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ARTICLE *in* RAPID COMMUNICATIONS IN MASS SPECTROMETRY · JANUARY 1995

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RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. 9, 1031–1034 (1995)

Ion Exchange and Purification of Carbohydrates on a Nafion®† Membrane as a New Sample Pretreatment for Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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A Nafion membrane was tested as a new tool for a fast and easy sample pretreatment for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) measurements. This membrane material was used for ion exchange and carbohydrate purification with minimal quantities of sample solution. An ion exchange method for carbohydrates was developed and demonstrated on a Dextran sample. The cations of the original sample were replaced within minutes by potassium or cesium ions. Another feature of this membrane was the fact that proteins and peptides adsorb on the acid surface. This behavior was investigated as a purification step for carbohydrates with high peptide and protein impurity concentrations. It was found that oligosaccharide libraries from rat- and mouse IgG mass spectra can be obtained clearly in the presence of high peptide impurities.

Since the introduction of matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), this method has been developed into a fast and sensitive analytical technique.[1–3] Especially for peptides and proteins, MALDI is the method of choice to get mass information. Another feature is the low quantity of sample solution that is needed for a MALDI investigation. Sometimes however, measurements become difficult because of high salt or buffer concentrations and the presence of certain impurities. The right choice of matrix and additives can improve these measurements, but this approach is limited to just a few applications. Another way of solving the problem is sample pretreatment. This often makes a dramatic change in the quality of MALDI spectra. Here, methods for a fast and easy sample pretreatment are needed that can be applied to 1 to 5 μL of sample solution, in order to fit MALDI requirements. One illustrative example is droplet dialysis[4,5] on a floating membrane for desalting small quantities of sample. By this means, a droplet of a Dextran sulfate solution was easily desalted and then identified with MALDI-MS.[6] Another method was described by Lubman *et al.*[7] using a Nafion film substrate directly on the probe tip to improve the MALDI spectra of oligonucleotides and proteins. These experiments show that a film substrate of Nafion can be produced directly on the probe tip. However, forming a Nafion film directly on the probe tip can cause some disadvantages. The reproducibility is afflicted and the MALDI spectrum in the negative mode often produces many peaks which originate from Nafion. Thus, membrane purification outside the probe tip will produce better quality mass spectra. In our laboratory, a Nafion membrane has been used for ion exchange and for suppression of the peptide signals in crude carbohydrate mixtures.

†®: Trademark of Du Pont.
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CCC 0951–4198/95/111031–04

Received 26 July 1995
Accepted 27 July 1995

EXPERIMENTAL

Materials and mass spectrometry

All MALDI results were obtained with an LDI-1700 instrument from Linear Scientific, Reno, NV, USA; now part of Hewlett-Packard, Palo Alto, CA, USA. The nitrogen laser had an average power between 4 and 10 μJ. In general, the vacuum of the flight tube was observed to be 10^{-7} to 10^{-6} Torr. The Nafion-117® membrane was purchased from Aldrich, Milwaukee, USA. The desalting membrane (MF, pore size 0.0025 μm, diameter 25 mm) was produced by Millipore, Bedford, USA. The oligosaccharide libraries of IgG from rat and mouse were purchased from Oxford Glyco Systems, Oxford, UK. Maltoheptaose was received from Merck, Darmstadt, Germany, and all other chemicals from Fluka, Buchs, Switzerland. As DHB/HIC matrix a mixture of 2,5-dihydroxy benzoic acid (0.2 M) and 1-hydroxy isoquinoline (0.06 M) with a mass ratio of 3:1 in water/acetonitrile was used.[6] This DHB/HIC matrix was found to give the best results for carbohydrates.[6]

The samples were dissolved in water at concentrations of approximately 1 μM and were mixed with matrix (1:1; v/v). After briefly vortexing approximately 0.6 μL of this mixture, it was transferred to the probe tip and dried under vacuum for fast crystallization (4 to 8 s) for best results.[8] All spectra were obtained by accumulating 10 to 50 laser shots. The carbohydrates were determined as alkali metal adduct ions. Ionization with protons or doubly charged ions were not observed.

Sample pretreatments

The Nafion-117 membrane was regenerated in concentrated hydrochloric acid at 80° or nitric acid for two hours. After washing the membrane with deionized water, all sulfate groups on the membrane were saturated with protons (H-Nafion). Regenerating the mem-

branes many times did not produce any changes in the experimental results. For ion exchange experiments this regenerated H-Nafion was put into an alkali metal salt solution with a concentration of approximately 1 M. After 10 min, all protons were exchanged by cations. Using potassium chloride, for example, a K-Nafion membrane was produced.

A piece of the membrane with 25 mm diameter was fixed in a small home-made nylon holder to provide a flat membrane. The nylon holder allows the bottom side of the membrane to have contact with a water reservoir. On the top side of the Nafion membrane 3 to 10 μL sample solution were deposited. However, the membrane could be saturated with different cations, such as lithium, sodium, potassium, cerium and protons. In these cases the water reservoir was filled with the corresponding 100 mM salt or acid solution. Mostly, after one minute, all ions were exchanged. Then the droplet was recovered by a micro-pipette. The resulting solution was treated as a normal analyte solution for a MALDI-MS investigation.

For separating peptides from carbohydrates, approximately 2 μL to 5 μL of an analyte solution were deposited on the H-Nafion membrane from a micro pipette. Fixed in the holder, the membrane was allowed to contact the water reservoir which contained 0.1% trifluoroacetic acid (TFA). After 3 to 5 minutes the Nafion had adsorbed all peptides from the sample solution and the pure carbohydrates could be measured with MALDI.

A pretreatment for sample desalting was performed on a dialysis membrane (MF).[4,5] As described by the manufacturer, the membrane was allowed to float freely with the hydrophilic side on a pure water surface. 5–20 μL of the sample solution were deposited onto the membrane (hydrophobic side). After 30 min to 3 h, the sample droplet was removed again with a micropipette. The resulting solution was used without further pretreatment.

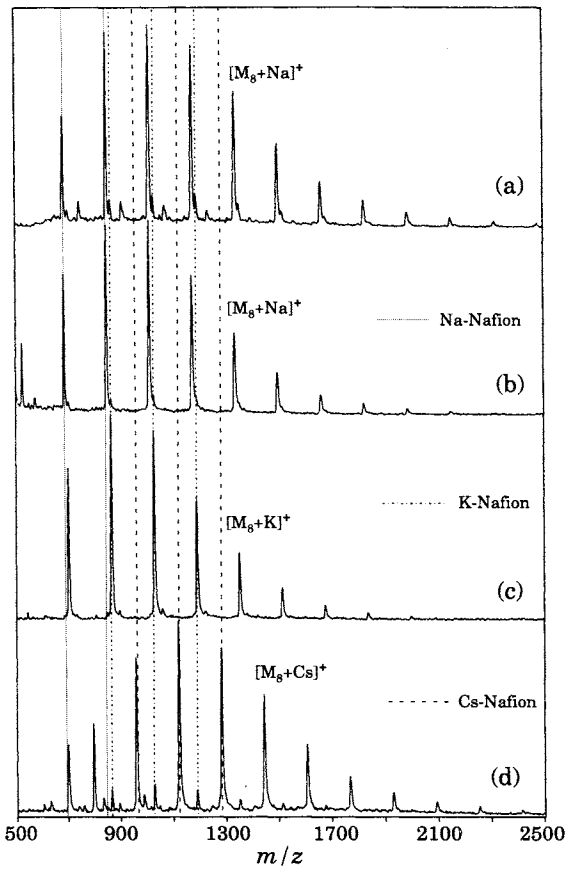

Figure 1. Positive-ion spectra of Dextran (molecular weight distribution around 1200 Da) with DHB/HIC as matrix. The spectrum at the top (a) shows the Dextran without any sample pretreatment, the spectra below show the dextran after ion exchange on the alkali-saturated Nafion membranes: (b) Na–Nafion, (c) K–Nafion and (d) Cs–Nafion.

RESULTS AND DISCUSSION

Ion exchange

In general, MALDI is able to ionize carbohydrates only after cationization with alkaline ions. Therefore, it is important that the investigated carbohydrate sample contains only one kind of alkaline ion. However, in the normal case a sample solution contains sodium as well as potassium ions. Thus the mass spectrometer shows two carbohydrate peaks: $[M+Na]^+$ and $[M+K]^+$. A spectrum from a complex mixture of carbohydrates is tedious to interpret, because one has to know, which peak belongs to a potassium or sodium adduct of one given complex oligosaccharide and which peak belongs to another carbohydrate. Also, it is known[6] that potassium ions give a three-fold higher oligosaccharide signal than sodium ions. In these cases it is helpful to exchange an alkaline ion mixture for a single ion in a fast and easy way.

For this purpose an ion exchange method with a Nafion membrane that can be used in the microliter range was applied. Nafion is a perfluorinated cation-exchange polymer whose active sites consist of sulfonic acid groups and the end of the side chains. Its chemical and physical stability are close to Teflon®. The Nafion membrane is permeable to many cations and polar compounds, but it is impermeable to anions and nonpolar compounds. A few microliters of sample solutions were deposited on the surface of the membrane for ion exchange over a period of 1–5 min. Because the membrane is highly permeable to cations, the alkali metal ions moved rapidly through the membrane in both directions. As a result, the alkaline ions of the solution will be replaced by the alkaline ions of the reservoir. A 5 μL sample solution containing sodium ions can be ion-exchanged with potassium ions in approximately one minute. Our experiments showed that the best Nafion material was a Nafion-117® membrane.

As an example, the mass spectra of Dextran with a molecular weight distribution around 1200 Da are given in Fig. 1 and the performance of the Nafion-117® membrane as a cation exchanger is demonstrated. In Fig. 1(a), the positive ion mass spectrum shows Dextran 1200 with DHB/HIC as matrix and without any sample pretreatment. Here, the saccharide molecules are ionized by the addition of sodium ions (large peaks) and by a lesser amount of potassium ions (small peaks at +16 Da). The peaks between the oligosaccharide ladder are impurities. The other spectra show the same sample solution after pretreatment on (b) sodium-, (c) potassium- and (d) cesium-saturated Nafion membranes. The shift of ion intensity distribution is clearly manifested. After treatment on a Cs-Nafion membrane, the sample still showed a low concentration of potassium ions (Fig. 1(d)). These

peaks can be seen between the more intense $[M_n + Cs]^+$ peaks. The time of ion exchange on the Nafion membrane was approximately one minute. Because of the lower ion mobility of cesium ions, the time on the membrane was too short for a complete ion exchange. Applying the sample over an extended exposure time on the membrane causes the potassium peaks in the spectrum to disappear. It should be noted that, in the same way, all cations can be exchanged with lithium ions. In our laboratory this ion exchange method was also applied successfully to other compounds like polystyrene sulfate. In this way and using (H-Nafion), the MALDI spectrum could be improved dramatically.

Separation of carbohydrates from peptides

Another application of the Nafion-117® membrane is the possibility of adsorbing proteins and peptides on an H-Nafion surface. To show this effect, the spectra of a synthetic mixture of maltoheptaose and angiotensin I with a concentration of 1 μM are given in Fig. 2. Figure 2(a) shows the mass spectrum of this mixture without any sample pretreatment. The signals of maltoheptaose and angiotensin I can be determined easily. As it is known, diammonium hydrogen citrate (DAHC)[9] suppresses alkali ions in MALDI-MS spectra by complex formation. With this additive, the carbohydrate signals can also be suppressed, because these molecules can only be ionized in MALDI by the formation of alkali ion adducts and protonated carbohydrates are not seen. If all alkali ions form a complex with the citrate ions, there are no more cations available to ionize oligosaccharide molecules. In this way, the signals of maltoheptaose could be 'switched off'. Figure 2(c) shows the solution of maltoheptaose and angiotensin I after purification on the H-Nafion membrane. Within 5 min, all molecules of angiotensin I were adsorbed. Hydrolyses of peptides and oligosaccharides were not observed in this period of time. The small peaks in the spectra on the left and on the right side of the spectrum were carbohydrate impurities. Now the peptide peak is 'switched off'.

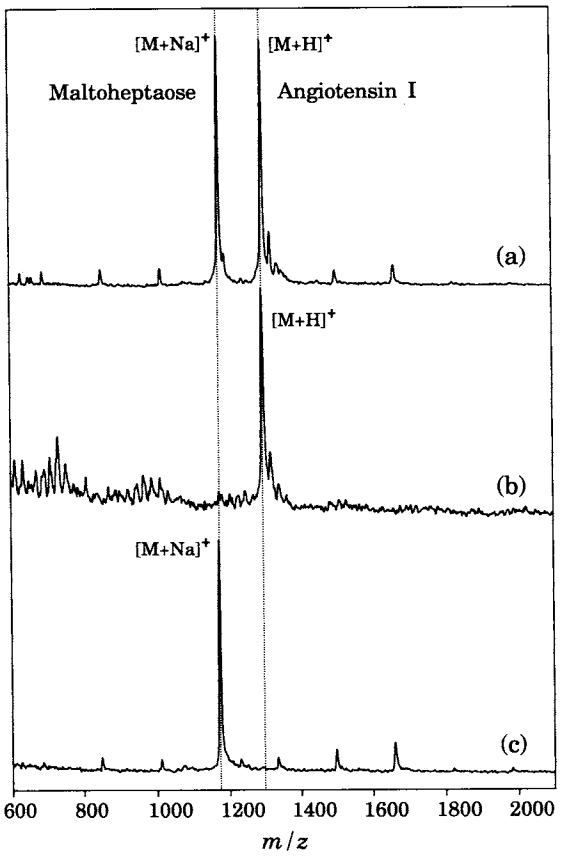

Figure 2. Positive-ion spectra of a mixture of maltoheptaose and angiotensin I. DHB/HIC was used as matrix. The spectrum at the top (a) shows the mixture without sample pretreatment, the spectrum below (b) shows the same solution with diammonium hydrogen citrate (DAHC) as an additive to the matrix (1:2). The saccharide peaks are suppressed. The spectrum at the bottom (c) shows the oligosaccharide/peptide solution after purification on the Nafion membrane; here the peptide was completely adsorbed on the Nafion.

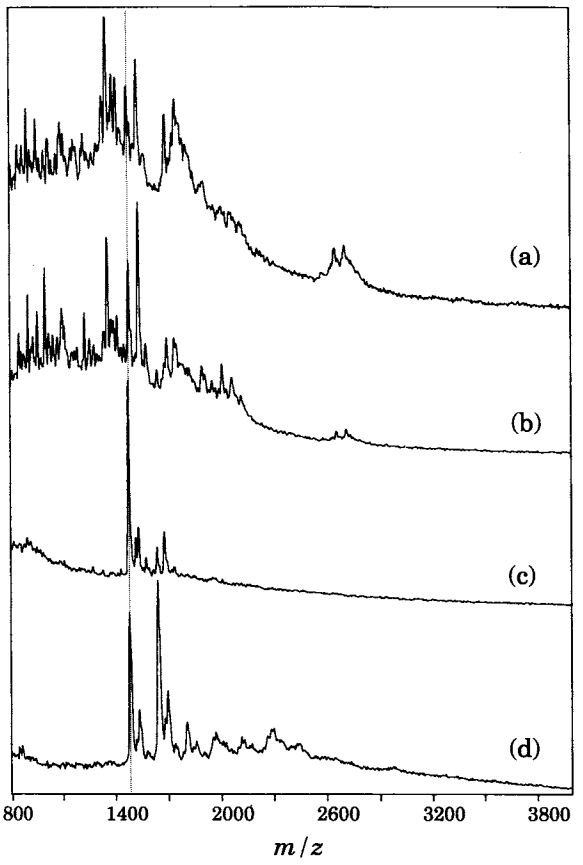

Figure 3. Positive-ion spectra of an oligosaccharide library from rat IgG with DHB/HIC as matrix. The spectrum at the top (a) was measured without sample pretreatment. The spectrum below (b) shows the signals of the same solution after desalting by droplet-dialysis. Spectrum (c) shows the oligosaccharide library after purification for 5 min on H-Nafion. Only the oligosaccharide peaks are visible. The spectrum at the bottom (d) shows the oligosaccharides from mouse IgG after the same sample pretreatment.

An important example of a carbohydrate/peptide separation is the purification of oligosaccharide libraries from glycoproteins. The upper spectrum in Fig. 3(a) shows the oligosaccharide library of rat IgG without any sample pretreatment. However, it was not possible to find any clear information about these molecules in this mass spectrum. The peaks of the carbohydrates cannot be identified by their molecular weights because there are too many peptide impurities in the sample, which overlap the carbohydrate signals. Desalting the sample on a cellulose membrane produces only a small improvement. Figure 3(b) shows the result after purification by droplet dialysis for one hour.[4,5] Most of the peaks are now better resolved and the base line has

become more horizontal, but it is still not possible to recognize the carbohydrate peaks of the oligosaccharide library. After purification on the H-Nafion membrane for only 5 minutes, improvements are seen, and the carbohydrates can readily be investigated. The sample is now purified from all peptide impurities, which have been adsorbed on the surface of the Nafion. Figure 3(c) shows only one major peak that fits perfectly with the expected mass value for this oligosaccharide library. The spectrum gives a mass of m/z 1485.8 for [M + Na]. The calculated molecular weight of the bi-antennary oligosaccharide was 1463.37 Da. The small peaks in the spectrum are other oligosaccharides.[11] The spectrum at the bottom (Fig. 3(d)) shows the oligosaccharide library of mouse IgG after the same Nafion purification step. Here, the major peak has the same mass as the oligosaccharide of the rat IgG. Details will be given in a future publication.[10] The signals between 1900 and 2500 Da are sialylated oligosaccharides that can be detected in the negative mode as highly resolved peaks (data not shown). In our laboratory, no hydrolysis of carbohydrates and libraries was observed after the short interaction time used with the acid Nafion membrane. In an additional experiment, it was determined that it takes several days of contact with the acid Nafion membrane to identify hydrolysis products of maltoheptaose. In summary, these experiments have demonstrated a new analytical method of sample pretreatment for matrix-assisted laser desorption/ionization. It should be noted that this method of adsorbing peptides cannot work with concentrations $>10^{-5}$ M of impurities, because in this case the limited surface of the sample droplet on the membrane would be saturated.

CONCLUSION

With these few examples of sample pretreatment of Nafion membranes, it is demonstrated that this technique in combination with MALDI-MS represents a powerful tool in the analytical investigations of carbohydrates. The advantages of this method are the low volumes of sample solution required, the fast reaction times and the availability of appropriate improvements prior to the MALDI measurements. Ion exchange on Nafion membrane is fast and easy to perform. By this means, peak splitting of the carbohydrate peaks by sodium and potassium adducts is avoided. This improves the peak intensity and makes the spectrum easier to interpret. This method of sample pretreatment does not seem to be limited to carbohydrates since ion exchange on an H-Nafion membrane is often useful for other chemical compounds (e.g. polystyrene sulfonate 100000).

Similar advantages result from membrane separation of carbohydrates from peptides and proteins on Nafion. In this application it is possible to adsorb all peptide molecules on a Nafion surface as a sample pretreatment step. Subsequently, with MALDI-MS investigations only the carbohydrate peaks appear, even if they were present in a lower concentration than the peptide impurities. Here, the peptide signals are completely suppressed. This method is very useful in the analytical investigation of oligosaccharide libraries from glycoproteins, as has been demonstrated with the examples from rat IgG and mouse IgG. In the future, sample pretreatment using the smallest possible quantities and advanced materials will have a great impact on MALDI-mass spectrometry.

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