Analysis of Oligonucleotides by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS; Karas et al., 1987; Karas and Hillenkamp, 1988) is one of the most useful techniques available for determining biomolecule mass. MALDI-MS offers high mass accuracy, good sensitivity, simplicity, and speed, all of which make it suitable for the analysis of oligonucleotides. Conceptually, sample analysis using MALDI-MS is relatively straightforward: the analyte of interest is mixed with an appropriate matrix and spotted on a sample plate, and the plate is placed in the instrument for analysis. Normally, singly charged ions of oligonucleotides are observed, which makes MALDI-MS spectra very easy to interpret and simplifies the analysis of mixtures (Fig. 10.1.1). Nowadays commercial MALDI instruments, usually interfaced with time-of-flight (TOF) mass analyzers, are easy to operate and are routinely used for many types of oligonucleotide analysis. A complementary approach to oligonucleotide analysis is electrospray ionization mass spectrometry (ESI-MS); this approach is presented, along with a comparison of the two techniques, in *UNIT 10.2*.

At present MALDI-MS is utilized routinely for the analysis of 120-mer and smaller oligonucleotides. Polymerase chain reaction (PCR) and restriction enzyme digestion products larger than 120-mers have not yet been routinely mass analyzed, although 500-mer and larger oligodeoxynucleotides have been ionized and observed (Tang et al., 1994a; Liu et al., 1995a; Berkenkamp et al., 1998). Poor ionization efficiencies and fragmentation during the desorption/ionization process have been blamed for the lack of success with higher-molecular-weight oligonucleotides (Nordhoff et al., 1993; Schneider and Chait, 1993; Zhu et al., 1995). However, MALDI-MS has become a useful method to rapidly identify and characterize oligonucleotides up to 120-mers, and may soon replace the combination of Maxam-Gilbert chemical degradation and subsequent polyacrylamide gel electrophoresis (PAGE) for sequence characterization of these samples. Only high femtomole to low picomole per microliter amounts of oligonucleotides are needed for an analysis. Because MALDI-MS can be used to analyze mixtures, synthetic oligonucleotides (usually <30-mers) can be characterized by their failure sequences (Keough et al., 1993; Limbach, 1996). Exonuclease digestion has also proven to be a versatile alternative approach for determining the sequences of moderate-length oligonucleotides (Pieles et al., 1993; Limbach, 1996; Smirnov et al., 1996). A still unproven, but promising, strategy for determining oligonucleotide sequences is the analysis of prompt and metastable fragment ions (Nordhoff et al., 1995; Juhasz, 1996).

Because of MALDI-MS's potentially greater accuracy and speed of analysis compared to electrophoresis-based methods, considerable effort has been expended on DNA sequencing reaction (Sanger reaction) product readout (Murray, 1996). One strategy to overcome the ionization and fragmentation limitations is to replace normal nucleotides with nucleotide analogs (7-deaza- and 2′-fluoropurines) that are more stable during MALDI-MS analysis and are tolerated by the polymerase(s) used during chain extension and termination reactions (Kirpekar et al., 1995; Schneider and Chait, 1995; Tang et al., 1997b). Another potential application of MALDI-MS is the analysis of PCR products as a means to characterize disease-specific genetic mutations (Bai et al., 1994b; Ch'ang et al., 1995; Hurst et al., 1996; Wada and Yamamoto, 1997). Affinity probes based on hybridization between complementary strands of oligonucleotides to capture specific oligonucleotides fragments (e.g., DNA sequencing fragments, PCR products) for poten-

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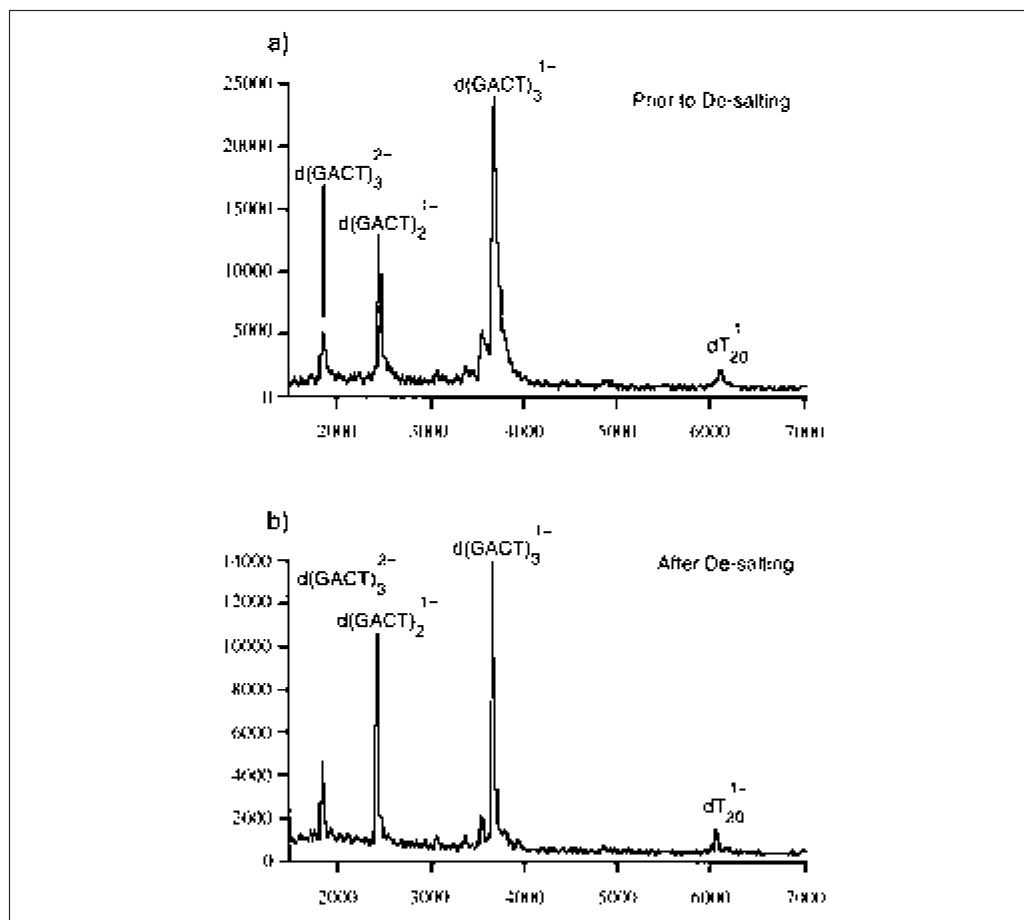

Figure 10.1.1 MALDI mass spectrum of oligonucleotide mixture (**a**) prior to desalting and (**b**) after desalting with ammonium-activated cation-exchange resin beads.

tial automated MALDI-MS analysis are being developed (Jurinke et al., 1997; Little et al., 1997; Ross and Belgrader, 1997; Ross et al., 1997).

In this unit the practical aspects of oligonucleotide analysis by MALDI-TOF MS are discussed, with protocols provided for certain specific procedures. Sample preparation—including purification of the oligonucleotide, preparation of the matrix and co-matrix, and preparation of the sample for analysis—is discussed in detail. Protocols for oligonucleotide purification using ammonium-activated cation-exchange resin beads (see Basic Protocol 1), spin columns (see Basic Protocol 2), C_{18} reversed-phase cartridges (see Alternate Protocol 1), and molecular-weight-cutoff filters (see Alternate Protocol 2) are included. Also covered are steps for preparing matrix and co-matrix solutions (see Basic Protocol 3) and the matrix/analyte samples for analysis (see Basic Protocol 4).

In addition, an introduction to the instrumentation and its calibration is provided, along with discussions of the technique's application to molecular-weight measurement, oligonucleotide sequencing—including a method for exonuclease-based sequencing of oligonucleotides using MALDI-TOF MS (see Basic Protocol 5)—and analysis of PCR reactions.

OLIGONUCLEOTIDE PURIFICATION AND DESALTING

The most important criterion for successful MALDI-MS analysis of oligonucleotides is sample preparation. Oligonucleotide sample contaminants *must* be reduced or removed prior to analysis. The primary challenge to accurate molecular-weight measurements of oligonucleotides and nucleic acids is the presence of salt adducts, which must be reduced or completely removed. In solution, the phosphodiester backbone is completely ionized at pH >1, and the solvent acts as a dielectric shield to reduce the repulsive Coulombic charging. During the ionization process, this Coulombic protection is lost, which eventually results in the adduction of any cations that may be present in the sample solution. These adducts shift the ion signal to higher *m/z* values and interfere with accurate determination of molecular weights (Fig. 10.1.1).

Sources of Contamination

Cations can arise from any source with which the oligonucleotide sample comes into contact, such as solvents, buffers, the mass spectrometer sample probe, solution storage containers, and pipet tips. To reduce cation concentration, ultrapure water—water that has been doubly distilled and deionized (e.g., "Nanopure" water, purified using a Barnstead/Thermolyne Nanopure system)—should always be used. Organic solvents to be used in preparing matrix solutions may need to be desalted by distillation or using activated ion-exchange resin beads, such as Bio-Rad AG501-X8(d) resin beads (Simmons and Limbach, 1998).

Often the sample holder can be a source of contaminants. The sample probe and plates must be cleaned carefully before use so that cross-contamination with the previous sample will not interfere with the newly deposited oligonucleotides. Sonicating and washing in organic solvents, such as methanol and acetone, effectively removes most matrices and analytes. Further, stainless steel sample plates can be cleaned by polishing followed by acid oxidation in a dilute inorganic solution such as 0.1 N nitric acid. The sample plates should be rinsed with ultrapure water to eliminate residual cations. Nonmetallic sample substrates, such as nitrocellulose and Nafion films, can be used as alternatives; these have been demonstrated to reduce salt adducts and enhance the ion signal of oligonucleotides, especially for those of >100-mer size (Bai et al., 1994a,b, 1995; Liu et al., 1995a).

Many buffers simply interfere with ion formation without forming intense adduct peaks. Investigation of the tolerance limits for several common buffers (Shaler et al., 1996) determined that oligonucleotides could be analyzed in the presence of up to 10 mM salt or 500 mM buffer in the sample solution, and that positive ions were less affected than negative ions by impurities.

Purification Methods

Table 10.1.1 summarizes the numerous oligonucleotide purification schemes available. The most common approaches are presented as protocols within this unit. In all cases, after purification, the sample may be analyzed immediately or stored (at –20°C) for future use. Optimal MALDI-MS results will be obtained if the sample is analyzed immediately after purification. A popular and useful method is to treat both the sample and matrix solutions with cation-exchange resin beads, such as Bio-Rad AG50W-X8 resin beads (see Basic Protocol 1; Nordhoff et al., 1992). Although these beads are usually obtained from the manufacturer in the free acid form, the ammonium-activated form is preferred for MALDI-MS; a procedure for activating the beads is described below (see Support Protocol 1).

A simple method to remove minor cations (at concentrations <10 mM) is to add an ammonium salt, such as ammonium citrate, tartrate, or fluoride, to the matrix solution

(Currie and Yates, 1993; Pieles et al., 1993; Zhu et al., 1996b, Cheng, 1996). Except with 2′,4′,6′-trihydroxyacetophenone (THAP), the concentration of ammonium salt should be ≤10 mM (see section on Matrix/Analyte Preparation); otherwise, the matrix will not crystallize well, limiting the ability to find "hot spots" that yield abundant ion signal. A method that has been useful for desalting oligonucleotides in ESI-MS (see *UNIT 10.2*) has been adapted for MALDI-MS (Simmons and Limbach, 1997, 1998). In this approach organic bases, such as triethylamine or piperidine, are used as co-matrices—i.e., are combined with the matrix; these organic bases have high cation affinities and serve to reduce or eliminate cation adduction at levels exceeding those obtainable with cation-exchange resin beads for extremely salty samples. The organic bases should be treated with ion-exchange beads, such as Bio-Rad AG501-X8(d) resin beads, before use to eliminate impurities.

A number of vendors (Millipore, Amersham Pharmacia Biotech, Boehringer Mannheim, Amicon) offer prepackaged purification systems. These purification devices are typically 0.5- to 2.5-mL microcentrifuge tubes containing coated particles or filters that retain the oligonucleotide and allow contaminants to be washed off the sample.

C_{18} reversed-phase cartridges (see Alternate Protocol 1) utilize the strong binding affinity of oligonucleotides for the stationary-phase packing material in the presence of 2 M triethylamine as the means of trapping the sample for purification. The oligonucleotides are eluted with 20% aqueous acetonitrile. Sephadex G-25 spin-column purification (see Basic Protocol 2) is a very useful, quick method (~5 min) to eliminate most salts and buffers if the oligonucleotide is larger than a 10-mer; generally one pass through the column yields satisfactory results. Spin columns must be equilibrated in ultrapure water before desalting. Molecular-weight-cutoff filters or membranes (see Alternate Protocol 2) retain oligonucleotides above a certain molecular weight and allow contaminants to be washed through. The membrane or filter is then inverted and the oligonucleotide is eluted and lyophilized.

For samples that contain a high concentration of inorganic impurities (such as PCR products), reversed-phase high-performance liquid chromatography (HPLC), anion-exchange chromatography, microdialysis, or ethanol precipitation (see *UNIT 10.2*) followed

Table 10.1.1 Common Approaches for Desalting and Purifying Oligonucleotides and Matrixes Prior to MALDI-TOF MS Analysis

Means of purification (and selected manufacturers)	Reference(s)
Oligonucleotides	
Cation-exchange resin beads	Nordhoff et al. (1992); Basic Protocol 1
Ammonium salt co-matrices	Pieles et al. (1993); Basic Protocol 3
Organic base co-matrices	Simmons and Limbach (1997); Basic Protocol 3
C_{18} reversed-phase cartridges (Waters, Perkin Elmer, Glen Research)	Alternate Protocol 1
Sephadex G-25 spin columns (Amersham Pharmacia Biotech, Boehringer Mannheim)	Roskey et al. (1996); Basic Protocol 2
Molecular-weight cutoff filters (Amicon, Millipore, Gelman Sciences)	Shaler et al. (1995); Alternate Protocol 2
Microdialysis	
HPLC	
Matrices	
Recrystallization	
Cation-exchange resin beads	Nordhoff et al. (1992); Basic Protocol 3

by lyophilization are reasonable purification techniques. Several appropriate HPLC buffers for oligonucleotide purification are presented in Table 10.1.2.

Biotin-linked oligonucleotides can be purified using affinity chromatography (Jurinke et al., 1997); this approach is often suitable (and preferred) for enzymatic digestion products.

BASIC PROTOCOL 1

Cation-Exchange Resin Purification of Oligonucleotides

Ammonium-activated resin beads are used to reduce salt contamination of oligonucleotide mixtures prior to MALDI-MS analysis. This method can also be used to desalt matrix solutions.

Materials

Oligonucleotide sample solution, 100 to 400 µM in ultrapure water
Ammonium-activated cation-exchange resin beads (see Support Protocol 1), either dry or slurry

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Place oligonucleotide sample in a test tube and add 10 to 20 mg of resin per 20 µL sample solution.
2. Let mixture stand 5 to 20 min. Decant supernatant.
3. The sample is now ready for analysis (see Basic Protocol 4).

   Overnight incubation of an oligonucleotide sample with resin beads should be avoided, as this leads to degradation of oligonucleotide spectra (Fitzgerald et al., 1993a).

   Best results are obtained if the sample is analyzed immediately, but it may instead be stored at –20°C for later analysis.

Table 10.1.2 Common HPLC Buffers Suitable for Oligonucleotide Purification

Buffer	Preparation
Reversed phase	
A = 25 mM triethylammonium bicarbonate (TEAB), pH 6	Add 7 mL triethylamine to container and fill with water to 2 L; bubble CO_2 gas (from sublimation of dry ice) through solution for ~1.5 hr until pH reaches 6
B = 40% acetonitrile (AcCN)	Add 80 mL AcCN to container and fill with water to 2 L
Anion exchange	
A = 25 mM TEAB/20% (v/v) AcCN, pH 6.4	Add 7 mL triethylamine and 400 mL AcCN to container and fill with water to 2 L; bubble CO_2 gas through solution for ~2.5 hr until pH reaches 6.4
B = 1 M TEAB/20% (v/v) AcCN, pH 7.6	Add 280 mL triethylamine and 400 mL AcCN to container and fill with water to 2 L; bubble CO_2 gas through solution for at least 2.5 hr until pH reaches 7.6

SUPPORT PROTOCOL 1

Ammonium Activation of Cation-Exchange Resin Beads

This protocol describes the procedure for converting free-acid cation-exchange resin beads to the preferred ammonium form for use in Basic Protocol 1.

Materials

Ammonium acetate, HPLC grade
Ultrapure (e.g., Nanopure) water
Cation-exchange resin beads in free-acid (H-) state (e.g., Bio-Rad AG50W-X8 beads)

1. Add 30 g ammonium acetate to 250 mL ultrapure water. Stir for 1 hr.
2. Transfer the supernatant solution into a small container.
3. Add resin beads to solution, ensuring that the solution completely covers the beads and that the mixture can be stirred easily. Stir on a stir plate overnight.
4. Filter excess solution from resin beads and air-dry the beads if desired. Transfer the beads to a closed container and store at room temperature for future use.

   The resin beads can be used for up to 12 months after activation.

BASIC PROTOCOL 2

Spin-Column Purification of Oligonucleotides

Oligonucleotides >10-mers can be purified prior to MALDI-MS analysis using a spin column. Alternative methods using C_{18} reversed-phase cartridges and molecular weight cutoff filters are presented below (see Alternate Protocols 1 and 2). Sephadex G-25 spin columns are efficient (95% salt free, 85% average recovery) and fast (<5 min).

Materials

Microspin column (e.g., Sephadex G-25)
Ultrapure (e.g., Nanopure) water
Oligonucleotide sample solution, 500 to 1000 μM in ultrapure water

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Resuspend the resin in the microspin column by vortexing gently.
2. Loosen the cap of the column and open bottom closure.
3. Place the column in a 1.5-mL microcentrifuge tube for support.
4. Prespin the column for 1 min at recommended *g* force.

   If the centrifuge is marked in rpm (revolutions per minute), using the following equation to convert the force (× g) to rpm:

$$\text{RCF (in } g) = \left(\frac{1.12 \times r \times \text{rpm}}{1000}\right)^2$$

   where r = radius in millimeters measured from center of spindle to bottom of rotor bucket.

5. Discard the solution to waste in the 1.5-mL tube support. Resuspend the resin in 200 μL ultrapure water to rinse off residual impurities and spin 1 min at the same speed.

6. Place the column in a new 1.5-mL tube and slowly apply the sample to the center of the angled surface of the compacted resin bed, being careful not to disturb the resin bed. Do not allow any of the sample to flow around the sides of the bed.

7. Spin the column for 2 min at the recommended *g* force.

   The purified sample will collect at the bottom of the support tube.

8. Lyophilize the purified sample. After lyophilization, the sample is ready for analysis (see Basic Protocol 4).

   Best results are obtained if the sample is analyzed immediately, but it may instead be stored at –20°C for later analysis.

ALTERNATE PROTOCOL 1

C_{18} Reversed-Phase Cartridge Purification of Oligonucleotides

This protocol describes the use of oligonucleotide purification cartridges, commonly used to purify machine-synthesized trityl-on oligonucleotides, for desalting oligonucleotides.

Materials

Oligonucleotide purification cartridge
Acetonitrile (AcCN), HPLC grade
2.0 M triethylammonium acetate (TEAA), HPLC grade
10 nmol oligonucleotide sample, dry
Ultrapure (e.g., Nanopure) water

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Slowly pass first 5 mL acetonitrile, then 5 mL of 2 M TEAA, through the purification cartridge to a waste container.

2. Dissolve the oligonucleotide sample in 1 to 3 mL ultrapure water, 0.1 M TEAA, or other aqueous solution.

   The loading solution should not contain any organic solvents or ammonium hydroxide.

3. Pass this solution through the oligonucleotide purification cartridge at a rate of ~1 drop per second exiting the cartridge, and collect the eluate.

4. Pass the eluate through the cartridge a second time.

5. Pass 10 mL ultrapure water through the cartridge to waste.

6. Elute and collect the desalted oligonucleotide by slowly passing, drop by drop, 1 mL of 50% acetonitrile in ultrapure water through the cartridge.

7. Lyophilize the desalted sample. After lyophilization, the sample is ready for analysis (see Basic Protocol 4).

   Best results are obtained if the sample is analyzed immediately, but it may instead be stored at –20°C for later analysis.

ALTERNATE PROTOCOL 2

Molecular-Weight-Cutoff-Filter Purification of Oligonucleotides

This protocol describes the procedure for purifying oligonucleotides larger than 25-mers prior to MALDI-MS analysis. It is essential to concentrate the sample to a small volume prior to desalting.

Materials

Molecular-weight-cutoff-filter column
Oligonucleotide sample solution, 100 to 400 µM in ultrapure water
Ultrapure (e.g., Nanopure) water

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Select a filter column with nucleotide cutoff equal to or smaller than the size of the nucleic acid to be desalted (consult the manufacturer's instructions).
2. Insert the sample reservoir into a microcentrifuge tube or into the tube supplied by manufacturer.
3. To concentrate the sample, pipet up to 500 µL of sample solution into the reservoir. Spin under the conditions (time and *g* force) recommended by the manufacturer, being careful not to exceed the recommended force.
4. To exchange salt, dilute the concentrated sample to 500 µL with ultrapure water. Spin under the conditions (time and *g* force) recommended by the manufacturer. To reduce salt concentration further, repeat this step.
5. Remove reservoir from vial and invert into a new vial (save and store filtrate at –20°C until sample has been analyzed).
6. Spin for 2 min at 500 to 1000 × *g* to recover oligonucleotide sample in the vial.
7. Remove reservoir.
8. Lyophilize sample in vial. After lyphilization, the sample is ready for analysis (see Basic Protocol 4)

   Best results are obtained if the sample is analyzed immediately, but it may instead be stored at –20°C for later analysis.

MATRIX/ANALYTE PREPARATION

Matrix and Co-Matrix Selection

The essence of MALDI-MS is the matrix—typically a small acidic organic compound that has a relatively high molar absorptivity (ε_λ) at the wavelength of the laser. Common MALDI-MS matrices have molar absorptivities on the order of 10^3 to 10^5 M^{-1} cm^{-1}. A large number of potential matrices have been investigated over the years, with a relatively small number becoming the standards for MALDI-MS of oligonucleotides (Fig. 10.1.2).

3-Hydroxypicolinic acid (3-HPA) has been the most useful oligonucleotide matrix, as it consistently results in the least fragmentation (Wu et al., 1993). Picolinic acid (PA) and

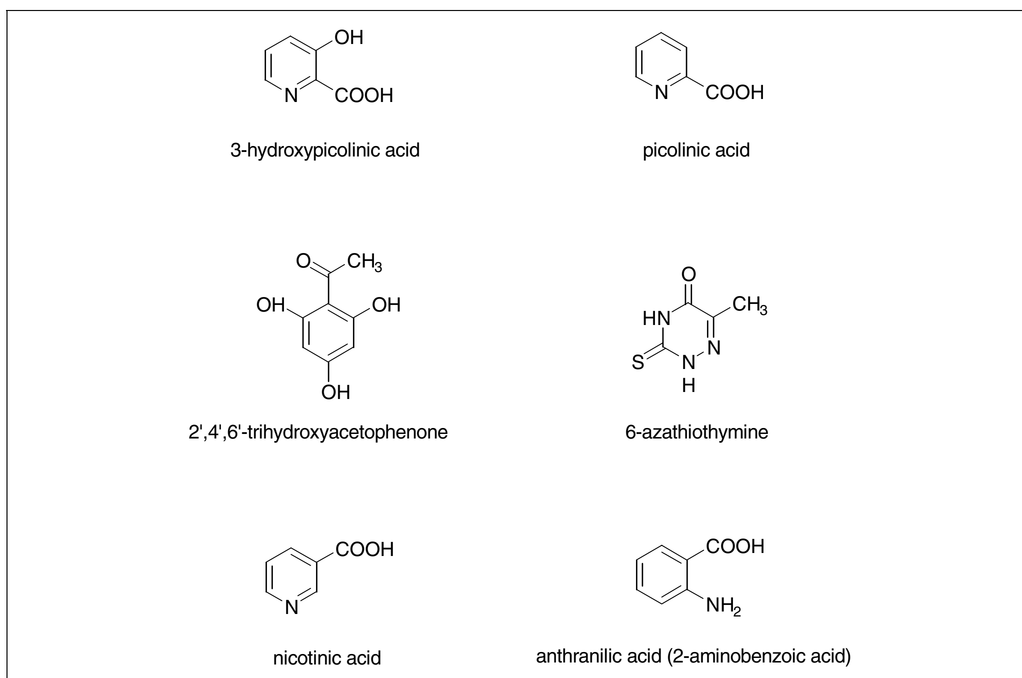

Figure 10.1.2 Chemical structures of the matrices most commonly used for analyzing oligonucleotides with MALDI-TOF MS.

3-aminopicolinic (3-APA) acid have also been investigated (Tang et al., 1994b), but are less practical choices, because PA works best at 266 nm and 3-APA is still not commercially available (Juhasz et al., 1996). However, a mixture of PA and 3-HPA is often used for larger oligonucleotides (Liu et al., 1995a). Other commonly used matrices include 2′,4′,6′-trihydroxyacetophenone (THAP; Pieles et al., 1993; Zhu et al., 1996a), a mixture of anthranilic acid and nicotinic acid (AA/NA; Nordhoff et al., 1992), and 6-aza-2-thiothymine (ATT; Lecchi et al., 1995). These are useful for smaller oligonucleotides, up to 25 bases in length (Nordhoff et al., 1996). The popular peptide and protein matrices, such 2,5-dihydroxybenzoic acid (DHBA; Strupat et al., 1991) and sinapinic acid (SA; Beavis and Chait, 1989), should not be used as they yield intense prompt and metastable fragmentation that limits the molecular ion abundance (Parr et al., 1992; Schneider and Chait, 1993).

BASIC PROTOCOL 3

Matrix and Co-Matrix Preparation

Fresh matrix solutions should be used within a week of preparation, before they start to degrade, and it is best to prepare them daily before use. Because most matrices have to be dissolved in suitable solvents prior to mixing with the analyte solution, the solvent(s) should be chosen based on the solubilities of the matrices. In all cases, a large molar excess of matrix to analyte (10^3 to 10^5:1) is used when preparing oligonucleotides for analysis. This protocol describes the procedure for preparing MALDI-MS matrix solutions.

Note: Optimal results are obtained when the highest-purity matrix and co-matrix are used.

Materials

One of the following matrices:
- 3-Hydroxypicolinic acid (3-HPA; Aldrich or Fluka)
- 3-Hydroxypicolinic acid and picolinic acid (Aldrich or Fluka; 3-HPA/PA)

2′,4′,6′-Trihydroxyacetophenone (THAP; Aldrich or Fluka)
6-Aza-2-thiothymine (ATT; Aldrich)
Co-matrix: dibasic ammonium citrate or imidazole (Aldrich or Fluka)
Organic solvent, HPLC grade: 100% methanol (for THAP) or 50% aqueous acetonitrile (for all other matrices)
Ultrapure (e.g., Nanopure) water
Ammonium-activated cation-exchange resin beads (see Support Protocol 1)

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Weigh one of the following amounts of matrix and place in a 1.5-mL tube:

   0.03 g 3-HPA;
   0.03 g 3-HPA and 0.0075 g PA;
   0.05 g THAP; *or*
   0.04 g ATT.

2. Add 500 µL of appropriate organic solvent to matrix (Table 10.1.3). Vortex for 30 sec. Except for THAP (which can be used directly), allow solutions to settle and remove supernatant for use with sample.

   Matrix solution should be used quickly after preparation. Storage for >3 days is not recommended.

3. Add ~50 mg of ammonium-activated cation-exchange resin beads to matrix solution. Stir gently and allow to stand 30 min. Remove supernatant for use with sample.

   Resin beads are included in matrix to reduce the formation of salt adducts. Using resin beads is strongly recommended when highest-purity matrix (purity >98%) is not available.

4. Optionally, prepare co-matrix solution by adding either 0.23 g dibasic ammonium citrate or 0.20 g imidazole to 10 mL water and vortexing until dissolved.

   Ammonium citrate solution should be made fresh prior to use; storage for >3 days is not recommended. Imidazole solution can be stored with Bio-Rad AG501-XG(d) resin beads for up to 3 months.

   Co-matrices are included in samples to reduce salt-adduct formation. Ammonium citrate is recommended for samples that are not expected to contain nonvolatile (e.g., sodium or potassium) salts, while imidazole is recommended for samples known to contain nonvolatile salts. Use of co-matrices is recommended even if the sample has been previously

Table 10.1.3 Guidelines for Preparation of UV Matrix Solutions

Matrix	Solution
3-Hydroxypiconilic acid (3-HPA)	Saturated (60 g/L) 3-HPA in 50:50 (v/v) acetonitrile/water or in 25:75 (v/v) methanol/water
3-Hydroxypiconilic acid/piconilic acid (PA)	Saturated 3-HPA and 15 g/L PA in 50:50 (v/v) acetonitrile/water
2′,4′,6′-Trihydroxyacetophenone (THAP)	100 g/L THAP in 50:50 (v/v) methanol (or ethanol)/water or in 100% methanol
6-Aza-2-thiothymine (ATT)	80 g/L ATT in 50:50 (v/v) acetonitrile/water

purified, as it tends to improve the quality of the mass spectral results, possibly through a cooling mechanism during the desorption process (Simmons and Limbach, 1998).

BASIC PROTOCOL 4

Matrix/Analyte Sample Preparation

Typically oligonucleotides up to 20-mers in size can be analyzed at concentrations down to the low femtomoles per microliter. To analyze larger oligonucleotides, more concentrated solutions—as much as 5 nmol/µl—are required. It is not critical that the sample, matrix, and co-matrix (if used) be mixed prior to spotting on the sample plate, although this is often done. A "two-layer" sample deposition method, similar to the sample preparation method for peptides suggested by Vorm et al. (1994), is widely used for oligonucleotides. In this protocol, the matrix solution is first deposited on the sample plate and allowed to dry. The oligonucleotide solution, with or without additional matrix solution, is then added on top of the dried matrix. The sample may be air-dried or drying may be assisted by blowing a slow stream of gas (house air or nitrogen) at room temperature over the sample.

Backbone-modified oligonucleotides in which the phosphodiester backbone is neutralized (e.g., methylphosphonates) or nonexistent (e.g., peptide nucleic acids) should be prepared and analyzed with MALDI-MS matrices used for peptides or protein analysis (Keough et al., 1993, 1996; Butler et al., 1996; Ross et al., 1997). If the backbone-modified oligonucleotide is not neutralized (e.g., phosphorothioates; Schuette et al., 1995; Polo et al., 1997), the oligonucleotides should be prepared as described for unmodified oligonucleotides.

This protocol details the procedure for preparing MALDI-MS sample solutions. If 2′,4′6′-trihydroxyacetopherone (THAP) is used as the matrix, follow steps 2b and 4b in place of steps 2a and 4a.

Materials

Ultrapure (e.g., Nanopure) water
Matrix solution (see Basic Protocol 3)
Co-matrix solution (optional; see Basic Protocol 3)
Oligonucleotide sample, dry, lyophilized or as a 100- to 400-µM solution in ultrapure water

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. If sample is in solid form, dissolve oligonucleotide sample in ultrapure water to a concentration of 100 to 400 µM.

   Lower concentrations are appropriate for small (<25-mer) oligonucleotides and higher concentrations are necessary for large (>50-mer) oligonucleotides.

2a. To a 0.5-mL tube, add 4 µL matrix solution, 1 µL oligonucleotide solution, and (optionally) 0.5 µL co-matrix solution.

2b. *Alternative procedure for THAP:* To a 0.5 mL tube, add 4 µL matrix solution and 1 µL co-matrix solution.

3. Mix by withdrawing and expelling the solution with the pipet 10 times.

4a. Spot 2 µL of solution on the sample plate and dry. To allow homogeneous crystallization, do not disturb the spotted samples after they start to crystallize.

4b. *Alternative procedure for THAP:* Spot 2 µL of matrix/co-matrix solution on the sample plate. Immediately spot 1 µL of the sample solution on top of the matrix solution on the sample plate before the matrix begins to crystallize. To allow homogeneous crystallization, do not disturb the spotted samples after they start to crystallize.

IMPORTANT NOTE: *Do not load the sample plate into the mass spectrometer before the plate is dry. Good signal can be obtain anywhere around the edges of the crystallized spot.*

INSTRUMENTATION

The Role of the Matrix

It is now agreed that the matrix plays several roles in promoting the formation of intact molecular ions from the sample being analyzed: analyte isolation, absorption of laser radiation, analyte desorption, and analyte ionization. The ability of the matrix to serve as a solvent for the analyte is critical to the success of MALDI-MS. The matrix has molar absorptivities much higher than the analyte, so the laser energy is preferentially deposited in (absorbed by) the matrix. This process reduces subsequent fragmentation of the analyte, improving the production of intact molecular ions. While the exact matrix characteristics that facilitate analyte desorption are still the subject of investigation, it is presumed that the laser energy absorbed by the matrix allows desorption of the first few layers of the sample. Current research suggests heats of sublimation are an important consideration in analyte desorption (Vertes et al., 1993; Bencsura et al., 1997). One of the more contentious issues in MALDI-MS is the role of the matrix in the ionization process. The scenario most commonly reported involves photoionization of the matrix molecule, which can then react, by one of a number of possible pathways, to yield the analyte ion (Ehring et al., 1992). A number of investigations aimed at better understanding the exact mechanism of analyte ion formation have been performed, and presently there is no consensus whether any one particular ion-formation pathway dominates the process (Dreisewerd et al., 1995; Gimon-Kinsel et al., 1997). Most likely, analyte ion formation is influenced largely by the chemical properties of the analyte and matrix used, and more than one pathway may lead to the ultimate formation of the analyte ions detected in the mass spectrum.

Laser Source

Pulsed-nitrogen lasers ($\lambda = 337$ nm) are most commonly used in MALDI-MS and are the standard lasers available on commercial instruments. IR lasers were used in some early studies of MALDI-MS, and have recently received additional attention because they give results strikingly similar to those of UV-MALDI lasers (Niu et al., 1998). In some cases, IR lasers are more successful than UV lasers at characterizing higher-molecular-weight samples (Berkenkamp et al., 1997, 1998).

Operationally, laser fluences on the order of 10^6 to 10^7 W/cm^2 with spot sizes around 10 to 100 µm are common in MALDI-MS. The best results are achieved by working at or just above the threshold irradiance necessary to generate analyte signal. (Threshold laser-power density is the minimum laser-power density necessary to obtain reproducible signal.) To achieve the appropriate laser irradiance, various means of attenuating the laser output are typically employed; these are common accessories on commercial instruments.

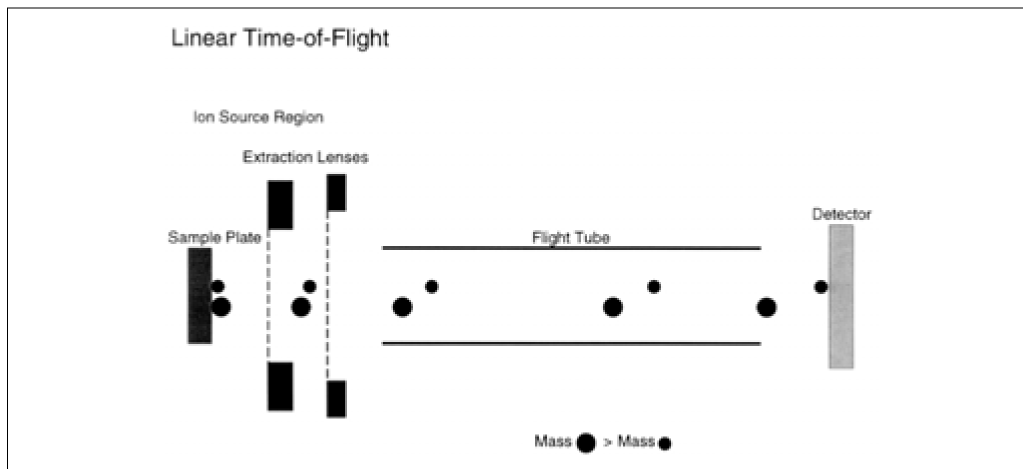

Figure 10.1.3 Schematic diagram of a linear MALDI-TOF mass spectrometer.

Time-of-Flight Mass Analyzer

The most common instrument configuration is a MALDI ion source coupled to a time-of-flight (TOF) mass analyzer (Fig. 10.1.3). Separation of ions of different *m/z* values in TOF mass spectrometry is accomplished by accelerating the ions through a short 20- to 30-kV electric field, allowing them to drift through a field-free region, and measuring the total flight time from ion formation to impact on an electron-multiplier detector. Lower-mass ions have shorter flight times than higher-mass ions and thus, by calibrating the ions' flight times through the instrument using standards of known mass, a mass spectrum can be obtained.

An ion's flight time is proportional to the square root of the mass-to-charge ratio of the ion and is proportional to the length of its flight path, as shown in the following equation:

$$t = \sqrt{\frac{m}{2zeV_{\mathrm{acc}}}}\,L$$

where t is the ion's flight time in seconds, m is its mass in kilograms, z is the number of charges on the ion, e is the fundamental charge (1.6×10^{-19} C), V_{acc} is the accelerating voltage in the ion source in volts, and L is the total flight path in meters.

The mass spectral resolution (R) between two ions, m and $m + \Delta m$, is proportional to the flight time of the ion divided by twice the time interval of ion arrival at the detector:

$$R = \frac{m}{\Delta m} = \frac{t}{2\Delta t}$$

As is evident from this equation, longer flight paths typically result in higher mass-spectral resolution than do shorter flight paths.

To avoid detector saturation by the matrix ions present in large excess in the sample, most commercial instruments are equipped with a low-mass ion gate. Low-mass matrix ions are deflected by an electrode that has a low potential of the same polarity as the ions and that is located in front of the detector, or the detector is turned on immediately after these low-mass ions reach it.

The most important instrumental factor limiting MALDI mass analysis of oligonucleotides is the instability of gas-phase oligonucleotide ions, which results in a continuous

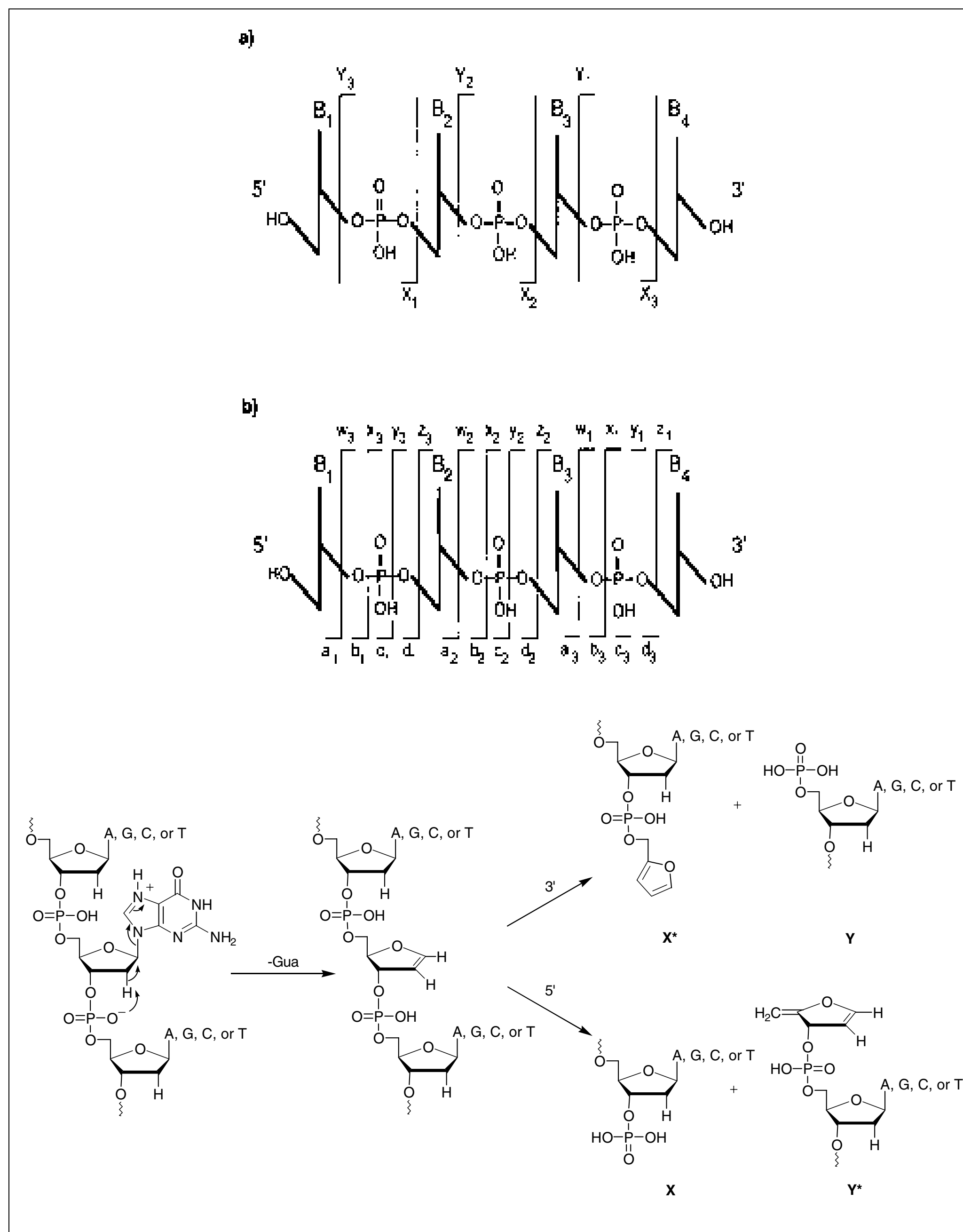

Figure 10.1.4 (**a**) Nordhoff (Nordhoff et al., 1995) and (**b**) McLuckey (McLuckey et al., 1992) nomenclature for the dissociation products of oligonucleotides. The McLuckey nomenclature is generally followed in the literature because of its applicability to ESI-MS as well as MALDI-MS. (**c**) Prevalent oligonucleotide fragmentation mechanism in UV-MALDI-MS (Nordhoff et al., 1995). Base protonation initiates base loss at purine sites as a result of cleavage at the N-glycosidic bond, which leads to subsequent cleavage of the phosphodiester backbone at the 3′-CO bond. Fragment ions here are denoted using the Nordhoff nomenclature.

fragmentation process beginning immediately as the ions are formed and continuing until they are detected (Fig. 10.1.4). There are essentially four differing time scales for desorption/ionization-induced dissociations in MALDI time-of-flight mass spectrometry (MALDI-TOF MS): prompt, fast, fast metastable, and metastable.

1. Prompt dissociations occur on a time scale equal to or less than that of the desorption event.

2. Fast dissociations occur after the desorption event, but before or at the beginning of the acceleration event.

3. Fast metastable decays occur on the time scale of the acceleration event.

4. Metastable decays occur after the acceleration event, during the field-free flight time of the ion.

In addition, the initial kinetic energy distribution and the presence of salt adducts can reduce the quality of mass-spectral results. To date, instrumentation improvements are able to reduce the deleterious effects of kinetic energy spread and ion formation. As mentioned above, reduction of the fragmentation processes can be achieved through proper matrix selection or through modification of the oligonucleotide structure. Adduct peaks can only be eliminated by appropriate purification techniques prior to mass analysis.

The sensitivity, resolution, and mass accuracy depend on the particular TOF configuration used. MALDI was first coupled with basic linear TOF (L-TOF) instruments (Fig. 10.1.3), in which ions are continuously accelerated for less than a few hundred nanoseconds. Less fragmentation is observed than in other instrument configurations: fragmentation that occurs after ions leave the acceleration region and enter the field-free region will not be detected, because the L-TOF instrument cannot differentiate the parent and fragment ions. A significant disadvantage of continuous-extraction L-TOF is that it cannot reduce peak broadening occurring from the initial velocity distribution of ions. Typically (e.g., see Wu et al., 1994) this results in poor resolution and mass accuracy, with oligonucleotides larger than 50-mers yielding resolutions on the order of 20 to 60 FWHM (full-width half-maximum).

Time-lag focusing, commonly referred to as delayed extraction (DE), was coupled to TOF as a means of reducing the initial velocity spread of ions while they are still in the accelerating region of the mass spectrometer (Colby et al., 1994; Brown and Lennon, 1995; Vestal et al., 1995). In the DE mode of operation, a second electrostatic gate is included in the ion-source region. During the laser pulse, this electrode is held at a potential sufficient to prevent the acceleration of the laser-desorbed ions into the field-free region. After a suitable delay period, which is mass dependent (Vestal et al., 1995), the ions are accelerated into the field-free region and analyzed as usual. DE L-TOF instruments achieve very reasonable resolution (~1000 FWHM) and lower mass errors (~0.01% to 0.1%) for oligonucleotides up to 50-mers (Juhasz et al., 1996).

A common instrumental approach for increasing the flight path, which has the added advantage of addressing a higher-order ion-focusing problem, is the addition of an electrostatic mirror at the end of the flight tube that reverses the ion's direction and refocuses it toward the detector. This "reflectron" TOF (re-TOF) mass analyzer increases the path-length of the ion, yielding longer flight times and hence higher mass-spectral resolution. The electrostatic ion mirror compensates for the initial kinetic energy spread of the ions, thereby improving the focusing of ions of a single m/z more effectively at the detector (Fig. 10.1.5). In addition, delayed-extraction can be combined with a reflectron time-of-flight mass analyzer to yield a "high-performance" MALDI mass spectrometer with resolution approaching 15,000 FWHM and part-per-million mass errors. The use of a reflectron can resolve metastable dissociations, but unless certain parameters are adjusted (Spengler, 1997), these ions appear as uninformative broad peaks.

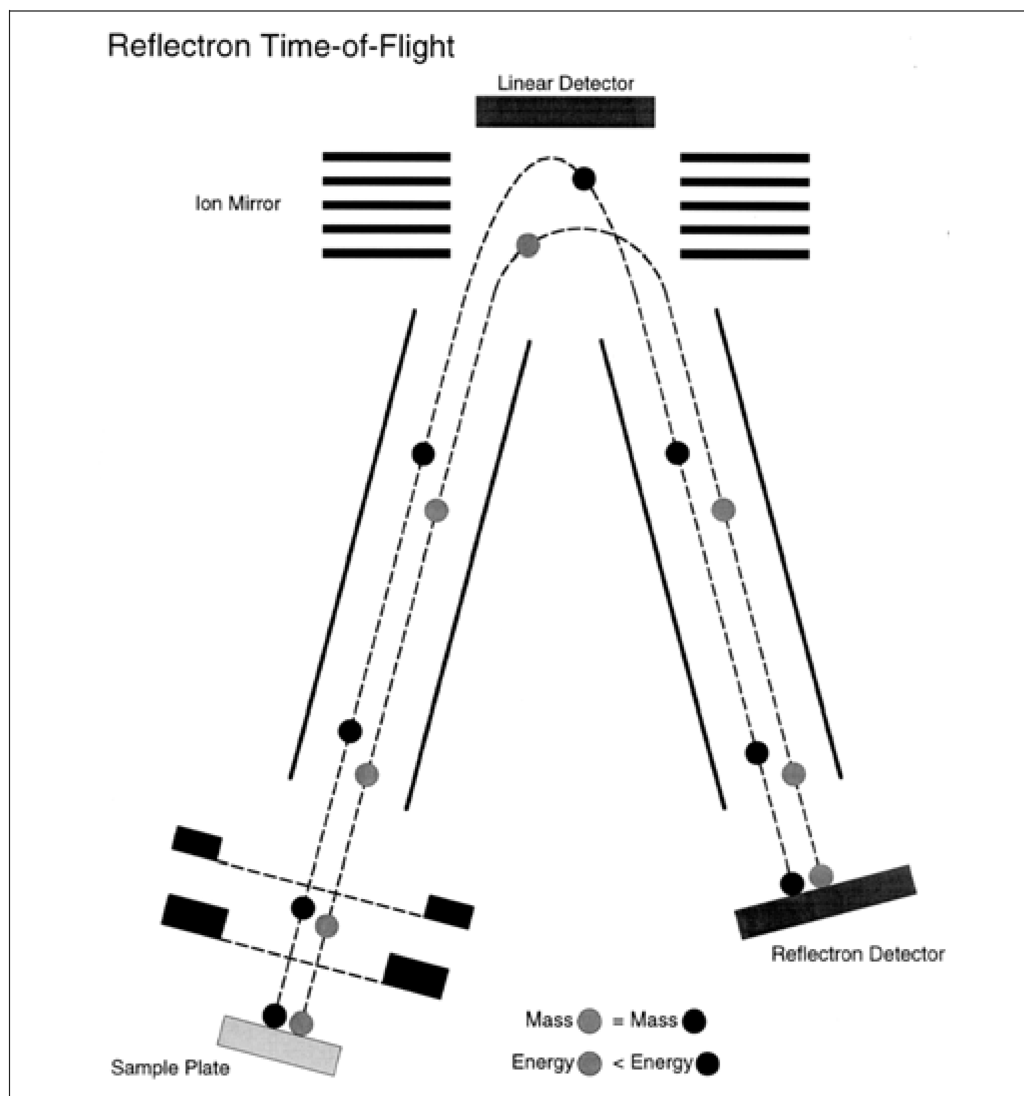

Figure 10.1.5 Schematic diagram of a reflectron MALDI-TOF mass spectrometer. Higher-energy ions penetrate the ion mirror further than lower-energy ions, allowing them to be focused to a common point at the detector.

INSTRUMENT CALIBRATION

The mass axis in TOF-MS is normally calibrated via a two-point calibration procedure. Calibration can be performed externally or internally; the latter provides higher mass accuracies. As is typical in TOF-MS, the two calibrant masses should bracket the mass(es) of interest. For small oligonucleotides (<10-mers), matrix peaks can serve as the lower-mass calibration point. Doubly charged molecular ions often appear at abundances significant enough to serve as a check for the mass calibration. For larger oligonucleotides, the two calibration masses must be from ions other than those from the matrix. Peptides and proteins are not suitable as calibrants for oligonucleotides. The concentration of the calibrants should be the same as the concentration of the analytes, especially when internal calibration is used. Because the initial ion velocities depend on the laser power density (Spengler and Bokelmann, 1993) and the matrix (Juhasz et al., 1997; see also references therein), internal calibration is necessary in L-TOF-MS, because this configuration cannot correct for the initial velocity distribution of the ions. On the other hand, DE-TOF and re-TOF instruments can mitigate those factors and external calibration

yields satisfactory results; internal calibration can still be used, however, and will provide better accuracy.

Caution should be exercised when assigning accurate molecular weights of the calibrants. In L-TOF, the average molecular weight should be used, because this configuration will not resolve the ^{13}C isotope peaks. The centroid of a peak (the mass value at the center of the peak) is preferred to the peak top when assigning molecular weights to ions whose isotopes have not been resolved. The centroid at 50% of the peak height may be used to avoid including trace cation-adduct peaks in the molecular weight assignment. Peak tops may be used to assign molecular weights of calibrants for instrument configurations that resolve the ^{13}C isotope peaks. Calibrants producing abundant cation adducts should be avoided to minimize errors in molecular-weight assignments.

MOLECULAR WEIGHT DETERMINATION USING MALDI-MS

MALDI-MS analysis of oligonucleotides is typically performed in the negative-ion mode, due to its higher sensitivity, higher resolution, and lower fragmentation compared to positive-ion-mode analysis (Stemmler et al., 1995; Tang et al., 1997a). The best mass-spectral data is obtained at near-threshold laser-power densities. Threshold laser-power density varies by matrix (Wu et al., 1993; Gusev et al., 1995) and is usually higher for oligonucleotide analysis than for peptide analysis (Wu et al., 1993). Laser power higher than threshold may produce higher oligonucleotide-ion abundance. However, as mentioned previously, the fragmentation and peak resolution are dependent on the laser-power density, and operating at laser powers significantly above threshold may degrade the quality of the mass spectrum. One should always be aware that signal abundance will vary from spot to spot within the same sample, due to the heterogeneous matrix/analyte crystals. The large shot-to-shot variability typically precludes the use of MALDI-MS in quantitative analysis, except under special experimental conditions (Bruenner et al., 1996).

OLIGONUCLEOTIDE AND NUCLEIC ACID SEQUENCING USING MALDI-MS

MALDI-MS is becoming the method of choice for rapid and accurate analysis of smaller synthetic oligonucleotides after the products are purified (Keough et al., 1993; Wang and Biemann, 1994), and is suitable as a quality control method in oligonucleotide synthesis (Ball and Packman, 1997). Sequence information from synthetic oligonucleotides can be obtained using several different approaches, of which the two most successful are failure-sequence analysis and exonuclease digestion—both dependent on the formation of a "mass ladder" (Limbach, 1996; Nordhoff et al., 1996) and hence well-suited to MALDI-MS. Sequence information arising from the formation of a mass ladder is obtained by determining the mass difference between successive peaks in the mass spectrum (Table 10.1.4 and Fig. 10.1.6). As is the case for all sequence-determination

Table 10.1.4 Characteristic Mass Losses for Naturally Occurring Deoxynucleotides (dX) and Ribonucleotides (rX)[a]

Nucleotide	Mass loss	Nucleotide	Mass loss
dA	313.27	rA	329.27
dG	329.27	rG	345.27
dC	289.25	rC	305.25
dT	304.26	rU	306.26

[a]Seen during failure sequence analysis or exonuclease digestion. All mass values are atomic weight based.

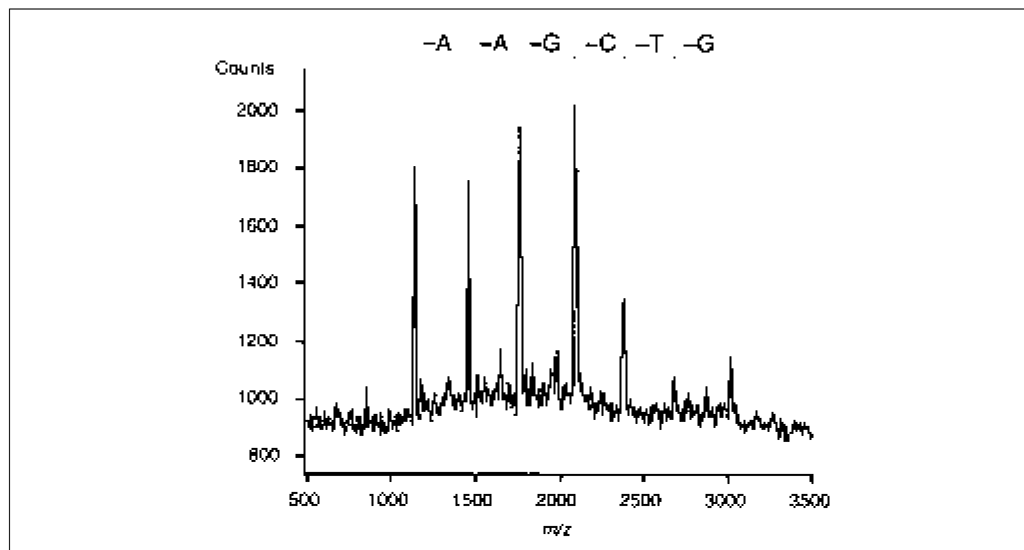

Figure 10.1.6 MALDI mass spectrum of 3′-to-5′ exonuclease digestion of d(GCCCAAGCTG). After 25 min of digestion, nearly all of the oligonucleotide sequence can be determined.

methods that rely on the mass measurements of successive *n*-mers, oligodeoxynucleotides are easier to characterize than oligoribonucleotides due to the relatively large differences in mass among the four oligodeoxynucleotide residues. Because of the small mass difference between U- and C-containing nucleotides (only 1 u), ribonucleotide analysis requires a higher mass accuracy to correctly distinguish Us from Cs. Furthermore, all mass-ladder methods have a distinct advantage for sequence determination because it is the difference in two mass measurements that results in the desired information—the identity of the nucleotide residue.

Detection of failure sequences from the original synthesis step is one of the simplest methods for characterizing the chain length and sequence of synthetic oligonucleotides (Keough et al., 1993; Butler et al., 1996; Juhasz et al., 1996). This simple and straightforward method takes advantage of the fact that automated solid-phase synthesis of oligonucleotides, especially those containing modified internucleotide linkages such as methylphosphonates or phosphorothioates, is not 100% efficient. The mass spectrum will therefore contain a series of peaks that correspond to the final product and to each failure sequence, each of which differs in mass by the appropriate nucleotide residue value. The sequence of the oligonucleotide is determined in the 5′-to-3′ direction from the mass ladder of the synthesis failure products.

The use of exonucleases to generate mass ladders of oligonucleotides that are suitable for analysis by mass spectrometry is now a standard method for sequencing small to moderate-length oligonucleotides—up to 30-mers for L-TOF without DE and 50-mers for DE-TOF (Pieles et al., 1993; Limbach, 1996). Unlike the failure-sequence analysis method described above, this approach is suitable for the sequence analysis of naturally occurring oligonucleotides. Two exonucleases are commonly used. Phosphodiesterase I, or snake venom phosphodiesterase (SVP), is a 3′-to-5′ exonuclease that is inhibited by a 3′-terminal phosphate and will generate 3′-to-5′ sequence information, while phosphodiesterase II, or calf spleen phosphodiesterase (CSP), is a 5′-to-3′ exonuclease that is inhibited by a 5′-terminal phosphate and will generate 5′ to 3′ sequence information.

SVP digestion of an oligonucleotide is generally more rapid than CSP digestion and is not inhibited by the presence of base- or sugar-modified nucleosides. CSP is

inhibited by 2′-*O*-methyl-modified nucleosides (Pieles et al., 1993). Such inhibition may be advantageous when base- and sugar-methyl-modifications must be distinguished in the overall sequence. Digestion with SVP would locate methyl-modified nucleosides, and digestion with CSP would distinguish between a base or sugar modification. Typically, complete sequence coverage of moderate-length (15 to 50 nt) oligonucleotides can be achieved within a 20- to 40-min reaction period, depending on the enzyme and digestion conditions used (Bentzley et al., 1996; Tolson and Nicholson, 1998).

BASIC PROTOCOL 5

Oligonucleotide or Nucleic Acid Sequencing Using Sequential Exonuclease Digestion and MALDI-TOF MS

This protocol describes the procedure for exonuclease sequencing of oligonucleotides or nucleic acids using MALDI-TOF MS. SVP, which cleaves oligonucleotides in the 3′-to-5′ direction, should be used to generate 3′ sequence information while CSP, which cleaves in the opposite direction, should be used if 5′ sequence information is required. During the first few minutes of analysis, information can be gathered on the first five or so nucleotides in the sequence. After longer reaction times, sequence information can be obtained for nucleotides further along the chain (Limbach, 1996; Smirnov et al., 1996). The ultimate time for analysis is dependent on the size of the oligonucleotide and the rate of digestion, and is determined empirically.

The buffer solutions used here are compatible with the matrices, and therefore matrix desalting is typically not required. Sample purification should be performed as needed just prior to sequencing.

Materials

5-μg oligonucleotide sample, lyophilized
Ultrapure (e.g., Nanopure) water
50 g/L dibasic ammonium citrate, pH ~6.4 (unadjusted)
10% (v/v) aqueous ammonium hydroxide
1×10^{-4} U/μL phosphodiesterase I (from snake venom; SVP; Sigma) or 1×10^{-3} unit/μL phosphodiesterase II (from calf spleen; CSP; Sigma or Worthington Biochemical) in ultrapure water
Matrix (see Basic Protocol 3)
Dry ice

CAUTION: RNA samples are easily degraded by adventitious nucleases. If RNA is to be purified or analyzed, all laboratory equipment must be sterilized prior to use. Use of gloves is recommended during preparation of nucleic acid samples and solutions to avoid contamination of glassware and other equipment. Autoclaved distilled water and the highest available grade of salts must be used during solution preparation.

1. Dissolve oligonucleotide sample in ultrapure water to a concentration of 100 to 400 μM (200 μM is optimum).

2. *If performing 3′-to-5′ sequencing using SVP:* Adjust ammonium citrate solution to pH 9.4 with 10% aqueous ammonium hydroxide.

3. Mix 4 to 5 μL phosphodiesterase and 1 μL ammonium citrate solution with 5 μL of oligonucleotide solution in a microcentrifuge tube. Close the tube and vortex the reaction mixture.

4. Place reaction mixture in 37°C water bath and allow temperature to equilibrate.

5. Every 10 min, remove a 1- to 2-µL aliquot of the reaction mixture.

6a. Quickly mix the aliquot with appropriate volume of matrix solution and place in dry ice to quench reaction. Either immediately spot samples on the sample plate *or* retain them to be tested all together after all digestion aliquots are obtained.

6b. Alternatively, analyze the reaction mixture aliquot immediately by MALDI-TOF MS.

Aliquots must be either quenched or analyzed immediately to limit further enzymatic digestion.

Future Developments: Full-Scale Oligonucleotide Sequencing

As mentioned earlier, there are essentially four differing time scales for desorption/ionization-induced dissociations in MALDI-TOF MS: prompt, fast, fast metastable, and metastable. In theory, dissociations occurring during any one of these time scales will generate fragment ions that could be used to determine the sequence of the oligonucleotide. In practice, unless certain instrumental parameters are manipulated, most of these fragments result in a broadening of the molecular ion peak with a concomitant loss of resolution and sensitivity.

Because of these drawbacks and because this sequencing method provides little control over the extent of fragmentation, there are few reports of the use of desorption/ionization-induced fragmentation to determine oligonucleotide sequences. As mentioned earlier, instrument configurations will to a large extent determine the type of fragment ions that can be detected in the mass spectrum. Currently, the use of desorption/ionization-induced fragment ions for sequence determination of oligonucleotides is limited. The mechanisms of prompt fragment-ion production have been studied in some detail (Fig. 10.1.4; Nordhoff et al., 1995; Zhu et al., 1995), and fragmentation patterns appear to be similar to those found in ESI–tandem mass spectrometry (ESI-MS/MS) studies of oligonucleotides (see *UNIT 10.2*), suggesting that routine use of this method for sequence determination may be possible in the near future.

As mentioned previously, analogs of nucleotides are more stable than natural oligonucleotides in MALDI-TOF MS. 7-Deazapurines have a carbon instead of a nitrogen at position 7 in the nucleobase—thought to be the site at which the protonation leading to oligonucleotide fragmentation occurs in the gas phase (Nordhoff et al., 1993; Schneider and Chait, 1993). The modification on 2′-fluoro-nucleotides makes the N-glycosidic bond stronger, preventing nucleobase loss (Zhu et al., 1995). Although DE-TOF MS has been used for the complete sequence assignment of the desorption/ionization-induced fragments of an 11-mer oligodeoxynucleotide (Juhasz et al., 1996), the fragmentation mechanisms are not understood and no other oligonucleotides have been characterized using this approach.

There has been considerable interest in the use of MALDI-TOF MS to replace current gel-based separation and radioactive or fluorescent detection schemes for enzymatically synthesized oligonucleotides. By overlapping four sets of Sanger sequencing products that are terminated at guanosine, adenosine, cytidine, and thymidine, respectively, the sequence of the oligonucleotide of interest can be determined. In the first report of this approach (Fitzgerald et al., 1993b), a mock sequencing experiment was performed in which machine-synthesized oligodeoxynucleotides, corresponding to the expected nested set of oligodeoxynucleotides in a Sanger approach, were analyzed using MALDI-TOF MS. The sequence of a potential DNA fragment could be determined up to 18 bases past the primer (a 35-mer overall) before the signal ceased to be observed.

Because of the buffers and other components in these reaction mixtures, however, the use of MALDI-MS to analyze actual Sanger dideoxy-termination sequencing reactions has proven to be much more difficult. Shaler et al. (1995) characterized the products from a dideoxy reaction catalyzed by a modified T7 DNA polymerase system. Working with picomole quantities of material, they were able to read the sequence out to the 19th base past the 12-mer primer. Using a 21-mer as the primer, the complete sequence could be determined for a 45-base synthetic template. Roskey and coworkers have analyzed the primer extension products from the Sanger reaction of a 50-base template with a 13-base primer; the use of a higher-resolution DE-MALDI-TOF mass spectrometer for the analyses afforded sequence information out to the 32nd base past the primer (Roskey et al., 1996). Mouradian and coworkers have performed mass spectrometric analysis of the primer-extension products that are generated from the M13 bacteriophage DNA template that is commonly used in actual Sanger sequencing (Mouradian et al., 1996; Roskey et al., 1996) and obtained sequence information out to the 18th base past the primer. Clearly more work is needed in this area before mass spectrometry in combination with the Sanger method becomes a viable alternative to traditional gel- or capillary-based separation and detection schemes.

ANALYSIS OF POLYMERASE CHAIN REACTION PRODUCTS USING MALDI-MS

A promising application of MALDI-MS is in the identification of DNA fragments amplified using the polymerase chain reaction (PCR). PCR allows selected regions of DNA extracted from a variety of sample sources to be amplified to a detectable level. PCR primers may be designed to produce amplified DNA products of anywhere from ten to hundreds of nucleotide residues. One impediment to high-accuracy analysis of PCR-amplified fragments is the presence of buffers, primers, and nucleoside triphosphates, which can interfere with the mass-spectral analysis.

A number of investigators have analyzed PCR-amplified fragments by MALDI-MS (Limbach, 1996). The combination of PCR and mass spectrometry shows the potential to be used as a rapid and accurate method for DNA diagnostics. Applications have included the analysis of human and bacterial DNA fragments. MALDI mass spectrometric analysis of PCR-amplified fragments has been used to distinguish a three-base deletion site in the cystic fibrosis transmembrane conductance regulator, which is characteristic of patients with cystic fibrosis (Ch'ang et al., 1995), and to distinguish genetic polymorphisms in human DNA (Liu et al., 1995b). *Legionella pneumophila*, the causative agents of Legionnaire's disease, has been identified using MALDI-MS (Hurst et al., 1996).

SUMMARY

The speed and sensitivity of mass spectrometric analyses make this an attractive approach to analyzing oligonucleotides and nucleic acids. With the advent of "soft" ionization techniques such as MALDI-MS and ESI-MS, the ability to analyze these molecules in their intact form has brought mass spectrometry into the forefront as a viable means for characterizing them. MALDI-MS is well-adapted for rapid characterization of oligonucleotides because of its relatively high tolerance of sample impurities, ease of spectral interpretation (resulting from the fact that predominantly singly charged ions are formed), and ability to handle complex mixtures of oligonucleotides. The rapid pace of technological development and the expected developments in understanding and reducing the causes of oligonucleotide fragmentation, should result in significant experimental advances in this area of analysis in the near future.

LITERATURE CITED

Bai, J., Liu, Y.-H., Cain, T.C., and Lubman, D.M. 1994a. Matrix-assisted laser desorption/ionization using an active perfluorosulfonated ionomer film substrate. *Anal. Chem.* 66:3423–3430.

Bai, J., Liu, Y.H., Lubman, D.M., and Siemieniak, D. 1994b. Matrix-assisted laser desorption/ionization mass spectrometry of restriction enzyme–digested plasmid DNA using an active Nafion substrate. *Rapid Commun. Mass Spectrom.* 8:687-691.

Bai, J., Liu, Y.-H., Liang, X., Zhu, Y., and Lubman, D.M. 1995. Procedures for detection of DNA by matrix-assisted laser desorption/ionization mass spectrometry using a modified Nafion film substrate. *Rapid Commun. Mass Spectrom.* 9:1172-1176.

Ball, R.W. and Packman, L.C. 1997. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as a rapid quality control method in oligonucleotide synthesis. *Anal. Biochem.* 248:185-194.

Beavis, R.C. and Chait, B.T. 1989. Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of peptides. *Rapid Commun. Mass Spectrom.* 3:432-436.

Bencsura, A., Navale, V., Sadeghi, M., and Vertes, A. 1997. Matrix-guest energy transfer in matrix-assisted laser desorption. *Rapid Commun. Mass Spectrom.* 11:679-682.

Bentzley, C.M., Johnston, M.V., Larsen, B.S., and Gutteridge, S. 1996. Oligonucleotide sequence and composition determined by matrix-assisted laser desorption/ionization. *Anal. Chem.* 68:2141-2146.

Berkenkamp, S., Menzel, C., Karas, M., and Hillenkamp, F. 1997. Performance of infrared matrix-assisted laser desorption/ionization mass spectrometry with lasers emitting in the 3-µm wavelength range. *Rapid Commun. Mass Spectrom.* 11:1399-1406.

Berkenkamp, S., Kirpekar, F., and Hillenkamp, F. 1998. Infrared MALDI mass spectrometry of large nucleic acids. *Science* 281:260-262.

Brown, R.S. and Lennon, J.J. 1995. Mass resolution improvement by incorporation of pulsed ion extraction in a matrix-assisted laser desorption ionization time-of-flight mass spectrometer. *Anal. Chem.* 67:1998-2003.

Bruenner, B.A., Yip, T.T., and Hutchens, T.W. 1996. Quantitative analysis of oligonucleotides by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 10:1797-1801.

Butler, J.M., Jiang-Baucom, P., Huang, M., Belgrader, P., and Girard, J. 1996. peptide nucleic acid characterization by MALDI-TOF mass spectrometry. *Anal. Chem.* 68:3283-3287.

Ch'ang, L.Y., Tang, K., Schell, M., Ringelberg, C., Matteson, K.J., Allman, S.L., and Chen, C.H. 1995. Detection of ΔF508 mutation of the cystic fibrosis gene by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 9:772-774.

Cheng, S.-W. and Chan, T.-W.D. 1996. Use of ammonium halides as co-matrices for matrix assisted laser desorption ionization studies of oligonucleotides. *Rapid Commun. Mass. Spectrom*. 10:907-910.

Colby, S.M., King, T.B., and Reilly, J.P. 1994. Improving the resolution of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by exploiting the correlation between ion position and velocity. *Rapid Commun. Mass Spectrom.* 8:865-868.

Currie, G.J. and Yates, J.R., III 1993. Analysis of oligodeoxynucleotides by negative-ion matrix-assisted laser desorption mass spectrometry. *J. Am. Soc. Mass Spectrom.* 4:955-963.

Dreisewerd, K., Schürenberg, M., Karas, M., and Hillenkamp, F. 1995. Influence of the laser intensity and spot size on the desorption of molecules and ions in matrix-assisted laser desorption/ionization with a uniform beam profile. *Int. J. Mass Spectrom. Ion Processes* 141:127-148.

Ehring, H., Karas, M., and Hillenkamp, F. 1992. Role of photoionization and photochemistry in ionization processes of organic molecules and relevance for matrix-assisted laser desorption ionization mass spectrometry. *Org. Mass Spectrom.* 27:472-480.

Fitzgerald, M.C., Parr, G.R., and Smith, L.M. 1993a. Basic matrices for the matrix-assisted laser desorption/ionization mass spectrometry of proteins and oligonucleotides. *Anal. Chem.* 65:3204-3211.

Fitzgerald, M.C., Zhu, L., and Smith, L.M. 1993b. The analysis of mock DNA sequencing reactions using matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 7:895-897.

Gimon-Kinsel, M., Preston-Schaffter, L.M., Kinsel, G.R., and Russell, D.H. 1997. Effects of matrix structure/acidity on ion formation in matrix-assisted laser desorption ionization mass spectrometry. *J. Am. Chem. Soc.* 119:2534-2540.

Gusev, A.L., Wilkinson, W.R., Proctor, A., and Hercules, D.M. 1995. Improvement of signal reproducibility and matrix/comatrix effects in MALDI analysis. *Anal. Chem.* 57:1034-1044.

Hurst, G.B., Doktycz, M.J., Vass, A.A., and Buchanan, M.V. 1996. Detection of bacterial DNA polymerase chain reaction products by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 10:377-382.

Juhasz, P., Roskey, M.T., Smirnov, I.P., Haff, L.A., Vestal, M.L., and Martin, S.A. 1996. Applications of delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry to oligonucleotide analysis. *Anal. Chem.* 68:941-946.

Juhasz, P., Vestal, M.L., and Martin, S.A. 1997. On the initial velocity of ions generated by matrix-assisted laser desorption ionization and its effect on the calibration of delayed extraction time-of-flight mass spectra. *J. Am. Soc. Mass Spectrom.* 8:209-217.

Jurinke, C., van den Boom, D., Collazo, V., Luchow, A., Jacob, A., and Köster, H. 1997. Recovery of nucleic acids from immobilized biotin-streptavidin complexes using ammonium hydroxide and applications in MALDI-TOF mass spectrometry. *Anal. Chem.* 69:904-910.

Karas, M., Bachmann, D., Bahr, U., and Hillenkamp, F. 1987. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Processes* 78:53-68.

Karas, M. and Hillenkamp, F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* 60:2299-2301.

Keough, T., Baker, T.R., Dobson, R.L.M., Lacey, M.P., Riley, T.A., Hasselfield, J.A., and Hesselberth, P.E. 1993. Antisense DNA oligonucleotides II: The use of matrix-assisted laser desorption/ionization mass spectrometry for the sequence verification of methylphosphonate oligodeoxyribonucleotides. *Rapid Commun. Mass Spectrom.* 7:195-200.

Keough, T., Shaffer, J.D., Lacey, M.P., Riley, T.A., Marvin, W.B., Scurria, M.A., Hasselfield, J.A., and Hesselberth, E.P. 1996. Detailed characterization of antisense DNA oligonucleotides. *Anal. Chem.* 68:3405-3412.

Kirpekar, R., Nordoff, E., Kristiansen, K., Roepstorff, P., Hahner, S., and Hillenkamp, F. 1995. 7-Deaza purine bases offer a higher ion stability in the analysis of DNA by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 9:525-531.

Lecchi, P., Le, H.M.T., and Pannell, L.K. 1995. 6-Aza-2-thiothymine: A matrix for MALDI spectra of oligonucleotides. *Nucleic Acids Res.* 23:1276-1277.

Limbach, P.A. 1996. Indirect mass spectrometric methods for characterizing and sequencing oligonucleotides. *Mass Spectrom. Rev.* 15:297-336.

Little, D.P., Cornish, T.J., O'Donnell, M.J., Braun, A., Cotter, R.J., and Köster, H. 1997. MALDI on a chip: Analysis of arrays of low-femtomole to subfemtomole quantities of synthetic oligonucleotides and DNA diagnostic products dispensed by piezoelectric pipet. *Anal. Chem.* 69:4540-4546.

Liu, Y.-H., Bai, J., Liang, X., Lubman, D.M., and Venta, P.J. 1995a. Use of a nitrocellulose film substrate in matrix-assisted laser desorption/ionization mass spectrometry for DNA mapping and screening. *Anal. Chem.* 67:3482-3490.

Liu, Y.-H., Bai, J., Zhu, Y., Liang, X., Siemieniak, D., Venta, P.J., and Lubman, D.M. 1995b. Rapid screening of genetic polymorphisms using buccal cell DNA with detection by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 9:735-743.

McLuckey, S.A., Van Berkel, G.J., and Glish, G.L. 1992. Tandem mass spectrometry of small, multiply charged oligonucleotides. *J. Am. Soc. Mass Spectrom.* 3:60-70.

Mouradian, S., Rank, D.R., and Smith, L.M. 1996. Analyzing sequencing reactions from bacteriophage M13 by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 10:1475-1478.

Murray, K.M. 1996. DNA sequencing by mass spectrometry. *J. Mass Spectrom.* 31:1203-1215.

Niu, S., Zhang, W., and Chait, B.T. 1998. Direct comparison of infrared and ultraviolet wavelength matrix-assisted laser desorption/ionization mass spectrometry of proteins. *J. Am. Soc. Mass Spectrom* 9:1-7.

Nordhoff, E., Ingendoh, A., Cramer, R., Overberg, A., Stahl, B., Karas, M., Hillenkamp, F., and Crain, P.F. 1992. Matrix-assisted laser desorption/ionization mass spectrometry of nucleic acids with wavelengths in the ultraviolet and infrared. *Rapid Commun. Mass Spectrom.* 6:771-776.

Nordhoff, E., Cramer, R., Karas, M., Hillenkamp, F., Kirpekar, F., Kristiansen, K. and Roepstorff, P. 1993. Ion stability of nucleic acids in infrared matrix-assisted laser desorption/ionization mass spectrometry. *Nucleic Acids Res.* 21:3347-3357.

Nordhoff, E., Karas, M., Cramer, R., Hahner, S., Hillenkamp, F., Lezius, A., Kirpekar, F., Kristiansen, K., Muth, J., Meier, C., and Engels, J.W. 1995. Direct mass spectrometric sequencing of low pmol amounts of oligodeoxynucleotides up to 21 bases by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* 30:99-112.

Nordhoff, E., Kirpekar, F., and Roepstorff, P. 1996. Mass spectrometry of nucleic acids. *Mass Spectrom. Rev.* 15:67-138.

Parr, G.R., Fitzgerald, M.C., and Smith, L.M. 1992. Matrix-assisted laser desorption/ionization mass spectrometry of synthetic oligodeoxyribonucleotides. *Rapid Commun. Mass Spectrom.* 6:369-372.

Pieles, U., Zürcher, W., Schär, M., and Moser, H.E. 1993. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: A powerful tool for the mass and sequence analysis of natural and modified oligonucleotides. *Nucleic Acids Res.* 21:3191-3196.

Polo, L.M., McCarley, T.D., and Limbach, P.A. 1997. Chemical sequencing of phosphorothioate oligonucleotides using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* 69:1107-1112.

Roskey, M.T., Juhasz, P., Smirnov, I.P., Takach, E.J., Martin, S.A., and Haff, L.A. 1996. DNA Sequencing by delayed extraction-matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* 93:4724-4729.

Ross, P.L. and Belgrader, P. 1997. Analysis of short tandem repeat polymorphisms in human DNA by matrix-assisted laser desortpion/ionization mass spectrometry. *Anal. Chem.* 69:3966-3972.

Ross, P.L., Lee, K., and Belgrader, P. 1997. Discrimination of single-nucleotide polymorphisms in human DNA using peptide nucleic acid probes detected by MALDI-TOF mass spectrometry. *Anal. Chem.* 69:4197-4202.

Schneider, K. and Chait, B.T. 1993. Matrix-assisted laser desorption mass spectrometry of homopolymer oligodeoxyribonucleotides. Influence of base composition on the mass spectrometric response. *Org. Mass Spectrom.* 28:1353-1361.

Schneider, K. and Chait, B. 1995. Increased stability of nucleic acids containing 7-deaza-guanosine and 7-deaza-adenosine may enable rapid DNA sequencing by matrix-assisted laser desorption mass spectrometry. *Nucleic Acids Res.* 23:1570-1575.

Schuette, J.M., Pieles, U., Maleknia, S.D., Srivatsa, G.S., Cole, D.L., Moser, H.E., and Afeyan, N.B. 1995. Sequence analysis of phosphorothioate oligonucleotides via matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Pharmaceut. Biomed. Anal.* 13:1195-1203.

Shaler, T.A., Tan, Y., Wickham, J.N., Wu, K.J., and Becker, C.H. 1995. Analysis of enzymatic DNA sequencing reactions by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 9:942-947.

Shaler, T.A., Wickham, J.N., Sannes, K.A., Wu, K.J., and Becker, C.H. 1996. Effect of impurities on the matrix-assisted laser desorption mass spectra of single-stranded oligodeoxynucleotides. *Anal. Chem.* 68:576-579.

Simmons, T.A. and Limbach, P.A. 1997. The use of a co-matrix for improved MALDI-TOFMS analysis of oligonucleotides. *Rapid Commun. Mass Spectrom.* 11:567-572.

Simmons, T.A. and Limbach, P.A. 1998. Influence of co-matrix proton affinity on oligonucleotide ion stability in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Am. Soc. Mass Spectrom.* 9:668-675.

Smirnov, I.P., Roskey, M.T., Juhasz, P., Takach, E.J., Martin, S.A., and Haff, L.A. 1996. Sequencing oligonucleotides by exonuclease digestion and delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Anal. Biochem.* 238:19-25.

Spengler, B. 1997. Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. *J. Mass Spectrom.* 32:1019-1036.

Spengler, B. and Bokelmann, V. 1993. Angular and time resolved intensity distributions of laser-desorbed matrix ions. *Nuclear Instruments and Methods in Physics Res. B.* 82:379-385.

Stemmler, E.A., Buchanan, M.V., Hurst, G.B., and Hettich, R.L. 1995. Analysis of modified oligonucleotides by matrix-assisted laser desorption/ionization fourier transform mass spectrometry. *Anal. Chem.* 67:2924-2930.

Strupat, K., Karas, M., and Hillenkamp, F. 1991. 2,5-Dihydroxybenzoic acid: A new matrix for laser desorption-ionization mass spectrometry. *Int. J. Mass Spectrom. Ion Process.* 111:89-102.

Tang, K., Taranenko, N.I., Allman, S.L., Chang, L.Y., and Chen, C.H. 1994a. Detection of 500-nucleotide DNA by laser desorption mass spectrometry. *Rapid Commun. Mass Spectrom.* 8:727-730.

Tang, K., Taranenko, N.I., Allman, S.L., Chen, C.H., Ch'ang, L.Y., and Jacobson, K.B. 1994b. Picolinic acid as a matrix for laser mass spectrometry of nucleic acids and proteins. *Rapid Commun. Mass Spectrom.* 8:673-677.

Tang, W., Nelson, C.M., Zhu, L., and Smith, L.M. 1997a. Positive ion formation in the ultraviolet matrix-assisted laser desorption/ionization analysis of oligonucleotides by using 2,5-dihydroxybenzoic acid. *J. Am. Soc. Mass Spectrom.* 8:218-224.

Tang, W., Zhu, L., and Smith, L.M. 1997b. Controlling DNA fragmentation in MALDI-MS by chemical modification. *Anal. Chem.* 69:302-312.

Tolson, D.A. and Nicholson, N.H. 1998. Sequencing RNA by a combination of exonuclease digestion and uridine specific chemical cleavage using MALDI-TOF. *Nucleic Acids Res.* 26:446-451.

Vertes, A., Irinyi, G., and Gijbels, R. 1993. Hydrodynamic model of matrix-assisted laser desorption mass spectrometry. *Anal. Chem.* 65:2389-2393.

Vestal, M.L., Juhasz, P., and Martin, S.A. 1995. Delayed extraction matrix-assisted laser desorption time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 9:1044-1050.

Vorm, O., Roepstorff, P., and Mann, M. 1994. Improved resolution and very high sensitivity in MALDI-TOF of matrix surfaces made by fast evaporation. *Anal. Chem.* 66:3281-3287.

Wada, Y. and Yamamoto, M. 1997. Detection of single-nucleotide mutations including substitutions and deletions by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 11:1657-1660.

Wang, B.H. and Biemann, K. 1994. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of chemically modified oligonucleotides. *Anal. Chem.* 66:1918-1924.

Wu, K.J., Steding, A., and Becker, C.H. 1993. Matrix-assisted laser desorption time-of-flight mass spectrometry of oligonucleotides using 3-hydroxypicolinic acid as an ultraviolet-sensitive matrix. *Rapid Commun. Mass Spectrom.* 7:142-146.

Wu, K.J., Shaler, T.A., and Becker, C.H. 1994. Time-of-flight mass spectrometry of underivatized single-stranded DNA oligomers by matrix-assisted laser desorption. *Anal. Chem.* 66:1637-1645.

Zhu, L., Parr, G.R., Fitzgerald, M.C., Nelson, C.M., and Smith, L.M. 1995. Oligodeoxynucleotide fragmentation in MALDI/TOF mass spectrometry using 355-nm radiation. *J. Am. Chem. Soc.* 117:6048-6056.

Zhu, Y.F., Chung, C.N., Taranenko, N.I., Allman, S.L., Martin, S.A., Haff, L., and Chen, C.H. 1996a. The study of 2,3,4-trihydroxyacetophenone and 2,4,6-trihydroxyacetophenone as matrices for DNA detection in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 10:383-388.

Zhu, Y.F., Taranenko, N.I., Allman, S.L., Martin, S.A., Haff, L., and Chen, C.H. 1996b. The effect of ammonium salt and matrix in the detection of DNA by MALDI-TOF mass spectroscopy. *Rapid Commun. Mass Spectrom.* 10:1591-1596.

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