Affordable small molecules as promising fluorescent labels for biomolecules: application in RNA-FISH probes

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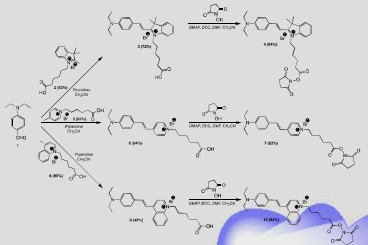
Introduction

Fluorescent labels are indispensable in various modern scientific applications, including direct and indirect immunochemistry, fluorescence microscopy, histochemistry, flow cytometry and fluorescence in situ hybridization (FISH) [1,2]. Small fluorescent labels offer significant practical benefits, enabling the optimization of fluorescence signals through the attachment of multiple fluorophores to a single biomolecule [3,4]. The commonly used fluorescent labels are prohibitively expensive for regular use in routine applications and most of them have small Stokes shifts. In this work we present three new small molecules (Figure 1), as promising fluorescent labels for biomolecules, obtained through an efficient, straightforward, and cost-effective synthetic strategy. Additionally, we evaluate the fluorescent three new small molecules labels as potentially effective fluorescent labels for biomolecules. Six new fluorescent oligonucleotide probes have been obtained, three directed to the rRNA region of eukaryotic cells (EUK516) and three to the rRNA region of prokaryotic cells (EUB338). The developed fluorescent probes were tested on microorganisms belonging to the culture collection of the Laboratory of Biodegradation and Biotechnology of the HERCULES Laboratory (University of Évora), showing effective performance as RNA-FISH probes. Density functional theory and time-dependent density functional theory calculations were carried out to gain insights into the observed photophysical properties. These findings evidenced the applicability of these new small molecules in labeling of biomolecules and bioimaging.

Discussion

In this work, we develop a simple and effective synthetic strategy to produce new inexpensive small molecules as promising fluorescent labels for biomolecules, using 4-diethylamino-benzaldehyde (**1**) as a starting material. The synthetic routes followed for the preparation of the fluorescent labels for biomolecules **4**, **7** and **10** are shown in **Scheme 1**. The aldol condensation reaction allowed the synthesis of intermediates **3**, **6** and **9,** with electron-donating groups (EDGs) and effective electron-accepting groups (EWGs), which would increase both the π-delocalization and the push-pull nature of the chromophore. The extension of the π conjugated system in these molecules is essential to an effective intramolecular charge transfer (ICT) process of the emissive excited state, improving their photophysical properties, such as high molar extinction coefficients and, in the case of compounds **4** and **7**, large Stokes shifts. The possible elimination of the spectral overlap between absorption and emission in fluorophores with large Stokes shifts, allows to eliminate the quenching process and reduce interference, providing effective detection of the fluorescence emission.



Scheme 1. Structure of the synthesized fluorescent labels.

The photophysical properties of the synthesized compounds **4**, **7** and **10** were studied, and their absorption and emission properties, as well as fluorescence quantum yields, are summarized in **Table 1**.

Table 1. Spectroscopic properties of 4-diethylamino-benzaldehyde derivatives.

Compounds	λ_{abs}[a] (nm)	λ_{em}[b] (nm)	Stokes shift (nm)	ε[c] ($cm^{-1}M^{-1}$)	Φ_f
1	342	385	43	19400	0.06
4	556	597	41	90000	0.57
7	490	604	114	64000	0.30
10	560	665	105	60000	0.02

[a] Absorption maxima in acetonitrile.
[b] Emission maxima in acetonitrile.
[c] Molar extinction coefficient at longest wavelength transition.
[d] Fluorescence quantum yield in ethanol, determined using 7-diethylamino-4-methylcoumarin (Φ_F = 0.73 in ethanol) as standard.

The 5'-AC6 amino-modified oligonucleotide sequences used for the development of these newly fluorescent probes were EUK516-mod (5' ACCAGACTTGCCCTCC-3') and EUB338-mod (5'-GCTGCCTCCCGTAGGAGT-3'). These oligonucleotide sequences target the rRNA regions of eukaryotic and prokaryotic cells, respectively. This methodology involves chemically labeling oligonucleotides with fluorescent labels, creating a covalent bond between the fluorescent labels (**4**, **7**, and **10**) and commercially available 5'-AC6 amino-modified oligonucleotide sequences (EUK516-mod or EUB338-mod) via the amino group situated at the 5' terminus.

Three of these new probes were designed to target the rRNA region of eukaryotic cells (designated as EUK516-(**4**), EUK516-(**7**) and EUK516-(**10**)), while the remaining three were tailored for the rRNA region of prokaryotic cells (labeled as EUB338-(**4**), EUB338-(**7**) and EUB338-(**10**)) (Fig. 1).

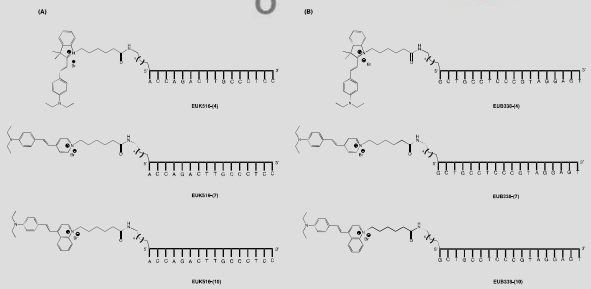


Figure 1. Fluorescent oligonucleotide probes: **(A)** Oligonucleotide probes complementary to eukaryotic cell RNA; **(B)** Oligonucleotide probes complementary to prokaryotic cell RNA.

Percentage of fluorescent cells from *Saccharomyces cerevisiae*. The study examined the efficacy of different synthesized oligonucleotide probes in labeling *Saccharomyces cerevisiae* (SC) cells. Probes EUK516-(**4**) and EUK516-(**7**) showed similar fluorescent cell percentages as the commercial probe EUK516-Cy3, indicating high specificity for SC cell labeling (Fig. 2). However, EUB338-(**10**) resulted in significantly fewer fluorescent cells, indicating limited specificity for labeling SC cells. These findings confirm the effectiveness of custom probes in specifically labeling *Saccharomyces cerevisiae* cells, offering potential alternatives to commercial probes.

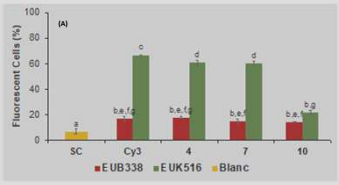


Figure 2. Percentage of fluorescent cells **(A)** from *Saccharomyces cerevisiae* cells labeled with EUK516 and EUB338 probes with different markers (**4**, **7**, **10** and **Cy3**) and their respective blank. In each assay 1000 cells were analyzed in triplicate. Values represented in A correspond to the average of flow cytometry measurements and error bars to standard deviation (± SD). In each plot, different letters located over the error bars indicate significant differences ($p<0.05$).

Percentage of fluorescent cells from *Bacillus*. The experiment involved hybridizing *Bacillus* (BA) cells with three different synthesized oligonucleotide probes. Overall, all probes increased the percentage of fluorescent cells compared to unlabeled cells. Probe EUB338-(**4**) showed high specificity similar to the commercial probe (Cy3). Although EUB338-(**7**) and EUB338-(**10**) resulted in lower fluorescent cell percentages, it still demonstrated respectable specificity in labeling BC cells (Fig. 3).

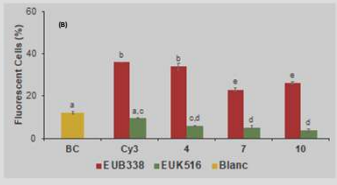


Figure 3. Percentage of fluorescent cells **(B)** from *Bacillus* cells labeled with EUK516 and EUB338 probes with different markers (**4**, **7**, **10** and **Cy3**) and their respective blank. In each assay 1000 cells were analyzed in triplicate. Values represented in A correspond to the average of flow cytometry measurements and error bars to standard deviation (± SD). In each plot, different letters located over the error bars indicate significant differences ($p<0.05$).

Conclusions

According to the results obtained in this study, it was concluded that: i) the fluorescent 4-dietylamino-benzaldehyde derivative labels serve as suitable components for tagging biomolecules containing primary amine groups. Specifically, they demonstrate remarkable effectiveness in producing single-fluorescent-labeled oligonucleotides that incorporate amino-modified nucleotides with high yields; ii) the resulting fluorescent oligonucleotide probes demonstrated remarkable efficacy as RNA-FISH probes, enabling the specific detection of microbial cells. These findings lay the groundwork for exploring numerous other potential applications for these amino-reactive fluorescent labels; iii) The EUK516-(**4**) and EUB338-(**4**) probes showed the greatest specificity for labeling both yeasts and bacteria. This highlights their exceptional effectiveness as RNA-FISH probes, enabling the precise identification of microbial cells.

References

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