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# Cyanines as New Fluorescent Probes for DNA Detection and Two-Photon Excited Bioimaging

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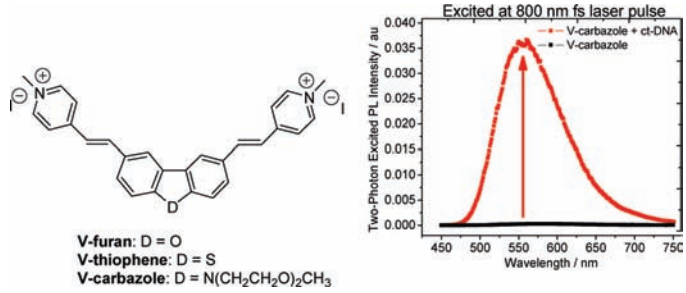
## ABSTRACT




**A series of cyanine fluorophores based on fused aromatics as an electron donor for DNA sensing and two-photon bioimaging were synthesized, among which the carbazole-based biscyanine exhibits high sensitivity and efficiency as a fluorescent light-up probe for dsDNA, which shows selective binding toward the AT-rich regions. The synergetic effect of the bischromophoric skeleton gives a several-fold enhancement in a two-photon absorption cross-section as well as a 25- to 100-fold enhancement in two-photon excited fluorescence upon dsDNA binding.**

There has been considerable interest in the development of facile and reliable methods to detect nucleic acids owing to their potential applications in bioanalytical, clinical, and forensic analysis.[1] Fluorophores that show an increase in fluorescence intensity upon binding with DNA have been used to stain and quantify nucleic acids in gel electrophoresis as well as in staining cell nuclei for microscopic bioimaging.[2] In addition, fluorescent dyes that possess high two-photon absorption (TPA) properties may be useful in two-photon excited fluorescence (TPEF) microscopic imaging.[3] As the two-photon absorption process is quadratically intensity-dependent, the excitation is confined to a small volume in the focal plane, which can provide very high spatial resolution with greatly reduced photobleaching and photodamage to the sample speciment during imaging. Furthermore, the use of an infrared laser source improves light

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penetration depth in tissues, thus allowing deeper tissue imaging. However, most of the currently used one-photon excited biological fluorophores show only low TPA cross sections ($\sigma$) and/or TPEF brightness ($\Phi_{FL}\sigma$).[4] Moreover, most of the highly active TPA molecules known are not biocompatible as they are often water-insoluble or form nonfluorescent aggregates in aqueous media. Consequently, highly sensitive two-photon excited fluorophores for DNA are rather rare.[5]

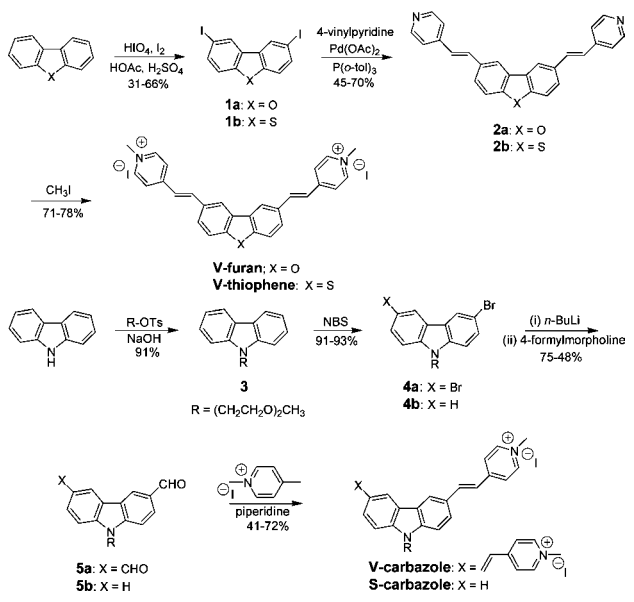
To enhance the water solubility and promote binding propensity with the negatively charged DNA[6] as well as induce the large two-photon absorption properties,[7] novel bis-cationic donor−acceptor fluorophores based on electron-donating fused aromatics, including dibenzothiophene, dibenzofuran, and carbazole, were designed and investigated for DNA detection and two-photon excited bioimaging. In this contribution, we have demonstrated that multifunctional carbazole-based biscyanine is a highly sensitive and efficient fluorescent light-up probe for double-stranded DNA (dsDNA) with a binding selectivity toward the AT-rich region. The synergetic effect of the bischromophore leads to a large enhancement in the TPA cross-section of biscyanine as compared to that of monocyanine. In addition to the several-fold increase in TPA cross-section, the carbazole-based cyanines exhibit 25−100-fold enhancement in TPEF upon dsDNA association, highlighting their potential as highly sensitive TPEF probes for DNA detection and fluorophores for bioimaging. Initial TPEF imaging study of the carbazole-based cyanine fluorophores in HeLa cells showed bright images with high contrast at low laser powers and specific subcellular localizations with no discernible photobleaching effects.

Palladium-catalyzed Heck coupling of 2,7-diiododibenzofuran or 2,7-diiododibenzothiophene and 4-vinyl-pyridine using Pd(OAc)$_2$/2P(*o*-tol)$_3$ as a catalyst was used as a key step to synthesize dibenzofuran- and dibenzothiophene-based biscyanines, respectively (Scheme 1). Di-iodination of dibenzofuran using $HIO_4$ and $I_2$ in acetic acid gave diiodo-product, **1a** in 66% yield. Palladium-catalyzed Heck-coupling of the diiodide **1a** and 4-vinylpyridine using Pd(OAc)$_2$/2P(*o*-tol)$_3$ as a catalyst afforded bis-product **2a** in 45% yield. Subsequent methylation carried out in DMF in the presence of $CH_3I$ at 90 °C gave dibenzofuran-based biscyanine, **V-furan**, in 71% yield. Following the same reaction sequence, di-iodination of dibenzothiophene (31%), Heck-coupling of the diiodide compound with 4-vinylpyridine (70%) and methylation (78%), and dibenzothiophene-based biscyanine, **V-thiophene** was also synthesized.

On the other hand, the Knoevenagel reaction of carbazolyl-3,6-dialdehyde and *N*-methyl-4-methyl-pyridium iodide was used to synthesize carbazole-based biscyanine **V-carbazole** and monocyanine **S-carbazole**. Alkylation of carbazole with ethylene glycol tosylate in the presence of NaOH gave alkylated carbazole **3a** in an excellent yield. Dibromination of **3a** in the presence of NBS gave alkylated 3,6-dibromocarbazole, **4a**, in 91% yield. Double formylation of **4a** via lithiation bromide exchange at low temperature followed by the subsequent reaction with *N*-formylmorpholine afforded carbazolyl-3,6-dialdheyde **5a** in 48% yield. Knoevenagel reaction of the dialdehyde **5a** and *N*-methyl-4-methylpyridium iodide in the presence of piperidine in ethanol afforded carbazole-based biscyanine, **V-carbazole** in a good yield (72%). **S-carbazole** was also prepared in a similar fashion. All the newly synthesized biscyanines were fully characterized with $^1$H NMR, $^{13}$C NMR, MS, and elemental analysis and found to be in good agreement with the proposed structures. Thermogravimetric analysis showed that these biscyanines possess high decomposition temperatures ranging from 314 to 343 °C.

All the biscyanines are highly soluble in aqueous solution with no aggregation. The aqueous solutions of **V-carbazole** obeyed Beers's law up to a concentration of $1 \times 10^{-4}$ M. As shown in Figure 1, all the biscyanines show very similar absorption characteristics, featuring a strong, broad, and structureless low-energy absorption band ($\lambda_{max}^{abs}$) at around 369−436

**Scheme 1.** Synthesis of Fused Aromatic-Based Cyanines



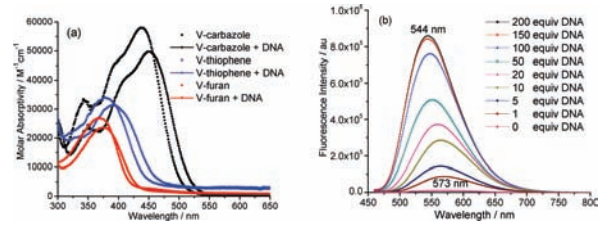




**Figure 1.** (a) Absorption spectra of biscyanines without and with 5 equiv of calf thymus DNA (ct-DNA) measured in pH 7 phosphate buffer solution. (b) Fluorescence spectra of **V-carbazole** upon addition of various concentrations of ct-DNA.

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**Table 1.** Summary of Photophysical Measurements of **S-Carbazole**, **V-Carbazole**, **V-Thiophene**, and **V-Furan** Measured in Buffer Solution

| | $\lambda_{max}^{abs}$/nm ($\varepsilon_{max}$ $10^4$/M$^{-1}$cm$^{-1}$) | $\lambda_{max}^{em}$ [a]/nm | $\Phi_{FL}$ [b,c] | $\tau$ [d]/ns | $\lambda_{max}^{abs}$/nm ($\varepsilon_{max}$ $10^4$/M$^{-1}$ cm$^{-1}$) [e] | $\lambda_{max}^{em}$ [a,f]/nm | $\Phi_{FL}$ [a,b] | $\tau$ [c,f]/ns |
|---|---|---|---|---|---|---|---|---|
| | without ct-DNA | | | | with addition of ct-DNA | | | |
| **S-carbazole** | 416 (4.03) | 573 | 0.02 | 1.95 | 418 (3.79) | 563 | 0.07 | 2.37 |
| **V-carbazole** | 436 (5.81) | 573 | 0.0029 | 0.78 | 451 (4.98) | 548 | 0.13 | 2.34 |
| **V-thiophene** | 375 (3.40) | 535 | 0.031 | 0.48 | 391 (3.13) | 511 | 0.093 | 2.18 |
| **V-furan** | 369 (2.69) | 467 | $<10^{-3}$ | –[f] | 374 (2.36) | 449 | $<10^{-3}$ | –[g] |

[a] Excited at the absorption maximum [b] Using coumarin 6 ($\Phi_{420} = 0.78$) as a standard for **S-carbazole** and **V-carbazole** and quinine sulfate monohydrate ($\Phi O_{350} = 0.58$) as a standard for **V-thiophene** and **V-furan**. [c] Average of two independent measurements. [d] Fluorescence lifetime [e] 5 equiv of calf thymus DNA (ct-DNA) added. [f] 100 equiv of ct-DNA added. [g] Too low to be determined reliably.

nm in phosphate buffer solution corresponding to the $\pi-\pi^*$ transition due to their large molar absorptivities (Table 1).

Upon excitation at their $\lambda_{max}^{abs}$, these biscyanines showed a very weak emission at ~467−573 nm (fluorescence quantum yield, $\Phi_{FL} = 0.001-0.031$) in sharp contrast to the moderate emission obtained in an organic solvent ($\Phi_{FL} = 0.001-0.16$ in DMSO). There are large Stokes shifts between the absorption and emission spectra (Δ98−160 nm) among these biscyanines, indicating that their first excited states, arising from the intramolecular charge transfer, are highly stabilized by the aqueous medium.

Upon addition of calf thymus DNA (ct-DNA) to the biscyanine buffered solutions, significant red shifts of the absorption spectra ($\Delta\lambda_{max}^{abs} = 5-16$ nm) with a concomitant decrease in the absorptivities were observed (Figure 1a), indicating an association of these V-shaped cationic dyes with DNA. Remarkably, despite very weak emission in a buffer medium, there is a progressive and dramatic increase in fluorescence intensity together with blue shift of emission spectra upon titration of these biscyanines with ct-DNA. (Figure 1b). The fluorescence enhancement is attributed to the large reduction in the nonradiative decay of the photoexcited biscyanines due to the restricted rotation of the central C−C bond upon DNA binding. This explanation is consistent with the observed increase in the fluorescence lifetimes of these biscyanines upon addition of ct-DNA (e.g.: **V-carbazole**, $\tau = 0.78$ ns, increased to $\tau = 2.34$ ns in the presence of ct-DNA) and in the $\Phi_{FL}$ in a viscous solvent (e.g.: $\Phi_{FL}$ of **V-carbazole** in 100 g/L of polyethyleneglucol-5000 = 0.016).[5b] It is important to note that **V-carbazole** exhibits much stronger (77-fold) fluorescence enhancement at saturation with ct-DNA than **V-thiophene** (2-fold) and **V-furan** (5-fold). There is also ~40 times increase in the fluorescence quantum yield ($\Phi_{FL}$) for **V-carbazole** at saturation with ct-DNA, increasing from 0.003 without ct-DNA to 0.13 at saturation, highlighting its potential as a fluorescence light-up probe for DNA detection. To ascertain the application of **V-carbazole** for DNA quantitation, fluorescence titration was performed using the pcDNATM6.2-GW/EmGFP-miR-neg plasmid DNA (Supporting Information). Linear variation of fluorescence intensity was observed from 60 ng/L of DNA down to a few ng/L at a dye concentration of $10^{-7}$ M with a slope of 1189. Thus, this dye offers a straightforward way to quantify DNA concentration by fluorescence technique.

The detailed binding characteristics of the biscyanine, **V-carbazole**, with DNA were investigated, together with its monocyanine counterpart, **S-carbazole**, for comparison, using various defined-sequence oligonucleotides. **S-carbazole** shows similar spectral responses upon DNA binding which included a red absorption peak shift, a blue emission peak shift, a decrease in fluorescence lifetime, and an increase in fluorescence quantum yield, albeit to a lesser extent supporting the merit of using the bis-cationic approach for DNA detection. In these fluorescence titrations, both cyanines exhibited higher sensitivity toward dsDNA than the corresponding single-stranded DNA (ssDNA), particularly for **V-carbazole**. They also show much larger fluorescence enhancement toward AT-rich dsDNA, suggesting selective binding toward the AT-rich regions. Weaker fluorescence enhancement on GC-rich sequences might be caused by fluorescence quenching properties of guanine. The binding associations ($K_b$) of **V-carbazole** with DNA $d[AT]_5$:$d[TA]_5$ and DNA $d[A]_{10}$:$d[T]_{10}$ as estimated by the nonlinear curve fitting analysis are $8.3 \times 10^5$ M$^{-1}$ and $20.5 \times 10^5$ M$^{-1}$, respectively, which are much larger than that obtained with DNA $d[C]_{15}$:$d[G]_{15}$ ($K_b = 1.5 \times 10^5$ M$^{-1}$). These results are consistent with the fact that the environments of the AT-rich sites in dsDNA are relatively electronegative, which favors binding interaction with the positively charged dyes. On the other hand, the exocyclic $NH_2$ substituent of guanine in the GC base pair regions has been reported to hinder effective binding of a dye.[8] In contrast, the binding associations of **S-carbazole** with DNA $d[AT]_5$:$d[TA]_5$ and DNA $d[A]_{10}$:$d[T]_{10}$ are $1.1 \times 10^4$ M$^{-1}$ and $4.7 \times 10^4$ M$^{-1}$, respectively, which are substantially smaller than those of **V-carbazole**. These findings are also in good agreement with

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the plasmid DNA unwinding assay,[9] which showed that **S-carbazole** is a DNA-intercalator while **V-carbazole** exhibits no DNA-intercalating property and thus prefers to bind within the minor groove (Supporting Information).

Both carbazole-based cyanines exhibit interesting two-photon-induced photoluminescence at around 575−578 nm upon excitation at 800 nm femtosecond (fs) laser pulses in buffered solution, which are slightly red-shifted as compared to the one-photon emission spectra because of reabsorption effect, suggesting that the same emissive states are involved in the two excitation processes. The TPA cross sections ($\sigma$) and the two-photon excited spectra of the carbazole-based cyanines were determined by the two-photon induced fluorescence method without and with ct-DNA in phosphate buffer solution.[10] Figure 2 shows the two-photon excitation

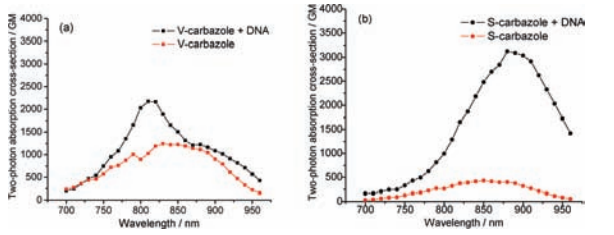


**Figure 2.** Two-photon excitation spectra of (a) **V-carbazole** and (b) **S-carbazole** without and with 200 equiv of ct-DNA in $10^{-5}$ M buffered solution.

spectra of **V-carbazole** and **S-carbazole** with $\sigma_{max}$ reaching 1241 GM at 830 nm for **V-carbazole** and 431 GM at 850 nm for **S-carbazole,** respectively, demonstrating the synergetic effect of the bis-chromophoric skeleton in enhancing the nonlinear optical properties. There are also dramatic enhancements (25−100-fold) of TPEF brightness of these cyanines upon addition of ct-DNA up to 284 GM and 218 GM for **V-carbazole** and **S-carbazole**, respectively, which are among the highest TPEF brightness values reported with DNA bioaffinity.[5b] In the presence of 200 equiv of ct DNA, the fluorescence quantum yields were enhanced, and the TPA cross sections increased greatly up to 2170 GM at 810 nm and 3127 GM at 880 nm for **V-carbazole** and **S-carbazole**, respectively. The present results on fluorescence light-up upon DNA binding are of great interest in fluorescence bioassays, which can take great advantages of the two-photon excitation and enable bioaffinity experiments without separations in a complex medium with subpicomolar sensitivity.[11]

An initial TPEF microscopic study of the carbazole-based cyanine fluorophores in Hela cells was carried out using 800 nm fs laser pulses. Figure 3a depicts the TPEF image of HeLa cells incubated with $10^{-6}$ M **V-carbazole** (10 mW laser power at sample) showing good contrast and brightness.

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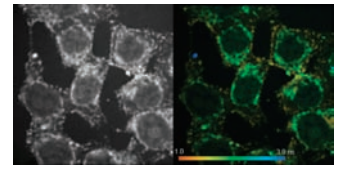

**Figure 3.** (a) TPEF image of **V-carbazole** in HeLa cells excited at 800 nm fs laser pulses using 10 mW laser power. (b) FLIM image of the same cell measured by the TCSPC method with lifetime distribution.

Fluorescence lifetime imaging microscopic (FLIM) experiments showed that this dye exhibits good photobleaching resistance and specific localization in the cell cytoplasm as measured by the time-correlated single photon counting (TCSPC) method (Figure 3b). Apparently, the nuclear penetration of **V-carbazole** was not very efficient, resulting in a brighter signal seen at the cytoplasm than in the nucleus. Nevertheless, two distinct subcellular environments, indicated by the fluorescence lifetimes, were observed for this dye: in the perinuclear region, $\tau = 2.0$ ns, and in the heterogeneously stained cytoplasm, where $\tau = 1.2$ ns. Interestingly, the TPEF image of **S-carbazole** in Hela cells was obtained with a lower laser power (5 mW) than that for **V-carbazole** producing the same quality image, indicating the higher sensitivity of **S-carbazole** for TPEF bioimaging (Supporting Information).

In summary, we have synthesized a series of cyanine fluorophores based on fused aromatics as an electron donor for DNA quantitation and two-photon bioimaging, among which the carbazole-based biscyanine exhibits high sensitivity and efficiency as a fluorescent light-up probe for dsDNA. The bis-chromophoric skeleton gives a several-fold enhancement in the TPA cross-section and 25- to 100-fold enhancement in TPEF brightness upon dsDNA binding, making these carbazole-based cyanines highly sensitive TPEF probes for DNA detection in fluorescence bioassays. The TPEF images of the carbazole-based cyanine fluorophores in HeLa cells exhibit good brightness and high contrast at low laser powers. These cyanine dyes also show specific subcellular localization without detectable photobleaching effects. Further studies are underway to determine the specific subcellular localizations of these dyes.

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**Supporting Information Available:** Detailed synthetic procedures, results of physical characterization, and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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