

REAL-TIME MONITORING OF PROTEIN COMPLEXES REVEALS THEIR QUATERNARY ORGANIZATION AND DYNAMICS

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SUMMARY

The dynamics of protein complexes are crucial for their function, yet are challenging to study. Here we present a nanoelectrospray mass spectrometry approach capable of simultaneously providing structural and dynamical information for protein complexes. We investigate the properties of two novel Small Heat Shock Proteins and find these proteins to exist as dodecamers composed of dimeric building blocks. Moreover, we show that these proteins exchange dimers on the timescale of minutes, with the rate of exchange being strongly temperature-dependent. As these proteins are expressed in the same cellular compartment, we anticipate that this dynamical behavior is crucial to their function *in vivo*. Furthermore, we propose that the approach used here is applicable to a range of non-equilibrium systems and is capable of providing both structural and dynamical information necessary for functional genomics.

INTRODUCTION

Understanding the function of proteins requires information about three basic properties: their structure, their interactions, and their dynamics. Mass spectrometry (MS) presents an established approach towards the study of the first two facets. Primary structural details, regarding sequence and post-translation modifications, are the focus of numerous MS-based proteomics studies (Aebersold and Mann, 2003). Furthermore, given that the vast majority of proteins perform their cellular roles in the form of higher-order assemblies (Sali et al., 2003), and that aspects of their quaternary structure can be maintained in the gas phase (Ruotolo and Robinson, 2006), the application of MS to the direct study of protein complexes is an extremely exciting avenue of research (Benesch et al., 2007; Heck and van den Heuvel, 2004; Sharon and Robinson, 2007). Structural genomics, however, remains largely the domain of X-ray crystallography, nuclear magnetic resonance, and electron microscopy (Sali et al., 2003). Though these approaches have the ability to provide high-resolution structural information, the time required to collect data hampers their application to the study of the dynamic properties of protein complexes. By contrast, as the speed of analysis is rapid, MS is well suited to the study of non-equilibrium states (Bothner et al., 2000; Fabris, 2005; Lee et al., 1989; van den Heuvel et al., 2005), and therefore presents an attractive technology for monitoring the reactions non-covalent protein complexes in real time.

One of the earliest studies which reported the ability of electrospray (ESI) to preserve noncovalent interactions upon injection into a mass spectrometer followed the time-course of an enzymatic reaction as it proceeded in the ESI capillary (Ganem et al., 1991). The application of such an online MS monitoring approach, adapted by employing nanoelectrospray (nESI) (Fligge et al., 1999), has in the

last few years been extended to studying the dynamics of multimeric proteins (Aquilina et al., 2005; Sobott et al., 2002). An alternative strategy is to perform such time-resolved experiments in an off-line mode, whereby a reaction mixture is sampled repeatedly rather than continuously. Such an approach has previously been used in several studies, including monitoring the assembly (Fändrich et al., 2000; Stockley et al., 2007), and dynamics (Keetch et al., 2005), of protein complexes. In these cases the dead-time, the time between mixing of components and detection, is ~1min. Early time-points have become accessible using an ESI capillary mixer (Wilson and Konermann, 2003), but until this technology can be transferred to a nESI platform the applicability to the study of macromolecular assemblies is limited (Benesch et al., 2007). Continuous monitoring of a reaction mixture by means of nESI suffers from the difficulty of maintaining a stable flow over the duration of the experiment, and because prolonged electrospraying can induce electrochemical changes in the solution within the capillary which may interfere with the reaction kinetics (van Berkel et al., 1997). Repeated manual sampling of a reaction can also prove problematic as there is a limit on the number of time-points obtainable, dictated by the experimentalist's capacity, and, the capillary-to-capillary irreproducibility can lead to an impairment in data quality (Keetch et al., 2003).

Here we present an automated nESI methodology for rapid repeated monitoring of the dynamics of protein complexes in real time. This approach obviates many of the difficulties associated with the real-time monitoring of reactions by MS described above, and is capable of providing reproducible data for a wide range of dynamic species. We detail the development of the method and demonstrate the advantages of the technology by application to the quantitative monitoring of the kinetics of an enzymatic digestion reaction. We subsequently examine the dynamics of two previously uncharacterized Small Heat Shock Proteins (sHSPs) HSP18.1 and HSP17.6 from *Arabidopsis thaliana*.

The sHSPs are a widely diversified family of molecular chaperones that are thought to prevent irreversible protein aggregation by holding destabilized substrates soluble for subsequent refolding by ATP-dependent chaperones (Haslbeck et al., 2005; Narberhaus, 2002; van Montfort et al., 2002). The importance of these proteins is evidenced by them being found in almost all organisms, and their implication in a range of disease states including cataract, cancer, myopathies, motor neuropathies, and neurodegeneration (Horwitz, 2003; Sun and MacRae, 2005; Welsh and Gaestel, 1998). We find HSP18.1 and HSP17.6 to be composed of twelve non-covalently bound subunits, an oligomeric form found in other cytosolic class-I plant sHSPs (Siddique et al., in press). Moreover, when incubated together we find these proteins exchange dimeric units on the timescale of minutes, and that this reaction is faster at higher temperatures. We conclude therefore that these dodecameric proteins are composed of dimeric building blocks, and are highly dynamic. Since these two proteins, and other close relatives, are expressed at high levels in the same cellular compartment *in vivo* during heat shock (Siddique et al., in press), it appears that the sHSPs form a diverse and dynamic chaperone network to enable cellular tolerance of stress conditions. Furthermore, we believe the approach described here to be generalized and consequently holds much promise for allowing the mining of both the dynamical and structural information necessary for understanding protein function.

RESULTS

AUTOMATED REAL-TIME NESI-MS

We have previously described the application of a robotic chip-based nESI platform (van Pelt et al., 2002) to the study of protein complexes (Keetch et al., 2003). Briefly, this platform functions by aspirating sample from a specified well on a multi-well plate with a disposable pipette tip, and delivering it to a nESI emitter. An electric potential and slight back pressure of gas are then applied in order to initiate and maintain electrospray. After sample infusion, the tip is discarded, and the process can be repeated (van Pelt et al., 2002). The platform can also perform various handling procedures, including the mixing of reactants and control of their temperature. A major advantage of this system is the improved reproducibility between different nESI emitters (Keetch et al., 2003). In order to adapt this system for our purposes we first calibrated and characterized the various stages of the robotic process (Supplementary Figure 1). Subsequently we delineated a generalized reaction scheme for automated real-time reaction monitoring with this platform (Supplementary Figure 2). Sample-containing solution is deposited into a sample well in the robot. If desired, one or more reagents are then automatically added to this initial solution, before the robot delivers an aliquot or preset volume to the mass spectrometer for a certain period of time. After this time has elapsed, the sampling cycle may be repeated as often as desired. The minimum amount of time between sampling events is ~32s, but this can be extended by inputting a waiting time. The nESI conditions (set on the robot) and MS conditions (set on the mass spectrometer) can be varied for each separate delivery. A useful extension is the ability to monitor several reaction mixtures in tandem. These could be the same reaction in several different wells, so as to generate improved statistics, or different reactions altogether, to improve overall duty cycle.

There are numerous reactions that proteins undergo in the cell. Among the most important of these involve enzymes and their substrates, and we therefore chose such a system to characterise our method. The enzyme, trypsin, and substrate, cytochrome C (CytC), were incubated together, and three aliquots deposited onto the sample plate of the robot, which was programmed to sample each well repeatedly, over a 5hr period (Fig. 1A). The total-ion chromatogram displays ‘blocks’ of signal, each corresponding to a separate infusion. These blocks are grouped in threes, due to the sequential sampling of the wells. A high degree of consistency was found in the intensity of signal within each group, evidencing the high level of reproducibility between emitters on the nESI chip. The total ion current, however, increases over the course of the reaction, suggesting that more species are observed as the reaction progresses. Mass spectra obtained at the beginning, middle, and end of the time-course are inset (Fig. 1A). At the first time-point, only three major peaks are observed, corresponding to the 5+, 6+ and 7+ charge states of intact CytC. After 300mins these peaks are no longer observed: instead the spectrum is dominated by peaks at lower m/z corresponding to tryptic peptides, the most intense of which were identified as CytC₈₀₋₈₆, CytC₂₈₋₃₈, CytC₁₄₋₂₂ (Supplementary Table 1). At intermediate times however, both these reactant and product species can be observed simultaneously. Moreover, a species corresponding to a 9.5kDa fragment (CytC₁₋₇₉) which is not observed in either the initial or final spectrum can be identified, demonstrating how this real-time approach can be used to detect transient intermediates that would not be identified in studies performed at equilibrium.

In order to get a quantitative description of the reaction we plotted the relative intensities of the most intense peaks corresponding to reactants, intermediates, and products in single MS scans as a function of time (Fig 1B). The very small deviation between measurements demonstrates the excellent

reproducibility afforded by this approach. Exponential decay of the intact CytC is observed, with a concomitant sigmoidal increase of the tryptic peptide products. The intermediate ions show an initial increase, followed by gradual decay as they are themselves digested. From these data a rate constant of $7.6 \times 10^{-3} \pm 0.6 \times 10^{-3} \text{ min}^{-1}$ was extracted, which is consistent with previously reported timescales (Stone et al., 2001), and current models of enzymatic digestion (Srividhya and Schnell, 2006). As the rate of many biological reactions is temperature dependent, we tested the thermo-control capabilities of the platform by performing the same experiment at a range of temperatures up to 50°C (Fig. 1C). At elevated temperatures the reaction proceeded much more rapidly: at 37 °C, 45 °C and 50 °C the reaction was complete in ~84 and ~48 and ~23mins, with rate constants of $2.5 \times 10^{-2} \text{ min}^{-1}$, $5.7 \times 10^{-2} \text{ min}^{-1}$ and $1.6 \times 10^{-1} \text{ min}^{-1}$, respectively. Even at the highest temperature monitoring transient intermediates was still possible (Supplementary Figure 3). This increase in rate of digestion with temperature is a result of more molecules overcoming the activation energy barrier, and, furthermore, by assessing the temperature-dependence of the rate constants we extracted an activation energy of 88.8 kJmol^{-1} (Figure 1C, inset).

In order to investigate the difference between the repeated sampling used above and a continuous sampling approach we set the robot to perform both these monitoring approaches on a CytC digest reaction in 33% methanol (Fig 1D). This solution condition induces a molten-globule form of CytC (Bychkova et al., 1996) which is particularly susceptible to acid-induced denaturation (Konermann and Douglas, 1997; Konermann et al., 2001). When this digestion reaction was monitored continuously, i.e. as an uninterrupted infusion through a single nanospray emitter, it reached completion within 30mins. However, with repeated sampling, i.e. multiple short infusions through a new nozzle every time, the rate of reaction was much slower such that ~85% of the protein remained undigested after the 30mins,

and took an order of magnitude longer to reach completion (Fig. 1D). We attribute these differences to prolonged electrospraying in the case of continuous sampling causing a pH drop in the aqueous solution within the emitter (van Berkel et al., 1997), which results in a further destabilization of the CytC (Konermann and Douglas, 1997; Konermann et al., 2001). The resulting increase in frequency and magnitude of local protein unfolding events leads to a consequent increased susceptibility to digestion. This shows, therefore, that continuous sampling, depending on solution conditions, may be unreliable. By contrast repeated sampling is not affected by electrochemical changes that might arise from prolonged electrospraying. As such the automated nESI platform we have described and characterized here provides a means for producing reliable and reproducible kinetic data. Furthermore, as using nESI is essentially a prerequisite for the routine analysis of macromolecular assemblies by means of MS (Benesch et al., 2007), this system has the considerable advantage of being applicable to the study of such species.

DYNAMIC SUBUNIT EXCHANGE OF THE SHSPS

Several members of the sHSPs family have been shown to be very dynamic, in that oligomers are capable of freely exchanging subunits (Haslbeck et al., 2005; Narberhaus, 2002; van Montfort et al., 2002). Moreover, as is the case for the mammalian α -crystallins, this exchange can also occur between closely related protein oligomers, and it is the resulting hetero-complexes which are the functional entities *in vivo* (Horwitz, 2003). In *Arabidopsis thaliana* 19 genes encode sHSPs, with several members being localised in the same cellular compartments (Siddique et al., in press). Here we apply our automated nESI MS approach to two cytosolic class-I sHSPs from *Arabidopsis thaliana*, HSP17.6 and HSP18.1. nESI mass spectra of these proteins display principal charge state series centered around 6900m/z, corresponding to masses of 210258Da and 216301Da for HSP17.6 and HSP18.1 respectively

(Fig. 2A). Additional peaks around 2200m/z are also observed in both cases. These correspond to charge states of monomeric species, their low charge state suggesting that they arise from a solution-phase equilibrium with the oligomers (Benesch et al., 2003). Comparison with the spectra of the proteins under denaturing conditions reveals that both are composed of twelve non-covalently bound subunits.

Incubating the two proteins at an equimolar ratio at room temperature for 2hrs before analysis resulted in the spectrum shown in the upper panel of figure 2B. The lower panel shows the overlaid spectra of the individual proteins. The equilibrated mixture however gave a very different spectrum, suggesting the occurrence of a subunit exchange reaction. In order to assist with the assignment of the peaks arising from this heterogeneous ensemble we simulated spectra for the 31+ charge state of the different candidate heterododecamers. The composite peaks that would be expected for unrestricted exchange of monomeric subunits to an equilibrium position given by a binomial distribution of equimolar components (top panel), and with the added constraint that the exchange units were dimers (lower panel), are shown in figure 2C (see Experimental Procedures). These simulations are compared with an expansion of the 31+ region of the experimental data (middle panel). Clearly the profile of the peaks observed is not fitted by monomeric exchange, but is very well represented by dimeric exchange. Differences between the relative intensities of the different peaks in the modeled and experimental data likely arise from a fractionally greater proportion of HSP18.1 over HSP17.6 in the reacting solution. Overall, however, the persistence of dimeric exchange on the timescale of this experiment shows that the dimer interfaces are not compromised on the timescale of the experiment.

In order to monitor the kinetics of this subunit exchange reaction we employed our automated nESI approach. Figure 3A shows the evolution of the spectra obtained over a 60min time course at 24°C, for the 31+ charge state (*cf.* Fig 2C). At the first time-point, 2mins, the dominant peaks correspond to the homododecamers 12:0 and 0:12 (expressed as the number of subunits of HSP17.6:HSP18.1). Some signal arising from the 10:2 and 2:10 heterododecamers is also observed. After 6min, the signal corresponding to these heterododecamers has increased, and 8:4 and 4:8 are also clearly observed. After 20 minutes almost no homododecamers are observed, and a significant population of 6:6 is observed. At the end of the time course the signal has converged so the most prevalent species is 6:6, reflecting the equimolar mixture of reactant sHSPs.

The intensity of the peaks corresponding to 12:0 and 0:12 are plotted as a function of time in the upper panel of figure 3B. The abundance of the homododecamers follows an exponential decay function for approximately the first 15mins of the reaction, well represented by first order kinetics. By plotting the natural logarithm of this decay we get a first-order rate constant of 0.16min^{-1} (inset). Repeating the experiment, but at 30°C, results in the reaction reaching completion after approximately 20mins with a rate constant of 0.40min^{-1} . The lower panel of figure 3B shows the relative abundance of the different heterododecamers formed. The plots corresponding to 10:2 and 8:4 both go through maxima, at approximately 8min and 22min respectively whereas that for 6:6 rises steadily to a maximum. At 30°C the reaction profile is unchanged except in its time-frame. Overall, these observations are consistent with a reaction wherein individual dimeric units are incorporated in a sequential fashion, and dissociation of the oligomers is rate-determining (Sobott et al., 2002). As such this rapid experiment has provided details as to oligomer stoichiometry (dodecamers), structural composition (robust dimeric building blocks), and dynamics (rapid sequential dimeric exchange).

DISCUSSION

Here we have described a robust and versatile automated nESI approach for studying the dynamics of protein complexes, allowing the quantitative monitoring of their reactions which occur on the timescale of minutes. The reproducibility due to the chip-based nESI, as well as the ability to perform measurements in triplicate, results in a precision of measurement not readily attainable by conventional nESI approaches. In the first instance we demonstrated the applicability of this platform for monitoring enzyme kinetics, by monitoring digestion of CytC by trypsin. Rate constants were determined, and, moreover, a proteolysis intermediate, CytC₁₋₇₉, was observed. This raises the intriguing possibility of determining the relative susceptibility of different cleavage sites to digestion, and thereby glean information as to the accessibility and flexibility of certain polypeptide regions. Such a real-time approach amounts to an extension of the limited proteolysis approach (Hubbard, 1998), and holds exciting potential for probing both structure and conformational dynamics of proteins and their complexes.

Subsequently we applied this reaction monitoring strategy to the subunit exchange of two previously uncharacterised and closely related sHSPs from *Arabidopsis thaliana*. nESI-MS analysis revealed these proteins to be dodecameric, and their subunit exchange reaction was successfully monitored at both 24°C and 30°C, even in the latter case when the reaction was complete within a few minutes. The apparent adherence to first-order kinetics suggests that dissociation of the oligomers is the rate-limiting step in the exchange reaction, a feature also observed in other members of this protein family (Bova et al., 1997). Furthermore, a rate constant at 24°C of 0.16min⁻¹ is very similar to that observed for a different pair of dodecameric plant sHSPs (Sobott et al., 2002), and highlights the remarkably dynamic

nature of members of this protein family. Moreover the extremely high resolution of separation afforded by MS allowed the monitoring of the relative populations of the heterododecamers formed. We therefore determined that subunit exchange is achieved by the sequential incorporation of dimeric units of one sHSP into a dodecamer of the other. This dynamical observation allows us to draw the structural conclusion that the protein dodecamers must be composed of dimeric units. Such dimeric ‘building blocks’ have been suggested as being a common feature of sHSPs (Haslbeck et al., 2005; van Montfort et al., 2002), but, interestingly, there is a marked contrast with previous subunit exchange data for two other dodecameric plant sHSPs, *Pisum sativum* HSP18.1 and *Triticum aestivum* HSP16.9, where heterododecamers composed of an odd number of each of the components (e.g. [HSP18.1]₉[HSP16.9]₃) were also observed (Sobott et al., 2002). This difference implies variability in the interfaces between subunits across even these evolutionarily closely related sHSPs. As the subunit interfaces are thought to be at least partly responsible for the substrate binding function of these proteins (Haslbeck et al., 2005; van Montfort et al., 2002), this suggests that adaptation has occurred in these interfacial areas of the sequence to regulate substrate specificity.

This ability to elucidate information as to oligomeric organization via real-time nESI subunit exchange monitoring is very attractive. Other strategies have been employed in combination with nESI to achieve this goal, notably gas phase dissociation (Benesch et al., 2006; Benesch and Robinson, 2006), or destabilization in solution either through temperature regulation (Benesch et al., 2003) or the addition of perturbants (Hernández et al., 2006). A major advantage of our approach is that it involves the monitoring of the protein complexes in their native state, with the proviso of an MS-compatible buffer. As such potentially non-specific interactions are avoided. Furthermore, the subunit exchange is non-dissociative, in the sense that intact oligomers rather than sub-oligomeric species in solution are

being monitored. Sub-oligomeric units may be elucidated, independent of their stability when not incorporated in the intact oligomer. This advantage is exemplified in the results in figure 2A, which reveal only dodecameric and monomeric forms of the sHSPs. The presence of dimeric substructure is only revealed upon performing the subunit exchange experiment.

Other methods have been used for the study of subunit exchange reactions, including fluorescence resonance energy transfer (Bova et al., 1997), native gel electrophoresis (van den Oetelaar et al., 1990), and affinity chromatography (Schneider et al., 2001). These suffer from either requiring the use of a potentially invasive tag, providing an ‘average’ of the whole reaction mixture, from a poor resolution of separation, or a combination thereof. In contrast, the high-resolution separation afforded by an MS approach allows the relative quantitation of the different species within heterogeneous ensembles (Aquilina et al., 2003). We have demonstrated here that it is the real-time monitoring of these populations which allows the elucidation of dynamical and structural details. Furthermore, monitoring the exchange of a protein complex with its isotopically labelled but isostructural equivalent obviates the need for a tag (Keetch et al., 2005), and represents a completely generalized strategy for future investigations. As such the approach described here provides an exciting robust and universal method for the study of the subunit exchange reactions and non-equilibrium states of protein complexes in general.

SIGNIFICANCE

The sHSPs are a family of molecular chaperones found in almost all organisms studied to date. Detailed structural studies on these proteins are comparatively scarce, due to the frequently polydisperse and dynamic character of these proteins (Haslbeck et al., 2005; Horwitz, 2003; Narberhaus, 2002; van Montfort et al., 2002). In fact it appears that this dynamic nature, particularly their ability to exchange subunits and form hetero-oligomeric species, may itself be crucial to their function *in vivo* (Haslbeck et al., 2005; Horwitz, 2003; Narberhaus, 2002; van Montfort et al., 2002). Here we have developed a robust and universal nESI-MS approach for monitoring the reactions of protein complexes in real time, and applied it to the subunit exchange of two sHSPs from *Arabidopsis thaliana*. By quantifying the relative populations of the different homo- and hetero-oligomeric species as the reaction proceeds, this report represents the first detailed study of a subunit exchange reaction between two species that are found in the same cellular compartment *in vivo*.

Through the simultaneous determination of both structural and dynamical properties of these proteins, showing them to be highly dynamic dodecamers with dimeric substructure, this study also demonstrates the versatility of nESI-MS. Functional genomics is concerned with combining structural and dynamical information so as to understand the roles and mechanisms of action of proteins and their non-covalently bound complexes in the cell. Most established structural genomics approaches however are not well suited to studying the dynamics of protein complexes as they are performed at equilibrium or on quenched states. nESI-MS has however not only become an established structural genomics approach (Robinson et al., 2007), but also, as we have shown here, by generating a large amount of

information in real time is capable of providing complementary dynamical insights crucial to understanding protein function.

EXPERIMENTAL PROCEDURES

AUTOMATED NESI- MS

All mass spectral measurements were performed using a Nanomate HD nESI system (Advion BioSciences Ltd, NY) (van Pelt et al., 2002). Mass spectra were recorded either on an LCT or Q-ToF II mass spectrometer (both Waters, UK). Spectra were calibrated externally using 33mgml⁻¹ cesium iodide. Data were acquired and processed with MassLynx software (Waters, UK), and are shown with no background subtraction.

ENZYME-SUBSTRATE REACTION

50µL of 100µM Horse-heart CytC (Sigma C-2506) in water, was combined with 50 µL of 200mM ammonium bicarbonate and 6.25µL of 0.5mgml⁻¹ sequence-grade modified trypsin (Promega V5113), giving a final enzyme:substrate ration of ~1:38.

Mass spectra were obtained on the Q-ToF II in positive ion mode, with sample cone: 180V, extractor cone: 10V, ion transfer stage pressure: 6.5×10^{-3} mbar, quadrupole analyser pressure: 5.5×10^{-3} mbar and ToF analyser pressure 4.7×10^{-7} mbar. nESI was achieved with a spray voltage of 1.65kV and a head pressure of 0.4psi (28mbar) set on the NanoMate.

To extract kinetic information each single 5s acquisition time-point was analysed, without smoothing. Relative abundances were assessed from the peak heights of the species being monitored (see Supplementary Table 1). Line-fitting was achieved using SigmaPlot 2001 (SPSS Science, IL), and error bars represent three standard deviations from the mean.

SUBUNIT EXCHANGE REACTION

HSP18.1 and HSP17.6 were expressed in *Escherichia coli* and purified as described previously (Basha et al., 2006). Samples were buffer exchanged into 200mM ammonium acetate by using a Superdex 200HR10/30 column (GE Healthcare). To monitor the subunit exchange kinetics in real time, the proteins were combined at a molar ratio of 1:1, to give a final protein concentration of 10 μ M (monomer) for each component, according to UV absorbance at 280nm.

Mass spectra were obtained on the LCT in positive ion mode, with sample cone: 150V (individual components) or 200V (subunit exchange experiment), extractor cone: 5V, ion transfer stage pressure: 7.93mbar and ToF analyser pressure: 1.1×10^{-6} mbar. nESI was achieved with a spray voltage of 1.8kV and a head pressure of 0.55psi (38mbar) set on the NanoMate. The reaction mixture was sampled 34 times over the 1hr experiment, with each 1 μ L aliquot being electrosprayed for 1min.

Simulated spectra were constructed using SigmaPlot 2001 as described previously (Sobott et al., 2002). Theoretical intensities of the different dodecamers were calculated based on a binomial distribution of the components HSP18.1 and HSP17.6 at a ratio of 1:1, assuming an equal preference for each

composition (top panel Fig 2). A second simulation was performed with the restriction that the exchanging units were homodimers (lower panel Fig 2). Kinetics were monitored by signal-averaging all scans in each separate infusion, and the peak heights of the 31+ charge states of the various dodecamers were monitored. Data was normalized to the final relative distributions dictated by the binomial distribution, and kinetic parameters were extracted using SigmaPlot 2001.

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FIGURE LEGENDS

FIGURE 1

Automated nESI monitoring of the tryptic digestion of CytC. **A** Total-ion chromatogram of CytC digestion monitored in triplicate over 300mins. **Inset** are spectra obtained at the beginning (blue), middle (red) and end (green) of the time-course. At the beginning the predominant species corresponds to full length CytC (blue circles), and at the end numerous peptides are observed, the most prominent being CytC₈₀₋₈₆, CytC₂₈₋₃₈, and CytC₁₄₋₂₂ (green squares). Halfway through the reaction an intermediate fragment, CytC₁₋₇₉, (red triangles) can also be detected. Plotting the relative abundances of these, **B**, allows the quantitative monitoring of the digestion reaction. The amount of CytC decreases exponentially, enabling the determination of first-order rate constants. Monitoring this reaction, specifically the disappearance of intact CytC, at different temperatures 24°C (orange), 37°C (dark orange), 45°C (red) and 50°C (dark red), **C**, demonstrates how the reaction velocity increases at higher temperatures. From the Arrhenius plot, **inset**, the activation energy and pre-exponential factor can be determined. In protein-destabilizing solution conditions, a different reaction profile for the disappearance of intact CytC is determined when the solution is sampled continuously versus repeatedly, **D**. This is likely to be due to pH effects in the emitter associated with prolonged electrospraying, and highlights the benefits of the repeated sampling methodology advanced here.

FIGURE 2

nESI-MS analysis of *Arabidopsis thaliana* HSP17.6 and HSP18.1. **A** Mass spectra of both HSP17.6 (lower panel, red) and HSP18.1 (upper panel, blue) display dominant charge state series around

6900 m/z , corresponding to dodecamers. Some signal is observed around 2000 m/z , corresponding to solution-phase monomers. **B** The mass spectrum of an equilibrated mixture of these proteins (upper panel) is significantly different to the overlay of the individual HSP17.6 (red) and HSP18.1 (blue) (lower panel), suggesting that a subunit exchange reaction has occurred. **C** Comparison of the experimental data for the 31+ charge state after equilibration (middle panel) with simulation of theoretical spectra assuming complete and unconstrained exchange (upper panel), and complete exclusive exchange of dimeric units (lower panel) suggests that the exchanging unit is a dimer and allows assignment of the peaks. The coloured key refers to the number of subunits of HSP17.6:HSP18.1 comprising the different dodecamers.

FIGURE 3

Real-time monitoring of subunit exchange of HSP17.6 and HSP18.1. **A** Monitoring of the 31+ charge state range (see Fig. 3) at 24 °C shows the evolution of the different sHSP dodecamers. Only species containing even numbers of the two components are observed. Labelling is as in figure 3C. The homododecamers decay exponentially, and faster at 30°C (white circles) than 24°C (black circles), **B**. From the first part of the reaction first-order rate constants can be obtained (inset). Plotting the relative abundance of the heterododecamers 10:2 (purple triangles), 8:4 (blue diamonds) and 6:6 (green squares) shows that the exchange reaction occurs via sequential incorporation of dimeric units.

FIGURE 1

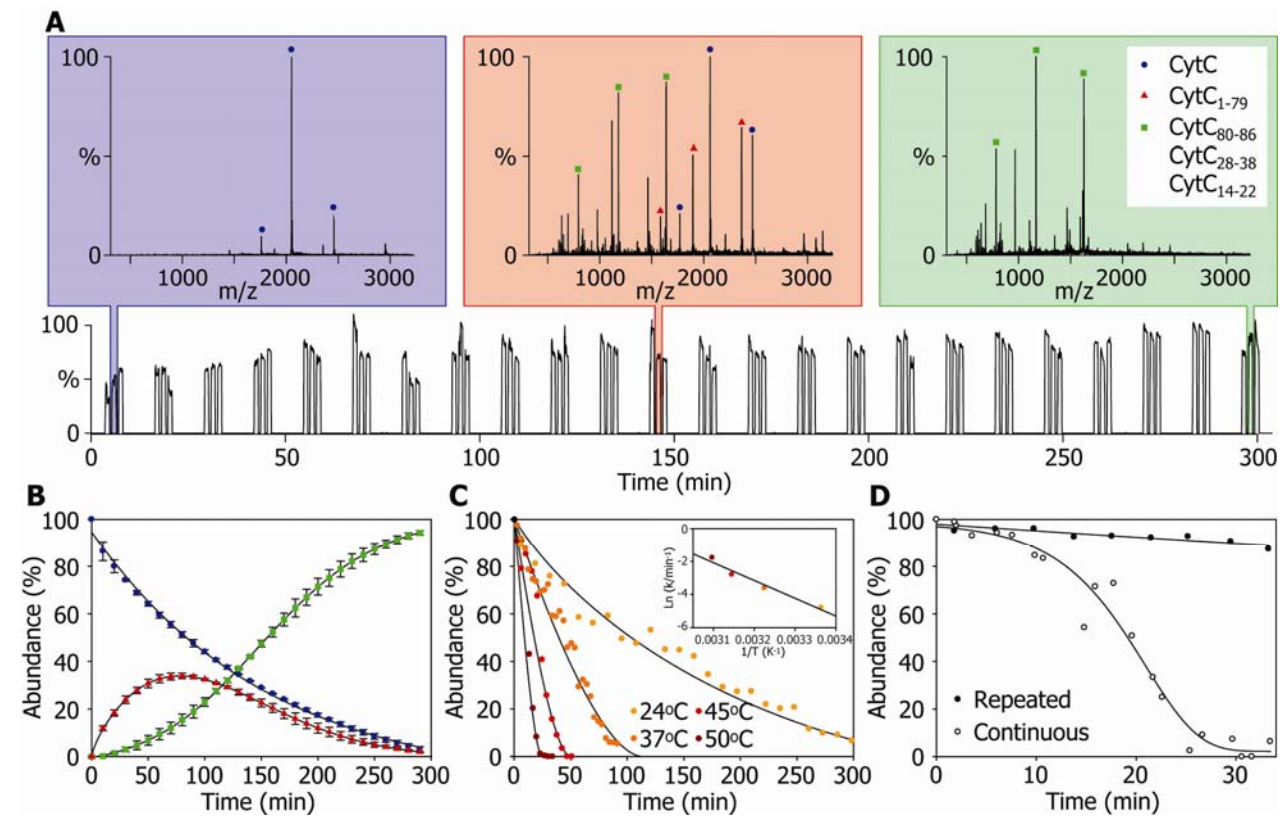


FIGURE 2

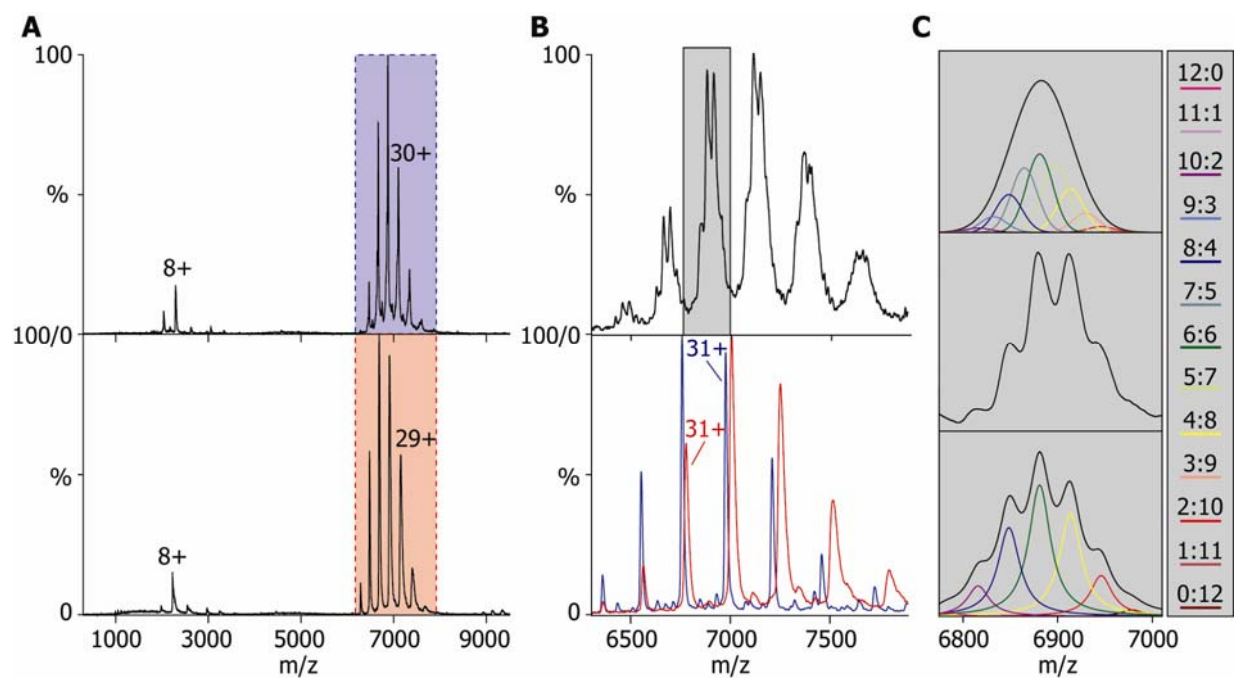
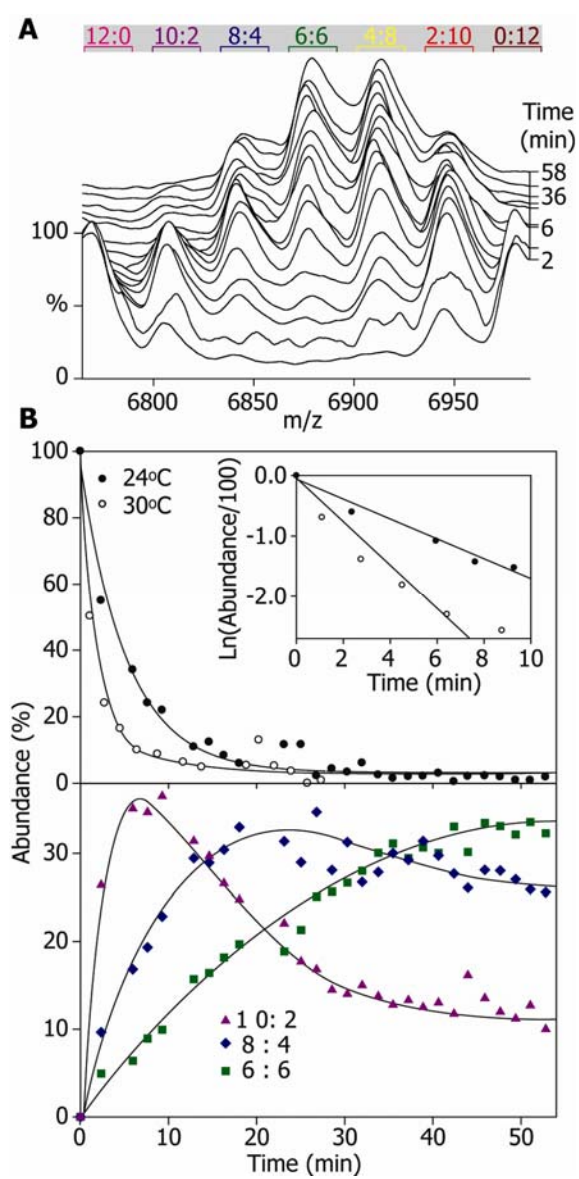
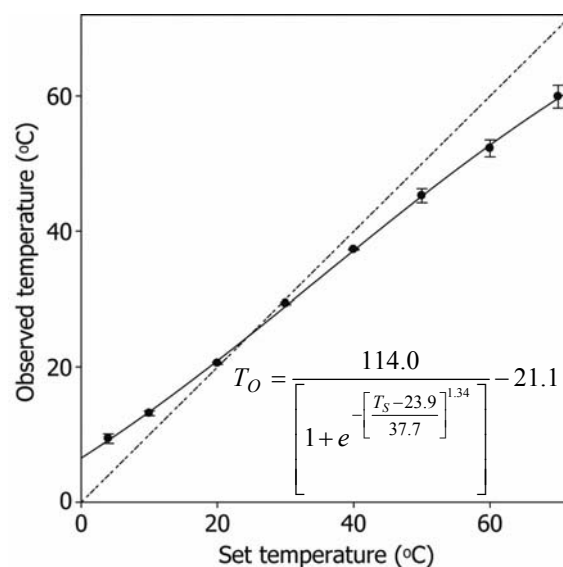


FIGURE 3



SUPPLEMENTARY FIGURE 1

CHARACTERIZATION OF AUTOMATED NESI PLATFORM PARAMETERS



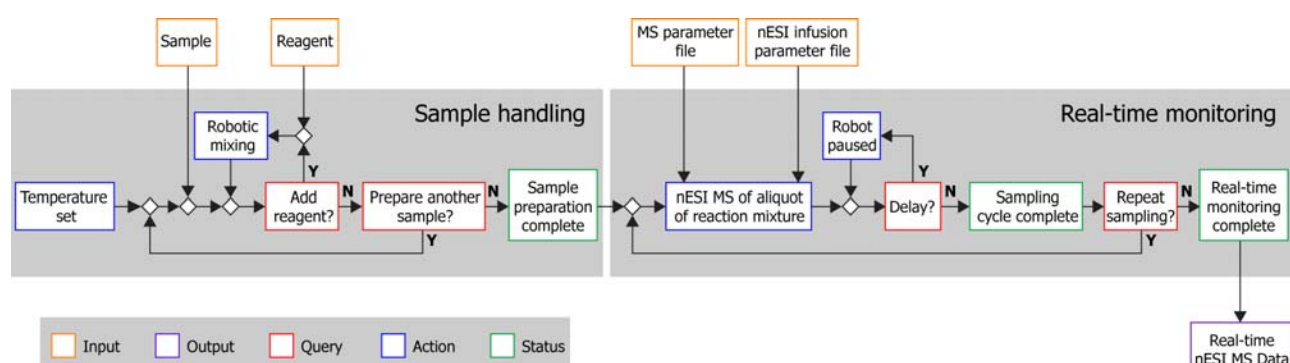
Temperature calibration of nESI platform. The correlation between the temperature set in the control software (T_S) and that measured with a thermocouple in a central sample well containing 100 μ L of water (T_O) follows a sigmoidal relationship well fitted by the inset equation (obtained using SigmaPlot 2001, SPSS Science, IL).

Action	Time (s)
Aspirate and dispense into adjacent well (10 μ L)	29 \pm 2
First mixing cycle (10 μ L)	22 \pm 2
Additional mixing cycle (10 μ L)	18 \pm 2
Dead-time before first infusion	34 \pm 2
Delay between consecutive infusions	35 \pm 2

Time parameters. The time it takes for the robot to perform different actions was assessed. Each action was monitored 10 times, with the mean and limits reported.

SUPPLEMENTARY FIGURE 2

AUTOMATION SCHEME

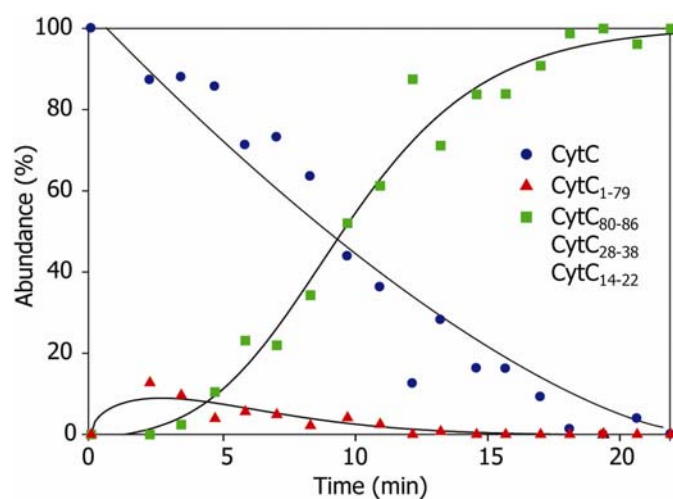


Schematic showing a generalized reaction scheme for automated nESI-MS reaction monitoring.

The robotic platform can be used for sample handling as well as automated nESI infusion into the mass spectrometer. In essence, sample-containing solution which has been deposited onto a multi-well plate can be manipulated by controlling its temperature and the addition of reagent(s). An aliquot of the prepared solution is then infused into the mass spectrometer for a set amount of time. This sampling can then be repeated such that a time series is obtained. The flexibility afforded by the software makes this platform widely applicable for the study of macromolecular dynamics.

SUPPLEMENTARY FIGURE 3

MONITORING OF FAST REACTIONS



Digestion of CytC at elevated temperature. The digestion of CytC by trypsin was monitored at 50°C, and the reaction was complete in ~20min. The data represent a single experiment. Even at this reaction velocity reactants, products, and, moreover, transient intermediates, were successfully monitored and relatively quantified.

SUPPLEMENTARY TABLE 1

ASSIGNMENT OF CYTC TRYPTIC DIGESTION

Species	Sequence mass (Da)	Charge state (+)	Calculated m/z	Observed m/z
CytC	12359.8	5	2472.9	2473.1
		6	2061.0	2061.1
		7	1766.7	1766.7
CytC ₁₋₇₉	9466.4	4	2367.6	2367.7
		5	1894.3	1894.3
		6	1578.7	1578.8
CytC ₁₄₋₂₂	1633.6	1	1634.6	1633.8
CytC₂₈₋₃₈	1167.6	1	1168.6	1168.8
CytC ₈₀₋₈₆	778.4	1	779.4	779.5

Peak assignment of CytC digestion. Nine major peaks were monitored for the digestion of CytC: three corresponding to the reactant (blue), three for an intermediate species (red), and three different final product peptides (green). Only the most intense of the latter (bold) was used for the extraction of kinetic parameters so as to not over-represent the products' contribution to the total ion current.