Polyvinylidene difluoride (PVDF): an interface for gel electrophoresis and matrix-assisted laser desorption/ionization mass spectrometry

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Introduction

Gel electrophoresis is arguably the best and most general applicable protein separation technique in use today [1]. Virtually every biochemistry and molecular biology laboratory uses gel electrophoresis regularly. With the recent development of matrix-assisted laser desorption/ionization (MALDI) [2] and electrospray ionization [3], mass spectrometry (m.s.) is the best technique available to determine molecular masses of proteins, especially in the 5000-150 000 Da range. Gel electrophoresis is also used for molecular mass determinations. In this case, the molecular masses are mobility based and mass accuracy can be generously estimated to be only ±20%. This does not compare favourably with ±0.1% for m.s. based determinations [4]. Using gel electrophoresis, two proteins such as cytochrome c (molecular mass 12360 Da) and lysozyme (molecular mass 14306 Da) may be hard to distinguish on the basis of molecular mass. However, they are very easily distinguished by m.s.

After gel electrophoresis, separated proteins are often electroblotted onto a more robust support for antibody probing or Edman sequencing. One of the most popular membrane materials is PVDF (polyvinylidene difluoride). The analytical potential of combining gel electrophoresis with m.s. has been noticed by several groups, and samples have been successfully presented on several kinds of membranes for plasma and laser desorption [5-12]. The ability of commercial MALDI mass spectrometers to raster the laser across the flat sample holder suggested to us that PVDF and other membranes could be used to interface gel electrophoresis and m.s.

This report discusses the steps involved in obtaining biochemically useful mass spectra from proteins, and their reaction products, on PVDF with a 337 nm u.v. laser.

Application of protein to PVDF

PVDF is very hydrophobic; hydrophobic interactions form the basis for the strong adsorption of proteins on these membranes. Since most protein solutions are water based, the application of proteins to PVDF requires more than one step. Its manufacturers recommend first wetting PVDF with methanol and then displacing the methanol with aqueous solutions. While PVDF is water wet, protein solutions can be applied either by pipette or by electroblotting. The two buffers most commonly used for this purpose are Towbin buffer (25 mM Tris, 192 mM glycine, pH 8.3) and 10 mM Caps [3-(cyclohexylamino)-1-propanesulfonic acid], pH 11, 10% methanol (v/v). We have used 20–50 mM NH₄HCO₃ and 0.1% trifluoroacetic acid solutions as well as Towbin and Caps to apply protein to the following PVDFs: Westran (Schleicher & Schuell, Keene, NH, U.S.A.), Immobilon-P (Millipore, Bedford, MA, U.S.A.), Immobilon-PSQ, and ProBlott (Applied Biosystems, Foster City, CA, U.S.A.). The two latter high capacity PVDFs have 0.2 µm pores while the first two have 0.45 µm pores. The buffer salts accompanying the protein during electroblotting...
Applying or pipette application can be removed from PVDF by rinsing with copious amounts of water, either while the PVDF is still water wet or after it dries. Placing (pipetting) 5–50 pmol of protein in a small, tight spot on PVDF, we have achieved good MALDI spectra. Thus far, we have carried protein through gel electrophoresis and electroblotting at the 1 nmol level [4].

Application of matrix to sample on PVDF

Many of the matrices for 337 nm MALDI-m.s. are small organic molecules that are not very water soluble. So, when a protein is embedded in PVDF, a solvent is needed that will spread the matrix on the protein but not degrade the PVDF. It is also important not to remove the protein from PVDF or move the protein on the PVDF while applying the matrix. Using cytochrome c, a dark red compound, we have observed that acids plus organic solvents and bases plus organic solvents move protein on PVDF. These combinations can wash protein off the membrane, change a tight band into a large diffuse one, or change a spot into a ring of protein. When applying matrices, such as α-cyano-4-hydroxycinnamic acid and sinapinic acid in organic solvents, to protein on PVDF, care needs to be taken to minimize changes in spot size. Organic solvents that wet PVDF but do not degrade it include methanol, acetonitrile and toluene. Matrices that can be used include α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, sinapinic acid, 6-aza-2-thiopyrimidine and p-nitroaniline.

Using these guidelines for applying protein and matrix to PVDF, the MALDI mass spectrum shown in Figure 1 was obtained from 38 pmol of rabbit muscle triose-phosphate isomerase on PVDF with α-cyano-4-hydroxycinnamic acid as matrix. This spectrum shows the 26609 Da subunit. The active form of triose-phosphate isomerase is a homodimer [13] that is clearly not preserved in the denaturing conditions used.

Mapping chemical and protease reaction products on PVDF

The triose-phosphate isomerase spectrum from PVDF shown in Figure 1 was obtained using a 337 nm (u.v.) laser and a matrix:analyte ratio of approx. 5000:1. In the absence of PVDF, α-cyano-4-hydroxycinnamic acid also produces an abundance of protein M2H+2 ions, which can be useful for checking calibration. The number of MH+ ions formed, relative to M2H+2 ions, and peak width depends on laser power, matrix and matrix:analyte ratio, as they do when PVDF is present. The calibration line for spectra from PVDF is different to the line for spectra of samples deposited on stainless steel (the geometry changes), but the accuracy is the same, better than 0.1% [4]. The most important point to be made here, however, is that the mass
accuracy from MALDI-m.s. is vastly superior to that from gel electrophoresis.

When applying protein of known concentration to PVDF by pipette, it is easy to calculate the amount transferred. It is another matter to estimate the actual amount the MALDI experiment consumes per laser shot. When PVDF is stained with Coomassie Blue after MALDI-m.s., the spot shows no visual evidence of laser impact. Since most of the protein is still on the membrane, there is enough left over for chemical- and enzyme-catalysed reactions, followed by another MALDI experiment with the same piece of PVDF.

The ions in the spectrum shown in Figure 2 are the products of two chemical reactions and one protease digestion, carried out sequentially on the membrane. For this series of experiments, 38 pmol of rabbit muscle triose-phosphate isomerase in 10 mM Caps buffer was applied to Immobilon-P50 (5 cm × 0.5 cm). Before the spot dried, dithiothreitol (10 μg/μl, 1 μl) in Caps buffer was added and allowed to react for 5 min. At that time, iodoacetamide (100 μg/μl, 1 μl) was added and allowed to dry (approx. 30 min); PVDF goes from grey to white on drying. The PVDF was rinsed with large amounts of water to remove the excess reagents and the Caps buffer. The PVDF was then taped (at the ends only) to a Kratos sample slide. The spot was wetted with methanol (1 μl); the methanol was then displaced with 50 mM Tris/HCl buffer, pH 9.1 (4 μl). Next, 2 μl of endoproteinase Lys-C (approx. 5 μg/μl) (Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added followed by more Tris.HCl (2 μl). The spot was allowed to dry (approx. 30 min) and was then rinsed with copious amounts of water to remove the buffer salts. The matrix, 6-aza-2-thiothymine was dissolved in methanol to give a 100 mM solution. To the spot was added 2 μl of the solution, and the spectrum shown in Figure 2 was obtained.

The peptides mapped are identified by amino acid residue positions in square brackets as predicted by the amino acid sequence [13] and the known specificity of endoproteinase Lys-C. Forty-nine per cent of the amino acids were mapped. Three peptides contain alkylated cysteine residues. The peaks at m/z 1138, 2450 and 2544 are all 57 higher than predicted for the non-derivatized peptides, [59–68], [33–54], and [194–218]. The utility of these sorts of maps for locating chemical modifications and post-translational modifications is well known [14]. While not all the amino acid residues are accounted for in the data shown, it is possible that different matrices, matrix:analyte ratios and laser powers could characterize additional peptides. MALDI-m.s. of mixtures are known

Figure 2
MALDI mass spectrum of triose-phosphate isomerase after on-membrane reduction and alkylation and on-membrane digestion with endoproteinase Lys-C

The matrix is 6-aza-2-thiothymine. The peptides mapped are marked by square brackets with the amino acid positions in the original protein given.
to desorb only some of the components of complex mixtures \[15\]. This process appears to generate a sufficient number of peptide ions for database searching \[16-18\].

It should be noted that the whole procedure required only 4 h from the application of triosephosphate isomerase to the PVDF to the MALDI-m.s. of the endoproteinase Lys-C products of the protein. This is considerably less than the time that would be required to carry out the derivatization and proteolysis in a test tube using larger volumes of solvents and intermediate lyophilization steps. The commonly used proteases, trypsin, endoproteinase Lys-C and chymotrypsin (all with maximum activity in the 7–9 pH range), have been used with PVDF.

**Locating samples on PVDF**

Thus, if one knows where the protein and/or its products are on PVDF and pays attention to the hydrophobic nature of PVDF, matrices can be applied and mass spectra obtained. Locating protein on a piece of PVDF is a bit of a challenge, especially if it has been deposited by electrophoresis: gels generally change shape during the electrophoresis process; PVDF is white and proteins are often colorless. To locate lanes of proteins on PVDF without staining, prestained standards can be run in adjacent lanes. Once the lane of interest is identified, then the scheme illustrated in Figure 3 can be used to locate protein. A strip of PVDF is taped at each end to a flat sample holder and covered with matrix. The matrix needs to be applied as small concentrated drops to minimize protein movement on the PVDF and to achieve a reasonable matrix:analyte ratio. The Kratos MALDI III mass spectrometer (Manchester, U.K.) uses a 1 cm × 7 cm stainless steel sample holder whose size matches a standard mini-gel lane. The PVDF strip is then moved through the laser beam and spectra collected. The ion intensity in the mass range specified tells which laser shots produced protein ions and hence identifies protein positions. Since MALDI-m.s. detects picomolar amounts of protein easily, its sensitivity is in the microgram range in which gel electrophoresis routinely operates.

**Conclusion**

To make the best advantage of both the separation power of gel electrophoresis and the mass accuracy of MALDI mass spectrometry, high yield electrophoresis is needed. Unfortunately, protein losses during electrophoresis can be very high \[7\]. The variables in electrophoresis, i.e. membrane, buffer, current and run time, need to be optimized for each protein of interest.

The time required for gel electrophoresis and electrophoresis is long compared with the time needed to acquire a MALDI mass spectrum. By running several lanes at once, gel electrophoresis and electrophoresis efficiency is maximized. Since protein bands on PVDF are easily stored between filter papers in a plastic bag, mass spectra can be collected at the experimenter’s convenience. Our experience has shown that a variety of useful information about proteins isolated by gel electrophoresis can be obtained rapidly using MALDI-m.s. This information includes mobility, molecular mass and proteolytic peptide maps.

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Electrospray ionization mass spectrometric analysis of subunits of NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria

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Introduction

Complex I (NADH:ubiquinone oxidoreductase) is the first enzyme in the respiratory chain of mitochondria. It is in the inner membrane and catalyses the transfer of electrons from NADH to ubiquinone. For each electron pair transferred from NADH to ubiquinone, four protons are pumped out of the mitochondria, helping to generate the proton electrochemical potential gradient across the inner membrane. (For recent reviews see [1,2].)

Complex I from bovine mitochondria is a large, membrane-bound, multisubunit structure. It binds one FMN cofactor per complex and contains at least five iron-sulphur clusters. Together they form the electron transfer pathway between NADH and ubiquinone. Bovine complex I is an assembly of at least 41 different proteins. Seven hydrophobic subunits, known as ND1–ND6 and ND4L, are encoded in mitochondrial DNA, and a further 34 subunits are products of the nuclear genes. Assuming that one copy of each subunit is present in the complex, its 41 subunits contain at least 7955 amino acids and have a total molecular mass greater than 910 kDa [2,3]. The primary structures of the 34 nuclear encoded subunits have been determined [4,5]. They have revealed relationships between complex I subunits and other proteins of known function, thereby giving clues to their functions. Together with other biochemical and spectral data, they provide details about the locations of redox centres and of the organization of subunits [2,6].

The sequence analysis of this extremely complicated enzyme was aided greatly by the development of two techniques, which together represent a powerful advance in the analysis of protein sequences. These developments are, first, a rapid protein sequencing strategy based on PCR that requires only 18 amino acids to be determined by direct sequencing on the protein of interest [4] and, secondly, the advent of electrospray ionization mass spectrometry (e.s.i.-m.s.), which enabled the molecular masses of the intact proteins to be determined accurately. This article will emphasize the role of e.s.i.-m.s. as an integral part of the sequencing strategy, and also its invaluable role in the localization of the subunits in specific subcomplexes made from complex I (M. Finel, J. M. Skehel, I. M. Fearnley and J. E. Walker, unpublished work) [6]. In many cases, the molecular mass measurement provided important verification of the protein sequences, and in addition it allowed post-translational modifications to be identified.

The rapid protein sequencing strategy

The strategy for obtaining protein sequences was developed so that the subunits of complex I could