Anal. Chem. **2006,** *78,* 459−469

Synthesis of a Polyelectrolyte and Its Applications in Laser Desorption/Ionization

Naotaka "Taka" Kitagawa*

Strata-Trans, P.O. Box 8249, Fremont, California 94536

A novel laser energy-absorbing polymer derivatized from an energy-absorbing molecule has been synthesized. This polymer effectively eliminates the matrix addition step and the use of requisite small organic chemical matrixes from MALDI. The resulting polyelectrolyte-assisted laser desorption/ionization method has been proven to have some exceptional advantages over current MALDI methods. First, the matrix addition step from the LDI analytical process was eliminated. Second, the low molecular weight signal from energy-absorbing molecules was minimized by covalently linking the energy-absorbing molecules to the backbone of the polymer and other ionization-enhancing pendant groups on the backbone. Finally, the mass resolution of LDI was enhanced by minimizing chemical background noise throughout the spectrum. Polymeric linkage of energy-absorbing molecules aligns them with each other in an ordered structure, similar to MALDI matrix crystallization. Using the designed polymer film, laser desorption/ionization for proteins and peptides were successfully performed without the addition of any matrix. Analytical results for the synthesized monomer and polymers will be also presented.

Mass spectrometry allows us to rapidly and accurately identify a substance on the basis of its mass. Chemists have long used mass spectrometry for the identification and characterization of small- and medium-sized molecules. The method is so sensitive that it is possible to trace very small quantities of each type of molecule. Doping and drug tests, food quality control, and environmental analysis are examples of areas where mass spectrometry is now in routine use.

The potential use of mass spectrometry in the study of macromolecules has long attracted scientists. During the 1970s, a number of successes were achieved in the conversion of macromolecules to ions in gas phase, a process termed "desorption technology". Today, there are two prominent techniques for causing proteins to enter the gas phase, without losing their structure and form. In the electrospray method, the sample is sprayed into a strong electrical field, yielding small, charged, freely hovering ions.[1] The other method uses an intense laser pulse. If pulsed under suitable conditions, energy-absorbing molecules absorb some of the energy of the laser pulse and become released as free ions. Tanaka[2] and Yoshida[3] showed that the protein molecules could be ionized using "soft laser desorption" (SLD). Oligomers of lysozyme containing up to seven monomeric units have also been ionized by his group, using a pulsed N_2 laser and a matrix of a metal powder, finely dispersed in glycerol. A laser pulse strikes the sample, which is in a solid or viscous phase. When the sample absorbs the energy from the laser pulse, it is blasted into small bits. The molecules are released as intact hovering molecule ions with low charge. The charged species are then accelerated by an electrical field, separating particles by molecular weight and charge as they fly. Accurate molecular weights of the particles can be obtained from their flight time from desorption to detection. They developed the method of sample preparation ("ultrafine metal plus liquid matrix method") to be able to form high-mass molecular ions of up to at least 34 kDa for laser ionization mass spectrometry. The applicability of laser technology to biological molecules was thus demonstrated.

Karas and Hillenkamp quickly perfected this principle and introduced the matrix-assisted laser desorption/ionization (MALDI) method of desorption and ionization.[4] To generate protonated molecules in gas phase, a large excess of matrix material is mixed with the analyte molecules. A submicroliter volume of the mixture is then pipetted onto a metal substrate and allowed to dry. The resulting solid is then irradiated by nanosecond laser pulses, usually from a small N_2 laser. The matrix is typically a small organic molecule with a peak absorbance at the wavelength of the laser employed. Work with biomolecules generally requires the use of α-cyano-4-hydroxycinnamic acid (4CHCA) or dihydroxybenzoic acid (DHB) as matrixes. Matrixes differ in the amount of energy they impart to the biomolecules during desorption and ionization and hence, the degree of fragmentation that they cause.

Since the MALDI methodology requires a large amount of chemicals to be mixed with analytes, these extra chemicals will be simultaneously exposed to the laser and desorbed. All of the ionized species, whether desirable or not, end up at the detector. As a result, both the matrix fragments and the target analytes will register as peaks on the mass spectra. The matrix fragments cause high background noise and make the detection of target molecules difficult, particularly in the low molecular weight range. The mass range below 1000 Da is often completely obscured by

* E-mail: tkit@novabeads.com.

(1) Watson, J. T. *Introduction to Mass Spectrometry*, 3rd ed.; Lippincott-Raven Publishers: New York, 1997.

(2) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. *Anal. Chem.* **1988**, *2*, 151−153.

(3) Yoshida, T.; Tanaka, K.; Ido, Y.; Akita, S.; Yoshida, Y. *Shitsuryo-bunseki* **1988**, *36*, 59−69.

(4) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299−2301.

10.1021/ac051253j CCC: $33.50
Published on Web 12/07/2005

matrix ions saturating the detector. Proteins generally undergo fragmentation to some extent during MALDI, resulting in broad peaks and loss of sensitivity; consequently, MALDI is mostly applied to the analysis of peptides.

Besides attempts to improve the equipment mechanically and electronically, there have been a few attempts to eliminate the matrix addition step from the MALDI-MS methodology. Hutchens et al. disclosed "surface array of energy absorbing polymer" by modifying the surface of a commercially available polymer, poly(1-octadecane-*co*-maleic anhydride), in order to covalently attach a UV energy-absorbing molecule, 4CHCA, on the surface.[5] Using the modified polymer, they were able to successfully desorb and ionize biomolecules. The concept is illustrated in U.S.Patent 5894063, 1999, as Surface Enhanced Neat Desorption (SEND).[6] The sample probe consists of a layer of energy-absorbing molecules on its surface. The analyte is applied to the layer of energy-absorbing molecules, where it can then be desorbed by laser and detected in the mass spectrometer.

A method was developed using etched porous silicon to trap analytes deposited directly on the surface and laser irradiation to vaporize and ionize them without any additional matrix.[7] The experimental protocol for desorption/ionization on silicon (DIOS) involves the generation of porous silicon from flat crystalline silicon by using a simple galvanostatic etching procedure. This procedure yields a micrometer-thick porous layer with nanocrystalline architecture that exhibits bright photoluminescence upon exposure to ultraviolet light. DIOS is useful for a variety of biomolecules. Peptides generate a good signal from the deposition of 700 amol of material and allow the analyte to be analyzed even in a saturated salt solution.

To attain more control over the structure of the porous surface than traditional electrochemically etched silicone, aerogel systems were explored as matrix-free substrates for laser desorption.[8] Aerogels are produced by cross-linking a colloidal dispersion of dissolved monomeric precursors. Resorcinol−formaldehyde aerogels were characterized as organic matrix-free substrates for laser desorption on a quadrupole ion trap instrument. The data indicate that the aerogel systems are capable of acting as organic matrix-free substrates for laser desorption mass spectrometry. The resulting mass spectra are similar to MALDI, but without the organic matrix background noise.

Cuiffi et al. reported a method for desorption/ionization based on a novel columnar/void network deposited silicon thin film.[9] The columnar/void network silicone films are deposited by plasma-enhanced chemical vapor deposition using an electron cyclotron resonance high-density plasma source. Poly(ethylene terephthalate) and glass substrates were coated with the silicone film. They demonstrated that these nanostructured deposited films enable molecular detection in the range of 0−6000 Da with little or no mass chemical noise.

Lin and Chen[10] presented a novel method for direct desorption/ionization of analytes from sol−gel-derived film. A common MALDI matrix, DHB, was incorporated into a sol−gel polymeric structure. The sol−gel-derived DHB thin film can assist the mass analysis of analytes by laser desorption/ionization with an interference-free background in the mass spectra.

Thus, the SLD methodology evolved from the use of inorganic and organic energy-absorbing materials to limited modifications of the probe surface to add laser energy-absorbing characteristics, as well as application of a limited number of inorganic and organic polymers as laser energy-absorbing materials. These developments in SLD definitely suggest that matrix cocrystallization with analytes may not be required to assist in their desorption and ionization.

This report describes the successful synthesis of monomers that exhibit MALDI matrix characteristics when polymerized. Starting with 4CHCA and methacryloyl chloride, reactive groups were attached to 4CHCA through the Schotten−Baumann reaction scheme. The monomer was polymerized in an organic solvent to perform a solution polymerization via free-radical polymerization. The resulting polymer was used to coat a MALDI probe with a polymeric film to be used for laser irradiation. The newly created polymer film could successfully desorb and ionize biomolecules less than 100 000 Da in size from the polymer surface by laser irradiation, without the addition of MALDI matrix. Though the synthesized monomer alone (α-cyano-4-methacryloyloxybenzoic acid, 4CHCAMA) performed poorly as a matrix, the polymerized product exhibited matrix desorption and ionization characteristics.

The materials and technology of Ciphergen Biosystems, Inc. were used in conducting the research discussed herein.

EXPERIMENTAL SECTION

Experimental Scheme: Monomer Synthesis to Polymer Synthesis. The synthesis of 4CHCAMA monomer in Figure 1 was performed through the Schotten−Baumann reaction, an acylation of alcohol with acyl halides in aqueous alkaline solution.[11] The reaction is robust with good yield. The resulting monomer is a crystalline solid. Purity of more than 99% can be achieved by serial recrystallization. The application of the reaction scheme to acrylate monomers was described previously.[12] The reaction works well with most MALDI matrixes having hydroxyl groups.

Materials and Methods. The chemicals used were as follows: 4CHCA (Sigma-Aldrich, Milwaukee, WI), acetone (VWR, West Chester, PA), methacryloyl chloride (Aldrich), hydrochloric acid (37% ACS reagent, Aldrich), potassium hydroxide (pellets, ACS reagent, Aldrich), acetic acid (99.7% ACS reagent, Aldrich), acrylic acid (99%, Aldrich, inhibited with hydroquinone), 3-(trimethoxysilyl)propyl methacrylate (98%, Aldrich), lauroyl peroxide (97%, Aldrich), 1-pentanol (99% ACS reagent, Aldrich), and 1-butanol (purity 99.4%, Aldrich).

All-in-one peptide mix consisted of five peptides: [Arg8]-vasopressin (MW 1084.25, 4.43 fmol/μL), somatostatin (MW 1637.9, 2.97 fmol/μL), bovine insulin B-chain (MW 3495.94, 10.00 fmol/μL), human insulin (MW 5807.65, 9.77 fmol/μL), and hirudin

(5) Voivodov, K. I.; Ching, J.; Hutchens, T. W. *Tetrahedron Lett.* **1996**, *37*, 5669−5672.
(6) Hutchens, T. W.; Yip, T. T. Surface-enhanced neat desorption for desorption and detection of analytes. U.S. Patent 5,894,063, 1999.
(7) Wei, J.; Buriak, J. M.; Siuzdak, G. *Nature* **1999**, *399*, 243−246.
(8) Belford, M. W.; Yost, R. A. Aerogels as novel polymeric matrixes for laser desorption. In Proceedings of the 50th ASMS Conference on Mass Spectrometry and Allied Topics; American Society for Mass Spectrometry: University of Florida, 2002.
(9) Cuiffi, J. D.; Hayes, D. J.; Fonash, S. J.; Brown, K. N.; Jones, A. D. *Anal. Chem.* **2001**, *73*, 1292−1295.
(10) Lin, Y. S.; Chen, Y. C. *Anal. Chem.* **2002**, *74*, 5793−5798.
(11) Sonntag, N. O. V. *Chem. Rev.* **1953**, *52*, 237−416.
(12) Kitagawa, N. Liquid crystals: synthesis of p-hydroxybenzoic acid derivatives and characterization of their mixtures by DTA and polarizing microscopy. Master's thesis, University of Massachusetts Lowell, MA, 1969.

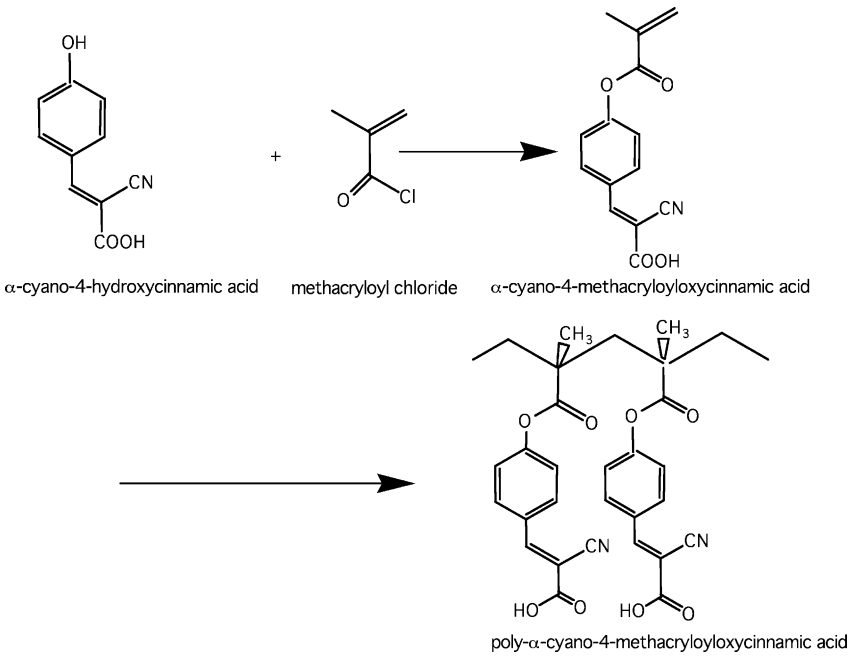

Figure 1. Synthetic procedure for derivation of polymeric 4CHCAMA from 4CHCA.

BKHV (MW 7033.61, 6.57 fmol/μL)] in a solution of 10 mM ammonium acetate, 25% acetonitrile, and 1.25% trifluoroacetic acid.

Melting points of synthetic products were determined by Digital Melting Point Apparatus IA9200 (made by Electrothermal Engineering, Essex, U.K.). NMR data were collected on a Bruker DPX200 console. Mass spectrometry was performed by means of a PBS II ProteinChip Reader LDI-TOF.

Synthetic Procedures. (1) Synthesis of 4CHCAMA. In a 100-mL three-necked round-bottom glass reactor equipped with a magnetic stirrer, 4CHCA (~3.2 g, 0.017 mol) was added to a solution of 2.00 g (~0.036 mol) of potassium hydroxide in 35 g of water and 30 g of acetone, and the reactor was placed in an ice bath. Methacryloyl chloride (~2.35 g, 0.023 mol) was placed separately in a dropping funnel and set onto the three-necked glass reactor. The methacryloyl chloride was added dropwise into the reactor slowly. The reaction was continued in an ice bath for 2 h. The resulting reaction mixture was acidified with dilute aqueous hydrochloric acid. The precipitate was filtered off and dried in vacuo. The dried filter cake was dissolved in 10 mL of glacial acetic acid (from Aldrich) and cooled in a refrigerator (~4 °C) overnight. The recrystallized material was dried and dissolved in methanol (from Aldrich). The methanol solution was placed in a freezer overnight. The resulting crystals were filtered off and dried in vacuo. The yield was 0.9 g (~26%). Melting point: 190−192 °C. (Figure 3).

(2) Synthesis of α-Cyano-4-acryloyloxycinnamic Acid (4CHCAAA). In a 100-mL three-necked glass reactor equipped with a magnetic stirrer, 4CHCA (~1.7 g, 0.009 mol) was added to a solution of 2.00 g (~0.036 mol) of potassium hydroxide in 25 g of water and 4.5 g of acetone and the reactor was placed in an ice bath. In a separate dropping funnel, 1.7 mL (~1.89 g, 0.021 mol) of acryloyl chloride was set onto the three-necked glass reactor. The acryloyl chloride was added dropwise into the reactor. The reaction was continued in an ice bath for 2 h. The resulting reaction mixture was acidified with dilute aqueous hydrochloric acid. The precipitate was filtered off and dried in vacuo. The dried filter cake was dissolved in 9 mL of glacial acetic acid and cooled in a refrigerator overnight. The recrystallized material was dried and dissolved in methanol and was stored at −4 °C overnight. The crystals were filtered off and dried in vacuo. The yield was 0.7 g (~50%). Melting point: 187−191 °C. (Figure 3)

Polymerization of 4CHCAMA. (1) Polymerization of 4CHCAMA. In a small glass bottle, 26.00 mg of 4CHCAMA (prepared as described above) was mixed with 1 mL of 1-pentanol (Aldrich) over a mildly heated water bath until the solution became clear. To the monomer solution, 4 μL of lauroyl peroxide 1-pentanol solution (~5% solution) was added and swirled by hand. The bottle was placed in an oven after purging with nitrogen gas and kept at 92 °C for 20 h. A 1-μL sample of the resulting polymer solution was placed on each spot of an 8-spot NP20 ProteinChip array, and the chip was dried in a vacuum oven at ~80 °C for 5 h under vacuum to remove the solvent, leaving a polymer film on the array.

(2) Copolymerization of 4CHCAMA with Acrylic Acid. In a small glass bottle, 26.45 mg of 4CHCAMA (prepared as described above) was mixed with 1 mL of 1-butanol (Aldrich) over a mildly heated water bath until the solution became clear. To the monomer solution, 1 μL of lauroyl peroxide (5% solution in 1-pentanol) and 20 μL of inhibitor-depleted acrylic acid monomer were added and the mixture was swirled by hand. The bottle was placed in an oven after purging with nitrogen gas and kept at 92 °C for 20 h. A 1-μL sample of the resulting polymer solution was placed on each spot of an 8-spot NP20 ProteinChip array, and the chip was dried in a vacuum oven at ~80 °C for 5 h under vacuum to remove the solvent, leaving a polymer film on the array.

(3) Copolymerization of 4CHCAMA with Acrylic Acid and Trimethoxysilyl Methacrylate. In a small glass bottle, 26.45 mg of 4CHCAMA (prepared as described above) was mixed in 1 mL of 1-pentanol (Aldrich) over a mildly heated water bath until the solution became clear. To the monomer solution, 1 μL of lauroyl peroxide (5% solution in 1-pentanol), 20 μL of inhibitor-depleted acrylic acid monomer, and 20 μL of trimethoxysilyl acrylate

Elemental Analysis				
	%C	%H	%N	%O
4CHCA Calculated	63.49	3.73	7.49	25.37
Found	63.76	3.76	7.47	25.75
4CHCAMA Calculated	65.37	4.31	5.44	24.88
Found	65.06	4.20	5.45	25.47

Figure 2. Elemental analysis of 4CHCA and monomeric 4CHCAMA performed by Desert Analytics, Tucson, AZ. The results suggest a purified 4CHCAMA monomer.

Melting Point Characterization	
4CHCA	242°C
4CHCAMA	189–192°C
4CHCAAA	187–191°C
poly-4CHCAMA	144°C - melting starts
homopolymer	197°C - discoloring starts
(polymerized	240°C - rubber-like
in 1-Pentanol)	240°C - brown color
	252°C - charred

Figure 3. Melting point characterization of 4CHCAMA and poly-4CHCAMA checked by Digital Melting Point Apparatus IA9200 (made by Electrothermal Engineering). 4CHCAMA homo-polymer prepared in 1-pentanol via free-radical polymerization using lauroyl peroxide for 20 h at 92 °C.

(improves adhesion of polymer to the array surface) was added and the mixture was swirled by hand. The bottle was placed in an oven after purging with nitrogen gas and kept at 92 °C for 30 h. A 1-μL sample of the resulting polymer solution was placed on each spot of an 8-spot NP20 ProteinChip array, and the chip was dried in a vacuum oven at ~80 °C for 5 h under vacuum to remove the solvent, leaving a polymer film on the array.

RESULTS AND DISCUSSION

Elemental Analysis. 4CHCAMA synthetic product was subjected to elemental analysis to confirm purity. Elemental analysis was performed by Desert Analytics (Tucson, AZ). Stoichiometric ratios as determined by elemental analysis closely matched theoretical values, suggesting that 4CHCAMA had been purified and properly isolated from the 4CHCA starting material (Figure 2).

Melting Point Characterization. 4CHCA and monomeric and polymeric 4CHCAMA were subjected to melting point characterization using a Digital Melting Point Apparatus IA9200 (Electrothermal Engineering), to further confirm purity of the synthetic product. The results are shown in Figure 3.

NMR Analysis. 4CHCAMA monomer product was subjected to ^{1}H NMR analysis to determine its chemical structure. NMR spectral analysis was performed on a Bruker DPX200 console (Bruker BioSpin, Manning Park, MA). ^{1}H NMR peaks (ppm): 3.3 (s, 3H), 5.85 (s, 1H), 6.25 (s, 1H), 7.35−8.25 (dd, 4H), 8.32 (s, 1H). Results confirm expected structure of the methacrylate moiety present in 4CHCAMA (Figure 4).

FT-IR Spectral Analysis of 4CHCA and Monomeric and Polymeric 4CHCAMA. The hydroxyl group peak of 4CHCA starting material at 3296 cm^{-1} disappears in the 4CHCAMA monomer (Figure 5). This peak can be used to confirm the purity of the 4CHCAMA monomer product. Two distinctive ester linkage peaks at 1726 and 1697 cm^{-1} and a vinyl group peak at 952 cm^{-1} are present in the 4CHCAMA monomer. The vinyl group peak at 952 cm^{-1} disappears in the polymerized product (poly-4CHCAMA).

Mass Spectrometric Analysis of Monomeric 4CHCAMA. Monomeric 4CHCAMA was prepared as described in above, then dissolved in methanol, and pipetted onto a gold-plated ProteinChip array. The low molecular weight region was scanned by means of a PBS II ProteinChip Reader. Two distinctive peaks for the monomer at 257.7 (4CHCAMA, MW 257.24) and 239.6 cm^{-1} (dehydrated 4CHCAMA) appeared (Figure 6). Those at 279.5 and 295.5 cm^{-1} are likely sodium and potassium adducts of 4CHCAMA, respectively. Negligible signal was observed for starting material (4CHCA, MW 189.17).

Desorption/Ionization Activity of Poly-4CHCAMA. (1) 4CHCAMA Homopolymer. 4CHCAMA polymer solution was prepared as described in above. A 1-μL sample of all-in-one peptide mix was applied to each spot and scanned on a PBS II spectrometer in order to check the polymer's LDI characteristics. The components of the peptide mixture were desorbed, ionized and resolved. The resulting mass spectrum is shown in Figure 7.

(2) 4CHCAMA/Acrylic Acid Copolymer. 4CHCAMA/acrylic acid copolymer solution was prepared as described above. A 1-μL sample of all-in-one peptide mix was applied to each spot and scanned on a PBS II spectrometer in order to check the polymer's LDI characteristics. The components of the peptide mixture were desorbed, ionized and resolved. The resulting mass spectrum is shown in Figure 8.

(3) 4CHCAMA/Acrylic Acid/Trimethoxysilyl Methacrylate Copolymer. 4CHCAMA/acrylic acid/trimethoxysilyl methacrylate copolymer solution was prepared as described above. A 1-μL sample of all-in-one peptide mix was applied to each spot and scanned on a PBS II spectrometer in order to check the polymer's LDI characteristics. The components of the peptide mixture were desorbed, ionized, and resolved. The resulting mass spectrum is shown in Figure 9.

(4) 4CHCAMA/Acrylic Acid/Trimethoxysilyl Methacrylate Copolymer. Effect of Dilution. 4CHCAMA/acrylic acid/trimethoxysilyl methacrylate copolymer solution was prepared as above, but diluted to 12% of its original concentration using 1-pentanol prior to deposition on the surface of a ProteinChip array and drying in a vacuum oven. A 1-μL sample of the diluted polymer solution was deposited on each spot of an 8-spot NP20 ProteinChip array and dried in a vacuum oven at 50 °C for 1 h. The resulting amount of polymer deposited on each spot was calculated to be 12 nmol. A 1-μL sample of all-in-one peptide mix was applied to each spot and scanned on a PBS II spectrometer in order to check the polymer's LDI characteristics. The components of the peptide mixture were desorbed, ionized, and resolved. The resulting mass spectrum is shown in Figure 10.

(5) 4CHCAMA/Acrylic Acid/Trimethoxysilyl Methacrylate Copolymer. Doping with 4CHCA. 4CHCAMA/acrylic acid/trimethoxysilyl methacrylate copolymer solution was prepared as described above. A 1-μL sample of all-in-one peptide mix was applied to each spot and scanned on a PBS II spectrometer in order to check the polymer's LDI characteristics. The components of the peptide mixture were desorbed, ionized. and resolved. The resulting mass spectrum is shown in Figure 11.

As modeled in Figure 12, when free 4CHCA is present in the polymer solution prepared, the free molecules will probably be wedged with the copolymer of 4CHCAMA with acrylic acid (some oligomers) through hydrogen bonding described above. Consequently, some repeating units will appear in the lower mass region separated by approximately 189 and 171 m/z, which correspond

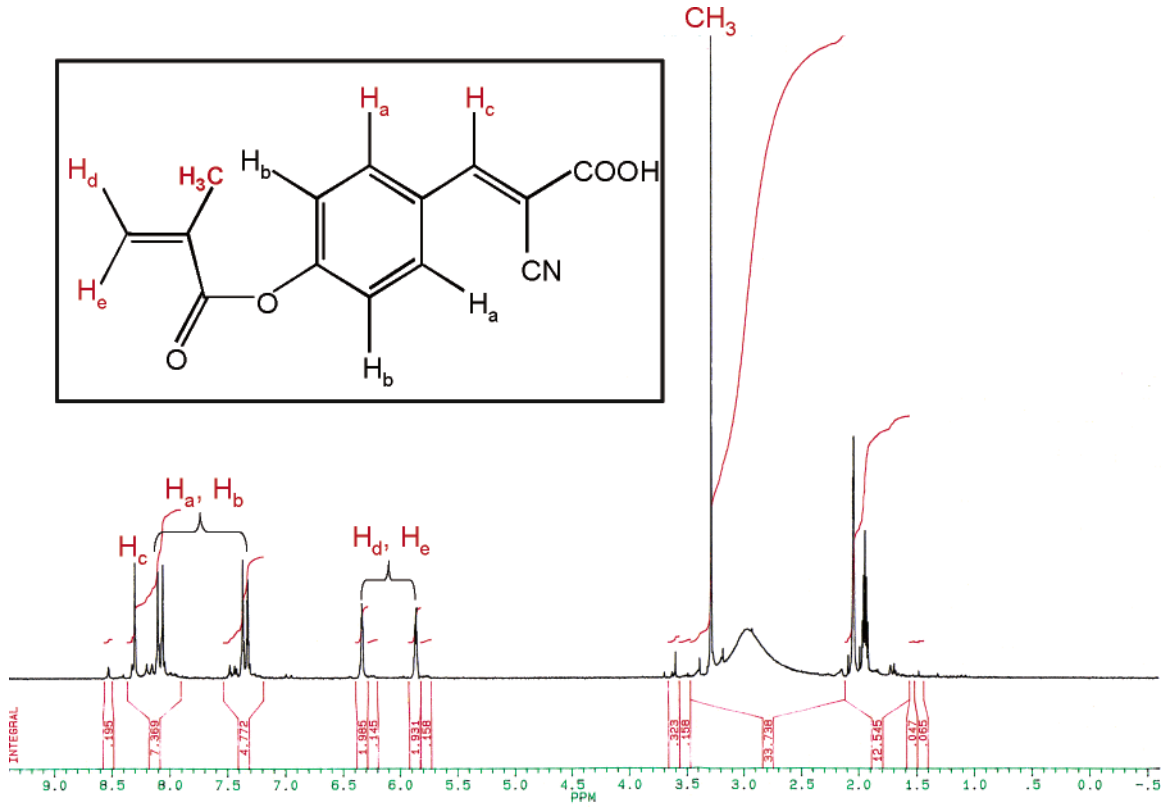

Figure 4. NMR Spectra of α-cyano-4-methacryloyloxycinnamic acid. Spectra obtained by Bruker DPX200 console. Creation of H_d, H_e singlets, and CH_3 peaks from methacrylate group signifies monomer formation.

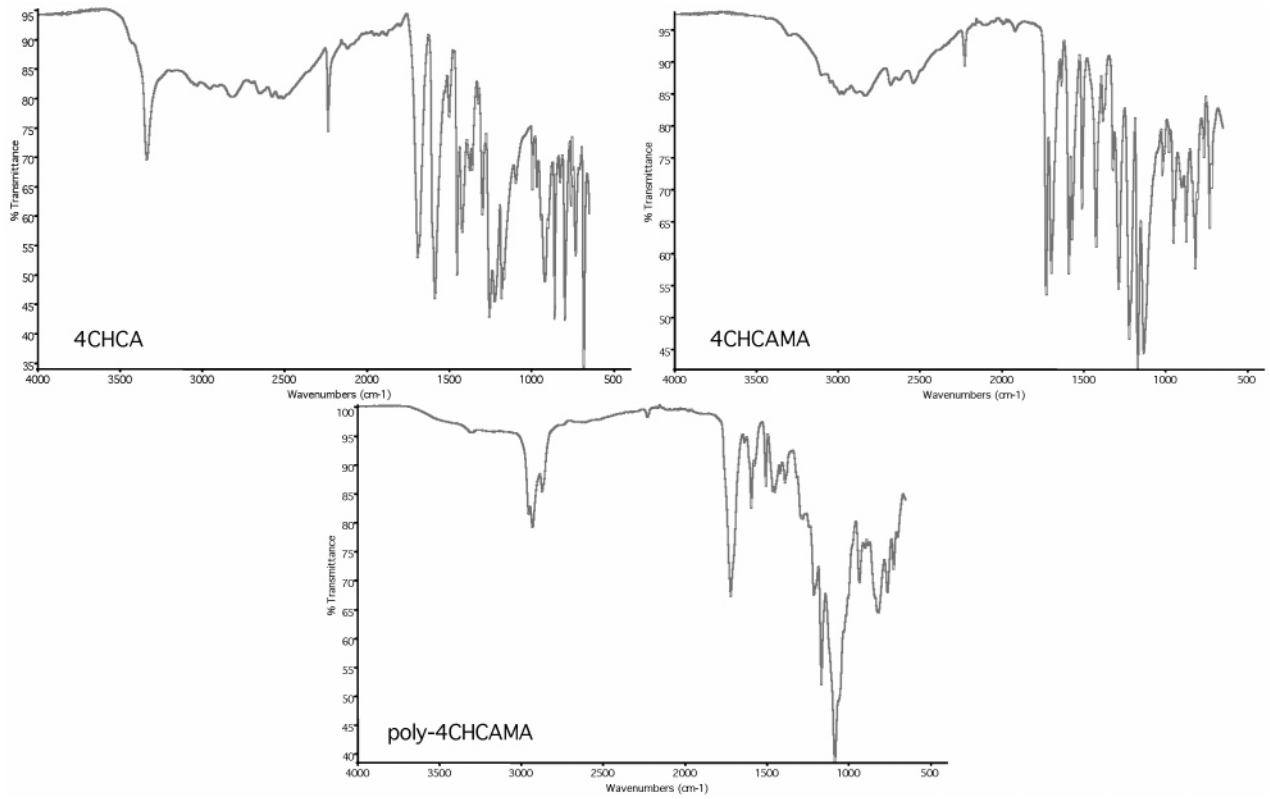

Figure 5. FT-IR spectrum for 4CHCA, 4CHCAMA, and poly-4CHCAMA respectively by Omnic FT-IR (made by Thermo Nicolet Corp.).

to the molecular weights of 4CHCA and dehydrated 4CHCA, respectively. The FT-IR spectrum (Figure 13) clearly shows the reappearance of the 4-hydroxyl −OH peak at ~3898.

(6) Poly(α-cyano-4-methacryloyloxycinnamic acid) (4CHCAMA) Dried at High Temperature. The poly-4CHCAMA copolymer (described previously) was deposited and dried in a

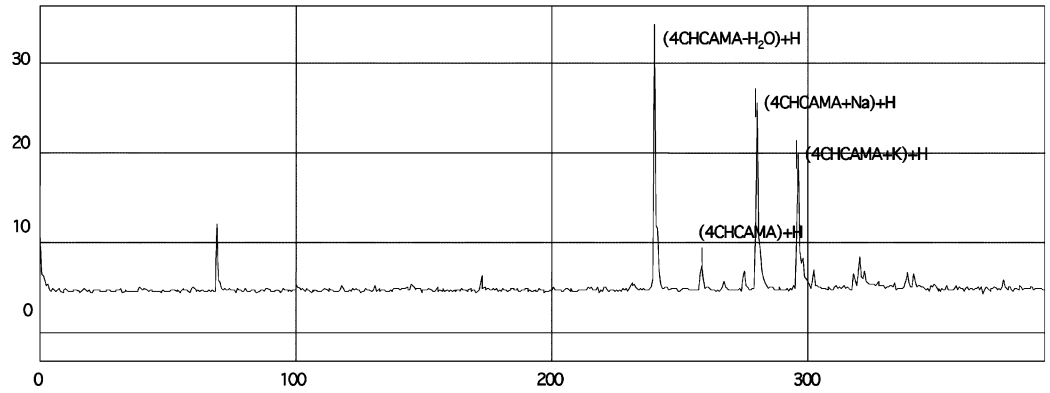

Figure 6. Mass spectrum of monomeric 4CHCAMA in low molecular weight region.

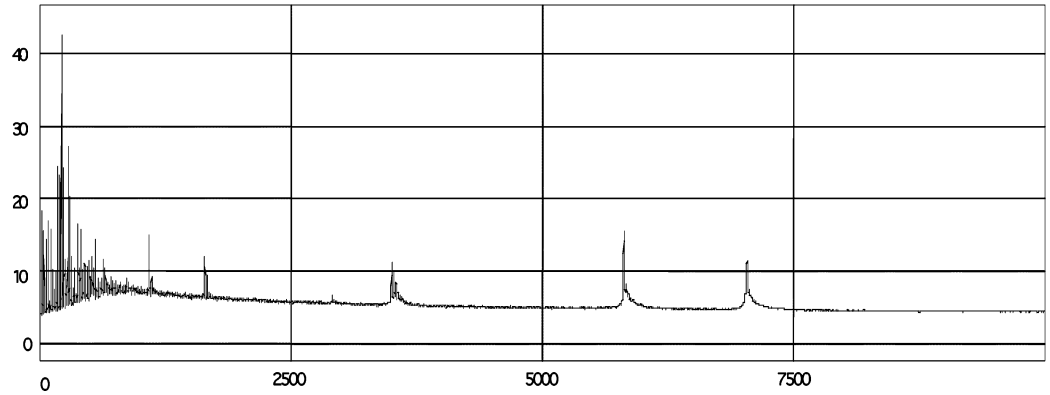

Figure 7. Homopolymer of 4CHCAMA. All-in-one peptide mix analyzed by ProteinChip Reader PBS II.

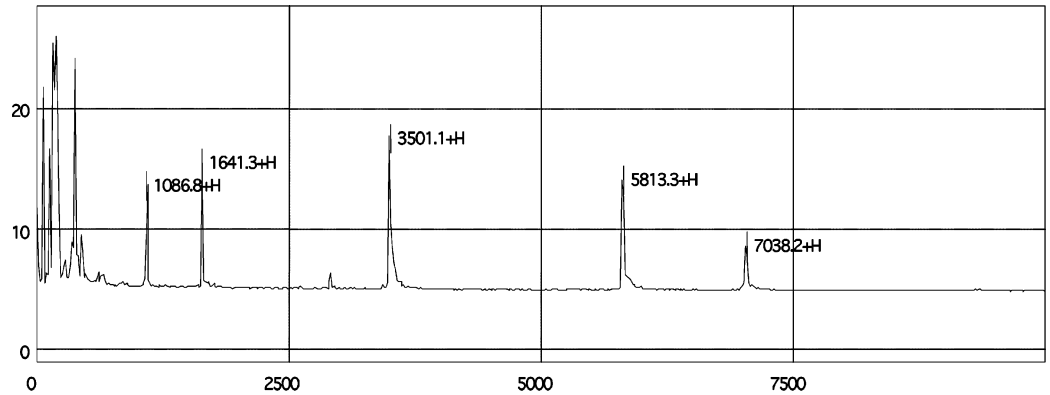

Figure 8. Copolymer of 4CHCAMA and acrylic acid. All-in-one peptide mix analyzed by ProteinChip Reader PBS II.

vacuum oven at 95 °C for 42 h to drive off residual free 4CHCA, if any, in the polymer. The resulting spectra using all-in-one peptide mix is shown in Figure 14.

Possible Mechanism for Polyelectrolyte-Assisted Laser Desorption/Ionization. The following are possible reasons why this polymer is successfully able to desorb and ionize peptides by means of laser irradiation.

(1) Electron Induction Effects. After the attachment of reactive pendant groups on 4CHCA, the resulting monomer loses its matrix characteristics. However, when the resulting monomer is polymerized, the polymer exhibits matrix desorption/ionization behavior. This interesting finding can probably be explained by so-called "inductive effects" of pendant groups.

In synthetic organic chemistry, structural effects are commonly divided into three broad categories: inductive (or electrostatic), resonance (or conjugation), and steric effects to correlate structure and reactivity. Even though this division is not based on thermodynamics, it is, in general, congenial with the thinking of organic chemists. By and large, no perfect quantitative treatment of the influence of structure on reactivity will be based on such a division. However, this categorization can be applied to explain the current molecular excitation phenomena in the laser desorption/ionization process.

Groups such as $-$OR or $-$CH$=$CH$_2$ with higher electron affinities than hydrogen are said to exhibit negative inductive ($-I$) effects. Importantly, polymerization of the monomer will dilute the inductive effects of the pendant groups by providing a polymeric backbone as an electron source for electron withdrawing groups.

There are two independent actions that must be consummated at the site of analytes to have a successful soft laser desorption. The analyte must be ionized (which is a chemical action) and

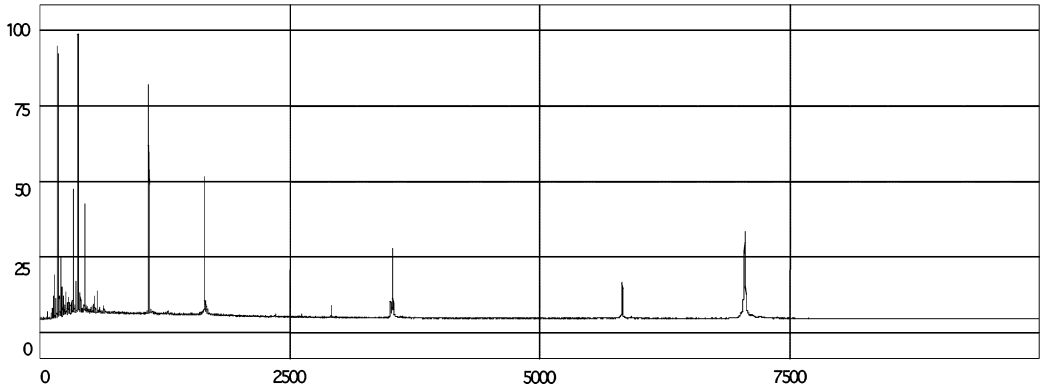

Figure 9. Copolymer of 4CHCAMA, acrylic acid, and 3-(trimethoxysilyl)propyl methacrylate. All-in-one peptide mix analyzed by ProteinChip Reader PBS II.

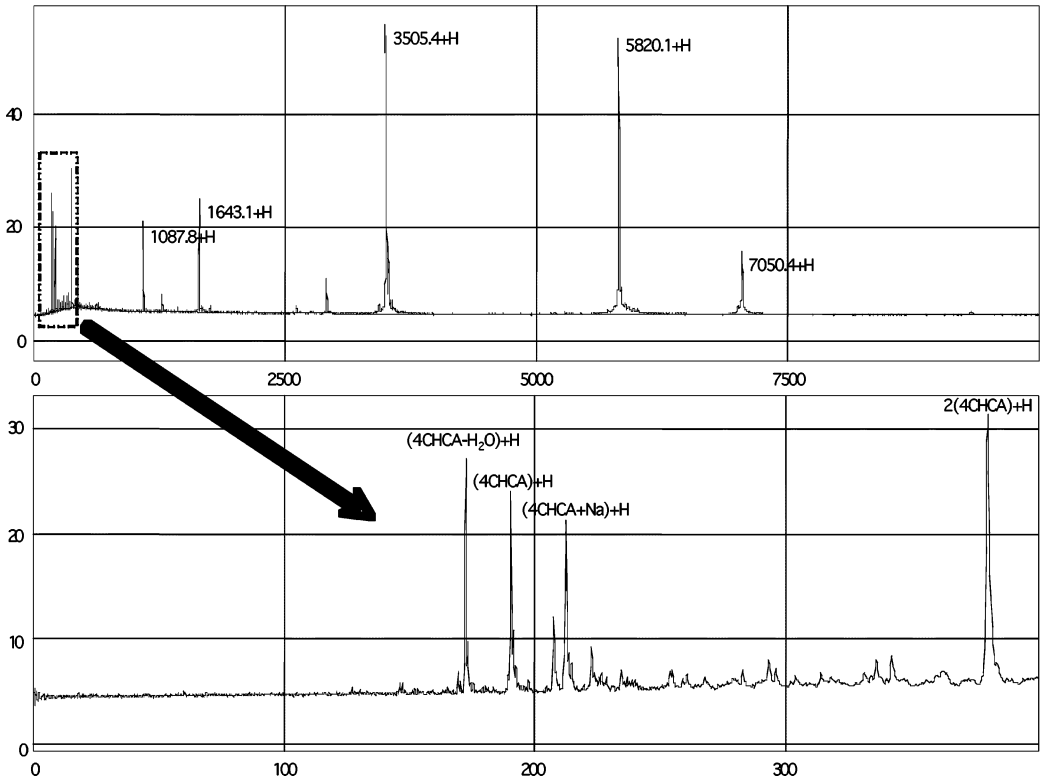

Figure 10. Copolymer of 4CHCAMA, acrylic acid, and 3-(trimethoxysilyl)propyl methacrylate diluted to 12% prior to deposition on spot. All-in-one peptide mix analyzed by ProteinChip Reader PBS II. Note diminished signal from low molecular weight products.

desorbed (which is a physical action). To ionize analytes, free ions must be available at the site. It is not likely that only ionization of analytes is sufficient to cause desorption. It is common knowledge that an exceptionally large source of energy is required to motivate a static substance into a dynamic state or, in other words, to induce physical movement.

When the laser excites 4CHCA molecules, neighboring protons are excited and become available for analyte ionization. The increase in local negative charges likely contributes to an "explosion event" because of repulsion. This explosion event would express a substantial amount of energy, currently thought to be required for desorption of analytes to occur. (Figure 15).

If one examines 4CHCA, which is one of the most useful matrixes in MALDI, it will be easy to understand which part of 4CHCA is responsible for the ionization of analytes. It may be reasonable to assume that the source of protons is the carboxyl groups. The two oxygen atoms on the carboxyl groups are positioned symmetrically[13] and both oxygens share the proton, which allows the proton to have mobility between them (Figure 16). Of course, there is no reason to believe that carboxyl groups are the only source of protons. Protons can be supplied by other groups, such as methyl groups, or even phenyl groups, where no carboxyl group is present.

In a crystalline state, these carboxyl groups often form homodimers.[13] Heterodimers between carboxyl groups on analytes and matrix molecules also makes cocrystallization with biomolecules easier.[14] Those carboxyl groups are likely aligned in the same plane, which will place carboxyl groups adjacent to each

(13) Gray, G. W. *Molecular Structure and Properties of Liquid Crystals*; Academic Press Inc.: London, 1962.
(14) Blumstein, A.; Kitagawa, N.; blumstein, R. *Mol. Cryst. Liq. Cryst.* **1971**, *12*, 215−227.

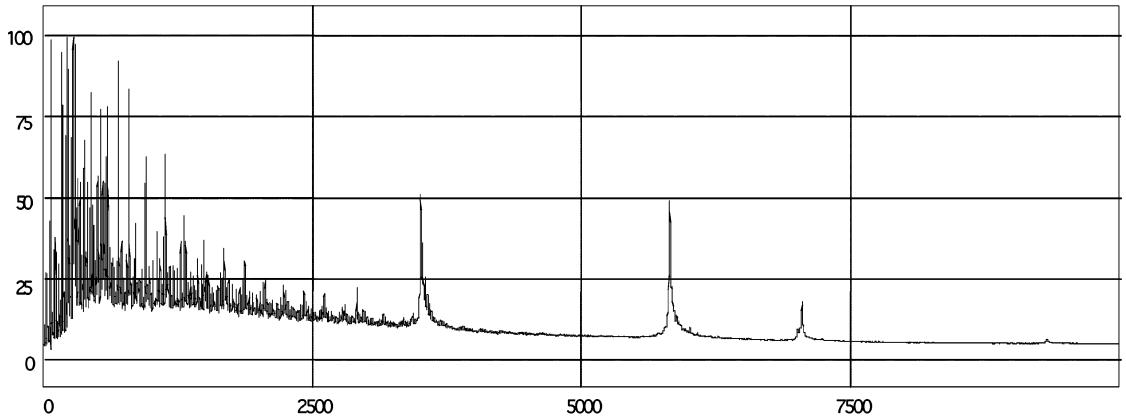

Figure 11. Copolymer of 4CHCAMA, acrylic acid, and 3-(trimethoxysilyl)propyl methacrylate doped with 4CHCA. All-in-one peptide mix analyzed by ProteinChip Reader PBS II.

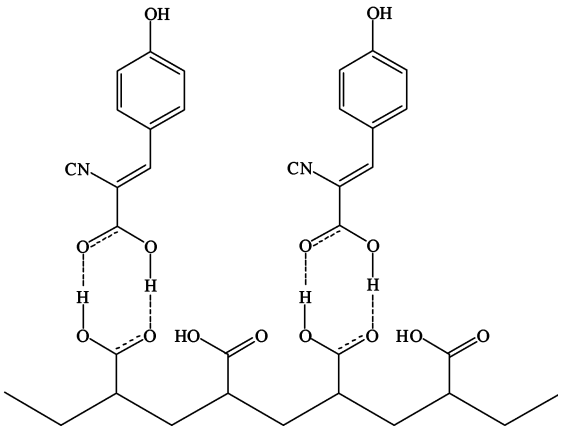

Figure 12. Model of 4CHCA interaction with polymeric 4CHCAMA and an acrylic acid backbone.

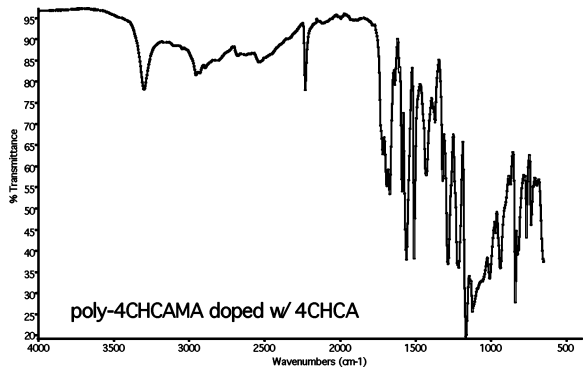

Figure 13. FT-IR spectrum for poly-4CHCAMA with free 4CHCA doped in.

other.[12,13] The configuration allows free protons to form a "proton cloud" suspended by the electron cloud plane. Consequently, any analyte biomolecule in proximity to the proton cloud can be readily protonated, since the crystalline matrix can share the burden of proton loss. As soon as the aligned carboxyl pendant groups lose enough protons from the system, oxygen atoms on the carboxyl pendant groups become negatively charged and produce repulsion forces between neighboring groups. This repulsion force can induce a physical rearrangement, which becomes a necessary driving force for desorption of analytes. The repulsion force created by electron deficiency may sometimes result in an explosion of the matrix crystals, an event that can even be visually observed.

(2) Polyelectrolyte Theory. The explosion of the molecules may become vigorous when the initial molecular alignment is orderly, since the intermolecular force between each molecule is stronger. In other words, crystallization is not an absolute requirement but merely an ideal condition for a successful MALDI desorption/ionization. Indeed, polymerization imposes a similar order upon the matrix molecules as pendant groups covalently attached to a polymer backbone.

As long as those MALDI matrix electrolytes are aligned in an ordered state, with electron-bearing pendant groups arranged in a row, the repulsive forces normally liberated by explosion will probably lead to vibration along the polymer backbone, which assists in desorption of protonated analytes in positive mode.[13] The desorption behavior of polyelectrolytes thus becomes very similar to that of crystallized matrix molecules.

What was produced in these initial experiments is a linear polyelectrolyte consisting of 4CHCAMA. The copolymer of 4CHCAMA and acrylic acid introduced later is also a polyelectrolyte (Figure 17).

The characteristics of linear polyelectrolytes, which show increase in viscosity on dilution of the polyelectrolyte solution, have been well characterized by Flory.[15]

By incorporating ionic materials into a polymer chain, the resulting material will obtain combined characteristics of electrolytes and polymers. It shows large conductivity in solution, and thermodynamic measurements give further evidence of ionic dissociation. The distance between polyelectrolyte molecules in dilute solution may be greatly expanded by the electrostatic repulsion between its charged groups, an effect that manifests itself in a very large intrinsic viscosity. The principle can be applied to both cationic polyelectrolytes and anionic polyelectrolytes.

Since we are dealing with analogues to poly(acrylic acid), let us consider an anionic polyelectrolyte, the polymer units of which are negatively charged. The negative charges are locked into the matrix of the polyelectrolyte (the polyelectrolyte domain) and, as such, cannot diffuse. The associated counterions (in this case, protons) are mobile and diffuse into the solvent (outside the

(15) Flory, P. J. *Principles of Polymer Chemistry*; Correl University Press: New York, 1953.

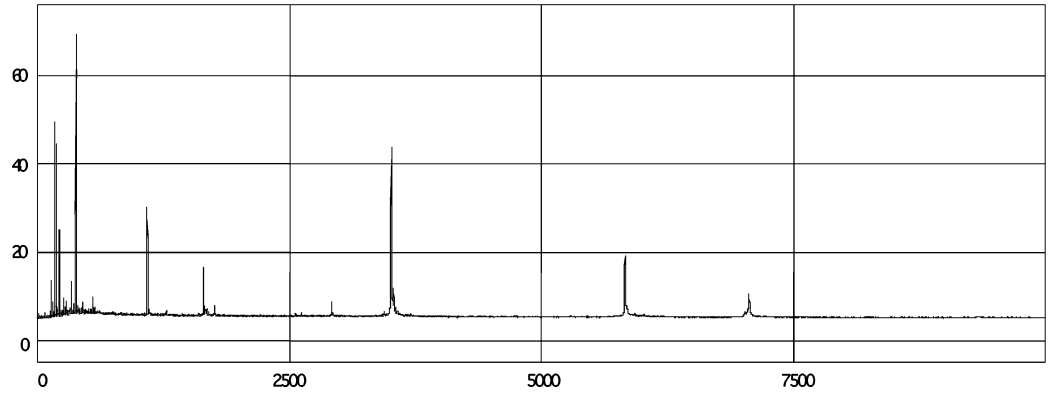

Figure 14. Poly-4CHCAMA in 1-pentanol solution deposited on ProteinChip NP20 and dried in a vacuum oven at high temperature (95 °C) for 42 h, to drive off any residual 4CHCA. All-in-one peptide mix analyzed by ProteinChip Reader PBS II.

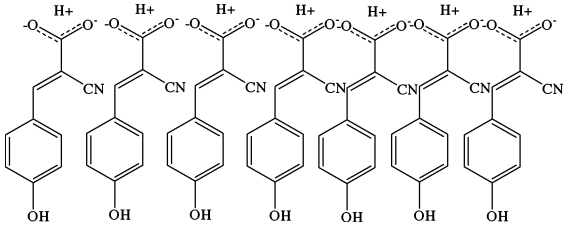

Figure 15. Model of alignment of 4CHCA in crystalline matrix.

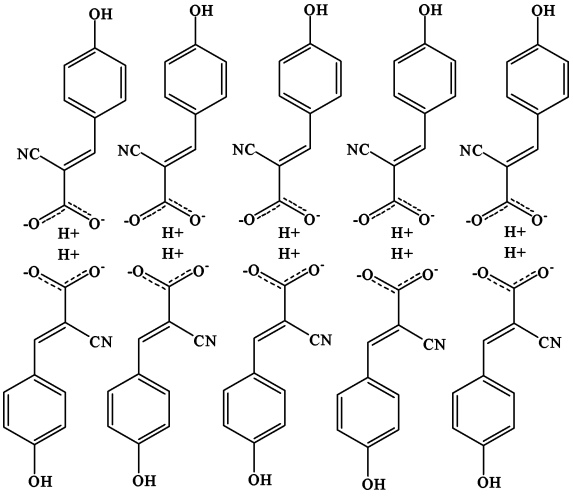

Figure 16. Model of carboxyl group alignment of 4CHCA in crystalline matrix.

polyelectrolyte domain). Protons will continue to diffuse until the potential within the molecule decreases relative to the solution, and further loss of protons is consequently discouraged. The system eventually reaches a state of equilibrium, at which the potential is just enough to support the difference in the concentrations of ions inside and outside the polyelectrolyte domain.

In a sense, one can look at this scenario as an osmotic event, occurring across a "hypothetical membrane" at the interface of polyelectrolyte and solution. If we assume that the condition of neutrality may be applied to the single molecule in solution, that is, that the stoichiometric excess of negative charge within the polyelectrolyte is negligible compared to its net (fixed) charge, the problem reduces to one of Donnan equilibrium across the hypothetical membrane. The mobile counterions of the polyelectrolyte exert a large osmotic pressure on the hypothetical membrane. The polymer molecule should therefore swell enormously with water if a substantial portion of it is ionized.

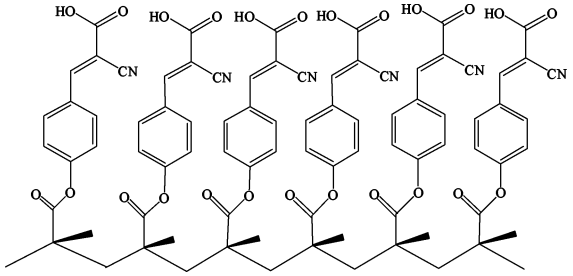

Figure 17. Model of polymerized 4CHCAMA.

A typical application for polyelectrolyte is the use of poly(acrylic acid) in superabsorbent polymeric materials. Linear poly(acrylic acid) in aqueous solution will increase its intrinsic viscosity when the solution is diluted. As the solution is diluted, the distance between the mobile ions and the polymer backbone becomes large. The polymer molecules no longer fill all the space, and intervening regions extract some of the mobile ions. Consequently, the polymer backbone becomes negatively charged and creates a repulsion force, which forces the chain to stretch further, thus making the solution viscous.

The scenario described above can explain polyelectrolyte behavior during laser desorption/ionization of biomolecules. In the ionization process, the mobile ions (protons) are taken away by biomolecules, leaving behind a net negative charge in the matrix (polyelectrolyte). A similar repulsion force must develop in a solid phase, semisolid phase, or even in liquid phase such as in crystals, polymers, and liquid substances as long as they lose substantial protons (or electrons) from the system.

Thus, the exact phenomenon described above regarding polyelectrolytes in solution can also be applied to the currently developed polymer having energy-absorbing molecules. Poly-4CHCAMA is an electrolyte, and counterions (mobile ions) are removed during laser desorption/ionization of biomolecules. As described above, even though the electrolytes are not in a solution but in a solid phase or semimelted phase, a similar repulsion force should develop between those charged pendant groups. In other words, net charges develop in the domains of the polymer molecules, causing them to expand. However, the polymer backbone chains are not as mobile in solid phase as they would be in solution. The repulsion force created by the removal of counterions must be relieved somehow. It is natural to imagine that the pendant groups may start vibrating. This vibrational movement is important to desorb the analytes to desorb from the

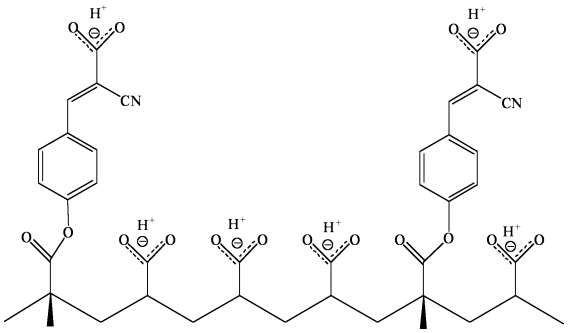

Figure 18. Model of copolymer of 4CHCAMA and acrylic acid.

polymer surface. Indeed, this movement can be so strong that some of the weaker chemical linkages may break, freeing the pendant groups in some cases.

As mentioned previously, there are two major events that must take place to have a successful laser desorption/ionization of biomolecules. One is ionization of analytes and the other is desorption of charged analytes. Both steps most likely take place at the vicinity of the polyelectrolyte. Another reasonable assumption is that the protons on carboxyl groups are the ones that ionize the analytes, since the pair of oxygen atoms are situated in symmetrical positions and the protons can bind to either oxygen, as far as carboxyl groups are concerned. This makes the protons more mobile and facilitates formation of a charged hydrogen plume.

The degree of polyelectrolytic behavior of the 4CHCAMA polymer variants in solution appears to coincide with their desorption performance. This suggests that a similar mechanism between linear electrolytes in solution and electrolytes upon laser irradiation is at work. In solution, empirical results indicate that poly(acrylic acid) yields a viscous solution upon dilution in aqueous solution. Poly(methacrylic acid), on the other hand, does not yield as viscous a solution as that of poly(acrylic acid) (results not shown). Interestingly, a copolymer of 4CHCAMA and methacrylic acid does not enhance the spectrum as much as that of a copolymer of 4CHCAMA and acrylic acid. Finally, the additional electron-donating $-CH_3$ group present on methacrylic acid is a positive inductive group ($+I$), which makes it harder for protons to leave the carboxyl groups. Acrylic acid does not have this moiety but hydrogen atoms instead, making it easier for protons to leave. An idealized structural figure is shown in Figure 18 to describe the argument.

CONCLUSION

MALDI is a powerful soft laser desorption/ionization tool to be used in biopolymer analysis. Ever since its inception, many scientists across many disciplines began a monumental search for new matrixes and an understanding of the MALDI mechanism. However, the MALDI method itself had been developed empirically, and despite its widespread use, the factors that determine success or failure of MALDI experiments are not yet fully understood. A useful matrix is believed to provide four major functions. The first is to isolate analyte molecules by dilution within the preparation, to prevent analyte aggregation. Second, the matrix has to absorb energy via electronic (ultraviolet or UV-MALDI) or vibrational (infrared or IR-MALDI) excitation. Third, disintegration of the condensed phase has to take place without excessive destructive heating of the embedded analyte molecules. Last, but not least, an efficient ionization of analyte molecules has to be provided.[16] It is interesting to note that Dreisewerd's review of the MALDI mechanism[17] did not emphasize a particular need of crystalline state for MALDI matrixes.

Karas and Kruger[16] explained similarly that the matrix effect was described to be threefold: first, a controllable energy transfer to the condensed-phase matrix−analyte mixture inducing a "uniform and soft desorption;" second, a promotion of ionization by chemical reactions; and third, to generate "favorable prerequisites" by isolating analyte molecules in an excess of matrix. The descriptions provided by both authors above fit into the polyelectrolyte model put forth in this current paper.

Dreisewerd[17] also did not clearly express the need for a phase explosion in MALDI matrixes. The assessment of phase explosion as a relevant process for UV-MALDI is somewhat complicated by the fact that inhomogeneous molecular microcrystals are prepared in almost all cases. It is likely that the phase explosion phenomenon may be the result of the repulsion forces created in the solid or semisolid phase by electron charges among matrix molecules that donated their counter charges to analyte ionization, as described in the previously mentioned polyelectrolyte theory.

It is believed that compounds with labile protons, such as carboxylic acids, are good MALDI matrixes in the positive ion mode because they are easily able to protonate neutral analyte molecules in the plume. The theoretical description discussed in the previous section about the anionic polyelectrolyte characteristics can also be applied to cationic polyelectrolytes. One of the classic examples is quaternized poly(4-vinylpyridine), which is an amine-containing polymer.[15] After all, losing counterions in a dilute solution and creating a repulsion force is the same phenomenon as seen in poly(acrylic acid).

Consequently, the exact same explanation given for poly(acrylic acid) applies to polyquaternized amines. The polyelectrolyte phenomenon can likely be applied to all MALDI matrixes having oxygen-, nitrogen-, sulfur-, etc.-containing compounds, which have a lone pair of electrons, including coordinated π-orbital-containing compounds, since they have some ability to bear either negative charge or positive charge in the structures by either losing protons or electrons, respectively. Assuming that the creation of repulsion force is needed for laser desorption of analytes to occur, it makes sense that pendant groups with lone pair electrons should have better matrix characteristics.

First, in order for polyelectrolytes to be useful in the laser desorption/ionization process, the polyelectrolytes must have laser energy-absorbing molecules on the polymeric backbone. Second, the polyelectrolyte must be capable of ionizing analytes. Third, electrolyte pendant groups must be aligned in such a way as to encourage repulsion forces when like charges are created among the pendant groups through the ionization of analytes.

The fact that laser desorption/ionization of biomolecules has been performed using a noncrystalline, linear polymer or copolymer having covalently attached energy-absorbing molecules suggests that crystals may not be required for soft laser desorption/ionization of biomolecules and suggests that the simple alignment

(16) Karas, M.; Kruger, R. *Chem. Rev.* **2003**, *103*, 427−440.
(17) Dreisewerd, K. *Chem. Rev.* **2003**, *103*, 395−426.

Approaches to Soft Laser Desorption/Ionization		
Chemical resuspension		
Cobalt/glycerol	liquid suspension	Tanaka 1987[2, 3]
MALDI	crystalline matrix	Karas & Hillenkamp 1988[4]
LDI probe surface modifications		
SEND	Surface-bound polymer	Hutchens & Yip 1999[5, 6]
DIOS	Porous silicon	Wei et al. 1999[7]
Silicon film	Plasma-enhanced chemical vapor deposition	Cuiffi et al. 2001[9]
Colloidal suspensions		
SGALDI	DHB integrated into inorganic sol-gel	Lin & Chen 2002[10]
Aerogels	Colloidal suspension of resorcinol & formaldehyde	Belford & Yost 2002[8]
Solid-phase organic polymer		
PELALDI	Amorphous linear polymer of 4CHCA and acrylic acid	Kitagawa 2002

Figure 19. Evolution of approaches to soft laser desorption/ionization.

of the matrix molecules or pendant groups on polymers is a more important factor in successful soft laser desorption/ionization.

As described in the introduction of this paper and the descriptive Figure 19, the soft laser desorption/ionization methodology initiated by Tanaka, Karas, and Hillenkamp has evolved into the major force in mass spectrometric analysis of biological molecules. In particular, organic chemical matrixes introduced by Karas et al. have been exceptionally useful in the field. Since then, many attempts to improve the methodology, such as DIOS, silicon films, SGALDI, SEND, and aerogels, expanded the methodology further in the field of polymeric materials. However, such development has been limited to either some surface modification of polymers or use of sol−gel technology, which is a colloidal suspension.

None of these developments are purely associated with polymerization of monomers through a free-radical polymerization process to amorphous, organic polymers for use in LDI. Polymeric film formers that exhibit MALDI matrix characteristics have been created by synthesizing monomers with energy-absorbing molecules in their structure. The synthesized monomer can easily be copolymerized with other commercially available monomers possessing other useful chemical properties (i.e., chromatographic affinities). Such copolymers made with the novel synthesized monomer can also desorb/ionize biomolecules upon laser irradiation.

There were two major reasons why this technology could have easily failed. First, one of the vital parts of the matrix—the hydroxyl group of 4CHCA—would be transformed into a linkage for a reactive group. Second, the cocrystallization of matrix with analyte, which had been a requirement of conventional MALDI, would be disabled through the polymerization process of creating an amorphous polymer. The fact that analytes are successfully desorbed and ionized with a polymeric matrix may refine our understanding of the mechanism of the MALDI desorption/ionization process, since desorption/ionization could take place without crystallization. It is also interesting that the purified monomer, α-cyano-4-methacryloyloxycinnamic acid, is not a good matrix for conventional MALDI methodology, even though the monomer can cocrystallize with analyte. However, upon polymerization, this monomer exhibits powerful matrix desorption/ionization characteristics.

The versatility of the monomer derived from a MALDI matrix cannot be emphasized enough, since the monomer can be a building block for polymeric matrixes for future research to create useful polymeric matrixes to improve the soft laser desorption/ionization methodology.

The fact that most MALDI matrixes are compounds with carboxyl groups may not be a simple coincidence. If physical movement of matrix molecules is required for desorption of biomolecules, such movement could be accomplished via the polyelectrolyte mechanism described herein. Carboxyl groups can more readily produce charged groups than other pendant groups, such as nitrogen-based compounds. However, this certainly does not rule out the usefulness of other laser energy-absorbing compounds without carboxyl groups for soft laser desorption/ionization. Any compound with the ability to bear electronic charge and ability to ionize analytes could have potential for use in soft laser desorption/ionization. The question becomes how easy it will be for a given compound to bear charges and simultaneously ionize biomolecules.

The author acknowledges that all configurations used in this paper are aimed to describe the hypothetical mechanism of the function of polyelectrolytes in laser desorption and ionization. In real world usage, the actual polymer chains are expected to be entangled in a random fashion. However, some orderly configurations, as described in this paper, can be observed in some regions of the chains. In doing so, it is possible that the proposed mechanism can assist in explaining the MALDI mechanism, which has not been clearly defined to date.

ACKNOWLEDGMENT

The author expresses his appreciation to Ciphergen Biosystems, Inc., for allowing the publication of this work.

Received for review July 14, 2005. Accepted November 4, 2005.

AC051253J