New 4-styrylcoumarin derivatives as potentials fluorescent labels for biomolecules: application in RNA-FISH probes

Raquel Eustáquio[1], Ana Teresa Caldeira[1,2,3], Sílvia Arantes[1], António Candeias[1,2,3], António Pereira[1,2,3]

[1] Laboratório HERCULES, Instituto de Investigação e Formação Avançada, Universidade de Évora, 7000-809 Évora, Portugal. [2] Departamento de Química e Bioquímica, Escola de Ciências e Tecnologia, Universidade de Évora, 7000-671 Évora, Portugal. [3] City U Macau Chair in Sustainable Heritage, Instituto de Investigação e Formação Avançada, Universidade de Évora, 7000-809 Évora, Portugal.

Introduction

The study focuses on fluorescence microscopy, a sensitive imaging technique widely used in fields like cellular biology, medicine, and environmental sciences. It leverages multiple fluorescent labels to visualize biomolecules, enabling multicolored images that identify specific components within complex structures and study their interactions.[1] Amino-reactive fluorescent labels are commonly used due to their easy integration into biomolecules, supporting various applications like fluorescence in situ hybridization, cell tracing, and immunochemistry.

Current fluorescent labels are costly and often have small Stokes shifts, limiting their efficiency. Coumarin derivatives, known for their biological activity, emerge as promising, cost-effective, and highly luminescent fluorophores with substantial Stokes shifts.[2] The study aims to evaluate synthesized 4-styrylcoumarin derivative labels as potential fluorescent labels for biomolecules.[3,4] This evaluation involves testing twelve new fluorescent oligonucleotide probes—six targeting the rRNA region of eukaryotic cells (EUK516) and six targeting the rRNA region of prokaryotic cells (EUB338). The assessment will be conducted on microorganisms from the culture collection of the Laboratory of Biodegradation and Biotechnology at the HERCULES Laboratory, University of Évora.

Discussion

In this research, we assess the potential of the 4-styrylcoumarin derivative labels we've synthesized using the 7-diethylamino-4-methylcoumarin as a cost-effective starting material (Fig. 1), as they show promise as effective fluorescent labels for biomolecules. 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 (**1**, **2**, **3**, **4**, **5**, and **6**) and commercially available 5'-AC6 amino-modified oligonucleotide sequences (EUK516-mod or EUB338-mod) via the amino group situated at the 5' terminus.

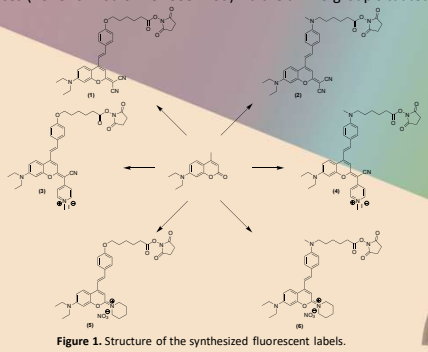

Figure 1. Structure of the synthesized fluorescent labels.

Six of these new probes were designed to target the rRNA region of eukaryotic cells (designated as EUK516-(**1**), EUK516-(**2**), EUK516-(**3**), EUK516-(**4**), EUK516-(**5**), and EUK516-(**6**)), while the remaining six were tailored for the rRNA region of prokaryotic cells (labeled as EUB338-(**1**), EUB338-(**2**), EUB338-(**3**), EUB338-(**4**), EUB338-(**5**), and EUB338-(**6**)) (Fig. 2).

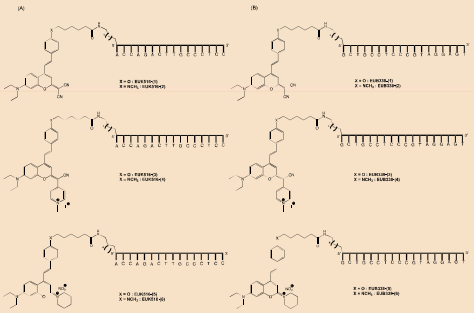

Figure 2. Fluorescent oligonucleotide probes: **(A)** Oligonucleotide probes complementary to eukaryotic cell RNA; (B) Oligonucleotide probes complementary to prokaryotic cell RNA.

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-(**3**) and EUK516-(**5**) showed similar fluorescent cell percentages as the commercial probe EUK516-Cy3, indicating high specificity for SC cell labeling. On the other hand, probes EUK516-(**1**), EUK516-(**4**), and EUK516-(**6**) also labeled SC cells effectively, displaying percentages close to the commercial probe. Although EUK516-(**2**) resulted in lower fluorescent cell percentages, it still demonstrated respectable specificity in labeling SC cells. These findings confirm the effectiveness of custom probes in specifically labeling *Saccharomyces cerevisiae* cells, offering potential alternatives to commercial probes.

Percentage of fluorescent cells from *Bacillus*. The experiment involved hybridizing *Bacillus* (BA) cells with six different synthesized oligonucleotide probes. Overall, all probes increased the percentage of fluorescent cells compared to unlabeled cells. Notably, EUB338-(**6**) displayed a significant increase in fluorescent cells compared to a commercial probe, indicating exceptional specificity for labeling BA cells. EUB338-(**2**) and EUB338-(**3**) also showed high specificity similar to the commercial probe, while EUB338-(**1**) and EUB338-(4) had reduced intensity but still labeled cells effectively. However, EUB338-(**5**) resulted in significantly fewer fluorescent cells, indicating limited specificity for labeling BA cells.

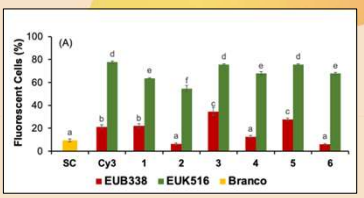

Figure 3. Percentage of fluorescent cells **(A)** from Saccharomyces cerevisiae cells labeled with EUK516 and EUB338 probes with different markers (**1**, **2**, **3**, **4**, **5**, **6** and Cy3) and their respective blank. In each assay 1000 cells were analyzed in triplicate. Values represented in 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).

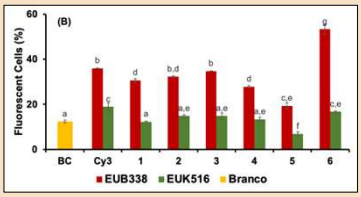

Figure 4. Percentage of fluorescent cells **(B)** from *Bacillus* cells labeled with EUK516 and EUB338 probes with different markers (**1**, **2**, **3**, **4**, **5**, **6** and Cy3) and their respective blank. In each assay 1000 cells were analyzed in triplicate. Values represented in A correspond 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-styrylcoumarin 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 proportion of fluorescent cells in the *Bacillus* bacteria cell probes exhibited a notable decrease when compared to the probes used with *Saccharomyces cerevisiae* cells; **iv)** several of the synthesized probes exhibited a proportion of fluorescent cells that matched or exceeded that of the commercial fluorescent label (Cy3). This highlights their exceptional effectiveness as RNA-FISH probes, enabling the precise identification of microbial cells.

References

1. Sednev, M.V., Belo, V.N., and Hell, S. W., Methods Appl. Fluoresc., 2015, vol. 3, no. 4, p. 042004.
2. Ren, T.B., Xu, W., Zhang, W., Zhang, X.X., Wang, Z.Y., Xiang, Z., Yuan, L., and Zhang, X. B., *J. Am. Chem. Soc.*, 2018, vol. 140, no. 24, pp. 7716-7722.
3. Barot, K.P., Jain, S.V., Kremer, L., Singh, S., and Ghate, M.D., *Med. Chem. Res.*, 2015, vol. 24, pp. 2771-2798.
4. Bojtár, M., Kormos, A., Kis-Petik, K., Kellermayerand, M., and Kele, P., *Org. Lett*., 2019, vol. 21, no. 23 pp. 9410-9414

Acknowledgments

The authors acknowledge financial support to FCT—Foundation for Science and Technology, I.P.—within the scope of the projects UIDB/04449/2020 (HERCULES Lab), UIDB/04033/2020 (CITAB), ART3mis (2022.07303.PTDC/FCT) and Old Goa Revelations project (2022.10305.PTDC/FCT) and also through the PhD Grant UI/BD/153584/2022. Acknowledges additionally the City University of Macau endowment to the Sustainable Heritage Chair & Sino-Portugal Joint Laboratory of Cultural Heritage Conservation Science supported by the Belt and Road Initiative.