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Photocleavable peptide–DNA conjugates: synthesis and applications to DNA analysis using MALDI-MS

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ABSTRACT

The synthesis and characterization of photocleavable peptide–DNA conjugates is described along with their use as photocleavable mass marker (PCMM) hybridization probes for the detection of target DNA sequences by matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry. Three photocleavable peptide–DNA conjugates were synthesized, purified, and characterized using HPLC and denaturing gel electrophoresis, as well as IR-MALDI and UV-MALDI. The hybridization properties of the conjugates were also studied by monitoring their thermal denaturation with absorption spectroscopy. No significant difference in the melting temperature (T_m) of the duplexes was observed between the unmodified duplex and the duplex in which one strand was modified with the photocleavable peptide moiety. These conjugates were evaluated as hybridization probes for the detection of immobilized synthetic target DNAs using MALDI-MS. In these experiments, the DNA portion of the conjugate acts as a hybridization probe, whereas the peptide is photoreleased during the ionization/desorption step of UV-MALDI and can serve as a marker (mass tag) to identify a unique target DNA sequence. The method should be applicable to a wide variety of assays requiring highly multiplexed DNA/RNA analysis, including gene expression monitoring, genetic profiling and the detection of pathogens.

INTRODUCTION

Most established methods for the detection of specific sequences in DNA are based on the use of hybridization probes containing labels that can be directly (radioactivity, fluorescence) or indirectly (hapten, enzyme) detected (1). In the case of highly multiplexed DNA assays, one important limitation of this approach is the maximum number of different labels that can be simultaneously detected. The time required for work-up and read-out is a second limitation. Mass spectrometry offers a potential solution to these problems (2), since it can rapidly and simultaneously detect the presence of many analytes based on differences in their mass. To date, direct MALDI-MS analysis has been applied to DNA sequencing (3,4), RNA sequencing (5,6), analysis of DNA tandem repeats (7) and detection of single nucleotide polymorphisms (SNPs) using PNA probes (8,9).

For the purpose of MALDI-MS DNA multiplex assays, the use of a photocleavable peptide–oligonucleotide conjugate is attractive since the peptide portion can serve as a label with unique mass (e.g. a mass marker). To date a variety of peptide–oligonucleotide hybrids have been described. Conjugation of a peptide moiety to an oligonucleotide can improve certain properties of antisense or therapeutic oligonucleotides, such as target binding properties, cellular uptake or exonuclease stability (10,11). Oligonucleotide–polyamide conjugates were also used as PCR primers (12), as carriers for non-radioactive labels (13,14) and in combinatorial chemistry (15). Peptide–oligonucleotide heteroconjugates have also been studied by mass spectrometry (16).

Several methods for the synthesis of peptide–DNA conjugates have been described that are based on either solution coupling or solid support synthesis. Solution coupling usually relies on a very specific chemical reaction between a sulfhydryl group and a maleimide moiety (10,16–21) whereas solid support synthesis relies on synthesis of the peptide moiety on a support followed by coupling to a specialty linker and then appending the DNA sequence (22–27).

Recently, we described the synthesis of photocleavable biotin (PC-biotin) DNA/RNA conjugates (28) and their analysis by UV-MALDI and IR-MALDI (29). We have shown that the nitrogen laser pulse (337 nm) normally used for UV-MALDI can be used to cleave the photocleavable linker between biotin and oligonucleotide. This method allows for affinity purification of the nucleic acid analyte through biotin–streptavidin interaction and photorelease of the unmodified nucleic acid.

In the present paper we describe the synthesis and characterization of photocleavable peptide–DNA conjugates as well as their evaluation as photocleavable mass marker (PCMM) hybridization probes for the detection of target DNA sequences via the use of MALDI-MS. The outline of the general approach for the detection of DNA sequences via

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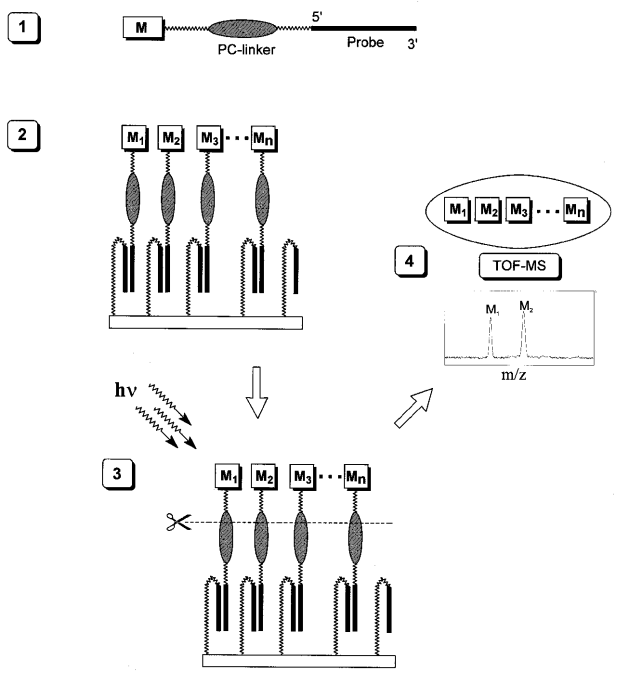

Figure 1. Schematic for the detection of target DNA/RNA sequences using photocleavable mass marker (PCMM) probes. (1) Structure of PCMM probe; (2) hybridization of a pool of PCMM probes to target DNA/RNA followed by stringent washes; (3) cleavage of the mass marker (M) molecules from hybridized PCMMs; (4) detection of marker molecules in a time-of-flight mass spectrometer.

photocleavable mass markers is presented in Figure 1. The photocleavable conjugates consist of a probe portion (DNA, RNA or PNA) which interacts with the immobilized target sequence (DNA or RNA). A photocleavable linker connects the probe portion of the conjugate to a marker molecule M, which is detected by mass spectrometry after the UV-MALDI desorption laser pulse has induced its photorelease. The marker M is designed to have a unique molecular weight, and serves as the sequence identifier. In a typical scenario (Fig. 1), a set of hybridization probes labeled with different mass markers is first hybridized to the immobilized target DNA/RNA of interest. After stringent washes to remove the non-hybridized probes, the matrix is applied and the substrate is subjected to UV-MALDI analysis. In the mass spectrum signals corresponding to several marker molecules M are detected, thereby identifying the sequences present in the target. The photocleavage step can also be performed using an external light source prior to MALDI analysis.

MATERIALS AND METHODS

Fmoc-protected peptides (>95% purity grade) were custom synthesized by Bio-Synthesis Inc. (Lewisville, TX). *N*-hydroxybenzotriazole (HOBt) and benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) were obtained from Novabiochem (La Jolla, CA). Tetramethylguanidine and 3-hydroxypicolinic acid were obtained from Aldrich (Madison, WI). Glycerol and succinic acid were obtained from Fluka (Buchs, Switzerland). All other chemicals were obtained from Perseptive Biosystems (Framingham, MA).

Oligodeoxyribonucleotide syntheses were carried out on a 1 μmol scale using an Expedite 8909 synthesizer (Perseptive Biosystems, Framingham, MA). For the preparation of photocleavable peptide–DNA probes, the DNA was modified on the 5′-end with PC-aminotag phosphoramidite (30). The columns were then removed from the synthesizer and treated with 2 ml of 20% tetramethylguanidine in anhydrous acetonitrile for 20 min using two syringes. The support was washed with 5 × 2 ml of acetonitrile and dried under vacuum for 15 min. The solution of Fmoc-protected peptide (5 μmol), PyBOP (10 μmol), HOBt (10 μmol) and diisopropylethylamine (DIPEA) (10 μmol) in 500 μl of DMF was then incubated with the PC-aminotag-modified DNA on a solid support for 2 h. The support was washed with DMF (3 × 2 ml), acetonitrile (3 × 2 ml) and dried under vacuum for 10 min. The PC-peptide–DNA conjugate was then cleaved from the solid support, deprotected with ammonia and purified by RP-HPLC as previously described (28). The resulting conjugates were characterized by analytical HPLC, denaturing PAGE and MALDI-MS. The yield of each purified conjugate was in the range 20–25 OD_{260}, which corresponds to a 17–22% yield based on the 1 μmol synthesis scale. Photocleavage reactions in solution for PAGE and HPLC analysis were carried out as described previously (28).

The sequences of the DNA and peptides used for the preparation of photocleavable mass marker probes were: PCMM-1, d(CACGTACAGGATGTACAG) and GRALGVFV; PCMM-2, d(TCGAGAGGTACATCGTG) and GRALGVFL; PCMM-3, d(AAGCGGTACGAGTAGCA) and GRALGVFF.

Immobilized, fully complementary target oligodeoxyribonucleotides for the hybridization experiments were synthesized on a DMT-C_{27} solid support with a hexaethylene glycol spacer at the 3′-end (Penninsula Laboratories, Belmont, CA) on a 0.2 μmol scale. After synthesis and deprotection, the support (~5–6 mg) was transferred to a centrifuge tube, washed with water (5 × 300 μl) and finally resuspended in 500 μl of hybridization buffer. For the hybridization experiment, a 10 μl aliquot of target suspension (~4 nmol) was hybridized with 10 pmol of the PC-peptide–DNA in 50 μl of hybridization buffer. Hybridization was carried out in 25 mM NaCl, 20 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5, using the following steps: denaturation at 72°C for 10 min; annealing at 72–37°C over 30 min. The CPG beads were then washed with ice-cold water (4 × 50 μl).

UV-MALDI-MS measurements were carried out on a Voyager DE-TOF mass spectrometer (PE Biosystems, Framingham, MA) with delayed ion extraction and a nitrogen laser (337 nm). IR-MALDI spectra were obtained on a laboratory built linear instrument (31) equipped with delayed ion extraction (U_{acc} = 20 kV) and a Q-switched Er:YAG laser (λ = 2.94 μm). For UV-MALDI measurements, 3-hydroxypicolinic acid (0.3 M 3-HPA in H_2O) was used as a matrix. For IR-MALDI, 0.3 M succinic acid (in H_2O) or glycerol was used (31). For measurements on PCMMs hybridized to immobilized DNA, an aliquot of the bead suspension (0.5–1.0 μl) was co-deposited with the matrix (dried droplet). Mass spectra were externally calibrated with peptide mixtures.

Thermal denaturation studies of the oligonucleotides were performed in a stoppered 1 cm path length quartz cell on a Hewlett-Packard 8543 spectrophotometer equipped with a temperature controller. The concentration of each strand was

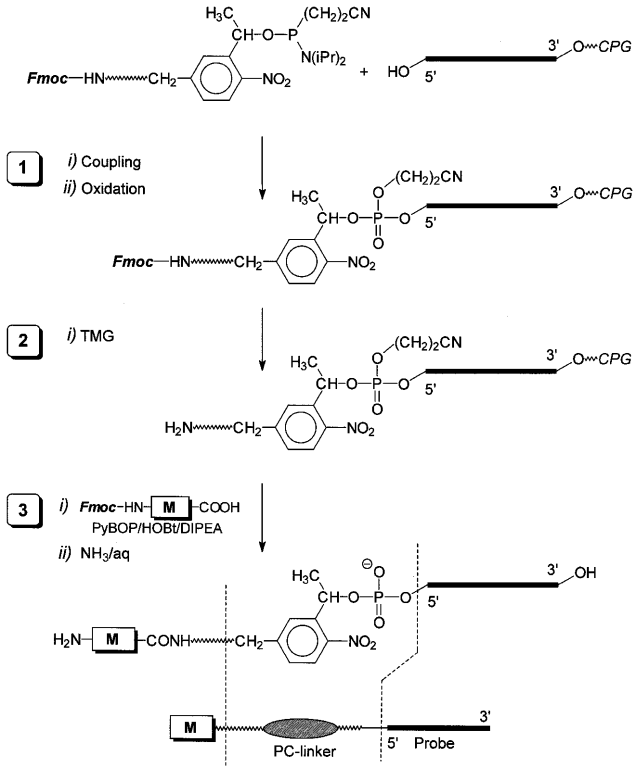

Figure 2. Synthesis of photocleavable peptide–DNA conjugates. (1) Conjugation of PC-aminotag phosphoramidite with DNA sequence; (2) removal of the Fmoc group with tetramethylguanidine solution; (3) coupling of the Fmoc-protected peptide (marker M) to the photocleavable amino group on the 5′-end of DNA followed by cleavage from solid support and deprotection with ammonia.

1.5 µM, the buffer used was 0.1 M NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH 7.0. Samples were initially annealed by heating to 90°C for 5 min and then cooling to 5°C at 1°C/min. The T_m curves were measured from 5 to 90°C with a ramping speed of 1°C/min, the solution being equilibrated at each temperature for 2 min. Standard HP software was used for the determination of T_m values. Each T_m value represents an average of nine measurements.

RESULTS

Synthesis of PC-peptide–DNA conjugates

The synthesis outline of the photocleavable (PC) peptide–DNA conjugates is presented in Figure 2. First, the DNA sequence was assembled on a solid support and modified on its 5′-end with the PC-aminotag phosphoramidite [Fig. 2, step **1**] (30). This results in modification of the 5′-phosphate with the photocleavable linker which is terminated with the Fmoc-protected amino group. In the next step **2**, the Fmoc group was selectively removed with tetramethylguanidine (TMG) solution under anhydrous conditions. TMG treatment typically caused 5–10% cleavage of the 3′-OH succinate linkage. In the next step **3**, the DNA chain with the free amino group on the photocleavable linker was coupled with the Fmoc-protected peptide (marker M), which was pre-activated with PyBOP/HOBt/DIPEA. The

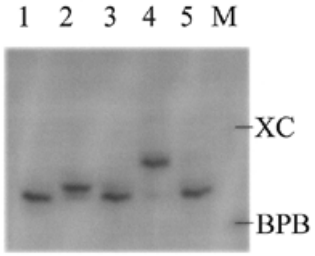

Figure 3. PAGE analysis of photocleavable peptide–DNA markers. Lane 1, 17mer oligonucleotide of PCMM-1; lane 2, same 17mer modified with PC-aminotag; lane 3, 17mer modified with PC-aminotag and irradiated; lane 4, PCMM-1; lane 5, PCMM-1 irradiated; lane M, dye markers xylene cyanol (XC) and bromophenol blue (BPB).

PC-peptide–DNA conjugate was then cleaved from the solid support and deprotected with concentrated ammonia. This treatment also removed the Fmoc group on the N-terminal amino group of the peptide.

After preparative RP-HPLC purification the photocleavable peptide–DNA conjugates were analyzed by RP-HPLC and denaturing PAGE, as well as MALDI-MS. In RP-HPLC (see Materials and Methods for details), the PC-peptide–DNA conjugates are characterized by retention times in the range 31–32 min (data not shown), presumably due to hydrophobicity imparted by the photocleavable linker and peptide moieties. For sequences modified with the PC-aminotag only, the retention times observed are in the range 16–18 min. After photocleavage and loss of the peptide or aminotag moiety, the retention time of the DNA portion decreases to ~11–12 min, which is characteristic of the unmodified oligonucleotide (data not shown).

Figure 3 shows the results of a denaturing PAGE analysis of one of the PC-peptide–DNA conjugates (PCMM-1). For comparison, unmodified DNA as well as DNA modified with the PC-aminotag were also included in this analysis. The introduction of the photocleavable aminotag moiety onto the DNA (lane 2) causes a small retardation on the gel when compared with unmodified DNA (lane 1). After photocleavage and removal of the PC-aminotag linker moiety (lane 3), retardation is no longer observed. For PC-peptide–DNA (lane 4), a significant mobility shift is observed due to the presence of the peptide moiety. After irradiation with near-UV light (lane 5), the mobility is comparable with that of the unmodified DNA (lane 1), which indicates complete loss of the peptide moiety due to photocleavage. Weak bands at the position of cleaved DNA are also visible in samples that were not subjected to UV irradiation (lanes 2 and 4). These are most likely due to stray light photocleavage. Similarly, for the UV-irradiated sample (lane 2), a weak band of non-cleaved conjugate is observed, most likely due to incomplete photocleavage.

Evaluation of PC-peptide–DNA conjugates as hybridization probes: thermal denaturation studies

In order to evaluate the hybridization properties of PC-peptide–DNA conjugates, a series of thermal denaturation experiments was performed. These studies involved thermal melting measurements for unmodified duplexes as well as for duplexes

in which one strand was modified with the PC-aminotag linker or with PC-peptide. The results of these experiments are presented in Table 1. This data shows that conjugation of PC-aminotag or PC-peptide to the DNA moiety has only a small effect on the T_m of the duplexes. No significant change is observed in the melting temperature for probes PCMM-1 and PCMM-3 upon modification with PC-aminotag or PC-peptide, whereas for probe PCMM-2 a small increase (+3°C) of the T_m is observed for modification with both PC-aminotag and PC-peptide. These results are consistent with earlier studies, indicating a T_m increase which depends on the number of arginine residues conjugated to an oligonucleotide (20,32).

Table 1. Melting temperatures (T_m, °C) of three photocleavable mass marker probes (PCMMs)

5′-Terminus	5′-OH	5′-H_2N-X-PC	5′-peptide-X-PC
PCMM-1	59.5 ± 0.8	59.9 ± 0.7	59.8 ± 0.7
PCMM-2	58.6 ± 0.4	61.3 ± 0.4	61.4 ± 0.4
PCMM-3	61.5 ± 0.6	61.3 ± 0.5	61.9 ± 0.4

Column 2, unmodified probe; column 3, probe modified with PC-aminotag; column 4, probe modified with PC-peptide. Standard errors are given. For details see Materials and Methods. X, spacer arm; PC, photocleavable linker

Evaluation of PC-peptide–DNA conjugates as hybridization probes using MALDI-MS

The synthesized photocleavable peptide–DNA conjugates were initially analyzed in solution by both UV-MALDI and IR-MALDI-MS. The molecular masses determined in these experiments are presented in Table 2. As an example, Figure 4 shows the spectra obtained from PCMM-2. In the UV-MALDI spectrum (Fig. 4a), a signal of the intact conjugate was observed at 6439.8 Da as well as signals corresponding to the products of the photocleavage reaction (DNA at 5332.1 Da and peptide mass marker at 1108.2 Da). The signal of the cleaved mass marker is the base peak in this spectrum. For both the signal of the intact conjugate and the cleaved mass marker,

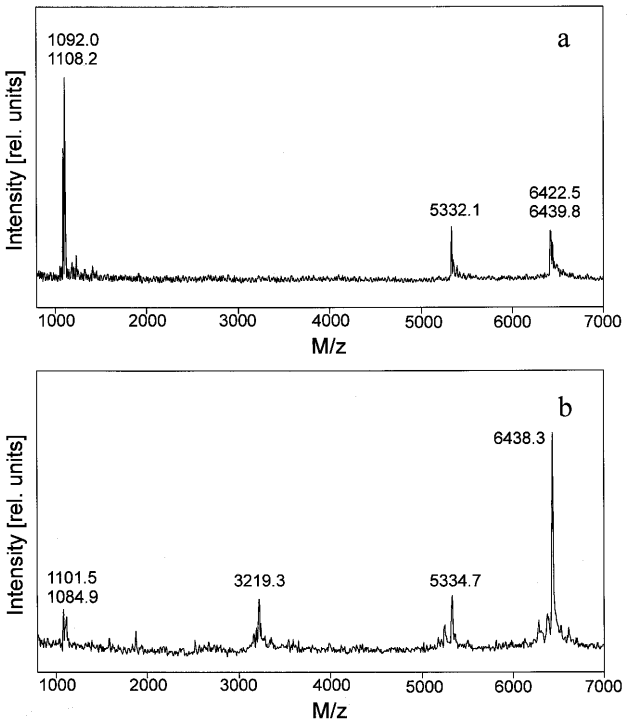

Figure 4. MALDI-MS spectra of PCMM-2 from solution: (**a**) UV-MALDI-MS; (**b**) IR-MALDI-MS.

satellite peaks at $\Delta m/z$ = –16 Da were observed at 6422.5 and 1092.0 Da, respectively. These satellite peaks are absent in the signal of the cleaved DNA portion of the conjugate observed at 5332.1 Da. Thus, the presence of these peaks is most likely due to a loss of oxygen from the intact conjugate as well as cleaved peptide mass marker. These satellite peaks were also observed when the sample was irradiated with an UV lamp prior to MALDI analysis (data not shown).

In order to demonstrate that the release of mass markers is due to photocleavage caused by the UV-MALDI, we also

Table 2. Measured and calculated molecular masses (Da) for photocleavable mass markers (PCMMs) using UV- and IR-MALDI

Conjugate		Calculated masses $(M+H)^+$	Measured masses (UV-MALDI)	Measured masses (IR-MALDI)
PCMM-1	$(M+H)^+$; $(M-O+H)^+$	6704.97; 6688.84	6709.1; 6692.5	6707.2; no
	$(DNA+H)^+$	5613.66	5615.9	5617.8
	$(peptide+pcl+H)^+$; $(peptide + pcl-O+H)^+$	1092.31; 1076.18	1094.90; 1078.7	1093.8; 1075.8[a]
PCMM-2	$(M+H)^+$; $(M-O+H)^+$	6436.79; 6420.87	6439.8; 6422.6	6438.3; no
	$(DNA+H)^+$	5331.46	5332.1	5334.7
	$(peptide+pcl+H)^+$; $(peptide+pcl-O+H)^+$	1106.34; 1090.42	1108.2; 1092.0	1101.5; 1084.9[a]
PCMM-3	$(M+H)^+$; $(M-O+H)^+$	6488.84; 6473.39	6489.9; 6473.9	6491.6; no
	$(DNA+H)^+$	5349.49	5349.8	5351.80
	$(peptide+pcl+H)^+$; $(peptide+pcl-O+H)^+$	1140.36; 1124.91	1141.6; 1125.0	1134.5; 1119.9[a]

pcl, photocleavable linker; no, not observed.
[a]Very small signals, limited mass accuracy.

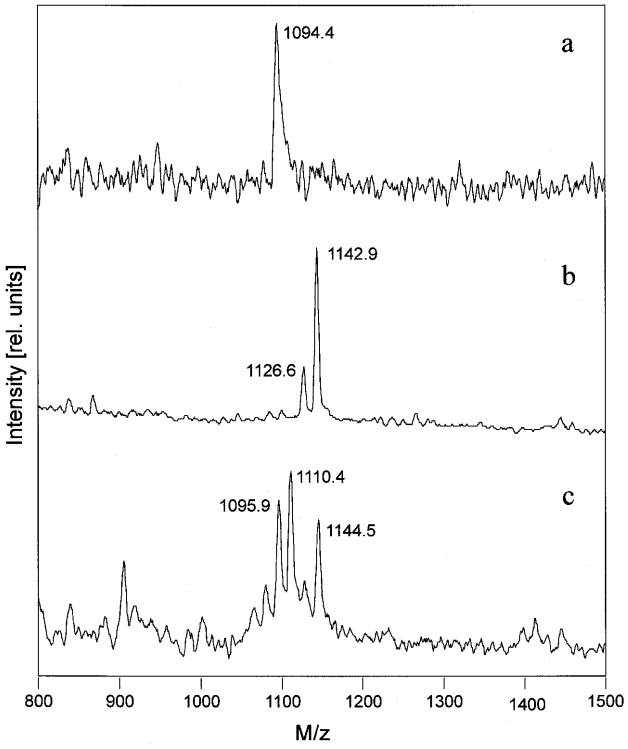

Figure 5. UV-MALDI analysis of PCMMs hybridized to DNA targets immobilized on CPG beads: (**a**) PCMM-1 hybridized to its target sequence; (**b**) mixture of three probes (PCMM-1, PCMM-2 and PCMM-3) incubated with target complementary to PCMM-3; (**c**) mixture of three probes (PCMM-1, PCMM-2 and PCMM-3) hybridized to target containing all three complements.

analyzed the conjugates by IR-MALDI-MS (Fig. 4b). The base peak observed in the IR-MALDI spectrum of PCMM-2 at 6438.3 Da corresponds to the uncleaved conjugate. Low intensity signals corresponding to cleaved DNA at 5334.7 Da and peptide mass marker are also observed in this spectrum. The signal observed at 3219.3 Da corresponds to the doubly protonated ion of uncleaved conjugate. In general, low intensity signals corresponding to the cleaved peptide and DNA were occasionally observed in the IR-MALDI mass spectra. Their origin is unclear, although both solution photolysis by stray light prior to sample preparation and collision-induced fragmentation might contribute here. The possibility that some cleavage reaction also takes place upon IR laser desorption, although unlikely, cannot be completely ruled out. Notably, no $[M-16]^+$ satellite peaks were observed in the IR-MALDI spectra (Table 2). We also note that IR- and UV-MALDI mass spectra were acquired on different instruments, so a direct comparison of both absolute and relative signal intensities is difficult.

In the next series of experiments, photocleavable peptide–DNA probes were hybridized to three synthetic fully complementary oligodeoxyribonucleotides (17–18 nt) immobilized on CPG beads. After stringent washes, aliquots of the CPG bead suspension were deposited on the MALDI target, mixed with matrix and analyzed by UV-MALDI-MS. The results of these experiments are presented in Figure 5. Figure 5a shows the spectrum of PCMM-1 hybridized to its complement on CPG beads. In this spectrum, a signal of the cleaved peptide mass marker at 1094.4 Da is also observed (corresponding to the loss of 16 Da, most probably oxygen).

The selectivity of detection was studied in a competition experiment where immobilized target DNA complementary to PCMM-3 was incubated with an equimolar solution of all three conjugates, washed, and then subjected to UV-MALDI-MS. The result of this experiment is presented in Figure 5b. As seen, only a signal of the mass marker from PCMM-3 at 1142.9 Da is observed in the spectrum, demonstrating both selectivity of hybridization and the absence of non-specific adsorption of the photocleavable peptide–DNA conjugates to the CPG beads. As in the case of solution analysis, a satellite peak at 1126.6 Da (loss of 16 Da) is also observed.

In a different experiment, designed to evaluate the possibility of multiplex detection of several target sequences, a CPG-immobilized oligonucleotide containing sequences complementary to all three PCMMs studied was used. The three immobilized target sequences were separated from each other by 2 nt. The CPG support was incubated with an equimolar solution of all three PCMMs, subjected to stringent washes and analyzed by UV-MALDI-MS. The result of this experiment is shown in Figure 5c. Signals from all three cleaved peptide mass markers at 1095.9, 1110.4 and 1144.5 Da are observed, as expected. Furthermore, the relative signal intensities were found to be independent of the arrangement of the target sequences within the immobilized strand (data not shown). This result clearly demonstrates the feasibility of the described approach to a highly multiplexed detection of target DNA/RNA sequences.

In summary, for all three immobilized PCMM–target duplexes analyzed, signals of the cleaved markers (peptides) are observed in the UV-MALDI spectra. Similarly to mass spectra obtained from solutions of PCMMs alone, satellite peaks at $\Delta m/z = -16$ Da are observed to a varying degree. Additionally, weak signals from the uncleaved conjugate or the cleaved DNA were occasionally observed in the mass spectra obtained from these preparations (data not shown). These latter signals are probably due to a partial duplex denaturation under MALDI sample preparation conditions. Furthermore, the mass accuracy in mass spectra obtained from CPG bead preparations is less than optimal in this m/z range. This effect can be explained by the very heterogeneous sample morphology. The time-of-flight and mass determination depend critically on the starting position of the ions in the electric field of the ion source. The former varies due to the non-uniform thickness of the bead preparations. However, in all cases the mass accuracy is sufficient to unequivocally detect specific hybridization.

DISCUSSION

In this paper the synthesis, characterization and application of photocleavable peptide–DNA conjugates to the detection of target DNA sequences by UV-MALDI has been described. The conjugates were prepared by first assembling the DNA sequence, followed by modification of the 5′-end with the PC-aminotag phosphoramidite, and then subsequent coupling of the Fmoc-protected peptide. This synthetic strategy has several advantages: (i) the solid support synthesis enables easy removal of excess reagents; (ii) the strategy can be applied to a wide variety of molecules containing a carboxyl group, including peptides; (iii) the chemical bond between the peptide and DNA portions of the conjugate is a peptide bond, stable

under MALDI-MS conditions; (iv) the conjugates are easily purified by RP-HPLC with reasonable yields (17–22%).

The photocleavable mass markers (PCMMs) described in this paper have several properties that make them very useful for multiplex assays, including the following. (i) The peptide portion serves as a mass marker with unique molecular mass. The 20 standard amino acids as well as unnatural analogs provide the possibility of synthesis of numerous marker molecules with easily resolvable mass differences. (ii) The DNA portion of the conjugate acts as a hybridization probe. The hybridization properties of such conjugates depend on the number of cationic residues in the peptide moiety (10,32) and can be easily controlled. (iii) The photocleavable linker connecting the DNA and peptide moieties is known to exhibit rapid and efficient photocleavage (28) when exposed to near-UV light and is also easily cleaved during UV-MALDI (33).

The use of PCMMs described in this paper avoids limitations such as metastable fragmentation or cation adduct formation, which are inherent in the direct detection of oligonucleotide hybridization probes (34), or PCR products (35). Future experiments will be directed towards the development of specific applications relying on multiplex detection of photocleavable mass markers cleaved from hybridization probes or PCR amplicons, as well as evaluation of other polymers as unique mass markers.

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