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5-Formyluracil as a Multifunctional Building Block in Biosensor Designs

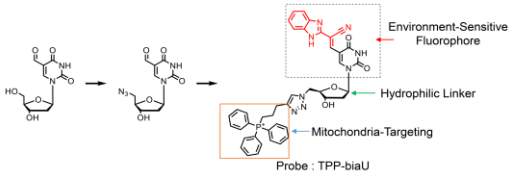
Chaoxing Liu[1, ‡], Guangrong Zou[1, ‡], Shuang Peng[1], Yafen Wang[1], Wei Yang[1], Fan Wu[1], Zhuoran Jiang[1], Xiong Zhang[1], and Xiang Zhou[1, *]

Abstract: 5-Formyluracil (5fU), which is known as a vital natural nucleobase, is widely present in organisms. Despite the recent development of sensor designs for organic fluorescent molecules for selective targeting applications, biocompatible and easily operated probe designs that are based on natural nucleobase modifications have rarely been reported. In this paper, we introduce the idea of 5fU as a multifunctional building block to facilitate the design and synthetic development of biosensors. The azide group was derived from the sugar of a nucleoside, which can be further used in the selective binding of cells or organelles through click chemistry with alkynyl-modified targeting groups. The aldehyde group of 5fU can react with different chemicals to generate environmentally sensitive nucleobases that have obvious characteristics, which precious reactants cannot achieve for selective fluorogenic switch-on detection of a specific target. We first synthesized 5fU analogues that had aggregation-induced emission properties, and then we used triphenylphosphonium as a mitochondria-targeting group to selectively image mitochondria in cancer cells and mouse embryonic stem cells. Additionally, the reagents exhibit a high selectivity for reaction with 5fU, which means that the method can also be used for the detection of 5fU. Combining the two characteristics, the idea of 5fU as a multifunctional building block in biosensor designs may potentially be applicable in 5fU site-specific microenvironment detection in future research.

5-Formyluracil (5fU), which plays an essential role in genomic features, has drawn wide attention in many related fields.[1] 5fU is present at approximately 1 to 10 moieties per 10^6 nucleobases in many tissues and cells.[2] Exposure to UV light,[3] Fenton-type reagents,[2a] ionizing radiation,[4] reactive oxygen species attack[5] or enzyme oxidation[6] can generate 5fU, which results in the introduction of gene mispairing[7], perturbations of DNA function,[8] the modulation of protein-DNA interactions[9] and the alteration of DNA structures.[10] Chemical tools that selectively tag 5fU to generate environmentally sensitive nucleobases are used in the detection of this important biomarker. For example, hydrazine,[2a, 11] amidoxyl,[12] phenylenediamine[13] and indantrione derivatives[14] can undergo highly effective reactions with aldehydes of 5fU. Additionally, the generated products, such as NBDU,[11] naphthalimide derivatives,[13, 15] 2,3,3-trimethylindole derivatives[14] and benzothiazole derivatives,[3] are all sensitive to specific targets. The design of 5fU analogues bearing changing characteristics is promising to produce effective and biocompatible biosensors (Scheme S2).

Biosensors that are based on DNA are continuously being developed in many fields and show great potential applications in biomarker detection, disease therapy, nanotechnology and computing.[16] Nucleobases possess great specificity for high-throughput oligonucleotide synthesis, have a long life and are remarkably compact and easy to use for specific target detection.[17] However, most research has been developed on aptamer beacons, nanoparticles and artificial bases.[18] Studies on the potential applications of the natural modification of nucleobases are rare.[19]

In this paper, we introduce the new concept of 5fU as a multifunctional building block to facilitate the design and synthetic development of biosensors. In Scheme 1, the 5'-OH of the sugar of 5-formyl-2'-deoxyuridine is replaced by the azide group, which is further used in selectively binding cells or organelles via click chemistry with alkynyl-modified targeting groups. The sugar in the uridine is an excellent hydrophilic linker. The aldehyde in the 5fU can conjugate with different chemicals to generate various environmentally sensitive fluorescent nucleobases for the detection of specific species. To verify the feasibility of this concept of creating 5-formyluracil-based probes for selective targeting applications, we chose triphenylphosphonium (TPP) as a mitochondria-targeting group. TPP,[20] peptides[21] and quaternized pyridine moieties[22] are always used in mitochondrial delivery vehicles to deliver probes to the mitochondrion because the positive charges are attracted to the negative potential of the mitochondrial membrane (typically a potential of -180 mV).[23] Mitochondria play vital roles in cell signalling, the regulation of calcium ions, the triggering of cell death and cellular metabolism.[24] Mitochondrial dysfunctions have been linked to atherosclerosis, Alzheimer's disease, Parkinson's, acute and chronic degenerative cardiac myocyte death, neuronal death, and cancer.[24c, 25] Research on mitochondrial targeting applications has attracted widespread attention in many fields.[21a, 26] For an environmentally sensitive fluorophore, in this paper, we used many chemicals and found a first nucleobase modification based on 5fU, which can undergo aggregation-induced emission (AIE). (2-benzimidazolyl)acetonitrile conjugates with 5fU to generate an AIE fluorogen with a rotor structure that has very weak fluorescence in dilute solutions but becomes highly emissive in the aggregated state.[27] In this design, the probe (Scheme 1) can selectively accumulate in cell mitochondria and induce its fluorescence.



Scheme 1. Strategies of 5fU as a multifunctional building block to design a 5-formyluracil-based probe bearing AIE characteristics for mitochondria-targeting applications.

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To verify the feasibility of our design, 5-formyl-2'-deoxyuridine was reacted with (2-benzimidazolyl)acetonitrile in a methanol acetic acid solution to generate 5-formyl-2'-deoxyuridine and (2-benzimidazolyl)acetonitrile adduct (biaU) (Figure 1a). The absorbance and fluorescence emission properties of biaU were acquired in various buffer solutions (Figure S1). The differences in the AIE characteristics and multifluorescence emissions of the probe in different conditions might be that intramolecular proton transfer together with the intramolecular hydrogen bonds served as noncovalent conformational locks to restrict intramolecular rotation processes, resulting in strong fluorescence (Scheme S1).[28] Due to the hydrophilic nature of the nucleoside skeleton, biaU is soluble in polar solvents, such as THF, and is slightly soluble in water. The AIE characteristic of biaU was studied in a THF/water solution. It has an absorption maximum at 375 nm and emits weakly with a maximum peak at 430 nm in THF. In a mixture of THF-water (v:v = from 1:80 to 1:95), biaU clusters into nanoaggregates and emits a strong blue fluorescence (Figure 1b). Then, we replaced the 5'-OH of the sugar of the 5-formyl-2'-deoxyuridine with an azide group. Afterwards, we added a TPP group via click chemistry with pent-4-yn-1-yltriphenylphosphonium iodide to produce a mitochondria-targeting AIE fluorogen based on 5fU (TPP-biaU) (Figure 1c). To demonstrate that the TPP-biaU probe has AIE characteristics under physiological conditions, fluorescence spectra were measured in a PEG-200/PBS solution. The probe has an absorption maximum at 375 nm and weakly emits with a maximum peak at 430 nm in PBS. In a mixture of PEG-200/PBS (v: v = 99: 1), TPP-biaU clusters into nanoaggregates and emits a strong blue fluorescence (Figure 1d). The difference in this blue light can be observed with different contents of PEG-200 in PEG-200/PBS solution under UV light (Figure 1e). With a high PEG-200 fraction, which is a crowded environment, significant fluorescence enhancement was observed with the formation of TPP-biaU aggregates due to the activation of the AIE process. Dynamic light scattering (DLS) studies showed the presence of aggregates in water with a particle size of 96.55 nm for the biaU probe (Figure S2) and the presence of aggregates in PEG when there was a particle size of 116.4 nm for the TPP-biaU probe (Figure S3). The aggregation behaviour of the biaU probe in water (Figure S4a) and TPP-biaU in PEG (Figure S4b) was also supported by the UV-Vis results, which showed the appearance of a levelled-off tail with an increase in water and PEG fraction, respectively. We also investigated the solid-state emission behaviour of biaU and TPP-biaU (Figure S5) when excited at 375 nm. The absolute quantum yield of solid state biaU was calculated as 11.63%, and that of TPP-biaU was calculated as 8.26%. All these data demonstrate the observed fluorescence changes of biaU, reflecting an AIE.

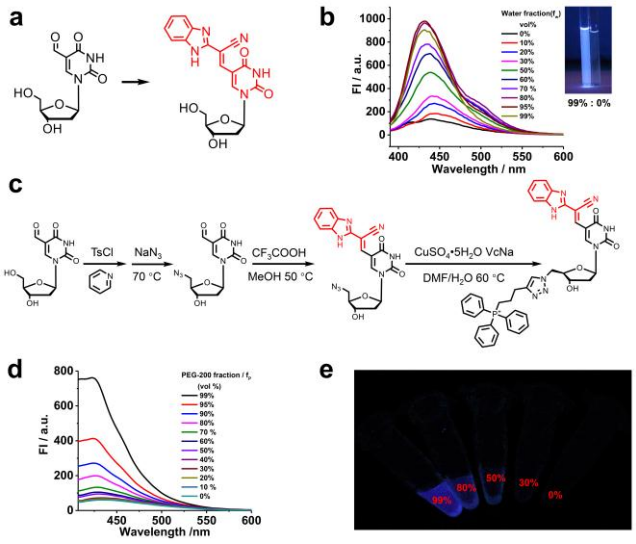

Figure 1. Synthesis and detection of AIE nucleobase based on 5fU. (a) Synthesis of 5-formyl-2'-deoxyuridine and (2-benzimidazolyl)acetonitrile adduct (biaU) (b) Fluorescence emission spectra (λ_{ex}: 375 nm, λ_{em}: 430 nm) of biaU (20 μM) in a mixture of THF-water solution with different water fractions (fw). (c) Synthesis of mitochondria-targeting AIE fluorogen based on 5fU (TPP-biaU). (d) Fluorescence emission spectra (λ_{ex}: 375 nm, λ_{em}: 430 nm) of TPP-biaU (20 μM) in a mixture of PEG-200/PBS solution with different PEG-200 fractions (fp). (e) Probe TPP-biaU in the different PEG-200 fractions of PBS buffer under UV light at 365 nm (from left to right, PEG-200/PBS =99%, 80%, 50%, 30%, 0%).

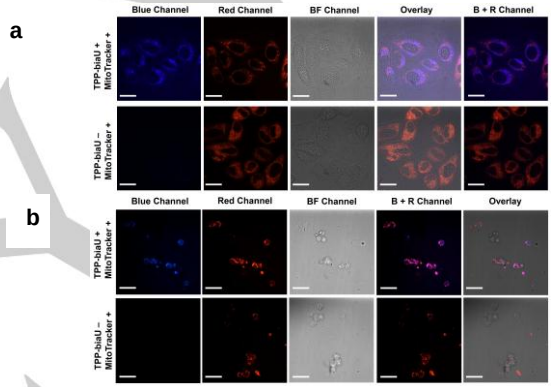

Figure 2. (a) Fluorescent images of HeLa cells stained with TPP-biaU (blue light) and overlapped with MitoTracker (red light). (b) Fluorescent images of mESCs stained with TPP-biaU (blue light) and overlapped with MitoTracker (red light). All images have the same scale bar of 24 μm.

Next, an MTT cell viability assay was used to study the cytotoxicity of TPP-biaU. It showed almost no cell toxicity to HeLa cells, even at a high concentration (500 μM) after 24 hours (Figure S6a) or even up to 72 hours of incubation (Figure S6b). We investigated the stability of TPP-biaU in the presence of cellular components (such as cellular reductants, proteins, etc.), which is very important for its bio-application. We studied the fluorescence emission spectra of TPP-biaU performed in cell medium and cell lysates, which revealed that TPP-biaU is not influential in the presence of cellular components (Figure S12). Then, we incubated TPP-biaU and MitoTracker (a red mitochondrial stain) with HeLa, MCF-7 and CAL-62 cells. The intracellular localization of TPP-biaU in the HeLa, MCF-7 and CAL-62 cells was compared. For the HeLa cells, the blue fluorescence signal overlapped well with the red fluorescence signal from the MitoTracker, as shown via confocal microscopy analysis (Figure 2a). Pearson correlation coefficients were used to quantify the overlap between the MitoTracker emission and TPP-biaU emission, which was calculated to be 0.89 (Figure S10a). For the MCF-7 and CAL-62 cells, the blue fluorescence signals also overlapped well with the red fluorescence signal

from the MitoTracker (Figure S8, S9) at high Pearson correlation coefficients (0.910 and 0.895, respectively) (Figures S10c and S10d). Therefore, TPP-biaU is capable of targeting and switching its fluorescence upon aggregation in mitochondria, which shows that is has a high selectivity and a lower cytotoxicity; however, there was no fluorescence in cell-culture media. Afterwards, a signal change in cells incubated with TPP-biaU was observed upon continuous scanning by the laser light, confirming that TPP-biaU involves less photobleaching (Figure S7).

Furthermore, we performed fluorogenic switching on the mitochondria in mouse embryonic stem cells (mESCs). As far as we know, there are no reports on targeting mitochondria in mESCs. However, the mitochondrial function plays a vital role in the early differentiation potential and proliferation of embryonic stem cells.[29] The lack of chemical tools available to study mitochondria in mESCs limits our understanding of the roles of mitochondria in mESCs. TPP-biaU and MitoTracker were treated with the mESCs. The intracellular localization of TPP-biaU in mESCs was compared. The blue fluorescence signal overlapped well with the red fluorescence signal from the MitoTracker at a high Pearson correlation coefficient of 0.947 (Figure S10b), as shown via the confocal microscopy analysis (Figure 2b). These confocal microscopy analyses confirmed that the TPP-biaU reagent can also fluorogenically switch on mitochondria in both carcinoma cells and normal cells, suggesting more future applications for TPP-biaU. The successful imaging with TPP-biaU in various cells inspired us to investigate the overall performance of the probe via flow cytometry. The fluorescence intensity of the experimental group (cells treated by TPP-biaU) was much higher than that of the control group (cells untreated by TPP-biaU) (Figure S11).

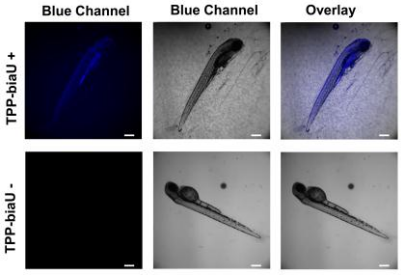

Figure 3. Images of living zebrafish embryos stained with TPP-biaU. incubated (above) or untreated with 300 μM TPP-biaU for 3 h at 28 °C. All images share the same scale bar of 200 μm.

Encouraged by the above results, we aimed to evaluate the usage of the probe in living zebrafish embryos, in view of the excellent sensing performance of TPP-biaU and the feasibility of our novel sensor design based on natural nucleobases. Zebrafish embryos at the 72-h stage were incubated with 300 μM of TPP-biaU in zebrafish embryo medium at 28°C for 3 h. After vital staining, TPP-biaU covered the surface of whole embryo with strong blue fluorescence (Figure 3).

In addition, the nucleoside in our case served as a linker between the fluorophore and the delivery module. 5fU served as a chemical building block for many sensitive probe designs. Previous chemical reagents did not have the characteristics of the detection target. However, reagents can be reacted with 5fU to generate special environmentally sensitive nucleobases that can be used in further applications. Additionally, the reaction was highly selective. Thus, we also used chemical reagents to selectively detect 5fU. For example, we found that (2-benzimidazolyl)acetonitrile could efficiently tag 5fU with a high yield (Figure S13b) and with selectivity (Figure S13c, S14) under warm conditions (NaOAc buffer, pH 5.0, 37°C, 6 h), in contrast to other aldehyde modifications present in DNA. The inherent chemical properties of (2-benzimidazolyl)acetonitrile make it highly selective for 5fU in both nucleosides and oligonucleotides, resulting in an extremely high yield under warm conditions (Figures S13, S14, and S15). We also explored the remarkable development of the selective detection of 5-formyluracil using this reagent via the AIE effect (Figure S16). The limit of detection is calculated as 157 nM (Figure S17). Compared to previous reports,[1e] the chemicals presented here had the advantages of warm reaction conditions, high yield, great selectivity, easy modified structure, the generation of an AIE nucleobase and low toxicity. The biotin derivative (2-benzimidazolyl)acetonitrile bears the potential ability to enrich 5fU and map it in genomic DNAs. Combining the two characteristics, 5fU can serve as a multifunctional building block for the design of biosensors for potential applications in future research of 5fU site-specific microenvironment detection (Figure 4).

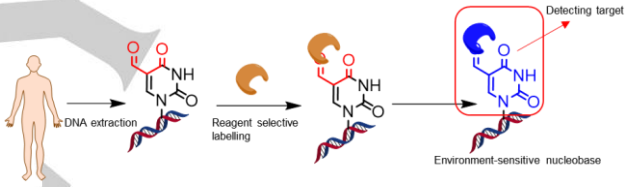

Figure 4. Strategies for potential applications in the 5fU site-specific microenvironment detection method.

In summary, we have demonstrated the new concept of 5fU as a multifunctional building block in the design and synthetic development of biosensors. To confirm that this concept is useful, outstanding and wide-ranging, we developed a series of 5fU derivatives that exhibit good fluorescence turn-on properties for the selective targeting of mitochondria via click chemistry with triphenylphosphonium. The findings presented here provide a new way of thinking and a method for designing probes that selectively bind cells or organelles to detect targeted species via environmentally sensitive nucleobases. As far as we know, this is the first nucleobase to bear AIE properties from a natural nucleobase. The probe has useful characteristics, including a good biocompatibility, water solubility, nontoxicity and minimal photobleaching. This new molecular design concept is based on a natural nucleobase modification method, which possesses general and universal characteristics. Additionally, the reagent is highly selective towards reactions with 5fU, making the design also useful for the detection of 5fU. These successful applications demonstrated that our new sensor design concept based on natural nucleobase modifications is reasonable and has wide application prospects in the fields of molecular design,

cell imaging, biochemistry manipulation and analytical chemistry. This could have a large impact on probe design in the future.

Acknowledgements

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Keywords: 5-formyluracil • probe • mitochondria-targeting • building blocks • AIE

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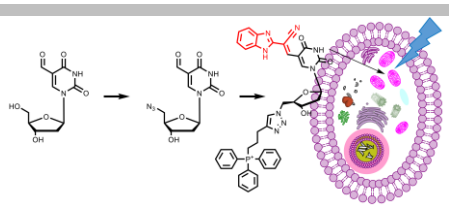

An idea of 5-formyluracil as a multifunctional building block to design a 5-formyluracil-based probe bearing AIE characteristics for mitochondria-targeting applications was introduced.

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