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Radiation damage to biological macromolecules: some answers and more questions

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Research into radiation damage in macromolecular crystallography has matured over the last few years, resulting in a better understanding of both the processes and timescales involved. In turn this is now allowing practical recommendations for the optimization of crystal dose lifetime to be suggested. Some long-standing questions have been answered by recent investigations, and from these answers new challenges arise and areas of investigation can be proposed. Six papers published in this volume give an indication of some of the current directions of this field and also that of single-particle cryo-microscopy, and the brief summary below places them into the overall framework of ongoing research into macromolecular crystallography radiation damage.

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It is now widely recognized that radiation damage (RD) during macromolecular crystallography (MX) experiments is a mainstream concern for structural biologists, since it can be a major limiting factor in structure determination and in obtaining high-resolution information, as well as sometimes compromising the biological interpretation of observed electron density. Concerted research into the character and progression rates of radiation damage in MX has now been ongoing for nearly fifteen years (see, for instance, papers from the second, third, fourth, fifth and sixth radiation damage workshops in special issues of the *Journal of Synchrotron Radiation* in 2002, 2005, 2007, 2009 and 2011, respectively). Although much more is now understood and there are answers to some of the questions posed in these earlier articles, there are still many areas where further investigation and more thorough characterization could greatly benefit the practising crystallographer. To obtain useful statistically significant results, RD experiments must involve more than one sample investigated under nominally the same conditions: this means that the studies have been both labour intensive and time consuming. However, the recent widespread and ongoing automation at synchrotron beamlines involving robotic crystal mounting from liquid-nitrogen dewars, and implementation of data collection and processing pipelines, as well as the staggering increase in detector speed, has opened up new and exciting possibilities for multi-sample systematic studies which are now bearing fruit. Additionally, many complementary methods are increasingly being employed in concert with crystallography to gain a deeper understanding of

the processes involved in RD progression [see, for example, X-ray-excited optical luminescence of protein crystals (XEOL) (Owen *et al.*, 2012a) and a recent special issue entitled 'Protein Structure and Function in the Crystalline State: From X-ray to Spectroscopy' of *Biochimica et Biophysica Acta* (BBA, 2011)].

For some aspects of RD, the accumulated knowledge from such research is now allowing practical recommendations for optimizing crystal dose lifetime to be suggested and tested experimentally. Five papers published in this volume give an indication of some of the current directions of the field, and the brief summary below places them into the overall framework of ongoing research into MX radiation damage. The remaining paper describes progress in combining the individual frames of a cryo-electron microscopy exposure series, in order to reduce the beam-induced blurring, a phenomenon caused by beam-induced particle movements.

The vast majority of systematic radiation damage studies have so far been carried out at 100 K, the temperature at which approximately 95% of all synchrotron MX datasets are currently collected because the radiation sensitivities of macromolecular crystals is reduced by up to two orders of magnitude (Garman, 2010) compared with at room temperature (RT). However, there is currently renewed interest in RD effects at RT since RT data collection at synchrotrons is at the beginnings of a renaissance, due to a number of factors such as the facility to mount crystallization plates directly on some goniometers (Jacquemet *et al.*, 2004), the availability of convenient devices to control the humidity (Sanchez-Weath-

erby *et al.*, 2009) and the recent discovery that RD could be outrun at RT (Owen *et al.*, 2012b; Warkentin *et al.*, 2013) using the latest generation of pixel detectors (*e.g.* Broennimann *et al.*, 2006). In fact a series of case studies showing the power of this *in situ* RT approach was published recently (Axford *et al.*, 2012). In addition, RT collection is used for protein crystals which do not tolerate cryo-cooling (*e.g.* virus crystals) and also because biologically relevant conformational heterogeneity can be preserved (Fraser *et al.*, 2011). Studies of RD at RT started in 1962 with the seminal work of Blake and Phillips (Blake & Phillips, 1962), but were followed by comparatively few investigations [all work until 2007 reviewed by Southworth-Davies *et al.* (2007)] until more recently when the renewed interest in RT data collection has resulted in a number of important observations and developments.

In this issue, Warkentin *et al.* (2013) provide a comprehensive review of recent progress made in describing global radiation damage at RT and how it evolves as the temperature is decreased down to cryo-conditions. A transition in radiation sensitivity has been observed at 200 K (Warkentin & Thorne, 2010), at which both protein and the surrounding solvent undergo a coupled glass transition (Weik & Colletier, 2010). Above 200 K, global radiation damage is thus dominated by diffusive motions in the protein and the solvent (Warkentin & Thorne, 2010) and a ‘dark progression’ of damage is observed when X-rays are turned off (Warkentin *et al.*, 2011). As opposed to data collection at 100 K where only small dose-rate effects have been observed at the flux densities currently used [global (Owen *et al.*, 2006; Sliz *et al.*, 2003), specific (Leiros *et al.*, 2006)], a dose-rate effect is present at temperatures above 200 K and, for example, half of the global damage can be outrun at 260 K by collecting data in ~ 1 s at a dose rate of 680 kGy s^{-1} (gray: energy absorbed per mass of crystal, $\text{Gy} = \text{J kg}^{-1}$) (Warkentin *et al.*, 2012a). At even higher dose rates (*i.e.* 1 MGy s^{-1}) a significant fraction of global damage can be outrun even at RT (Owen *et al.*, 2012b). Consequently, further increases in synchrotron-radiation brilliance and detector readout speeds are predicted (Warkentin *et al.*, 2013; Owen *et al.*, 2012b) to raise the RT dose limit close to the 30 MGy (dose to reach 0.7 of the initial diffraction intensity) value determined for data collection at 100 K (Owen *et al.*, 2006). Synchrotron-based data collection at RT is also the cornerstone of time-resolved Laue crystallography, a technique which aims to provide structural snapshots of conformational changes after reaction initiation in crystalline proteins. A recent paper addressed RT RD in time-resolved Laue studies on photoactive yellow protein crystals (Schmidt *et al.*, 2012). The authors found that refinement of a kinetic reaction mechanism was only possible from data collected up to 40% of the dose limit applicable to determining reliable static structures.

A further RT study is presented in this issue (Leal *et al.*, 2013), in which a comprehensive analysis of the RD rates at dose rates between 0.05 and 300 kGy s^{-1} for 15 different model proteins using previously established automated techniques (Leal *et al.*, 2011) was carried out. The decay of diffracted intensity as a function of dose was explored using a

two-parameter model which at RT is found to be resolution independent and to give a linear increase in Debye–Waller factors. The authors introduce a new global damage metric called the normalized half-dose, which is a modified version of the conventional $D_{1/2}$ (the dose required to reduce the intensity of the diffraction to half of its original value). This new metric is found to be a better descriptor of the intensity decay since $D_{1/2}$ was observed to be dependent on the B -factor, B_0 , of the first wedge of data, with higher B_0 giving higher $D_{1/2}$ due to the fact that the weak high-resolution reflections were not present even at the start for these cases. The usual $D_{1/2}$ is thus normalized here to $D_{1/2}^N$ corresponding to the decay of the sum of intensities when adjusted to a starting value representing a standard overall B -factor of 20 \AA^2 . $D_{1/2}^N$ is reported to vary by a factor of more than ten over the range of crystal types studied. In the study, no dose-rate effects were observed over the range investigated, in contrast to some previous studies within the same dose-rate range [*e.g.* Rajendran *et al.* (2011): 1.3 to 8.4 kGy s^{-1}]. Interestingly, a correlation was established between crystal sensitivity at RT and solvent content with the approximate relationship $D_{1/2}^N \propto (\text{solvent content})^{-1} - 1$. The normalized $D_{1/2}^N$ will be calculated in *BEST* (Bourenkov & Popov, 2010).

RT crystallography is also attracting renewed attention owing to the recent exciting and promising results emerging from using X-ray free-electron lasers (XFELs) on biological samples, where outrunning the damage has now been successfully achieved on a grand scale. The latest experiments impressively expand the field beyond the seminal paper of Chapman *et al.* (2011) which first proved that the diffraction-before-destruction concept (Neutze *et al.*, 2000) is applicable to protein structure determination [reviewed by Spence *et al.* (2012), Schlichting & Miao (2012)]. In these so-called serial femtosecond crystallography experiments (SFX) performed at RT, several tens of thousands of diffraction images are collected, each one from a small (typically $1 \mu\text{m}$) protein crystal. Short (5–100 fs) and very brilliant X-ray pulses from an XFEL are used with the crystals streaming across the beam path in a free jet. The dose absorbed (as calculated using *RADDOSE* v2) by each crystal during the XFEL pulse equals or even exceeds (Boutet *et al.*, 2012; Kern *et al.*, 2012) the experimental dose limit of 30 MGy determined for one or several complete datasets in MX at 100 K (Owen *et al.*, 2006). It should be noted that since *RADDOSE* currently does not take into account the possibility of photoelectrons escaping from the irradiated crystal, it has limited applicability in estimating absorbed doses for XFEL experiments, during which this escape of energy is highly probable from the nano-crystals used. SFX has now even successfully been applied to structure determination from *in vivo* grown protein crystals (Redecke *et al.*, 2012; Koopmann *et al.*, 2012). Furthermore, time-resolved pump–probe SFX (Neutze & Moffat, 2012) is beginning to be successfully applied (Aquila *et al.*, 2012) and might eventually provide molecular movies of proteins at work with femto-second time resolution.

Returning to more conventional MX experiments, one of the possible strategies for extending the crystal dose lifetime is

to add radical scavengers either at the crystallization stage (co-crystallization) or by soaking crystals prior to data collection at RT or 100 K. Studies to test this idea in MX date back to 1974 with attempts to use styrene to prolong immunoglobulin crystal lifetimes using styrene (Zaloga & Sarma, 1974). Results from ongoing investigations to determine the efficacy of many different scavengers using different global RD metrics [diffraction intensity loss of dataset n relative to the first dataset (I_n/I_0), relative B -factor increase (B_{rel}) and decay R -factor increase (R_d)] as well as their effect on final electron density maps have produced contradictory results, with very little agreement between studies conducted by different research groups. Only one scavenger has been reported to be effective in modifying global damage by more than a factor of two, and that is 1,4-benzoquinone at RT (Barker *et al.*, 2009) monitored by overall diffraction intensity decay and another, sodium nitrate, reduced the specific damage to disulfide bonds by a factor of five at 100 K (De la Mora *et al.*, 2011) as observed in electron density maps. However, neither of these results could be reproduced in experiments which monitored global damage to four different protein crystal types soaked in 19 different small-molecule compounds *via* the increase in B_{rel} of repeated 5° wedges of data, although sodium nitrate was found to be slightly effective (factor of two or less) at RT on some of the crystal types (Kmetko *et al.*, 2011). The latter study found none of the compounds to be effective at 100 K and that six of the compounds in fact sensitized the crystals at RT.

A paper in this issue (Allan *et al.*, 2013) examines the possible reasons for these disparate results and provides a table detailing all the scavengers which have given inconsistent results in MX, as well as a supplementary table containing all studies to date. Identified causes of experimental disagreement are that conclusions as to efficacy can depend on the global damage metric used to analyse the data and inaccuracies in calculating the dose against which these metrics are plotted. Additional confounding features in these studies are an observed large variation in results from the same type of crystal treated in the same way under the same beam conditions, and the influence of the different mother liquors, some of which themselves have scavenging or sensitizing properties. The authors report RT microspectrophotometry results showing that combinations of mother liquor constituents can produce new absorption peaks absent for the individual components. One conclusion from the work is thus that a clear understanding of the radiation chemistry in crystallization solutions and at various pHs would better inform our understanding of the rates of radiation damage. Taken together, the various factors summarized above indicate that, unless a scavenger is found repeatedly to have an efficacy of greater than a factor of two in reducing global damage rates, it is unlikely to be of general utility in MX.

An interesting question arises out of MX scavenger studies: if the rate of damage can be modified at 100 K by chemical additives, is the concept of an upper dose limit for crystals after which the biological information is compromised because of radiation damage a valid one? Such a limit was

suggested by Henderson (1990) to be a $D_{1/2}$ of 20 MGy by analogy with observed destruction rates in electron crystallography, and a $D_{1/2}$ of 43 MGy has been experimentally determined for MX [although 30 MGy ($= D_{0.7}$) was suggested as the maximum to avoid compromised biological results]. Here, Allan *et al.* suggest that indeed this limit will pertain in the absence of electron scavengers in the crystallization solution or cryo-buffer, since electrons and holes are presumably the only significantly mobile species in an amorphous glass at 100 K.

Development of methods for correcting data for RD effects after the data have been collected has attracted some effort in recent years. However, apart from zero-dose extrapolation (Diederichs *et al.*, 2003), standard protocols are not yet available in the data-reduction software packages for these to be routinely used, since a realistic radiation decay model is required that can be incorporated into them. In this issue, Borek *et al.* (2013) present a conceptual radiation decay model involving matrix singular value decomposition (SVD) in reciprocal space to separate the effects of global and specific damage for multiple complete datasets. Since the structural changes alter the structure factors, RD affects the scaling and merging of data collected at different absorbed doses. Using SVD, the authors have proposed a model with two components: one is a resolution-dependent decay correction and the other is a uniform-per-unique-reflection term to account for specific radiation-induced changes. They have tested and validated the suggested model by applying SVD to sequential datasets (between 9 and 25 per crystal) collected at 100 K from one crystal each of three different proteins and two crystals of another protein which were soaked in either gold or platinum compounds. The scaling B -factors of the datasets are used as a proxy for the dose, assuming that the B -factor increases linearly with dose at 100 K (Kmetko *et al.*, 2006). The model was found to perform well, and offers a first step in being able to carry out post-experiment correction for RD to improve the quality of resulting structures.

In the above study, the B -factor was used to determine the dose, the metric against which global and specific damage indicators are normally plotted. This method was used because the calculation of the actual absorbed dose remains problematic in MX, since it requires knowledge of both the X-ray beam parameters (size, profile, flux, energy) and crystal properties (size, unit cell, number of amino acids per unit cell, number of heavier atoms per unit cell, concentration of buffers). In addition, *RADDOSE* (Murray *et al.*, 2004; Paithankar *et al.*, 2009; Paithankar & Garman, 2010), the widely used software for dose calculations, was designed for experimental situations in which the crystal was typically smaller than the beam, so that the crystal was totally bathed in the beam as it was rotated. Although in *RADDOSE* beam profiles can be characterized as 'top hat' or Gaussian in shape, the calculation for the latter case returns the worst-case scenario (dose at the position irradiated by the peak flux). For beams with small full width at half-maxima (FWHM), as are now available at a number of synchrotrons, the true dose profile can be very inhomogeneous across the irradiated

volume. As a first step in addressing this challenge, a paper in this issue (Zeldin *et al.*, 2013) describes simulations of the spatial dose profiles for two different dose-spreading data-collection strategies, helical scanning and translational, for four different generic crystal shapes (cuboid, plate, short and long needles) and a range of Gaussian beam FWHM. The results are compared with the dose profile obtained using a 'standard' data collection protocol. For these simulations, a new software package has been developed, *RADDOSE-3D*, which includes crystal rotation and also provides a range of new dose metrics suggested by the authors to better describe the differential irradiation of crystals. Analysis of these metrics, in particular the peak dose per unit diffraction, indicates that for the cases presented the optimum strategy is always to use as uniform (top hat) a beam as possible. In addition, as is already common practice, the beam size should be matched in both dimensions to the crystal, or a helical scan should be performed with a beam which is narrow along the rotation axis and matched to the crystal size in the perpendicular axis. Further development of this simulation software and its release to the community should enable dose-spreading data-collection protocols to be more easily optimized in the future, particularly if the actual beam profile and true crystal topography can be input into the calculation in real time on the beamline.

A different approach, Monte Carlo simulation, has contributed to the debate on the optimum wavelength for MX experiments. It has been used to explore data-collection efficiency [defined as the number of data of prescribed quality, in terms of resolution and $I/\sigma(I)$, that can be collected per unit volume of a given crystal] at 100 K as a function of incident X-ray energy between 5 and 80 keV (Fourme *et al.*, 2012). The simulations took into account the escape of photoelectrons from the crystal surface (which *RADDOSE* currently does not), and found that there would be an advantage for micrometre-sized crystals irradiated between 24 and 41 keV, in broad agreement with previous work (Cowan & Nave, 2008). Experimentally, the authors combined their own data for chicken egg-white lysozyme crystals irradiated at 18 keV and 33 keV with that from a previous study carried out at nine energies between 6.5 and 33 keV (Shimizu *et al.*, 2007). After corrections for the variation in detective quantum efficiency (DQE) with energy were applied to all the data, it was found that the dose required to collect a dataset of a particular resolution and signal-to-noise ratio decreased with increasing photon energy and the data collection efficiency increased by a factor of eight if the detector were ideally efficient. This was a larger gain than was predicted by the Monte Carlo simulations, and the authors commented that further experiments are needed to confirm the conclusions of their study, but that pixel detectors with good efficiencies at high X-ray energies will be vital to realise the potential gains in the future.

New detectors are also an important factor in the current revolution in the information that can be obtained from cryo-electron microscopy (cryoEM), which is becoming an increasingly important technique for elucidating the structure and function of macromolecular assemblies. Low-dose cryo-

electron crystallography is now a mature technique and has produced the highest resolution EM structures to date, including some of membrane proteins at resolutions better than 3 Å (Fujiyoshi, 2013). Tremendous improvements have been made in single-particle (SP) cryoEM, from which near-atomic-resolution density maps have been generated for icosahedral viruses without the need for crystallization (Zhou, 2011; Chang *et al.*, 2012). Substantial progress has also been made in determining structures of non-icosahedral proteins, either by employing SP cryoEM or cryo-electron tomography. Hardware as well as software advances have been essential to obtain such improvements. The new generation of cryo-electron microscopes which have now been available for some years have played a crucial role: a further leap forward is expected (Faruqi & Henderson, 2007) from the new generation of fast high-DQE direct-detection detectors. The first novel structure utilizing such a detector has just been reported (Bai *et al.*, 2012). These detectors provide a unique opportunity for improving our understanding of and, ultimately, our treatment of apparent specimen motions induced by the high-voltage electron beam during the measurements. It was shown recently (Brilot *et al.*, 2012) that large viral particles, suspended in a thin layer of vitrified ice spanning a hole in perforated carbon films, move in patches upon beam exposure, and can rotate up to a few degrees. In this issue, Karimi Nejadasl *et al.* (2013) use CCD detector exposure series (Karimi Nejadasl *et al.*, 2011; Karuppasamy *et al.*, 2011) to study beam-induced specimen movements and present non-rigid registration schemes to correct for them.

Previously, it has been shown that specific X-ray-induced radiation damage could be converted into an advantage and utilized for structure determination (Nanao & Ravelli, 2006). Recently, an analogue of this was presented for cryoEM: the inner body of a bacteriophage could be structurally investigated with the aid of radiation damage (Wu *et al.*, 2012). At low dose, the inner body cannot be distinguished from its surrounding DNA owing to the low contrast. However, at higher doses, the inner body proteins disintegrate with a characteristic 'bubblegram' whereas the surrounding DNA appears less sensitive to radiation damage. The use of the location and orientation of the bubblegrams enabled the authors to calculate a three-dimensional reconstruction of the inner body from previously recorded low-dose images. Interestingly, the inner body did not show radiation-induced bubbling in the absence of DNA.

Papers in this special issue predominantly deal with global radiation damage. However, progress has also been made over the last two years in describing and understanding specific radiation damage, in particular within active sites of fluorescent proteins (Royant & Noirclerc-Savoye, 2011), metalloproteins (De la Mora *et al.*, 2012), in a protein-inhibitor complex (Koch *et al.*, 2011) and at 100 K *versus* 300 K (Warkentin *et al.*, 2012b). Advances have also been made in the use of specific UV (Panjekar *et al.*, 2011) or X-ray (de Sanctis & Nanao, 2012) damage for radiation-induced phasing (Ravelli *et al.*, 2003; Nanao & Ravelli, 2006). Modelling specific damage might profit from recent advances in model-

ling atomic disorder in protein crystal structures. These include ensemble refinement with time-averaged restrained molecular dynamics simulations (Burnley *et al.*, 2012), systematic sampling of electron density around the dihedral angles of protein side chains [program *RINGER* (Lang *et al.*, 2010)] and automatic modelling of discrete heterogeneity by fitting multi-conformers [*qFit* WEB server (van den Bedem *et al.*, 2009)].

Given the progress in MX RD over recent years, what can be concluded regarding which questions have been answered and which questions remain?

Among the overall aims of the research is to establish a better understanding of how to optimize the dose lifetime of macromolecular crystals so that solid guidance can be provided to practicing crystallographers. Additionally, there is a need to ensure that RD artifacts are recognized so that reliable biological information is obtained from structure determinations.

The various studies summarized above have provided answers to several questions, especially regarding temperature-dependent damage rates and a new model for RT RD decay. These advances will allow for much improved planning of RT experiments. The fact that damage is seen to be outrun for RT synchrotron data collection opens up new possibilities, especially as even faster pixel detectors become available.

Computational and conceptual advances have resulted in a radiation decay model which can describe both the global and specific damage at 100 K by SVD analysis. It is to be hoped that this model will soon be implemented in commonly used data-reduction software, since, as the radiation-induced non-isomorphism will be reduced by the post-data acquisition corrections, its incorporation in the processing could mean success for an otherwise failed structure solution. However, this procedure will be a non-trivial challenge since it involves a combined experimental and computational feedback mechanism.

Comparative studies of RD rates to crystals rely heavily on robust knowledge of the dose, the metric against which the chosen observables of crystal degradation are measured. There are now well established protocols for beam flux and beam profile calibrations, and these data are becoming routinely available at most beamlines. In conjunction with improvements in *RADDOSE*, experimenters should thus soon be able to more easily maximise the amount of useful data that can be collected from their crystals, particularly when using microbeams. However, this will require information on crystal topology to be conveniently available during the experiment, which is a challenge still to be overcome.

Remaining pertinent questions for RD researchers include: what is the origin of global and of specific damage; understanding UV *versus* X-ray damage (are they of different origin and do they reach same end points?); what are the fingerprints electrons leave on structures in cryoEM; what are the underlying reasons for the fact that different metrics of global damage gives different results; is there a metric that is so far untested: a metric that would be a 'make or break' for structure solution would be ideal; how much effect on dose lifetime

does the radiation chemistry in the interstitial spaces of a protein crystal have; is it worth continuing the search for effective scavengers; would new scavengers be useful for EM; can we arrive at a coherent framework for interpretation of dose-rate data at RT; can we understand dose-rate effects in EM versus MX; and, finally, how far can XFELs take us along the road to beating MX RD?

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