Optimization of peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) method for the detection of bacteria: the effect of pH, dextran sulfate and probe concentration

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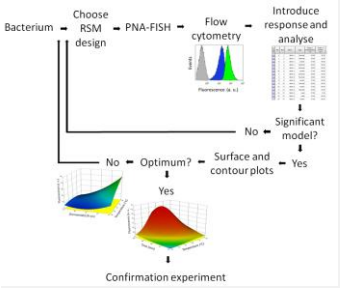

Fluorescence *in situ* hybridization (FISH) appeared in the 1980's and is nowadays widely used in the field of microbiology. FISH is affected by a wide variety of abiotic and biotic variables and their interplay. This is translated into a wide variability of FISH procedures that can be found in the literature. The aim of this work is to study the effects of pH, probe and dextran sulphate concentration in the FISH protocol. For this, response surface methodology (RSM) was used to optimize FISH protocol for gram-negative (*E. coli* and *P. fluorescens*) and gram-positive bacteria (*L. innocua*, *S. epidermidis* and *B. cereus*), for these 3 parameters. The obtained results show a clear distinction between the two groups: higher pH (>9) combined with lower dextran sulphate concentration (<2.5% [w/v]); for Gram-negative bacteria and pH from 6.5 to 9 together with higher dextran sulphate concentrations (>7% [w/v]), for Gram-positive bacteria. The optimal probe concentration was the same for both groups (300 nM). These results seem to result from an interplay of pH and dextran sulphate ability to influence the probe concentration and migration inside the bacteria.

Introduction

Fluorescence *in situ* hybridization (FISH) first appeared in the 1980's through the work of Bauman and co-workers [1]. FISH is a very straightforward technique that consists essentially in hybridizing an oligonucleotide probe to its complementary sequence obeying to Watson-Crick hydrogen-bonding [2,3]. FISH is widely used in the field of microbiology [4], namely in the identification, quantification and characterization of phylogenetic defined microbial populations in complex environments [5]. From the beginning until now, different types of probes have emerged and peptide nucleic acid (PNA) molecules have shown to be a DNA mimic with recognized superior hybridization features [6]. Because of this, PNA probes were quickly introduced in FISH studies for the detection of microorganisms [7,8,9,10].

The efficiency of FISH hybridization is affected by a wide variety of abiotic and biotic variables and their interplay [11,12,13]. Variables such pH, dextran sulphate and probe concentration, were never fully studied and their impact on FISH hybridization was never systematically assessed. pH from 6.5 to 7.5 is usually used in the hybridization step of FISH, but it is known that higher pH values in hybridization solutions produce more stringent conditions [14]. This is probably due to the denaturing effect of alkaline conditions (pH >9) on nucleic acids, known to deprotonate guanine, thymine and uracil bases [15], causing a destabilization on the established hydrogen bonds between Watson-Crick base pairs. Probe concentration affects the rate at which the first few base pairs are formed (nucleation reaction), which is an important limiting step in hybridization. Therefore, the higher the concentration of the probe, the higher the annealing rate [14]. Dextran sulphate in aqueous solution is strongly hydrated [14], meaning that less volume of solvent is available to the probe [16], causing an apparent increase in probe concentration, which is translated into higher hybridization rates [17].

The implementation of a FISH methodology usually requires an initial optimization [18] which is currently performed as a trial and error procedure. In order to better understand the hybridization efficiency of nucleic acids in

bacteria, a systematic protocol is lacking [12]. Santos and co-workers [12] have successfully started a pioneering approach of FISH optimization, applying response surface methodology (RSM), a modeling technique that has become popular in recent years for optimization studies [19]. RSM allows model the effects of different factors, evaluating not only the influence of each factor but also their interaction.

This work aimed to continue that FISH modeling effort, through optimization of a universal PNA EUB338 probe for bacteria quantification by PNA flow-FISH, focusing on the hybridization pH, probe and dextran sulphate concentration. The effect of these variables and their interplay on the fluorescence intensity was studied for different bacteria, through RSM.

Methods

In this study, the bacterial species used were *Escherichia coli* CECT 434, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* CECT 910, *Staphylococcus epidermidis* RP61A and an isolated *Bacillus cereus*.

A PNA-FISH protocol for suspension based on a previous study [12] was performed, taking into account the conclusions found by Santos and co-workers [12] and the factors subjected to modulation in this study: pH, dextran sulphate and probe concentration, according to the tables 1 and 2. The range and levels of these variables were defined according to previous studies [10,21] and the results obtained within this study. The hybridization was performed for all species at 60 ºC. *E. coli*, *P. fluorescens*, *L. innocua* and *S. epidermidis* were subjected to a 55 min hybridization with 5.5% (v/v) formamide in the hybridization solution. *B. cereus* was subjected to a hybridization step of 110 min in the presence of 49.5% (v/v) formamide. *L. innocua*, *S. epidermidis* and *B. cereus* were also tested with a lower molecular weight dextran sulphate, 10 kDa, instead of the usual one with 500 kDa. EUB338 (5'-TGCCTCCCGTAGGA-3'), developed by Amann and co-workers [20], was used as a probe. It recognizes a conserved region of the 16S rRNA in the Bacteria domain. Each experiment was performed in triplicate.

The average fluorescence intensity obtained after PNA-FISH was quantified by flow cytometry with a minimum of 20,000 events falling into the defined bacterial gate.

RSM was employed according to the strategy depicted in the Graphical Illustration [12]. Central composite designs (CCD) were set up using the statistical software Design Expert® 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) to estimate the coefficients of the model. The average intensity values obtained were fitted into a quadratic model. The interaction of the three independent variables and their effect on the fluorescence intensity was inspected by constructing the response surface and contour plots.

E. coli and *P. fluorescens* were successfully modeled using the experimental range presented in table 1; while *L. innocua, S. epidermidis* and *B. cereus* were successfully modeled using the experimental range presented in table 2. A confirmation experiment was done to infer about the likelihood of the optimization parameters obtained by the statistical modeling.

Table 1. Experimental levels of variables tested using *E. coli* and *P. fluorescens*.

Variables	Range and level				
	-α	-1	0	+1	+α
x_1[a]	5.9	7.3	9.3	11.3	12.6
x_2[b]	0.00	1.00	2.50	3.90	4.95
x_3[c]	32	100	200	300	368

[a] pH [b][Dextran sulphate] (%w/v) [c][PNAEUB338] (nM)

Table 2. Experimental levels of variables tested using *L. innocua*, *S. epidermidis* and *B. cereus*.

Variables	Range and level				
	-α	-1	0	+1	+α
x_1[a]	4.5	5.7	7.5	9.3	10.5
x_2[b]	0.00	4.05	10.00	15.95	20.00
x_3[c]	32	100	200	300	368

[a] pH [b][Dextran sulphate] (%w/v) [c][PNAEUB338] (nM)

Results

This work has optimized the temperature, time and formamide concentration in the hybridization solution, recurring to a factorial design, in 3 gram--positive species and in 2 gram-negative species. A successful optimization of FISH protocol was achieved for all five tested species, leading to the optimum hybridization conditions summarized in table 3. Analyzing the results it is possible to notice that the optimal probe concentration was 300 nM, the highest considered in the model prediction. This was expected, since the probe concentration affects the nucleation reaction and the higher the probe concentration the faster the annealing between the target and the probe [14].

Interestingly, analyzing the results of the optimal pH and dextran sulphate (500 kDa) concentration, it is possible to distinguish 2 different tendencies. High pH (>9) and low dextran sulphate concentration (<2.5%) were found to be favorable to gram-negative species (*E. coli* and *P. fluorescens)*, while more moderate pH (6.5 to 9) and higher dextran sulphate concentration (>7%) favored gram-positive species (*L. innocua*, *S. epidermidis* and *B. cereus*).

Table 3. Optimum pH, dextran sulphate (500 kDa and 10 kDa) and PNA EUB338 probe concentration in hybridization predicted through the RSM models for the tested bacteria. The predicted and obtained fluorescence values in those conditions are shown.

Bacteria	Optimum conditions			Predicted Fluorescence (a. u.)	Obtained Fluorescence (a. u.)
	pH	Dextran (% w/v)	Probe (nM)		
B. cereus (500 kDa)	6.92	11.70	300	37.9	30.6 ± 1.4
B. cereus (10 kDa)	8.09	12.16	287	23.5	*
E. coli (500 kDa)	9.87	1.93	300	37.1	37.7 ± 1.5
P. fluorescens (500 kDa)	10.83	2.32	300	98.2	171.7 ± 8.3
L. innocua (500 kDa)	8.36	7.94	300	24.9	21.6 ± 0.2
L. innocua (10 kDa)	9.14	10.52	300	65.9	*
S. epidermidis (500 kDa)	8.56	12.84	300	18.0	17.4 ± 1.6
S. epidermidis (10 kDa)	>9.30	15.43	300	32.3	*

* not evaluated (the objective was only to compare the optimum protocol obtained with dextran 500 and 10 kDa)

The difference in the optimum dextran sulphate concentration obtained may be related to the peptidoglycan thickness of the bacteria. It is known that gram-positive bacteria are harder to permeabilize than gram-negative bacteria [22], mainly due to its thicker peptidoglycan layer. So, in order to facilitate the entry of the probe inside the cell, we theorized that a higher probe gradient between the inside and the outside of the bacteria should be obtained.

Regarding the pH, we can see that higher pH values are favored, probably due to the denaturating effect on nucleic acids [15], allowing a better accessibility of the probe to the target. Moreover, it is interesting to notice that the order of optimal pH for each strain is inversely related to the thickness of the peptidoglycan layer, with the exception of *L. innocua* and *S. epidermidis* [23,24]. A deleterious effect of high pH on bacterial cellular membranes and cell walls has been described [25,26,27]. However the possible membrane/cell wall damages caused by high pH values cannot explain the results obtained here. More precisely, higher cell wall thickness would support higher hybridization pH; however the opposite was observed (Table 3).

Nevertheless, the effect of pH can be seen in relation to the dextran sulphate. Different pH will affect the degree of ionization of dextran sulphate, affecting its viscosity, molecule swelling, osmotic coefficients, among others [28]. Dextran solutions viscosity show a pattern like a parabolic curve, increasing with ionization (higher pH) until a plateau, declining further from that point. Although pH affects the viscosity of dextran solutions, the main parameter affecting it is dextran concentration and molecular weight [29]. In fact a solution with dextran 500 kDa and 10 kDa at 10% (w/v) presents a viscosity of 30 m.Pa.s and 2 m.Pa.s, while at 20% (w/v) the value rises to 200 m.Pa.s and 8 m.Pa.s, respectively.

Taking this into account, we theorized that high pH is favored in PNA-FISH protocol and for gram-positive bacteria, with a thicker cell wall, a higher gradient of probe is needed to have a successful hybridization. Those conditions are however limited to the viscosity of the hybridization solution produced. Too high viscosity and the diffusion of the probe inside the cell became hindered, too low and the probe gradient would not be enough to produce a positive hybridization.

In order to test this hypothesis we repeated the protocol, for gram-positive species, using a dextran sulphate with lower molecular weight, 10 kDa. We expected that using it, the hybridization solutions produced will be less viscous, allowing protocol optimization to be achieved with higher pH and dextran concentrations. The results in table 3 were in line with what was expected, corroborating the formulated hypothesis.

Conclusions

This work intended to, for the first time, study the effects of pH, dextran sulphate and probe concentration in PNA-FISH protocol. We were able to successfully optimize the conditions enunciated before for the 5 bacterial species tested, using a PNA EUB338 probe through RSM.

Briefly, the optimal probe concentration was the same for all 5 species, 300 nM. A clear difference for gram-positive and gram-negative bacteria protocols regarding pH and dextran sulphate concentration was observed. Gram-negative bacteria have an optimal signal with high pH (>9) and low dextran sulphate concentration (<2.5%). For gram-positive more moderate pH (6.5 to 9) and higher dextran sulphate concentration (>7%), are beneficial. Those results arise manly from the interplay of peptidoglycan thickness, probe concentration and dextran sulphate characteristics with ionization.

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