanopure water (2.0 L) using a pre-swollen semi-permeable membrane (cutoff 12,000 – 14,000 Da) until free ligand was completely removed. Micelles were characterized by dynamic light scattering and atomic force microscropy. The successful conjugation of ligands on to the surface of micelles was verified by analyzing the ligand amount of the surface modified micelles.

Quantification of conjugated Alexa Fluor 488 and loaded 7-Et-CPT

The concentration of the dyes was quantified by fluorescence intensity measurements (BMG Labtech POLAR star optima). A micelle solution was freeze-dried to give a solid, which was redissolved in a mixture of chloroform and DMSO (1:1, v/v) for fluorescence intensity measurements (for Alexa Fluor 488: excitation 485 nm, emission 520 nm; for 7-Et-CPT: excitation 360 nm, emission 450 nm). Standard curves of Alexa Fluor 488 or 7-Et-CPT were employed for quantification.

Quantification of conjugated mannoside

Sugar analysis was performed on DIONEX ICS-3000 HPAEC chromatograph using deionised water and 200 mM NaOH as an eluent. Sample preparation: 1–2 mg of sample and D-(+)-mannose were treated with 2 M TFA in water (250 μL) for 4 h at 100 °C. Sample and standard were spin dried, redissolved in water (500 μL) and filtered. Sample concentration was then determined based on the calibration curves of mannose standards

Quantification of conjugated peptide

L-8800 Hitachi amino acid analyzer was employed for analyzing peptide content of the samples after 6 N HCl hydrolysis for 24 h at 110 °C.

Quantification of conjugated biotin

The amount of conjugated biotin on micelles was quantified by using Fluorescence Biotin Quantitation Kit from Thermo Scientific. Briefly, the fluorescence intensity of the dye of avidin significantly increases when the weakly interacting quencher HABA (4'-hydroxyazobenzene-2-carboxylic acid) is displaced by the biotin. The premix fluorescence dye labeled avidin with HABA (DyLight Reporter) is added to the micelle solution containing conjugated biotin. Because of its higher affinity for avidin, biotin displaces the HABA, allowing the avidin to fluoresce. The fluorescence intensity was measured on a BMG Labtech POLAR star optima reader. The amount of biotin is measured in a microplate by comparing the fluorescence to a biocytin standard curve.

Dynamic light scattering (DLS) and zeta potential measurements

Dynamic light scattering (DLS) and Zeta Potential measurements were performed on a Zeta Potential and Particle Size Analyzer (ZetaPALS, Brookhaven Instruments Corp., US). For DLS measurement, dust-free vials were used for the aqueous solutions, and measurements were carried at 25 °C at an angle 90°. Zeta potential measurements were carried at 25 °C with PALS Zeta Potential Analyzer using a Smoluchowski Zeta Potential Model.

AFM Experiments

Instruments

An Agilent 5500 AFM system equipped with an inverted light microscope (ILM) system (Agilent, Chandler, AZ) was used. An Agilent multi-purpose AFM scanner was applied for scanning an area of 10 μm^2. CS-10 silicon AFM probe is from Nanoscience Instruments. Silicon cantilever tips with a nominal spring constant of about 0.1 N m^{-1} were for all experiments. The images were recorded in aqueous solution under Magnetic AC (MAC) mode AFM.

In-situ imaging of micelles attached to a modified MICA substrate

The MICA substrate was chemically functionalized with amino groups by treatment with 3-aminopropyltriethoxysilane (APTE, 98%, Sigma-Aldrich) under vapor condition, which was expected to facilitate the attachment of micelles. A micelle solution (200–400 μL, ~0.1 mg mL^{-1}) was administered to the mica substrate and after an incubation time of 1 h, the surface was imaged in solution without rinsing.

In situ imaging of micelles on an unmodified gold substrate

A gold substrate was annealed by hydrogen flame to remove possible contaminations and attain a flat surface. A micelle solution (200–400 μL, 0.1 mg mL^{-1}) was applied to the surface and after an incubation time of 1h, the surface was imaged without rinsing.

In situ imaging of micelles on a ConA-modified gold substrate

A solution of lipoic acid-modified ConA in 0.01 M Tris buffer (200 μL, 5 μg mL^{-1}) was applied to an annealed gold surface, which was incubated at 4 °C for 18 h, then the gold surface was gently rinsed with Tris buffer to remove unbound ConA. A micelle solution (200–400 μL, 0.1 mg mL^{-1} in 0.01 M Tris buffer) was introduced onto the ConA modified surface for 1 h at room temperature after which it was gently rinsed and imaged in Tris buffer. Next, a ConA or BSA solution in 0.01 M Tris buffer (200 μL, 5 μg mL^{-1}) was slowly injected into the liquid well to observe the real time change of the micelles while imaging.

Biological Experiments

Cell culture conditions

Human breast adenocarcinoma MCF-7 cells (ATCC) were cultured in Minimum Essential Medium, Eagle (ATCC), adjusted to contain L-glutamine (2 mM), Earle's balanced salt solution, sodium bicarbonate (1.5 g L^{-1}), non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM) and supplemented with penicillin (100 u mL^{-1}) / streptomycin (100 μg mL^{-1}; Mediatech), fetal bovine serum (FBS, 10%; Hyclone) and bovine insulin (0.01 mg mL^{-1}; Sigma). Cells were maintained in a humid 5% CO_2 atmosphere at 37 °C.

Measurement of cytotoxicity

On the day of the exposure assay, exponentially growing MCF-7 cells were plated as 2,000 cells/well in 180 μL in 96-well tissue culture plates (Nunc). Cells were then incubated with micelle preparations or free 7-Et-CPT by adding 10× solutions in cell culture medium (20 μL) to give a final volume of 200 μL/well for 24 h or 72 h. Control cells were given only cell culture medium (20 μL). Cells incubated for 24 h were given fresh medium (200 μL) and were further cultured for 48 h. The viability was measured by quantifying the cellular ability to reduce the water-soluble tetrazolium dye 3–4,5-dimethylthiazole-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble formazan salt as follows. At 68 h, MTT (5 mg mL^{-1} in PBS, 20 μL/well) was added to the wells and the cells were further incubated for 4

h. At 72 h the supernatant was carefully removed and the water-insoluble formazan salt was dissolved in DMSO (100 μL/well). The absorbance was measured at 560 nm using a microplate reader (BMG Labtech). Data points were collected in triplicate and expressed as normalized values for untreated control cells (100%).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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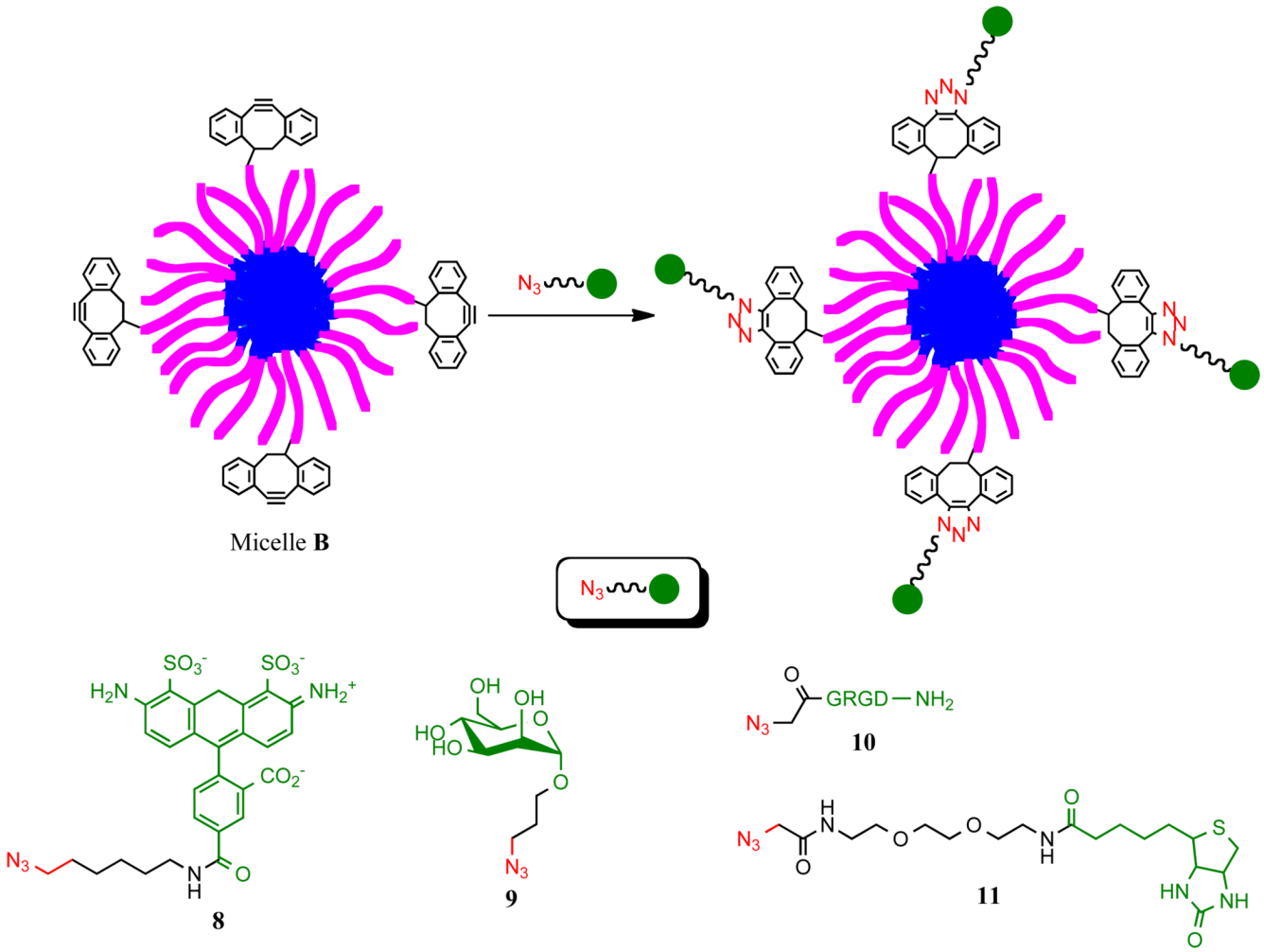


Figure 1.
Molecules containing an azido group for modifying micelles.

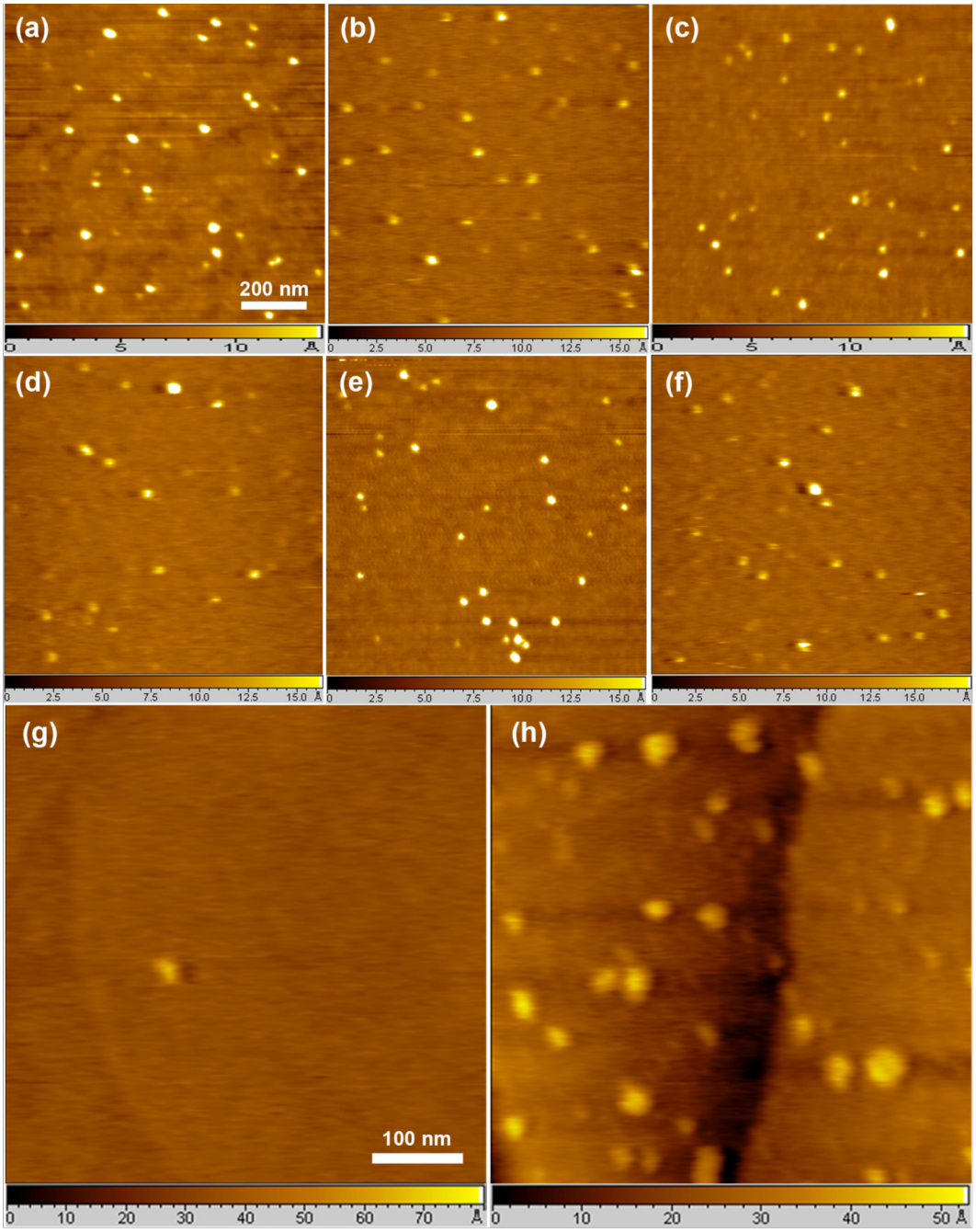


Figure 2.

Topographical images of micelles. (a–f), micelle solutions on the MICA substrate with surface treated by 3-aminopropyltriethoxysilane (APTE) under vapor condition: (a) micelles **A**; (b) micelles **B**; (c) micelles **C**; (d) micelles **D**; (e) micelles **E**; (f) micelles **F**. (g, h), micelle solutions on the gold substrate: (g) micelles **A**; (h) micelles **F**.

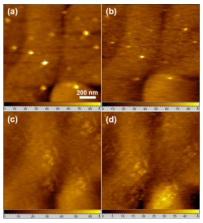


Figure 3.
Real-time topographical images of micelles **D** attached to immobilized ConA. (a) Images of micelles. (b–d) Images of the micelles after addition of ConA at (a) 15, (b) 30 and (c) 45 min.

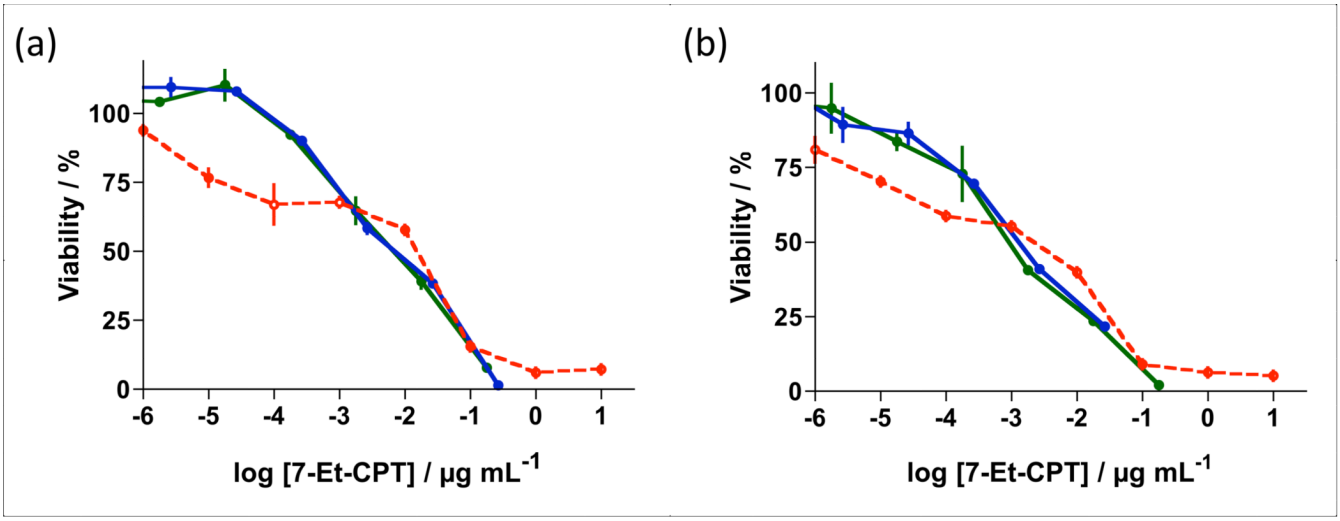
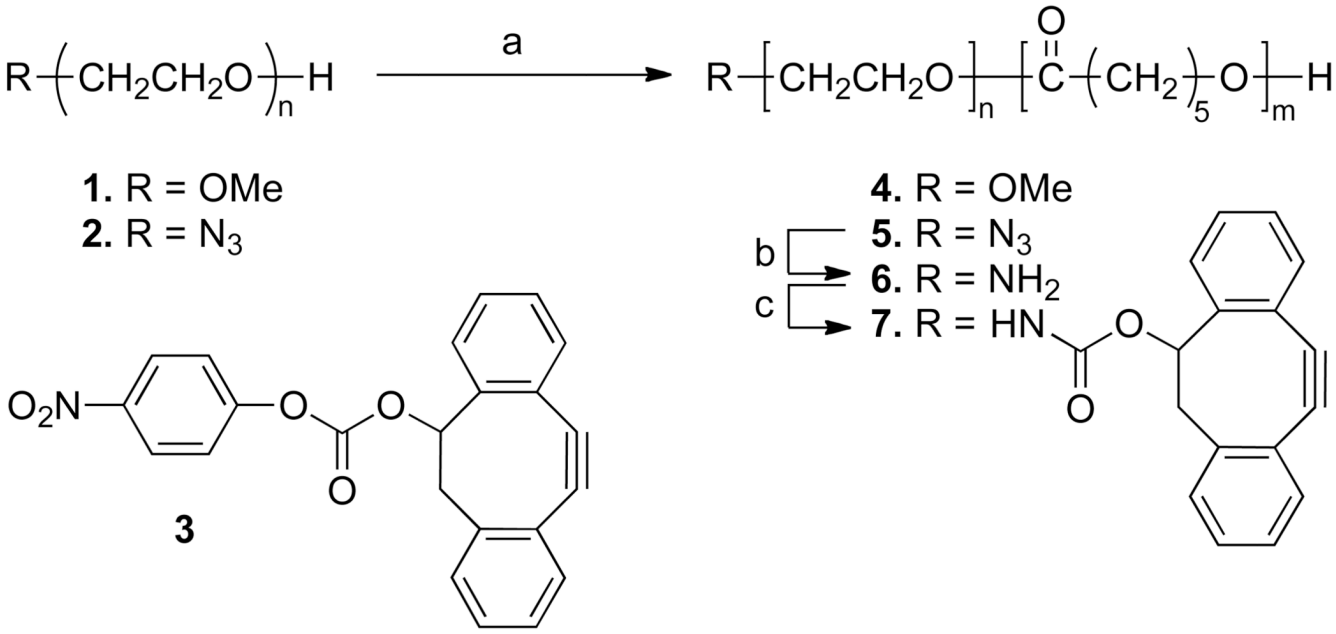


Figure 4.

Cytotoxicity assessment of 7-Et-CPT-loaded micelles **G** and **H**. Human MCF-7 were incubated with micelle preparations **G** (blue) and **H** (green) and free 7-Et-CPT (red) at the indicated concentrations of 7-Et-CPT. (a) After an incubation of 24 h, the medium was replaced and incubation was continued for 48 h. (b) The cells were incubated for 72 h without replacement of medium. Cell viability was assessed by the ability of cells to reduce MTT to its insoluble formazan salt. Cell viability values (*n*=3) were normalized for untreated control cells (100%).



Scheme 1.
Chemical synthesis of block copolymer **4** and **7**. Reagents and conditions. (a) caprolactone, SnOct, 130 °C; (b) H_2, Pd/C; (c) 3, Et_3N, CH_2Cl_2.

Table 1

Composition and physical properties of micelle preparations.

Micelle Type	Polymer 4 (Weight ratio)	Polymer 7 (Weight ratio)	Surface Modification	Loading	Mean Diameter (nm)	Zeta Potential (mV)
A	100%	0	-	-	35.3±1.5	−30.6±1.7
B	90%	10%	-	-	32.1±1.8	−29.7±2.7
C	90%	10%	**8**	-	37.7±2.4	−36.2±2.3
D	90%	10%	**9**	-	39.2±2.1	−32.9±1.9
E	90%	10%	**10**	-	41.0±2.7	−40.4±2.3
F	90%	10%	**11**	-	39.1±2.5	−39.8±2.4
G	100%	0	-	7-Et-CPT	42.4±3.1	−36.5±3.5
H	90%	10%	**10**	7-Et-CPT	43.8±3.5	−38.6±3.2

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