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Water-Soluble Tetraphenylethene Derivatives as Fluorescent "Light-Up" Probes for Nucleic Acid Detection and Their Applications in Cell Imaging

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Abstract: The detection of nucleic acids, such as DNA and RNA, plays a significant role in genetic engineering, forensics, and bioinformatics. Traditional nucleic acid probes are mainly intercalators, which are potential mutagens, or groove binders that show high preference only for double-stranded DNA. We herein present two versatile fluorescent probes for nucleic acid detection and visualization. The nonemissive tetraphenylethene derivatives (TTAPE) are induced by DNA/RNA to emit, thereby showing a novel phenomenon of aggregation-induced emission (AIE). This kind of "light-up" property enables the quantitation and visualization of nucleic acids in aqueous solution and electrophoretic gels, respectively. The cationic TTAPE can penetrate cells with a compromised plasma membrane easily but cannot enter live cells with an intact membrane, thus making them useful for the differentiation between dead and live cells. On account of the high binding affinity to DNA, TTAPE can selectively label the chromosomes and nuclei in fixed cells, which provides a simple and fast method for the observation of cell mitosis. Owing to their AIE characteristics, the dye molecules aggregate in DNA-rich regions and exert appreciable quantum efficiency as well as superior photostability.

Keywords: aggregation · electrostatic interactions · fluorescent probes · nucleic acids · phenylethene

Introduction

Fluorescent (FL) probes are powerful analytical tools, particularly in the field of biochemical and biomedical research.[1] While the use of inorganic quantum dots and fluorescent nanoparticles offers a variety of advantages, such as improved photostability and high luminescence quantum efficiency, small organic dye molecules offer the opportunity to vary the chemical and photophysical properties of fluorescent probes by a deliberate modulation of the chemical structures of the fluorophores and a systematic variation of their substitution patterns to generate an almost unlimited range of potential applications.[2–4]

In particular, fluorescent probes with emission that is activated upon interaction with biomacromolecules, such as proteins and DNA, are useful markers in proteomics and genomics because they enable the visual observation of the biological species and the tracking of their related events ("light-up" probes).[1a,5] Several probes for nucleic acid detection based on FL enhancement have been developed; these include ethidium bromide (EB), Hoechst dyes, acridizinium salts, cyanine derivatives, and ruthenium complexes.[6–11] Through intercalation between two adjacent base pairs or binding to the minor grooves of the DNA chains, their fluorescence can be triggered. Among them, EB is a universal nucleic acid stain in molecular biology laboratories; it is used especially for gel electrophoresis because of its sensitivity and low cost.[6] However, EB is suspected to be a very strong mutagen or carcinogen. There are some alternatives to EB in the laboratory, such as SYBR-based dyes.[11c] These dyes have been found to be less carcinogenic. However, they are lipophilic and have to be suspended in organic solvents such as DMSO, which can rapidly pass through skin. Thus, it is desirable to develop a "safe" probe for the quantitation and visualization of nucleic acids.

Most of the conventional FL dyes aggregate when dispersed in aqueous media or bound to biomacromolecules in large quantities.[12] Self-quenching often accompanies the aggregation of the dyes, thereby resulting in a drastic reduc-

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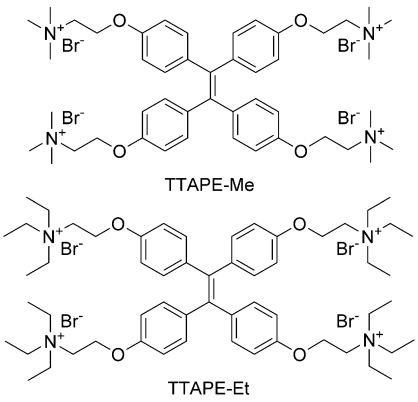
tion in their FL signals. This aggregation-caused quenching (ACQ) has been a thorny problem in the development of efficient bioprobes and biosensors.[13] Recently, we and others have observed a phenomenon of aggregation-induced emission (AIE) that is exactly the opposite of the ACQ effect: a series of nonemissive dyes such as siloles and tetraphenylethenes (TPE) are induced to emit intensely by aggregate formation.[14] Through a series of designed experiments and theoretical calculations, we proposed the restriction of intramolecular motions as the main cause for the AIE effect.[14b] We fabricated light-emitting diodes based on the AIE luminophores and obtained outstanding results.[15–17] To explore the potential biological applications of the TPE luminophores, in this study we synthesized water-soluble cationic TPE derivatives (TTAPE) and investigated their utilities for nucleic acid detection.

In aqueous solution, the nonemissive TTAPE molecules became highly luminescent upon binding to DNA or RNA through electrostatic attraction on account of their multiple positive charges. By taking advantage of the "light-up" effect, they were employed for the quantitative analysis of DNA and RNA in solution, visualization of DNA bands in electrophoretic gels, and imaging of fixed cells. The aggregates of these dyes exerted excellent resistance to photobleaching relative to isolated small organic molecules. They could easily penetrate the cells with compromised plasma membranes and yet will not enter the nucleus of live cells, thus making them useful for discriminating between dead and live cells. A methyl thiazolyl tetrazolium (MTT) assay revealed that these dyes are cytocompatible and pose no threat of toxicity to living cells.

Abstract in Chinese:
核酸的檢測，包括脫氧核糖核酸及核糖核苷酸的檢測，在基因工程，犯罪取證及生物信息學中都起着極其重要的作用。傳統的核酸探針主要分為兩類。一類通過嵌入鹼基對而實現其對核酸的特異性染色。但這種特性也令該類化合物成為潛在的誘變劑。另一類探針傾向於與核糖核酸的溝槽結合。但此類探針僅對雙鏈的脫氧核糖核酸有響應。在此，我們報導兩例用於核酸檢測及顯像的多功能熒光探針。這兩種探針是四苯基乙烯的衍生物（簡稱TTAPE），具有聚集誘導發光特性。一旦接觸核酸，原本不發光的探針即被點亮。這種"點亮"式的特性使之能對水溶液中或者電泳膠中的核酸進行定量和顯像。由於TTAPE帶有正電荷，可以輕易地穿透有損傷的細胞膜，但卻不能穿透具有完整細胞膜的活細胞，所以它們可以用來區分死細胞及活細胞。TTAPE對脫氧核糖核酸有極強的親和力，可對固定後細胞的染色體和核仁進行特異性染色。該方法簡單而快速，亦可用於細胞分裂的觀察。得益於它們的聚集誘導發光特性，這些聚集於富含脫氧核糖核酸的區域的染料展現出了良好的發光效率和光穩定性。



Results and Discussion

Nucleic Acid Detection in Solution

To explore the potential biological applications of the AIE luminophores, we synthesized two water-soluble TPE derivatives according to our previous publications.[16a,b] 1,1,2,2-Tetrakis[4-(2-bromoethoxy)phenyl]ethene was quaternized by either trimethylamine or triethylamine to furnish the salt 1,1,2,2-tetrakis[4-(2-trimethylammonioethoxy)phenyl]ethene tetrabromide (TTAPE-Me) and 1,1,2,2-tetrakis[4-(2-triethylammonioethoxy)phenyl]ethene tetrabromide (TTAPE-Et), respectively. Both TTAPE dyes are soluble in water and buffer solutions (up to 50 mg mL^{-1}). The addition of methanol, acetonitrile, tetrahydrofuran (THF), and 1,4-dioxane to their aqueous solutions barely causes the dye molecules to aggregate and to enhance their light emission, which is possibly due to the amphiphilic nature of the ammonium moieties. However, an increase in the viscosity and decrease in the temperature of the TTAPE solutions can increase their quantum efficiencies, which suggests that they are both AIE-active.[16b] Such external perturbation restricts their intramolecular motions, which blocks the nonradiative relaxation channels and hence turns on their light emission.

The restriction of intramolecular motions can also be accomplished in the molecular recognition process of biomacromolecules. Thus, we investigated the interaction of the cationic AIE dyes with nucleic acids. Here we used calf thymus DNA (ctDNA) and RNA from torula yeast as models for the spectrometric titrations. A dilute solution of TTAPE-Me in phosphate-buffered saline (PBS) solution (pH 7.0) is virtually nonluminescent. Addition of a small amount of ctDNA to the aqueous solution turns on its emission. Increasing the DNA concentration further enhanced the FL intensity but caused no change in the spectral profile (Figure 1A). The FL intensity recorded at 470 nm increases rapidly at low ctDNA concentration and is gradually saturated when the ctDNA concentration becomes higher (Figure 1B). The change of (I/I_0-1) versus ctDNA concentration can be

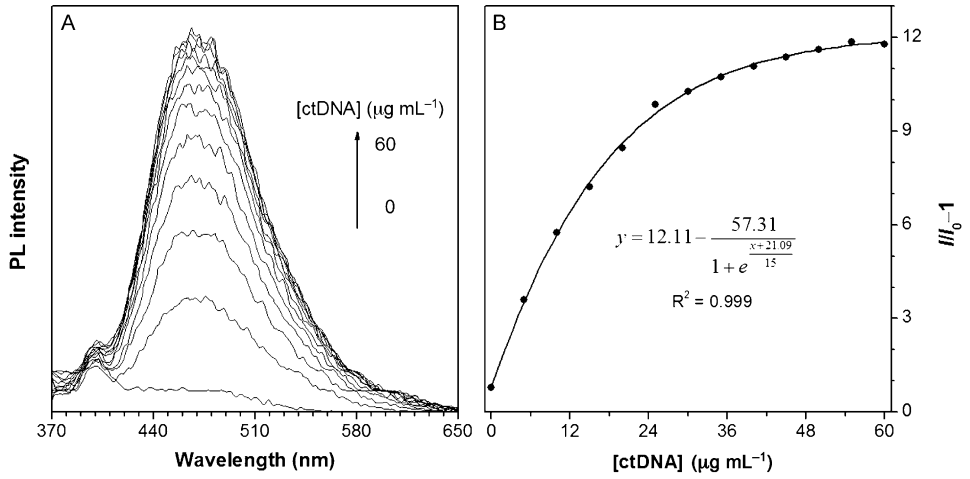


Figure 1. A) Fluorimetric titration of ctDNA to an aqueous solution of TTAPE-Me in PBS (pH 7.0). B) Plot of I/I_0-1 at 470 nm versus the ctDNA concentration. I_0 = emission intensity in the absence of ctDNA. [TTAPE-Me] = 5 μM; λ_{ex} = 350 nm.

satisfactorily fitted to the Boltzmann function as shown in Figure 1B.

The FL process of TTAPE-Me can also be activated upon binding to RNA. With an increase in the RNA concentration, the emission intensity of TTAPE-Me increases, accompanied by a gradual blueshift of the emission maximum from 463 to 443 nm (Figure 2A). The plot of the FL intensity at 470 nm as a function of RNA concentration shows that the emission keeps rising with increasing RNA concentration without reaching a maximum, thus indicating a wide dynamic range of RNA detection (Figure 2B). In the RNA concentration range of 0–125 μg mL^{-1}, the plot displays a good linear relationship with an R^2 value of 0.997.

TTAPE-Et exhibits a similar FL "turn-on" property when binding to DNA and RNA. Its FL intensity of TTAPE-Et increases swiftly at low DNA concentration and reaches a plateau when the DNA concentration is higher than 60 μg mL^{-1}. The plot of (I/I_0-1) values versus the ctDNA concentration can be expressed by the Hill function as shown in the Supporting Information. The change in FL intensity of TTAPE-Et in the presence of RNA is given in the Supporting Information. Similar to TTAPE-Me, the (I/I_0-1) value increases monotonically with increasing concentration. A linear relationship is established at low RNA concentrations (0–20 μg mL^{-1}).

The above results demonstrate the utility of TTAPE for the detection and quantitation of nucleic acids in aqueous media. In buffer solutions, the cationic dye molecules spontaneously bind to the negatively charged DNA and RNA mainly driven by electrostatic forces to form TTAPE/nucleic acid complexes. When docked on the surfaces of the biopolymers, the intramolecular motions of the dye molecules are restricted, which impedes their radiationless transitions and activates their FL processes. Due to the AIE nature, the emissions of the TTAPE–nucleic acid complexes are intensified with increasing dye concentration.[16a] This is remarkable as conventional FL probes suffer from the ACQ problem at high dye concentrations.

Nucleic Acid Visualization in Gels

The FL "turn-on" property of TTAPE upon binding to DNA and RNA prompted us to examine whether they can be used as nucleic acid stains for gel electrophoresis. Figure 3 shows the gel images of electrophoresed oligonucleotides after staining with TTAPE solution. The DNA bands become visible under UV illumination. The detection limit of TTAPE-Me can be as low as 0.25 μg per lane (lane 2, Figure 3A), whereas that of TTAPE-Et is around 1.0 μg per lane (lane 4, Figure 3B). In general, TTAPE-Me performs better than TTAPE-Et in terms of sensitivity. This is understandable because the trimethylammonium groups in TTAPE-Me are less bulky than the triethylammonium functionalities in TTAPE-Et. This

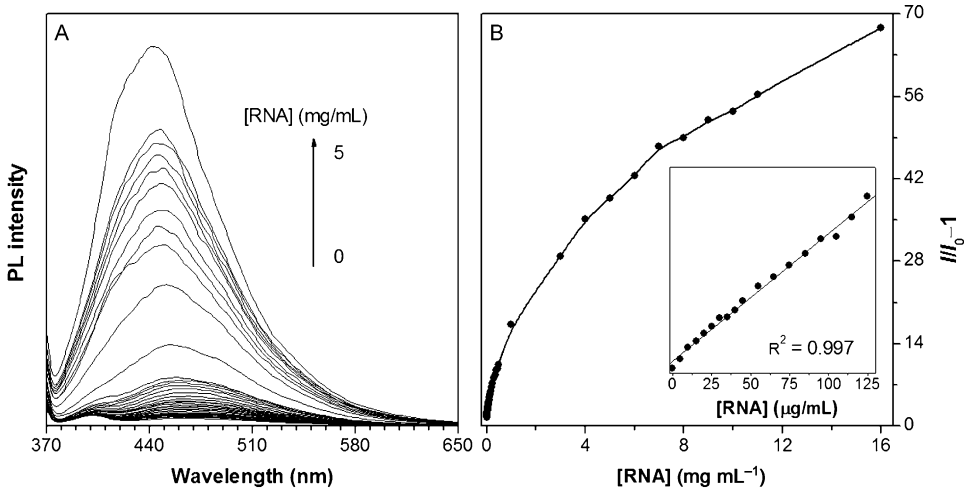


Figure 2. A) Fluorimetric titration of RNA to an aqueous solution of TTAPE-Me in PBS solution (pH 7.0). B) Plot of I/I_0-1 at 470 nm versus the RNA concentration. I_0 = emission intensity in the absence of RNA. Inset in (B): Linear region of the binding isotherm. [TTAPE-Me] = 5 μM; λ_{ex} = 350 nm.

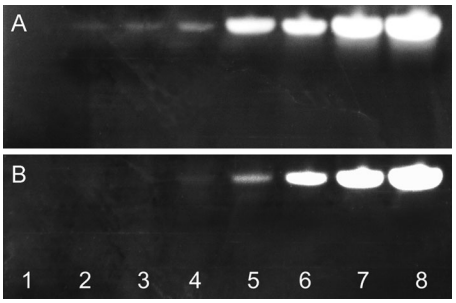


Figure 3. Staining of oligonucleotides in PAGE by A) TTAPE-Me and B) TTAPE-Et. Concentrations of oligonucleotide in lanes 1–8: 0, 0.25, 0.5, 1.0, 5.0, 10.0, 25.0, and 50.0 μg. Concentration of dyes: 10 μM. Staining time: 30 min.

barely shields the positively charged nitrogen atoms and hence strengthens its interaction with the negatively charged DNA strands.

Agarose gel electrophoresis is commonly used for the separation of DNA with high molecular weight. The visualization of DNA in agarose gels can be achieved either by prestaining, in which an aliquot of dye is premixed with the agarose solution prior to casting, or by staining the gel in a bath of dye solution after electrophoresis. The Supporting Information shows the gel prestained by TTAPE-Me after electrophoresis. The DNA bands are clearly discernible under UV illumination. However, the background is relatively strong, probably because the intramolecular motions of the dye molecules are restricted in the gel matrix, thus making them emissive. Under the electric field, the negatively charged DNA strands and the positively charged dye molecules migrate in opposite directions. The dye molecules might bind to the DNA strands and accumulate on their DNA surfaces, which will further intensify their fluorescence. On the other hand, the agarose gels can also be poststained like polyacrylamide gels. Gels are soaked in either TTAPE-Me or TTAPE-Et solution. The DNA bands can be visualized by TTAPE with low background noise (see the Supporting Information). The dye molecules are only weakly adsorbed on the surface of the gel and thus can be easily removed to decrease the background noise.

To examine whether the TTAPE dyes are "safe" staining agents, cytotoxicity assays were performed with HeLa cells on the basis of reduced activity of MTT.[18] Living HeLa cells were exposed to different concentrations of TTAPE buffer solutions for 48 hours, after which the percentages of the viable cells were quantified. The MTT assay revealed that the cell viability was not significantly altered even when up to 80 μM of TTAPE was added to the culture medium (see the Supporting Information). In other words, the dyes are cytocompatible without interfering with the metabolisms of the living cells.

Nuclear and Chromosomal Staining

The cationic AIE dyes exhibit high affinity for nucleic acids and thus can be applied for the quantitation and visualization of nucleic acids in solution and in gels, respectively. The "light-up" property and their excellent biocompatibility encouraged us to utilize them for cell imaging. However, the two dyes do not permeate to the cell nucleus. We thus developed them as DNA counterstains for chromosome labeling in fixed cells. Human breast cancer cells (MDA-MB-435) in metaphase were arrested by demecolcine and spread on microscopic slides followed by incubation with 50 μM TTAPE for 3 minutes at room temperature. As TTAPE dyes are practically nonfluorescent in the aqueous medium, no washing is required. The Supporting Information shows the FL images of chromosomes and the nuclei of cells stained by TTAPE. The DNA-rich regions are lit up by the blue fluorescence of TTAPE. As discussed above, TTAPE-Me shows a higher affinity for DNA than TTAPE-Et on account of the less bulky ammonium claws. Thus, it exhibits a better performance as a nucleic acid stain than TTAPE-Et.

Photostability is an essential parameter for determining the suitability of a fluorophore for imaging applications.[17c] Both fluorophores possess excellent photostability in solution (see the Supporting Information). For imaging, a slice of metaphase cells stained by TTAPE-Me was chosen for the photostability test (Figure 4). After continuous UV illumination for 90 seconds, emission from the dye-stained chromosomes and nucleus was still visible. The fluorescence was weakened clearly when the total exposure time was prolonged to 5 minutes. However, the genetic material could still be seen upon manual modulation of the image contrast. Although the photobleaching resistance of TTAPE-Me is not as good as that of quantum dots (QDs), it is much better than that of some fluorescent proteins (FPs) and typical organic fluorophores, the emissions of which are easily bleached upon UV exposure for less than 1 s and 1–10 s, respectively.[19]

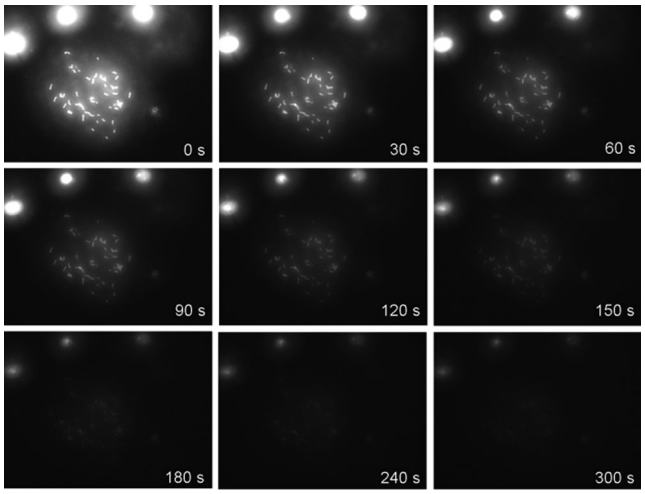


Figure 4. Fluorescent images of nuclei and chromosomes of MDA-MB-435 cells stained by TTAPE-Me under continuous illumination. [TTAPE-Me] = 50 μM.

The improved photostability of TTAPE might originate from its AIE characteristics. Driven by electrostatic attraction, the cationic dye molecules prefer to agglomerate in the DNA-rich regions, such as the nucleus and chromosomes. Since conventional fluorophores suffer from the ACQ effect in the aggregated state, they are commonly utilized as isolated single molecules in very dilute solutions. The small number of the dye molecules in dilute solutions can be quickly photobleached when a laser beam is used as the excitation light source. By contrast, the AIE effects of TTAPE permit the use of their solutions at high concentration or aggregates in poor solvents to increase the brightness and the resistance to photobleaching when exposed to UV irradiation. Only emissions of the dye molecules on the surface of the aggregates are quenched. The inner parts of the aggregates will be shielded from the harmful species that cause photobleaching.

Drosophila melanogaster offers an outstanding opportunity for studying the structure of chromosomes on account of the polyene nature of the chromosomes found in its salivary glands. In these glands, repeated rounds of DNA replications have occurred without mitosis, thus forming a giant chromosome with specific banding patterns along their length. The banding patterns of the chromosomes contain information about chromosomal alterations such as deletions, transpositions, duplications, and so on.[20] The isolated salivary glands from the larvae were fixed, stained with TTAPE, and squashed. As shown in Figure 5, only the chromosomes in the squash can be visualized with the help of the fluorescence from TTAPE. More significantly, the bands on the chromosomes can be resolved for further interpretation of the genetic information.

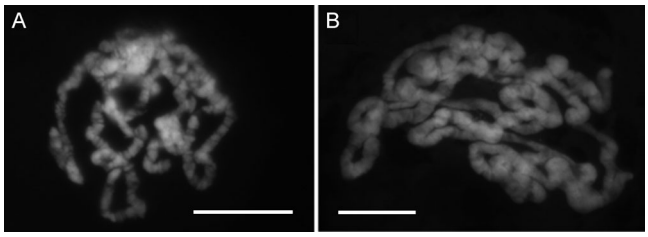


Figure 5. Polytene chromosomes from *Drosophila* salivary gland stained by A) TTAPE-Me and B) TTAPE-Et. [TTAPE-Me] = 5 mM; [TTAPE-Et] = 1 mM. Scale bars, 20 μm.

In addition to animal cells, we performed the staining experiments using plant cells. Onion (*Allium cepa*) root-tip cells in different phases of mitotic cell division can be clearly observed by the emission from TTAPE-Me (Figure 6). Intact nuclei are captured in the interphase. When entering the prophase, the nuclear membrane disintegrates and frees the chromosomes into the surrounding cytoplasm. The chromosomes align along the center plane in the metaphase and the dye molecules aggregate on the chromosomes, thereby making them highly emissive. During anaphase, the sister chromatids begin to migrate toward the opposite poles of the cells, and the picture shown here presents the cell in the early anaphase. At telophase, the chromosomes begin to cluster together, thereby facilitating the formation of a new nuclear membrane. The molecules of TTAPE-Me show a high affinity for DNA and thus can readily light up the chromosomes and nucleus with high efficiency. The method provided here is simple and fast for the observation of cell mitosis and it will be more useful when counterstaining with other fluorophores for multicolor FL imaging.

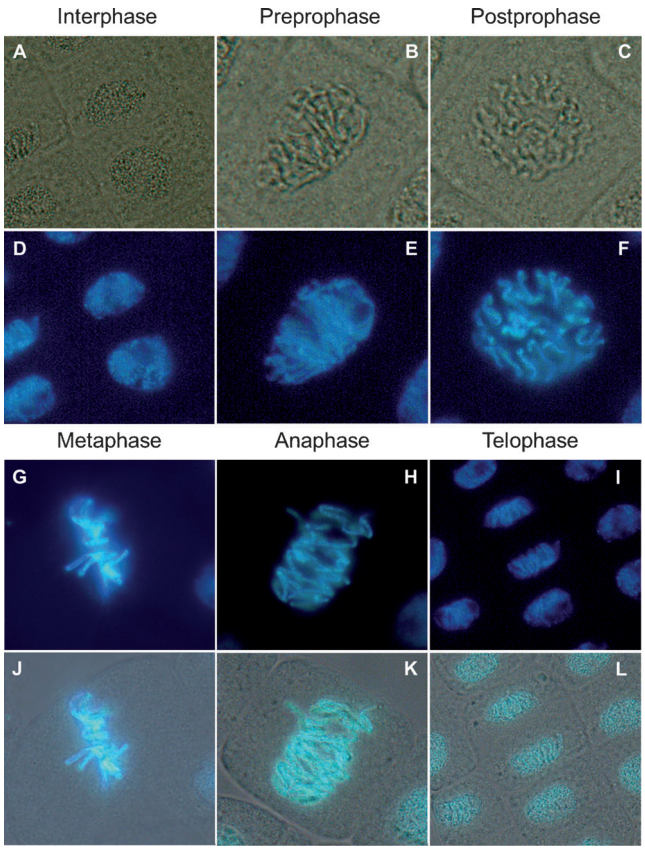


Figure 6. A–C) Phase contrast, D–I) fluorescence, and J–L) merged images of cells from onion root tips at different stages of the cell cycle stained by TTAPE-Me. [TTAPE-Me] = 50 μM.

Live/Dead Cell Differentiation

The TTAPE molecules can easily penetrate cells with a compromised plasma membrane and cluster in the nucleus but will not cross the nuclear membrane of live cells. This is especially useful for dead-cell staining. Cell necrosis was induced by addition of hydrogen peroxide to the medium. As can be seen from Figure 7A and C, only dead cells exhibit bright blue fluorescence. From the phase contrast images, it is not easy to discriminate between dead and live cells under mitosis. However, upon addition of TTAPE, the dead cells can be identified and quantitated with ease under UV illumination (Figure 7B and D).

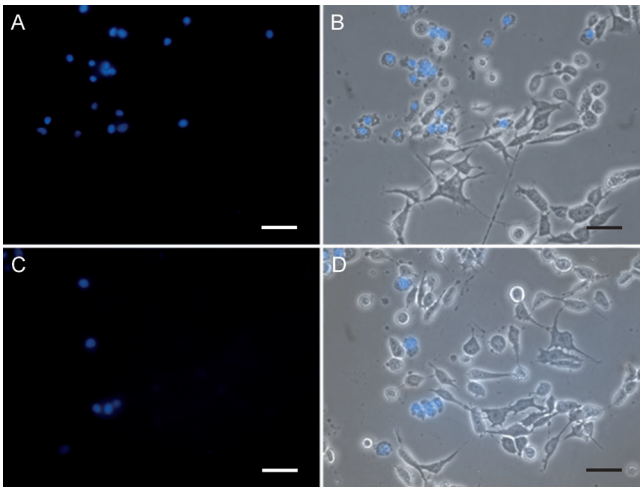


Figure 7. A, C) Fluorescent and B, D) merged images of MDA-MB-435 cells treated with hydrogen peroxide stained by A, B) TTAPE-Me and C, D) TTAPE-Et. [TTAPE] = 5 μM. Scale bars: 25 μm.

Conclusion

In this study, two water-soluble TPE derivatives have been synthesized and their biological applications have been explored. When binding to the negatively charged DNA and RNA driven by electrostatic forces, the emissions of the cationic TTAPE molecules are turned on due to the restriction of their intramolecular motions. Such "light-up" properties enable them to act as FL probes for quantitative analysis of nucleic acids in solution and visualization of DNA bands in gels. Unlike conventional nucleic acid stains, the aggregates of TTAPE show a high affinity for DNA-rich regions such as chromosomes and the nucleus, and they exhibit intense fluorescence and superior photostability. All these attributes make them especially useful for time-lapse microscopy. TTAPE can easily penetrate cells with compromised plasma membranes and yet will not enter the nucleus of live cells, which is indicative of their potential applications as simple and quantitative one-step dead-cell indicators for epifluorescence and confocal laser-scanning microscopes, fluorometers, fluorescence microplate readers, and flow cytometers. Further structural optimization to improve the sensitivity of the AIE dyes for nucleic acid detection is in progress in our laboratories.

Experimental Section

Materials and Methods

TTAPE-Me and TTAPE-Et were prepared according to our previously published procedures.[16a,b] Calf thymus DNA (ctDNA) and RNA from torula yeast were purchased from Sigma–Aldrich and used as received. Oligonucleotides are purchased from Invitrogen (Carlsbad, CA). Phosphate-buffered saline (PBS) with a pH of 7.0 was purchased from Merck. Water was purified with a Millipore filtration system and sterilized by autoclave at 121 °C for 20 min. All the experiments were performed at room temperature unless otherwise specified.

DNA and RNA solutions were prepared in PBS (1.0 mg mL^{-1}) and filtered through a 0.45 μm filter. The actual concentrations were determined by UV photometry by using the extinction coefficient ε_{260} = 6600 M^{-1} cm^{-1}. Stock solutions (5.0×10^{-4} M) of TTAPE were prepared in PBS. Fluorescence titration was carried out by sequentially adding aliquots (10 μL) of DNA or RNA solution to dilution solutions (10 mL) of TTAPE. The mixtures were vortexed prior to the measurements.

Nuclear and Chromosomal Staining

Human breast cancer cells (MDA-MB-435) were cultured in a minimum essential medium (MEM) that contained 10 % fetal bovine serum and antibiotics (100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) in a 5 % CO_2 humidity incubator at 37 °C. To obtain cells arrested at the metaphase of the cell cycle, subconfluent monolayer cells were subjected to treatment with 0.1 μg mL^{-1} Demecolcine (Sigma) for 3 h. The arrested cells were re-suspended in 0.075 M KCl hypotonic buffer for 20 min and then fixed with methanol/acetic acid (3:1). An aliquot of cell suspension (15–20 μL) was dropped onto a frozen slide, and then the slide was placed under a fumehood to evaporate the fixatives. The sample was incubated with 50 μM TTAPE for 5 min at room temperature and then washed with PBS before the imaging study.

A *Drosophila melanogaster* larvae was picked up with a dissecting needle, rinsed with water, and placed on a microscope slide. A pair of salivary glands was isolated by dissecting it with two needles and soaking it in 0.7 % NaCl for 10 min. The glands were then re-suspended with 1 N HCl for 2 min to fix the tissue, then rinsed with water twice. An appropriate amount of TTAPE was used to stain the glands for 5 min. The excess amount of dye was removed by rinsing with water. A coverslip was placed on top of the sample, and the chromosomes were squashed before examination under a fluorescence microscope.

Onion bulb (*Allium cepa*) was purchased from a local market and rooted in water. When the roots were 2 to 3 cm long, the tips were cut and soaked in 1 N HCl solution for 5 min. Afterwards, the root tips were rinsed with water and carefully transferred to the center of a clean microscope slide with a drop of water. The root tips were covered with a cover slip and squashed by pushing down the cover slip. The root tips were spread out to a diameter about 0.5–1 cm. The slides were stained by TTAPE-Me (50 μM) and blotted to remove the excess amount of stain before mounting on the microscope.

Acknowledgements

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