Pseudo 3D Single-Walled Carbon Nanotube Film for BSA-Free Protein Chips

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Rapid progress in genomics and proteomics has inspired many scientists to develop techniques for efficient screening of specific biological interactions, such as DNA hybridization, antigen–antibody recognition, and potential drug molecule–protein interactions.[1] Microarray technology combined with fluorescence detection is one of the most popular tools for the high-throughput analysis of biological events and has been the cornerstone of many scientific advances.[2–7] In contrast to the success of DNA microarray chip applications,[8] protein chips have suffered from several critical issues, including loss of molecular conformation and, therefore, protein activity after immobilization on substrates. Another issue of concern is orientation control of proteins during immobilization, which is believed to have a significant influence on their biological activities. A great many improvements have been made by introducing site-specific linker molecules to the terminal groups of proteins, which preferentially adsorb onto a chemically modified substrate while the active sites of the proteins are directed towards the incoming targets.[4c, 9–10] The suppression of nonspecific binding (NSB) during the assay is also one of the most important issues.[4e, 11] Among the possible approaches to prohibiting NSB, the addition of bovine serum albumin (BSA) has been most popularly used. Conventionally, BSA is introduced after the immobilization of probe proteins in order to fill the unreacted areas of the substrate, which are potential sites for the NSB of target proteins. However, this space-filling concept is difficult to apply when the size of probe protein (or peptide) is very small because BSA treatment will significantly shield the immobilized small proteins (or peptides) and result in inefficient specific binding with the target proteins. In order to overcome this problem, Schreiber et al. have suggested an approach in which small proteins are immobilized on a preformed monolayer of BSA activated with linker molecules, such as *N*-hydroxysuccinimide (NHS) groups.[6] In this case, however, another technically important chemical treatment step is required to quench the unreacted NHS-activated BSA. BSA treatments also frequently lead to ambiguous results, mostly arising from the size and intrinsic NSB characteristics of BSA itself. Therefore, the development of a BSA-free system is a necessary and a challenging subject for the realization of highly efficient protein chips.

Herein, we report the successful demonstration of a BSA-free protein chip with a 1,1′-carbonyldiimidazole-activated Tween20-functionalized (CDI–Tween20) high-yield single-walled carbon nanotube (SWNT) film: CDI–Tween20/SWCN. Tween20 has a similar chemical structure to poly(ethylene glycol) (PEG), except for a long alkyl chain moiety. Tween20 and its derivatized forms have been reported to be good "interfacing molecules" between carbon nanotubes and proteins in terms of efficiently preventing NSB and simultaneously facilitating the immobilization of proteins onto carbon nanotubes.[12–13] Our strategy is to passivate CDI–Tween20 on both SWNT and bare hydroxy-terminated SiO_2/Si surfaces such that no further treatment is required for NSB prevention. After the passivation, SWNTs are ready to accommodate probe proteins by covalent coupling through the CDI groups. At the same time, the reaction of CDI with hydroxyl groups of the SiO_2/Si surface covalently attaches Tween20 to the surfaces where no SWNTs are present, thus effectively preventing NSB in all areas. Furthermore, SWNT substrates might help preserve protein conformation. Due to its high aspect ratio and pseudo 3D network structure, SWNT film could significantly reduce the contact area for protein immobilization, leading to minimal shape deformation of protein molecules.

A schematic representation of SWNT film-based microarray protein chip preparation is shown in Figure 1a. A successful growth of the high yield of pure SWNTs was confirmed by a simple resistance measurement (5–40 kΩ) as well as by atomic force microscope (AFM, Digital Instruments, Nanoscope III, Figure 1b).

As a new platform for protein chip application, it was necessary to confirm that the formation of protein microspots on the CDI–Tween20/SWNT substrate was reproducible and homogeneous. The spotting conditions were optimized from several test spottings (4×4 array) with Cy3–IgG (20 μg mL^{-1}) with respect to several variables such as concentration, incubation time, and washing conditions. Successful immobilization and reproducible formation of protein spots were confirmed by observing fluorescent dots with a laser scanner after 3 hours' incubation (Figure 1c). Protein immobilization on the CDI–Tween20/SWNT surface was also investigated by AFM, which clearly showed IgG molecules (60 μg mL^{-1}) immobilized on the SWNT sidewalls (Figure 1d). Note that SWNTs grown in low yield were used for visual clarity.

The optimal concentration for the immobilization of probe protein was determined from the fluorescence versus concentration curve (Figure 2 and Figure S1 in the Supporting Information). The change in fluorescence intensity was monitored from the specific bindings of Cy3–IgG (20 μg mL^{-1}) to the SpA spotted at various concentrations. Figure 2 shows that saturation occurs at around 250 μg mL^{-1}. This concentration was used for the probe protein immobilizations in future experiments.

For the study of specific and nonspecific interactions on BSA-free SWNT film substrates, biotin–BSA + Cy5–SA and SpA

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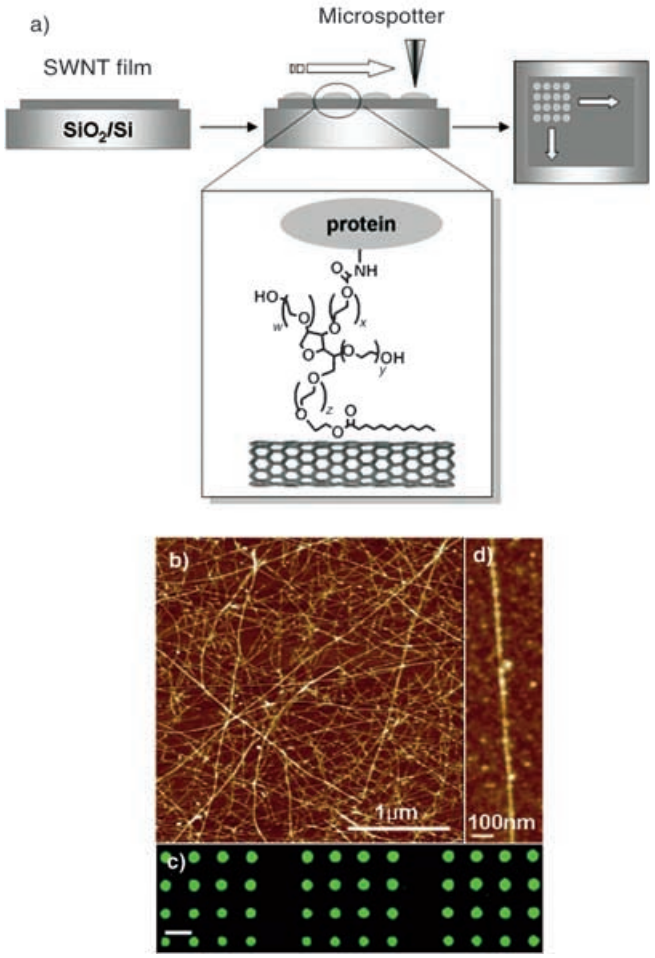

Figure 1. Immobilization of proteins on CDI–Tween20/SWNT substrates. a) A schematic view of protein microarray formation on a CDI–Tween20/SWNT substrate. b) AFM image of the high yield of SWNT film grown by the CVD method. c) Fluorescence image of Cy3–IgG spotted on a CDI–Tween20/SWNT substrate. Three 4×4 spot arrays are shown for the confirmation of reliable spot formation; scale bar = 500 μm. d) AFM image of IgG immobilized on a single SWNT.

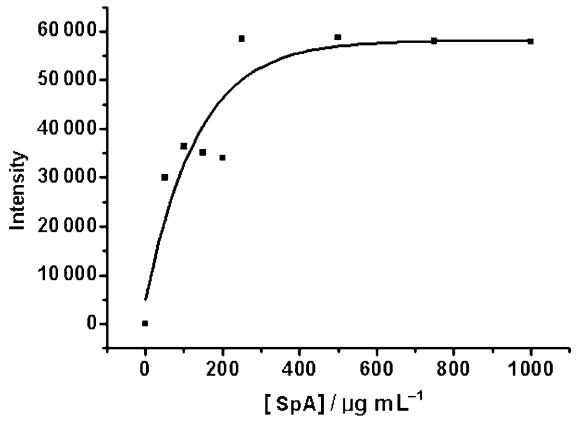

Figure 2. Fluorescence intensity-concentration curve obtained from specific binding of Cy3–IgG (20 μg mL^{-1}) and SpA immobilized at various concentrations. This graph indicates that SpA immobilization reaches saturation at approximately 250 μg mL^{-1}.

+ Cy3–IgG pairs were investigated. Biotin–BSA was first spotted as a 4×4 array onto the top region of the CDI–Tween20/SWNT substrate, followed by SpA spotting onto the bottom region. The sample was then incubated for 3 h before being transferred to react with Cy5–SA (20 μg mL^{-1}) for 1 h. As shown in Figure 3a, only biotin–BSA spots (top region) show

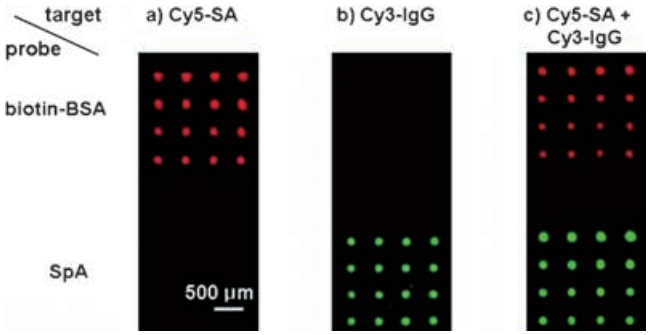

Figure 3. Fluorescence images after reaction of substrates containing biotin–BSA (top region) and SpA (bottom region) with Cy5–SA, Cy3–IgG, and a mixed solution of Cy5–SA and Cy3–IgG.

red fluorescence owing to their specific binding to Cy5–SA, while the SpA spots remain dark (bottom). The opposite is observed when the substrate is treated with Cy3–IgG solution (20 μg mL^{-1}). Only SpA spots show green fluorescence, which originates from specific binding to Cy3–IgG (Figure 3b, bottom) while the biotin–BSA spots remain dark (top). The results show that the specific/nonspecific binding-discrimination efficiencies are very high. For instance, the fluorescence intensity count for the specific binding of SpA and Cy3–IgG is about 60 000, but lower than about 100 for the nonspecific binding of biotin–BSA and Cy3–IgG.[14] Note that there is no significant improvement of specific/nonspecific binding discrimination even when these experiments are performed in the presence of BSA (2% in PBS).

Cross reactivity was also investigated by immersing a substrate containing the same arrays of probe proteins, that is, biotin–BSA at the top region and SpA at the bottom region, into a solution of Cy5–SA and Cy3–IgG (1:1) at final concentrations of 20 μg mL^{-1} each. As shown in Figure 3c, both the red spots from Cy5–SA/biotin–BSA and the green spots from Cy3–IgG/SpA specific bindings are well resolved. All these results suggest that the immobilized proteins on CDI–Tween20/SWNT substrate specifically bind to their counterparts.

To test the limits of our BSA-free SWNT film, specific recognition of small biomolecules was also examined. For this demonstration, we used 3×FLAG/Cy3–antiFLAG and biotin/Cy5–SA pairs. In a similar manner to the previous experiments, 3×FLAG and biotin were spotted onto the top and bottom regions of the substrate, respectively. After treatment with Cy5–SA (Figure 4a), Cy3–antiFLAG (Figure 4b), and a mixture of the two (20 μg mL^{-1} each; Figure 4c), it can be clearly seen that the target proteins recognize their probe biomolecules selectively.

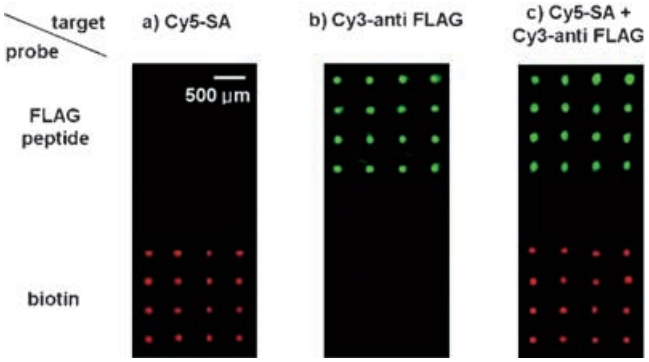

Figure 4. Fluorescence images after reaction of substrates containing 3×FLAG (top region) and biotin (bottom region) with Cy5–SA, Cy3-antiFLAG, and a mixed solution of Cy5–SA and Cy3-antiFLAG.

The probe proteins and small biomolecules immobilized on the CDI–Tween20/SWNT substrate show high specificity for the target proteins with negligible NSB. These results suggest that the SWNT/CDI–Tween20 combination is highly effective in coupling proteins as well as in NSB prevention in the absence of BSA. In order to investigate the critical effects of SWNT and CDI–Tween20 on the successful fabrication of SWNT film-based protein chips, we performed several control experiments. The results show that 1) a high yield of SWNT is critical for the reliable formation of protein spots. No well-defined fluorescent spots were observed on a bare SiO_2/Si substrate or on a low-density SWNTs functionalized with CDI–Tween20 (200–500 kΩ, Figure S2 in the Supporting Information) and 2) efficient functionalization of CDI–Tween20 on SWNTs plays another important role because high-yield SWNT films without CDI–Tween20 show large increases in background signals with high cross reactivity from NSB (Figure S3 in the Supporting Information).

Our BSA-free SWNT-based protein chip was also directly compared to a state-of-the-art protein chip substrate, SuperAmine (ArrayIt). When small peptide FLAG molecules were immobilized on SuperAmine slides then treated with Cy3–anti-FLAG in the absence of BSA, the spot/background fluorescence intensity ratio was substantially decreased, more than twice that of when BSA was applied (Figure S4 in the Supporting Information). While BSA indeed provides efficient NSB prohibition, it also diminishes fluorescent spot sizes (>75% in area); this is one of the previously discussed drawbacks of using BSA, the shielding of small biomolecules.

We propose the following mechanism for the successful suppression of NSB achieved by simple treatment with CDI–Tween20. CDI–Tween20 on SWNTs readily reacts with incoming proteins by covalent coupling. Any unreacted CDI–Tween20 molecules adsorbed on SWNTs are simultaneously quenched by water during the 3 h incubation period under 80% humidity. Hence, the NSB of proteins on SWNTs is sufficiently prohibited (Figure 5). Although the portion is very small, there are still bare SiO_2/Si regions prone to NSB, since SWNTs do not fully cover the substrate. As shown in Figure 5, the bare SiO_2/Si surface is also protected by covalent coupling of the imidazolyl carbamate group of CDI–Tween20 to the hydroxyl groups on the SiO_2/Si surface during the CDI–Tween20 coating period.

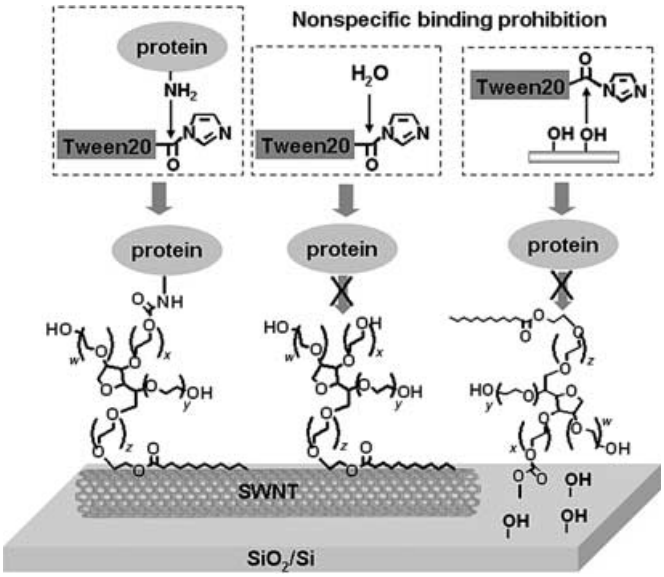

Figure 5. Functionalization of SWNT on SiO_2/Si substrates with CDI–Tween20. 1) Covalent coupling of probe proteins to SWNTs (left). 2) Nonspecific binding (NSB) prohibition on SWNT (middle). 3) NSB prohibition on bare SiO_2/Si containing hydroxyl groups (right).

Therefore, it finally forms a NSB-repelling Tween20 layer. Note that the hydrophobic chain of the CDI–Tween20 molecule is much smaller than the hydrophilic branches.

In summary, high-yield CVD-grown SWNT films have been successfully demonstrated as efficient substrates for microarray protein chip applications. A simple one-step treatment with CDI–Tween20 on SWNT films allows both the immobilization of probe proteins and suppression of NSB very efficiently without the need for additional BSA. A high specific/nonspecific protein-binding discrimination ratio has been obtained not only from the conventional probe–target protein interactions but also from the small probe biomolecule–target protein interactions. With its simple preparation and BSA-free prevention of NSB, the CDI–Tween20/SWNT protein chips could provide opportunities for extensive and detailed studies of diverse biomolecular interactions.

Experimental Section

High-yield SWNT growth on SiO_2/Si substrate: The growth of SWNTs on the SiO_2/Si substrate containing iron oxide nanoparticles was performed by chemical vapor deposition (CVD) at 950 °C for 5 min under a controlled gas flow rate of 1000/500/20 sccm of CH_4, H_2, and C_2H_4, respectively. For the preparation of iron oxide catalyst nanoparticles, Fe^{III} hydroxylamine nanoclusters were spontaneously formed on a SiO_2/Si substrate by immersion in deionized water (10 mL) to which $FeCl_3 \cdot 6H_2O$ (10 μL, 10 mM) and $NH_2OH \cdot HCl$ (100 μL, 40 mM) were sequentially added.[15] This reaction was performed at room temperature for 3 min. The iron oxide nanoparticles were then prepared by calcining Fe^{III} hydroxylamine nanoclusters at 800 °C in air.

Preparation of CDI–Tween20/SWNT substrate: 1,1′-Carbonyldiimidazole-activated Tween20 (CDI–Tween20) was prepared by the reaction of Tween20 (2.25 mL; Bio Basic Inc., USA) and 1,1′-carbon-

yldiimidazole (1.09 g; Aldrich) in DMSO (6.08 mL) at 40 °C for 2 h.[16] In order to remove excess CDI and DMSO, the product solution was extracted with diethyl ether (Aldrich) to result in yellow precipitate. Diethyl ether and residual DMSO were removed by evaporation in vacuo overnight. CDI–Tween20 was then functionalized on the side walls of SWNTs (CDI–Tween20/SWNT) through van der Waals interaction by simply soaking a SWNT film substrate into the diluted CDI–Tween20 solution (1 wt% in water) for 1 h.

Preparation of biotinylated BSA: Biotinylated BSA (biotin–BSA) was prepared by the reaction of BSA (40 mg; Sigma) with EZ-Link® Sulfo-NHS-LC-Biotin (1 mg; Pierce, USA) in phosphate buffered saline (PBS; 0.5 mL) at 25 °C for 1 h, then dialyzed against *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 20 mM)/NaOH at 4 °C.

Concentration control of proteins: Probe proteins including protein A (SpA, derived from *Staphylococcus aureus*, Zymed®), biotinylated BSA(biotin–BSA), EZ-Link Biotin-LC-PEO-Amine (biotin, Pierce) and 3×FLAG® peptide (N-Met-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C, Sigma) were diluted to 250 $\mu g\,mL^{-1}$ in PBS (10 mM, pH 7.4). Target proteins, Cy3-labeled rabbit anti-mouse immunoglobulin G (Cy3–IgG, Zymed®), Cy5-labeled streptavidin (Cy5–SA, Zymed®), and ANTI-FLAG M2 monoclonal antibody-Cy3 conjugate (Cy3-antiFLAG, Sigma) were diluted to 20 $\mu g\,mL^{-1}$ in PBS buffer (10 mM).

Spotting and detecting proteins: Probe proteins were spotted onto CDI–Tween20/SWNT substrates in a 4×4 spot array by using a protein microarray dispenser (Cartesian). The samples were then incubated for 3 h and washed with PBS buffer (10 mM, pH 7.4) for 1 min, then with deionized water. After being dried under N_2, the substrates were treated with target protein (20 $\mu g\,mL^{-1}$) for 1 h followed by washing with PBS buffer and deionized water for 9 min. Protein spot arrays were characterized by monitoring the fluorescence intensities and the spot shapes with a laser scanner (arrayWoRx®). During the experiments, humidity and temperature were controlled at 80% and 20–25 °C, respectively.

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