Synthesis of Wavelength-Shifting Fluorescent DNA and RNA with Two Photostable Cyanine–Styryl Dyes as the Base Surrogate Pair

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Two nucleic acid building blocks were synthesized, consisting of two photostable green- and red-emitting cyanine–styryl dyes and (S)-3-amino-1,2-propanediol as a substitute for the ribofuranoside, and incorporated as base-pair surrogates by using automated phosphoramidite chemistry in the solid phase. The optical properties and, in particular, the energy-transfer properties were screened in a range of DNA duplexes, in which the “counter bases” of the two dyes were varied and the distance between the two dyes was enlarged to up to three intervening adenosine–thymidine pairs. The DNA duplex with the best optical properties and the best red/green emission ratio as the readout bore adenosine and thymidine opposite to the dyes, and the two dyes directly adjacent to each other as the base surrogate pair. This structural arrangement can be transferred to RNA to obtain similarly fluorescent RNA probes. Representatively, the positively evaluated DNA duplex was applied to verify the fluorescence readout in living HeLa cells by using fluorescence confocal microscopy.

1. Introduction

Molecular imaging is a potent technique to visualize intracellular structures and to observe biomolecules inside cells in real time.[1] Labeling nucleic acids with bright fluorescent probes for molecular imaging is important with respect to both the central functions of DNA and RNA inside cells and the delivery of exogenous nucleic acids into cells and organisms.[2] From an organochemical point of view, the advantage of nucleic acids is that they are synthesized from building blocks by using automated chemistry in the solid phase. Fluorescent labeling can be achieved by providing the corresponding fluorophores directly as DNA building blocks,[3] or through reactive building blocks and post-synthetic labeling.[4, 5] With respect to the cationic character of a significant majority of potent DNA- and RNA-labeling dyes as base surrogates, the 2-deoxy-ribofuranosides had to be replaced by a linker that lacks the hydrolytically labile glycosidic bond and, hence, provides sufficient chemical stability. We applied (S)-3-amino-1,2-propanediol,[3d] where-as other groups used d-threoninol[5, 6] or cyclopentanes[7] for the same purpose. This approach worked for several dyes, including ethidium,[7] BODIPY,[8] thiazole orange and red,[9] and other functional molecules.[10] In particular, thiazole orange and red were combined in an inter-strand manner to promote an efficient energy transfer between them.[10] This DNA and RNA base-substitution approach allowed us to develop wavelength-shifting fluorescent probes (“DNA and RNA traffic lights”) as a powerful tool for molecular imaging,[11] evidenced especially for siRNA constructs.[12] The thiazole-derived dyes, however, show a low photostability that limits their imaging applicability.[13] Recently, we developed a broad range of structurally related cyanine–styryl dyes with blue, green, yellow, and red emission and, more importantly, with significantly improved photostability.[14] Herein, we report on the synthesis of two new DNA/RNA building blocks, 5 and 6, based on the photostable green- and red-emitting cyanine–styryl dyes 1 and 2, their incorporation into DNA and RNA double strands (D1 and D2), and their energy-transfer properties (Scheme 1) in vitro and in living cells.

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Scheme 1. Principle of wavelength-shifting fluorescent DNA and RNA “traffic lights”: Excitation of the green emitting dye 1 (D1) in the double strand leads to efficient energy transfer to dye 2 (D2) and yields red emission, whereas the single strand shows green fluorescence.
2. Results and Discussion

The synthesis of the two nucleic acid building blocks is straightforward, starting from the hydroxypropylated dyes 1\textsuperscript{(15)} and 2\textsuperscript{(16)} that were synthesized according to our previously published protocols (Scheme 2). The reaction with carbonyldimidazole links the dyes to the tritylated (5)-3-amino-1,2-propanediole 3 through a carbamate group. Phosphitylation of the remaining secondary hydroxy groups of the intermediate compounds 4 and 5 yield the phosphoramidites 6 and 7, respectively. Those were dissolved in dichloromethane (0.1 m) and applied for automated oligonucleotide synthesis. Compared to commercially available building blocks, the coupling time for 6 and 7 was drastically enhanced with intervening washing steps\textsuperscript{(17)} for RNA synthesis. CPG columns with 2'-acyl-protecting groups were applied, which require a special cleavage protocol. To avoid degradation of the dyes during DNA/RNA cleavage and workup, the commercially available "ultramild" cleavable building blocks were used\textsuperscript{(18)}.

All synthesized oligonucleotides were purified by using semi-preparative HPLC and identified with MALDI-TOF mass spectrometry (see the Supporting Information). The two dyes were placed in a diagonally oriented fashion as an energy transfer-based base surrogate pair. This dye arrangement and the sequences of DNA0–XY and RNA0 were derived from our recent siRNA construct with thiazole orange as the energy donor and thiazole red as the energy acceptor, which was used for molecular imaging of siRNA delivery and processing in live cells\textsuperscript{(12)}. To evaluate the best environment for energy transfer between the two dyes, the “counter bases” X and Y were systematically varied (A, G, T, or C). Moreover, the distance between energy donor (dye 1) and acceptor (dye 2) was increased from 1 to 3 intervening A–T pairs in DNA1–DNA3.

The UV/Vis absorption of all doubly modified DNA and RNA duplexes shows the presence of the fluorophores with absorption bands for D\textsuperscript{1} at around 470 nm and for D\textsuperscript{2} at around 550 nm (see the Supporting Information). The extinction and absorption maxima of both chromophores, D\textsuperscript{1} and D\textsuperscript{2}, as base surrogates show significant differences among the duplexes DNA0–XY, and the alterations are more distinct for D\textsuperscript{1}. This also yields differences in fluorescence readout, as further discussed below. D\textsuperscript{1} was selectively excited at 435 nm, and the fluorescence intensities I\textsubscript{535} and I\textsubscript{610} at the dye-typical wavelengths 535 nm (D\textsuperscript{1}) and 610 nm (D\textsuperscript{2}), respectively, gave information on the energy-transfer efficiency between the two dyes. The best “traffic lights” with respect to the structural variations (counter bases X/Y and distance between D\textsuperscript{1} and D\textsuperscript{2}) identified by a low fluorescence intensity I\textsubscript{535} and a high intensity I\textsubscript{610} and, thereby, a high contrast I\textsubscript{535}/I\textsubscript{610} as a characteristic red/green fluorescence readout. Additionally, the enhancement factor f for the red fluorescence was calculated as the major hybridization readout, according to the “traffic light” concept (see Scheme 1). f is the fluorescence ratio I\textsubscript{535}/I\textsubscript{610} of the doubly modified double strands (D\textsuperscript{1} and D\textsuperscript{2}) divided by the fluorescence ratio I\textsubscript{535}/I\textsubscript{610} of the singly modified single strands (D\textsuperscript{1} only). The complete screening of these optical properties (Table 1) revealed that A and T are the best counter bases for both D\textsuperscript{1} and D\textsuperscript{2}; in particular, DNA0–TA, DNA0–TT, and DNA0–AT show emission color contrasts of I\textsubscript{535}/I\textsubscript{610} = 12–34 and enhancement factors of f = 72–212 as fluorescence readouts. It also became obvious that C and G are not suitable as counter bases, neither as X nor as Y. In particular, the fluorescence of the duplexes DNA0–XC all show low intensities for the acceptor.

As already described previously\textsuperscript{(19)} ground-state interactions between two dyes interfere with the energy-transfer process between them. In particular, excitonically coupled dimers of both dyes do not yield an efficient energy transfer, because this requires the selective excitation of the uncoupled energy donor (D\textsuperscript{1}) in the neighborhood of the uncoupled and, thus, unexcited acceptor (D\textsuperscript{2}). The absorption maxima \(\lambda_{\text{max}}\) of D\textsuperscript{1} and D\textsuperscript{2} and their ratios \(\lambda_{\text{max}}\textsuperscript{D1}/\lambda_{\text{max}}\textsuperscript{D2}\) give more detailed information on this issue. If the distance \(\Delta\lambda\) between the two absorption maxima exceeds 84 nm, fluorescence quenching of D\textsuperscript{2} is observed, but no efficient energy transfer to D\textsuperscript{2}. In these cases, the overlap of the D\textsuperscript{1} emission and the D\textsuperscript{2} absorption, which is crucial for an efficient energy transfer, is low. For instance, this scenario is obvious for DNA0–AC with \(\Delta\lambda = 102\,\text{nm}\) and I\textsubscript{535}/I\textsubscript{610} = 2.4. Absorption ratios \(\lambda_{\text{max}}\textsuperscript{D2}/\lambda_{\text{max}}\textsuperscript{D1}\) higher than 1.1 indicate excitonic interactions between the dyes that result in higher extinction coefficients for D\textsuperscript{2} and lower ones for D\textsuperscript{1}. These effects are most pronounced in the duplex DNA0–GG. The absorption ratio is \(\lambda_{\text{max}}\textsuperscript{D2}/\lambda_{\text{max}}\textsuperscript{D1} = 1.61\) and the emission shows almost no energy transfer and mainly quenching. The fluorescence readouts are I\textsubscript{535}/I\textsubscript{610} = 1.1 and f = 4.0, which are the lowest values in these DNA series. This interpretation of ground-state interactions between the dyes was representationally supported by additional optical spectroscopic experiments with DNA0–GG (see the Supporting Information). With an excess of the D\textsuperscript{2}-containing single strand (1.2 equiv), the
green fluorescence of $D^1$ is significantly reduced, whereas the red fluorescence of $D^2$ is not enhanced, but also reduced. Accordingly, it is clearly not the lack of the $D^4$-containing strand that limits the energy transfer in DNA0–GG. The absorption and excitation spectra further support the ground-state interpretation. With an excess of the $D^2$-containing strand, the extinction of $D^1$ is reduced (although the amount of $D^2$-containing strand is higher!), which can be similarly observed in the excitation spectra. Interestingly, all duplexes of the series DNA0–XG show the influence of the base environment by a significant red shift of both the absorption and the emission (ca. 5 nm) of $D^2$, indicating stacking interactions with G as counter base Y.

The determination of the melting temperatures ($T_m$) revealed that the duplexes DNA0–XY with $XY = \{\text{AT, TA, CG, and GC}\}$ are higher than the others, which may indicate base pairing of the two “counter bases”, thereby controlling the optical properties of the two dyads. The extinction of $D^1$ in the singly modified single strands is significantly lower than in the corresponding doubly modified duplexes. The absorption ratio $A_{max/D^1}/A_{max/D^2}$ of the two dyads in two complementary singly modified single strands that were subsequently annealed to the duplex additionally helped to elucidate the best choice of the counter bases X and Y for these dyads. Here, the best “traffic lights” were identified by the $A_{max/D^1}/A_{max/D^2}$ ratio of the double strand if it was twofold higher than the corresponding ratio in the separated single strands. These observations are particularly valid for DNA0–AT with a ratio of 2.04. This apparently optimal duplex with the best optical properties shows an excellent fluorescence readout with $I_{610}/I_{535} = 34$. Together with $f = 212$ and the highest fluorescence intensity of all duplexes, especially an acceptor quantum yield of $\Phi_e(D^1) = 35\%$, it best fits the requirements for our “traffic lights” and shows a high potential for successful in vivo imaging experiments.

If the interpretation that excitonically coupled fluorophores interfere with energy transfer, it looked reasonable to separate $D^1$ and $D^2$ by additional base pairs. This was realized in DNA1–DNA3 by using the best counter base combination XY = AT (Figure 1). Interestingly, both critical readouts were diminished in these duplexes; the $I_{610}/I_{535}$ ratio dropped from 34 for DNA0–AT over 3.1 for DNA1, 4.4 for DNA2 to only 1.9 for DNA3, $f$ dropped from 212 to 12. Over such short distances, the relative orientation of the two dyads may control the energy transfer, as studied by Wilhelmsson et al.\textsuperscript{[19]} and Asanuma et al.\textsuperscript{[29]} Clearly, DNA0–AT already contains the best possible arrangement and least excitonically coupled fluorophore pair and, thus, shows the best fluorescence readout. With the knowledge on the best energy-transfer arrangement of $D^1$ and $D^2$ as base surrogates in DNA double strands, RNA0 and RNA1 were prepared with sequences in analogy to DNA0–AT and DNA1. The fluorescent readout values of these two RNA duplexes were lower compared to their DNA counterparts, owing to the different A-type conformation of double-helical RNA. But, in particular, RNA0 showed excellent fluorescent readout values with $I_{535}/I_{610} = 20$ and $f = 99$ that make this RNA duplex a promising candidate for in vivo fluorescent imaging.

Representative cell experiments were performed to demonstrate the imaging potential of duplex DNA0–AT in living cells. 3 x $10^4$ HeLa cells were transfected with 15 pmol of DNA0–AT and ScreenFect\textsuperscript{[20]} for 24 h and imaged by confocal fluorescent microscopy using an excitation wavelength of 488 nm (Figure 2). To demonstrate the energy transfer, the emission of the energy donor $D^1$ ($\lambda_{em} = 490–530$ nm) and the emission of the energy acceptor $D^2$ ($\lambda_{em} = 650–790$ nm) were separately de-
3. Conclusions

Two new DNA/RNA building blocks were synthesized, which carry the cyanine–styryl dyes 1 and 2 that were previously evaluated as very photostable alternatives to thiazole orange and thiazole red, and (S)-3-amino-1,2-propanediol as a substitute for the ribofuranoside. By using these building blocks, both dyes were successfully incorporated into DNA and RNA through automated solid-phase chemistry. Excitation of the green-emitting D1 in the double strand led to an energy transfer to D2 and yielded red light emission, whereas the single strand showed only green fluorescence. This fluorescent readout was characterized by the red/green emission color contrast I610/I535 and the fluorescence enhancement factor f. The screening comprised of duplexes, in which the counter bases X and Y to D1 and D2, respectively, were varied and the distance between the dyes was enlarged from 0 to 3 intervening AT base pairs. The best readout was obtained for the combination with X = A as counter base to D1 and Y = T as counter base to D2. Separating the dyes as a base surrogate pair by additional A–T base pairs did not improve the readout. In particular, DNA0–AT showed excellent fluorescence readout with I610/I535 = 212, and an acceptor quantum yield of ϕa(D2) = 35%. This duplex did not only suit the requirements of our “traffic light” concept for in vivo imaging experiments, but also showed significantly better fluorescence properties compared to our previous DNA and RNA “traffic lights” based on thiazole orange and thiazole red as the base surrogate pair. In comparison to our recently published post-synthetic modification of the 2′-position of uridine (in the ribo and arabinofuranoside configuration), the quantum yields of the base surrogate approach (as presented herein) were significantly higher. An additional advantage in comparison to the post-synthetically modified DNA and RNA samples was the avoidance of copper (I) that may interfere with cellular experiments, owing to its cytotoxicity. This offered the application for in vivo imaging experiments, which was represented and demonstrated for DNA0–AT. Fluorescence confocal microscopy revealed that the energy transfer could also be obtained in living cells. In summary, the two cyanine–styryl dyes 1 and 2 as base pair surrogates in DNA as well as in RNA efficiently form an energy-transfer pair, giving rise to a very promising tool for molecular imaging.

Experimental Section

All experimental details are described in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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