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Radiation damage to macromolecules: kill or cure?

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Radiation damage induced by X-ray beams during macromolecular diffraction experiments remains an issue of concern in structural biology. While advances in our understanding of this phenomenon, driven in part by a series of workshops in this area, undoubtedly have been and are still being made, there are still questions to be answered. Eight papers in this volume give a flavour of ongoing investigations, addressing various issues. These range over: a proposed new metric derived from atomic *B*-factors for identifying potentially damaged amino acid residues, a study of the relative damage susceptibility of protein and DNA in a DNA/protein complex, a report of an indication of specific radiation damage to a protein determined from data collected using an X-ray free-electron laser (FEL), an account of the challenges in FEL raw diffraction data analysis, an exploration of the possibilities of using radiation damage induced phasing to solve structures using FELs, simulations of radiation damage as a function of FEL temporal pulse profiles, results on the influence of radiation damage during scanning X-ray diffraction measurements and, lastly, consideration of strategies for minimizing radiation damage during SAXS experiments. In this short introduction, these contributions are briefly placed in the context of other current work on radiation damage in the field.

Keywords: X-ray radiation damage; macromolecular crystallography; FEL; SAXS; radiation damage induced phasing; simulations.

Interest in radiation damage to macromolecules during structural experiments has not abated over the last few years, since there remains a need to understand both the parameters that affect radiation damage progression (the 'kill') and also the artifacts produced by it. Although there is now a growing body of literature pertaining to this topic (see, for example, the special issues of the *Journal of Synchrotron Radiation* arising from papers presented at the 2nd to 7th International Workshops on Radiation Damage to Biological Crystalline Samples, published in 2002, 2005, 2007, 2009, 2011 and 2013, respectively), clear foolproof methods for experimenters to routinely minimize damage have yet to emerge. Additionally, radiation damage is also a concern and limiting problem in other methods used in structural biology such as electron microscopy, SAXS and scanning X-ray diffraction. However, the recently available free-electron lasers (FELs) have presented the possibility and promise that samples will give 'diffraction before destruction': is this indeed the 'cure' for the challenges of radiation damage?

For the majority of macromolecular crystallographers, using a FEL is not yet a realistic expectation. For them, radiation damage to their samples is likely to become an increasingly observed phenomenon, since much smaller X-ray beams with very high flux densities are becoming available due to upgrades in both electron storage rings and the synchrotrons that feed them. These fourth-generation synchrotrons are engendering even more interest in research into radiation damage and its deleterious effects.

In this special issue, there are eight papers which were presented at the 8th International Workshop on Radiation Damage to Biological Crystalline Samples held at the EMBL Hamburg in April 2014. The first two cover radiation damage investigations carried out at synchrotrons and the next four describe studies related to data collection at FELs, detailing, respectively: indications of specific X-ray damage to ferrodoxin crystals, diffraction data analysis, phasing of structures, and simulations of radiation damage effects. These are followed by an account of the detection of damage to focused ion beam (FIB) milled samples investigated by X-ray scanning, and, lastly, by a discussion on mitigation strategies in small-angle X-ray scattering (SAXS) experiments.

Radiation damage to macromolecular crystals is observable in both reciprocal space (global damage) and real space (specific damage). Diffraction quality is compromised (high-resolution reflections fade with X-ray exposure, scaling *B*-factors increase and the unit cell expands) while, in the elec-

tron density, susceptible residues lose definition and metal centres are reduced. Generally specific damage occurs in a reproducible and clearly defined order as a function of dose, starting with the reduction of metal centres, followed by elongation and scission of disulfide bonds, and then decarboxylation of aspartates and glutamates. However, residues of the same type do not show damage at the same rate. Attempts to link the differential damage progression to various physiochemical parameters such as solvent accessibility and pK_a have resulted in limited and sometimes contradictory outcomes. In an effort to identify the parameters affecting the order of damage, Gerstel *et al.* (Gerstel *et al.*, 2015) suggest a new damage metric, B_{damage}, derived from atomic *B*-factors in a Protein Data Bank (PDB) file by accounting for the correlation of *B*-factor with packing density, and use this metric to carry out a large-scale survey of over 2000 different structures in the PDB. The metric was first validated using pairs of low-dose datasets separated by a burn, using six test cases previously collected for radiation-induced phasing (RIP) investigations by Nanao *et al.* (2005). Gerstel *et al.* conclude that differential damage rates are weakly correlated with accessibility and, for disulfide bonds, also with their conformation (spiral, hook or staple), but not with the local secondary structure around them. They postulate that a metric such as B_{damage} could be used in future to detect signs of damage in structures already deposited in the PDB: if this possibility can indeed be realised it would certainly be of great benefit.

A large amount of effort has been expended in radiation damage research to identify suitable metrics to plot against the absorbed dose in order to characterize the damage progression. In reciprocal space the predominant metrics currently used are, firstly, the decrease in the summed intensity of the diffraction of an entire dataset, of a repeated wedge or of individual reflections and, secondly, the change in the scaling *B*-factor. The absorbed dose has frequently been calculated using the software *RADDOSE*, which uses the elemental composition of the unit cell to calculate the appropriate X-ray absorption coefficient, and the beam characteristics (energy, flux, size, profile) to estimate the energy lost by the beam in the crystal, and hence the dose (energy lost per unit mass). *RADDOSE* originally did not take into account the crystal rotation in the beam, and also it reported the maximum dose absorbed at the peak of the beam profile. This was a good estimate for a crystal which was smaller than the X-ray beam, and thus completely immersed in a top-hat or near top-hat shaped beam, but did not give an accurate overall dose value for a crystal which was larger than the beam or irradiated by a Gaussian-shaped beam. A completely re-engineered version of the code, *RADDOSE-3D*, has now been released which provides a time- and space-resolved dose distribution map for a cuboid or spherical crystal rotating in an X-ray beam (Zeldin *et al.*, 2013*a*). In order to develop a more representative average dose metric than the maximum dose or the average dose over the whole crystal, Zeldin *et al.* (2013*b*) have suggested and experimentally validated a new dose metric, diffraction weighted dose (DWD). DWD combines information from the aggregation of dose within each volume element of the crystal up to a given time, with the way the crystal is being exposed at that moment. To validate the metric, three X-ray beams with different dimensions and different profiles were used on cubic insulin crystals to show that plotting the diffracted intensity decay against DWD gave remarkably similar dose to half-intensity values, whereas maximum dose and several other average dose metrics gave very scattered results. In the future, the use of DWD as the *x*-axis in radiation damage investigations should allow measurements from different researchers using varied beam conditions to be more readily comparable.

Bury *et al.* (2015) have used this new DWD metric against which to plot the specific structural damage to a biologically relevant protein–DNA complex (C. Esp1396I) at 100 K over a 2–45 MGy dose range. A generally applicable computational method was developed to analyse the thousands of difference peaks observed, allowing the general features of specific radiation damage to be extracted. This followed the expected pattern on the protein but, strikingly, the DNA component was determined to be far more resistant to specific damage than the protein. This raises the interesting question of whether the DNA is intrinsically less sensitive to radiation than the protein, or rather that the protein is more efficient than the DNA at scavenging the mobile electrons at 100 K and this is 'protecting' the DNA from damage.

In terms of understanding the detailed radiation chemistry of specific structural damage, an analytical model of disulfide bond breakage has been proposed and experimentally validated by Sutton *et al.* (2013). In a multi-method investigation, electron paramagnetic resonance and UV–vis microspectrophotometry, as well as X-ray diffraction, were used to obtain rate constants for various steps in the reduction of the four disulfide bonds in chicken egg-white lysozyme crystals. Another study using the complementary methodologies of high-resolution X-ray crystallography and online microspectrophotometry investigated the damage rates to bacteriorhodopsin (bR) crystals at 100 K and demonstrated that this protein undergoes structural alterations at doses of only 0.06 MGy, a dose 20 times below that previously thought to cause damage to the bR active site, and far below the doses usually considered 'safe' for structural studies carried out at 100 K (Borshchevskiy *et al.*, 2014).

Collecting data at 100 K compared with at room temperature (RT) is generally considered to give an increase in crystal lifetime of around a factor of 70 (Nave & Garman, 2005), although reported values for model test proteins vary somewhat. In a recent study of 70S ribosome crystals, data were collected from one crystal at 100 K and another at 300 K. The observed increase in lifetime and resolution of data collected at 100 K compared with 300 K (half doses of 64 MGy at 3 Å resolution and 150 kGy at 5 Å resolution, respectively), once the adjustments for the different resolutions were made, was around ten times greater than that observed for model proteins. The authors suggest that this increase in lifetime cannot solely be explained by a reduction in the rate of radiation damage. The hypothesis put forward is that the 70S

ribosome crystals diffract to significantly better resolution at 100 K because they undergo cooling-induced ordering that improves their diffraction quality (Warkentin *et al.*, 2014). It is interesting to note that the ribosome has a large RNA component, which may behave similarly to the DNA in the DNA–protein complex described above and reported by Bury *et al.* (2015).

Examination of X-ray induced specific damage has also resulted in some new mechanistic biological insights. For instance, direct evidence for a peroxide intermediate and a reactive enzyme–substrate–dioxygen configuration in a cofactor-free oxidase has been obtained. *In crystallo* Raman spectroscopy and high-resolution crystallography in conjunction with supporting QM/MM calculations were used to unambiguously show how uricase catalyses uric acid degradation *via* a C5(S)-(hydro)peroxide intermediate. At 100 K, O_2 was released and then trapped *in situ* by the breakage of the intermediate C5–OO(H) bond at low X-ray dose (Bui *et al.*, 2014). This study is a good example of the positive side of radiation damage and also of the use of complementary techniques in structural biology.

Several studies on methods to mitigate radiation damage in macromolecular crystallography (MX) have attracted recent interest. One suggestion, supported by experimental evidence, is that, if RT measurements at synchrotron sources are made fast enough, there can be a $\sim$100 ms lag phase before intensity loss is observed, effectively outrunning the damage (Owen *et al.*, 2014). New hardware in the form of the PILATUS series of detectors (*e.g.* PILATUS3 300K, frame rate 500 s^{-1}, each frame 2 ms with 0.95 ms dead-time in between) provides a means to collect data much faster than before and allows this outrunning effect to be observed. This is a very promising result since it might allow essentially undamaged structures to be determined even at RT. Further evidence for the RT dose rate effect has been provided by an experimental and modelling study of the RT photoreduction of manganese ions in the oxygen-evolving complex of photosystem II, using time-resolved X-ray emission spectroscopy with wavelength-dispersive detection. This protein is known to be particularly radiation sensitive. A model was developed and fitted to the experimentally observed rate constants which was then used to infer the behaviour of the system at other incident wavelengths and different dose rates. Trends indicating later onset of damage at higher dose rates and when using a pink beam were predicted (Davis *et al.*, 2013).

Another mitigation strategy, again experimentally tested, is that a vertical submicrometre line-focus beam (0.7 µm full width at half-maximum) of 18.6 keV X-rays can give a mitigation factor of 4.4 $\pm$ 0.4 when compared with the damage caused by a wider beam under conditions of equal exposure and equal protein crystal volume (Finfrock *et al.*, 2013). The decrease in damage rate is due to the escape of the photoelectrons, determined here to have a penetration depth of 5 $\pm$ 0.5 µm, from the irradiated crystal volume. Such improvement due to the escape of photoelectrons from the beam volume has also been shown previously for a circular beam (Sanishvili *et al.*, 2011).

In the future, optimization of data collection strategies will be particularly important to fully realise the potential of the newly available microbeams being provided for MX. The information and experience gained from such studies as those mentioned above will be of vital importance to inform practice at fourth-generation synchrotron sources. Several of these will be coming on line soon and will deliver much higher X-ray flux densities, although will be nowhere near those provided by the available FELs.

X-ray FELs produce short (tens of fs) and extremely intense X-ray pulses with a peak brilliance exceeding that obtained at third-generation synchrotron sources by ten orders of magnitude. The basic concept behind FEL-based crystallography is a 'diffract-before-destruct' approach (Neutze *et al.*, 2000), in which the femtosecond pulse provides a diffraction pattern before radiation damage destroys the crystal. The sample is replenished and diffraction data are collected in a serial way on microcrystals (Chapman *et al.*, 2011). By applying this so-called serial femtosecond crystallography [SFX; for a review see Schlichting (2015)], protein structures can be determined at high resolution (Boutet *et al.*, 2012). Alternatively, macrocrystals can be used by translating and rotating them across the beam to collect serial images (Hirata *et al.*, 2014; Suga *et al.*, 2015; Cohen *et al.*, 2014). As a complement to synchrotrons, FELs promise to revolutionize structural biology by allowing the structure of radiation-sensitive proteins to be solved from tiny microcrystals and their dynamics to be studied with up to femtosecond time resolution (see a recent special issue of *Philosophical Transactions of the Royal Society B* entitled 'Biology with free-electron X-ray lasers'). In the majority of cases, SFX experiments are being carried out at RT, thus preserving the flexibility of the biological macromolecule at physiological temperatures that is altered in cryo-crystallographic experiments (Weik & Colletier, 2010; Fraser *et al.*, 2011).

The absorbed X-ray dose per crystal in an SFX experiment can exceed many-fold the dose limit for cryo-cooled crystals in a synchrotron MX experiment (30 MGy; Owen *et al.*, 2006) without signs of global (Chapman *et al.*, 2011) or specific radiation damage (Boutet *et al.*, 2012). A cure for the daunting curse of radiation damage in MX thus seems within reach. Indeed, metallo-protein structures showed damage when data were collected on large crystals at 100 K at synchrotron sources but not when collected at a FEL (Hirata *et al.*, 2014; Suga *et al.*, 2015) with the same cumulative dose (less than 30 MGy). However, when collecting SFX data at a FEL at much higher doses [*e.g.* up to 3 GGy (Lomb *et al.*, 2011), *i.e.* well above the 400 MGy limit below which diffraction is mainly from pristine atoms (Chapman *et al.*, 2014)], global radiation damage has been observed (Lomb *et al.*, 2011; Barty *et al.*, 2012) as well as indications of local damage (Lomb *et al.*, 2011). Such global radiation damage has been suggested to originate from X-ray induced atomic disorder that eventually turns off Bragg diffraction before the end of the incident FEL pulse (Barty *et al.*, 2012). A paper in this issue (Nass *et al.*, 2015) now for the first time provides indications of specific radiation damage to a crystalline protein at high resolution

as a result of FEL pulses. The authors deliberately chose to expose ferredoxin microcrystals, containing two [4Fe–4S] clusters, to unattenuated 80 fs pulses from the FEL at Stanford. SFX data sets were collected with an absorbed dose per crystal of up to 30 GGy. In addition, traditional rotation data sets were collected from ferredoxin macrocrystals at a synchrotron source, with cumulative doses that were six orders of magnitude lower. Difference electron density maps calculated between the synchrotron and the FEL data show reduced electron density of the iron atoms in the FEL data. Most interestingly, this effect is stronger in one of the two clusters, indicative of differential specific radiation damage due to the slightly different geometries of the clusters.

Nass *et al.* (2015) also carried out plasma code calculations, indicating that most of the diffraction signal obtained with 80 fs pulses comes from the first 10–30 fs. Using the same plasma code for calculations, another manuscript in this issue presents simulations of how the temporal profile of a FEL pulse affects global radiation damage (Jönsson *et al.*, 2015). The authors conclude that a front-loaded FEL pulse, with most photons early in the pulse, maximizes the diffracted intensity.

On the timescale of a femtosecond FEL pulse, the diffracting crystal remains immobile and a so-called *still image* is collected. In contrast to Bragg-spot intensities retrieved from traditional oscillation images collected from a macrocrystal on synchrotron sources, the partiality of intensities in still SFX images is unknown. Unknown partialities are the major reason why tens of thousands of indexed still images are usually required per SFX data set, so that typically the integrated structure factors are determined by taking the mean of the large number of observations in a so-called Monte Carlo approach (Kirian *et al.*, 2011). A paper in this issue (Sauter, 2015) presents a method to correct experimentally measured still images for partiality by using post-refinement, under the simplified assumption that the X-rays are monochromatic. Together with alternative approaches to estimate partiality (Kabsch, 2014; White, 2014), the paper by Sauter thus gives direction to the quest of reducing the required number of indexed images in serial crystallography.

Almost all protein structures determined so far from SFX data have relied on molecular replacement for phasing. SFX data have been shown to be accurate enough to measure the weak anomalous signal from naturally occurring sulfur atoms, yet the signal has been too weak for SAD phasing (Barends *et al.*, 2013). So far, only a single protein structure has been determined *de novo* by SFX, making use of the strong anomalous signal from gadolinium atoms for experimental SAD phasing (Barends *et al.*, 2014). Also a modified version of MAD in the high-intensity regime has been suggested for potentially providing experimental phases in SFX at FELs (Son *et al.*, 2013). In this issue, Galli *et al.* propose a method for phasing SFX data based on the radiation-induced ionization of sulfur atoms (Galli *et al.*, 2015). Analogously to the parent RIP method at synchrotron sources (Ravelli *et al.*, 2003), the high-intensity radiation-induced phasing (HI-RIP) suggested by Galli *et al.* exploits the change in sulfur scattering factors between data sets collected with high and low photon fluence. The simulations imply that HI-RIP, under the experimental conditions currently available at FEL facilities, could indeed be used to determine substructures and produce interpretable electron density maps. While experimental proof has yet to be provided, HI-RIP suggests a way to make positive use of radiation-induced changes that are generally an undesirable aspect of MX.

Method developments in synchrotron- and FEL-based MX are complementary and benefit each other. Since early 2014, several publications have reported the implementation of serial crystallography at synchrotron sources (Gati *et al.*, 2014; Stellato *et al.*, 2014; Heymann *et al.*, 2014; Nogly *et al.*, 2015; Botha *et al.*, 2015; Coquelle *et al.*, 2015) in which the total absorbed X-ray dose is spread over a very large number of crystals. Most importantly, serial synchrotron crystallography can be carried out at RT by delivering crystals to the X-ray beam in a capillary (Stellato *et al.*, 2014), or on a microfluidic chip (Heymann *et al.*, 2014), or by using slowly flowing lipidic cubic phase (LCP) (Nogly *et al.*, 2015) or high-viscosity extrusion (Botha *et al.*, 2015) sample injectors. RT crystallography avoids the use of cryo-protectants, allows for time-resolved kinetic experiments and provides macromolecular structures that have not had their conformational flexibility altered by cryo-cooling (Fraser *et al.*, 2011). Furthermore, we expect serial crystallography at third- and fourth-generation synchrotron sources to provide fine opportunities for systematically studying specific and global radiation damage to biological macromolecules at RT over a wide range of doses and dose rates.

In 2014, the UNESCO International Year of Crystallography was celebrated to mark 100 years of diffraction and both *Nature* and *Science* included specially commissioned papers reviewing the field of macromolecular crystallography. These included reviews of the current state and possible future trends in the field (Garman, 2014; Miller, 2014) and a lively debate of the relative merits of synchrotron and FEL data collection for structure solution (McSweeney & Fromme, 2014). The next few years will surely put these perspectives to the test.

This Special Issue also includes two papers covering aspects of the challenge of radiation damage in complementary structural biology methods. Storm *et al.* (2015) employ focused ion beam (FIB) milling using gallium ions in combination with scanning electron microscopy to prepare precisely aligned softwood (Norway spruce) samples for scanning X-ray diffraction (SXD) experiments. Horizontal and vertical scanning with a less than 100 nm $\times$ 100 nm sized X-ray beam (ID13, ESRF) allowed high spatial resolution to be achieved to investigate the cell wall structure of the wood. Storm *et al.* (2015) observed that the gallium ions from the FIB milling had penetrated at least 1 μm into the sample, causing dislocations to the order of the cellulose structure in the process. Biological samples are known to suffer severe radiation damage during SXD, and here a combination of these dislocations and the enhanced X-ray absorption due to the incorporated gallium ions contributed to the high fading rate of the detected

diffraction intensity. As in all the experiments described thus far in this introduction, the importance of understanding the effects of damage on the results is yet again reinforced.

Another method in structural biology which has risen in application and utility in the last few years is small-angle X-ray scattering (SAXS). Due to vastly improved dedicated hardware at synchrotron beamlines and more sophisticated automated processing software such as *ATSAS* (Petoukhov *et al.*, 2012), SAXS is now commonly used to define the molecular envelopes and complement both electron microscopy and X-ray diffraction structural information. However, radiation damage is also a perennial problem in SAXS measurements, causing aggregation of the protein and thus obscuring the signal from non-interacting protein molecules. Common strategies for minimizing the problem are either to oscillate the sample or to continuously flow new material through the beam path. Cryo-SAXS is also an option, although it is complicated by the necessary addition of a cryo-protectant reducing the contrast between the weak sample signal and the solution. Recent SAXS measurements of the improvement of lifetime of samples irradiated at 100 K rather than at RT have shown that the maximum tolerable dose is ∼100 MGy (Meisburger *et al.*, 2013), nearly five orders of magnitude larger than the limit of ∼400 Gy at RT (Kuwamoto *et al.*, 2004). These compare with the MX values for crystals of ∼500 kGy at RT (Southworth-Davies *et al.*, 2007) and 30 MGy at 100 K (Owen *et al.*, 2006). A critical examination and evaluation of radiation damage mitigation approaches for SAXS is given by Jeffries *et al.* (Jeffries *et al.*, 2015), based on experimental observations born from operating the high-brilliance SAXS beamline [P12: 5.1×10^{12} photons s^{-1}, 10 keV, 200 (V) µm × 110 (H) µm FWHM beam size at the sample position] at PETRA III in Hamburg. This beamline is equipped with both hardware and software which enable automated sample handling and SAXS data acquisition. Among the strategies discussed for reducing the rate of damage are flow-enabled samples *versus* static ones, beam attenuation and the use of solution additives.

Although there are no papers in this Special Issue discussing radiation damage in electron microscopy (EM) measurements, it should be noted that this field is currently undergoing a revolution due to the development of new faster and more sensitive detectors which directly, rather than indirectly, detect the electrons [*e.g.* the K2 Summit camera (Gatan)]. These are allowing an impressive extension of existing EM capabilities, and have enabled, for instance, the 3.2 Å resolution structure of *Escherichia coli* β-galactosidase (465 kDa) to be solved using single-particle cryo-EM (Bartesaghi *et al.*, 2014). Interestingly, by examining the electron density in structures obtained at various electron doses ranging from 10 to 30 e^{-} Å^{-2}, the authors noted that negatively charged residues such as aspartates and glutamates suffered greater radiation damage, as is also observed in MX. These developments in EM, providing structures at much higher resolution nearing that obtained in MX, open up the possibility of even greater complementarity between the two fields and of obtaining structures of large proteins, or complexes thereof, that cannot be crystallized.

In conclusion, it is clear from the above brief survey that there remains much scope for further studies to inform both experimental practice and the interpretation of the resulting structures so that radiation damage can become a widely recognized and understood facet of structural biology. These experiments on macromolecular crystals will certainly involve more 'kill', and, it is to be hoped, some 'cure' too.

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