Experimental determination of the radiation dose limit for cryocooled protein crystals

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Communicated by Douglas C. Rees, California Institute of Technology, Pasadena, CA, February 6, 2006 (received for review November 9, 2005)

Radiation damage to cryocooled protein crystals during x-ray structure determination has become an inherent part of macromolecular diffraction data collection at third-generation synchrotrons. Generally, radiation damage is an undesirable component of the experiment and can result in erroneous structural detail in the final model. The characterization of radiation damage thus has become an important area for structural biologists. The calculated dose limit of $2 \times 10^7$ Gy for the diffracting power of cryocooled protein crystals to drop by half has been experimentally evaluated at a third-generation synchrotron source. Successive data sets were collected from four holoferritin and three apoferritin crystals. The absorbed dose for each crystal was calculated by using the program RADDose after measurement of the incident photon flux and determination of the elemental crystal composition by micro-particle-induced x-ray emission. Degradation in diffraction quality and specific structural changes induced by synchrotron radiation then could be compared directly with absorbed dose for different dose/dose rate regimes: a 10% lifetime decrease for a 10-fold dose rate increase was observed. Remarkable agreement both between different crystals of the same type and between apoferritin and holoferritin was observed for the dose required to reduce the diffracted intensity by half ($D_{1/2}$). From these measurements, a dose limit of $D_{1/2} = 4.3 \pm 0.3 \times 10^7$ Gy was obtained. However, by considering other data quality indicators, an intensity reduction to $h_{n2} = \ln 2 \times h_0$, corresponding to an absorbed dose of $3.0 \times 10^7$ Gy, is recommended as an appropriate dose limit for typical macromolecular crystallography experiments.

Results

The effect of radiation damage was investigated by monitoring a number of parameters as a function of the absorbed dose. The diffracting power of the crystals, as assessed by summing the scaled intensities of all of the reflections in the data set, $I_{\text{Tot}}$, both over all resolution ranges and also in the outermost resolution shell (taken as 4 Å to the limit of diffraction), and the $R_{\text{meas}}$ and Wilson B values. From this data analysis, the effects of radiation damage can clearly be seen as a function of the cumulative dose at the midpoint of the data set ($D_{\text{CM}}$) for all seven crystals studied (see Tables 2–4, which are published as supporting information on the PNAS web site). To calculate this cumulative dose, the beam and crystal were characterized as detailed below.

The beam profile on ID14-4 has been determined to be a top-hat shape (13), and the photon flux was calibrated by using a photodiode. Different attenuations were used in the experiments to allow investigation of possible dose rate effects, the flux being varied from $0.3 \times 10^{12}$ to $3.9 \times 10^{11}$ photons per s (values shown in Fig. 1), always with a $100 \times 100-\mu$m beam as calibrated by using a yttrium/aluminum garnet (YAG) screen viewed by an on-axis camera.

Conflict of interest statement: No conflicts declared.

Abbreviation: microPIXE, micro-particle-induced x-ray emission.

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PNAS | March 28, 2006 | vol. 103 | no. 13

www.pnas.org/cgi/doi/10.1073/pnas.0600973103

4912–4917
After the collection of micro-particle-induced x-ray emission (microPIXE) elemental maps (Fig. 2), the stoichiometric ratios of sulfur, cadmium, and iron were measured by collecting point spectra on both apoferritin and holoferritin liquid protein samples and apoferritin and holoferritin crystals. No iron was detected in any of the apoferritin samples; the lowest detectable limit was 0.07 iron atoms per monomer. Knowledge of the sulfur content (two cysteines and three methionines) of ferritin allowed the number of iron and cadmium atoms per monomer to be calculated, starting with the iron to sulfur ratio present in the commercial holoferritin protein. From this ratio, the cadmium and additional sulfur per monomer in the crystals (from the crystallization buffer) could be quantified. For the apoferritin crystals, the cadmium to solvent sulfur ratio from the holoferritin crystal was used, but to obtain the number of each species per monomer (Table 1), the values were scaled to take into account the volume of the inner 24-mer ferritin shell normally occupied by the iron. The iron content of holoferritin, measured to be 1,750 and 1,766 atoms per 24-mer, respectively, for the two batches of protein used, is remarkably consistent and compares favorably with previous work that reported an average of 2,000 iron atoms per 24-mer for horse spleen ferritin (14).

To make a direct comparison with the calculated Henderson dose limit of $2 \times 10^7$ Gy, at which diffraction intensity is predicted to be halved ($D_{1/2}$), data set quality was initially assessed in terms of a reduction of the diffracting power of the crystals. As reflections measured in successive data sets are collected over the same region of reciprocal space, $I_{\text{Tot}}$ provides a convenient method of quantifying the intensity reduction. $I$ also can be measured over different resolution ranges, allowing the effect of radiation damage on low- and high-resolution reflections to be differentiated. The summed intensity of the reflections, $I_{\text{Tot}}$, was normalized to 1.0 for the first data set (i.e., $I_{\text{Tot}} = I_{\text{Tot}}/I_0$), to allow easy comparison between different crystals. The decay of $I_{\text{Tot}}$ with dose was fitted with a linear function for all seven crystals, giving correlation coefficients of not less than 95%. The rate of decay was observed to be remarkably consistent, not only for the four holoferritin (Fig. 3) and three apoferritin (Fig. 4) crystals, but for all seven crystals. The average $D_{1/2}$ is $4.3 (\pm 0.3) \times 10^7$ Gy, where the error takes account of the variance of the individual measurements and the errors associated with the dose calculation.

The overall error is dominated by the uncertainties associated with the computation of the absorbed dose. The experimental parameters affecting this calculation can be divided into three components.

The first of these components relates to the photodiode calibration. This calibration depends on both an accurate knowledge of the silicon diode thickness, so that the energy loss in it can be properly calculated, and also on the calibration, which correlates the in-line diode readings with the current observed in the calibrated diode. Both these errors are small (<3%) compared with the second source of error: the uncertainty in the microPIXE elemental composition determination. The error on stoichiometric ratios determined by this method is known from measurements on standards and is typically ±15% (15). In this case, different points measured on the same crystal gave results with a standard error of 7% (changing the iron content in RADDOS by 7% changes the absorption coefficient of the crystal by 4%).

The third component of error is due to the rotation of the crystal in the beam; if the crystal is bigger than the x-ray beam, new, previously unirradiated material is brought into the beam resulting in a larger irradiated volume and thus a lower absorbed dose. Currently, RADDOS does not account for this effect. In our case, the small rotation range required to obtain complete data

![Fig. 1](image1.png)  
**Fig. 1.** Bar chart showing the incident photons per second and the absorbed dose per second for the four holoferritin and three apoferritin crystals studied. The difference in the relative size of the photons per second bar compared with the dose per second bar for the two crystal types highlights the necessity of taking careful account of the crystal composition when calculating the absorbed dose.

![Fig. 2](image2.png)  
**Fig. 2.** Elemental areal concentration microPIXE maps ($500 \times 500 \mu m$) obtained by scanning a 1-μm-diameter proton beam in x and y across a holoferritin crystal (holo1): sulfur (Left), iron (Center), and cadmium (Right) distributions. The iron is localized in the protein crystal, whereas because of the presence of ammonium sulfate in the solvent, the sulfur is more spread out.

<table>
<thead>
<tr>
<th>Element</th>
<th>Holoferitin</th>
<th>Apoferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur (protein)</td>
<td>5 (120)</td>
<td>5 (120)</td>
</tr>
<tr>
<td>Sulfur (solvent)</td>
<td>120.7 (2,897)</td>
<td>154.7 (3,713)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>8.9 (213.6)</td>
<td>11.3 (271)</td>
</tr>
<tr>
<td>Iron batch 1</td>
<td>72.9 (1,750)</td>
<td>&lt;0.07 (&lt;1.7)</td>
</tr>
<tr>
<td>Iron batch 2</td>
<td>73.6 (1,766)</td>
<td>&lt;0.07 (&lt;1.7)</td>
</tr>
</tbody>
</table>

Table 1. Results of the microPIXE elemental composition determinations on apoferritin and holoferritin crystals
results in a correspondingly small irradiated volume correction, depending on the crystal shape and initial orientation (<5% here). Hence, the cumulative doses were not corrected for this effect.

An additional correction associated with the physics of the energy loss calculation carried out in RADDose is necessary. The program currently does not take into account that for heavier elements, atoms excited by a K-shell photoelectron emission can decay by fluorescence as opposed to the Auger effect. Depending on their energy, these fluorescent x-rays may have a high probability of escaping from the sample. The overall energy deposited in the crystal is thereby reduced, thus lowering the absorbed dose.

For elements with Z < 20, the probability of fluorescence is <10%, so the concomitant energy loss can be neglected. However, for the iron in the holoferritin crystals, 34% of K-shell excitations produce fluorescent x-rays, ~80% of which can escape from a 200-μm-thick crystal. Thus, for the holoferritin crystals, this effect reduces the effective photoelectric cross section of the iron atoms, and our doses have been correspondingly corrected.

Although there is good agreement between our results for D_{1/2} from the seven crystals investigated, it should be noted that the mean D_{1/2} obtained for the apoferritin crystals [4.8 (±0.2) × 10^7 Gy] is systematically higher than that observed for holoferritin crystals [4.0 (±0.2) × 10^7 Gy]. This difference is not thought to be due to temperature rises in the crystals: by using the lumped model described by Kuzay et al. (16), RADDose predicts a temperature rise of <5 K for holoferritin at the highest dose rate used. Previous measurements using a white beam of flux density of 2 × 10^15 photons per s/mm² found no evidence of beam heating in a 100 K crystal sample (17). Two effects may be responsible for the measured dose limit difference: first, the uncertainty in the holoferritin dose calculation, which is greater because of the large iron content, and second, the dose is a parameter that takes only the deposited energy into account but none of the possible chemical processes.

The different values of D_{1/2} can be compared to investigate any dependence on the dose rate. We find that for both apoferritin and holoferritin, the higher the dose rate, the lower the resulting D_{1/2}. For apoferritin, a 10-fold increase in the dose rate (0.4 × 10^4 to 4.0 × 10^4 Gy/s) gives a 10% reduction in D_{1/2} (5.1 × 10^7 to 4.6 × 10^7 Gy, respectively). For holoferritin, two crystals were measured at dose rates <1.6 × 10^4 Gy/s and two >5 × 10^4 Gy/s, giving means for D_{1/2} of 4.2 × 10^7 and 3.7 × 10^7 Gy, respectively, showing a 10% reduction in D_{1/2} for a 3-fold increase in dose rate. The R values for all resolution ranges [R_{mean(all)}], for the highest-resolution shell [R_{mean(hr)}], and the Wilson B values all increased with dose as expected (Tables 2–4). The rise in R_{mean(all)} is initially slow for doses up to ~3 × 10^7 Gy but then rises steeply in the high-dose region (>3 × 10^7 Gy). R_{mean(hr)} rises faster than R_{mean(all)} because of the faster rates of decay of higher-resolution reflections. The Wilson B values also increase, as the intensities of the higher-resolution data fall, and the intensity distribution as a function of resolution is thus modified. The mosaicism did not show consistent behavior between crystals, with some values decreasing and others increasing slowly with dose.

The unit cell volumes of all of the holoferritin and apoferritin crystals increased linearly with dose in the low-dose region (<3.0 × 10^7 Gy), albeit at different rates even between crystals of the same type, in agreement with previous observations (18, 19). In the high-dose region, changes in the unit cell volume (ΔV) become nonlinear and unpredictable, as reported in ref. 20. Because of the variability between crystals, and the nonlinear behavior of ΔV in the high-dose region, this parameter was not used in establishing a dose limit.

Refined models were assessed both in terms of the changes in electron density and also by the Debye–Waller parameters (B values), which quantify the static and dynamic disorder in a crystal. Inspection of the 2F_o − F_c electron-density maps allowed the structure from each data set to be grouped according to the degree of damage. The most susceptible residues/sites are as follows: disulfide bridges (not present in ferritin), aspartates, glutamates, tyrosines, and methionines (5–7). Fig. 5 shows the gradual loss of density around a glutamate and arginine from a neighboring monomer and also illustrates a “control” residue: a threonine that retains its density as a function of dose. This example highlights the effect of local environment, because arginines are not generally considered susceptible residues, but here the damage appears to be affected by the proximity of the amorphous iron and the glutamate. The electron density obtained from the individual data sets was classified as follows: first signs of damage (no “lost” side-chain atoms, but general reduction in atom definition), one residue damaged, two or three residues damaged, and lastly, the majority of susceptible residues damaged. These categories were used to identify data sets as...
good, usable, marginal, and questionable, respectively. The results of this analysis are presented in Fig. 6.

The relative increase in atomic $B$ value between the first and last data sets for the apo1 and holo1 crystals also was analyzed as a function of amino acid type (data not shown). For both the apo and holo structures, the largest increase (at least 1.2 times the average increase of all residues) in $B$ value was for asparagines (12 per monomer), methionines (3 per monomer), threonines (6 per monomer), and tyrosines (6 per monomer). The contrast between these and those susceptible residues previously reported (aspartates, cysteines, glutamates, and methionines) suggests that the residue environment plays a large part in determining how residues respond to a large absorbed dose.

The number of water molecules as a function of dose also was monitored, and it was found that this function showed a biphasic behavior, with a slow decrease in the low-dose region ($<3 \times 10^7$ Gy), followed by a much faster decrease in the high-dose region (data not shown).

**Discussion and Conclusions**

The results presented above allow a direct comparison to be made between the calculated dose limit, which is predicted to destroy half the diffracting power of the crystal held at 77 K, of $2 \times 10^7$ Gy, and our experimental observation of $4.3 \times 10^7$ Gy for this limit. Experience of researchers in electron microscopy was that a total flux of 5 electrons per Å$^2$ destroys half the diffraction from a 2D crystal (21). Henderson (11) calculated that this value corresponded to an absorbed dose of $5 \times 10^7$ Gy and then postulated that in the first part of the depth–dose curve the dose could be two to three times less, thus arriving at his limit of $2 \times 10^7$ Gy. However, it is now generally accepted that biological samples in electron microscopy can withstand a total flux density of $\sim 10$ electrons per Å$^2$ (Catherine Venien-Bryan, personal communication), which would give a revised calculated limit of $4 \times 10^7$ Gy, consistent with our experimental result.

It should be noted that the implicit assumption made in Henderson's calculation of the dose limit was that the damage inflicted on the sample is directly proportional to the energy deposited, irrespective of the mechanisms by which electrons and x-rays lose energy. Thus, our experimentally determined dose limit is based solely on the premise that loss of diffracting power is directly proportional to the energy deposited in the crystal and takes no account of any radiation chemistry effects such as specific structural damage to amino acids involved in crystal contacts (22). After their experiments on room-temperature myoglobin crystals, Blake and Phillips (1) proposed a general
and decay model, later generalized by Hendrickson (23), whereby the unchanged fraction of the crystal (A₁) decreased linearly with dose, and the severely disordered part (A₂) changed exponentially as a function of resolution. This model results in an intensity decrease obeying the relationship

\[ I(t)/I(0) = A_1(t) + A_2(t) \exp(-B_2 \sin^2 \theta / \lambda^2), \]

where B₂ is a measure of the disorder, λ is the x-ray wavelength, and θ is the Bragg angle. For crystals held at cryotemperatures, a linear model of damage rate with dose (i.e., the linear decay of total diffracted intensity) also has been found to be appropriate (e.g., refs. 20 and 24), and this postulate is confirmed in our case by the high-correlation coefficients of linear fits to the intensity–dose plots presented in Figs. 3 and 4.

However, an experimental dose limit should not be calculated solely in terms of an overall reduction in diffracting power. Although loss of intensity provides an easily measured metric, of more importance is the point at which the quality of the data becomes unacceptable because biological information is lost. Accordingly, diffraction data also have been assessed in terms of changes in the high-resolution diffraction limit, Rₚ, Wilson B values, and the number of damaged residues (Fig. 6).

The time at which data collection should be stopped, or the decision on when the data quality becomes unacceptable, is a subjective process that is largely governed by consideration of the purpose for which the data are being collected. This subjective approach becomes problematic when trying to set a global experimental dose limit. Limits must be set on changes in the parameters to determine a specific point when the data becomes “usable,” “marginal,” or “questionable.” One of the most widespread statistical indicators of diffraction quality is the R value; Rₚ offers a quantity that assesses the data quality independently of data redundancy. Here, overall Rₚ values of <0.10%, 10–15%, 15–20%, and >20% were deemed to indicate good, usable, marginal, and questionable data, respectively. These limits should be lowered if, for example, obtaining phases from sulfur single-wavelength anomalous dispersion data are the aim of the experiment.

If different resolution shells are chosen, D₁₂ changes: for instance, for holo1, the resolution shell of 2.6–2.5 Å gives D₁₂ of 2.1 × 10⁷ Gy compared with 3.6 × 10⁶ Gy over all resolution bins. Conversely, for the 20.0–10.0 Å shell, D₁₂ is 2.0 × 10⁷ Gy (data not shown). Thus, if collection of high-resolution data is the experimental objective, the tolerable dose will be significantly reduced. This finding concurs with the Blake and Phillips model outlined above, which predicts that the rate of intensity decay is resolution dependent (term in A₂) and has been confirmed previously at cryotemperatures in a study of the intensity decay of a hen egg white lysozyme crystal (20).

In addition to the data-reduction statistics discussed above, inspection of the electron density provides the final test of whether the quality of the data obtained is acceptable for the purposes of the experiment. From analysis of the electron density in terms of the number of damaged residues, and from the other data quality indicators, we conclude that by the time D₁₂ has been reached, the integrity of the biological information will be compromised. The implication of this observation is that a lower dose limit could usefully be defined, and from the results presented above, we suggest that an upper limit of 3 × 10⁷ Gy is an appropriate value. Figs. 3 and 4 can be used to convert this dose to an easily monitored on-line metric, i.e., a reduction in diffracting power to an intensity of I₀₂ = I₀ × ln², giving I₀₂ ≈ 0.7 × I₀. It should be noted that this is an upper dose limit and that some crystals may decay much faster because of the varying susceptibility of crystal contacts (22) and protein-specific features that have been shown to affect how a protein crystal behaves under cryoconditions, such as solvent content and distribution (25).

For apoferritin and holoferritin, D₁₂ corresponds to the absorption of 10⁷ photons per unit cell (i.e., 27 photons per 24-mer sphere). The rate at which these photons are absorbed has a measurable effect on the rate of damage, with higher rates giving slightly reduced lifetimes. This observation has implications for optimization of data collection practice at third-generation synchrotron beamlines. It is possible that this dose/dose-rate effect, which has been reported (26), is because of the higher density of tracks, which both lowers the probability of electron-hole recombination and increases the likelihood of multiple oxidations or reductions (Bill Bernhard, personal communication).

In conclusion, an experimental dose limit has been measured for seven crystals of two different species with very different elemental composition: one a typical protein and the other having a particularly high metal content. Our dose calculations have allowed us to compare these crystal types directly and to propose an experimental limit based on both the data collection statistics and the quality of the electron density maps. The mean dose required to reduce the diffracting power by half was 4.3 × 10⁷ (±0.3) GY, but an experimental upper dose limit of D₁₂ = 3.0 × 10⁷ GY is proposed, corresponding to the dose that gives I₀₂. Above this dose, the biological information extracted from the macromolecular structure is likely to be compromised, and further data collection would be unproductive.

**Methods**

**Materials.** Ferritin is an iron storage protein of monomeric mass 19.8 kDa (174 aa), occurring in a spherical 24-mer conformation. This spherical shell, of internal diameter 78 Å, contains a variable number of iron(III) atoms, from 0 in the case of apoferritin to 4,500 (holoferritin) (27). Equine spleen apoferritin and holoferritin were purchased from Sigma and diluted to 20 mg/ml with 0.1 M NaCl. Cubic crystals of both apoferritin and holoferritin, of size ~200 μm and space group F432 (a = b = c = 181 Å), were grown from identical conditions by using a well solution of 0.6 M (NH₄)₂SO₄ and 10 mM CdSO₄. Crystals were cryoprotected by replacing 40% of the water in the well solution with glycerol and soaking for up to 30 min.

**Data Collection and Processing.** Crystals were loop-mounted and flash-cooled in a 100 K dry nitrogen stream. Sequential complete data sets [up to 12 at doses of between 1 and 4 MGy (holo3) per data set] were collected from each crystal on beamline ID14-EH4 at the European Synchrotron Radiation Facility (Grenoble, France) at beam energies of 13.2 keV (1 eV = 1.602 × 10⁻¹⁹ J) (0.939 Å) and 12.7 keV (0.976 Å). Typically, each data set consisted of 30° of data taken starting at an oscillation angle determined by the strategy option of MOSFLM (28); these parameters were kept constant for each sequential data set. Between each data set, crystals were exposed to controlled periods of unattenuated beam (inflicting doses of between 2 and 10 MGy). Normally, in macromolecular crystallography, crystals would be discarded well before the diffraction intensity is reduced by a factor of 2. However, as the calculated dose limit has been defined in terms of a halving of the diffracting power, in our experiments crystals were exposed to the beam until this criterion had been well exceeded. Data were processed by using MOSFLM and SCALA (29). After x-ray data collection, the crystals were allowed to warm up to room temperature and then mounted onto 2-μm-thick mylar microPIXE sample holders directly from the cryoloop.

**Flux Calibration.** The integrated intensities recorded by the internal beamline diode in the header of each image were converted into the number of incident photons as follows. A Keithley
picoameter was used to record the induced current in a calibrated silicon PIN photodiode (S3204-09; Hamamatsu Photonics, Hamamatsu City, Japan) positioned at the crystal position with a range of beam attenuators in place. These currents then were converted into photon fluxes by considering the energy deposited within the silicon diode and thus could be directly related to the corresponding internal beamline diode readings. This calibration was carried out for each experimental run, so that any drifts could be monitored. To account for variations in the beam current during the course of the experiment, measured total intensities for each data set were normalized by using the ratio of the total flux for the first data set to the flux for that data set.

MicroPIXE. Knowledge of the atomic composition of a protein crystal is essential if the absorbed dose is to be accurately calculated. The microPIXE technique allows stoichiometric crystal is essential if the absorbed dose is to be accurately calculated. MicroPIXE (30) was used to determine the x-ray emission spectra (15). This emission is induced by using a highly focused (1-μm diameter) 2.5 MeV proton beam which can be scanned in x and y across the dried sample held in vacuum. Initially, a coarse scan (500 × 500 μm) was collected to determine the position of the protein sample, and then several point spectra were recorded across the sample. Detection of Rutherford backscattered protons allows the sample thickness and matrix composition to be accurately determined. Concentrations of all of the elements of interest in the protein sample were then extracted from the x-ray spectrum by using the sulfur concentration, known from the number of methionines and cysteines per monomer, as an internal calibration.

Because sulfur and cadmium were present in the crystallization conditions, the sulfur and cadmium content of both the commercially purchased protein solutions and the crystals used for data collection, in addition to the iron content, was quantified by irradiation could then be observed and analyzed as a function of absorbed dose.

Dose Calculations. The dose absorbed by a crystal in an x-ray beam is a function of the beam characteristics (energy, profile, flux, size) and of the crystal properties (composition, size, irradiated volume). The program RADDOSE (30) (program available from E.F.G. upon request) uses all these parameters to calculate the absorption coefficient of the sample, defining the average composition of an amino acid as 5C + 1.35N + 1.5O + 8H. It thus obtains a value for the absorbed dose by summing the energy lost by the x-ray beam by means of photoelectric and Compton interactions in the sample. The absorption coefficients of apoferritin and holoferitin crystals at 13.2-kV incident x-ray energy were calculated to be 0.67 and 1.43 mm⁻¹, respectively.

Structure Refinement and Analysis. Structure refinement, starting with Protein Data Bank ID code 1IER (2.26-Å resolution; R value = 0.187) (31) was carried out against each data set by using rigid-body minimization, followed by restrained refinement in REFMAC (32). Models were refined to the resolution limit of the first data set (see Tables 2–4) typically to R values < 0.20 and Rfree values < 0.25, and with satisfactory rms deviations from ideal bond lengths and bond angles (data not shown). These values necessarily deteriorated as the absorbed dose increased, and concomitantly the data quality decreased.

2Fo − Fc electron density maps were viewed and overlaid in COOT (33). Changes in the electron density, specific structural damage to particular residues, and increasing B values induced by irradiation could then be observed and analyzed as a function of absorbed dose.

We thank Bill Bernhard, Norman Charnley, Geoff Grime, and Andrew Leslie for useful discussions; Martin Noble and Raimond Ravelli for valuable comments on the manuscript; Sean McSweeney, Raimond Ravelli, Robert Southworth-Davies, and Martin Weik for help with data collection; the Surrey Ion Beam Centre for microPIXE facilities; and the European Synchrotron Radiation Facility for beam time. R.L.O. is supported by a Biotechnology and Biological Sciences Research Council studentship. E.R.-P. was supported by the Programa de Apoyos para la Superación del Personal Académico, Universidad Nacional Autónoma de México.