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One-step immobilization of aminated and thiolated DNA onto poly(methylmethacrylate) (PMMA) substrates

F. Fixe,[ab] M. Dufva,[c] P. Telleman[c] and C. B. V. Christensen[c]

[a] *INESC, Microsistemas e Nanotecnologias (INESC-MN), Rua Alves Redol, 9, 1000-029 Lisboa, Portugal*

[b] *Center for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*

[c] *Microarray Group, MIC – Department of Micro and Nanotechnology, Technical University of Denmark, Ørsteds Plads, Bld. 345 east, DK-2800 Lyngby, Denmark*

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Direct immobilisation of modified DNA oligonucleotides (aminated or thiolated) onto a plastic substrate, poly(methylmethacrylate), (PMMA) is described. Using the methyl esters present on non-modified PMMA, it was possible to establish a covalent bond between the electron donor of a DNA probe and the C terminal ester of the PMMA substrate. Since the procedure consists of a single brief wash in isopropanol or ethanol, the procedure is simple and enviromentally friendly. The new immobilization strategy was characterized by analysing DNA microarray performance. The new procedure resulted in probe- and hybridization densities that were greater or equivalent to those obtained with commercially available surfaces and other procedures to immobilize DNA onto PMMA. The described chemistry selectively immobilized the DNA *via* terminal thiol or amine groups indicating that probe orientation could be controlled. Furthermore, the chemical bond between the immobilized DNA and the PMMA could endure repeated heat cycling with only 50% probe loss after 20 cycles, indicating that the chemistry could be used in integrated PCR/microarray devices.

Introduction

Microarrays have become a very powerful tool in the field of biological, medical and nanoscience research, with a wide range of applications, mainly due to the highly parallel throughput in processing and analyzing of different kinds of samples like mRNA, cDNA and proteins.[1–4] These so-called biochips are replacing labour-intensive and reagent-consuming, gel-based methods. DNA microarrays can be produced, essentially in two different ways: by on-chip synthesis or by immobilization of pre-synthesized DNA on the selected substrate. The on-chip synthesis method, requires sophisticated robotic equipment and financial resources.[5–6] and that is why the immobilization procedure is less expensive and more established in research laboratories and in commercial applications.[7]

One of the most important parameters regarding robustness and fidelity of biochips is the stability and the accessibility of the DNA immobilized on the surface. Pre-synthesized DNA can be covalently immobilized onto various solid supports like glass, oxidized silicon, membranes, crystalline silicon and plastics.[7–19] Plastic polymers are attractive materials since they allow low production costs as well as the possibility to integrate microelectronics technology onto the chips.[20–23] The use of polymers for the construction of analytical microdevices is an emerging area of importance due to the impact that such miniaturized devices will have on different areas like diagnostics in medicine and environmental monitoring.[24–26]

Polypropylene is one of the most used polymers for the construction of these microdevices.[27] PMMA is also gaining popularity, because of the ability to chemically modify the surface,[20,28–29] like yielding aminated surfaces enabling attachment of biomolecules, *e.g.*, enzymes and oligonucleotides.

In this report we describe a new and simple way of immobilizing DNA onto PMMA surfaces, without previous chemical treatment of the surface.

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Materials and methods

Materials

All chemicals and solvents used were of analytical grade and were purchased from Sigma (Vallensbæk, Denmark), or Merck (Albertslund, Denmark), unless stated otherwise, and used without additional purification. All oligonucleotides were ordered from TAGCopenhagen (Copenhagen, Denmark), and purified by HPLC by the manufacturer.

PMMA cleaning step

PMMA sheets were obtained from Röhm, (Darmstadt, Germany). Microarray substrates of 75 × 25 mm were obtained by cutting the PMMA with a CO_2 laser (Synrad Inc., Mukilteo, WA, USA). After cutting the PMMA, surfaces were cleaned by soaking the substrate in isopropanol (IPA) or ethanol, for 10 min, and rinsed thoroughly with MilliQ water. After this cleaning step the PMMA surface was used for direct immobilization of 5′-end aminated or thiolated DNA probes.

PMMA modification

Two different procedures were used to chemically modify PMMA; as proposed by Bulmus *et al.*[30] and by aminolysis.[29] Both protocols are described in Fig. 1, and result in aminated PMMA surfaces. The aminated surfaces were further activated by sulfo-EMCS as described below.

Glass silanization

Glass slides (75 × 25 mm) were first cleaned and oxidized in nitric acid (5%) for 1 h at 90°C and washed three times in MilliQ water for 5 min each. The silanization protocol,[31] was done using APTES (3-aminopropyltriethoxysilane) 8% (v/v), pH 3.0, for 2 h at 80 °C. After washing with MilliQ water (twice, 5 min each), substrates were dried for 16 h at 115 °C.

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DNA microarrays

DNA was spotted onto the different substrates using a Cartesian PixSys 5500 spotting robot (Huntingdon, UK) equipped with a Stealth pin (Telechem, Sunnyvale, CA, USA) depositing approximately 0.6 nL per spot on the substrates. The size of the spots ranged from approximately 20 to 100 μm, separated by 300 μm, depending on the hydrophobicity of the surface.

Immobilization of DNA probes to non-activated PMMA substrates.

After the PMMA cleaning step described above, DNA probes ((probe-NH_2: 5′-NH_2-ATG CAA AGC CCG ATG ACG-Cy3-3′) or (probe-SH: 5′-SH-ATG CAA AGC CCG ATG ACG-Cy3-3′)) were dissolved in borate buffer (100 mM, pH 11.5) and spotted onto the substrates as described above. The substrates were incubated from 15 min to 24 h in a humidified chamber at 30 °C. The incubated slides were washed with 5× SSC + 0.1% Tween 20 for 15 min, rinsed with MilliQ H_2O, and dried.

Immobilization of DNA probes to sulfo-EMCS activated PMMA or glass substrates

The aminolyzed (aminated) PMMA surfaces were activated using sulfo-EMCS as described previously[31] (Fig. 1C). Briefly, the primary amino groups on the substrate (Fig. 1A and B) were reacted with the NHS ester moiety present on the cross-linker sulfo-EMCS for 2 h (pH 9), to yield maleimide reactive groups. After substrate surface cross-linking, the DNA probes were spotted and incubated for 2 h in a humidified chamber at 30 °C. The incubated slides were washed with 5× SSC + 0.1% Tween 20 for 15 min, rinsed with MilliQ H_2O, and dried.

Hybridization

Non-activated spotted PMMA microarrays were pre-hybridized using 2% BSA in PBS (10 mM), for 2 h at room temperature.

Activated and spotted PMMA and glass microarrays were blocked using sodium borohydrate (Na_2BH_4) or 2% BSA in PBS (10 mM), for aldehyde- and maleimide-activated substrates, respectively.

Complementary DNA target (5′-end Cy5-labeled: CGT CAT CGG GCT TTG CAT) and non-complementary target (5′-end Cy5-labeled: GCC AGG AAT ACC CAG TCA) solutions were prepared (concentration range between 10 nM and 1 μM) in 6× SSC + 0.6% SDS + sperm ssDNA + 2% BSA. Hybridization was carried out under coverslips in a humidified chamber (30–40 °C) for 30 min to 5 h. To remove the non-hybridized DNA target the coverslip was removed, and the surfaces were washed in 0.1× SSC + 0.5% SDS for 15 min, followed by 5 min in 0.1× SSC solution.

DNA immobilization and hybridization quantification

The slides were scanned using a ScanArray Lite scanner (Packard BioScience, Billerica, MA, USA) and the spots were quantified using Optiquant™ quantification software (Packard Bioscience). Standard curves were prepared by diluting fluorescent Cy3-labeled amino and thiolated DNA probes in 0.1 M borate buffer, pH 11.5, to a final concentration ranging from 0.02 μM to 5 μM. Each dilution was spotted in 10 replicates on non-modified PMMA,

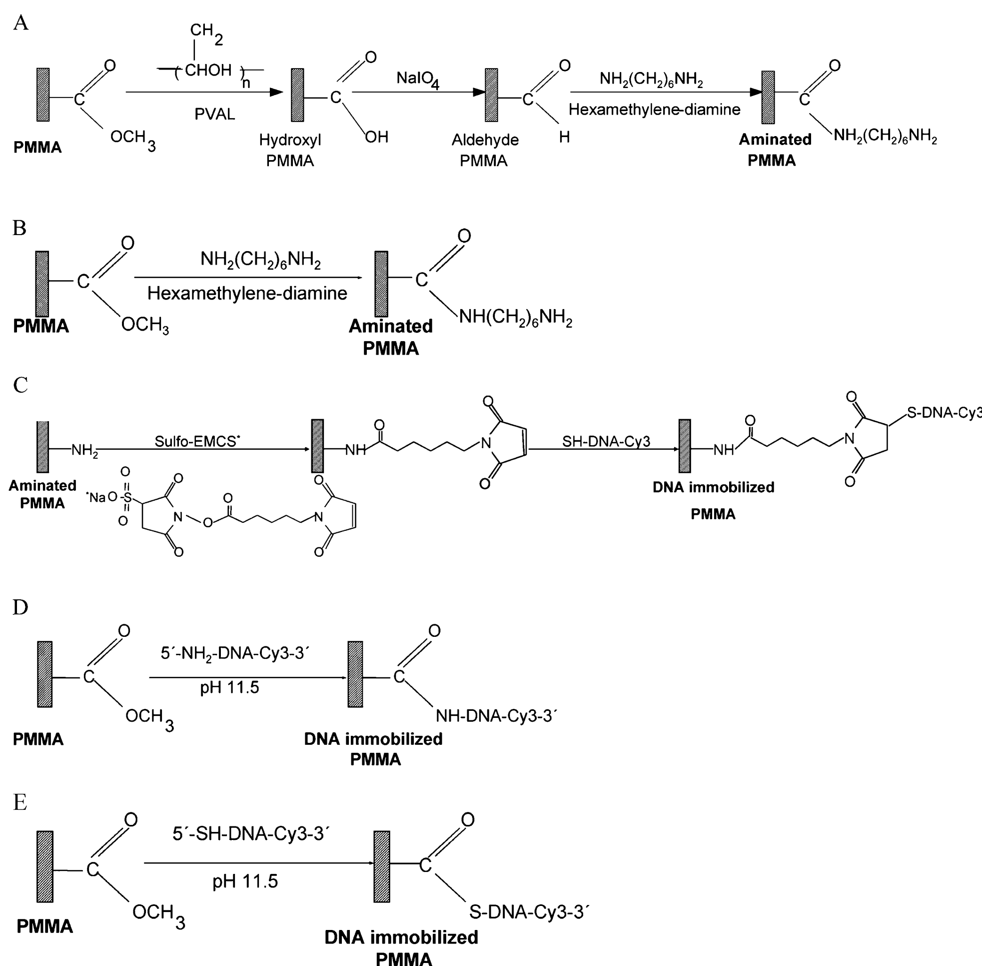

Fig. 1 Schematic outline of reactions used for chemical modification of PMMA surfaces and for immobilizing DNA on PMMA. (A) Aminolysis, as described by ref. 29. (B) Chemical modification, as described by ref. 30. (C) Immobilizing thiolated DNA to aminated PMMA surfaces. The aminated PMMA is reacted with the NHS ester group of the heterobifunctional crosslinker sulfo-EMCS and subsequently the maleimide portion of the sulfo-EMCS is reacted with 5′-end thiolated DNA probe, to form a covalent bond between DNA and PMMA substrate. (D) Immobilization of 5′-end aminated or (E) thiolated DNA probes to PMMA slides, using a buffer solution with pH 11.5.

chemically-modified PMMA and glass. The same procedure was adopted for Cy5-labeled DNA targets, but the range of dilutions for the DNA target was between 5 nM and 1 μM.

PCR heat cycling

To evaluate the stability of the probe immobilization, DNA microarrays were subjected to 5, 10, 15 and 20 cycles of PCR (MJ-research, PTC-200, Merck). The microarrays were confined with a Gene frame (Abgene, Epsom, UK) filled with PCR buffer and $MgCl_2$ solution (Roche, Hvidovre Danmark). PCR parameters were: 94 °C for 30 s, 54 °C for 30 s and 72 °C for 45 s. After PCR cycling the microarrays were rinsed thoroughly with MilliQ water and the hybridization reaction was performed as described above.

Results and discussion

The most widely used approach to functionalize solid supports with biomolecules is by means of introducing amine groups onto the surface. The utility of amines stems from their high nucleophilicity and the existence of a wide range of amine-based coupling chemistries.[32] The most known chemistry to achieve amino-terminated surfaces is silanization and it is well described on glass.[33] Until now, PMMA has been modified by silanization procedures,[30] and aminolysis,[28–29] to yield aminated PMMA sheets. The chemistry proposed by Bulmus *et al.*[30] (Fig. 1A) involves a first step with PVAL to promote the acidic hydrolysis of the esters. The hydroxyl groups are then converted to aldehyde groups by periodate oxidation and finally reacted with an amino compound to yield an amine through the addition of a nitrogen nucleophilic group.[34] The aminolysis chemistry described in Fig. 1B[29] is based on the nucleophilic addition–elimination that esters undergo at their acyl carbon atoms when treated with primary or secondary amines.[35]

Here, we have explored whether a non-modified PMMA surface can be used for direct immobilization of 5′-end aminated or thiolated oligonucleotides (Fig. 1D and E). These reactions are based on the aminolysis reaction of esters in the presence of electron donators, at basic pH conditions.[35] The binding of DNA to PMMA is most likely an SN2 reaction in which the primary amino groups as well as the thiol groups on the DNA are involved in a nucleophilic attack on electron-poor carbon on the surface esters of the PMMA. Both reactions result in elimination of a methanol from the ester and the reactions follow a second-order kinetics.

This novel approach for immobilization of DNA to PMMA was compared to aminated PMMA surfaces obtained by silanization and aminolysis (Fig. 1A–B), and further activated by sulfo-EMCS (Fig. 1C). To that end, DNA probes (concentrations ranging from 0 to 20 μM) were spotted on PMMA slides prepared by the different methods (Fig. 1) and the immobilization reaction was allowed to proceed for 2 h. Immobilization kinetics studies showed that after 1 h the surface was saturated with 5′-end modified probes on PMMA (data not shown). Both aminated and thiolated probes could be immobilized onto sulfo-EMCS activated PMMA and directly onto non-activated PMMA. The maximum probe attachment was achieved using DNA concentrations of 8 μM or higher in the spotting buffer (Fig. 2). The direct immobilization of aminated and thiolated DNA to PMMA resulted in similar or higher immobilization densities compared to the other two immobilization chemistries (Fig 1A, B and C) relying on sulfo-EMCS crosslinking (Fig. 2).

The variance of the hybridization signal obtained from the direct coupling chemistry onto PMMA appeared higher (CV = 25%), suggesting that the direct coupling chemistry is less stable than the other two chemistries tested. However, the variance observed previously using the aminolysis and the method by Bulmus *et al.*[30] for coupling DNA to PMMA was 20%[29] indicating that all three coupling methods are similar in performance. We have, however, noticed that coupling reactions fail occassionally, one explanation being a variable amount of free ester on the surface of the PMMA. Such variation of ester density could explain the discrepancy between the observed hybridization variances for the different chemistries.

To demonstrate the specificity of the immobilization chemistry, increasing concentrations of capture probes (0, 2, 5, 8, 10 and 20 μM) without the reactive group on the 5′-terminus, were spotted on non-modified PMMA slides. After 2 h of immobilization, the amount of non-functionalized probes on the surface was 0.20 ± 0.04 pmol cm^{-2}, independent of the initial DNA concentration present in the spotting solution (4–6% of the signal as compared to that obtained from thiolated/aminated DNA probes). These results suggest that immobilization of thiol or amino modified oligonucleotides is specific to the thiol or amino modification, and that the nucleic acid in itself does not to a large extent contribute to the immobilization (data not shown). This also suggests that the DNA protrudes from the PMMA surface.

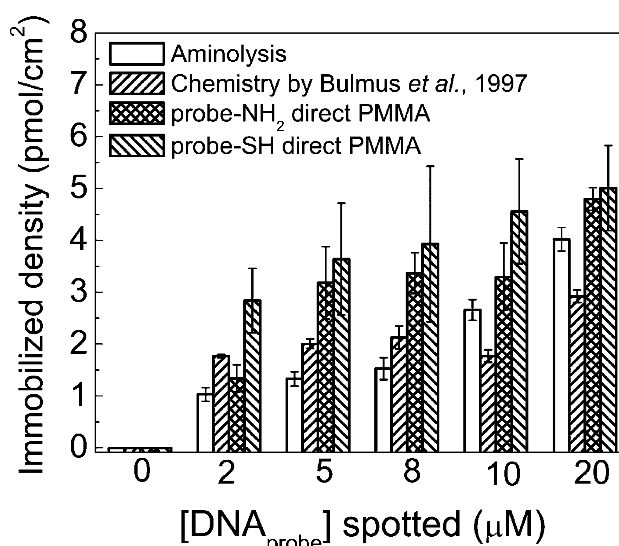

Fig. 2 Immobilized DNA densities on different PMMA surfaces. The different surfaces were spotted with aminated or thiolated DNA probes and incubated for 2 h. The slides were washed and scanned and the fluorescent signals were quantified and compared to a standard curve to obtain the probe densities on the surface as described.[29] The bars represent average probe density values from three independent experiments and the error bars represent standard deviations.

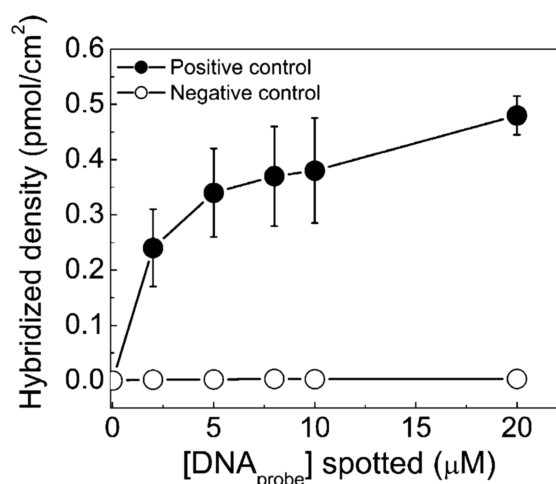

Fig. 3 Specificity of hybridization on non-activated PMMA surfaces. The 5′-end thiolated DNA probes were immobilized for 2 h and hybridized with 0.2 μM target solution for 2 h. The specificity of the DNA probes was tested by hybridization with a complementary (closed circles) and with a non-complementary (open circles) DNA target solution. The signal from the negative control represents less than 1% from the signal obtained with a complementary DNA target. Hybridized density values are from three independent experiments and the error bars represent standard deviations.

The specificity of the DNA probes directly attached to PMMA was evaluated by immobilizing thiolated DNA for 2 h on non-activated PMMA slides and subsequently hybridizing with a complementary and a non-complementary DNA target for 2 h using a 0.2 μM target DNA solution. Hybridization of the non-complementary DNA target (the negative control) did not result in significant signal after hybridization, proving the specificity of the hybridization (Fig. 3). The signal-to-noise ratio observed between the signal from the positive control and the negative control was between 140 and 160. It should be mentioned that 0.2 μM DNA target saturated the hybridization signal, since 0.5 μM and 1 μM target solutions did not increase the hybridization signal. Furthermore, the hybridization kinetics were studied for 24 h and showed that after 2 h of hybridization, the signal reaches a plateau (data not shown).

In order to evaluate the performance of non-activated PMMA as a substrate for DNA microarrays, the hybridization signals were compared with the hybridization signals obtained on aminolyzed PMMA or silanized glass both crosslinked with sulfo-EMCS prior to spotting. The 5′-end thiolated probes were immobilized for 2 h and subsequently hybridized with complementary DNA for 2 h. The hybridization efficiency was calculated as a ratio of the probe density and the hybridization density as described in Figs. 2 and 3 respectively. The hybridization efficiency obtained on non-activated PMMA is essentially the same as on aminated PMMA and silanized glass (8–10%, Fig. 4A). The hybridized density increases linearly with the amount of immobilized probes (Fig. 4B), for the three types of substrates studied, but the percentage of immobilized probes on the surface that are available to form hybrids only increases for immobilized densities up to 1–3 pmol cm^{-2} (Fig. 4C). Above 3 pmol cm^{-2} of immobilized probes the hybridization efficiency was constant or started to decrease as for the silanized glass substrate. Since the density of immobilized probes (3 pmol cm^{-2}) represents only 2% of a close-packed full monolayer of ssDNA (150 pmol cm^{-2}) considering that the molecules are cylinders with 20 Å of diameter and are oriented perpendicular to the plane of the surface), the hybridization efficiency should be determined not only by steric effects or probe density, but also by thermodynamic conditions between the immobilized probe and the DNA target in solution and also by the spatial orientation of the capture probe after being immobilized. The morphology of the spot after hybridization with a Cy5-labeled target to thiolated DNA probes immobilized on non-activated PMMA, aminolyzed PMMA and silanized glass are shown in Fig.

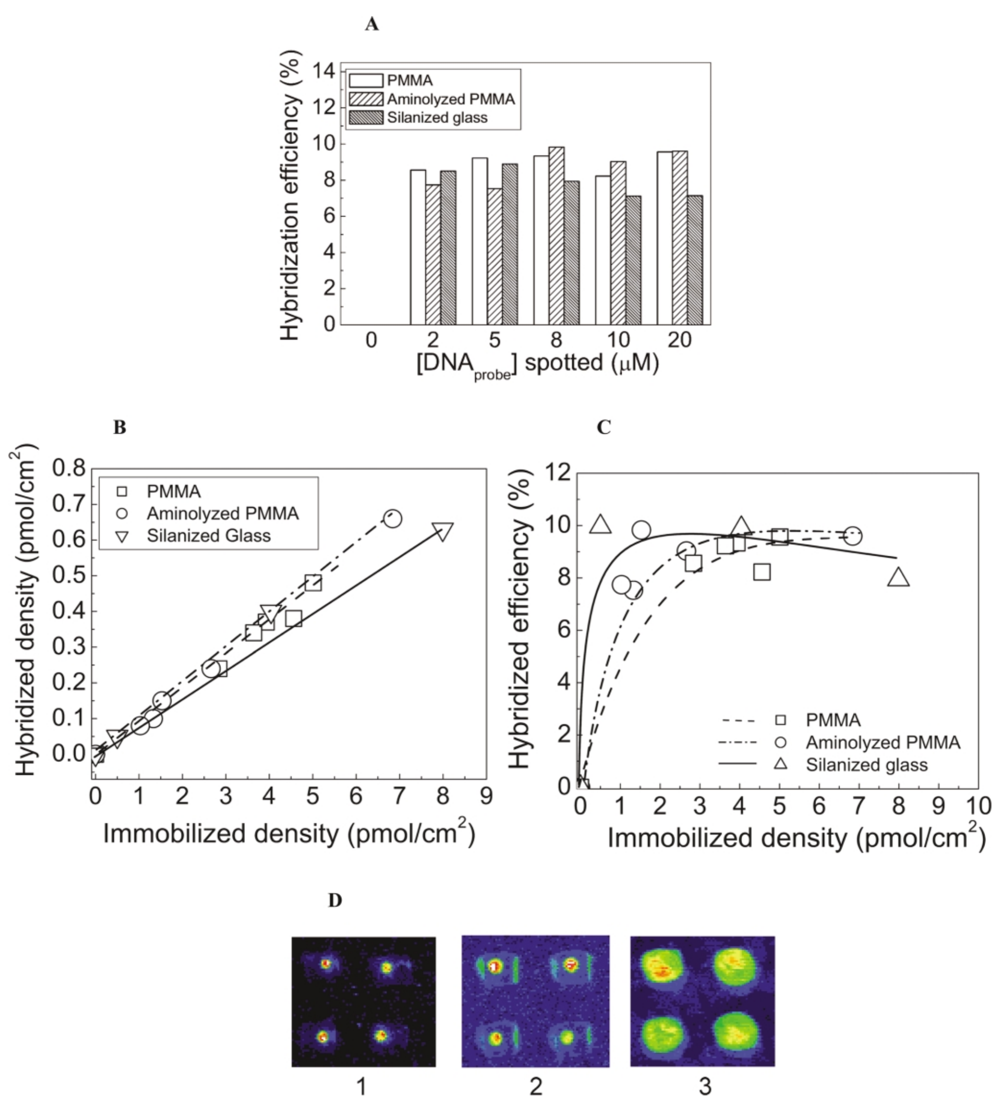

Fig. 4 Comparison of hybridization signals obtained on different substrates using different chemistries. 5′-thiolated DNA probes at different spotting concentrations were immobilized on sulfo-EMCS modified slides in the case of aminolyzed PMMA and silanized glass. On non-activated PMMA slides, the probes were directly reacted with the surface. The probes were immobilized for 2 h and were subsequently hybridized with 0.2 μM of target-Cy5 for 2 h. (A) Dependence of hybridization efficiency, achieved on surface non-activated or aminolyzed PMMA substrates and on silanized glass, on the the initial DNA probe concentration. (B) Hybridization density and (C) efficiency as a function of the density of immobilized probes on surface non-activated PMMA, aminolyzed PMMA and silanized glass. (D) Spot morphology obtained after hybridization on surface non-activated PMMA (1, spotsize = 20 μm), on PMMA modified by aminolysis (2, spotsize = 45 μm) and on silanized glass (3, spotsize = 225 μm).

4D. The spots on non-activated PMMA surfaces are smaller than on activated PMMA or glass, however, showing a faint print pin image around each spot. This is probably due to the high hydrophobicity of the unmodified PMMA substrate, condensing the spots immediately after printing. Futhermore, the backround appears to be lowest on the non-modified PMMA surfaces (Fig. 4D).

The heat stability of the DNA probes directly immobilized onto non-activated PMMA was evaluated by submitting the DNA microarrays to 0, 5, 10 and 20 heating cycles. After the heat cycling, microarrays were hybridized with a complementary Cy5-labeled target and the hybridization density was determined. After 20 PCR cycles the maximum hybridization loss observed was 60% (Fig. 5) indicating that the modified oligonucleotide probes are covalently immobilized to the PMMA surface. The expermient also demonstrates that direct attachment of DNA to unmodified substrates can be used in chips having a combined PCR and hybridization chamber.

Conclusion

We have here reported a new and straightforward method for immobilizing thiolated or aminated oligonucleotide probes directly onto the surface of non-activated PMMA. The covalent bonding between the substrate and the incoming DNA is presumably through the methyl esters of PMMA and the electron donor atom present on the 5′-end of the nucleic acid. The immobilized probes are relatively stable during PCR conditions also suggesting a covalent bond between the DNA and the PMMA surface. This new approach for DNA immobilization was compared to DNA microarrays made using activated PMMA substrates and silanized glass. The hybridization density obtained, as well as the hybridization efficiency, was equal to the other substrates. Furthermore, the non-activated PMMA had significantly lower background.

Since aminated as well as thiolated DNA could be attached to the non-activated PMMA, other molecules containing these reactive groups *e.g.* proteins, could be immobilized directly onto PMMA using the described protocol.

PMMA is an attractive material for fabricating microfluidic devices. A simple and high-performance technique to attach DNA to PMMA is particularly useful for integrating the microarray technology into disposable microfluidic devices. Future work will combine the immobilization and microfluidic techniques to develop devices with a large range of applications and advantages over the traditional glass/silicon-based DNA microarrays.

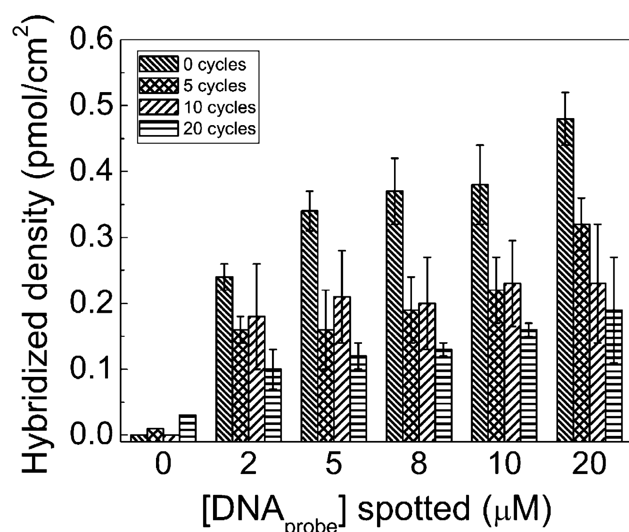

Fig. 5 Heat cycling stability of probes attached to non-activated PMMA slides. The 5′-thiolated DNA probes were immobilized for 2 h and subjected to 0, 5, 10 and 20 PCR heat cycles. Subsequently, the microarrays were hybridized with a complementary target DNA for 2 h using a DNA target solution of 0.2 μM. For each condition the hybridization signal was quantified and plotted as a function of the concentration of the DNA probes in the initial spotting solution (2, 5, 8, 10 and 20 μM). The bars represent average probe density values from three independent experiments and the error bars represent standard deviation.

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