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DNA-Ag cluster as the sensor of BODIPY isomers and HepG-2 cells

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The isomers of BODIPY exhibit different cytotoxicity in the cancer cure process. A type of homoduplex structure of DNA-Ag NCs was prepared to distinguish the para-substituted-BODIPY (C2) from the meta-substituted-BODIPY (C1) by the fluorescent quenching constants. Intercalation of C2 to the homoduplex and effective energy transfer in the conjugated system formed by the coordination of Ag in DNA-Ag NCs with the N of two pyridines were found to be two key factors for the DNA-Ag NCs to recognize the special isomers of BODIPY and therefore enhance the emission of C2. The high sensitivity of this kind DNA-Ag NCs to total protein from HepG-2 cells make it be a potential sensor for cancer cells with high expression of hypoxia-inducible factor.

Introduction

The DNA-Ag nanoclusters (DNA-Ag NCs), as a new type of fluorescent marker, have attracted significantly attention in biolabelling and chemical/biological sensing due to their fluorescence controll property, highly fluorescent sensitivity, 

For example, a multi-DNA-Ag NCs based on the template containing a recognition sequence and C-rich oligonucleotides had been constructed to detect the change of HIF (Hypoxia-inducible factor) in cancer cell. The fluorescence intensity changes upon the interaction between target substance and oligonucleotides template, which enable a sensitive, in situ detection of the analyte using fluorescence spectroscopy, so DNA-Ag can be used in the detection of single-stranded DNA binding protein (SSB) and complementary DNA.

All of these assays feature fluorescent DNA-Ag NCs that can be synthesized by varying the nucleobase sequence. Because of the high binding affinity of silver toward sulfur, DNA-Ag NCs have been used for detection of biothiols and thiolk containing pharmaceutics.

Xiaoda Yang reported a sensitive method for determination of N-acetylcysteine (NAC) based on the red fluorescence quenching of oligonucleotide-protected silver nanoclusters (Ag NCs) because DNA-template of Ag NCs was partly replaced by NAC.

However, there is no report on the fluorescence DNA-AgNCs to distinguish organic isomers so far. Currently, the fluorescence sensing mechanism of the synthesized DNA-Ag NCs probe could be concluded as follow: 1). Conformation change induced by the target, such as detecting of K+, H+, Cu2+ and hemin; 2). Interfering with the original silver core, for instance, GSH (glutathione) can be detected due to the disturbance of its original core by forming Ag-S bond, 3). Blocking the electron transfer in the DNA-Ag system, such as the detection of single-strand DNA binding protein, hypoxia-inducible factor, aptamer, RNA based on the binding to the partial of DNA template. For example, the anti-thrombin aptamer nucleic acid sequence, which was linked to CdSe/ZnS QDs, was blocked by a complementary nucleic acid to form a duplex. The intercalation of doxorubicin into the blocked duplex structure led to the electron transfer quenching of the QDs. Based on the intrinsic electron-hole transport properties, special conformation and accompanying conformation changes when interacted with drugs, DNA-AgNCs may be used as a probe to sense the special isomer of organic compounds.

4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazas-indacene (BODIPY) derivatives represent a unique class of fluorophores, which has found important applications in chemical biology and biomolecular chemistry. 

Two fluorescence chemosensors based upon conjugation of BODIPY and di(2-picolyl)amine through two benzyl groups as spacers has been found to selectively sense copper(II) ions and imaging mitochondria in vitro. However, the two analogous compounds exhibit different cytotoxicity to HepG-2 cells. Generally, the emission quenching mechanism of EB-DNA system (EB, Ethidium Bromide) is used to evaluate the intercalation of anticancer compounds to DNA. BODIPY derivatives have long-wavelength absorption and fluorescence emission. The interaction of anticancer drug with DNA is very important to analysis of drug cytotoxicity. So, it is possible to use DNA-Ag NCs as a fluorescent probe to analyze the cytotoxicity.
constructed, which are based on a special sequence DNA 5'-C3AC3AC3GC3A-CTACGTGCT-3'. It is interesting to find that the fluorescent DNA-Ag NCs show different fluorescence response on two isomers of BODIPY derivatives (C1 and C2, Scheme S1), which have different anticancer activities. A new mechanism for the fluorescence sensing, has been suggested. Moreover, the near-infrared fluorescent DNA-Ag NCs show high sensitivity for the total protein from HepG-2 cells due to the specific homoduplex structure of DNA-Ag NCs.

Results and discussion

Characterization of DNA-based silver nanoclusters

A new kind of DNA-based silver nanoclusters has been successfully synthesized using 5'-C3AC3AC3GC3A-CTACGTGCT-3' as a template. The size of DNA-Ag NCs is in the range of 0.5–2 nm (Fig. S1A), the main emission maximum is at 770–800 nm exciting at 730–750 nm (Fig. 1). The UV absorption peaks of Ag NCs are at 350, 540 and 720 nm. The peaks at 350 nm and 720 nm correspond to the distinguishing absorption of Ag NCs, while the peak at 540 nm is from the surface Plasmon resonance of silver nanoparticles. The CD spectra show that the free DNA contains a positive band at 286 nm and a negative bands around 245–260 nm (Fig. 2). It is reported that the negative band at 245 nm and the positive bands around 260–280 nm is characteristic bands of B-form, and the antiparallel G-quadruplexes are characterized by a positive band at 290 nm and a negative band at 260 nm. So we deduce the free DNA is a B-form DNA with C-, G- quadruplexes. Then, the conformation of DNA has changed greatly after the synthesis of DNA-Ag NCs.

The positive band become smaller and the negative band become deepening. The former indicates the presence of guanine–guanine stacking and the destroyed of C-quadruplexes caused by Ag-N bonds between the Ag and DNA which was demonstrated by the peak at 240 cm⁻¹ in the Raman spectra (Fig. S1B). The latter indicates the inter-strands adenine–adenine interaction mediating a duplex formation. Also, the slightly acid buffer (citrate buffer pH 5.0) and the sequence GpA (5'-CTACGTGCT-3') are important for the formation of the parallel homoduplex. So, the main conformation of DNA in the DNA-Ag NCs is parallel homoduplex. The synthesis process of DNA-Ag NCs can be deduced as scheme 1. When heating the DNA solution, the hydrogen bond of base pairs on DNA would break, resulting in a free state DNA. So a high loaded-Ag⁺ DNA complexes were obtained after the temperature shifting from 71(Tm of the template oligonucleotides) to 25 °C. The newly formed complex of DNA-Ag⁺ was reduced to DNA-Ag by NaBH₄ and then gathered to form fluorescence homoduplex-DNA-Ag NCs at 4°C.

![Scheme 1](image)

**Scheme 1** The synthesis chart of DNA-Ag NCs: **a** mix DNA and Ag⁺ at 71°C, 1 min, Ag⁺ (200 µM); **b** cooling to 25°C, 3 h; **c** adding NaBH₄ (400 µM), and then kept it at 4°C in the dark for 3days.

In order to study whether there is a energy transfer between substituted BODIPY (C1 and C2) and DNA-Ag NCs, we have measured fluorescence sense of DNA-Ag NCs for 8-[di(2-picolyl)amine-3-benzyl]-4,4-difluoro-1, 3, 5, 7-tetramethyl - 4 - bora - 3a, 4a-diaza-s-indacene (C1) and 8-[di(2-picolyl)amine-4-benzyl]-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (C2) (Fig. S2). The two compounds (C1 and C2) have effect on the emission DNA-Ag NCs at 770 nm. The quenching constants of C1, C2 are about 0.179, and 0.84 respectively, indicating that C2 decreased the emission much more greatly than C1. So the synthesized fluorescent probe can be used to distinguish the isomers in the range of 4-80 µM (Fig. S3).

In order to study the mechanism, the effect of DOBIPY derivatives with the 8-(N-benzyl-8-hydroxyquinolinium)-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene chloride (C3) and 8-(N-benzyl-4-acetylpyridium)-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene chloride (C4) on the fluorescence had been tested. It is interesting to found that that all of them (C3-C5) show weak effect on the emission DNA-Ag (770 nm) and even much smaller than C1. The quenching constants of them are 0.085, 0.063 and 0.01 respectively (Fig.3A). So, we deduce that BODIPY section in C2 or pyridyl groups itself are not the main reason for the quenching. According to the Hammett equation, the para-electron substitution is better than the meta-substitution. 

![Fig. 1](image)

**Fig. 1** UV-vis absorption spectrum and fluorescence spectra of fluorescent DNA-Ag NCs: **a** is the UV-vis absorption spectrum, the NCs; **b** and **c** are the excitation and emission fluorescence spectra, respectively, λ ex/em = 560/617 nm, 730/770 nm.

![Fig. 2](image)

**Fig. 2** CD spectra of DNA-Ag NCs in 0.5 mM citrate buffer (pH 5.0): (solid line) no Ag; (dash line) DNA-Ag NCs.

The sensitivity for BODIPY derivatives and mechanism

The fluorescence of DNA-Ag NCs was then used to detect the DNA and total protein of HepG-2 cells. The fluorescence quantum yield of the DNA-Ag NCs is 0.053, which was measured by a comparison method with fluorescein as the standard. To compare the quenching effect of different protein, we used these protein in the 0.1 mM citrate buffer (pH 5.0) and the DNA-Ag NCs were added to the mixture. The quenching constants of DNA-Ag NCs in the presence of different proteins were obtained and the results are shown in Table 1. The quenching constant of DNA-Ag NCs was calculated by the formula of Stern-Volmer quenching constant. The results showed that the quenching constant of DNA-Ag NCs was increased with the increase of protein concentration.
in facilitating the electron transfer which leads to the quenching of the luminescence of the quantum dots,\textsuperscript{13,19} therefore, we deduce that the para-substituted group (di(pyridyl)methyl amine) in benzyl group of C2 lead to a higher electron transfer efficiency from the DNA-Ag NCs to the BODIPY section than the meta-substitued of C1, resulting the emission at 770 nm (Scheme 2 ). The positive (275–285 nm) and negative (245 nm) bands in CD spectra move up integrally, reflecting the change of spiral in double helix resulting from the embedding of the compounds C1 or C2 (Fig. S4 A, D).\textsuperscript{20d} In order to study the action mode of the compounds, the induced circular dichroism (ICD) of the compounds had been tested. In ICD spectra of DNA-Ag-C1 system (Fig. S4 B, C), the multi positive peaks around 300–450 nm are caused by the binding of C1 to the major groove of DNA-Ag NCs which can be bonded in multi directions.\textsuperscript{20e} The affinity of C1 to the major groove by the format of Genin dimers or oligomers was further demonstrated by the appearance for one negative band at 495 nm and one positive band at 505 nm (Fig. S4 C).\textsuperscript{20d} However, the C2 can intercalate to the DNA-Ag NCs with the appearance of one negative band at 395 nm in the ICD spectrum of DNA-Ag-C2 complexes (Fig. S 4E).\textsuperscript{20e} So the quenching constants may be affected by the action mode of the compounds to DNA-Ag NCs.

![Image](https://example.com/image.png)

**Scheme 2** The different electron transfer between meta-dpa-BODIPY (C1) and para- dpa-BODIPY (C2).

Next, the influence of DNA-Ag NCs on the fluorescence of the compounds has also been tested. The results are shown in Fig.3B. The emission of C1, C2 and C3 increased with the addition of DNA-Ag NCs indicating different energy transfer from DNA-Ag NCs to electron donating group BODIPY (C1, C2, C3). The significant increasing emission of C2 at 508 nm is leaded by the quenching of DNA-Ag (at 770 nm) by transferring energy to C2. However, the decreasing emission of C4 indicates the influence of electron withdrawing group (carbonyl group) in BODIPY on the energy transfer between BODIPY section and DNA-Ag NCs. For the sake of a better understanding of the transfer mechanism, we make the following speculation: In addition to the cytosine’s N3 atom, the Ag can coordinate to the both N atoms of the pyridines from the dpa-DOBIPY, forming the conjugated system, which can facilitate the energy transfer from the DNA-Ag NCs to C1/C2. This conjugate system is one important factor for the increasing emission of C1/C2 with two pyridines, which is two times larger than that of C3 with one pyridine (Scheme 3).

![Image](https://example.com/image.png)

**Scheme 3** The conjugate system formed by DNA-Ag NCs and compounds.

**The sensitivity for HIF in total protein from HepG-2 cell**

Hypoxia-inducible factor (HIF) is the core transcription factor in regulating the oxygen homeostasis and plays an important role in the formation of cancer cell.\textsuperscript{21b} It is well known that most of cancer cells are in an oxygen deficient environment due to the exuberant metabolism. So HIF-1 is more highly expressed in most cancer cells than in normal cell, especially for HepG-2 cells. However, its amount is still not enough to be tested by an efficient method other than electrophoresis which is complicated and expensive. So, it is important and necessary to develop a simple and economic method to detect HIF. The DNA sequence containing 5’-RCGTG-3’ was identified as HIF-1 specific binding sites on the Hypoxia response element (HRE) of many different metabolic related enzyme genes.\textsuperscript{21b, c} The sensitivity of synthesized DNA-Ag NCs had been studied. The fluorescence emission of DNA-Ag NCs decreased with the increasing concentration of total protein from HepG-2 (Fig. S5). Although both of the total protein from HepG-2 and normal cell can decrease the emission of DNA-Ag NCs, the fluorescence quenching constant of total protein in HepG-2 cells is 0.27009, which is obviously larger than that (0.1106) in normal cells (Fig. 4A). So, the designed DNA-Ag NCs can detect the HIF in total protein from HepG-2. In addition, the sensitivity of different DNA-Ag has been compared. As shown in Fig.4B, the quenching constants of 10 µg/mL total protein from HepG-2 cells for DNA-Ag NCs in this report (0.27009) is more than 10-, 15-fold comparing with the multi-DNA-Ag NCs in the former report (0.02326).\textsuperscript{4} The sensitivity of DNA-AgNCs for HIF has been improved significantly. Based on the binding mode between 5’-RCGTG-3’ and HIF, two G-residue in 5’-CTACGTGCT-3’ binding to the major groove of HIF,\textsuperscript{21d} and the special homoduplex structure, we deduce that the formation of homoduplex DNA make the G-residue extruded and facilitate it binding to HIF. So, the fluorescent sense was enhanced finally.
A near-infrared fluorescent DNA-Ag NCs with parallel homoduplex conformation and an emission at 770–800 nm has been constructed using 5'-kC3AC3AC3GC3AkCTACGTGCTk3' as a template. The para-electron donating group, intercalation of C2 to the homoduplex and the conjugated system, which are formed by the coordination of Ag in DNA-AgNCs with the N of two pyridines from C2, make the emission of C2 at 508 nm formed by the coordination of Ag in DNA-AgNCs with the N of DNA-Ag NCs for sensing HIF. Overall, this kind of DNA-Ag NCs can serve as a probe to discriminate C2 from C1 based on the different fluorescence quenching constants of the probe. Based on the different toxicity for C1 (IC50 > 100 μM) and C2 (IC50 11 μM) to HepG2 cells (It has been reported, the result was shown in Fig. S6), we deduce that the DNA-Ag NCs may be used as a probe to explore the possible toxicity mechanism of anticancer BODIPY derivatives. Moreover, the sensitivity of this kind of DNA-Ag NCs for total protein from HepG2 cells with high expression of HIF, has been improved significantly, laying a foundation for the wildly use of DNA-Ag NCs for sensing HIF. Overall, this kind of DNA-Ag NCs open a new application area for the noble metal nanoclusters in sensing cancer cells or as a probe to explore the interaction of the drug with DNA.

**Experiment**

**Material**

DNA oligomer was purchased from Sangon Biotech. Its base sequence was 5'-C3AC3AC3GC3A-CTACGTGCT-3'. Silver nitrate (99%, A.C.S. reagent), citrate acid, sodium hydroxide, NaBH4 (power, 96%) were all obtained from Sinopharm Chemical Reagent Co. Ltd. 8-[di(2-picolyl)amine-3-benzyl]-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (C1), 8-[di(2-picolyl)amine-4-benzyl]-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (C2) were synthesized as reported. The synthesis procedure of 8-(N-benzyl-8-hydroxylquinolinium)-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene chloride (C3) and 8-(N-benzyl-4-acetylpyridinium)-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene chloride (C4) were shown in supported materials. 4,4'-dimethyl-2,2-dipyridine (C5) was purchased from Sigma Aldrich Co. Ltd. The structure of C1, C2, C3, C4, C5 were shown in Scheme S1. Water was purified with a Millipore Milli-Q system (25°C: 18.2 MΩcm, 7.2×102 N m⁻¹).

**Synthesis of DNA-Ag NCs**

Silver clusters were synthesized by combining DNA (5'-C3AC3AC3GC3A-CTACGTGCT-3') and Ag⁺ solutions in a 10 mM citrate buffer at pH = 5. The final concentration of AgNO₃ and DNA is 200 μM and 13 μM, respectively. Then the DNA-Ag⁺ was heated to 71°C, and stayed for 2 min, followed by slow cooling to the room temperature. The whole cooling process is about 2 h. An aqueous solution of NaBH₄ was added to give a final concentration of 2 BH₄⁻/Ag⁺ at room temperatures, and the resulting solution was vigorously shaken for 1 min, kept standing for 3 days in the dark at 4°C.

**Characterization**

The electronic absorption spectrum was record using a UV-2450 UV-visible spectrophotometer at room temperature. Photoluminescent emission spectra were measured on an American’s Varian Carry Eclipse spectrophotofluorometer. TEM was performed at room temperature on a JEOL JEM-200CX transmission electron microscope using an accelerating voltage of 200 kV. Circular dichroism (CD) spectra were measured on Jasco J-815 spectropolarimeter at room temperature. The resonance Raman spectra of the samples were obtained in the 100-1800 cm⁻¹ range using a Jobin-Yvon Ramanor HG-2S double monochromator operating in the second order of gratings and exciting by 532 nm excitation line with the average laser power of 5 mW.

**Extraction of the total protein in the cells.**

First, 2×10⁶ cells, which are normal cells and HepG-2 cell, were collected and centrifuged at 1,000 g, washed by PBS. Then the precipitation was lysed in 100 μL precooled lysis buffer containing 0.5% Triton X-100, 100 mM Tris–HCl, 150 mM NaCl, 0.1 U/mL aprotinin for 30 min on ice and centrifuged at 12,000 g for 2 min. The supernatant collected is the total protein.

**Analysis of compounds and HIF**

1μM DNA-Ag NCs in solution of citrate buffer (10 mM, pH 7.0) and varying quantity (as shown in figures) of (C1), (C2), (C3), (C4) and (C5), and total protein from normal cell (WRL-68) and HepG-2 were kept at room temperature for 10 min. Then the mixtures were subjected to fluorescence measurement.

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**Notes and references**

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