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Mismatch discrimination in fluorescent in situ hybridization using different types of nucleic acids

Fontenete Silvia & Barros Joana & Madureira Pedro & Figueiredo Céu & Wengel Jesper & Azevedo Nuno Filipe

F. Silvia · B. Joana · A. N. Filipe (✉)

LEPABE—Laboratory for Process Engineering, Environment,
Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
e-mail: nazevedo@fe.up.pt

F. Silvia, F. Céu IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, 4200-465 Porto, Portugal

F. Silvia, W. Jesper

Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, 5230 Odense M, Denmark

F. Silvia, M. Pedro ICBAS, Institute of Biomedical Sciences Abel Salazar, University of Porto, 4050-313 Porto, Portugal

B. Joana
INEB, Instituto de Engenharia Biomédica, 4150 Porto, Portugal

M. Pedro
IBMC, Institute for Molecular Biology and Cell Biology, 4150 Porto, Portugal

F. Céu
FMUP, Faculty of Medicine of Porto University, 4200-319 Porto, Portugal

Abstract

In the past few years, several researchers have focused their attention on nucleic acid mimics due to the increasing necessity of developing a more robust recognition of DNA or RNA sequences. Fluorescence in situ hybridization (FISH) is an example of a method where the use of these novel nucleic acid monomers might be crucial to the success of the analysis. To achieve the expected accuracy in detection, FISH probes should have high binding affinity towards their complementary strands and discriminate effectively the noncomplementary strands. In this study, we investigate the effect of different chemical modifications in fluorescent probes on their ability to successfully detect the complementary target and discriminate the mismatched base pairs by FISH. To our knowledge, this paper presents the first study where this analysis is per- formed with different types of FISH probes directly in biological targets, *Helicobacter pylori* and *Helicobacter acinonychis*. This is also the first study where unlocked nucleic acids (UNA) were used as chemistry modification in oligonucleotides for FISH methodologies. The effectiveness in detecting the specific target and in mismatch discrimination appears to be improved using locked nucleic acids

(LNA)/2′- *O*-methyl RNA (2′OMe) or peptide nucleic acid (PNA) in comparison to LNA/DNA, LNA/UNA, or DNA probes. Further, the use of LNA modifications together with 2′OMe monomers allowed the use of shorter fluorescent probes and increased the range of hybridization temperatures at which FISH would work.

Keywords FISH · Oligonucleotides · Nucleic acids · Microbiology · Mismatch discrimination

Introduction

It has been shown that fluorescent in situ hybridization (FISH) is a very sensitive and specific method for microbial identification in clinical, industrial, and environmental samples (DeLong et al. 1989; Sekiguchi et al. 1999; Kempf et al. 2000). The success of this methodology relies on the specificity of oligonucleotide sequences to their complementary tar- get. Ideally, oligonucleotides should hybridize with fully complementary DNA or RNA sequences and fail to hybridize with sequences that contain one or more mismatches (Yan et al. 2012). However, it has been shown that for DNA sequences, this discrimination is often difficult to achieve (Kubota et al. 2006). More recently, other types of synthetic nucleic acids such as peptide nucleic acid (PNA) and locked nucleic acids (LNA) have been used in FISH with very promising results (Guimaraes et al. 2007; Mook et al. 2007; Tavares et al. 2008; Campbell and Wengel 2011; Almeida et al. 2013; Cerqueira et al. 2013). To understand the specificity of these novel molecules towards DNA and RNA, several thermal dissociation studies have been conducted to analyze the effects of mismatches on duplex stability (Owczarzy et al. 2011; Yan et al. 2012; Matsumoto et al. 2013). However, this type of analysis has always been performed with naked DNA in standard chemical solutions. Therefore, it is not necessarily predictive of what occurs during a FISH procedure in which a chemically modified probe hybridizes to ribosomal RNA (rRNA) within a cellular microenvironment (Cerqueira et al. 2008).

In here, we evaluate the mismatch discrimination potential of different types of DNA/RNA mimics used frequently in the literature for FISH, such as PNA, LNA, and 2′-*O*-methyl RNA (2′OMe), and also of a molecule that has not been previously used in FISH, named unlocked nucleic acids (UNA). Mismatch discrimination was assessed by both thermal dissociation studies and by FISH experiments. As a case study, we tested the discrimination of the sequences for two closely related *Helicobacter* spp., *Helicobacter pylori* and *Helicobacter acinonychis* (Marshall et al. 1984; Eaton et al. 1993). In fact, our group has been focused on the development of several FISH methodologies to detect *Helicobacter* species, namely using peptide nucleic acids (PNA) (Guimaraes et al. 2007; Cerqueira et al. 2011, 2013), locked nucleic acids (LNA), and 2′-*O*-methyl RNAs (2′OMe) (Fontenete et al. 2013). The novel oligonucleotides were purposely designed for a specific and conservative region in the 16S rRNA of *H. pylori* that is also present, with a single mismatch, in *H. acinonychis*. Therefore, we evaluate how a single mismatch in the DNA or RNA target influences the hybridization stability and also report on the ability of different probes to discriminate between *H. pylori* and *H. acinonychis*, at a large range of temperatures.

Materials and methods

Probe design

For mismatch discrimination, it was necessary to find a sequence which was identical within the two *Helicobacter* species with the exception of a single nucleotide. The 16S rRNA target region was selected based on a search conducted at the 16S rRNA database of the Ribosomal Database Project II (RDP-II), version 10 (http://rdp.cme.msu.edu/) The ribosomal sequence *5′-ATTACTGGAGAGACTAAGC-3′*, which allowed the detection of *H. pylori*, was selected. The 16S sequence of *H. acinonychis* is similar except for the G (underlined) that in *H. acinonychis* is an A. We used different types of nucleic acids targeting *H. pylori*: LNA, 2′OMe, unlocked nucleic acid (UNA), PNA, and DNA (Table 1). Different designs of oligo- nucleotide probes were tested to find the best discrimination possible based on earlier published criteria to improve mismatch discrimination (Kumar et al. 1998; You et al. 2006; Langkjaer et al. 2009; Pasternak and Wengel 2010, 2011; Owczarzy et al. 2011). As DNA has a restricted flexibility of oligonucleotide design, only one probe was designed (Table 1). The length of this probe was selected based on the analysis of the Gibbs free energy ($\Delta G°$), which, according to Yilmaz et al.(Yilmaz et al. 2006), must be lower than −13. 5 kcal/mol. The probe used in this study has a $\Delta G°$ of −16.63 kcal/mol. Similarly, PNA also has a restricted flexibility of probe design as PNA cannot be mixed with nucleotide monomers like DNA, LNA, or UNA. In this case, and because there are no guidelines related to the minimum $\Delta G°$, we based our design on our extensive experience in working with these probes (Guimaraes et al. 2007; Cerqueira et al. 2011, 2013). In contrast, LNA, 2′OMe, and UNA bases can be positioned anywhere within an oligonucleotide sequence, which means that the design is much more flexible and that probe fine- tuning is possible (You et al. 2006). As a general rule, these probes were designed with a triplet of LNA-modified nucleotides positioned at the center of the mismatch site to improve the discrimination (You et al. 2006; Owczarzy et al. 2011). The use of a higher density of LNA residues in each probe is an- other important parameter to improve mismatch discrimination (You et al. 2006). In the case of HyP_LNA/2′OMe probes (1 and 2) (Table 1), every second base was LNA modified as previously reported (Kierzek et al. 2005).

Oligonucleotide synthesis and purification

The DNA oligonucleotide (HyP_DNA) attached to fluoresce- in phosphoramidite (FAM) was purchased from Sigma- Aldrich (St. Louis, USA). The PNA (HyP-PNA) probe was synthesized by Panagene (Daejeon, South Korea), and the probe N terminus was attached to an FAM molecule via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker. Both probes were HPLC purified to reach a purity of >90 %.

The remaining oligonucleotides were synthesized on an Expedite DNA synthesizer (PerSpective Biosystems, Expedite 8909 instrument) using standard phosphoramidite chemistry in 1.0 μmoL scale. The synthesized oligonucleotides were deprotected and cleaved from the solid support by treatment with 32 % (*v*/*v*) aqueous ammonia solution for 12 h at 55 °C (UNA1_HPy, UNA2_HPy, UNA3_HPy, LNA1_HPy, and LNA2_HPy oligonucleotides) or with 98 % (*v*/*v*) aqueous methanol/ammonia solution 7 N in methanol (1:1) for 2 h at RT, followed by an incubation with 32 % (*v*/*v*) aqueous ammonia solution for 12 h at 55 °C (2′ OMe1_HPy and 2′OMe2_HPy oligonucleotides). DNA and LNA monomers are commercially

available from Proligo Reagents and Exiqon (Copenhagen, Denmark), respectively. UNA and 2′OMe monomers are commercially available from RiboTask (Langeskov, Denmark). All oligonucleotides were purified by reversed phase HPLC (RP-HPLC) and characterized by ion exchange HPLC conditions (IE-HPLC) on a Dionex system HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). The purified oligonucleotides were precipitated by acetone, and their purity (>90 %) and composition were verified by IE-HPLC and MALDI-TOF analyses, respectively.

For thermal denaturation studies, unmodified DNA [HyP_DNA_Target: 5′-d(GCTTAGTCTCTCCAGTAAT)-3′] and RNA oligonucleotides [HyP_RNA_Target: 5′-r(GCUU AGUCUCUCCAGUAAU)-3′] were purchased from Sigma- Aldrich and Integrated DNA Technologies (Leuven, Belgium), respectively. Also, the DNA sequence 5′-d(ATTA CTGGAGAGACTAAGC)-3′ (HyP_DNA_Ref) that was used as a reference in thermal denaturation studies was purchased from Sigma-Aldrich.

Thermal denaturation studies

The thermal denaturation studies were performed following published protocols (Christensen et al. 2001; Perlikova et al. 2014). Melting curves of fully complementary and of one- mismatch-containing oligonucleotide duplexes were recorded on a PerkinElmer LAMBDA 35 UV/Vis spectrometer equipment with a PTP 6 (Peltier Temperature Programmer) element (Massachusetts, USA). One micromolar of each strand was used in different types of melting buffers: medium salt buffer with 110 mM Na^+ (100 mM NaCl, 10 mM NaH_2PO_4, and 0.1 mM EDTA; pH 7.0); low salt buffer with 30 % formamide and 10 mM Na^+ (10 mM NaCl, 5 mM EDTA, 50 mM Tris- HCl, and 30 % (*v*/*v*) formamide; pH 7.5); and a high salt buffer with 4 M of urea and 900 mM Na^+ (900 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, and 4 M of urea; pH 7.5). The medium buffer is a standard buffer used in this type of studies. The low and the high salt buffers were used to mimic the buffers used in FISH experiments with bacterial cells. Each sample was mixed and the resulting complexes denatured by heating to 85 °C during 5 min; samples were then cooled to the starting temperature of the experiment. Quartz optimal cells with a path length of 1.0 cm were used. Melting temperatures (Tm values) were determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs. temperature for medium salt buffer and high salt buffer with 4 M urea and A_{270} vs. temperature low salt buffer with 30 % (*v*/*v*) formamide). Absorbance was monitored at 270 nm in the low salt buffer because of the inherent absorbance of formamide at 260 nm (Sadhu et al. 1984). A temperature range from 13 to 15 °C to 80–85 °C and a ramp of 1.0 °C/min were used. Reported Tm values are an average of two measurements with- in ±0.5 °C.

Bacterial strains and culture conditions

H. pylori 26695, obtained from the American Type Culture Collection (ATCC 700392, VA, USA) and *H. acinonychis* strain 90-119, obtained from the Health Protection Agency Culture Collections (HPA Culture Collections 12686, Salisbury, UK), were subcultured every 48 h in trypticase soy agar (TSA) supplemented with 5 % (*v*/*v*) sheep blood (Becton Dickinson GmbH, Germany) and incubated at 37 °C under microaerobic conditions using a GENbox microaer (bioMérieux, Marcy l′Étoile, France). Cell concentration was obtained by optical density (O. D.),

and each initial culture was diluted in saline buffer in order to obtain a final concentration of 10^6 total cells/mL.

Optimization of probe hybridization conditions

Different hybridization conditions were used, depending on the nucleic acid composition, length, and chemical nature of each probe. Therefore, we studied a large temperature range of 25–70 °C in pure cultures of *H. pylori*. For specificity analysis, we tested these probes for mismatch discrimination using *H. acinonychis* at temperatures for which probes showed higher sensitivity. All probes were optimized in FISH standard conditions (hybridization temperature, salt concentration, pH, and formamide/urea concentration). To optimize the DNA- FISH protocol, we used different types of model protocols for DNA-FISH (Krimmer et al. 1999; Moreno et al. 2003), and we performed several adjustments for all steps. After fixation and cell disaggregation of each suspension (as previously described (Fontenete et al. 2013)), an extra permeabilization step was performed by adding 30 μL lysozyme (2 mg/mL in 10 mM Tris-HCl (pH 8)) during 1 h at 37 °C. The fixed cells (100 μL) were resuspended in equal volume of the hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.001 % (*v*/*v*) sodium dodecyl sulfate (SDS), 30 % (*v*/*v*) formamide, and 400 nM of probe) and incubated at different temperatures for 90 min. Samples were centrifuged at 16,873*g* for 5 min, resuspended in 500 μL of washing solution (0.64 M NaCl, 5 mM Tris-HCl [pH 7.5], 0.01 % (*v*/*v*) SDS, pH 7), and incubated at the same temperature of hybridization for 20 min. Samples were again centrifuged at 16,873*g* for 5 min and resuspended in saline buffer. To remove aggregates, samples were filtered by a sterile filter with 10 μm pore size (CellTrics®) and were directly analyzed using flow cytometry.

The hybridization procedure for the PNA probe was similar to that used for the LNA probes (Guimaraes et al. 2007; Fontenete et al. 2013) however using a different type of hybridization buffer as described in Table 2. The hybridization method in suspension for LNA probes (LNA+DNA, LNA+2′ OMe, and LNA+UNA) was based on procedures described by Fontenete et al. (Fontenete et al. 2013).

The ability of the oligonucleotides to discriminate the mismatch base pairs was then assessed by fluorescence intensity quantification studies (flow cytometry).

Evaluation of rRNA level

To confirm if the percentage of rRNA is similar in both of *Helicobacter* species, an universal peptide nucleic acid (PNA) EUB338 probe (5′Alexafluor 488-TGCCTCCCGT AGGA-3′) which recognized a conserved region of the 16S rRNA in the domain bacteria (Amann et al. 1990) was used as a model probe . FISH experiments were performed as de- scribed above for PNA probes at 57 °C. Each experiment was performed in triplicate.

Flow cytometry and data analysis

Flow cytometry analysis was performed using an EPICS XL flow cytometer containing a low-power air-cooled 15 mW blue (488 nm) argon laser. Data analysis was performed with the EXPO32ADC software (Beckman Coulter, Brea, USA). For each sample, 20,000 events were collected. All experiments were repeated in two runs (using three biological replicates of cultures

for each run), and negative controls without probe were included for each type of protocol in every analysis. Flow cytometric analyses of samples were per- formed based on both scattering signals (forward scatter and side scatter) and FL-1. FAM fluorescence was detected on the FL-1 channel (BP530/30). For all detected parameters, amplification was carried out using logarithmical scales.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) by applying the Tukey multiple comparison test, using SPSS® statistics 17.0 (Statistical Package for the Social Sciences (SPSS), Chicago, USA) or the Microsoft Office Excel® (Microsoft Corporation, Redmond, USA). Results were expressed as mean values. Differences were considered significant when $p<0.05$.

Results

Melting temperature analysis

The Tm values for the probes bound to fully complementary and single-mismatched targets are shown in Table 3 for the DNA target and in Table 4 for the RNA target. Because FISH hybridizations are performed in buffers of various compositions, we used different types of buffers with different ionic environments (different salt concentrations) and denaturing agents. In general, we observed an increase of Tm at higher salt-concentrated solutions and destabilization in the duplex strands when formamide was used. For oligonucleotides of similar lengths, LNA/2′OMe displayed the highest thermal stability in all buffers studied. LNA/DNA oligonucleotides also allowed the formation of relatively stable duplexes. The PNA oligonucleotide also showed a high stability in all buffers. The use of UNA monomers affected negatively the stability of the duplexes and as such the lowest melting temperatures in all buffers were obtained for these oligonucleotides (Table 4).

The specificity of the LNA and PNA oligonucleotides was confirmed by testing their ability to discriminate against mismatches in single-mismatched DNA and RNA strands (Tables 3 and 4, respectively). Modified oligonucleotides exhibited better mismatch discrimination than the DNA oligomers, as confirmed by the higher ΔTm values. HyP_UNA/LNA oligonucleotides showed a high capacity of mismatch discrimination in medium salt buffer (110 mM), and in some cases, no hybridization in the buffers with FA and urea was found with the single-mismatched strand. LNA/2′OMe oligo- nucleotides had the highest discrimination capacity in the high salt buffer with 4 M of urea, even though HyP_LNA/DNA and PNA oligonucleotides also presented a strong specificity for this sequence.

Mismatch discrimination analysis in bacteria by flow cytometry studies

To analyze the efficiency of the probes in bacterial cells, we tested each probe in a range of temperatures between 25 and 70 °C measuring the fluorescence signal by flow cytometry. All FISH conditions used were the standard for each type of oligonucleotide; therefore, we used different buffers depending on whether we were using DNA, PNA, or LNA probes.

The hybridization experiments were first performed with *H. pylori* as a target, in order to understand at which temperatures we could obtain a more efficient detection. The optimal hybridization temperature for each probe was considered as the temperature at which the hybridization with the microorganism of interest provided the strongest fluorescent signal and more specificity (weakest signal for the microorganism with the mismatch).

The subsequent experiments analyzed the capacity of mismatch discrimination. As a result, we performed FISH experiments with each probe in *H. acinonychis*, which had a single mismatch in the rRNA comparatively to *H. pylori*. As an example, Fig. 1 shows the results obtained for HyP_LNA/2_OMe1 for both microorganisms at different temperatures. From Fig. 1, we conclude that the hybridization temperature where the fluorescence intensity is higher for *H. pylori* is 45 °C, whereas this hybridization temperature for *H. acinonychis* is 30 °C. This appears to indicate a ΔTm for this probe of 15 °C, which compares with the ΔTm of 18.1 °C obtained in the thermal dissociation studies for the buffer used for LNA probes (900 mM Na+ buffer with 4 M urea). While these values are not very different, further studies would have to be performed to confirm that thermal dissociation studies provide an acceptable prediction of differences in hybridization temperatures between fully complementary and single- mismatched targets in FISH experiments.

The results of the FISH experiments for the optimal hybridization temperatures of *H. pylori* using the different probes can be analyzed in Fig. 2. The fluorescence intensity of the HyP_LNA/2′OMe1 and HyP_LNA/2′OMe2 probes is significantly higher in *H. pylori* than in *H. acinonychis* (p=0.028 and p=0.00, respectively). The difference between the detection in *H. pylori* than *H. acinonychis* is also statistically significant in HyP_PNA (p=0.001). On the other hand, no statistically significant differences were observed in terms of fluorescence intensity between the bacteria in study with HYP_DNA probe, HyP_LNA/UNA probes, and HyP_LNA/ DNA probes (p>0.05).

In the specific conditions used for each experiment, LNA/ 2′OMe oligonucleotides showed the highest fluorescence signal of the oligonucleotides analyzed. The difference in fluorescence intensity is statistically significant (p<0.05) between these probes and HyP_LNA/UNA and HyP_LNA/DNA2 probes. However, the PNA oligonucleotide represents a good alternative for these types of studies, since it showed a higher sensitivity and specificity at lower hybridization temperatures. LNA/DNA oligonucleotides showed distinct results, which were dependent on the length of the oligonucleotide. The shorter probe had similar results to the PNA oligonucleotide in terms of fluorescent intensity; however, the 11 mer oligo- nucleotides showed a very low capacity of hybridization with the target. Although the data from the melting behavior of this probe was very similar to those of the 9 mer LNA/DNA probe, the hybridization in the bacteria revealed very different results. The same behavior was observed for the HyP_DNA probe.

For the evaluation of the rRNA level of each species of *Helicobacter*, we used EUB338 probe which targets both species. It was observed similar gates (percentage of cells identified by EUB338 probe) and comparable AUF for both bacteria (47.9±9.8 and 45.8±5.3 for *H. pylori* and *H. acinonychis*, respectively). Therefore, there are not significant differences in rRNA levels between these bacteria.

Discussion

The detection of microorganisms by FISH has significantly changed with the introduction of several types of synthetic oligonucleotides that possess a higher capacity of discrimination and affinity towards complementary sequences (Buchardt et al. 1993; Obika et al. 1997; Koshkin et al. 1998; Majlessi et al. 1998; Guimaraes et al. 2007; Guga and Koziolkiewicz 2011). Therefore, it is very important to discuss which type of nucleic acid mimics is more suitable for each specific situation, such as the recognition of mismatches. To tackle this issue, in this study, we analyzed the performance of different types of probes to discriminate mismatches, not only by thermal melting analysis of naked nucleic acids but also for specifically targeting bacterial cells with a FISH methodology.

To analyze the relationship between different types of probes and specificity, we performed melting studies for all probes with DNA and RNA targets. The use of different tar- gets is important because even though the FISH methodology is more frequently described for RNA targets, there are also several studies using modified probes which use DNA as a target, e.g., (Celeda et al. 1994; Matthiesen and Hansen 2012). In accordance with the literature, a higher Tm was observed at higher salt-concentrated solutions (Owczarzy et al. 2004). Higher concentrations of NaCl allow the repulsion between the negatively charged oligonucleotides to be increasingly compensated by the positive counterions. However, this effect is smaller in one-mismatch duplexes because the two strands are further apart (Mishra et al. 2013). On the other hand, the use of formamide and urea was expected to allow for the destabilization in the duplex strands leading to lower melting temperatures (Yilmaz and Noguera 2007). However, this de- stabilization did not decrease the Tm to the same extent in the high salt buffer (Table 4). As expected, we observed a large difference in Tm for all probes bound to fully complementary oligonucleotides vs. oligonucleotides containing one mismatch. The difference between the Tm values for each oligo- nucleotide was in agreement with the results published earlier (Filichev et al. 2004; Kaur et al. 2008; Pasternak and Wengel 2010). LNA/2′OMe and PNA showed high stability in all buffers, comparatively to LNA/DNA oligonucleotides. It has already been demonstrated that LNA and 2′OMe have different impacts on duplex stability, in spite of sharing a *C3′-endo* sugar pucker conformation (Yan et al. 2012). In general, we observed an increase of thermal duplex stability in the following order: HyP_LNA/UNA probes<DNA probe<HyP_LNA/ DNA probes < HyP_PNA = HyP_LNA/2′OMe probes (Table 4).

Usually, Tm is used as an indication for the hybridization temperature because it is at this temperature half of the nucleic acid strands are forming a duplex and the other half are single stranded (SantaLucia et al. 1996). However, the hybridization temperature determined by experimental procedures in vitro is dependent of all biological parameters and chemical com- pounds involved in the hybridization step of a FISH procedure. These factors can be a cause for the absence of a pattern between these temperatures (Fontenete et al. in press). In fact, when we compare the discrimination temperature difference obtained by thermal studies with FISH under the same buffer conditions (Table 4), we can observe that statistically significant differences may arise ($p< 0.05$). Actually, it is highly unpredictable how each probe will hybridize in experiments with bacteria, because there are several factors that can affect the efficiency of the hybridization (Cerqueira et al. 2008). For instance, the diffusion through the extracellular membrane can be different for each probe (Politz et al. 1998). Therefore, we used the same standard parameters for all studied probes, and the experiments were performed under presumed optimal conditions. We have

also assessed for the first time the use of HyP_LNA/UNA probes in FISH experiments. However, our results show that the use of UNA leads to a large destabilization in the duplex which affects the hybridization in bacteria (Table 4 and Fig. 2). Therefore, the use of UNA modifications in FISH probes may require the use of longer probes.

In general, nucleic acid mimics can indeed provide better mismatch discrimination than standard DNA probes in FISH, as it has been suggested previously (Stender et al. 2002; Guimaraes et al. 2007; Mishra et al. 2012, 2013). PNA with a low number of bases can broadly provide a good discrimination. Some studies have shown that LNA can increase single-base mismatch discrimination in bulk solutions (You et al. 2006). However, the design flexibility in LNA and 2′ OMe oligonucleotides appears to indicate that the better performance should typically be obtained with these probes.

In this study, we showed that the analysis of probes using native targets allows a more realistic approach as it accounts for all variables that can interfere during the hybridization process. Methods that use only naked DNA for studying or comparing probes do not analyze the factors inside the cells that can interfere with the hybridization, e.g., molecular crowding and the presence of several solutes. As such, when several probes are compared, it is fundamental to analyze this behavior in terms of permeabilization or access to the target. For example, a molecule can have adequate thermodynamic parameters, but its chemistry may not be compatible with its use in a biological target. Therefore, approaches that combine naked and native targets contribute for a more complete and realistic study.

As a final conclusion, both LNA/2′OMe and PNA probes are able to discriminate mismatches with a high signal-to- noise ratio, but LNA/2′OMe probes allow for a greater flexibility in probe design. Nonetheless, as probe design and optimum hybridization conditions vary for each type of probe, it is impossible to have an absolute certainty on which type of nucleic acid is better for mismatch discrimination. As such, for each specific sequence and mismatch, testing many possible probe designs and hybridization conditions with probes consisting of PNA and LNA/2′OMe is still advisable. Future work will focus on reassessing the impact of mismatches in a larger number of microorganisms which contain different types of cell wall, like Gram-positive bacteria and yeast cells.

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Conflict of interest

JW is a cofounder of RiboTask ApS which offers LNA/2′-OMe-RNA probes for RNA targeting. NFA is a cofounder of Biomode SA which develops molecular methods for the rapid detection of microorganisms.

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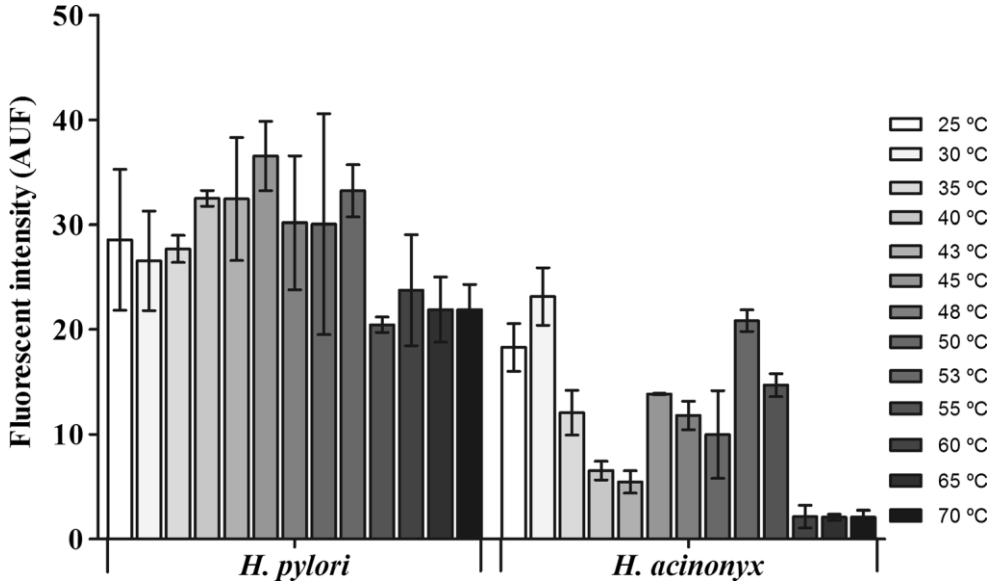

Fig. 1 The effect of the temperature on the specificity of HyP_LNA/2_OMe1 probe. Histogram showing the distribution of fluorescence intensity with the temperature in *H. pylori* and *H. acinonychis*

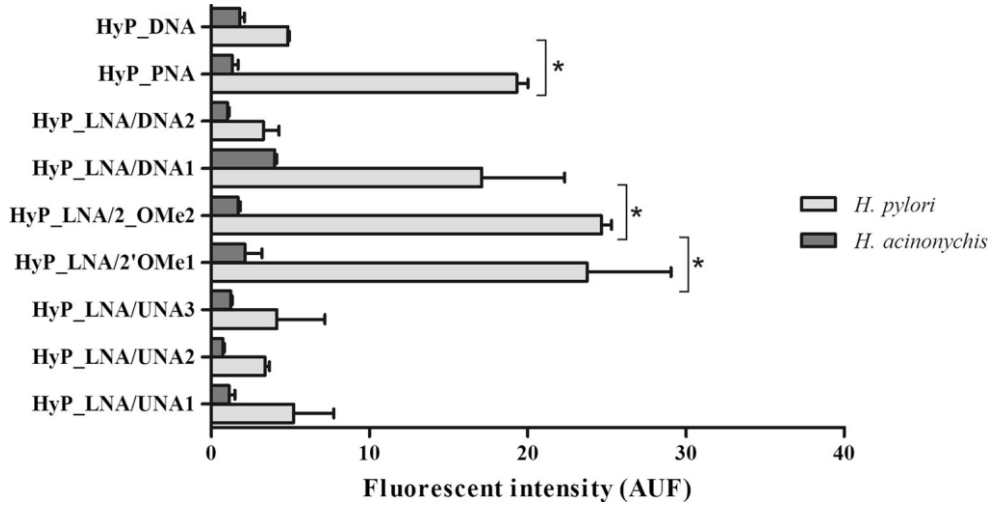

Fig. 2 FISH detection of *H. pylori* and *H. acinonychis* by cytometry at the optimal hybridization temperature. Quantification of the mean fluorescence intensity of each probe in two independent experiments. The optimal hybridization temperature for each probe is described in Table 4. The fluorescent signal intensity is expressed in arbitrary fluorescent units (AUF). The symbol *asterisk* (*) indicates the difference between *H. pylori* and *H. acinonychis* which is statistically significant ($p<0.05$)

Table 1 Sequence of the different oligonucleotide probes used in the present study. LNA nucleotide monomers are represented with L superscript, 2′-OMe-RNA monomers are represented with m superscript, DNA monomers are represented with italic *d*, and UNA monomers are represented with u and in boldface letters

Name	Sequence (5′FAM-3′)
HyP_DNA	*d*(TTACTGGAGAGACTAAG)
HyP_PNA	CTGGAGAGACT
HyP_LNA/UNA1	$T^{L}G^{L}$**uG**$A^{L}G^{L}$**A**u**G**$A^{L}C^{L}$
HyP_LNA/UNA2	$C^{L}T^{L}$**uG**$G^{L}A^{L}G^{L}A^{L}$**G**u**A**$C^{L}T^{L}$
HyP_LNA/UNA3	$C^{L}T^{L}$**G**u**G**$A^{L}G^{L}$**A**u**G**$A^{L}C^{L}T^{L}$
HyP_LNA/2′OMe1	$U^{m}G^{L}G^{m}A^{m}G^{L}A^{m}G^{L}A^{m}C^{m}$
HyP_LNA/2′OMe2	$C^{m}U^{L}G^{m}G^{m}A^{L}G^{m}A^{m}G^{L}A^{m}C^{m}U^{m}$
HyP_LNA/DNA1	T^{L}*d*GG^{L}*dA*G^{L}*d*AG^{L}*d*AC^{L}
HyP_LNA/DNA2	C^{L}*d*T*d*G*d*G$A^{L}G^{L}A^{L}$*d*G*d*A*d*CT^{L}

Table 2 Differences between hybridization buffers used for each type of probe

Probe	Denaturant	[NA^{+}] (mM)	Tris-HCl (mM)	Other reagents
HyP_DNA	30 % (*v*/*v*) FA 0.001 % (*v*/*v*) SDS	900	20	–
HyP_PNA	30 % (*v*/*v*) FA	10	50	0.1 %(*v*/*v*) Triton-X 10 % (*v*/*v*) dextran sulphate 5 mM of EDTA
HyP_LNA	4 M urea	900	50	

FA formamide

Table 3 Melting temperatures, Tm (°C), and mismatch (C> T/U) discrimination temperature difference. ΔTm in different buffers for DNA complementary target in 110 mM Na^+ buffer[a], 10 mM Na^+ buffer with 30 % (*v*/*v*) formamide, and 900 mM Na^+ buffer with 4 M urea

	T_m at total [Na^+] indicated					
	110 mM		10 mM		900 mM	
Name	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
Reference	52.3	6.9	37.4	1.3	56.6	8.2
HyP_DNA	48.9	8.4	33.5	6.9	51.5	8.9
HyP_PNA	64.6	15.3	42.7	16.5	60.9	17.2
HyP_LNA/UNA1	17.4	nb	15.3	nb	42.1	nb
HyP_LNA/UNA2	38.5	17.9	21.8	9.7	41.6	21.3
HyP_LNA/UNA3	35.5	14.2	20.5	NT	34.2	14.9
HyP_LNA/2′OMe1	59.1	12.1	47.6	17.3	70.8	10.2
HyP_LNA/2′OMe2	63.51	12.2	51.7	15.5	78.3	13.1
HyP_LNA/DNA1	57.5	20.3	44.6	17.3	60.7	13.2
HyP_LNA/DNA2	53.2	15.0	44.2	21.2	62.0	14.1

nb no binding could be detected

Table 4 Melting temperatures, Tm (°C), mismatch (C > T/U) discrimination temperature difference (ΔTm), and hybridization temperatures TH (°C) at which each probe had highest sensitivity and specificity. ΔTm in different buffers for RNA complementary oligonucleotide: 110 mM Na^+ buffer[a], 10 mM Na^+ buffer with 30 % (*v*/*v*) formamide and 900 mM Na^+ buffer with 4 M urea

	T_m at total [Na^+] indicated						Hybridization temperature
	110 mM		10 mM		900 mM		900 mM
Name	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m	T_H
Reference	49.3	3.3	35.2	5.1	52.6	9.1	–
HyP_DNA	44.5	10.0	30.2	9.35	46.6	9.9	70
HyP_PNA	67.6	13.2	44.6	15.6	62.4	11.4	45
HyP_LNA/UNA1	32.4	nb	32.1	nb	35.9	16.8	53
HyP_LNA/UNA2	53.7	13.8	38.1	15.3	59.6	13.3	53
HyP_LNA/UNA3	50.7	12.9	36.1	14.9	54.9	13.4	48
HyP_LNA/2′OMe1	69.4	13.3	57.8	15.9	78.9	18.1	60
HyP_LNA/2′OMe2	75.4	12.8	62.3	13.2	78.2	17.6	65
HyP_LNA/DNA1	66.4	13.5	54.2	14.1	71.4	11.5	43
HyP_LNA/DNA2	62.1	12.8	47.9	14.4	67.4	13.9	40

nb no binding could be detected