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Locked Nucleic Acid Based Beacons for Surface Interaction Studies and Biosensor Development

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DNA sensors and microarrays permit fast, simple, and real-time detection of nucleic acids through the design and use of increasingly sensitive, selective, and robust molecular probes. Specifically, molecular beacons (MBs) have been employed for this purpose; however, their potential in the development of solid-surface-based biosensors has not been fully realized. This is mainly a consequence of the beacon's poor stability because of the hairpin structure once immobilized onto a solid surface, commonly resulting in a low signal enhancement. Here, we report the design of a new MB that overcomes some of the limitations of MBs for surface immobilization. Essentially, this new design adds locked nucleic acid bases (LNAs) to the beacon structure, resulting in a LNA molecular beacon (LMB) with robust stability after surface immobilization. To test the efficacy of LMBs against that of regular molecular beacons (RMBs), the properties of selectivity, sensitivity, thermal stability, hybridization kinetics, and robustness for the detection of target sequences were compared and evaluated. A 25-fold enhancement was achieved for the LMB on surface with detection limits reaching the low nanomolar range. In addition, the LMB-based biosensor was shown to possess better stability, reproducibility, selectivity, and robustness when compared to the RMB. Therefore, as an alternative to conventional DNA and as a prospective tool for use in both DNA microarrays and biosensors, these results demonstrate the potential of the locked nucleic acid bases for nucleic acid design for surface immobilization.

Over the past decade, DNA/RNA-based analytical methods have been extremely important in molecular biology, disease diagnosis, and gene expression studies. Biosensors, such as DNA microarrays and/or DNA chips, have gained popularity because they allow the measurement and detection of a high number of samples using a fast and simple setup. Moreover, biosensors allow for real-time detection of nucleic acid molecules and gene expression changes.[1−6] However, these results can only be achieved by efficient, reproducible and stable immobilization of DNA probes onto specific surfaces.[1−7] Most DNA biosensors are based on the detection of DNA−DNA and DNA−RNA hybrids with the DNA probe immobilized on a transducer surface. Of the many varieties of DNA sensors, the most common are (*i*) electrochemical-based sensors which involve the immobilization of the DNA probe onto an electrode surface such that target molecules are detected by changes in electron transfer[8,9] and (*ii*) fluorescence-based sensors where DNA probe immobilization is usually performed on a glass surface such that target molecules are detected upon changes in the fluorescence intensity when hybrids are formed.[3,10,11] Out of the many fluorescent-based DNA probes, molecular beacons (MBs)[12] are the most effective probes for DNA biosensors as they have high sensitivity and can be used for detection without separation. In addition, because of their hairpin structure, MBs have better selectivity than linear DNA probes in sensing target nucleic acids. MBs are single-stranded oligonucleotide probes that contain a loop-and-stem structure.[12] The loop portion of the beacon contains the sequence complementary to a target (usually 15−30 bases), whereas the two stems are complementary to each other (5−7 bases). A quencher is covalently attached at the end of one stem, while a fluorophore is covalently attached to the end of the other. Because the fluorophore and quencher are in close proximity, MBs do not fluoresce until hybridization with the target nucleic acid sequence. In the presence of a target, the hairpin structure of MBs undergoes conformational changes that force the fluorophore and quencher moieties to separate, thus restoring fluorescence intensity. MB probes have been studied as DNA probes for DNA biosensors with the aim of overcoming some drawbacks inherent in single-

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stranded DNA probes[3,13−15] and DNA array applications.[3,4,6,7,16−19] Specifically, because of their inherent signal transduction mechanism, MBs provide superior properties over single-stranded linear DNA probes as probes for surface biosensors.

In solution, MBs possess many attractive properties, such as high sensitivity, selectivity, and DNA detection that can be performed in real-time, as the unbound probes do not need to be separated out from the bound probes. However, the use of MBs for surface hybridization has been limited, mainly because fluorescence enhancement of the immobilized MBs decreases significantly when compared to that of MBs in solution. Thus, whereas MBs can typically achieve fluorescence enhancements above 25-fold in solution,[3,14,15,17,18] these values can in some cases drop to 2- to 5-fold once immobilized.[12,20] This behavior is mainly a result of interactions between the MB and the surface which partially disrupt the loop and, consequently, destabilize stem structure. This can cause inefficient quenching,[18] which is reflected in high fluorescence background signal, thus affecting the overall sensitivity of the MBs.[14,15] Some published studies have reported ways of improving MB performance on the surface. For example, to have a more liquid−liquid environment, instead of liquid−solid interface, polyacrylamide gel and agarose gel have been used for MB immobilization.[2,17] In other reports, physicochemical parameters, such as pH and ionic strength, as well as the distance of the MB to the surface, have been investigated, although with limited improvement.[18] Despite these advances, further development of new DNA probes with a higher stability on the surface, which, at the same time, allow a simple design, low background signal, and high hybridization efficiency, is still needed to recreate the performance and sensitivity of MBs in solution. In addition, improving the surface performance of MBs will be easily transferable to biotechnologies based on immobilization of nucleic acid probes on surfaces such as DNA arrays and protein arrays based on aptamers.

In this study, we have optimized the design of molecular beacons by incorporating locked nucleic acids (LNAs) to create a highly stable MB and therefore improve the performance of these probes on a solid surface. LNA is a nucleic acid containing a bicyclic furanose unit locked in an RNA mimicking sugar conformation.[21] The methylene bridge that connects the 2′-oxygen and the 4′-carbon of the ribose ring confers higher structure rigidity to the LNA base pair, thus preventing potential interactions with the surface and other molecules that might be present in the solution. Furthermore, LNAs contain a structure resembling RNA, which allows a superior affinity and specificity toward the

Table 1. Molecular Beacons and Target Sequences

Regular Molecular Beacon (RMB)	
No PEG	5’-Biotin Cy3 CCT AGC TCT AAA TCA CTA TGG TCG CGC TAG G-BHQ2-3’
6 PEG units	5’-Biotin PEG_6 Cy3 CCT AGC TCT AAA TCA CTA TGG TCG CGC TAG G-BHQ2-3’
12 PEG units	5’-Biotin PEG_{12} Cy3 CCT AGC TCT AAA TCA CTA TGG TCG CGC TAG G-BHQ2-3’
Locked Molecular Beacon (LMB)	
No PEG[a]	5’-Biotin Cy3 CCT AGC TCT AAA TCA CTA TGG TCG CGC TAG G-BHQ2-3’
6 PEG units	5’-Biotin PEG_6 Cy3 CCT AGC TCT AAA TCA CTA TGG TCG CGCTAG G -BHQ2-3’
12 PEG units	5’-Biotin PEG_{12} Cy3 CCT AGC TCT AAA TCA CTA TGG TCG CGCTAG G -BHQ2-3’
Target	5’-GCG ACC ATA GTG ATT TAG AGC TAG G-3’
Mismatch	5’-GCG ACC ATA GTG AAT TAG AGC TAG G-3’

[a] Red letters represent LNA bases. [b] Blue letter represents the mismatch base.

target strand when compared to that of DNA.[22] Other attractive properties of LNA bases are high resistance against degradation and thermostability, which is reflected in low background signal, and efficient target hybridization.[23,24] These properties typically allow LNA bases to increase the sensitivity and selectivity of any molecular probes, thus allowing the development of LNA-based microarrays for miRNA profiling.[25−28] In spite of these advantages, the hybridization kinetics of the fully modified LNA MB is slower when compared to that of the DNA MBs. This apparent flaw, which can be attributed to the slow stem dehybridization rate,[24] is discussed in detail below; however, it can be noted here that the hybridization kinetics of LNA MBs can be improved substantially by decreasing the percent of LNA bases in the beacon’s design.[24] Therefore, considering the advantages that molecular beacons can offer over conventional DNA probes, we have studied the performance of beacons incorporating LNA bases in the structure after immobilization onto a glass surface in terms of stability, background signal, thermodynamics, kinetics, selectivity, and sensitivity. Specifically, we have synthesized a MB with a combination of DNA and LNA bases, and it has been compared with MB containing only regular DNA bases. This newly designed LMB can potentially be used for a more sensitive DNA array detection and other biotechnological applications.

MATERIALS AND METHODS

Probe Preparation. The MBs, cDNA, and mismatch targets were synthesized and are listed in Table 1. All the DNA and LNA reagents for the synthesis of the beacons and targets were purchased from Glen Research. The probes and DNA complementary targets were synthesized with an ABI3400 DNA/RNA

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synthesizer (Applied Biosystems, CA). Blackhole quencher 2 (BHQ2) core pore glass (CPG) was used for all MB synthesis; Cy3 phosphoramidite was used to label the probes with Cy3, and Spacer phosphoramidite 18, which incorporates PEG as linker into the beacons. A biotin phosphoramidite was introduced for labeling at the 5′ terminus of the MBs. MBs that contained LNA bases were synthesized using LNA phosphoramidites. LMBs were prepared by alternating LNA and DNA bases in both, the stem and the loop (every other base). This design was chosen to take advantage of LNA bases without compromising the hybridization rate. In addition, targets were prepared in such a way that the complementary sequence was complementary not only to the target but also to the stem as well, to ensure that the beacons open in the presence of the targets. Deprotection of the probes was performed using overnight incubation with ammonium hydroxide at room temperature. The solution resulting from deprotection was precipitated in cold ethanol. Subsequently, the precipitates were dissolved in 0.5 mL of 0.1 M triethylammonium acetate (TEAA, pH 7.0) for further purification with reverse phase high-pressure liquid chromatography (RP-HPLC). RP-HPLC was performed on a ProStar HPLC Station (Varian, CA) equipped with a fluorescent and a photodiode array detector using a C-18 reverse phase column (Econosil, C18, 5 μM, 250 × 4.6 mm) from Alltech (Deerfield, IL). The product collected from the HPLC was vacuum-dried and then added with TEAA for a second round of HPLC. The final product was collected, vacuum-dried, and dissolved in 200 μL of acetic acid (80%) for 20 min, followed by 200 μL of cold ethanol and then vacuum-dried. Quantification of the probes and targets was performed using a UV−vis Spectrometer (Cary Bio-300, Varian, CA).

Immobilization of the MBs on the Glass Surface. The microscope slides and coverslips used in the experiments were obtained from Fisher (optical borosilicate glass with a size of 18 × 18 mm and 0.13 to 0.17 mm thickness). The surfaces were cleaned with a 3:1 ratio of conc. H_2SO_4 to H_2O_2 (30%) to remove organic impurities. Subsequently, the treated microscope slides were washed thoroughly with deionized water and dried with compressed nitrogen. Strips of double-sided tape (3M) were placed 3 mm apart on a microscope slide, and a cover glass was placed on top. Channels were filled by capillary action. Solution exchange was performed by simultaneously pipetting solution at one end and withdrawing fluid from the other end with P8 filter paper (Fisher).[29] All the steps were performed at room temperature, where both RMB and LMB form a stable hairpin structure in the absence of target (in solution, the melting temperatures of the beacons are ~56 °C for RMB and >85 °C for LMB[24]). The channels were washed twice with 10 mM phosphate buffer (PBS) at pH 7.4 before use. Next, 5 μL of 1 mg·mL^{-1} of avidin was incubated in the channels for 5 min. The excess of avidin was removed with PBS washes (20 μL, three times). The biotinylated MB (2.5 μM in PBS buffer) was incubated with avidin-treated channels for 10 min, and the excess of MB was washed away with washing steps of PBS buffer. A solution of the cDNA (cDNA, 10 μM prepared in 20 mM Tris, 50 mM of NaCl, and 5 mM of $MgCl_2$, pH 7.5) was incubated with the immobilized MB and the fluorescence was monitored at different times depending on the experiment. An end-point fluorescence was determined after 10 min of incubation. Several images were collected at every step to obtain an average fluorescence, and the procedure was repeated and measured several times. The sensitivity of the MB was tested by measuring the fluorescence enhancement at different target concentrations (from 1 nM to 100 μM).

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Stability of the MBs at Different Temperatures. The stability of the MBs at different temperatures was studied by incubating the glass slide on a surface block dry bath (Barnstead Thermolyne type 17600 Dri-Bath) for the temperatures ranging from 20 to 50 °C. The samples tested at 4 °C were kept in the refrigerator prior to use to achieve the desired temperature. To prevent any light damage to the probes, aluminum foil was used to cover them. All the steps were performed under the conditions previously described. Images of the glass slide were taken at every step as detailed earlier.

Stability of the MBs in Complex Matrixes. The stability of the system in complex matrixes was studied by performing the experiments in cell lysate and serum media. Cell lysate solution was prepared following commercial specifications from Cell Signaling Technology, Inc. (Danvers, MA). Cell lysate was kept at −20 °C until analysis. Fetal Bovine Serum (FBS, heat-inactivated, GIBCO) was obtained from Invitrogen (Carlsbad, CA). For this set of experiments, buffer, cell lysate, and FBS were incubated for 10 min with the immobilized MBs. In addition, target solutions were prepared in buffer, cell lysate or FBS for a final concentration of 10 μM and subsequently incubated with the immobilized beacon. Then, fluorescence measurements were performed following the same immobilization conditions previously described.

Fluorescence Imaging. Fluorescence imaging was performed with a confocal microscope setup consisting of an Olympus IX-81 inverted microscope with an Olympus Fluoview 500 confocal scanning system and a green HeNe laser (543 nm), with a photomultiplier tube (PMT) for detection. The images were taken with a 10×/0.30 NA objective. Cy3-labeled MBs were excited at 543 nm and collected at 560 nm with a long pass filter. The data collected from the confocal microscope were analyzed using the Fluoview analysis software.

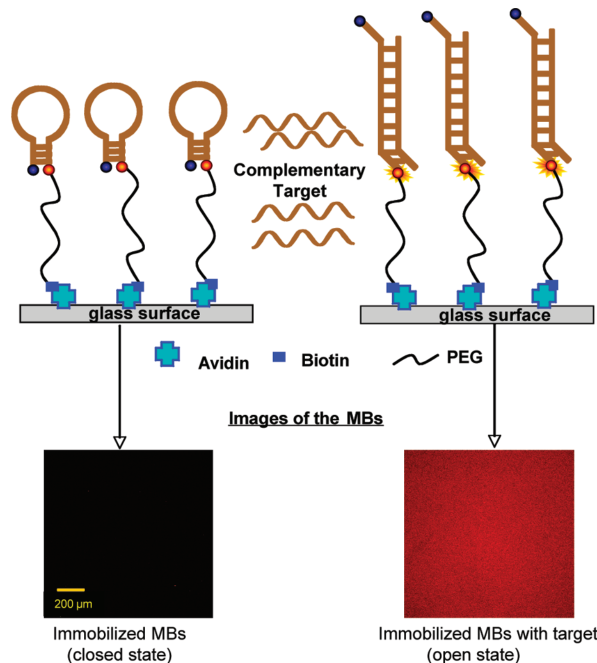
To make sure the images were obtained from the brightest point of the channel, the microscope was focused onto the surface of the microslide, followed by a zeta section scan. The zeta scan allowed us to capture the brightest image at a specific zeta position. Subsequently, all the images from avidin, immobilized MB, and target addition were taken at the same position for that particular channel. This position may vary for each channel; therefore, the zeta position was calculated with each immobilization.

The data collected from the images were analyzed, and the fluorescence enhancement was calculated using the following equation:

$$\text{Fluorescence Enhancement} = (S_{\text{MB open}} - B_{\text{avidin}})/(S_{\text{MB close}} - B_{\text{avidin}})$$

where $S_{\text{MB open}}$ is the signal of the beacon in the open state form or hybridized with the target, $S_{\text{MB close}}$ is the signal of the MB in the closed state form or unhybridized, and B_{avidin} is the background fluorescence intensity corresponding to the immobilized avidin in buffer solution. An average of the fluores-

Scheme 1. Schematic Representation of the Molecular Beacon onto a Glass Surface[a]



[a] Images in the closed and open state posses a value of 500 and 2500 counts, respectively, at the fixed conditions used to measure the fluorescence.

cence was determined by taking several images from the same channel.

Kinetics experiments were performed using confocal microscopy by taking images of the hybridization with the target every 3 s for 10 min. Each image was analyzed by taking the average fluorescence intensity at the determined zeta position.

RESULTS AND DISCUSSION

Molecular Beacon Design and Surface Immobilization. In previous reports, fully modified LNA beacons have shown higher sensitivity but relatively slower hybridization rates than their counterpart DNA.[23,24] However, by decreasing the percentage of LNA bases in the stem and the loop, LMBs can retain the affinity and the stability of the LNA bases without compromising the kinetics and hybridization rate of the assay. Thus, LMBs with alternating LNA and DNA bases in both the stem and the loop (every other base) were utilized for this study. In addition, it is well-known that LNA bases have a strong affinity with complementary LNA bases.[22] Therefore, targets were prepared such that the cDNA sequence was not only complementary to the loop of the MBs, but to the stem as well (also known as shared stem target).

In this study, MBs were immobilized on a glass surface for surface hybridization kinetics studies and for biosensor development. To achieve this, the avidin−biotin interaction was selected. The biotin molecule was attached at the 5′ end of the MB followed by the poly(ethylene glycol) (PEG) linker (see Scheme 1). PEG was chosen as a linker to increase the distance of the MBs to the glass surface. In addition, PEG possesses great solubility and hydrophilicity and can be easily incorporated into DNA sequences using standard phosphoramidite chemistry. Furthermore, PEG has no charge, which results in limited electrostatic interactions and minimizes the precipitation or aggregation of the probes at the glass surface.[30] Finally, since PEG adds flexibility to an otherwise rigid oligonucleotide structure, PEG linker facilitates a more efficient immobilization onto the solid surface and further hybridization with target molecules.

Table 2. Effect of Different PEG Units on the Background Intensity and the Overall Fluorescence Change after Target Addition

probes	background intensity (a.u.)	fluorescence enhancement
	RMB	
0 PEG	510 ± 14	2.8 ± 0.4
6 PEG	608 ± 113	7.9 ± 0.6
12 PEG	2540 ± 220	1.2 ± 0.1
	LMB	
0 PEG	200 ± 14	2.8 ± 0.6
6 PEG	258 ± 6	25 ± 5
12 PEG	239 ± 28	6.1 ± 2

The length of the PEG is a key factor in our MB design, and care was taken to ensure that the linker was neither too short nor too long, either of which can affect hybridization efficiency. Therefore, sets of LMBs and RMBs with different distances between the biotin group and the sequence were initially designed by incorporating 0, 6, and 12 units of PEG. The sequences of the MBs are summarized in Table 1.

One of the major reasons why LMBs can be used for effective surface studies and biosensor development is their low background signal which is a result of the rigid structure of LNA bases in the MB. We evaluated the initial background fluorescence of the beacons in the absence of cDNA, as well as the fluorescence change after target addition. The results are summarized in Table 2. For the RMB, the background fluorescence slightly increased when 6 units of PEG were incorporated in the sequence. However, for the RMB with 12 PEG, the resulting background was ∼5-fold higher than MBs containing zero and 6 PEG linker units. On the other hand, for the LMB, the background intensity remained the same and stable, regardless of the length of the linker. Most importantly, the background intensity of LMB was found to be lower than that of RMB (around 2.5 times). Moreover, the signal was comparable to the one obtained from the system background (avidin-coated surface, ∼160 ± 10). These data support previous reports, which concluded that destabilization of regular DNA-based MB occurs after immobilization, resulting in incomplete quenching which is reflected in high background signal.[3,18] This is one of the major limitations of using MBs for surface immobilization. Nevertheless, the addition of LNA bases into the beacon for hybridization studies brings stability and minimizes surface-glass interactions. Also, LNA bases in the stem keep the fluorophore/quencher moieties in close contact, which is reflected in the low background signal.

On the other hand, higher fluorescence change of the RMB after target addition was obtained when a PEG linker was incorporated between the biotin and the sequence, reaching a maximum signal after the addition of 6 PEG units (∼ 8-fold enhancement signal). This result proves that PEG increases the distance to the surface and facilitates the accessibility of the molecular beacon for an efficient hybridization with the target. Similarly, LMB with 6 PEG units produced the highest fluores-

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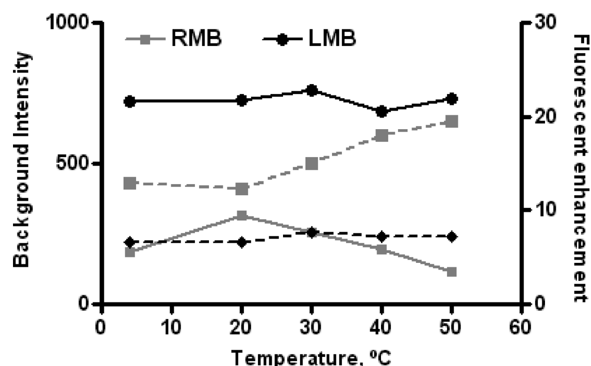

Figure 1. Temperature effect on the stability of the MBs immobilized onto the surface. Black straight line: fluorescence enhancement in LMB; black dashed line: background intensity of LMB; gray straight line: fluorescence enhancement in RMB; gray dashed line: background intensity of RMB.

cence enhancement upon target addition (up to 25-fold signal enhancement) compared to 2.8 and 6.1 for 0 and 12 PEG units, respectively. Thus, considering that similar background intensities were observed for LMB with different PEG units, we can conclude that signal enhancement, in this case, results from a more efficient hybridization with the target when the 6-PEG linker is used. This may, therefore, also represent the optimum probe-to-surface distance. The addition of more PEG units (12 units) results in a longer and perhaps a more flexible linker. However, the extended length and higher bulkiness of the linker could, at the same time, hinder the accessibility of the target hampering an efficient hybridization, resulting in overall lower signal enhancement. Considering these results, the probes with 6 PEG units were selected for further experiments.

Thermal Stability of the Beacons. To prove the improved stability and robustness of the sensor using LNA bases, the influence of incubation temperature was investigated. Figure 1 shows the background intensities of the beacons before target addition and the signal enhancements of the MBs after addition of the target sequence at 4, 20, 30, 40, and 50 °C. As can be observed in the graph, the highest signal enhancement for RMBs was recorded at 20 °C, with a 9.4- fold enhancement. Subsequent temperature increases caused a decrease in the signal enhancement of the RMBs to 3.4-fold, as a result of a significant and gradual increase in the overall background signal at higher temperatures. These results suggest thermal destabilization of the beacon structure, thus compromising the stability of the probe-target complex. In addition, this increase in temperature is likely to unzip the bases of the stem and thereby decrease the rigidity of the RMB, forcing the beacon to melt and form into fluorescent random coils.[20] The random coil forces the fluorophore and quencher to separate, which causes an increase of background intensity. In contrast, LMBs showed better stability, as can be observed from the steady background throughout the entire array of temperatures studied. This resulted in a stable signal enhancement in the whole range of temperatures, which, in all cases, was ~22, but also clearly higher than the results for RMB, as demonstrated in the previous experiments. This improved robustness is a result of the intrinsic stability of LNA bases, bringing rigidity to the probe, which is necessary to maintain the beacon in a closed state form, even at higher temperatures. These results also confirm previous data obtained regarding the higher melting temperature of the beacon containing the LNA bases.[24] Thus, by the addition of the LNA bases in the beacon design, we have demonstrated that MBs with higher stability and reproducibility can be obtained, thus increasing the versatility of the sensor for extended applications.

Hybridization Kinetics of the MBs. The kinetics properties of the LMBs are relatively different from those of the RMBs. Depending on the percentage of LNA bases, hybridization efficiency in these MBs can be greatly affected, usually by slowing down the hybridization rate with the target DNA. To study and compare the hybridization kinetics of both molecular beacons with the target onto the glass surface, the fluorescence enhancement was monitored in the confocal microscope every 3 s for 10 min after addition of the target. As shown in Figure 2, the fluorescence signal of RMB reached hybridization equilibrium within 3 min, whereas LMBs slowly increased the fluorescence signal over time. This behavior, already observed in previous work, can result from the presence of LNA−LNA base-pair in the stem of the LMB.[24] In this study, LMBs possessed three LNA−LNA pairs in the stem (50%), which provide great stability and affinity to the beacon. This base-pairing percentage made stem opening difficult and unfavorable, slowing down the hybridization rate with the target, which is consistent with, and supports, our results. To know how LMB and RMB hybridization rates vary, we calculated the initial rate for hybridization of the beacons from the linear fraction of the hybridization kinetics (inset Figure 2). In this manner, the rate of each beacon was determined by the slope of the linear graph of the fluorescence intensity versus time. The slope of the RMB is ~7 times higher than that of the LMB, quantitatively confirming that RMB possesses faster hybridization rate. Interestingly, this hybridization rate can be drastically accelerated with the replacement of some LNA bases in the stem of the LMB by DNA bases (i.e., decreasing the percentage of LNA in the stem); on the other hand, this reduction of LNA bases can also result in decreasing the stability of LMB.[24] Notwithstanding these results, we believe that the hybridization rate exhibited by the LMB containing 50% LNA and DNA, despite being slower than RMB, is still very reasonable for nucleic acid−based biosensors since it can completely open the beacon in just a few minutes. Thus, the performance of the biosensor is minimally compromised, or even less, considering the improvement achieved in terms of signal enhancement and stability when LNA are incorporated in the probe.

Sensitivity of the Immobilized MBs. In DNA arrays and biosensors, it is important to determine the minimum concentration of target molecules that can provide a measurable signal. Thus, in the following experiment, we investigated the sensitivity of the immobilized MBs by testing different concentrations of target, ranging from 1 nM up to 100 μM. The same concentration of MBs was used to immobilize on the surface (initial concentration of 2.5 μM). However, it should be noted that only the initial concentration of MBs used for immobilization was known. As expected, the signal enhancements of both the LMB and the RMB were observed to be proportional to the target concentration, and the saturation point for each beacon was 5 μM (see Figure 3) with detection limits (DL) for RMB and LMB of 25 nM and 7.5 nM, respectively. Evidently, the high background intensity of the RMBs (around 2.5 times higher) limits the amount of target that

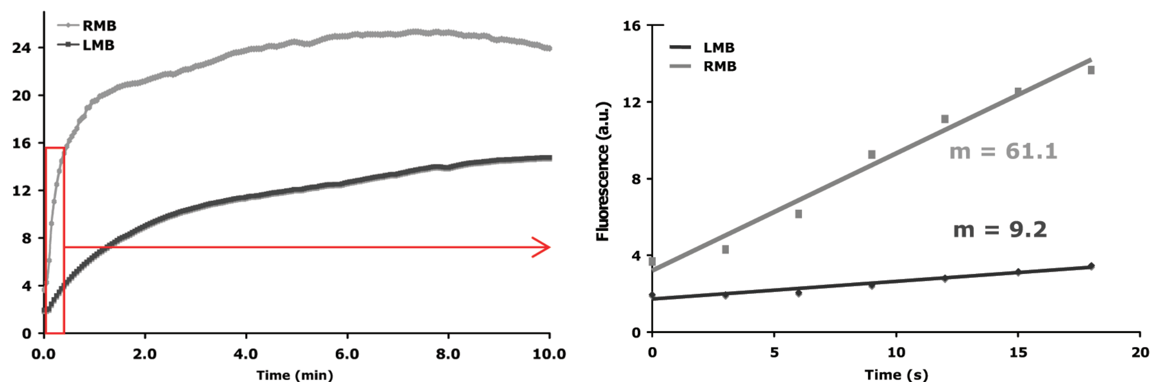

Figure 2. Hybridization kinetics of RMB and LMB immobilized onto the surface after target addition (10 μM). Right figure shows the initial rate of the immobilized beacons. The curves correspond to the average of three independent experiments. The *Y* axis represents fluorescence (arbitrary units), and the values have been divided by 100 to clarify the graphics.

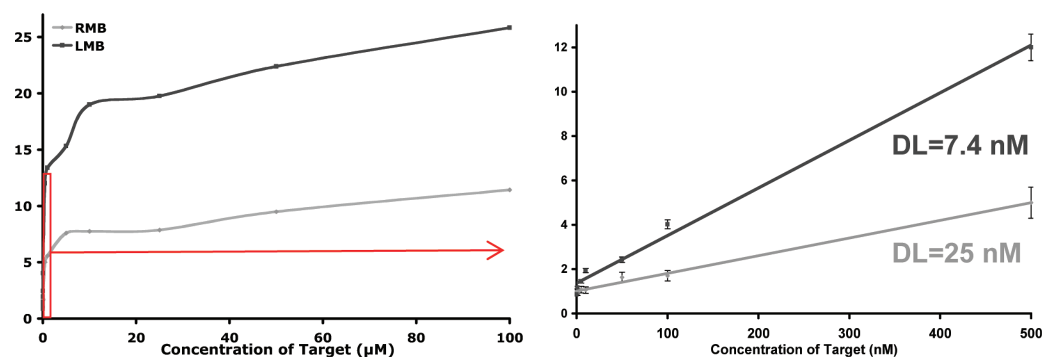

Figure 3. Comparison of the influence of target concentration on hybridization of RMBs and LMBs. Each point corresponds to the average of three measurements. The detection limit (DL) was calculated from the expression DL = $\text{mean}_{\text{blank}} + 3\text{SD}_{\text{blank}}$ (graphic on the right).

is detectable. On the other hand, the lower background intensity of the LMBs allows the detection of the target, even at low concentrations. In addition, it has been documented that LNA bases possess higher affinity[22] for DNA bases than DNA itself, which could also explain the lower detection limit of the LMBs.

Biosensor selectivity is critical, particularly in DNA-based diagnostic devices. Thus, the selectivity of MBs immobilized onto the glass surface was also investigated, and for this purpose, a sequence with a single base mismatch was synthesized and evaluated. The mismatch was positioned in the loop and complementary to a DNA base (see Table 1), not a LNA base, in the two beacons. The signal enhancement was obtained for the perfect match and mismatch targets to compare the detection capabilities of RMB and LMB. Similar to what was observed in solution,[23] LMB showed a slightly superior selectivity to that of the RMB (see Figure S1 in Supporting Information), proving that this pattern was also maintained when the probe is immobilized on a glass surface. The better discriminatory capability of the LMB can be directly attributed to the high affinity of the LNA bases toward DNA target molecules.[31]

MB Sensitivity in the Presence of Complex Matrixes. Another important aspect in the study of RMBs and LMBs to be used in surface immobilized biosensors is the ability of probes to detect a target in complex matrixes. This is especially important in the early detection of various genetic diseases where rapid and sensitive detection is needed. Ideally, the MB signal should only light up from the detection of target molecules. However, this is not always the case since degradation of the MB can occur because of DNA binding proteins and nucleases that are found inside the cells, resulting in false positive signals. Moreover, these proteins can hinder the ability of the MB to bind with the targets, thereby decreasing overall signal enhancement. Therefore, it is imperative to evaluate the performance of the molecular beacon-based biosensor in complex matrixes. Two different media, fetal bovine serum (FBS) and cell lysate, were selected, and the results are summarized in Figure 4. These media were selected because they contain many proteins, amino acids, sugars, and lipids that

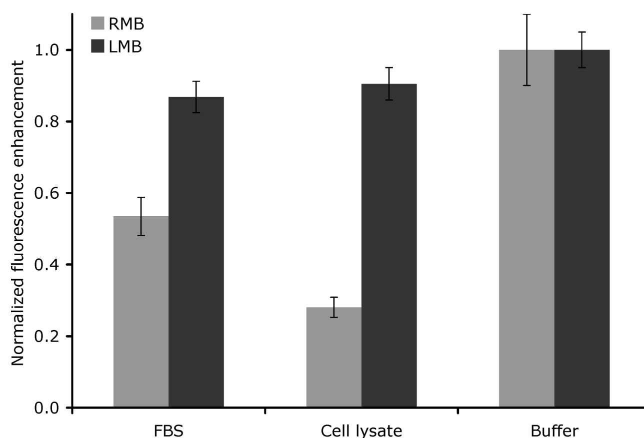

Figure 4. Normalized fluorescence enhancement of the immobilized RMB and LMB after treatment of the target with fetal bovine serum (FBS), cell lysate, and PBS buffer solution.

(31) Mouritzen, P.; Nielsen, A. T.; Pfundheller, H. M.; Choleva, Y.; Kongsbak, L.; Moller, S. *Expert Rev. Mol. Diagn.* **2003**, *3*, 27–38.

can potentially interfere with hybridization efficiency and/or degrade the MB. As expected, the data revealed a decrease in the signal enhancement of the RMB in the presence of FBS, as well as cell lysate. The background intensity of the RMBs did not change significantly (data not shown), which indicates that both the FBS and cell lysate solutions impede the ability of RMBs to bind with the target. Also, it is possible that degradation of the loop sequence has taken place since the DNA bases in the loop are more exposed and, therefore, more prone to degradation than the DNA bases in the stem. This effect is not as significant in LMBs since the signal enhancement appears to be unaffected after treatment with FBS and cell lysate. The degradation of the loop is less likely because of the inherent resistance of the LNA bases to degradation typically caused by nucleases. Nonetheless, endogenous materials can still block hybridization with the target, which, to some degree, explains the reduction of the signal enhancement observed for the LMB. Overall, these results show that LMBs on solid surface retain the ability to detect the target in complex matrixes.

CONCLUSION

In this paper, we have developed a surface-immobilized MB, which possesses superior stability for surface hybridization studies. The incorporation of LNA bases in the beacon design allows us to take combined advantage of the sensitivity of the MBs and the high binding affinity and stability of the LNAs bases. Moreover, the incorporation of a PEG linker with optimum length has considerably improved the target hybridization. Overall, the introduction of these modifications in the MB sequence it has made possible to achieve a signal-to-background of 25-fold, considerably better than the previously best reported values of only 5.5-fold with regular MBs immobilized probes. The biosensor developed with these MBs was highly stable and robust, as well as selective and sensitive, resulting in a promising design for use in a wide variety of biological and biotechnology applications. Future investigation will address the immobilization of multiple LMBs on solid surfaces, targeting different gene sequences for robust biosensor assay development.

SAFETY CONSIDERATIONS

Piranha solution is a dangerous and highly corrosive substance. It must be handled extremely carefully under the hood and using protective equipment. Fresh solutions should be prepared right before use, and should not be stored in a closed container.

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SUPPORTING INFORMATION AVAILABLE

Further details are given in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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