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ORIGINAL RESEARCH

Binding fullerenol $C_{60}(OH)_{24}$ to dsDNA

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Abstract: The first $C_{60}(OH)_{24}$-DNA complex and its fluorescence enhancement is reported. The enhanced fluorescence intensity of fullerenol $C_{60}(OH)_{24}$ is in proportion to the concentration of DNA in the range of 1×10^{-9} to 8×10^{-5} molL^{-1} and the detection limit was 1.3 ng mL^{-1}. Fullerenol $C_{60}(OH)_{24}$ binds significantly to the phosphate backbone of native dsDNA and to base-pairs within the major groove of sodium salt of dsDNA.

Keywords: nanomedicine, fullerenol, DNA complexation, fluorescent probe

Introduction

Nanoscale materials seems to offer great opportunities for biomedical applications such as therapeutic and diagnostic tools.[1–9] Biomedical applications under development include drug delivery systems targeted to the brain and cancer tissues, gene transfection, and intravascular nanosensor and nanorobotic devices for imaging and diagnosis.[3,9]

In this context, the biological activities of fullerene derivatives have attracted much attention in the past 25 years.[10–17] As potent free-radical scavengers and antioxidants[18–23] the water-soluble polyhydroxylated [C_{60}]fullerenes, fullerenols, exhibit an exciting range of biological activities as glutamate receptor antagonists,[24] antiproliferative,[25–27] neuroprotective,[28–31] and anticancer agents.[32–37]

Knowing the ways fullerenols interact with proteins and nucleotides is a prerequisite for understanding their biological effects at membrane penetration and the intracellular level, only two studies deal with their binding to proteins[38,39] and their interaction with DNA has not been reported to date.

On the other hand, the solution-based assays and quantitative analysis of nucleic acids are critical in current biochemistry and biomedical science. Throughout the years, a number of fluorimetric methods for the determination of nucleic acids have been developed with ethidium bromide,[40–42] lanthanide cations,[43–45] ruthenium complexes,[46–48] and asymmetric cyanine dyes as fluorescence probes.[49–51]

Despite the prominence of fullerenes in bionanotechnology, the exploration of their fluorescent properties in solution remains still at a very early age. Several studies have been devoted to dsDNA/single-walled carbon nanotube hybrid systems,[52,53] but only very few deal with their fluorescent proprieties[54,55] when dispersed in aqueous solution.

Herein, we are happy to report the first complexation of dsDNA with $C_{60}(OH)_{24}$ in aqueous media in the absence of a buffer in physiological pH-range.

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Materials and methods

C_{60} (99.5+%) was purchased from MER Corp (Tuscon, AZ, USA). KOH (99.99%, semiconductor grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DNA (low molecular weight, salmon sperm) was purchased from Fluka (St. Louis, MO, USA). All other reagents were purchased from Sigma-Aldrich.

Fluorescence spectroscopy was performed with a Perkin Elmer LS55 spectrometer Perkin Elmer, Wellesley, MA, USA). To prepare fluorescence samples, the only operation was the mixing of two solutions before fluorescence measurements. X-ray photoelectron spectroscopy (XPS) measurements were carried out using a Leybold LHS 10 spectrometer (Leybold, Cologne, Germany).

To the best of our knowledge, most of these fullerenols are not pure $C_{60}(OH)_n$, but a complex mixture of products. For instance, those synthesized through sulfuric/nitric acid,[56] hydroboration,[57] or nitronium chemistry[58] afforded products with average composition of $C_{60}O_x(OH)_y$. The so-called fullerenols prepared by alkaline polyhydroxylation of C_{60} under phase transfer conditions[59] are not simply $C_{60}(OH)_x$, but stable radical anions with the molecular formula, $Na^+_n[C_{60}O_x(OH)_y]^{n-}$,[60] and the fullerenol obtained by alkaline hydrolysis of $C_{60}Br_{24}$ is not $C_{60}(OH)_{24}$ as claimed by Bogdanovi and Dvordjevic,[37,61] but $C_{60}(ONa)_8(OH)_{16}$.[62] In the light of this, many biomedical studies involving fullerenols species in the literature may need to be reconsidered. The pure fullerenol $C_{60}(OH)_{24}$ used in this study was prepared by a modified method of alkaline hydrolysis of $C_{60}Br_{24}$,[62] followed by demetallation of the obtained $C_{60}(OK)_8(OH)_{16}$ with a cation exchange resin and exhaustive purification by dialysis.

Representative procedure for synthesis of $C_{60}(OH)_{24}$

All experiments were performed with Schlenk techniques under argon and protected from light. According to the literature, before the synthesis of the polyhydroxylated fullerene, bromofullerene $C_{60}Br_{24}$ was synthesized first.[62] In the synthesis of the $C_{60}(OH)_{24}$, to a sonicated (40 W, 15 min) suspension of $C_{60}Br_{24}$ (200 mg, 0.075 mmol) in de-aerated water (100 mL), fresh KOH (200 mg, 3.57 mmol) was added under argon protection and stirred for 10 days at room temperature. After the reaction was completed, the resulting dark-brown solution was passed to a centrifuge at 4000 rpm for 30 min and the supernatant was brought to dryness in a rotavapor apparatus at 40 °C. The dark-brown residue was dissolved in 50 mL of deionized water, stirred with ion exchange resin AMBERJET™ 1200[H] (Rohm and Haas Company, Philadelphia, PA, USA) (20 mL) for 8 h and subjected to dialysis (Spectra/Por® 1000 D; Spectrum Laboratories, Rancho Dominguez, CA, USA) for four days. Finally, the dialyzed solution was brought to dryness in a rotavapor apparatus at 60 °C and dried at 80 °C and 10^{-4} Torr for 24 h. The fullerenol thus obtained contained 24 hydroxyl groups as characterized by elemental analysis, Fourier transform infrared (FT-IR) spectroscopy, and XPS spectroscopic measurements.

Elemental analysis

Calculated for $C_{60}H_{24}O_{24}$: C, 63.82; H, 2.12. Found: C, 63.66; H, 1.98. FTIR (KBr): ν max, 3436 (–OH), 1605 (C = C), 1430 (δ –OH), 1095, 1046 (ν C-OH), 1018, 994, 825, 877, 570, 530 cm^{-1}.

XPS analysis

C1s components: % C = C (284.6 eV) 59.77 (clcd. 60); % C-OH: (285.8 eV) 39,76 (clcd. 40); O/C = 0.42 (clcd. 0.40).

Results and discussion

The fullerenol water solution, exhibited different maxima depending on the concentration (Figure 1a). In the range of 1.6×10^{-5} to 4.4×10^{-5} $molL^{-1}$ one fluorescent maximum was observed at 469 nm, while two fluorescence maxima where found for lower concentrations located at 469 nm and 492 nm at $\lambda_{ex} = 420$ nm.

These emission profiles of fullerenol at different concentrations provided the baseline for understanding perturbation upon interaction with dsDNA. As shown in Figure 1a, the most appropriate concentrations of fullerenol in water for fluorescence measurements at $\lambda_{ex} = 420$ nm are within the range of 1.6×10^{-5} to 4.4×10^{-5} $molL^{-1}$ ($\lambda_{em} = 469$ nm).

As can be seen from Figure 1b, the fluorescence intensity of fullerenol alone is dependent on its concentration in the range of 4.4×10^{-5} to 1.6×10^{-5} $molL^{-1}$ according to a very significant linear relationship.

Figure 2 shows the fullerenol and DNA emission spectra recorded at different excitation wavelengths. Inspection of how fluorescence emission spectra of fullerenol and DNA change as a function of excitation wavelength yield additional supporting information on the appropriate fullerenol excitation wavelength suitable for the fluorescence investigation of $C_{60}(OH)_{24}$ – DNA complex in aqueous media. One can observe that for a concentration higher than 1.6×10^{-5} $molL^{-1}$, the emission spectra of fullerenol do not overlap with emission spectra of DNA when excited at 340, 360, 380, and

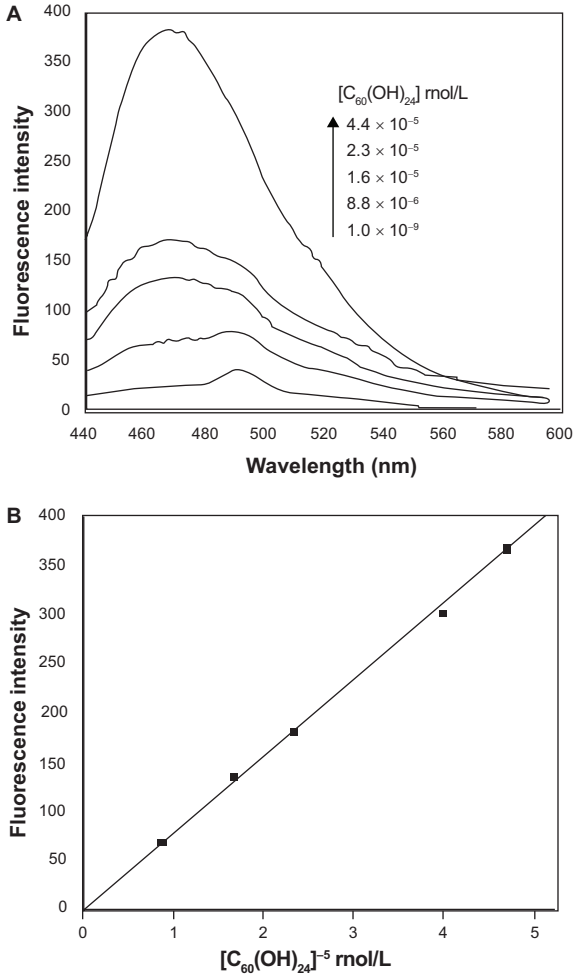


Figure 1 Fluorescence data of $C_{60}(OH)_{24}$ in aqueous media. **A**) Fluorescence emission spectra of $C60(OH)_{24}$ after 5 min incubation in water, with excitation at 420 nm. **B**) Plot of fluorescence intensity versus $[C_{60}(OH)_{24}]$, with excitation at 420 nm; average standard error, 3.76%.

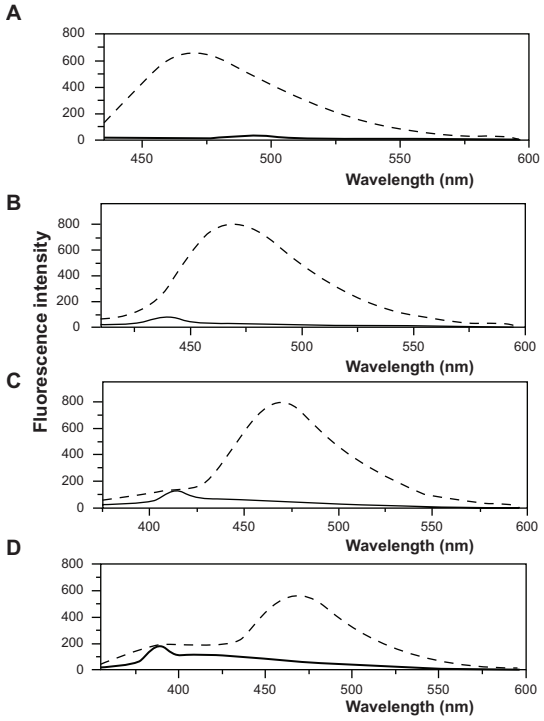


Figure 2 Fluorescence emission spectra of fullerenol (- - -) and dsDNA (——) for different excitation wavelength. **A**) λ = 420 nm. **B**) λ = 380 nm. **C**) λ = 360 nm. **D**) λ = 340 nm. $[C_{60}(OH)_{24}] = 1 \times 10^{-4}$ molL^{-1}; [DNA] = 1×10^{-4} molL^{-1}.

420 nm, respectively. Apparently, all these fluorescence excitations should be suitable for a fluorescence study of DNA-fullerenol interaction. However, the emission maxima of fullerenol at concentrations $<1.6 \times 10^{-5}$ molL^{-1} (Figure 1a) and DNA (Figure 2a) overlap at 492 nm when recorded at $\lambda_{ex} = 420$ nm. This is the reason why, to cover a large concentration range (1^{-9}–4.5^{-5} molL^{-1}) of fullerenol, the fluorescence excitation at 420 nm and emission at 469 nm were used for fluorescence intensity measurements in this work.

In Figure 3a, the emission spectra of DNA-fullerenol complexes, with constant DNA concentration and increasing fullerenol content are shown. It can been seen that increasing the concentration of the fullerenol results in a strong increase in fluorescence intensity of fullerenol from 50 to 500 nm, without causing any perceptible shifts of the fluorescence maximum at λ = 469 nm. In order to establish the DNA binding affinity of fullerenol, these fluorescent-enhancing data were plotted (Figure 3b) according to the equation (6) derived from the equilibrium equation (1):

$$n[C_{60}(OH)_{24}] + [DNA] \underset{}{\overset{K}{\rightleftharpoons}} [C_{60}(OH)_{24} - DNA] \quad (1)$$

$$K = [C_{60}(OH)_{24} - DNA]/[C_{60}(OH)_{24}]^n [DNA] \quad (2)$$

$$[C_{60}(OH)_{24} - DNA] = K[C_{60}(OH)_{24}]^n [DNA] \quad (3)$$

$$\log [C_{60}(OH)_{24} - DNA] = \log K + n\log [C_{60}(OH)_{24}] + \log [DNA] \quad (4)$$

$$\log [C_{60}(OH)_{24} - DNA]/[DNA] = \log K + n\log [C_{60}(OH)_{24}] \quad (5)$$

$$\log F\text{-}F_1/F_0 = \log K + n\log [C_{60}(OH)_{24}] \quad (6)$$

where F_1 is the fluorescence intensity from the fullerenol in the absence of DNA (Figure 1b), F_0 is the fluorescence intensity from the DNA in the absence of fullerenol at 467 nm for $\lambda_{ex} = 420$ (Figure 2a), F is the fluorescence intensity from the DNA-fullerenol complex in the presence of different concentrations of the fullerenol (Figure 3b) and n is the number of associated molecules of fullerenol with one base pair of DNA. From the linear plot for $(\log(F\text{-}F_1)/F_0)$ vs $(\log[C_{60}(OH)_{24}])$ (Figure 5), according to equation (1), the values of K and n were estimated to be 6×10^5 M^{-1} and 0.8 ± 0.2, respectively.

In order to evaluate the range of [DNA] determination, the binding of fullerenol to DNA was characterized

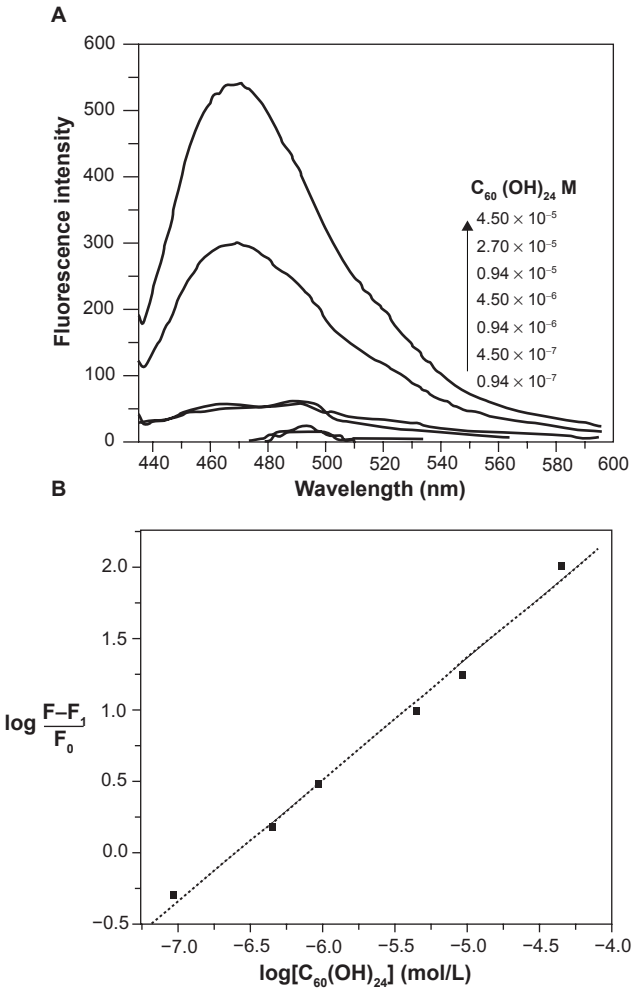


Figure 3 Fluorescence data of $C_{60}(OH)_{14}$ in the presence od dsDNA. **A**) Fluorescence emission spectra of fullerenol (——) in the presence of dsDNA (- - -) with excitation at 420 nm, as a function of fullerenol concentration; [dsDNA] = 6.31 × 10^{-5} molL^{-1}. **B)** Plot for DNA-$C_{60}(OH)_{24}$ system as a function of fullerenol concentration in the range of 0.94 × 10^{-7} to 4.5 × 10^{-5} molL^{-1}; [dsDNA] = 6.31 × 10^{-5} molL^{-1}; average standard error, 0.17%.

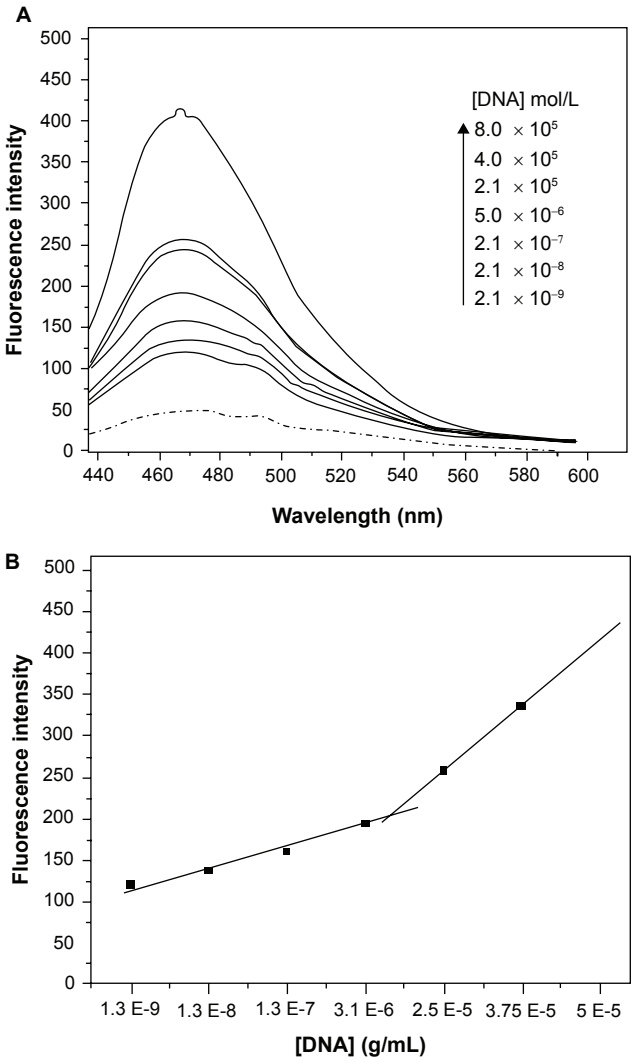


Figure 4 Dependence of fluorescence intensity of $C_{60}(OH)_{24}$ on dsDNA concentration. **A**) Fluorescence spectra of fullerenol (– · –) with increasing concentration of DNA (——) with excitation at 420 nm ; **B**) Plot of fluorescence intensity of fullerenol as a function of [dsDNA] in the concentration range of 1.3 × 10^{-9} to 4.4 × 10^{-6} molL^{-1}; [$C_{60}(OH)_{24}$] = 4.5 × 10^{-5} molL^{-1}; average standard errors: 2.69% for 2.5E^{-5}–5E^{-5} region and 10.80% for 1.3E^{-9}–3.1E^{-6} region.

through fluorescence emission titration of fullerenol. The enhancement of the fluorescence intensity of fullerenol with DNA at increasing concentrations is shown in Figure 4. One can observe that even for nanoscale concentration of DNA the fluorescence intensity of fullerenol increases from 25 to 100 (Figure 4a). The plot in Figure 4b is broken down into two regimes corresponding to ranges from 1.3 × 10^{-9} to 3.1 × 10^{-6} gL^{-1} and 2.5 × 10^{-5} to 5 × 10^{-5} gL^{-1}. The low [DNA] range in the plots of Figure 4b (detection limit = 1.3 ng/mL) are close to what can be accomplished with current available fluorescence probes, ie, Hoechst 33258 (20 ng/mL) and YO-PRO-1/YOYO-1 (0.5–2.5 ng/mL). In addition, from the shape and intensity of emission spectrum recorded for [DNA] = 2.1 × 10^{-9} molL^{-1} (Figure 4a), it is useful to point out that the sensitivity can be extended into lower regions.

As regards the chemical interactions between fullerenol and the DNA target, the electrostatic and intercalative binding are ruled out and hydrogen-bonding interaction can only be taken under consideration. Earlier studies[38,39] have pointed out that hydrogen-bonding plays the main role in the interaction between fullerenols and proteins. Thus, the major groove binding of fullerenol through the hydrogen-bonding between its hydroxyl groups and free or bridged –NH_2 in base-pairs of DNA is predictable.

Taking into account that phenols can interact with phosphates,[63] the hydrogen bonding between fullerenol and phosphate backbone of DNA can be also suggested as a possibility for the binding of fullerenols with DNA.

From the linear plot in Figure 3b according to equation (1) the value of n was estimated to be 0.8 ± 0.2. This value

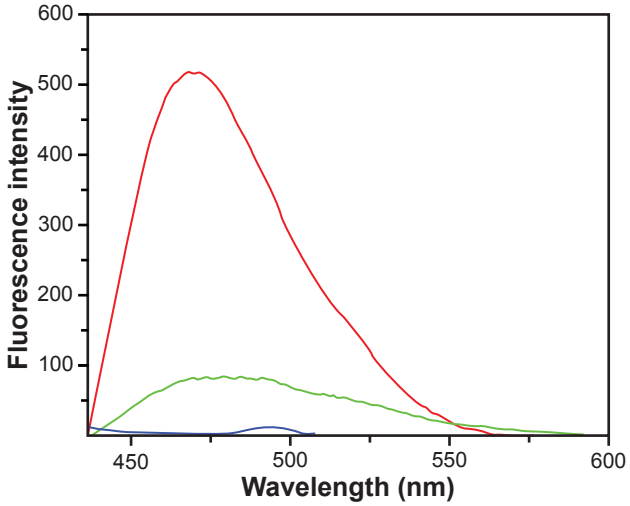
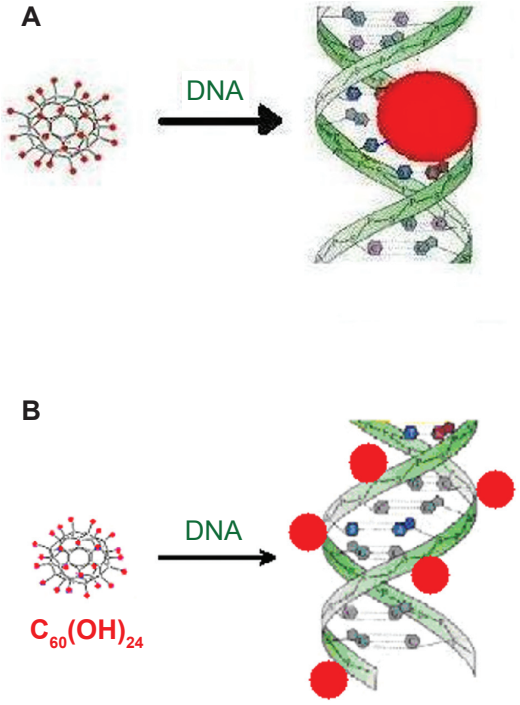


Figure 5 Emission spectra of $C_{60}(OH)_{24}$ (red line), DNA-sodium salt (blue line) and $C_{60}(OH)_{24}$ – DNA-sodium salt complex (green) line.

accounts for a single fullerenol molecule associated with a base pair of DNA. An important question is whether the fullerenol binds significantly to the phosphate backbone of DNA in addition to nonintercalative groove binding to base-pairs of DNA.

Because of its diameter (9.8 Å) the globular three-dimensional $C_{60}(OH)_{24}$ molecule does not fit the minor groove of DNA (6 Å). The width of the major groove (12 Å) is larger than 9.8 Å and thus the fullerenol molecule can fit snugly according to a nonintercalation model as shown in Scheme 1a.

Indeed, upon binding to sodium salt of DNA (Figure 5), the fluorescence intensity of fullerenol does not increase, but decreases, which suggests that only in the absence of hydrogen bond-forming P-OH moieties of DNA, fullerenol binds to base-pairs into the major groove of DNA, according to a nonintercalative model, which strongly change its average local environment. The perceptible shift of the emission maximum of fullerenol also support a strong change of its average local environment. We can thus conjecture that, under the present experimental conditions with native dsDNA, hydrogen binding to phosphate backbone to the outside of dsDNA helix is the main binding mode of fullerenol C60$(OH)_{24}$ to DNA, as shown in Scheme 1b.



Scheme 1 Binding fullerenol $C_{60}(OH)_{24}$ to dsDNA. **A**) Binding fullerenol to the major groove of sodium salt of dsDNA. **B**) Binding fullerenol to the outside of the dsDNA.

Conclusions

Fullerenol $C_{60}(OH)_{24}$ binds to phosphate backbone to the outside of native dsNNA and to base-pairs within major groove of sodium salt of dsDNA. The fluorescence of fullerenol $C_{60}(OH)_{24}$ is highly enhanced by dsDNA due to the binding of the probe to DNA in a nonintercalative way. Because of its high binding affinity ($K = 10^5\ M^{-1}$) and sensitivity (1.2×10^{-9} g/mL) towards DNA, there are good prospects that $C_{60}(OH)_{24}$ will be used as versatile fluorescent probe for DNA quantification. In addition to its high sensitivity, other advantages of this fullerenol-based method include its simplicity, nontoxicity, and rapidity.

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