# Preparing Protein Microarrays by Soft-Landing of Mass-Selected Ions

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# Preparing Protein Microarrays by Soft-Landing of Mass-Selected Ions


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**Intact, multiply protonated proteins of particular mass and charge were selected from ionized protein mixtures and gently landed at different positions on a surface to form a microarray. An array of cytochrome c, lysozyme, insulin, and apomyoglobin was generated, and the deposited proteins showed electrospray ionization mass spectra that matched those of the authentic compounds. Deposited lysozyme and trypsin retained their biological activity. Multiply charged ions of protein kinase A catalytic subunit and hexokinase were also soft-landed into glycerol-based liquid surfaces. These soft-landed kinases phosphorylated LRRASLG oligopeptide and D-fructose, respectively.**


Patterning of biological macromolecules onto surfaces in the form of microarrays ("chips") produces a sample format suited to automated analysis for biological activity (*1*–*4*). The archetypal case is the widely used DNA chip, where a high spot density (10,000 spots/cm$^2$) is created by automated procedures that supply the constituent nucleotides to preselected positions. It is widely expected that the development of proteomics and the application of combinatorial chemistry methods in drug discovery will mandate similar sample formats for studies of proteins and other biological compounds. Techniques developed for deposition of macromolecules onto solid supports include microdispensing (*5*), electrospray deposition (*6*), robotic printing (*7*), stamping (*8*), and ink jet deposition (*9*).

Ion soft-landing is another method with potential value in chip production (*10*). The soft-landing of molecular ions onto surfaces, first proposed (*11*) in 1977, was later demonstrated (*12*) with low–kinetic energy (typically 5 to 10 eV) mass-selected ion beams at polyfluorinated self-assembled monolayer (SAM) surfaces. Mass spectrometric analysis was used to confirm the presence of sterically bulky soft-landed organic ions such as *N*,*N*-dimethylisothiocyanate. Simple organic cations (*13, 14*) and a 16-nucleotide double-stranded DNA (*15*) (mass ~ 10 kD) have also been soft-landed intact onto surfaces, as have metal clusters (*16*). In some of these cases there is evidence that the molecular entity on the surface is the ion (*12*); in others, it is the corresponding neutral molecule. In one striking experiment, virus particles were ionized, crudely mass-selected in a time-of-flight instrument, and showed evidence of activity after deposition on a collector plate (*17*).

The use of mass spectrometry as a separation method is expected to provide high selectivity because components having different molecular formulae, including isotopomers, can be separated and individually soft-landed. This should include separate glycoforms of proteins, individual polysulfonated forms, and other posttranslationally modified variants [the mass difference due to acetylation of an immunoglobulin G antibody can be resolved with a mass resolution of 3000]. In principle, too, the technique should exhibit good spatial resolution because ion beam spots of micrometer dimensions are attainable. Potential disadvantages are the relatively small amounts of ionized material and the possibility of alteration of delicate biological molecules in the course of ionization, mass analysis, or soft-landing.

To investigate the possibility of soft-landing of mass-selected multiply charged protein ions, we ionized mixtures of up to four well-studied proteins—cytochrome c, lysozyme, insulin, and apomyoglobin (*18*)—by electrospray ionization (ESI). The proteins were selected individually by mass/charge ratio ($m/z$) and deposited onto various surfaces (*19*, *20*). The experiments were performed with two instruments: a commercial SSQ-710C (Thermo Finnigan, San Jose, California) quadrupole mass spectrometer that was modified by addition of a surface and its surface moving stage and a custom-built instrument that used a Thermo Finnigan linear ion trap (LIT) (*21*) shown in Fig. 1. This latter instrument includes an ESI ion source, a high-capacity LIT, wave form capabilities for ion isolation, a radial detector for mass analysis, and facilities for low-energy transfer of trapped, mass-selected ions onto specific spots on an electronically movable target. In comparison with the SSQ instrument, the LIT provides considerably higher ion flux (on the order of $10^9$ to $10^{10}$ s$^{-1}$), the opportunity to check the spectrum of the material to be landed by in situ mass analysis, and more control over the landing energy. The landed proteins were redissolved into a methanol/water (1:1) rinse solution. The rinse solutions were examined by ESI with an LCQ Classic mass spectrometer (Thermo Finnigan). In separate experiments, matrix-assisted laser desorption ionization (MALDI) was used to examine the deposited proteins in situ (*22*).


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Figure 2B shows the ESI mass spectrum of a mixture of the four proteins, indicating the regions of the mass spectrum selected for soft-landing. A photograph of the landed material is shown in Fig. 2A. No ion beam focusing was used in these preliminary experiments. Figure 2, C to F, shows the ESI spectra resulting from analysis of rinse solutions for each spot. These spectra contain only multiply charged ions of the corresponding protein (in the high-mass range, $m/z > 500$) and show no evidence for fragmentation or cross-contamination. A total of 480 μl of a solution ($10^{-7}$ to $10^{-6}$ M in each protein) was sprayed, and the amounts of proteins recovered (in the 10-ng range) indicate that multilayer deposition occurs and that neutralization occurs subsequently to landing. These quantities do not represent detection limits, and recovered amounts of proteins are roughly estimated as 5% overall transfer and landing efficiency for cytochrome c and apomyoglobin, but only 0.5% for lysozyme. Subsequent experiments revealed that neither the landing efficiency nor the collection of native proteins depends on the selected charge state of the proteins. However, the landing energy (and type of surface) was a critical parameter, and fragmentation ("crash landing") was observed at higher energies (*23*).

To investigate the biological activity of soft-landed proteins, we separated a mixture of two enzymes, trypsin and lysozyme, with the SSQ-710C mass spectrometer. The pure proteins were soft-landed together with two blanks generated by landing from a mass/charge region ($m/z$ 200 to 210) containing no protein ions. In this initial experiment, extracellular enzymes containing no prosthetic groups were chosen to ensure their structural stability throughout the ionization and soft-landing process. The bioactivity of landed lysozyme was tested with hexa-*N*-acetyl chitohexaose (Seikagaku, Tokyo, Japan) as substrate. Cleavage products of the substrate were detected by MALDI; data collected in the reflectron mode are shown in Fig. 3. The specific activity of soft-landed lysozyme was determined from two parallel soft-landing experiments. $[\text{Lysozyme} + 8\text{H}]^{8+}$ ions were soft-landed on a gold substrate for 1 hour, and the landed material was quantified by ESI-MS from the intensity of the lysozyme ions with charges from 7 to 11. Quantitative results were obtained by comparison with ion intensities from a series of calibration experiments. Landed material from the parallel experiment was assayed with hexa-*N*-acetyl chitohexaose (Seikagaku) as a substrate, using ESI-MS for quantification. The amount of landed lysozyme was 1.8 ± 0.3 ng as determined by direct quantification and 2.0 ± 0.1 ng based on the bioassay. From these results we conclude that lysozyme retains its bioactivity virtually completely when soft-landed under these experimental conditions.

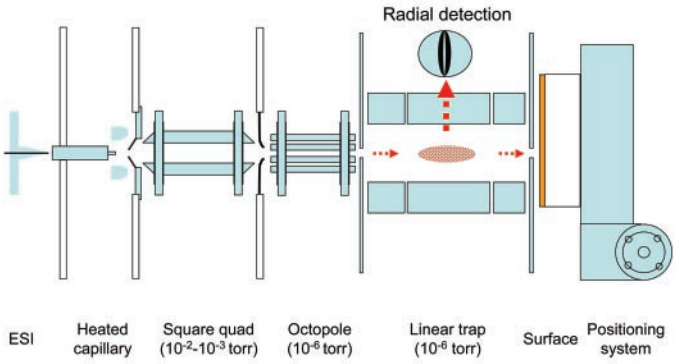


**Fig. 1.** Ion soft-landing instrument including ESI ion source and Thermo Finnigan linear ion trap with capabilities for radial mass analysis, trapping of ions of selected $m/z$, and axial deposition of the selected ions onto the target surface.

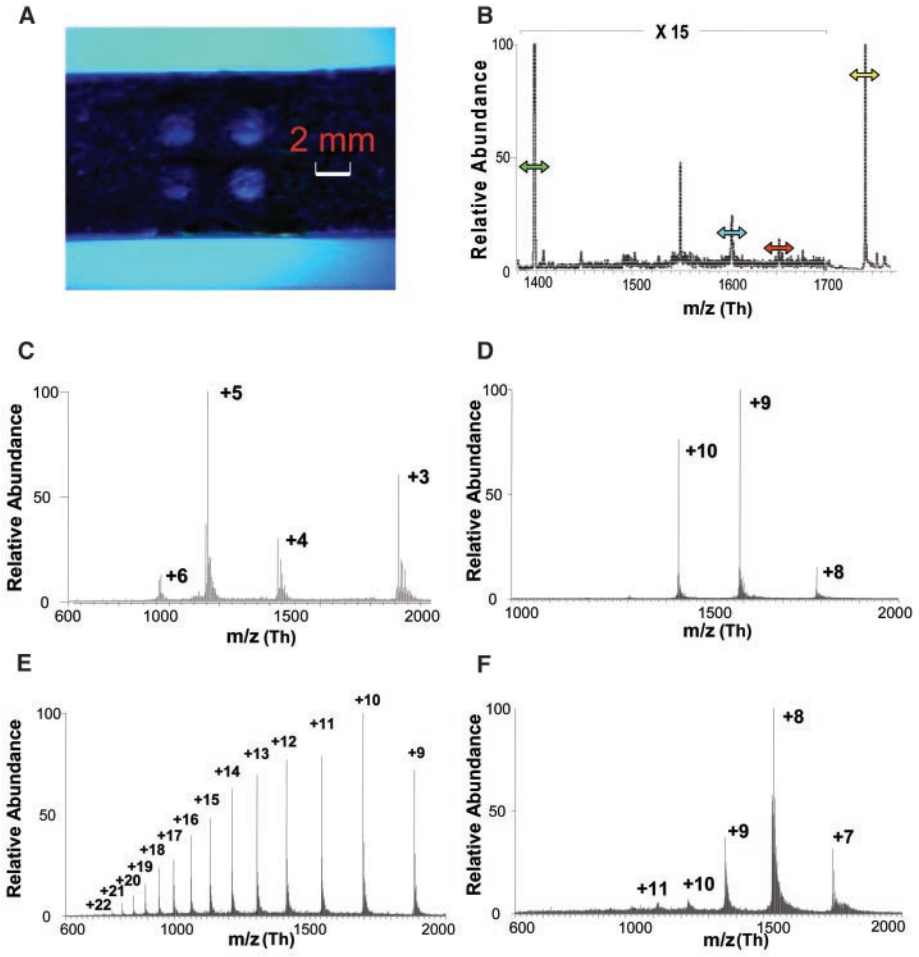


**Fig. 2.** (**A**) Photograph (in blue light) of a microarray of four proteins soft-landed onto a gold substrate; each spot is 1 mm in radius. (**B**) ESI mass spectrum of a mixture of 2.5 μg $\text{ml}^{-1}$ cytochrome c (molecular mass 12,360 daltons), 2.5 μg $\text{ml}^{-1}$ lysozyme (molecular mass 14,316 daltons), 2.5 μg $\text{ml}^{-1}$ insulin (molecular mass 5734 daltons), and 2.5 μg $\text{ml}^{-1}$ apomyoglobin (molecular mass 16,951 daltons) in methanol/water 1:1 used for soft-landing. The ions of +9 charge state of cytochrome c ($m/z$ 1359), +11 charge state of lysozyme ($m/z$ 1301), +4 charge state of insulin ($m/z$ 1398), and +15 charge state of apomyoblobin ($m/z$ 1135) were selected for ion soft-landing based on a window of 5 $m/z$ units. Nominal charge landed for each protein was on the order of $10^{-6}$ C. Mass spectra of the soft-landed proteins after rinsing the spots: (**C**) insulin, (**D**) lysozyme, (**E**) apomyoglobin, and (**F**) cytochrome c.

The bioactivity of landed trypsin was tested by digesting cytochrome c on the surface. Characteristic tryptic fragments of cytochrome c were detected in situ by MALDI. The blank spots did not show any bioactivity, which indicates that neutral species produced in the ion source did not have access to the surface. In another experiment performed un-

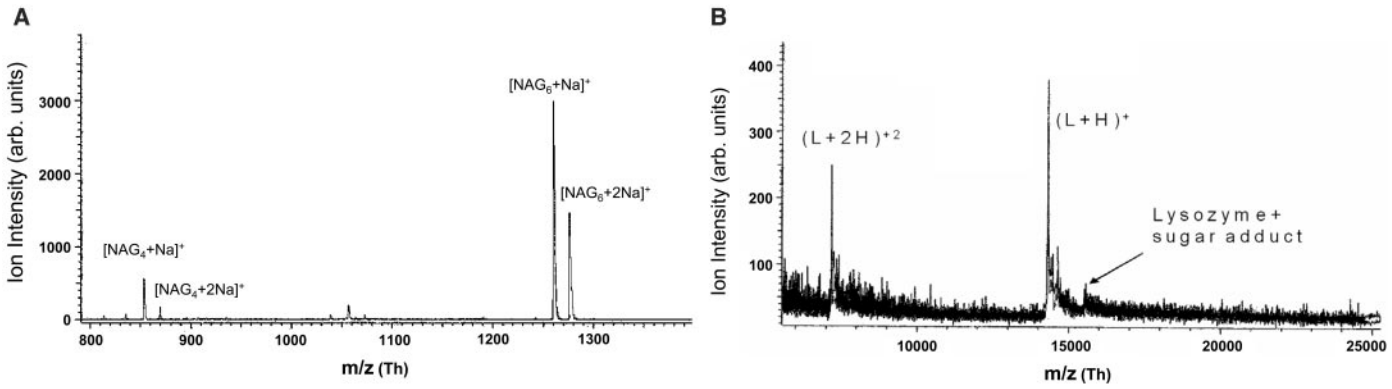


**Fig. 3.** (**A**) Low-mass MALDI spectra showing sodiated hexa-*N*-acetylchitohexaose ($NAG_6$) and a cleavage product (tetra-*N*-acetylchitotetraose) ($NAG_4$) formed during in situ tests of biological activity. (**B**) High-mass MALDI spectrum showing the singly and doubly charged ions of intact lysozyme (L) and the enzyme-substrate complex.

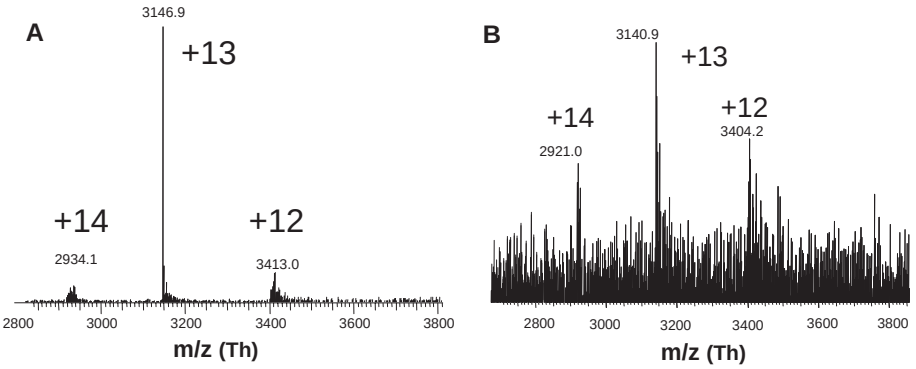


**Fig. 4.** Electrospray mass spectra of PKAc (molecular size 40,856 daltons) (**A**) used for soft-landing and (**B**) recovered from soft-landing on a gold surface by rinsing the surface with 50 μl of ammonium acetate buffer (0.02 M).

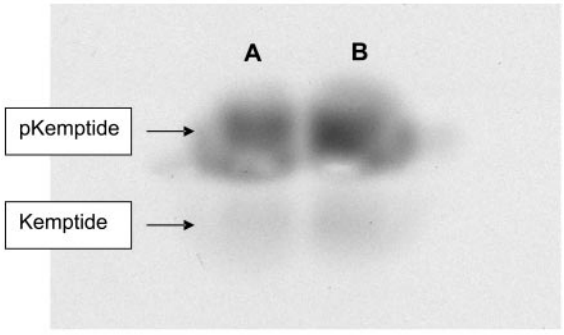


**Fig. 5.** Electropherogram from the assay of landed PKAc (**B**) and of a positive control experiment (**A**) using Promega PepTag assay (catalog number TB132). PKAc molecular ions of all charge states (Fig. 4A) were soft-landed into a glycerol surface for 1 hour. Enzymatic activity was assayed based on phosphorylation of fluorescently labeled Kemptide in the presence of ATP and $Mg^{2+}$ and electrophoretic separation of labeled Kemptide and phospho-Kemptide. For the positive control experiment, 1 ng of enzyme was used.

der similar landing conditions, the bioactivity of trypsin was tested with neurotensin as substrate. After a 24-hour digestion of neurotensin with soft-landed trypsin, characteristic tryptic fragments of the substrate at *m/z* 516 and 661 were detected by ESI-MS. We confirmed that these ions were tryptic fragments by performing collision-induced dissociation and comparing the data with the authentic spectra of the tryptic fragments. These results indicate that enzymes can retain their activity through the ionization, mass selection, and soft-landing processes. It is unclear whether the proteins unfold while undergoing this series of events and refold when being resolvated or if they retain their secondary-tertiary structure throughout.

An application of this method in protein purification is illustrated by an experiment in which an impure sample of lysozyme was subjected to ESI and the +10 charge state of the ionized molecule was selected and soft-landed into a glycerol/fructose liquid surface under high vacuum. Ultrafiltration of the liquid matrix and reexamination by ESI gave a spectrum that showed excellent signal/noise separation and a charge state distribution characteristic of lysozyme (*20*). In other experiments, we have shown that these viscous liquid matrices are suitable for measuring bioactivity of some enzymes—for example, lysozyme and trypsin.

Traditional chemical and biochemical analysis could be performed either before or after soft-landing. For example, lysozyme was detected in situ by MALDI after landing onto SAM surfaces bearing carboxyl and pyridine *N*-oxide functional groups, and trypsin was landed into a liquid surface and in situ analysis performed by cleaving cytochrome c to give characteristic peptide fragments. Glycerol-based liquid surfaces provide a suitable soft-landing medium for protein ions because they offer the possibility of resolvation upon landing. Several proteins lose activity irreversibly upon dehydration, and this phenomenon might be avoided by the use of carbohydrate additives (*24*).

The feasibility of soft-landing of delicate proteins with high biological relevance was tested by landing different kinases into vacuum- and protein-compatible liquid surfaces (*20*). Bovine protein kinase A catalytic subunit (PKAc; molecular mass 40,856 daltons) and yeast hexokinase (molecular mass 52,360 daltons) were ionized and soft-landed onto various surfaces. Figure 4, which shows the mass spectrum of PKAc before and after soft-landing, demonstrates that the protein can be landed intact. PKAc ions putatively assigned to the original folded structure were mass-selected and soft-landed into a glycerol/fructose/water 65:35:5 liquid surface containing adenosine 5′-triphosphate (ATP) Mg salt and the substrate Kemptide, i.e., LRRASLG. Analysis of the liquid surface after incubation by ESI-MS/MS showed the presence of phospho-Kemptide (LRRApSLG), demonstrating retention of biological activity. Similar experiments done with hexokinase in the presence of fructose yielded fructose-6-phosphate (*25*). The specific activ-

ity of the soft-landed PKAc was estimated on the basis of the phosphorylation assay (Fig. 5) and estimated ion currents. This rough estimate gave values of ~50% of the specific activity of the original enzyme preparation in several different experiments. In this series of experiments sensitive, intracellular enzymes were ionized by electrospray under mild conditions (physiological pH, aqueous media), and mass-selected multiply charged ions were transferred to another hydrophilic medium represented by the glycerol liquid surface. The fact that these kinases survived this process and retained their biological activity is evidence that soft-landing of active enzymes is feasible for various eukaryotic (e.g., human) cytosolic proteins.

Mass spectrometry represents a separation method that is complementary to chromatography or electrophoresis. Traditional methods may fail to separate species having highly similar structures, but mass spectrometry should be successful as long as the molecular weights are different. Although the ionization of complex protein mixtures can suffer from suppression effects (*26*) in either ESI or MALDI, these will be minimized when the analytes are highly similar, as in the case of mixtures of synthetic (*27*) and biological (*15*) polymer congeners like polyclonal antibodies, glycoproteins, and polysulfonated or phosphorylated products of posttranslational modification. Collection of "fractions" in a soft-landing experiment can be performed easily, by moving either the target (as demonstrated) or the ion beam on the surface to yield an array of the separated components of the initial mixture. These arrays could also provide tools for combinatorial chemistry–based methods, like identification of target molecules (*28*).

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18. A four-protein mixture of apomyoglobin, cytochrome c, insulin, and lysozyme (0.0025 mg/ml each) in 1% acetic acid in 1:1 MeOH:$H_2O$ (v/v) was used for soft-landing of the four-spot array. Proteins were obtained from Sigma (St. Louis, MO).
19. Various surfaces, including aluminum, gold, various functionalized SAM surfaces, and some liquid surfaces, have been used as substrates for ion soft-landing. The data shown in this report are for gold and various liquids.
20. Details of protein-purification methods and of the bioassays performed are available on *Science* Online.
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22. MALDI sample preparation was carried out by applying 1 μl of saturated sinapinic acid solution in 0.1% aqueous trifluoroacetic acid/acetonitrile 2:1 onto the landed material. The dried sample was analyzed with a Bruker Reflex III instrument, in the positive ion mode, with delayed extraction. Singly and doubly protonated ions of lysozyme, cytochrome c, and apomyoglobin were detected in the linear mode.
23. Characteristic fragments of lysozyme were detected on the surface by MALDI in the case of COOH-terminated SAM at 10 eV/charge collision energy.
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# Simultaneous Fluorescence and Raman Scattering from Single Carbon Nanotubes


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**Single-molecule fluorescence spectroscopy was used to determine the electronic properties of individual single-walled carbon nanotubes. Carbon nanotube structure was determined simultaneously from Raman spectroscopy. Fluorescence spectra from individual nanotubes with identical structures have different emission energies and linewidths that likely arise from defects or the local environment. Unlike most other molecules studied to date, the fluorescence intensity or spectrum from a single nanotube unexpectedly did not fluctuate.**


Single-walled carbon nanotubes (SWNTs) are synthesized as mixtures of metallic and semiconducting tubes (*1*). Their individual structures can be characterized by two integers [(*n*,*m*)] that define both their diameter and chirality (*2*); if ($n - m$) is not divisible by 3, the nanotubes are semiconducting. Recently, the photoluminescence of SWNT mixtures suspended in surfactant micelles in water was characterized as arising from band-gap fluorescence from semiconducting tubes with different structures (*3*, *4*). Such a spectrum (Fig. 1A) (*5*) contains overlapping fluorescence features. However, ensemble averaging obscures the true spectral linewidths and the details of the band shape. These optical properties are likely needed for the development of SWNT photonic applications, such as nanometer-scale, integrated electroluminescent devices (*6*).


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We measured the electronic structure of individual SWNTs using single-molecule photoluminescence spectroscopy. Although the spectra from individual SWNTs with identical diameters were similar, they exhibited a distribution of peak positions and linewidths not observed in ensemble studies of isolated SWNTs (*3*, *4*, *7*). Unlike most single molecules (*8*) or semiconductor nanoparticles (*9*), the fluorescence for SWNTs does not show any spectral or intensity fluctuations.

Spatially isolated individual SWNTs were achieved by spin-coating ~75 μl of the SWNT suspension onto a glass coverslip (*5*). Fluorescent samples are known to contain short (~200 nm long) SWNTs isolated in micelles (*3*). Thus, we expected the spin-coating procedure to result in single SWNTs well dispersed on a thin surfactant layer. Indeed, atomic force microscopy measurements revealed predominantly short SWNTs (with lengths of 200 to 300 nm) on top of residual surfactant patches a few nanometers thick. Optical characterization of SWNT coverage was achieved through confo-