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Highly sensitive multiplexed DNA detection using multi-walled carbon nanotube-based multicolor nanobeacon

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ABSTRACT

A highly sensitive and selective multi-walled carbon nanotube (MWCNT)-based multicolor fluorescent nanobeacon is developed for multiplexed analysis of DNA in homogeneous solution. In this work, three different dye-labeled DNA hairpins were adsorbed on MWCNTs surface via π-stacking, which brings the dyes and MWCNTs into close proximity and leads to the quenching of fluorescence of the dyes. When target DNAs were added to the solution, the target DNAs specifically hybridize with the probes to form stable DNA duplexes, which weakens the interactions between the probes and MWCNTs, and results in the fluorescence recovery of the dyes. By using three 15-mer DNA fragments as proof-of-principle analytes, the proposed method showed good analytical performance. The limits of detection obtained were in the range of 35–42 pM. Moreover, this method also exhibits an excellent ability to discriminate between single nucleotide polymorphisms.

1. Introduction

Molecular beacons (MBs) are specifically designed stem-loop-structured oligonucleotides, enabling real-time analysis of target nucleic acids via observation of fluorescence alteration [1,2]. Typically, MBs are composed of a DNA hairpin structure that are dual-labeled by a fluorophore and a quencher at the two opposite ends, which brings an attached fluorophore/quencher pair into close proximity, leading to fluorescence quenching. Hybridization to a complementary target opens the stem-loop structure, and separates the fluorophore from the quencher, resulting in the fluorescence restoring of the fluorophore [3]. Compared with linear probes, MBs possess several advantages, such as signal-on sensing and high sequence specificity resulting from the conformational constraint offered by the stem-loop structure [4,5]. Because of these unprecedented merits, MBs have been widely applied in real-time quantitative PCR [6], genetic screening [7], the study of enzymatic processes [8], biochip construction [9], detection of single-nucleotide polymorphisms [10], messenger-RNA monitoring in living cells [11,5] and biosensor design [12]. Although the substantial progress was accomplished, MBs remain still to be improved in many facets including sensitivity and stability improvement [13].

The coupling of the unique optical, electronic and catalytic properties of nanomaterials with high specificity of biomolecular recognition events has been used to develop new-style tools for bioanalysis [14–17]. For example, gold nanoparticles [18–20], semiconductor quantum dots [21], nano-C_{60} [22], nanowires [23] and graphene [24] have extensively been used for development of biosensors. Recently, carbon nanotubes (CNTs) have emerged as one of the most extensively studied nanomaterials due to its unique and excellent optical, electronic, thermal and mechanical properties [25,26]. Specifically, the extraordinary and distance-dependent fluorescence quenching property of CNTs has been used to design various sensors for assaying biomolecules in homogeneous solution [27–31]. Tan and co-workers reported the use of single-wall carbon nanotubes (SWNTs) as a superquencher to develop a biosensing platform for the detection of DNA based on the conformation alteration of dye-labeled DNA for its release from SWNTs upon the recognition binding with target sequence [29]. Similarly, a resuable fluorescent sensor based on fluorescence quenching of SWNTs for highly sensitive and selective detection of silver ion and cysteine was reported [32]. A multi-walled carbon nanotube (MWCNTs)-based molecular aptamer beacon for the analysis of adenosine triphosphate was also described [33]. More recently, Zhang et al. reported an one-pot fluorescence assay method for detecting multiple analytes based on noncovalent assembly of SWNTs and aptamers [34]. Although substantial progress was accomplished, to the best of our knowledge, no study has been reported the development of a CNT-based multicolor nanobeacon for multiplexed detection of nucleic acids in homogenous solution.

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Developments in DNA sensing methodology are of great importance, owing to their versatile applications in gene expression profiling, clinical disease diagnostics and treatment [35]. A variety of methods such as G-quadruplex based hybridization chain reaction [36], the curcumin encapsulated in nanoparticle-assembled microcapsules [37], graphene-based molecular beacon [24], the copper tetraphenylporphyrin complex [38], fluorescence resonance energy transfer (FRET) between quantum dots and graphene oxide [39] and molecular beacon-functionalized gold nanoparticles [40] have been developed for the detection of DNA. Here we report the use of MWCNTs to developing a novel multicolor nanobeacon for sensitive, selective and multiplexed analysis of DNA in homogeneous solution. The proposed nanobeacon combines the high specificity of hybridization reactions of DNAs with the unique fluorescence quenching property of MWCNTs. The introduction of MWCNTs here makes assay much easier to achieve multiplex detection compared with conventional fluorescence methods. Moreover, owing to the ultrahigh fluorescence quenching ability of MWCNTs, the proposed method showed higher sensitivity than that of traditional MBs. In addition, the present assay requires only one labeled fluorophore, which is simple and cost-effective compared to traditional MBs. With the use of three 15-mer DNA fragments as model analytes, the proposed method showed high sensitivity and specificity, and promises a sensitive platform for the multiplexed analysis of nucleic acids.

2. Experimental

2.1. Reagents and apparatus

All DNA oligonucleotides were purchased from Sangon Biological Sangon Biotechnology Co. Ltd. (Shanghai, China) and purified using high-performance liquid chromatography. The sequences of involved oligonucleotides were given in Table 1. H1 was used for assaying T1 and T4, H2 was used for detecting T2 and T5, and H3 was used for analyzing T3 and T6. MWCNTs were purchased from Shenzhen Nanotech Port Co. Ltd. (Shenzhen, China). All DNA oligonucleotides were diluted in Tris–HCl (10 mM, pH 8.0) buffer solution to give stock solutions of 100 μM DNA. The hybridization reaction buffer was phosphate-buffered saline (137 mM NaCl, 2.5 mM Mg^{2+}, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.4). Fluorescence spectra were measured on a LS-55 fluorescence spectrophotometer (Perkin–Elmer, USA).

2.2. Pretreatment of MWCNTs

Since oxidized CNTs contain highly delocalized π electrons, the surface of CNTs can be easily functionalized through π−π interactions with compounds that possess a π-electron-rich structure [13]. The commercial MWCNTs were first oxidized and purified as described previously [41]. Briefly, the MWCNT (200 mg) was first refluxed in HNO_3 (150 mL 2.0 M) for 2 days. After being kept overnight, the clear solution above the suspension was removed. The suspension was centrifuged at 14000 rpm for 30 min, and the clear solution over the precipitates was removed. The resulting precipitates were oxidized by 40 mL of HNO_3/H_2SO_4 ($VHNO_3:VH_2SO_4=1:3$) in an ultrasonic bath for 4 h. Then, the suspension was diluted 10-fold by water and allowed to stand for 12 h at room temperature. After removal of the clear solution over the precipitates, the remaining solution was filtered through a 0.45 μm filtration membrane and then washed with water to neutral pH. The resulting precipitates were kept in an oven at 50 °C for 5 h to remove the water. The oxidized MWCNTs were redispersed in water to give a final concentration at 1 mg/mL.

Table 1
DNA sequence used in this work.

Oligonucleo-tides name	Sequences (5' to 3') description	Description
FAM-H1	FAM-CAGAG ACG ACT G TT TCC GCG CTCTG	Stem underlined
ROX-H2	ROX-CTCCT GAG GCA GT A TTG CGG AGGAG	Stem underlined
Cy5-H3	Cy5-CCTAC GCC ATA GCC CTC CCG GTAGG	Stem underlined
T1	CGC GGA AAC AGT CGT	Complementary DNA
T2	CCG CAA TAC TGC CTC	Complementary DNA
T3	CGG GAG GGC TAT GGC	Complementary DNA
T4	CGC GGA ATC AGT CGT	Mismatch underlined
T5	CCG CAA TTC TGC CTC	Mismatch underlined
T6	CGG GAG GCC TAT GGC	Mismatch underlined

2.3. Preparation of human sera samples

Human blood samples were kindly provided by the No. 5 People's Hospital (Guilin, China). Human blood samples were centrifuged at 2000 rpm for 15 min to obtain serum. These samples were stored at −20 °C until analysis, and diluted 100 fold with phosphate-buffered saline (137 mM NaCl, 2.5 mM Mg^{2+}, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.4) before analysis.

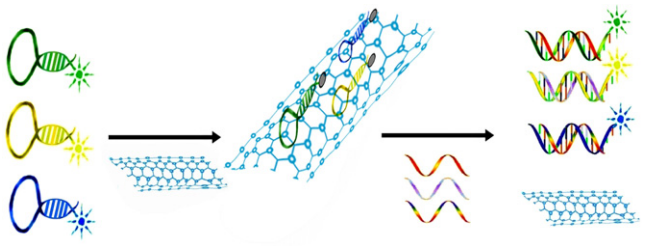
2.4. Fluorescent assays

In a single-analyte sensing format, the working solutions containing 50 nM FAM-labeled H1 and 40 μg/mL MWCNTs in 990 μL hybridization reaction buffers was first incubated for 1 h at room temperature. After that, 10 μL of target T1 or its mismatched target solution was added to the above solution and further incubated for 4 h at room temperature. Fluorescence emission from the resulting solution was measured at 520 nm. For multiplexed detection, 990 μL of working solution containing three kinds of dye-labeled hairpin-structured oligonucleotides (each 50 nM) and 40 μg/mL of MWCNT were first incubated for 1 h at room temperature. Then a mixed solution of three different DNA targets (10 μL) was added to the working solution and further incubated for 4 h at room temperature. The fluorescence signals were measured at three wavelengths: 520, 607 and 665 nm by exciting the sample solutions at 493 nm for fluorescein amidite (FAM), 570 nm for 6-carboxy-X-rhodamine (ROX), and 645 nm for cyanine dye 5 (Cy5). All experiments were repeated three times, and each sample solution was measured five times. Slits for both the excitation and the emission were set at 5 nm for all dyes.

3. Results and discussion

3.1. Assay principle

Scheme 1 outlines the working principle of the MWCNT-based multicolor nanobeacon for multiplexed detection of DNA. In a single-color mode, FAM-labeled hairpin-structured oligonucleotide (FAM-H1) acting as signaling probe is first adsorbed onto MWCNTs by means of π-stacking interactions between nucleotide bases and the carbon nanotube sidewalls [42], forming a single-color nanobeacon. This adsorbtion process brings the reference dye FAM to the close proximity of MWCNTs, which leads to the quenching of the fluorescence of the dye FAM. In the presence of specific target, the target hybridizes with the signal probe, and forms a DNA/DNA duplex which releases from the surface of MWCNTs, leading to fluorescence recovery. In the multicolor mode, three different hairpin-structured oligonucleotides (H1, H2 and H3) that labeled at their 5' terminus



Scheme 1. Schematic illustration of MWCNTs-based multicolor nanobeacon for multiplex detection of DNA.

with different fluorescence dyes are mixed at equal molar ratio and co-adsorbed on MWCNTs surface, forming a multicolor nanobeacon. The choice of three fluorescence dyes relies on the consideration of their spectral properties, with H1 labeled with FAM, H2 with ROX, and H3 with Cy5. In the absence of the target DNAs, the multicolor nanobeacon stays in the "OFF" state, all dyes are in close proximity to MWCNTs, which results in significant fluorescence quenching due to the energy transfer effect. The common absorption spectrum of a typical MWCNT sample spans a wide range of wavelengths (approximately 500–900 nm) [44], significantly overlapping the photoluminescence spectra of various fluorophores. This allows FRET to occur. FRET involves the transfer of energy from a photoexcited donor fluorophore to a ground state acceptor MWCNT in the proximity of the donor [45,13]. This type of energy transfer can occur when a donor photon is not radiated but is reabsorbed by an acceptor. When the multicolor nanobeacon is exposed to the target DNAs, specific hybridization reactions occur and form stable DNA/DNA duplex which is released from the MWCNTs substrate, resulting in the fluorescence recovery. By monitoring the increase in the fluorescence intensities of the respective dyes, multiple DNA targets were simultaneously detected. Remarkably, this new MWCNTs-based multicolor nanobeacon need only one labeled fluorophore for each target, which is simple and cost-effective compared with traditional MBs. Moreover, the use of MWCNTs as both a "nanoscaffold" for the single-strand oligonucleotides and a "nanoquencher" for the fluorophores makes it much easier to achieve multiplex detection with a high sensitivity compared with traditional MBs.

3.2. *Fluorescence quenching efficiency of MWCNTs*

To confirm the quenching efficiency of MWCNTs to the fluorescence of dye-labeled hairpin-structured oligonucleotides, the change of fluorescence intensity for three dye-labeled hairpin-structured oligonucleotides (FAM-H1, ROX-H2, and Cy5-H3) caused by MWCNTs were investigated. In these experiments, different concentrations of MWNTs were incubated with the dye-labeled hairpin-structured oligonucleotides, and the fluorescence intensities of FAM at 520 nm, ROX at 607 nm and Cy5 at 665 nm were measured. As show in Fig. 1, FAM-H1, ROX-H2 and Cy5-H3 (each 50 nM) have strong fluorescence emissions in the absence of MWCNTs. However, upon incubation with MWCNTs, the fluorescence intensity of all dye-labeled hairpin-structured oligonucleotides decreased with the increase of the concentration of MWCNTs, and the fluorescence quenching was well correlated to the relative amount of dye-labeled hairpin-structured oligonucleotides adsorbed on the MWCNT surface, which was due to more dyes brought closer to the quencher MWCNTs. It was also found that the fluorescence intensity remained almost unchanged when the concentration of MWCNTs were larger than 40 μg/mL. The maximal quenching efficiencies were about 99.0% for FAM, 98.9% for ROX, and 98.6% for Cy5.

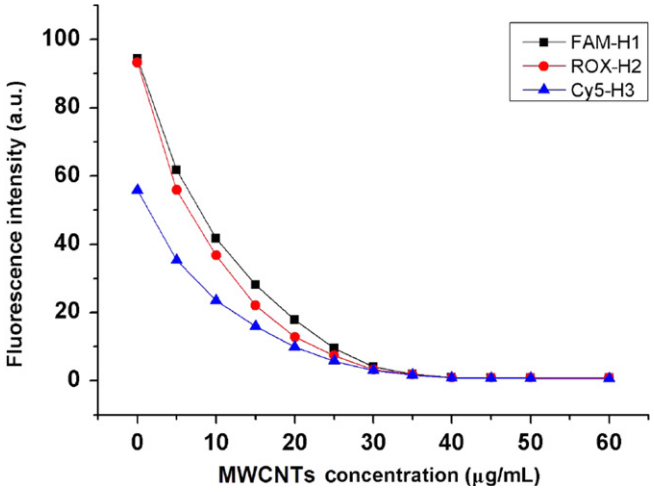


Fig. 1. Effect of MWCNTs concentration on the quenching efficiency of the fluorescence.

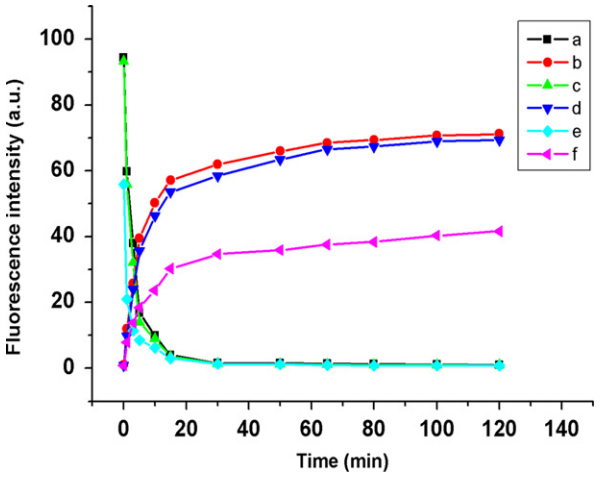


Fig. 2. Effect of incubation time on the fluorescence quenching (curve a, c, e) and fluorescence restoration (curve b, d, f).

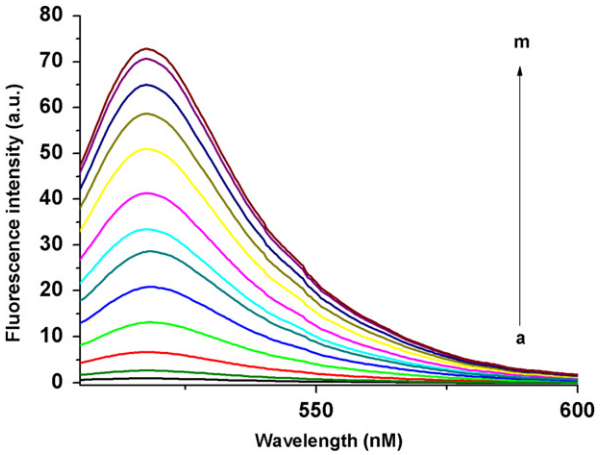


Fig. 3. Fluorescence spectra for FAM-labeled single-color nanobeacon in the presence of T1 at different concentrations: a. 0; b. 0.2; c. 0.5; d. 1; e. 2; f. 5;. G. 8; h. 20; i. 50; j. 100; k. 200; l. 500; m. 800.

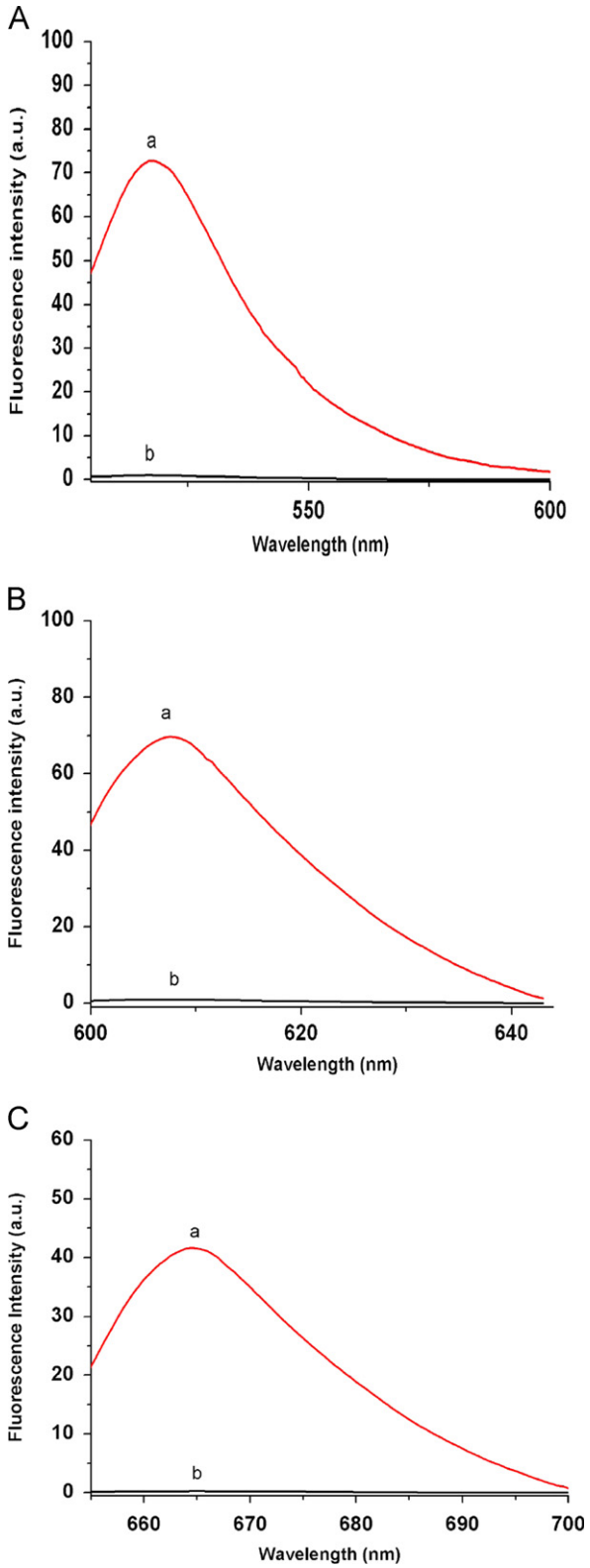


Fig. 4. Fluorescence spectra for a multicolor nanobeacon in the absence (curve b) and presence (curve a) of 800 nM of target: (A) T1; (B) T2 and (C) T3.

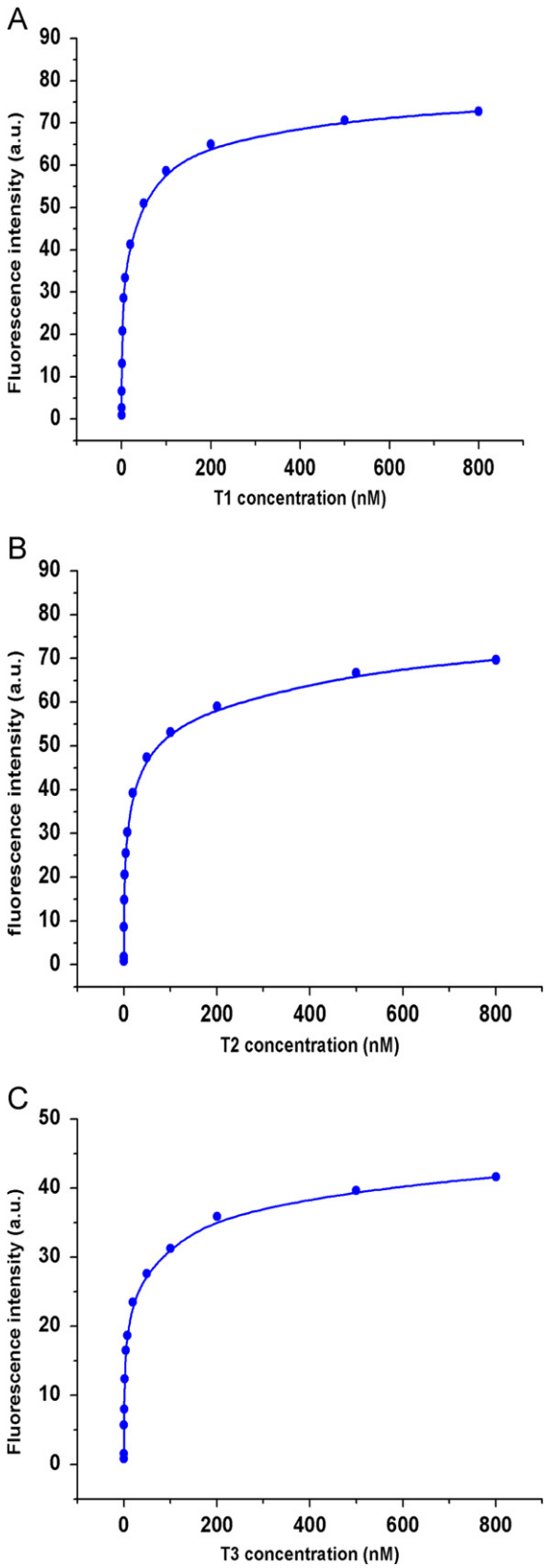


Fig. 5. Plots of fluorescence intensity versus the concentration of perfect complementary target: (A) FAM-H1; (B) ROX-H2 and (C) Cy5-H3.

3.3. Kinetic characterization of fluorescence quenching and fluorescence restoration

To understand the kinetic properties of the fluorescence quenching of dye-labeled hairpin-structured oligonucleotides by MWCNTs and the subsequent fluorescence restoration after adding the specific targets, we monitored the fluorescence intensity as a function of time. Fig. 2 shows the fluorescence quenching of FAM-H1, ROX-H2 and Cy5-H3 in the presence of MWCNTs as a

Table 2
Comparison of methods for the analysis of DNA.

Method	Linear range (nM)	Detection limit (nM)	References
Gold-nanoparticle-based multicolor nanobeacons for sequence specific DNA analysis	0.50–200	—	[43]
Molecular beacon-functionalized gold nanoparticles as probes in dry-reagent strip biosensor for DNA analysis	0.25–50	0.050	[40]
FRET between quantum dots and graphene oxide for sensing biomolecules	50–1500	12	[39]
Graphene-based molecular beacon for homogeneous DNA detection	1.0–120	1.0	[24]
Fluorometric detection of ssDNA by G-quadruplex based hybridization chain reaction	20–120	4.0	[36]
MWCNT-based multicolor Fluorescent nanobeacon for Multiplexed analysis of DNA	0.20–800	0.042 0.038 0.035	This work

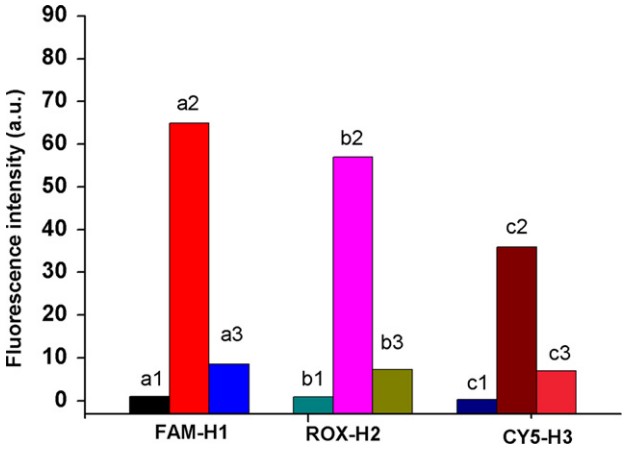


Fig. 6. Specificity of the nanobeacon for DNA detection. (a1/b1/c1): FAM-H1/ROX-H2/Cy5-H3 (50 nM) + MWCNTs (40 μg/mL); (a2/b2/c2): FAM-H1/ROX-H2/Cy5-H3 (50 nM) + MWCNTs (40 μg/mL) + T1/T2/T3 (each 200 nM); (a3/b3/c3): FAM-H1/ROX-H2/Cy5-H3 (50 nM) + MWCNTs (40 μg/mL) + T4/T5/T6 (each 200 nM).

function of incubation time. As can be seen, the fluorescence intensity exhibits a rapid reduction in the first 5 min for all dye-labeled hairpin-structured oligonucleotides, and a slow decrease over a period of 15 min. After 30 min, the fluorescence intensity reaches least value and remained unchanged. However, in the presence of the specific targets, with the formation and release of the double-stranded DNA from MWCNTs, the fluorescence intensity exhibits a rapid increase in the first 20 min, followed by a slow process over a period of 60 min. The best fluorescence restoration was achieved after 4 h incubation time. Therefore, the incubation time of 4 h was selected for further applications.

3.4. Single-color sensing mode

The MWCNT-based single-color nanobeacon was evaluated by fluorescence assays of single DNA target. In the absence of the target, the single-color nanobeacon only exhibited low background fluorescence (Fig. 3). However, upon addition of perfect complementary target (T1), the nanobeacon was turned on in response to the hybridization of FAM-H1 with the target T1, which led to a significant increase in the fluorescence of FAM at 520 nm. The fluorescence enhancement was attributed to the release of the formed DNA/DNA duplex from MWCNTs surface, and weakens the interaction of FAM–MWCNTs. Furthermore, as the concentration of the target T1 was higher, the fluorescence of FAM was intensified (Fig. 3). It should be pointed out that the fluorescence intensity stopped increasing when the concentration of T1 reached 800 nM. These results indicated that the single-color nanobeacon could be used to detect a single DNA target by monitoring the fluorescence signal change of the fluorophore.

3.5. Multicolor sensing mode

The performance of the MWCNTs-based multicolor nanobeacon was evaluated by multiplexed fluorescence detection of three different DNA targets, T1, T2 and T3. In the absence of the DNA targets, the multicolor nanobeacon emited very low background fluorescence at all three wavelengths corresponding to the dyes FAM (λ_{max}=520 nm), ROX (λ_{max}=607 nm), and Cy5 (λ_{max}=665 nm). It was found that the addition of a specific DNA target turned on the nanobecon and led to significantly enhanced fluorescence emission at the respective wavelength. When a solution containing all three DNA targets was assayed, fluorescence emission at all three wavelengths was intensified. That is, T1 enhanced the emission of FAM-H1 at 520 nm, T2 enhanced the emission of ROX-H2 at 607 nm, and T3 enhanced the emission of Cy5-H3 at 665 nm (Fig. 4). The fluorescence enhancement was attributed to the formation of the DNA/DNA duplexes and releasing them from the surface of MWCNTs. The fluorescence enhancement at the respective wavelength was shown to be dependent on the concentration of the specific DNA targets. Fig. 5 shows the calibration curves corresponding to the fluorescence increase of the respective dyes upon different concentrations of the specific DNA targets. As the concentration of the specific DNA targets increased, the fluorescence of the respective dyes increased. The increase in fluorescence intensity was in the range of 52–74 times for the different dyes, which reflected the difference in hybridization efficiency of each target with its dye-labeled probe, the different ratio of each hairpin DNA at the MWCNTs surface, and the difference in quantum yields of each dye. This method enables the analysis of the DNA targets with detection limits corresponding to 42 pM T1, 38 pM T2 and 35 pM T3, which is more sensitive than most of the methods reported for

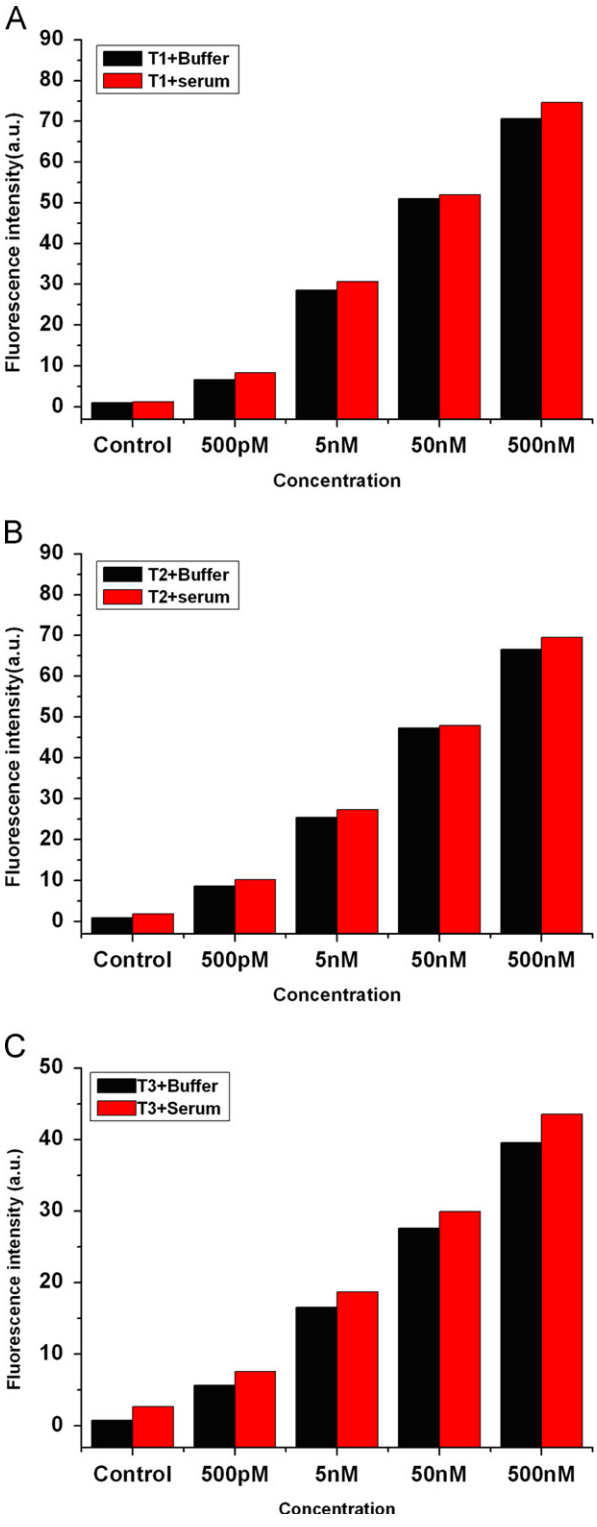


Fig. 7. Results obtained from the testing of human sera samples spiked with DNAs T1(A), T2 (B) and T3 (C) (diluted in 1:100 ratio) and DNAs in buffer.

DNA assays (Table 2). The results indicated that the proposed method could be used for the multiplex detection of DNAs with high sensitivity.

3.6. DNA detection specificity

The designed MWCNTs-based multicolor nanobeacon was highly selective since each target DNA only perfectly hybridized with the specific dye-labeled hairpin probe, leading to the increase in the fluorescence of the corresponding dye. With this performance, the multicolor nanobeacon might show an ability to discriminate the perfect target from the mismatched one. Thus, three single-base mismatch DNA targets were tested. As show in Fig. 6, only the specific targets led to a dramatic fluorescence enhancement toward corresponding dyes, while all the single-base mismatch DNA targets led to only minimal fluorescence increase. These results demonstrated that designed MWCNT-based multicolor nanobeacon was highly specific, and exhibited an excellent ability to discriminate between single nucleotide polymorphisms (SNPs).

3.7. Target DNA detection in human serum samples

To demonstrate the feasibility of the practical application of the proposed method, we detected the target DNA in real samples (healthy human serum). The 100 fold diluted serum sample was spiked with 500 pM, 5 nM, 50 nM and 500 nM target DNA, and then analyzed. As shown in Fig. 7, comparable responses were obtained for the detection of target DNA in both buffer and real samples. The fluorescence signal in serum samples were slightly higher than the fluorescence signal measured in buffer solutions. These results indicated the potentiality of the proposed method for real biological sample analysis.

4. Conclusion

In summary, we have developed a novel MWCNT-based multicolor nanobeacon for multiplexed analysis of DNAs. To the best of our knowledge, this work is the first to utilize MWCNTs for a multiplex nucleic acid assay. By taking advantages of high specificity of hybridization reactions of DNAs and the super fluorescence quenching efficiency of MWCNT, the proposed nanobeacon exhibits a high sensitivity toward target DNA with the detection limit of 35–42 pM, which are more sensitive than most of the DNA assays reported. Moreover, this method also exhibits an excellent ability to discriminate between single nucleotide polymorphisms.

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