Tetrahedron Letters 54 (2013) 2627–2632

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

# Sensing of biomolecules and label-free discrimination of DNA containing a triple T–C/T–G mismatch pair with a fluorescence light-up probe, triazolylpyrene ($^{TNDMB}$Py)

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ARTICLE INFO

*Article history:*
Received 24 December 2012
Revised 6 March 2013
Accepted 7 March 2013
Available online 16 March 2013

*Keywords:*
Triazolylpyrene
Fluorescence light-up probe
Sensing biomolecules
ct-DNA
BSA
Label free mismatched DNA detection

ABSTRACT

Binding to the minor groove of calf-thymus DNA (ct-DNA) and strong binding in hydrophobic pocket of bovine serum albumin (BSA), switched-on the fluorescence of smart fluorescent probe, triazolylpyrene ($^{TNDMB}$Py). Also, a novel label-free strategy was adopted to detect DNA base mismatch via the generation of distinct fluorescence signal at visible region utilizing the same probe



Monitoring various structures, functions, and dynamics of biological molecules such as DNAs and proteins and their surroundings with an ideal microenvironment sensitive fluorescence probe is an emerging research area in biological science and engineering for understanding biological events associated with interbiomolecular interactions.[1] In such a probing scenario, when the fluorophore comes into interaction with biomolecules, the fluorophores' emission property may be so modulated as to enable visual observation of biomacromolecular species.[1e–h] Several fluorescent bioprobes, thus, have been reported based on the fluorophores' properties like intercalation, stacking, and/or groove binding for DNA detection with the generation of enhanced fluorescence signal.[2] On the other hand, protein binding abilities of several chromophores also have been studied via enhanced fluorescence signal generation.[3] However, many of the explored fluorophores were found to suffer from self quenching of emissions that makes them inefficient biosensors.[4] Therefore, there is a need to design probes which would show low or zero emission in buffer but upon interaction with biomolecules it could fluoresce drastically.

As a part of our ongoing research efforts on the design of solvatochromic fluorescent molecules via click reaction, we, recently have reported triazolylpyrene as a dual emissive highly solvatochromic fluorophore (Fig. 1a).[5] We have now observed that in buffer (2% DMF was added in each case to completely solubilize the probe) of various pHs, it showed only a faint ICT emission ($\lambda_{em}$ = 525 nm). We envisaged that the pseudo aromatic 1,2,3-triazole residue of the probe might significantly enhance the interaction ability via stacking/H-bonding/electrostatic interaction with the amino acid residues in a protein and/or with the aromatic bases in DNA. This would allow one to gather information on the protein's/DNA's microenvironments. Our probe consists of an *N*,*N*-dimethyl aminophenyl donor linked triazole functionality which served as an electron donor to effectively modulate the emission response of electronically coupled pyrene and also can provide an interaction site for biomolecules (Fig. 1a). This idea along with the intense emission of the probe in low polar media compared to buffer drew our attention to exploit it for the possible detection of ct-DNA and BSA protein which are easily available biomolecules with widespread applications and biological importance.[2,3]

Herein, we report our probe triazolylpyrene as an efficient fluorescent light-up biosensor for the detection of DNA and protein.

Before going to study the interactions with DNA/protein, we wanted to establish that the fluorescence at 525 nm is only due to ICT emission. Thus, we subsequently studied the spectral parameters over a broad range of probe concentration. Figure 1 showed the absorption and fluorescence spectra of the probe over the concentration range of 10–70 μM in a buffer of pH 7.0. The absorption, excitation, and fluorescence spectra did not change in band position. Moreover, the absorbance, and fluorescence intensities depend linearly on the concentrations of the probe in solution

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http://dx.doi.org/10.1016/j.tetlet.2013.03.029

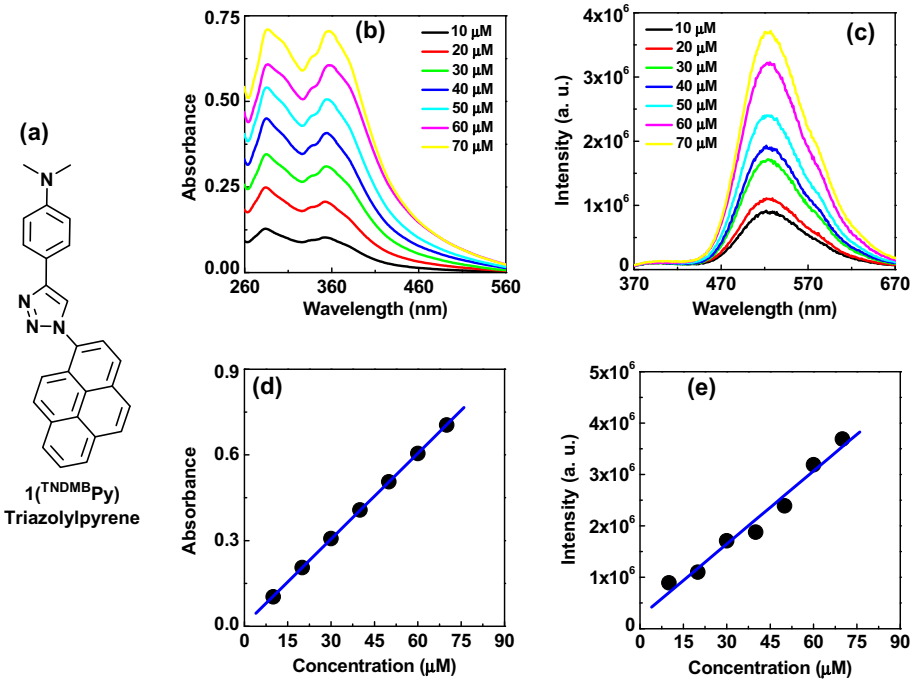


**Figure 1.** (a) Chemical structure of the probe **1** ($^{TNDMB}$**Py**). UV–visible (b) and emission spectra (c) of various concentrations of **1** in phosphate buffer of pH 7.0. Plots of absorbance (d) and emission intensity (e) versus concentration of the probe ($\lambda_{ex}$ = 345 nm).

(Fig. 1d and e) suggesting that the triazolylpyrene existed in a monomeric state and no aggregation formed. We have also studied the absorption of the probe of various concentrations in the presence of a fixed amount of biomolecule, BSA and plotted the absorbance versus concentration of the probe. The plot showed a good linear relationship (see Supplementary data) that would allow us to fit fluorescence titration curve data quantitatively to derive the association constants of probe-protein/DNA complex with no harm.

The strong emission at a long wavelength region (520 nm) in the presence of biomolecule in buffer again might be confused with the possibility of aggregation induced emission.[6] However, our observation in emission behavior of a dioxane solution of the probe when titrated with water did not reflect any aggregation induced emission (see Supplementary data). Thus, increasing % of water led to a decrease in emission along with a red shift of the band at 520 nm of the probe. The band totally died down at 20% water and the LE band at 380 nm started appearing. The LE band intensity reached maximum at 70% water content. When a large amount (80% and 100%) of water, a poor solvent of the probe, was added to the dioxane solution (with the final concentration of the mixture being adjusted to 10 μM) the LE band intensity again started to decrease with a negligible appearance of only LE band in 100% water under identical measurement conditions. These results suggested that the emission was not from an aggregate state but from the monomeric probe (see Supplementary data).

We attributed further that the emission at 520 nm was due to the ICT emission. However, the structure-less emission at longer wavelength could also be a characteristic to the excimer emission of pyrene.[7] Therefore, to establish the ICT emission we measured emission spectra of the probe of varying concentrations and plotted emission intensities versus concentrations at two different wavelengths of emission. If the emission at 520 nm was due to excimer emission, only this emission should increase compared with the emission at 380 nm (monomer emission) at the higher concentration; otherwise both the emissions would increase. Thus, we checked this event and we could say that the long wavelength emission at around 520 nm was due to ICT emission and not due to the characteristic excimer emission of pyrene. The proof of our assignment came from a study of concentration dependent emission of both the bands in 10% water in dioxane solvent and evaluation of the changes in intensity of both the LE (~380 nm) and ICT (~520 nm) bands. This solvent system was so chosen as to get both the emissions prominently. However, in this solvent system, the ICT band was appearing at 570 nm and the LE band centered at 393 nm. If the emission at 570 nm was excimer emission, it should increase compared with the emission at 393 nm at higher concentrations. However, we observed that the intensity of both the emission bands increased linearly as the concentration of the probe increased supporting our assignment of the band at 570 as ICT emission (see Supplementary data).

After establishing the long wavelength emission band as ICT emission and linear dependency of absorption and emission to the probe concentration either in the absence or in the presence of biomolecule, we next turned our attention to exploit this fluorophore's emission property to the study of possible interaction with protein/DNA biomolecules.

To examine the biomolecule sensing ability of our probe, first, we investigated the interaction behavior with ct-DNA by UV–visible and fluorescence spectroscopy in a buffer (pH 7.0) at 25 °C. Thus, the absorption maxima of triazolyl pyrene located at 355 nm showed a clear hyperchromicity with a minimal shift in absorption wavelength as ct-DNA was added gradually. This indicated a probable external groove binding characteristics of our probe (see Supplementary data).[8] From the fluorescence titration spectra it was evident that upon addition of increasing concentrations of ct-DNA, the fluorescence intensity ($\lambda_{em}$ = 525 nm) of triazolyl pyrene significantly increased and reached to a maximum at 300 μM concentration of ct-DNA compared to 50 μM probe concentration. This result indicated a well-defined binding of the probe with ct-DNA (Fig. 2a). The association constant of triazolyl pyrene with ct-DNA was determined by a Benesi–Hildebrand plot (Fig. 2b) which was found to be 7.2 × $10^3$ $M^{-1}$ with free energy of

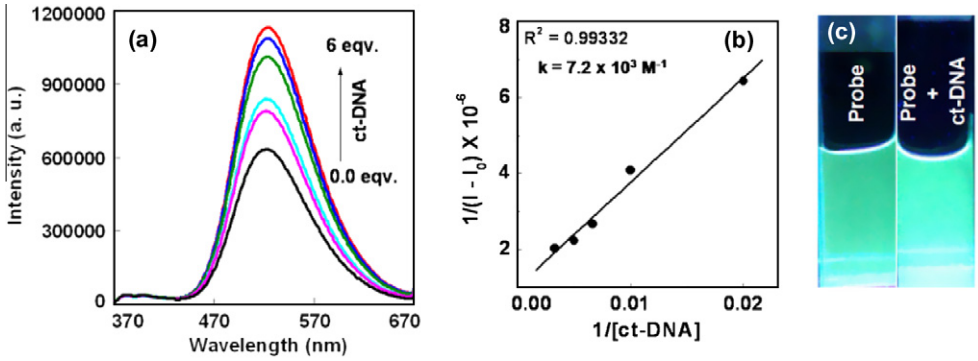


**Figure 2.** (a) Fluorescence titration of probe with various concentrations of ct-DNA. (b) Benesi–Hildebrand plot. (c) Fluorescence image under UV-transilluminator (254 nm) of only probe, in the presence of ct-DNA. All the experiments were carried out in 5 mM phosphate buffer, 5 mM NaCl, pH 7.0, rt, $\lambda_{ex}$ = 345 nm.

binding, $\Delta G = -5.24$ kcal/mol. The enhancement of fluorescence emission was also clear from a change of color of the probe solution from that of ct-DNA-probe solution when irradiated at 254 nm under a transilluminator (Fig. 2c).

Thermal denaturation experiment indicated a little destabilization of ct-DNA upon binding with the probe possibly because of steric clash near the groove. However, CD spectral measurement in the presence of the probe revealed an unperturbed B-form DNA conformation of ct-DNA. These results indicated that the probe possibly bind to a groove of ct-DNA (see Supplementary data).[8] To investigate the binding mode of the probe with ct-DNA, whether it is an intercalator or a groove binder, both the ethidium bromide (EB) and Hoechst 33258 displacement experiments were carried out. Ethidium bromide is a well-known example of an intercalating agent.[9] Fluorescence intensity of EB increases when it binds with DNA because of intercalation. In a competing scenario, if our probe, $^{TNDMB}$**Py**, intercalates with ct-DNA, then it would be a competitive binding agent with EB and the fluorescence intensity of EB bound with ct-DNA would decrease with an increase in probe concentration. However, we did not observe any significant fluorescence change of EB-ct-DNA complex upon increase in probe concentration. Thus, this observation implied that the probe may be a groove binder (see Supplementary data).[10] To establish the groove binding property of the probe, fluorescence titration experiment with Hoechst 33258 was carried out. Hoechst 33258 is a minor groove binding agent to double-helical DNA.[11] Like EB the fluorescence intensity of Hoechst 33258 increased upon binding with DNA. The crystal structure of Hoechst 33258 bound with DNA complex showed that Hoechst 33258 bound to a minor groove of DNA. If the probe binds in minor groove, then again it would be a competitive binding agent of Hoechst 33258 and it would replace Hoechst 33258. Thus, the fluorescence intensity of Hoechst 33258 bound with ct-DNA would decrease with an increase in probe concentration. Unlike with EB, we observed a significant decrease in fluorescence intensity ($\lambda_{em}$ = 468 nm) of hoechst 33258 of hoechst 33258-ct-DNA complex with an increase in probe concentration (Fig. 3a). The Multi Gaussian fitting spectra clearly shows the decrease in Hoechst emission with an increase in probe concentration (Fig. 3b). This result indicated that the probe was likely to be a minor groove binder of ct-DNA. As the excitation wavelengths ($\lambda_{ex}$ ≈340 nm) of both hoechst 33258 and the probe were quite same, we observed another emission band ($\lambda_{em} \approx 520$ nm) dominated over the parent hoechst's band ($\lambda_{em} \approx 468$ nm) in high concentrations of the probe (40–50 μM). The appearance of this band was due to the binding of the probe with ct-DNA and possibly because of energy transfer between Hoechst and the probe triazolyl pyrene (see Supplementary data).[11b] Hoechst had a significantly lower quantum yield when free in solution, in comparison to that when bound to ct-DNA. Therefore, both resonance energy transfer and release of Hoechst from ct-DNA probably were the main cause of decrease in emission intensity of Hoechst. Finally, the competition between the probe and hoechst for association with ct-DNA indicated that the probe triazolyl pyrene bound to the DNA minor groove.[12] The groove binding event of the probe was further supported by an insignificant change of fluorescence anisotropy/polarization (Fig. 3b).[13] Amber* optimized geometry of the model DNA-probe complex also supported the minor groove binding event of the probe (see Supplementary data).[14]

Motivated by the detection of ct-DNA with an enhanced fluorescence signal and to explore the versatility of our probe we next investigated the detection of protein, BSA spectroscopically.[1,3] Thus, upon addition of increasing amounts of BSA to the probe's solution, we observed a hyperchromicity with very little (7 nm) blue shift of the absorbance band as was clear from the UV–vis. spectra (see Supplementary data). To get further insight into the binding properties and to investigate the protein sensing ability of the probe, we then investigated the fluorescence changes upon addition of various concentrations of BSA to the solution of the probe upon excitation at 344 nm. Thus, with increasing concentrations of BSA, both the fluorescence intensity and quantum yield of the fluorophore increased with a blue shift of 41 nm of emission maximum (Fig. 4a). The fluorescence enhancement was maximized upon addition of 2.5 equiv of BSA. This result indicated a clear binding of the probe in more hydrophobic pocket of BSA which was also evident from the intrinsic emission behavior of the probe in various solvents of varying dielectric constants (see Supplementary data).[3,5,15] The binding constant of triazolyl pyrene with BSA was also determined fluorimetrically by a Benesi–Hildebrand plot which was found to be $5.1 \times 10^4$ $M^{-1}$ with an experimental free energy of binding, $\Delta G = -6.41$ kcal/mol (Fig. 4b). The blue shift of emission wavelength was also clear from a change of color of probe solution from yellowish green to bright blue color of probe-BSA solution when irradiated at 254 nm under a transilluminator (Fig. 4c). An increase in the % α-helicity, as was indicated by CD spectra of BSA in the presence of our probe, could be attributed to conformational adjustments on complex formation (see Supplementary data).

The lipophilic nature of the probe and the above results indicated that it would preferentially bind to the hydrophobic subdomain of BSA, thus might involve in energy transfer with Trp unit.[15] The possibility of energy transfer process from Trp residue of BSA to the probe molecule was satisfied primarily by the spectral overlap between the fluorescence emission spectrum of BSA and the UV–visible absorption spectra of the probe (see Supplementary data). Thus, upon excitation at donor wavelength (280 nm of Trp of BSA), we observed quenching of fluorescence

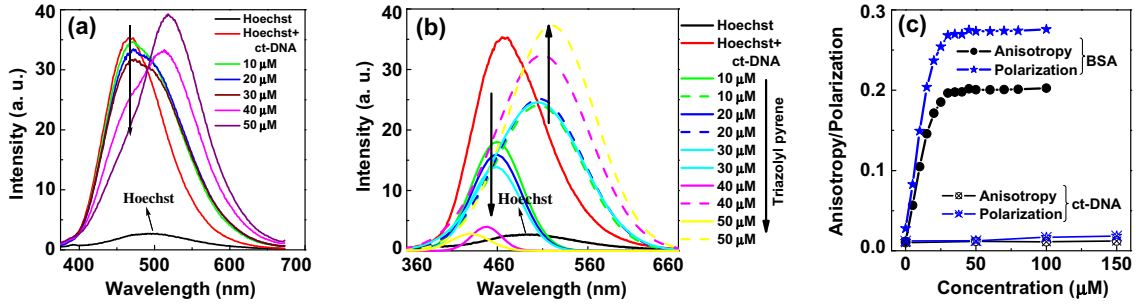


**Figure 3.** (a) Emission spectra of hoechst, hoechst-ct-DNA complex, and hoechst-ct-DNA complex titrated with various concentrations of $^{TNDMB}$**Py** ($\lambda_{ex}$ = 340 nm). (b) Multi Gaussian fitting of spectra '**a**' to show clear decreases in Hoechst emission with an increase in [$^{TNDMB}$**Py**]. Solid lines and dotted lines are the contribution of Hoechst's and $^{TNDMB}$**Py**'s emission respectively in each concentration; [Hoechst] = 10 μM, [ct-DNA] = 50 μM and [$^{TNDMB}$**Py**] = 10, 20, 30, 40, and 50 μM. (c) Fluorescence anisotropy change of $^{TNDMB}$**Py** in the presence of various concentrations of ct-DNA and BSA. All the experiments were carried out in 5 mM phosphate buffer, 5 mM NaCl, pH 7.0, rt, $\lambda_{ex}$ = 345 nm.

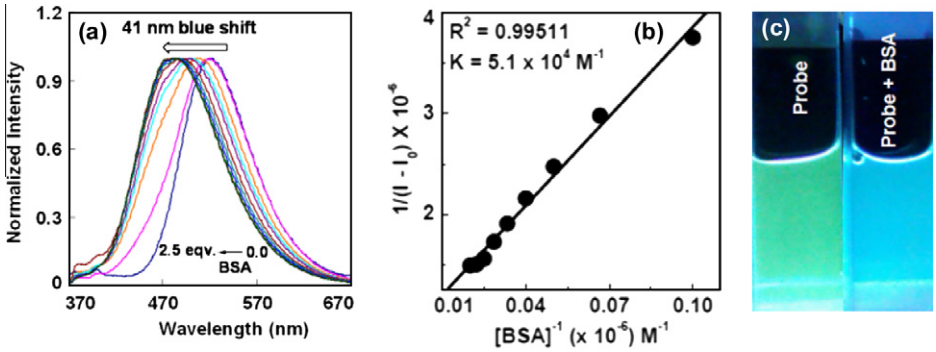


**Figure 4.** (a) Fluorescence titration of probe with various concentrations of BSA. (b) Benesi–Hildebrand plot. (c) Fluorescence image under UV-transilluminator (254 nm) of only probe, in the presence of BSA. All the experiments were carried out in 5 mM phosphate buffer, pH 7.0, rt, $\lambda_{ex}$ = 345 nm.

of BSA and enhancement of fluorescence of the acceptor probe at long wavelength (490–514 nm). This result indicated the occurrence of a FRET process (see Supplementary data). We calculated the Förster distance of energy transfer which was found to be $R_0$ = 24.4 Å with an energy transfer efficiency of 62.3%.[15]

The occurrence of FRET process was also supported by a molecular docking calculation which clearly showed that the probe was located in the vicinity of tryptophan (Trp-237) and remained surrounded by other hydrophobic amino acids of the hydrophobic pocket of sub-domain IIA in site-I of BSA (see Supplementary data). The free energy of binding ($\Delta G$) of $^{TNDMB}$**Py–BSA** complex obtained from the docking calculation was found to be in (−7.72 kcal/mol) good agreement with our experimental value. That the probe involved in tight binding inside the hydrophobic pocket of BSA and experienced a highly restricted rotational motion, was further evident from an enhancement of fluorescence anisotropy of the probe from 0.018 in free aqueous buffer to 0.201 in the presence of 2.5 equiv of BSA (Fig. 3b).[16,17] This large fluorescence enhancement in case of BSA is quite good for practical applications.

Therefore, it is clear from the above findings that the probe triazolylpyrene was efficient in sensing biomolecules via the generation of enhanced fluorescence intensity. The low fluorescence intensity of the probe in phosphate buffer in the absence of biomolecules was not because of poor solubility of the probe in buffer but might be attributed to radiationless channel assisted by intermolecular hydrogen bonding present in aqueous solution. However, in the presence of biomolecules the non-radiative channels were possibly blocked to a great extent and less effective as the probe bound more and more with protein's hydrophobic pocket leading to a fluorescence switch-on signal of enhanced intensity and quantum yield. The same explanation possibly holds true in case of ct-DNA induced fluorescence enhancement of the probe where the probe experienced restricted radiationless channel inside the groove of ct-DNA.

To explore the versatility of our probe, we also studied the detection ability of short DNA duplex containing triple mismatched base pairs. DNA base mismatch is frequent among the most common forms of DNA lesions which when left unrepaired by the base mismatch repair machinery can lead to deleterious mutations that are dangerous to cellular survival and replication.[18] Therefore, the detection and targeting of such specific small deformation in DNA are of paramount importance for the design of new diagnostics and chemotherapeutics. Several methods have been developed to detect mismatch DNA with fluorescent probe.[19] However, conceptual design of label-free detection which we present here is a novel, simple, cost effective, and rapid strategy (Fig. 5).

Spectroscopic evidences of minor groove binding of the probe with ct-DNA and Amber* optimized geometry of the probe with a model DNA sequence (see Supplementary data) using MacroModel led us to think of the detection of short oligonucleotide duplex with our probe via just 'Mix & Read' strategy. Our concept relies on the groove binding of the probe assisted by strong interaction possibly via triazolyl nitrogens in the vicinity of a deformed/mismatched position of the DNA duplex. We envisaged that the triazolyl pyrene might involve in stacking interaction along the groove close to the deformed (mismatched) site (Fig. 6a). Such kind of groove binding and recognition of DNA base mismatch were also shown by imidazole–imidazole pair.[19a] Because of such groove binding we expected that the probe would experience restricted radiationless channel inside the groove that would lead to the

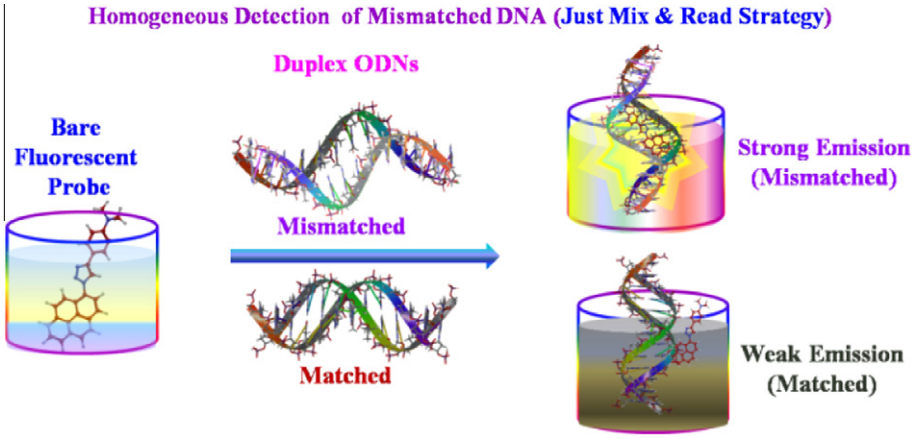


**Figure 5.** Schematic presentation of our concept of mismatch DNA detection.

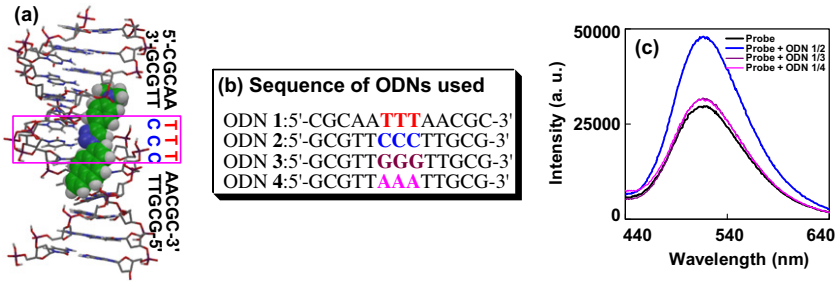


**Figure 6.** (a) Amber* optimized geometry of probe-mismatched DNA showing groove binding. (b) Sequences of ODNs used. (c) Fluorescence response of $^{TNDMB}$**Py** in the presence of various mispaired ODNs at pH 7.0, 50 mM sodium phosphate, 0.1 M sodium chloride, room temperature. Excitation wavelength was, $\lambda_{ex}$ = 345 nm. Concentration of each single strand and the probe, $^{TNDMB}$**Py** was 2.5 μM.

generation of enhanced fluorescence signal. Thus, we could detect deformed DNA duplex with a light-up fluorescence response from bare fluorescent probe, $^{TNDMB}$**Py**.

We also surmised that depending on the sequence at the deformed (mismatched) site the probe might be able to generate a distinct fluorescence signal allowing one to possible discrimination of different mismatched duplex DNAs. Because of quenching nature of G-bases the fluorescence of probe in a triple **T/G** containing short DNA duplex might not show any enhancement. However, the fluorescence enhancement could be the result when the probe would bind to a triple **T/C** mismatch containing DNA. Therefore, we could discriminate **T/C** mismatch DNA from **T/G** mismatch DNA via the generation of a distinct and enhanced fluorescence signal (Fig. 5).

From the fluorescence spectra it was evident that an enhancement of fluorescence compared to probe's emission was observed upon addition of 2.5 μM of probe in duplex ODN **1**/**2** containing triple **T**/**C** mispair (Fig. 6b). On the other hand, duplex ODN **1**/**3** containing triple **T**/**G** mispair or a fully matched duplex ODN **1**/**4** led to no change in emission of bare fluorescent probe, $^{TNDMB}$**Py**. These results indicated that our probe was able to discriminate triple **T**/**C** mispair from a triple **T**/**G** mispair duplex via the generation of an enhanced and distinct fluorescence signal (Fig. 6c). Though the discrimination was not high, our preliminary result might be an inspiration to the design of such fluorescent small molecule to bring efficient discrimination. Currently we are focusing in this aspect.

In conclusion, we have successfully shown, for the first time, that the click chemistry derived triazolyl fluorophore serves as a versatile fluorescent light-up probe for protein and DNA detection in aqueous media. The probe bound efficiently to the minor groove of ct-DNA and the hydrophobic pocket of BSA protein. These modes of binding were supported by fluorescence anisotropy experiment. Also, the probe was able to detect DNA base mismatch with a generation of distinct fluorescence signal. All the spectral evidences open up a multitude of possibilities for using our probe, triazolylpyrene as a fluorescent light-up bioprobe. Furthermore, because the groove binding event in DNA and strong interaction with BSA are important in consideration of drug-DNA/drug-protein interaction, our probe might find application in studying such events. Our preliminary study on the label-free fluorescent light-up sensing and discrimination of DNA base mismatches revealed that the probe would be very useful and might find future applications for the detection of and targeting DNA lesions with a less laborious and cost-effective label-free way. We are currently exploring a detailed study in the context of sensing G-quadruplex DNA with the same probe.

## Acknowledgments

Authors thank the Department of Science and Technology [DST: SR/SI/OC-69/2008], Govt. of India, for a financial support.

## Supplementary data

Supplementary data (photophysical spectra, docking/macromodel structures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.03.029.

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