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Short communication

Analysis of biopolymers by size-exclusion chromatography–mass spectrometry

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1. Introduction

Size-exclusion chromatography (SEC) is a powerful analytical method that allows separation of analytes in a size-dependent mode. The introduction of new columns that feature virtually no limit in the molecular mass of the species to be analyzed, low interaction with the analytes, along with ruggedness, reproducibility, efficiency and short analysis time, has made it possible to analyze any class of compounds by SEC regardless their chemical composition [1]. Now that SEC offers the possibility to size-separate a wide range of polymeric species, a complete exploitation of SEC requires interfacing to detection techniques that feature similar properties, e.g., the ability to track analytes regardless of their structure and composition. Conventional detectors that have been interfaced to SEC monitor one specific parameter of the analyte, such as: UV absorption, light scattering or refractive index [2–4]. These detectors are usually not specific enough to track a single compound within a mixture. Isotope tags could represent an ideal way of labeling a specific molecule without changing its chemical properties. If stable isotopes are to be used rather than radioisotopes, occupational and environmental risk and biological limits associated with the use of radioactivity could be overcome. Thus, a detector able to monitor the difference between a labeled compound and its unlabeled counterpart could fulfil the requirements of selectivity for the 'ideal' SEC detector.

In the last few years our laboratory has implemented an innovative mass spectrometric technique named chemical reaction interface mass spectrometry (CRI-MS) [5]. In brief, CRIMS allows the analyst to monitor the elemental and isotopic composition of any organic molecule that CRI-MS quantitatively transforms into a set of specific, low-molecular mass, reaction products. Moreover, CRI-MS provides unique parameters for the characterization of each molecular species (e.g., the $^{13}C/^{12}C$ isotope ratio or the presence of an unusual element, e.g., S or Cl in a molecule) [6]. CRI-MS has already been shown to be a practical tool for metabolic and pharmacokinetic studies of stable isotope-labeled compounds [5,7].

The goal of this work was to interface SEC with CRI-MS. Direct LC–MS interfacing has become quite easy to achieve [8]. However, SEC of biopolymers often requires mobile phases containing non-volatile salts, which are not suitable for MS analysis. Beside this, any given type of MS ionization process has a field of application that does not allow the analysis of every class of biomolecules in an unlimited range of M_r. Here with SEC–CRI-MS any class of biopolymers is first separated in size-dependent mode using volatile mobile phases and then

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analyzed with a detection system unaffected by the nature or the size of the analytes under investigation.

2. Experimental

2.1. Materials and methods

Solvents employed for the chromatographic separations (water, methanol, tetrahydrofuran) were obtained from EM Science (Gibbstown, NJ, USA) at the highest available grade of purity.

Heparin from bovine intestinal mucosa was obtained from Sigma (St. Louis, MO, USA) and used without further purification. $^{13}C/^{15}N$-labeled rat growth hormone (rat GH) was produced in our laboratories by feeding GH_3 rat pituitary adenoma cells (ATCC, Manassas, VA, USA) with $^{13}C/^{15}N$-labeled culture medium (Celtone, Martek Bioscience, Columbia, MD, USA) as detailed previously [9].

2.2. Chromatographic conditions

Silica-based SynChropak columns (GPC 300, 250×4.6 mm I.D., particle size 5 μm; GPC linear 250×4.6 mm I.D., particle size 7 μm) obtained from Keystone Scientific (Bellefonte, PA, USA) were used for SEC. For the analysis of nucleic acids and polysaccharides the mobile phase was a solution of 100 m*M* ammonium acetate at pH 6.5. For proteins the mobile phase consisted of 1.5 *M* triethylammonium formate, made by adding triethylamine to a solution of 1.5 *M* formic acid to reach pH 3 and supplemented with tetrahydrofuran (THF) to a final ratio buffer–THF of 2:1 (v/v). SEC of proteins was performed at 40°C, all other separations were done at room temperature.

2.3. CRI-MS analysis

A conventional HPLC–CRI-MS set-up is schematically represented in Fig. 1 and has been described in detail elsewhere [10]. Briefly, it consists of an HPLC pump SSI 222C (SSI Instruments, State College, PA) connected to an Extrel C50/400 (ABB Extrel, Pittsburgh, PA, USA) mass spectrometer via a Vestec-Universal Interface (UI) (Perseptive BioSystems, Framingham, MA, USA) that removes the solvents employed for the chromatographic separation. Located downstream from the UI and before the MS system, a microwave-powered chemical reaction interface (CRI) quantitatively transforms the analytes into a set of low-M_r products by reaction with a specific gas, e.g., SO_2 (Matheson Gas Products, East Rutherford, NJ, USA) or NF_3 (Air Products, Allentown, PA, USA). The chemical nature of the products obtained is related to the elemental composition of the analyte and the nature of the reactant gas. For example, when SO_2 is used all the C atoms in the analytes are transformed to CO_2 molecules (M_r 44), whereas if NF_3 is used, all the C atoms are transformed into CF_4 and detected as CF_3^+ (m/z 69). Products of the CRI-MS reaction enter the MS system where they are ionized in electron impact mode and analyzed by the quadrupole analyzer.

The UI is designed to give the best performance at a flow of 1 ml/min. On the other hand, analytical SEC separations are better if carried out at lower flow-rates (0.1–0.5 ml/min). To achieve efficient chromatographic separations without compromising the UI performance we carried out the chromatography at 0.2–0.3 ml/min and added water (0.7–0.8 ml/min) to the eluate through a three-way mixer (Alltech, Deerfield, IL, USA).

3. Results and discussion

The main problem in interfacing liquid chromatography to any mass spectrometric technique is the achievement of chromatographic conditions suitable for the mass spectrometer. Therefore, it is essential that the mobile phases contain only volatile species. Obviously, the solvent should be able to dissolve the analyte without causing chemical denaturation and still work for the chromatographic separation. Experiments aimed at finding the optimal chromatographic system yielded mobile phases that fulfil the requirements.

For the analysis of nucleic acids (data not shown) or polysaccharides, a mobile phase of 100 m*M* ammonium acetate (pH 6.5) in water worked well. Fig. 2 shows an example of multiple elemental monitoring applied to SEC–CRI-MS analysis of a polysaccharide (heparin) using NF_3 as the reaction gas [11]. This analytical configuration allows the

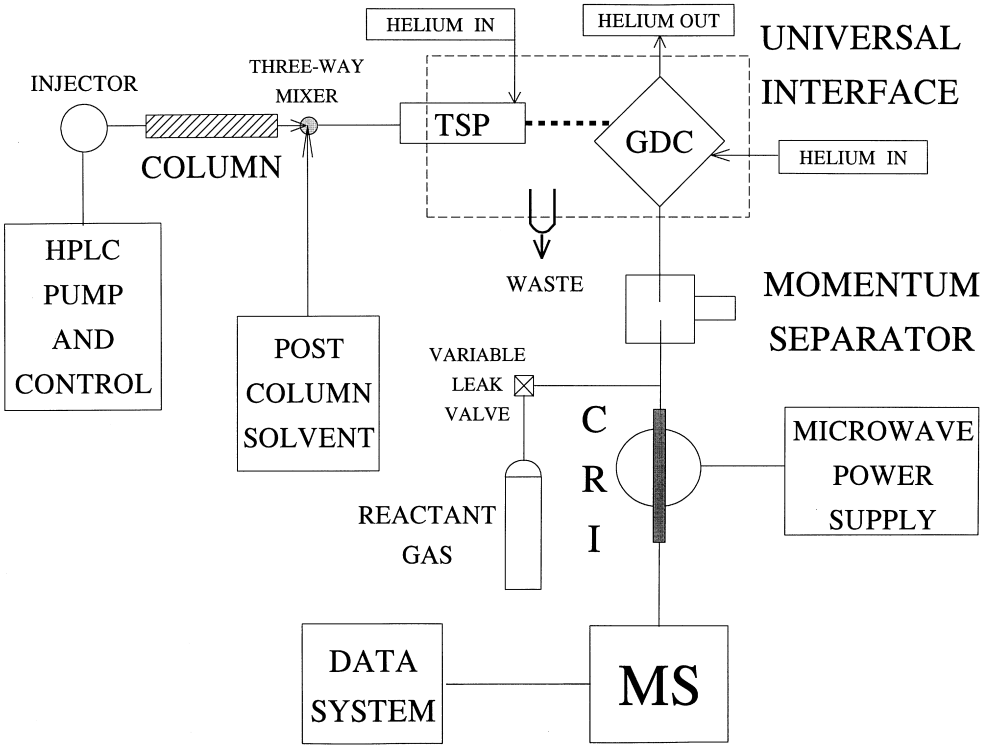

Fig. 1. HPLC–CRI-MS set up. The outflow from the SEC column (0.2–0.3 ml/min) is mixed with water in a three-way mixer to yield a flow of 1 ml/min, as needed for properly operating the Universal Interface. At this flow-rate, the analyte solution is thermosprayed (TSP) into the desolvation chamber, where it is then dried by a stream of helium. The helium stream then carries the analyte into the gas diffusion chamber (GDC) where volatile molecules (residual solvents) diffuse out through a membrane and therefore are removed from the involatile molecules (analytes) that continue downstream. The helium propels the analyte through a momentum separator and into the microwave-powered chemical reaction interface (CRI). Here, analytes react quantitatively with the reactant gas (SO_2, NF_3) in a helium plasma to produce stable low-M_r compounds that are detected by the mass spectrometer (MS).

simultaneous detection of C and S. The upper chromatogram in Fig. 2 corresponds to the 'carbon channel' (i.e., the signal obtained monitoring CF_3^+ at m/z 69). The lower chromatogram shows the 'sulfur trace' (m/z 127 corresponding to SF_5^+). Heparin, which is a mixture of sulfated hexose and hexosamino polymers with an average M_r of 16 000, is eluted at 8 min and shows a peak in both chromatograms. The peak at retention time 11.5 min corresponds to a low-M_r (<500) C-containing, S-free species yet to be identified.

Separation of proteins by SEC is a challenging task. Common procedures employ high concentrations of nonvolatile buffers to overcome interactions between the stationary phase and protein as well as protein–protein interactions [12]. We find that 1.5 *M* triethylammonium formate (TEAF) at pH 3.0 in water–tetrahydrofuran 2:1 (v/v) is a good mobile phase for protein analysis by SEC. This mobile phase is well tolerated by the chromatographic columns that we used, and has acceptable volatility (days of use have not presented a problem for any component of the SEC–CRI-MS system). All the standard proteins tested, so far (among them: thyroglobulin, collagen, transferrin, bovine serum albumin, carbonic anhydrase, lysozyme, cytochrome *c*, insulin, tryptophan) were detected by SEC–CRI-MS.

Our specific interest is in measuring ^{13}C-enriched biopolymers. Fig. 3 shows one example where CRI-MS was used to selectively detect ^{13}C-labeled rat growth hormone spiked in horse serum. For this analysis SO_2 was used as the reactant gas. The upper

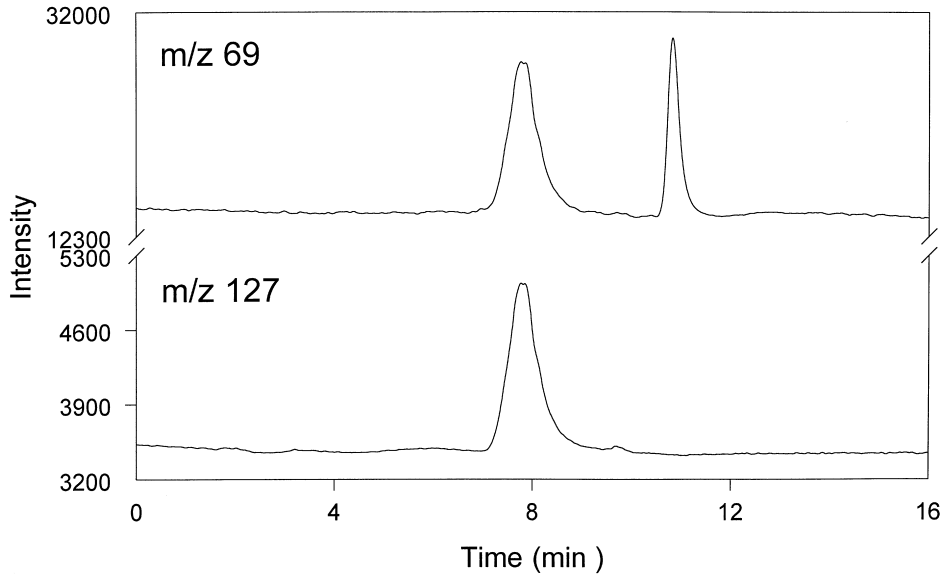

Fig. 2. Analysis of heparin by SEC–CRI-MS. Heparin (50 μg) was separated by SEC with a SynChropak GPC linear column, mobile phase 100 m*M* ammonium acetate, pH 6.5, at a flow-rate of 0.3 ml/min. For the CRI-MS detection NF_3 was employed as the reaction gas. Under these conditions, the 'carbon trace' is monitored as CF_3^+ at m/z 69 (upper chromatogram), whereas the 'sulfur trace' is monitored as SF_5^+ at m/z 127 (lower chromatogram). Heparin (that contains C and S) was detected in both channels at a retention time of 8 min.

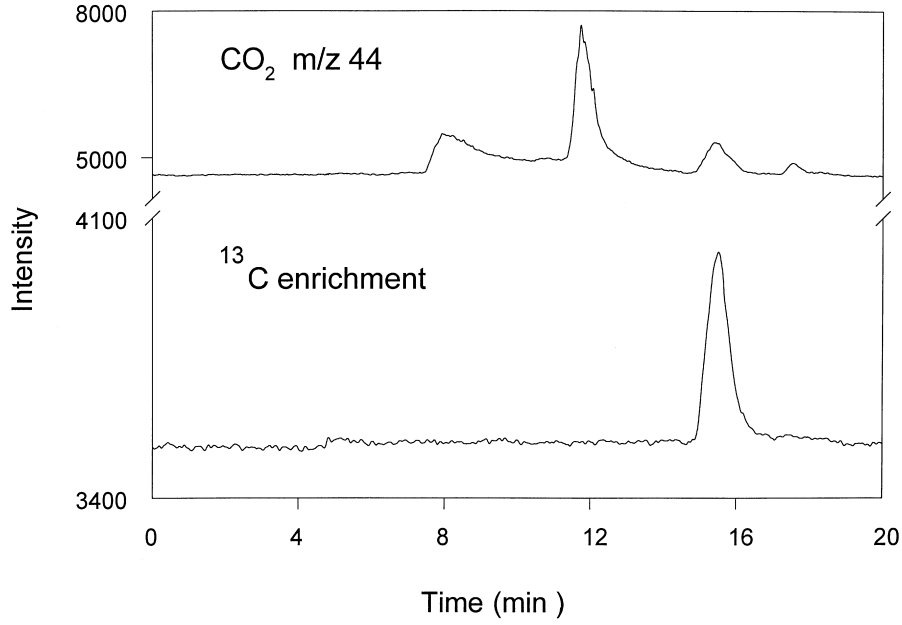

Fig. 3. Analysis of labeled proteins by SEC–CRI-MS. ^{13}C-labeled rat growth hormone was spiked in horse serum. SEC analysis was performed using a SynChropak GPC 300 column with a mobile phase consisting of 1.5 *M* triethylammonium formate in water–tetrahydrofuran (2:1) at a flow-rate of 0.2 ml/min at 40°C. SO_2 was employed as reactant gas for the CRI-MS detection. The upper chromatogram is the 'carbon trace' (monitored as CO_2 at m/z 44). The lower chromatogram is the '^{13}C-enriched trace', showing only ^{13}C-labeled molecules, from which the background of naturally occurring ^{13}C has been subtracted.

chromatogram in Fig. 3 shows the trace in the general carbon channel. The lower chromatogram shows the profile obtained by subtracting the natural abundance 'background' of ^{13}C (1.19% of ^{12}C) from the m/z 45 trace. Hence, the lower chromatogram represents only those peaks having enrichment of ^{13}C. Regarding sensitivity, use by our group of unenriched proteins with their natural 1.19% abundance of ^{13}C, we have established the potential to analyze less than 100 ng of a ^{13}C-containing biopolymer by SEC–CRI-MS (data not shown).

4. Conclusions

In this paper we report successfully interfacing SEC to CRI-MS. The optimal combination between a general chromatographic method (SEC) and the universal detection capability of CRI-MS allows the analysis of various classes of compounds including: proteins, peptides, nucleic acids and polysaccharides. In our laboratory this technique appears to be extremely promising for metabolic studies of materials labeled with stable isotopes.

Acknowledgements

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