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The role(s) of MS in drug research and development

In a flash of light: X-ray free electron lasers meet native mass spectrometry

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During the last years, X-ray free electron lasers (XFELs) have emerged as X-ray sources of unparalleled brightness, delivering extreme amounts of photons in femtosecond pulses. As such, they have opened up completely new possibilities in drug discovery and structural biology, including studying high resolution biomolecular structures and their functioning in a time resolved manner, and diffractive imaging of single particles without the need for their crystallization. In this perspective, we briefly review the operation of XFELs, their immediate uses for drug discovery and focus on the potentially revolutionary single particle diffractive imaging technique and the challenges which remain to be overcome to fully realize its potential to provide high resolution structures without the need for crystallization, freezing or the need to keep proteins stable at extreme concentrations for long periods of time. As the issues have been to a large extent sample delivery related, we outline a way for native mass spectrometry to overcome these and enable so far impossible research with a potentially huge impact on structural biology and drug discovery, such as studying structures of transient intermediate species in viral life cycles or during functioning of molecular machines.

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Introduction

Efficient drug design relies to a large extent on the knowledge of molecular structures of protein drug targets. With the current broad portfolio of structural biology methods, biomolecular structures are being deposited to repositories daily. However, there are classes of proteins, most notably membrane proteins such as G-protein coupled receptors (GPCRs), but also intrinsically disordered proteins, which have a huge potential for drug discovery and development, but are signif-

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icantly underrepresented in structure databases [13]. This is largely due to their heterogenic and dynamic nature, which causes significant experimental problems or puts many of these systems beyond the reach of conventional structural biology methods completely [4]. Another prime example would be transient low abundant protein species, such as those temporarily formed in the course of the assembly and disassembly of viral capsids during the viral life cycle [5] or large enzyme complexes in action. As these proteins and protein complexes cannot generally be natively purified and are mostly thermodynamically unstable, such intermediates cannot be crystallized and because of their low abundance, they also pose problems for methods like cryo electron microscopy (EM).

To tackle such challenging systems, which nevertheless promise a wealth of information for biology as well as plenty of possible targets for drug development, integrative structural biology approaches as well as new biophysical and structural biology methods are needed. Mass spectrometry (MS) as one of such techniques has for many years been contributing to the drug discovery and integrative structural biology efforts [68]. Apart from its broadly known applications in various omics techniques, especially hydrogen/deuterium exchange MS and native MS have found their place in (bio)pharmaceutical industry for studying structural dynamics and stoichiometries of biotherapeutics and antibody-drug conjugates [9,10]. Also, various structural MS approaches have increasingly been used to probe challenging complex samples such as viruses [11,12], which are prime human pathogens as only too well demonstrated during the ongoing COVID-19 pandemic. Furthermore, in recent years, X-ray free electron lasers (XFELs) have entered the field delivering X-ray radiation with unparalleled brilliance and record numbers of photons in short pulses, opening up completely new, exciting avenues for science.

This perspective will briefly review the operating principle behind XFELs and their relevance and immediate uses for structural biology and drug discovery, as well as describe current challenges encountered while pushing the boundaries for single molecule diffraction experiments. Further, it will specifically focus on how MS can help alleviate some of these problems and discuss how new techniques based on XFELs in combination with native MS can open up new scientific approaches.

XFELs as a structural biology technology

Though their operating principle was first theoretically conceived already in the 1970s [13], it took several decades for XFELs to be built. First instruments with higher photon energies started operations in early 2000s working initially in UV and soft X-ray wavelengths, while LCLS (Linac Coherent Light Source) in the US came online in 2009 as the worlds first facility delivering radiation in hard X-ray region (close to 1 æ wavelength) [14]. Since then, several user facilities have opened around the world (Table 1). Notably, the European XFEL close to Hamburg in Germany, which is the brightest X-ray source built so far, produced its first flashes of light and started user operations in 2017 [15,16].

While XFELs are similar to synchrotrons in that they both use highly accelerated electrons to produce light of tunable wavelengths, they differ significantly in both the way they operate and in the properties of generated radiation. In brief, synchrotrons keep accelerated electrons on quasi-stable orbits inside their storage rings and output almost continuous light by making electrons turn repeatedly in a periodic magnetic lattice of so-called undulators. On the other hand, XFELs use distinct electron pulses from a linear particle accelerator. The electrons are passed through multiple magnetic undulators, significantly longer than used in synchrotrons, where the turning electrons irradiate photons based on their energy and the properties of the magnetic field. The photons co-propagate with the electrons and through constant photon-electron interactions, the electrons further self-organize in a process called micro-bunching into very narrow bands with high density, spaced according to the wavelength of the produced light. This forms a positive feedback loop,

Table 1. Overview of hard X-ray FEL facilities worldwide.

Facility	Location	Operational	E_{max} [GeV]	Wavelength [nm]	Pulses per second	Peak SASE brilliance [photons/s/mm^2/mrad^2/0.1%bw]
European XFEL	Germany	2017	17.5	0.05–4.7	27 000	5 × 10^{33}
LCLS	USA	2009	14.3	0.15–4.4	120	2 × 10^{33}
LCLS-II[a]	USA	2020/2022	15/4	0.05–1.24/0.18–6.2	120/929 000	1.5 × 10^{33}/0.12 × 10^{33} [b]
PAL-XFEL	South Korea	2016	10	0.06–10	60	1.3 × 10^{33}
SACLA	Japan	2011	8.5	0.06–0.3	60	1 × 10^{33}
SwissFEL	Switzerland	2017/2021	5.8	0.1–5	100	1 × 10^{33}
SHINE	China	expected 2025	8	0.05–3	–1 000 000	–1 × 10^{33}

Four facilities are currently open to user experiments. In late 2020, LCLS finished the first phase of its upgrade to LCLS-II with plans for two different accelerator technologies in parallel use, while SHINE in Shanghai is under construction. Not all parameters can be achieved simultaneously. E_{max} – maximal operational electron energy at the end of the linear accelerator, bw – spectral bandwidth.

[a] Depending on the accelerator in use (normal-conducting/super-conducting).

[b] Without seeding; up to 13 × 10^{33}/1.8 × 10^{33} with self-seeding.

which ultimately, in a process called SASE (self-amplified spontaneous emission), leads to most electrons emitting highly coherent, laser-like light with a very narrow band of amplified wavelengths (on the order of 0.5% bandwidth). For the detailed physics, interested readers are kindly referred to the great overview paper by Margaritondo & Rebernik Ribic [17], the book An Introduction to Synchrotron Radiation: techniques and applications by Phil Willmott [18] or for a true in-depth look into the theory The Physics of Free Electron Lasers by Saldin, Schneidmiller and Yurkov [19]. Here, it is only crucial to note that the SASE process in XFELs gives rise to radiation with wavelengths down to hard X-ray range, delivered in very short pulses (on the order of femtoseconds), which are however extremely bright.

The unique properties of the light generated by XFELs and above all their high peak brilliance, which can be a billion times higher than for the best modern third and fourth generation synchrotrons [20], have opened up whole new research avenues for physics, material science as well as for structural biology. For the latter, two main science drivers behind the development of hard XFELs have been serial crystallography and single particle diffraction approaches, which will be discussed in more detail in the following sections.

Serial femtosecond crystallography at XFELs

The first major scientific success of XFELs in structural biology has been the development of serial femtosecond crystallography (SFX). As the name suggests, XFELs produce X-ray pulses bright enough to obtain sufficient diffraction signal from crystals smaller than a micrometer, not amenable to standard synchrotron-based approaches. [21] As the radiation damage induced in such tiny crystals at room temperature is immense, they are delivered into the beam sequentially for just a single shot diffraction, usually in a fast continuous jet of liquid, commonly formed by gas dynamic virtual nozzles [22]. Also, more advanced designs and jet injectors compatible with viscous media, such as lipidic cubic phase, are available as covered in recent reviews [4,23].

As the data processing and also achievable resolutions are comparable to standard crystallographic workflows, the method has been readily applied to drug discovery purposes as described in detail elsewhere [4]. Moreover, the short XFEL pulses are of high interest for monitoring fast reactions in molecules, especially in combination with pump-probe excitation by a laser pulse to ultimately enable studying the course of biochemical reactions in the form of molecular movies [24,25]. Using very small protein crystals of a few hundred nanometers in size makes even mixing experiments possible, where reactants or ligands diffuse into the crystal directly before diffraction using mixing nozzles [2628]. While SFX is the most mature XFEL structural biology approach to date and pushed the boundaries of crystallography towards harder-to-crystallize species, it nevertheless relies on crystallization, which still poses a problem for many biologically relevant species. Hence, as discussed next, efforts are also underway to circumvent this obstacle completely.

Single particle diffractive imaging at XFELs

The second major scientific driver for the structural biology applications of XFELs has been the possibility to circumvent the need for stable crystals and to allow imaging of biomolecules in a time resolved fashion without the need for their cryogenic preservation. This should eventually lead to directly observing the details of biochemical reactions on time scales significantly faster than accessible by cryo-EM. [29,30] Theoretical feasibility of the XFEL diffractive single particle imaging (SPI) approach was first shown through simulations by Neutze et al. in 2000 [31]. In this seminal paper, the authors simulated the behavior of lysozyme upon irradiation by XFEL light. They observed that the radiation dose delivered to a protein particle by an XFEL pulse is indeed extreme and eventually turns the particle into hot plasma due to extensive photoionization, bond fragmentation and ensuing Coulombic explosion [31]. While the whole process gets even more complex when electronic damage is explicitly considered [3234], it has been accepted that when the X-ray photons are delivered in a very short pulse on the order of femtoseconds, the photons can scatter from the particle before the onset of significant radiation damage [31].

This diffraction before destruction process was first demonstrated experimentally by Chapman et al. in 2006 at FLASH soft X-ray FEL in Hamburg, where a non-periodic cartoon drawing nanofabricated into a thin support was obliterated in a single 25 fs X-ray pulse, but provided enough diffracted photons to faithfully reconstruct the 2D image without observable radiation damage [35]. Further, with the construction of first hard XFELs, the first proof-of-principle single particle diffraction was obtained in 2011 on 400 nm mimivirus particles [36]. The results suffered from low number of acquired diffraction patterns, thus severely limiting the resolution of the reconstructed electron density to only 125 nm with the highest resolution signal observed up to 32 nm [37]. More importantly though, the study showed that it indeed is possible to obtain diffraction from single biomolecular assemblies and that the data can be processed into 3D electron density volumes [36,37].

At this point, it is important to briefly reflect on how structural results are obtained from single particle diffraction. For 3D objects, similarly as in single particle cryo-EM, many snapshots of particles from different angles are necessary. These are later aligned and reconstructed into an electron density map using dedicated computational approaches such as the currently most popular EMC (expand-maximize-compress) algorithm [38,39], multi-tiered iterative phasing (MTIP) [40] or others. As mentioned before, individual

particles are destroyed upon irradiation by XFEL pulses. Hence, similarly to SFX, SPI needs a constant delivery of sample into the interaction region of the experiment. Crucially however, SPI does not rely on the amplification of angle-specific diffraction maxima by a crystal lattice (Bragg peaks), but instead provides a weak but continuous scattering similar to SAXS. Therefore, there is ultimately enough data in the patterns to directly retrieve the phase information avoiding the phasing problem encountered in crystallography [37].

Since the first SPI experiments at XFELs, more datasets have been appearing with increasing structural resolution. For example, using careful hit classification to select only the best hits, an SPI dataset [41] obtained from 70 nm PR772 bacteriophage, yielded a 3D reconstruction down to 7.89 nm resolution primarily limited by detector geometry at the LCLS facility (Fig.1A) [29]. Also, in another experiment, low background data on rice dwarf virus have been collected with signal ultimately discernible down to 5.9 æ detector limit, however this dataset contained an insufficient number of diffraction patterns for 3D reconstruction [42]. As is apparent from the examples listed here, the structural resolution obtained from SPI experiments at XFELs has been an open issue. Extensive simulation efforts indicate that with sufficient numbers of even noisy diffraction patterns and structurally homogeneous particles, existing algorithms should be able to reconstruct electron densities with near atomic resolution (Fig.1B) [4348]. Also, full start-to-end simulations of the European XFEL facility conclude that even for smaller proteins this should be feasible with optimized beam parameters and the current beamline design [34,49]. However, this goal has so far remained elusive experimentally.

Already in 2014 an international SPI consortium was formed, which summarized many of the outstanding obstacles on the way to 3 æ resolution in XFEL SPI experiments [51]. The current status and bottlenecks have also recently been reviewed in detail by Bielecki et al. [52] Apart from the pulse repetition rate and photon flux delivered to the experiment, which should be already close to the necessary range at the second generation XFELs such as the European XFEL [45] or the currently upgraded LCLS-II [53], one of the major obstacles to obtaining sufficient amounts of diffraction patterns has been the sample introduction into the beam [30,51,54]. The appropriate method needs to deliver a fast-replenishing stream of homogeneous particles into the interaction region of a vacuum beamline chamber. Further, it needs to do this with minimal background as extensive scattering caused by liquid or gas introduced with the sample have been identified as one of the major factors negatively influencing the performance and resolution of 3D reconstruction [45].

Liquid jets, as used for SFX, unfortunately do not work well for SPI as the water jet creates very strong background scattering, drowning the signal from particles of interest [54]. Therefore, the majority of SPI experiments have so far been

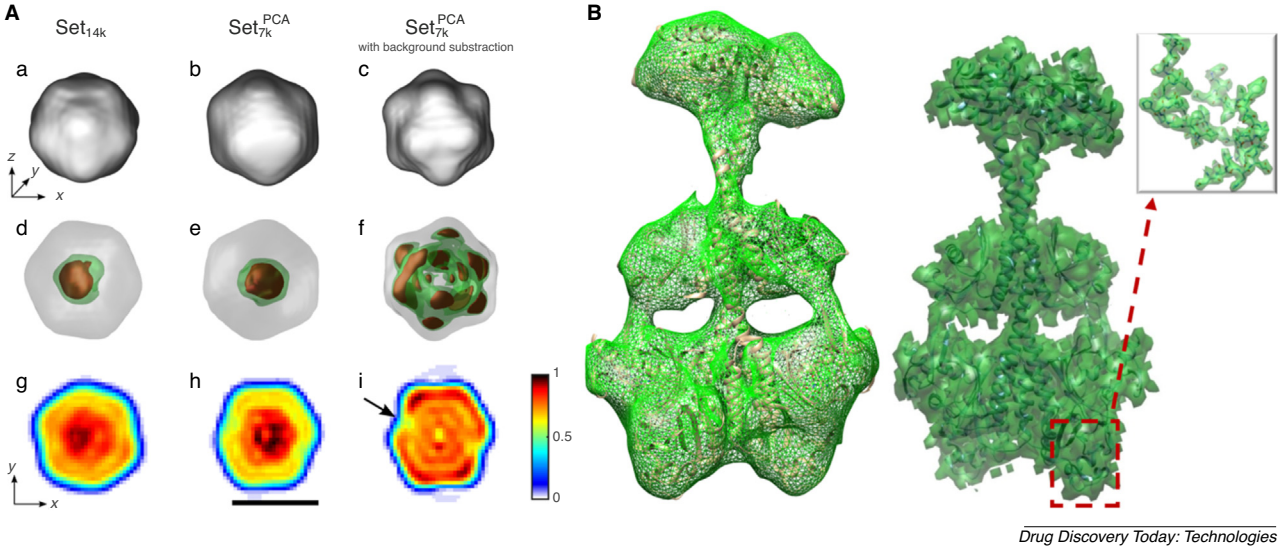

Fig. 1. Three dimensional structure reconstruction from XFEL SPI data.
(A) Experimentally achieved reconstruction of PR772 bacteriophage capsid. [29] Data obtained at LCLS were extensively filtered for ‘high quality single hits’ with gradually more stringent criteria providing better structural resolution down to 7.8–9 nm (a–c). Internal electron density isosurfaces and slices through the center of the particle are shown in (d–f) and (g–i), respectively. Arrow indicates detected deviation from icosahedral symmetry and a low density region in internal arrangement also detected by other analyses [40,50]. **(B)** Reconstruction of a bacteriophytochrome dimer structure (159 kDa) from simulated noisy diffraction patterns using realistic XFEL beam parameters. [48] Even for such a relatively small protein, reconstruction down to 10 æ and 3 æ was proven to be feasible from 38.000 (left) and 66.000 (right) patterns, respectively. (A) Reproduced with permission of the International Union of

performed with aerodynamically focused aerosol injectors [55]. In these devices the sample is dispersed into a mist of droplets, which are propelled and gradually focused by sheath gas flow into a tiny aerosol stream entering the diffraction region. However, albeit the most successful SPI sample introduction method so far, it has been found that the 3D volumes of particles reconstructed from such experiments exhibit much rounder features than expected [29,40]. This effect was attributed to residual non-volatile contaminants from sample buffers concentrating and drying down on the surface of the particles during droplet evaporation, which was shown to strongly depend on the initial size of the aerosol droplets [56]. Expanding on earlier attempts [55], Bielecki et al. modified the existing design of the aerosol injector to accommodate an electrospray ionization (ESI) nozzle producing smaller initial droplets. This significantly decreased the thickness of the contaminant crust, however to interface with the purely aerodynamical guiding of the aerosol, the design also features a ^{210}Po α particle source to remove any charges from the initial electrospray droplets [56]. While clearly beneficial to limit the drying artifact formation, the design still offers room for improvement - for example in reducing the background gas admission into the interaction region as significant amounts of both nitrogen and CO_2 are needed for nebulization and decharging, respectively [56]. Further, improvements in terms of sample consumption would be desirable as the aerosol injector delivers sample continuously and cannot be efficiently pulsed. Hence, research into new means of sample delivery into the XFEL beam can make a real breakthrough for SPI efforts at XFELs.

Native MS for single particle imaging at XFELs

Mass spectrometry has long been part of drug discovery processes in the form of proteomics and solution based studies of protein dynamics [57,58]. Moreover, native MS has become accepted as a powerful technique to transfer non-covalent protein complexes into the gas phase without perturbing their interactions and thus enabling studies of protein stoichiometries, binding and also conformations even though in a low resolution fashion [59,60]. Native MS is applicable for a broad range of molecular systems of all sizes from small isolated proteins up to huge multi-megadalton assemblies like viral capsids [61,62]. Moreover, the technique has also successfully tackled samples considered as notoriously difficult such as membrane proteins [63,64] or highly heterogeneous and dynamic systems of viral assembly intermediates [61,6567]. Finally, while not inherently claimed by its definition [59], it becomes accepted from experimental, spectroscopic and simulation work, that when handled properly, many proteins and their complexes can retain their 3D structure during native MS analyses, at least over the time scale of the experiments [6870]. Of great help in studying this has been the coupling of native MS with ion mobility, which can provide conformational information on the gas phase ions inside a mass spectrometer [71]. Furthermore, so-called soft landing experiments have been performed, where ions transmitted through a mass spectrometer have been gently landed on EM grids and were shown to have retained their conformation [72,73] or even their enzymatic activity [74] and infectivity in the case of viruses [75,76].

While so far ESI has only been used in SPI experiments with post-ionization charge neutralization, this effectively removes a potentially useful handle, which could be leveraged for manipulations of particles and their focused delivery into the XFEL interaction region for diffraction [77]. Further, apart from merely guiding ions from one place to another, MS can offer more advanced techniques of ion handling. For instance, ions can be temporarily accumulated in an ion trap and released in synchronization with the arrival of XFEL pulses. Ion trapping is expected to provide higher particle densities in the X-ray interaction region and thus better hit probability, while limiting the amount of sample wasted by accumulating the ions during periods of dark time in between the XFEL pulse trains. The dark time always constitutes a significant proportion of the X-ray delivery time. This is especially true at the European XFEL, where just 0.6 ms long high-repetition rate pulse trains of up to 2700 pulses each are interspersed by 99.4 ms of dark time [78]. During this period, no diffraction patterns are obtained and any particles delivered into the interaction region are wasted, unless used for other diagnostics purposes.

Mass spectrometry is also known for its ability to select and isolate ions in the gas phase based on their mass-over-charge ratio (*m/z*) or even their shape and conformation in mass filters and ion mobility devices, respectively. Quadrupolar mass filters typically use a combination of radiofrequency (rf) and DC voltages to enable only ions of specific *m/z* to pass through on a stable trajectory. While these rf-fields are normally harmonically oscillating, traps and mass filters driven by digital waveforms are being developed. [79,80] This is interesting because with a fixed frequency of harmonic oscillator, bigger particles can only be efficiently trapped by increasing the wave amplitude, soon reaching limits with glow discharge between electrodes. With digital mass filters, on the other hand, bigger particles can be handled by decreasing the freely adjustable frequency of the waveform, while keeping the amplitude constant [79], which should prove much more efficient for very high *m/z* species, currently relevant for SPI.

Also, controlled acceleration of ions and their gentle collisions with a neutral gas such as nitrogen, argon or xenon have long been used to control the amount of activation of ions in the gas phase and their level of desolvation. This could be a very useful feature for SPI efforts, where improper desolvation has been one of the factors introducing unnecessary diffractive noise and confounding high-resolution 3D reconstruc-

tion efforts [56]. However, completely stripping all water molecules from a gas phase ion may not always be the best approach. Especially for smaller proteins some residual adducted water molecules are often beneficial to keep their solvent-like structure temporarily kinetically trapped in the gas phase and prevent unfolding or retain protein-ligand interactions mediated by water molecules or hydrogen bonding [8183]. Also, XFEL SPI simulations indicate an optimum between better diffraction due to water-induced small protein stabilization and the level of induced diffractive noise [84]. It remains less clear to which extent this holds for viral particles and other huge assemblies, which were shown to be rather robust and of correct overall shape in the gas phase, even with less than one water molecule per subunit [85]. Nevertheless, means of controlling the level of desolvation and non-volatile buffer adduction will in any case prove beneficial to SPI efforts.

Finally, as a more challenging but potentially highly beneficial feature, ions with a dipole moment have been observed to orient in strong electric fields in variants of ion mobility MS [86,87]. This could provide a way to pre-orient molecules along their dipole axis in the gas phase just prior to imaging, which would significantly simplify computational demands for diffraction pattern orientation and electron density reconstruction [51,88]. Theoretical simulations addressing this topic demonstrated that (a) even partial pre-orientation would benefit computational reconstruction efforts and (b) depending on their size and dipole moment, protein orientation could be achieved in fields technically feasible and on time scales preventing the unfolding of the particle structure [88].

Perspectives for future science with native MS and XFELs

As discussed in the previous section, native MS offers very desirable features for SPI sample introduction at high-repetition rate XFELs. Importantly for the prospects of this technique, a preliminary study has shown that native MS can indeed generate sufficient ion fluxes for SPI experiments [89]. Hence, we are working within the MS SPIDOC consortium on exploiting native MS as sampling technique for SPI at XFELs (Fig.2). The prototype instrument is being designed around nanoflow ESI with a digitally-driven mass filter and ion trap to initially enable accumulation and selection of high mass particles such as virus capsids, which diffract strongly in SPI experiments. Further, ion mobility and high voltage dipole orienting modules are included to further conformationally select ions and to experimentally attempt to pre-orient ions, respectively. Finally, a time-of-flight mass analyzer with an ion detector is included in the design to enable online monitoring of the electrospray process and efficiency of ion manipulations.

If the native MS-based sample delivery approach proves successful, we envisage a whole spectrum of novel scientific applications opening up for SPI at XFELs. With its ability to select only particles of interest for analyses from a complex mixture, native MS should alleviate the challenge of sample heterogeneity, which has been identified as a bottleneck for SPI efforts [52,90]. This will also ultimately enable studies on transient species, which are only fleetingly present during the course of biochemical reactions, and are therefore inherently instable and resistant to purification and crystallization. As such species often only appear in tiny amounts, they tend to be very challenging for cryo-EM and its conformational classification algorithms, which can *ex post* sort observed particles into conformational classes and purify them computationally. However, this comes at high cost, as (if at all possible) the necessary size of the EM dataset expands dramatically for very low abundant species [91].

In the case of transient intermediates, of particular interest for both structural biology and rational drug design would be viruses and their (dis)assembly processes. These occur at key points during viral lifecycle and their successful completion is critical for the virus to multiply. The process is fine-tuned to facilitate assembly and mutations in the responsible proteins are hence rare. Therefore, the intermediates are interesting targets for pharmacological interference [5]. The process has notably been studied by native MS and ion mobility before, however, directly obtaining details of atomic structures of the intermediate states has been impossible so far [62,92,93] (Fig.3). Further on viruses, we can envisage studies of conformational changes occurring through structural cross-talk between a viral protein capsid and its genomic cargo, which plays an important role governing the packaging of viral particles [94]. Studies of these effects would on one hand necessitate 3D reconstruction algorithms not utilizing particle symmetry, first of which however already exist [29,40,50]. On the other hand, it can also be argued that such asymmetry could potentially induce sufficient dipole moment in the particle to enable its defined orientation prior to imaging, which could in turn help with aligning collected diffraction patterns.

Apart from viruses, direct visualization of biologically active transitional states of enzymes and structural proteins would in many cases enable proper rational design of drugs instead of blind screening. As a prominent example, one could think of the sophisticated machinery of a ribosome. During its functioning, a ribosome undergoes complex conformational changes which contribute to its active sites being extremely flexible [95]. As ribosomes due to their critical importance in protein synthesis have long been targeted by many antibiotics [96] and have also been recognized as a potential drug target for other diseases such as cancer [97], high resolution structural information on its transient states could be of high importance for designing new antibiotics

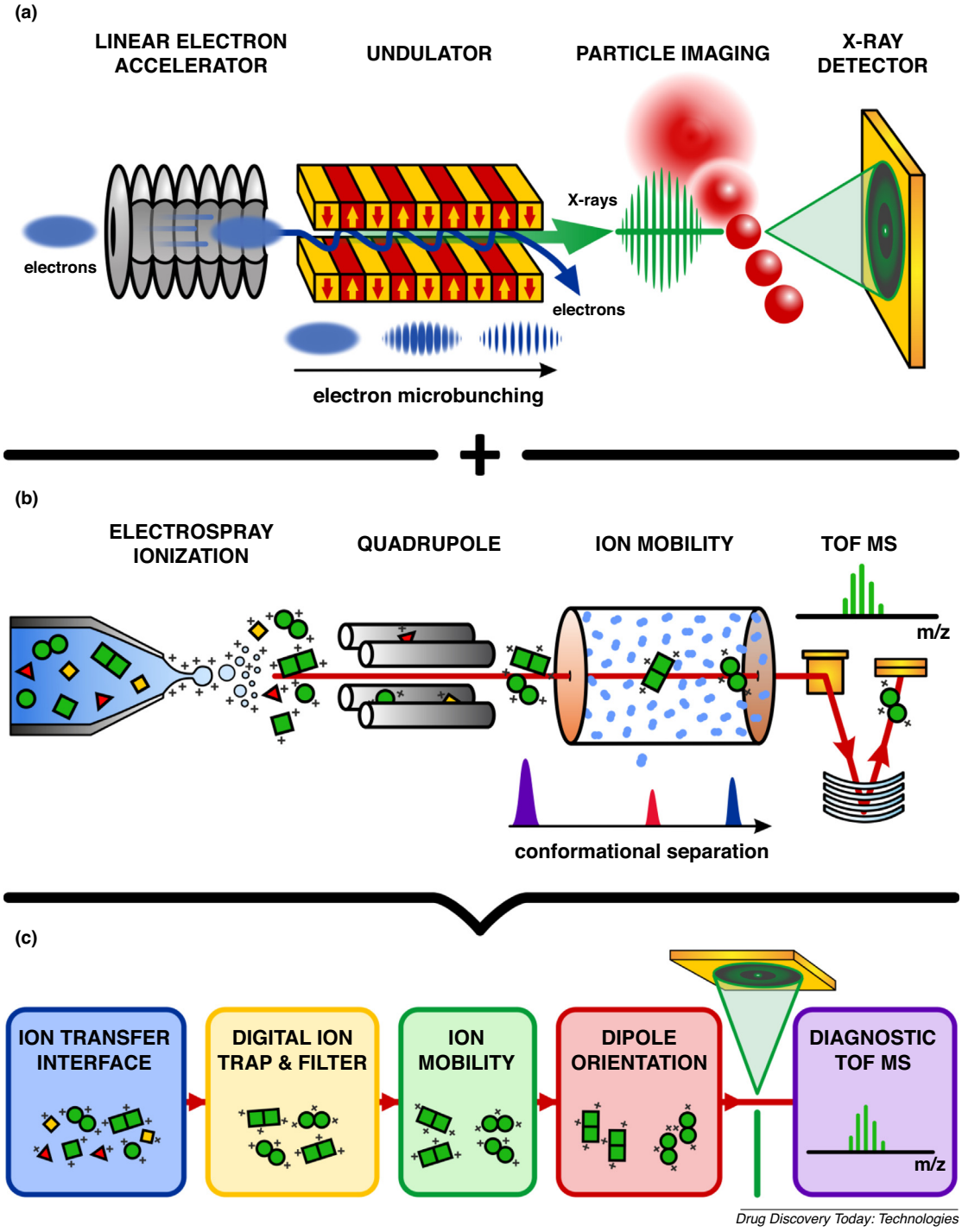

Fig. 2. Native MS meets XFELs – a schematic representation.
(A) XFEL-based single particle imaging. Electrons from a particle accelerator are used to create strong pulses of X-rays for imaging a stream of particles in a "diffraction before destruction" process. **(B)** Native MS allows a mass- and conformationally-selective analysis of proteins. A protein of interest in its two

and better drugs. Further, other notoriously challenging protein systems have been known to be amenable to studies by native MS alone or in combination with ion mobility. These techniques can provide information on the overall stoichiometry of such proteins and their conformation, but lack the structural resolution to pinpoint atomic details of their interaction with ligands or inhibitors, which SPI would be able to contribute. An example of such proteins could be GPCRs [98] or intrinsically disordered proteins [99], which are both prominent drug targets [100102]. However, they can adopt

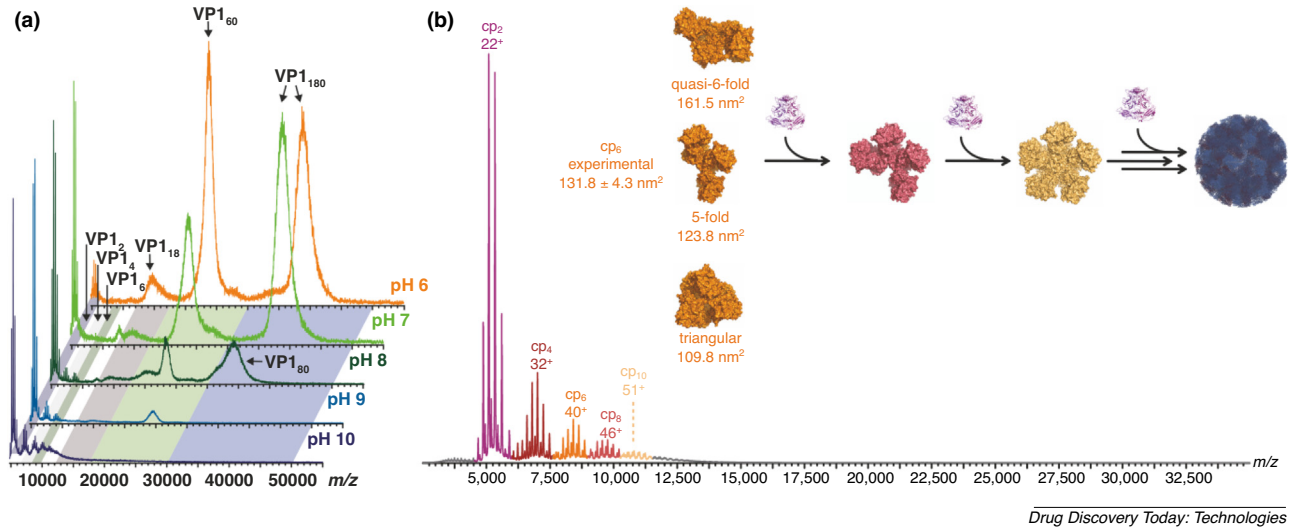

Fig. 3. Viral capsid assembly intermediates studied by native MS and ion mobility.
(A) Norovirus-like (GI.1 West Chester) particles display varying stability depending on solution pH as shown by native MS. A range of capsid formation intermediate products are visible as oligomers of the major capsid protein VP1. **(B)** Norovirus capsid intermediates probed by native MS and ion mobility under non-assembly conditions. Inset: experimental ion mobility collision cross section values for hexamer ions helped to propose the assembly pathway

highly flexible conformations, which quickly interconvert in solution, making them hard to study. Here, the ability to image molecular structures in a conformationally pre-selected manner would be beneficial. While assemblies of GPCRs are with their molecular size of about 100150 kDa somewhat too small for SPI at XFELs at least for the moment, and have thus so far been primarily studied (with atomic resolution) at XFELs by SFX [103], single particle diffraction from proteins of similar and smaller sizes has been simulated at realistic XFEL beam conditions and may become accessible experimentally in the future [48].

Last but not least, research in native MS has also developed in the direction of analyzing samples without their complete purification from cell lysates [104106] or released directly from natural membrane environment [64]. While these approaches are pioneering and need further optimization and benchmarking, one can imagine that if they eventually could be leveraged by a native MS-based sample introduction technique for SPI at XFELs, the options for biomolecular imaging would be moved to a whole different level.

Conclusions

With several world-class facilities available for user operation around the world and more forthcoming, hard XFELs hold a great promise for extremely fast, time-resolved studies of biomolecular structure and dynamics. This is especially true for modern high-repetition rate facilities like the European XFEL or LCLS-II, which provide extremely bright photon pulses at unparalleled speed. While serial crystallography approaches are generally mature enough at XFELs, sample introduction methods for single particle imaging still need further development to enable achieving near-atomic resolutions. We are convinced that if high-resolution diffractive imaging efforts at XFELs are to be successful, a sample introduction method based on mass spectrometry, leveraging the charges of ions to deliver a background free, mass- and conformationally separated particle beam into the X-ray interaction region, has the best chance of success. Finally, while so far the construction of hard XFELs necessitated huge investments to build and operate large dedicated experimental facilities, current developments of laser wakefield acceleration technology [107,108] could, if the numbers of photons per pulse can be significantly increased, eventually result in far more compact future designs, avoiding several kilometers-long tunnels or halls with electron accelerators, potentially making XFEL experiments and diffractive SPI more accessible. As such, we expect there may indeed be a flash of light visible at the end of the tunnel.

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Conflict of interest

The authors declare no conflict of interest.

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