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A NOVEL MASS SPECTROMETRIC PROCEDURE TO RAPIDLY DETERMINE THE PARTIAL STRUCTURE OF HEPARIN FRAGMENTS

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SUMMARY: The molecular weight and degree of sulfation has been obtained for di-, tetra- and hexasaccharide fragments of heparin obtained by enzymatic depolymerization of porcine mucosal heparin. The sodium salt form of the sulfated oligosaccharide is adsorbed onto an immobilized cationic surfactant film which is inserted directly into the mass spectrometer. Analyses are routinely obtained on 25-50 μg samples in less than an hour. This approach provides rapid confirmatory structural information that is complementary to existing methodologies.

Heparin has long been recognized for its anticoagulant activities. More recently it has been recognized that it possesses multiple biological activities including its ability to cause tumor regression (1), inhibit complement activation (2) and interact with plasma lipoproteins and lipoprotein lipase which has intriguing implications in the treatment of atherosclerosis (3). The heparin molecule is a polydisperse sulfated copolymer of N-acetylglucosamine and iduronic or glucuronic acid having a molecular weight in the range of 6-25 megadaltons. Evidence indicates that in many cases the structural requirements for its multiple biological activities may involve fragments as small as a tetrasaccharide. These fragments are obtained by heparinase digestion of the macromolecule producing a heterogeneous mixture of saccharide fragments of various size and degree of sulfation. To study the structure-function relationships, the saccharide fragment must be purified and characterized. This is accomplished by a combination of chemical, enzymatic, electrophoretic and spectroscopic methods, including ^{13}C and ^{1}H NMR (4). Since this requires a considerable commitment of resources, few structures have been determined unequivocally. We have developed a rapid assay based upon mass spectrometry that can be used to structurally screen potential biologically active fragments. The molecular

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weight and degree of sulfation can be obtained on a total sample size of less than 100 μg in several hours. With this data it is possible to determine whether a large scale effort to completely characterize the molecule should be initiated.

Since heparin fragments are of comparatively low molecular weight, they should be amenable to analysis by mass spectrometry. However, this class of compounds has been difficult to analyze by any MS method because of the multiple anionic functional groups and the lability of the N-sulfate linkages. Carr and Reinhold have shown that the molecular weight and sugar sequence can be obtained for chondroitin sulfate fragments, a structurally related glycosaminoglycan, up to the level of an octasaccharide by FABMS (5). Application of this methodology to heparin fragments was not as successful; fewer structurally significant fragment ions were observed and therefore the sequence could not be determined (6). In both cases, it was not straightforward to identify the molecular ion because of the addition of multiple alkali metal ions. Attempts to eliminate this problem by desalting resulted in an overall decrease in ion production. Problems of this type were even more severe using ^{252}Cf-PDMS because of the constraint of working with solid films of the molecules in contrast to the liquid matrix commonly used in FABMS. Strong intramolecular interactions and impurities in the solid matrix, e.g. salts, buffers, etc. appear to be responsible for the attenuation of molecular ion yields (7). In an effort to eliminate these problems a program was initiated to investigate the use of various solid films which would preferentially adsorb the desired analyte (8,9,10). Recently we reported on the use of an immobilized cationic surfactant as a selective matrix for the adsorption of RNA fragments (11).

The surfactant used in these experiments, tridodecylmethylammonium chloride (TDMAC), was chosen because it was one of several surfactant coatings used to prepare heparinized plastic surfaces. This methodology was used to prevent blood coagulation when the plastic surfaces were used in surgical applications. The mechanism for the binding of the surfactant to the plastic polymer involves the interaction of the hydrophobic end of the surfactant with the polymer surface. This leaves the cationic head available to bind the

heparin through an ion-pair interaction with the anionic functional groups thereby producing a heparinized surface. We exploited the property of the immobilized TDMAC for binding polyanionic biopolymers in developing a better method for the analysis of nucleic acid fragments. When ions of the RNA fragments adsorbed on TDMAC films were formed, we observed that the $TDMA^+$ cation displaced the alkali metal counterion in the salt producing molecular ions having a much higher molecular weight than the sodium salt. Despite the relatively high molecular weight of these ions, yields were significantly enhanced compared to those obtained using the usual deposition methods (11).

In the next test of this approach we examined the heparinized TDMAC film. Analysis by ^{252}Cf-PDMS of the adsorbed heparin macromolecule on the TDMAC surface did not yield any structurally significant information. In this paper, however, we show that when small heparin fragments are adsorbed using the same protocol and the films analyzed by ^{252}Cf-PDMS, intense molecular ions are produced. From the fragmentation pattern it is also possible to assess the number of anionic functional groups. This information could not be obtained when the samples were prepared by the usual solid phase deposition methods.

MATERIALS AND METHODS

Materials: Aluminized Mylar foil was purchased from Alan-Tol Industries. TDMAC was obtained from Polysciences. The heparin fragments were a gift from R.J. Linhardt, University of Iowa; the chondroitin fragment was a gift from H.E. Conrad, University of Illinois, Urbana-Champaign.

Methods: Aluminized Mylar foils were impregnated with TDMAC by the procedure previously reported (11). Aqueous solutions of the heparin or chondroitin fragments (sodium form) were applied to the surfactant surface. After 5 min the foil was rinsed in water then dried by spinning at 3000 rpm for 30 s. The amount of adsorbed heparin was determined by measuring the UV absorbance of the unbound heparin in the rinse at 232 nm. For the disaccharide fragment, approximately 25-30 μg was bound to the surfactant surface when 50-100 μg was applied to the film.

A description of the ^{252}Cf-PDMS instrument and data acquisition system appears in a prior publication (12). This instrument was designed and constructed in our laboratory (CJM, RDM). Similar results have also been obtained on the commercially available BIN-10K (Bio-Ion Nordic AB) ^{252}Cf-PDMS instrument by one of us (IJ). The acceleration voltage was +10 or +12 kV and -10 kV.

RESULTS AND DISCUSSION

The positive ion spectra of di-, tetra-, and hexasaccharide fragments from heparin adsorbed on the TDMAC surface are shown in Figure 1. In each

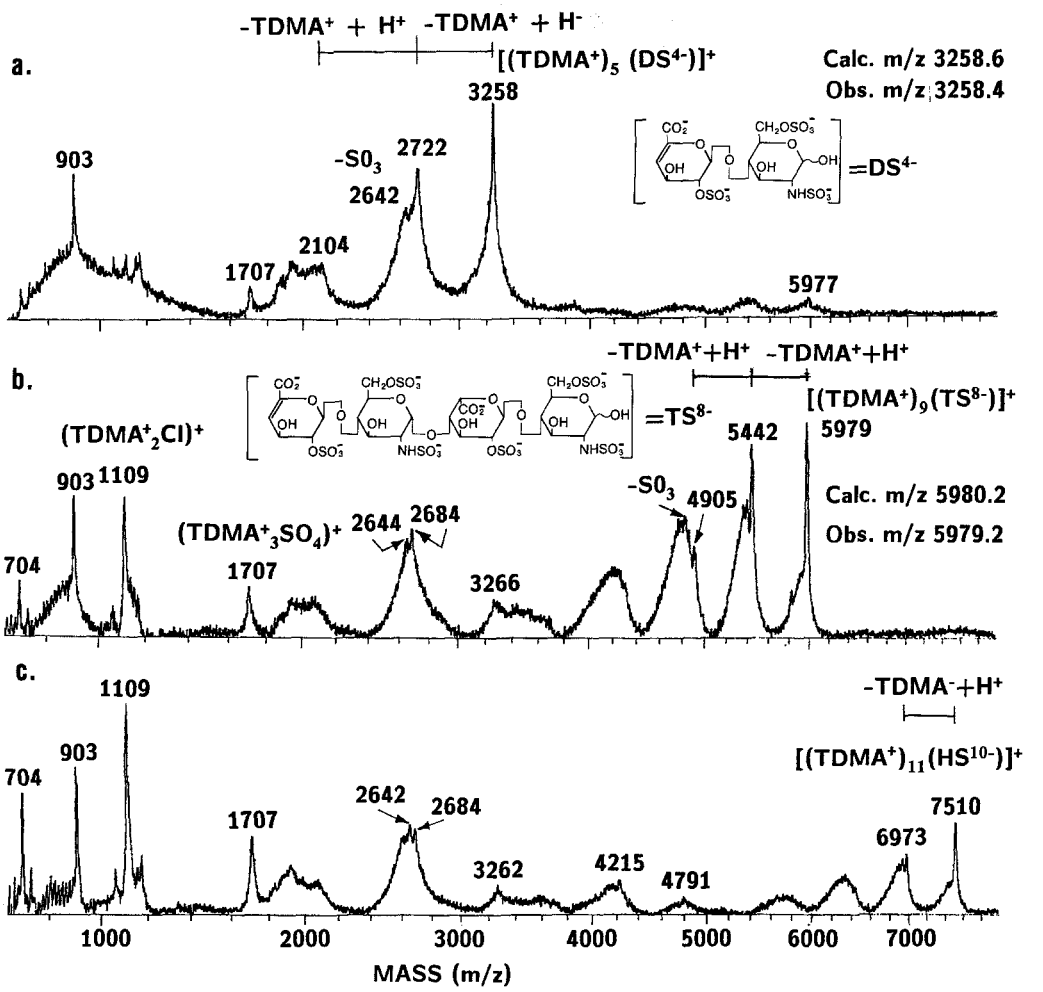

Figure 1. ^{252}Cf-PDMS positive ion spectra of heparin di-, tetra-, and hexasaccharide fragments adsorbed on TDMAC. The structures and calculated and observed masses of the $TDMA^+$ salt are indicated for the di-, and tetrasaccharide fragments.

case the molecular ion was formed by the addition of one more $TDMA^+$ ion than the total number of negative charges. The spectra are unusual in appearance since metastable ions were transmitted and detected as well as the stable components. Therefore, the spectra are a composite of very narrow peaks and broad metastable distributions, sometimes superimposed. Even though it is difficult to obtain meaningful masses of these distributions, we have observed that the number of distributions correspond to the number of negatively charged functional groups on the polysaccharide. The fragmentation pattern generally resulted from sequential losses of the $TDMA^+$ groups, and, in some

cases, small pieces of the molecule such as the SO_3^- ion. Sequential fragmentation at each glycosidic linkage was not detected. The spectrum of the hexasaccharide indicates that there are two additional negatively charged groups on this heparin fragment compared to the tetrasaccharide and that the HS^{-10} anion has a mass of 1602.5 ± 1. Although this structure has not been fully characterized, the molecular weight is consistent with available data. Dimer ions were observed for all three fragments however, only the disaccharide dimer, $[(TDMA^+)_9(DS^{4-})_2]^+$, falls within the mass range plotted in Figure 1. The $(TDMA_2Cl)^+$ ion, prominent in the tetra- and hexasaccharide spectra, is observed when there is incomplete exchange of the sulfated polysaccharide on the TDMAC surface, usually as a result of incomplete coverage. Below m/z 600, the three spectra are identical to the TDMAC spectrum reported in a previous publication (11) and do not contain any structurally significant fragment ions of the polysaccharide. The method is also applicable to the analysis of synthetically prepared heparin fragments and chondroitin-4-SO_4 and chondroitin-6-SO_4 fragments from whale cartilage. These spectra contained the same general features as the heparin fragments in Figure 1.

Representative negative ion spectra of the heparin disaccharide fragment and a chondroitin-6-SO_4 tetrasaccharide, both bearing four anionic functional groups, are shown in Figure 2. Molecular ions were formed by the addition of one less $TDMA^+$ ion than the number of negatively charged groups on the polysaccharide. This provided additional verification of the molecular weight. As in the positive ion spectra, fragment ions were formed by sequential losses of the attached $TDMA^+$ groups. In addition, the chondroitin spectrum contained fragment ions corresponding to cleavages at the glycosidic linkages. The peaks at m/z 1831 and 1725 correspond to the loss of the nonreducing and reducing terminal monosaccharides respectively. The m/z 1295 and 1183 are the same fragments containing one less $TDMA^+$ group. The peak at m/z 1012 is the disaccharide fragment. The mass is the same for the reducing and nonreducing termini. The monosaccharide fragments have not been identified. In the m/z 100-500 mass range extensive fragmentation of the carbohydrate backbone was produced in both cases. The spectra of the various heparin fragments are almost superimposable in this mass range but as can be seen from

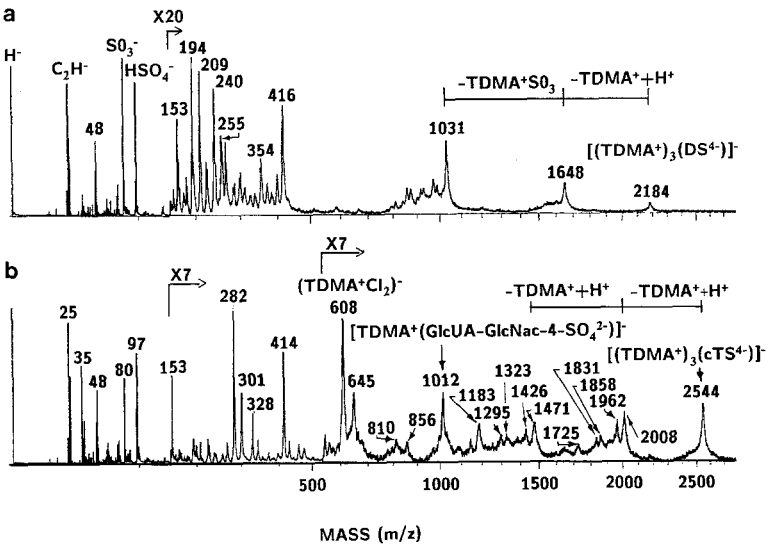

Figure 2. ^{252}Cf-PDMS negative ions spectra of a) a heparin disaccharide fragment and b) a chondroitin-4-SO_4 tetrasaccharide fragment. The structure of the heparin fragment is given in figure 1a. The structure of chondroitin fragment is given by $(GLcU\rightarrow GalNAc\text{-}4\text{-}SO_4)_2^{2-}$. This sample did not completely cover the TDMAC surface and therefore, the Cl^- and $(TDMA^+Cl^-_2)^-$ ions were present in the spectrum.

Figure 2, the chondroitin and heparin fragments are distinctly different. The identities of some of these ions have been determined. More structural information may be available from the spectra, but additional known structures must be analyzed before the assignments can be made with confidence.

CONCLUSIONS

We have demonstrated that ^{252}Cf-PDMS can provide rapid structural information on glycosaminoglycans adsorbed on an immobilized cationic surfactant. Analyses are routinely made with a total of 25 to 50 μg; samples can be prepared, analyzed and interpreted in a matter of hours. The relatively high molecular weight of the $TDMA^+$ salt posed no problem for the ^{252}Cf-PDMS method which is unrivaled with regard to its mass range and ease of operation. The simplicity of this approach and the advantages of this instrumentation are well suited to clinical applications, particularly where sample throughput and reliability are important factors.

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REFERENCES

(1) Folkman, J., Langer, R., Linhardt, R.J., Haudenschild, C., and Taylor, S. (1983) Science 221, 719-725.
(2) Sharath, M.D., Merchant, Z.M., Kim, Y.S., Rice, K.G., Linhardt, R.J., and Weiler, J.M. (1985) Immunopharmacology 9, 73-80.
(3) Brown, W.V., Wang-Iverson, P., and Paterniti, Jr., J.R. (1980) "Chemistry and Biology of Heparin", pp 175-186, Lundbald, R.L., Brown, W.V., Mann, K.G., and Roberts, H.R., Eds., Elsevier/North Holland, New York.
(4) Merchant, Z.M., Kim, Y.S., Rice, K.G., and Linhardt, R.J. (1985) Biochem. J. 229, 369-377.
(5) Carr, S.A., and Reinhold, V.N. (1984) J. Carbohydr. Chem. 3, 381-401.
(6) Reinhold, V.N., Carr, S.A., Green, B., Petitou, M. and Choay, J., Carbohydr. Res., submitted for publication.
(7) Macfarlane, R.D. (1982) Acc. Chem. Res. 15, 268-275.
(8) Jordan, E.A., Macfarlane, R.D., Martin, C.R., and McNeal, C.J. (1983) Int. J. Mass Spectrom. Ion Phys. 53, 345-348.
(9) Macfarlane, R. (1986) "Mass Spectrometry in the Health and Life Sciences", pp 85-98, Burlingame, A.L., and Castagnoli Jr., N., Eds., Elsevier Science Publishers B.V., Amsterdam.
(10) Macfarlane, R.D., McNeal, C.J., and Martin, C.R. (1986) Anal. Chem. 58, 1091-1097.
(11) McNeal, C.J., and Macfarlane, R.D. (1986) J. Am. Chem. Soc. 108, 2132-2139.
(12) Macfarlane, R.D. (1983) Anal. Chem. 55, 1247A-1254A.