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Developing ICP-MS/MS for the detection and determination of synthetic DNA-protein crosslink models via phosphorus and sulfur detection

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Abstract

Various endogenous and exogenous agents drive the un-directed formation of covalent bonds between proteins and DNA. These complex molecules are of great biological relevance, as can derive in mutations, but are difficult to study because of their heterogeneous chemical properties. New analytical approaches with sufficient detection capabilities to detect and quantify these compounds can help to standardize study models based on synthesized standards. The use of atomic spectrometry can provide quantitative information on the DNA-protein cross-link reaction yield along with basic stoichiometry of the products, based on internal elemental tags, sulfur from Cys and Met amino acids, and phosphorus from the DNA. A new instrumental approach to remove isobaric and poly-atomic interferences from $^{31}\text{P}^+$ and $^{32}\text{S}^+$ was developed recently, with state-of-the-art for interference removal that captures $^{31}\text{P}^+$ in Q1; it reacts with O_2 in an octopole collision-reaction cell generating $^{47}\text{PO}^+$, therefore allowing detection in Q3 without $^{31}\text{NOH}^+ / ^{48}\text{Ca} / ^{47}\text{Ti}$ interferences. Similarly, $^{32}\text{S}^+$ is reacted to $^{48}\text{SO}^+$, eliminating the polyatomic interferences at $m/z=32$. In conjunction with the high resolving power of high-

performance liquid chromatography (HPLC), this newer technology was applied by to the product purification of a DNA-protein cross link model and some preliminary structural studies.

Keywords

Bioanalytical methods; Speciation; Spectroscopy/instrumentation; Nucleic acids (DNA | RNA)

Introduction

It is well known that genomic DNA can be transiently associated with various structural, regulatory, repair, and transcription-involved proteins. The dynamic and reversible association between DNA and proteins guarantee the accurate expression and propagation of genetic information [1]. However, various endogenous and exogenous agents, including free radicals from metabolism [2], chemotherapeutic agents (e.g., cisplatin) [3], and toxins, such as hexavalent chromium, result in DNA-protein cross-links (DPCs). Compared with other DNA lesions, DPCs are distinctive in that they are very bulky, meaning they strongly impair replication, transcription, and repair. In addition, DPCs are poorly structurally characterized due to their complex nature. Importantly, adding analytical capabilities associated with their detection and improving the characterization of synthesized DPCs to model their formation will allow a better understanding of their properties and structures.

Inductively coupled plasma mass spectrometry (ICP-MS) has been a valuable tool to study a variety of complex molecules based on internal or external elemental tags, with the advantage of being a species-independent detector, which allows quantifying by using simple inorganic standards. The complex reaction between DNA and proteins, even at the level of model standards, possesses a challenge for product's characterization, even at the most fundamental level, reaction yield and stoichiometry of the products. In this regard, the current methods for characterization include several indirect techniques, such as alkaline elution [4], nitrocellulose filter binding [5], SDS/K⁺ precipitation [6], and comet assays [7]. These methods only provide the information on relative amounts of DPCs and yield neither quantitative information nor stoichiometric characterization of the adducts. Additionally, ¹²⁵I- [8] and fluorescein post-labeling [9] are very powerful for the direction and quantitative analysis of DPCs, even though they require the purification of chromosomal DNA. What is required is absolute quantification with the ability to identify the exact type of DPC formed. ICP-MS in theory is capable of this, but detection of metalloids and non-metals that are poorly ionized in the plasma source due to their high ionization potential remains a challenge. Important for DPCs are sulfur and phosphorus that suffer from isobaric and polyatomic interferences [10]. High-resolution ICP-MS instruments can be used to separate the isobaric interferences that affect S and P, based on a mass difference of 0.0178 to 0.0416 Da, but these instruments are very costly and less robust on its operation. In order to remove the isobaric interferences on a single quadrupole-based ICP-MS, a reaction with oxygen has been explored in the past with promising results, but two issues stand in the way of this approach: the matrix-dependent reactivity of the analytes and the spectral interferences from ⁴⁷Ti⁺, ⁴⁸Ca⁺, and ⁴⁸Ti⁺ that cannot be distinguished from the oxidation products of P and S at *m/z* ⁴⁷PO⁺ and ⁴⁸SO⁺. Most recently, the implementation of an ICP-

MS/MS shows the possibility of eliminating most of the limitations in analyzing P and S via reacting them with O₂. By filtering all m/z ions except at 31 or 32 at Q1, the reaction takes place at the octopole collision-reaction cell in a much cleaner environment than in a single quadrupole instrument. And because $m/z=47$ and 48 are filtered in the first quadrupole, Ti and Ca ions, respectively, no interferences at Q3 are present from ⁴⁷Ti or ⁴⁸Ca to reach the detector, only the reaction products of P or S oxidation. This mass shift operation mode has been presented previously by Fernandez et al. and Balcae et al. [11, 12]. This opens the possibility of using the matrix-independent and compound-independent detection and quantification capabilities of the ICP-MS/MS to analyze DPCs through the detection of sulfur and phosphorus. Furthermore, by coupling ICP-MS/MS detection with high-performance liquid chromatography (HPLC or LC), we can achieve nearly simultaneous separation and detection [13]. Gel filtration can be used to purify the crude extract and to obtain stoichiometry information for the products. Following with the use of capillary HPLC allows the use of organic-based mobile phases as eluents, necessary for the use of reversed phase columns. With this combination, capLC-ICP-MS/MS, the DPCs, its precursors and its byproducts can be separated with high efficiency and detected at sub-ng levels in a highly specific manner.

Optimization of the instrumental parameters

To optimize the instrument sensitivity and detection limits, the O₂ flow rate was systematically changed from 0 to 50 % of 1 ml while keeping the analytes concentration constant (ATP or Cys) at 200 ppb with respect to P or S (Fig. 1). We followed both $m/z=31$ and 47 for P while $m/z=32$ and 48 for S. A blank solution was used to compare the instrumental response to the analyzed m/z vs. the standard solution at different O₂ flows. The signal to noise ratio was manually calculated with a blank solution at each point of the capLC ramping procedure. It was found that at the standard solution, as O₂ increases, the signal for ³¹P⁺ decreased until reaching a minimum. The non-zero minimal value likely reflects detection of poly-atomic interferences. A simultaneous increase at $m/z=47$ was attributed to the increase in the ⁴⁷PO⁺ formation. The maximum S/N value was observed at 30 % of 1 ml min⁻¹ of O₂ (the manner this flow is described by the instrument). The blank solution showed a small decrease at $m/z=31$, while $m/z=47$ went from no-counts to less than 1000 cps, which can be attributed to P impurities in the formic acid or water.

For sulfur, the plot looks different than the one for P, with $m/z=32$ being in the tens of millions cps, and virtually identical for the blank and for the 200 ppb S standard all along the O₂ ramping. This means that the level of interference for $m/z=32$ is much more intense than that for $m/z=31$ under current conditions. Only at $m/z=48$ a difference was observable between the blank and the 200 ppb S standard. Two orders of magnitude in the difference was found vs. to the more than three for P. This observation can be attributed to the immense signal from the nitrogen-, oxygen- and hydrogen-based interferences, which can exceed the peak filtering capacity of a quadrupole-based ICP-MS, along with the higher level of contamination in the reagents and water used for the blanks.

This substantial difference with background removal for P and S directly affects the limits of detection (LOD) for these elements under the MS/MS mode.

The lenses were tuned with a solution containing 20 ppb of Y at a flow rate of 10 $\mu\text{L min}^{-1}$ by using a syringe pump connected to the capillary nebulizer with a 50 μm internal diameter capillary to maximize the signal of $^{89}\text{Y}^+ \rightarrow ^{105}\text{YO}^+$. The argon carrier gas flow was set to 0.9 L/min, which is lower than normal, but necessary for capillary LC systems. With these tune parameters, the instrument shows a signal with a $^{105}\text{YO}^+$ target above 130,000 cps, while for $^{89}\text{Y}^+$, it was below 2000 cps, yielding a 98 % conversion to $^{105}\text{YO}^+$.

Detection of laboratory synthesized DPCs—The separation of synthesized DPCs from the reactants is becoming an increasingly important problem due to the low yield of the cross-linking reactions. However, the high sensitivity and low limit of detection available with the sulfur and phosphorus detection with the ICP-MS/MS is promising for purification proposes. Size exclusion chromatography (SEC) coupled with ICP-MS/MS was the technique used for purification of the synthesized DPC. Also, desalting and changing to a volatile solvent was achieved. To determine the retention time of the reactants and products, the two main reactants (oligonucleotide and RNase) and the solution from the cross-linking reaction were injected into the instrument separately. From the chromatogram in Fig. 2a, it may be seen that the protein itself provides a $^{48}\text{SO}^+$ signal at a retention time of 15 to 20 min, while DNA gives a signal of $^{47}\text{PO}^+$ at *ca.* 17 min. DPC gives signals for both sulfur and phosphorus at a single retention time earlier than both of the reactants due to its higher hydrodynamic radius. By collecting this fraction, crosslink product purification can be achieved, and by comparing with the peak area applied in the following equation, the mole ratio of DNA to protein in forming the DPCs is determined to be *ca.* 0.8, Eq. 1.

$$\frac{\text{moles of DNA}}{\text{moles of protein}} = \frac{\text{Area}(P) \times \text{Area}(S \text{ STD.}) \times 12 \times 32}{\text{Area}(S) \times \text{Area}(P \text{ STD.}) \times 27 \times 31} \quad (1)$$

Molar ratio calculation performed with the DNA and protein parts involved in DPCs, after sensitivity adjustment.

Tandem LC-ICP-MS/MS—Capillary reverse phase LC with a 0.5 mm i.d. column was coupled to ICP-MS/MS for separation and detection of the trypsin-treated DPC. Acetonitrile was used as the eluting mobile phase, and because the microflow produces only a small organic flow to the ICP-MS/MS, it minimizes the carbon deposits on the interface and plasma instability that would occur with normal column bore analytical LC flow.

Detection of a DPC would occur by detecting a chromatographic signal with both $^{47}\text{PO}^+$ and $^{48}\text{SO}^+$ signals. To estimate sensitivities, we injected 2 μL of ATP and cysteine standards. Cysteine-based S concentrations ranged from 50 to 2500 ppb and ATP based P standards ranged from 0 to 1000 ppb. It was found that the limit of detection for S as cysteine was 5.5 ppb. The limit of detection was calculated as three times the standard deviation of the blank intensity at the base line of the chromatograms, and then divided by the slope of the calibration curve, based on the height of the chromatographic signals from the standards. The limit of detection for P as ATP was 0.1 ppb. In addition, signal increased linearly as the *R* values were greater than 0.999 in both cases. Thus, this method provides unrivaled performance with higher sensitivity and lower backgrounds for sulfur and

phosphorus detection and can achieve the separation and detection with small volume samples.

Capillary reverse phase LC-ICP-MS/MS detection of DPCs after trypsin

proteolysis—The ICP-MS/MS coupled with capillary RPLC provides the capability for separation and detection of DPCs post-trypsin proteolysis. Trypsin cleaves peptide chains primarily at the carboxyl side of lysine or arginine, except when either is followed by proline, which makes it attractive for further mass spectrometric analysis. Once the DPC has been cleaved, capillary LC with a 0.5 mm i.d. C18 column was used to separate the generated peptides, one of which was attached to the oligonucleotide residue. To simplify the problem of identifying the peptide involved, the ICP-MS/MS chromatographic signal of the pure protein digest was compared with the DPC digest. As can be seen from Fig. 3a, the inorganic materials elute in the void volume between 0 and 5 min. The S signal for the DPC was low, as a very small amount was purified and injected to the system. But the co-elution of an S containing peptide and the P containing oligonucleotide can be observed at 48.5 min. This signal can be correlated to the large S signal at 51.5 min from the digested protein, by taking into account that the addition of the polar nucleotide residue will decrease the retention time. Given the poorer detection limit of S compared with P, it is not surprising that the intact oligonucleotide, containing 27 P atoms, shows a much larger signal than the sulfur containing peptide. The peptide involved in the DPC is amenable to two ways of identification. The retention time for the protein-derived peptide (51.5 min from Fig. 3a) was identified by LC-MS analysis, in a traditional bottom up proteomic approach. And secondly, the elution order of the S containing peptides produced from tryptic digestion of the protein was estimated by taking account the hydrophobicity and confirmed by semi-quantitatively comparing the S content of each peptide according to the predicted elution order. These, notwithstanding, the aim of this study are to report a new approach to improve the characterization and purification steps at the synthesis for a DPC model. When the proposed sequence NGQTNCYQSYSTMSITDCR matches with the elution order and contains three atoms of S, from cysteine and methionine, assured by the S ICP-MS/MS signal, its identity is only suggested and not confirmed.

The identity of the peptide-oligonucleotide is currently under study for direct analysis by traditional LC-MS with electrospray source, but is impaired by ionization problems, as the DNA fragment contains a large number of negatively charged moieties. Therefore, a simplification of the DPC model is required by removing the phosphate chain and leaving just the base attached to the peptide—this approach is under current investigation.

Conclusion

The improved capabilities for P and S detection of an ICP-MS/MS were successfully applied to assist the synthetic DPC purification and characterization. From the purification of reaction products with a stoichiometry estimation, to the final assignment of the protein fragment associated with the DNA moiety, the ICP-MS/MS was a very useful tool, especially with SEC or capRPLC for sample introduction. The possibility of following the S and P related signal of tryptic digested proteins and genetic material opens a new window of opportunity for protein studies and facilitates the quantification DNA-protein conjugates

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Fig. 1.

Reaction cell optimization for $^{47}\text{PO}^+$ and $^{48}\text{SO}^+$. **a** Dotted lines represent the response to $m/z=31$ of the blank (*empty square*) and 200 ppb P standard as ATP (*filled square*), while continuous lines represent the signal for $m/z=47$ of the blank (*empty triangle*) and 200 P standard as ATP (*filled triangle*). **b** Dotted lines represent the response to $m/z=32$ of the blank (*empty square*) and 200 ppb S standard as Cysteine (*filled square*), while continuous lines represent the signal $m/z=48$ of the blank (*empty triangle*) and 200 S standard as Cysteine (*filled triangle*). The abscissa axis corresponds to the oxygen flow in ml min^{-1} , while the ordinate axis represents the signal response in a logarithmic scale

Fig. 2. SEC-ICP-MS/MS chromatograms for **a** ribonuclease A, SO^+ signal in black; **b** 27mer-nucleotide, PO^+ signal in blue; and **c** DPC after cross-linking reaction merged SO^+ signal in black and PO^+ signal in blue

Fig. 3. CapLC-ICP-MS chromatograms from tryptic digested samples **a** SO^+ RNase A digest; **b** SO^+ signal of DPC digest, *in black*, the reagent blank and *in red*, the digested DPC; **c** PO^+ signal of DPC digest, *in black*, the reagent blank and *in red*, the digested DPC. The indicated signal at 48.5 min in **b** correspond to the peptide fragment associated to the oligonucleotide as it co-elutes with the P signal from the oligonucleotide moiety