Supporting Information

Proximity Enzymatic Glyco-Remodeling Enables Direct and Highly Efficient Lipid Raft Imaging on Live Cells

Jing Tao, Xiaofei Yu, Yuna Guo, Guyu Wang, Huangxian Ju, and Lin Ding*

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Chemistry and Biomedicine Innovation Center (ChemBIC), Nanjing University, Nanjing 210023, P. R. China

Corresponding Author:

*E-mail: dinglin@nju.edu.cn. Phone/Fax: +86-25-89681927

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Experimental Section

Materials and Reagents. Gold(III) chloride trihydrate ($HAuCl_4$), sodium citrate, TWEEN® 20, *O*-(2-carboxyethyl)-*O*′-(2-mercaptoethyl)heptaethylene glycol (SH-PEG$_7$-COOH), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), Cholera toxin B subunit (CTxB), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), tris(hydroxymethyl)aminomethane (Tris), fluorescein-5-thiosemicarbazide (FTZ), Alexa Fluor™ 647 hydrazide (hydrazide-AF647), biotin hydrazide, 7-ketocholesterol (7KC), methyl-β-cyclodextrin (mβCD) and OptiPrep™ density gradient medium (OptiPrep) were purchased from Sigma-Aldrich Inc. (USA). CTxB was dialyzed through dialysis membrane (MW 3500) for 3 days before use. Galactose oxidase (GO), goat serum, D-(+)-galactose (D-Gal), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (4×), Western transfer buffer (10×), Tris buffered saline (TBS) (20×), block buffer (1×), and sodium borate decahydrate were purchased from Shanghai Sangon Biotechnology Company, Ltd. (China). Phosphate-buffered saline (PBS, pH 7.4, containing 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, 1.41 mM KH_2PO_4), MCF-7 cells, A549 cells, MDA-MB-231 cells, trypsin and RPMI-1640 were purchased from KeyGen Biotech Company, Ltd. (China). Anti-biotin mouse monoclonal antibody was purchased from Abcam Trading Co., Ltd. (USA). Anti-annexin II rabbit monoclonal antibody was purchased from Huabio Biotech Company, Ltd. (China). 4-Morpholineethanesulfonic acid (MES) buffer (50 mM, pH 5.5) and Triton X-100 cell lysis buffer were purchased from Leagene Biotech Company, Ltd. (China). Potassium

ferricyanide ($K_3[Fe(CN)_6]$), sodium phosphate monobasic dihydrate, sodium phosphate dibasic dodecahydrate and dithiothreitol (DTT) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (China). Alexa Fluor™ 647 conjugated Cholera toxin B subunit (CTxB-AF647), NuPAGE™ 4-12% Bis-Tris protein gels, NuPAGE™ MOPS SDS running buffer (10×), Amplex™ Red Galactose/Galactose Oxidase Assay Kit and fetal bovine serum (FBS) were purchased from Invitrogen™ Thermo Fisher Scientific (USA). Aniline and potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]$) were purchased from Shanghai Macklin Biochemical Co., Ltd. (China). ECL luminescent working solution was purchased from Bioelite Co., Ltd. (China). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, HRP conjugated goat anti-mouse IgG and BCA Protein Assay Kit were purchased from CWBIO biotechnology Co., Ltd. (China). Dialysis membranes (MW 3500) were purchased from Yuanye Biotechnology Co., Ltd. (China). Cell Counting Kit-8 (CCK-8) was purchased from EnoGene Biotechnology Co., Ltd. (China). Boric acid was purchased from Nanjing Chemical Reagent Co., Ltd. (China). Phosphate buffer (PB, 10 mM, pH 7.5), borate buffer (50 mM, pH 8.5), Tris-HCl (50 mM, pH 7.5), 1× TBST (TBS containing 0.1% TWEEN® 20) and other aqueous solutions were prepared using ultrapure water (≥18 MΩ, Milli-Q, Millipore).

Apparatus. The UV-vis absorption spectra were obtained on a UV-3600 UV-VIS-NIR spectrophotometer (Shimadzu, Japan). Transmission electron microscopy (TEM) images were obtained using a JEM-2100 projection electron microscope (JEOL, Japan). Dynamic light scattering (DLS) analysis was carried out on a 90Plus Particle Size Analyser (Brookhaven, USA). The fluorescence images of cells were acquired on a SP8

STED 3× confocal laser scanning microscope (CLSM) (Leica, Germany). Cell number was calculated with Countess® II Automated Cell Counter (Thermo Fisher Scientific, USA). Protein transfer to PVDF membranes was performed on a Trans-Blot Turbo Transfer System (Bio-Rad, USA). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on XCell SureLock™ Mini-Cell (Thermo Fisher Scientific, USA) and imaged on a Bio-Rad ChemDoc XRS facility (Bio-Rad, USA). BCA protein quantification, galactose oxidase (GO) activity assay, and CCK-8 cell viability assay were performed on a Multiskan FC microplate reader (Thermo Fisher Scientific, USA). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) data were obtained on a 4800 plus MALDI TOF/TOF analyzer (AB sciex, USA). For analysis of the concentration of gold nanoparticles, an ELAN 9000 inductively coupled plasma mass spectrometer (ICP-MS, PE, USA) was used. For density gradient centrifugation, a Beckman Coulter OPTIMA L-100XP ultracentrifuge (100 Ti Rotor) was used.

Cell Culturing. MCF-7, MDA-MB-231 and A549 cells were separately cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicilin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified chamber containing 5% CO_2.

Preparation of Gold Nanoparticles (AuNPs). AuNPs with a diameter of 20 nm were prepared by the classic sodium citrate reduction method with a little modification.[1] Briefly, to a boiling $HAuCl_4$ solution (0.01%, 200 mL), 3.98 mL of 1% sodium citrate was immediately added under stirring, and the mixture was subjected to boiling for 10

min. Then the heat source was removed and the solution was stirred for another 15 min. The concentration of the as-prepared AuNPs was estimated to be 17.9 nM by ICP-MS.

Optimization of Probe Recognition Conditions. To optimize the probe concentration for recognizing lipid rafts, after seeding and blocking, the MCF-7 cells were allowed to incubate in 100 μL PBS containing 50 mM $K_4[Fe(CN)_6]$ and GO-AuNP-CTxB (G:C ratio is 12.0:1.0) of different concentrations (4.5, 8.9, 17.9 and 26.8 nM) at 4 °C for 30 min. Then the cells were subjected to the same reactivation, FTZ reaction and imaging procedures as mentioned in **PEGR-Based Lipid Raft Imaging on Live Cells**.

To optimize the probe recognition time, after seeding and blocking, the MCF-7 cells were allowed to incubate in 100 μL PBS containing 17.9 nM GO-AuNP-CTxB (G:C ratio is 12.0:1.0) and 50 mM $K_4[Fe(CN)_6]$ at 4 °C for different periods of time (10, 20, 30 and 40 min). Then the cells were subjected to the same reactivation, FTZ reaction and imaging procedures as mentioned in **PEGR-Based Lipid Raft Imaging on Live Cells**.

CTxB Competition Assay. To demonstrate CTxB-dependent PEGR, after seeding and blocking, the MCF-7 cells were allowed to incubate in 100 μL PBS containing 17.9 nM GO-AuNP-CTxB (G:C ratio is 12.0:1.0), 50 mM $K_4[Fe(CN)_6]$, and 10 μg/mL CTxB at 4 °C for 30 min. Then the cells were subjected to the same reactivation, FTZ reaction and imaging procedures as mentioned in **PEGR-Based Lipid Raft Imaging on Live Cells**.

Fractionation of Cell Lysates from Labeled Cells by Density Gradient

Ultracentrifugation. The MCF-7 cells were seeded in culture dishes (10 cm), and cultured in a 37 °C incubator for 24 h. Then the cells were subjected to blocking and PEGR treatment with the solution volume being 10-fold of that for cell experiments in confocal dishes. After incubation in PBS (1 mL) containing 10 mM aniline, 100 μM biotin hydrazide, and 5% FBS for 1 h at 4 °C, the cells were washed gently with PBS for three times. Then the cells were treated by 1 mL of DTT (200 mM) at 4 °C for 1 h. After washing three times with pre-chilled PBS, 600 μL of Triton X-100 cell lysis buffer was added to lyse the cells. Then the cells were scraped off and incubated at 4 °C for 30 min with a shaking operation every 10 min. After centrifugation (950 g) at 4 °C for 15 min, the supernatant was collected for subsequent density gradient centrifugation using OptiPrep as the separation medium.[2,3] Briefly, a 1550 μL mixture of lysate and OptiPrep with a final OptiPrep concentration of 40% (vol/vol) was placed at the bottom of a 6.2 mL ultracentrifuge tube. Then Optiprep solutions of concentrations of 30%, 20%, 5%, with the same volume of 1550 μL, were layered sequentially. After centrifugation (100,000 g) at 4 °C for 4 h, 10 equal fractions with 600 μL each were collected from top to bottom of the tube. The lysates from control MCF-7 cells, with all treatment steps performed except the omission of incubation with GO-AuNP-CTxB probe, were also subjected to OptiPrep-based fractionation. The collected fractions were used as the control in the following Western blot (WB) experiments.

WB analysis. The fractions 2 to 10 collected from treated and untreated MCF-7 cells, respectively, by density gradient centrifugation were subjected to WB analysis. The protein concentration was determined by BCA. Each sample of 30 μL (with the same

protein concentration) was mixed with 10 μL SDS-PAGE sample buffer (4×) and boiled at 100 °C for 5 min. The proteins in the samples were separated by NUPAGE 4-12% Bis-Tris GEL pregel electrophoresis, and transferred onto a PVDF membrane. The membranes were immersed in block buffer (1×) and shaken at 70 rpm for 2 h. After three-time washing with 1× TBST, the membranes were respectively immunoblotted with anti-biotin antibody and anti-annexin II antibody at 4 °C overnight in 1× TBST containing 3% BSA. The membranes were then washed three times with 1× TBST, incubated with HRP conjugated goat anti-mouse IgG or HRP conjugated goat anti-rabbit IgG in 1× TBST containing 3% BSA at r.t. for 1 h, and washed three times again with 1× TBST. Then the ECL luminescent working solution was evenly placed on the surface of the membranes and the membranes were imaged on a Bio-Rad ChemDoc XRS imaging system.

Monosaccharide Inhibition Assay. To demonstrate the Gal/GalNAc remodeling specificity, MCF-7 cells, after incubation in 100 μL PBS containing 17.9 nM GO-AuNP-CTxB (G:C ratio is 12.0:1.0) and 50 mM $K_4[Fe(CN)_6]$ at 4 °C for 30 min and subsequent washing, were allowed to incubate in 100 μL PBS containing 10 mM $K_3[Fe(CN)_6]$ and 20 mg/mL D-Gal at 4 °C for 30 min. Then the cells were subjected to the same FTZ reaction and imaging procedures as mentioned in **PEGR-Based Lipid Raft Imaging on Live Cells**.

Cell Viability Analysis. 100 μL of MCF-7 cells (1×10^4) were cultured in wells of 96-well plates for 24 h. After removing the culture medium, the cells were subjected to blocking, GO-AuNP-CTxB (or AuNP-PEG_7-COOH, GO-AuNP) incubation, and

reactivation procedures as mentioned in **PEGR-Based Lipid Raft Imaging on Live Cells**. Then the cells were incubated with RPMI-1640 complete medium in a 37 °C incubator for 5 h, followed by addition of CCK8 (10 μL per well). After incubation for 4 h, the absorption at 450 nm was measured, and the cell viability (%) was calculated by the equation: cell viability (%) = (average OD value for treated cells/average OD value for control cells) × 100%.

CTxB-AF647 Based Lipid Raft Imaging on Live Cells. After seeding and blocking, MCF-7 (or MDA-MB-231, A549) cells were allowed to incubate with 10 μg/mL CTxB-AF647 in an ice-water bath for 30 min, followed by three-time washing with PBS and CLSM imaging.

Statistical Analysis. All data were representative results from at least three independent experiments and average ± standard deviation were shown. Statistical analysis was performed using the Student's *t*-test. $0.01<*p<0.05$ and $***p<0.001$ were considered statistically significant, $p>0.05$ was considered statistically not significant (NS).

Supporting Figures

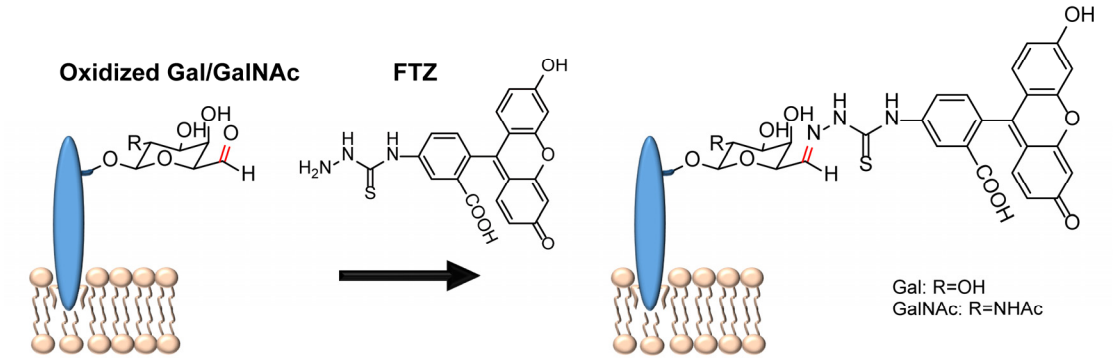

Figure S1. Scheme for the reaction mechanism of the oxidized Gal/GalNAc in raft domains with FTZ.

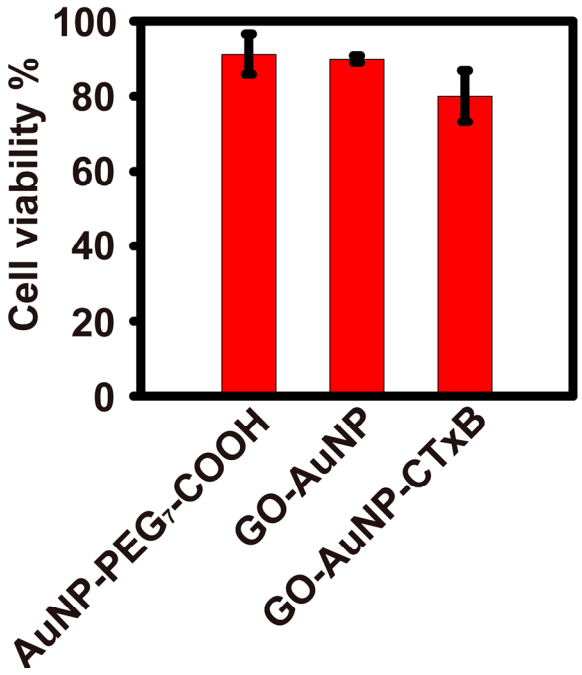

Figure S2. Probe cytotoxicity assay. MCF-7 cell viability after incubation with AuNP-PEG_7-COOH, GO-AuNP and GO-AuNP-CTxB in the presence of $K_4[Fe(CN)]_6$, respectively, followed by $K_3[Fe(CN)_6]$ reactivation and further 5-h incubation. Data are represented as average ± standard deviation (from three independent measurements).

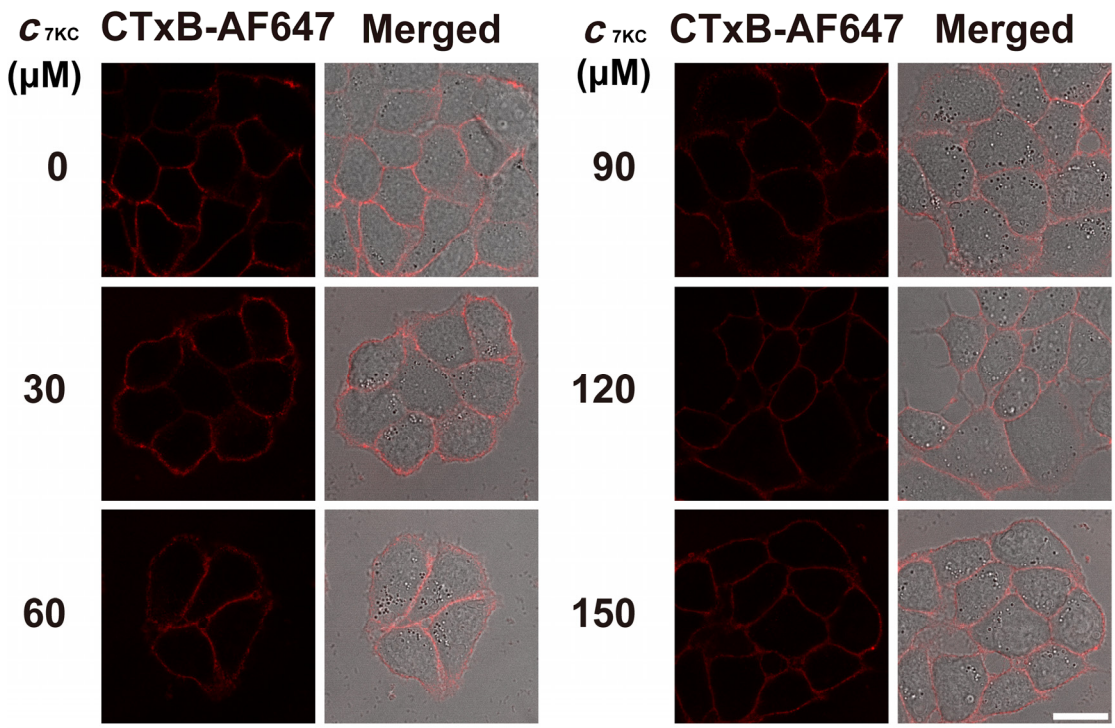

Figure S3. Optimization of 7KC concentration for destroying raft order. CLSM images of MCF-7 cells after incubation with 7KC of different concentrations at 37 ºC for 30 min followed by incubation with CTxB-AF647 of 4 μg/mL in an ice-water bath for 30 min. Scale bar: 20 μm.

The concentration of 7KC was optimized by using CTxB-AF647 (a commercially-available fluorescent lipid raft marker)[4] to monitor the accompanying variation of lipid rafts, and 120 μM was selected when minimum binding of CTxB-AF647 was observed.

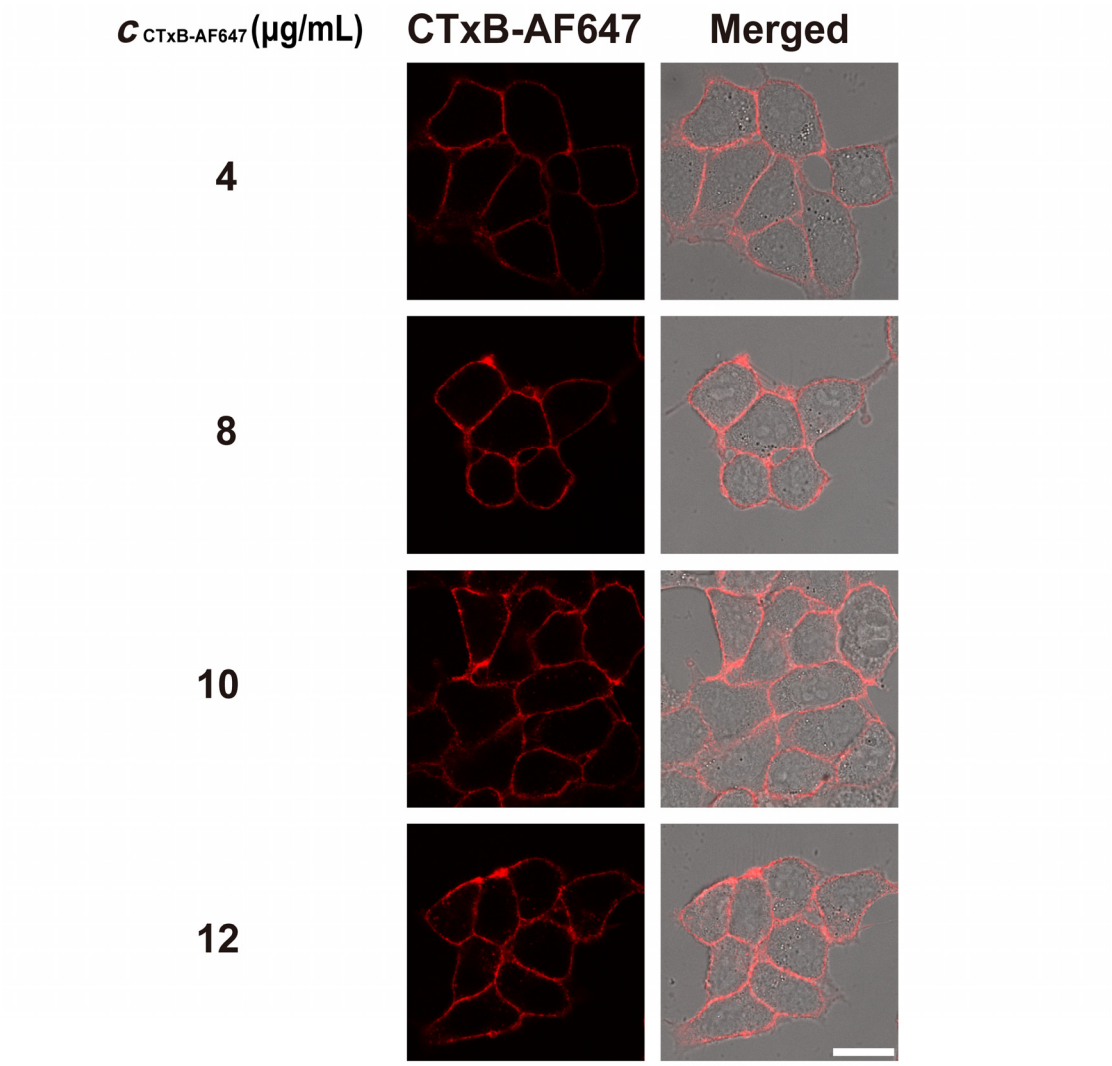

Figure S4. Optimization of CTxB-AF647 concentration for labeling lipid rafts. CLSM images of MCF-7 cells after incubation with CTxB-AF647 of different concentrations in an ice-water bath for 30 min. Scale bar: 20 μm.

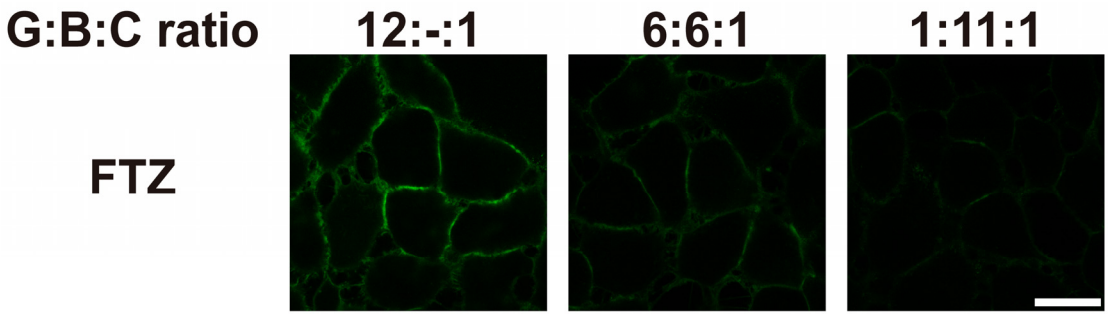

Figure S5. CLSM images of MCF-7 cells after PEGR and FTZ reaction using counterpart probes (GO (BSA)-AuNP-CTxB) prepared with different ratios of GO, BSA and CTxB (G:B:C ratio). Scale bar: 20 μm.

References

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(3) Setiadi, H.; McEver, R. P. Clustering Endothelial E-selectin in Clathrin-coated Pits and Lipid Rafts Enhances Leukocyte Adhesion under Flow. *BLOOD* **2008**, *111*, 1989-1998.

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