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ARTICLE TYPE

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

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5 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-substitued-BODIPY (C2) from the meta-substitued-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
10 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
15 fluorescent marker, have attracted significantly attention in bio-labelling and chemical/biological sensing due to their fluorescence controll property, highly fluorescent sensitivity.[1,2,3] For example, a multi-DNA-Ag NCs based on the template containing a recognition sequence and C-rich oligonucleotides
20 had been constructed to detect the change of HIF (Hypoxia-inducible factor) in cancer cell in vitro.[4] 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
25 DNA-Ag can be used in the detection of single-stranded DNA binding protein (SSB) and complementary DNA.[5] 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
30 detection of biothiols and thiol- containing pharmaceutics.[6] Xiaoda Yang reported a sensitive method for determination of N-acetylcysteine (NAC) based on the red fluorescence quenching of oligonucleotide-protected silver nanoculsters (Ag NCs) because DNA-template of Ag NCs was partly replaced by NAC[7].
35 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
50 target, such as detecting of K^+, H^+, Cu^{2+} and hemin; [8,9,10] 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, [5,6] 3). Blocking the electron transfer in the DNA-Ag system, such as the detection of single-strand
55 DNA binding protein, hypoxia-inducible factor, aptamer, RNA based on the binding to the partial of DNA temple. [1,4,11,12]. Electron transfer quenching mechanism has been implemented to develop quantum dots (QD)-based aptasensors.[13] For example, the anti-thrombin aptamer nucleic acid sequence, which was
60 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
65 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-diaza-s-indacene (BODIPY) derivatives represent a unique class of fluorophores,
70 which has found important applications in chemical biology and biomolecular chemistry. [14] 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
75 two analogous compounds exhibit different cytotoxicity to HepG-2 cells.[15] Generally, the emission quenching mechanism of EB-DNA system (EB, Ethidium Bromide) is used to evaluate the intercalation of anticancer compounds to DNA. [16a, b] BODIPY derivatives have long-wavelength absorption and fluorescence
80 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. Here, a new type of near-infrared fluorescent DNA-Ag nanoclusters (NCs) with emission at 770~800 nm has been

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constructed, which are based on a special sequence DNA 5'-C3AC3AC3GC3A- CTAC<u>GTG</u>CT-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.
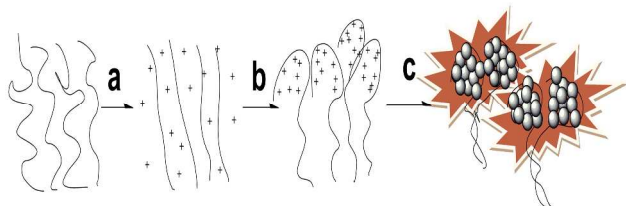
Scheme 1 The synthesis chart of DNA-Ag NCs: **a** mix DNA and Ag$^+$ at 71°C, 1min, Ag$^+$ (200 μM); **b** cooling to 25°C, 3 h; **c** adding NaBH$_4$ (400 μM), and then kept it at 4 ^{o}C in the dark for 3days.

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, [17a] while the peak at 540 nm is from the surface Plasmon resonance of silver nanoparticles. [17b] The CD spectra show that the free DNA contains a positive band at 286 nm and a negative bands at 210 nm, which is the characteristic band of C-quadruplexes, and also contain the negative bands around 240-260 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 later indicates the inter-strands adenine–adenine interaction mediating a duplex formation.[18] Also, the slightly acid buffer (the citrate buffer pH 5.0) and the sequence GpA (5'-CT<u>ACG</u>TGCT-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 ^{o}C. The newly formed complex

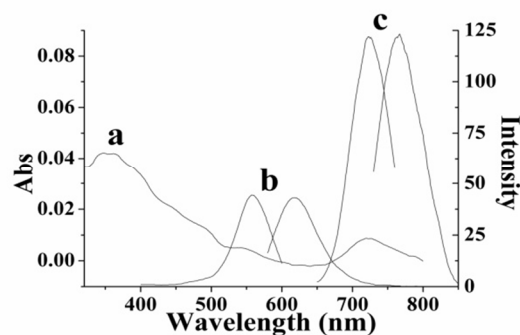of DNA-Ag$^+$ was reduced to DNA-Ag by NaBH$_4$ and then gathered to form fluorescence homoduplex-DNA-Ag NCs at 4 ^{o}C

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
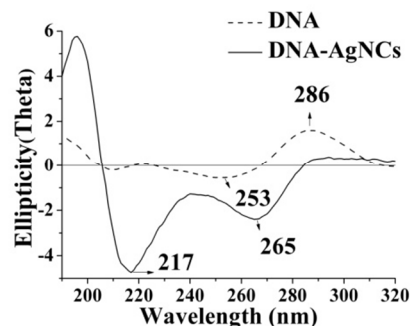

Fig. 2. CD spectra of DNA-Ag NCs in 0.5 mM citrate buffer (pH 5.0): (solid line) no Ag; (dash line) DNA-Ag NCs.

The sensitivity for BODIPY derivatives and mechanism

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-hydroxylquinolinium) -4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza- s-indacene choloride (C3) and 8-(N-benzyl-4-acetylpyridium)-4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza- s-indacene   chloride (C4) and 4,4'-dimethy -2'2-dipyridine (C5) on the fluorescence had been tested. It is interesting to found that that all of them (C3-C5) show weak effect on the emission of 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

in facilitating the electron transfer which leads to the quenching of the luminescence of the quantum dots,[13],[19] therefore, we deduce that the para-substituted group (di(pyridylmethyl) 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-substituted of C1, resulting the emission at 770 nm (Scheme 2). The positive (275~285nm) 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). [20b] 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.[20c] 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).[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).[20e]   So the quenching constants may be affected by the action mode of the compounds to DNA-Ag NCs.
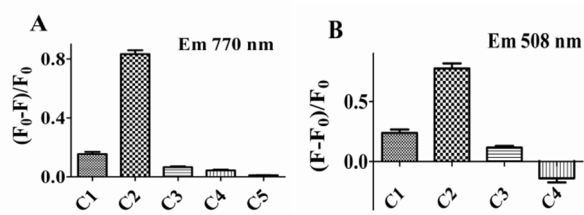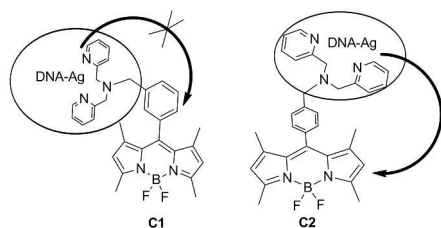

Fig. 3 The quench constant of DNA-Ag NCs (1μM) at 770 nm (ex. 730 nm) in the presence of compounds (C1, C2, C3, C4, C5), the ratio of compound and DNA1-Ag is 6:1. B. the fluorescence increase of compounds (2 μM) at 508 nm, ex.460 nm by adding DNA-Ag NCs, the ratio of compound and DNA-Ag NCs is 2:1.

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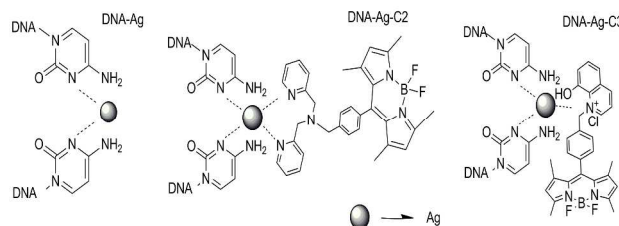
Scheme 3 The conjugate system formed by DNA-Ag NCs and compounds.

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.[21a] 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 gene.[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).[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,[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.
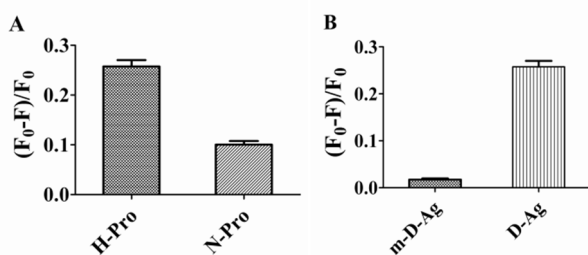
Fig. 4 A The quenching constants of fluorescence 1uM DNA-Ag NCs in the presence of total protein from HepG-2 cells (H-Pro) (10 µg/mL) and normal cell (N-Pro) (10 µg/mL). **B** The quenching constants of fluorescence DNA-AgNCs in the presence of total protein from HepG-2 cells (H-Pro) (10 µg/mL). The adding volume of total protein is 5 uL. D-Ag is DNA-Ag NCs in this paper; m-D-Ag is multi-DNA-Ag NCs.

Conclusions

A near-infrared fluorescent DNA-Ag NCs with parallel homoduplex conformation and an emission at 770~800 nm has been constructed using 5'-C3AC3AC3GC3A-CTACGTGCT-3'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 increased and the emission of DNA-AgNCs at 770 nm decreased greatly by facilitating the energy transfer from the DNA-Ag NCs to C2. The interaction of C1 to the major groove by the Genin dimers has weak effect on emission at 770 nm of DNA-Ag NCs. So, the synthesized 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 ($IC_{50} > 100$ µM) and C2 (IC_{50} 11 µM) to HepG-2 cells (It has been reported, [15] 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 HepG-2 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, $NaBH_4$ (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,[15] The synthesis procedure of 8-(N-benzyl-8-hydroxylquinolinium) -4,4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a,4a-diaza-s-indacene choloride (C3) and 8-(N-benzyl-4-acetylpyridium)-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℃: 18.2 MΩcm, 7.2×10^{-2} 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 $AgNO_3$ 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 $NaBH_4$ was added to give a final concentration of 2 BH_4^-/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 a American's Varian Carry Eclipse spectrofluorometer. 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, 2×10^6 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 precooling lysate 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

1uM 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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