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Application of circular dichroism and magnetic circular dichroism for assessing biopharmaceuticals formulations photo-stability and small ligands binding properties

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ABSTRACT

Synchrotron radiation circular dichroism (SRCD) is a powerful tool for photo-stability assessment of proteins. Recently our research has been interested in applying SRCD to develop screening methodologies for accelerated photo-stability assessment of monoclonal antibody formulations. Despite it was proven to be reliable and applicable within a wide range of salts and excipients containing solutions, the presence of far-UV (<260 nm) strong absorbing species (*e.g.*, sodium chloride, histidine, arginine) in common formulations completely prevent the analysis. Herein, we propose a new method based on CD coupled with magnetic CD (MCD) to address the problem and offer an additional versatile tool for monitoring the photo-stability. This is done by assessing the stability of the samples by looking at the near-UV band, as well as giving insights in the denaturation mechanism. We applied this method to four mAbs formulations and correlated the results with dynamic light scattering data. Finally, we applied MCD in ligand interaction to key proteins such as lysozyme, comparing the human with the hen enzyme in the binding of *N*,*N*′,*N*″-triacetylchitotriose.

1. Introduction

Among the factors affecting protein stability, light exposure is a major issue for bio-pharmaceutics development. Light exposure leads to denaturation and degradation and eventually loss of activity. The optimisation of a methodology of stability assessment including fast and reproducible photo-stability is therefore a requirement. Light sources with constant and controlled photon-flux and with precise and tuneable emission spectrum would be required to ensure reproducibility and reliability. In these respects, the high photon-flux available at synchrotron radiation circular dichroism B23 beamline (Diamond Light Source Ltd., Didcot, Oxfordshire) represents a resourceful innovation for bio-formulation stability assessment. Moreover, circular dichroism (CD) is able to give direct information on secondary structures which exhibits characteristic signatures in the farUV (below 260 nm) (Greenfield, 2006; Kelly et al., 2005). However, the farUV range is sometime unaccessible, particularly in presence of high salt (*e.g.*, chloride) concentration and excipients, such as guanidinium derivatives (*e.g.*, arginine) and histidine with high absorption in the far-UV (<250 nm). Nonetheless, proteins present other absorptions that could account for changes in the structure. The 260–320 nm CD bands due to aromatic side-chains (mostly tryptophans and tyrosines) and disulphide bonds are exploited to assess changes occurring in the tertiary structure due to either the presence or absence of rotational freedom of the aromatic side-chain causing decreased or increasing the optical activity respectively. Both can contribute to the overall intensity of the CD spectrum. Complementary to circular dichroism, magnetic circular dichroism (MCD) is a related technique where the optical transitions are induced by an external magnetic field (Faraday effect). MCD has been widely applied in the study of porphyrines, (Kalman et al., 1973; Kobayashi and Nakai, 2007) paramagnetic metal complexes, (Barrett et al., 1986; Modine et al., 1971) metal nano-particles, (Zaitoun et al., 2001) other biological relevant organics, (Voelter et al., 1968) indole derivatives, (Albinsson et al., 1989; Sprinkel et al., 1975) haem, (Brittain et al., 1978; Pond et al., 1999a,b,c,c) and proteins (Barth et al., 1971, 1972b; McFarland and Coleman, 1972). For proteins in particular, tryptophan residue has been widely targeted

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for MCD investigations. Notably, the 293 nm positive contribution in the near-UV region is mainly due to tryptophan contributions, with negligible opposite contributions due to the 275 nm tyrosine band, and it has been shown to display low dependency on the tertiary structure (Barth et al., 1971). Only above 15/1 ratio between tyrosine and tryptophan the 293 nm peak is distorted by a negative contribution from tyrosine (Barth et al., 1972b). However, corrections can be taken into account since all these contributions are linearly combined. The possibility to detect tryptophan in proteins independently by conformations can be applied using MCD for the evaluation of the overall protein concentration as reported by Barth et al. (1971, 1972a,b),),). A similar aim for MCD has been already employed with uro-porphyrines (Kalman et al., 1973).

Here, we demonstrate the application of MCD to assess proteins photo-stability of cetuximab, a monoclonal antibodies, in combination with CD in the near-UV giving useful information on the behavior of bio-pharmaceutical upon light exposure. First, we will apply MCD for determining the tryptophan content and exposure to solvent, applying it on several commercialised model proteins, such as hen egg lysozyme, human serum albumin, bovine serum albumin, myoglobin and human serum immunoglobulin G, concanavalin A, α-lactalbumin and avidin, in comparison with the effects of denaturants. Then, we will apply it to the detection of the effects of light exposure, comparing the MCD results with the CD and dynamic light scattering. Finally, we will show how MCD could complement CD measurements to account for binding interactions between a protein and ligands. In particular, we chose the binding of hen lysozyme to *N*,*N*′,*N*″-triacetylchitotriose (NAG), an inhibitor of enzymatic activity towards the cleavage of glycosidic bonds.

2. Materials and methods

2.1. Samples preparation

5 mL of a solution of cetuximab (commercial name Erbitux) has been dialyzed using Pur-A-Lyzer™ G2 (Sigma–Aldrich) dialysis tubes for 24 h in water. The solution was then concentrated using Vivaspin 500, 3–5 kDa MWCO (GE) in a centrifuge (21,000 rpm, 15 min). The concentrated solutions (about 40–50 mg mL^{-1}) were then diluted in the chosen buffers to a concentration of ∼1–1.5 mg mL^{-1}. Hen egg lysozyme (Sigma L6876), human serum albumin (HSA, Sigma A3782), bovine serum albumin (BSA, Sigma A9085), equine skeletal muscle myoglobin (Sigma M0630) and human serum immunoglobulin G (IgG – Sigma I4506), concanavalin A (Type VI, Sigma L-7647), α-lactalbumin (Type III, Sigma L-6010) and avidin (Sigma A-6128) and human lysozyme (Sigma L-1667) were stored at −20 °C before use. *N*,*N*′,*N*″-triacetylchitotriose was purchased by Sigma (90,251 Fluka). All measurements were recorded in water and concentrations measured either with Bradford assay and absorbance measurement ($\varepsilon^{280\,nm}$ = 150,000, 36,000, 35,700, 43,824, 13,980, 210,000, 35,034, 29,642, 94,460 mol^{-1} L cm^{-1} respectively) employing an IMPLEN nanophotometer P330. Salts for excipient preparation were bought from Sigma. Tween® 20 was bought from Thermo Scientific.

2.2. Bradford assay

The Bradford test (BT) was performed on a Perkin Elmer Lambda 950 UV/VIS spectrometer equipped with a PTP 1 + 1 Peltier system with bovine serum albumin (BSA) and bovine γ-globulin (BGG) standards purchased from Fisher Scientific. Coomassie Blue G-250 1X solution was purchased from Fisher Scientific and was added to each standard of required concentration, mixing vigorously, equilibrating for 5–10 min at room temperature, centrifuging and reading the absorbance at 595 nm (1 cm plastic cell, 25 °C). Each measurement was repeated in quadruplicate with a 1 cm disposable plastic cell on four different solutions. All the spectra were subtracted from the baseline (Coomassie 1X at the same concentration in water). The results were compared with an absorbance reading at 280 nm.

2.3. CD and MCD

Magnetic circular dichroism measurements were performed on the OLIS module B unit at B23 beamline at Diamond Light Source, Harwell Research and Innovation Campus, Didcot, Oxfordshire, UK. For UV-denatured samples the CD spectra were collected on an applied photophysics Chirascan Plus spectropolarimeter equipped with a fluorescence detector. The module B OLIS instrument for CD/MCD was provided with a sample chamber room able to support a 1.4 T OLIS magnet. The spectra for model proteins were recorded in the 340–260 nm wavelength range, with a 1 cm quartz cell. For photo-denaturation samples, the spectra were collected in a 1 cm/100 μL cell. A 1 mm or 0.5 mm slit were employed with 1 sec of integration time. MCD spectra were recorded in both field directions (N/S and S/N), subtracted (S/N–N/S) ensuring a positive value at 293 nm. Spectra are reported un-corrected for the field magnitude.

2.4. Sample irradiation

Controlled photo-denaturation of samples were performed with a Peqlab box, equipped with five 60 W lamps emitting at 254 nm. The mAbs were irradiated at ∼0.7 mg·mL^{-1} concentration solution (concentration measured with Bradford test and

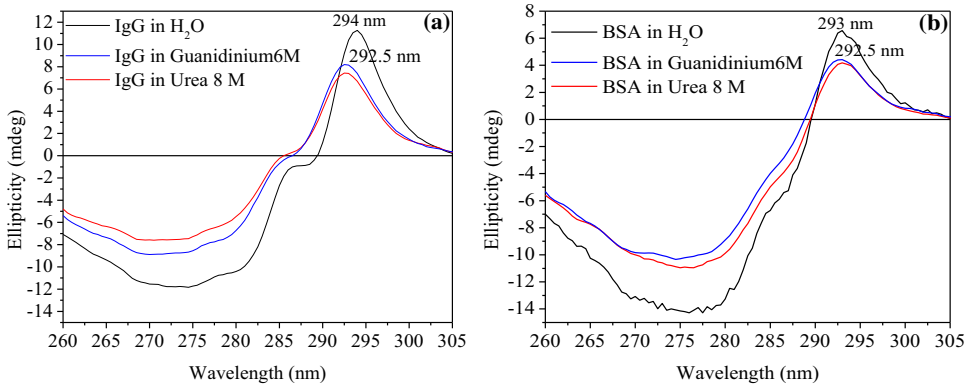

Fig. 1. (a) MCD of IgG 1 mg mL^{-1} water, guanidinium 6 M or urea 8 M. (b) MCD of BSA 2.4 mg mL^{-1} water, guanidinium 6 M or urea 8 M. 1 cm cell, 4 scans. Spectra divided by 2.