

An immunoassay using biotinylated single-walled carbon nanotubes as Raman biomarkers†

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A new immunoassay with biotinylated single-walled carbon nanotubes as persistent, non-photobleaching Raman biomarkers demonstrated excellent sensitivity and specificity.

Organic fluorophores have been widely used to tag antibodies in typical immunological assays including an immuno-fluorescent staining. However, photobleaching of the chromophore limits the life time and long-term stability, resulting in the disability of researchers to investigate specimens repeatedly and over long periods.^{1,2} Nano-scale particles such as quantum dots^{1,3} and carbon nanotubes^{1,4} have been investigated to overcome this hurdle. Quantum dots are approximately 100 times more resistant to photobleaching than organic fluorophores. However, they exhibit a limited life time in aqueous solutions (approximately 7–12 days)⁵ and their signal decreases under prolonged excitation.¹ Single-walled carbon nanotubes (SWNTs) show persistent, non-photobleaching Raman scattering with some of the highest known cross-sections for single molecules^{1,4,6} enabling the detection of the signal from a single carbon nanotube.⁷ Single-stranded DNA (ssDNA)-bound SWNT probes have been employed to tag stem cells¹ and to detect particular sequences of DNA with excellent sensitivity and selectivity.⁴

Here we present an immunological assay in which antibodies are non-covalently labeled with SWNTs *via* biotinylated ssDNA. The high affinity of the biotin-avidin complex, which is known as one of the strongest non-covalent bindings between protein and its ligand, was utilized to combine antimouse IgG with SWNTs. The target antigen is human cytomegalovirus (HCMV)-encoding protein UL44 which is confined in HCMV-infected human fetal lung fibroblast (HFLF). HCMV is one of the common agents of congenital infections and is known to cause fatal opportunistic infection in immunocompromised patients such as AIDS.^{8–10} The first part of the paper is a confirmation of tethering a SWNT-ssDNA-biotin probe to a primary antibody (SCMVM34), which is specific to the target antigen (HCMV-UL44), and the second part describes probing the

amount of the antigen in cell extracts by obtaining the characteristic Raman band of the nanotube probe complex.

Fig. 1 shows a schematic of the assay mechanism. The sequence of oligonucleotide is 5'-d(GT)₁₅-3', and the 3'-end is pre-modified with biotin. The SWNT-ssDNA-biotin probes were prepared utilizing a similar protocol developed by Zheng *et al.*¹¹ (see ESI for experimental details†). Previous investigations revealed that the ssDNA with d(GT)_n, *n* = 10–45, effectively wraps and disperses SWNTs *via* π -stacking.^{11,12} The SWNT-ssDNA-biotin probe was stable for several months without showing any aggregation. Non-covalent binding between biotin-(GT)₁₅ and SWNTs provides individual isolation of nanotubes and conserves their intrinsic electronic/optical nature. It is expected that multiple biomolecules wrap a nanotube since the length of the tube is significantly longer than that of biotin-conjugated ssDNA.

SCMVM34 is a monoclonal antibody and used as a primary antibody. Dot blotting was carried out to prove specific binding between the probe complex (SWNT-ssDNA-biotin) and a primary antibody (SCMVM34). Six 100-fold diluted samples of SCMVM34 were dotted onto a nylon membrane, which has porous structures with a binding capacity of 100 $\mu\text{g}/\text{cm}^2$. Fig. 2 shows a schematic of SCMVM34 dotted on the nylon membrane (2 μl each spot). A blank spot with 2 μl of PBS was also prepared as a control. Phosphate buffered saline (PBS) was loaded instead of SCMVM34. The nylon membrane was treated in skim milk (2 wt%) for 1 hour after dotting to prevent non-specific binding of other molecules. In the next step, biotin-conjugated antimouse IgG (secondary antibody), avidin and SWNT-ssDNA-biotin were incubated with a molar ratio of 2 : 1 : 2 for 1 hour at room temperature. The incubation was carried out under mild shaking conditions. Avidin acts as a coupler and has four identical biotin binding subunits. In a statistical point of view, both biotin-conjugated antimouse IgG and biotin-ssDNA-SWNT are expected to bind equally to the biotin acceptor sites of avidin. Finally, the membrane was washed three times for 15 min each with PBS-T

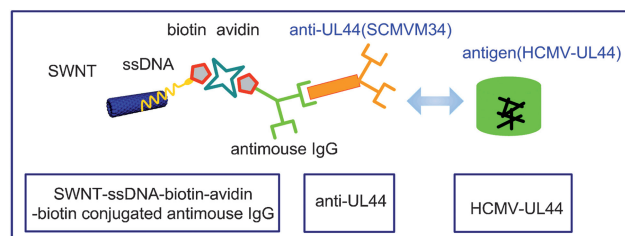


Fig. 1 A step-by-step conjugation procedure of the SWNT probe complex *via* biotin-avidin interaction. A target antigen is UL44 in HCMV-infected HFLF. A primary antibody is SCMVM34 and a secondary antibody is antimouse IgG.

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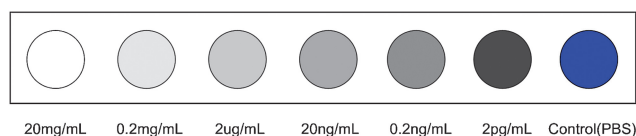


Fig. 2 A schematic of SCMVM34 prehybridized on the nylon membrane. Six 100-fold diluted samples of SCMVM34 were dotted on the nylon membrane and PBS was loaded on the blank spot as a control.

solution. The washing process was carried out in a shaker at room temperature.

Fig. 3a shows Raman spectra from the nylon membrane after dot blotting (Kaiser Optical RXN1, 785 nm excitation). The tangential mode (or G peak) of nanotubes at a Raman shift of 1593 cm^{-1} , caused by stretching along C–C bonds of graphene,¹³ is used to benchmark specific binding of the nanotube probe. The G peak intensity increased as the concentration of SCMVM34 increased from 0.2 ng/mL to 20 mg/mL. The signal was negligible at 2 pg/mL, and the blank spot did not show any signal. The area under the G peak was integrated from 1585 to 1615 cm^{-1} , and the results are shown in Fig. 3b. The detection limit of SCMVM34 was 0.2 ng/mL, which is 10^6 times better than that of the conventional color reaction technique as shown in Fig. 3c. The color reaction was performed using a similar protocol published by Hwang *et al.*¹⁰ Biotin-conjugated antimouse IgG and avidin-conjugated peroxidase were serially reacted and followed by the treatment of substrate with diaminobenzidine and hydrogen peroxide. The reaction time was selected so that the background color change was minimal while the specific color change was apparent. The characteristic Raman band did not diminish under a prolonged exposure on the excitation laser as previously reported¹⁴ (ESI†). The complex formation between SWNT-ssDNA-biotin and SCMVM34 was also carried out in multi-steps. Biotin-conjugated antimouse IgG was first reacted with SCMVM34 on the nylon membrane. Avidin was reacted in the next step, and SWNT-ssDNA-biotin was reacted in the last step. This cross-checking experiment provided an almost identical trend (ESI†).

The interaction between biotin-tethered SWNTs and SCMVM34 (1 mM) was further verified by fluorescence modulation in aqueous

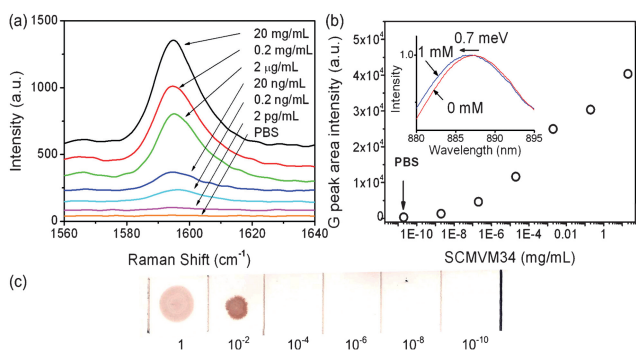


Fig. 3 Monitoring of a reaction between a primary antibody (SCMVM34) and the probe complex (SWNT-ssDNA-biotin-avidin-biotin-conjugated antimouse IgG): (a) Raman G mode profiles of nanotubes bound to SCMVM34 on the nylon membrane; (b) integrated area intensity under the G mode; (c) color reaction. The detection limit was 0.2mg/mL.

solution. The pre-conjugated SWNT-ssDNA-biotin-avidin-biotin-conjugated antimouse IgG complex showed strong fluorescence in the region of infrared, indicating individual suspension of nanotubes as shown in Fig. 4.¹⁴ There was a slight decrease in the fluorescence intensity after the interaction. The agents such as DNA backbones and amino acid building blocks of protein cause ion binding to SWNTs which can lead to a decrease in fluorescence. Biomolecules adsorbed onto the sidewall of SWNTs also change surface coverage and modulate the dielectric environment. This results in a shift in the emission energy from the bandgap of nanotubes by a solvatochromic mechanism.^{15–17} The inset in Fig. 4 shows a blue shift ($\sim 0.7\text{ meV}$) for the (6,4) tube upon the binding event carried out in a quartz cuvette for 4 hours at room temperature. The maximum intensity, before and after the reaction, was normalized in the inset. The blue shift was also observed for (7,5) and (6,5) at the excitation wavelength of 785 nm. The binding of SCMVM34 to the secondary antibody occurred distant from nanotube surface due to a linker layer in-between, such as avidin ($\sim 5\text{ nm}$) and secondary antibody ($\sim 8\text{ nm}$). The blue shift in fluorescence suggested that the long-range interaction (approximately by 13 nm) between large biomolecules resulted in a modulation of dielectric environments around the SWNTs.

HCMV-infected HFLF was grown in Dulbecco's modified Eagles' medium (ESI†). After 2 days of incubation, HFLF was collected by centrifugation and underwent a decomposition procedure to expose UL44 protein inside HCMV-infected cells to foreign analytes. The amount of extracted HFLF protein was roughly $150\text{ }\mu\text{g/mL}$. *In vitro* tracing of UL44 was performed using the similar protocol described above. Six HFLF droplets ($2\text{ }\mu\text{L}$ each) were prepared by diluting the reference sample and loaded onto the nylon membrane. A blank spot with PBS was also prepared as a control. After skim milk (2 wt%) blocking, SCMVM34 ($2\text{ }\mu\text{L}$, 0.2 mg/mL) was subsequently added to the membrane and incubated for 1 h. In the next step, the SWNT-ssDNA-biotin, avidin and biotin-conjugated antimouse IgG were reacted with the membrane at a molar ratio of 2 : 1 : 2. The nanotube concentration in the incubator was $6\text{ }\mu\text{g/L}$. Finally, the membrane was thoroughly washed using PBS-T. Fig. 5a shows G modes from the nylon membrane, and integrated areas are provided in Fig. 5b. Raw Raman data are provided in the ESI.† The intensity decreased as the concentration of extracts of HCMV-infected HFLF decreased.

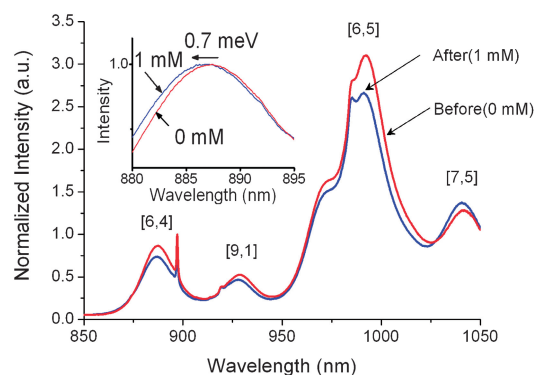


Fig. 4 Fluorescence modulation upon interaction between the SWNT-ssDNA-biotin-avidin-biotin-conjugated antimouse IgG complex and SCMVM34 in an aqueous solution. Observed shifted energies are as follows: (6,4) $\approx 0.7\text{ meV}$, (6,5) $\approx 0.74\text{ meV}$, (7,5) $\approx 0.15\text{ meV}$. The inset shows a blue shift ($\sim 0.7\text{ meV}$) for the (6,4) tube after normalizing the maximum intensity.

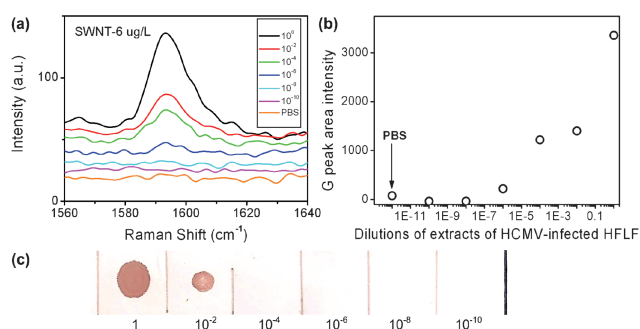


Fig. 5 Targeting of an antigen (UL44 in HCMV-infected HFLF) using the nanotube probe complex (SWNT-ssDNA-biotin-avidin-biotin-conjugated antimouse IgG); SCMVM34 was used as a primary antibody: (a) Raman G mode profiles on the NC membrane; (b) integrated area intensity under the G mode (from 1585 to 1615 cm^{-1}); (c) color reaction for HCMV. The detection limit was 1 : 100 dilution of the extract.

The Raman signal was negligible when the reference sample was diluted 10^8 times whereas the signal from the color test disappeared when the sample was diluted 10^4 times (Fig. 5c). Fig. 5 clearly shows the improved sensitivity, which is on the order of 150 pg/mL , compared with the colorimetric method. More rigorous comparison with the ELISA method or other optical labels, such as plasmonic (Au nanoparticles) labels and quantum dot labels, needs to be carried out in the future. The cytotoxicity of SWNTs is controversial depending on experimental conditions.^{18,19} The dot blotting method is designed to probe the target antigen in cell extracts. Therefore, the cytotoxic effect is minimal.

In conclusion, we have demonstrated a new immunoassay using biotinylated SWNTs as Raman biomarkers. Biotin-conjugated antimouse IgG was linked to nanotubes through the biotin-avidin interaction. The nanotube probe could detect a primary antibody (SCMVM34) and an antigen (UL44) in HCMV-infected HFLF with excellent sensitivity and specificity. The biotinylated nanotube platform can be readily expanded to a variety of biomolecular probes via the strong, versatile biotin-avidin reaction.

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