Optimizing locked nucleic acid/2'-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2'OMe-FISH) for bacterial detection

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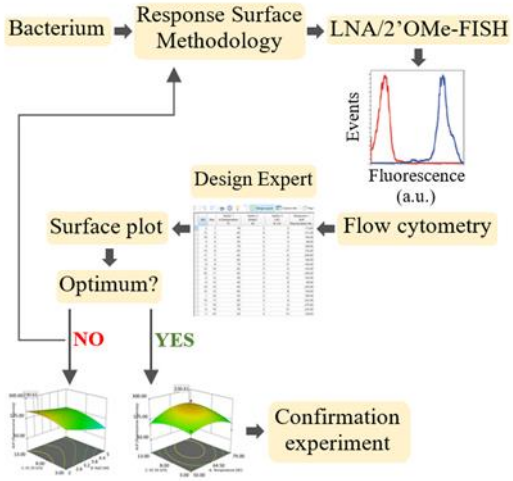

Despite the successful application of locked nucleic acid/2'-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2'OMe-FISH) procedures for bacteria detection, there is a lack of knowledge on the properties that affect hybridization. Such information is crucial for the rational design of the protocols, especially in multiplex assays. Hence, this work aimed to evaluate the effect of 3 essential factors on the LNA/2'OMe hybridization step - hybridization temperature, NaCl concentration and type and concentration of denaturant (formamide, ethylene carbonate and urea). This optimization was performed for 3 Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Citrobacter freundii*) and 2 Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus epidermidis*), employing the Response Surface Methodology and an Eubacteria probe. In general, it was observed that high NaCl concentrations (from 2 to 5M) are beneficial, regardless of denaturant. Urea, formamide and ethylene carbonate are suitable denaturants for FISH applications; but urea provides similar fluorescence signals among the different bacteria. Overall, the results indicate that 2 M of urea, 4 M of NaCl and 62 °C of hybridization temperature would be a proper starting point for multiplex LNA/2'OMe-FISH procedures.

Introduction

Fluorescence *in situ* hybridization (FISH) is one of the most well-established molecular biology techniques used for the rapid and direct detection, localization and quantification of microorganisms in many fields of microbiology (e.g. 1-3). Recently, the combination of the FISH method with nucleic acid mimics, such as peptide nucleic acid (PNA), locked nucleic acid (LNA) and 2'-O-methyl RNA (2'OMe), has shown to have advantages compared to DNA-FISH [3]. For instance, introducing LNA monomers at every third position of 2´OMe probes is a common approach used to improve FISH experiments in terms of affinity and sensitivity (e.g. 2). This also allows for a thorough control of the thermodynamic parameters, facilitating multiplex approaches (detection of multiple targets simultaneously). Despite the obvious advantages of LNA/2'OMe molecules in terms of improving the accuracy, stability, robustness and simplicity of the FISH, there is an absence of studies that have analyzed the impact of denaturant and salt concentration on the LNA/2′OMe-FISH efficiency. This information is very important to find the more suitable hybridization conditions for bacteria detection either in an individual or in a multiplex assay, and to move towards a tailored design of hybridization experiments.

Methods

The optimization of the hybridization conditions may be time-consuming due to the large number of factors that affect the FISH efficiency. As such, the effect and the interplay of hybridization temperature, NaCl and denaturant (formamide, ethylene carbonate and urea) on LNA/2'OMe-FISH was studied using an universal LNA/2′OMe probe for the Eubacteria domain (5'mT*lG*lC*mC*lT*mC*mC*lC*mG*mT*lA*mG*mG*lA *3'; "l" - LNA; "m" - 2'OMe; * - phosphonothioates backbone) based on Amann et al. (1990) [4], through Response Surface Methodology (RSM). RSM, a mathematical and statistical tool, was applied to model the data obtained from 3 Gram-negative (*E. coli CECT 515*, *C. freundii SGSC 5345* and *P. aeruginosa PAO1*) and 2 Gram-positive bacteria (*E. faecalis* CECT 184 and *S. epidermidis* RP61A). Hence, to evaluate the effect of the three factors on the fluorescence intensity of LNA/2'OMe-FISH method, the hybridizations were performed in suspension based on Azevedo et al. (2015) [2], followed by signal quantification using flow cytometry.

The standard central composite designs (CCD) were set up for each bacterium, using the statistical software Design Expert®11 (Stat-Ease Inc., Minneapolis, USA) to estimate the coefficients of the model. In designs, the hybridization temperature (*x1*), denaturant concentration (*x2*) and salt concentration (*x3*) were considered the independent variables and the fluorescence intensity was the response. To find the optimum hybridization conditions for all five species in the study, the obtained fluorescence values were fitted to a quadratic model. Each model was analyzed using analysis of variance (ANOVA) to test the significance and adequacy of the model. Finally, the optimum conditions within the experimental range that maximize the fluorescence intensity were estimated using the optimization function of the Design Expert®11. The value estimated for the optimum conditions was, then verified for each bacterium on a confirmation experiment, in triplicate.

Results and Conclusion

In this work, different species were selected to include bacteria with different characteristics, including different cell wall structures and compositions. Furthermore, three denaturant agents were included, because even though the formamide is more frequently used in FISH methodology, there are also studies using less hazardous compounds as denaturant agents, including urea [e.g. 2] and ethylene carbonate [e.g. 5]. Most of the quadratic models obtained for each bacterium were highly significant ($p<0.05$), confirming the adequacy of the model fits. Furthermore, the coefficients of determination, R^2 (0.70-0.90) confirmed a good fit between predicted values and the

experimental data points. Hence, using the successful modelling of the hybridization temperature, NaCl and denaturant concentration, we have been able to obtain the optimum hybridization conditions that lead to the maximum intensity fluorescence for all the five bacteria (Table 1). These optimum ranges presented in Table 1 will provide guidelines on compromise conditions for each variable.

Surprisingly, observing Table 1, the optimal NaCl concentration, ranging from ~2M to ~5M, is higher than those used in conventional LNA/2′OMe-FISH protocols (0.9 M) (e.g. 2). Optimal values were not related with the type of denaturant. In fact, NaCl is highly important in the hybridization to stabilize the repulsive interactions of LNA/2'OMe-rRNA duplexes. These electrostatic repulsions can be reduced by increasing the NaCl concentration that will stabilize the negative charges of the duplex, allowing the binding of LNA/2′OMe probes to the rRNA target.

Regarding the denaturants, the use of urea provided values of fluorescence more homogenous, with *S. epidermidis* values being in lines with the fluorescence values obtained for the other species. Urea is a chaotropic agent that has been studied for its effect on permeabilization of cells [6] and destabilization of proteins and nucleic acids [7], which might enable a higher accessibility of the probe to the target. Moreover, another main observation is related with the fact that, when urea was applied in LNA/2′OMe-FISH, the ranges of the optimal urea concentration are overlapped (e.g. 1 M to 4 M of urea for *E. coli* and *E. faecalis*; 0.6 M to 2 M of urea for *P. aeruginosa*, *C. freundii* and *S. epidermidis*) (Table 1), which simplifies the design of any multiplex approach.

In conclusion, urea and high salt concentrations seem to be an adequate choice to balance fluorescence signal among species and to reach an universal hybridization solution for multiplex assays. However, according to the properties of the target bacteria some minor adjustments should be performed in optimal hybridization conditions to improve the efficiency of the hybridization. This study gives general recommendations for, at least, the starting point on optimization experiments of LNA/2'OMe-FISH method would include approximately 2 M of urea, 4 M of NaCl and 62 °C of hybridization temperature.

Table 1. Optimal ranges of hybridization temperature, NaCl and denaturant concentration predicted through the RSM models for the tested bacteria. The predicted and experimental fluorescence values are also shown. Ranges have been stablished assuming a fluorescence intensity of at least 85% of the maximum value.

Denaturant	**Bacteria**	**Temperature (°C)**	**[NaCl] (M)**	**[Denaturant] (M; % v/v)**	**Predicted fluorescence (a.u.)**	**Obtained fluorescence (a.u.)***
Formamide	*E. coli*	51.41-76.08	0.03-1.99	1.18-4.40	296.13-348.87	369.14
	P. aeruginosa	56.40-74.19	2.11-4.92	4.27-12.80	344.46-405.25	225.30
	C. freundii	50.00-79.00	2.25-5.00	9.00-17.69	113.76-133.83	141.26
	E. faecalis	50.00-69.29	2.66-5.00	22.00-32.81	287.84-338.63	131.15
	S. epidermidis	62.00-82.00	2.00-5.00	3.00-13.00	47.51-55.89	296.62
Ethylene carbonate	*E. coli*	55.53-77.88	2.00-5.00	3.13-12.42	278.02-327.08	346.03
	P. aeruginosa	53.77-78.09	2.03-3.42	4.02-12.71	201.12-236.61	291.57
	C. freundii	57.58-79.00	2.00-4.89	3.76-13.00	266.56-313.59	353.14
	E. faecalis	46.00-63.00	2.00-5.00	1.00-4.00	170.34-200.40	126.34
	S. epidermidis	62.00-82.00	2.90-5.00	0.00-2.00	106.68-125.50	254.37
Urea	*E. coli*	50.13-60.04	2.41-5.01	1.41-4.09	200.40-235.76	253.87
	P. aeruginosa	47.12-58.12	2.45-5.01	0.67-2.37	395.90-465.75	324.35
	C. freundii	50.21-63.29	2.42-4.57	0.63-2.08	183.51-215.89	225.82
	E. faecalis	50.13-79.87	2.59-5.01	1.41-4.09	345.03-405.92	205.58
	S. epidermidis	64.16-82.91	3.37-5.01	0.61-1.84	188.04-221.22	314.17

*The obtained fluorescence was evaluated using the optimum hybridization temperature, denaturant and salt concentration predicted through the RSM models for each bacterium.

Acknowledgements

This work was financially supported by: Project Coded-FISH PTDC/DTP-PIC/4562/2014 – POCI-01-0145-FEDER-016678 - funded by FEDER funds through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) and by national funds through FCT - Fundação para a Ciência e a Tecnologia, I.P.; Post-doctoral fellowship SFRH/BPD/121601/2016; Project POCI-01-0145-FEDER-006939 (Laboratory for Process Engineering, Environment, Biotechnology and Energy – UID/EQU/00511/2013), funded by European Regional Development Fund (ERDF) through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI), and by national funds through FCT – Fundação para a Ciência e a Tecnologia.

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