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Characterization of Noncovalent Complexes of Polyelectrolytes by Mass Spectrometry

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ABSTRACT: Various ions of poly-L-lysine and polystyrene sulfonic acid were successfully observed first within a matrix-assisted laser desorption/ionization mass spectrometer and subsequently in an electrospray ionization mass spectrometer. The successful ionization of these polymers allowed for studies to be performed on the noncovalent interactions between the two. Noncovalent interactions were effectively studied on both instruments, but further quantified using tandem mass spectrometry found within the MALDI system and ion-mobility mass spectrometry found within the electrospray ionization system. As a result of employing the use of ion-mobility, various different stoichiometries and charges of polyelectrolyte complexes could be distinguished.

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i. Introduction

Mass spectrometry is an important family of analyses commonly used in analytical chemistry. Although there are various different configurations of mass spectrometers, including electrospray ionization and matrix assisted laser desorption/ionization varieties, each one is based on a single principle of measuring a mass-to-charge (m/z) ratio of ions.[1] By finding the m/z differences between the most intense peaks, a resulting m/z number is indicative of a repeating unit within the compound being analyzed.[1] This is especially useful when analyzing polymeric substances as most produce an orderly polymeric distribution within the spectrum where the highest intensity peaks indicate the most common polymer within a sample.[1] Once the identity of a peak is discovered, further analysis can be done using the internal capabilities of mass spectrometers including tandem mass spectrometry and ion-mobility. Through the use of tandem mass spectrometry, a certain compound can be isolated and fragmented into numerous daughter

compounds.[1] These daughter compounds can give information to the composition of the parent compound, which may be helpful in elucidating a structure. Ion-mobility is useful in discovering multiply charged compounds as it creates a spectrum which may easily show changes in m/z visualized as a band of intensity (Fig 1).

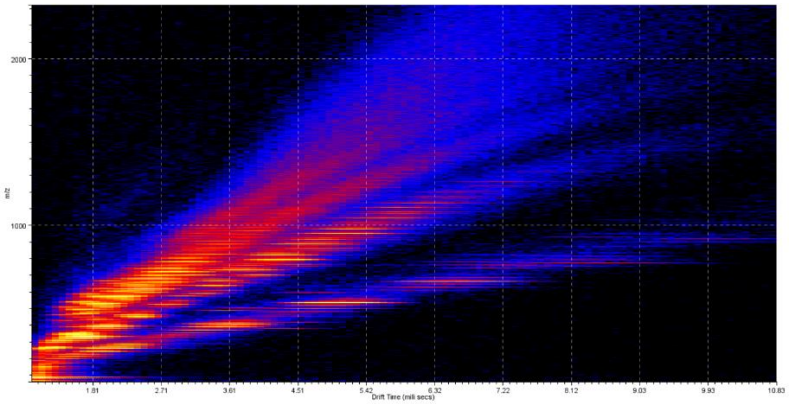

Fig 1. ESI Ion-mobility spectrum of a 5:1 molar ratio of poly-L-lysine and polystyrene sulfonic acid in negative ion mode. Each band represents a differently charged group of noncovalently interacting compounds with singly charged complexes found in the top-most band and increasing in charge by one as m/z in each band increases.

In this study, different analytical techniques including MS/MS and ion-mobility were used to study the interactions between poly-L-lysine and polystyrene sulfonic acid (Fig. 2) within an ESI and MALDI mass spectrometer. The polymers of these two compounds usually carry a net charge – negative for polystyrene sulfonic acid and positive for poly-L-lysine. Thus it is theorized that their polymers could potentially bond with each other noncovalently due to electrostatic interactions between their large groupings of charges.

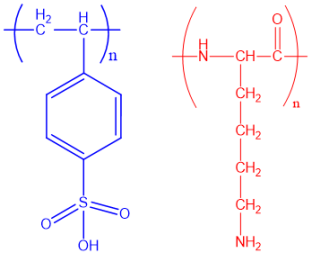

Fig 2. Polystyrene sulfonic acid (left, 184.019 Da/monomer) and poly-L-lysine (right, 128.095 Da/monomer)

These types of noncovalent interactions have many possibilities for real-world applications, but one of the most innovative applications is in the field of tissue engineering. In this field, biosynthetic tissues are created to replicate animal tissue for use in surgery and implantations. The difficulty in creating biosynthetic tissues is that the body may reject unnatural foreign materials, which can lead to infection. However, tissue engineers

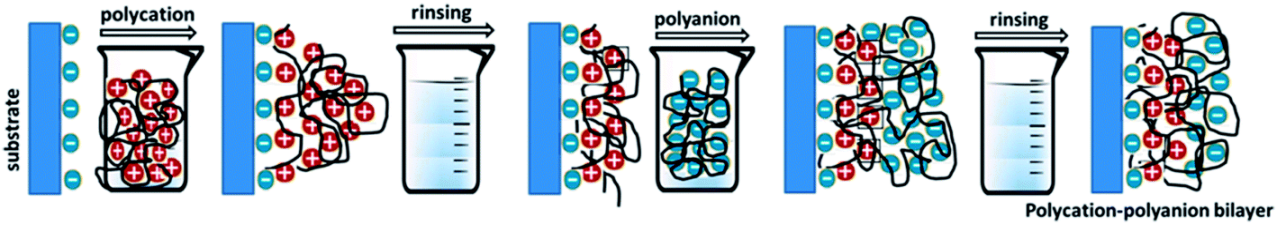

Fig 3. Joseph, N.; Ahmadiannamini, P.; Hoogenboom, R.; Vankelecom, F. J. ***Layer-by-layer preparation of polyelectrolyte multilayer membranes for separation****. Polym. Chem.* ***2014****, 5, 1817.*

have made recent discoveries, which show that complex polyelectrolyte systems that consist of a biopolymer and a synthetic polymer can be more readily accepted into the body than a synthetic by itself. [2][3] In order to create such polyelectrolyte systems, polymers of opposing charges are stacked upon one another using a layer-by-layer method (Fig. 3) and are bound via noncovalent interactions. This stacked scaffold is usually more readily accepted into the body and can then be used for drug delivery, tissue growth, biosensors and more.[4] By examining the

binding properties of polystyrene sulfonic acid and poly-L-lysine, the results show potential usefulness of incorporating the two compounds into a multilayer system for tissue engineering as lysine is a very common amino acid found throughout the body and may help increase the compatibility of polystyrene-containing polyelectrolytes.

ii. Materials and Methods

The poly-L-lysine, polystyrene sulfonic acid salts, and alpha-cyano-4-hydroxycinnamic acid matrix were obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). Methanol and acetonitrile were acquired from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). MALDI analysis was performed on a Bruker UltraFlex III MALDI tandem time-of-flight (ToF/ToF) mass spectrometer (Bruker Daltonics, Billerica, MA), while ESI analysis was performed on a Waters SYNAPT HD Q/TOF mass spectrometer (Waters, Milford, MA).

For MALDI analysis, a solution of alpha-cyano-4-hydroxycinnamic acid matrix with a 10 mg/mL concentration in a 1:1 (v/v) mixture of water/acetonitrile. Solutions of poly-L-lysine and polystyrene sulfonic acid were prepared similarly to the matrix in a 1:1 (v/v) mixture of water/acetonitrile but at a 1 mg/mL concentration. When preparing the sample plate, the solutions were mixed in a 5:1 (v/v) ratio of matrix to sample. 1.0 μL of these mixtures were spotted onto the sample plate and ionized using a Nd:YAG laser emitting at 355nm. Mass spectra were only acquired in negative ion mode.

For ESI analysis, poly-L-lysine and polystyrene sulfonic acid were prepared in a 7:3 (v/v) mixture of water/methanol in a 100 μM concentration. These samples were then combined in a 5:1 poly-L-lysine to polystyrene sulfonic acid ratio and injected into the instrument at a 5 μL/min flow rate. All mass spectra were acquired in both positive and negative ion mode with the

following parameters: a capillary voltage of 2.7 kV, sampling cone voltage of 45 V, extraction cone voltage of 2.7 V, source temperature of 60°C, and desolvation temperature of 150°C with a constant backing pressure of 2 mbar. After initial data collection, ion-mobility was also performed in order to discover higher-charged noncovalent complexes.

iii. Results and Discussion

Initial testing was performed on the individual polymers, polystyrene sulfonic acid and poly-L-lysine within the MALDI mass spectrometer using an alpha-cyano-4-hydroxycinnamic acid matrix, which was truncated from the spectrum. Both positive and negative ion modes of polystyrene sulfonic acid produced spectra, however negative ion mode produced a spectrum with higher intensity (Fig 4). The peaks in this spectrum can be seen as a polymeric distribution of polystyrene sulfonic acid with a hydrogen adduct, having an m/z difference of ~184. As with polystyrene sulfonic acid, spectra of poly-L-lysine were produced in positive and negative ion mode, with negative ion mode producing a spectrum with higher intensity (Fig 5). The peaks in this spectrum can also be seen as a polymeric distribution of poly-L-lysine, having an m/z difference of ~128. Due to the beneficial nature of negative ion mode in generating data, the remaining testing on the MALDI instrument was performed in negative ion mode. Various mixtures of the two samples were tested, with a 5:1 molar ratio mixture of poly-L-lysine to polystyrene sulfonic acid producing the most viable spectrum (Fig 6). At lower m/z, the intensity polystyrene sulfonic acid completely overwhelms any signals of poly-L-lysine and is the predominant compound measured and a polymeric distribution is formed. However, at m/z greater than 1500, complexes of polystyrene sulfonic acid and poly-L-lysine can be recognized (Fig 7). From this figure it was found that polystyrene sulfonic acid contained sodium adducts, as

well as hydrogen. Various stoichiometric ratios of polystyrene sulfonic acid and poly-L-lysine complexes were formed at measurable ratios as high as [S_6+L_{12}]. The most stable, intense ratios were formed when the molar ratio of polystyrene sulfonic acid to poly-L-lysine is roughly 1:2 - ratios that are roughly 1:1 did not form very intense complexes.

Using the most intense peak from Figure 7, [S_5+L_9] at 2148.08 m/z, an MS/MS spectrum was taken using fragmentation to study the daughter compounds (Fig 8). The most intense resulting daughter compound was found at 977.83 m/z. This equates to a net loss of 9 poly-L-lysine units from the parent compound, leaving 5 units of polystyrene sulfonic acid with hydrogen adducts remaining. This test is crucial in understanding the behavior of polyelectrolyte complexes as polystyrene sulfonic acid and poly-L-lysine appear to separate under the high energy used in fragmentation, but do not readily break apart into smaller polymer chains while separation is occurring. This allows for the calculation of stoichiometric ratios within the complex to be easily accomplished.

Testing of the 5:1 molar ratio was continued on an electrospray ionization mass spectrometer in both positive and negative ion mode. Figure 9 shows the first measurement of complex formation in positive ion mode. Having a net positive charge, poly-L-lysine is the predominant polymer found at lower m/z at different charges due to the nature of ionization within the ESI nebulizer. The ion-mobility data associated with this spectrum shows that at higher m/z, polyelectrolyte complexes form up to quadruple-charged positive ions. When looking at the spectrum from 500 m/z onward (Fig 10), many complex peaks can be seen carrying doubly positive charges. Interestingly, in comparison to MALDI results, ESI seems to produce more stable complexes at 1:1 polystyrene sulfonic acid to poly-L-lysine molar ratios in doubly charged states. When examining the spectrum of triply positive charged complexes (Fig 11), that ratio

changes back to a 1:2 molar ratio as seen in MALDI results. Finally, when examining the spectrum of quadruple positive charged complexes the molar ratio is also approximately 1:2, but with significantly larger complexes formed of up to $[S_{14}+L_{16}]^{+4}$ being formed. The increase of complex size is likely due to a larger charge distribution of positive poly-L-lysine allowing for stronger binding to polystyrene sulfonic acid.

Figure 13 shows the first measurement of complex formation in negative ion mode. Having a net negative charge, polystyrene sulfonic acid is the predominant polymer found at lower m/z. The ion-mobility data within negative ion mode is much different than in positive ion mode, showing only up to doubly negative charged complexes. When examining the spectrum from 500 m/z onward (Fig 14), many complex peaks can be seen carrying doubly negative charges. As with result from positive ion mode, doubly negative charged complexes appear to bind best in 1:1 molar ratios. However when examining triply charged complexes (Fig 15), there is no clear molar ratio that produces the most stable complexes as a 1.4:1 and 1.6:1 ratio produce the most intense peaks with 1:1 ratios being much less intense.

Fragmentation of a complex, $[S_4+L_5]^{2-}$ at 725.3420 m/z in negative ion mode produces a very intense peak at 396.0390 m/z (Fig 16), indicating a loss of 4 monomer units of polystyrene sulfonic acid. A less intense peak appears at 657.5154 m/z, indicating the loss of 5 monomer units of poly-L-lysine. Fragmentation of a complex, $[S_4+L_5]^{2+}$ at 727.8795 m/z in positive ion mode produces a very intense peak at 330.2761 m/z (Fig 17), indicating a loss of 5 monomer units of poly-L-lysine. Interestingly, only during this fragmentation a complex is formed from the loss of one unit of poly-L-lysine resulting $[S_4+L_4]^{2+}$ being produced at low intensity.

iv. Conclusion

Be examining the results, it is clear the poly-L-lysine and polystyrene sulfonic acid form polyelectrolyte complexes regardless of ionization method, whether it be MALDI or ESI. These polyelectrolyte complexes show stability that may be potentially useful in tissue engineering and polyelectrolyte multilayer development. The most common ratios for complex formation are 1 mole of polystyrene sulfonic acid for every 2 moles of poly-L-lysine in MALDI negative ion mode, and ESI positive ion mode. In ESI positive ion mode, the complex strongly benefits by having a higher overall positive charge, up to 4+, and can create much larger complexes as a result. There does not appear to be a clearly superior molar ratio from ESI negative ion mode testing. In this format, not only do complexes have unusual binding ratios, but they do not bind in nearly the large size that their positive ion counterparts do. Fragmentation of the produced polyelectrolyte complexes gives very useful information in discerning the identity of the parent complex. The parent complexes will readily fragment into discrete polymers of polystyrene sulfonic acid and poly-L-lysine with negligible amounts of smaller complex formation and decomposition occurring.

v. References:

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Appendix 1: Safety Considerations

Due to the potential hazards involved in any chemical setting, appropriate personal protective equipment should be worn at all times. During the course of this experiment, protective eyewear and nitrile gloves were used. Fume hoods should be used for the preparation of experimental materials in order to minimize exposure to any substances.

1. Exposure To poly-L-lysine:
    - Poly-L-lysine may be harmful if inhaled. Areas of skin which come in contact should be flushed with soap and water. Eyes should be flushed for at least 15 minutes. If ingested, do not induce vomiting.
2. Exposure to polystyrene sulfonic acid:
    - Polystyrene sulfonic acid may be harmful if inhaled. Areas of skin which come in contact should be flushed with soap and water. Eyes should be flushed for at least 15 minutes. If ingested, do not induce vomiting.
3. Exposure to alpha-cyano-4-hydroxycinnamic acid:
    - Alpha-cyano-4-hydroxycinnamic acid may be harmful if inhaled. Areas of skin which come in contact should be flushed with soap and water. Eyes should be flushed for at least 15 minutes. If ingested, do not induce vomiting.
4. Exposure to methanol
    - Methanol may be harmful if inhaled. Areas of skin which come in contact should be flushed with soap and water. Eyes should be flushed for at least 15 minutes. If ingested, do not induce vomiting. Contact a medical professional immediately under any circumstances of contact.
5. Exposure to acetonitrile

- Acetonitrile may be harmful if inhaled. Areas of skin which come in contact should be flushed with soap and water. Eyes should be flushed for at least 15 minutes. If ingested, do not induce vomiting.

Appendix 2: Calculations

Sample calculation for the determination of noncovalent complex constituents: since poly-L-lysine and polystyrene sulfonic acid can form many different stoichiometric ratios, finding which ratio a certain peak denotes is essential. This process is done using MS/MS fragmentation and is started by finding the most intense peak believed to be a noncovalent complex and noting its m/z. For example, a peak at 2148.08 may be fragmented resulting in a daughter compound with the highest intensity at 977.83 m/z (Fig 8). The difference between the original 2148.08 m/z and daughter 977.83 m/z indicates a loss of approximately 9 monomer units of lysine. The remaining 977.83 m/z of the daughter molecule can then be divided by the mass of a polystyrene sulfonic acid monomer unit to yield the amount of polystyrene sulfonic acid within the complex – 5 in this case. Thus the ratio of compounds within the complex is [S5+L9].

Appendix 3: Figures

Each spectrum was either acquired by a MALDI or ESI mass spectrometer, as noted in each figure. In each figure, the noncovalent complexes formed are listed above the intensity peak in the format [Sx+Ly] where S is polystyrene sulfonic acid and L is poly-L-lysine. For ion mobility, the drift time vs m/z chart is labeled with a number corresponding to a set of singly or multiply charged noncovalent complexes. For MS/MS in-source fragmentation, the parent complex is denoted by a red and blue diamond with its daughter compounds appearing at lower m/z.