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ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800426v · Source: PubMed

Anal. Chem. **2008,** *80,* 5892–5898

DNA Detection Using a Triple Readout Optical/AFM/MALDI Planar Microwell Plastic Chip

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A ready-to-spot disposable DNA chip for specific and sensitive detection of DNA was developed. Plastic copolymeric substrate chemistry was optimized to selectively couple the target DNA with the active chip surface. At the same time, the developed substrate limits the unspecific adsorption of probe DNA molecules or additional polar contaminants in the test samples to the chip surface. The combination of glycidyl and *n*-butyl methacrylates was found to best fit the requirements of the assay. The fabricated DNA microarrays have mechanical properties similar to those of the glass or silicon substrates and, at the same time, provide chemically reactive surfaces that do not require lengthy chemical modification. An additional advantage of the plastic microchip is its compatibility with different analytical readout techniques, such as mass spectrometry (MALDI-TOF/MS), optical detection (fluorescence and enzyme-induced metal deposition), and imaging techniques (atomic force microscopy). These multiple readout techniques have given us the ability to compare the sensitivity, selectivity, and robustness of current state-of-the-art bioanalytical methods on the same platform exemplified by successful DNA-based detection of human cytomegalovirus. The obtained sensitivity for enzymatically enhanced silver deposition (10^{-15} M) surpasses that of conventional fluorescence readouts. In addition, the assay's dynamic range (10^{-6}–10^{-15} M), reproducibility, and reliability of the DNA probe detection speaks for the silver deposition method. At compromised sensitivity (10^{-9} M), the length of the DNA probes could be checked and, alternatively, DNA single point polymorphisms could be analyzed.

The use of microarray-based technology is growing rapidly and has had considerable impact on genomic and proteomic research.[1,2] Crucial factors in microarray technology are the surface chemistry of the substrate enabling the defined immobilization of the capture molecules[3,4] and the sensitivity/selectivity of the readout associated with the instrument.[5]

Previously, glass, silicon, and quartz have been the primary DNA microarray substrates due to their low background signals during fluorescence readout.[6–8] Nevertheless, the chemical groups on the surface of the glass, silicon, and quartz solid structures are not suitable for directly immobilizing biomolecules; hence, recent chemical modification protocols as well as thin-layer coatings have been applied to the surface of glass, silicon, and quartz to bind DNA, RNA, proteins, etc. In addition, new platforms are constantly being developed to improve the ability of molecules to bind to the surface.[9]

Furthermore, the progress in the field of DNA microarray can be described in terms of (a) throughput and (b) sensitivity. The first category refers to the huge leaps that have been made in the fabrication process concerning spot densities on silicon substrates, either in situ[10] or ex situ.[11,12] Nevertheless, as mentioned above, scientists are still interested in exploring flexible and cost-effective substrate materials with which to fabricate high-throughput and reproducible DNA microarrays;[3,13] the ideal substrate is one whose material provides inherent coupling functionality.

Recently introduced materials and technologies have increased the sensitivity and reproducibility of DNA detection. New labeling methods based on enzymatic silver deposition (i.e., enzyme metallography) can overcome the deficiencies of fluorescence labels, in particular their short lifetimes.[9] To diagnose genetic

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10.1021/ac800426v CCC: $40.75
Published on Web 06/21/2008

diseases and detect infectious agents, labels such as metal nanoparticles,[14–17] enzymes,[9,18–20] and quantum dots[21,22] are usually preferred, due to their rapid and simple synthesis, reproducibility, and stability. In addition, the sensitivity and robustness of DNA chips have also increased due to the use of different readout detectors, for example, electrochemical approaches,[16,17,20] light-scattering,[15,23] surface plasmon resonance,[24] atomic force microscopy,[25] and label-free methods (e.g., MALDI-MS).[26–29]

The human herpes virus 5 or cytomegalovirus (CMV) has the largest genome (~235 kb) of the human herpes virus family, which includes herpes simplex (1 and 2), Epstein–Barr, and varicella-zoster virus.[30,31] Although CMV is not highly contagious, it is the most common congenital infection (80% of the world population is infected)[32] and is particularly hazardous in immunocompromised individuals[31,32] and pregnant women (e.g., 10% of infected newborns with CMV exhibit permanent mental retardation and auditory damage).[32,33]

Thanks to developments in the field of miniaturization in recent decades, biosensors, chips, and in particular microarrays have shown great capability to analyze viral DNA.[7,8,13] In the case of CMV, microarray technology is starting to replace traditional methods such as ELISA and latex agglutination, because microarrays allow multiplexed, parallel analysis. DNA microarrays can monitor the viral load of CMV-infected patients using only viral cultures from urine, throat swabs, or tissue samples.[32–36]

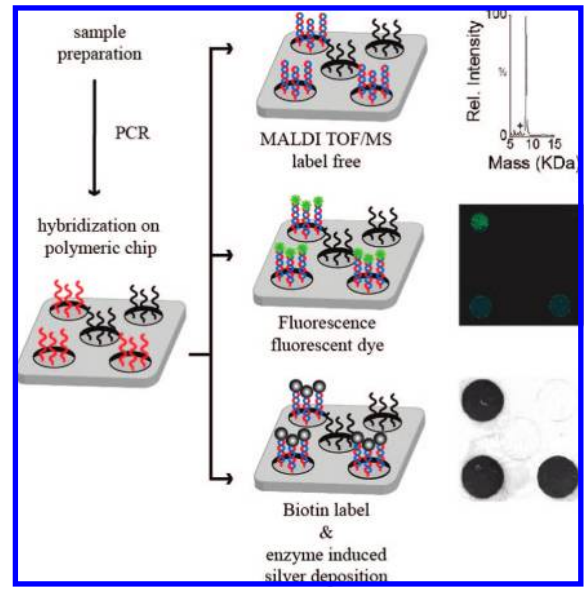

Figure 1. Schematic workflow of the multiple readouts compatible with the copolymeric planar DNA microarray chip (DNA chip). The multiple readout methods shown here are (a) MALDI-TOF/MS, (b) fluorescence, and (c) enzymatic silver enhancement.

Matrix-assisted laser desorption/ionization time-of-flight mass analyzers (MALDI-TOF/MS) have become effective tool for DNA studies,[34–36] because in contrast to fluorescence, they directly measure a physical property of the DNA (i.e., mass), thus reducing the number of false positive signals. Hence, a microarray system that couples both optical and mass spectrometry readout formats promises to be extremely sensitive and selective. Nevertheless, the use of silicon-, glass-, or quartz-based microarrays is clearly not economically optimal because of the costly material and fabrication techniques involved compared to those required by polymer microstructures that are equal quality in terms of structure resolution and planarity of the surface.[37–39]

In this paper, we describe the development of a low-cost material that can be readily reacted with biomolecules—in this case, ssDNA—to generate high-throughput microarray platforms (Figure 1). As a proof of concept, CMV DNA sequence was used to illustrate the performance of the copolymeric DNA chip. The following work extends our current research on the copolymeric plastic chips (i.e., pMALDI chips) that have so far been used for protein analysis by MALDI-MS.[40]

EXPERIMENTAL SECTION

Chemicals and DNA Sequences. Methyl methacrylate, butyl methacrylate, glycidyl methacrylate, and 2-(methacryloyloxy)ethyldimethyl(3-sulfopropyl)ammonium hydroxide (all containing hydroquinone monomethyl ether as stabilizer), Ultrasensitive Streptavidin–Peroxidase Polymer (S2438), and 3-hydroxypicolinic acid were purchased from Sigma. Benzoin methyl ether and

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Table 1. DNA Sequences of Probes and Targets Used in This Work

function	abbrev	length	sequence 5′−3′	modification
probe	0mis	30	TTT TTT CAG CAT GTG CTC CTT GAT TCT ATG	5′-aminohexyl
	NC	35	**ACT GAC TGA CTG ACT GAC TGA CTG GGC GGC GAC CT**	5′-aminohexyl
	3mis	30	TTT TTT CAG CAT **TAT** CTC CTT GAT TCT ATG	5′-aminohexyl
	1mis	30	TTT TTT CAG CAT G**G**G CTC CTT GAT TCT ATG	5′-aminohexyl
probe control	B-0mis	30	TTT TTT CAG CAT GTG CTC CTT GAT TCT ATG	5′-aminohexyl, 3′-biotin
	F-0mis	30	TTT TTT CAG CAT GTG CTC CTT GAT TCT ATG	5′-aminohexyl, 3′-FITC
	B-NC	35	**ACT GAC TGA CTG ACT GAC TGA CTG GGC GGC GAC CT**	5′-aminohexyl, 3′-biotin
	F-NC	35	**ACT GAC TGA CTG ACT GAC TGA CTG GGC GGC GAC CT**	5′-aminohexyl, 3′-FITC
	B-3mis	30	TTT TTT CAG CAT **TAT** CTC CTT GAT TCT ATG	5′-aminohexyl, 3′-biotin
	B-1mis	30	TTT TTT CAG CAT G**G**G CTC CTT GAT TCT ATG	5′-aminohexyl, 3′-biotin
target	30mer	30	CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA	
	B-30mer	30	CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA	5′-biotin
	F-30mer	30	CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA	5′-FITC
	20mer	20	CAT AGA ATC AAG GAG CAC AT	
	B-20mer	20	CAT AGA ATC AAG GAG CAC AT	5′-biotin
	40mer	40	GGG GGG GGG GCA TAG AAT CAA GGA GCA CAT GCT GAA AAA A	
	B-40mer	40	GGG GGG GGG GCA TAG AAT CAA GGA GCA CAT GCT GAA AAA A	5′-biotin

activated alumina (Grade CG20) were obtained from Polysciences (Warrington, PA). The horseradish peroxidase silver enhancement kit, EnzMet reagent kit, was purchased from Nanoprobes Inc. (Yaphank, NY). Oligonucleotides were obtained from MWG-Biotech Inc. (High Point, NC) and had the sequences reported in Table 1. Randomly sized human DNA samples extracted from female placenta cell nuclei and *Escherichia coli* protein cell lysates were from our laboratory stock.

Sample Preparation. All stock solutions (100 μM) were prepared in double-deionized MilliQ water (ddH_2O; Millipore, Bedford, MA). The phosphate buffer solution (PBS) consisted of 0.01 M potassium monobasic phosphate, 0.137 M NaCl, and 0.003 M KCl (adjusted to pH 7.4 with sodium hydroxide). A sodium chloride/sodium citrate (SSC) buffer solution was used for the hybridization and washing steps and consisted of 0.3 M sodium citrate buffer, 3 M sodium chloride, adjusted to pH 7 with HCl.

Apparatus. A MALDI Micro MX mass spectrometer (Waters/Micromass, Manchester, UK) was used in linear (positive and negative ion) mode for the DNA analysis. The instrument operated with 5 kV set on the sample plate, −12 kV on the extraction grid; pulse and detector voltage were 1.95 and 2.35 kV, respectively. A nitrogen laser (337 nm, 5 Hz, 50 μJ/pulse) was used for ionization. MassLynx v4.0 software (Waters) served for data acquisition, and each spectrum was composed of 10 laser pulses. The pMALDI chip was fixed to a standard metallic plate with adhesive tape and introduced at the source. A mixture containing 20-, 30-, and 40-base-long ssDNA fragments was used to calibrate the mass spectrometer (using the m/z values given by the quality control department of Eurofins MWG for these ssDNAs). The signal from the 30-base-long ssDNA served as an external lock-mass reference.

^{1}H NMR spectra were recorded with Bruker AVANCE DRX 500 nuclear magnetic resonance spectrometer (Bruker, Billerica, MA) at 500.13 MHz. All spectra were measured in $CDCl_3$. The proton chemical shifts in the NMR spectrum are given in δ values relative to tetramethylsilane $\delta = 0.0$. Typically, 256 scans were accumulated per spectrum.

Chip Fabrication. The rapid prototyping of the copolymeric DNA microarrays is similar to that used for copolymeric plastic MALDI (i.e., pMALDI) chips; [40–45] the dimensions of the pMALDI chip and the well are 42 × 56 × 0.2 mm, and 2.5 (diameter) × 0.03 mm (depth), respectively. The copolymerized plastic chips were fabricated using a modified atmospheric molding procedure in which three-dimensional sample zone arrays were fabricated on a silicon substrate and used to template polymeric chips. The masters were prepared from 100-mm-diameter positive doped, <100> orientation, silicon wafers using soft photolithography and wet chemical etching. The complete fabrication procedure has been described elsewhere.[41] The atmospheric molding in situ polymerization technique yields a negative copy of the silicon substrate in a thin sandwich mold. A thin aluminum spacer and a poly(tetrafluoroethylene) seal tape was used between the silicon substrate and the glass cover plate to prepare ∼0.2-mm-thick chips. The decreased thickness of the chip dramatically reduced previously observed charging effects.[44] The molding and demolding process of prepared copolymeric plastic DNA chips (Figure 2) was straightforward, as was the process previously reported for other plastic MALDI chips.[41–45]

Conjugation of the DNA Probes to the Epoxide-pMALDI Chip. Prior to immobilizing the ssDNA oligonucleotide to the surface, the copolymer was cleaned, rinsed with ddH_2O, and dried under a stream of nitrogen. A piece of the copolymeric DNA microarray was cut with scissors and dissolved in $CDCl_3$. The presence of the characteristic proton signals for the epoxide group (2.38, 2.63, and 3.13 ppm) and their relative intensity when compared to the $-OCH_2-$ signal of the backbone side chain guarantee the quality of the chip even after long-term storage at room temperature in a high-humidity environment.

The probe immobilization is based on a nucleophilic ring-opening reaction (Figure 2). The ssDNA probes used for our studies were 30-base ssDNA with different complementarities, full match, 1-base mismatch, 3-bases mismatch, and a longer non-complementary ssDNA probe (0mis, 1mis, 3mis, and NC, respectively) to the target CMV DNA sequences (Table 1). Moreover, labeled control probes were used to confirm the degree of labeling of the copolymeric DNA chip surface (Table 1).

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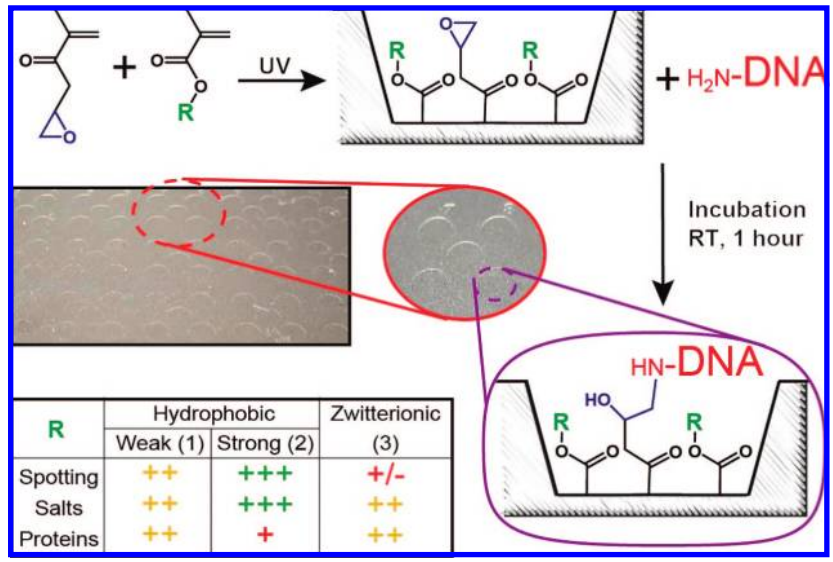

R	Hydrophobic		Zwitterionic
	Weak (1)	Strong (2)	(3)
Spotting	++	+++	+/-
Salts	++	+++	++
Proteins	++	+	++

Figure 2. Schematic representation of the copolymerization reaction formed reactive surface and the subsequent conjugation of an amino-linked DNA probe to the chip surface. The inset table shows the benefits (i.e., spot quality and tolerance to contaminants such as salts and proteins) associated with the copolymerization of different backbone monomers with glycidyl methacrylate. Quality scale: excellent (+++), very good (++), good (+), unsatisfactory (±). The R in the table represents (1) methyl, (2) butyl, and (3) 2-ethyl-2,2-dimethyl-(3-sulfopropyl)ammonium hydroxide, respectively.

The probe immobilization involved the spotting, in each microwell, of 2.0 μL of 10 μM single-stranded label-free or labeled-probe DNA (Table 1) in 1× Arrayit spotting buffer (Telechem, Sunnyvale, CA). The DNA chip was incubated for either 1 h at 50 °C or 8 h at 37 °C; subsequently, the remaining probe-spotting solution was flushed away with ddH_2O and dried under nitrogen. The remaining active surface was blocked with 50 mM ethanolamine in 0.1 M Tris and 0.1% SDS, pH 9, for 15 min. Finally, the chips were flushed with ddH_2O and dried under nitrogen.

DNA Probe Array Hybridization. For the optical and AFM detection, the biotin-modified ssDNA targets of different sizes (i.e., B-20mer, B-30mer, and B-40mer, Table 1) were used. MALDI-TOF/MS measurements were done with label-free ssDNA targets of sizes equivalent to those employed for AFM and optical detection (i.e., 20mer, 30mer, and 40mer, Table 1). Regardless of the detection method used, the ssDNA target (labeled or nonlabeled) was dissolved in 1 × SSC + 0.1% SDS and incubated with the DNA chip at least for 1 h in a humidity chamber at 37 °C. This was followed by two washing steps, first in 2 × SSC for 5 min and then in 0.2 × SSC for 5 min, and a drying step under a stream of nitrogen.

Gold Nanoparticle Deposition for AFM Analysis. For the streptavidin gold nanoparticle (5-nm gold nanoparticles; British Biocell, Cardiff, UK) labeling, 100 μL of a 1:100 dilution of the original nanoparticle solution was dissolved in PBS with 0.1% BSA. Each chip was incubated with this solution for 1 h at 37 °C in a humidity chamber. Afterward, the chips were washed six times for 5 min each in PBS with 0.05% Tween 20 and then briefly rinsed with distilled water to remove any excess of chloride ions.

Silver Deposition for Optical/AFM Analysis. Based on the streptavidin−peroxidase polymer, the substrates were incubated with a 1:1000 dilution of the original solution in PBS with 0.05% Tween 20. A 100 μL portion of this solution was applied and incubated at 20 °C for 1 h on the chip. Each pMALDI chip was then washed six times for 5 min in PBS containing 0.05% Tween 20 and then for 5 min in ddH_2O to remove any excess of unbound enzyme complexes and chloride ions that could interfere with the silver deposition reaction. The enzyme-induced silver deposition was performed using the EnzMet reagent kit. The chips were incubated with the EnzMet reagent kit for 1, 2, 3, or 5 min. Longer reaction times were used to test whether they improved the strength of the signal. However, incubation times longer than 5 min led to an increase of nonspecific signals (i.e., background) and were therefore not used. To avoid the inactivation of the enzyme, the EnzMet reagents were applied immediately after the washing step. The reaction was stopped by rinsing with ddH_2O to wash away the kit solution. After the enzyme-induced silver deposition is stopped, the enzyme becomes inactive because it is trapped by the deposited silver.

MALDI-TOF/MS for Label-Free ssDNA Analysis. The pMALDI chip was washed six times for 5 min each in PBS containing 0.05% Tween 20 and then briefly in ddH_2O to remove any excess of buffer and surfactants. Although this treatment was not necessary, it was done to maintain a similar sample handling protocol for the different analytical techniques. Subsequently, the double-stranded DNA was denatured by adding 2 μL of ddH_2O to each microwell and increasing the temperature to 98 °C for 10 min. To prevent water from evaporating, the whole microarray was placed inside a humidity chamber. To avoid the reassociation of single-stranded DNA, 1.2 μL of 3-hydroxypicolinic acid (25 mg/mL), dissolved in water/acetonitrile (1:1), was immediately added by aspirating and dispensing the solution to each well, and the analyte/matrix mixture was left to crystallize at room temperature.

Safety considerations. *Alkyl methacrylates and benzoin methyl ether are toxic substances. Other used chemicals are irritants. Accidental inhalation, ingestion, or skin contact with these chemicals should be avoided. UV light may cause damage to skin and eyes; protective goggles and gloves should be used. The polymerization should be carried out in a ventilated fume hood.*

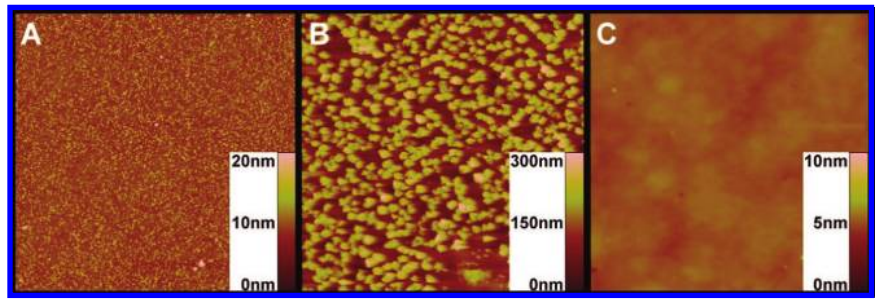

Figure 3. AFM images of the positive control measurements (10 μm × 10 μm) using (A) control probe (B-0mis) labeled with gold nanoparticles, (B) biotin-labeled control probe (B-0mis) and subsequent peroxidase-enhanced silver deposition after 5 min, and (C) peroxidase-enhanced silver deposition after 5 min on a BMA-based nonreactive DNA chip. DNA probe concentration was 10 μM. The color-coded scales of surface roughness are given in the right-hand corners of the individual images.

RESULTS AND DISCUSSION

The microarray for detecting human CMV DNA presented in this paper relies on one common ready-to-spot inexpensive substrate in combination with parallel MALDI-MS, transluminiscence/AFM, and fluorescence detection (Figure 1). To our knowledge, the present protocol represents the first example of how different state-of-the-art DNA readouts can be integrated and combined with a low-cost and ready-to-spot copolymeric microarray substrate.

Material Optimization for Copolymeric DNA Microarray Chips. The material chemistry of the plastic DNA microarray chip was extensively studied. As the epoxide group was previously found to be the most suitable linking unit for immobilizing DNA onto glass and silicon substrates,[9] the chips were prepared by copolymerizing (Figure 2) commercially available glycidyl methacrylate (i.e., epoxide-containing monomer) with a backbone monomer such as methyl methacrylate (backbone 1 in Figure 2; weakly hydrophobic), butyl methacrylate (backbone 2 in Figure 2; strongly hydrophobic), or methyl methacrylate−[2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (backbone 3 in Figure 2; zwitterionic).

Because of the different backbone monomers employed in the fabrication of the DNA chips, different surface properties were obtained. To test the relationship between copolymer composition and the MALDI-MS, AFM, and optical transluminescence signals, the quality of the spot and its robustness, i.e., tolerance of nonspecific adsorption of common interferences present in biological studies such as salts, random DNA sequences, and proteins, were measured (inset table in Figure 2).

To evaluate the quality of the spot (i.e., wettability), the surface coverage was characterized using optical and scanning probe microscopy, and the signal intensity of the DNA ions from recorded mass spectra (MALDI-MS signals depends very much on this parameter).

Similarly, the robustness of the DNA detection was evaluated using a common sample hybridization protocol to detect CMV DNA target sequences that have dissolved in solutions of increasing complexity, such as spotting buffer (containing only salts as interference), randomly sized human placenta DNA extracts (containing salts and noncDNA as interference), and *E. coli* cell lysates (containing salts, noncDNA, lipids, and proteins as interference).

Based on MALDI-MS measurements and bright-field (optical) microscopy (i.e., which measures the light transmitted after the DNA target molecules are silver-labeled on the surface), glycidyl methacrylate/butyl methacrylate (EBMA, i.e., strongly hydrophobic, inset table in Figure 2) was selected for further use. To further optimize EBMA substrates for MALDI-MS, optical and scanning probe microscopy measurements, it was necessary to ensure a homogeneous distribution of the epoxide groups on the surface of the plastic substrates. Therefore, monomers (glycidyl methacrylate/butyl methacrylate) were copolymerized using different molar ratios: 1:13.3, 1:26.6, and 1:39.9. Furthermore, in addition to each monomer ratio, different concentrations of two different 30-base pairs capture CMV−DNA probes, a ssDNA probe, and ready labeled-probe (0mis and B-0mis, respectively), and a 35-base pair noncomplementary sequence probe (NC) were dispensed manually into each well (1.5 μL/well).

Using bright-field and scanning probe microscopy readouts (i.e., transluminescence and atomic force microscopy, respectively), we concluded that a 10 μM DNA probe immobilized on a 1:13.3 ratio pMALDI chip provided the most efficient and homogeneous distribution of active recognition sites on the polymer surface.

These results are partially illustrated in Figure 3. After the chip surface was treated with biotin-labeled probe (B-0mis, 10 μM), the probe was labeled with gold nanoparticles (Figure 3A) or silver deposition (5 min) by peroxidase-based enhancement (Figure 3B). Surface images obtained at random positions demonstrated that the sites of reaction were equally distributed across the surface. Moreover, the measurement of different heights of the formed structures in Figure 3 shows that enzymatic labeling (Figure 3B) gives bigger particles (~300 nm) than does labeling using gold nanoparticles (5 nm, Figure 3A). Figure 3C shows the control for the silver deposition labeling method, according to which a biotin-labeled probe (B-0mis) was applied to a pure butyl methacrylate surface (AFM signal not higher than 10 nm). Therefore, owing to its better signal-to-noise ratio, the horseradish peroxidase-based labeling system was preferred over the gold nanoparticle labeling system for further experiments.

Sensitivity Comparison among State-of-the-Art Techniques. A comparison based on the sensitivity between MALDI-TOF/MS and optical measurements (using FITC-labeled and enzymatically labeled targets) is shown in Figure 4A. The dynamic range of the silver enhancement kit depends on the incubation time of the peroxidase enzyme (Figure 4A shows the signal for 5-, 3-, and 1-min incubation). Moreover, while FITC labels tended to photobleach, only negligible signal decay during measurements

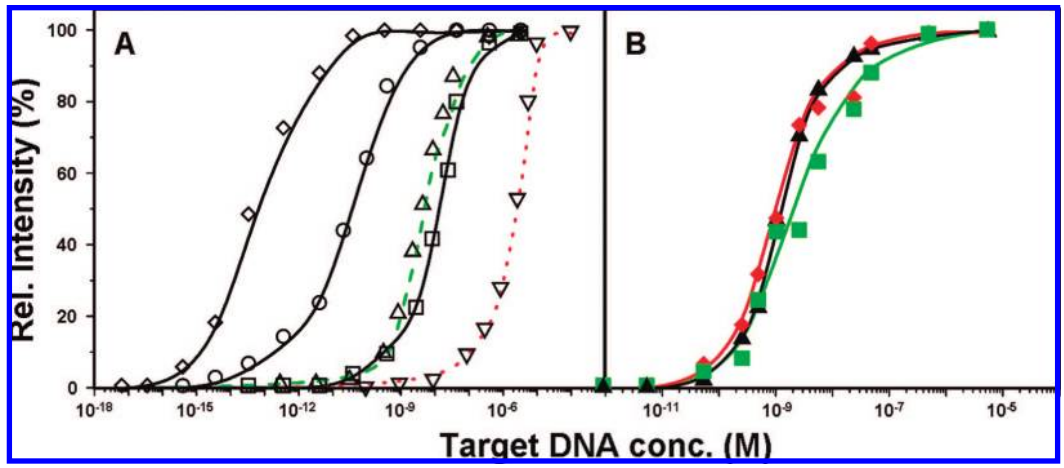

Figure 4. Comparison of calibration plots using horseradish peroxidase-based (HRP) DNA detection (i.e., transluminiscence) at different incubation times, FITC-based (i.e., fluorescence), and MALDI-TOF/MS DNA detection. Five-minute HRP incubation (◆), 3-min HRP incubation (●), 1-min HRP incubation (■), FITC (▲), and MALDI-TOF/MS (▼). (B) Reproducibility of the HRP DNA detection, using a 0mis probe and B-30mer target, in different environments: nonbackground (▲), human placenta DNA purified extract (◆), and *E. coli* cell lysates (■) using 2-min incubation time.

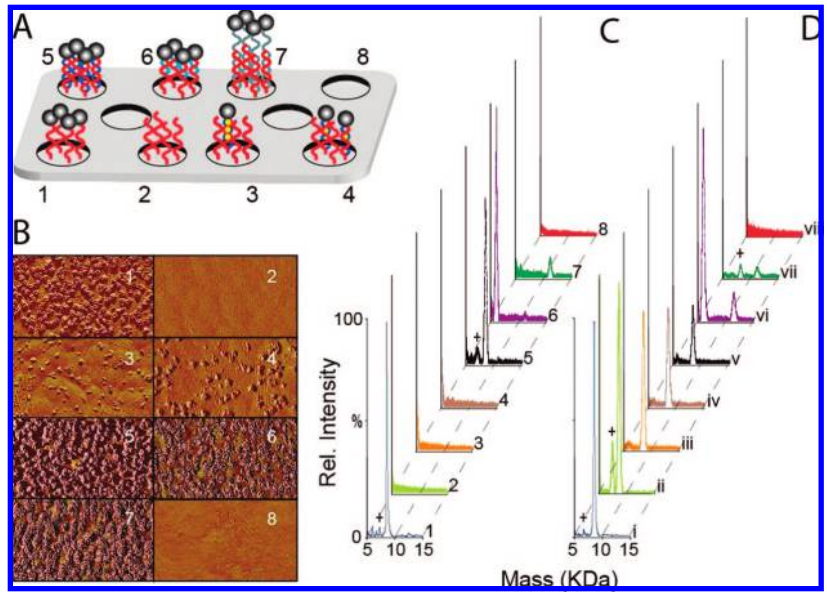

Figure 5. Reliability and validity of CMV DNA detection with two complementary readout techniques (AFM and MALDI-TOF/MS, (B) and (C), respectively). Wells 2−5 compare the hybridization efficiency of a 30-base oligomer target (30mer) to different ssDNA probe sequences: NC, and 30-base probes with 3-mismatches (3mis), 1-mismatch (1mis), and complementary sequence (0mis). Wells 6 and 7 compare the hybridization efficiency of a 30-base oligomer probe (0mis) to target ssDNA samples of different lengths with perfect complementarity to the probe: 20mers and 40mers, respectively. Well 1 contains a positive control (B-0mis for AFM and 30mer for MALDI-MS); well 8 is a blank. Roman numerals (D) represent MALDI-TOF/MS spectra of the nonbound target DNA from the respective immobilization step for the Arabic-numbered well; "+" represents $[M + H - 5A]^+$ fragment ions of the DNA.

was observed for the enzymatically deposited silver layers. Hence, the limit of detection is lower and the overall dynamic range given by horseradish peroxidase silver enhancement kit on the copolymer DNA chip is much greater (5 fM−5 μM) than that previously reported for the optical detection of CMV−DNA using either enzymatically labeled[25] and FITC-labeled targets on glass slides[46] or prestructured MALDI-MS sample supports for enhancing ssDNA detection.[29]

Robustness of the CMV Detection Based on a Plastic DNA Chip. The robustness of the optical microscopy measurements using enzymatically labeled target CMV ssDNA on the copolymerized (EBMA) is presented in Figure 4B. After 2 min of silver enhancement, consecutive calibration plots were made to detect CMV DNA on the plastic DNA chip in a spotting solution ($n = 5$, RSD = 16.7) in the presence of randomly sized human placenta DNA samples ($n = 3$, RSD = 7.1) and *E. coli* cell lysates ($n = 3$, RSD = 23.9).

Achieving Reliability in DNA Identification Using Multiple Readout Formats. Figure 5 compares the reliability and validity of the methods. For this experiment, the grid (Figure 5A) was divided in two sets: (a) Spots 3−5 contain different 30-base ssDNA probes, which were hybridized with a 30-base ssDNA target (30mer, Table 1). The nomenclature used to describe the probe in each well depends on the number of mismatches in their oligonucleotide sequence: 3 mismatches (spot 3), 1 mismatch (spot 4), or completely complementary (spot 5). The oligonucle-

otide sequence for these probes can be found in Table 1 (3-mis, 1-mis, and 0-mis, respectively). (b) Spots 6−7 contain the same completely complementary probe sequence used in spot 5 (0-mis, Table 1), but instead of hybridizing to the 30mer CMV ssDNA target sequence, as in the case of spot 5, the spots were used to study the recognition of two target ssDNA molecules that possess a complementary sequence but different lengths, i.e., a 20-base-long ssDNA target (spot 6) and 40-base-long ssDNA target (spot 7). The oligonucleotide sequence for these targets can be found in Table 1 (referred to as 20mer and 40mer, respectively).

The efficiency of the MALDI-MS, transluminescence and AFM measurements was evaluated using as references the signal from spot 5, and the positive (B-0mis in Table 1 for AFM and 30mer in Table 1 for MALDI), negative (noncomplementary probe, NC in Table 1) and blank controls, which were located in spots 1, 2, and 8, respectively.

The transluminescence and AFM readouts were able to differentiate the hybridization events in case a, providing qualitative information for the hybridization of the target to the complementary DNA (0mis, TTTTTTCAGCATGTGCTCCTTGATTCTATG), 1-mismatch (1-mis, TTTTTTCAGCATG**G**GCTCCTTGATTCTATG) and 3-mismatch (3-mis, TTTTTTCAGCAT**TAT**CTCCTTGATTCTATG) sequences. However, transluminescence and AFM measurements failed to differentiate the binding events in case b, since 20mer (**CATAGAATCAAGGAGCACAT**) and 40mer (GGGGGGGGGG-**CATAGAATCAAGGAGCACATGCTGAAAAAA**) target ssDNA share the same recognition sequence with the 30mer (**CATAGAATCAAGGAGCACATGCTGAAAAAA**) and hence provide similar enzymatic responses (i.e., leading to a high number of possible false positive identifications).

On the other hand, MALDI-TOF/MS instruments operating in linear mode have an accuracy of 0.2% in relation to the mass measurement. Thus, the expected mass errors, ~20 Da for typical m/z 10 000 values used here, are smaller than the addition or subtraction of the smallest nucleoside monophosphate, deoxycytidine monophosphate (289.2 Da). This makes MALDI-TOF/MS a good readout instrument for obtaining information regarding the size of the DNA sequence, which cannot be obtained in terms of matching sequence identity only. Moreover, the sensitivity for DNA MALDI-TOF/MS has been reported to be in the low-femtomole range when hydrophobic substrates are used to preconcentrate the sample DNA in a discrete spot in combination with a decreased amount of matrix.[29]

Therefore, using a readout method such as MALDI-TOF/MS, it was possible to differentiate the lengths of bound DNA (Figure 5C). Additionally, in the case of MALDI-TOF/MS analysis, the nonbound target DNA present after the hybridization step could still be analyzed (Figure 5D). For this analysis, the sample was preconcentrated either in an anion-exchange pMALDI chip (e.g., one containing a quaternary amine functionality on its surface)[42] or on a dialysis membrane and later spotted to hydrophobic (e.g., one containing *n*-butyl group) pMALDI chip[41]and subsequently measured using MALDI-TOF/MS as was the case for Figure 5D.

Unfortunately, although the plastic-based substrates used in this work enhanced the sensitivity of MALDI-MS instruments in comparison to commercial available metallic targets, our MALDI-TOF/MS instrument failed to reach the limit of detection achieved by the enzymatic-labeled transluminiscence assay (Figure 4A). Therefore, in situ DNA amplification (e.g., in situ PCR amplification) prior to MALDI-TOF/MS measurement is required.

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CONCLUSION

A versatile, ready-to-spot, disposable UV-photopolymerized plastic substrate for gold and silver nanoparticle-labeled and label-free ssDNA detection based on optical, scanning probe microscopy and mass spectrometry was developed. Unlike the conventional silicon, glass, or quartz DNA chip substrates previously used, the key advantage of this novel substrate is that the plastic material exhibits an inherent tendency to immobilize DNA, obviating the need for additional activation steps. Active functional epoxide groups are stable for months, allowing the chips to be produced before they are actually spotted with DNA probes. Moreover, the copolymerized DNA chip can be molded in different geometries, and with or without structures on the surface, to fit to different readout instruments; hence, the fabrication protocol could be easily modified to prepare medium- to high-density chips of from 10 000 to 50 000 wells with the desired shape/size patterns and surface properties; in such chips, the activity of the surface could be probed by infrared spectroscopy or by ^{1}H NMR.

Although a standard DNA sample was used to illustrate the capabilities of this novel DNA microarray substrate, the composition of the copolymeric DNA chips can be further tailored to prevent proteins from adhering to the chip (Figure 2 inset table). Eliminating the binding of nonspecific proteins to the chip surfaces will make the study of specific DNA−protein interactions feasible.

Currently, we are developing an in situ PCR amplification using a robotic pipetting device from Scienion AG to increase the analysis through-put and to extend the dynamic linear range of the MALDI-TOF/MS measurements toward lower concentrations. Furthermore, we foresee diverse applications for the fast and reliable detection of human pathogens, single nucleotide polymorphisms, and microRNA detection. Finally, this platform can be applied in areas not currently covered by commercial DNA chips, where the benefits of orthogonal readouts (sensitivity and robustness) are important, such as the study of infectious agents of toxicological or ecological relevance.

ACKNOWLEDGMENT

A.J.I. gratefully acknowledges financial support by the International Max Planck Research School "The Exploration of Ecological Interactions with Molecular and Chemical Techniques". The funding of this project by the Max Planck Society is equally acknowledged. The authors thank Emily Wheeler for editorial assistance, Steffen Harzsch for his support with the LIF measurements, and Jürgen Kroymann for helpful discussions. A.J.I. and T.S. contributed equally to the paper.

Received for review February 29, 2008. Accepted May 23, 2008.

AC800426V