Effective Cell Capture with Tetrapeptide-Functionalized Carbon Nanotubes and Dual Signal Amplification for Cytosensing and Evaluation of Cell Surface Carbohydrate

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A novel electrochemical cytosensing strategy was designed based on the specific recognition of integrin receptors on a cell surface to arginine–glycine–aspartic acid–serine (RGDS)-functionalized single-walled carbon nanotubes (SWNTs). The covalent conjugation of the RGDS tetrapeptide to SWNTs was proved with Raman and FT-IR spectra. The conjugated RGDS showed a predominant ability to capture cells on the electrode surface by the specific combination of RGD domains with integrin receptors. With the use of BGC-823 human gastric carcinoma cells (BGC cells) as a model, the cell surface mannosyl groups could specifically bind with horseradish peroxidase labeled concanavalin A, producing an electrochemical cytosensor. On the basis of the dual signal amplification of SWNTs and enzymatic catalysis, the cytosensor could respond down to 620 cells mL⁻¹ of BGC cells with a linear calibration range from 1.0 × 10³ to 1.0 × 10⁷ cells mL⁻¹, showing very high sensitivity. The dual signal amplification could be further used to evaluate the mannosyl groups on the cell surface, and the mannosyl groups on a single living intact BGC cell were detected to correspond to 5.3 × 10⁷ molecules of mannose. This strategy presents a promising platform for highly sensitive cytosensing and convenient evaluation of surface carbohydrates on living cells.

Carbohydrates are the third information-carrying biomolecules of growing interest next to the genome and proteome.¹ Accumulating evidence has indicated that carbohydrates have roles in cell growth and development, cell signal transduction, immune recognition/response, and cell–cell communication.² We Abnormal glycosylation has been associated with many diseases such as rheumatoid arthritis, viral and bacterial infections, and cancers.³,⁴ Especially, tumor-associated alterations of cell surface carbohydrates play a crucial role in escaping from the immune response and metastasis of carcinoma cells.⁵,⁶ Thus, the characterization of carbohydrate expression on the cell surface has become an important subject for understanding their role in disease development and providing diagnostic tools to guide treatment.⁷ However, the elucidation of carbohydrate functions in living organisms has still been far behind the genome and proteome due to the lack of toolboxes for the reliable identification of carbohydrate expression on cell surfaces.

Several methods including mass spectrometry, high-performance liquid chromatography, and nuclear magnetic resonance have been used for carbohydrates detection.⁸‐¹² Although these techniques can reveal molecular details, they require expensive equipment, advanced technical expertise, and a great need of time for destructive sample preparation¹³ and are difficult to use to evaluate the intact cell surface glycans. Recently, lectin microarrays have been developed as a glycan profiling tool to recognize cell surface carbohydrates.¹⁴ The detection process involves the fluorescent labeling of cells. But the issues of active-site accessibility and lectin denaturation in the surface immobilization format have been far behind the genome and proteome due to the lack of toolboxes for the reliable identification of carbohydrate expression on cell surfaces.

¹–² Sharon, N.; Lis, H. Glycobiology 2004, 14, 53R–62R.
transducer with nanobiotechnology using peptide-functionalized single-walled carbon nanotubes (SWNTs) to develop a novel methodology for conveniently and sensitively quantifying the carbohydrate expression status on living cell surface.

Many peptides containing arginine–glycine–aspartic acid amino acids (RGD) have been applied in biomodification of synthetic materials,19 cancer-cell-specific targeting,20 construction of tumor cell microchips,21 and detecting histamine.22 The RGD tetrapeptide was a recognized minimal adhesion domain of most extracellular matrix proteins (ECM) to bind selectively with integrin receptors in all the cellular adhesion phenomena.23–25 In this work, an arginine–glycine–aspartic acid–serine (RGDS) tetrapeptide was employed to functionalize SWNTs, and the resulting novel RGDS–SWNTs nanocomposite was then used as nanoscale anchorages to effectively capture cells on an electrode surface via the specific binding between cell surface integrins and RGD domains. Due to the large surface area and excellent electrical conductivity of SWNTs,26,27 the biocompatible integrins and RGD domains. Due to the large surface area and excellent electrical conductivity of SWNTs,26,27 the biocompatible nanocomposite not only presented abundant RGD domains for cell capture but also played a role of signal amplification in following electrochemical detection.28

The mannosyl groups on the captured cell surface could be specifically recognized by horseradish peroxidase labeled concanavalin A (HRP–ConA).29 Therefore, by coupling with another signal amplification based on an enzymatic catalytic reaction of HRP toward the oxidation of o-phenylenediamine (o-PD) by H2O2,30 a highly sensitive method was developed for monitoring the cell capture (Scheme 1), leading to a highly sensitive cytosensor for quantitation of cell numbers. Furthermore, a method for evaluating the expression of mannosyl groups on a living cell surface was also proposed. The cell capture showed extremely high efficiency. With the use of BGC-823 human gastric carcinoma (BGC) cells as a model, the cytosensor could capture the cells from the suspension containing down to 6 BGC cells. This work provided an important protocol for cytosensing and quantitative evaluation of cell surface carbohydrate sites. The proposed methods possess potential applications in clinical diagnosis and therapy of human cancers and the elucidation of carbohydrate functions in living organisms.

**EXPERIMENTAL SECTION**

**Materials and Reagents.** HRP–ConA and bovine serum albumin (BSA) were obtained from Sigma (U.S.A.). RGDS tetrapeptide was obtained from Shanghai Sangon Biotechnical Engineering Technology and Services Co., Ltd. (China). SWNTs (<2 nm diameter) were purchased from Shenzhen Nanotech Port Ltd. Co. (China). N-Hydroxysuccinimide (NHS) and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Aldrich (U.S.A.). Mannose with analytical grade was from Sinopharm Chemical Reagent Co. (China). All other reagents were of analytical grade. The 0.01 M pH 7.4 phosphate-buffered saline (PBS) contained 136.7 mM NaCl, 2.7 mM KCl, 87 mM Na2HPO4, and 14 mM KH2PO4. All aqueous solutions were prepared using ≥18 MΩ ultrapure water (Milli-Q, Millipore).

SWNTs of a certain mass were dispersed in 30% HNO3 and then refluxed for 24 h at 140 °C to obtain carboxyl group-functionalized SWNTs. The resulting suspension was centrifuged, and the sediment was washed with deionized water until the pH reached 7.0. Then, the oxidized SWNTs were dispersed in deionized water to a concentration of 1 mg mL⁻¹.

**Cell Line and Culture.** The BGC cell line was kindly provided by Affiliated Zhongda Hospital of Southeast University, Nanjing, China. BGC 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% CO2. At the


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**Scheme 1. Preparation and Enzyme-Catalyzed Analysis of the Designed Cytosensor**

logarithmic growth phase, the cells were trypsinized and washed twice with sterile 0.01 M pH 7.4 PBS by centrifugation at 1000 g for 10 min. The sediment was then resuspended in 0.01 M pH 7.4 PBS containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ to obtain a homogeneous cell suspension. The presence of divalent cations was to ensure the effective binding between cell surface integrins and RGDS on the cytosensor. The cell number was determined using a Petroff–Hausser cell counter (U.S.A.).

Preparation of the Cytosensor and Cell Capture. A glassy carbon electrode (GCE, 3 mm diameter) was polished to a mirror using 0.3 and 0.05 µm alumina slurry (Buehler) followed by rinsing thoroughly with deionized water. After successive sonication in 1:1 nitric acid, acetone, and deionized water, the electrode was thoroughly washed with deionized water. After successive sonication in using 0.3 and 0.05 µm alumina slurry (Buehler) followed by rinsing thoroughly with deionized water, 10 µL of 1 mg mL$^{-1}$ carboxylic group-functionalized SWNTs solution was dropped on the pretreated GCE and dried in a desiccator. The SWNTs-coated GCE was then immersed in a solution containing 2 mM EDC and 5 mM NHS for 1 h. After the activated SWNTs/GCE was thoroughly rinsed with deionized water, 10 µL of 1 mg mL$^{-1}$ RGDS was immediately dropped on its surface and then incubated for 2 h to yield an RGDS–SWNTs-modified GCE. Following a rinse with 0.01 M pH 7.4 PBS, 10 µL of BGC cell suspension at a certain concentration was dropped on the RGDS–SWNTs/GCE surface and incubated at 37 °C for 2 h (incubation step I). After carefully rinsing with 0.01 M pH 7.4 PBS to remove the noncaptured cells, the obtained BGC/RGDS–SWNTs/GCE was used for subsequent assay.

As control a glutathione-functionalized SWNTs/GCE (GSH–SWNTs/GCE) was prepared with a procedure similar to that for the RGDS–SWNTs/GCE. The RGDS-modified GCE (RGDS/GCE) was prepared by covalently binding RGDS to an oxidized GCE that was first activated with EDC and NHS.

Enzyme-Amplified Electrochemical Analysis. All electrochemical measurements were performed on a CHI 660 electrochemical analyzer (CHI Co., TX) with a conventional three-electrode system comprising of platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference, and different electrode system comprised of platinum wire as auxiliary electrode, chemical analyzer (CHI Co., TX) with a conventional three-electrode system. The cyclic voltammetric behavior was examined with a LEO-1550 scanning electron microscope (SEM, LEO, Germany).

RESULTS AND DISCUSSION

Cyclic Voltammetric Behavior. The cyclic voltammograms of GCE, SWNTs/GCE, RGDS–SWNTs/GCE, BGC/RGDS–SWNTs/GCE, and HRP–ConA/BGC/RGDS–SWNTs/GCE in 0.1 M pH 7.0 PBS did not show any detectable signal (Figure 1). The presence of SWNTs on the GCE surface resulted in an excessive increase in the background current due to the increased surface area. Upon addition of o-PD and H$_2$O$_2$ into PBS the cyclic voltammogram of HRP–ConA/BGC/RGDS–SWNTs/GCE exhibited a pair of stable and well-defined redox peaks at −0.510 and −0.585 V (Figure 1, short dash line), which corresponded to the redox of 2,2′-diaminoazobenzene, the enzymatic product. The electrochemical signal was directly related to the coverage of the immobilized HRP, which depended on both the expression of mannosyl groups on the cell surface and the amount of cells captured on the cytosensor surface. Thus, quantifying the cells and evaluating the expression of mannosyl groups on a single living cell surface could be carried out by monitoring the enzyme-amplified electrochemical signal.

Characterization of RGDS–SWNTs/GCE. The covalent modification of SWNTs with RGDS tetrapeptide was confirmed by Raman spectra (Figure 2A). Both spectra of oxidized SWNTs (curve a) and RGDS–SWNTs (curve b) showed the characteristic peaks of SWNTs at ~1580 cm$^{-1}$ (tangential modes) and ~1354 cm$^{-1}$ (disorder mode). The relatively increased intensity of the disorder mode, which was diagnostic of disruptions in the hexagonal framework of the SWNTs, provided direct evidence of the covalent modification of SWNTs. The positions of the radial breathing mode peak before and after covalent modification by RGDS occurred at 173 and 168 cm$^{-1}$, respectively. The red-shift indicated the increase in the size of SWNTs due to the covalent conjugation of the RGDS tetrapeptide to the SWNTs.

The FT-IR spectrum of oxidized SWNTs displayed two peaks at 1718 and 1586 cm\(^{-1}\) (Figure 2B, curve a), which could be assigned to the carbonyl stretch mode of COOH and COO\(^{-}\). After covalent conjugation of the RGDS tetrapeptide to the SWNTs, the vibration of amide I and amide II of the RGDS tetrapeptide could be observed at 1645 and 1533 cm\(^{-1}\) (Figure 2B, curve b), whereas the peaks assigned to the carbonyl stretch mode decreased greatly, indicating the effective reaction of the carboxylic acid groups of SWNTs with the amino groups of the RGDS.

The SEM image of the RGDS–SWNTs-modified GCE showed a uniformly distributed SWNTs film with large surface area (Figure 3). The apparent diameter larger than the real diameter resulted from the aggregation of SWNTs in bundles. This resulting film not only offered a biocompatible surface but also acted as an analogue of extracellular matrix to induce cell adhesion effectively by the specific interaction between the abundant RGD domains and the integrin receptors on the cellular membrane surface.

**Capture of Cancer Cells.** Most cancer cells can express abundant integrin family on their surfaces. Thus, BGC-823 human gastric carcinoma cells were chosen as a model to construct a cytosensor. To demonstrate the ability of the RGDS–SWNTs/GCE to capture cancer cells, after incubation step 1 with BGC cells an enzyme-catalyzed electrochemical analysis was carried out by further incubating the cancer cells captured cytosensor with HRP–ConA solution and then detecting the electrochemical response in the presence of H\(_2\)O\(_2\) and o-PD. After incubation with 10\(^3\) or 10\(^6\) cells mL\(^{-1}\) of BGC cell suspension the RGDS–SWNTs/GCE showed much higher DPV peak current than GCE, SWNTs/GCE, and GSH–SWNTs/GCE (Figure 4). The small responses at these control electrodes resulted from the nonenzymatic reduction of H\(_2\)O\(_2\) or nonspecific adsorption of a small amount of BGC cells, which could bind a small amount of HRP–ConA. The potential effects of the improved dispersion and distribution of SWNTs caused by the covalent functionalization of RGDS on the cell physical adhesion could be ruled out by comparing the amperometric responses at SWNTs/GCE and GSH–SWNTs/GCE, which showed similar peak currents and were much lower than that at BGC/RGDS–SWNTs/GCE, indicating the low possibility of the physical adsorption of HRP–ConA onto the SWNTs due to the blocking of BSA. High cell concentration (10\(^6\) cells mL\(^{-1}\)) led to more cells being nonspecifically adsorbed on the electrode surface than at 10\(^3\) cells mL\(^{-1}\), thus showing higher response. At the concentration of 10\(^3\) cells mL\(^{-1}\), the peak current at the RGDS–SWNTs/GCE was about 4 times that at GCE and GSH–SWNTs/GCE, showing significant difference. This indicated that even at a low cell concentration the RGDS–SWNTs/GCE could effectively capture cancer cells due to specific binding between the cellular membrane integrin receptors and RGD domains on RGDS–SWNTs/GCE. Although RGDS/GCE could also specifically capture the cancer cells, the much lower peak current indicated that the presence of SWNTs obviously increased the RGD domain density on the cytosensor surface, thus amplifying the detection signal.

**Optimization of Detection Conditions.** The incubation time was an important parameter for both capturing cells on the
The DPV peak current absolutely relied on the amount of HRP–ConA conjugated on the cytosensor, which was directly related to both the number of captured BGC cells and the quantity of mannosyl groups on each cell surface. In order to detect the quantity of these mannosyl groups, a method was designed by...
partly blocking the mannose-specific binding sites of HRP–ConA with mannose solutions at different concentrations for 1 h. The resulting HRP–ConA solutions were then used to incubate the cytosensors. The mannose-blocked HRP–ConA could not conjugate the mannosyl groups of the captured BGC cells. Thus, at the same BGC/RGDS–SWNTs/GCE the obtained peak currents were lower than those obtained with nonblocked HRP–ConA solution. The latter could be calculated from eq 1 and the number of BGC cells captured on the cytosensor.

The decrease of peak current ($\Delta i_p$) was related to the mannosyl sites un conjugated by HRP–ConA on the cell surface, which could be expressed as the amount of mannose ($m$) used to block HRP–ConA. The plot of $\Delta i_p$ versus the amount of mannose showed a linear relation in the range of 0.2–1.0 pmol ($R = 0.988$, $n = 5$) (Figure 7B):

$$\Delta i_p (\mu A) = -0.97m \text{ (pmol)} + 0.08$$ (2)

On the other hand, the decrease of peak current could be converted into the number of captured BGC cells according to eq 1. Thus, the mannosyl sites on each captured BGC cell could be calculated from eqs 1 and 2 to correspond to $5.3 \times 10^7$ molecules of mannose.

**CONCLUSIONS**

A novel nanocomposite of RGDS tetrapeptide covalently functionalized SWNTs has been designed to construct an electrochemical cytosensor. The RGD domains in the nanocomposite can specifically recognize the integrin receptors on cellular membrane, leading to effective capture of living cells. By combining the specific binding of mannosyl groups on cellular membrane to HRP–ConA with dual signal amplification of SWNTs and an enzymatic reaction, a highly sensitive method to quantify BGC cells is thus proposed. A novel strategy for electrochemically evaluating the surface carbohydrate expression of living intact cells has been for the first time developed with the designed cytosensor. This strategy integrates the advantages of nanotechnology, bioconjugate techniques, enzyme amplification, and electrochemical detection and provides a significant tool for monitoring the change of surface carbohydrate expression occurring in the process of cancer disease progression and other biological processes. We anticipate that this method can be expanded readily for profiling cell surface carbohydrates and miniaturized for high-throughput detection of glycomic structures to meet the challenges in unraveling the complex mechanisms underlying biological processes related to carbohydrates.

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