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Journal of Structural Biology 172 (2010) 161–168

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Separating and visualising protein assemblies by means of preparative mass spectrometry and microscopy

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ARTICLE INFO

Article history:
Received 20 November 2009
Received in revised form 3 March 2010
Accepted 4 March 2010
Available online 15 March 2010

Keywords:
Soft-landing
Heterogeneity
Polydispersity
Electron microscopy
Atomic force microscopy
Mass spectrometry
GroEL

ABSTRACT

Many multi-protein assemblies exhibit characteristics which hamper their structural and dynamical characterization. These impediments include low copy number, heterogeneity, polydispersity, hydrophobicity, and intrinsic disorder. It is becoming increasingly apparent that both novel and hybrid structural biology approaches need to be developed to tackle the most challenging targets. Nanoelectrospray mass spectrometry has matured over the last decade to enable the elucidation of connectivity and composition of large protein assemblies. Moreover, comparing mass spectrometry data with transmission electron microscopy images has enabled the mapping of subunits within topological models. Here we describe a preparative form of mass spectrometry designed to isolate specific protein complexes from within a heterogeneous ensemble, and to 'soft-land' these target complexes for *ex situ* imaging. By building a retractable probe incorporating a versatile target holder, and modifying the ion optics of a commercial mass spectrometer, we show that we can steer the macromolecular ion beam onto a target for imaging by means of transmission electron microscopy and atomic force microscopy. Our data for the tetradecameric chaperonin GroEL show that not only are the molecular volumes of the landed particles consistent with the overall dimensions of the complex, but also that their gross topological features can be maintained.

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1. Introduction

Determining the structure of proteins and the assemblies they form is proceeding through a combination of high-throughput structural genomics, and evolving structural biology approaches (Chandonia and Brenner, 2006; Sali and Kuriyan, 1999). The enduring need for ever more sophisticated structural techniques stems from the fact that many protein assemblies exhibit heterogeneity, form transient interactions, or contain intrinsically unstructured or trans-membrane regions which frustrate traditional approaches. Over the past few years it has become apparent that no single experimental method provides sufficient capabilities to determine the structure of all systems of interest. Consequently hybrid approaches need to be developed which integrate the benefits of several methodologies (Cowieson et al., 2008; Robinson et al., 2007; Sali et al., 2003; Steven and Baumeister, 2008). Here we describe a novel approach designed for the structural biology of heterogeneous protein complexes: the combination of mass spectrometry (MS) with both atomic force microscopy (AFM) and transmission electron microscopy (TEM).

The centre-piece of this new methodology is preparative gas-phase separation by means of nanoelectrospray (nESI) MS. Resulting from the ability to maintain large protein assemblies intact in the gas phase, MS is an emergent approach for elucidating both quaternary architecture and dynamics of multiprotein complexes (Benesch et al., 2007; Heck, 2008; Heck and van den Heuvel, 2004; Sharon and Robinson, 2007). Particularly pertinent is the high resolution of separation afforded by MS. For instance, in the case of the α-crystallins, which resist traditional high-resolution structural characterization due to their inherent polydispersity (Haley et al., 1998), separation and consequently identification of the constituent oligomers is possible by means of MS (Aquilina et al., 2003).

Recently the selective deposition of individual proteins and peptides by 'soft-landing' within a mass spectrometer has been

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doi:10.1016/j.jsb.2010.03.004

demonstrated (Gologan et al., 2006; Laskin et al., 2008). Moreover, studies have shown that in the case of proteins and viruses, biological activity can be retained after the landing and collection processes (Gologan et al., 2004; Ouyang et al., 2003; Siuzdak et al., 1996; Volny et al., 2005). In parallel to these discoveries, experimental evidence has emerged that the topology of large proteins can be preserved, at least on the quaternary level, upon introduction into the gas phase (Ruotolo and Robinson, 2006). Here we extend these observations to soft-land a specific protein complex, after selection of the appropriate mass to charge *(m/z)* value, for direct visualisation by means of both AFM and TEM.

2. Materials and methods

2.1. Instrument modification

We modified a quadrupole time-of-flight (Q-ToF) mass spectrometer (Q ToF 2, Waters, Milford, MA, USA) which had previously been customised for the transmission, dissociation, and detection of macromolecular assemblies (Sobott et al., 2002). The retractable probe was based on the sample transfer unit of a Tecnai Polara electron microscope (FEI, Hilsboro, OR), and customised in house. The probe inserts into the ToF housing of the mass spectrometer, with the standard line-of-sight detector having been replaced with a smaller electron multiplier (Miniature Channeltron, Burle Industries Inc, Lancaster, PA, USA). The probe is brought to rough vacuum separate to the pumping system of the mass spectrometer by an oil-free scroll pump (Edwards). This reduces contamination dramatically, but ultimately further anti-contamination measures such as the incorporation of a cold trap might prove beneficial. The focussing and steering lenses were designed and machined in house, and ion trajectories were simulated using SIMION (Scientific Instrument Services, Ringoes, NJ, USA).

2.2. Mass spectrometry

Escherichia coli GroEL (C7688, Sigma) was purified and buffer exchanged into 200 mM ammonium acetate pH 6.8 as described previously (Freeke et al., 2009). The solution was adjusted to a final concentration of 2 μM tetradecamer, using a theoretical extinction coefficient (Pace et al., 1995). nESI MS experiments were performed using conditions chosen to maintain the oligomers intact in the gas phase, according to a previously described protocol (Hernández and Robinson, 2007). Spectra were analysed using MassLynx software (Waters, Milford, MA, USA) and are shown with minimal smoothing and no background subtraction.

2.3. Visualisation of soft-landed material

After deposition, GroEL was visualised by using either AFM or TEM. Tapping-mode AFM analyses were performed using a Multimode atomic force microscope (Digital Instruments, Santa Barbara, CA). Silicon cantilevers at a drive frequency of ~300 kHz and a specified spring constant of 40 N/m (MikroMasch, Portland, OR) were used. TEM images were obtained on a Tecnai 10 electron microscope (FEI, Hilsboro, OR), after negative staining with uranyl acetate (2% *w*/*w*) (Sigma). Carbon-coated grids with well-defined holes (Quantifoil, Jena, Germany) were used to verify that the target holder was correctly positioned.

3. Results

3.1. A mass spectrometer for selective deposition of protein assemblies

Our approach involves the modification of a Q-ToF mass spectrometer to allow MS purification in the gas phase, and subsequent deceleration and landing of the ions for imaging. We have chosen to modify a mass spectrometer previously customised for high mass operation. The quadrupole mass filter within this instrument is capable of ion selection up to 32,400 *m/z* (Sobott et al., 2002), corresponding to an intact molecular mass of ≈6.3 MDa (Stengel et al., 2010). After selection in the quadrupole, ions of interest are directed toward a target inserted into the vacuum system (Fig. 1A). We elected to introduce the collection device in-line with the principal ion beam axis of the instrument, after the time-of-flight orthogonal extraction source. This arrangement allows us to use the Q-ToF in its normal mode of operation, while taking advantage of the relatively low sampling efficiency (20–25%) of the asynchronously sampled orthogonal acceleration ToF (Rauschenbach et al., 2006). Consequently we are able to detect and mass measure ions simultaneous to their deposition on the target.

To accommodate the custom-built microscopy probe, we replaced the standard line-of-sight detector relative to the quadrupole filter with a compact electron multiplier detector, and introduced a vacuum interlock system (Fig. 1A). The probe is fully retractable into a separately pumped vacuum housing. We designed the target-holder to accommodate both 1.2 cm diameter mica discs and 3.05 mm diameter carbon-coated grids for subsequent AFM and TEM analysis, respectively, of soft-landed material. To ensure that the ions are appropriately directed we introduced steering and focussing lenses in the region between the orthogonal extraction source and the ion collection target. Simulations of the ion trajectories demonstrate that, when properly shielded from the large potentials placed within the ToF flight tube, these ions optics are capable of guiding the selected ions for soft-landing (Fig. 1B).

To locate the ion beam within the instrument we inserted a 0.5 mm diameter aperture into the direct insertion probe where the landing target is typically located. Translating the aperture to defined locations and monitoring the ion current on the in-line detector allows us to obtain an approximate cross-sectional profile of the ion beam (Fig. 1C). The beam is roughly elliptical with dimensions ≈2.0 × 1.5 mm. By adjusting the voltage bias on the steering lens we are able to direct the ion beam laterally (Fig. 1D). These experiments confirm our simulation results and demonstrate that we can successfully focus, direct and manipulate the ion beam.

3.2. Mass spectrometric deposition and ex situ AFM analysis of a protein assembly

To examine the feasibility of our combined MS, EM and AFM approach we required a protein complex which had been well characterised by the three methods independently. For this purpose we selected the tetradecameric molecular chaperone GroEL due to its successful study by means of MS (Rostom and Robinson, 1999; van Duijn et al., 2005), AFM (Yokokawa et al., 2006), and TEM (Chen et al., 1994; Ludtke et al., 2008). A mass spectrum of GroEL displays a single charge state series, centred at ≈11,500 *m/z* for the 70+ charge state (Fig. 2A). Mass determination from these peaks gives a mass of 803.2 kDa, consistent with 14 times the subunit mass of GroEL with some residual solvent and buffer molecules adhering to the complex (McKay et al., 2006).

For the AFM imaging of GroEL particles we initially carried out a control experiment in which we applied an aqueous solution of GroEL directly to the freshly-cleaved mica surface. After air drying, we performed tapping-mode AFM on the GroEL adsorbed onto the mica and observed numerous particles (Fig. 2B, black). As expected the GroEL, after adsorption onto dry mica, appears as flattened ring-like structures. The next step was therefore to carry out AFM measurements on GroEL particles after soft-landing in the mass spectrometer.

For MS purification and deposition of GroEL ions for subsequent AFM analysis we used the quadrupole mass filter to select two

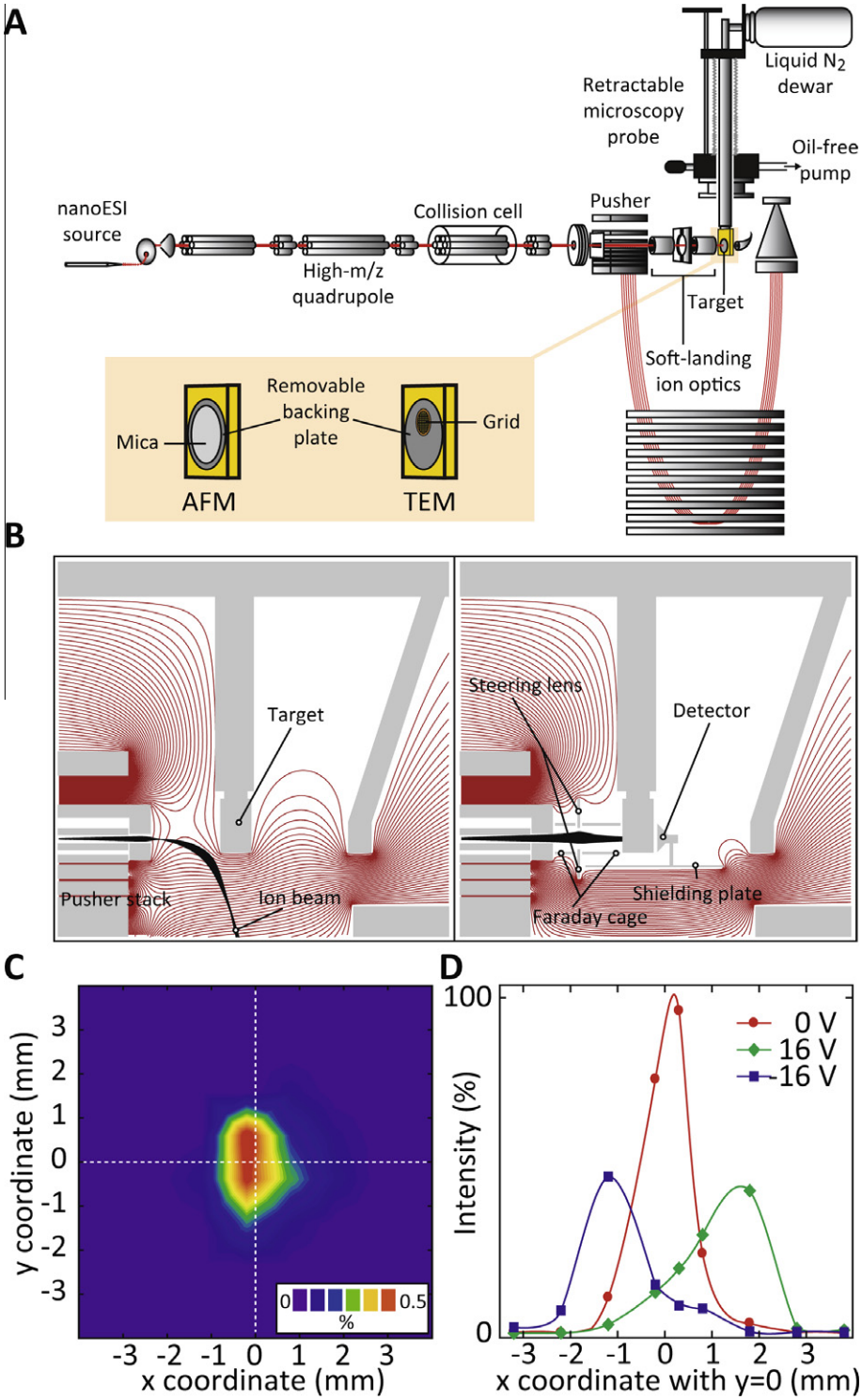
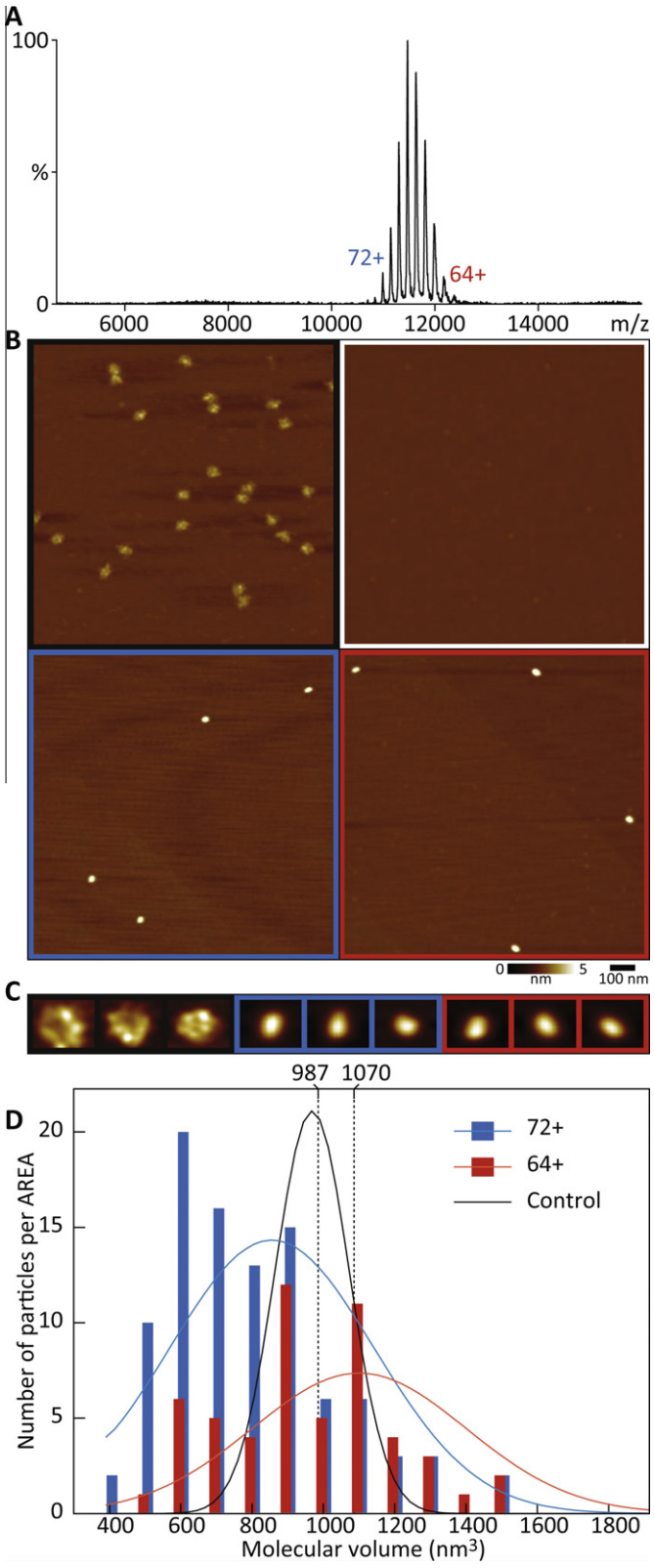


Fig. 1. Modified instrumentation for preparative MS. (A) A schematic diagram of the modified Q-ToF instrumentation used in these studies. Additions include a lens stack following the ToF orthogonal extraction source (soft-landing ion optics), an interlock system for direct insertion of the soft-landing target into the vacuum system (retractable microscopy probe and target) and a miniature line-of-sight detector for ion beam imaging and diagnostics. The target holder can accommodate either a mica disc or EM grid (inset). (B) SIMION electric field simulations showing the area of the vacuum system surrounding the landing stage. If the path of the ions is not shielded from the high voltages placed on the ToF region of the instrument (left) then ions do not reach the landing stage. Efficient collection is simulated using the ion optical set-up described here (right). (C) Image of the ion beam at the soft-landing target recorded by intercepting the ion beam with a disc containing a 0.5 mm aperture. Some distortion of the image is expected due to aperture effects. (D) Directing the ion beam using deflection plates within the ion optics set-up described. Efficient deflection of the ions both to the left and the right is achieved using a 16 V bias on the appropriate optical element.

different charge states that are well separated on the *m/z* scale. The 72+ and 64+ charge states of the GroEL tetradecamer were focussed towards the mica surface with a landing voltage (equal to the entrance voltage into the ToF minus that applied to the target) of 20 V. AFM imaging of the target surface after 15 min of deposition shows the presence of soft-landed particles (Fig. 2B, blue and red). The number of observed particles correlates with the deposition time, suggesting that they arise from the transmitted ions

(Supplementary Fig. 1). Moreover, repeating the landing experiment with the quadrupole mass filter selecting an *m/z* region where no GroEL signal is observed (5000 *m/z*), results in no particles being detected by AFM (Fig. 2B, white). To further verify that the soft-landed particles on the mica surfaces were GroEL, we carried out a tryptic digestion on the mica surface after deposition of GroEL particles, and subsequent mass spectrometry analysis. A number of tryptic peptides derived from the GroEL sequence are revealed (see Supplementary Fig. 2). Therefore it is clear that the particles observed are selected GroEL ions, and are not background ions or neutrals, as these would drift to the mica independent of the selected mass range.



Close comparison between the GroEL applied to the mica directly and that landed within the mass spectrometer reveals some interesting differences (Fig. 2C). After deposition by soft-landing, the GroEL particles appear of narrower diameter than the control, having lost the ring-like topology. To examine the particles in detail we chose to calculate their molecular volumes as, by minimising the contribution from adsorption effects, it is the most reliable indicator (Ratcliff and Erie, 2001; Schneider et al., 1998). Quantification of the molecular volumes (Barrera et al., 2008b) of particles applied directly to the mica surface gives a Gaussian distribution of molecular volumes centered at 968 ± 18 nm^3 (Fig. 2D). This volume is consistent with the calculated volume for GroEL according to its molecular mass (987 nm^3) (Schneider et al., 1998), and atomic properties (1070 nm^3) (Voss and Gerstein, 2005). After 15 min of landing, monomodal Gaussian distributions of molecular volumes centred on 855 ± 24 and 1100 ± 33 nm^3 were obtained for the 72+ and 64+ charge states, respectively (Fig. 2C). Therefore, with the volumes of the landed GroEL matching well with those of the control, we can conclude that the particles we observe correspond to intact GroEL tetradecamers.

One possible cause of the distortion to the overall topology could be that the energy used for landing the GroEL particles is simply too high. To investigate this possibility, we varied the landing energy ranging from 2.7 to 65 V and studied the effects on the soft-landed GroEL particles by using AFM. Particle count was decreased dramatically at lower landing energies, but the particle morphology for the GroEL particles observed by AFM was however largely unaffected (data not shown). This leads us to conclude that the landing energy is unlikely to be cause of the deformation of the topological features. Interestingly, there is a difference observed in the distribution of volumes arising from the deposition of the two GroEL charge states, with the 64+ ions being slightly larger. This variation in size between different charge states has been observed in ion mobility MS experiments of protein assemblies (Ruotolo et al., 2005), including GroEL (van Duijn et al., 2009). This has been attributed to the higher charge states undergoing more activation at a set acceleration voltage than their lower charged counterparts, and hence being able to access collapsed or contorted conformations (Benesch, 2009; Ruotolo and Robinson, 2006). It is plausible that similar activation effects are responsible for the differences in topology we observe here.

3.3. TEM analysis of a soft-landed protein assembly

It is also possible that soft-landing onto the mica surface used for the AFM could cause distortion of the GroEL particles. To investigate this, and to get a more detailed view of the structural differences that occur as a result of landing, we decided to use TEM. We began by performing a set of tests to verify the GroEL ion beam was being

Fig. 2. AFM imaging of the soft-landed GroEL tetradecamer following mass analysis and separation *in vacuo*. (A) A mass spectrum of the GroEL tetradecamer showing the charge states selected for landing. (B) Images of GroEL particles collected at atmospheric pressure, prior to introduction into the mass spectrometer (black outline). Images of soft-landed mass-selected GroEL tetradecamer ions (72+, blue; and 64+, red). No particles are observed when a region of the spectrum is selected where no ion current is observed (white). (C) Close-ups of different particles from the control, and landed experiments (same colouring as in B) show an alteration in topology of GroEL after transmission and landing in the mass spectrometer. (D) Histograms of the distributions of GroEL molecules adsorbed on mica before (control experiment, black line, area of 16 μm^2) and after mass analysis and selection *in vacuo* (72+ charge state, blue bars, area of 68 μm^2; 64+ charge state, red bars, area of 72 μm^2). Lines represent Gaussian fits of the data.

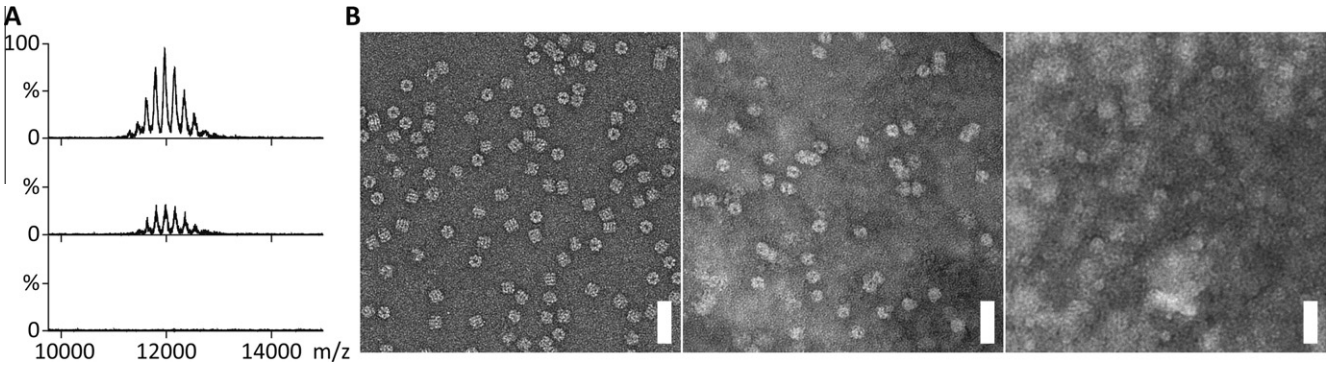


Fig. 3. TEM images of soft-landed GroEL tetradecamer. (A) Prior to soft-landing the line-of-sight detector is used to align the ion beam. Spectra are acquired with the soft-landing target containing no grid (top), grid with holes (middle, 50% projected transmission efficiency), and a carbon-coated grid (bottom, 0% projected transmission efficiency). Taken together, the data shown suggests the ion beam is aligned with the landing stage. (B) Images showing GroEL deposited from solution (left) by nano-electrospray deposition at atmospheric pressure outside the mass spectrometer (middle) and after passing through the vacuum system (right). Scale bars are 50 nm. GroEL tetradecamers are clearly visible in all images, demonstrating their successful transferral through the mass spectrometer and soft-landing prior to TEM imaging.

properly directed at the TEM grid. Firstly, we acquired a mass spectrum with the soft-landing target in place but without a grid, allowing particles to pass through to the detector. A standard spectrum of the GroEL assembly was recorded (Fig. 3A, top). Introducing a carbon grid with holes cuts the transmission efficiency effectively by 50%. This is reflected in the intensity of the GroEL spectrum recorded (Fig. 3A, middle). Finally, with the carbon-coated grid in place, the transmission of all GroEL ions is effectively prevented from reaching the in-line detector. This is consistent with the 0% transmission efficiency observed in our mass spectrum, with all particles having been intercepted by the grid (Fig. 3A, bottom). The attenuation of the ion beam that we observe in these experiments is in accord with the proper alignment of the landing stage within the ion beam and consequently the landing of GroEL tetradecamers on the EM grid.

For the negative stain TEM experiments we began by carrying out two control experiments. In the first experiment GroEL was applied to the TEM grid directly from solution and in the second nanoflow ESI was used to deposit GroEL assemblies outside of the mass spectrometer at atmospheric pressure. After staining, TEM analysis shows characteristic end and side views, revealing the symmetry and topology of the GroEL tetradecamer (Fig. 3B, left and middle). Subsequently, we transmitted GroEL ions through the mass spectrometer and deposited the entire GroEL charge state envelope onto a carbon-coated EM grid for a period of 15 min. After negative staining and TEM, GroEL tetradecamer particles are clearly observable (Fig. 3B, far right). Some distortion of the topological features of the complex is evident in our images, although to a lesser extent than was observed by AFM (Fig. 2B). Concurrently, our particle density, on the order of 45 μm^{-2} for this 15 min deposition, is higher than that measured in the AFM experiments. Together, these results could indicate that the carbon-coated TEM grids provide a 'softer' surface relative to mica for soft landing. Alternatively the hydration provided by the application of aqueous negative stain prior to TEM might restore some of the native structural features of the tetradecamer. Although the origin of this distortion is not known, it is clearly possible to obtain negative stain TEM images of GroEL after transmission through the mass spectrometer. This is an exciting result, and one which provides much promise for further experimentation and optimisation.

4. Discussion

4.1. Preservation of quaternary structure in the gas phase

The data presented here clearly demonstrate the principle and practice of coupling microscopy technologies with preparative MS. After their ionisation, a narrow band of GroEL tetradecamer ions have been selected with a quadrupole mass filter and soft landed onto a surface. Our results rule out the influence of both neutrals from the nESI source and background particles from the vacuum system, since control experiments, including tryptic digestion and particle counting experiments, verify the presence of GroEL tetradecamer on the target. AFM experiments reveal that, though the particles appear somewhat deformed, their molecular volumes of the particles are consistent with those anticipated for the GroEL tetradecamer. Negative stain TEM images further validate the overall integrity of the assembly and reveal interesting topological differences for the particles landed on mica and EM grids.

It is possible that this artefactual distortion of the overall structure could occur during the transition from solution to gas-phase. While there is evidence that protein complexes can retain their quaternary organisation during this phase transition, detailed knowledge of their structure is limited. Ion mobility MS measurements of collision cross-section on intact GroEL provide gross measurements of the structure that are consistent with the native state topology. Moreover, the ability of GroEL to subsume substrate proteins, without appreciably increasing its collision cross-section, suggests the retention of a cavity within the *apo*-complex (van Duijn et al., 2009). Little detailed structural information is available however due to the large size of the monomer units that comprise the complex and the relatively low resolution of current commercial ion mobility instrumentation (Ruotolo et al., 2008). We are currently investigating factors that stabilise protein complexes in the gas phase, specifically charge-reduction (Pagel et al., submitted for publication) and small-molecule adduction strategies (Freeke et al., 2009), to reduce the propensity for deformation of the topological features.

4.2. Minimising undesirable surface interactions

One of the advantages of using AFM for our experiments is that it gives a direct report of the material on the mica surface, and does not rely on additional staining or labelling. However, apparently due to the absence of solvent, the particles observed by using AFM appear significantly distorted. In an effort to prevent this deformation of the protein complex we will investigate the use of modified surfaces (Gologan et al., 2006; Laskin et al., 2008) to decrease their effective landing energy. It appears that the hydration afforded by the application of aqueous heavy metal stain may result in the landed material adopting conformations closer to the native state. Conversely, the negative-staining process itself has been shown to cause significant alteration of structure (Boisset et al., 1995), particularly in the case of protein complexes less

robust than GroEL. As such extending the technology presented here to allow stain-free TEM analysis is a future goal.

One attractive possible approach could be the adaptation of the standard cryoEM protocol, by landing into a liquid layer on a perforated carbon-coated grid, removing the target from vacuum and plunge-freezing, and then imaging the vitrified particles through the holes in the carbon film. To ensure the liquid phase survives long enough within the vacuum of the mass spectrometer, an instrument modified to function at increased pressure, or solution additives such as sucrose or glycerol could be employed. Though high concentrations of such small molecules can cause a significant loss of image contrast (Grassucci et al., 2007), successful reconstructions can nevertheless be obtained (Sato et al., 2001).

An alternative route would still involve soft-landing of the particles onto a solid surface, but with the replacement of the amorphous-carbon coating of standard EM grids with a featureless substrate. Materials such as graphene are emerging as attractive candidates for specimen support in EM experiments (Pantelic et al., 2010; Wilson et al., 2009). Graphene is remarkably thin and uniform (Geim and Novoselov, 2007; Meyer et al., 2007), and hence would minimize unwanted electron scattering. Furthermore, its elasticity and strength (Lee et al., 2008) could prove useful attributes in creating a 'softer' landing surface. The benefits provided by a substrate such as graphene would be particularly pertinent for the study of small and fragile protein assemblies, as their separation is especially challenging using image processing techniques.

4.3. Combining in vacuo and in silico separation for EM of heterogeneous protein assemblies

Here, by examining different charge states of the GroEL, we have demonstrated the ability to selectively deposit species separated within the mass spectrometer for *ex situ* imaging. Although the GroEL tetradecamer is robust, monodisperse, and hence well suited to characterisation by means of EM, many protein complexes function *in vivo* as polydisperse ensembles of oligomeric states (Stengel et al., 2010; Tilley et al., 2005). Such heterogeneous complexes defy the classical notions of structural characterisation, as each individual state, as well as the dynamic interplay between them, likely dictates function (Jaffe, 2005). Furthermore other sources of heterogeneity, including conformational changes, both along a reaction pathway (Saibil, 2000) and at equilibrium (Baldwin and Kay, 2009; Smock and Gierasch, 2009); or the binding of ligands to a subset of the protein assemblies (Halic et al., 2004) can provide significant obstacles for EM.

A number of *in silico* particle sorting and classification techniques have been developed recently, dramatically improving the application of EM to heterogeneous assemblies (Elad et al., 2008; Gao et al., 2004; Heymann et al., 2004; Scheres et al., 2007; White et al., 2004; Zhang et al., 2008). However, these approaches still encounter difficulties in situations where the multiplicity of states is great and the differences in size are small. The separation afforded by our preparative MS approach described here is extremely high, and we estimate that protein assemblies varying in mass by as little as a few percent could be deposited individually for EM imaging and single-particle analysis (Fig. 4). Even in cases when only enrichment, rather than complete purification, is possible, performing subsequent image sorting will nevertheless be made more tractable.

5. Conclusions

A number of stimulating possibilities are highlighted by the initial results presented here of the soft-landing and subsequent imaging of the GroEL tetradecamer. Ultimately we envisage a situation whereby the *in vacuo* and *in silico* approaches will combine to afford high-fidelity reconstructions of protein assemblies within heterogeneous ensembles (Fig. 4). An exciting potential application is prompted by the recent discovery that membrane complexes, containing both trans-membrane and cytoplasmic subunits, can be released intact from micelles in the gas phase (Barrera et al., 2008a). This could allow selection of membrane complexes of

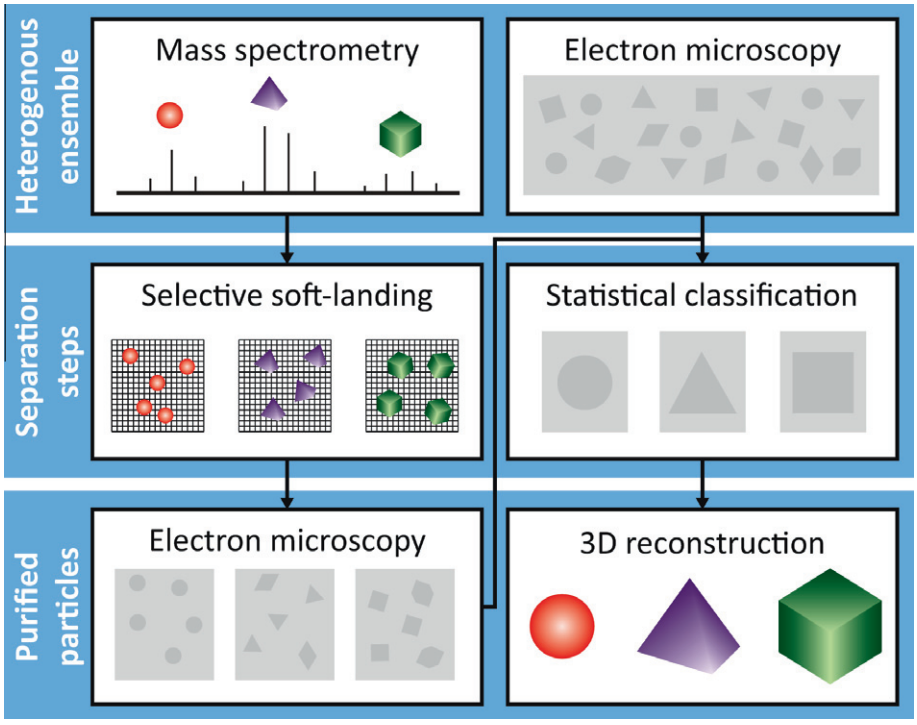


Fig. 4. Scheme for how the preparative MS approach described here can integrate with existing single-particle analysis techniques. This hybrid approach, benefitting from both *in vacuo* and *in silico* particle sorting, will allow the 3D reconstruction of the protein assemblies comprising heterogeneous ensembles.

interest and subsequent imaging, without the presence of the high concentrations of detergent that are usually necessary for their solubility. Overall, therefore, we believe that combining imaging with mass spectrometry has the capability to enrich lowly populated species, to target oligomers from a background of polydisperse assemblies, and to visualise intact membrane protein complexes, largely devoid of their micellar background. As such this hybrid approach has the potential to contribute to the investigation of a large variety of systems refractory to traditional structural biology techniques.

Acknowledgments

We thank all past members of the CVR group for their involvement during the timescale of this development. Specifically we thank Todd Mize, Frank Sobott, Michal Sharon, Min Zhou, Michael Edwardson and Robert Henderson (all University of Cambridge); Daniel Clare (Birkbeck College); Jason Wildgoose and Robert Bateman (both Waters UK Ltd); and Gatan for input at various stages of this project. Funding from the BBSRC, NSERC, EPSRC, and the EU 6th and 7th Framework Programmes '3D Repertoire' and 'PROSPECTS' is also gratefully acknowledged. JLPB is a Royal Society University Research Fellow, BTR a Waters Research Fellow, and CVR a Royal Society Professor.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2010.03.004.

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