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Charge-Neutral Morpholino Microarrays for Nucleic Acid Analysis

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Abstract

A principal challenge in microarray experiments is to facilitate hybridization between probe strands on the array with complementary target strands from solution while suppressing any competing interactions that the probes and targets may experience. Synthetic DNA analogs, whose hybridization to targets can exhibit qualitatively different dependence on experimental conditions than for nucleic acid probes, open up an attractive alternative for improving selectivity of array hybridization. Morpholinos (MOs), a class of uncharged DNA analogs, are investigated as microarray probes instead of DNA. Morpholino microarrays were fabricated by contact printing of amino-modified probes onto aldehyde slides. In addition to covalent immobilization, MOs were found to efficiently immobilize through physical adsorption; such physically adsorbed probes could be removed by post-printing washes with surfactant solutions. Hybridization of double-stranded DNA targets to MO microarrays revealed a hybridization maximum at intermediate ionic strengths. The decline in hybridization at lower ionic strengths was attributed to an electrostatic barrier accumulated from hybridized DNA targets, while at higher ionic strengths it was attributed to stabilization of target secondary structure in solution. These trends, which illustrate ionic strength tuning of forming on-array relative to solution secondary structure, were supported by a stability analysis of MO/DNA and DNA/DNA duplexes in solution.

Keywords

surface hybridization; microarrays; morpholinos; DNA; immobilization; PNA

INTRODUCTION

DNA microarrays have established themselves as versatile tools for life science research.[1; 2; 3; 4; 5] Despite their breadth of applications, however, microarray technologies continue to be limited by cross-interactions that compete with the desired recognition between “probe” oligonucleotides immobilized on the array and complementary sample “target”

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Supplementary Material. Supplementary material describing measurement of solution melting curves and of morpholino mass spectra can be found in the online version.

sequences in solution. These cross-interactions are typically suppressed through dedicated processing steps such as thermal denaturation of samples to reduce interactions among solution strands that would otherwise interfere with array hybridization, and post-hybridization washing of arrays to break apart probe/target pairs that are not perfectly sequence-matched.

Another critical consideration that determines selectivity of probe/target array hybridization over other interactions is choice of probe material. Conventional DNA probes benefit from a refined synthetic capability and an established database of physical properties and predictive tools. However, a fundamental constraint with DNA probes is that optimization of experimental conditions to select out the desired probe/target association is difficult. This is because changes in conditions (e.g. ionic strength, temperature) tend to shift all nucleic acid interactions, whether at the surface or in solution, in the same direction instead of separating them. Another drawback of DNA probes is that they create a negatively charged surface repulsive to target molecules [6; 7; 8; 9] and, therefore, penalize rather than favor probe/target interactions relative to solution associations among strands. Hybridization to DNA probes can also exhibit complex kinetics, including overshoots and multiple plateaus,[10; 11; 12] suspected to derive from an interplay of competing interactions at the surface that can complicate data interpretation. Often, surface hybridization kinetics can be significantly slower than in solution.[13; 14; 15]

An ideal probe type would provide contrast in interactions that allows preferential selection of on-array probe/target hybridization over other interactions that may be available, would not pose an electrostatic barrier to target hybridization, and would exhibit simple, predictable kinetic behavior. Microarrays prepared with non-ionic probes, such as peptide nucleic acids (PNAs) and morpholinos (MOs) (Figure 1) [16; 17], could offer a solution. The charge neutrality of MOs and PNAs alters electrostatics of their hybridization with DNA so that, relative to DNA/DNA hybridization, dependence on salt concentration is eliminated (e.g. MO/DNA duplexes) [18; 19] or even slightly reversed (e.g. PNA/DNA duplexes).[20] Such qualitatively different behavior provides a tunable contrast in interactions; e.g. low ionic strengths can be used to increase selectivity of hybridization to MO or PNA microarrays by destabilizing associations among nucleic acids in solution. For example, recently Zu *et al* reported that nanoparticles functionalized with MO probes were effective in hybridizing with hairpin-forming DNA targets under dilute salt conditions.[21] Similarly, microarrays based on uncharged probes should allow significant hybridization even at lower ionic strengths [18; 22; 23; 24] because, at the outset of an experiment, the microarray is uncharged and thus free of an electrostatic barrier to hybridization.

PNA probes continue to be explored for microarray applications [25; 26; 27; 28; 29; 30] as well as other bioanalytical technologies.(e.g. see [31]) The exceptionally high binding affinity of PNAs renders them perfect for identifying shorter target sequences with excellent single mismatch sensitivity, such as for analysis of single nucleotide polymorphisms (SNPs). [28; 29; 30] However, PNA oligomers are subject to length (i.e. typically less than 16 residues) and composition restrictions due to synthetic difficulties [32; 33; 34] and, for longer lengths in this range, their potential for cross reactivity with mismatched sequences becomes a concern.[19] As such, PNAs are not a good choice for applications requiring 20mer or longer probes, as in gene expression profiling, where such longer lengths are needed to uniquely identify a genome site.

Compared to PNAs, MOs are easily prepared at longer lengths and are significantly more soluble in aqueous solutions, generally at the mmol L⁻¹ scale for 25mer sequences, or about a 100-fold higher than for PNAs.[19] The DNA-affinity of MOs is lower than that of PNAs, a beneficial attribute when longer probe lengths are needed for higher sequence specificity

as the moderate affinity decreases cross-hybridization to mismatched sequences. Compared to DNA probes, the affinity of MOs toward DNA is comparable to that of DNA/DNA under moderate salt conditions (e.g. $\sim 0.1 \text{ mol L}^{-1}$), while it exceeds DNA/DNA affinity at lower ionic strengths.[18; 19] Surface hybridization properties of MO probes are just beginning to be understood. Recent studies using DNA targets revealed that initial hybridization kinetics are consistent with a sequential adsorption process.[15] Moreover, in contrast to DNA/DNA surface hybridization which is strongly suppressed in the high probe coverage limit, coverage of MO probes exerted a much milder effect presumably due to precipitation of the probes onto the solid support as a thin, desolvated layer, thus reducing steric constraints to target hybridization.[18] In the area of diagnostic applications, MO probes have been used in label-free approaches based on nanopore and nanowire conductance,[35; 36; 37] interfacial capacitance,[38; 39], electrocatalysis,[40] and formation of nanoparticle-assemblies.[21]

The present study reports on adaptation of MO probes to microarray technologies. Conditions for printing, immobilization, hybridization and washing of morpholino microarrays on aldehyde slides [41; 42] were considered and optimized. Hybridization behavior of morpholino microarrays to double-stranded DNA targets was characterized as a function of probe coverage and ionic strength, and observed trends were interpreted in terms of the relative stability of MO/DNA and DNA/DNA duplexes as derived from solution melting measurements. The impact of surfactant additives on MO/DNA hybridization was also considered.

MATERIALS AND METHODS

Materials

Monosodium phosphate, disodium phosphate, sodium carbonate, trifluoroacetic acid, and α -cyano-4-hydroxycinnamic acid were purchased from Sigma-Aldrich. Sodium borohydride (NaBH_4), sodium dodecyl sulfate (SDS) and Tween 20 were purchased from Fisher Scientific. Morpholino oligomers were obtained from Gene Tools LLC, and DNA oligodeoxyribonucleotides were from Integrated DNA Technologies. All sequences are provided in Table 1. The probe sequences were derived from the macrophage infectivity potentiator (Mip) gene of *Legionella pneumophila* Philadelphia 1 (GenBank accession number AE017354). Probes with fluorescein modification were shifted by one position relative to unlabeled probes to decrease G-related quenching effects. All probes and targets were diluted with deionized water ($18.2 \text{ M}\Omega \text{ cm}$) to $200 \mu\text{mol L}^{-1}$ before storage at -20°C . MO and DNA concentrations were confirmed spectrophotometrically. Commercial SuperAldehyde 2 microarray slides (Arrayit Corp.) were stored at room temperature in vendor-sealed packaging as recommended. SecureSeal hybridization chambers (Grace Bio-Labs) were used for microarray hybridizations.

Solution melting measurements

Solution melting temperatures T_m for MO/DNA and DNA/DNA duplexes were determined using a $0.2^\circ\text{C min}^{-1}$ scan rate, as described in the Supplementary Material. These data were used to look for surfactant/duplex interactions, and to provide insight into how microarray hybridization is affected by stability of solution secondary structure.

Microarray fabrication

Microarrays were printed on a SpotArray 72 microarray contact printing system (Perkin-Elmer, Fremont, CA) using TeleChem Stealth pins. Each MO probe solution was spotted at least twenty times. Spotting concentrations that were tested included 1, 5, 10, 20, 30, and $40 \mu\text{mol L}^{-1}$ probe. Initial spotting from $1 \mu\text{mol L}^{-1}$ concentrations did not yield measurable extents of immobilization, an outcome attributed to loss of probes to adsorption on the

labware used in handling and holding of probe solutions. All subsequent studies therefore used the range from 5 to 40 $\mu\text{mol L}^{-1}$. Various buffers, including deionized water, were tested as spotting media. After printing, slides were dried and stored for 22 hours at 23 °C, at a humidity of 30 % or less.

Next, slides were washed to remove excess, unbound probes. Experience showed that to avoid immobilization of excess probes around spot edges it was necessary to perform the wash step concomitantly with deactivation of any aldehydes remaining on the slides. Deactivation was performed by first washing the slides with a NaBH_4 solution which, in addition to reducing the imine bonds between probes and slides, also converted unreacted aldehydes to hydroxyl groups. The NaBH_4 wash consisted of a 5 min rinse in 50 ml of reducing solution prepared from 38 ml of 0.1 mol L^{-1} pH 7.0 phosphate buffer, 12 ml of absolute ethanol, and 0.14 g of NaBH_4 . Next, slides were rinsed in deionized water for 2 min, followed by a 5 min surfactant rinse with 0.05 % (w/v) Tween 20 in deionized water, and another 2 min rinse with deionized water. All rinse steps were performed under gentle mixing on a rotator (Fisher Scientific, Suwanee, GA). Lastly, slides were dried under a nitrogen stream.

Microarray hybridization

Hybridization samples consisted of 1:1 solution mixtures of TD2 Cy5-labeled DNA targets and complementary PD1 DNA strands (Table 1), prepared at 0.2 $\mu\text{mol L}^{-1}$ concentration of each strand in hybridization media ranging from deionized water up to 1 mol L^{-1} pH 7.0 phosphate buffer, with no other additives. Microarray slides were placed inside hybridization chambers which were filled with the target solution. Adhesive tape was used to seal each of the chamber portals, and the chambers were inserted into a hybridization oven at 37 °C for 16 hours without shaking, after which the solution was pipetted out and the chambers were disassembled. Hybridized slides were rinsed at room temperature in deionized water for 1 min, dried with nitrogen gas, and scanned.

Microarray analysis

Microarray slides were scanned on a ScanArray Express scanner (Perkin-Elmer, Fremont, CA) at 95 % laser power, PMT sensitivities between 40 % and 80 %, and scanning resolution of 5 μm . Scanner channels 1 and 4, corresponding to 633 nm and 488 nm, were used for Cy5 and fluorescein, respectively. Images were processed with the ScanArray software. Initial location of spots was acquired from the spotter input file and was automatically aligned with the microarray image by the software. During image analysis pixels belonging to spots and background were defined as follows. Three concentric circles were defined for each spot, consisting of a “spot circle” containing only “lit” pixels, a background inner circle excluding any pixels from the spot, and a background outer circle whose size was kept sufficiently small so as to exclude lit pixels from any of the neighboring spots. Pixels within the spot circle were used as signal while pixels within the annular region defined by the background inner and background outer circles were used as background. Pixel intensities were imported into Excel, and the averaged background intensity for each spot was subtracted from the corresponding averaged spot intensity. Results from replicate spots were combined to provide final averaged hybridization intensities and standard deviations.

Measurement of MO mass spectra

Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (see Supplementary Material) was used to compare NaBH_4 -treated with untreated MO probes to consider the possibility that, during washing of printed arrays, NaBH_4 may have

reduced bonds (e.g. –C=N-bonds on nucleic bases) other than the imine used for immobilization.

RESULTS AND DISCUSSION

Solution studies

Solution melting studies were used to test prospective hybridization buffers for selectivity for MO/DNA over DNA/DNA hybridization. Buffers that selectively destabilize DNA/DNA hybridization can be used to reduce secondary structure in the sample, thus improving availability of sample sequences and reducing secondary structure bias on array hybridization.[43; 44] Buffers tested consisted of pH 7.0 sodium phosphate buffers of various concentrations (0, 0.012, 0.037, 0.11, 0.33, and 1 mol L⁻¹), with and without presence of SDS (0.1 % w/v). SDS is often used to suppress nonspecific adsorption of DNA targets to solid supports. Additional experiments tested solutions (0.05 % w/v) of the nonionic surfactant Tween 20 in deionized water. Melting temperatures T_m were determined as described in the Supplementary Material.

Figure 2A shows that DNA/DNA melting exhibited expected dependence on buffer concentration C_B , with higher ionic strengths corresponding to greater stability of duplexes and thus higher T_m values. Duplex formation was suppressed in deionized water (0 mol L⁻¹). The dependence of T_m on C_B was minimally affected by SDS, Figure 2B, except at the lowest ionic strength of 0.012 mol L⁻¹ for which the ~ 0.0035 mol L⁻¹ concentration of SDS contributed significantly to the total Na⁺ concentration. These trends are better visualized in Figure 2E which plots the DNA/DNA T_m values (red curves) versus C_B , and also demonstrates good consistency with values predicted from DINAMelt [45] (green curve).

In contrast to DNA/DNA melting, MO/DNA T_m values were nearly independent of ionic strength in the absence of SDS, Figure 2C, as also recently observed for a different sequence.[18] The weak, if any, dependence of T_m on C_B is consistent with absence of electrostatic repulsion between strands due to charge neutrality of morpholinos. The decreased hyperchromicity change accompanying melting in 1 mol L⁻¹ buffer may reflect weakened base stacking interactions, as for PNA/DNA melting.[20] Strikingly, and in contrast to DNA/DNA melting, addition of SDS caused the T_m to acquire a dependence on ionic strength, accompanied by pronounced variations in hyperchromicity, Figure 2D. Plotting T_m against buffer concentration (Figure 2E, black dashed line) shows that, in the presence of SDS, the relationship between T_m of MO/DNA duplexes and C_B was step-like.

The effect of SDS on T_m suggests that SDS associates with unhybridized morpholino strands. SDS possesses a twelve carbon hydrophobic tail that could interact with the somewhat hydrophobic MOs. More direct evidence comes from SDS-PAGE experiments in which the SDS-containing carrier imparted mobility to otherwise immobile MO oligomers, producing smeared bands. The unusual zig-zag variation of T_m with buffer concentration (Figure 2E) suggests that the degree of complexation depends on ionic-strength. Such dependence may be expected since SDS is negatively charged, so that already associated SDS molecules would electrostatically oppose additional binding, with discrete addition of further molecules only if certain ionic strengths are exceeded. Such a physical mechanism could explain the step-like variation in T_m with ionic strength, Figure 2E. Association of SDS to MO strands would be expected to also affect base stacking, explaining the observed variations in hyperchromicity of melting. Lastly, SDS/MO interactions should lower the T_m of MO/DNA duplexes, in agreement with Figure 2E, since hybridization of SDS-decorated MO strands with DNA would be penalized by presence of the negative SDS charge, while possibly also requiring energetically-costly displacement of bound SDS.

Addition of 0.05 % w/v Tween 20, a nonionic surfactant, also suppressed MO/DNA hybridization, decreasing T_m from 66 °C to 52 °C in deionized water. This result provided further evidence of the tendency for surfactants and morpholinos to associate.

In summary, optimal conditions for favoring MO/DNA over DNA/DNA hybridization were low ionic strengths and the absence of surfactants. These conditions were therefore selected for further testing in microarray hybridizations.

Microarray fabrication

For printing, amine-terminated MO probes were dissolved in various spotting media including pH 9.0 sodium carbonate buffers at concentrations from 0.05 to 0.8 mol L⁻¹, 1:1 mixtures of these buffers with dimethyl sulfoxide (DMSO), pure deionized water, and 0.1 mol L⁻¹ sodium phosphate buffers at pH from 4 to 9. Fluorescein-labeled PM2 probes (Table 1) were used to visualize the resultant spot morphology. Morpholinos printed well from all aqueous solutions (e.g. columns A through F, Figure 3), but addition of 50 % DMSO by volume to an aqueous buffer interfered with immobilization (e.g. columns G and H, Figure 3). The various print conditions produced MO spots with a diameter of 100 to 120 μm.

Following printing, slides were washed with a solution containing NaBH₄ to reduce the imine bonds between probes and surface aldehydes, while deactivating any remaining aldehyde groups to hydroxyls. Due to concern that NaBH₄ may reduce other bonds, such as –C=N-bonds on nucleic bases, MALDI-TOF mass spectrometry was used to compare NaBH₄-treated with untreated PM0 probes to look for hydrogen incorporation. PM0 probes were kept for 15 min in the NaBH₄ reducing solution, followed by purification by two passages through a NAP 10 column filled with deionized water. To maintain the same processing conditions, untreated probes were also passed twice through a NAP 10 column. The resultant mass spectra were dominated by the same features for both treated (Figure 4A) and untreated (Figure 4B) probes, with main peaks appearing at mass/charge (m/z) ratios of around 5070, 5092, and 5114, in reasonable agreement with the probe molecular mass without (5068 Da) and with one (5091 Da) or two (5114 Da) complexed sodium atoms. Not surprisingly, the NaBH₄ treated sample exhibited more extensive association with Na⁺. Had significant incorporation of hydrogen taken place, additional peaks would be expected on the high m/z sides of the main peaks with separations of about 2 in m/z (e.g. corresponding to reduction of an imine bond). Comparison of Figures 4A and 4B indicates that, even after a three-fold longer exposure than used in array fabrication (15 vs 5 min), NaBH₄ treatment did not significantly modify the MO probes.

Fabrication of microarrays revealed a strong propensity of MO probes for adsorption. This propensity was evidenced by printing of PM3 probes, which were identical to PM2 but without a terminal amine for immobilization (Table 1); hence, PM3 probes were not expected to immobilize. Nevertheless, printing of PM2 and PM3 side-by-side produced PM3 probe intensities about 40 % of those of PM2 spots, as measured after NaBH₄ reduction and a 2 min wash with 0.1% w/v SDS in deionized water. Hybridization of thus prepared arrays to complementary, Cy5-labeled TD2 targets resulted in target intensities from PM3 spots about 17 % of those from PM2 spots, demonstrating that printed PM3 probes were also capable of significant hybridization. Compared to DNA probes, the lower water solubility of MO oligomers is expected to enhance their adsorption, an interpretation also consistent with electrochemical measurements which concluded that unhybridized MO layers under aqueous buffers existed in a collapsed, desolvated state.[38; 39]

Various washes were tested for removal of physisorbed probes including deionized water at temperatures of up to 75 °C, solutions of SDS (0.1 % w/v) or Tween 20 (0.05% w/v) in deionized water, and organic solvents including DMSO and acetonitrile. Washes were

performed on slides that had been printed, dried overnight, and treated with NaBH_4 . To better distinguish covalent from physical immobilization, slides were printed with PM2 and PM3 probes from 0.1 mol L^{-1} phosphate buffers of various pH, as well as from deionized water. Covalent immobilization should exhibit dependency on pH since reactivity of the terminal MO amines increases at higher pH. All printing solutions used $10 \text{ } \mu\text{mol L}^{-1}$ probe concentrations. After washing, arrays were hybridized for 16 h at 37°C to $0.1 \text{ } \mu\text{mol L}^{-1}$ solutions of Cy5-labeled TD2 target in 0.1 mol L^{-1} pH 7.0 phosphate buffer.

The most successful procedure for removal of physisorbed probes was a 5 min wash with 0.05 % solution (w/v) of Tween 20 in deionized water. Tween 20 is a nonionic surfactant often employed as a washing agent in immunoassays and as a solubilizing agent for membrane proteins.[46; 47] As shown in Figure 5A, hybridization of slides washed with Tween 20 produced strong hybridization signals from PM2, but not PM3, spots that were printed side-by-side and hybridized simultaneously. Moreover, Figure 5B illustrates that PM2 hybridization intensities exhibited the expected pH dependence for covalently immobilized probes, increasing for spots printed from higher pH buffers. These results indicate that PM2 probes with a terminal amine immobilized at coverages significantly higher than otherwise identical PM3 probes that lacked a terminal amine. The much reduced hybridization intensity from PM3 spots moreover confirmed that Tween 20 washing was effective for removing physically adsorbed MO probes. Based on these results, a post-printing wash with Tween 20 was incorporated as a standard step in microarray fabrication.

The influence of spotting concentration on probe coverage was examined by printing PM2 probes from deionized water at concentrations from 5 to $40 \text{ } \mu\text{mol L}^{-1}$, and imaging the probe fluorescein intensity after reduction with NaBH_4 and a Tween 20 wash. Typically, probe intensities increased with deposition concentration, as for slides #1 through #3 in Figure 6. However, as illustrated by slides #4 and #5, for some slides in a batch printed together with the same solutions and pins the dependence of probe intensity on spotting concentration was suppressed. When observed such deviations were usually symptomatic of an entire slide. This observation prompted adoption of PM2 spot intensities as a control on consistency of slide preparation. For this purpose slides were printed with PM2 control spots at the various concentrations, in addition to PM1 spots used for hybridization. Only those slides whose PM2 intensities exhibited the expected increase with printing concentration and, at each spotting concentration, were within 20 % when compared between slides were passed for further use.

Hybridization studies

Microarray hybridizations were used to test how array hybridization competes with solution DNA/DNA associations. For these experiments sample solutions contained TD2 DNA targets and their fully complementary PD1 DNA sequences in a 1:1 stoichiometry, at a $0.2 \text{ } \mu\text{mol L}^{-1}$ concentration of each strand, with no other sequences added; under these conditions targets were free from solution interactions other than with themselves or their perfectly complementary sister strands. Slides were printed with sub-arrays of both fluorescein-labeled (PM2) and unlabeled (PM1) probes from 5, 10, 20, 30 and $40 \text{ } \mu\text{mol L}^{-1}$ probe concentrations in deionized water. The slides were dried for 22 h, followed by NaBH_4 reduction and a Tween 20 wash as described above. Consistency of slide preparation was judged by comparing probe intensities from the PM2 control spots, as discussed in connection with Figure 6; only those slides that met the selection criteria were used for hybridization.

Hybridizations were performed to the sub-arrays of unlabeled PM1 probes to avoid possibility of cross-talk between probe and target labels. All hybridizations were for 16 h at 37°C in pH 7.0 phosphate buffers at concentrations C_B of 0.012, 0.037, 0.11, 0.33 and 1

mol L⁻¹, as well as in deionized water. Following hybridization, slides were washed (1 min, deionized water) and scanned at 45 %, 60 % and 80 % PMT sensitivity, all at 95 % laser power. Further analysis was performed on images taken at 60 % sensitivity. Since washing of slides prior to scanning presumably perturbed any equilibrium that may have been realized, analysis was restricted to a comparison of trends. Control experiments for nonspecific target adsorption, using a noncomplementary morpholino probe (5'-NH₂-TTTTAAATTCTGCAAGTGAT), produced about a 30-fold lower intensity than hybridizations to complementary probes.

As shown in Figure 7A, hybridization increased monotonically with probe spotting concentration from 0 to 40 μmol L⁻¹, regardless of ionic strength. Since higher spotting concentrations are expected to result in greater probe coverages, this trend indicates that higher probe coverages resulted in greater surface capacity for hybridization. As observed elsewhere for hybridization of MO probes to single-stranded DNA targets,[18] at sufficiently high coverages ($\sim 5 \times 10^{12}$ cm⁻² for 20mer probes) hybridization can become approximately independent of probe coverage; the data in Figure 7A suggest that printing at up to 40 μmol L⁻¹ probe concentrations has not yet reached this limit.

The dependence on ionic strength, Figure 7B, was more complex. Considering data for the 10 μmol L⁻¹ spots as typical (Figure 7B, green points), as ionic strength increased from 0 mol L⁻¹ the hybridization signal rose to peak at 0.037 mol L⁻¹, then sharply decreased as buffer concentration rose further. A similar trend was observed for probes spotted at the other concentrations. The initial increase with buffer concentration shows that ionic strength assisted MO/DNA surface hybridization, a trend in contrast to solution data for which ionic strength had minimal effect (Figure 2C). The influence of ionic strength, for surface hybridization, is attributed to a buildup of an electrostatic hybridization barrier from previously bound targets. An increase in ionic strength mitigates this barrier, so that greater extents of hybridization can be realized before the barrier becomes significant to suppress further binding.

The pronounced decrease in hybridization above C_B of 0.037 mol L⁻¹ can be understood by recalling the solution hybridization data in Figure 2E. The solution melting temperatures show that, at these higher C_B values, DNA/DNA duplexes are stabilized relative to same sequence MO/DNA duplexes. Therefore, under these conditions hybridization of DNA targets to an MO microarray becomes unfavorable because the targets have a thermodynamically more attractive option to hybridize with complementary DNA in solution. Consistent with this interpretation, a decrease in MO/DNA surface hybridization at higher C_B was not observed for single-stranded DNA targets;[18] i.e. in the absence of competition from solution hybridization. Based on the above results, optimal ionic strengths for MO microarrays are expected to be at or below that of the 0.037 mol L⁻¹ phosphate buffer.

CONCLUSIONS

This study described fabrication of microarrays based on morpholino nucleic acid analogues, and suggested optimization of array performance by tuning the balance between on-array versus solution interactions. Quill pin printing of morpholinos from various aqueous buffers onto aldehyde slides produced spots with good uniformity. The most convenient approach, which was adopted for array fabrication, was to print morpholinos directly from deionized water. In addition to covalent immobilization of amino-terminated morpholinos to aldehyde slides, morpholinos were shown capable of stable physical adsorption. Physically adsorbed morpholinos could be efficiently removed with a wash incorporating the nonionic surfactant Tween 20.

Not surprisingly, hybridization of morpholino microarrays required readjustment of protocols developed for DNA microarrays. Morpholinos are uncharged and more hydrophobic than DNA; these differences in physical characteristics can lead to unexpected outcomes. For example, solution measurements showed that in the presence of charged surfactants MO/DNA hybridization acquired a dependence on ionic strength and that surfactants tended to suppress MO/DNA hybridization, an effect attributed to complexation between the surfactant and MO oligomers. Addition of surfactants to hybridization cocktails, often implemented for DNA microarrays, should therefore be carefully reconsidered for MO microarrays.

Another difference is that DNA microarrays cannot be operated under conditions that would denature secondary structure in the sample, since the microarray reaction would then also be suppressed. In contrast, by functioning under low ionic strengths, MO microarrays can operate under conditions that destabilize secondary structure in solution and therefore improve sample availability, as well as moderate biasing of array hybridization due to base pairing in solution. This benefit was confirmed by studies in which DNA targets were hybridized to MO microarrays in the presence of complementary DNA in solution. These studies also revealed a need for balancing minimization of solution secondary structure, favored by low ionic strengths, with maintenance of sufficient ionic strength to screen the electrostatic barrier to hybridization that accrues from bound targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Comparison of DNA, PNA and morpholino oligomer structures. B = adenine, cytosine, guanine, thymine. The morpholino structure also shows the terminal modification used for immobilization.

Fig. 2.

Melting curves, measured on cooling, for (A) DNA/DNA duplexes in phosphate buffers without SDS, (B) DNA/DNA duplexes in phosphate buffers with 0.1 % (w/v) SDS, (C) MO/DNA duplexes in phosphate buffers without SDS, and (D) MO/DNA duplexes in phosphate buffers with 0.1 % (w/v) SDS. Legends indicate buffer concentration in molarity of phosphate groups. DNA/DNA duplexes consisted of PD1 and TD1 oligodeoxyribonucleotides, while MO/DNA duplexes were prepared from PM1 and TD1 (Table 1). The sets of melting curves shown in (A)-(D) have been aligned at the low temperature limit by vertical translation, followed by normalization of each curve in a set to the highest hyperchromicity change between the random coil and duplex states observed for that set. This normalization preserves the relative hyperchromicity change between curves. (E) Summary of T_m values for all investigated conditions, together with DINAMelt predictions for PD1/TD1 DNA duplexes. For MO/DNA curves, the arrow indicates deionized water conditions.

Fig. 3.

Spot morphologies for fluorescein-labeled PM2 probes, printed from various buffers. The image was taken after the NaBH_4 reduction step, at 95 % laser power and 50 % PMT sensitivity. **Column A:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.05 mol L^{-1} carbonate buffer (CB), pH 9.0; **B:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.1 mol L^{-1} CB, pH 9.0; **C:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.2 mol L^{-1} CB, pH 9.0; **D:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.4 mol L^{-1} CB, pH 9.0; **E:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.8 mol L^{-1} CB, pH 9.0; **F:** $20 \mu\text{mol L}^{-1}$ PM2 from water+DMSO (v/v=1); **G:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.1 mol L^{-1} CB+DMSO (v/v=1); **H:** $20 \mu\text{mol L}^{-1}$ PM2 from 0.2 mol L^{-1} CB +DMSO (v/v=1).

Fig. 4. MALDI-TOF mass spectra of (A) untreated and (B) NaBH_4 -treated PM0 probes.

Fig. 5. Hybridization of single-stranded TD2 targets to a microarray of alternating rows of PM2 and PM3 spots printed from 0.1 mol L^{-1} phosphate buffers of different pH, at $10 \text{ } \mu\text{mol L}^{-1}$ probe concentration. **(A)** Scanned image, corresponding to the Cy5 target channel at 45 % PMT sensitivity and 95 % laser power. **(B)** Mean spot intensities of each row in (A), with error bars showing standard deviation based on 20 spots per row.

Fig. 6.
Slide selection: slides #1 through #3 satisfy the criterion of acceptable variation between slides.

Fig. 7.

Hybridization of PM1 morpholino microarrays to solutions containing a 1:1 stoichiometric mixture of TD2 and PD1 DNA strands, where PD1 is fully complementary to TD2. **(A)** Mean hybridized intensity of 20 spot replicates as a function of printed probe concentration, at various buffer strengths C_B . **(B)** Same data as (A), but plotted as a function of C_B , at various printed probe concentrations. Settings: 95 % laser power, 60 % PMT sensitivity.

Table 1

Probe and target sequences.

Abbr.	Type	Sequence	Purpose
PM1	MO	5' -NH ₂ -GTAGCTAATGATGTGGCATCGGTTG	unlabeled probe for study of array and solution hybridization
PM2	MO	5' -NH ₂ -TAGCTAATGATGTGGCATCGGTTGC-Fluorescein	fluorescent probe for study of array fabrication
PM3	MO	5' -TAGCTAATGATGTGGCATCGGTTGC-Fluorescein	fluorescent immobilization control (no amine group)
PM0	MO	5' -CATACTTCGATCGAT	oligo for MALDI-TOF controls
PD1	DNA	5' -GTAGCTAATGATGTGGCATCGGTTG	probe for study of solution hybridization
TD1	DNA	5' -CAACCGATGCCACATCATTAGCTAC	target for study of solution hybridization
TD2	DNA	5' -CAACCGATGCCACATCATTAGCTAC-Cy5	fluorescent target for study of array hybridization