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Competition-based transfer of carbohydrate expression information from cell-adhered surface to a secondary surface

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Experimental

Materials. Concanavalin A (Con A), bovine serum albumin (BSA), mannan, methyl mannopyranoside and silver enhancer kit including enhancement solutions A and B were purchased from Sigma-Aldrich Inc. (USA). 3-Glycidoxypropyltrimethoxysilane (GPTMS) and adipic dihydrazide (ADH) were from Alfa Aesar China Ltd. $AuCl_3HCl•4H_2O$ (Au% > 48%) and sodium citrate were obtained from Shanghai Chemical Reagent Co., Ltd. (China). N-acetylglucosamine (GlcNAc) with analytical grade was from Sinopharm Chemical Reagent Co., Ltd (China), and swainsonine (SW) was from Merck KGaA (Darmstadt, Germany). Sylgard 184 silicone elastomer and curing agent were purchased from Dow Corning (Midland, MI). Fluorescein-labeled Con A was from Vector Laboratories (Burlingame, USA). Phosphate buffer saline (PBS, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4. All other reagents were of analytical grade. All aqueous solutions were prepared using ultra-pure water (≥ 18 MΩ, Milli-Q, Millipore).

Apparatus and characterization. Glass slides after silver enhancement were scanned using a HP scanjet 2400 scanner. The scanning parameters were listed as follows: highlights, 225; shadows, 6; midtones,

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2.20; Output level white, 240; output level black, 0. The UV-vis absorption spectra were obtained with a UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Kyoto, Japan). The morphology of different monocrystalline silicon substrates was observed under an Agilent 5500 atomic force microscopy (AFM, U.S.A.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at Mini-PROTEAN 3 cell (Bio-Rad, USA). The fluorescent image of mannan-modified glass slide after incubation with fluorescein-labeled Con A was obtained using an EC3 Imaging System (UVP, U.S.A.).

Cell culture and treatment. BGC-823 cells were kindly provided by Affiliated Zhongda Hospital of Southeast University, Nanjing, China, and cultured in a flask in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. The cell cycle period and the interphase of BGC-823 cells are 41 and 40 h, respectively. At the logarithmic growth phase, the cells were trypsinized and washed twice with culture medium by centrifugation at 1000 rpm for 6 min. The sediment was re-suspended in the culture medium to obtain a homogeneous cell suspension. Cell number was determined using a Petroff-Hausser cell counter (USA). SW-treated BGC-823 cells were obtained by incubating the cells in culture medium in the presence of 2 μg mL^{-1} SW for 2 days.

Preparation and SDS-PAGE of nanoprobe. Gold nanoparticles (AuNPs) with a diameter of 13 nm were prepared by the citrate-reduction method. After trisodium citrate solution (1%, 10 mL) was added to a boiling and rapidly stirred $HAuCl_4$ solution (1 mM, 100 mL), the mixture was kept boiling and stirred for 20 min, and then cooled to room temperature to obtain AuNPs. The prepared AuNP solution (7.6 nM) was stored at 4 ^{o}C. 1 mg Con A dissolved in 200 μL 0.02 M pH 8.0 PBS was added dropwise to 4 mL AuNPs of pH 8 under stirring. After 10 min, the mixture was stabilized by gradually adding BSA solution until reaching the final concentration of 1%. After stirring for another 10 min, the obtained nanoprobe was washed thrice by centrifugation (15000 g, 40 min, 4 ^{o}C) and re-suspended with 1.0 mL of 2 mM pH 8.0 PBS containing 0.1 M Ca^{2+}, 0.01 M Mn^{2+} and 0.8% NaN_3. The purified nanoprobe (28 nM) was stored at 4 ^{o}C for further use.

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The coexistence of Con A and BSA on AuNPs was identified by SDS-PAGE. After centrifugation of the nanoprobe solution (13000 g, 30 min, 4 °C), the sediment was collected and dissolved by SDS reducing buffer, and then heated at 100 ^{o}C for 2 min. The resultant solution was loaded into wells of gel to perform electrophoresis at 110 V for 60 min. All the equipment assembly, solution preparation and operation process were according to the Mini-PROTEAN 3 Cell Instruction Manual (Bio-Rad, USA). After the electrophoresis was completed, the gel was stained with Coomassie blue and photographed by SensiAnsys gel imaging analysis system (Peiqing, Shanghai, China).

Preparation of carbohydrate modified glass slides. Glass slides were firstly dipped in a piranha solution (30% hydrogen peroxide and 70% sulfuric acid) for 24 h. After washed thoroughly with water and dried under a stream of nitrogen, each glass slide was silanized by incubating with 400 μL GPTMS overnight in a sealed container at room temperature (Scheme S1).[1] After rinsed with acetone and dried under a stream of nitrogen, the slides were immersed in 0.2 M ADH in the mixture of ethanol and water (1:1, v/v, pH 3) for 24 h under 60 ^{o}C. After rinsed with ultra-pure water and dried, 5 μL of 0.2 mg mL^{-1} solutions of mannan (for the rightmost 7 spots) and methyl mannopyranoside (for the leftmost spot) in 0.2 mM pH 5.0 PBS were dropped at the defined locations, respectively, to form an 8-spot droplet line of carbohydrate. Microwave radiation was used to achieve high reaction efficiency between ADH and carbohydrate,[2] using a domestic Galanz microwave oven with a radiation frequency at 2.45 GHz. The 6-min radiation process with the power level set at 330 W was performed twice. The unbound carbohydrate was washed away using 10 mM pH 7.4 PBS containing 0.01% Tween 20 and then rinsed with water. After dried under a stream of nitrogen, the slides were blocked with α-methoxy-*ω*-formyl poly(ethylene glycol) (60 mg mL^{-1}) for 1 h at 37 ^{o}C,[3] and then sodium borohydride solution (0.33 mg mL^{-1}) for 5 min at room temperature. Afterwards, the glass slides were washed by ultra-pure water and dried.

The successful immobilization of mannan on glass slides could be demonstrated by dropping 5 μL methyl mannopyranoside (0.2 mg mL^{-1}) at the leftmost spot and 5 μL mannan with desirable concentrations in 0.2 mM pH 5.0 PBS at the rightmost 7 spots of ADH-modified glass slide, respectively. After microwave radiation, blocking and washing procedures, 10 μL of 20 μg mL^{-1} fluorescein-labeled

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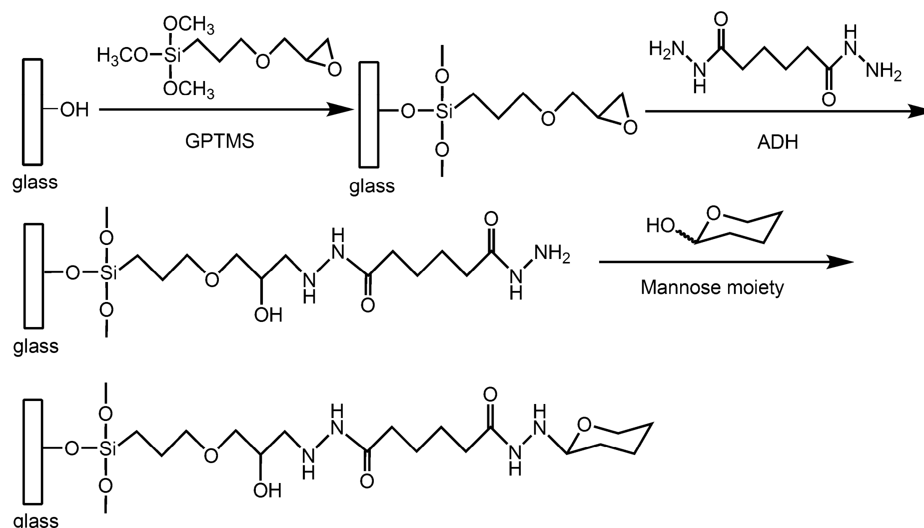
Con A in pH 8.0 PBS containing 1 mM Ca^{2+}, 1 mM Mn^{2+} and 1% BSA was dropped at each carbohydrate spot, and incubated for 1 h at 37 °C. After washed with PBS and water, the slide was dried using N_2 stream. The fluorescent image of the slide was obtained using the EC3 Imaging System with SYBR Green as emission filter.



Scheme S1 Schematic representation of the modification procedure for carbohydrate-assembled slide.

Preparation of poly(dimethylsiloxane) (PDMS) sheet. PDMS membrane was firstly prepared by coating a mixture of silicone elastomer and curing agent (10:1, w/w) on a glass plate. Its thickness was estimated to be 0.5 mm. The prepared PDMS membrane was cut to pieces with the same size as the carbohydrate modified glass slides. A line of 8 holes with diameter of 4 mm was then formed at the defined location on the PDMS pieces by simply drilling with a puncher. The formed PDMS sheets were used for both preparation of the cell-adhered glass slides and fabrication of the competitive device.

Preparation of cell-adhered glass slides. After covering the PDMS sheet on a clean glass slide to form a line of wells and sterilizing under a UV-lamp, 20 μL drops of BGC cells in culture medium with desirable concentration were added into the rightmost 6 wells of the 8-well line. After incubation for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2 to allow the adhesion of cells to the glass slide, the slide was washed thrice with PBS to remove the excess non-adhered cells, and the PDMS sheet was peeled off.

Competitive assay. After covering the carbohydrate modified slide with the PDMS sheet, the formed wells were filled with 14 nM nanoprobe containing 1 mM Ca^{2+}, 1 mM Mn^{2+} and 1% BSA. Then a cell-

1 adhered slide was covered on the top of the PDMS sheet, and the two slides were clamped to prevent
2 solution leakage. After incubating the device at 37 ^{o}C for 1 h, the carbohydrate modified slide was
3 detached from the device, and washed with PBS containing 0.01% Tween 20, NaCl-free PBS and water,
4 respectively. The slide was then quickly dried using nitrogen stream, and was ready for silver
5 enhancement and data analysis.

6 **Silver enhancement and data processing**. Silver enhancement was performed by immersing the slide in
7 a 1:1 mixture of silver enhancement solutions A and B for 2 min. After rinse with water and dried, this
8 process was performed again. For quantification of the grey extent of the spots, the slide was scanned
9 using a scanner, and the resultant image was quantified by reading the greyscale intensity using Adobe
10 Photoshop software. The greyscale here represented the mean shades of gray of the chosen field, varying
11 from black at the weakest intensity (0) to white at the strongest (255). Considering that stronger silver
12 signal showed darker spot, which corresponded to lower value of greyscale intensity, this work used
13 relative greyscale intensity *I* to quantify the silver signal of spots by subtracting the greyscale intensity
14 value of the mannan spot from that of the control spot. The control spot was the leftmost methyl
15 mannopyranoside-treated spot on the carbohydrate-modified slide, which corresponded to the cell-free
16 spot on the cell-adhered slide in the competition step. After this data processing, stronger silver signal
17 corresponded to higher *I*. For more distinct visualization, the grayscale data of cell-based assay were also
18 presented in multicolor scale using GenePixPro software for graph conversion.

19 Characterization of nanoprobe.

20 UV-vis spectroscopy was employed to demonstrate the successful preparation of the nanoprobe. As
21 shown in Fig. S1a, AuNPs exhibited their characteristic absorption peak around 520 nm. After co-
22 assembly of the Con A and BSA on AuNPs, the adsorption peak of the obtained nanoprobes displayed a
23 red-shift, owing to the environmental change around the surface of AuNPs, indicating the successful
24 conjugation of proteins on AuNPs, which could be verified using SDS-PAGE (Fig. S1b). The resultant

polypeptides showed two distinct bands at around 30 kD and 60 kD, corresponding to Con A monomer and BSA, respectively.

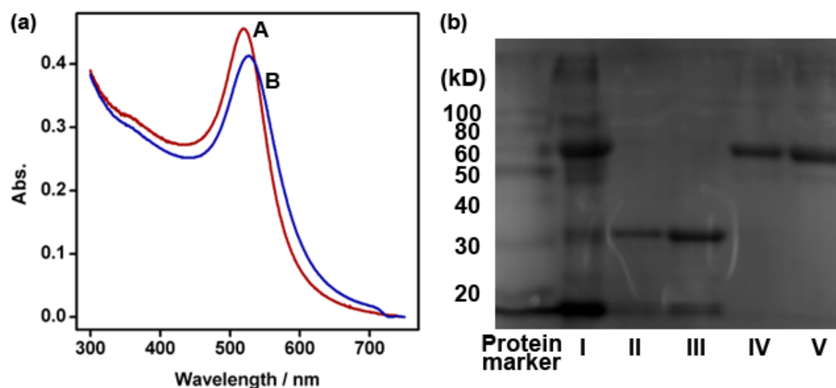


Fig. S1 (a) UV-vis absorption spectra of 1.9 nM AuNPs (A) and 1.7 nM nanoprobe (B). (b) SDS-PAGE stained with Coomassie blue. Wells: protein ruller II, nanoprobe (I), 5 μg Con A (II), 10 μg Con A (III), 5 μg BSA (IV) and 10 μg BSA (V).

Characterization of carbohydrate modified glass slide.

Fig. S2 Fluorescence image of carbohydrate modified glass slide after incubation with fluorescein-labeled Con A in the presence of Ca^{2+} and Mn^{2+}. The spots from II to VIII correspond to mannan immobilization concentrations of 2×10^{-6}, 2×10^{-5}, 2×10^{-4}, 0.002, 0.02, 0.2, 2 mg mL^{-1}, and spot I corresponds 0.2 mg mL^{-1} methyl mannopyranoside.

Optimization of mannan and nanoprobe concentrations.

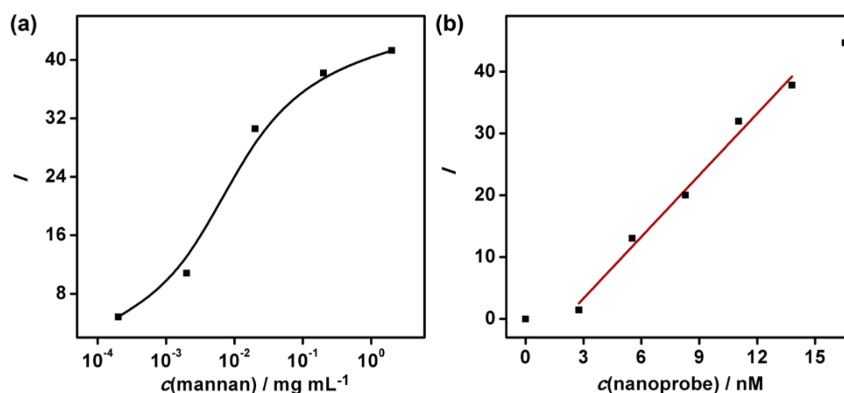
At different mannan concentrations, the obtained carbohydrate modified spots were subjected to incubation with 14 nM nanoprobe for 1 h. After subsequent washing, silver enhancement and scanometric quantification, the relationship between the *I* value and mannan concentration was shown in Fig. S3a. The mannan concentration of 0.2 mg mL^{-1} was chosen for fabrication of the mannan-modified spots.

1 Different concentrations of nanoprobe were used to incubate with mannan modified spots, which were

2 obtained using 0.2 mg mL^{-1} mannan for coupling with ADH. With the increasing nanoprobe

3 concentration, the I increased (Fig. S3b). Because high concentration of nanoprobe led to the increase of

4 chance for non-specific interaction, the nanoprobe concentration of 14 nM was used for competition step.



5

6 ***Fig. S3*** (a) Plot of I *vs* mannan concentration for coupling to ADH modified spots, and (b) plot of I *vs*

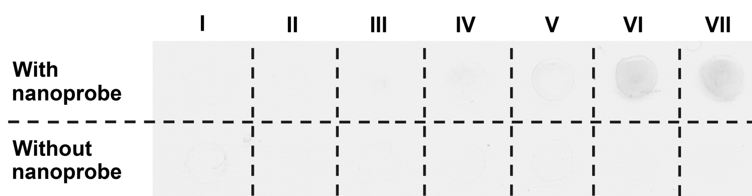
7 nanoprobe concentration.

8 ## Cell surface mannose expression detected without competition.

9 When the cell-adhered slide was directly incubated with 14 nM nanoprobe solution without

10 competition with the carbohydrate modified surface, very weak silver signal could be observed at cell

11 concentrations greater than 5×10^5 cells mL^{-1} (Fig. S4).



12

13 ***Fig. S4*** Scanometric images of cell-adhered slides with (*top*) and without (*bottom*) incubation with 14 nM

14 nanoprobe after silver enhancement. The spots from I to VII correspond to cell concentrations of 0,

15 5.0×10^3, 5.0×10^4, 2.5×10^5, 5.0×10^5, 2.5×10^6 and 5.0×10^6 cells mL^{-1}, respectively.

16 Because the spot without nanoprobe incubation did not display observable signal (bottom line in Fig.

17 S4), the grey signal could be attributed to the specific recognition of nanoprobe to cell surface

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carbohydrate. The sensitivity without competition process was obviously lower than the competition-based strategy, indicative of the advantage of the proposed method.

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