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DNA sequence detection based on Raman spectroscopy using single walled carbon nanotube

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A Biosensor for detection of DNA sequence in bacterium *Bacillus anthracis* has been developed using Raman spectrum of single walled carbon nanotubes (SWNTs). We have utilized the fact that being hydrophobic in nature single strand of target DNA (diseased DNA) gets wrapped over SWNT surface forming single stranded DNA–SWNT complex. The sensing ability of this sensor has been studied using the dependency of G peak intensity (in the Raman spectrum of SWNTs) on the covered surface of SWNTs. When a DNA having a sequence complementary to that of the target DNA is added to DNA–SWNT complex, hybridization between these sequences takes place. This results in large covered surface area of SWNT and reducing the intensity of G peak. A slight red shift in the G peak has also been observed. The intensity of G peak depends on the exposed area of SWNT to the excitation beam. On the other hand with noncomplementary DNA, no significant change in intensity of G peak is observed. Finally, results were cross-checked by gel electrophoresis.

[**Key words:** Raman spectrum; Probic DNA; Complementary DNA; Gel electrophoresis; Single walled carbon nanotube; Noncomplementary DNA]

Medical diagnostics based on deoxyribonucleic acid (DNA) sequence detection has recently become very popular because it is a low cost, fast and reliable technique. It uses a very small amount of DNA (only few nanomoles) and does not require conventional methods such as polymerase chain reaction (PCR), gel electrophoresis etc. (1–4), which are generally very expensive and time consuming. The basic principle of conventional diagnostics is based on detection of antibodies of different diseases in the patient where the structure of antibodies and the corresponding antigens are to be determined (5–7). The techniques for detecting antibody are different and specific for each disease. Often the cost of medical diagnostics in this manner becomes far more expensive than the treatment itself. The analytical methods most commonly used for sequence detection through DNA hybridization are optical, piezoelectric (8) and electrochemical. Optical detection, particularly based on Raman spectroscopy from solution-based system is advantageous due to high sensitivity and selectivity.

In our previous work, we have worked on Fluorescence Resonance Energy Transfer (FRET) based biosensing technique (5,9) where we used a water soluble cationic conjugated polymer, poly [(9,9-di(6,6′-N,N′trimethylammonium) hexylfluorenyl-2,7-diyl)-alt-co-(1,4-phenylene)] di-bromide salt, PFP having emission in the blue wavelength region and peptide nucleic acid (PNA) attached with a fluorescein dye as a probe (PNAC*). Hybridization of PNAC* with single stranded DNA (ssDNA) (having a sequence complimentary to that of PNA) makes the hybridized system negatively charged, which is electrostatically attracted toward the positively charged PFP molecules. When the distance between PFP (donor) and PNAC*/DNA (acceptor) complex is close to Forster critical radius, excitonic energy is transferred from donor to acceptor molecules provided that the emission spectrum of the donor and absorption spectrum of the acceptor have good overlap (5). The distance between donor and acceptor is controlled by varying their concentrations. We found that when FRET occurs, the blue emission of the polymer gets almost completely quenched and that of the acceptor increases by ~20 times. However, the problem with this sensor is that the PNA has to be labeled with fluorescent dyes, which makes the technology relatively expensive and cumbersome. We therefore switched over to single walled carbon nanotubes (SWNTs) based biosensors where labeling of the dye is not required for DNA sequence detection.

A DNA–SWNT sensor works on the principle that both ssDNA (known sequence) and SWNT are hydrophobic in nature (in case of ssDNA, one surface is hydrophobic and the other surface is hydrophilic). Consequently, the ssDNA segment gets very quickly adsorbed and wrapped on the SWNT surfaces. The adsorbing ssDNA molecules help in dispersion of SWNTs individually. However, it is found by some workers (10) that only ssDNA sequences of repeats of alternating G and T, i.e., poly (GT) n with $n \geq 10$ can efficiently disperse SWNTs henceforth termed as probic DNA [$(GT)_{15}$ in our case)]. For detection of the sequence of target DNA (*Bacillus anthracis* in the present case); it (target DNA) is attached to probic DNA. Combined assembly of probic DNA–target DNA is referred as pDNA. The next step is to put the ssDNA having a sequence

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complementary to target DNA. If the sequences of two DNA are complementary, they will hybridize. If noncomplementary (nDNA) sequence of ssDNA is introduced in the solution, hybridization of DNA does not take place.

In the present work, we have utilized the Raman spectrum of SWNTs for detection of DNA sequence. The Raman spectrum of the SWNTs contains three characteristic modes namely Radial Breathing Mode (RBM), D mode and G mode. RBM corresponds to radial expansion and contraction of the nanotube where all the carbon atoms are moving in phase (acoustic phonons) in the radial direction (11) and its frequency (cm^{-1}) depends on the diameter of the nanotube given by the relation (12,13).

$$\nu_{RBM} = [223/d] + 10 \quad (1)$$

where, ν_{RBM} is the RBM frequency and d is the corresponding diameter (in nanometers). In case of large diameter nanotubes acoustic phonons will have small amplitude for harmonic oscillations that results in faster oscillations, i.e., higher frequency and hence, low Raman Shift. Similarly in small diameter tubes acoustic phonons will have large amplitude for harmonic oscillations, which results in lower frequency and hence, large Raman Shift. Another mode in SWNTs is the G mode where neighboring atoms are moving in opposite directions (surface optical phonons) along the surface of the tube as in 2D graphite (11). The third mode is the D mode which signifies presence of impurities in sample. Ideally D mode should be forbidden and nanotube sample should be free from structural defects (14).

In particular we have used change in G mode of the Raman spectrum of SWNT as a proof of hybridization of pDNA on SWNT surface. Due to stretching in C–C bonds of graphene, SWNTs give a Raman line between 1592 cm^{-1} and 1596 cm^{-1} (G peak) (15). The intensity of this Raman line decreases with the increase in DNA concentration, i.e., with the increase of covered surface area of SWNT. In our case, when complementary DNA (cDNA) is added to the system it will hybridize with pDNA–SWNT complex and the covered surface area of SWNT will increase resulting in a significant decrease in the intensity of G line. On the other hand if nDNA is added to the system, the intensity of G line will not show any significant decrease. Intensity of G line is systematically studied as a function of concentration of cDNA. Besides the change in the intensity of G Raman peak there is a small red shift ($\sim$2 cm^{-1}) in the G mode of Raman spectrum (16) when cDNA is added to the pDNA–SWNT complex.

The main advantage of this sensor is that the wrapping of pDNA around individual SWNT is by aromatic interactions (17) and the pDNA–SWNT complex is highly stable. SWNTs are defiant to photo bleaching and therefore the sensor has a long functioning life. This bio-nano system will ultimately lead to new generation of sensors with high biological recognition capabilities. We used this technique to sense the unknown DNA sequence of known antigen (in our case *B. anthracis*, which causes anthrax disease) in an inexpensive and simple manner. We have further cross-checked our results by conventional gel electrophoresis method. Transmission electron microscopy (TEM) has been used to characterize pDNA–SWNT composites deposited on carbon coated Cu grids. Electron micrographs provide proof for wrapping of pDNA around individual SWNT and bundle of SWNTs.

MATERIALS AND METHODS

The SWNT samples and ssDNA primers were commercially procured from Cheaptubes (Brattleboro, VT, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The procured SWNT samples have diameter ranging from 1.0 nm to 2.0 nm and length from 5 to 30 μm. The sequences of procured ssDNA samples are as shown in Table 1. Bold text in Table 1 shows the bases in cDNA which have mutated. The DNA primer was synthesized at Sigma–Aldrich with the help of an instrument called nucleic acid synthesizer where each base is added via computer control of the reagent delivery. DNA synthesis requires four steps viz. de-blocking, base condensation, capping and oxidation for attaching every single base (i.e., A, G, T or C) to the primer. All steps are repeated until all desired bases have been added to the oligonucleotide to obtain a DNA primer of desired sequence.

TABLE 1. List of oligonucleotides used in the experiment.

Serial no.	Oligonucleotide	Sequence
1	ssDNA probe	5′-$(GT)_{15}$–(GTAAATGGTGTTAGGGTTGC)-3′ (50 mer)
2	cDNA	5′-(GCAACCCTAACACCATTTAC)-3′ (20 mer)
3	nDNA	5′-(CGTTGGGATTGTGGTAAATG)-3′ (20 mer)
4	15 base pair mismatch	5′-G**ACT**C**AA**T**GGC**G**TTA**G**ACTG**-3′ (20 mer)
5	5 base pair mismatch	5′-**CG**AACCCTAA**G**ACCATTT**TG**-3′ (20 mer)
6	1 base pair mismatch	5′-GCAACCCTA**G**CACCATTTAC-3′ (20 mer)

Experiments were performed using Millipore Milli-Q deionized water (18.0 MΩ cm resistivity). For preparing pDNA–SWNT hybrid, an aqueous solution containing pDNA with concentration 1 mg/ml was prepared in TRIS buffer (pH 7.4). The pH value as mentioned was calibrated by a micro pH meter (Labindia Phan pH meter). To this solution, SWNTs were added keeping their concentration as 1 mg/ml and the resultant solution was sonicated using probe type sonicator (Sonics Vibra-Cell 20 kHz ± 50 Hz) operating at 22% of its maximum speed for about 10–50 min to obtain a homogeneous suspension of SWNTs in the solution in an ice beaker. Sonication rate was one pulse per second. This solution was then centrifuged for 30 min at 15,000 ×*g* in a micro-centrifuge to remove nondispersible SWNTs and other accompanying impurities. The Raman spectrum of this solution was studied using micro-Raman Renishaw spectrometer. Spectral resolution of this instrument is better than 1 cm^{-1} (close to 1/1000 cm^{-1}) using 2400 lines/mm and spatial resolution is 1 micron using 100x objective. The sample was excited using 785 nm laser. All the measurements were performed at room temperature. To this solution, cDNA and nDNA primers were added separately with molar concentration of 500 nM and 600 nM (optimized for best results) and their effect on the Raman spectrum of the SWNTs was studied after annealing the solutions at 50°C for about 30 min. The annealing improves hybridization of DNA and therefore the temperature and duration of annealing were optimized after many trials and errors.

Gel electrophoresis experiment Native polyacrylamide gel electrophoresis (PAGE, 12%, Acr:Bis = 29:1) was carried out in 5× TBE buffer. 5× TBE buffer was made using tris(hydroxymethyl) aminomethane (Tris, 89 mM), ethylenediaminetetraacetate (EDTA, 2 mM), and boric acid (89 mM), pH 8.0. The polyacrylamide gel (12%) was prepared by 4.8 ml of 30% acrylamide (29:1), 4.8 ml of Milli-Q, 2.4 ml of 5*TBE, 200 μl of 10% APS and 10 μl of TEMED. The so prepared gel was poured in gel caster and different wells were made in it. Subsequently samples were loaded into the wells and this arrangement ran overnight at 10 V/cm at room temperature.

RESULTS AND DISCUSSION

The Raman spectrum of the SWNTs dispersed in probic DNA solution is shown in Fig. 1. All the three modes of SWNTs (namely

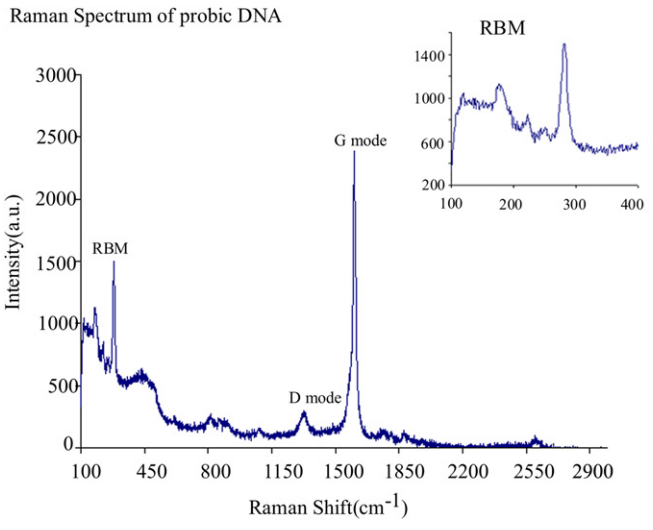

FIG. 1. Raman spectra of pDNA–SWNT hybrid when excited by red laser (785 nm).

TABLE 2. Diameter distribution of SWNT in the present sample calculated using RBM mode.

Serial no.	ν_{RBM} (cm^{-1})	Diameter (nm)
1	119.692	2.032965
2	138.885	1.730225
3	175.963	1.343673
4	222.816	1.047854
5	250.527	0.927131
6	281.407	0.821644

RBM, D mode and G mode) are observed at wavelengths 100–300 cm^{-1}, 1308 cm^{-1} and 1605 cm^{-1} respectively. By RBM mode (using Eq. 1) the diameters of SWNTs present in our sample were calculated and are shown in Table 2. The RBM mode shows six distinct peaks corresponding to the six different diameters of SWNTs in the sample. The positions of the peaks are shown upto 3rd place of decimal which is in the limit of resolution of the instrument (1/1000 cm^{-1}). From Table 2 it is found that large diameter tubes have low Raman Shift and narrow diameter tubes have high Raman Shift. It may be noted that RBM band at 450 cm^{-1} is quite broad. This shows that there is wider distribution of narrow-tube diameter.

It can be seen from the Fig. 1 that the intensity of D mode is very low, which indicates that our samples are almost free from structural defects. Further the tangential mode (G peak) caused by stretching along C–C bonds of graphene is found to be strongest in our sample (11).

The Raman spectrum of cDNA–pDNA–SWNT solution is shown in Fig. 2. It is observed that in presence of cDNA the intensity of G peak decreases as compared to that of pDNA only. Further as the cDNA concentration increases (from 500 nM to 600 nM) the intensity of G peak decreases and a slight red shift is also observed in the G peak (as shown in inset of Fig. 2). However this shift is too small of the order of 1 cm^{-1} and it is difficult to draw any conclusion on this red shift. A possible cause of this red shift may be that DNA wrapping does not permit oscillations to be completed. More the DNA wrapping faster will be oscillations hence higher frequency and low Raman shift or red shift. However difficulty in this explanation is that if it is true, i.e., damping of oscillator is fast the spectral bandwidth must be broadened which is not the present case. Therefore not much importance can be given to red shift in the present case. On the other hand, when nDNA is added, no significant change in the G peak is observed.

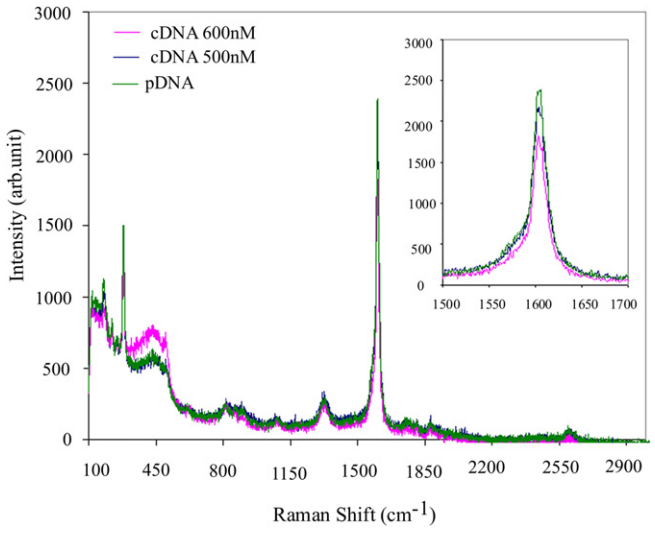

FIG. 2. Raman spectra of pDNA–SWNT hybrid in presence of cDNA.

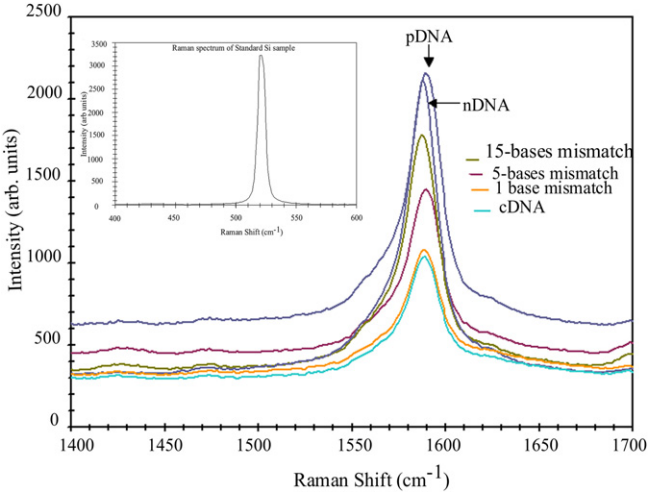

FIG. 3. Raman spectra of pDNA–SWNT, nDNA–pDNA–SWNT, cDNA–pDNA–SWNT, 15 base pair mismatch-pDNA–SWNT, 5 base pair mismatch-pDNA–SWNT and 1 base pair mismatch-pDNA–SWNT hybrids.

Before the Raman Spectrum studied for our sample, we calibrated the instrument with the help of a standard Si sample provided with the instrument. The peak position of Raman band for Si should be at 520 cm^{-1} and is shown in inset of Fig. 3. The corresponding intensity of this Raman peak is also shown. To correct the intensity of the Raman spectra, the intensity of all other Raman peaks was compared with the intensity of the standard Si sample.

In order to indicate the accuracy of this method and to exclude the possibility of a false detection, we also presented the results of 1 base pair mismatch, 5 base pair mismatch and 15 base pair mismatch. It is found (Fig. 3) that the intensity of G peak for 1 base pair mutated sequence is very close to intensity of cDNA while intensity for 15 base pair mutated sequence is close to ncDNA. It can be concluded from Fig. 3 that as the mismatch in pDNA and DNA to be tested decreases, the intensity of G peak decreases.

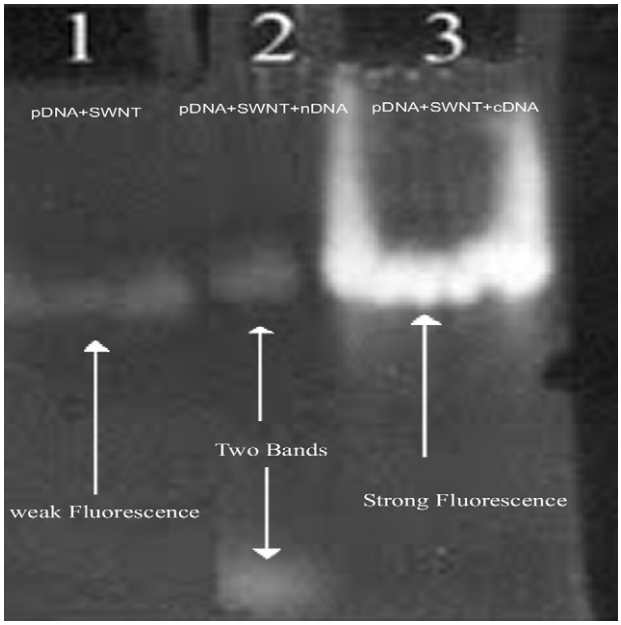

FIG. 4. Result of gel electrophoresis of pDNA–SWNT, nDNA–pDNA–SWNT and cDNA–pDNA–SWNT hybrids.

TABLE 3. Experimental evidence of hybridization from gel electrophoresis.

Lane	Sample	Inference
1	Probic DNA + SWNT	Weak fluorescence
2	Probic DNA + SWNT + nDNA	Two bands
3	Probic DNA + SWNT + cDNA	Strong fluorescence (one band)

To confirm the hybridization of cDNA with SWNT–pDNA probe, we have used conventional gel electrophoresis technique. Fig. 4 reports the gel electrophoresis (12% polyacrylamide) result of our samples; it confirms the hybridization of cDNA and pDNA on the SWNT surface. Different wells are loaded with different samples and are summarized in Table 3. Lanes 1–3 contain SWNT with pDNA, SWNT–pDNA complex with nDNA and complex with cDNA, respectively. Lane 1 shows a glowing band corresponding to the position of pDNA–SWNT complex. In lane 2 two bands are observed one band at same position as that of pDNA–SWNT and another band is observed far away from pDNA–SWNT. This band corresponds to nDNA, which does not hybridize itself with SWNT–DNA complex. Being lighter in weight it moves swiftly and covers a larger distance toward anode. Lastly lane 3 shows a single band corresponding to SWNT–pDNA–cDNA complex. As its weight is similar to that of SWNT–pDNA complex the band is found near to that of lane 1. Absence of second band ensures DNA hybridization, which is in accordance with the observed high luminescence, an evidence of DNA hybridization in gel electrophoresis technique.

To visualize the wrapping of pDNA over the surface of the nanotube TEM measurements were made on our samples. TEM images of pDNA–SWNT composite are shown in Fig. 5. The image shows both individual and bundles (~25 nanotubes) of SWNTs. It can also be observed from Fig. 5 that edges of the carbon nanotubes are not sharp, which can be attributed to the wrapping of ssDNA over the walls of SWNT.

It can be concluded that pDNA–SWNT hybrid can be used to detect a particular sequence of DNA specific to a disease, in an inexpensive and reliable manner. Higher will be the DNA concentration, lower will be the intensity of G peak and is an obvious evidence of DNA hybridization on SWNT surface.

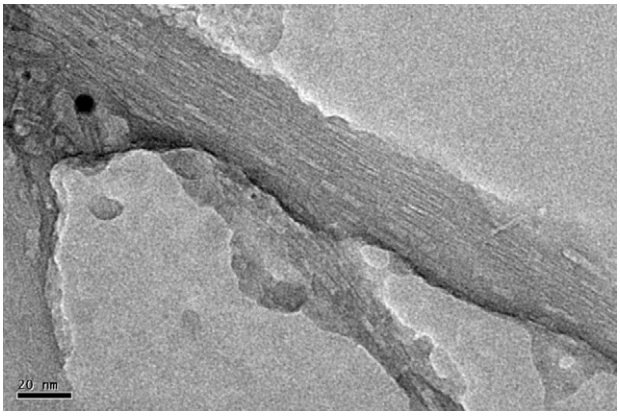

FIG. 5. TEM image of pDNA–SWNT showing apparent wrapping of pDNA around an SWNT bundle.

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