Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Tug-of-war: molecular dynamometers against living cells for analyzing sub-piconewton interaction of specific protein with cell membrane

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Experimental section

Materials and reagents. S60HS silica bubbles (average diameter 30 μm, desnsity 0.6 g/mL) were purchased from 3M Company (USA). Dibenzocyclooctyne-PEG_4-maleimide (MAL-DBCO), succinimidyl-4,4′-azipentanoate (Dia-NHS), N,N-diisopropylethylamine (DIPEA), N,N-dimethylformamide (DMF), 2-bromopalmitic acid (2-BP), O-(propargyloxy)-N-(triethoxysilylpropyl) urethane, $CuSO_4$, Triton X-100, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), dimethylsulfoxide (DMSO), palmitic acid (15-yne) and ascorbic acid were purchased from Sigma-Aldrich Inc. (USA). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG) was obtained from MedChemExpress Inc. (USA). (4-[[Bis-(1-tert-butyl-1H-[1,2,3] triazol-4-ylmethyl)-amino]-methyl]-[1,2,3]triazol-1-yl)-acetic acid (BTTAA) was obtained from Click Chemistry Tools (USA). Alexa Fluor 555 azide was acquired from Thermo Scientific (USA). FITC conjugated soybean agglutinin (SBA) was provided by Vector Laboratories (USA). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and silica microspheres (diameter: 10 and 20 μm) were obtained from Tokyo Chemical Industry Co. Ltd. (Shanghai, China). (3-Glycidoxypropyl) methyldimethoxysilane was purchased from Adamas Reagent Ltd (Shanghai, China). MCF-7 cells, RPMI 1640 medium and phosphate buffered saline with a pH of 7.4 (PBS) were obtained from KeyGen Biotech. Co. Ltd. (Nanjing, China). Goat serum was obtained from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China). All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (≥ 18 MΩ /cm, Milli-Q, Millipore).

Apparatus. Fluorescence spectra were recorded on an F-7100 fluorescence spectrophotometer (Hitachi, Japan). The gel electrophoresis was performed on the Electrophoresis Analyser (Bio-Rad, USA) and imaged on a Bio-Rad ChemDoc XRS (Bio-Rad, USA). Confocal laser scanning microscopic (CLSM) imaging was performed on a SP8 STED 3X confocal laser scanning microscope (Leica, Germany). Optical tweezer experiments were performed on Aresis Tweez250Si (Aresis, Slovenia). Mass spectrometric analysis was performed on a MALDI-7090 MALDI-TOF mass spectrometer (Shimadzu, Japan). Contact angles were detected on an OCA30 contact angles analyzer (Dataphysics Instruments Gmb). Flow cytometric analysis was performed on a FACSAria II flow cytometer (BD, USA). The numbers of cells and MDMs were determined using a Countess II FL Automated Cell Counter (Life Technologies, USA).

DNA sequences (5′-3′). All DNA with following sequences were customized from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China):

FAM-aptamer for MUC1 (Apt): H_2N-GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTT/iFAMdT/TTTTTTTTTTTTTT-SH;

random FAM-DNA (Ran): H_2N-XXXXXXXXXXXXXXXXXXXXXXXXXTTTTTTTTTT/iFAMdT/TTTTTTTTTTTTTT, X means random base. The molecular weight of the Ran sequence is approximate to the Apt;

DNA complementary to Apt (Com-Apt): AAAAAAAAAAAAAAAAAAAAAAAAACCAGGGTATCCAAAGGA TCAACTGC;

azide-Cy3-C3: azide-TTTTTTTTTTTTTT/iCy3dT/TTTTTTTTTTTTTT-C3 spacer;

azide-Cy3-C6: azide-TTTTTTTTTTTTTT/iCy3dT/TTTTTTTTTTTTTT-C6 spacer;

azide-Cy3-C9: azide-TTTTTTTTTTTTTT/iCy3dT/TTTTTTTTTTTTTT-C9 spacer;

azide-Cy3-C12: azide-TTTTTTTTTTTTTT/iCy3dT/TTTTTTTTTTTTTT-C12 spacer;

azide-Cy3-C18: azide-TTTTTTTTTTTTTT/iCy3dT/TTTTTTTTTTTTTT-C18 spacer.

Preparation of MDM. 0.01 g of S60HS silica bubbles were firstly immersed in 1.5 mL of 5 M H_2SO_4 at room temperature overnight to obtain hydroxyl surface. The hydroxyl bubbles were washed with water twice, dried in air and added to 1 mL of (3-glycidoxypropyl) methyldimethoxysilane solution (5% in dichloromethane) to obtain epoxy-silylated bubbles by shaking overnight. After washing with ethanol three times and desiccation, the epoxy-silylated bubbles were incubated with 0.8 mL of 1 mg/mL DSPE (containing 10 mg/mL sodium dodecyl sulfate) and shaken for 6 hours to obtain DSPE modified bubbles, which were washed with water for three times. The DSPE modified bubbles were then dried in air and dispersed into 1 mL n-decane/isobutanol (10:1, v/v). After 2 μL of azide-Cy3-Cx (50 μM) with different alkane chain lengths was added into the dispersion respectively, and full mixed, the bubbles were collected by buoyancy and further treated with 1 mg/mL DSPE in n-decane/isobutanol (10:1, v/v) to obtain different types of MDM.

Contact angle tests of functionalized silica bubbles. The dispersion of functionalized silica bubbles was dripped onto a slide covered with double-sided tape. After desiccation, the functionalized silica bubbles were dried softly by nitrogen to obtain a film of functionalized silica bubbles. A drop of water was dripped onto the film and photographed by contact angles analyzer to analyze the contact angles. The contact angle tests of the functionalized 10 μm silica microspheres was performed with the same procedure.

Quantification of azide-Cy3-Cx on MDM. The amounts of azide-Cy3-Cx assembled on MDM were quantified by measuring the remained azide-Cy3-Cx in above mixed solutions with the fluorescence intensity of Cy3 and the corresponding calibration curves. Here the amounts of MDM were acquired with a Countess II FL Automated Cell Counter.

Cells. MCF-7 cells were cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2.

Preparation of FA and Dia-Ran. 20 μL of 100 μM Apt was mixed with 2 μL of 10 mM Dia-NHS in DMF, 2 μL of 10 mM MAL-DBCO in DMF and 1.5 μL of 10 mM DIPEA in DMF to react with shaking for 48 hours in the darkness at room temperature. The mixture was then purified by ultrafiltration with PBS for three times to get FA. Dia-Ran was prepared by incubating 20 μL of 100 μM Ran with 2 μL of 10 mM Dia-NHS and 1.5 μL of 10 mM DIPEA in DMF at room temperature for 48 hours, and then purified by ultrafiltration with PBS for three times.

CLSM measurement of embedding force. MCF-7 cells were firstly seeded on 10 mm single-well confocal dishes and cultured overnight. After washing with PBS, the cells were blocked with 10% goat serum in PBS at 4 °C for 30 min, incubated with FA (1.0 μM in PBS) at 37 °C for 30 min, washed with PBS for three times, and then exposed under the UV light at room temperature for 15 min to bind FA to target protein on cell membrane. Afterward, different types of MDMs (about 2.2×10^6 bubbles/mL, dispersed in 100 μL RPMI 1640 medium supplemented with 10% fetal bovine serum) were added to the dishes seeded with FA labeled cells, respectively, which were inverted for the contact of MDMs with cells to bind azide on MDMs with DBCO on cells at 37 °C for 8 min. The dishes were then inverted back to normal position to separate MDMs and cells by buoyancy at 37 °C for 2 min. The above process of inversion was repeated for three times for full contact of MDMs with cells. After the cells were washed with PBS for three times, CLSM imaging was performed to measure the amount of FA remained on cell membrane by FAM fluorescence, which could be semi-quantitatively converted to the embedding force

by comparing the fluorescence change upon alkane chain variation with the "ruler" obtained by the optical tweezer analysis.

Flow Cytometric Analysis of Embedding Force. 500 μL MCF-7 cells (10000 cells/mL) was firstly blocked with 10% goat serum-containing PBS at 4 °C for 30 min, and incubated with 1 μM FA in PBS at 37 °C for another 30 min. Afterward, the cells were exposed under the 365 nm UV light for 15 min to obtain FA labeled cells, which were then treated with different types of MDMs (about 8.8×10^5 bubbles/mL) in FBS-containing RPMI 1640 medium at 37 °C by 3 cycles of continual inversion on a vertical rotator for 55 min to bind the FA to MDMs and centrifugation at 1800 rpm for 5 min to perform the tug-of-war. Lastly, the cells were dispersed in PBS for flow cytometric analysis.

"Ruler" for Embedding Force Measurements. The embedding force "ruler" was obtained by using DSPE bilayer modified silica microspheres (10 μm in diameter, SM10) as cell mimic and azide-Cy3-Cx modified silica microspheres (20 μm in diameter, SM20) as MDM mimic for optical tweezer analysis. The MDM mimic was prepared by immersing 10 mg SM20 in 5 M H_2SO_4 overnight and then 1 mL 5% O-(propargyloxy)-N-(triethoxysilylpropyl) urethane in dichloromethane with shaking overnight to obtain alkynyl-silylated SM20, which was further dispersed in 100 μL mixture of 60 μM BTTAA, 25 μM $CuSO_4$, 0.5 μM azide-Cy3-Cx and 250 μM sodium ascorbate to incubate with shaking in the dark at room temperature for 2 hours. The cell mimic was prepared by incubating the epoxy-silylated SM10 in 0.8 mL of 1 mg/mL DSPE (containing 10 mg/mL sodium dodecyl sulfate) with shaking for 6 hours.

The optical tweezer analysis was performed by fixing DSPE bilayer modified SM10 with an optical trap and moving alkane chain modified SM20 right and left at a speed of 1 μm/s with another optical trap to contact the fixed SM10. The horizontal force acted on SM10 was obtained by detecting the tiny displacement of SM10 during the contact. The force caused by the contact of alkane chain modified SM20 was gained through subtracting the force in X direction on SM10 before the contact from that after the contact. The hydrophobic interaction force which represented the insertion of one or multiple alkane chains was obtained for semi-quantitative evaluation of the embedding force by eliminating the force caused by unmodified SM20.

Inhibition to Palmitoylation or O-glycosylation of MUC1. 2-BP was used to inhibit the palmitoylation of MUC1 by incubating MCF-7 cells seeded on confocal dishes in 100 μM 2-BP and 100 μM palmitic acid (15-yne) in 1640 (containing 10% FBS) for 8 hours. The latter could metabolically label the palmitoyl groups to further conjugate Alexa Fluor 555 azide through click chemistry, which was performed by fixing the labeled cells with cold methanol at -20 °C for 10 min, permeabilizing them with 0.1% Triton X-100/PBS at room temperature for 5 min, and then incubating these cells in 400 μL PBS containing 1.0 mM TCEP, 1.0 mM $CuSO_4$ and 0.1 mM Alexa Fluor 555 Azide at room temperature for one hour.

The O-glycosylation of MUC1 was inhibited by treating seeded MCF-7 cells with 2.5 mM BAG for 48 hours. After washing with PBS, the cells were blocked with 10% goat serum-containing PBS at 4 °C for 30 min, and treated with 20 μg/mL FITC-SBA in PBS at 37 °C for 30 min to perform CLSM imaging.

Supporting Figures

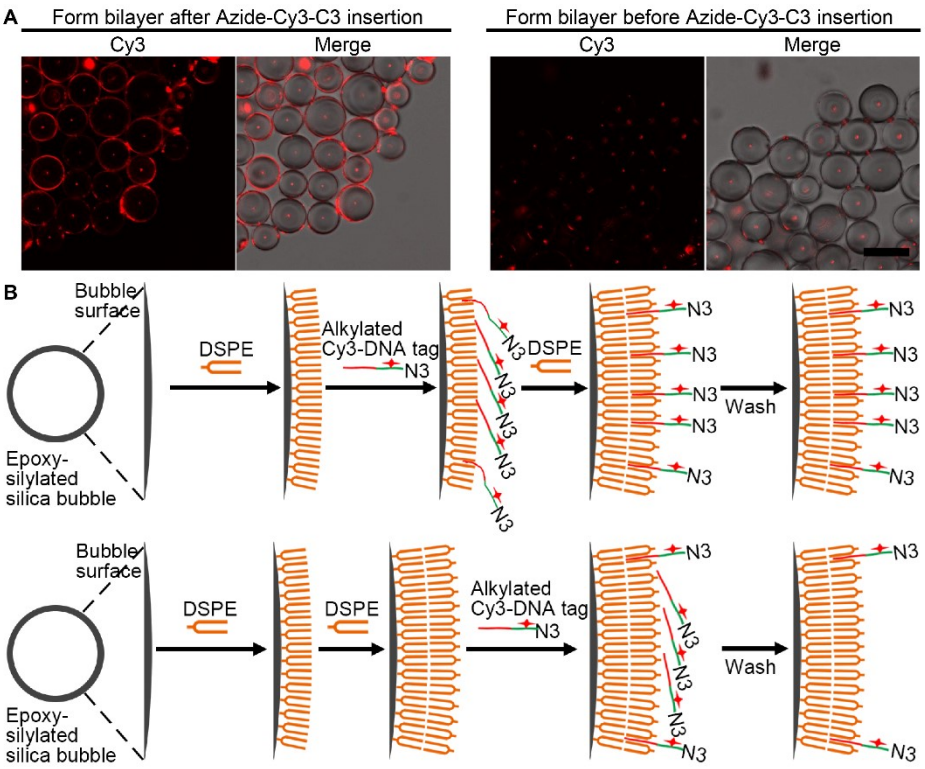

Fig. S1. Mechanism of MDM assembly. (A) Confocal laser scanning microscopic (CLSM) images. Scale bar: 50 μm. (B) Assembly procedure of MDMs by forming bilayer after or before azide-Cy3-C3 insertion.

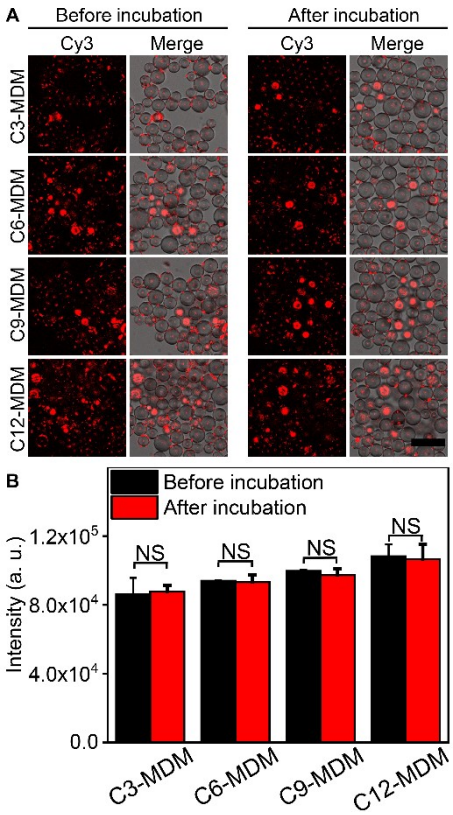

Fig. S2. Stability of MDMs. (A) CLSM images of MDMs before or after incubated in 1640 medium at 37 °C for 30 min. Scale bar: 100 μm. (B) Statistical analysis of Cy3 fluorescence in (A) with t test. NS, not significant. Error bars represent s.d. of 3 independent experiments.

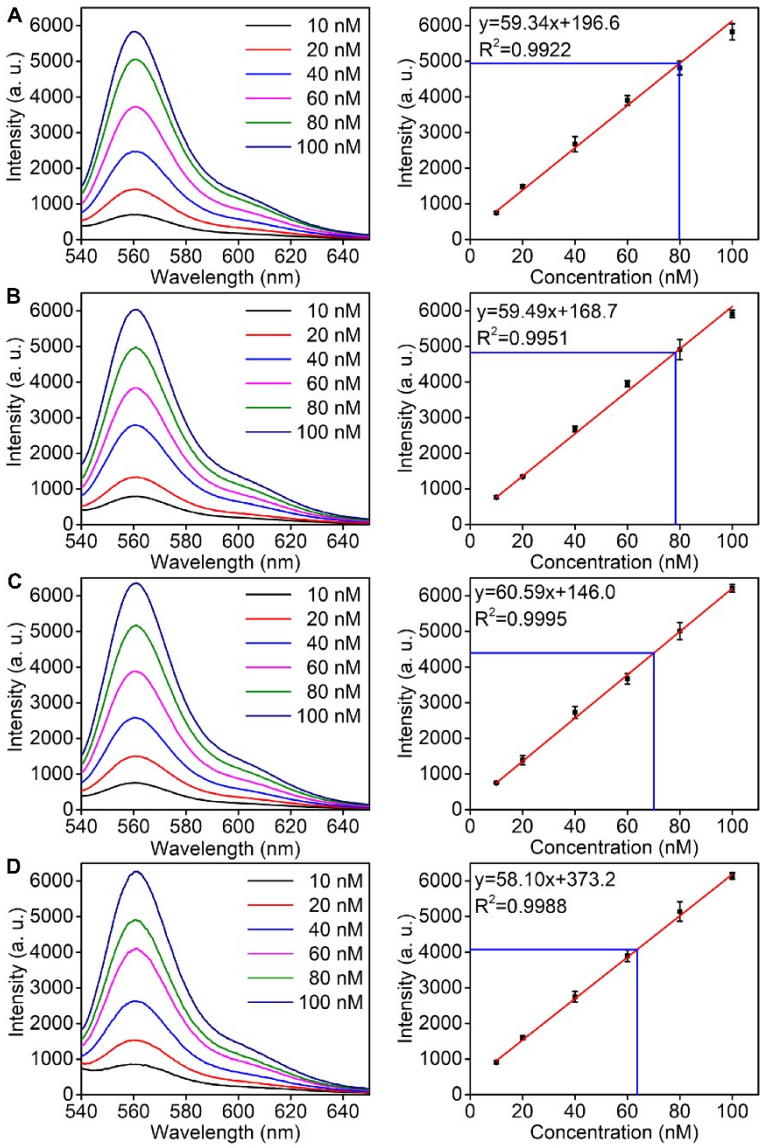

Fig. S3. Fluorescence spectra and calibration curves of azide-Cy3-Cx. Fluorescence spectra (left) and calibration curves (right) of (A) azide-Cy3-C3, (B) azide-Cy3-C6, (C) azide-Cy3-C9 and (D) azide-Cy3-C12. Error bars represent s.d. (n=3 independent experiments). Blue lines represent the fluorescence intensities and corresponding concentrations of azide-Cy3-Cx remained in incubation solutions for assembly of 1.1×10^6 C3-MDM, C6-MDM, C9-MDM and C12-MDM. The coverages of azide-Cy3-Cx on different types of MDMs are obtained by subtracting the remained amount from the added amount of azide-Cy3-Cx.

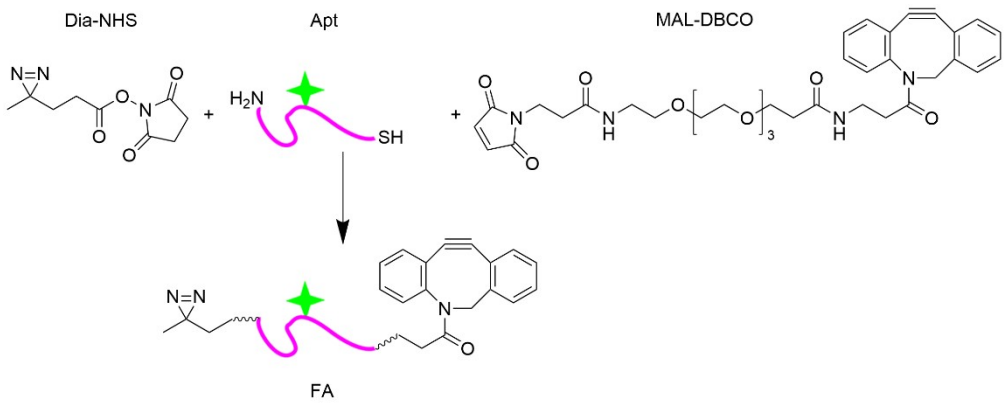

Fig. S4. Schematic illustration of FA preparation.

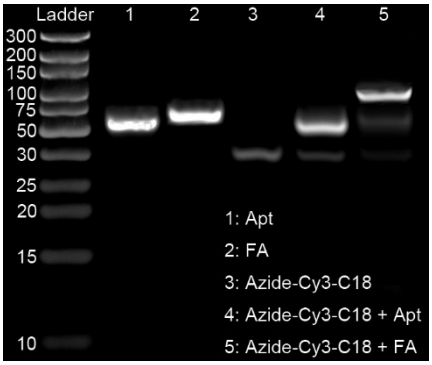

Fig. S5. Characterization of FA by gel electrophoresis. Native PAGE analysis of dibenzocyclooctyne (DBCO) functionalized carboxyfluorescein (FAM)-aptamer (FA) and its conjugate with Azide-Cy3-C18.

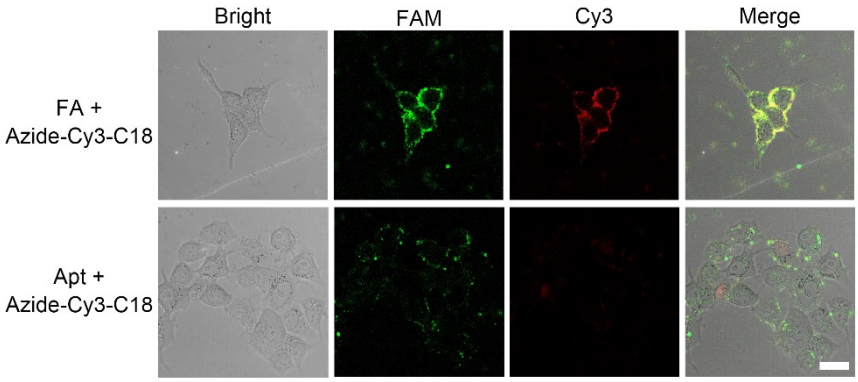

Fig. S6. Click function of FA on cell surface. CLSM images of MCF-7 cells incubated with 1 μM FA or FAM-aptamer (Apt) and then 1 μM Azide-Cy3-C18 at 37 °C for 30 min. Scale bar: 25 μm.

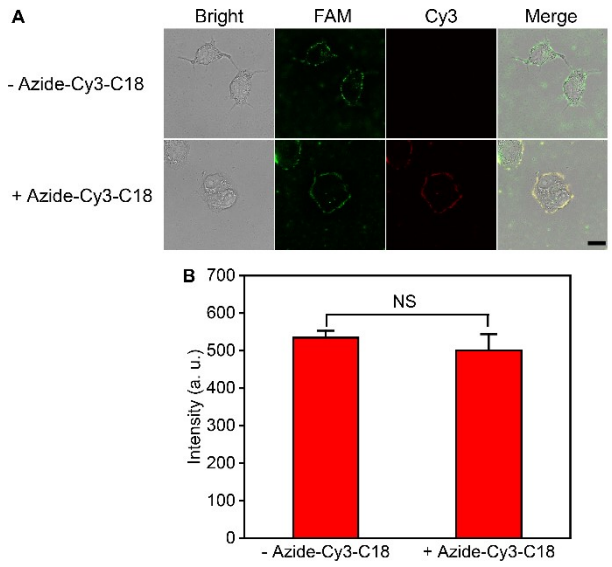

Fig. S7. Exclusion of Förster resonance energy transfer. (A) CLSM images of FA-treated MCF-7 cells incubated with or without 1 μM Azide-Cy3-C18 at 37 °C for 30 min. Scale bar: 15 μm. (B) Statistical analysis of the fluorescence intensities of FAM from (A), which was performed with the t test (NS, not significant). Error bars represent s.d. (n=3 independent experiments).

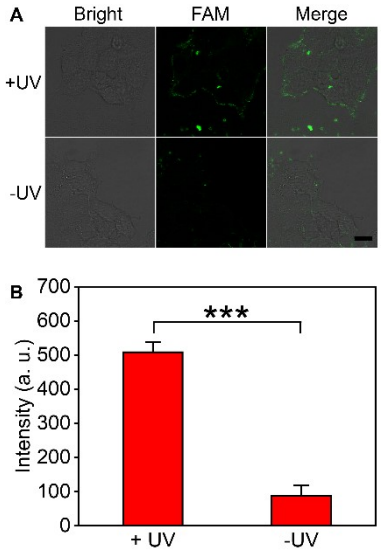

Fig. S8. Covalent crosslink function of FA on cell surface. (A) CLSM images of FA treated MCF-7 cells with or without UV irradiation after incubation in 1 μM DNA complementary to Apt at 37 °C for 30 min. Scale bar, 15 μm. (B) Statistical analysis of the fluorescence intensities of FAM from (A), which was performed with the t test (***$p < 0.0005$). Error bars represent s.d. (n=3 independent experiments).

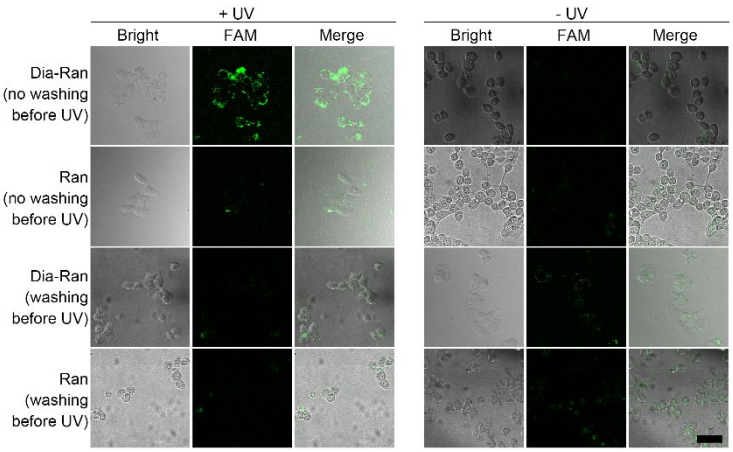

Fig. S9. UV irradiation mediated covalent anchor of diazine group. CLSM images of MCF-7 cells incubated with diazine-modified random sequence (Dia-Ran) or random sequence (Ran) and then treated with washing or/and UV light irradiation. Scale bar, 60 μm.

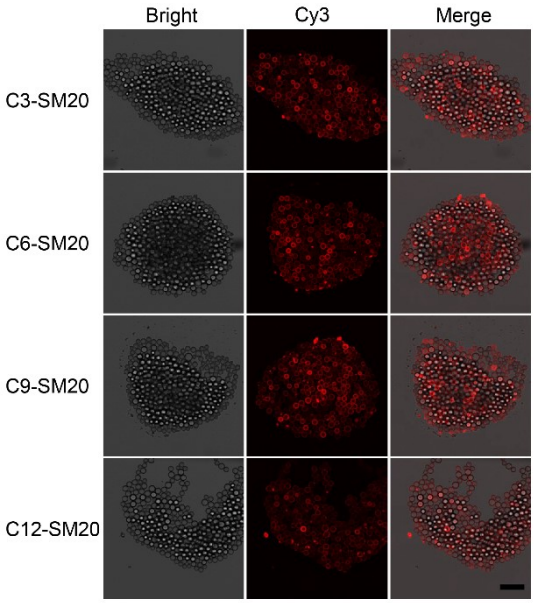

Fig. S10. CLSM images of azide-Cy3-Cx modified silica microspheres (20 μm). Scale bar, 100 μm.

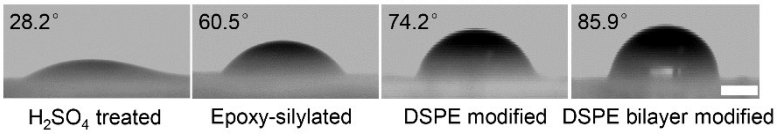

Fig. S11. Characterization of DSPE bilayer modified silica microspheres (10 µm) by contact angle analysis. Contact angle images of the thick films of H_2SO_4 activated, epoxy-silylated, DSPE modified, and DSPE bilayer modified silica microspheres (10 µm). Scale bar, 500 µm.

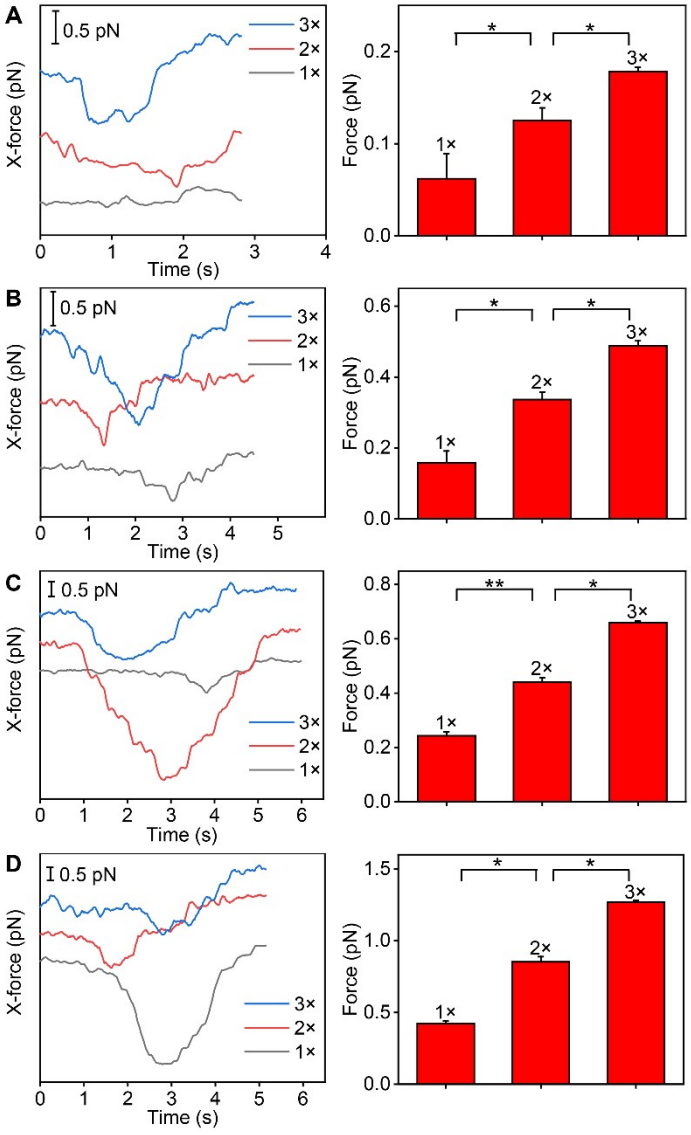

Fig. S12. Integer multiple forces in X direction measured with optical tweezer for (A) C3, (B) C6, (C) C9, (D) C12 alkane chains modified SM20 acting on DSPE bilayer modified SM10. Right column: after deducting the blank, and error bars represent s.d. of 3 independent experiments (*p<0.05; **p<0.005).

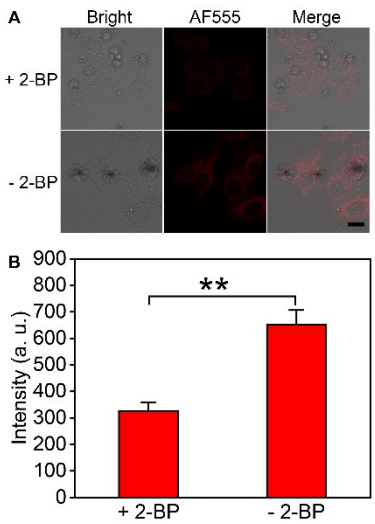

Fig. S13. Characterization of the inhibition of palmitoylation. (A) CLSM images of MCF-7 cells treated with 100 μM 2-BP and 100 μM palmitic acid (15-yne) and then labeled with Alexa Fluor 555, and MCF-7 cells treated with 100 μM palmitic acid (15-yne) without 2-BP and then labeled with Alexa Fluor 555. Scale bar, 15 μm. (b) Statistical analysis of the fluorescence intensities of AF555 from (a), which was performed with the t test (**$p < 0.005$). Error bars represent s.d. (n=3 independent experiments).

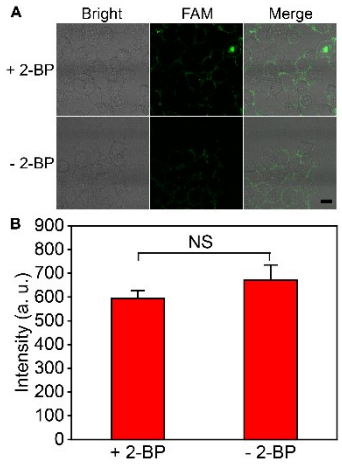

Fig. S14. The label of FA to palmitoylation-inhibited cells. (A) CLSM images of MCF-7 cells treated with 100 μM 2-BP and then labeled with FA, and cells directly labeled with FA. Scale bar, 15 μm. (B) Statistical analysis of the fluorescence intensities of FAM from (A), which was performed with the t test (NS, not significant). Error bars represent s.d. (n=3 independent experiments).

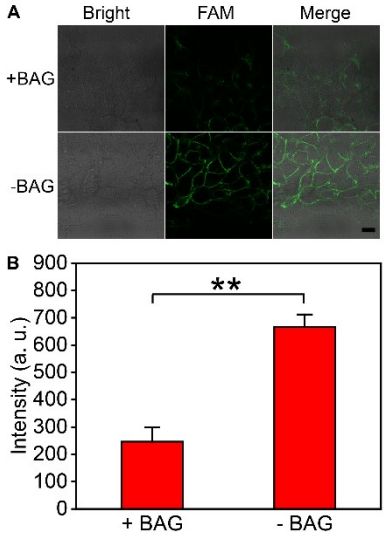

Fig. S15. Characterization of the inhibition of O-glycosylation. (A) CLSM images of MCF-7 cells treated with 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG) and then labeled with FITC conjugated soybean agglutinin (SBA), and cells directly labeled with FITC conjugated SBA. Scale bar, 15 μm. (B) Statistical analysis of the fluorescence intensities of FAM from (A), which was performed with the t test (**$p < 0.005$). Error bars represent s.d. (n=3 independent experiments).

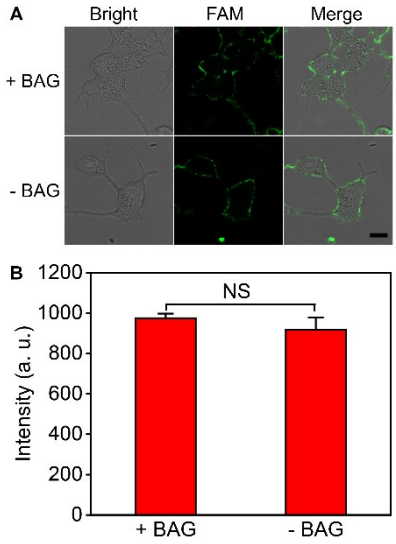

Fig. S16. The label of FA to O-glycosylation-inhibited cells. (A) CLSM images of MCF-7 cells treated with 2.5 mM BAG and then labeled with FA, and cells directly labeled with FA. Scale bar, 15 μm. (B) Statistical analysis of the fluorescence intensities of FAM from (A), which was performed with the t test (NS, not significant). Error bars represent s.d. (n=3 independent experiments).

Supporting Table

Table S1. Hydrophobic interaction forces between different alkane chains and DSPE bilayer obtained with optical tweezer.

Optical tweezer measurements	C3	C6	C9	C12
Hydrophobic interaction force (pN)	0.06 ± 0.03	0.16 ± 0.03	0.24 ± 0.01	0.42 ± 0.02