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Matrix-assisted laser desorption/ionization mass spectrometry of immobilized duplex DNA probes

Kai Tang, Dongjing Fu, Sven Kötter¹, Robert J. Cotter², Charles R. Cantor³ and Hubert Köster^{1,*}

Sequenom Inc., 101 Arch Street, Boston, MA 02110, USA, ¹University of Hamburg, Department of Biochemistry and Molecular Biology, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany, ²Johns Hopkins University School of Medicine, Department of Pharmacology and Molecular Science, 725 North Wolfe Street, Baltimore, MD 21205, USA and ³Boston University, Center for Advanced Biotechnology, 36 Cummington Street, Boston, MA 02215, USA

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ABSTRACT

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry was used to analyze short DNA duplex probes with one strand immobilized on solid supports (streptavidin-coated magnetic beads or controlled pore glass beads). Only the non-immobilized strand could be detected. Partial denaturation was found when the duplex probes were mixed with 3-hydroxypicolinic acid, ammonium citrate matrix. The strategy has several applications, such as fast DNA sequence analysis and DNA diagnostics.

INTRODUCTION

Two promising techniques that could revolutionize DNA sequence analysis are sequencing by hybridization (1–4) and mass spectrometry. An enhanced version of sequencing by hybridization, termed positional sequencing by hybridization (PSBH) has been developed which uses duplex probes containing single-stranded five base 3' overhangs instead of simple single-stranded probes (5).

A different PSBH approach, which uses the same kind of duplex probes to capture DNA targets and then performs conventional Sanger sequencing on such immobilized duplex-template complexes, has recently been developed (6). When translated to a DNA chip format the advantage of this approach is a significant increase in speed of sample preparation, due to the simultaneous capture of many target DNAs, while the drawback is its dependence on gel electrophoresis to separate the sequencing ladders. On the other hand, mass spectrometry, especially matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) offers the advantage of separating a mixture of biomolecules in a fraction of milliseconds without gel electrophoresis and labeling (7–15). The detection range of MALDI-TOFMS for DNA molecules has been extended to 500 bases long (16). These advances demonstrate that MALDI-TOFMS could offer considerable promise as a replacement for gel electrophoresis and labeling in DNA sequencing processes.

In this report we demonstrate that immobilized duplex DNA probes can be strand separated, directly desorbed and detected by MALDI-TOFMS. The immobilized strand of the duplex remains intact and bound to the support, while the non-immobilized strand is desorbed and detected in the MALDI process. Based on these experiments we propose a new strategy for DNA sequencing that combines DNA chip technology, Sanger sequencing and MALDI-TOFMS. The method could promise DNA sequencing at unparalleled speed and would also be of value for DNA diagnostic applications.

MATERIALS AND METHODS

Preparation of duplex DNA on streptavidin-coated magnetic beads

Streptavidin-coated magnetic beads (0.5–2 mg; Dynal, Oslo) were pre-washed and suspended in 50–200 µl 2× binding and washing (B&W) buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2M NaCl). Biotinylated (at either the 5'- or 3'-end) single-stranded DNA (200–1000 pmol) was then added and the mixture was shaken at room temperature for 1 h. The supernatant containing the unbound oligonucleotide was removed and the beads were washed twice with 2× B&W buffer and resuspended in 50–200 µl either B&W or TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) buffer and stored at 4°C. The binding capacity was estimated to be 100–200 pmol oligonucleotide/mg beads.

To prepare the duplex probe 0.5 mg of beads containing the immobilized single-stranded DNA was resuspended in 50 µl B&W or TE buffer. Then a total of 200 pmol complementary oligonucleotide (shorter or longer than the immobilized strand) in an equal volume of the same buffer was added. The 100 µl mixture was heated to 70°C and slowly cooled to room temperature. This annealing process was performed at an ~1:2 ratio of immobilized strand and its ligand and it usually took ~1 h. After annealing the supernatant containing excess ligand was removed, the beads with immobilized duplex were washed twice in either B&W or TE buffer, re-suspended in 50 µl of the same buffer solution and kept at 4°C. A total amount of ~50–100 pmol DNA duplex was estimated to be immobilized on the beads. For

* To whom correspondence should be addressed

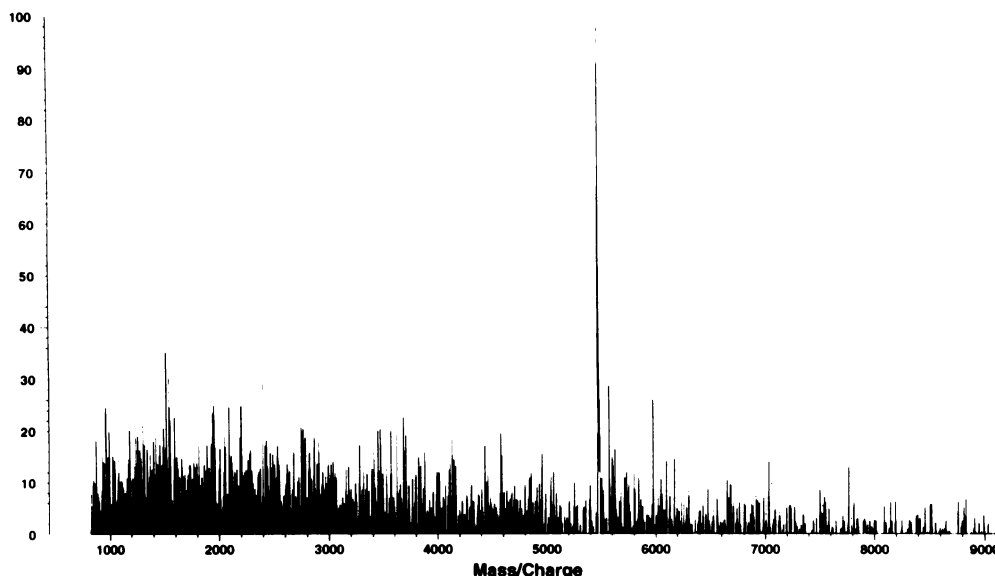


Figure 1. MALDI-TOFMS spectrum of duplexes on streptavidin-coated magnetic beads. A 23mer (NB 21) was immobilized and a 18mer (CM1) was annealed. Only the 18mer was observed. The total sample loading was estimated to be ~2–5 pmol. The spectrum was obtained with a linear TOF mass spectrometer with a resolution of 900. A 0.5 μ l matrix (0.7 M 3-HPA, 0.07 M ammonium citrate, dibasic) was used.

the preparation of mixtures of immobilized duplexes the amount of beads, the volume of buffer and the amount of total DNA ligands were increased proportionally by two or four times.

Preparation of duplex DNA on CPG beads

Duplexes on controlled pore glass (CPG) beads were prepared as follows. The CPG beads (500 Å, 74–125 μ m, 51 m²/g surface area) were functionalized with 3-glycidoxypentyl trimethoxysilane and hexane-1,6-diol in analogy to a described procedure (17). A 55 base deoxynucleotide (5'-AGC TAG TCA TGT AAT GCA GGT CCT ACA GTC AAT GGC CGT AAT GAC CGT TAT TTT T-3') was synthesized on 13.8 mg CPG *in situ* using β -cyanoethyl phosphoramidite chemistry (18,19), a DNA synthesizer (MilliGen Model 7500) and the more base-labile 4-*t*-butylphenoxyacetyl group for protection of the exocyclic amino group (20,21). Deprotection of the amino and phosphate protecting groups was accomplished by a 2 h treatment with methanol saturated with ammonia at room temperature. Loading of the CPG beads with the 55mer was determined spectrophotometrically through the 4,4'-dimethoxytrityl (DMT) cation at 498 nm and found to be 8.7 μ mol/g. For hybridization of oligodeoxynucleotides to the immobilized 55mer 1 nmol CPG-bound 55mer was incubated with 100 μ l hybridization solution made from 10 μ l oligomer (~1 nmol) in TE buffer, 10 μ l 10 \times hybridization buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂ and 250 mM NaCl) and 80 μ l pure water in a microspin centrifuge (Roth; filter pore size 0.45 μ m) at 65°C for 10 min and annealed at 37°C for 30 min. After centrifugation the filtrate was measured in a UV spectrophotometer. The CPG-bound duplex was then washed three times with 80 μ l ice-cold 50 mM ammonium citrate (dibasic); each filtrate was monitored by UV absorbance at 260 nm. The amount of oligodeoxynucleotides in the filtrate after the

third wash was negligible. The beads with the immobilized duplex were air dried at 20°C and stored at -22°C. In a control experiment CPG beads without the immobilized 55mer were used. After hybridization and identical treatment no oligonucleotide could be detected by MALDI-TOFMS.

MALDI-TOFMS sample preparation

To analyze the duplex on streptavidin-coated magnetic beads the stored beads with immobilized duplex were washed twice with 0.2 M ammonium citrate and resuspended in 5 μ l washing solution. Then 0.5 μ l suspension were pipetted onto the target probe of the mass spectrometer and immediately mixed with 0.5 μ l 3-hydroxypicolinic acid (3-HPA; 0.7 M in 50% acetonitrile solution) matrix solution (22). The mixture was dried at ambient temperature and then introduced into the mass spectrometer for analysis. In most cases the beads with immobilized duplex could also be washed with and resuspended in pure water. In this case 0.7 M 3-HPA with 70 mM ammonium citrate in 50% acetonitrile was used as the matrix. The use of ammonium citrate was for cation exchange to remove possible Na⁺ and K⁺ ions on the DNA backbone (14).

For mass spectrometric analysis of the duplex on CPG beads several dried CPG beads were put directly on the target probe of the mass spectrometer. Then 0.5 μ l matrix solution (0.7 M 3-HPA with 10% ammonium citrate in 50% acetonitrile) were added onto the spot and allowed to dry.

Two mass spectrometers were used: a Kratos/Shimadzu (Manchester, UK) Kompact MALDI/III linear reflectron TOF mass spectrometer with a 20 keV ion source, operated in the linear mode; a Finnigan MAT (Bremen, Germany) Vision 2000 reflectron TOF mass spectrometer, a reflectron with a 5 keV ion source and 20 keV

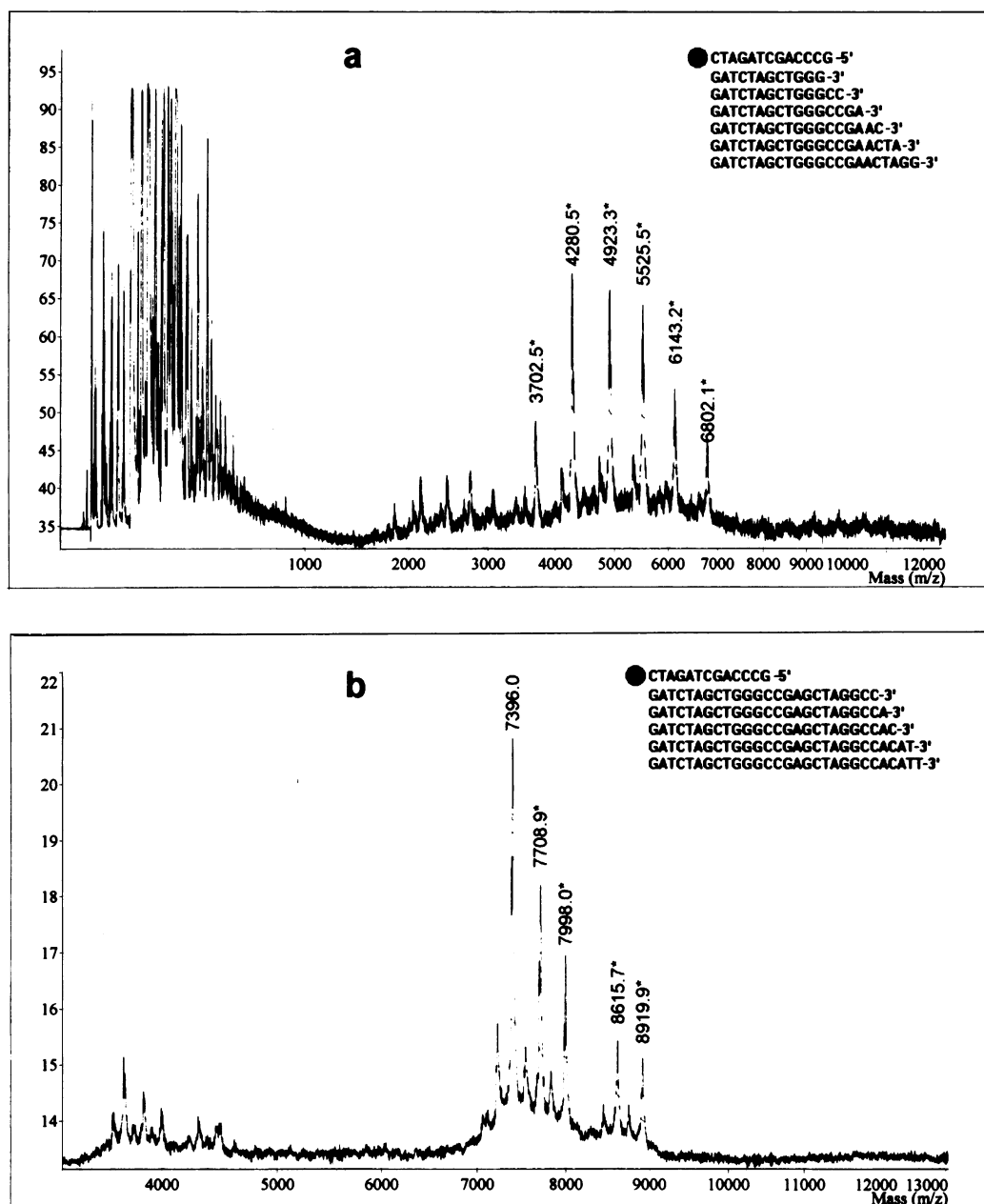


Figure 2. MALDI-TOF spectra of (a) 12mer, 14mer, 16mer, 18mer, 20mer and 22mer and (b) 24mer, 25mer, 26mer, 28mer and 29mer annealed to a 13mer immobilized at the 3'-end to the streptavidin-coated magnetic beads. Aliquots (0.5 μ l) of the 5 μ l bead suspension, containing ≤ 4 pmol for each duplex, were loaded with 0.5 μ l of matrix (0.7 M 3-HPA, 0.07 M ammonium citrate). The theoretical $[M+H]^+$ values are 3702.4, 4280.8, 4923.2, 5525.6, 6143.0 and 6801.4 in (a) and 7395.8, 7709.0, 7998.2, 8615.6 and 8919.8 in (b) respectively.

post-acceleration. Both mass spectrometers used nitrogen lasers for MALDI. All spectra were taken in the positive ion mode.

RESULTS AND DISCUSSION

The denaturation of duplex DNA in MALDI

Initial tests were made to observe the behavior of immobilized duplex DNA under standard MALDI conditions. A 5'-end biotinylated 23mer (NB21, 5'-biotin-GAT GAT CCG ACG CAT CAG AAT TT) was immobilized on streptavidin-coated magnet-

ic beads. A complementary 18mer (CM1, 5'-CTG ATG CGT CGG ATC ATC-3') was annealed. Under standard MALDI conditions the immobilized duplex was denatured and only the annealed strand (the 18mer) was observed in the mass spectrum (Fig. 1). This result was expected, given the previous finding that double-stranded DNA was denatured in the MALDI process (23–25). The immobilized strand (the 23mer) was not desorbed, indicating that the binding between biotin and streptavidin is strong enough to survive any possible cleavage. Although the sample crystals were not homogeneously distributed, which

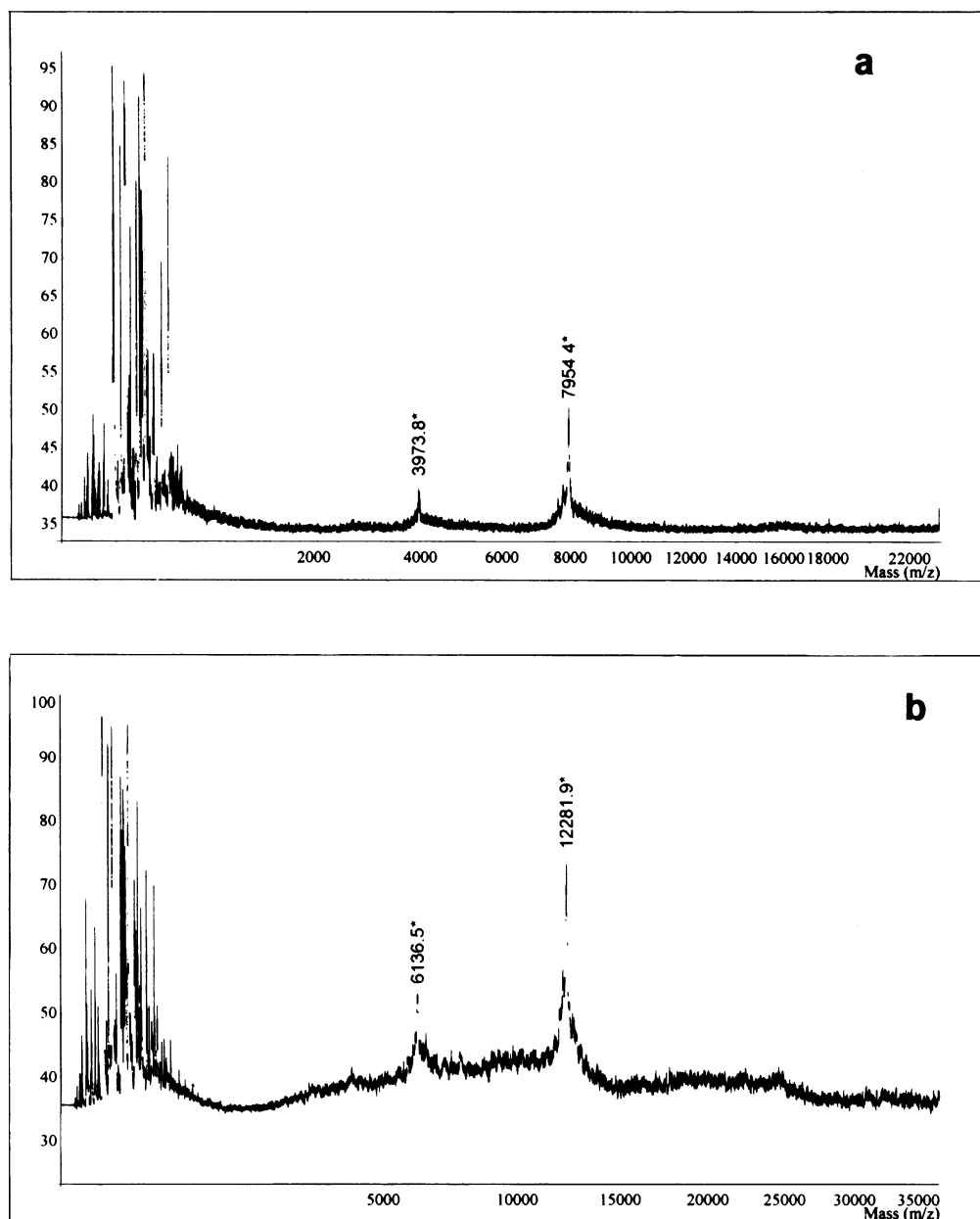


Figure 3. MALDI-TOF spectra of duplex DNA on CPG beads. One strand (a 55mer) was immobilized to which (a) a 26mer with a DMT protecting group at the 5'-end or (b) a deprotected 40mer was annealed. The 26mer was observed without the DMT group. In both cases two CPG beads, containing ~2–4 pmol of the duplex were loaded with 0.5 μ l matrix (0.7 M 3-HPA, 0.07 M ammonium citrate).

means hunting for good spots is still necessary, a good spot, once found, would last at least 20 laser shots with irradiance slightly above the desorption threshold, to yield a spectrum with good signal-to-noise ratio. The threshold irradiance for desorption was comparable with that required in normal MALDI-TOFMS of oligodeoxynucleotides of similar length with a 3-HPA, ammonium citrate matrix. Comparable with what has been seen in previous MALDI of polydeoxynucleotides, the mass resolution varied from spot to spot, ranging from 400 to 900 for the desorbed 18mer in the linear TOF mass spectra.

In order to understand the cause of denaturation a simple test was made for the duplex. After washing the immobilized duplex

three times with pure water the beads containing the bound duplex were resuspended at room temperature in 5 μ l matrix solution (0.7 M 3-HPA, 0.07 M ammonium citrate), mixed with gentle shaking and then the tube was placed in a magnetic particle concentrator (DynaL MPC). The supernatant, containing the matrix and possibly denatured 18mer (CM1), was removed from the tube and 1 μ l pipetted onto the sample holder of the mass spectrometer for analysis. The remaining beads were further washed twice and resuspended in 5 μ l pure water. An aliquot of the suspension (0.5 μ l) was mixed with 0.5 μ l 3-HPA, ammonium citrate matrix as usual. In MALDI-MS analyses the 18mer was observed both in the matrix supernatant and on the remaining

beads. This indicates that partial denaturation occurred when the duplex was mixed with the 3-HPA, ammonium citrate matrix. Good spots yielding a decent 18mer signal were relatively rare in the matrix supernatant sample compared with the sample containing the remaining beads, indicating that only a small amount of the duplex on the beads was denatured. These results are consistent with the previous finding that double-stranded DNA denatures at a level of ~10% when mixed with 3-HPA matrix (23). With a pH of 4.5 the 3-HPA, ammonium citrate matrix can serve as a protonation reagent for the deoxynucleotides. The protonated bases destabilize hydrogen bonding between the two strands, therefore, it is reasonable that short duplex probes partially denature upon mixing with 3-HPA, ammonium citrate matrix. Further experiments are needed to clarify this point.

Alternative immobilization and analysis of duplex mixtures

A duplex can also be biotinylated and immobilized on the 3'-end of one strand. For example, a 3'-end biotinylated 13mer (DF3-0, 5'-GCC CAG CTA GAT C-biotin-3') was immobilized and a 29mer (DF3, 5'-GAT CTA GCT GGG CCG AAC TAG GCC ACA TT-3') was annealed. This duplex gave the signal of the annealed 29mer (spectrum not shown). No immobilized 13mer was observed. For this kind of duplex configuration two mixtures of duplexes were made by annealing complementary strands of different lengths to the same 3'-end immobilized 13mer. In one mixture the complementary strands were a 12mer, 14mer, 16mer, 18mer, 20mer and a 22mer. In the other mixture the complementary strands were a 24mer, 25mer, 26mer, 28mer and a 29mer. The mass spectra of these mixtures of duplexes also showed the expected result (Fig. 2a and b), except for a decrease in signal intensity with oligomer length. The reason for this decrease is not clear at this moment. It could result from the yield of annealing in the duplex mixture preparation or from the desorption/ionization efficiency of the duplex mixture. The theoretical $[M+H]^+$ values are given in the figure caption. Mass accuracies of better than 0.01% were normally obtained.

MALDI-TOFMS of duplex DNA on CPG beads

Duplex DNA can also be immobilized on CPG beads and desorbed by MALDI in the same way as from streptavidin-coated magnetic beads. As an example, a 55mer (5'-AGC TAG TCA TGT AAT GCA GGT CCT ACA GTC AAT GGC CGT AAT GAC CGT TAT TTT T-3') was synthesized on a CPG support, to which a 26mer (5'-DMT-TAA CGG TCA TTA CGG CCA TTG ACT GT) with a DMT protecting group was annealed. The duplex loading was estimated at a level of 1–2 pmol/CPG bead after three washing steps. As expected, the annealed 26mer was observed and the immobilized 55mer was not desorbed (Fig. 3a). However, it is remarkable that the mass of the 26mer observed corresponded to the oligomer without the DMT protecting group, indicating that the protecting group was stripped off during laser desorption or that it had been cleaved in the acidic matrix solution. Deprotected complementary strands up to a 40mer were also tested with satisfactory results (Fig. 3b). CPG beads have a larger surface area than streptavidin-coated magnetic beads. The density of immobilized DNA molecules can be much higher when CPG beads are used (1 nmol oligonucleotide/mg CPG beads, compared with 100–200 pmol/mg in the case of streptavidin-coated magnetic beads).

Compared with the streptavidin-coated magnetic beads, which have a much smaller size (~2.8 μm) and form a homogenous but microscopically rough surface, with matrix crystals distributed mainly on the outside ring area, the CPG beads are much larger (74–125 μm), with matrix crystals surrounding each bead. A good spot was usually found at the interface area of the matrix crystal and the beads. This may indicate that denaturation occurs mainly in laser desorption, rather than upon adding the matrix solution. The quality of mass spectra taken for duplexes of similar size from magnetic beads and CPG beads was very similar, indicating that the mechanisms of desorption of duplexes from both kinds of beads are the same.

In both situations hunting for good spots is still necessary. This problem could be solved by modifying the surface conditions for both kinds of beads and controlling the crystallization process of the matrix solution. If the spot-to-spot reproducibility could be improved, the method could be ready for automation.

Potential applications in molecular biology

Desorption of the annealed strands complementary to the immobilized strands of duplexes opens a new strategy for DNA sequencing. Genomic DNA can be digested into small fragments using various kinds of restriction enzymes. Duplex probes, immobilized at the 3'-end of one strand and providing all possible (4⁵) five base single-stranded 3' overhangs on the complementary strand, can be arranged on a chip to capture the digested DNA fragments. The strand containing the five base overhang also serves as a primer for subsequent Sanger sequencing. A restriction site could be positioned such that most of the known primer sequence is cut off prior to mass spectrometry. Thus actual and valuable sequence information could be obtained, even if only short Sanger ladders are produced and analyzed. After the sequencing reaction the ladders can be, for example, restricted, washed and then desorbed spot by spot and mass separated directly from the chip. Elution of the ladders from off the support is not necessary. Sequencing of all DNA fragments can be carried out in parallel. Since primer labeling, gel electrophoresis and autoradiography are replaced by MALDI-TOFMS, the speed of sequencing could be increased by a few orders of magnitude. The design of customized DNA chips for diagnostic detection and identification of specific DNA sequences by MALDI-TOFMS can also be envisioned.

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