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Tandem Mass Spectrometry of Multiply Charged Polymers: Using MS3 Strategies for Full Structural Characterization of Polyacrylamides

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ABSTRACT: Multiple stages of CAD and ETD fragmentation methods were performed using an electrospray ionization mass spectrometer in order to show that multiple fragmentation stages give information that a single fragmentation method could not give. Through the analysis of two end group functionalized polyacrylamides, NIPAM-COOH and NIPAM, it was found that combination of two different ion activation methods (CAD and ETD) is especially effective in areas where a single fragmentation method is not enough for full characterization of the structure. As a result of MS^3 strategies that have been applied in this study, structural information such as side chain functional groups, end group functionalities as well as backbone sequence information on poly(N-isopropylacrylamide) samples are obtained.

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i. Introduction

In mass spectrometry, a compound is taken and converted into a gas-phase ion by an electrical charge (Balogh, 2009). From these ions, a spectrum is then generated that displays the mass to charge ratio, which is able to then be analyzed to find out the charge of the ions, as well as the mass of the compound (Balogh, 2009). The mass of the compound can be found by taking the value of a peak on the spectrum (in m/z), and multiplying that value by the charge of the peak (the z value), which will give an m value for the specific peak. These values can be used to confirm masses of specific products, or to confirm masses of repeating units. Other strategies in mass spectrometry can be used to find out more about a compound's structure. By using MS/MS techniques the molecular ion can be isolated and fragmented, and further interpretation of these fragment ions can reveal more information about the molecular structure. There are several specific ways in which molecules can be activated for fragmentation. Two of those methods that were used in this study are 1. Electron Transfer Dissociation (ETD), where fluoranthane anions that generate react analyte cations which results in electron transfer from anion species to the molecular cation. This process results either charge reduction or fragmentation depending on the electron attachment site on the molecule 2. Collisionally Activated Dissociation (CAD), where activation of the analyte ion is done by collisions with inert gas molecules. Energy generated by these collisions is distributed along the molecule which eventually results in cleavage of labile bonds. Because of the difference in activation mechanism these fragmentation methods results in different fragmentation patterns that can provide complementary information about the structure (Wesdemiotis, 2012). One can do a single type of fragmentation to find out how the molecule fragments in CAD or ETD, as these methods are established techniques that are powerful for discovering structural information of precursors, but these methods will only give limited information (Hart-Smith, 2014). A combination of two fragmentation strategies (MS^3) can be done to find even more information than a single fragmentation can do.

In this study, the two poly(N-isopropyleneacrylamide) (pNIPAM) samples with different end group functionalities were analyzed by tandem mass spectrometric techniques for full characterization of the structure and investigation of fragmentation mechanisms of poyleacrylamides. The two structures are shown below (fig. 1).

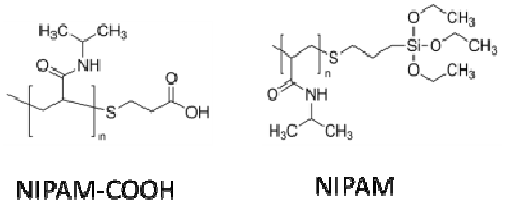

Fig. 1: The structures of the two poly(N-isopropyleneacrylamide) structures. NIPAM-COOH contains a carboxy;ic acid end group, while NIPAM contains a triethoxysilane end group.

ii. Materials and Methods

The p(NIPAM) samples and were obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, MO), the MeOH and THF solvents were also acquired from Sigma Aldrich (Sigma Aldrich, St. Louis, MO), and all of the experiments are performed on (Bruker Daltonics, Billerica, MA).

Sample solutions of NIPAM, and NIPAM-COOH, were prepared with 1mg/mL concentrations in MeOH/THF in 1:1 v/v ratio (Sigma Aldrich, St. Louis, MO). From these solutions, 0.005 mg/mL solutions of each separate solid were prepared and injected to the instrument with a flow rate of 180 µl/h. MS/MS experiments were done on singly-

charged, doubly-charged, and triply-charged ions with CAD and ETD methods. For CAD experiments isolation width was kept at 1.0 and the amplitude of the excitation RF field was kept in between 0.23 and 0.30 (arbitrary units), depending on the precursor ion isolated. For ETD experiments, fluoranthane radical anions (reagent ions) were produced in a negative chemical ionization (nCI) source filled with methane buffer gas (2.0-2.6 bar). Ion trap conditions for maximizing the generation and transmission of reagent anions are as follows: reagent ion ICC 100,000, ionization energy 70 eV, emission current 2.0 µA, and reagent remove cutoff *m/z* 210. After accumulation of both type of species in the ion trap, ion/ion reaction time and cutoff parameters are arranged within 50-170 and 150-170, respectively. These values are optimized during MS^3 experiments to keep the isolated fragment ion abundance as high as possible for the next fragmentation stage.

iii. Results and Discussion

When the first spectrum for NIPAM-COOH was taken (fig.2), a spectrum was found with many noticeable singly-charged peaks with a difference of m/z 113.1 between peaks. Also, several doubly-charged peaks were found with a difference of m/z 56.6, and triply charged peaks were found with a difference of m/z 37.7 between peaks. As a salt was not used to ionize the polymer, it was assumed that main distribution of ions was protonated. However, when the mass of the end group of a singly-charged peak was calculated, it was found the mass of the end group was 127.9676 Da when a repeating unit was added to the leftover mass of the removal of all repeating units. In order to remedy this issue, it was then assumed that sodium adducts are formed, rather than hydrogen. With this change, the end groups were found to have a total mass of 106.0087 Da. As the carboxylic acid functionalized end group of NIPAM-COOH had a mass of 105.0009 Da (ω-end), it was found that the α-end group had a mass of 1.0078g, which was the exact mass of a hydrogen molecule. By looking at this data it determined that the measured mass of the end group matched the mass of the proposed end group structure.

After the basic structure of the molecule was identified, CAD tandem MS studies of both singly and multiply charged ions were carried out. Other than a sodium loss for the 2^+ and 3^+ charged molecules, the losses observed on three spectra (fig.3, fig. 4, and fig. 5) showed similar fragmentation patterns. Each of the peaks showed a removal of m/z 59, which indicated the cleavage along the side chain resulting in the loss of C_3H_9N. In addition, the loss that was observed in each spectra as m/z 42 difference also indicated cleavage along the side chain resulting in the loss of a propene group. The other fragment which was characteristic to CAD spectra was the loss of m/z 106, which would be exact with the mass of the end group of mass of 105.009, with a single hydrogen added to it. After this removal, each spectrum showed continual removal of the C_3H_9N group, followed by the propene group from the side chains.

ETD fragmentations of 2+ and 3+ charged ions were carried out. As results were between spectra similar, only one of the spectra (fig. 6) was shown in the report for analysis. One of the major fragmentation occurred after electron attachment was the loss of a $-H^{\cdot}$. According to the results that is obtained from the precursor of m/z 641.4, after e^- transfer either a $H^{\cdot}$, or an isopropyl radical from the side chain was cleaved off depending on the e- attachment site. In addition, e- can also be captured by one of the sodium adducts, resulting in charge reduction by sodium loss. Based on the intensity of the peaks, there was a larger amount of $H^{\cdot}$ loss than there was sodium or isopropyl radical loss. The strongest fragmentation that was observed in this spectrum was the loss of a C_4H_8NO group which indicated the cleavage of the whole side chain, which happened after charge reduction by $H^{\cdot}$.

Three separate fragment ions within an ETD spectrum were isolated and collisionally activated for further fragmentation. The first MS^3 spectrum (fig. 8) showed loss of m/z 106.1 and 127.9, as well as several backbone cleavages. The loss of m/z 106.1 indicated the loss of the end group, as it was in the single stage CAD data. The fragment ion, which was the result of a m/z 127.9 loss, suggested the cleavage of an end group with sodium attached on the acid end. After this loss was an array of peaks that showed the presence of backbone cleavages within the group of repeating units. Multiple peaks of continuous ~113 Da differences indicated that there are multiple points that a backbone cleavage can happen within this molecule. This fragmentation pattern was a result of internal fragmentations along the backbone of the structure. Interpretation was based on methods of previous experimentation, and fragments were labeled based on structure after cleavage (Wesdemiotis, 2009). The complete interpretation of these fragments provided backbone sequence information (fig. 7 and 8). The second of the three spectra (fig. 9) showed similar peaks to the first spectrum, except for three things. Two main fragmentations were observed, with m/z 59.1 and 42 differences, which indicated the cleavages along the side chains were the same ones that were observed in single stage CAD spectra. The second thing that was noticed on this spectrum was the missing 106.1 loss. This proved that the sodium adduct was located at the acid end and cleaved off with the end group (m/z 127.9). The third characteristic of the spectra that differed from the first spectrum was the distribution of backbone cleavages, as charge reduction of $-H^{\cdot}$ was not observed, and the only backbone cleavages observed were cleavages of z_n (fig. 7 and 9). The third spectrum (fig. 10), with a second precursor, showed similar losses to the second MS^3

spectrum (fig. 9), but with a 106.1 loss for the end group rather than the 127.9 loss. This was due to the fact that one of the sodium adducts has already been lost from the first precursor. This gives further evidence that the 127.9 losses of the previous two spectra were from an end group-sodium complex. Backbone losses for this spectrum were similar to the losses observed in the second spectrum.

The same strategies for MS/MS and MS3 experiments were carried out on NIPAM. The main spectrum (fig. 11) showed similar characteristics to the NIPAM-COOH spectrum, doubly and triply sodiated ions are observed throughout the spectrum. The only difference was the additional singly-charged distribution. It has a repeating unit mass of 113.1 Da, but its end group has a mass of 72.1008+113.1n Da. More tests are needed for complete identification of the structure of the end group of this minor distribution.

CAD on 1^+, 2^+, and 3^+-charged ions were carried out to investigate their fragmentation pattern. The 1^+-charged polymer spectrum, much like the NIPAM-COOH fragmentations, losses of a propene, m/z 59 C_4H_8NO group, and the large end group were observed (fig. 12). As for the 2^+ and 3^+ charged spectra, the only fragmentation that was observed was the loss of one sodium adduct (fig. 13). A second peak showed a loss of 332.5 m/z within the 2^+ spectrum, which was unable to be calculated as a loss from the original molecule, and may have been a fragment of an overlapping peak with the precursor ion 650.9. As overlap has been noted in previous fragmentation experimentation, it was assumed that this was the case observed (Wesdemiotis, 2009). A CAD-CAD MS^3 analysis was performed on the sodium loss peak of the 2^+ precursor. As a result of this fragmentation, the spectrum comes out exactly like the NIPAM-COOH spectra (fig. 14).

The ETD results for NIPAM, much like the 2^+ and 3^+ CAD spectra, showed an intense fragment ion contribution to one of the sodium adducts. The other major loss that was seen within this peak was a proton loss through the loss of the end group with triethoxysilane. As in the 2+ and 3+ spectra for NIPAM CAD, there were no other fragments observed in the spectrum. These results showed that probability of e- capture by Na+ was high enough that it surpassed any other possible fragmentation (fig. 14).

The MS^3 for NIPAM, much like the MS^3 for NIPAM-COOH, was done through ETD-CAD fragmentations. The first spectrum, with a 2^{nd} precursor of an end group loss (fig. 15), showed losses of 59 and 87.2, which were characteristic of cleavages on the side chain. As with the ETD-CAD results of NIPAM-COOH extensive amounts of backbone cleavages were observed, indicated by 113.1 differences between the fragment peaks. Just like NIPAM-COOH, this allows the interpretation of the full sequence of the backbone, and fragments were labeled in a similar manner. The second spectrum, with a 2^{nd} precursor of a sodium loss (fig. 16), showed losses exactly like the spectrum of NIPAM-COOH MS^3 with a 2^{nd} precursor of a sodium loss (fig. 10), which includes propene and C_4H_8NO group losses, an end group loss, including the cleavages along the backbone itself. Both of these spectra, in the same manner as NIPAM-COOH, were labeled based on multiple cleavages, and each provided complete backbone sequence information (fig. 14, 15, and 16).

iv. Conclusion

By looking at the results, it can be clearly pointed out that both CAD and ETD ion activation methods can provide a large amount of information, but one method alone may not be enough. Specific fragmentations can surpass more informative fragment ions from being observed in the spectra, in this case further fragmentation stages are needed. It has been shown that using both CAD and ETD methods for a MS^3 experiment, full characterization polyacrylamides including the side chain modifications, end group functionalities and full backbone sequence coverage can be achieved. With the knowledge that was gained from this study, a few things can be done from here. Further CAD/ETD fragmentation combinations can be done from here to investigate further similarities and differences between the fragmentation mechanisms of the two molecules. As the mechanisms would not only give further evidence to prove the fragmentations, it can provide better understanding on gas phase ion/ion reactions. Finally, the methods that have been applied in this study can be used for characterization of more complex copolymeric system of polyacrylamide materials.

v. References:

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Appendix 1: Safety Considerations

As eye and skin safety is incredibly important due to high risk of exposure, gloves and protective eyewear should be worn at all times in a laboratory environment. When handling solids and solutions, use care to prevent exposure; however, if there is exposure with any of the compounds within this experiment, specific measures need to be taken to contain the exposure, and to prevent further exposure. These measures are shown below.

1. Exposure To NIPAM-COOH:
    a. If inhaled: Move person to an area with fresh air. If the person is not breathing, use an artificial respirator in order to induce breathing.
    b. If contacted to skin: Remove any eyewear/contacts, and wash the area and surrounding area of contact with soap and water for a prolonged amount of time.
    c. If contacted to eyes: Flush the eyes with the nearest eye washing station for 15 minutes.
    d. If ingested: Rinse mouth with water. If the person who ingested the solid is unconscious, do not give anything orally. If swallowed, call a physician and follow instructions.
2. Exposure to NIPAM:
    a. If inhaled: Move person to an area with fresh air. If the person is not breathing, use an artificial respirator in order to induce breathing.
    b. If contacted to skin: Remove any eyewear/contacts, and wash the area and surrounding area of contact with soap and water for a prolonged amount of time. If continued irritation, contact physician.
    c. If contacted to eyes: Flush the eyes with the nearest eye washing station for 15 minutes.
    d. If ingested: Rinse mouth with water. If the person who ingested the solid is unconscious, do not give anything orally. If swallowed, call a physician and follow instructions.
3. Exposure to THF:
    a. If inhaled: Move person to area with fresh air. If the person is not breathing, use an artificial respirator to induce breathing. If serious inhalation, loosen any tight clothing, and bring to fresh air.
    b. If contacted to skin: Wash the area and surrounding area of contact with soap and water for a prolonged amount of time. Remove any clothing that has been touched by the liquid, and make sure clothing is washed before worn again.
    c. If contacted to eyes: Remove any eyewear/contacts, and wash the area and surrounding area of contact with soap and water for a prolonged amount of time.
    d. If ingested: Rinse mouth with water. If the person who ingested the solid is unconscious, do not give anything orally. If swallowed, call a physician and follow instructions.
4. Exposure to MeOH
    a. If inhaled: Move person to an area with fresh air. If the person is not breathing, use an artificial respirator in order to induce breathing.
    b. If contacted to skin: Remove any eyewear/contacts, and wash the area and surrounding area of contact with soap and water for a prolonged amount of time.
    c. If contacted to eyes: Flush the eyes with the nearest eye washing station for 15 minutes.
    d. If ingested: Rinse mouth with water. If the person who ingested the solid is unconscious, do not give anything orally. If swallowed, call a physician and follow instructions.

For each of the compounds, if irritation continues, or victim becomes unresponsive, contact a physician and follow their instruction.

When using each solution, ensure use of well-ventilated environment, as inhalation is the greatest safety risk in this experiment. Do not breathe directly over solutions, as doing so will cause inhalation of harmful vapors. When disposing of solutions, place each solution in a well-ventilated, designated waste hood. Ensure that each solution is open to the air so that vaporization of solutions can be facilitated. If solution is spilled in the lab, move away from the area of spill, and wait until evaporation of solution before continuing experiment.

As glass may be used in experimentation, use extreme care in handling any glass instrumentation, as it is easily broken. If glass is broken, use care in cleaning up. Immediately clean up the glass and DO NOT use hands to clean up glass. Ensure all glass is cleaned up and thrown away in proper containers. If glass is broken with a solution in it, evacuate the immediate area, and wait until all solution is evaporated before cleaning glass.

There is relatively little risk when running the HCT Ultra II quadrapole ion trap mass spectrometer; however, there may be risk in cleaning the ESI source. When cleaning the source, ensure that no fumes are breathed in, as the solution that will be used to clean the machine is the solvents that were used to dissolve the compounds. See the information above for inhalation of MeOH and THF before cleaning the ESI source.

Appendix 2: Calculations

Calculation for solutions: Though solution concentration does not have to be exact, it is important to dilute the solutions to 0.005 mg/mL, as higher concentrations may cause intensity of peaks to contain values greater than what can be observed. To do this, a 1 mg/mL sample was created, and then diluted to 0.005 mg/mL. Calculations are based on 1.1 mg solid obtained.

- mL solution needed: 1.1 mg * (1mg/1mL) = 1.1 mL solution needed

From this calculation, the solution could be diluted to a 0.005 mg/mL solution.

- $m_1v_1 = m_2v_2$
- 1.0M * 1.0mL = 0.005M * v_2
- v_2 =0.005 mL stock solution needed.
- $v_{solvent} = 1.0 - v_2$
- $v_{solvent}$ = 0.995 mL

Calculations for peak loss: In order to calculate loss of mass in Da between losses, the difference between two peaks were taken and then multiplied by the charge of the peaks. The example of a 59.1 loss was used from fig. 3.

- Loss = $[(m/z)_1-(m/z_2)]*n^+$
- Loss = $[468.0(Da/1^+)-408.9(Da/1^+)]*1^+$
- Loss = 59.1 Da

Appendix 3: Figures

Each spectrum was acquired by an HCT Ultra II quadrapole ion trap mass spectrometer equipped with ESI source. The precursor peak is denoted by the * symbol, and losses are denoted by subtraction of letters corresponding to structure (top of spectra). For backbone cleavages, losses are not noted, but peak structure is noted by letter and repeating unit. These fragments can be seen by observation of fig. 7 for NIPAM-COOH, and fig. 16 for NIPAM. Figures are shown in landscape format, to ensure each detail can be seen. Each spectrum figure gives the precursor peak mass, as well as charge of precursor. Figure 1 is not in this appendix, as it is already contained in the introduction.