The remarkable advances in structural biology in the past three decades have led to the determination of increasingly complex structures that lie at the heart of many important biological processes. Many of these advances have been made possible by the use of X-ray crystallography using synchrotron radiation. In this short article, some of the challenges and prospects that lie ahead will be summarized.

The first crystal structures of macromolecules were determined about half a century ago by Perutz, Kendrew and their colleagues. Even after the realization that isomorphous replacement could yield phase information for protein crystals [1], it still took another decade to obtain the first atomic structures. Although better computing and the advent of area detectors improved the situation considerably over the next two decades, macromolecular crystallography remained a multiyear effort.

However, beginning around 1980, the situation began to change dramatically. The idea that synchrotron radiation, normally a ‘waste product’ for high-energy physicists, could be used to produce very high-intensity beams for diffraction studies had led to the construction of several beamlines designed for macromolecular crystallography, beginning with the one at DESY in Hamburg [2]. High-intensity beams led to significantly higher radiation damage. Such damage was greatly minimized by the advent of cryo-crystallography, involving data collection below 100 K [3]. The use of cryo-crystallography with accurate image plate detectors that could scan in situ [4,5] meant that often a complete dataset could be collected from a single crystal in an automated manner and without interruption.
Prior to ca 1980, essentially all macromolecular crystals were obtained by purification from native sources. However, the use of overexpression techniques, especially those based on the T7 promoter [6], allowed large amounts of a given protein to be made by overexpression of its gene under inducible control in the bacterium *Escherichia coli*, allowing production of proteins that would normally be toxic to the host. This greatly increased the range of problems that could be studied, as natural abundance of a particular protein was no longer a limitation. Subsequently, the development of other expression systems more suitable for eukaryotic proteins expanded the range further.

Unlike rotating-anode X-ray generators, synchrotrons provide X-ray beams whose wavelength can be tuned with exquisite precision. This allowed diffraction experiments to be carried out at wavelengths at absorption edges where the scattering properties of special atoms can vary significantly, allowing phase determination by a combination of anomalous and dispersive signals, in the so-called multiwavelength anomalous diffraction (MAD) method [7–9]. The utility of MAD in conjunction with the K edge of selenium [10] was greatly enhanced by using overexpression methods for the general incorporation of selenomethionine into proteins [11]. Such incorporation was made possible by the overexpression systems produced by molecular biologists, showing how developments in synchrotrons and molecular biology went hand-in-hand to facilitate macromolecular crystallography. As a result, by using cryo-crystallography, a selenomethionine-labelled protein crystal and synchrotron radiation, it became possible to solve protein structures within days of collecting data from a single crystal. Given sufficient resolution, automatic programmes could even build much of the structure without human intervention [12,13], reducing structure determination to hours or even minutes following data collection [14].

The ease of protein structure determination for many problems has led to the impression that structural biology is now routine.

While that may be true for a certain class of problems, many problems continue to pose great difficulty at various stages. Some of these are the difficulty of obtaining well-diffracting crystals as the complexes become larger or are membrane-bound; extreme radiation sensitivity; sufficiently large crystal size for weakly diffracting crystals with large unit cells; and heterogeneity within and among crystals. Fortunately, there has been progress in all areas, and, in particular, the staff at the Diamond synchrotron have been forward-looking in tackling these problems. Although I mention examples specific to Diamond, there is considerable exchange of ideas among synchrotron staff internationally, so these advances should be seen as part of a worldwide effort.

The use of robotics allows many thousands of crystallization conditions to be examined with relatively small volumes for each drop. In combination with better expression systems for both bacterial and eukaryotic proteins, this improves the chances of obtaining good crystals, especially for very large complexes that may have conformational heterogeneity in solution, as well as for membrane proteins. While robotics is widespread, the establishment of a research complex at Harwell close to the Diamond synchrotron to provide access to such facilities to those users who may not have it is a welcome step.

A particular problem with robotics is that screening the very large number of crystallization conditions for potentially useful crystals is problematic. So *in situ* screening at Diamond, which allows potential crystals to be examined in an X-ray beam directly in the drop in the crystallization tray, has the potential to speed up this process. In the most advanced implementation of this, it is possible to actually collect and combine data from crystals *in situ* [15] and this was used to determine the structure of an entire virus [16]. By combining data from many crystals and limiting the dose on any particular one, it is thus possible to overcome radiation sensitivity.

*In situ* crystallography may not work with a class of problems involving very weak diffraction combined with radiation sensitivity at non-cryogenic temperatures. Here, both the current and planned microfocus beamlines can make it possible to combine data from many small crystals. These beamlines also make it possible to deal with a problem that was previously ignored, namely detection of heterogeneity within a crystal, thus allowing data collection on the best or most isomorphous parts of a crystal [17,18], resulting in overall better data quality.
A feature that is making synchrotrons more accessible is remote data collection, in which crystals are sent in a particular assembly to the synchrotron, and data are collected by the users remotely. This requires sophisticated robotics and robust control systems at the synchrotron, and also requires that beam and crystal alignment can be done in a standard way. It will require staff at the synchrotron to be available for the inevitable mishaps. The implementation of such data collection at Diamond has been very successful [19]. Finally, it is worth pointing out that Diamond has made a significant investment in state-of-the-art detectors, and in particular has installed the highly accurate and sensitive direct photon PILATUS detectors [20] on all of the macromolecular crystallography beamlines. This helps to ensure that the best possible data can be collected.

These advances have resulted in an increasing number of macromolecular structures being solved at Diamond only a few years after the commissioning of the first beam lines for users (http://biosync.sbkb.org/stats.do?stats_sec=RGNL&stats_focus_lvl=SITE&stats_site=DIAMOND). Perhaps more important than the total number of structures being solved are the number of truly challenging projects being undertaken there. In addition to the outstanding examples in the accompanying articles in this volume, there are many others, including large macromolecular complexes [21,22], membrane proteins [23–26] and virus structures [16,27].

Structure determination is now often being carried out by biologists who are not experts in diffraction methods. For routine problems, ‘FEDEX crystallography’, in which expert staff at the synchrotron screen and collect data on crystals, will ensure that ‘data will be collected by professionals, not amateurs’ [28]. Diamond should seriously consider implementing some form of this in the future.

At the same time, many structures that specifically focus on major biological questions will continue to be challenging, involving larger and more unstable complexes of proteins and/or nucleic acid, or membrane proteins (or both). There is thus potentially a conflict in the requirements of those problems that can be done in a ‘high-throughput’ mode and those that require extensive work. It is important that Diamond maintain versatile capabilities that can help with both classes of problems. Thus, for large unit cells, microfocus beamlines may not necessarily be optimal, as it may be more advantageous to expose as much of the crystal volume to the beam as possible. This requires producing large, homogeneous beams that nevertheless have low divergence. Ideas to use longer wavelengths for using absorption edges of sulfur or M edges of heavy metals such as uranium impose technical demands on the instruments and need to be explored.

The synchrotron community also needs to keep in mind developments beyond traditional synchrotron radiation. Recent results using an X-ray free electron laser (XFEL) technique show that it is possible to obtain crystallographic data and even solve structures from nanocrystals [29]. It is not yet clear how widely applicable XFEL will be, but early results show that, if well-ordered microcrystals can be obtained, the technique is promising.

Whereas XFEL requires large dedicated facilities similar in scale of investment to synchrotrons, cryo-electron microscopy (cryoEM) with the use of highly sensitive direct electron detectors is already capable of producing near-atomic resolution structures with no crystals at all [30,31]. In almost the inverse of crystallography, it is easier to determine structures by cryoEM for larger particles rather than smaller, because of the greater signal and the resulting ability to align individual particles. It is not yet clear that cryoEM can be pushed to resolutions much beyond 3 Å, which may be important for detailed understanding of chemical steps. However, for large complexes, such high resolutions are rarely reached even with crystallography. Crystallography and cryoEM may thus have complementary advantages. Finally, I have not mentioned NMR methods at all here, but they too are now providing structural information for increasingly larger proteins.

In summary, the progress in structural biology in the last two decades has been truly remarkable. In large part, this has been due to X-ray crystallography using synchrotron radiation. The developments at the Diamond synchrotron, in particular, have been very welcome by the UK structural biology community. At the same time, development in other areas such as cryoEM and
possibly XFEL will add to the arsenal of structural biologists to tackle the increasingly difficult problems that lie at the heart of fundamental biological processes.

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References


