Single-molecule fluorescence detection of a tricyclic nucleoside analogue†

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Fluorescent nucleobase surrogates capable of Watson–Crick hydrogen bonding are essential probes of nucleic acid structure and dynamics, but their limited brightness and short absorption and emission wavelengths have rendered them unsuitable for single-molecule detection. Aiming to improve on these properties, we designed a new tricyclic pyrimidine nucleoside analogue with a push–pull conjugated system and synthesized it in seven sequential steps. The resulting C-linked 8-(diethylamino)benzo[b][1,8]naphthyridin-2(1H)-one nucleoside, which we name ABN, exhibits $\epsilon_{442} = 20 000 \text{ M}^{-1} \text{ cm}^{-1}$ and $\Phi_{\text{em},540} = 0.39$ in water, increasing to $\Phi_{\text{em}} = 0.50–0.53$ when base paired with adenine in duplex DNA oligonucleotides. Single-molecule fluorescence measurements of ABN using both one-photon and two-photon excitation demonstrate its excellent photostability and indicate that the nucleoside is present to > 95% in a bright state with count rates of at least 15 kHz per molecule. This new fluorescent nucleobase analogue, which, in duplex DNA, is the brightest and most red-shifted known, is the first to offer robust and accessible single-molecule fluorescence detection capabilities.

Introduction

Single-molecule fluorescence studies of biological molecules have a unique capacity to provide mechanistic insights into the relationships between structural dynamics and function, which are lost to averaging in ensemble measurements.1–3 Most of these studies have used extrinsic fluorophores, which can potentially interfere with the native biomolecular behavior and obscure local structural details.4–7 An ideal approach in this regard would be the use of intrinsically fluorescent biomolecules, prepared by the synthetic introduction of only minimal changes.8 It remains, however, a major challenge to attain adequate brightness and photostability in this approach.

Fluorescent nucleobase analogues (FBAs) have been a mainstay of biophysical studies of nucleic acid structure and dynamics because they can be placed precisely in a desired sequence and are less structurally perturbing than proximally tethered fluorophores.9–10 They are available with a range of fluorescent properties and, in response to base pairing and stacking, their fluorescence may be quenched (e.g. 2-amino-purine), retained (e.g. tC and iG) or turned on (e.g. DEmtC).11–14 However, few nucleobase analogues have extinction coefficients $>10^4$ with $\Phi_{\text{em}} > 0.3$; most are approximately an order of magnitude dimmer than conventional fluorophores such as Alexa Fluor 488 and rhodamine B.8,9,15–17 This lack of brightness has rendered them largely unsuitable for single-molecule fluorescence studies.16–18 Furthermore, with the current rapid development of spatially-resolved transcriptomics and genomics, there is clearly a future need for fluorescent analogues that can act as effective single-molecule probes.21,22

Conventional fluorophores typically exhibit superior optical properties to fluorescent nucleobase analogues, primarily because of their larger extinction coefficients—sometimes in excess of $10^5$—but they need not be larger molecules.23,24 In structures such as rhodamine B, there is a prominent push–pull character, but the benzene carboxylic acid is twisted out of plane and does not contribute significantly to the photophysical properties. The fluorescent, tricyclic core is similar in size to many of the common fluorescent nucleotides, but the most important difference is that push–pull motifs are underrepresented in existing FBAs.8,9,25 The required positions of heteroatoms for the Watson–Crick face and the glycosidic bond make incorporation of this motif challenging.

In this work, we hypothesized that, by redesigning a fluorescent, tricyclic cytidine analogue to include a push–pull motif, a substantial enhancement of brightness could be obtained (Fig. 1). A comparison of DEmtC with rhodamine B shows that the
Results and discussion

Synthesis

The synthesis of ABN starts with the construction of the bicyclic ring 2-chloro-7-(diethylamino)quinoline-3-carbaldehyde 2 by the reaction of 3-(diethylamino)acetanilide with the Vilsmeier reagent. The electron donating nature of the diethylamino group renders selective formation of the singly formylated product difficult to achieve, but careful temperature control allows for an adequate yield at multi-gram scale. The quinoline ring 2 undergoes s_{E}Ar and cyclization with sodium azide to give a tricyclic compound 3 and the tetrazole ring is then opened reductively by triphenylphosphine in 2 N HCl at reflux to give 2-amino-7-(diethylamino)quinoline-3-carbaldehyde 4. Adding the Wittig reagent ethyl 2-bromo-2-(triphenylphosphoranylidene) acetate 8 (synthesized by the bromination of ethyl (triphenylphosphoranylidene)acetate) to compound 4 yields the brominated tricyclic nucleobase precursor 5. A Heck reaction of 5 with 3',5'-O-TBS dihydrofuran 10 using palladium acetate and triphenylarsine followed by desilylation with acidic trifluoroacetic acid gives 3'-keto nucleoside 6. This Heck reaction is selective for the β face, owing to the sterical influence of the 3'-O-TBS group, as usual in the synthesis of C-ribosides. Stereoselective reduction of 6 with sodium triacetoxylborohydride completes the ABN nucleoside 7. The β configuration of the anomeric C is verified by its hydrogen’s coupling constants J_{H1,H2} = 5.9 and 10.0 Hz. A comparison of 13C NMR shifts—the carbonyl at δ = 165.0 ppm for ABN in CD_{3}OD is especially diagnostic—with published data for simpler 1,8-naphthyridin-2(1H)-one nucleoside analogues indicates that ABN is present only in the thymidine-like tautomeric form as shown (Fig. 1), to the limit of detection by NMR. Computational prediction of the NMR spectra (MP2/cc-pVDZ/COSMO) confirms this assignment (see ESIF). B3LYP and MP2 calculations using three different basis sets, with and without solvation, predict the T-like tautomer to be 10.3–13.8 kcal mol^{-1} more stable than the cytidine-like tautomer (see the ESIF for details and a molecular model). Dimethoxytritylation followed by 3'-phosphoramidite installation under standard conditions prepares the nucleotide for solid-phase oligonucleotide synthesis (Fig. 2; see the ESIF).

Oligonucleotide design and preparation

To assess ABN’s photophysical properties and natural base mimicry in oligonucleotides, we designed and prepared a hairpin ODN1 and two 10-mer sequences ODN4 and ODN7 that provide a representative set of local environments (Fig. 3). The hairpin was designed to place ABN at the third position of a six-residue loop, a site that is not conducive to base stacking and is expected to leave the nucleobase predominantly solvent exposed. OD4 and OD7 were selected to provide a first look at neighboring base effects on ABN’s fluorescence. By annealing these ODNs to matched and mismatched complements, the base pairing of ABN and its effects on fluorescence can be measured.

Photophysical properties

Steady-state measurements. Steady-state absorption and fluorescence measurements of ABN in water, 1× PBS buffer (pH 7.4), and in 50% DMSO in water were recorded. The emission quantum yield for ABN in water was determined to be Φ_{em} = 0.09 (see ESIF for details).

Electronic property calculations. M06-2X/6-311G(d,p) calculations of the electronic structure of ABN and its environment-dependent analogues were carried out in order to model the effect of mismatches and solvation on the photophysical properties of ABN. The M06-2X functional was chosen based on previous photophysical studies, 

Fig. 1 Push–pull motifs are hallmarks of bright organic fluorophores, but are rare in fluorescent nucleosides. The redesign of a nucleoside motif to include this motif significantly increases ε and Φ_{em}, enabling single-molecule detection.

Fig. 2 Synthesis of the ABN nucleoside analogue. Reagents, conditions, and yields: (a) DMF, POCl3, 50 °C, 20 min (15%). (b) NaN3, DMF, 90 °C, 18 h (85%). (c) PPh3, 2 N HCl, reflux, 2 h (70%). (d) NaOEt, ethanol, 70 °C, 4 h. (e) AsPh3, Pd(OAc)2, Bu3N, 60 °C, 18 h. (f) TBAF, AcOH, rt, 1 h. (g) NaBH(OAc)2, AcOH, CH3CN, 0 °C, 1 h (9% over 4 steps).
base pairing and stacking in stranded and duplex DNA oligonucleotides to determine how spanning 500 lengths ranging from 310

Recorded emission spectra in water using excitation wave-

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Table 2. ABN increases its aqueous solution (Fig. S20

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3.0

ABN is brighter than any other known FBA when present in oligonucleotides.

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ABN in dioxane (black) and water (red). The integral areas of emission spectra are normalized to brightness $\varepsilon \cdot \Phi_{\text{em}}$.

Temperature-dependent circular dichroism measurements of all six duplexes are consistent with the B-DNA conformation with only minimal perturbation (Table 2; see all CD spectra in the ESI†). Except in the ODN1 hairpin, where ABN is expected to be mostly solvent exposed, the melting temperatures of the duplexes are typically somewhat depressed as compared with their natural counterparts. Duplex stability is lowest when ABN has 5'-G and 3'-C neighbors, an observation consistent with other tricyclic FBAs, especially those that are electron-rich.14 The observed melting temperatures provide little indication of whether ABN is a better T- or C-surrogate. Solution NMR studies of the free nucleoside and computation clearly indicate a preferred tautomer with an acceptor-donor-acceptor hydrogen bonding pattern as in thymine, as discussed above. It is possible that ABN forms wobble base pairs with G or base pairing with G drives tautomerism to a C-like donor–acceptor hydrogen bonding pattern (Fig. S22†).

The Stokes shift is shortened in ABN:G pairs, resulting mostly from red-shifted absorption relative to what is observed

Table 2 Steady-state photophysical data for ABN in DNA oligonucleotides

| Oligo$^a$ | $\lambda_{\text{abs, max/nm}}$ | $\lambda_{\text{em, max/nm}}$ | $\Phi_{\text{em}}$ | $T_m/^\circ\text{C}$ | $\Delta T_m$ $^b$/°C |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ODN1      | 450             | 530             | 0.55            | 63.6 ± 0.6      | +3.1            |
| ODN1:ODN2 | 440             | 530             | 0.53            | 61.8 ± 0.4      | −4.7            |
| ODN1:ODN3 | 468             | 523             | 0.40            | 61.3 ± 0.6      | +1.6$^c$        |
| ODN4      | 452             | 549             | 0.49            | —               | —               |
| ODN4:ODN5 | 440             | 525             | 0.51            | 39.9 ± 0.3      | −0.5            |
| ODN4:ODN6 | 470             | 523             | 0.29            | 41.3 ± 0.3      | −7.3$^f$        |
| ODN7      | 532             | 532             | 0.62            | —               | —               |
| ODN7:ODN8 | 532             | 532             | 0.50            | 34.3 ± 0.3      | −14.1           |
| ODN7:ODN9 | 532             | 532             | 0.55            | 37.9 ± 0.3      | −11.8$^f$       |

$^a$ Oligonucleotide sequences are given in Fig. 3. $^b$ $\Delta T_m = T_m$ for ABN-containing duplex listed in the table row – $T_m$ for the corresponding duplex with canonical thymidine in place of ABN. $^c$ Comparison with $T_m$ for the corresponding natural duplex with a C-G base pair. $^d \lambda_{\text{em, max}}$ is concentration-dependent; see Fig. S18.
in ABN:A pairs. Computational studies (B3LYP/cc-pVDZ) predict that the C-like tautomer will absorb at approximately 45 nm longer wavelength than the T-like tautomer (see ES†). These calculations are consistent with a tautomeric base pair with G that retains high fluorescence but is indicated by changed \( \lambda_{\text{em}} \). The absorption spectra of ODN7 alone and in the ODN7:ODN8 and ODN8:ODN9 duplexes are concentration dependent (Fig. S18†). This dependency indicates a significant potential for change in the local environment around ABN and possibly the analogue’s tautomeric state in these sequence contexts.

**Single-molecule fluorescence measurements**

Given the very attractive bulk-level photophysical properties of ABN in solution, we next investigated its potential as a single-molecule probe. Recent studies have demonstrated multiphoton excitation as a promising approach to the sensitive detection of fluorescent base analogues.\(^{19,20}\) Two-photon excitation (TPE) in solution, we next investigated its potential as a single-molecule analogue DMA\(^{19b}\)U was detected as a free nucleoside at the single-molecule level for the first time \( \text{via} \) multiphoton excitation with a brightness of \( \sim 7 \text{ kHz} \) per molecule following three-photon excitation.\(^{28}\) Using an experimental setup consisting of a broadband ultrafast laser with dispersion compensation,\(^{19,20}\) we found that ABN could be optimally excited \( \text{via} \) a 2P process (Fig. 5A). The 2P brightness was measured using fluorescence correlation spectroscopy (FCS) and found to match that of DMA\(^{19b}\)U at 7 kHz per molecule (Fig. 5B). Importantly, unlike DMA\(^{19b}\)U, which was predominately (96%) in a dark state, a controlled dilution suggests that ABN is exclusively in a bright state, which we attribute to the single tautomer observed by NMR (see above).

The red-shifted absorption profile, high 2P brightness and predominance of the bright state made ABN a promising candidate for single-molecule detection \( \text{via} \) 1P excitation. We found that spatially isolated individual molecules of ABN randomly dispersed on a glass coverslip could be readily visualized using single-molecule total internal reflection fluorescence (smTIRF) microscopy (Fig. 6A and Movie S1†). Furthermore, these fluorescent puncta underwent single-step photobleaching under constant irradiation (Fig. 6B), consistent with the idea that ABN has suitable optical properties to be used as a single-molecule fluorescent nucleoside. By quantifying >2000 individual trajectories, we were able to determine the mean total detected photon value of 5300 ± 1800 photons per molecule; furthermore a mean of 1500 ± 600 photons were detected per 100 ms integration from each molecule at a power density of 0.2 kW cm\(^{-2}\). Combined, these data suggest that the mean total on-time of ABN was 0.35 ± 0.18 s under these conditions. The similarities in brightness following 1P and 2P excitation (15 kHz and 7 kHz per molecule, respectively), show that ABN has excellent photostability under both excitation regimes.

**Conclusions**

The design of this new fluorescent nucleoside analogue ABN, centered around a push–pull motif common in bright organic fluorophores, has provided an unprecedented combination of high brightness and long absorption and emission wavelengths while retaining a Watson–Crick face. Fluorescence is further enhanced when the compound is present in single-stranded and duplex oligonucleotides. ABN’s robust photophysical properties and tautomeric stability allow detection of single molecules of the ABN nucleoside using either 1P or 2P at convenient excitation wavelengths for both. These results place ABN as the most promising fluorescent nucleoside analogue to
date for single-molecule studies of nucleic acid structure and dynamics. A forthcoming full study will elucidate the finer details of this analogue’s fluorescent properties, base pairing, tautomerism, and local structural perturbations in a variety of neighboring base sequences.

Conflicts of interest

There are no conflicts to declare.

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