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Molecular Beacon CNT-based Detection of SNPs

V P Egorova¹, H V Krylova², I V Lipnevich², A A Veligura¹, B G Shulitsky³, L.Y. Fedotenkova¹

¹Belarusian State Pedagogical University, 18 Sovetskaya Str, 200005 Minsk, Belarus

²Physics Department, Belarusian State University, 4 Nezavisimosti Ave., 200030 Minsk, Belarus

³Belarusian State University of Informatics and Radioelectronics, 6 P. Brovki Str, 200013 Minsk, Belarus

E-mail: alla.veligura@mail.ru

Abstract. An fluorescence quenching effect due to few-walled carbon nanotubes chemically modified by carboxyl groups has been utilized to discriminate Single Nucleotide Polymorphism (SNP). It was shown that the complex obtained from these nanotube and single-stranded primer DNA is formed due to stacking interactions between the hexagons of the nanotubes and aromatic rings of nucleotide bases as well as due to establishing of hydrogen bonds between acceptor amine groups of nucleotide bases and donor carboxyl groups of the nanotubes. It has been demonstrated that these complexes may be used to make highly effective DNA biosensors detecting SNPs which operate as molecular beacons.

1. Introduction

SNP is the most prevailing mutation of genome. A large number of SNPs makes them unique genetic markers for preventive medicine and biomedical diagnostics. The molecular beacons (MB) technique is one of the most common used methods to detect SNPs. Using of the MB to discriminate SNPs has substantial disadvantages such as the presence of residual fluorescence and exposure to intracellular nucleases. This problem can be solved by using nanostructured materials to modify MBs. Single stranded deoxyribonucleic acid (ssDNA) can form a stable complex with individual single-walled nanotubes (SWCNTs) or multi-walled nanotubes (MWCNTs) by the aromatic interactions between nucleotide bases and SWCNT or MWCNT sidewalls. It has been shown that the carbon nanotubes (CNTs) are effective quenchers of fluorescent oligonucleotides which are labelled by dye molecules [1]. The fluorescence is quenched by touching the rings of the dye molecule bound to the FAM-oligonucleotide, with the surface of CNTs [1–3]. If the hybridization of the FAM-oligonucleotide labelled by fluorophore FAM (fluorescein phosphoramidites) with complementary ssDNA target in solution occurs then the fluorescence is recovered. Stacking $\pi-\pi$ -interaction reduces the distance between the surfaces of FAM-rings and CNTs to such an extent, that the charge transfer occurs to acceptor CNTs. Complexes – non-modified CNT/linear probe DNA are less effective than MB based on Fluorescence Resonance Energy Transfer with chemically linked fluorescent and quenching [1].

DNA selectively disperse different CNTs, with unfilled coating CNTs. In order to uniformly distribute the DNA on CNTs, the last are functionalized by polymers. Probe DNA chemically linked to the functionalized by polymer MWCNT is placed transversely to the CNT surface. It makes it possible discrimination of SNP [4]. Unfortunately, it does not prevent the folding of DNA with



subsequent compactification into globules and inefficient charge transfer, that sets a nanomole limit for concentration detection [3, 4]. Preferential adhesion of oligonucleotide sequence $(GT)_n$ to SWCNT bundle in comparison with other oligonucleotides allowed to use $(GT)_n$ as reporter sequences chemically bound to ssDNA target. Since the bundle are not selected by the ssDNA targets, then the last are located transversally to SWCNT surface and are homogeneously distributed. It makes possible to perform hybridization. In paper [5] it has been suggested that because of the dependence of the light absorption coefficient on the density of the hybrids, the intensity of Raman scattering in SWCNTs will be reduced linearly with density of the hybrids. However ssDNA complexes with unmodified and non-functionalized SWCNTs do not allow to discriminate SNP. It was shown that DNA single strands self-assemble on carbon nanotubes (CNTs) with the formation of electron-dense layers of the oligonucleotide [6].

In this study, we have utilized the fluorescence quenching effect due to few-walled CNTs (FWCNTs) chemically modified by carboxyl groups to develop a new approach which allows us to identify SNP. We show in this paper that the complex ssDNA/FWCNT is formed due to stacking interactions between the hexagons of CNTs and aromatic rings of nucleotide bases as well as due to establishing of hydrogen bonds between acceptor amine groups NH of nucleotide bases and donor carboxyl groups of the nanotubes. Hydrogen bonds, orienting aromatic rings in a plane transversal to the surface of CNT, prevent $\pi-\pi$ -interactions to minimize the distance between the aromatic rings and the surface of CNTs in the complex. The inclination of planes of nucleotide bases allows not only self-assembling of the oligonucleotide shell of complex, but an epitaxial growth of oligonucleotide molecular crystallites on electron-dense self-assembling oligonucleotide. DNA strands of epitaxial layers in the complex are vertically oriented, contrary to horizontally oriented DNA strands of self-assembling oligonucleotide. Therefore, as we will demonstrate the hybridization of probe DNA with DNA target can take place on complex ssDNA/FWCNT.

2. Materials and methods

ssDNA primer dye-labeled sequence 5'-FAM-GCCATATACTCTCCTTGGTGACA-3' (fluorescent oligonucleotide FAM-ON1) is used as DNA probe. For the heteroanalysing we utilize oligonucleotide targets 5'-TGTCACCAAGGAGAGTATATGGC-3' (ON2) and 5'-GTAGGCTTGGGAATAGTACGA-3' (ON4), complementary and non-complementary to the ON1, as well as single-base mismatched target 5'-TGTCACCAAGGCGAGTATATGGC-3' (ON5) were synthesized at «Praymteh» (Minsk, Belarus).

We will utilize FWCNTs with diameters ranging from 2.0 to 5 nm and length of ~ 50–100 μm obtained by chemical vapor deposition method [7]. Then, FWCNTs were covalently modified by carboxyl groups. We used the suspension of carbon nanotubes obtained by dispersing of FWCNTs (from 400 to 600 mg) in 400 ml of TE buffer or deionized water with resistivity 18.2 $\text{M}\Omega\cdot\text{cm}$. A complexification is performed by ultrasound treatment of a mixture from fluorescent FAM-ON1 with FWCNTs, suspended in TE buffer at various concentrations. Preliminary, it was added 100 μL of suspension of various concentrations of nanotubes in TE buffer to 13.5 μL of oligonucleotide FAM-ON1 ($c_{\text{ssDNA}} = 0.5 \text{ pmol}/\mu\text{L}$) in TE buffer. The reaction mixture was kept in a cuvette at room temperature or sonicated for 1 to 10 minutes (22 kHz). The structural and functional properties of the resulting FWCNT/FAM-oligonucleotide complexes was characterized by using transmission electron microscopy (TEM), PAGE electrophoresis, and the method of heterostructure analysis (HA).

Electron microscopy images are obtained by a transmission electron microscope JEM-100CX TEM (JEOL, Japan) at an accelerating voltage of 100 kV.

The electrophoresis was performed in polyacrylamide gel in 0.5-x 0.09 M TBE buffer (pH 8.0) at room temperature during 45 min. Control of the electrophoretic system electrical parameters which are reset by power, was carried out on the equipment Electrophoresis Power Supply EPS 601 (GE Healthcare Bio-Sciences AB, Sweden). PAGE electrophoresis was repeated at least 3 times.

3. Results and Discussion

3.1. *Structural analysis* The original carbon nanotubes are shown in figure 1a. TEM images of complexes FWCNT/ssDNA in figures 1b and 2 demonstrate that FWCNTs were covered by electron-dense layers of self-assembled oligonucleotide. As one can see in figure 1b, molecular crystallites can grow epitaxially on the surface of electron-dense layers of complexes FWCNT/ssDNA fabricated in deionized water. The TEM images of complexes FWCNT/ssDNA fabricated in TE buffer was used to estimate the dependence of thickness l_{DNA} of DNA layer included in the complexes, upon FWCNT concentration c_{FWCNT} , used at fabrication of these complexes. Measured values of thickness of the oligonucleotide shell in the complex are presented in table 1.

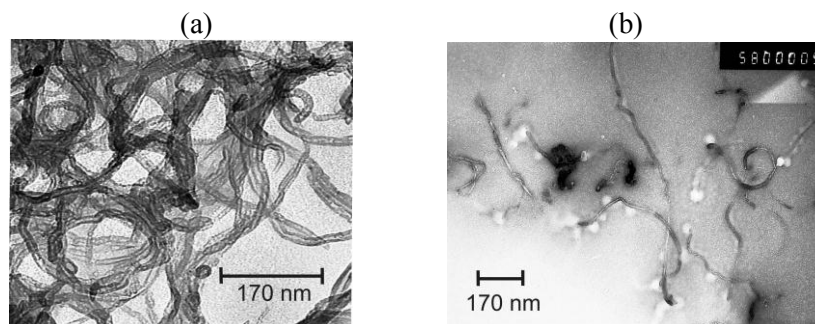


Figure 1. (a) TEM-image of original FWCNTs. (b) TEM-image of complexes ssDNA/FWCNT in deionized water.

According to table 1, l_{DNA} takes on the highest values at low c_{FWCNT} equals to 0.05 mg/ml. Due to the remoteness, stacking interaction between the upper monolayers of the self-assembled oligonucleotide and CNT is a weak. Therefore, at these concentrations the epitaxial growth of molecular ssDNA crystallite does not occur, and, respectively, there always is the ssDNA probe which is not linked with CNTs (figure 2a). At increasing c_{FWCNT} (0.1 mg/ml and more) the thickness l_{DNA} of FWCNT/DNA is decreases, since the weakly bound oligonucleotide of top monolayers of the complexes is redistributed to other CNTs. For l_{DNA} equal to 2.5 nm and less the increased stacking interaction of self-assembled ssDNA with CNT is able to hold the oligonucleotide molecules involved in the process of epitaxial growth of monocrystals as one can see in figures 1b and 2b. At high value c_{FWCNT} equals to 0.15 mg/ml due to the decrease l_{DNA} to value 1.2 nm the stacking interaction increases in such an extend DNA and holds the epitaxial DNA polycrystallites (figure 2c).

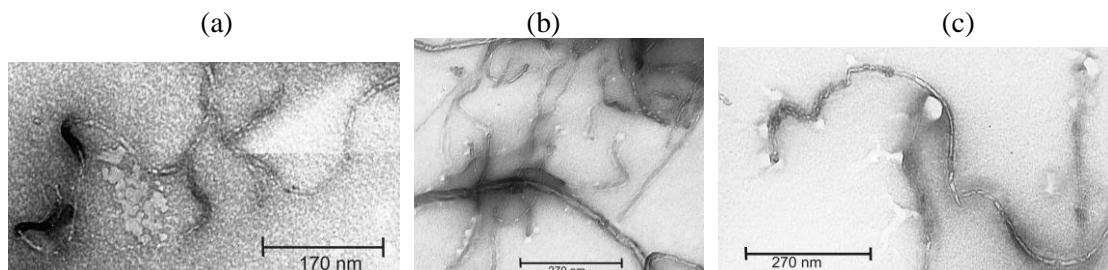


Figure 2. TEM-image of complexes ssDNA/FWCNT which are fabricated at FWCNT concentrations 0.05 (a), 0.1 (b), and 0.15 (c) mg/ml in TE buffer.

3.2. *Fluorescence-Quenching.* The influence of temperature on complexation was estimated assuming inversely proportional dependence of ionic conductivity of the solution on the temperature T , by analogy with the electrical conductivity of solids [8]:

$$\sigma = q^2 Nk / (DT) \tag{1}$$

where q is the electric charge of an ion, k is the reaction rate of ion with counterion, D is a diffusion coefficient, N is the ion density. Using (1), relative temperature of electrophoresis was calculated by the formula

$$\frac{T_m}{T_1} = \frac{W_m I_1^2}{W_1 I_m^2} \tag{2}$$

where $T_m, I_m, W_m, m = 1, 2, 3$ are temperature, electric current and power of m -th electrophoresis, respectively. Three electrophoresis with parameters listed in table 2 are represented by the curves with symbols «●», «■» и «◆» in figure 3 for relative temperatures $\{T_n/T_1\} = \{0.59, 1, 1.26\}$ respectively. As one can see from figure 3, up to concentrations $c_{FWCNT} \sim 0.08$ mg/ml inclusive, the increase of T leads to increase in luminescence intensity I and, respectively, the number of free molecules of FAM-ON1. It is caused by probability decrease of π - π -bonding of a selected oligonucleotide with FWCNT at increase of T due to the increase of fluctuations intensity.

Table 1. Thicknesses l_{DNA} of ssDNA layer in complexes FWCNT/ssDNA in depend on FWCNT concentration c_{FWCNT} utilized at fabrication of these complexes.

$c_{FWCNT}, \text{mg/ml}$	l_{DNA}, nm
0.05	5.3, 3.7
0.1	3.7, 2.5
0.15	3.7, 2.5, 1.25

Table 2. Electrophoresis parameters in depend on the relative temperature at voltage 100 V.

No.	I_n, mA	W_n, W	T_n/T_1
1	19.1	2	1
2	30.5	3	0.59
3	17.0	2	1.26

At high concentrations $c_{FWCNT} \sim 0.1$ and 0.15 mg/ml, when FAM-ON1 molecules start to interact each other, the decrease of relative temperature from 1 to 0.59, contrary, leads to the increase of I , because low diffusion mobility of FAM-ON1 prevent fast relaxation of FWCNT/ FAM-ON1 complex to an equilibrium state with a minimum of the free energy by minimizing interparticle orientational interaction in the oligonucleotide coating, as a result of which part of FAM-ON1 molecules do not have time to link with FWCNTs. However, very high temperatures are also undesirable at high c_{FWCNT} , because they disturb the equilibrium state with the minimum of the free energy.

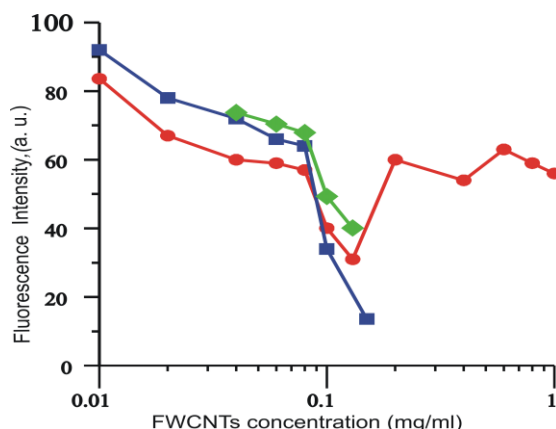


Figure 3. The intensity dependence of fluorescence of FAM-ON1 on FWCNT concentration c . Lines with symbols «●», «■» и «◆» denote dependencies for relative temperatures 0.59, 1, and 1.26 respectively.

At concentration $c_{\text{FWCNT}} \sim 0.15$ mg/ml and fixed value of c_{FAM} (FAM-ON1 concentration) the mean number of free oligonucleotide molecules, which are not linked with carbon nanotubes, decreases practically to zero with formation of FWCNT/ON1 complex (see curve with symbols «■» in figure 3). However, in accord with figure 3 (curve with symbols «●»), effective quenching of FAM-fluorescence in mixture of FWCNTs and ON1 occurs not at all concentrations the oligonucleotide ($c_{\text{FWCNT}} \geq 0.15$ mg/ml), but only for some optimal thicknesses of oligonucleotide coating $l_{st(1)}$, $l_{st(2)}$, $l_{st(3)}$ of carbon nanotube, corresponding to $c_{\text{FWCNT}} \approx 0.15, 0.4, 0.9$ mg/ml. This testifies the existence of a discrete set of FWCNT/ON1 complex sizes with thermodynamically stable structure.

3.3. Hybridization Assays. The method of HA have demonstrated that the presence of complementary DNA target in the solution leads to the dissociation of the FAM-oligonucleotide/FWCNT complexes due to the hybridization with homoduplexes formation. As consequence, the recovering of the fluorophore fluorescence occurs. The intensity of the fluorescence is much more than the fluorescence intensity for free FAM-oligonucleotides. The presence of the target SNP oligonucleotide sequence drops both the fluorescence intensity of the fluorophore and the electrophoretic mobility of heteroduplexes containing single mismatched base.

Figure 4 demonstrates a fluorescence intensity diminishing for FAM-oligonucleotide due to the formation of oligonucleotide hybridization duplex ($c_{\text{FWCNT}} = 0.04$ mg/ml). Forming homoduplexes ON2/FAM-ON1 (lane 6 in figure 4a,b) leaves the surface of ON1/FWCNT complexes, because on DNA-electrophoregram this homoduplexes emits in the field of molecular weights of freely formed homoduplexes (lane 5 in figure 4a,b).

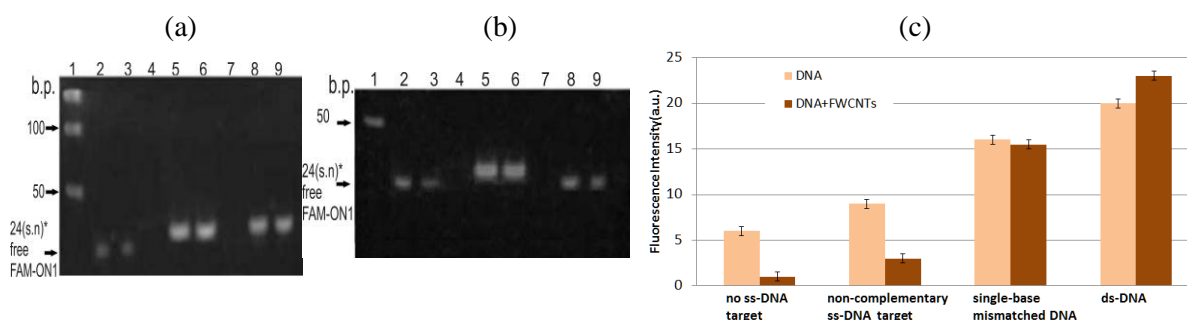


Figure 4. (a) Electrophoregram of DNA hybridization with nucleotide, containing single mismatch. Lanes: 1 – DNA-marker; 2 – DNA probe (fluorescent-oligonucleotide, FAM-ON1); 3 – FAM-ON1/FWCNT complex; 4 – complementary oligonucleotide (ON2) as DNA target, 5 and 6 – FAM-ON1/ON2 homoduplex without and with FWCNT, respectively; 7 – nucleotide with SNP (ON5) as DNA target; 8 and 9 – FAM-ON1/ON5 heteroduplex without and with FWCNT, respectively. (b) Electrophoregram of DNA hybridization with complementary and noncomplementary oligonucleotides. Lanes: 1 – DNA-marker; 2 – fluorescent-oligonucleotide (FAM-ON1) as DNA probe; 3 – FAM-ON1/FWCNT complex; 4 – complementary DNA target (oligonucleotide ON2), 5 and 6 – FAM-ON1/ON2 homoduplex without and with FWCNT, respectively; 7 – noncomplementary DNA target (oligonucleotide ON4); 8 and 9 – FAM-ON1/ON4 heteroduplex without and with FWCNT, respectively. (c) Intensity of FAM-ssDNA (light bars) and complexes FAM-ssDNA/FWCNT (dark bars) fluorescence in the absence of DNA target (left), at the presence non-complementary ssDNA target ON4 and single–base mismatched target ON5 (in center), and at the presence of perfect complementary ssDNA (right). Molecular weight FAM-ON1 of order 24 is given in single-nucleotides (s.n.).

According to figure 4c the FAM-oligonucleotide fluorescence quenching by few-walled CNTs stipulates the significant luminescence intensity differences of homoduplexes, heteroduplex, and free fluorescent oligonucleotide FAM-ON1 in comparison with their fluorescence without quenching.

Since the mobility of homoduplexes with and without FWCNTs are practically undistinguishable, the cooperative binding causes a detachment of epitaxial oligonucleotide along the crystal planes. Remaining in the monocrystal oligonucleotide remains bound to self-assembled FAM-ON1 of FAM-ON1/FWCNT complexes.

The method of HA have demonstrated that the presence of complementary target-DNA in the solution leads to the dissociation of the FWCNT/FAM-oligonucleotide complexes due to the hybridization with homoduplexes formation. As consequence, the recovering of the fluorophore fluorescence occurs. The intensity of the fluorescence is much more than the fluorescence intensity for free FAM-oligonucleotides. The presence of the target SNP oligonucleotide sequence reduces both the fluorescence intensity of the fluorophore and the electrophoretic mobility of heteroduplexes containing a single mismatch.

4. Conclusion

Thus, FAM-oligonucleotide/FWCNT complexes may be used to make highly effective DNA biosensors detecting SNPs which operate as molecular beacons.

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