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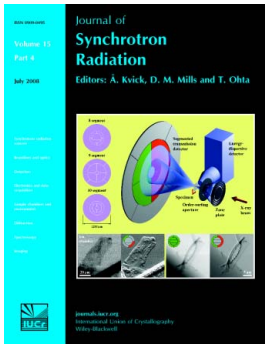

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Light flux density threshold at which protein denaturation is induced by synchrotron radiation circular dichroism beamlines

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New high-flux synchrotron radiation circular dichroism (SRCD) beamlines are providing important information for structural biology, but can potentially cause denaturation of the protein samples under investigation. This effect has been studied at the new CD1 dedicated SRCD beamline at ISA in Denmark, where radiation-induced thermal damage effects were observed, depending not only on the radiation flux but also on the focal spot size of the light. Comparisons with similar studies at other SRCD facilities worldwide has lead to the estimation of a flux density threshold under which SRCD beamlines should be operated when samples are to be exposed to low-wavelength vacuum ultraviolet radiation for extended periods of time.

Keywords: protein denaturation; radiation damage; synchrotron radiation circular dichroism spectroscopy; photon flux; vacuum ultraviolet wavelengths.

1. Introduction

The first synchrotron radiation circular dichroism (SRCD) beamline was developed in the early 1980s at the SURF II ring at the National Institute of Standards and Technology (Sutherland *et al.*, 1980) and moved to the National Synchrotron Light Source (NSLS) at Brookhaven in 1982. Until recently, however, only a small number of SRCD stations have been in operation at synchrotrons around the world, notably the stations at the NSLS, station 3.1 at the SRS Daresbury, and UV1 at the Institute for Storage Ring Facilities (ISA) in Denmark. A common feature of the first-generation instruments was the relatively low light flux, usually of the order of 10^{11} photons s^{-1}, resulting in a flux density of the order of 10^{10} photons s^{-1} mm^{-2} for a spectral bandwidth of about 1 nm. This is similar to the flux of conventional laboratory-based commercial CD instruments at 220 nm for a comparable spectral bandwidth. However, whereas conventional instruments display a rapid decrease in flux at lower wavelengths such that the CD signal cannot be effectively measured below about 190 to 185 nm, the synchrotron radiation light source flux remains constant at this level well into the VUV, thus allowing the measurement of aqueous samples down to 170 nm or below. Like conventional CD spectroscopy, SRCD spectroscopy has found increasing utility in structural studies of biomolecules, especially proteins. Initial concerns about the effect of low-wavelength radiation on protein integrity were shown to be unfounded (Orry *et al.*, 2001) until a second generation of beamlines came into operation. Station 12.1 at the SRS (Clarke & Jones, 2004) currently has the highest flux of all such beamlines, in the region of $10^{12–13}$ photons s^{-1} for spectral bandwidths of about 1 nm, typically used for studies of biomolecules in solution. This would be ideal for fast time-resolved studies, but has noticeable effects upon many proteins when acquiring spectra for structural analyses. Normally users would average three consecutive scans of the same sample before subtracting the baseline, thereby ensuring reproducibility and allowing the calculation of error bars (Miles & Wallace, 2006). However, in the time taken to run a scan from 280 nm to 170 nm, a high proportion of proteins are found to be compromised so that subsequent scans of the same sample do not produce identical spectra. In all cases a decrease in spectral magnitude and a change in shape of the curve reminiscent of denaturation is seen and further analysis confirmed that denaturation is taking place (Wien *et al.*, 2005). This is most likely due to the heating of water molecules internal to the protein (because there is no overall change in temperature as detected by a thermal probe, the effect is not due to the bulk water being heated) rather than free-radical attack which would cause irreversible damage. The problem of radiation-induced denaturation also renders the beamline operating at full intensity unsuitable for studies of protein thermal stability or any protocol that requires a spectrum to be taken before conditions are altered *in situ* (such as the addition of a ligand) so that a second spectrum can be measured for comparison.

2. Materials and methods

Human serum albumin (HSA) was purchased from Sigma-Aldrich. It was dissolved in deionized water at a concentration of $\sim$8 mg ml^{-1}. The solutions were centrifuged to remove scattering artifacts such as those due to dust particles or any undissolved material that might be present and then degassed to remove any dissolved oxygen (Miles & Wallace, 2006). Samples were examined in circular demountable 0.0015 cm pathlength Suprasil cells (Hellma UK), which had been previously calibrated using interferometry methods, at 298 K over the wavelength range from 280 to 165 nm, using a 1 nm interval. Consecutive repeats (with no delay time between spectra) were collected for the HSA samples at each of the beamlines [CD1 (ISA), UV1 (ISA), U11 (NSLS), 4B8 (Beijing Synchrotron Radiation Facility, BSRF) and CD12 (SRS)].

Flux measurements were undertaken using calibrated photodiodes (Fig. 1), and the spot sizes were measured using a piece of paper inserted into the beam at the position of the sample cell.

Table 1
Flux, flux density and spot size of various SRCD beamlines.

The last two rows show the effects of the beams on the lifetime of HSA, where the time that it remains stable is defined as undergoing less than a 5% change in the peak near 190 nm with respect to the initial scan.

	SRCD station, location				
	UV1, ISA	4B8, BSRF	CD1, ISA	U11, NSLS	CD12, SRS
Slit size (mm)	0.5	0.8	0.4	1.0	4.4
Horizontal beam size at slit (mm)	5	40	5	5	10
Spectral bandwidth (nm)	0.5	1.0	0.6	0.32	1.0
Scan speed (nm min^{-1})	22	12	22	15	16
Maximum flux at 200 nm ($\times 10^{11}$ photons s^{-1})	1.5	0.6	4.5	30	>250
Spot size at sample (mm^2)	10	6	12	4	25
Flux density ($\times 10^{11}$ photons s^{-1} mm^{-2})	0.125	0.1	0.37	7.5	20
Time HSA remains stable (min)	>3600	>180	35	$\sim$20	<12
Number of scans before significant signal decay observed	25+	14+	7	2	1

3. Results and discussion

When commissioning the new SRCD beamline, CD1, at ISA, Denmark, human serum albumin, a very thermally sensitive protein, was used to monitor the potential for the beamline to induce denaturation of protein samples (Miles *et al.*, 2007). The original design focused a flux of 4.5×10^{11} photons s^{-1} onto a spot at the sample of 2 mm $\times$ 1 mm. Thus the light flux density was 2.3×10^{11} photons s^{-1} mm^{-2}. This was found to cause rapid denaturation of HSA after one scan. However, by moving the sample chamber out of the focal point so that the spot size was increased to 2 mm $\times$ 6 mm (flux density 0.4×10^{11} photons s^{-1} mm^{-2}), the protein retained its integrity for seven scans and, since the total flux was unaltered, the signal-to-noise ratio was not reduced. It was found that other proteins (myoglobin, conconavalin A and lysozyme), each of which displayed some evidence of denaturation after six to eight scans when exposed to the original light intensity, remained stable for 20 scans when the intensity was reduced. A comparison between the light intensity at 200 nm and the effect of radiation-induced denaturation of HSA was examined at several other SRCD beamlines (see Table 1 and Fig. 2). The severe effect of irradiating HSA at 190 nm on CD12 is shown in Fig. 3, where the rapid decrease in the 190 nm CD signal was followed over time. Studies conducted at U11, the high-intensity SRCD beamline at the NSLS, indicate that denaturation is significant only at wavelengths less than about 220 nm (although there is detectable degradation at CD12 in wavelengths even as high as 220 nm); the effect increases in severity with decreasing wavelength (Wien *et al.*, 2005). This issue also needs to be considered when using conventional CD

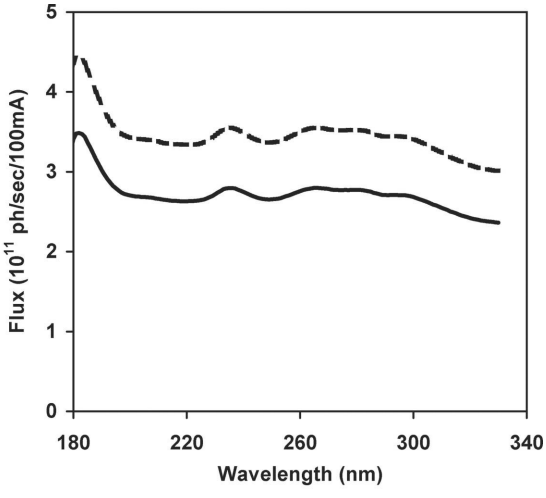

Figure 1
Photon flux at the sample position on beamline CD1 at ISA normalized to 100 mA ring current. The effect of changing the exit slit size (0.4 mm, solid line; 0.5 mm, dashed line) is shown.

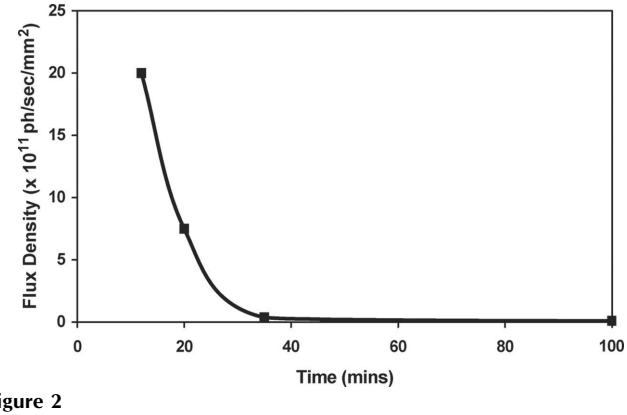

Figure 2
Plot of flux density *versus* time that the sample remains stable, showing a rough 'threshold' for degradation.

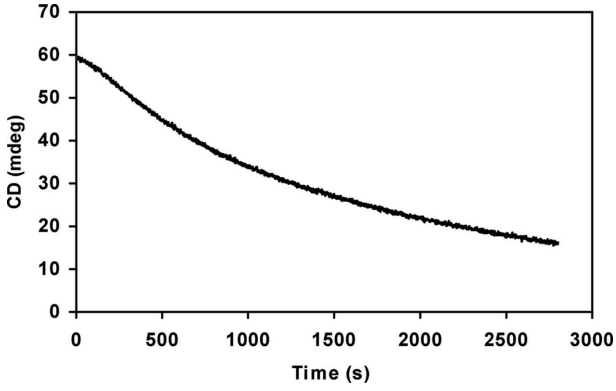

Figure 3
CD signal of HSA at 190 nm as a function of time on the beamline which most severely affects the sample (CD12).

instruments such as the Chirascan (Applied Photophysics, UK) with high flux (reported by the manufacturer to be $>10^{13}$ photons s^{-1} at 190 nm); for this instrument, degradation of HSA was significant after exposure for >60 min (data not shown).

The effects can be reduced either by changing the baffle or slit settings to decrease the light on the sample (Janes & Cuff, 2005) but at the cost of lowering the signal-to-noise ratio, by changing the spot size to decrease the light density as noted above, or by decreasing the time constant or response time, which will also adversely affect the signal-to-noise ratio. Alternatively, and more optimally, these empirical observations suggest that to minimize damage to protein samples during experiments that require extended exposure of a sample to the beam the beamline should be designed such that the flux density of the beam at the sample should not be above $\sim 0.4 \times 10^{11}$ photons s^{-1} mm^{-2}.

Fortunately, experiments such as stopped-flow SRCD, which have the greatest need for high photon fluxes, are not affected because each sample is only exposed to the beam for a short time (in the millisecond time range) only once before being discarded.

4. Conclusion

In summary, the effects of radiation-induced thermal denaturation on protein samples in high-flux SRCD beamlines can be significant and need to be considered in the design of new, and the operation of existing, high-intensity beamlines.

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