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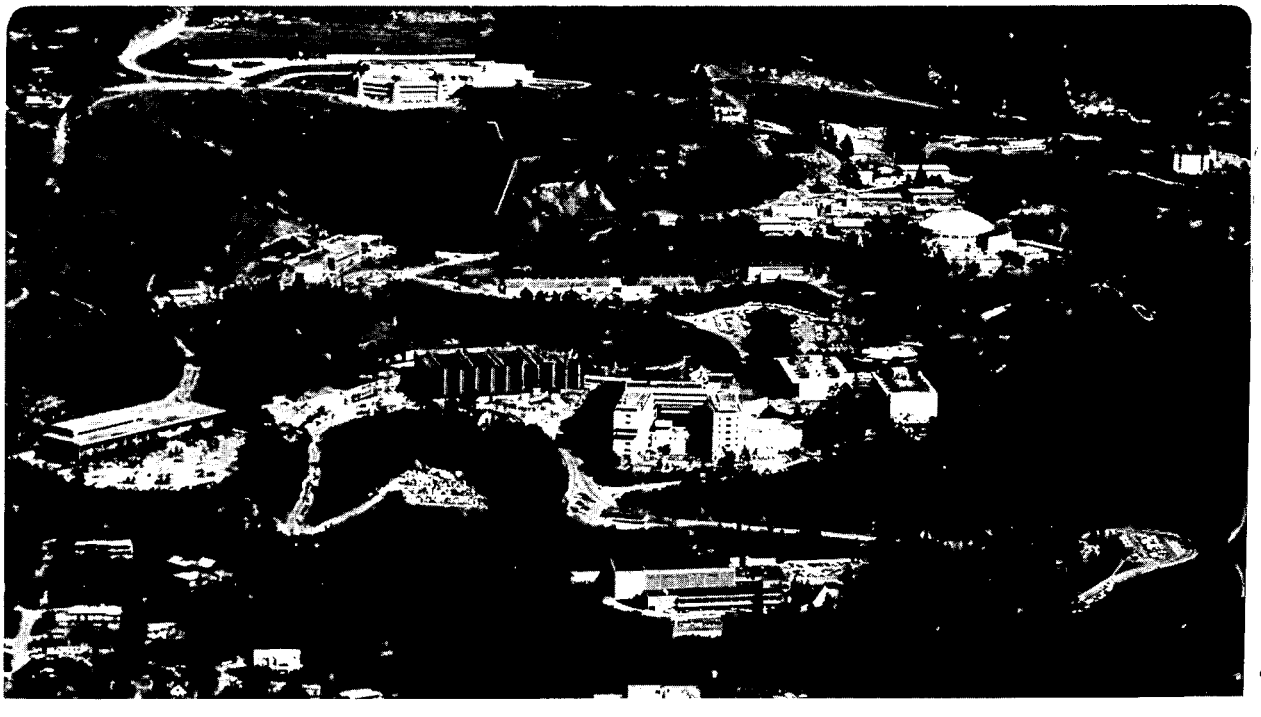

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Abstract

We present results obtained with a novel mass spectrometer capable of determining the mass of multiply charged electrospray ions generated from samples of macromolecules in the megadalton (MDa) size range. The instrument utilizes a sensitive amplifier which can detect the charge on a single ion as it passes through a tube detector. A velocity measurement of an ion with known electrostatic energy provides the ion's mass to charge ratio. Simultaneous detection of the ion charge permits a mass assignment to be made for each ion. Electrospray ions of DNA and polymer molecules with masses greater than 1 X 10^6 Da and charge numbers (z) in excess of 425 e^- are readily detected in this mass spectrometer. The weights of small particles were also measured. The on-axis single ion detection configuration provides a duty cycle of nearly 100% and extends the practical application of electrospray mass spectrometry to the analysis of very large molecules with relatively inexpensive instrumentation.

Introduction

Much of the progress in the field of mass spectrometry over the last ten years has been marked by an ever increasing mass range made possible by new ionization techniques. Electrospray ionization (ESI) appears to be unlimited by the size of molecules that can be ionized and macromolecular ions with masses up to 100,000 Da are now analyzed routinely in most mass spectrometers which can be interfaced to an ESI source. Perhaps as important as ESI's ability to generate high mass molecular ions in the gas phase without fragmentation, is its ability to create multiply charged ions. These ions have m/z valves sufficiently low to allow their analysis in instruments with mass ranges normally limited to a couple of thousand Da for singly charged ions. In interpreting results of their pioneering molecular beam experiments with ESI of 400,000 Da polystyrene molecules, Dole and co-workers concluded that such massive ES ions carried, at most, the charge of only five electrons.[1] Later, when Fenn and his colleagues combined ESI with a mass spectrometer they discovered that polyethylene oxide molecules, as well as other macromolecules such as proteins, could hold many more charges (on average about one charge per 1000 Da).[2,3] Nohmi and Fenn eventually recorded ES mass spectra of polymer ions with molecular weights as high as 5 million and as many as 5000 charges.[4] Beuhler and Friedman obtained similar high mass spectra of proteins with molecular weights up to half a million.[5] The actual molecular weights of the ions in the ESMS spectra could not be determined because the mass analyzers used lacked the resolution to identify a series of adjacent peaks corresponding to consecutive charge states. The experiments were further complicated by the distribution of molecular weights in most polymer samples. The extent of multiple charging, therefore,

effectively limited practical mass analysis with ESI to about 100,000 Da, and much less for polymer distributions. Chernusevich and co-workers, however, have recently determined the mass of protein complexes with MW's as large as 1.3 megadaltons (MDa) using an ESI time-of-flight instrument which enabled them to make measurements at relatively high values of m/z.[6]

A breakthrough in the analysis of high molecular weight compounds by ESMS was made by Smith and coworkers using a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer to trap single ions of megadalton DNA.[7,8,9] Individual electrospray ions of a few million molecular weight carried enough charge to be detected and isolated in the trapping cell of their instrument. These researchers trapped single ions long enough to observe shifts in their charge states caused by reaction with background gas molecules, and demonstrated that the resulting set of adjacent m/z "peaks" could be deconvoluted to yield the ion mass with high accuracy for ions with MW's up to 1 $x10^7$. They were not able to determine m by deconvoluting the spectrum from a large number of trapped ions, in part, because they could not be sure that several adjacent charge states of a single ion species were represented in the initial cloud of trapped ions. As an alternative to the charge state shift method, the Smith group determined the mass of individual ions by measuring their charge directly along with a value for each ion's m/z. Using this approach they were able to "weigh" individual ions of DNA with MW's as high as 1 $x10^8$ Da and more and than 35,000 charges![10] The system was sensitive enough to detect a single ion with only 30 charges. At present, the FTICR-MS is able to determine the charge on an ion with a precision of only $\pm$ 10% regardless of the absolute charge. The error, which results from uncertainties in the ion orbit, leads to a proportionate error in the mass determination.

The complexity and cost of the hardware required for FTICR mass spectrometry offers practical limitations to the widespread use of the technique for such applications as sizing megadalton DNA. In addition, the data from FTICR-MS of massive electrospray ions is not in the form of a true mass spectrum because the ions are analyzed one at a time. Data representative of the spectrum of masses in a sample is desirable, but collecting it requires considerable time by the present FTICR-MS methodology.

In seeking to apply the relatively simple technique of time-of-flight mass spectrometry (TOF-MS) to the study of massive ES ions, we realized that we too could exploit the large amount of charge on these ions by detecting and quantifying their charge individually. This measure, together with a value of m/z, allows a calculation of m for each ion. We refer to this general method of mass determination as charge detection mass spectrometry (CD-MS). Unlike FTICR-MS, which uses an antenna to sense the periodic signal induced by a cloud of nearby trapped ions, our approach employs a more efficient Faraday cage charge detector through which the ions pass. The ions are accelerated from a known electrostatic potential and are directed to fly through the center of a tube shaped electrode onto which an image charge equal to that of the ion is impressed. The amplitude of the image charge signal is proportional to z. The entrance and exit signals from the passage of each ion provides a measure of the ion velocity which, in turn, allows calculation of an m/z for each ion. With these two pieces of information we can assign a mass to each ion which passes through the tube.

Mass measurement of ions by simultaneous charge and velocity detection dates back over 30 years to the work of Shelton and co-workers who were interested in charging and sizing solid particles for cosmic dust experiments.[11, 12] They charged the iron microspheres in vacuum electrostatically and detected particles with as few as 10^4 charges. Hendricks later applied the system for sizing electrospray droplets.[13] Lue and Kelly made similar mass measurements of electrospray droplets using a quadrupole mass filter in conjunction with a charge sensitive pulse amplifier attached to a Faraday cup.[14] Like Hendricks, they generated the electrosprays in vacuum. More recently, the group of Keaton and Stradling, as part of their own simulations of micrometeoroid impacts, developed a particle mass spectrometer of the Shelton design with improved charge measurement sensitivity enabling them to weigh particles with as few as 1500 charges.[15, 16] All of the above studies involved particles or droplets and therefore are to be regarded as separate from studies involving macromolecules, the latter distinguished as concerning ions with distinct molecular weights.

The CD-MS systems for measuring particles and droplets mentioned so far did not demonstrate the sensitivity of charge detection we believed necessary for analyzing ES ions of megadalton DNA. Two recent investigations suggested that it might be possible to perform in flight charge detection with more precision than had been done previously. Vercoulen and co-workers used a metal cylinder electrode to measure charges as small as 5×10^{-17} C (z=300) on individual aerosol particles.[17] Park and Callahan recently reported experiments on the detection of the charge from packets of what they believed were as few as a hundred singly charged, laser desorbed ions in a TOF spectrometer by means of a charge sensing grid.[18] *Although they realized that a cylinder design for a non-destructive charge detector might be more sensitive, they apparently did not recognize the possible utility of their detector for the measurement of individual, highly charged electrospray ions.

Experimental Apparatus

The design of the electrospray ion source interface is based on that of Whitehouse and Fenn.[2] A glass capillary, two skimmers, and an orifice plate define four stages of pumping in the source. The electrospray needle is held at 1 to 3 cm away from the inlet to the vacuum system, a glass capillary, 18 cm long with a 0.5 mm bore. The exterior of the capillary is coated with metal at each end to help establish potentials for electrostatic focusing. The inlet end of the capillary is shrouded with a sheath gas flowing counter-current to the direction of ion drift. We typically use CO_2 heated to about 70^0C which promotes evaporation of the spray droplets and suppresses corona discharge at the electrospray needle. The exit of the capillary rests 4.0 mm from the first of two conical skimmers which are separated from each other by 15.0 mm (see Fig. 1). The opening in the first skimmer is 0.8 mm in diameter and that in the second skimmer is 1.0 mm in diameter. The first skimmer acts as a barrier between the first and second vacuum stages which are evacuated by two 15 c.f.m. mechanical pumps to background pressures of 1-2 torr and 80 - 150 millitorr, respectively. The third stage is evacuated by a diffusion pump to below 1×10^{-3} torr.

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* Park and Callahan use the term "inductive detector" for their grid because ions "induce" an image charge on it. The term capacitive detector more accurately describes this electrostatic principle which does not involve induction as the term is used in electronics.

A short einzel lens rests in the 20.0 mm space between the second skimmer and the inlet to the fourth vacuum stage, which is a 3.0 mm hole in a 1.0 mm thick plate. The fourth vacuum stage contains the analyzer region and is pumped by a turbo-molecular pump to 1.0×10^{-6} torr. The metal coated exit of the glass capillary and the skimmers act as electrostatic focusing lenses. Potentials as high as 330 volts may be set on these elements, while the inlet plate to the fourth vacuum stage is usually held at ground potential. With these lens potential settings, ions are accelerated to a kinetic energy whose value is roughly the average of the electrostatic potentials on the two skimmers. Alternatively, the inlet plate can be floated so that ions are accelerated primarily in the high vacuum region where they should experience fewer collisions with the background gas. Floating the inlet plate however, produces a lower ion transmission.

The source can be tuned to select massive ions while rejecting lighter ones by setting the capillary exit voltage below that of the first skimmer by 10 to 20 volts. Such selectivity is confirmed by the correlated response of the micro-channel plate ion detector, which responds to all ions, with the response of the charge detector, which only registers the highly charged massive ions. These experiments are described below. When electrospray ions of moderately sized proteins, such as Cytochrome C are generated from solutions of methanol-water, total ion currents of 0.1 to 1.0 pA are detectable in the analyzer region. Ions posessing at least 500 charges reach the analyzer region at rates up to 100 ions per second when concentrated (0.1 g/L) solutions are sprayed.

The charge detector assembly is patterned after that of Keaton and Stradling and is illustrated in figure 2.[16] It consists of a thin-wall brass charge pick-up tube 3.5 cm in length with a 6.35 mm bore. The bore of this tube is aligned with the ion beam axis allowing a fraction of the ions in the beam to pass through the detector unhindered. The tube is supported with teflon insulators inside a second metal cylinder approximately 3 cm in diameter and 5 cm long which provides electrical shielding. The hollow end caps of this larger cylinder are movable permitting adjustment of the gap between the end caps and the inner pick-up tube. Adjusting this gap will change the rise time of induced pulses. The outer cylinder is mounted firmly on a 2.5 cm O.D. hollow metal post which in turn is fastened to an electrically isolated vacuum flange equipped with electrical feedthroughs.

The signal induced on the pick-up tube is amplified by a low-noise charge sensitive preamplifier. As an ion enters the pick-up tube, it induces an equal and opposite charge on the tube. The pick-up tube is "grounded" through the high impedance input of a JFET transistor operated as a high gain negative feedback amplifier. The tube/shield assembly is designed to possess a minimum capacitance in order to maximize the voltage presented to the input of the transistor by a small charge. We also attempted to minimize the capacitance because thermally generated noise measured at the output of the preamplifier is directly proportional to the total capacitance at the transistor gate. The detector tube assembly and its electrical lead, along with a feedback capacitor and a 0.3 pF test capacitor, have a total capacitance of 4-5 pF. This total capacitance is matched by that of the JFET transistor input. The test capacitor allows a known amount of charge to be pulsed onto the pick-up tube for calibration purposes. The test pulses are generated with a shaping pulse generator so that the time dependent signal response can be determined as well.

The low noise JFET acts as the remote input stage to a preamplifier whose output is differentiated and integrated by a second pulse shaping amplifier. The resulting output for each ion is a double pulse signal typified by that shown in the top oscilloscope trace of Fig. 3. The leading positive amplitude pulse corresponds to the charge impressed on the cylinder by the ion as it enters the tube and the second negative amplitude pulse results as the ion exits. The time between the two pulses corresponds approximately to the flight time required for the ion to traverse the length of the pick-up tube.

The shaping amplifier improves the signal to noise ratio through pulse peaking time filtering. The peaking time is the time required for the signal to rise from the baseline to the peak of the pseudo-Gaussian pulse. It corresponds roughly to the upper frequency of a signal filter with a band pass. The filtering reduces much of the higher frequency "series" noise associated with the channel of the preamplifier input JFET, well as low frequency parallel noise sources. With a peaking time constant of 3.2 microseconds, the system exhibits a minimum noise level of 150 electrons r.m.s., or +/- 75 electrons. If the rise time of the actual signal is longer than peaking time, the measured pulse amplitude will be reduced. Pulse amplitude reduction can be accounted for in the assignment of a charge value, if the rise time of the induced pulse is known. Estimating rise time is discussed in the section on error sources.

The passage of an ion is captured by a LeCroy 9350 digital oscilloscope which not only records the entire waveform for each passing ion, but also calculates the time between the leading and trailing pulse and the amplitudes of the two pulses. The data are transferred to a desk-top computer where they are used to compute the charge and the mass of each ion. At present, signal processing and data transfer are rapid enough to allow the capture of up to 5 events per second. With the addition of dedicated hardware, the system could process 100 events or more per second. Because the beam does not require modulation or "chopping" to provide timing information, the duty cycle of this instrument is virtually 100%. In other words, each highly charged ion whose trajectory passes through the tube is detected, although only a small fraction of these ions is recorded at present. The transmission efficiency of the electrospray ion source, however, is quite low. The fraction of the total number of sample molecules ionized in the electrospray region reaching the analyzer stage of our instrument could at most 1 out of 10^4.

The mass-to-charge ratio of an ion is determined by time-of-flight techniques using ion velocity and acceleration voltage so that:

$$\frac{m}{q} = \frac{2V}{v_m^2} \qquad (1)$$

where m is the mass, q is the ion charge, V the electrostatic acceleration voltage, and v_m the measured ion velocity. In our system, a correction needs to be made to take into account the initial kinetic energy imparted to the ion by the free jet expansion of the gas prior to acceleration by the electric field. The ion's total kinetic energy is the sum of its initial kinetic energy and the electrostatic potential energy set by the skimmer lenses. It follows that the mass of an ion is given by :

$$m = \frac{2qV}{v_m^2 - v_g^2} \qquad (2)$$

where the ion velocity due to the gas expansion, v_g, is determined by grounding all electrostatic lenses and timing the passage of the ion through the detector. The value of v_g is usually about 10% that of v_m for ions of DNA in the 10,000 m/z range when V is set to 300 volts. Because $v_m^2 >> v_g^2$, uncertainties in v_g lead to a minimal error in the determination of m. This is less true for larger m/z ions. For example 315 nm polystyrene latex microspheres nebulized from solution by electrospray ionization and having masses around 1×10^{10} Da, increase their velocity by only 10% over v_g when accelerated with 300 V.

Some of the ions which fly through the charge detector strike a pair of micro-channel plates (MCP) positioned 40.0 cm behind the exit of the charge detection tube. A grounded grid lies directly in front of the MCP detector to ensure that the ions are not accelerated by the -2 kV on the first MCP until they are close to the plates. The MCP detector will respond to ions above a minimum charge to mass ratio which depends on the kinetic energy of the ion beam. The plates are not sensitive to the amount of charge on the detected ions. Massive DNA ions with m/z values up to 20,000 and an energy of at least 2000 ev/charge create pulses in our micro-channel plate detector. The MCP detector is used to determine the arrival time and flux of these ions.

An improved estimate for the ion velocity may be made by timing the flight of the ion over the 40 cm between the tube detector and the MCP detector instead of just the 3.5 cm length of the pick-up tube. By timing the ion's passage for a longer flight path, the precision of the velocity estimate should improve ten fold. However, at the moment the precision with which the velocity is determined using the pick-up tube alone is already ten times better than that of our charge measurement. Uncertainty in the ion charge is therefore the primary factor limiting resolution. Under some circumstances, we observe evidence in the MCP signal suggesting fragmentation of megadalton DNA ions. Often the signal on the MCP correlating to a DNA ion is characterized by as many as five pulses clustered around the expected arrival time of that ion. The charge detector in its present location cannot be used to distinguish between the passage of an intact ion and a few closely spaced fragment ions. The occurrence of multiple signals at the MCP is associated with the settings of lens voltages in the ion source and is most likely due to nozzle-skimmer dissociation. The effect of the source conditions on fragmentation needs to be studied further.

The MCP detector was used in conjunction with a retarding potential grid to determine the ion energy distribution in the beam. Two grids, in addition to the grounded grid already mentioned, were placed in front of the MCP detector. The first and third grids were electrically grounded while the second one held a retarding potential. This configuration of grids was intended to provide axial electrostatic repulsion of the beam while minimizing radial defocusing effects on the ion flux. Pulses from the MCP detector were amplified and registered by a rate meter. The arrival rate of ions to the detector could then be monitored as a function of retarding grid potential and the resulting data plotted as ion transmission vs. retarding voltage. Skimmer potentials were set to pass mostly massive ions, but a substantial fraction of the ion counts on the MCP detector did not correlate with signals from the charge detector and were thus due to light ions. The retardation was not performed in front of the charge detector because, although the

detector responds only to the large ions of interest, the signal from the pickup tube is sensitive to ion velocity.

All of the mass spectra shown were recorded in experiments with ions generated from positively charged electrosprays. Samples were pumped through a one meter length of 50 μm I.D. silica capillary at flowrates from 0.01 to 1.0 μL/min, depending on the solution conductivity. The fine bore capillary helps stabilize the electrospray at low flowrates. The flow is regulated, as suggested by De la Mora and Loscertales, by adjusting the pressure of a helium atmosphere above the sample reservoir.[19] At low flowrates, the initial electrospray droplets can be as small as 100 nm, but DNA samples with molecular weights under 10 million were readily ionized with low flowrate sprays, even though the DNA molecules, when extended, are several microns in length. Samples were prepared by diluting supplier's stock solutions (typically DNA at 0.5 mg/mL in 1-10 mM Tris, 1 mM EDTA buffer) ten to one hundred fold in autoclaved water. The stock solutions were typically reported to contain 1 mM NaCl. Samples included pBR322 , a 4363 base pair DNA plasmid (MW = 2.8 MDa) and pMSG-Cat DNA, (8405 base pairs, MW = 5.5 MDa) both obtained from Pharmacia Chemicals. Powdered polyethylene-oxide (PEG) with a nominal average MW = 7×10^6 was obtained from Aldrich Chemical Co. Col E1 Amp Plasmid DNA (MW = 7.3×10^6) and Lambda Phage DNA (MW = 31.5×10^6) were purchased from Sigma Chemical Co.

Results

We have obtained molecular weight information for several DNA samples with nominal molecular weights ranging from 2.8×10^6 to 31×10^6 Daltons. Mass to charge ratios for these ions ranged from 2000 - 12,000 m/z and measured charge states ranged from 500 to 5000 positive charges. A mass spectrum of 2.8 MD DNA (pBR322) is depicted in Fig. 4(a). This histogram format presentation of masses, after Keaton et al.,[15] represents the measurement of about 3000 individual ions sampled during a period of 20 minutes. The ion of the double stranded molecule is represented by the tallest peak of the histogram and it's centroid occurs around 2.8 MDa ($\sigma = \pm$ 200,000 Da). The smaller peak, whose centroid falls at roughly half the mass of the tallest one, corresponds to the single stranded molecule. The small ion populations at higher mass (greater than 3 MDa) may represent dimers of the single- and double-strands or molecules contaminated with solvent or other adducts. The distribution of charge for the ions in this experiment is histogrammed in Fig. 4(b). Note that the charge distribution is narrow ($\pm$ 10% FWHM) in comparison with the range in mass, with most of the ions processing between 600 and 800 charges. The m/z values presented in Fig. 4(c) range from 1500 to 5000. The distribution of charge states was restricted at the lower end by the threshold limit of charge detection set by the trigger level on the oscilloscope. The threshold is typically set at 90 mV which corresponds to a charge of around 425 electrons, about three times the background noise limit for charge detection.

The mass spectrum from a suspension of polyethylene-oxide (PEG) with a nominal average MW = 7×10^6 Da and prepared in 50% methanol water solution at a concentration of 0.01 g/L, is shown in Fig. 5. The mass distribution of the polymer exhibits a maximum at 5 million Da as well as a high mass tail. In other experiments, however, mass spectra indicated high mass tailing where none was expected. The cause of this unexpected tailing may be aggregation or

incomplete desolvation of sample ions. In addition, ions having less kinetic energy than expected will also be interpreted as having a higher than actual mass. This possibility is discussed in the section on sources of error. Figure 6 is a mass spectrum of Col E1 Amp Plasmid DNA, MW = 7.3×10^6. The main peak is centered around 6.9×10^6 Da, somewhat smaller than the expected value. We believe the discrepancy is due to a slight underestimation of the flight time of the ions resulting from the manner in which the oscilloscope processes the signals, or to an overestimation of the effective length of the pick-up tube. In the latter case, using an effective length of the tube in the calculation of velocity can serve as a way to calibrate the mass scale. Such a correction was applied to the other spectra shown and is discussed in more detail in the section on instrument resolution and sources of error.

The raw data used in the calculation of the mass spectrum of Col E1 Amp Plasmid DNA (Fig. 6) are plotted in the 2-D graph displayed in Fig. 7. Each point in the scatter plot represents data for a single ion. The mass of an ion should be roughly proportional to the ratio of the charge divided by the square of the ion velocity as shown in Eq. 1. Accordingly, ions possessing the same mass but different charge should lie along a curve relating flight time to charge. Such a relationship is represented by the crescent shaped cluster of ions at the center of the plot. A scatter plot relating mass and charge is presented in Fig. 8. The data for Fig. 8 was collected from a sample of pMSG-Cat DNA (MW = 5.5×10^6 Da) We believe the two distributions of ion mass, as seen in the spectrum of Fig. 4(a), represent the single- and double-stranded molecules. The sharp transition at the right edge of the scatter plot is due to ions with charge signals greater than the maximum amplitude range set on the oscilloscope during data acquisition. The two clusters of data show opposite trends with the higher mass ion group exhibiting a slightly decreasing charge with increasing mass. While we decline to venture an explanation for such trends, we believe the plot illustrates the unique type of data that CD-MS can provide. As yet we have not applied rigorous statistical analysis to our data. We expect that averaging of large numbers of ion counts will be essential to extracting the maximum accuracy in mass determination from CD-MS data.

A mass spectrum of Lambda Phage DNA (MW = 31.5×10^6 Da.) is exhibited in Fig. 9(a). The peak in the histogram is broader than expected considering that these ions hold two to three times the average charge of smaller DNA ions. The precision of CD-MS should improve in principle with more highly charged ions because the fractional uncertainty in the charge measurement decreases. We expected more precise charge measurements for these 31.5 MDa ions and thus a relatively narrow mass distribution, but instead observed a very wide mass distribution. It is possible that the ions formed reflect fragmentation as well as clustering of the Lambda Phage DNA. No evidence for molecule aggregation or fragmentation was found when the sample was analyzed by pulsed gel electrophoresis. Therefore, any fragmentation or clustering if present must have occurred in the ES ion source.

Figure 9(b) is a scatter plot of the 31.5 MDa DNA data along with a 100 point moving average of charge vs. mass. The fact that the charge per unit mass appears to be constant, as indicated by the general linear relationship between charge and mass, suggests that the massive DNA ions are more linear or extended, and not compact, or "balled up," in their configuration. We reason simply that the charge per mass of a large electrospray ion should be proportional to the ratio of the ion's surface area to its volume. For molecules with a linear geometry, this ratio remains constant as m increases. A Lambda Phage DNA molecule, when fully extended, is 16 μm long.[20]

The primary droplets formed in electrospray are typically no larger than a couple of micrometers in diameter. This dimensional incongruity evokes the question, first raised by Chen et al,[8] of how ions with linear dimensions larger than their host droplets can form. It may be that molecules of DNA have a compact shape inside the droplets but assume an extended shape after forming gas phase ions when the droplets evaporate. Fenn has proposed the intriguing hypothesis that large polymer molecules, extended by shear flows inside the Taylor cone meniscus at the tip of the spray needle, could fit inside the narrow filament of liquid which issues from the meniscus before it breaks into individual spray droplets.[21] In this scenario, polymer ions would "snake" out of the liquid cone tip in an extended form. The string-like ions presumably would not fragment as the liquid filament breaks apart.

The CD-MS instrument in principle is capable of weighing any airborne ion or particle which carries sufficient charge to be detected. All ESMS systems create residue particles from the non-volatile solutes present in evaporating droplets. Some of these particles, whose size depends primarily on the diameter of the spray droplets and the concentration of non-volatile solutes, can be sampled into the analyzer stages of a mass spectrometer. The charged residue particles lie out of the mass range of almost all mass spectrometers and, to our knowledge, have not been previously observed in a mass spectrum. Residue particles from electrospray droplets have been studied by Mann et al.[22] and De la Mora and Loscertales,[23] who used mobility and aerosol impaction methods to size them.

Figure 10 illustrates a size spectrum obtained with CD-MS of residue particles formed in an electrospray of a 50% methanol in water solution containing 0.2g/L of the protein Cytochrome C and 1% acetic acid at a flowrate of 1.0 mL/min. The mean particle mass observed for these conditions is about 1×10^{-19} kg and the mean charge is about 1×10^{-16} C. The mass of each particle was converted to a particle diameter assuming that the residues have a density of 1.5 g/cm^3. Knowing the electrospray flowrate and solution conductivity and using a previously established model,[19] we estimated that the initial spray droplets were around 1- 1.5 μm in diameter. Assuming that the droplets did not change their mass substantially due to Coulomb explosions before they evaporated, a protein concentration of 0.2 g/L would lead to residue particles of the size observed, namely 50-60 nm . The charge on a water droplet of that size at the Rayleigh stability limit is roughly 1×10^{-16} C, which is consistent with our observations and would be predicted by the theory of Loscertales and De la Mora for the evolution of evaporating electrosprays.[23]

Figure 11 is a size distribution histogram based on a mass spectrometric analysis of polystyrene latex microspheres with a nominal mean diameter of 314 nm ($\sigma = \pm$ 16 nm) and a specific gravity of 1.05. The particles were suspended in water at a concentration of 0.01% (w/v) which was electrosprayed at 1.0 μL/min. The measured size distribution for these particles was centered around 319 nm. If these particles were molecules their molecular weight would be ten billion. The mean charge on the particles was 5×10^{-16} C, which is only about 25% of that held by a water droplet of the same size charged to the Rayleigh limit. Ions with high m/z ($>10^5$) are not greatly accelerated by the 300 volt potential in our ion source. When m/z is on the order of 10^7, the ion velocity after electrostatic acceleration may only increase by a factor of 10% over v_g. In this regime, accurate determination of v_g, the velocity imparted to the ion by the gas jet alone, becomes critical. The mass analysis of particles, such as these, demonstrates that the electrospray

is capable of ionizing molecules and particles over a size range encompassing many orders-of magnitude. These findings suggest that major portions of chromosomes might be sized with CD-MS. A 300 nm particle is equivalent to 1.3×10^7 bp or about 30% of the smallest human chromosome.

Instrument Resolution and Error Sources

An error analysis of Eq. 2 which relates the ion mass to the charge q, flight time t, and electrostatic potential V, yields the simple result that the instrument resolution, $R = M/\Delta M$, is related to uncertainties in charge and flight time in the following manner:

$$R_q = q/\Delta q \quad \text{and} \quad R_t = t/2\Delta t \{1 - (v_g/v_m)^2\}$$

where R_q and R_t are the components of the overall resolution associated with charge and time measurement respectively. The overall resolution is given by $R^{-1} = (R_t^{-2} + R_q^{-2})^{1/2}$. When $v_g << v_m$, as is usually the case, R_t becomes $t/2\Delta t$, which is a commonly used definition of resolution in TOF-MS. Most amplified pulses induced by passing DNA ions have a 5 - 15 μs flight time and an amplitude of 90 to 350 mV. The r.m.s. noise level on the detector is typically 35 mV which corresponds to a charge of about 150 electrons. The timing jitter caused by the noise in the charge measurement ranges from 50 to 500 ns depending on the signal processing method used. One method finds the time interval between the trigger from the first peak and the time of the 50% rise of the trailing peak. The resulting value for t is strongly dependent on the amplitude of the peaks. Constant fraction discrimination should aid in timing measurements from peak arrivals and we plan to incorporate it in the next version of the instrument.

Another means for reducing the timing imprecision is to increase the flight time, which can be accomplished by using two or more detectors spaced a known distance apart. The combined use of pickup tube with the MCP detector for better timing was discussed in the experimental section. The use of two identical detectors, however, can reduce an additional error source related to determining velocity by timing the passage of an ion through only one detector. The error arises from the fact that the time between the "entrance" and "exit" pulses does not necessarily correspond to the time required by the ion to traverse the tube. Weinheimer analyzed the charge induced on a conducting cylinder due to a point charge passing through it.[24] He predicted that a point charge will impress 95% of its full value onto a cylinder of the dimension of our pick-up tube after it penetrates 5.0 mm past the entrance plane, or slightly less than one tube diameter. Therefore, the image charge signal should not necessarily be expected to have a maximum value when the ion is at the tube entrance. In our system the "effective" length corresponding to the time between the pulses was determined by experiment, and hence was regarded as a calibration parameter. The problem is circumvented if the ion passes through two identical detector tubes. In this case the time between pulse pairs should correspond exactly to the spacing between the two detectors, regardless of the pulse shape. We also used Weinheimer's analysis to estimate a rise time for detector pulses so that appropriate amplifier peaking times could be chosen.

In the present system ions having a flight time of 10 μs and measured with a 500 ns timing error correspond to a value for R_t of 10. As this is a worst case estimate for R_t, which is usually ten

times larger than R_q, higher resolution will primarily be gained via improvements in measuring ion charge. When the signal is only two or three times the noise level, $R_q = q/\Delta q$ may be no larger than 3. As discussed in the experimental section, the noise level on the signal is directly proportional to the stray capacitance at the input of the JFET transistor. The bulk of the input capacitance in our system comes from the pick-up tube. If the tube capacitance can be made smaller by making the tube shorter and narrower, the noise level could be decreased. Using a smaller tube, however, would cause the event rate to decrease because the tube would intercept a smaller fraction of the ion beam.

An obvious means of improving R_q is to increase the amount of charge carried by each ion. The average charge on the ions trapped by the Smith group was two to four times what we have measured on ions of comparable size.[7] DNA is negatively charged in solution and most ES-MS analyses of oligonucleotides, including those of Smith and co-workers with megadalton ions, have been performed with negatively charged ions. When a solution containing 7.3 megadalton DNA was electrosprayed in negative mode with SF_6 to suppress corona discharge, no appreciable increase in the average charge state was observed over that seen for the same sample when sprayed in positive mode. However, we do not consider the results of that preliminary attempt to be conclusive. We did however observe higher average charge states after a sample of 8.0 MDa DNA was repeatedly washed with 10.0 mM ammonium acetate solution in an ultrafiltration cartridge. This desalting procedure doubled the average positive charge per mass compared to the untreated sample. Solution conditions effecting charge state need to be explored further. We anticipate that future efforts to increase the amount of charge carried by the DNA ions reaching our detector will rely on negative mode spraying as well as implementation of a commercial electrospray ion source.

Additional degradation of mass resolution in our instrument results from the distribution in electrostatic energy of ions in the source. In the ideal situation all ions are accelerated from the same electrostatic potential established by the voltages on the lens elements in the source. Figure 12 is a plot of relative ion count rate from the MCP detector as a function of the retarding potential on the grid using 2.8 MDa DNA ions. The lens voltages in the source were set to exclude lighter ions generated in the spray. Under these conditions most of the pulses from the MCP correlated with the passage of massive ions through the charge detector, although a substantial fraction of the ion flux consisted of lighter ions, most likely small solvated species. The plot reveals that roughly 65% of the ions had electrostatic energies of 290 volts as expected. The remainder of the ions had lower energies distributed from 290 ev to 0 ev. The presence of low energy ions in a CDMS mass spectrum creates a high mass tail in the peaks, as observed in several of the spectra e.g. Fig. 6 and Fig. 9(a). A possible cause for the broad energy distribution is the high pressure in the third pumping stage through which the ions are accelerated. The kinetic energy of an ion emerging from a free jet in the presence of an axial electric field will be the sum of three energy components: the energy imparted to the ion from the motion of the gas, the kinetic energy associated with the drift of the ion relative to jet at the point where the ion's motion ceases to be controlled by gas collisions, and the ion's electrostatic potential energy at that point. For megadalton DNA electrospray ions in our instrument, acceleration by the gas comprises the smallest component (<1%) of the ion energy. A collection of multiply charged ions, electrostatically accelerated through the gas jet, will have widely varying mobilities due to the distribution of charge states. They may consequently emerge from our electrospray ion

source with a wider distribution of ion energies because of variation in their drift velocities in the jet. While an estimate of the ion energy distribution could be used to partially correct the mass distributions recorded in our instrument, implementation of an ion source with lower background pressures provides a more practical means of improving instrumental resolution.

Several ESI-TOF mass spectrometers employ pulsed orthogonal deflection of ions from a low energy ion beam to provide more monoenergetic ion packets for time-of-flight analysis. The same could be considered in a CDMS system where the passage of an ion through a charge detector could provide the trigger for subsequent deflection. Alternatively, the signal from a primary detector could provide the trigger for a subsequent acceleration in the axial direction, followed by timing with a secondary detector. At the moment, most of our efforts toward improving this CDMS system are focused on detecting ion charge with higher precision. In light of that goal, we recognize that a charge sensing Faraday cup, in conjunction with any of several mass spectrometric methods, including the one described in this paper, may provide alternative means for performing CDMS on massive electrospray ions and particles. We are also considering remeasurement techniques based on single ion trapping to improve the statistics of charge measurement as well as other more sensitive charge detection schemes, including multiple detector measurements.

Applications of CDMS

Electrospray ionization in conjunction with CDMS has the potential for allowing accurate sizing of DNA fragments in a fraction of the time required for pulsed slab-gel electrophoresis. At present it provides size information for mixtures of megadalton DNA with a mass resolution of R < 10. For example, a single 1.2 MDa fragment ion (2000 base pairs) possesses a charge of about 600 e- which can be measured with a precision of +/- 75 e-, or 12.5% (+/- 250 base pairs, R=4). With this resolution two species with MW's of 2.0 and 2.7 MDa could be resolved into two separate mass peaks. A single 12 MDa ion (20,000 base pairs) can hold up to 6000 unit charges. Measuring that charge with a precision of +/- 75 e- will, in principle, lead to a 1.2% uncertainty in the estimate of ion mass (+/- 250 base pairs, R=40). At the moment ion beam energy spread and sample preparation limit our resolution for ions greater than 10 MDa . We anticipate that improved methods of charge detection will increase resolution for single ion mass measurements by as much as a factor of 10. In contrast to the resolution, the mass accuracy of the calibrated instrument for measuring pure samples is relatively high, and is based on the averaging of many single ion mass measurements. A set of four separate mass assignments, each made by averaging 1000 ions of 7.3 MDa DNA from four different experiments indicated a mass of 7.44, 7.40, 7.29, and 7.34 MDa. The average was 7.36 MDa and had a coefficient of variation which was less than 1%.

The task of mapping and sequencing the human genome's 3 billion nucleotide base pairs requires a means of routinely sizing large and small fragments of DNA. Gel electrophoresis of large fragments above 10 kilobase pairs in length (> 6.4 MDa) has traditionally been a slow analysis method requiring from hours to days with only modest mass resolving power.[25] Recent electrophoresis experiments using capillaries have demonstrated dramatically decreased analysis times (~ 10 min.) but still require internal size standards for each run.[26, 27] TOF-MS creates data in digital form; an advantage when it comes to processing the data to extract size information. A

charge detection mass spectrometer with even low mass resolution (R ~ 50) could provide sufficiently accurate mass information to molecular biologists needing to size megadalton DNA fragments.

Molecules and particles of virtually unlimited mass can be ionized with electrospray, as has been previously demonstrated with electrosprays containing colloidal suspensions of solid particles,[28] as well as by data presented in this work. It is conceivable that CDMS will find a role in the analysis of a variety of fine particles of biological interest, including DNA fragments and chromosomes, viruses,[29] viral DNA, and other cellular constituents as well as entire cells. In addition to the ion mass, CDMS might provide information about particle shape and surface chemistry via the ion charge.

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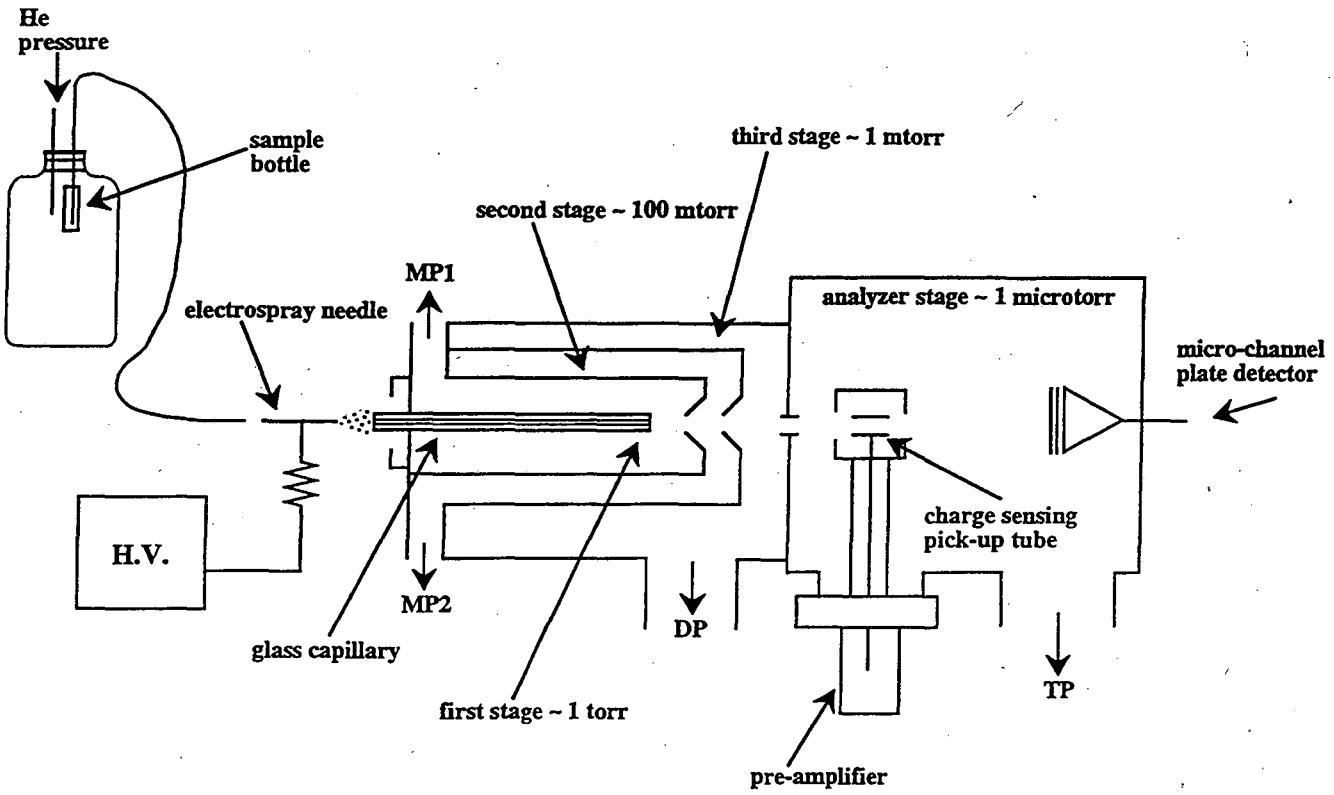


Fig.1 - Experimental layout. Typical lens voltage settings are 320 V on capillary nozzle, 300 V on skimmer 1, and 280 V on skimmer 2. Ions are accelerated primarily between skimmer 2 and the inlet plate orifice which is grounded. The distance from skimmer 2 to the center of the charge detector is 10 cm. The MCP detector is primarily used for diagnostic purposes.

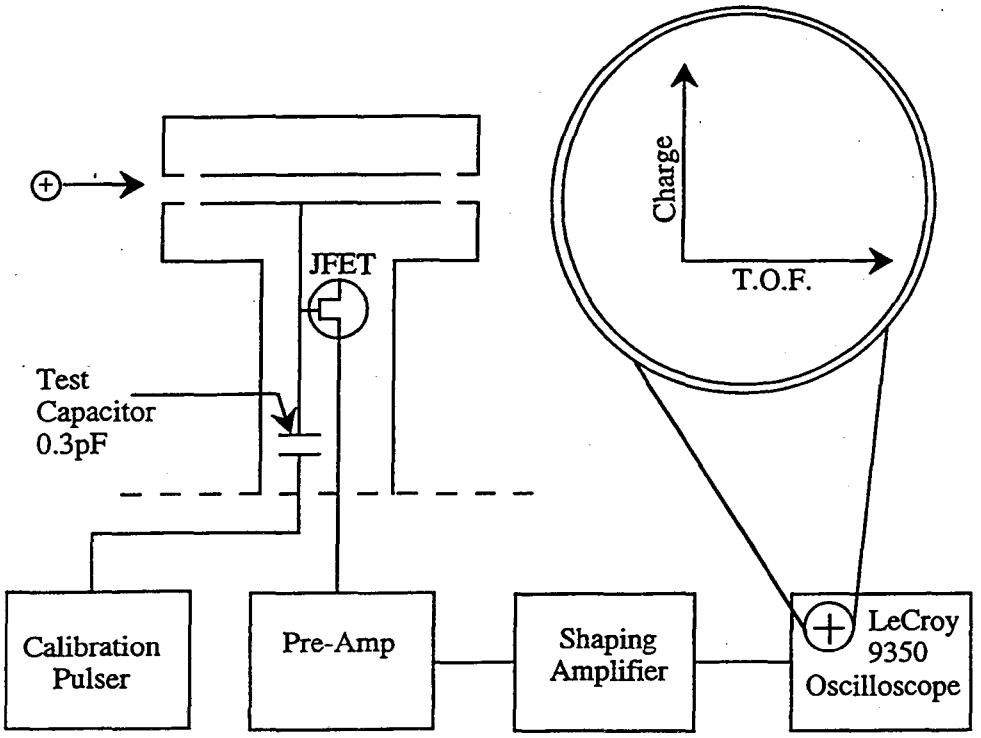


Fig. 2 - Charge detector and amplifier set up. The shielded 3.5 cm brass pick-up tube is connected to the input of a low noise transistor. The capacitance present at the input is calibrated with test voltage pulses across a known capacitance of 0.3pF. The signal from a passing ion yields both timing and charge information.

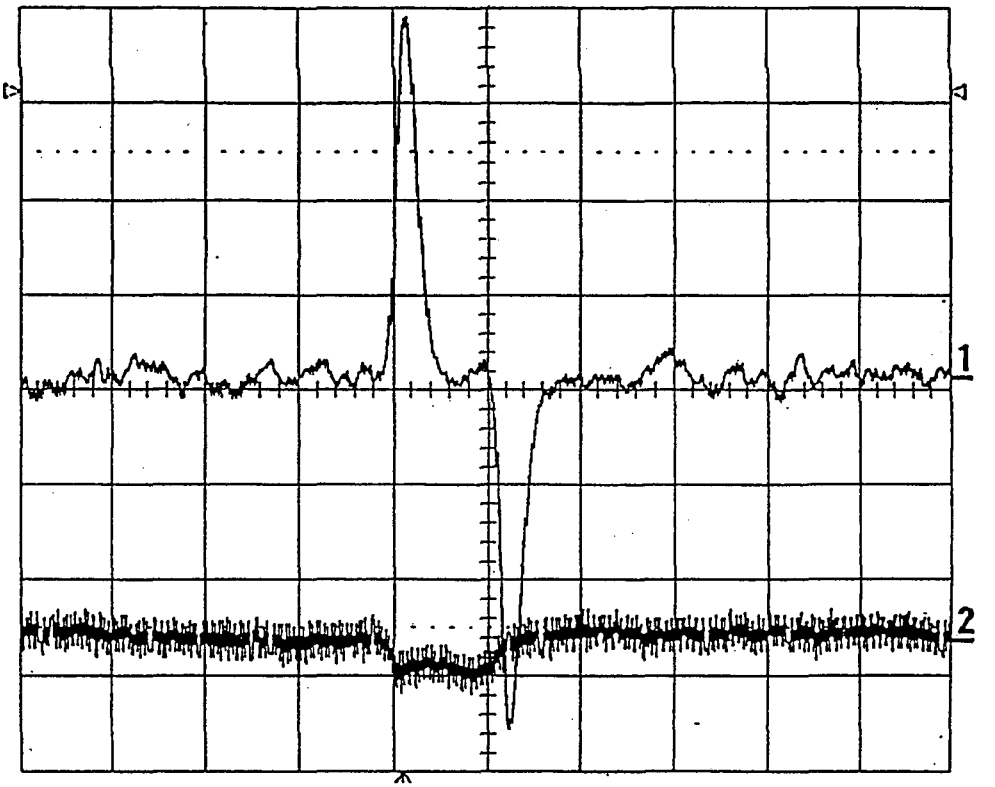


Fig. 3 - Oscilloscope traces of the signal from a single ion of DNA (sample MW = 6.4 MDa). The lower trace is the signal from the pre-amp (2 mV/div.) and the upper trace is the differentiated signal from the shaping amplifier (100 mV/div.). The time scale is 10 μs/div. Based on the top trace amplitude, the ion carries about 1900 charges (0.2e-/mV) and is traveling at a velocity of 2900 m/s. Lens settings: V_{cap}=200, $V_{skimmer\ 1}$ = 200, $V_{skimmer\ 2}$ = 150.

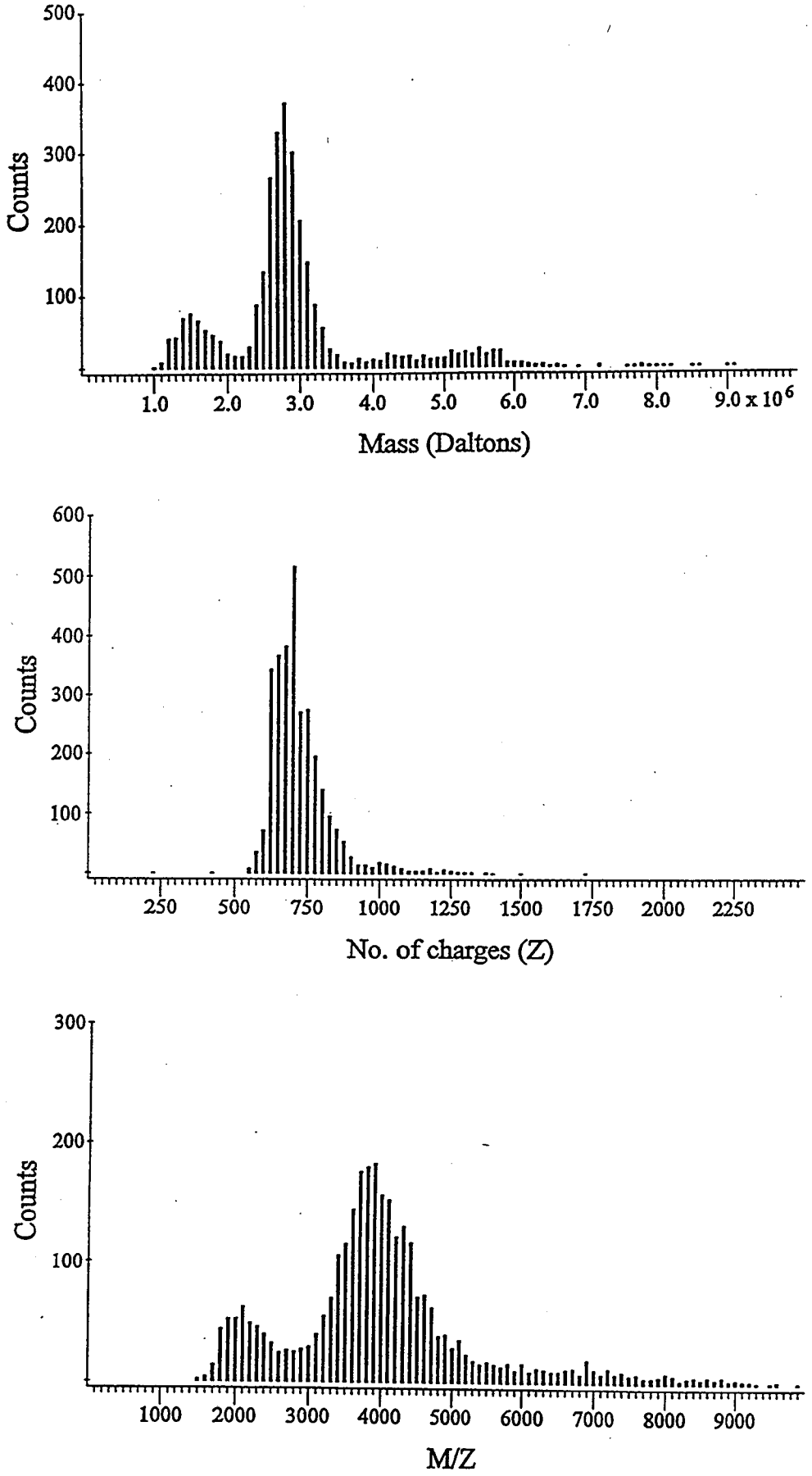


Fig. 4a-c - A histogram mass spectrum (4a) of 2.8 MDa pBR322 DNA showing evidence for single stranded and double stranded molecular ions. The associated distributions of positive charge (4b) and m/z (4c) indicate that most ions had between 600 and 800 charges, while m/z varied by a factor of 3.

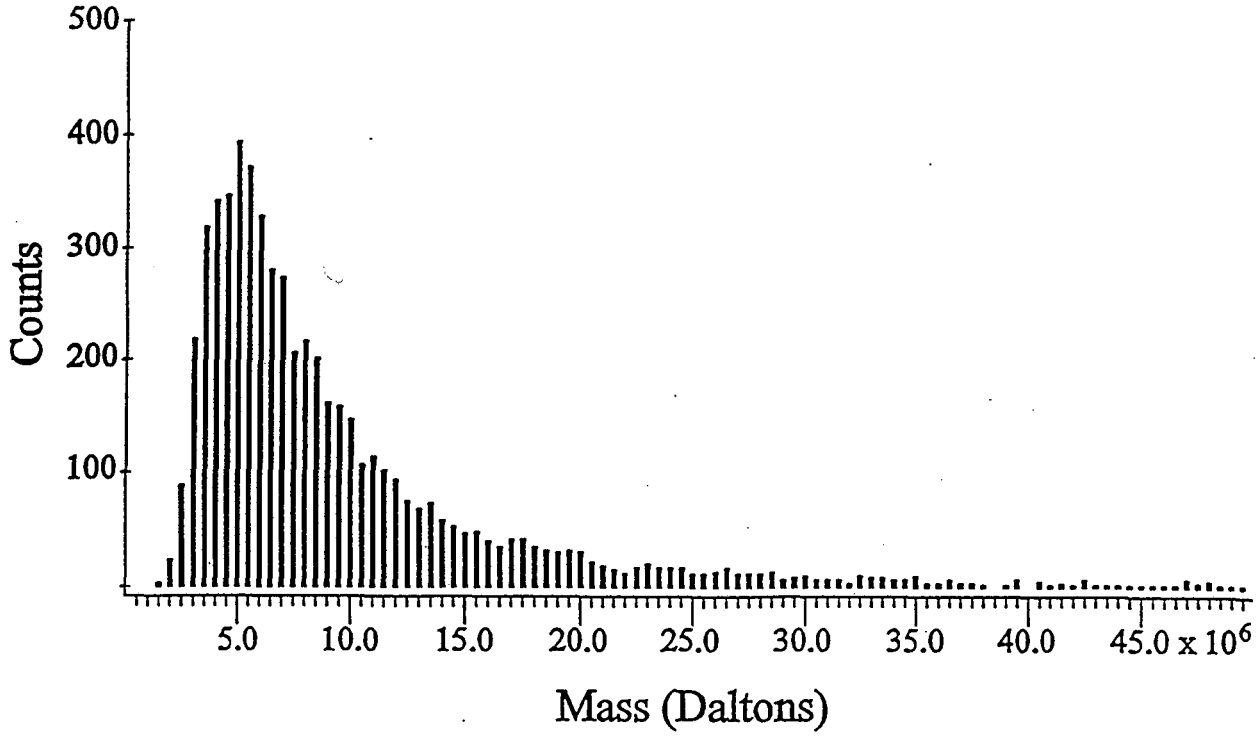


Fig. 5 - Mass spectrum of polyethylene oxide (nominal mean MW = 7 MDa). Positive mode electrospray of 0.01 g/L solution in 50% MeOH in water. The spectrum indicates that the sample mixture contained polymer molecules with masses out to 20 million Daltons.

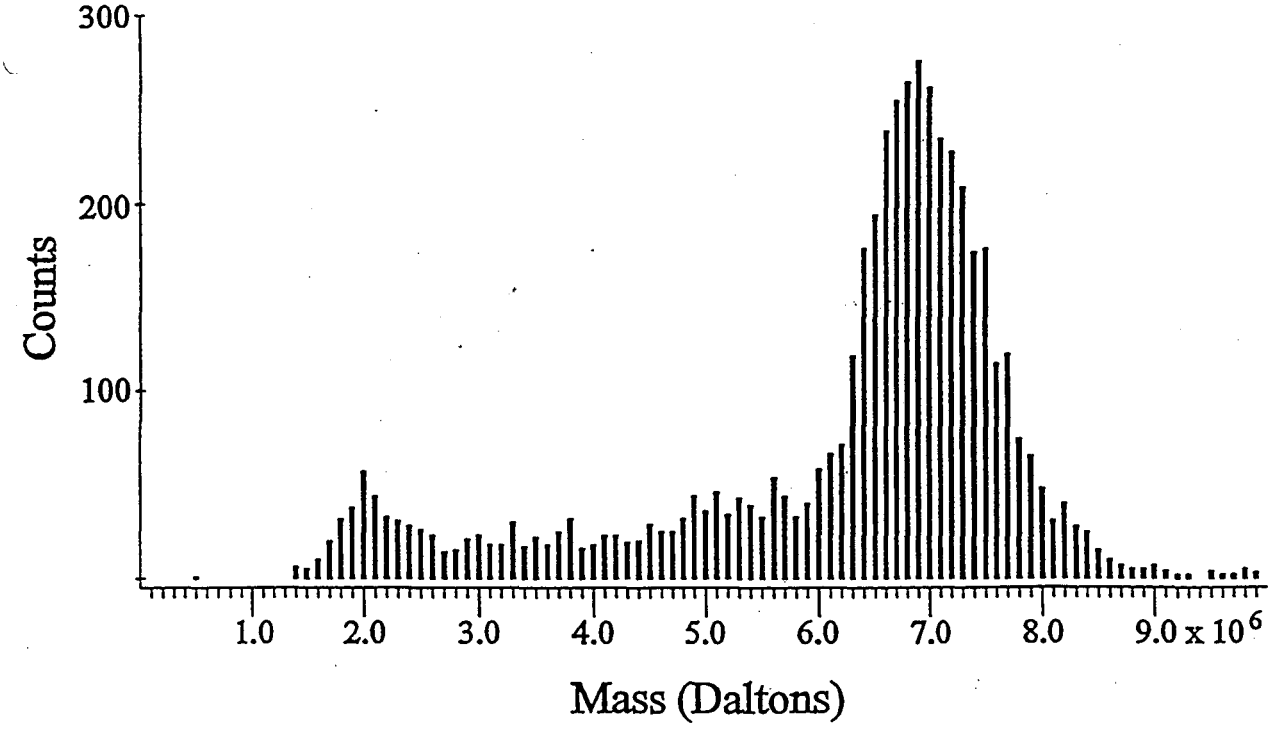


Fig. 6 - Electrospray mass spectrum of Col E1 plasmid DNA (MW=7.3 MDa). Data represent over 3000 single ion measurements recorded over a twenty minute period.

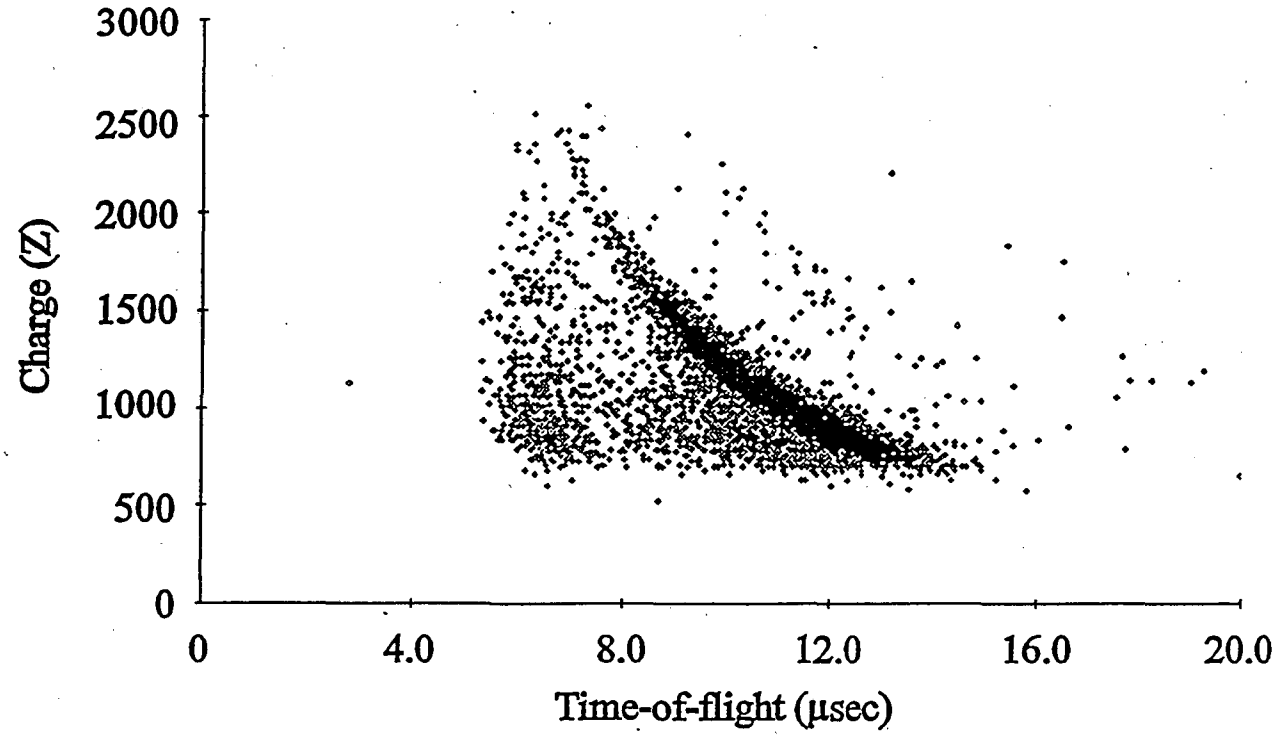


Fig. 7 - Raw data used to calculate the masses in Fig. 6. Ions with more charge take less time to pass through the pick-up tube while those of equal mass but having less charge take longer. The result is the crescent shaped cluster of ions corresponding to the main peak in Fig. 6.

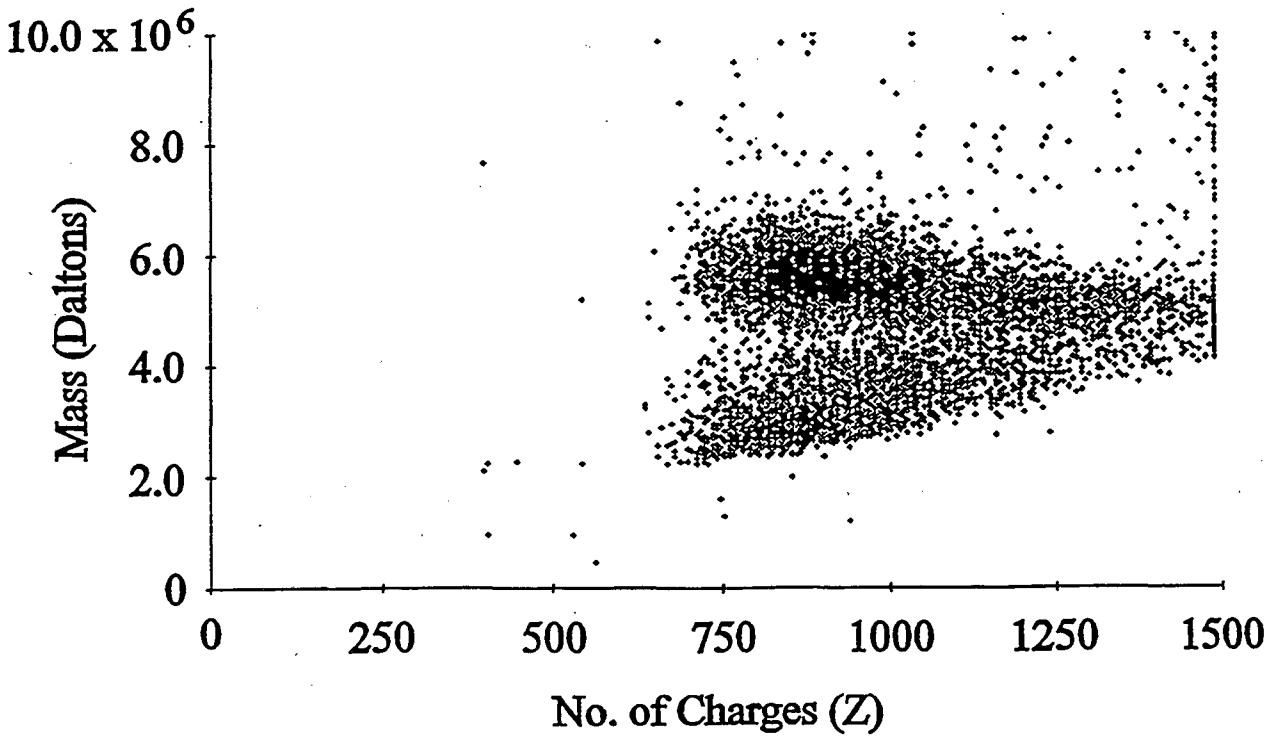
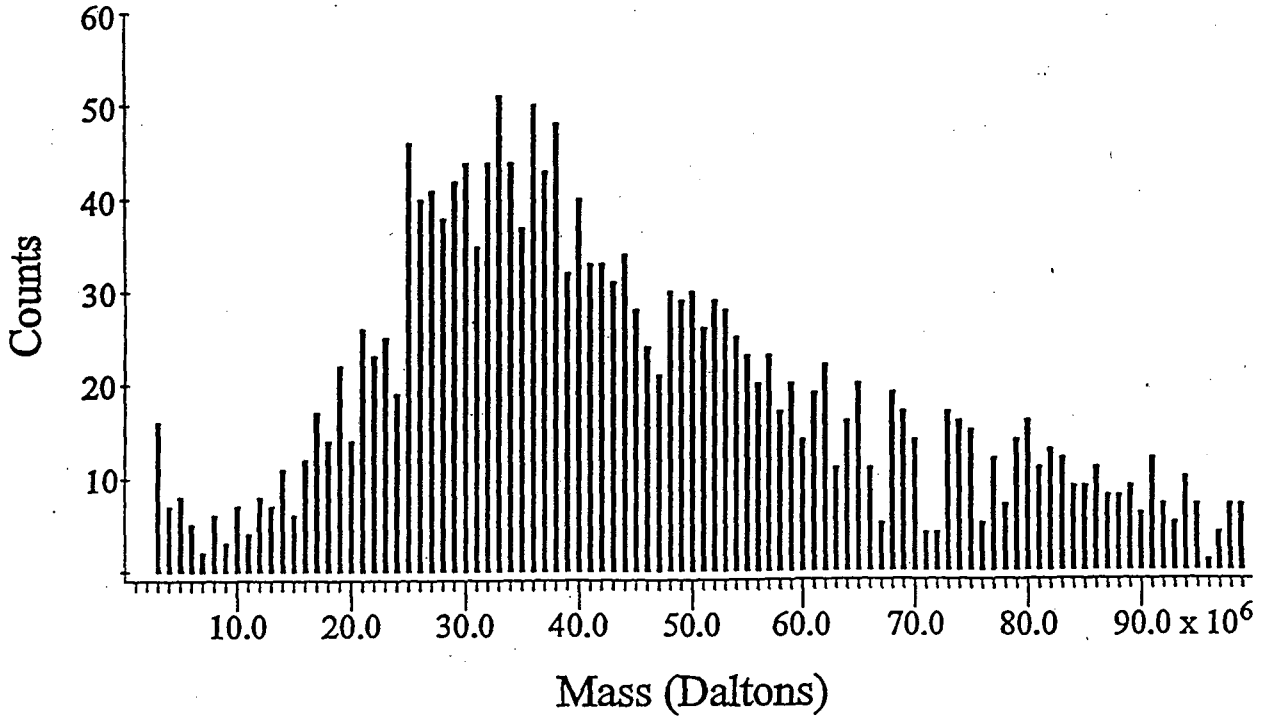


Fig. 8 - CD-MS can provide unique data sets as is illustrated in this scatter plot depicting the relationship between mass and charge. The line of points at right results from clipping at the maximum amplitude of the oscilloscope scale.



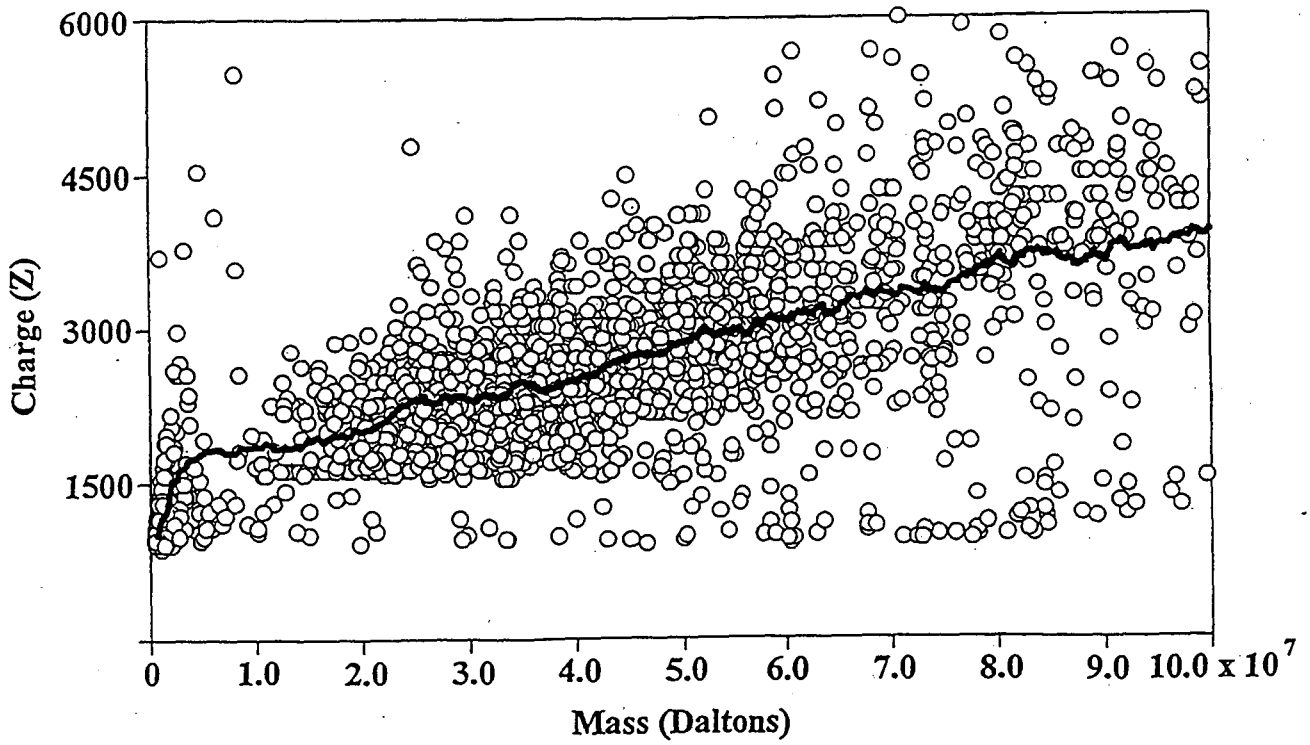


Fig. 9 a-b - Mass spectrum from Lambda Phage DNA (MW=31.5 MDa) showing broad mass distribution despite high charge state. The sample was not tested for purity and might have contained additional fragments of chromosomal DNA. A 100 point moving average of charge vs. mass relationship (b) shows linear trend, suggesting that these gas phase ions are elongated.

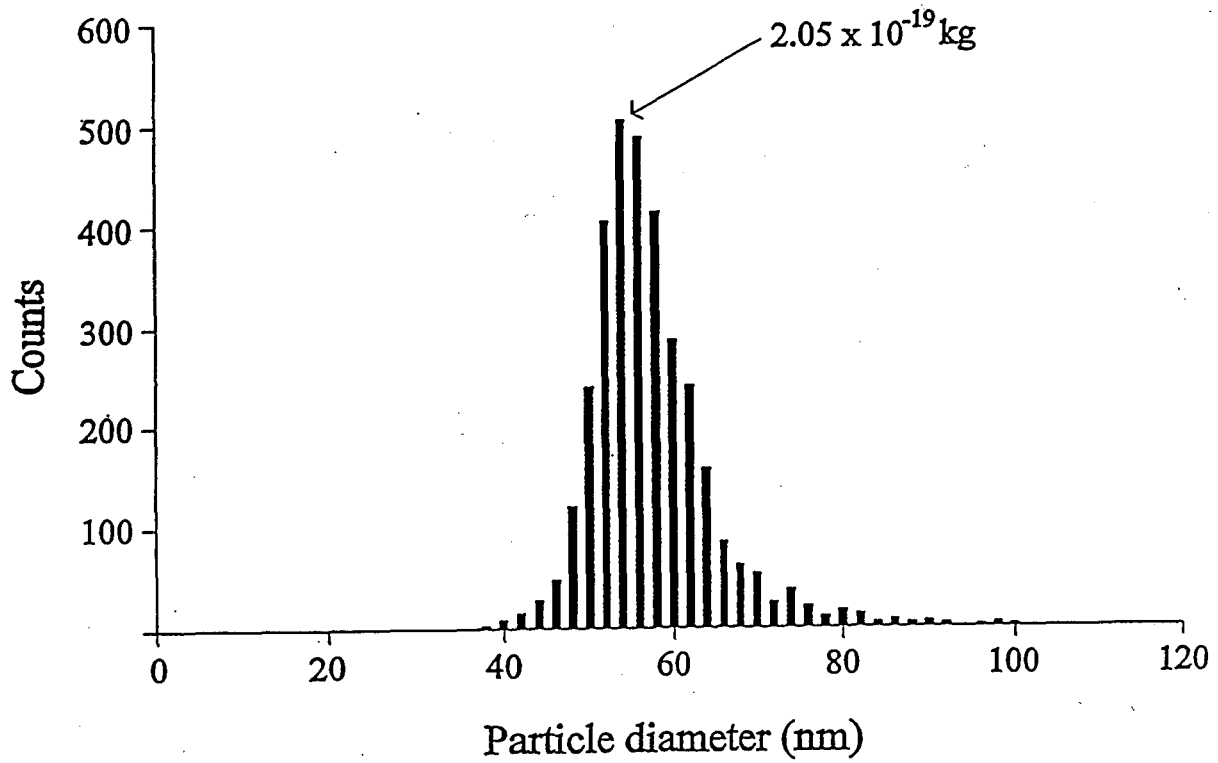


Fig. 10 - Size distribution from CD-MS mass measurement of residue particles from an electrospray of 0.2 g/L Cytochrome C solution. Evaporation of micron diameter initial droplets would result in residue particles of 50-60 nm assuming a particle density of 1.5 gr/cm^3. Mean particle mass equivalent to 100 MDa.

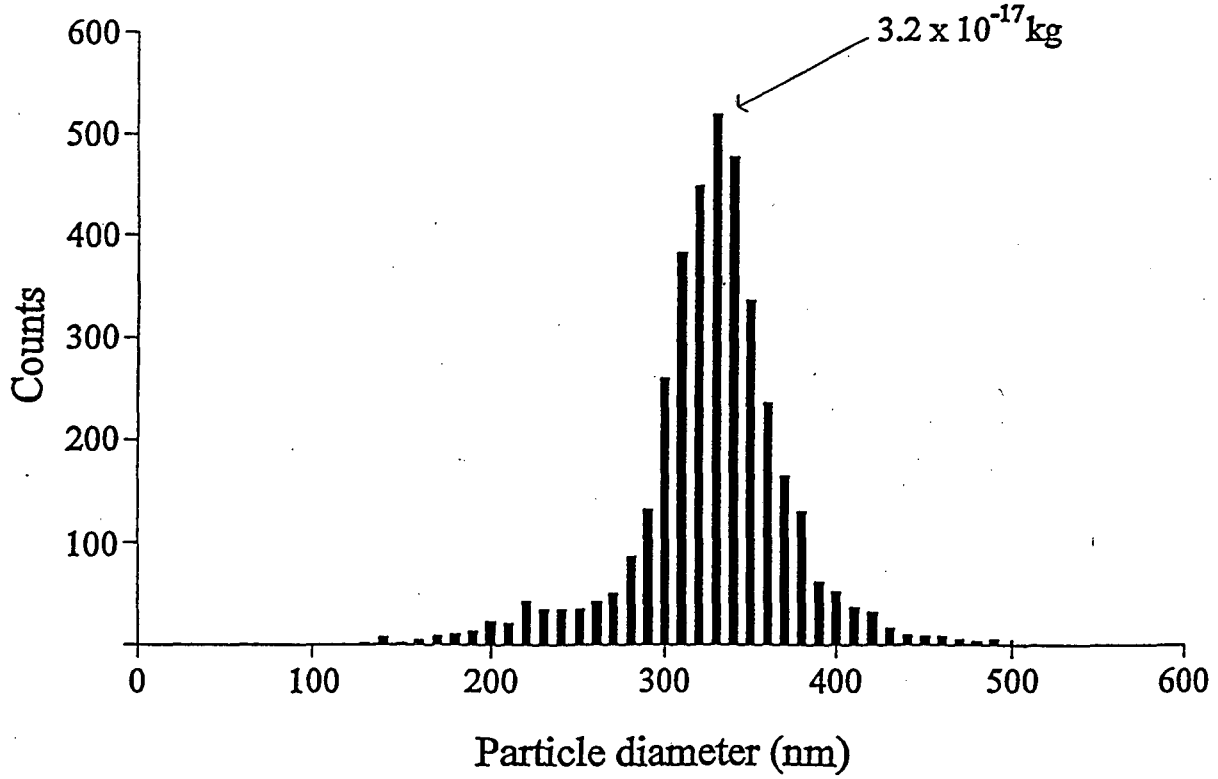


Fig. 11 - Size spectrum of polystyrene microspheres obtained by charge detection mass spectrometry. Particles had a nominal diameter of 314 nm (Duke Scientific) and had a density of 1.05 gr/cm^3. Equivalent mean mass is 1x10^{10} Da. Average z was 2500.

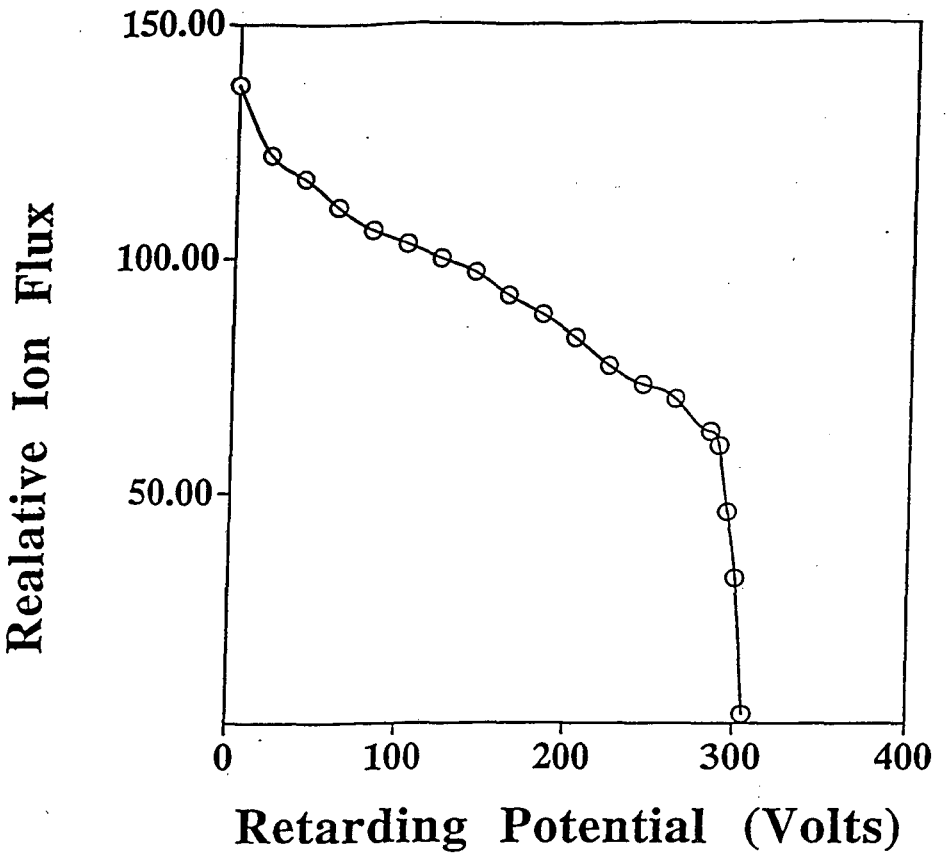


Fig. 12 - Retardation potential measurement of ion kinetic energy distribution for a beam containing primarily massive, highly charged DNA ions. Ions with less energy than the acceleration potential will appear to have higher than actual masses. Lens settings were : V_{cap}=265, $V_{skimmer\ 1}$ = 300, $V_{skimmer\ 2}$ = 280, $V_{inlet\ plate}$ = 0.

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