Immobilized molecular beacons: A new strategy using UV-activated poly(methyl methacrylate) surfaces to provide large fluorescence sensitivities for reporting on molecular association events

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Abstract

We have designed appropriately prepared solid supports consisting of poly(methyl methacrylate) (PMMA) that provide enhanced performance levels for molecular beacons (MBs) that are used for recognizing and reporting on signature DNA sequences in solution. The attachment of primary amine-containing MBs to the PMMA surface was carried out by UV activating the PMMA to produce surface-confined carboxylate groups, which could then be readily coupled to the MBs using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) chemistry. The fluorescence properties of the MBs covalently attached onto this UV-activated PMMA surface were evaluated and compared with the same MBs immobilized onto glass supports. We observed improved limits of detection for the solution complement to the MBs when immobilized onto PMMA, and this was attributed to both the lower autofluorescence levels exhibited by PMMA at the detection wavelengths used and the improved quenching efficiency of the MBs when in their closed hairpin configuration when strapped to a PMMA surface as opposed to glass. As an example of the utility of the PMMA-based immobilization strategies developed for MBs, we report on the analysis of complementary DNAs specific for fruitless (fru) and Ods-site homeobox (OdsH) genes extracted from Drosophila melanogaster fruit flies. The fra gene functions in the central nervous system, where it is necessary for sex determination and male courtship behavior, whereas the OdsH gene is involved in the regulation of transcription.

Keywords: Molecular beacons; PMMA supports; cDNA arrays

DNA/RNA detection probes that not only provide molecular recognition of unique structures but also report on the molecular association through a fluorescence transduction event have become very attractive assemblies in a variety of applications. An example of such probes is molecular beacons (MBs)\(^1\) [1], which are hairpin probes consisting of a loop and stem structure. The loop portion is the sequence recognition section of the MB, whereas the stem, which is composed of complementary sequences, hybridizes intramolecularly in the absence of the loop complement. On one end of the stem is a fluorophore, whereas the other stem possesses a quencher. On hybridization of the stems, the closed hairpin structure forms, placing in close proximity the fluorophore and quencher, producing quenching of the fluorescence reporter either through contact mediation or through energy transfer. When the

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\(^{1}\) Abbreviations used: MB, molecular beacon; cDNA, complementary DNA; dPEG, discrete polyethylene glycol; PMMA, poly(methyl methacrylate); fru, fruitless gene; OdsH, Ods-site homeobox gene; Act5C, Actin5C control gene; RT, reverse transcription; dNTP, deoxynucleoside triphosphate; Mes, 2-(N-morpholino)ethanesulfonic acid; NHS, N-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; IPA, isopropyl alcohol; ddH\(_2\)O, double-distilled water; SPAD, single-photon avalanche diode; RET, resonance energy transfer; mRNA, messenger RNA.

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recognition loop hybridizes to its complementary target sequence, the stem opens, restoring the fluorescence. These probes have found numerous applications, including detection of pathogenic retroviruses [2], discrimination of wild-type and single-point mutations [3,4], real-time monitoring of DNA/RNA in solution/living specimens [1,5], and single-nucleotide polymorphism detection [6].

Although extensive research involving MB probes has been performed in homogeneous phases, surface immobilization of MBs has not been reported extensively. Surface immobilization has the advantage of allowing spatially multiplexed detection with a probe that not only offers selective affinity for its complement but also reports on the molecular structure, obviating the need for labeling of the solution complement. The implementation of surface-immobilized MBs or other molecular probes configured into an array format also enables the seamless integration of this technology to various sample processing steps by the direct coupling of the array to a microfluidic system, which offers high levels of automation and reduced sample and reagent consumption, lowering assay costs [7]. An additional advantage of MBs, as compared with their linear nucleic acid probes, is their recognition specificity. By analyzing free energy phase diagrams of MBs in solution with matched and mismatched targets, structurally constrained MBs have been shown to distinguish mismatches over a wider range of temperatures than have unstructured (linear) probes [8,9].

Unfortunately, when MB probes are immobilized onto solid supports, they display lower sensitivities, where sensitivity here is defined as the ratio of fluorescence of the probe on binding to its complement but that of the probe in its closed (nonfluorescent) form, as compared with their solution-based counterparts. The lower sensitivity is due in part to inefficient quenching of the surface-attached MBs that is caused by increased nonspecific surface interactions with the solid support that destabilizes the hairpin structure. As such, a significant effort has been expended into exploring various solid supports and appropriate probe designs that provide better sensitivities for MBs. For example, MBs have been attached onto optical fiber core surfaces via biotin-avidin/streptavidin interactions [10–13]; however, higher background noise from both the closed (quenched) form of the MB and the cladding of the optical fibers was an issue in these reports.

Glass has been widely used as a solid support for the immobilization of MBs [10,14–16]. Unfortunately, glass suffers from well-documented problems due primarily to interfacial effects; the static charging experienced by glass surfaces can partially open the closed hairpin structure, resulting in high levels of fluorescence for the immobilized MB in its closed conformation. MBs have also been immobilized onto microwells/porous surfaces using agarose or polyacrylamide gel films, which provide a solution-like environment rather than the typical heterogeneous liquid–solid interface [17,18]. However, the use of these gel films slows the hybridization process due to diffusion-limited mass transport of the targets through the gel network.

Gold metallic surfaces have also been studied as viable substrates for the attachment of MB probes, where the gold serves the purpose of being a solid support as well as a quencher of the fluorescence [19–22]. Even though the MBs can be tethered easily onto gold surfaces via self-assembly of alkane thiols, nitrogen-based moieties along the probe’s DNA backbone can chemisorb to these same surfaces, resulting in nonspecific adhesion of the DNA to the gold surface. Nonspecific interactions of the solution target with the gold surface must be minimized by employing blocking agents to prevent the nitrogen-containing nucleotide bases from interacting directly with the gold surface [20]. Another artifact that affects the sensitivity, specificity, and hybridization kinetics of the MBs on gold is the nonuniform distribution of hairpin probes on the gold surface, causing surface-induced aggregation [20,23,24].

In another attempt to improve the sensitivity of surface-immobilized MBs, Zuo and coworkers designed MBs that contained a linker arm serving as a spacer as well as a restriction site within the loop structure [25]. These authors reported improvements in the fluorescence sensitivity of 5.2-fold compared with glass and an MB not containing a restriction site. Their results also showed that the loop of the immobilized MB did not open effectively on hybridization with complementary DNA (cDNA) targets.

The reaction thermodynamics and kinetics of surface-immobilized MBs differ from those in solution as a result of interfacial concentration gradients, species–interface interactions, and steric hindrance. For example, surface electrostatics greatly affects the binding parameters of surface-immobilized probes because of the wide distribution of probe–surface distances among these probes due to entropic effects, which can contribute to variations in hybridization melting temperatures [26–28]. For surface hybridization, nucleic acid targets can be repelled or attracted to the surface depending on the immobilization material as well. Electrostatic repulsion between single-stranded nucleic acid targets and the surface-immobilized probes (due to the high negative charge of nucleic acid oligomers) can result in Coulomb blocking of hybridization events. Tethered probes with long linker molecules have electrostatic interactions that can dominate short-range Van der Waals forces. Also, steric hindrance, which increases with increasing surface probe densities, can alter the hybridization efficiencies of surface-immobilized probes due to the presence of repulsive electrostatic interactions [29].

In the current work, we report on an immobilization strategy for MB probes to enhance their sensitivity when configured in a microarray format. The approach adopted a two-pronged strategy: (i) designing appropriate linker structures to minimize probe aggregation effects on the surface and (ii) using a support that provided simple and stable attachment chemistries and minimized electrostatic
effects. The MBs contained a C6 amino linker appended to their stems to aid in surface immobilization and also contained discrete polyethylene glycol (dPEG) cross-linkers. The dPEG cross-linkers are both extremely water/organic soluble and hydrophilic. The commonly used alkyl linker/spacers have the characteristic of being hydrophobic and can suffer from increased aggregation and/or precipitation effects at the surface [30]. The dPEG linkers decrease these artifacts greatly [30,31].

We also used a UV photomodification process as described previously [32–34] to activate a poly(methyl methacrylate) (PMMA) surface onto which the MBs were attached via carbodiimide coupling chemistry. The same MBs were also attached to glass surfaces using conventional siloxane-based chemistry, and the sensitivity of the MBs on glass and PMMA were compared rigorously. The performance of these surface-immobilized MBs was compared with their solution counterpart assays as well. As an example of the performance of this appropriately designed linker and support system for MBs, the loop sequences were used for the analysis of cDNAs generated from fruitless (fru) and Ods-site homeobox (OdsH) genes extracted from *Drosophila melanogaster* fruit flies. The *fru* gene functions in the central nervous system, where it is necessary for sex determination and male courtship behavior, whereas *OdsH* is involved in transcriptional regulation and plays a role in hybrid dysfunctions in spermatogenesis.

**Materials and methods**

**Preparation of cDNA target samples**

Three genes—*OdsH*, *fru*, and a control gene, *Actin5C (Act5C)*—were chosen for these investigative studies based on their potential influence on the spermatogenesis pathway, role in the sex determination pathway, and ubiquitous presence in many cell processes. cDNA from these genes was obtained from *Drosophila simulans* due to the species-specific alleles’ relevance in an unrelated study. To obtain the sequence of these genes in *D. simulans*, the sequence of *D. melanogaster* (obtained from FlyBase, www.flybase.org) was BLASTed (www.ncbi.nlm.nih.gov/BLAST) against the whole genome sequence of *D. simulans*. Primers were designed to bind to the *D. simulans* sequence, which flanked an intron and did not have any sequence similarity to other genomic DNA. This allowed a successful amplification of only the sequence of interest and also provided a means of differentiating cDNA amplification from DNA amplification. The PCR primer sequences were as follows:

*OdsH*-F: CTCATAGTTCATCCCCCACAGAG  
*OdsH*-R: AGCTATGTAATCGGCCCTTCAGAC  
*fru*-F: ATCCCCATCTCTACTTGAAGATGT  
*fru*-R: GAGGCCGTAGTTCAGATTGTGTTAT  
*Act5C*-F: GGATATCCGTAAGGATCTGTATGC  
*Act5C*-R: CCAAGACAAAGCAGTCTTCTTA

*D. simulans* stocks were maintained at 20 °C on a 12 h light:dark cycle. Virgin males were collected, aged 4 days, and then frozen at −80 °C between 1 and 2 h after “lights on” on the fourth day. RNA was extracted from 35 to 40 fruit flies using the Qiagen RNeasy Mini Kit (Valencia, CA, USA). Reverse transcription (RT) was performed using the reverse primer with MMLV Reverse Transcriptase and RNAsin from Promega (Madison, WI, USA). PCR amplification was performed in a 25-μl volume with 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 μM of each primer, 1 U of *Taq* polymerase, and 5 μl of cDNA template. Samples were amplified through 1 cycle at 95 °C for 5 min, 3 cycles at 94 °C for 1 min/56 °C for 30 s/72 °C for 30 s, 3 cycles at 94 °C for 1 min/53 °C for 30 s/72 °C for 30 s, 30 cycles at 94 °C for 1 min/50 °C for 30 s/72 °C for 30 s. This PCR protocol allows a single program to work for a variety of primer pairs. The successive reduction of annealing temperatures is referred to as a “touch down” procedure, which is useful when a primer is a good match to the template but has alternative (weaker) binding sites as well. High-stringency annealing steps favor binding only to the correct sites; however, at later thermal cycles when the mixture is dominated by PCR products, lower stringency annealing temperatures are less likely to result in binding at alternative sites [35]. The product was run on a 2% agarose gel with both a positive control and a blank (no RNA) negative control, and the appropriate-sized band was extracted and purified using the Qiagen Gel Extraction Kit. The isolated product was then used as the template for eight replicate rounds of PCR amplification using the same protocol as described above, except that 1 μl of template was used. The eight replicates were pooled and purified with the Qiagen PCR Purification Kit. The final product was then sequenced to confirm that the correct product was obtained, and the sequence was used to design the probe and target.

**Design of the MB probe**

The oligonucleotide sequences for both the probes and targets are given in Table 1. We designed two different MBs: one for *fru* (MB1) detection and the other for *OdsH* (MB2) detection. The beacons were labeled at their 5′ ends with a CY 5.5 fluorophore and at their 3′ ends with BHQ-3 dark quencher. Also, the stem was functionalized with a C6 amino linker (Fig. 1) for attachment to the solid support. The MBs were synthesized by Gene Link (Hawthorne, NY, USA) and used without further purification.

**Immobilization of MBs on solid substrates**

PMMA substrates (1 mm thickness) were obtained from Goodfellow (Berwyn, PA, USA), whereas aldehyde-functionalized glass substrates were purchased from Telechem International (Sunnyvale, CA, USA). 2-(N-Morpholino)ethanesulfonic acid (Mes), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)
carbodiimide hydrochloride (EDC), phosphate, and Tris buffer solutions were obtained from Sigma (Milwaukee, WI, USA). Deionized water (17.9 MΩ) from an E-pure water purification system (Barnstead, Dubuque, IA, USA) was used for preparation of all buffers and rinsing reagents.

MBs were covalently attached onto both PMMA and glass substrates using the appropriate linkage chemistry.

Table 1
Molecular beacon probes and target sequences

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<td>MB1</td>
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<td>T3</td>
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Note. MB1 and MB2 represent the molecular beacons used for these studies and their respective sequences. Lowercase letters denote the stem sequences of the beacons, whereas uppercase letters are the recognition loop sequences. T1 and T2 are target sequences complementary to MB1 and MB2, respectively. The underlined sequence is the section of the target complementary to the MBs. T3 is a noncomplementary sequence to both MB1 and MB2. The targets T1, T2, and T3 are cDNAs extracted from D. melanogaster for fru, OdsH, and Act5C genes, respectively. Actin is noncomplementary to both MB1 and MB2 and was used as a negative control in these studies.

Fig. 1. Structure of molecular beacon probes used for the detection of fru gene MB1 (A) and OdsH gene MB2 (B). Their stem structures possessed a C6 amino linker to aid in surface immobilization using a dPEG cross-linker (C).
For PMMA, the substrates were photoactivated by exposure to broadband UV radiation, which was performed using a UV station equipped with a UV light (500 W DUV, model UXM-501 MA, Ushio America, Cypress, CA, USA). The substrates were placed at a distance of 1 cm from the source for 20 min with a radiation intensity of 15 mW/cm². Following UV activation for approximately 30 min, the PMMA substrates were rinsed thoroughly with 2% isopropanol alcohol (IPA) and then with double-distilled water (ddH₂O), followed by drying under nitrogen gas. The PMMA slides were cross-linked with a dPEG spacer (aminom dPEG₁₂ acid, Quanta Biodesign, Powell, OH, USA) using carbodiimide coupling chemistry. The surfaces were incubated with 100 mM Mes containing 10 mM EDC/5 mM NHS for 30 min, followed by 100 μM of the dPEG for at least 5 h at room temperature, after which the slides were rinsed thoroughly in ddH₂O and dried with pressurized air. Therefore, in this step the amino group of the dPEG₁₂ was attached to the PMMA or glass surface, leaving the acid-functional group of the cross-linker available for attachment of the MB probe, which contained an amino group. Then the MBs were dissolved in 100 mM Mes (pH 5.5) containing 10 mM EDC/5 mM NHS to a final concentration of 100 nM, spotted onto the substrate by micropipetting 0.2 μl of the appropriate solution onto the solid substrate, and incubated for 2 h at room temperature. The spot diameter (obtained by multiplying the number of pixels across the spot by the scanning step resolution) was approximately 2000 μm. For glass, the aldehyde-functionalized slides were incubated with 100 μM dPEG₁₂ spacer in phosphate buffer at pH 8.3 for at least 5 h and then rinsed thoroughly in ddH₂O and dried with pressurized air. MBs were immobilized to the glass via the dPEG₁₂ cross-linker through its carboxy-functional group in a fashion similar to that outlined for PMMA.

Hybridization of MB probes to their targets

For solution-based assays, three different solutions were evaluated: 100 nM MB without target molecules, 100 nM MB with a 10-fold molar excess of noncomplementary targets, and 100 nM MB with a 10-fold molar excess of complementary target molecules (for target sequences, see Table 1). All assays were performed in a buffer containing 20 mM Tris·HCl, 10 mM MgCl₂, and 10 mM KCl at pH 7.5. The hybridization reaction was allowed to proceed for 30 min at room temperature, and fluorescence spectra were obtained (λex = 675 nm) using a Fluorolog-3 fluorimeter (Jobin Yvon, Edison, NJ, USA). Array-based hybridization was accomplished by incubating the slides containing the immobilized MB probes in a prehybridization buffer for 30 min at room temperature, facilitating the annealing of the stem of the probes. Then target solutions were placed onto the MB spots and allowed to incubate at room temperature for 2 h, followed by fluorescence scanning of the array surface.

Imaging of MB arrays

After hybridization, both glass and PMMA slides were imaged using a home-built near-infrared fluorescence scanner that has been described previously [34]. In brief, it consisted of a laser diode excitation source lasing at 670 nm with an optical output power of 10 mW (Thorlabs, Newton, NJ, USA). The excitation beam was passed through a neutral density filter (ND 0.6, Thorlabs) and a line filter (670DF20, Omega Optical, Brattleboro, VT, USA). A beam splitter (690DRLP, Omega Optical) was positioned at a 45° angle and focused onto the array surface using a 40 × high-numerical aperture (numerical aperture = 0.85) microscope objective (Nikon, Natick, MA, USA). The fluorescence was collected by the same microscope objective and was transmitted through the dichroic and finally through a filter stack consisting of a 700 ALP long-pass filter and a 720 DF20 band-pass filter (Omega Optical). After passing through the filters, the fluorescence was sent through a pinhole and focused onto a single-photon avalanche diode (SPAD). The entire detector was mounted on an X/Y microtranslational stage interfaced to a computer.

Results and discussion

MB design

Important design parameters associated with MBs are their probe and stem lengths and sequence content because at a given temperature they largely control the fraction of MBs in the three different conformational states: bound-to-target, stem–loop hairpin, and random coil [36]. In general, the loop sequence consists of 15 to 25 nucleotide bases, with the sequence content based on the target sequence and melting temperatures required. The stem typically has 4 to 6 nucleotide bases and is chosen to have no sequence homology to that of the target. It has been shown that longer stem lengths are accompanied by a lower target affinity and a decreased probe-target hybridization rate, whereas MBs with short stems have faster hybridization kinetics and improved target affinities but lower sensitivities when compared with MBs with longer stems [37]. On the other hand, MBs with longer loop lengths have improved target affinities and increased kinetic rates but also display reduced specificities for discrimination between fully matched and mismatched target–loop duplexes [37].

In this study, we designed our MBs with stems containing 5 bases and loops having either 25 nucleotide bases (Fig. 1A) or 22 nucleotide bases (Fig. 1B). Both MBs had a CY 5.5 fluorophore at their 5’ ends and a BHQ-3 (black hole quencher) at their 3’ ends. There are two possible quenching mechanisms that can be envisioned when these probes are in their hairpin configuration: contact quenching and energy transfer. Contact quenching occurs when there is a collision between the fluorophore and quencher, creating a disruption of the energy levels of the excited fluorophore and causing the quencher to dissipate the energy it
receives from the fluorophore as heat. Resonance energy transfer (RET) requires spectral overlap between the emission spectrum of the donor (fluorophore) and the acceptor’s (quencher) absorption spectra. We took RET into consideration by selecting the fluorophore and quencher that provided good spectral overlap. For surface immobilization, it was important to have a functional group to aid in the attachment of the MB to the solid support. We also designed our MBs to have a C6 amino linker on the stem. It was also highly desirable to have enough space between the solid support and the probes to enable the probes to be readily accessible to target molecules and also to minimize potential interactions between the MB probes and the surface given that this could destabilize the hairpin conformation. Therefore, we used a dPEG$_{12}$ spacer molecule (Fig. 1C) to keep the probes separated from the surface so as to minimize these artifacts and to avoid any possible steric effects hindering target accessibility to its complementary probe.

**Fluorescence of hybridized MBs in solution**

The results shown in Fig. 2 indicate that the fluorescence sensitivity (ratio of fluorescence of the probe on binding to its target with that of the probe in its closed [nonfluorescent] form) of these MBs when hybridized to their complementary DNA targets in solution was 16 for MB1 and 14 for MB2. The MBs incubated without targets or those incubated with noncomplementary targets showed minimal amounts of fluorescence.

**Immobilization of MBs onto PMMA and glass surfaces**

To capture complementary targets in an array format, MBs were immobilized onto PMMA and their sensitivities were compared with those of MBs immobilized onto glass supports. For PMMA, the immobilization was done by first activating the surface via exposure to UV irradiation, introducing a scaffold of carboxylate-functional groups on its surface that were then used for coupling to a bifunctional discrete polyethylene glycol cross-linker through carbodiimide coupling chemistry. This linker molecule consisted of a terminal amine group, which was used to form an amide bond with the surface through the UV-generated carboxylic acids for PMMA or the aldehydes of glass. The PEG$_{13}$ cross-linkers also contained a carboxylic acid group to allow tethering of the MB, which contained an amino group. These linker molecules kept the immobilized MBs spatially removed from the solid surface, improving their hybridization efficiencies [38,39].

Recently, Yao and Tan reported on the use of poly-T linker molecules to reduce MB associations with the glass surface to which they were attached [15]. They found that when a long poly-T (> 25 bases) was used, high negative charges eventually could repel the target DNA and reduce efficiency of duplex formation. The use of the dPEG linkers minimizes this electrostatic artifact because the PEG linker carries no charge at the pH values used for the work reported here.

Another factor we considered when immobilizing the MBs onto the surfaces was the fact that high probe densities typically reduce the binding efficiency of the probes to targets. Vainrub and Pettitt discussed these effects by studying interface electrostatic interactions for chip array hybridizations. In their work, high probe densities lead to high negative charges, resulting in strong repulsion between single-stranded nucleic acid targets and their surface-immobilized probes, giving rise to Coulomb blockage of hybridization [26–28]. Also, Peterson and coworkers described how probe density was a controlling factor for

![Fig. 2. Solution-based hybridization results for MB1 (A) and MB2 (B). Three solutions were used: 100 nM MB without target molecules, 100 nM MB with a 10-fold molar excess of noncomplementary targets, and 100 nM MB with a 10-fold molar excess of complementary target molecules. The solutions were incubated for 30 min in a hybridization buffer consisting of 20 mM Tris–HCl, 10 mM MgCl$_2$, and 10 mM KCl at pH 7.5. The fluorescence spectra were obtained using $\lambda_{ex} = 675$ nm.](image-url)
efficient target capture as well as for producing favorable kinetics for target–probe hybridization [29]. They demonstrated that hybridization depends strongly on probe density in both the efficiency of duplex formation and the kinetics of target capture, such that with low probe densities essentially 100% of the surface-immobilized probes were hybridized to their complementary targets with Langmuir-like binding kinetics, whereas hybridization efficiencies dropped to approximately 10% in the case of high probe densities. In either case (low or high probe density), binding saturation at a particular location of the array can lead to limited dynamic range for the expression profiling. To control the immobilization densities, we used low concentrations of probes (100 nM) and limited the immobilization times to less than 2 h. For our PMMA substrates and using carbodiimide attachment chemistry, probe densities were determined to be approximately $2.4 \times 10^{12}$ molecules/cm$^2$. These probe densities are comparable to those shown to yield high hybridization efficiencies ($2.0 \times 10^{12}$ molecules/cm$^2$) [29,40].

Following immobilization of the MBs onto glass or PMMA surfaces, they were incubated in a prehybridization buffer containing divalent (Mg$^{2+}$) cations to facilitate stem annealing so as to further reduce the background fluorescence of the unhybridized probes. Figs. 3A and B show fluorescence images obtained after hybridization with complementary oligonucleotide targets using glass and PMMA supports, respectively. Two different MBs were used in these studies: MB1 and MB2 (for the sequences of these probes, see Table 1). Both images indicated recovery of the fluorescence on binding with complementary targets. Interestingly, glass exhibited a much higher autofluorescence level at the excitation wavelength used in these studies than did PMMA. The autofluorescence arises from the substrate itself and is measured in areas on the surface where no MB is found. Fig. 4 gives the fluorescence sensitivities of the surface-immobilized MBs in comparison with the same probes used in solution. The fluorescence sensitivity found for the PMMA support was approximately 8, whereas that found for glass was 4 for both MB probes,

Fig. 3. Comparison of the fluorescence hybridization results for molecular beacons immobilized onto glass (A) and PMMA (B). Functionalized PMMA and glass substrates were used for coupling a bifunctional dPEG cross-linker through carbodiimide coupling chemistry. The solution complements we re synthetic oligonucleotides (see underlined sequences for T1 and T2 in Table 1) set at a concentration of 100 nM that were allowed to hybridize with the array in a humidified chamber for 2 h prior to fluorescence scanning. Fluorescence images were obtained before hybridization (left panels) and after hybridization to the immobilized MB probes with their fully complementary targets (right panels) for both MB1 and MB2.
but both substrates resulted in reduced sensitivities compared with their solution counterparts. In solution, the MBs encounter higher fluorescence sensitivity because they can bind freely with their targets compared with their constrained surface-immobilized counterparts. When MBs are in solution with their targets, they can exist in an open conformation (bound to targets) or a closed conformation (free of targets). This two-state model is an equilibrium process, with the closed state characterized by lower enthalpy than the open state due to base pairing and stacking. The opening rate depends on the unzipping energy of the hairpin probes [8,9,41]. In solution, these hairpin probes diffuse more freely and are unperturbed by surface interactions, hence the ease of interaction with target molecules. On the other hand, surface-immobilized probes lack this freedom. In addition, MBs that are immobilized onto solid surfaces have different electrostatic properties at the surface–liquid interface, affecting the local ionic strengths and making them differ from those in bulk solution as noted above.

Surface effects can also destabilize the stem structures of the immobilized MB probes, reducing their quenching efficiency when they are in their closed configuration. For the optical setup used in these assays, glass exhibited an auto-fluorescence background of 240,000 counts per image pixel, whereas PMMA exhibited a background of 40,000 counts per image pixel. When the auto-fluorescence background was subtracted from the intensities of the MB probes without targets (closed configuration) or from the intensities of these MBs after incubation with noncomplementary targets, the net signal was 40,000 for both surfaces. The lack of difference between these values indicates that both surfaces affect the closed configurations of the surface-immobilized MBs to the same degree, and this is not too surprising given that in both PMMA and glass a monolayer of the dPEG linker is formed over the underlying surfaces. However, on binding of the MB probes to their full complementary targets, glass exhibited an intensity of 160,000 counts per image pixel, whereas PMMA showed a value of 340,000 counts per image pixel (both were background subtracted). The lower MB sensitivity on glass surfaces could arise due to static charging. On the other hand, PMMA exhibited better fluorescence sensitivity due to its electrostatic surface effects (thermodynamic equilibrium distribution) that affect the probe–target binding strength near the surface in a more favorable manner. It has been shown that strong attraction of a probe–target duplex for the surface promotes duplex formation, whereas surface

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Fig. 4. Fluorescence sensitivity ratios for solution-based hybridization events and surface-immobilized MBs using glass and PMMA substrates.

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Fig. 5. Analysis of cDNAs generated from *D. melanogaster* fruit flies for the *fru* and *OdsH* genes. Array measurements were performed using the MB probes immobilized onto PMMA substrates. PMMA substrates were activated by exposure to UV radiation, followed by reaction with the dPEG cross-linker, and finally immobilization of the MB probes to the dPEG surface using a 100-nM solution. These probes were then used for hybridization with complementary and noncomplementary targets for 2 h at room temperature. Fluorescence images were obtained after hybridization of surface-immobilized molecular beacons without targets (A), with fully complementary targets (B), and with noncomplementary targets (C). The complementary targets T1/T2 and noncomplementary targets T3 (for their sequences, refer to Table 1) are cDNAs extracted from *D. melanogaster* fruit flies.
repulsion of the probe–target duplex will shift the hybridization equilibrium toward melting of the duplexes [27].

**Analytical sensitivity of MBs immobilized onto PMMA substrates**

When carrying out MB hybridization assays, it is desirable for the loop sequence to hybridize only to the specific sequence of interest and also to work within useful target concentrations. We immobilized MB probes onto PMMA surfaces, with the loop sequences corresponding to the OdsH gene (MB1) and the fru gene (MB2) of *D. melanogaster*. The target samples were prepared by creating cDNA from messenger RNA (mRNA) through RT. Before being used in the hybridization assays, the cDNAs were denatured for 5 min at 95 °C and then immediately cooled on ice. Fig. 5 shows the fluorescence images obtained after hybridization of MB probes immobilized onto PMMA surfaces without targets (Fig. 5A), on hybridization with cDNAs (Fig. 5B), and after hybridization with noncomplementary DNAs (Fig. 5C). The Act5C gene is noncomplementary to both MB1 and MB2 and therefore was used as a negative control in these studies (Table 1).

For both immobilized probes, those without targets (Fig. 5A) and with noncomplementary targets (Fig. 5C) did not show any significant fluorescence, whereas those with perfect complementary targets (Fig. 5B) exhibited strong fluorescence. The MB probes did bind to their target sequences, forming probe–target hybrids that were more stable than the stem hybrid. Only perfect complementary hybrids were sufficiently stable to force the stem hybrid to open, resulting in higher fluorescence.

The analytical sensitivity for reporting on the concentration of the solution complements using these immobilized MB probes was then determined using different concentrations of the targets and constructing calibration plots. For each MB probe, the experiments were performed in triplicate on different PMMA slides using a solution cDNA concentration ranging from 100 nM to 2.5 μM. Fig. 6 shows the calibration plots for the observed fluorescence intensities versus target concentrations for MB1 (Fig. 6A) and MB2 (Fig. 6B). Low target concentrations required longer reaction times to reach steady state, whereas increased target concentrations promoted more intermolecular duplex formation. The plots were linear, with correlation coefficients of $R^2 = 0.96$ for MB1 and $R^2 = 0.97$ for MB2.

The presence and abundance of targets within a sample usually are indicated by the intensity of the hybridization signal at the corresponding probe sites. Alternatively, the abundance of the targets can be obtained by RT–PCR-based assays, which are common methods for comparing mRNA levels in different sample populations. The mRNA levels are measured and normalized to reference genes, allowing each gene expression to be measured as a numerical value and enabling direct comparison between experiments. The relative abundance of the genes used in this study has been determined, and the normalized expression values obtained were 9.12 for fru mRNA, 3.43 for OdsH mRNA, and 11.24 for Act5C mRNA [42]. These results indicate that the fru mRNA is highly abundant in *D. simulans*, whereas the OdsH mRNA is near the threshold of detection of conventional arrays.

**Conclusion**

These studies were based on the concept that proper MB design and careful solid support choice with robust surface chemistries could lead to improved sensitivities of surface-immobilized MBs. We chose a surface modification procedure described previously in our laboratory [34] to produce functional scaffolds consisting of carboxylic acid groups that allowed the covalent attachment of amine-functionalized MB probes onto PMMA surfaces through carbodiimide coupling. These processes involved only broadband UV exposure of the polymer surface followed by carbodiimide coupling of amine-containing MB probes to the
surface (via an amide bond). Surface-bound probes require enough interstitial space to improve hybridization efficiency. Therefore, we employed dPEG cross-linker molecules to minimize any steric effects that might occur as well as to minimize surface aggregation effects. MBs immobilized onto PMMA showed higher fluorescence restoration than did those immobilized onto glass surfaces. In this study, PMMA was found to be a better substrate than glass for the designed probes and wavelengths used for their interrogation. The ability to perform quantitative assays using these types of MB probes and proper substrates will be a useful tool in gene expression analysis.

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