A label-free strategy for facile electrochemical analysis of dynamic glycan expression on living cells

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Experimental

Materials. Concanavalin A (Con A), wheat germ agglutinin (WGA), Dolichos bifows agglutinin (DBA), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 3′-Azido-3′-deoxythymidine (AZT) were purchased from Sigma-Aldrich Inc. (USA). Peanut agglutinin (PNA) was purchased from Medicago Inc. (Sweden). Fluorescein-labeled WGA (molar ratio of fluorescein/protein, F/P = 4.5), Con A (F/P = 5.7), PNA (F/P = 5.1), DBA (F/P = 2.2) were from Vector laboratories (Burlingame, USA). Mannose (Man) and *N*-acetylglucosamine (GlcNAc) with analytical grade were from Sinopharm Chemical Reagent Co., Ltd (China). Single-walled carbon nanohorns (SWNHs) were kindly provided by Prof. S. Iijima (Japan Science and Technology Agency). Single-walled carbon nanotubes (SWNTs, ≤ 2 nm in diameter; 5-15 μm in length) and multi-walled carbon nanotubes (MWNTs, 20-40 nm in diameter; 5-15 μm in length) were from Shenzhen Nanotech Co. (China). 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). SWNHs were dispersed in 30% HNO_3 and then refluxed for 24 h at 140 °C to obtain carboxylic group-functionalized SWNHs. The resulting suspension was centrifuged, and the sediment was washed with deionized water until the pH reached 6.0. Then, the oxidized SWNHs were dispersed in deionized water to a concentration of 0.5 mg mL^{-1}. Oxidized SWNTs and MWNTs were produced by the same procedure.

Cell culture and cell treatment. K562 cell line was kindly provided by the Affiliated Zhongda Hospital, Southeast University, Nanjing, China. K562 cells were 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 °C in a humidified atmosphere containing 5% CO_2. The cells in the exponential growth phase were collected and separated from the medium by centrifugation at 1000 rpm for 3 min, and then washed three times with a sterile pH 7.4 PBS. The sediment was re-suspended in the PBS to obtain a homogeneous cell suspension. Cell number was determined using a Petroff-Hausser cell counter (USA). AZT-treated K562 cells were obtained by incubating the cells in culture medium in the presence of 20 μM AZT for 3 h. The K562 cells were differentiated to erythroid lineages for 4 days by addition of 1 mM sodium butyrate (NaBu) in culture medium.

Electrode preparation. The glassy carbon electrode (GCE) was firstly polished with 1.0, 0.3, and 0.05 μm α-Al_2O_3 powder (Beuhler) successively. After sonication in water, the electrode was rinsed with deionized water and allowed to dry at room temperature. SWNH solution (6 μL, 0.5 mg mL^{-1}) was dropped on the pretreated GCE and dried in a desiccator to obtain SWNHs-coated GCE, which was then immersed in 8 μL PBS solution containing 2.5 mM EDC and optimal concentration of lectin for 3 h to yield lectin-immobilized electrode. Following a rinse with 0.01 M pH 7.4 PBS, the modified electrode was soaked in 50 mM pH 7.4 Tris-HCl buffer containing 1% BSA and 0.1 M NaCl for 30 min to block the surface. 12 μL of 2.0×10^6 cells mL^{-1} K562 cell suspension was dropped on the lectin-immobilized electrode and incubated at 25 °C for 1 h. After careful rinsing with 0.01 M pH 7.4 PBS to remove

noncaptured cells, the obtained electrode was ready for impedance measurement. For Con A-immobilized electrode, 1 mM Ca^{2+} and Mn^{2+} should be added to the recognition solution.

M**onosaccharide inhibition assay**. The lectin-immobilized electrode was preincubated with 12 μL 200 mM monosaccharide (Man or GlcNAc) dissolved in PBS for 1 h at 25 °C.[1] Then 2 μL of K562 cell suspension at 1.4×10^7 cells mL^{-1} was added to the monosaccharide solution on electrode surface and incubated for 1 h at 25 °C. After careful rinsing with PBS to remove the noncaptured cells and sugar, the electrode was used for impedance assay. Data shown are the percentage of control signals (the ΔR_{et} from monosaccharide-preincubated lectin modified GCE divided by the ΔR_{et} from PBS-preincubated lectin modified GCE).

Optimization of lectin modification concentration. Lectins were dissolved in 0.01 M pH 7.4 PBS containing 2.5 mg mL^{-1} EDC at different concentrations (from 0.25 mg mL^{-1} to 2.5 mg mL^{-1}), and then 8 μL of the mixture was deposited on SWNH-coated GCE. After coupling, BSA blocking, and cell capturing steps, the obtained cell-captured electrodes were subjected to impedance measurements.

Examination on the effect of cell concentration. To demonstrate the cell concentration-dependent signal change, the lectin-immobilized electrodes were incubated with 12 μL of K562 cell suspension at certain concentrations from 5×10^5 to 5×10^6 cells mL^{-1} for 1 h at 25 °C. After careful rinsing with 0.01 M pH 7.4 PBS, the electrodes were subjected to impedance measurements.

Comparison of the cell binding capacity. 0.5 mg mL^{-1} oxidized SWNHs, SWNTs and MWNTs were dropped on glass slides, respectively, and dried in a desiccator. The subsequent WGA immobilization, BSA blocking and cell incubation steps were the same as described above. The obtained slides were subjected to optical microscopic observation.

Flow cytometry analysis of glycan expression pattern on K562 cell surface. K562 cells were collected by centrifugation at 1000 rpm for 6 min at room temperature. After the cells were washed with cold PBS, they were resuspended in PBS at a concentration of 1×10^7 cells mL^{-1}. 50 μL cell suspension was then added to the mixture of 445 μL PBS and 5 μL 2 mg mL^{-1} fluorescein-labeled lectin. For fluorescein-

labeled Con A, 1 mM Ca^{2+} and Mn^{2+} were added to the recognition solution. After incubation for 30 min, the cells were collected by centrifugation at 1000 rpm for 6 min, washed with PBS, resuspended in 500 μL PBS, and assayed by flow cytometry. Unlabeled K562 cells were used as the negative control for estimation of autofluorescence, and relative cell-associated fluorescent intensity was obtained by subtraction of autofluorescence. For comparison purpose, the obtained relative cell-associated fluorescent intensity was standardized with the molar ratio of fluorescein/protein.

Benzidine staining experiment. 50 μL cell suspension at 1×10^6 cells mL^{-1} was mixed with 10 mL benzidine reagent containing 0.6% H_2O_2, 0.5 M acetic acid and 0.2% benzidine dihydrochloride. The percentage of benzidine staining-positive cells (blue cells) was determined by light microscopic examination of 100 cells per sample.[2]

Apparatus and characterization. The morphologies of SWNHs coated on GCE (3 mm diameter) and lectin-functionalized SWNHs/GCE were observed under a Hitachi S-4800 scanning electron microscopy (SEM, Japan) and a JEOL JEM-2100 transmission electron microscopy (TEM, Japan). Optical microscopic images were obtained by Nikon TE2000-U inverted fluorescence microscope (Japan). Infrared spectra were recorded on a Nicolet 400 Fourier transform infrared spectrometer (Madison, WI). Flow cytometry was carried out on a FACS Calibur flow cytometer (Becton Dickinson, USA). Electrochemical impedance spectroscopic measurements were performed on a PGSTAT30/FRA2 system (Autolab, Netherlands) in 10 mM pH 7.4 PBS containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 0.1 M KCl using a conventional three-electrode system with modified GCE as working, platinum wire as auxiliary, and saturated calomel electrode as reference electrodes. Typical impedance spectrum includes a semicircle portion and a linear line portion, which correspond to the electron transfer process and diffusion process, respectively. The diameter of the semicircle represents the electron-transfer resistance at the electrode surface. The impedance spectra were recorded within the frequency range of 5×10^{-2} – 10^5 Hz. The amplitude of the applied sine wave potential was 5 mV. All the experiments were performed at 25 °C. All R_{et} value was obtained from three independent experiments, and error bars represent the S.D. of the mean.

Infrared characterization of coupling of lectin to SWNHs[3]

After Con A coupling, two new absorption bands corresponding to amide band I (C=O stretching) and II (N-H bending) appeared at 1640 and 1525 cm^{-1}, respectively, and the peaks at 1723 and 1581 cm^{-1} attenuated.

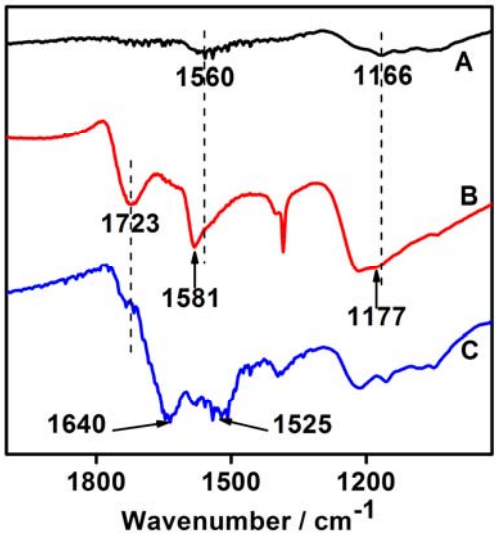

Fig. S1. Infrared spectra of (A) SWNHs, (B) oxidized SWNHs and (C) Con A-SWNHs conjugates.

SEM and TEM characterization of lectin coupling to SWNHs.

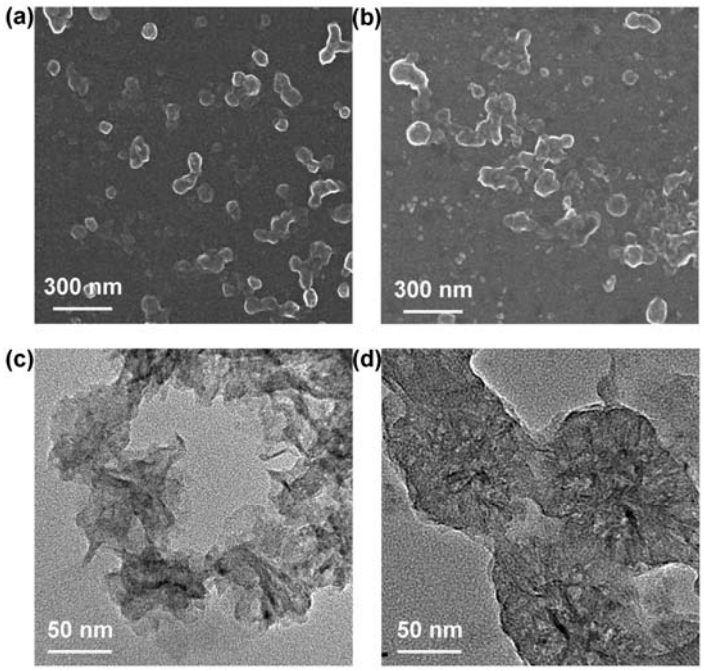

Fig. S2 SEM images of (a) SWNHs/GCE and (b) Con A-SWNHs/GCE, and TEM images of (c) SWNHs and (d) Con A-SWNHs conjugates.

Comparison of cell capture capacity of lectin-functionalized carbon nanomaterials

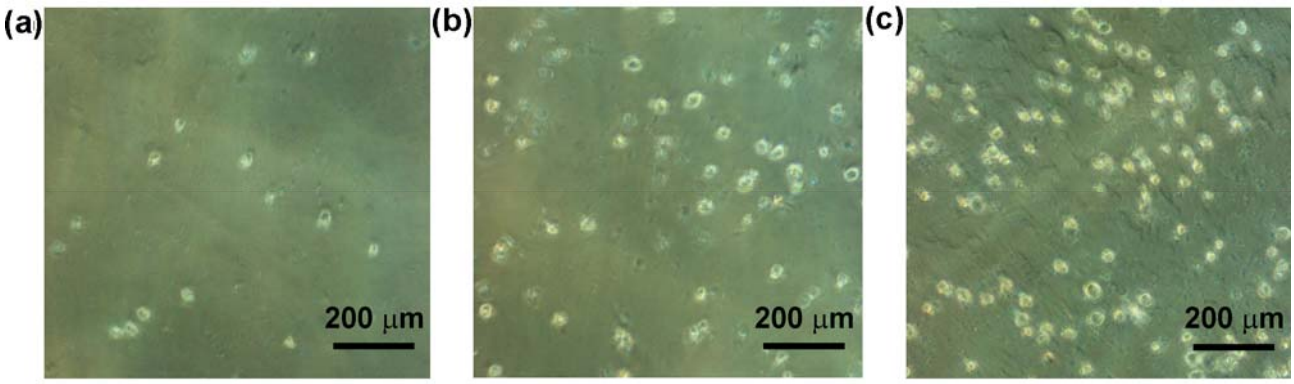

Fig. S3 Optical microscopic images of K562 cells captured on WGA-functionalized (a) single-walled carbon nanotubes, (b) multi-walled carbon nanotubes and (c) SWNHs on glass slides. The nanomaterial interface was fabricated by the same procedure as described in the Experimental section.

Table S1 Carbohydrate-binding specificities of the lectins used in this work[4]

Lectins	Origin	Binding specificity[a]	Optimized concentration for immobilization (mg mL^{-1})
Con A	*Canavalia ensiformis*	terminal α-Man, Manα3(Manα6)Man	2.0
DBA	*Dolichos biflorus*	GalNAcα-Ser/Thr (Tn), GalNAcα1-3GalNAc	0.5
PNA	*Arachis hypogaea*	Galβ1-3GalNAcα-Ser/Thr, Galβ1-3GalNAcβ1-4Galβ	1.0
WGA	*Triticum unlgari*	$(GlcNAc)_n$, multivalent Sia	1.5

[a] Man, mannose; GalNAc, *N*-acetylgalactosamine; Gal, galactose; Sia, sialic acid; GlcNAc, *N*-acetylglucosamine; Ser, serine; Thr, threonine.

Flow cytometric analysis of the glycan expression pattern on K562 cell surface

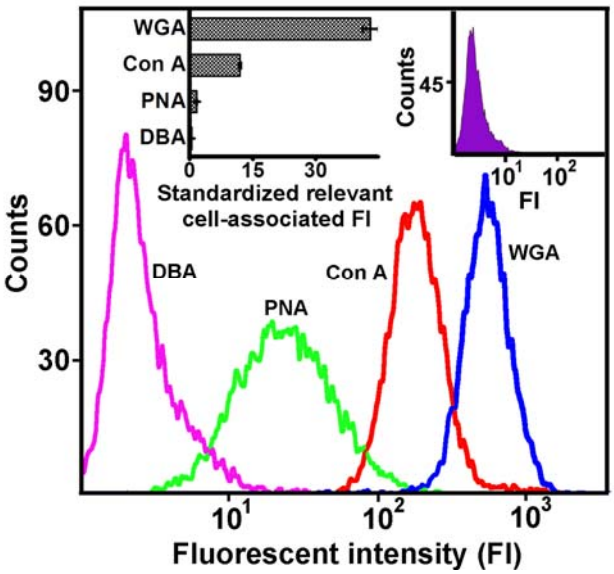

Fig. S4 Flow cytometric analysis of lectin-binding sites on K562 cells with fluorescein-labeled DBA, PNA, Con A, and WGA at room temperature. Inset: standardized relative cell-associated fluorescent intensity of K562 cells after incubation with fluorescein-labeled lectins (*left*), and autofluorescence of unlabeled K562 cells (*right*).

References

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