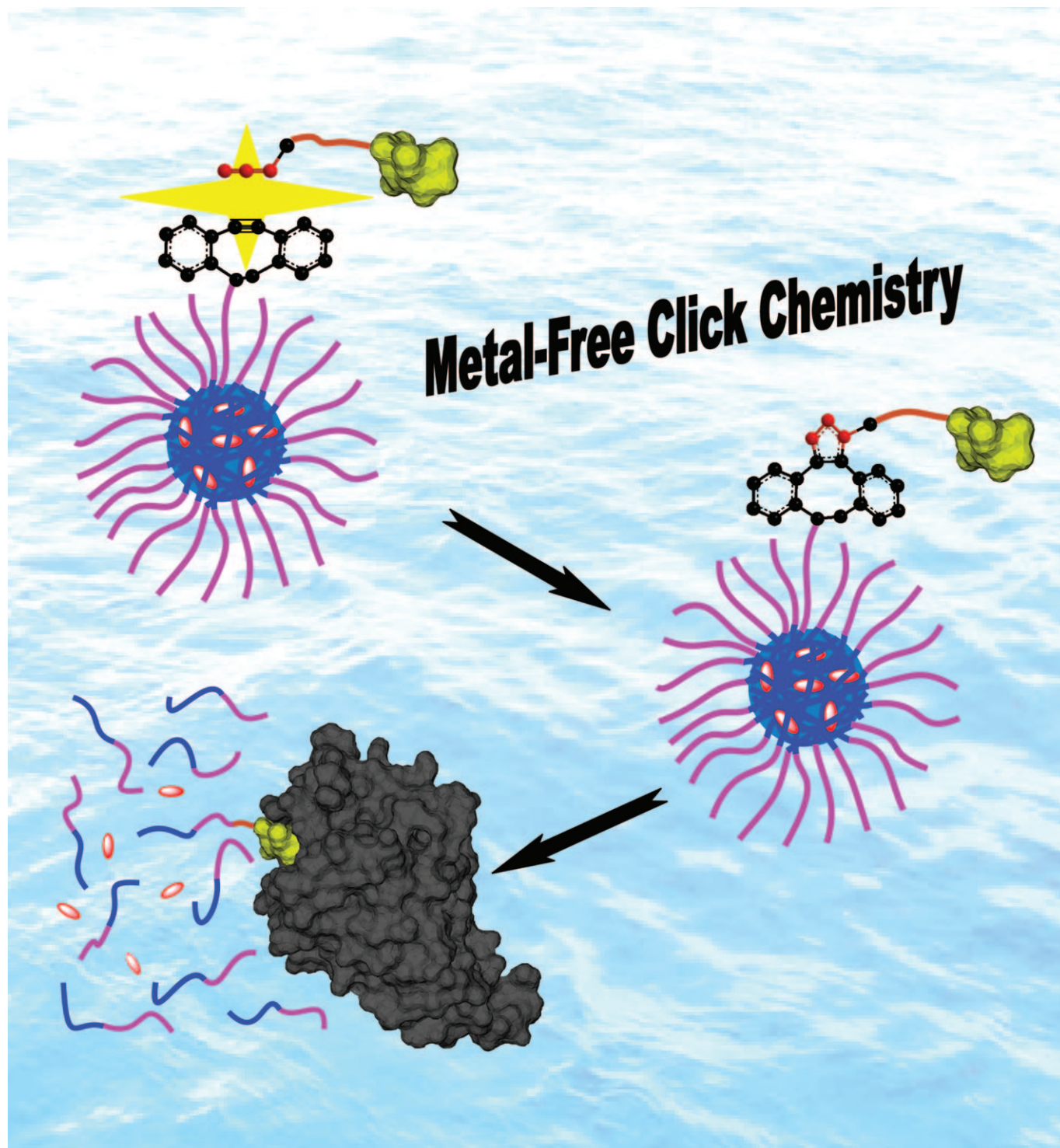


Surface Modification of Polymeric Micelles by Strain-Promoted Alkyne–Azide Cycloadditions

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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: bioconjugation • click chemistry • drug delivery • nanocarriers • scanning probe microscopy

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 a *trans*-activating transcriptional activator (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-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 -caprolactone) (PCL) that are modified at the polar PEO terminus with a 4-dibenzocyclooctynol (DIBO) residue can be employed for the controlled preparation of organomicelles. Such 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.

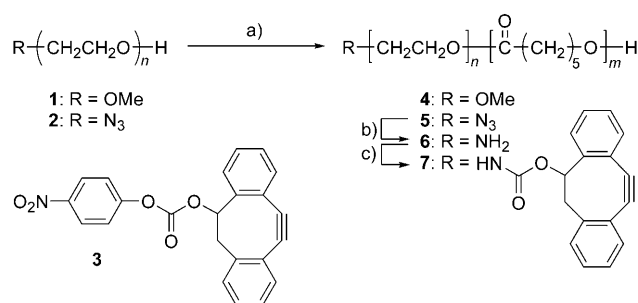
Thus, we developed an efficient approach for the preparation of amphiphilic block co-polymers **7**, which is composed of poly(ethylene oxide) (PEO) and poly(ϵ -caprolactone) (PCL) and modified at the polar PEO terminus with a DIBO residue (Scheme 1). Furthermore, block copolymer **4** was synthesized; this compound has a 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**, $n \approx 45$) or azido polyethylene glycol (**2**) with caprolactone in the presence of tin(II) 2-ethylhexanoate (SnOct) at 130°C for

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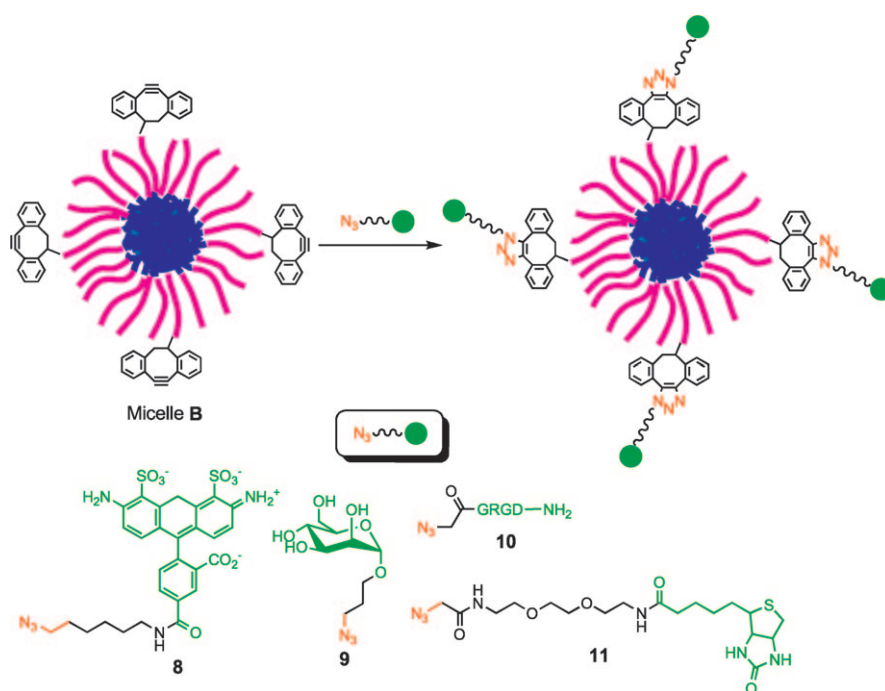
Scheme 1. Chemical synthesis of block copolymers **4** and **7**. a) Caprolactone, SnOct, 130 °C; b) H₂, Pd/C; c) **3**, Et₃N, CH₂Cl₂.

24 h gave **4** and **5**, respectively^[14] ($m \approx 26$, $M_n = 5000$, 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** (9:1, 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** (Scheme 2) 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.

ture, indicating that the fluorescein probe was covalently attached to DIBO of micelles **B**. Furthermore, TLC analysis of copolymers **4** and **7**, fluorescein probe **8**, and the micelles **B** and **C** 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** (Scheme 2) to give micelles **D–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 ma-



Scheme 2. Molecules containing an azido group for modifying micelles.

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

Table 1. Composition and physical properties of micelle preparations.

Micelle type	Polymer 4/7 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

resolutions and sensitivity. Images of the micelles in solution were obtained by the magnetic alternating current (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 1, a

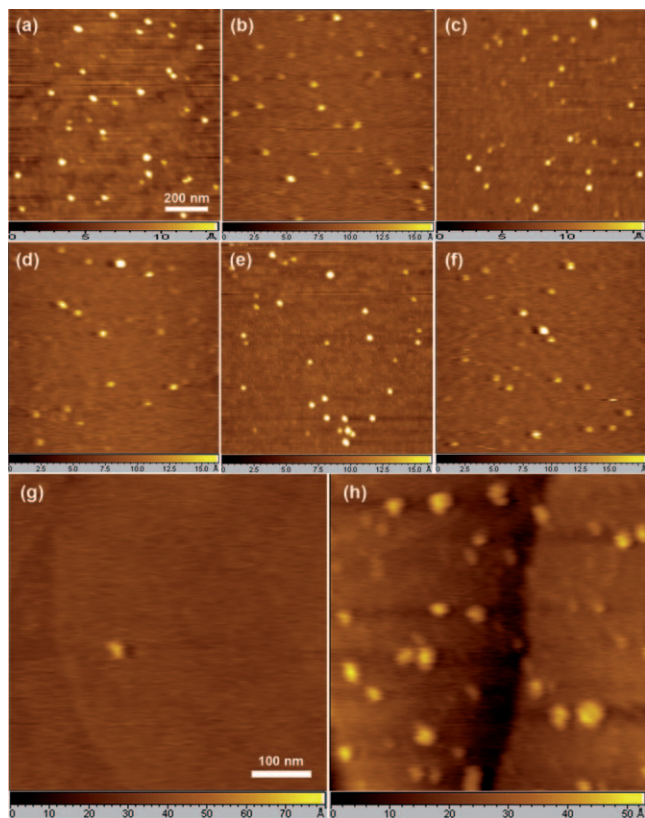


Figure 1. Topographical images of micelles. a)–f), micelle solutions on the mica substrate with surface treated by 3-aminopropyltriethoxysilane (APTE) under vapor condition: a) micelles **A**; b) micelles **B**; c) micelles **C**; d) micelles **D**; e) micelles **E**; f) micelles **F**. g, h), Micelle solutions on the gold substrate: g) micelles **A**; h) micelles **F**.

substantial number of micelles **A**–**F** had been absorbed onto the modified mica surface. The micelles had spherical morphologies with approximate diameters of 24–44 nm, which compared well with the results obtained by 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 mannoside binding lectin ConA. Thus, ConA was modified with thiols by treatment with the N-hydroxysuccinimide (NHS) active ester of lipoic acid.^[17] The chemically modified lectin was dialyzed and 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

in the Supporting Information). Next, a suspension of micelles **D** was exposed to the ConA-modified surface, which was imaged by AFM. Interestingly, a significant number of micelles were observed, which had similar morphologies as the micelles adhered to a mica surface (Figure 2a). The use

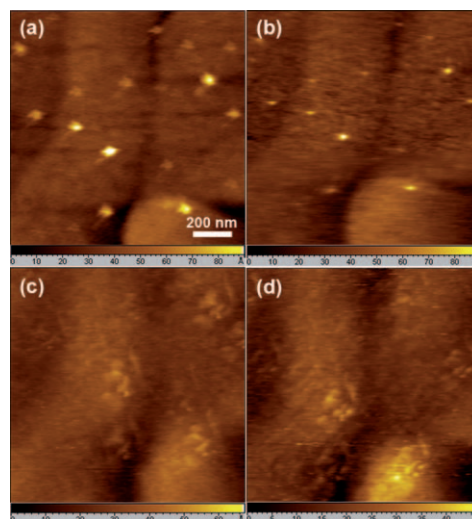


Figure 2. Real-time topographical images of micelles **D** attached to immobilized ConA. a) Images of micelles. b)–d) Images of the micelles after addition of ConA at b) 15, c) 30 and d) 45 min.

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** that 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 2b–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. Exposure times of 30 and 45 min led to only remnants of the micelles. No disassembly of the micelles was observed after exposure to bovine serum albumin (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; 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/CHCl₃ (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 3). The RGD peptides of micelles

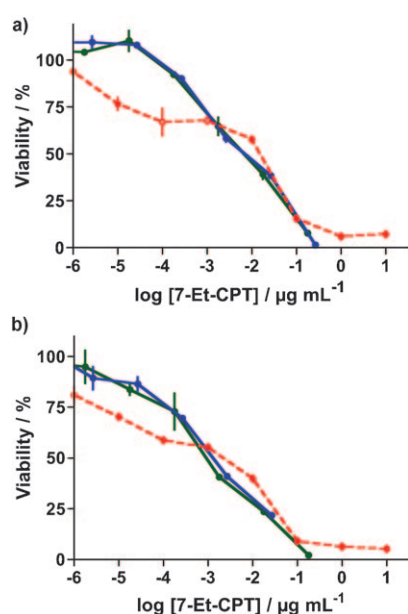


Figure 3. Cytotoxicity assessment of 7-Et-CPT-loaded micelles **G** and **H**. Human MCF-7 were incubated with micelle preparations **G** (blue) and **H** (green) and free 7-Et-CPT (red) at the indicated concentrations of 7-Et-CPT. a) After an incubation of 24 h, the medium was replaced and incubation 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 %).

E and **H** bind to $\alpha_v\beta_3$ -integrins on tumor endothelial cells.^[15c,d] 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 by 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 nanosize 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 semipermeable membrane (cutoff 12000–14000 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 semipermeable membrane (cutoff 12000–14000 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 equiv 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 12000–14000 Da) until the free ligand was completely removed. Micelles were characterized by dynamic light scattering and atomic force microscopy. 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 trifluoroacetic acid (TFA) in water (250 μ L) for 4 h at 100 °C. The sample and standard were spin-dried, redissolved in water (500 μ L), and filtered. Sample concentration was then determined based on the calibration curves of mannose standards.

Quantification of conjugated peptide: An 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 μ m². 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 conditions, which was expected to facilitate the attachment of micelles. A micelle solution (200–400 μ L, \approx 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 μ L, 0.1 mg mL⁻¹) was applied to the surface and after an incubation time of 1 h, 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 μ L, 5 μ g 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 μ L, 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 μ L, 5 μ g 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 μ g 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% CO₂ 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 μ L in 96-well tissue culture plates (Nunc). Cells were then incubated with micelle preparations or free 7-Et-CPT by adding 10X solutions in cell culture medium (20 μ L) to give a final volume of 200 μ L per well for 24 h or 72 h. Control cells were given only cell culture medium (20 μ L). Cells incubated for 24 h were given fresh medium (200 μ L) 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-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble formazan salt as follows. At 68 h, MTT (5 mg mL⁻¹ in phosphate buffered saline (PBS), 20 μ L per 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 μ L per 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 %).

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