Detection of Drug-Protein Complexes in Aqueous Serum using Two-Dimensional Infrared Spectroscopy

Contact *neil.hunt @york.ac.uk*

S. H. Rutherford
Department of Physics
University of Strathclyde, Glasgow, UK

G. M. Greetham
STFC Central Laser Facility
Rutherford Appleton Laboratory, Didcot, UK

M. Towrie
STFC Central Laser Facility
Rutherford Appleton Laboratory, Didcot, UK

A. W. Parker
STFC Central Laser Facility
Rutherford Appleton Laboratory, Didcot, UK

M. J. Baker
Department of Chemistry
University of Strathclyde, Glasgow, UK

N. T. Hunt
Department of Chemistry
University of York, York, UK

Introduction

The binding of small molecules to specific target proteins plays an important role in many areas, from biology and biochemistry to the design of drug molecules in the pharmaceutical sector. In this report, we focus on the binding of small molecule drugs to the serum albumin protein. Although not a specific target for drug molecules, albumin is the major proteinaceous component of blood serum, making up ~35-50 mg/ml. The main biological role of albumin is as a transport protein and it has been widely documented to bind to a variety of substrates including metal ions, steroids, hormones, glucose and pharmaceutical drugs. This means that the interaction of small molecules with albumin is an important factor in the pharmacokinetic behaviour of drugs.[1,2]

In the case of a drug administered via the bloodstream, the unbound fraction of the drug is likely to be predominantly responsible for the pharmacological action. It is therefore important to understand the thermodynamics and kinetics of the interaction of any new drug molecule with the components of blood serum, such as albumin. The ability to measure the extent of drug binding in serum samples with minimal processing would be a powerful tool in developing this understanding. This would be especially relevant in serum samples where fluctuations of the composition of the serum due to metabolism can influence albumin binding behaviour.

The structure of serum albumin consists of three domains (A,B,C), each featuring two sub-domains (I,II) made up of α-helices connected via random coil and inter-domain helixes.[3] The structure supports two major drug binding sites, Sudlow I which is located in subdomain IIA and Sudlow 2 is situated in subdomain IIIA. Importantly, environmental factors within blood serum such as the concentrations of fatty acids can lead to subtle transitions between different structural forms, with the changes involving relatively few of the residues of the complete protein. Furthermore, fatty acids can allosterically affect binding affinities at the two sites.[4,5]

Two-dimensional infrared (2D-IR) spectroscopy has shown considerable potential as a route to monitoring drug binding in serum. 2D-IR measurements of the amide I band of proteins have been shown to be sensitive to changes in secondary structures.[6,7] Furthermore, 2D-IR has been applied to characterise these changes quantitatively, with comparable accuracy to circular dichroism measurements,[8] and to identify changes in both structure and dynamics accompanying drug binding.[9,10]

Most importantly for work in serum samples, where water predominates, 2D-IR spectroscopy has recently been shown to be able to observe the protein amide I band by suppressing the water bending vibration, which obscures the amide I mode in IR absorption spectroscopy measurements.[11] This ability arises from the non-linear nature of the 2D-IR signal, which amplifies the high molar extinction coefficient protein amide I band relative to the weaker, per molecule, water bending vibrational mode. This method enables direct spectroscopic measurements of biological compounds in their physiological environment without the need for pre or post measurement water subtractions or isotopic substitution.

In this report, we demonstrate proof of concept for the use of 2D-IR spectroscopy to detect drug binding to serum albumin in blood serum, reporting changes in the amide I band of serum albumin caused by binding of the common drug molecules paracetamol, ibuprofen and warfarin.

Experimental

2D-IR spectra were recorded using the Fourier transform 2D-IR method on the LIFEtime laser system at the Central Laser Facility. The method and the spectrometer have been described in detail elsewhere.[12,13] Briefly, two Yb:KGW amplified laser systems operating at a pulse repetition rate of 100 kHz were used to pump optical parametric amplifiers equipped with difference frequency mixing of the signal and idler beams. The resulting mid-IR wavelength pulses had a temporal duration of ~200 fs; a central frequency of 1650 cm-1 and a bandwidth of ~100 cm-1.

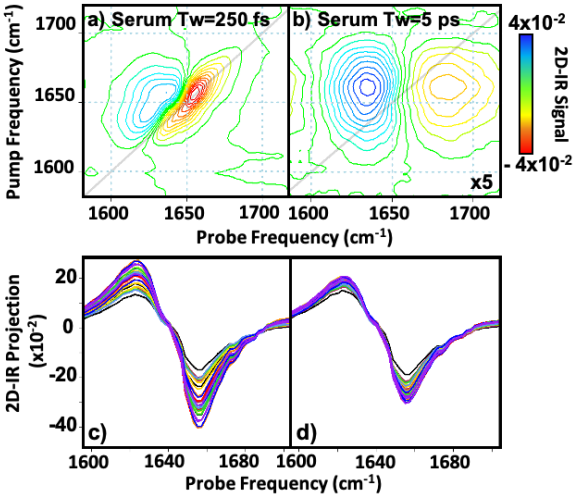

Figure 1: *2D-IR spectra of blood serum obtained with a waiting time (T_w) of a) 250 fs showing protein amide I peak and b) 5 ps showing peak due to water heating. c) and d) show 2D-IR spectra of serum spiked with a range of concentrations of paracetamol projected onto the probe frequency axis before (c) and after (d) normalisation.*

Samples were held in a transmission cell comprised of two CaF_2 windows without a spacer. The tightness of the sample holder was adjusted to obtain approximately consistent absorbance values of ~0.1 for the combination band of water located at 2130 cm^{-1}. For each sample, 2D-IR spectra were recorded at two waiting times of 250 fs and 5 ps (Figure 1(a,b). This allowed the spectral signature due to residual heating of the water by the laser pulses, which is delayed temporally relative to that from the protein content of the sample, to be used to normalise the signals for subsequent difference frequency analysis (Figure 1(c,d). A full description of this method has been published elsewhere.[14]

Samples were produced by adding differing concentrations of acetaminophen (paracetamol), ibuprofen and warfarin to pooled equine blood serum. For each drug, drug-protein molar ratios were created that spanned their clinically relevant range. For the data shown in this report, individual drug-protein solutions were obtained at a 2:1 molar ratio of drug to protein, except in the case of warfarin, 0.07:1, which is administered to patients at much lower concentrations. All chemicals were obtained from Sigma Aldrich and used without further purification.

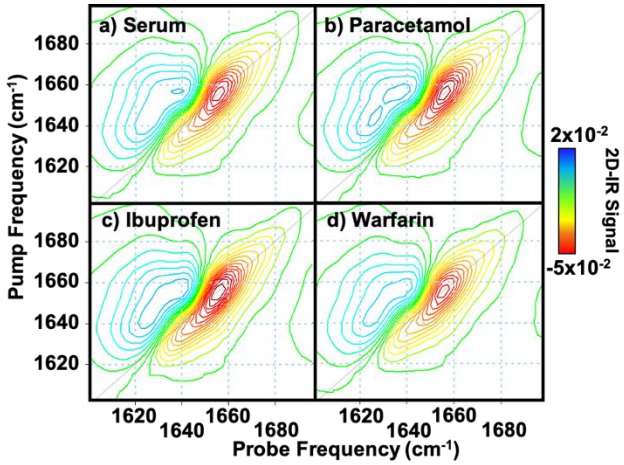

Figure 2: *2D-IR spectra of a) serum, and serum spiked with b) paracetamol 2:1, c) ibuprofen 2:1 and d) warfarin 0.07:1. All spectra are plotted using the same scale, see colour bar.*

Results and Discussion

The 2D-IR spectrum of blood serum in the amide I region is shown in Figure 2(a). This is consistent with previous studies and shows the v=0-1 transition of the amide I band on the diagonal of the spectrum (red) alongside the accompanying v=1-2 transition, shifted to lower probe frequencies. The amide I band has two main contributions located at 1655 cm^{-1} and 1640 cm^{-1}. The former has been assigned to serum albumin and the latter to the globulin fraction of the serum proteins. The separation in peak frequencies is due to the dominance of α helix and β-sheet in the secondary structures of these proteins respectively.

Addition of the drug molecules led to little significant variation in the overall shape of the amide I peak (Figure 2(b-d)), however, it is noted that the impact of binding is expected to be limited to the albumin fraction of serum and to have a subtle impact on the overall lineshape of the response from that protein.

In order to determine the impact of binding, each 2D-IR spectrum was normalised to the thermal water response[14] before a difference 2D-IR spectrum was created by subtracting the spectrum of pure serum from that of the serum with the drug added (Figure 3). In each case, it was determined that the contribution of the drug to the spectrum was negligible at the concentrations used.

Comparisons of the difference spectra produced from each of the three samples shows that each drug induces a different effect on the spectrum of serum albumin, with changes observed in both the 2D-IR lineshape and peak positions. Addition of both paracetamol and ibuprofen resulted in an increase in amplitude of the amide I peak (Figure 3(a,b), red) whereas addition of warfarin led to a decrease in signal amplitude (Figure 3(c) blue) upon binding. Furthermore, the ibuprofen difference spectrum peak appears elongated along the diagonal compared to the more circular lineshape observed for paracetamol data. This suggests that each drug induces a slightly different structural or dynamic change upon binding to the albumin.

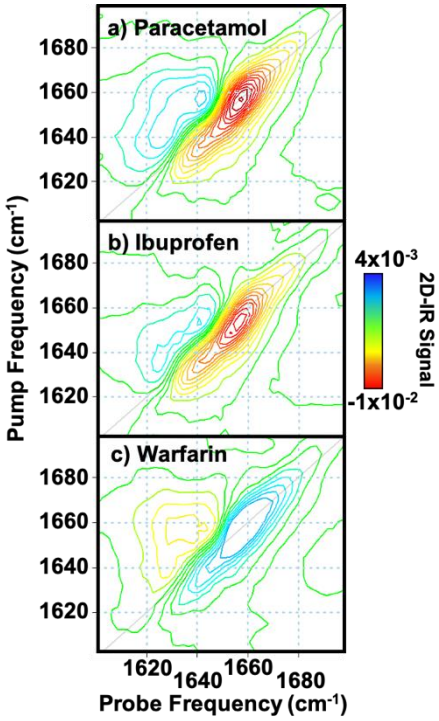

Figure 3: *Difference 2D-IR spectra following addition of a) paracetamol, b) ibuprofen and c) warfarin to blood serum. All spectra are plotted on the same scale, see colour bar.*

Conclusions

2D-IR spectroscopy of blood serum to which drug molecules have been added shows characteristic changes in the amide I region due to binding of the drugs to serum albumin. Further work is needed to clarify limits of detection and to assign the structural origins of the changes observed definitively. The ability to measure different structural responses due to binding of paracetamol, ibuprofen and warfarin, at physiological concentrations, suggest that the 2D-IR method has the potential to be used for screening of protein-drug combinations or for monitoring of serum concentrations of specific drugs.

Acknowledgements

We are grateful to the STFC Central Laser Facility for access to the ULTRA suite of spectrometers.

References

1. Fasano, M. *et al.*, *IUBMB Life* **2005**, *57*, 787.
2 .Sudlow, G. *et al.*, *Mol. Pharmacol.* **1975**, *11*, 824.
3. Kragh-Hansen, U. *et al. Biol. Pharm. Bull.* **2002**, *25*, 695.
4. Yamasaki, K. *et al.*, *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **1999**, *1432*, 313.
5. Spector, A. A., *J. Lipid Res.* **1975**, *16*, 165.
6. Baiz, C. R., *et al.*, *Analyst* **2012**, *137*, 1793.
7. Deflores, L. P. *et al.*,. *J. Am. Chem. Soc.* **2009**, *131*, 3385.
8. Minnes, L. *et al.*, *Anal. Chem.* **2017**, *89*, 10898.
9. Shaw, D. *et al.*, *Chem. Sci.* **2017**, *8*, 8384.
10. Shaw, D. J. *et al.*, *Sci. Rep.* **2017**, *7*, 1.
11. Hume, S. *et al.*, *Chem. Sci.* **2019**, *10*, 6448.
12. Greetham, G. M. *et al.*, *Appl. Spectrosc.* **2016**, *70*, 645.
13. Donaldson, P. M., et al., *J. Phys. Chem. A* **2018**, 122, 780.
14. Hume, S. *et al.*, *Anal. Chem.* **2020**, *92*, 3463.