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Immunoassay with Single-Walled Carbon Nanotubes as Near-Infrared Fluorescent Labels

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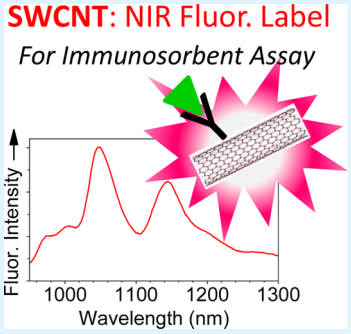
Supporting Information

ABSTRACT: The intrinsic photoluminescence of single-walled carbon nanotubes (CNTs) in the near-infrared (NIR) above 1000 nm makes them promising candidates for biological probes owing to low interference by bioorganic molecules and deep tissue penetration. We here demonstrate an immunoassay by using a NIR CNT labels conjugated to immunoglobulin G (IgG) antibodies. Most of the CNT-conjugated IgG was successfully immunoprecipitated with protein G-attached magnetic beads and eluted from them, which was confirmed by the NIR emission of the conjugated CNTs at 1000−1200 nm. The photoluminescence intensity of the CNT labels was strong enough to detect antigens at 600 pM by our simple procedures.



KEYWORDS: *carbon nanotube, near infrared fluorescence, immunoassay, IgG, protein G*

1. INTRODUCTION

In clinical tests, detection of weak signals is especially important for detection of low concentration biomolecules. An enzyme-linked immunosorbent assay (ELISA) is often employed for antigen detection, where background fluorescence that is generated from the combination of autofluorescence from serum components and nonspecific binding serum proteins limits detection sensitivity of an antigen by ELISA.

Near-infrared (NIR) fluorescence has attracted much more interests in diagnostic fields because of the lower background interference and the improved tissue penetration. Several NIR fluorescent molecules have been tried in enzymatic assays,[1] DNA sequencing,[2] capillary electrophoresis,[3] immunoassay,[4−6] and so on. For example, by using NIR fluorescent nanoparticles, alpha fetoprotein was successfully detected even in whole-blood samples,[5] which indicates a great advantage of NIR labels in clinical tests. Under these circumstances, novel NIR fluorescent molecules for biological label have so far been synthesized.[7−10]

Single-walled carbon nanotubes (CNTs) have received much attention because of their superior mechanical, optical, thermal, and electrical properties.[11,12] In particular, CNTs have been considered as ideal labels for biological imaging in vitro[13−16] and in vivo[17−20] because CNTs show photoluminescence in the wavelength region of 1000−1400 nm,[21] so-called the second NIR window.[22] In this wavelength region, indigenous tissue autofluorescence is much lower than that in the visible (400−750 nm) and shorter NIR (750−900 nm) regions, which enables clear vascular imaging with the CNT labels.[19] This benefit is further enhanced by a large Stokes shift between emission and excitation resonances of CNTs,[21] leading to further suppression of endogenous autofluorescence.[18] Moreover, CNT photoluminescence does not show photobleach under prolonged duration of excitation as compared with NIR fluorescent dyes and quantum dots.[23]

In the present work, to show the potential ability of the CNT labels in the immunological tests, we conjugated CNTs with immunoglobulin G (IgG) antibodies by using a linker molecule of phospholipid polyethylene glycol (PEG), and their immunoprecipitation using protein G magnetic beads was examined. The combination of IgG and protein G was adopted because they specifically bind with each other. The present results, using CNTs as the NIR fluorescent labels for the immunoassay, will pave a new way for medical applications of CNTs.

2. EXPERIMENTAL SECTION

CNT Dispersion with DSPE-PEG-NHS. CNTs (1 mg, CoMoCAT CG, Sigma Aldrich) were mixed with 10 mg of 3-(N-succinimidyloxyglutaryl) aminopropyl, polyethyleneglycol-carbamyl distearoylphosphatidyl-ethanolamine (DSPE-PEG-NHS, MW 2800; SUNBRIGHT DSPE-020SG, NOF Corporation, see Scheme 1) in a phosphate buffer (PB; 50 mM, pH 6.2, 10 mL), sonicated with a tip-type sonicator for 10 min, and centrifuged at 35 000 rpm at room temperature for 1 h. The centrifuge supernatant (ca. 9 mL) contained individual and thin

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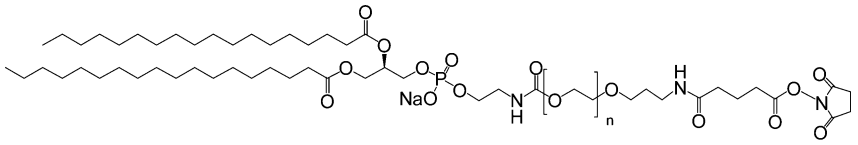
Scheme 1. Molecular Structure of DSPE-PEG-NHS (n = 45)



bundles of CNTs coated with DSPE-PEG-NHS, designated as CNT-PEG. To remove the excess DSPE-PEG-NHS, the supernatant (3.6 mL) was filtered (Nanosep 300K Omega, Pall Corporation) at 12 000 rpm for 10 min, washed with PB (pH 6.2, 1.2 mL) 5 times under the same centrifuge conditions, and the CNT-PEG was dispersed in PB (50 mM, pH8.0, 1.2 mL) for the reaction with IgG.

Preparation of CNT-PEG-IgG. The CNT-PEG in PB (pH 8.0, 1.2 mL) was mixed with rabbit IgG (Sigma, I5006, 60 μg) and left at room temperature for 1.5 h for the reaction. After the reaction, the excess IgG was removed by filtration (12 000 rpm, 10 min), followed by the washing of the reactant (CNT-PEG-IgG) with PB (50 mM, pH 8.0, 1.2 mL) for 3−5 times until the optical absorption spectra of the filtrate did not show peaks of IgG at 280 nm. The washed reactant of CNT-PEG-IgG was re-dispersed in PB (50 mM, pH 8.0, 1.2 mL) and stored at 4°C.

Immunoprecipitation (IP) Experiments. In IP of the CNT-PEG-IgG, the protein G magnetic beads (PrG-beads; 40 μL, Bio-Adembeads Protein G, Ademtech) were used. According to the manufacturer's intruction, PrG-beads were washed twice with, and dispersed in, D-PBS(-) + 0.5% Tween20 (40 μL) before IP. Then, the CNT-PEG-IgG solution (40 μL) was mixed with PrG-beads at room temperature for 30 min and left at 4°C for about 20 h. After the IP treatment, 20 μL of the dispersion of CNT-PEG-IgG/PrG-beads were taken and washed with PB (50 mM, pH 8.0, 20 μL) twice followed by re-dispersion in PB (50 mM, pH 8.0, 10 μL). From another 20 μL of the CNT-PEG-IgG/PrG-beads dispersion, the CNT-PEG-IgG/PrG-beads were collected with a permanent magnet. The collected CNT-PEG-IgG/PrG-beads were washed with PB (50 mM, pH 8.0, 20 μL) twice, and the CNT-PEG-IgG was eluted from the beads. The elution process entailed dispersion in a solution of PB (50 mM, pH8.0, 5 μL) and 10% SDS solution (5 μL), followed by boiling at about 100 °C for 20 min. The obtained elution solution was taken out by removing the beads with a permanent magnet. After elution, the beads were washed with PB twice and were redispersed in PB (50 mM, pH 8.0, 10 μL) for the photoluminescence measurement.

Two control experiments were carried out. First, to confirm that CNT-PEG did not attach to PrG-bead, the similar IP processes were carried out using CNT-PEG without IgG. In the second, to confirm that IgG specifically attached to CNT-PEG-NHS, the similar IP processes were performed using CNT-PEG-OCH_3 (No reactive group, N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl- sn-glycero-3-phosphoethanolamine, sodium salt, SUNBRIGHT DSPE-020CN, NOF Corporation) and IgG.

Characterization. The photoluminescence spectra of the CNTs were measured from 950 to 1300 nm with MicroHR spectrometer and Symphony InGaAs detector (Horiba JY) under the optical microscope (Olympus BX51) at room temperature (excitation wavelength = 660 nm). The sample solutions were dropped in Sekisui micro-plates, which were made of microscope slide and cover glass with three sides shielded and the one side open (UR157S, Sekisui Polymatech; 12 mm long, 10 mm wide, and 0.07 mm deep). The focal point was adjusted to a middle depth.

The optical absorption spectra of CNTs were measured with a UV-3150 spectrometer (Shimadzu).

The Raman spectra were measured with T64000 spectrometer (Horiba JY) with an optical microscope (Olympus BX51) at room temperature (excitation wavelength = 514.5 nm). The specimens for the Raman measurements were placed on a microscope slide and dried.

The structure of the CNT-PEG was observed with transmission electron microscope (TEM, Topcon 002B) at an acceleration voltage of 120 kV. For the structure observations, the original CNTs were dispersed in water with DSPE-PEG (CNT: DSPE-PEG = 1:0.5 in weight) by a tip-type sonicator for about 10 min and an aliquot of the dispersion was dropped onto a TEM microgrid.

3. RESULTS AND DISCUSSION

The synthesis procedures and the characterizations of the NIR CNT labels are firstly described. Figure 1 shows a TEM image

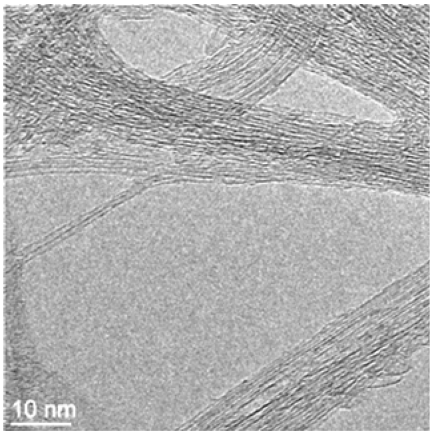


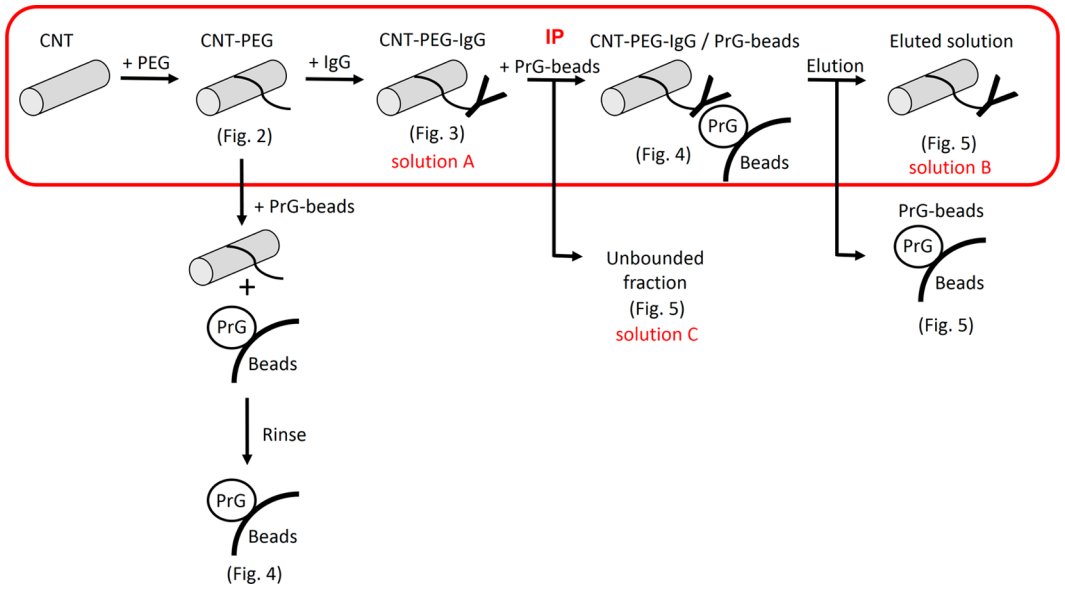
Figure 1. Typical transmission electron microscope image of CNTs.

of the original CNTs, in which the single-walled cylindrical structures were clearly observed. The resonance Raman spectra were characteristic of CNTs with diameters of about 0.8−0.9 nm (see Supporting Information 1).

The IgG was labeled with CNTs by two steps (Scheme 2): First, CNTs were coated with DSPE-PEG-NHS (Scheme 1), which was designated as CNT-PEG. Second, IgG was combined with the NHS group to obtain CNT-PEG-IgG.

The obtained CNT-PEG and CNT-PEG-IgG were characterized by photoluminescence and resonance Raman spectroscopy. The CNT-PEG was homogeneously and stably dissolved in phosphate buffer solution (PB) (pH 6.2). Two major photoluminescence peaks at about 1045 nm and 1145 nm were observed from the solution, which are assignable to (7, 5) and (7, 6) CNTs with diameters of 0.82 nm and 0.89 nm,

Scheme 2. Flowchart of the Present Experiment[a]



[a]Corresponding figure numbers are indicated in parentheses.

respectively (Figure 2a).[24] Lower peaks observed at 980 nm and 1000 nm can be attributed to (8, 3) and (6, 5) CNTs,

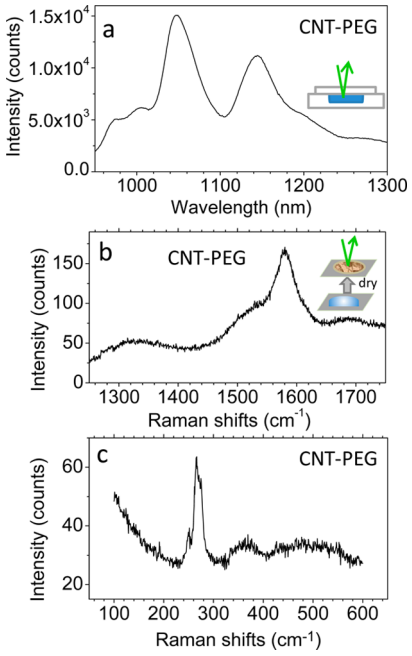


Figure 2. Spectroscopic characterizations of CNT-PEG. (a) Photoluminescence spectrum (excitation wavelength = 660 nm) of CNT-PEG dispersed in a PB solution and (b, c) Raman spectra (excitation wavelength = 514.5 nm) of CNT-PEG measured after drying its PB solution dropped on a quartz glass plate.

respectively (Figure 2a). The spectral widths of the peaks were 10−20% broader than those of individually dispersed CNTs, suggesting that the CNTs were more or less bundled in the dispersion. Furthermore, the spectral broadening might be caused by the ionic species in the solvent of the PB (pH 6.2).

The Raman spectrum of CNT-PEG was measured after drying, which showed the G band at 1580 cm^{-1} (Figure 2b) and a peak of radial breading mode at ~270 cm^{-1} (Figure 2c).[25] The CNT diameter estimated from the observed frequency was ~0.9 nm.[25] The Raman spectrum also showed a weak D band (1340 cm^{-1})[26] and a broad shoulder of the Fano line[27] at the lower energy side of the G band (Figure 2b). The Fano line appears when CNTs with small diameters (<1 nm) have a metallic band structure. The weak D band indicates that the quantity of the amorphous carbon[26] and/or the number of structure defects in the CNT are small.[28]

The obtained CNT-PEG-IgG was well dispersed in PB (pH 8.0). The shape of the photoluminescence spectrum observed from the CNT-PEG-IgG solution (Figure 3a) was almost identical to that of CNT-PEG in PB (pH 6.2) (Figure 2a). The resonance Raman signals of the CNT-PEG-IgG (Figures 3b, 3c) also show similar spectral features to those of the CNT-PEG (Figure 2b, c). The similarities in the photoluminescence and the Raman spectra indicate that the spectroscopic features of CNT were not influenced by the process of IgG conjugation with the PEG chain.

By using the obtained CNT-PEG-IgG, the IP was tested as shown in Scheme 2. The IP of CNT-PEG-IgG on Protein G-attached magnetic beads (CNT-PEG-IgG/PrG-beads) was performed. Panels a, c, and d in Figure 4 are the photoluminescence and the Raman spectra of the resultant CNT-PEG-IgG/PrG-beads, respectively, which shows close similarities to those of the CNT-PEG-IgG (Figures 3a-3c). Thus it is apparent that the optical properties of the CNTs were not changed by the PrG-beads.

To verify the IP reaction between CNT-PEG-IgG and PrG-beads, we carried out the following two control experiments. First, CNT-PEG without IgG was used for the reaction (Scheme 2, first vertical branch). After IP with PrG-beads, the resultant solution did not show any photoluminescence of the CNT as expected (Figure 4b). This indicates that IgG is necessary to couple with protein G. Further, the same IP procedure was conducted by using CNT-PEG-OCH_3 instead of CNT-PEG-NHS because CNT-PEG-OCH_3 is nonreactive with IgG. The photoluminescence spectrum of CNT-PEG-CH_3 dispersed in PB slution is shown Figure 5a. On the other

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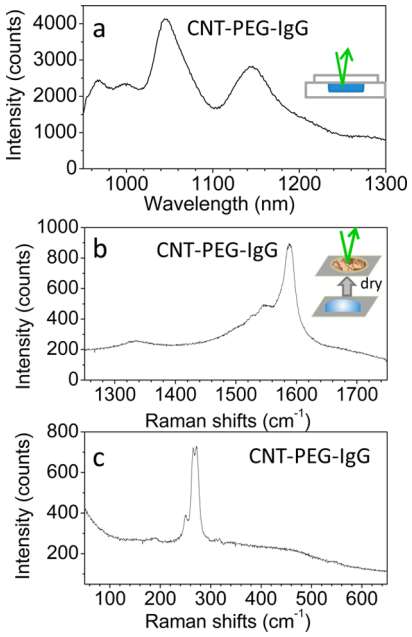

Figure 3. Spectroscopic characterizations of CNT-PEG-IgG. (a) Photoluminescence spectrum (excitation wavelength = 660 nm) of CNT-PEG-IgG in PB solution and (b, c) Raman spectra (excitation wavelength = 514.5 nm) of CNT-PEG-IgG measured after drying its phosphate buffer solution dropped on a quartz glass plate.

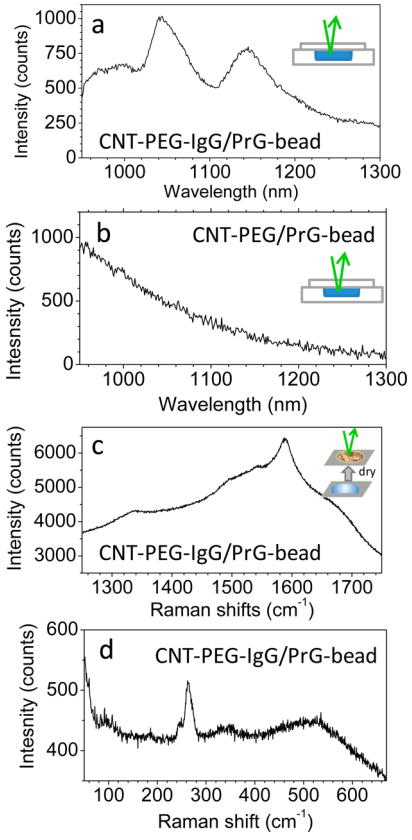

Figure 4. Photoluminescence spectra (excitation wavelength = 660 nm) of (a) CNT-PEG-IgG/PrG-beads dispersed in a buffer solution and (b) CNT-PEG/PrG-beads dispersed in a buffer solution. (c, d) Raman spectra (Excitation wavelength = 514.5 nm) of CNT-PEG-IgG/PrG-beads measured after drying its phosphate buffer solution dropped on a quartz glass plate.

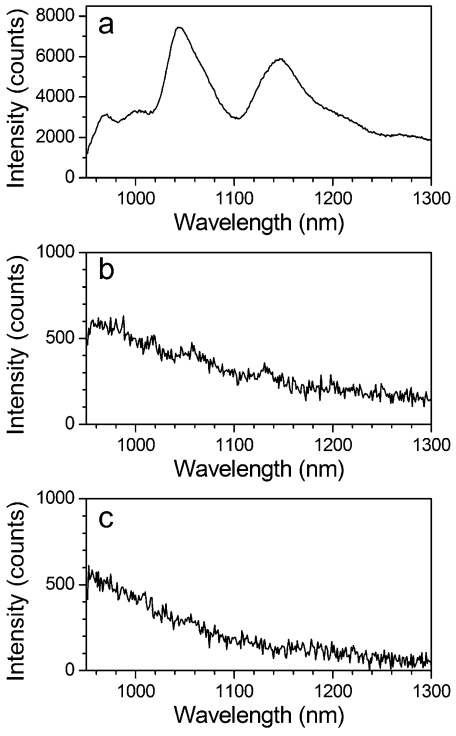

Figure 5. Photoluminescence spectra (excitation wavelength =660 nm) of (a) CNT-PEG-OCH_3/IgG in PB solution, (b) PrG-beads after the treatment with CNT-PEG-OCH_3/IgG , and (c) the solution eluted from the immunoprecipitated PrG-beads.

hand, no photoluminescence signal was observed from the CNT-PEG-OCH_3/IgG/PrG-beads solution (Figure 5b) as well as the eluted solution (Figure 5c) after the IP process. The results showed that CNT-PEG without the reactive groups of NHS was not bound to IgG, therefore was not selectively immunoprecipitated with PrG beads.

To ensure the usefulness of the CNT label in an immunoassay, the CNT-PEG-IgG was eluted from CNT-PEG-IgG/PrG-beads (Scheme 2). The characteristic photoluminescence signals of the CNTs were successfully observed from the eluted solution (Figure 6a). On the other hand, the photoluminescence spectrum of PrG-beads after the elution did not show peaks characteristic of CNTs (Figure 6b), suggesting that almost all of the CNT-PEG-IgG was eluted from the beads.

We also measured the photoluminescence spectrum of the unbounded fraction after IP (Scheme 2). The photoluminescence peaks of CNTs were clearly observed (Figure 6c). The photoluminescence intensity ratio of CNTs in the solution before IP (solution A, see Scheme 2), in the elution solution (solution B), and in the residual solution after IP (solution C), was 8.7:1:4.8. Here, the photoluminescence intensities were estimated from the total area of four deconvoluted peaks (see Supporting Information 2). The value of 8.7 and the sum of 5.8 (= 1 +4.8) are reasonably close values when considering the proteins can attach to the walls of containers, pipets, etc. Assuming that the IP residual solution contained only CNT-PEG, the CNT number ratio in the eluted solution and in the unbounded fraction after IP, 1:4.8, would be representative of the number ratio of CNT-PEG-IgG:CNT-PEG. The ratio of 1:4.8 means that the reaction yield of CNT-PEG-IgG from CNT-PEG (see Scheme 2) is ~17 %. Improvement of the reaction efficiency remains as a future work.

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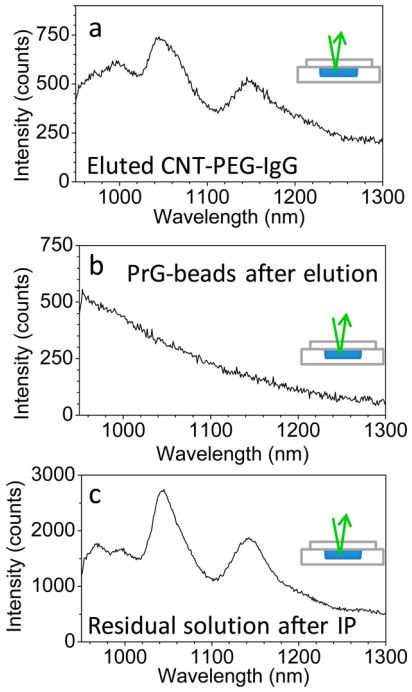

Figure 6. Photoluminescence spectra (excitation wavelength = 660 nm) of (a) CNT-PEG-IgG eluted from protein G beads, (b) the beads after the elution, and (c) the residual solution after the immunoprecipitation of CNT-PEG-IgG with protein G beads.

Finally the detection limit of CNT immunoassay is discussed. Under our experimental condition, the CNT photoluminescence intensity was 175 counts at 1045 nm for 1 nM CNT dispersion, where the average molecular weight of CNT was assumed to be 600 K (see Supporting Information 3). This suggests that, if IgG combine with certain antigens perfectly (100% yield reaction), the detection limit would be 600 pM, as the lowest possible counts of the photoluminescence signal was about 100 counts in our experimental conditions. One of the biggest problems of CNT fluorophores is their lower quantum yield ($\sim 1 \times 10^3$).[21] The detection limit should be improved by using the oxidized CNTs with stronger emission[29] and/or the metal-enhanced fluorescence technique.[30] The detection limit of antigens in the CNT immunoassay would be further improved by employing the certain chirality CNTs such as (7, 5) and (7, 6) that emit photoluminescence at 1000−1300 nm. This is possible by applying recently developed technique of sorting CNTs by the chiralities.[31,32]

In the present experiment, we demonstrated that the CNT label linked with IgG antibodies was successfully immunoprecipitated by protein G magnetic beads. To detect biomarkers in clinical trials, an antibody-conjugated CNT labels should couple with a specific antigen. Further study along this line is now in progress by the present research group.

4. CONCLUSION

In conclusion, the IgG antibody was fluorescently labeled with CNT (excitation 660 nm; emission 1000−1200 nm) by conjugating with DSPE-PEG-NHS, the linker molecule. The number percent of the CNT-PEG that reacted with one IgG molecule was estimated to be about 17 % based on the CNT photoluminescence intensities of the residual solution after IP and the IP elution solution. The PrG-beads after elution did not show the CNT photoluminescence, indicating that the almost all of the CNT-PEG-IgG molecules were eluted from the magnetic beads. Successful IP reaction was also confirmed by the control experiment, where CNT-PEG without IgG was not precipitated with PrG-beads. The detection limit of antigen by the CNT-PEG-IgG in this study was estimated to be 600 pM. These results ensure a potential usefulness of CNT as a NIR label in immunoassays.

■ ASSOCIATED CONTENT

Supporting Information

The Raman spectra of original CNTs; Control experiments for confirming that IgG specifically attached to CNT-PEG-NHS; curve fitting of photoluminescence spectra of CNTs; calibration lines of CNT absorption and photoluminescence intensity. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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