ORGANIC LETTERS

2009
Vol. 11, No. 19
4302−4305

Nucleic Acid-Induced Aggregation and Pyrene Excimer Formation

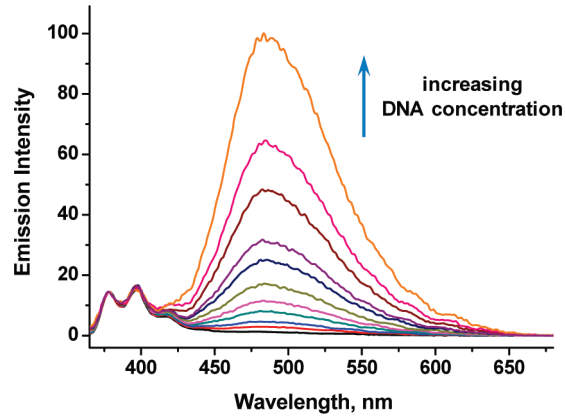
Ruixing Zhang,[†,‡] Dan Tang,[†,‡] Ping Lu,[†,‡] Xiangyu Yang,[†] Dongli Liao,[†] Yujing Zhang,[‡] Mingjun Zhang,[‡] Cong Yu,[*,†] and Vivian W. W. Yam[*,§]

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China, Graduate School of the Chinese Academy of Sciences, Beijing 100039, P. R. China, College of Animal Science and Veterinary Medicine, Jilin University, Changchun 130062, P. R. China, and Department of Chemistry, The University of Hong Kong, Hong Kong, P. R. China

congyu@ciac.jl.cn; wwyam@hku.hk

Received July 14, 2009

ABSTRACT



Nucleic acid was found to induce the aggregation of the positively charged pyrene probe (compound 1); as a result, strong pyrene excimer emission was observed. The intensity of the excimer emission was dependent on the concentration of the pyrene probe and the oligonucleotide length, sequence, and concentration. These results suggest a new strategy for label-free nucleic acid-based biosensing applications.

Sensing nucleic acids and the analysis of their sequence, structure, and interactions with regulating factors play a critical role in basic biological/biomedical research. The knowledge imparted by these studies has had a profound influence on diverse areas such as disease diagnostics and development of new drugs.[1] In recent years, various new nucleic acid sensing methods have been developed concomitant with the emergence of novel advanced materials and concepts.[2−10] However, in many cases, covalent labeling of the nucleic acid with a fluorescent probe is needed either for fluorescence resonance energy transfer (FRET) or fluorescence quenching studies. Herein we describe the use of aggregation-induced pyrene excimer fluorescence for label-free nucleic acid sensing studies.

It is well-known that a number of the planar, aromatic compounds such as pyrene can form excited dimeric structures known as excimers. As a result of excimer formation, a significantly red-shifted, very broad, and almost

[†] State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, and Graduate School of the Chinese Academy of Sciences.
[‡] College of Animal Science and Veterinary Medicine, Jilin University.
[§] Department of Chemistry, The University of Hong Kong.

(1) (a) Bloomfield, V. A., Crothers, D. M., Tinoco, I., Jr. *Nucleic Acids: Structures, Properties, and Functions*; University Science: Sausalito, 2000. (b) *Nucleic Acids in Chemistry and Biology*; Blackburn, G. M., Gait, M. J., Eds.; Oxford University Press, Oxford, 1996. (c) *The Nucleic Acid Protocols Handbook*; Rapley, R., Ed. Humana Press: Totowa 2000.

10.1021/ol901607g CCC: $40.75
Published on Web 09/01/2009

structureless emission band can generally be observed.[11] The use of excimer emission for various sensing applications is well documented, especially for studies of the physicochemical properties of synthetic polymers.[11c] Pyrene excimer formation has also been used in a number of cases for nucleic acid related applications, for example, selective sensing of potassium or construction of novel nanomaterials.[12] However, all these methods require that pyrene be covalently attached to the nucleic acid, which is synthetically quite demanding. As a result, such methods are time-consuming, laborious, and very costly.

We have synthesized a positively charged pyrene probe (compound **1**) (Figure 1). The probe shows considerable water solubility (>1 mM). When compound **1** was mixed with the oligonucleotides in an aqueous buffer solution, remarkable changes in the fluorescence and UV−vis absorption spectra were observed (Figure 2). For all the oligonucleotides tested, the spectra showed a significant decrease in emission from the monomeric form of the pyrene probe and the concomitant appearance of a red-shifted, broad pyrene emission band with a peak maximum at around 485 nm, which was assigned to pyrene probe excimer emission according to numerous literature reports.[11]

The relative intensity of the pyrene excimer emission varied significantly as a function of the amount of compound **1** that was added. Our results show that for a fixed poly(dA)$_{25}$ and poly(dT)$_{25}$ oligonucleotide concentration of 7.2 μM, the intensity ratio of pyrene probe excimer emission (485 nm) to monomer emission (377 nm) (the I_E/I_M value) changed considerably as the concentration of compound **1** was varied. The trends in the I_E/I_M values were quite similar (Figure S1, Supporting Information). As the concentration of compound **1** was increased, the I_E/I_M value became larger until the point at which the I_E/I_M value reached the maximum. A further increase of compound **1** concentration caused a decrease of the I_E/I_M value. Figure S2 (Supporting Information) shows the change in I_E/I_M value versus the change in concentration of compound **1** for poly(dG)$_{25}$ at pH 7.5 and for poly(dC)$_{25}$ at pH 5.0 and 8.5. It was found that the maximum I_E/I_M value for poly(dG)$_{25}$ was much larger than that for poly(dC)$_{25}$. For poly(dC)$_{25}$, the I_E/I_M value for pH at 8.5 was considerably larger than that at pH 5.0.

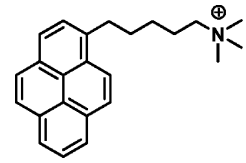


Figure 1. Structure of compound **1**.

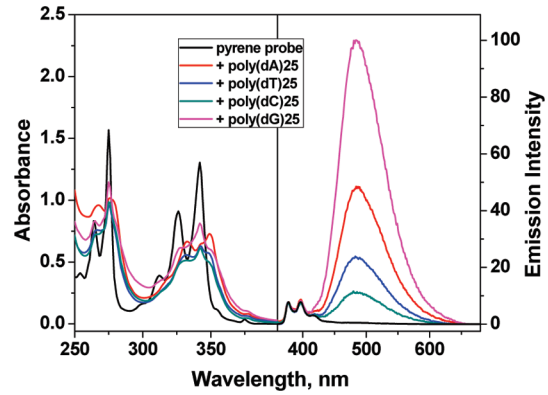


Figure 2. UV−vis absorption and fluorescence emission spectra of 180 μM of compound **1** alone and following its binding to 7.2 μM of simple oligonucleotide repeats in an aqueous buffer solution (MOPS, pH 7.5).

As Figure 2 shows, the I_E/I_M value varied significantly with the sequence of the nucleic acid added. Poly(dG)$_{25}$ gave by far the largest I_E/I_M value (12.88). Poly(dA)$_{25}$ and poly(dT)$_{25}$ also showed large I_E/I_M values (6.20 and 3.03, respectively), but the value of I_E/I_M was considerably smaller for poly(dC)$_{25}$ (1.46).

The I_E/I_M value also varied significantly with the concentration of the nucleic acid added. For example, when 3.6, 7.2, or 10.8 μM of poly(dA)$_{25}$ was mixed with compound **1** (Figure S3, Supporting Information), all three curves showed the same trend as the probe concentration was increased. The I_E/I_M value initially increased, reached the maximum, and then began to decrease. With the increase of the oligonucleotide concentration from 3.6 to 7.2 and 10.8 μM, the concentration of compound **1** needed to reach the maximum I_E/I_M value increased from 150 to 180 and 270

(2) (a) Thomas, S. W., III; Joly, G. D.; Swager, T. M. *Chem. Rev.* **2007**, *107*, 1339–1386. (b) Ho, H.-A.; Najari, A.; Leclerc, M. *Acc. Chem. Res.* **2008**, *41*, 168–178. (c) Achyuthan, K. E.; Bergstedt, T. S.; Chen, L.; Jones, R. M.; Kumaraswamy, S.; Kushon, S. A.; Ley, K. D.; Lu, L.; McBranch, D.; Mukundan, H.; Rininsland, F.; Shi, X.; Xia, W.; Whitten, D. G. *J. Mater. Chem.* **2005**, *15*, 2648–2656.

(3) Gaylord, B. S.; Heeger, A. J.; Bazan, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10954–10957.

(4) Tang, Y.; Feng, F.; He, F.; Wang, S.; Li, Y.; Zhu, D. B. *J. Am. Chem. Soc.* **2006**, *128*, 14972–14976.

(5) Park, S.-J.; Taton, T. A.; Mirkin, C. A. *Science* **2002**, *295*, 1503–1506.

(6) Zhang, J.; Song, S.; Zhang, L.; Wang, L.; Wu, H.; Pan, D.; Fan, C. *J. Am. Chem. Soc.* **2006**, *128*, 8575–8580.

(7) Li, H.; Rothberg, L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 14036–14039.

(8) Tan, W.; Wang, K.; Drake, T. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 547–553.

(9) Wang, M.; Zhang, D.; Zhang, G.; Tang, Y.; Wang, S.; Zhu, D. *Anal. Chem.* **2008**, *80*, 6443–6448.

(10) (a) Yu, C.; Wong, K. M.-C.; Chan, K. H.-Y.; Yam, V. W.-W. *Angew. Chem., Int. Ed.* **2005**, *44*, 791–794. (b) Yu, C.; Chan, K. H.-Y.; Wong, K. M.-C.; Yam, V. W.-W. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 19652–19657. (c) Yu, C.; Chan, K. H.-Y.; Wong, K. M.-C.; Yam, V. W.-W. *Chem.—Eur. J.* **2008**, *14*, 4577–4584. (d) Yu, C.; Yam, V. W.-W. *Chem. Commun.* **2009**, 1347–1349. (e) Yu, C.; Chan, K. H.-Y.; Wong, K. M.-C.; Yam, V. W.-W. *Chem. Commun.* **2009**, 3756–3758.

(11) (a) Birks, J. B. *Rep. Prog. Phys.* **1975**, *38*, 903–974. (b) De Schryver, F. C.; Collart, P.; Vandendriessche, J.; Goedeweeck, R.; Swinnen, A.; Van Der Auweraer, M. *Acc. Chem. Res.* **1987**, *20*, 159–66. (c) Winnik, F. M. *Chem. Rev.* **1993**, *93*, 587–614.

(12) (a) Langenegger, S. M.; Häner, R. *Chem. Commun.* **2004**, 2792–2793. (b) Okamoto, A.; Ichiba, T.; Saito, I. *J. Am. Chem. Soc.* **2004**, *126*, 8364–8365. (c) Nagatoishi, S.; Nojima, T.; Juskowiak, B.; Takenaka, S. *Angew. Chem., Int. Ed.* **2005**, *44*, 5067–5070. (d) Nakamura, M.; Ohtoshi, Y.; Yamana, K. *Chem. Commun.* **2005**, 5163–5165. (e) Malinovskii, V. L.; Samain, F.; Häner, R. *Angew. Chem., Int. Ed.* **2007**, *46*, 4464–4467.

μM, respectively. At the same time, the maximum I_E/I_M value increased from 2.60 to 6.14 and 8.67, respectively. In addition, a similar trend was observed with the concentration of compound **1** fixed and the concentration of poly(dA)$_{25}$ varied (Figure S4, Supporting Information). With increasing poly(dA)$_{25}$ concentration, the I_E/I_M value increased until it reached the maximum, after which a further increase of the oligonucleotide concentration caused a decrease of the I_E/I_M value.

The I_E/I_M value also depended on the chain length of the oligonucleotide (Figure 3). For example, for poly(dA), reduction of the oligonucleotide length from 25 bases to 20, 15, 10, and 5 bases caused a gradual decrease of the I_E/I_M value. With only five bases, poly(dA)$_5$ could not induce the aggregation of the probe, and hardly any excimer emission was observed.

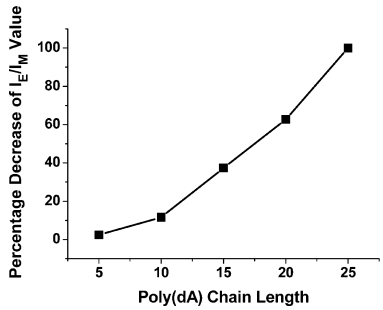


Figure 3. Percentage decrease of the I_E/I_M value when 180 μM compound **1** was mixed with poly(dA) of different chain lengths. The nucleic acid base concentration (dA) was kept constant at 180 μM.

A 25-base oligonucleotide poly(dM)$_{25}$ (5′-GAT CTG ACG GTT CAC TAA ACG AGC T-3′) was randomly selected. Poly(dM)$_{25}$ and the corresponding duplex DNA were tested for induced excimer emission (Figure S5, Supporting Information). It was found that both poly(dM)$_{25}$ and the duplex DNA showed trends similar to the other simple oligonucleotides. For duplex DNA, however, higher I_E/I_M values were generally observed.

Since nucleic acid is a polyanion, the results suggest that when nucleic acid was mixed with compound **1**, electrostatic attractive force between the positively charged trimethylammonium functional group in **1** and the negatively charged phosphate backbone in the oligonucleotide induced aggregation of compound **1** in the vicinity of the nucleic acid. That is, the local concentration of compound **1** around the nucleic acid increased. Equally important, the positive charge of compound **1** was largely balanced out by the negative charge of the nucleic acid. As a result, hydrophobic $\pi-\pi$ stacking interactions between the planar aromatic rings of the probe molecule dominated. As shown in Figure 2, UV−vis spectral changes of compound **1** upon mixing with the nucleic acid clearly indicate ground state aggregation of the probe molecule, which suggests that the observed excimer is largely static in nature.[11]

The intensity changes of the probe excimer emission upon mixing with the oligonucleotides were dependent on the concentration of the probe, as well as the type, concentration, and length of the oligonucleotides. At a fixed concentration of the oligonucleotide, an increase of the probe concentration increased the extent of probe binding to the nucleic acid and consequently increased the degree of probe aggregation. As a result, the I_E/I_M value increased. However, since the concentration of the nucleic acid was kept constant in the buffer solution, the total number of negative charges for probe binding in the assay system did not increase. The negative charges on the phosphate backbone of the nucleic acid were largely neutralized up to a certain point. A further increase of the probe concentration would not be expected to result in additional binding (that is, the probe reached a saturation concentration). Consequently, a decreased I_E/I_M value was observed (Figures S1−S3, Supporting Information).

The intensity of the induced excimer emission was dependent on the type of oligonucleotide used. This effect is most dramatically demonstrated in Figures 2 and S2 (Supporting Information). It is well-known that poly(dG) can form G-quadruplex structures as a result of the guanine base hydrogen bonding and $\pi-\pi$ stacking interactions among the four strands of poly(dG).[1] It is also well-known that poly(dC) can form i-motif structures (Figure S6, Supporting Information) under an acidic condition.[1] Poly(dG)$_{25}$ could form extended four-stranded structures, and the steep rise of charge density upon G-quadruplex formation would greatly enhance the aggregation of the probe molecule, and therefore, considerably larger I_E/I_M values were observed. In contrast, half of the cytosine bases must be protonated for i-motif formation. As a result, the total number of negative charges on the nucleic acid decreases by half, which resulted in decreased I_E/I_M values.

The intensity of the induced excimer emission was dependent on the concentration of the oligonucleotide (Figures S3 and S4, Supporting Information). A higher concentration of the oligonucleotide resulted in an increase in the number of electrostatic binding sites available for the probe molecule, so a higher I_E/I_M value is expected. However, at a fixed probe concentration, if the concentration of the oligonucleotide exceeded a certain threshold value, a further increase of its concentration resulted in a decrease of the I_E/I_M value. The reason is that if the concentration of the oligonucleotide is too high, the average number of probe molecules binding to each oligonucleotide decreases. This effectively "diluted" the number of probes binding to each oligonucleotide, and a decreased I_E/I_M value was observed (Figure S4, Supporting Information).

The induced excimer emission was dependent on the chain length of the oligonucleotide. A longer oligonucleotide would carry more negative charges and better induce probe aggregation. Therefore, a decrease of the I_E/I_M value was observed with the decrease in chain length of poly(dA) (Figure 3). For duplex DNA, although the total number of negative charges in the system was not increased, the

increased charged density resulted in higher I_E/I_M values (Figure S5, Supporting Information).

Because of the extensive aggregation of compound **1** upon binding to nucleic acid under our normal experimental conditions, conventional methods for binding constant evaluation could not be directly applied. However, under conditions of lower compound **1** concentration, a good linear fit was obtained. For binding of compound **1** to poly$(dN)_{25}$, the calculated binding constant was 1.6×10^5. The corresponding duplex gave a higher value of 3.1×10^5, which is consistent with the experimental results. In addition, Job's plot suggests that the binding stoichiometry between compound **1** and the nucleic acid base of poly$(dN)_{25}$ was about 1.4:1. If pure electrostatic interactions were involved, we would expect a binding stoichiometry of 1:1; thus, the results suggest that in addition to ionic interactions, hydrophobic interactions could also be involved in the binding process (see the Supporting Information).

As a proof-of-principle demonstration, we have utilized the nucleic acid induced excimer fluorescence for the detection of nuclease activity. A fixed amount of single-strand specific nuclease S1 was mixed with poly$(dN)_{25}$ [or poly$(dA)_{25}$] and incubated for a certain period of time. Compound **1** was subsequently added, and the emission spectrum was measured. The results show that strong excimer emission was observed with 0 min incubation time, indicating strong induced probe aggregation. Excimer emission gradually decreased with increasing incubation time and with no induced excimer emission able to be observed after 25 min of incubation (Figure S9, Supporting Information).

The results suggest that after prolonged incubation with nuclease S1, more single-stranded DNA was hydrolyzed. As mentioned above, little induced compound **1** aggregation could be observed with an oligonucleotide sequence shorter than five bases. Apparently, after degradation by nuclease for a certain period of time, short oligonucleotides and mononucleotides were produced, and the concentration of oligonucleotides with a length greater than five bases decreased. This resulted in a reduced degree of induced aggregation of the probe molecule, and therefore, reduced excimer emission was detected.

Pyrene excimer emission induced by nucleic acid can also be applied to the detection of single nucleotide polymorphism. The target sequence mixed with the mutant sequence (ssDNA-0 + ssDNA-2) (Supporting Information) and the target mixed with the complementary sequence (ssDNA-0 + ssDNA-1) were incubated at 37.5 °C for 15 min. Subsequent rapid cooling to −20 °C ensured that the mixture containing a complementary sequence produced normal duplex DNA, whereas the mutant could only give a mixture of single stranded oligonucleotides. The addition of nuclease S1 could completely remove all single strands in the reaction mixtures, but had no effect on the duplex DNA.

As shown in Figure S10 (Supporting Information), the mixture containing no mutation showed strong excimer fluorescence after nuclease digestion. In contrast, excimer fluorescence vanished with the mixture containing mismatched ssDNA-2. Mismatched sequences have a lower duplex melting temperature. By careful selection of a suitable annealing temperature, only the mixture containing the complementary sequences could have duplex DNA. At the same time, the mixture containing a single point mismatch had little duplex formation. Mismatched ssDNAs cleaved with nuclease S1 gave no induced pyrene probe aggregation, so no excimer emission was detected.

In conclusion, a positively charged pyrene probe (compound **1**) was synthesized. Oligonucleotides were shown to induce aggregation of compound **1** via electrostatic and hydrophobic interactions, and the induced $\pi-\pi$ stacking interactions between pyrene aromatic rings gave rise to strong excimer emission. We demonstrate that the induced excimer fluorescence is related to the concentration, length, sequence, and the secondary structure of the nucleic acid, as well as the concentration of the probe molecule. The induced excimer emission was furthermore used to monitor the DNA cleavage reaction by nuclease S1 and the detection of single-point mutation in DNA. We believe that the properties of the probe can be finely tuned, and that the assay conditions may be further optimized. We envision that our method could assist the future developments of basic biochemical research and practical applications.

Acknowledgment. This work was supported by the "Hundred Talents Project" (initiation support) of the Chinese Academy of Sciences and the National Natural Science Foundation of China (No. 20845006).

Supporting Information Available: Experimental details, synthesis and characterization of compound **1**, and UV−vis and fluorescence studies. This material is available free of charge via the Internet at http://pubs.acs.org.

OL901607G