Coherent Multidimensional Infrared Spectroscopy – application to the study of biomolecules under oxidative stress.

LAYS REZENDE VALIM GIERAKOWSKI

INSTITUTE OF CHEMICAL BIOLOGY
DEPT OF CHEMISTRY, IMPERIAL COLLEGE LONDON

Thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy and for the Diploma of the Imperial College London.

London, December 2017
DECLARATION OF
ORIGINALITY AND COPYRIGHT

I hereby certify that the material covered in this thesis, which I now submit for the award of Doctor of Philosophy and the Diploma of the Imperial College London, is entirely my own work unless otherwise cited or acknowledged within the body of the text.

Furthermore, I certify that this manuscript does not exceed 100,000 words and has not been previously submitted either in whole or in part towards another degree at this or any other university.

Signature:

Date:

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build up on it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.
"(...) You have brains in your head
You have feet in your shoes.
You can steer yourself
any direction you choose.

You're on your own. And you know what you
know.
And YOU are the guy who'll decide where to
go.

(...) You'll be on your way up!
You'll be seeing great sights!
You'll join the high fliers who soar to high
heights.

(...) Wherever you fly, you'll be best of the best.
Wherever you go, you will top all the rest.

Except when you don't.
Because, sometimes, you won't.

I'm sorry to say so
but, sadly, it's true
that Bang-ups and Hang-ups
can happen to you.

(...) You can get so confused
that you'll start in to race
down long wiggled roads at a break-necking pace
and grind on for miles cross weirdish wild space,
headed, I fear, toward a most useless place.
The Waiting Place...

...for people just waiting.
Waiting for a train to go
or a bus to come, or a plane to go
or the mail to come, or the rain to go
or the phone to ring, or the snow to snow
or the waiting around for a Yes or No
or waiting for their hair to grow.
Everyone is just waiting.

(...)NO!
That's not for you!

Somehow you'll escape
all that waiting and staying
You'll find the bright places
where Boom Bands are playing.

With banner flip-flapping,
one more you'll ride high!
Ready for anything under the sky.
Ready because you're that kind of a guy!

(...) And will you succeed?
Yes! You will, indeed!
(98 and 3/4 percent guaranteed).

KID, YOU'LL MOVE MOUNTAINS!" - Dr. Seuss, extracted from
"Oh! The places you'll go."
ACKNOWLEDGEMENTS

I would like to take a moment to thank my supervisors Prof David Klug, Prof Oscar Ces and Prof Keith Willison for their guidance throughout the past 6 years. Also, to Dr Julia Davies, Dr Frederic Fournier and Dr Katherine Kornau for everything that they have taught me. Dr Julia Davies has also contributed massively towards improving stability of our laser system and her guidance has allowed me to reach most of the outcomes presented in this thesis. I am also very thankful to our former and current group members and all the friends that I have met through the Institute of Chemical Biology. You have provided me with a listening ear every time I needed to talk through topics ranging from equipment issues to existential problems.

Last, but most certainly not least, I would like to express my sincere gratitude to my family. Thanks to Adrian Gierakowski for supporting me in my decision to move to London and for all the assistance given in those early days of my Higher Education. Huge thanks to my partner, Fred, and my gorgeous sister, Lorenna, for being by my side throughout all the ups and downs that I have gone through. And, with all my heart, I would like to thank my lovely boys, Erik and Marc. Being your mother has been the most rewarding adventure of my life

∞

I would like to dedicate this thesis to my mother, Cristianne, my grandmother, Elizabeth and my uncle, Carlos. You are the world’s best teachers and I would not have gotten this far without the many lessons I have learned from you.

This work has been supported by the Engineering and Physical Sciences Research Council (EPSRC) through a doctoral training studentship EP/J500239/1 and by the Proxomics project, through the EPSRC grant EP/I017887/1.
ABSTRACT

There is a growing body of evidence which suggests post-translational modifications occurring under oxidative stress (oxPTMs) play an important role in both human health and disease. The focus of the work described in this thesis has been on the use of coherent multidimensional spectroscopy (CMDS) to perform detection and quantification of oxPTMs in a label-free and non-destructive manner. Electron-Vibration-Vibration (EVV) two-dimensional infrared (2DIR) spectroscopy is a CMDS technique which is able to directly observe intra- and intermolecular interactions. As a result, EVV 2DIR spectroscopy is particularly useful for characterising (oxPTMs). EVV 2DIR spectroscopy employs one near-IR and two mid-IR picosecond excitation beams to probe vibrational couplings in a sample via a four-wave mixing process. This results in the spread of vibrational coupling information across two dimensions, which leads to spectral decongestion and the ability to directly analyse vibrational modes within complex molecules, such as proteins. Here, tyrosine (Tyr) nitration is used as a study model due to its importance in inflammatory diseases, amongst other pathologies. Results are presented for various nitration models and will demonstrate EVV 2DIR spectroscopy's ability to identify, relatively quantify and characterise the effect of nitration of tyrosine side-chains.
# Table of Contents

Acknowledgements .................................................. 5

Abstract ............................................................. 7

List of Tables ....................................................... 11

List of Figures ....................................................... 12

List of Abbreviations, Symbols and Nomenclature ................. 16

1 Introduction ....................................................... 17
   1.1 The role of oxidation in human health & disease ............ 19
   1.1.1 Reactive oxygen and nitrogen species .................... 19
   1.1.2 Redox homeostasis & the role of RONS in physiological processes 21
   1.1.3 Involvement of RONS in the development of pathologies .... 22
   1.2 Tyrosine nitration: characterisation & biological significance .... 24
   1.3 Analytical tools used in the identification of nitrooxidative biomarkers ... 26
      1.3.1 Mass spectrometry ........................................ 27
      1.3.2 Antibody-based methods .................................... 29
      1.3.3 Spectroscopic techniques ................................. 31
   1.4 Optical coherent multidimensional spectroscopy ............. 33
   1.5 EVV 2DIR spectroscopy ........................................ 35
      1.5.1 EVV 2DIR spectroscopy as a bioanalytical tool ............ 37
      1.5.2 Classical theory of EVV 2DIR spectroscopy ............... 39
      1.5.3 Quantum mechanical theory of EVV 2DIR spectroscopy .... 41
   1.6 Summary of research questions ................................ 43
   1.7 Thesis overview ............................................... 44

2 Methods ........................................................... 47
   2.1 Experimental Set-up of EVV 2DIR Spectroscopy ............... 49
   2.2 Acquisition and processing of EVV 2DIR data .................. 55
2.3 Theoretical Simulation of EVV 2DIR Spectra ........................................... 55
2.4 Sample preparation ........................................................................... 57
  2.4.1 Sample preparation of side-chain analogues ................................. 57
  2.4.2 Peptide ε protein sample preparation ........................................ 57
2.5 LC-MS analysis of peptides and proteins ........................................... 59

3 Experimental optimisations ................................................................. 63
  3.1 Non-resonant signal of substrates and windows ............................... 66
  3.2 Longitudinal positioning of samples .............................................. 71
  3.3 Improving the reproducibility of relative cross-peak energy intensities. 73
  3.4 Considerations when preparing liquid samples ............................... 79
  3.5 What to know when designing model peptides ............................... 80
  3.6 Conclusion & proposed future optimisations .................................. 81

4 Identification of tyrosine nitration by EVV 2DIR spectroscopy ............... 83
  4.1 Side-chain analogues ................................................................... 85
    4.1.1 Simulated 2DIR spectra .......................................................... 86
    4.1.2 Improving signal strength via an electronic resonance .............. 88
    4.1.3 Experimental 2DIR spectra .................................................... 90
  4.2 Peptide models ............................................................................ 92
    4.2.1 Spectral features of tyrosine ................................................... 95
    4.2.2 Spectral features of 3-nitrotyrosine ......................................... 96
  4.3 BSA as a model of protein-tyrosine nitration ................................... 98
  4.4 Conclusion ............................................................................... 104

5 Quantification of tyrosine nitration ....................................................... 107
  5.1 Detection of nTyr in peptide mixtures ............................................. 109
  5.2 Construction of calibration curves ............................................... 112
  5.3 Quantification of mixtures containing low percentages of nTyr ........... 113
  5.4 The limit of detection of EVV 2DIR spectroscopy ............................ 115
  5.5 Conclusion ............................................................................... 116

6 Conclusions and Outlook ..................................................................... 119
  6.1 Experimental limitations .............................................................. 121
  6.2 Future work .............................................................................. 122
    6.2.1 Study of oxidative markers in proteins .................................. 122
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2.2</td>
<td>Chemical mapping of oxidative markers</td>
<td>123</td>
</tr>
<tr>
<td>6.3</td>
<td>Work summary</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td><strong>List of References</strong></td>
<td>126</td>
</tr>
<tr>
<td><strong>Appendices</strong></td>
<td></td>
<td>136</td>
</tr>
<tr>
<td>A</td>
<td>Publications</td>
<td>136</td>
</tr>
<tr>
<td>A.1</td>
<td>L Rezende Valim et al 2014</td>
<td>136</td>
</tr>
<tr>
<td>A.2</td>
<td>Conference abstract</td>
<td>153</td>
</tr>
<tr>
<td>B</td>
<td>HPLC &amp; mass spectrometry traces of peptides</td>
<td>155</td>
</tr>
<tr>
<td>B.1</td>
<td>Trimer peptides</td>
<td>155</td>
</tr>
<tr>
<td>B.2</td>
<td>Heptamer peptides</td>
<td>164</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1.1 List of common ROS and RNS divided between radicals and non-radicals. ........................................ 20

1.2 Spectroscopic characteristics of tyrosine and 3-nitrotyrosine in the UV-Vis spectrum. ........................................ 25

1.3 Liouville pathways which can be accessed via EVV 2DIR spectroscopy ........................................ 42

4.1 Comparison between experimental and simulated cross-peak frequencies for unmodified Tyr and their vibrational assignments ........................................ 96

4.2 Comparison between experimental and simulated cross-peak frequencies for neutral nTyr and their vibrational assignments ........................................ 97

4.3 Comparison between experimental and simulated cross-peak frequencies for charged nTyr and their vibrational assignments ........................................ 97

4.4 Nitrated peptides detected in nBSA(ONO-O−) ........................................ 99

4.5 Nitrated peptides detected in nBSA(TNM). ........................................ 101

5.1 Linear fit coefficients of calibration curves extracted from peptide mixtures in pH 5.6. ........................................ 112

5.2 Linear fit coefficients of calibration curves extracted from peptide mixtures in pH 9.1. ........................................ 112

6.1 Example of proteins reported to be targets of tyrosine nitration. ........................................ 123
LIST OF FIGURES

1.1 Schematics of an energy level diagram for the EVV pathways . . . . . . . 35

1.2 Schematics of the excitation beams. . . . . . . . . . . . . . . . . . . . . 37

1.3 Schematics of the various possible models of tyrosine nitration . . . . . . 45

2.1 Schematics of excitation beams interacting with a sample . . . . . . . . 47

2.2 Schematics of Spectra Physics system set-up . . . . . . . . . . . . . . . 50

2.3 Example of energy profile of IR excitation . . . . . . . . . . . . . . . . . 53

2.4 Schematics of peptide and protein sample preparation . . . . . . . . . 58

3.1 Experimental EVV 2DIR spectra of various substrates . . . . . . . . 67

3.2 NRB signal as function of longitudinal position . . . . . . . . . . . . . . 68

3.3 Experimental EVV 2DIR spectra of 4M2NP at various time delays. . . 70

3.4 Diagram of longitudinal positioning of a liquid sample cell. . . . . . . 71

3.5 Experimental EVV 2DIR spectra of 4MP. . . . . . . . . . . . . . . . . . 72

3.6 Profilometry data of coffee-rings formed at pH 5.5. . . . . . . . . . . 74

3.7 Profilometry data of coffee-rings formed at pH 7.6. . . . . . . . . . . 75
3.8 Profilometry data of coffee-rings formed at pH 9.0. .......................... 76
3.9 EVV 2DIR signal variations across coffee-rings .............................. 77
3.10 Examples of peptide samples prepared at various pH values .............. 81

4.1 Schematics of a tyrosine side-chain being nitrated into 3-nitrotyrosine .. 83
4.2 Simulated one-dimensional IR spectra of 4MP, as well as neutral and charged 4M2NP. ................................................................. 86
4.3 Experimental and simulated FTIR spectra of 4MP. .............................. 87
4.4 Simulated EVV 2DIR spectra of 4MP, neutral 4M2NP and charged 4M2NP 89
4.5 Experimental 2DIR spectra of 4MP and 4M2NP solutions in C₂Cl₄. .... 91
4.6 Schematics of the chemical structure of Tyr, nTyr and nTyr⁻ .................. 92
4.7 Experimental EVV 2DIR spectra of heptamer peptides containing Tyr, nTyr and nTyr⁻ ................................................................. 94
4.8 Schematics of terminal capping of a peptide. .................................... 95
4.9 Spectra of BSA and nBSA at pH 9 .................................................. 102
4.10 EVV 2DIR spectrum of capped SPSnYSPS peptide. .......................... 103

5.1 Experimental EVV 2DIR spectrum of a mixture of peptides containing Tyr and nTyr ................................................................. 107
5.2 Experimental EVV 2DIR spectra of heptamer peptides mixtures at pH 5.6. 110
5.3 Experimental EVV 2DIR spectra of heptamer peptides mixtures at pH 9.1 111
5.4 Experimental EVV 2DIR spectra for peptide mixtures containing 10 and 5% of nTyr. .............................................. 114

5.5 Extracted ion chromatograms for peptide mixtures containing 5 and 10% of the nTyr peptide. .............................................. 115
List of Abbreviations, Symbols and Nomenclature

ELISA  Enzyme-linked immunosorbent assay.
2DIR   Two-dimensional infrared [spectroscopy].
4M2NP  4-methyl-2-nitrophenol.
4MP    4-methylphenol, also known as p-cresol.
\*NO   Nitric oxide.
ATP    Adenosine triphosphate.
BSA    Bovine serum albumin.
C\textsubscript{2}Cl\textsubscript{4} Tetrachloroethylene.
CMDS   Coherent multidimensional spectroscopy.
DOVE   Doubly vibrationally enhanced [spectroscopy], also known as EVV 2DIR spectroscopy.
EVV 2DIR Electron-vibration-vibration two-dimensional infrared [spectroscopy].
FWHM   Full width at half maximum.
FWM    Four-wave mixing.
GC-MS  Gas-chromatography coupled to mass-spectrometry.
H\textsubscript{2}O\textsubscript{2} Hydrogen peroxide.
HRP    Horseradish peroxidase.
IR     Infrared [spectroscopy].
LC-MS  Liquid-chromatography coupled to mass-spectrometry.
MnSOD  Manganese superoxide dismutase.
MS     Mass spectrometry.
nBSA   Nitratated bovine serum albumin.
NMR    Nuclear magnetic resonance.
NRB    Non-resonant background [signal].
oxPTM  Oxidative post-translational modification.
PM     Photomultiplier [detector].
PTFE   Polytetrafluoroethylene.
PTM    Post-translational modification.
RNS    Reactive nitrogen species.
RONS   Reactive oxygen and nitrogen species.
ROS    Reactive oxygen species.
SFG    Sum-frequency generation.
SOD    Superoxide dismutase.
TNM    Tetratnitromethane.
TRIVE  Triply vibrationally enhanced [spectroscopy].
Tyr    Tyrosine amino-acid.
This chapter provides an overview of the relevance of oxidation in human health and disease. The analytical tools currently employed in the study of oxidated biomolecules are discussed, as well as the motivations for exploring the capabilities of multidimensional infrared spectroscopy for the analysis of molecules subject to oxidative damage.

This thesis focuses on utilising a type of multidimensional spectroscopy named electron-vibration-vibration two-dimensional infrared (EVV 2DIR) spectroscopy. Thus, a summary of the theoretical and experimental background of EVV 2DIR spectroscopy is also provided in this chapter.
CHAPTER 1

Contents

1.1 The role of oxidation in human health & disease .................. 19
  1.1.1 Reactive oxygen and nitrogen species .......................... 19
  1.1.2 Redox homeostasis & the role of RONS in physiological processes 21
  1.1.3 Involvement of RONS in the development of pathologies ....... 22

1.2 Tyrosine nitration: characterisation & biological significance . 24

1.3 Analytical tools used in the identification of nitrooxidative biomarkers .................................................. 26
  1.3.1 Mass spectrometry ........................................... 27
  1.3.2 Antibody-based methods .................................. 29
  1.3.3 Spectroscopic techniques .................................. 31

1.4 Optical coherent multidimensional spectroscopy ................. 33

1.5 EVV 2DIR spectroscopy ........................................... 35
  1.5.1 EVV 2DIR spectroscopy as a bioanalytical tool ............... 37
  1.5.2 Classical theory of EVV 2DIR spectroscopy .................. 39
  1.5.3 Quantum mechanical theory of EVV 2DIR spectroscopy ...... 41

1.6 Summary of research questions ................................. 43

1.7 Thesis overview .................................................. 44
1.1 The role of oxidation in human health & disease

The term reactive oxygen species (ROS) is used to describe a number of oxidant molecules and radicals derived from molecular oxygen. Radicals display unstable configuration since they have an unpaired electron. As a result, radicals quickly react with other molecules or radicals to achieve a stable configuration.

ROS are by-products of normal cell metabolism in all aerobic species. They are constantly being formed as a result of biological reactions that use electron transfer [1]. A well known example is the production of adenosine triphosphate (ATP) in mitochondria. Oxidative phosphorylation, as well as other redox processes - such as activity of oxidases and reductases - are also known to generate ROS [1].

Redox homeostasis allows biological systems to neutralize the oxidizing agents to prevent damaging effects of ROS. However, a deregulation of redox signaling takes place when antioxidants fail to prevent the accumulation of ROS, which results in oxidative stress. In addition to ROS, reactive nitrogen species (RNS) are also major contributors to oxidative stress and are known to react and potentially disrupt the cellular functions of lipids, proteins and nucleic acids.

1.1.1 Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (RONS) include a range of radicals and non-radicals, such as the ones highlighted in Table 1.1, that have effects on multiple targets and pathways.

The hydroxyl radical (·OH) is reacts very close to its site of formation and is very biologically damaging. Superoxide radical (·O$_2^-$) may take a role in ·OH production by inactivating enzymes containining [Fe–S] clusters [2], which results in the release of
Table 1.1: List of common ROS and RNS divided between radicals and non-radicals.

<table>
<thead>
<tr>
<th>Radicals</th>
<th>Non-radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide anion</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>*O$_2^-$</td>
<td>Peroxynitrile</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>ONOO$^-$</td>
</tr>
<tr>
<td>*OH</td>
<td>Peroxynitrous acid</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>ONOOH</td>
</tr>
<tr>
<td>*NO</td>
<td>Nitroxy anion</td>
</tr>
<tr>
<td>Nitrogen dioxide</td>
<td>NO$^-$</td>
</tr>
</tbody>
</table>

Fe$^{2+}$ intracellularly. The synthesis of *OH then takes place from the catalytic conversion of H$_2$O$_2$ in the presence of Fe$^{2+}$ via Fenton reaction [3, 4]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + *OH$$

Superoxide radicals (*O$_2^-$) are produced by mitochondria and NADPH oxidases [5, 6, 7]. Additionally, the removal of *O$_2^-$ by superoxide dismutase leads to the formation of H$_2$O$_2$. Thus, *O$_2^-$ and H$_2$O$_2$ are continuously produced during aerobic metabolism. While *O$_2^-$ and H$_2$O$_2$ are less reactive than *OH, they play a role in the formation of other highly oxidising molecules. Nitric oxide (*NO) is also relatively unreactive but it is a widespread signaling molecule which regulates important physiological processes. It is produced from the metabolism of the amino acid L-arginine into L-citrulline via an enzyme-catalyzed process involving various types of nitric oxide synthases.

In the presence of oxidants including *O$_2^-$, H$_2$O$_2$ and transition metal centers, *NO metabolism may form secondary products such as peroxynitrite anion (ONOO$^-$) and nitrogen dioxide (*NO$_2$). ONOO$^-$ is a strong in vivo oxidant able to damage many biological molecules and is produced from the diffusion-controlled reaction between *O$_2^-$ and excess *NO.

ONOO$^-$ has a half life of 5–20 ms [8] and can oxidize lipids, methionine and tyrosine residues in proteins and DNA to form nitroguanine [9]. Direct oxidative reactions of sulphydryls, thiols and ascorbate take place via the generation of intermediates such as hydroxyl and nitrogen dioxide radicals. At biological pH, it exists in equilibrium with peroxynitrous acid, as highlighted by the following chemical equations [10]:

20
\[ \cdot \text{O}_2^- + \cdot \text{NO} \rightarrow \text{ONO}_2^- \]

\[ \text{ONO}_2^- + \text{H}^+ \rightleftharpoons \text{ONO}_2\text{H} \rightleftharpoons \text{ONO}_2\text{H}^+ \rightarrow \text{NO}_3^- + \text{H}^+ \]

\[ \text{ONO}_2^- + \text{H}^+ \rightleftharpoons \text{ONO}_2\text{H} \rightleftharpoons [\cdot \text{HO} + \cdot \text{NO}_2]^+ \rightarrow \text{NO}_3^- + \text{H}^+ \]

The most significant protein modifications caused by \( \text{ONOO}^- \) are glutathiolation and S-nitrosation of cysteine and nitration of tyrosine residues into 3-nitrotyrosine, making the latter a biomarker of peroxynitrite formation. Tyrosine nitration is discussed in depth later in this chapter.

1.1.2 Redox homeostasis & the role of RONS in physiological processes

Normally, endogenous and exogenous antioxidants continuously prevent and repair damages caused by RONS. Redox homeostasis, i.e. the balance between pro- and antioxidants, is crucial for various physiological processes [11, 12]. This is achieved by several mechanisms within the cell, such as scavenging enzymes, catalases and glutathione peroxidases [13, 14], which readily remove oxidants. For example, superoxide is rapidly removed by high concentrations of superoxide dismutases (SOD) and NO is removed by its rapid diffusion through tissues into red blood cells, where it is converted to nitrate by reaction with oxyhemoglobin.

Under redox homeostasis, RONS modulate intracellular transduction pathways and transcriptional factors involved in cell proliferation [15], differentiation and maturation [16, 17, 18]. Also, formation of RONS can destroy invading pathogens and may help modulate inflammatory responses [19, 20].

Evidence shows that, under physiological condition, ROS influence signal transduction pathways in various systems and cell types. A wide range of critical signaling molecules,
such as mitogen-activated protein (MAP) kinases, phosphatase and tensin homolog (PTEN), and protein tyrosine phosphatases have been shown to interact with ROS to initiate signaling in cellular processes such as proliferation and survival [21, 22]. They also play crucial roles in gene activation [23].

In the vascular system, ROS have been identified to regulate differentiation of vascular smooth muscle cells, participate in blood pressure control [22], control vascular endothelial cell proliferation and migration [21, 24].

More specifically, nitric oxide (\textsuperscript{•}NO) is known to take part in a wide range of physiological processes. For example, it is known to modulate blood flow and nonspecific host defence [25, 26]. It also acts as a messenger in the transduction pathways important for synaptic plasticity in the central nervous system [27].

Finally, peroxynitrite-driven oxidative modification can cause conformational changes of zinc finger family of transcription factors, such as p53. Since p53 functions as a stress-responsive transcription factor, modulation of its redox status via nitration of critical tyrosine residues in the DNA binding-domain appears to be an important determinant of cell fate [28, 29, 30].

1.1.3 Involvement of RONS in the development of pathologies

Uncontrolled formation of RONS may lead to oxidative modifications of biomolecules in all aerobic living species on Earth. Oxidative stress plays an important role in the development of chronic and degenerative illnesses, such as cardiovascular and neurodegenerative diseases, cell fate, chronic inflammation, cancer and ageing (reviewed in [27, 25, 31]).

In the cardiovascular system, large quantities of RONS cause the development and progression of vasculopathies, as well as congestive heart failure, atherosclerosis [32, 21]. Additionally, ROS-mediated reduction of disulfide bonds in proteins, specifically fibrino-
gen, may produce dysfunctional hemostatic clots, leading to thrombosis [33].

Neurodegenerative and psychiatric diseases have been linked to accumulation of RONS in the brain. Since the brain consumes a large amount of oxygen, while having less antioxidant activity than other organs, cerebral oxidative stress tends to rise with aging and has been shown to be responsible for the loss of cognitive and motor functions in several brain diseases.

Disruption of mitochondrial metabolism is often observed due to peroxynitrite. Inhibition of manganese superoxide dismutase (MnSOD) has been observed via manganese cation cathalysis [34] via the nitration of key tyrosine residues in the presence of peroxynitrite. MnSOD is the mitochondrial isoform of superoxide dismutase (SOD) and its inhibition results in impaired enzymatic activity leading to a positive feedback loop for enhanced mitochondrial peroxynitrite formation. This results in increased oxidative stress, which can then trigger apoptosis [34, 35]. Lipid peroxidation via hydroxyl radical and peroxynitrite can also damage cell membranes and lipoproteins [36].

Additionally, the literature has a wide range of evidence that high levels of both oxidation and inflammation can be found in chronic illnesses [37, 38, 39]. Environmental insults trigger ROS generation through acute inflammatory responses, while pro-inflammatory gene expression can be triggered by ROS themselves, which demonstrates an interdependent relationship between the two conditions. This is known to result in chronic inflammation, which may predispose the host to various pathologies, such as cancer, in an event termed inflammation-induced tumorigenesis. Two of the main examples of such events are the helicobacter pylori and human papillomavirus infections, which cause gastric and cervical cancers, respectively. DNA modifications, such as base and sugar lesions, strand breaks, DNA-protein cross-links, base-free sites and base-hydroxylation, can result from oxidative stress and are also considered an important event in cancer development [26].

Finally, protein aggregation has been shown to occur in a ROS-dependent manner [40, 41]. Since aging and age-associated diseases are accompanied by the accumulation of high-molecular protein aggregates, the proteasome, which is responsible for the degradation of
damaged and oxidized unfolded protein structures, is seen to be inhibited upon binding to such aggregates [42]. A vicious cycle is then created where increase in oxidative stress leads to a reduced level of degradation of newly formed oxidised proteins. For example, accumulation of nitrated α-synuclein is found in hallmark lesions of Parkinson’s disease [43].

1.2 Tyrosine nitration: characterisation & biological significance

Tyrosine nitration into 3-nitrotyrosine is a covalent oxidative post-translational modification (oxPTM) which has been used as a marker of nitroxidative stress present in a variety of human diseases. Protein nitration is mediated by peroxynitrite anion (ONOO−), which adds a nitronium (NO2+) molecule to tyrosine residues [44]. This lowers the pKa of tyrosine’s phenolic ·OH group from approximately 10 to 7.5, which may potentially result in conformational changes, loss of function through steric restrictions, variation of biological half-life of proteins and inhibition of other post-translational modifications (PTMs), such as phosphorylation of tyrosine residues important for signal transduction [45, 46, 47, 48, 49, 50, 51, 52].

Despite nitration being derived by non-discriminating chemical reactions, it has been reported that neither the number of tyrosine residues nor the abundance of a protein determines the protein target that is modified by nitrating agents in vitro [53]. Tyrosine nitration appears to be a selective process since only a relatively limited number of proteins display affinity for nitration reactions and only a few specific tyrosine residues are usually nitrated within these proteins [54, 55, 56, 51]. A few potential reasons are:

a. Species participating in nitration have short diffusion distances requiring close proximity to sources of nitric oxide synthesis and enzymatic catalysis (either self-catalysis or by peroxidases).

24
Table 1.2: Spectroscopic characteristics of tyrosine and 3-nitrotyrosine in the UV-Vis spectrum.

<table>
<thead>
<tr>
<th>Abs. peak (nm)</th>
<th>3-nitrotyrosine</th>
<th>tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>neutral charged</td>
<td>neutral</td>
</tr>
<tr>
<td>ε (M⁻¹cm⁻¹)</td>
<td>360 428</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>2200 4100</td>
<td>1360</td>
</tr>
</tbody>
</table>

Table shows data for tyrosine and 3-nitrotyrosine either with a protonated or deprotonated hydroxyl group (labelled neutral and charged, respectively). The wavelength of the absorption peaks and the extinction coefficients have been extracted from [60].

b. Site-specific tyrosine nitration is a metal catalyzed process requiring proximity to metal-binding sites since heme-containing proteins facilitate nitration via formation of ferryl intermediates [50].

There are several accounts of increased level of tyrosine nitration in conditions such as atherosclerosis, Parkinson’s disease, diabetes and inflammatory bowel disease. Increased levels of 3-nitrotyrosine in a variety of tissues have been identified in over 80 different pathologies. However, it is important to realise that other peroxynitrite-mediated PTMs, such as thiol oxidation, sulfoxidations of methionine and dityrosine formation may also alter cellular processes.

As mentioned earlier in this chapter, loss of activity of the mitochondrial enzyme MnSOD is thought to be due to the nitration of a specific tyrosine residue (Tyr34) located close to its active site [51]. Protein tyrosine nitration is also known to occur in the tumour suppressant p53. In silico experiments [57], as well as immunoprecipitation experiments performed on immortalized lymphocytes derived from AD patients [58], have demonstrated conformational changes in p53 as a result of tyrosine nitration.

While tyrosine nitration is thought to be an irreversible process, a rapid, reversible and target-selective cycle of denitration and nitration of tyrosine occurs in liver mitochondria of Sprague-Dawley rats, suggesting a regulatory role in redox signalling [59]. Proteins critical for energy and antioxidant homeostasis were identified as selective targets: manganese superoxide dismutase, enoyl-CoA hydratase and acetyl-CoA acyltransferase (which are proteins involved in fatty acid oxidation) and electron transport flavoprotein (a key electron acceptor for multiple pathways) [59].
Detection of 3-nitrotyrosine is very often achieved via mass spectrometry and antibody-based techniques, although HPLC or GC based spectrophotometry have also been described. UV/vis spectroscopy is widely used in order to obtain molecular mass of proteins via the absorption peak of tyrosine at 275nm. Given the pKa of the phenolic hydroxyl group in tyrosine is approximately 10, the intensity of this absorption peak can be readily measured across a wide range of pH. Upon nitration, however, this pKa lowers significantly to approximately pH 7.5, thus two forms of 3-nitrotyrosine are usually found: the phenolic form, with absorption at 428nm, and a phenolate form, with absorption at 360nm.

1.3 Analytical tools used in the identification of nitrooxidative biomarkers

Much effort has been placed on developing techniques for detection of protein post-translational modifications (PTMs), such as phosphorylation, acetylation, glycosylation, ubiquitination, oxidation, nitration, and nitrosylation. These PTMs are currently well known for their ability to alter protein function by playing roles in a variety of signalling pathways.

The use of proteomic tools, such as mass spectrometry (MS), has enabled the construction of a large knowledge database surrounding the causes and effects of protein modifications. However, oxidatively driven PTMs (oxPTMs), such as oxidation and nitrosylation of cysteine and nitration of tyrosine, occur at low copy numbers, which, due to low signal-to-noise ratios, leads to many difficulties in establishing their reproducible and unambiguous identification, quantification and characterisation. Thus, the development of robust characterisation tools is very important towards the elucidation of the molecular mechanisms surrounding protein damage due to oxidative stress.

Various techniques have been used to study oxPTMs, each having their own strengths
and being suitable for a given set of applications. Mass spectrometry is the gold standard in the analysis of oxidation, followed by immunoassays. A review of such techniques is provided in this section.

1.3.1 Mass spectrometry

Mass spectrometry (MS) analysis is an invaluable proteomics tool which is used to identify and quantify molecules in simple and complex mixtures. Samples are ionised through bombardment of high energy electrons and the resulting ions are accelerated through an electromagnet. The magnetic field is applied perpendicularly to the direction of ion motion, which allows the separation of the ions by their mass-to-charge ratio via deflection.

MS analysis is generally divided into top-down, where whole-protein characterisation is performed, and bottom-up, where characterisation is applied to protein fragments. Top-down MS analysis of whole proteins is currently limited in sensitivity and presents difficulties in handling complex samples. Top-down analysis is usually carried out by matrix-assisted laser desorption/ionisation (MALDI) or electrospray ionization (ESI) MS [61] but may at times require high-resolution instruments such as Q-TOFs, Orbitraps or FT-ICR MS [62]. Since whole proteins may acquire many different charges during the ionisation step, the resulting spectrum tends to contain a number of peaks corresponding to each different charge state. While this may help improve the mass accuracy of the analysis, it also means that only a limited number of proteins species can be simultaneously analysed before signals start to overlap, which leads to difficulties in spectral deconvolution [62]. As a result, most top-down studies have been conducted in vitro with low molecular weight proteins (up to 80 kDa) [63]. Calcium-modulated protein following in vitro incubation with lipopolysaccharide (LPS)-activated macrophage lysate has been successfully studied via top-down MS, leading to the detection and quantification of nitration levels in the sample [64].

Bottom-up MS analysis of enzymatically digested proteins (i.e. total digestion to a pep-
tide mixture is performed before MS analysis) is a common method which is extensively used to sequence and identify proteins. However, it does not provide information on the specific protein that has been modified or the modification site. More relevant to the study of oxPTMs, the most remarkable disadvantage of using bottom-up MS approach is the fact that proteins must be fragmented for analysis: both the digestion in solution and in-gel digestion have been shown to introduce artefacts such as methionine, cysteine or tryptophan oxidation. Thus, the fragmentation process leads to overestimation of the measured levels of oxPTMs. Finally, due to the digestion process, bottom-up analysis cannot be used to infer relationships between PTMs within an individual protein [62].

In order to lower background signal, protein characterisation by MS is typically performed in combination with separation techniques such as two-dimensional gel electrophoresis and two-dimensional liquid chromatography [62]. Other common separation techniques used in conjunction with MS are liquid- and gas-chromatography (LC-MS or GC-MS, respectively) [65].

Sample enrichment and chemical labelling via addition of chemical groups is also employed in MS studies. Label-free analysis is most common in standard proteomics as it requires less sample manipulation. However, enrichment via modification-specific chemical tagging has been regarded as an essential step towards improved selectivity in studies involving oxPTMs [62]. Both the targeted and non-targeted approaches rely on subsequent bioinformatic analysis of the spectra to assign a modification to a given amino acid [65].

With potentially thousands of individual MS/MS obtained from bottom-up experiments, automated data analysis is required [66]. To this date, many statistical search engines have been developed for identification of proteins. A few examples are Mascot, PEAKS, Sequest, ProteinPilot, Tandem, Ommsa and Phenyx (reviewed in [62]). While these engines work very well in general proteomics, issues have been found when trying to identify oxPTMs [67]. Mascot and Sequest have been identified as particularly reliant on user parameter input in order to correctly identify oxPTMs [62].
Search engines currently offer the ability to include a number of oxPTMs as possible modifications. However, only about 3 or 4 modifications can be computed in parallel [68], which can be limiting when heterogeneous oxidation has occurred. That can be particularly an issue with targets of peroxynitrite-driven oxPTM. These shortcomings have required the use of de novo sequencing as a data validation step for the identification of oxPTMs. While de novo sequencing allows the user to validate both the presence and location of the modification in given sequence, this is a time-consuming step which further increases the complexity of MS data analysis.

As briefly mentioned above, enrichment may be used in order to increase signal-to-noise ratios in analysis of oxPTMs. Immunoenrichment techniques, such as those using anti-nitrotyrosine antibodies, have been used to enrich proteins from biological samples [69], although this approach relies on the specificity of the utilised antibodies. Detection of protein bound nitrotyrosine via chemical tagging approaches usually require the reduction of the nitro group into an amine in order to facilitate binding to a wide range of tags that can be used as reporters in MS analysis [70].

1.3.2 Antibody-based methods

Antibody-based Western blot analysis, also known as immunoblotting, is a rapid assay for the detection and characterisation of individual proteins in a protein mixtures (e.g. cell lysates). In this approach, protein mixtures are solubilised and electrophoretically separated by gel electrophoresis, followed by the blotting step, which involves the transfer and irreversible binding to a substrate which can be either nitrocellulose, PVDF or nylon. The blotted proteins can then be detected via an array of methods.

The most common detection method for immunoblotting requires the blotted proteins to react with two antibodies. A primary antibody, which is specific to the protein of interest, is first introduced, followed by a secondary antibody, which is specific to the host species of the primary antibody and is used to amplify the signal of the first antibody. The
secondary antibody is usually conjugated with fluorescent or radioactive labels or enzymes that enable detection. Horseradish peroxidase (HRP) and alkaline phosphatase are the most commonly used labels for immunoblotting since they can be extremely sensitive when optimised with an appropriate substrate. Biotin-conjugated antibodies may also be used for this purpose. Whatever the detection method, the intensity of the signal is correlated with the abundance of the antigen on Western blot.

Differential densitometry of the associated chemiluminescent and/or fluorescent signals from the blots may lead to quantitave results in immunoblotting. However, this recent shift in the experimental methodology, data acquisition and interpretation has been associated with several pseudo-quantitative results being published. This calls for the development of strategies with the aim to improve confidence in the accuracy of quantitative western blots. A note of caution, however, is that the quality of a western blot analysis relies on accurately quantifying the signal intensity, which requires, amongst other things, the prevention of signal saturation. Additionally, enzyme reactions are short-term, so the detection step should take place promptly.

Due to ease of use and low costs, another widely used immunoassay is the enzyme-linked immunosorbent assay (ELISA). There are three major types of ELISA: indirect, sandwich and competitive. The latter two methods will be discussed since, unlike indirect ELISAs, they are able to detect antigens of very low concentration in the sample, which is particularly relevant in the study of oxPTMs.

Sandwich ELISA involves attachment of an antibody to a microplate in order to capture a target protein or analyte of interest. The microplates are then rinsed and incubated with a secondary antibody to allow detection. Competitive ELISA, on the other hand, relies on the use of immobilized antigen coating of the wells of a microplate. By adding the test sample mixed with HRP-conjugated antibody, the degree of competition can be measured through its proportional relationship to the concentration of soluble antigen in the sample. Therefore, the signal in each well has an inverse relationship to the amount of antigen. Accurate quantification of the antigen content in the test sample may be achieved by the inclusion of a serially diluted standard in the assay to generate a calibration curve of the
absorption.

ELISA is much faster and not as technically challenging as immunoblotting since the latter requires optimisation of the experimental conditions, such as protein isolation, buffers, type of separation, gel concentration, etc. Also, until recently, most western blots have provided, at best, a means for semi-quantitative analysis. However, unlike ELISA, immunoblotting allows separation of the protein mix by size, charge and/or conformation, due to the electrophoretic step. Also, detection of multiple modifications on the protein mixture under investigation can be achieved by stripping off the antibody from the membrane for further analysis with other antibodies, contrary to ELISA where only one type of antigen can be detected. Fluorescent blotting is growing in popularity as it allows the simultaneous detection of multiple proteins on a single blot.

Despite individual strengths, immunoassays rely on the specificity of the antibody-antigen interaction to identify a target protein in a complex protein mixture. They are, therefore, indirect characterisation tools and their sensitivity and specificity rely heavily on the properties of the employed antibodies.

1.3.3 Spectroscopic techniques

Nuclear magnetic resonance (NMR), as well as infrared (IR) and Raman spectroscopy are forms of spectroscopic techniques able to detect, as well as to probe, the interactions and dynamics in complex systems.

Attenuated total reflection (ATR) FTIR spectroscopy has been successfully used to study biological systems in contact with aqueous solution. Water molecules have large IR absorption in the fingerprint region, which usually makes it an unsuitable IR solvent. Since ATR limits the path-length to just 1-2 µm, it is then possible to study aqueous environments such as cells and tissues samples, as well as protein solutions. Due to overly congested 1DIR spectra, spectral identification tends to apply to large groups
of molecules. For example, by analysing Amide I band, information about the general protein environment may be gained but specific amino acid information is lost due to signal convolution.

UV-vis spectroscopy is used to analyse concentration of proteins containing aromatic residues by relating the absorbance at 280 nm to the sample concentration via Beer-Lambert law. UV-vis spectroscopy is particularly useful in studying tyrosine nitration, given it can efficiently determine the nitration degree of a purified protein sample without relying on hydrolysis or digestion, as it is the case with MS analysis. This is because 3-nitrotyrosine has an electronic transition which can be easily characterised by UV-vis spectroscopy, as previously summarised in table 1.2.

NMR, UV-Vis and circular dichroism spectroscopy study of the pH titration of two highly pure nitrated variants of horse heart cytochrome c have been carried out [71]. Nitration of the solvent-exposed tyrosine residues were observed and optical spectroscopy results have shown shifts of the alkaline transition to lower pH values as a result of nitration. NMR spectroscopy was then utilised to assess the conformational effects resulting from the protein’s pKa shift [71].

Since 1950s, developments in two-dimensional NMR have resulted in new techniques such as Correlation spectroscopy (COSY) and Nuclear Overhauser-effect spectroscopy (NOESY), which are widely used in the study complex protein structures, as well as dynamics, such as protein folding processes on μs time scales. Feynman showed that the concepts of NMR could theoretically be transferred to optical spectroscopies. Since then, 2DIR methods including pump probe and photon echo have been used to explore structure and dynamics of a wide range of systems. 2D Raman spectroscopy was also developed.

An advantage of using 2D optical methods is that they are able to probe molecular dynamics on a much shorter timescale than NMR. Thus, these various spectroscopies are able to provide complimentary analysis of protein dynamics.
1.4 Optical coherent multidimensional spectroscopy

Optical spectroscopy is possible due to the relationship between the sample's polarisation and the electric field of an exciting beam. The induced polarisation in the illuminated material will oscillate and produce an output electric field which is then used to gain information about the sample. The commonly encountered optical spectroscopies, such as IR and Raman, utilise low electric field intensities, leading to a linear dependence of the induced polarisation. However, by utilising high field intensities of pulsed lasers, non-linear polarisation can be induced, allowing excitation of modes which would not otherwise be allowed (involving two or more quanta).

Optical coherent multidimensional spectroscopy (CMDS) uses multiple excitation beams to excite sequential transitions of electronic and/or vibrational states in the sample. Coherences formed from pairs of the excited states emit coherent beams of light. Two-dimensional contour plots of the emitted signal allow visualisation of the states which are resonant with the excitation beams.

Very high spectral and temporal resolution may be achieved through CMDS. This allows information on both structure and dynamics to be simultaneously obtained. Thus, CMDS has greatly expanded the capabilities of molecular spectroscopy by enabling the study of a range of sample characteristics, such as conformation, H-bonding, metal binding, etc.

Photon echo, pump-probe and transient absorption spectroscopy are all examples of CMDS. While there are several types of coherent 2D spectroscopies, all of them rely on three components: a) sufficiently intense (> 100 MW/cm²) ultrafast tunable lasers are used to generate non-linear optical effects on the sample, b) optical components, such as mirrors and lenses, needed to guide, delay and manipulate properties of the input and output beams and c) an optical detection system, which can rely on a single element detector or an array detector attached to a monochromator. Signal detection follows a direction determined by conservation of momentum and a wavelength determined by conservation of energy. That is, phase-matching dictates the direction of the output
beam and the total set of possible output frequencies includes several combinations of the excitation frequencies. Delays between the excitation beams allows for the selection of a desired frequency combination.

Two-dimensional spectra are displayed as contour plots of the detected signal intensity as a function of two excitation frequencies. Whenever the two excitation frequencies are different, the signal is observed as off-diagonal peaks known as cross-peaks, which can be used to identify coupling between modes observed in the 1D spectrum.

By combining multiresonant nonlinear spectroscopy with two-dimensional plots, 2DIR spectroscopy is able to reduce congestion and improve spectral resolution. Additionally, the ability to independently tune the frequency of the excitation beams, as well as their polarization, relative direction, phase and relative timing, different information may be accessed about the sample.

Photon echo experiments, in particular with the use of two colour pulses, allows analysis of protein dynamics analogous to that of two-dimensional NMR techniques mentioned in the previous section.

Wright et al. have developed several mixed time-frequency CMDS, such as doubly and triply vibrationally enhanced (DOVE, also known as EVV 2DIR, and TRIVE, respectively) spectroscopy. By varying the delays between the excitation pulses in the frequency domain instrument, time-domain information, such as cross-peak lifetime, can be accessed. This approach uses picosecond pulses, which, relative to vibrational de-phasing times, are sufficiently short to measure kinetics.

Continued growth is expected both in future applications and capabilities of CMDS. If the field follows the path taken by multidimensional NMR, then the next major expansion will likely be into the third dimension, requiring additional frequency or temporal control of a third excitation laser to access new information about the sample.
Figure 1.1: Schematics of an energy level diagram for the EVV pathways.

The diagram depicts the four-wave mixing (FWM) of the three excitation laser beams at different pulse orderings. The time delay between successive laser pulses is typically chosen to be on the order of a few ps. Specific pathways may be selected by varying the pulse order. Time evolution occurs from left to right on the diagrams. Note that all pathways are fully coherent. The labels a, b represent normal vibrational modes and c represents an overtone mode given by a + b. The labels g and e indicate the ground state and a virtual electronic state, respectively. The last arrow shows the output of the FWM process.

1.5 EVV 2DIR spectroscopy

Electron-vibration-vibration two-dimensional infrared (EVV 2DIR) spectroscopy was originally developed by Wright et al., under the name of doubly vibrationally enhanced (DOVE) spectroscopy, and applied to the study of small compounds such as carbon disulphide CS₂ and acetonitrile [72]. This very important proof-of-concept has laid the groundwork for the development of EVV 2DIR spectroscopy as we have today in our group.

EVV 2DIR spectroscopy involves the detection of the emitted light resulting from the nonlinear four-wave mixing (FWM) of the excitation beams. By utilising three picosecond pulses, a sequence of coherences can be accessed within a sample’s available energy levels (Figure 1.1). Since two of the pulses are infrared and the other is of near-infrared frequency, the energy levels accessed are of vibrational and electronic nature, respectively.

The nonlinear polarisation resulting from the excitation beams is proportional to the number of molecules in the volume of interaction of the beams. By using homodyne detection, the FWM signal is detected as an intensity proportional to the modulus square
of the third-order polarization created in the sample. Thus, the detected signal will then be proportional to the square of the number of molecules.

Since pulse ordering dictates the order of excited coherences in the sample, the nonlinear polarisation from all coherence pathways coexist and interfere if the pulses are overlapped in time. Introducing time delays between the arrival of each input field and time ordering them allows the selection of a particular excitation pathway (Figure 1.1). Therefore, the temporal evolution of quantum states and the relation of different states to each other can be investigated.

EVV 2DIR pathways must involve at least one combination/overtone transition, which is only allowed in the presence of intra-/intermolecular mode coupling arising from mechanical and electrical anharmonicities in the molecule. This coupling requirement is a major strength because it isolates the spectral features that are associated with interacting moieties in the sample.

There are two EVV 2DIR pathways (EVV-IR$_\alpha$ and EVV-IR$_\beta$ in figure 1.1) that involve excitation of two vibrational transitions from the ground state followed by a non-resonant Raman transition (although it could be made resonant). The latter results from the scattering of the near-infrared beam from the induced polarisation generated by the first two beams. Thus, the third excitation beam provides a read-out of the coupling between the two resonant vibrational modes. Likewise, the EVV-Raman pathway involves a vibrationally enhanced Raman transition followed by a non-resonant Raman transition.

The resonances involved in EVV 2DIR pathways are illustrated by wave mixing energy level (WMEL) diagrams (Figure 1.1), which show the evolution of the coherences triggered by the sample's interaction with the excitation fields under phase-matching conditions. Liouville pathways are also provided in table 1.3. Note that the pathways are fully coherent because there are no intermediate populations.
Figure 1.2: Schematics of the excitation beams. The EVV 2DIR experiment uses a time sequence (a) of the three pulses where $\omega_\alpha$, $\omega_\beta$ and $\omega_\gamma$ are set to arrive at the sample with a time delay $T_{12}$ between the first two pulses and $T_{23}$ between the second and third pulse. By varying the time delay, pulse ordering is achieved to select specific sequence of coherences. EVV-IR$_\alpha$ pathway, which employs the pulse ordering $\omega_\alpha, \omega_\beta, \omega_\gamma$, leads to the generation of a four-wave mixing signal (b) of frequency $\omega_5 = -\omega_\alpha + \omega_\beta + \omega_\gamma$ and wave-vector $k_5 = -k_\alpha + k_\beta + k_\gamma$.

The EVV-IR$_\beta$ and EVV-Raman coexist under the same pulse ordering and their output coherences have opposite phase from each other. As a result, their FWM signal will cancel each other unless the mechanical anharmonicity of the system is large enough to shift the transition frequencies.

It is worth highlighting that the EVV-IR$_\alpha$ pathway only requires electrical anharmonicity to be present. This is created by dipole-dipole coupling from intermolecular interactions. This is particularly important as it allows cross-peaks between two interacting molecular systems to be monitored through this pathway. Additionally, structural characterisation is possible since dipole-dipole coupling has a well-defined dependence on the angles and distance between the dipoles. Thus this pathway can be of particular use for applications to biological samples.

1.5.1 EVV 2DIR spectroscopy as a bioanalytical tool

As mentioned earlier in this section, EVV 2DIR spectroscopy can only probe chemical groups which are in resonance with both IR excitation frequencies. This means that coupling is required in order for EVV 2DIR spectroscopy to be able to measure vibrational modes from the sample. As a consequence, significant spectral decongestion is observed as compared to one-dimensional IR spectroscopy. This is particularly important on the study of complex biological molecules such as proteins.
Where further spectral decongestion is required, the experiment may be designed such that the third excitation source will be in full resonance with a known electronic state of a molecule of interest, which will lead to considerably stronger four-wave mixing signals. By doing so, signal-to-noise may be increased significantly. This ability of turning EVV 2DIR spectroscopy into a triply resonant technique can prove very useful to the study of chromophores.

Additionally, since EVV-IR$_g$ pathway exists even in the absence of mechanical anharmonicity, intermolecular coupling in the absence of a chemical bond (through-space) can be characterised through the electrical anharmonicity of the sample [73]. 2DIR cross-peaks can be very sensitive to molecular structure and, in systems where electrical anharmonicity dominates, intermolecular interactions - such as the ones observed in many biological systems - can be investigated.

Finally, the EVV 2DIR signal is dependent on the polarisation of the excitation beams. So, spectra may be used to deduce the angle between molecules. Since the distance between two moieties can be measured through the intensity of an interaction cross-peak, by looking at the polarisation-dependence of the spectra, one may estimate the distances between the two interacting parts.

Klug and co-workers have so far applied EVV 2DIR spectroscopy to the detection of amino acids in short peptides by facilitating the quantification of the aromatic amino acids tryptophan, tyrosine, phenylalanine and the total methyl (CH$_3$) content of well-purified peptide and protein fractions [74, 75]. Bioinformatics have been used to demonstrate the ability of EVV 2DIR spectroscopy to distinguish between proteins based on their amino acid content [76, 75]. Additionally, preliminary work on chemical mapping of tissue sections has been carried out by spatially mapping the EVV 2DIR signal level of hematoxylin dye in H&E stained rat kidney sections [77, 75]. The goal of this project has been to further explore these capabilities in the context of biomolecules under oxidative stress.
1.5.2 Classical theory of EVV 2DIR spectroscopy

A light wave passing through a medium is known to exert forces on the valence electrons, resulting in a polarisation of the medium. In linear media and for low electric fields, this macroscopic polarisation is linearly dependent on the applied field. Optical phenomena such as refraction, absorption and diffraction are processes where the polarisation is linearly proportional to the applied field. However, for high fields, the polarisation displays a nonlinear response to the applied field, which results in phenomena observed in nonlinear optics.

For isotropic media (i.e. materials having the same optical properties in all directions), we can write the polarisation \( \vec{P} \) of the medium as a series expansion in powers of the net input electric field \( \vec{E} \) as shown below:

\[
\vec{P} = \varepsilon_0 \chi_\varepsilon \vec{E} = \varepsilon_0 (\chi^{(1)} \vec{E} + \chi^{(2)} \vec{E}_1 \vec{E}_2 + \chi^{(3)} \vec{E}_1 \vec{E}_2 \vec{E}_3)...
\]

where the susceptibility \( \chi_\varepsilon \) is also expanded as a series of adding coefficients, with the linear coefficient \( \chi^{(1)} \) being normally much larger than the nonlinear coefficients \( \chi^{(2)}, \chi^{(3)}, \text{etc} \).

As previously mentioned, EVV 2DIR spectroscopy is a four-wave mixing (FWM) technique, i.e. it utilises three excitation beams: \( E_\alpha, E_\beta \) and \( E_\gamma \). This process generates a third-order polarisation of the form:

\[
\vec{P}^{(3)} = \chi^{(3)} \vec{E}_{(\alpha)} \vec{E}_{(\beta)} \vec{E}_{(\gamma)}
\]

where \( \chi^{(3)} \) is the third-order susceptibility and \( \vec{E}_{(\alpha)}, \vec{E}_{(\beta)} \) and \( \vec{E}_{(\gamma)} \) are the electric field vectors of the excitation beams. Here, each of the beams are able to sequentially excite
a coherence in the molecule.

The third-order susceptibility is a universal property found in any material, regardless of its spatial symmetry, and is the lowest order nonlinearity which is present in centrosymmetric materials, where all the even-order nonlinear susceptibilities are zero. \( \chi^{(3)} \) is given as a sum of the mechanical and electrical anharmonicities arising from nonlinear terms in the potential energy surface and dipole moment, respectively.

The potential energy can be expressed as a Taylor-series expansion:

\[
V(x) = V(0) + \left( \frac{dV}{dx} \right)_0 x + \frac{1}{2} \left( \frac{d^2V}{d^2x} \right)_0 x^2 + ...
\]

where the lowest-order anharmonic terms of the potential energy surface represent the mechanical anharmonicity of the system.

By also expanding the transition dipole moment, the following expression can be written:

\[
\vec{\mu} = \vec{\mu}_0 + \left( \frac{d\vec{\mu}}{dx} \right)_0 x + \frac{1}{2} \left( \frac{d^2\vec{\mu}}{d^2x} \right)_0 x^2 + ...
\]

where the term \( \frac{d^2\vec{\mu}}{d^2x} \) represents the electrical anharmonicity of the system.

Since the detected FWM signal in EVV 2DIR spectroscopy is proportional to the intensity of the generated field, one can approximate its value to be proportional to \( \chi^{(3)} \) via the following relationship:

\[
I \approx |E|^2
\]

\[
EVV_{signal} \approx I \approx |\chi^{(3)}|^2
\]
1.5.3 Quantum mechanical theory of EVV 2DIR spectroscopy

While the classical treatment above provides a good phenomenological explanation, it is not a rigorously correct interpretation as it assumes the response is instantaneous. The more comprehensive calculation of such nonlinear phenomena requires a quantum interpretation.

Time-dependent quantum mechanics is used to describe quantum entanglement of two states. Coherences arise from quantum entanglement of two different states induced by an electromagnetic field perturbation. Successive interactions with the excitation fields create a sequence of coherences, which may be described via density matrix notation. If a and b are the two entangled quantum states, then the system can be observed via the probability density, which takes the form of the square of the total time-dependent wavefunction:

\[ |\Psi(x, t)|^2 = |c_a(t)\psi_a(x) + c_b(t)\psi_b(x)|^2 \]

\[ |\Psi(x, t)|^2 = c_a(t)c_a^*(t)\psi_a(x)\psi_a(x) + c_b(t)c_b^*(t)\psi_b(x)\psi_b(x) + |c_a(t)\psi_a(x)|^2 + |c_b(t)\psi_b(x)|^2 \]

Density matrix notation is generally used to identify the amplitude products from the equation above via \( \rho_{ij} = c_i c_j \), which can be referred by a simplified notation containing only the quantum state indices of the mixed states, i.e. \( \rho_{ij} = ij \). The time-dependent density matrix for a two-level system, such as the one described above, can be written as:

\[ \rho(t) = \begin{pmatrix} \rho_{aa} & \rho_{ab} \\ \rho_{ba} & \rho_{bb} \end{pmatrix} \]

where the off-diagonal elements of the matrix will take value of 1 if a coherence is created between the respective levels. A three-level system density matrix, as the one required
Table 1.3: Liouville pathways which can be accessed via EVV 2DIR spectroscopy

<table>
<thead>
<tr>
<th>Time-ordering</th>
<th>Pathways</th>
<th>Diagrams</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>EVV-IR$_\alpha$</td>
<td>$gg \rightarrow ga \rightarrow ca \rightarrow ea \rightarrow aa$</td>
</tr>
<tr>
<td>II</td>
<td>EVV-IR$_\beta$</td>
<td>$gg \rightarrow cg \rightarrow ca \rightarrow ea \rightarrow aa$</td>
</tr>
<tr>
<td>III</td>
<td>EVV-Raman</td>
<td>$gg \rightarrow cg \rightarrow bg \rightarrow cg \rightarrow gg$</td>
</tr>
</tbody>
</table>

to represent mixed states in FWM, is written as:

$$\rho(t) = \begin{pmatrix} \rho_{aa} & \rho_{ab} & \rho_{ac} \\ \rho_{ba} & \rho_{bb} & \rho_{bc} \\ \rho_{ca} & \rho_{cb} & \rho_{cc} \end{pmatrix}$$

For example, when in resonance with a vibrational mode in the molecule, the first excitation beam will excite a transition from ground state $g$ to a vibrational state $a$. This results in the excitation of a coherence $ga$. Each of the successive transitions, and their respective coherences, can be described by the subscripts in the corresponding density matrix element $\rho_{ij}$.

The resulting nonlinear field of interest for EVV-IR$_\alpha$ pathway, which is the focus of this work, radiates at frequency $\omega_\beta - \omega_\alpha + \omega_\gamma$ (as shown in Figure 1.1). Therefore, it will require the following sequence of coherence excitation: $gg \rightarrow ga \rightarrow ca \rightarrow ea \rightarrow aa$, where the vibrational state $c$ is a combination of state $a$ and a second vibrational state $b$ (i.e. $c = a + b$) or an overtone band of state $a$ (i.e. $c = 2a$). The virtual electronic state $e$ is generated through the samples interaction with the third beam $E_\gamma$.

Table 1.3 provides a summary of all coherences accessed by each of the three EVV 2DIR pathways.

Furthermore, each coherence oscillates spatially and temporally. If the coherence formed by three field interactions (e.g. $ea$ for the EVV-IR$_\alpha$ pathway) has a transition dipole moment, then the ensemble of oscillating coherences generates nonlinear contributions to the polarisation, which can be written as $P_{NL} = N\mu F \rho_{ea}$, where $N$ is the number density, $\mu$ is the transition dipole moment associated with the final emission, $\rho_{ea}$ is the third-order induced coherence (also represented by $ea$) and $F$ is the local field enhancement factor,
which relates the electric field of the excitation laser to the electric field within the sample [78]. The consequence of the expression for the nonlinear polarisation is that, for a three-level system, the third-order induced coherence is proportional to the dipole moment that generates the output field.

Quantum mechanically, the transition dipole moment of an individual molecule's coherence creates the polarisation of the molecule, which then launches an electromagnetic field at a frequency corresponding to the mixing of the frequencies of the excitation beams. In other words, the polarisability of individual moieties in the sample can be related to the average susceptibility and polarization density as classically described above.

Finally, if the $k$-vector of the electromagnetic field (in this case, given by the vector sum of the $k$-vectors of all three excitation beams) matches the polarisation wave vector, then the emitted field can constructively interfere and a signal will be observed. This event is known as phase matching, which is a requirement imposed by the conservation of momentum law.

1.6 Summary of research questions

In order to assess the suitability of EVV 2DIR spectroscopy as an analytical tool of oxidation/nitration of biomolecules, a series of basic, but important, questions had to be investigated. Below are the main research questions addressed in the work discussed in this thesis:

1. Can EVV 2DIR spectra be used to identify oxPTMs?

2. Does the current model utilised in the simulation of EVV 2DIR spectra provide a good tool to pre-assess the ability of EVV 2DIR spectroscopy to experimentally probe vibrational modes arising from oxPTMs?

3. Can quantification be performed to assess the levels of oxPTM within a sample?
4. What other ways can the strengths of EVV 2DIR spectroscopy be used to allow novel analysis of oxidation/nitration?

5. Can signature modes be measured from a wide range of systems, including small molecules such as peptides and lipids, as well as more complex biomolecules such as proteins and nucleic acids?

1.7 Thesis overview

The work discussed focuses on the EVV 2DIR spectroscopy study of various models of 3-nitrotyrosine, the nitrated form of tyrosine which results from reaction with peroxynitrite in vivo. The choice of tyrosine nitration stems from the fact that unmodified tyrosine - as well as other tyrosine PTMs, such as phosphorylation [77, 75] - had previously been characterised via EVV 2DIR spectroscopy [76, 75]. Additionally, 3-nitrotyrosine has been chosen due to its biological relevance and potentially favourable spectroscopic qualities.

Experimental methods and optimisations are discussed in Chapters 2 and 3. The latter addresses common issues, such as non-resonant background signal originating from a variety of substrates.

Chapter 4 assesses the ability of EVV 2DIR spectroscopy to identify nitration in a variety of models (Figure 1.3). Simulated spectra of tyrosine and 3-nitrotyrosine side-chain analogues are compared with experimental spectra performed on the same molecules in liquid phase. EVV 2DIR spectroscopy’s ability to differentiate between the two molecules is demonstrated and the work is extended to proteins. Bovine serum albumin protein is tested as a larger model for nitration and its spectra are discussed. Chapter 4 also reports a pH study demonstrating that EVV 2DIR spectroscopy is able to distinguish between charged and neutral 3-nitrotyrosine residues in short peptides.

Chapter 5 demonstrates EVV 2DIR spectroscopy is able to quantify the degree of nitra-
Figure 1.3: Schematics of the various possible models of tyrosine nitration. The work reported in this thesis covers models 1-3 and discusses future work regarding model 4.

tion within hepta-peptide mixtures. Various mixtures of known ratios are used to produce a calibration curve which is then shown to successfully enable the quantification of nitration degree in solutions containing 10% and 5% of 3-nitrotyrosine. Limit of detection is also discussed.

Finally, a critical discussion on the outlook of the use of EVV 2DIR spectroscopy to the study of oxPTMs is provided in chapter 6. Sensitivity limitations are discussed and a variety of improvements are proposed. Further discussion is also provided on the suitability of the technique for chemical imaging of tissue sections from chronic inflammation sites.
Chapter 2

Methods

This chapter aims to highlight important aspects of the experimental set-up used to perform EVV 2DIR spectroscopy and to describe how data acquisition and analysis have been carried out. Protocol used for sample preparation is also discussed.

Figure 2.1: Schematics of excitation beams interacting with a sample. Three pulsed lasers are focused onto the sample by an off-axis parabolic mirror to generate four-wave mixing signal observed in EVV 2DIR spectroscopy. The generated signal is then sent to a detector by a second parabolic mirror.
CHAPTER 2

Contents

2.1 Experimental Set-up of EVV 2DIR Spectroscopy .......................... 49
2.2 Acquisition and processing of EVV 2DIR data .............................. 55
2.3 Theoretical Simulation of EVV 2DIR Spectra ............................... 55
2.4 Sample preparation ............................................................. 57
   2.4.1 Sample preparation of side-chain analogues ......................... 57
   2.4.2 Peptide & protein sample preparation ............................... 57
2.5 LC-MS analysis of peptides and proteins ................................. 59
2.1 Experimental Set-up of EVV 2DIR Spectroscopy

Prof. D. R. Klug’s group has been utilising EVV 2DIR spectroscopy for over a decade and several publications highlighting the methodology are available in the literature [77, 74, 76, 79, 80]. The majority of the protocols and the optical design utilised here has been based on the development of a previous system by former group members D. J. Palmer and P. M. Donaldson. However, since its implementation, the current system has been used and modified by myself and following co-workers: K. Kornau, F. Fournier and J. A. Davies. This section aims to provide a thorough overview of the current apparatus.

P. M. Donaldson provides a very detailed account [81] on the development of the original apparatus and the protocols on which the current work is based. However, it is worth highlighting the key differences between the two systems in order to avoid confusion:

1. The newer commercial lasers have enabled a substantial increase of laser powers.

2. The original system had each beam focused at the sample by a different lens each, while the current system uses one off-axis parabolic mirror for all three beams. The introduction of reflective optics to focus and overlap the excitation beams spatially at the sample have simplified the optical set-up and eliminated potential spherical and chromatic aberration from taking place.

3. The newer system is fully enclosed from the output of the regenerative amplifier, allowing the entire system to be purged with N₂ during experiments in order to avoid absorption of excitation beams by molecules such as water vapour and CO₂. In contrast, the previous system did not purge the 790 nm beam.

4. While the excitation beams in the original optical set-up followed a collinear geometry, currently the a non-collinear geometry is used in order to select a phase-matching of interest, which maximises output signal while spatially separating it from the excitation beams.
The diagram depicts the instrumentation utilised to generate 790 nm picosecond laser. This diagram has been adapted from [75].

The optical apparatus described here is largely based on a Spectra-Physics laser system and is used to generate, amplify and tune the laser beams needed to perform EVV 2DIR spectroscopy. A mode-locked laser (Tsunami), which is pumped by a diode-pumped Nd:YVO₄ continuous wave (CW) laser (Millennia Pro) is set-up to produce a 790 nm output at approximately 35 fs duration, 80 MHz repetition rate and 450 mW of power. This is then used to seed a regenerative amplifier (Spitfire Pro XP), which in turn is pumped by a frequency-doubled Q-switched Nd:YLF laser (Empower). Via the regenerative amplifier’s chirped pulse amplification (CPA) set-up, which includes an optical pulse stretcher followed by a Ti:Sapphire amplifier and an optical pulse compressor, a 790 nm output is produced at approximately 1 ps duration, 1 KHz repetition rate and 2W of power (2 mJ per pulse).

The output of the regenerative amplifier is split into three optical paths, two of which are sent to optical parametric amplifiers (OPAs) in order to achieve the frequency conversion required to produce infrared wavelengths. Two Spectra Physics OPA-800C operating in difference-frequency mixing (DFM) mode are used for this purpose. Within each OPA, the input laser is split into three different paths, one of which is used to produce a white-light. Signal and idler beams are generated and amplified through the use of a nonlinear medium - a type II, angle-tuned, β-Barium Borate (BBO) crystal. During the first pass through the BBO (known as pre-amplification stage), a pre-pump beam (approximately 15% of the 790 nm beam entering the OPA) and white light beam combine to generate an idler and a signal beam. During a second pass (or power amplifier stage), a grating
is used to reflect the previously generated idler beam back to the BBO crystal, where it meets with a pump beam in order to achieve optical parametric amplification of the idler beam.

The signal and idler output wavelengths are determined by the angle of the BBO crystal. The grating angle is optimised for each generated idler frequency. Finally, the amplified signal and idler beam are difference frequency mixed by the use of a silver gallium sulfide (AgGaS$_2$) crystal. As such, three LabVIEW-controlled actuators, driven by a DC servo motor each (Newport LTA-HIS), are used to operate the positioning of the BBO crystal, the grating and the AgGaS$_2$ crystal to obtain a continuous range of DFM output. Communication between LabVIEW and the actuators is done through motion control cards (National Instruments).

While a variety of non-linear crystal configurations may be used to enable frequency tuning in different spectral regions, we have chosen DFM mode ($\omega_{\text{signal}} - \omega_{\text{idler}}$) for both OPAs since we were mostly interested in monitoring spectra at the fingerprint and overtone regions, more specifically 1300 – 1700 cm$^{-1}$ (5.3 - 7.7 $\mu$m) and 2700 – 3450 cm$^{-1}$ (2.9 - 3.7 $\mu$m). DFM mode is able to generate wavelengths from 3 to 10 $\mu$m, which provides good coverage of the spectral regions of interest.

Calibration of the OPA frequencies is initially achieved via a LabVIEW routine which scans the positions of the grating and non-linear crystals in the OPA and measures the wavelength and intensity of the output. A monochromator coupled to a liquid nitrogen cooled mercury cadmium telluride (MCT) detector (Infrared Associates) is used to monitor OPA output. An anti-reflection ZnSe window is used at the MCT detector. Communication between the monochromator and various LabVIEW routines is done through a GPIB card (National Instruments). A fourth-order polynomial fit is applied to the curve produced by monitoring the output wavelength as a function of actuator positions. The resulting polynomial coefficients enable remote control of the OPAs to any frequency within the calibrated range.

Additionally, the absorption spectrum of a 38 $\mu$m thick polystyrene calibration film trace-
able to NIST 1921b frequencies [82] is routinely used to fine-tune the frequencies produced by the OPAs prior to experiments. Any constant offset in frequency within 10 cm\(^{-1}\) may be corrected after data acquisition. However, larger/variable frequency offsets usually require OPA realignment and a new calibration curve.

In order to produce a clean, spatially uniform, Gaussian beam, \(\omega_\gamma\) is sent through two irises for spatial filtering. The first iris is used to select the most intense and uniform spatial region of the Gaussian beam, while the second is used to exclude any diffraction rings resulted from the use of the first spatial filter.

Once the near-IR beam is spatially filtered and the frequencies of the two IR beams are correctly tuned, each of the three excitation beams are then directed to telescopes which are used to collimate and expand the beams. The latter is done in order to enable tighter focus of the beams onto the sample via an off-axis parabolic mirror. Reflective optics are chosen to avoid the spherical and chromatic aberrations observed in transmissive optics. They are also conveniently able to focus all three excitation beams onto the sample at the same point, independently of the wavelength in the chosen spectral range.

Furthermore, the three beams are polarised parallel to the plane of propagation (PPP) and are focused into the sample in a non-collinear format, all set at the same height from the optical table. A manual actuator is used to adjust the horizontal position of the near-IR beam on the off-axis parabolic mirror in order to achieve a good phase-matching angle.

Spatial overlap and focus of the excitation beams is performed through a 100 \(\mu\)m pinhole which is placed at the sample stage. The pinhole diameter is chosen to match the \(1/e^2\) of \(\omega_\gamma\) beam, which is used as the reference beam for alignment purposes. As such, the X, Y and Z positions of the pinhole are set to be aligned with \(\omega_\gamma\). The alignment of the two IR beams is then finely adjusted so that both beams go through the pinhole. The focal point of the incident beams varies with the wavelength of the beams due to dispersion across lenses and slight differences in angles of the tuneable beams as they exit from the OPAs, which generate a spatial walk-off. To remedy such problem, following spatial
Figure 2.3: Example of energy profile of IR excitation beams through a pinhole placed on the sample position.

The graphs above illustrate a typical energy profile measured through a 100 μm pinhole across the frequency ranges of $\omega_\alpha$ m and $\omega_\beta$.

overlap, an iterative alignment process is carried on the optical path of the two IR beams in order to achieve near-symmetric energy profiles through the pinhole (Figure 2.3) for IR frequencies across the studied ranges (1300-1700 cm$^{-1}$ for $\omega_\alpha$ with $E_{\text{max}}$ = 800 nJ at 1500 cm$^{-1}$ and 2600-3400 cm$^{-1}$ for $\omega_\beta$ with $E_{\text{max}}$ = 2.0 μJ at 3000 cm$^{-1}$).

Once near-symmetric energy profiles are obtained through the pinhole, the latter is then removed and a CaF$_2$ window is positioned at the sample stage with its longitudinal position set at the focal point of the excitation beams and NRB signal is measured for excitation frequencies of 1500/3000 cm$^{-1}$.

The FWM signal ($\omega_\delta$) generated at the sample is collected by a second off-axis parabolic mirror and sent to a Peltier-cooled photomultiplier (PM) detector (Hamamatsu H7422-50). The gain of the PM is fixed at 0.9V throughout experiments and a neutral density filter wheel is used to attenuate signal levels exceeding the PM’s linear range. Two optical filters are fitted in front of the PM in order to exclude the near-IR excitation beam, as well as any signal coming from processes other than FWM. The detected signal is then sent to a gated integrator in order to improve signal-to-noise ratio. The integrated signal is then sent to LabVIEW through a Rack-Mounted BNC Terminal Block (BNC 2090, National Instruments) via a data acquisition card (National Instruments).
The non-resonant FWM signal from the CaF$_2$ window is generally detected even when the excitation pulses are not fully overlapped in time. In order to ensure temporal overlap of the pulses, delay stages located at each optical path are scanned iteratively so that their arrival time are set to give maximum non-resonant FWM signal. Generally, the arrival time of the $\omega_\gamma$ beam is fixed while the others are scanned.

While EVV 2DIR spectroscopy can undergo three different wave-mixing pathways (Figure 1.1), the experiments described in this thesis follow the non-parametric EVV-IR$\alpha$ pathway. This can be achieved by setting-up the delay stages of the incident beams to allow the $\omega_\alpha$ beam to arrive first at the sample, followed by $\omega_\beta$ and then $\omega_\gamma$.

As previously noted, the OPAs are optimised for a set range of frequencies. However, spatial and temporal overlap is done at the centre frequencies of these ranges ($\omega_\alpha/2\pi c = 1500$ cm$^{-1}$ and $\omega_\beta/2\pi c = 3000$ cm$^{-1}$). As a result of dispersion and beam walk-off, the travel time of the excitation beams will be dependent on their wavelength. In order to correct for such variations, a LabVIEW routine is used to construct delay calibration curves once T0 position (which corresponds to the time at which all beams are overlapped) is found for the centre frequencies. The routine involves stepping the OPA frequency and scanning the delay stage of the corresponding beam to obtain maximum signal through a CaF$_2$ window. A third-order polynomial fit is then applied to the calibration curve and the resulting coefficients are used to generate correction values for T0 position at any chosen frequency within the calibrated range.

In order to avoid absorption of the IR beams by atmospheric water vapour, the system is enclosed and purged with N$_2$ in order to maintain humidity levels below 3% during experiments. Data acquisition is done through a LabVIEW routine which steps the frequency of the IR beams while measuring the FWM signal collected through the PM detector. This routine enables the user to handle data acquisition parameters such as sampling rate and delays between the excitation beams. Unless otherwise stated, EVV 2DIR spectra discussed in this thesis have been obtained with acquisition rate of 100 samples per point and 5 cm$^{-1}$ step size.
2.2 Acquisition and processing of EVV 2DIR data

The EVV 2DIR data acquired through the experiments discussed in this thesis take the form of a tab-separated intensity matrix, where any given value corresponds to the measured signal intensity for a frequency pair $\omega_\alpha/\omega_\beta$. The column position of the signal intensity corresponds to the $\omega_\alpha$ frequency, while the row position corresponds to the $\omega_\beta$ frequency.

Sampling rates are often set at 100 and, occasionally, 500 samples per spectral point for regions showing weak cross-peak intensities. Despite the fact that signal averaging is performed during scans, in most cases, at least three separated spectra are obtained throughout an experiment in order to further average instrumental errors. Spectra are also smoothed using the shrinking/expanding algorithm in OriginPro (OriginLab Co.).

Unless otherwise stated, the data presented in this work have been normalised with respect to a reference cross-peak. Relative quantification is often required due to variations in the number of molecules interacting with the excitation beams, as well as variations of energy profiles of the excitation beams themselves. Data is then plotted as contour maps using a logarithmic scale in order to highlight weak cross-peaks.

Finally, if required, an offset in frequency might be added to account for small shifts in the calibration of the OPAs. This is done by continuously monitoring the absorption spectra of polystyrene film (Sigma-Aldrich) traceable to NIST 1921b frequencies [82].

2.3 Theoretical Simulation of EVV 2DIR Spectra

Simulated EVV 2DIR spectra have been produced by R. Guo for side-chain analogues of tyrosine and 3-nitrotyrosine amino acids [80] and provide essential information towards peak assignment of the EVV 2DIR spectra experimentally obtained for the tyrosine nitra-
tion models discussed in this thesis. Thus, in order to ensure completeness, this section will be used to describe the methodology employed by R. Guo to produce such spectra [83, 80].

Geometry optimisation is obtained via the software package GAUSSIAN 03. Density Functional Theory (DFT) is employed with B3LYP hybrid energy functional. By using both Becke's 3 parameter exchange correlation functional and Lee Yang and Parr dynamic correlation functional generally good results are achieved while maintaining computational efficiency [84, 85]. A large 6-311++G(p,d) basis set, as well as an ultrafine integral grid with tight cutoffs on forces and step size is aimed at achieving high numerical accuracy. The calculated harmonic frequencies are then scaled by 0.98.

After geometry optimisation, numerical calculation of the third-order susceptibility $\chi^{(3)}$ is performed as a function of the harmonic frequencies by following the method reported by Kwak et al. [86]. In summary, the second derivative of the dipole moment and the first derivative of the polarisability are calculated in order to evaluate the mechanical and electronic anharmonicity of the system.

Most terms of the density matrix are treated harmonically given they only involve one-quantum transitions. Since EVV-IRα pathway also involves coupling between two vibrational modes, resulting in overtone/combination transitions, numerical calculation of the potential energy surface is required in order to extract the cubic anharmonic coefficient. The result is then used to evaluate the first order perturbation expansion coefficients in the transition dipole matrix element (of the anharmonic oscillator wave function) corresponding to the two-quanta transition.

Each simulated cross-peak is then represented as a 2D Gaussian function with its full width at half maximum (FWHM) set to 20 cm$^{-1}$ and its centre frequency set to match the harmonic frequencies of the contributing IR bands. The signal intensity of the cross-peaks is assumed to be proportional to $\chi^{(3)}$ only, i.e. the intensities of the excitation beams are not taken into consideration.
2.4 Sample preparation

2.4.1 Sample preparation of side-chain analogues

Analysis of side-chain analogues has been carried out in liquid-phase. For that purpose, solutions of 4-methylphenol (4MP) and 4-methyl-2-nitrophenol (4M2NP) are prepared in anhydrous tetrachloroethylene (C₂Cl₄, Sigma Aldrich) at various concentrations. An in-house built sample cell containing IR-grade windows, such as CaF₂ and MgF₂, of 1 mm thickness or less, is loaded with the liquid sample and a polytetrafluoroethylene (PTFE) spacer is employed to create the desired path-length. Prior to use, IR windows are subject to a cleaning protocol as follows:

Window cleaning protocol: whenever possible, windows are placed in a glass staining jar and bath sonicated in isopropanol solution for 5 min. In cases of very thin windows (below 500 μm thickness) and specially in the case of MgF₂ windows, which tend to be quite brittle, it is advised to perform this step manually by using lens cleaning tissue soaked in isopropanol. Immediately prior to use, windows are then cleaned manually with lens cleaning tissue containing spectroscopic grade acetone. Outward circular motion is employed to avoid leaving residues on the material.

2.4.2 Peptide & protein sample preparation

Peptides and protein samples have been analysed in gel-phase. Sample preparation follows dilution of lyophilised peptide/protein in milliQ water and deposition of droplets onto a glass substrate (VWR borosilicate glass - cat no. 631-0146, thickness No. 1) in order to allow samples to dry at normal room conditions (Figure 2.4). An intermediate pH-adjustment step may be employed before sample deposition onto coverslip.
Figure 2.4: Schematic of peptide and protein sample preparation. The diagram depicts the process of depositing droplets of sample solution onto a substrate, which is usually a microscope coverslip. Droplets are then left to dry and generally form gel-phase coffee-rings, as indicated by the arrows.

Peptide synthesis was performed by Peptide Protein Research Ltd. and all samples were provided as lyophilized powders with purity greater than 98% (see Appendix B). The peptide powders were stored at −20 °C and allowed to thaw for 1 h before being dissolved in Milli-Q water to achieve the desired concentration. Solution pH was generally quite acidic upon addition of milliQ water. Where pH adjustment was required, NaOH solutions were used. A double junction pH probe was used to perform pH measurements since it has one additional salt bridge, which prevents reactions between the electrode solution and the sample from taking place.

Nitrated bovine serum albumin (nBSA) was purchased from Enzo Life Sciences. Nitration of tyrosine was achieved by treatment of bovine serum albumin (BSA) with tetranitromethane. An alternative nBSA sample, which has been produced by treatment with peroxynitrite, was purchased from Sigma-Aldrich. Certificate of analysis in both cases was lacking enough characterisation of nitration levels and required further analysis, which was conducted via mass spectrometry. Additionally, BSA samples were purchased as
lyophilized powder, fatty acid and globulin free, of at least 99% purity (Sigma Aldrich). Preparation of nBSA and BSA samples followed the same protocol as for peptides.

For EVV 2DIR experiments, droplets of each peptide (or protein) solution were deposited onto glass coverslips and dried under ambient conditions. Soluble peptides formed gel-phase coffee-ring structures, while less soluble peptides tended to dry as a powder film. Gel-phase samples were preferred as they allow peptides to remain hydrated. Also, powder films lead to experimental issues such as scattering of the excitation beams, as well as generation of other nonlinear processes such as sum-frequency generation.

2.5 LC-MS analysis of peptides and proteins

Model peptides, as well as Bovine serum albumin (BSA), protein and nitrated BSA (nBSA) have been analysed via LC-MS by Dr. K Tween Jensen from Aston University. This section highlights the analytical methods utilised for this study.

The peptides mixtures were diluted to 4 \( \mu \text{M} \) before separation. BSA and nBSA were dissolved in water and a volume equivalent to 100 \( \mu \text{g} \) was loaded onto a 10% SDS-PAGE gel. The proteins were visualised with Instant Blue stain (Expedeon) and in-gel digested.

The instrumentation used was common for both peptides and proteins and consists in an Ultimate 3000 system (Dionex, Camberley) connected to a 5600 TripleTOF (ABSciex, Warrington, UK) via a New Objective PicoTip emitter (FS360-20-10-N, Woburn, MA, USA) controlled by Chromeleon Xpress and Analyst software (TF v1.5, ABSciex, Warrington).

For both the peptides and proteins, the species were captured and desalted on a C18RP pre-column (C18 PepMapTM, 5 \( \mu \text{m} \), 5 mm x 0.3 mm i.d. Dionex, Bellefonte, PA, USA) by washing for 4 min with 2% aqueous acetonitrile (0.1% formic acid) at 30 \( \mu \text{L/min} \). The peptides were then separated on a C18 nano-HPLC column (C18 PepMapTM, 5 \( \mu \text{m} \), 75
$\mu$m i.d. x 150 mm, Dionex, Bellefonte, PA, USA) using a gradient elution running from 2% to 45% aqueous acetonitrile (0.1% formic acid) in 60 minutes and a final washing step running from 45% to 90% aqueous acetonitrile (0.1% formic acid) in 1 min. The system was then washed with 90% aqueous acetonitrile (0.1% formic acid) for 5 min and the equilibrated with 2% aqueous acetonitrile (0.1% formic acid).

The ionisation was achieved in both cases with spray voltage set at 2.4 kV, a source temperature set at 150 °C, with a dechustering potential of 50 V and curtain gas of 15. Survey TOF MS scans were collected in high-resolution positive ion mode and MS/MS data was collected using information-dependent acquisition (IDA).

The following criteria were used for the peptides: scan from 350 to 1250 Da for 500 ms. The three most intense ions with 1+ to 3+ charge states and a minimum intensity of 200 counts per second (cps) were chosen for analysis using dynamic exclusion for 10 s, a 500 ms acquisition time and rolling collision energy.

For the proteins, the parameters and criteria were as follow: scan from 400 to 1200 Da for 200 ms. The ten most intense ions with 2+ to 5+ charge states and a minimum of intensity of 500 counts-per-second were chosen for analysis, using dynamic exclusion for 20 s, 250 ms acquisition time and rolling collision energy.

The raw peptides data were analyzed with PeakView (ABSciex). Accurately prepared solutions were used to measure the relative signal intensities of the two peptides using the sum of the corresponding extracted ion chromatograms (XICs), of the Gaussian smoothed by 4 points, of the 1+ and 2+ charge states, and of the protonated and monosodium adduct of both charge states. The 3-nitrotyrosine-containing peptide signals were calculated to be approximately 2.61 times more intense than those of the native peptide; this factor was used to correct the data for the mixed peptides.

The protein data from chapter 5 was analysed using the Mascot search engine using the following criterion: fixed modification of carbamidomethyl, nitrotyrosine, as well as oxidized methionine, and searched against the Swissprot database using mammalian tax-
onomy. Both the protein and the fragment tolerances were set at 0.5 Da, choosing +2, +3 and +4 charge states and allowing for one missed cleave by trypsin. In a separate search, cysteine sulfonic acid and oxidation of tyrosine were included as variable modifications.
Chapter 3

Experimental Optimisations

It is our group’s efforts to continuously implement changes which will lead to increase in reliability of data, speed of operation, user-friendliness and robustness of experimental set-up.

This chapter focuses on the discussion of improvements made to the platform used to acquire EVV 2DIR spectra. Such platform involves not only the laser system itself, but also important aspects to our experiments, such as sample preparation.
## Contents

3.1 Non-resonant signal of substrates and windows ............... 66
3.2 Longitudinal positioning of samples .......................... 71
3.3 Improving the reproducibility of relative cross-peak energy intensities. .............................................. 73
3.4 Considerations when preparing liquid samples .................. 79
3.5 What to know when designing model peptides .................. 80
3.6 Conclusion & proposed future optimisations ................. 81
Since the early EVV 2DIR spectroscopy experiments, several members of Prof Klug’s group have worked towards implementing new capabilities in order to enable acquisition of larger spectral ranges, as well as to improve detection and reliability of the system. P. M. Donaldson developed most of the initial set-up of the laser system used for the work described in this thesis. He lists in his thesis [81] implementations such as OPA automation, improved detection scheme, online detection of laser energies and flexible software control of experimental parameters, amongst others, which have been crucial to the study of more complex molecules via EVV 2DIR spectroscopy.

Further optimisations, which have been performed by myself and Dr J. A. Davies, include:

1. Finer adjustment of longitudinal positioning of samples to allow better positioning with the focal point of beams.

2. Changes applied to the apparatus to allow wider range of wavelengths to reach the sample.

3. Protocol changes to monitor the excitation beams through a wide frequency range and to ensure energy profiles of the excitation beams were kept the same across experiments.

4. Full characterisation of non-resonant background signal from a variety of substrates in order to improve sample deposition protocol.

5. Also relevant to sample deposition protocol, a wide range of techniques were assessed as a way to eliminate coffee-ring formation in peptide/protein samples and to increase sample path-length.

**Disclaimer:** while software development in LabVIEW, which was required to support some of the improvements listed above, has been done solely by myself, all the remaining improvements listed here have been carried out in conjunction with Dr J. A. Davies.
3.1 Non-resonant signal of substrates and windows

Quite often samples will go through more than only one nonlinear effect. For example, sum-frequency generation (SFG) has often been detected in peptide samples of low solubility. This is believed to be due to the crystallisation of these peptides during the drying process of the sample. Formation of micro-crystals from less soluble peptide samples, with possible non-centrosymmetric structures, tend to favour second order processes such as SFG. Fortunately, since SFG is only function of one IR frequency and is then constant along one of our 2D IR axes, it is often easy to remove the SFG contributions from the EVV 2DIR spectra by applying simple data treatment. This is usually done by identifying areas in the spectrum where cross-peaks are absent. Their respective signals are then extracted from the signal intensity matrix in the form of 1D arrays and averaged into one background array. SFG signal observed along $\omega_3$ is removed by subtracting each column (vertical array) in the signal intensity matrix by the background array. However, one must note that, whenever possible, sample preparation should be optimised to eliminate SFG signal since its interference with the FWM signal prevents meaningful interpretation of spectral information such as cross-peak broadening.

Non-resonant background (NRB) signal is another process which tends to appear in EVV 2DIR spectra. It can be particularly problematic because its lifetime is comparable to the lifetime of some vibrational modes and it is not easily removed by the means of data processing. If the resonant four-wave mixing (FWM) signal is very large, then the NRB signal can often hide in the background. However, it may still be comparable to weaker features and might make quantification problematic. Therefore, it was particularly important to understand the sources of NRB signal, as well as how to minimise it.

Figure 3.1 shows NRB signal contributions arising from several substrates such as borosilicate glass slides, UV and IR grade fused silica and glass coverslips, as well as windows made of IR-grade crystals calcium and magnesium fluoride (CaF$_2$ and MgF$_2$, respectively). Glass materials, such as cover slips, have so far been the most commonly used substrates in the deposition of samples such as peptides and proteins. The rationale in
Figure 3.1: Experimental EVV 2DIR spectra of various materials. Figure shows spectra of: (a) CaF$_2$ window; (b) a microscope glass slide (VWR 631-0114, 1.0-1.2 mm thick); (c) MgF$_2$ window; (d) a cover slip (VWR borosilicate glass - cat no. 631-0146, thickness No. 1); (e) UV-grade fused silica (from Crystran); (f) IR-grade fused silica (from Crystran). Samples (a), (c) and (e) are 25 mm diam x 2 mm thick, while sample (f) is 25 mm diam x 3 mm thick. Time delays were set to 1.5/1ps.
employing non-IR materials as a sample substrate is that, by being behind the sample, their IR absorption does not affect our experiments. However, NRB signal from such surfaces can make it difficult to optimise the longitudinal alignment of samples.

From the EVV 2DIR spectra presented in Figure 3.1, it appears that IR-grade fused silica is, by far, the most appropriate substrate, only leading to minor NRB signal even though the material utilised was 3 mm in thickness. This would be particularly useful on studies of weak 2DIR features, which tend to be buried in the NRB signal for other substrates.

In order to further understand the nature of NRB signals on glass substrates, its dependence on the material thickness was analysed via EVV 2DIR spectroscopy. A No. 1 thickness coverslip (0.13 to 0.16 mm) and a glass slide of thickness ranging from 1 to 1.2 mm are compared in Figure 3.1-b and 3.1-d. While the increase in material pathlength is 10-fold, the observed NRB signal is less than two-fold. A possible reason for this dependence not following a squared-law proportionality expected in homodyne detected signal
could be the fact that the thickness of the window exceeded the length of overlap of the excitation beams. While the absorption of either excitation or FWM beams through the sample could generally also result in such behaviour, the IR and visible transparency of these materials (approximately 95% transmission) make it unlikely that absorption played a role in this observation.

We have hypothesised that the observed NRB signal was possibly originated at the surfaces of the substrate, rather than at its bulk. This hypothesis was tested by translating CaF$_2$ and MgF$_2$ windows longitudinally while monitoring NRB signal. Figure 3.2, which shows the detected signal as a function of time taken to scan the longitudinal axis, demonstrated that, at T0 (i.e. excitation beams are fully overlapped), the signal intensity for both CaF$_2$ and MgF$_2$ windows increases to a local maximum around the surfaces of the material. It was also observed that the timing between the excitation pulses affected the apparent source of the NRB signal. It is clear that, when all beams are overlapped in time, both the front and back surfaces of the substrate seem to generate NRB signal. However, at 1.5/1.0ps delay, only NRB signal from the back surface is observed.

Additionally to glass substrates, windows made of materials such as CaF$_2$ and MgF$_2$ are usually utilised when samples have to be loaded inside a sample cell. This may be required for liquid samples or even for peptide/protein samples where there is a requirement for humidity control. Early data on side-chain analogues suffered from strong NRB interference due to the use of CaF$_2$ windows. From the EVV 2DIR spectra in Figure 3.1, it was identified that MgF$_2$ was a much more suitable material and therefore, a change of protocol was employed to enable collection of data presented in this thesis.

Due to the fact that NRB is mainly observed towards the back surface of the material for time delays such as 1.5/1.0ps and above, thicker window materials have been preferred for the back of the sample cell in order to distance the source of NRB signal from the sample, thus providing a further spatial filter by removing the NRB source away from the focal point of the excitation beams.

While complete exclusion of NRB signal is rather difficult to accomplish and, to some
Figure 3.3: Experimental EVV 2DIR spectra of 4M2NP at various time delays. Figure shows spectra of 4M2NP solution in C$_2$Cl$_4$ with 100 μm path length at 66.7 mM concentration. Time delays between $\omega_\beta$ and $\omega_\alpha$ ($T_{23}$) were set to (a) 1.0ps and (b) 1.5 ps. The delay between $\omega_\alpha$ and $\omega_\beta$ ($T_{12}$), was set to $T_{12} = 1.5$, $T_{12} = 2.0$ and $T_{12} = 2.5$ (spectra from left to right) in order to compare the dependence of the NRB signal to $T_{12}$ vs $T_{23}$. 

70
extent, undesirable (given NRB signal aids with the longitudinal alignment of the sample), the analysis provided above sets up a framework for significantly reducing such interference to the EVV 2DIR signal.

Finally, it is generally understood that NRB signal can be further reduced by increasing the first time delay between the IR beams (Figure 3.3). However, this is usually limited by the life-time of the studied cross-peaks and is not generally feasible to increase it beyond a couple of picoseconds.

### 3.2 Longitudinal positioning of samples

Given the large changes in NRB signal observed by scanning the focus of the beams across the windows, it became clear that better control of the longitudinal axis alignment of samples was needed in order to increase reproducibility of experiments. The longitudinal axis lies along the focal point of the incident beams and is also referred as the Z-axis (Figure 3.4). The replacement of the manually-operated sample stage controller by a software-based control has since allowed much finer alignment of the sample. It is common practice to scan the sample’s longitudinal position for a maximum signal at a known cross-peak frequency. However, when NRB signals are comparable to fully resonant FWM signal, this protocol was often leading to the case where the signal was largely coming from NRB. As a result, the focal point was very often unknowingly set for maximum NRB signal.

As described in the previous section, separating fully-resonant FWM signal from other
contributions is still a laborious task. However, by implementing finely-tuned, software operated Z-axis adjustment, full user control has been made possible, with the ability to return to known positions and to finely step the sample for optimum alignment with the lasers focal point. Figure 3.5 shows spectra for the side-chain analogue 4-methylphenol (4MP) prior and after the implementation of software-controlled longitudinal positioning. Both spectra had been acquired at a longitudinal position which the user believed to lead to maximum signal. However, by finely stepping the axis, NRB became more easily separated from the FWM arising from the sample.

Finally, by extending the software-controlled axis positioning to all the three axes, it has become possible to swap samples around with high reproducibility of three-dimensional positioning. Prior to this, every time a sample had been removed, the axes positions were then lost.
3.3 Improving the reproducibility of relative cross-peak energy intensities.

We have learned that, when acquiring spectra across a large number of frequencies, it is particularly important to observe the signal ratio between reference cross-peaks, especially in peptides and proteins, since these ratios appear to be very dependent on the positioning of samples. The horizontal and vertical alignment of samples displaying coffee-ring topology is of particular concern because it appears that the curved geometry of the coffee-ring leads to large variances of relative cross-peak intensities. Also, longitudinal positioning appears to have an effect. It has since become routine to measure cross-peak signals across different X, Y and Z positions to be able to identify areas where the sample does not display an abrupt variation of cross-peak ratios as a function of its position. This, however, requires a laborious iteration between sample positioning and measurement of reference cross-peaks such as Amide I and CH$_2$ or CH$_3$ cross-peaks.

To further understand the variations of relative EVV 2DIR signal intensities, several sample preparation routes were explored. Firstly, samples were prepared at various pH and droplet volumes in order to understand the effect of those variables on the formation of coffee-rings. Figures 3.6, 3.7 and 3.8 demonstrate measured profiles of coffee-rings produced by drying BSA solutions of pH 5.5, 7.6 and 9.0 with droplet volumes of 0.5, 1.0, 3.0 and 5.0 $\mu$L. Note that the samples were pH adjusted with HCl, for pH 5.5, and NaOH, for pH 9.0. Profiles were measured using profilometry.

FWHM of coffee rings at pH 5.5 (Figure 3.6) were relatively lower than in the remaining samples. Also, while increases in coffee-ring width were observed as a function of droplet volume across the three samples, this was not a linear relationship due to the spreading of the droplet onto the surface.

Various sample preparation routes have been assessed in order to suppress coffee-ring formation and to limit the spreading of the sample across the surface, thus maximising
The labels and arrows on the plots show example profiles of each droplet volume, as well as their height and full width at half maximum, as indicated on the plots. Figures 3.6: Profiles of coffee rings formed at pH 5.2. Volumes of 0.5, 1.0, 2.0, and 5.0 μl of BSA solution were deposited onto a glass surface.
Figure 3.7: Profile of coffee-rings formed at pH 7.6. Volumes of 0.5, 1.0, 3.0 and 5.0 µL of BSA solution were deposited onto a glass surface and allowed to dry. Plots show example profiles of each droplet volume, as well as their height and full width at half maximum, as indicated on the labels.
Figure 3.8: Profile of coffee rings formed at pH 9.0. Volumes of 0.2, 1.0, 3.0, and 5.0 mL of BSA solution were deposited onto a glass surface and allowed to dry. Plots show example profiles of each droplet volume, as well as their height and full width at half maximum, as indicated.
Figure 3.9: EVV 2DIR signal variations across coffee-rings. Same volume of samples of BSA have been deposited onto a glass substrate and allowed to dry under room conditions. Profiles of coffee-rings formed from solutions of pH 7.6, 9.0 and 5.5 are seen on the lateral scans from left to right. The Amide I signal (at 1660/3320 cm$^{-1}$) is monitored in (a), while the methyl signal (at 1480/2950 cm$^{-1}$) is monitored in (b).

its path length. These include the use of surface-acoustic waves (SAW) and surface patterning of hydrophilic wells to reduce spreading of droplets. Unfortunately, none of these methods have achieved reproducible removal of coffee-rings.

To illustrate the variations observed in EVV 2DIR signal across a coffee-ring, the Amide I and methyl cross-peak signals were monitored across the lateral axis of the samples in Figure 3.9. It was found that the methyl signal was particularly prominent at the edges of the coffee-rings while the Amide signal was also observed towards the centre of the ring. Methyl signal was particularly irreproducible at pH 7.6, with one side of the ring emitting significantly different FWM signal from the other side.

While our efforts have not led to complete elimination of coffee-rings, we have observed that vibrating the substrate during the drying process leads to sample being also dried inside the coffee-ring, forming a doughnut-shaped topology. This can be very useful as it results in flatter areas, thus reducing variability in cross-peak relative signals. Future
work should explore the use of humidity cells to allow formation of hydrated samples.

Irrespective of the sample topology, it appears that the most prominent cause of the relative peak intensity variations arise from slight misalignment of the excitation beams as a function of frequency. To understand the root cause of this issue, one must know that the difference frequency generating (DFG) crystal, which is used to tune the frequencies of the IR-beams in the OPAs, requires a very large turning angle, which results in beam walk-off. While the introduction of larger, Z, mirrors and the adjustment of optics has ensured the beams are not clipped off at any point of their optical path, it is important to appreciate that beam-walk off becomes a severe problem when working at large spectral ranges such as the ones reported here (turning angle of approximately 70 degrees). As a result of this walk-off, the same beam will travel off-centre through telescope lenses at certain frequencies, leading to spherical aberrations for any beam frequencies other than the ones close to the central frequency, which is the frequency at which alignment is carried out.

Dr P. M. Donaldson has proposed ways to decrease walk off in his thesis [81]. He suggested replacing the DFG crystal but found a range of limitations in the various crystals he explored: a) AgGaSe₂: large tuning range but low conversion efficiency from 3-5 μm. b) GaSe: despite having good thermal properties, the narrow tuning angle range of required a high angle resolution for phase match, which was hard to achieve. Also, neither crystals can be coated, which lead to reflection losses.

Another crystal which has not yet been explored is LiGaS₂. It has a greater transparency range in the visible, which reduces two photon absorption and therefore allows more power through it. However, a possibly simpler alternative is, once signal and idler are filtered out from the beam, to add a motor to rotate a piece of IR transparent glass, such as ZnSe at Brewster angle, the opposite direction of the crystal rotation.

Whichever the route taken, it is important to address the beam walk-off and other optical aberrations arising from transmissive optics. This would lead to much higher energies reaching the samples at wider spectral ranges.
3.4 Considerations when preparing liquid samples

A few considerations were taken when designing the study of side-chain analogues:

1. A wide range of solvents were explored and $C_2Cl_4$ was chosen given its small one-dimensional IR absorption in the fingerprint and overtone regions, as well as the fact that no EVV 2DIR features existed either, which ensures no solvent cross-peaks would be overlapping with features from the side-chains. EVV 2DIR spectra of solvent alone were obtained in order to confirm the above. Solvents such as DMSO and methanol have significant IR absorptions.

2. Concentrations of the solutions should ideally remain within low optical density region in order to minimise distortions to the FWM signal originating from high absorption of the incident beams.

3. Due to solvents being rather volatile, the path-length and dimensions of the liquid cell were maximised to ensure the total volume of solution was sufficiently high so that evaporation of solvent over time would become negligible. By doing so, one could assume the concentration of the sample stayed the same across the whole experiment. Path-length of 50 $\mu$m appears to be adequate for sample cells of 22 to 25 mm diameter.

Once the sample preparation protocol had been established, several EVV 2DIR parameters were tested, from time-delays between incident beams, to sampling rate and sample positioning. The following are generally useful practices:

1. Longer time-delays are chosen in order to reduce non-resonant signal that may originate from the interaction between the incident beams and the windows in the sample cell. However, given certain coherences have short life-times, a compromise must be reached so that such features are still visible.

2. Due to issues, such as sample evaporation and laser instabilities, signal sampling rates (which determine the number of interactions between the incident light and the sample
upon which one chooses to average) had to be optimised to ensure signal averaging would not require excessively long time to produce the full range spectra without leading to limitingly low signal-to-noise ratios. With that in mind, the chosen approach here has been to obtain spectra at rates ranging from 2 to 10 points per second (this is equivalent to 500 and 100 interactions per point, respectively) and to average 3 spectra after data collection. As a result, frequent monitoring of experimental conditions is made possible, while higher signal averaging could still be achieved.

3. As previously mentioned, sample positioning has a very important role in reliability and reproducibility of the obtained spectra. Given liquid samples do not vary their path-length across X and Y, only the longitudinal (Z-) direction is of importance. Due to optical aberrations arising from transmissive optics, the focal point of the incident beams varies in frequency. Therefore, apart from making improvements to allow fine tuning and reproducible sample positioning, it became clear that our experimental protocol required the longitudinal alignment of the sample to be carried out at the same excitation frequencies across an experiment, i.e. at one given cross-peak only.

### 3.5 What to know when designing model peptides

As it will be discussed in the following chapters, model peptides serve as very useful models for EVV 2DIR spectroscopy studies. However, when designing a peptide sequence, one should ensure solubility will be sufficiently high to avoid issues with sample preparation. Here are a few important findings observed during this project:

1. Proline residues are particularly helpful with formation of gel-phase films upon drying. However, proline is known to result in constraints on the peptide backbone and, unlike other amino acids, its α amino group is not able to hydrogen-bond. Thus, it is advisable to keep these residues at least one amino acid away from the amino acid of interest.

2. Terminal capping reduces solubility of peptide but creates a better mimic of a protein
environment. It is usually good practice to use peptides with terminal capping. In
order to do so without sacrificing on solubility, larger peptides may be required in
order to allow introduction of soluble amino acids such as: arginine, lysine, asparagine,
aspartic acid.

3. Neutral amino acids may be used. Figure 3.10 clearly shows that replacing the slightly
hydrophobic amino acid alanine by the neutral amino acid serine is enough to dramat-
ically improve solubility.

4. Additionally, the required pH needs to be taken into consideration too. For example, a
pH titration of peptides followed by deposition onto a glass substrate and subsequent
drying at room conditions can be seen in Figure 3.10.

3.6 Conclusion & proposed future optimisations

This chapter summarised a variety of steps taken towards improving the overall repro-
ducibility, stability of the EVV 2DIR apparatus. A wide variety of materials has been
studied in order to learn about their NRB contribution. Based on the findings listed here,
a few changes in protocols had to be made in an attempt to improve the quality of the
acquired data.

In order to decrease the NRB signal contribution to the spectra of liquid samples, CaF$_2$ windows had to be replaced by MgF$_2$ ones, given the observed 3-fold decrease in NRB signal in these windows compared to the former. Additionally, since the NRB contribution is very short lived, it has been proposed that delays between the first two excitation pulses may help lead to drops of NRB signal at higher rate than FWM signal. However, the latter becomes a limiting factor since coherences and populations decay rather quickly and the output signal experiences a substantial decrease.

It was also shown that the curved topology of coffee-rings leads to variations in the relative cross-peak signals. It was found that larger volume droplets should be favoured due to their larger FWHM, which cause less prominent changes on signal ratios. Efforts to eliminate coffee-ring in peptide and protein samples have been made but still require further investigation.

Suggestions on how to address these and other issues are made throughout the chapter and will hopefully aid further optimisation, the most important one being the elimination of beam walk-off due to the wide tuning range of the DFM crystal in the OPAs.
Chapter 4

Identification of Tyrosine Nitration by EVV 2DIR Spectroscopy

Spectroscopic data from a variety of models of tyrosine nitration, including side-chain analogues and short peptide sequences, are presented. The primary goal of this study has been to identify vibrational signatures of nitration in EVV 2DIR spectra.

Spectral differences between side-chain analogues and peptide-bound amino acids are investigated in order to understand the effect of increased molecular complexity on the EVV 2DIR spectra.

This chapter also reports on the attempts to characterise nitration of protein-bound tyrosine. Bovine serum albumin is chosen as a model protein since it is often used as a nitration standard for bioanalytical methods such as immunoassays.

Figure 4.1: Schematics of a tyrosine side-chain being nitrated into 3-nitrotyrosine.
CHAPTER 4

Contents

4.1 Side-chain analogues ........................................... 85
  4.1.1 Simulated 2DIR spectra ................................... 86
  4.1.2 Improving signal strength via an electronic resonance ........ 88
  4.1.3 Experimental 2DIR spectra ................................. 90
4.2 Peptide models .................................................. 92
  4.2.1 Spectral features of tyrosine ............................... 95
  4.2.2 Spectral features of 3-nitrotyrosine ....................... 96
4.3 BSA as a model of protein-tyrosine nitration .................. 98
4.4 Conclusion ...................................................... 104
As reviewed in Section 1.2, Tyrosine (Tyr) nitration into 3-nitrotyrosine (nTyr) is an oxidative PTM (oxPTM) known to affect activity of several proteins [60], including the mitochondrial MnSOD [34, 87] and the transcription factor protein p53 [30, 88, 89, 90]. Nitration levels of Tyr are found to be particularly high in sites of chronic inflammation [39], such as those observed in neuropathologies like Alzheimer’s disease [56, 91].

Tyr has been analysed via EVV 2DIR spectroscopy in various peptides prior to the work described in this thesis [74, 76, 77]. This well characterised amino acid, which is known to be subject to various post-translational modifications (PTMs), such as phosphorylation, is also a target of nitration [8, 71, 92, 93, 94].

Quantitative analysis of nTyr is most commonly performed by mass spectrometry (MS) coupled to separation techniques, such as two-dimensional electrophoresis and liquid chromatography. Immunoassays such as Western blot and ELISA are also often used. However, the destructive nature of the sample preparation of MS, the required interpretation of the patterns of ions generated upon sample fragmentation, as well as the immunoassays’ dependence on the quality of utilised antibodies, mean that these analytical tools suffer from shortcomings that could be avoided by the use of optical spectroscopy.

Here, EVV 2DIR spectroscopy is investigated as a possible alternative tool for the identification of tyrosine nitration via the probing of vibrational signatures of nitration in samples including small molecules, peptides and proteins.

### 4.1 Side-chain analogues

In order to assess the ability of EVV 2DIR spectroscopy to probe spectral signatures of nTyr, a variety of models were characterised. With the goal to construct a bottom-up model for nTyr characterisation, side-chain analogues were first investigated and their experimental EVV 2DIR spectra were compared to simulated spectra in order to aid with cross-peak assignment.
Figure 4.2: Simulated one-dimensional IR spectra of 4MP, as well as neutral and charged 4M2NP. Absorption values are arbitrarily set. Vibrational mode assignments referred to by the labels have been carried out based on the analysis of DFT calculations performed on the software package Gaussian 03.

The primary aim of this study has been to model Tyr nitration with small molecules, both experimentally and via computational simulation. This should enable the validation of the theoretical model utilised to predict EVV signatures of molecules, as well as allow important information regarding spectral assignment to be gained. Also, by assessing the quality of the simulated spectra and by identifying a reliable theoretical model, valuable time could be saved by employing theoretical calculations as a pre-screening step for EVV 2DIR spectroscopy.

4.1.1 Simulated 2DIR spectra

Following the methods described in Section 2.3, simulated one- and two-dimensional infrared spectra\(^1\) have been produced for side-chain analogues of Tyr and nTyr: 4-methylphenol (4MP) and 4-methyl-2-nitrophenol (4M2NP), respectively. The hydroxyl

---

\(^1\)The simulated work presented here was produced by Dr. R. Guo, who was, at the time, working in Prof. D. R. Klug’s group at the Department of Chemistry, Imperial College London. This collaboration was supported by the Proxomics project, through the Engineering and Physical Sciences Research Council grant EP/I017887/1.
Figure 4.3: Experimental and simulated FTIR spectra of 4MP. Spectra were acquired on samples of 500 mM concentration and 25 μm path length. Solid lines show experimental data acquired from samples at different times. Dashed line corresponds to simulated data and its absolute absorption is arbitrarily set. Frequency labels are for the experimental data.

A group located at the Tyr phenolic ring is known to have a pKa of approximately 7.3 in nTyr. Thus, at physiological pH, one should expect both neutral and charged nTyr species to be present. On that account, simulations have been performed for both nTyr species.

This simplistic model of a single molecule in vacuum does not account for any intermolecular forces such as H-bonding and other interactions with surrounding molecules. Dephasing, which refers to the time in which the coherence caused by the excitation beam decays towards the initial vibrational state, is also neglected and the energy of excitation beams are assumed to be equal.

The calculated one-dimensional spectra shown in Figure 4.2 demonstrate that the vibrational modes for 4M2NP vary significantly, both from one another and from 4MP. The most prominent modes in 4MP, which involve the fundamental modes at 1514 and 1623 cm⁻¹ (Figure 4.3) were found to be strongly shifted upon nitration. Based on the analysis of the theoretical calculations of 4MP and 4M2NP, it appears that, upon nitration, these fundamental frequencies are shifted to 1494 and 1584 cm⁻¹ for the neutral 4M2NP sample. They seem to shift even further for the charged 4M2NP molecule (1421 and 1507
cm⁻¹, respectively). Also, the NO₂ group is believed to give rise to the vibrational mode observed at 1550 cm⁻¹ for the neutral 4M2NP and 1446 cm⁻¹ for the charged 4M2NP.

Figure 4.4 shows the simulated EVV 2DIR spectra for the two side-chain analogues containing a protonated phenolic hydroxyl group, as well as a spectrum for 4M2NP containing a deprotonated hydroxyl group. Labels are provided for cross-peaks of interest in order to assist discussion and will be referred in more detail later in this chapter. Cross-peak numbering was done from bottom left to top right.

4.1.2 Improving signal strength via an electronic resonance

A signal enhancement is expected when the wavelength of the third excitation beam of EVV 2DIR spectroscopy is tuned to be in resonance with an electronic transition of the sample. A signal increase of four orders of magnitude has been reported previously on samples displaying a suitable electronic transition [77]. Additionally, it was also shown that significant enhancement could be achieved even when the third excitation beam, which is set to a fixed wavelength on the near infrared (790 nm), was not completely matching the electronic resonance of interest. This was the case of protein bacteriorhodopsin, which has a chromophore with an electronic resonance at 570 nm [75, 77]. This suggests a quite high tolerance for wavelength mismatch with the electronic resonance. As a result, EVV 2DIR spectroscopy could lead to an amplification of signal via a weakly triply-resonant process (doubly vibrationally enhanced and singly electronically enhanced).

It is widely reported in the literature that Tyr has an electronic transition, which is observed at 280 nm, and that nitration leads to an additional, red-shifted, transition at 357 nm for the neutral nTyr residue and 428 nm for the charged nTyr residue [60]. It has been hypothesised that this red-shift could provide us with an enhancement in the EVV 2DIR cross-peak signals of the charged nTyr species. The rationale behind this idea is that, due to the wavelength of the third excitation beam being closer to the nTyr electronic transition, EVV 2DIR spectroscopy could potentially be, to an extent,
Figure 4.4: Simulated EVV 2DIR spectra of 4MP, 4M2NP neutral and charged 4M2NP. Results of theoretical simulations of EVV 2DIR spectra for a) 4MP, b) neutral 4M2NP and c) charged 4M2NP are presented. Diagonal line shows cross-peaks involving overtone bands.
weakly resonant with such electronic state. Simulated EVV 2DIR spectra of Tyr and nTyr side-chain analogues were initially used to assess such hypothesis.

Comparison between vibrational modes predicted by theoretical calculations and the ones obtained via FTIR is provided in Figure 4.3 for 4MP. A good agreement between theoretical and experimental spectra is observed. It is important to notice that prior to this work, a scaling factor of 0.96 was commonly applied to the calculated frequencies. However, it has since been found that 0.98 provided a more appropriate correction and, therefore, it has been utilised to produce the simulated spectra discussed in this thesis.

The EVV 2DIR spectrum for the protonated 4M2NP suggests an increase of oscillator strength of its vibrational modes, with a significantly higher signal enhancement (of approximately 3 orders of magnitude) being observed in the charged 4M2NP spectrum in Figure 4.4-c. While this appears in line with the aforementioned hypothesis, given that the theoretical calculations do not include dephasing times, it was important to first evaluate the experimental spectra before drawing any conclusions regarding the possible electronic enhancement of the EVV 2DIR signal for charged nTyr.

4.1.3 Experimental 2DIR spectra

In order to produce experimental spectra for the side-chain analogues, solutions were prepared in C$_2$Cl$_4$. Only the neutral form of 4M2NP was studied.

Experimental EVV 2DIR spectra are shown in Figure 4.5 for both side-chain analogues. The 4MP spectrum shows cross-peaks along the fundamental lines centred at approximately 1430, 1515 and 1600 cm$^{-1}$, which is also in check with the experimental FTIR data. The 4M2NP, similarly, shows cross-peaks involving the fundamental modes at 1325, 1420, 1500, 1545, 1575, 1640 cm$^{-1}$, all of which are in close proximity to the one-dimensional spectra.
**Figure 4.5:** Experimental 2DIR spectra of 4MP and 4M2NP solutions in C<sub>2</sub>Cl<sub>4</sub>. Spectra were acquired at different time delays in order to ensure best visualisation of the cross-peaks. Labels are provided for cross-peaks identified in the simulated spectra.

Despite spectral congestion, it is possible to experimentally observe Tyr2 and Tyr4 cross-peaks, for the 4MP spectrum, just as predicted. Additionally, nTyr3, nTyr4, nTyr9 can be clearly identified on the spectrum for 4M2NP (Figure 4.5).

While there is an overall good agreement between the experimental and simulated spectra, it is important to note that there are various simulated cross-peaks which are not observed experimentally. This is most likely due to these cross-peaks being short-lived. Therefore, a more meaningful theoretical model would require knowledge of dephasing time of each involved mode, which cannot be calculated.

Experimental EVV 2DIR spectra show considerable spectral congestion, indicating a very large number of vibrational couplings for these molecules. Attempts to alleviate such congestion, that is, to remove the streak-like features in the EVV 2DIR spectra
Figure 4.6: Schematics of the chemical structure of Tyr, nTyr and nTyr⁻. The phenolic hydroxyl group in the nTyr residue has a pKa of approximately 7 and will, therefore, lead to both neutral and charge species to coexist at physiological pH.

have not been successful. A special focus was put into reducing non-resonant background (NRB) signal arising from sample cell windows, as discussed in Section 3.1. NRB signal is known to affect lineshapes by interfering with resonant features [51,52] but despite a large reduction of NRB signal, the spectra obtained for side-chains still contained smeared cross-peaks. It appears that singly vibrationally enhanced (SIVE) processes are likely to play a role on the smearing of the IR lines, as observed in Figure 4.5. SIVE processes require the excitation of one vibrational state and, therefore, will be modulated by the energy profile of one of the IR beams.

I have refrained from dwelling too much on the assignment and analysis of the experimental side-chain cross-peaks due to the spectral congestion. Instead, short peptide sequences were then analysed and compared with the simulated EVV 2DIR spectra to produce a complete list of mode assignment\(^2\) for the observed cross-peaks.

### 4.2 Peptide models

In order to assess whether the experimental observations reported so far would be conserved in more complex and more biologically relevant samples, short peptides were synthesised with either a Tyr or a nTyr residue at the centre of the sequence.

Dried drops of 10mM Ac-SPSYSPS-NH₂ and Ac-SPS(nY)SPS-NH₂ peptides were anal-

\(^2\)The mode assignment was carried out in collaboration with Dr. R. Guo and Dr J. A. Davies, who were both, at the time, working in Prof. D. R. Klug's group at the Department of Chemistry, Imperial College London. This collaboration was supported by the Proxomics project, through the Engineering and Physical Sciences Research Council grant EP/1017887/1.
ysed. Given the pKa of the hydroxyl group in Tyr decreases to approximately 7.3 upon nitration (Figure 4.6), it was important to isolate the charged and the neutral species in order to identify their individual contributions to the observed spectra. For this purpose, peptide solutions were alliquoted and pH-adjusted to pH 9.1 and 5.6 via addition of NaOH solution. The dried spots remained the same colour as their respective solutions, suggesting that gel-phase samples retained their pH.

Figure 4.7 contains spectra for neutral Tyr 4.7.a, as well as neutral and charged nTyr 4.7.b-c. Three spectral features are common to all three spectra and are discussed below. EVV 2DIR spectra shown in this chapter have their intensity normalised against the CH$_2$ cross-peak intensity.

The acetyl end-cap on the N-terminus (Figure 4.8) of the peptides gives rise to the cross-peak present at 1710/3120 cm$^{-1}$ across all peptide samples. Also, the cross-peak labelled CH$_2$ (1460/2920 cm$^{-1}$) arises from couplings between CH$_2$ scissor fundamental and its overtone vibration coming from 11 CH$_2$ groups in the peptide: each serine residue contains one methylene group, while three are present in each proline residue, and one on the Tyr and its nitrated form.

Vibrational couplings arising from backbone motions have been observed experimentally via the cross-peak labelled as Amide I (centred at ~1640/3280 cm$^{-1}$) in Figure 4.7.a. The amide I cross-peak arises from the C=O stretch mode and its overtone. Amide I substructures having components at approximately 1620/3240, 1635/3270, and 1660/3320 cm$^{-1}$ are observed. These are thought to arise from structural inhomogeneity in the sample and, since their relative intensities vary dramatically among the spectra, it appears that the peptide conformation and/or aggregation changes upon nitration and is also dependent on the protonation state of the nTyr group.

Conventional 2DIR spectroscopic techniques have extensively studied the amide I band in order to analyse conformation and aggregation of peptides and proteins. Amide I substructures are widely reported in the literature [95] and, based on the available information, the component that appears at 1635/3270 cm$^{-1}$ can be assigned to $\beta$ strands,
where $a$ corresponds to: (a) neutral Tyr, (b) neutral Tyr and (c) charged Tyr.

Figure 4.2: Experimental EYF 2DIR spectra of capped SP-SPS peptide. Spectra are provided for peptides of sequence SP-SPS.
while the highest frequency component at 1660/3320 cm\(^{-1}\) can be assigned to either a short \(\alpha\) helix or a disordered structure. The first component at 1620/3240 cm\(^{-1}\) is in close agreement with frequencies reported to arise from a red-shift of the \(\beta\)-strand component due to proline residues [96]. Given the experimental conditions have been optimised to directly probe vibrational modes associated with the Tyr side chains, the optical density for the amide I band is too high to enable further analysis.

### 4.2.1 Spectral features of tyrosine

While various works have been published discussing the signature EVV 2DIR spectra of Tyr side-chains [74, 76, 77], the results described here aim to analyse such features in more detail and over a broader spectral range. The experimental spectrum (Figure 4.7) is also compared against the simulated EVV 2DIR spectrum of 4MP (Figure 4.4) in order to assign vibrational modes to the observed cross-peaks.

The cross-peaks labelled as Tyr2 and Tyr4 in Figure 4.7 refer to the strongest and the longest lived vibrational couplings in the Tyr side-chain. Tyr2 has been assigned to the coupling between the CH rock (19a) and the combination band between CH rock (19a) and CC stretch (8a). The Tyr4 cross-peak has been assigned to the coupling between CC stretch (8a) and its overtone. For further assignments, please refer to table 4.1 and appendix A.1.

Other weaker cross-peaks have been identified to arise from Tyr ring-modes, such as the
Table 4.1: Comparison between experimental and simulated cross-peak frequencies for unmodified Tyr and their vibrational assignments

<table>
<thead>
<tr>
<th>Cross-peak label</th>
<th>Experimental freq. (cm⁻¹)</th>
<th>Simulated freq. (cm⁻¹)</th>
<th>Vibrational assignment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr1</td>
<td>1520/3000</td>
<td>1514/3028</td>
<td>CH rock (19a)/2(CH rock (19a))</td>
</tr>
<tr>
<td>Tyr2</td>
<td>1520/3130</td>
<td>1514/3137</td>
<td>CH rock (19a)/CH rock (19a) + CC stretch (8a)</td>
</tr>
<tr>
<td>Tyr3</td>
<td>1595/3025</td>
<td>1599/2762</td>
<td>CC stretch (8a)/CC stretch (8a) + CC stretch (19b)</td>
</tr>
<tr>
<td>Tyr4</td>
<td>1600/3190</td>
<td>1623/3049</td>
<td>CC stretch (8b)/2(CC stretch (8b))</td>
</tr>
<tr>
<td>Tyr5</td>
<td>1615/2810</td>
<td>1623/3246</td>
<td>CC stretch (8b)/CC stretch (8b) + COH bend</td>
</tr>
</tbody>
</table>

* Value inside bracket corresponds to the aromatic mode assignment in Varsanyi notation [98].

combination band between CC stretch (8a) and CC stretch (19b) for Tyr3 at 1595/3025 cm⁻¹. For further assignment, refer to the supplementary table provided in Appendix A.1.

4.2.2 Spectral features of 3-nitrotyrosine

The most appreciable difference between the Tyr and nTyr spectra (Figure 4.7) is the significantly larger number of observed cross-peaks for the nitrated species. By analysing experimental spectra of peptides and side-chain analogues shown in previous section, together with information available in the literature [97] and further supported by the simulated EVV 2DIR spectra in Figure 4.4, a large portion of cross-peaks can be assigned to couplings involving the NO₂ asymmetric stretch, which experiences a large frequency change between the neutral and charged nTyr species. These can be observed experimentally along 1545 cm⁻¹ and 1420 cm⁻¹ for the neutral and charged nTyr peptides, respectively. Spectral assignment of the labelled nTyr cross-peaks can be found in tables 4.2 and 4.3.

While the EVV 2DIR spectra of nitrated peptides displays an abundance of observed aromatic modes, most features are still reasonably resolvable without much need for complex data handling. However, this may become an issue in more complex samples. Given the shift in the NO₂ asymmetric stretch due to hydroxyl deprotonation, the cross-peak labelled nTyr₃, although not very strong, provides a good choice for monitoring nitration of samples containing a variety of aromatic amino acids, as this would hopefully
Table 4.2: Comparison between experimental and simulated cross-peak frequencies for neutral nTyr and their vibrational assignments

<table>
<thead>
<tr>
<th>Cross-peak label</th>
<th>Experimental freq. (cm⁻¹)</th>
<th>Simulated freq. (cm⁻¹)</th>
<th>Vibrational assignment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTyr1</td>
<td>1430/2850</td>
<td>1419/2838</td>
<td>CC stretch (19b)/2(CC stretch (19b))</td>
</tr>
<tr>
<td>nTyr2</td>
<td>1430/2965</td>
<td>1419/2969</td>
<td>CC stretch (19b)/CC stretch (19b) + asymmetric NO₂ stretch</td>
</tr>
<tr>
<td>nTyr3</td>
<td>1490/3070</td>
<td>1494/3078</td>
<td>CH rock (19a)/CH rock (19a) + CC stretch (8b)</td>
</tr>
<tr>
<td>nTyr4</td>
<td>1540/3080</td>
<td>1550/3100</td>
<td>asymmetric NO₂ stretch/2/asymmetric NO₂ stretch</td>
</tr>
<tr>
<td>nTyr5</td>
<td>1545/2795</td>
<td>1550/2808</td>
<td>asymmetric NO₂ stretch/asymmetric NO₂ stretch + symmetric NO₂ stretch</td>
</tr>
<tr>
<td>nTyr6</td>
<td>1545/2900</td>
<td>1550/2931</td>
<td>asymmetric NO₂ stretch/asymmetric NO₂ stretch + CC stretch (14)</td>
</tr>
<tr>
<td>nTyr7</td>
<td>1545/2970</td>
<td>1550/2969</td>
<td>asymmetric NO₂ stretch/asymmetric NO₂ stretch + CC stretch (19b)</td>
</tr>
<tr>
<td>nTyr8</td>
<td>1585/3165</td>
<td>1584/3168</td>
<td>CC stretch (8b)/CC stretch (8b)</td>
</tr>
<tr>
<td>nTyr9</td>
<td>1630/3205</td>
<td>1638/3222</td>
<td>CC stretch (8a)/CC stretch (8a) + CC stretch (8b)</td>
</tr>
<tr>
<td>nTyr10</td>
<td>1635/2980</td>
<td>1638/3019</td>
<td>CC stretch (8a)/CC stretch (8a) + CC stretch (14)</td>
</tr>
</tbody>
</table>

* Value inside bracket corresponds to the aromatic mode assignment in Varsanyi notation [98].

Table 4.3: Comparison between experimental and simulated cross-peak frequencies for charged nTyr and their vibrational assignments

<table>
<thead>
<tr>
<th>Cross-peak label</th>
<th>Experimental freq. (cm⁻¹)</th>
<th>Simulated freq. (cm⁻¹)</th>
<th>Vibrational assignment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTyr'1</td>
<td>1365/2700</td>
<td>1361/2722</td>
<td>CH rock (3)/CH rock (3) + CC stretch (14)</td>
</tr>
<tr>
<td>nTyr'2</td>
<td>1375/2910</td>
<td>1361/2936</td>
<td>CH rock (3)/CH rock (3) + CO stretch</td>
</tr>
<tr>
<td>nTyr'3</td>
<td>1415/2745</td>
<td>1446/2750</td>
<td>asymmetric NO₂ stretch/asymmetric NO₂ stretch + CC stretch (14)</td>
</tr>
<tr>
<td>nTyr'4</td>
<td>1420/2845</td>
<td>1446/2867</td>
<td>asymmetric NO₂ stretch/asymmetric NO₂ stretch + CH rock (19a)</td>
</tr>
<tr>
<td>nTyr'5</td>
<td>1510/2845</td>
<td>1507/2811</td>
<td>CC stretch (8b)/CC stretch (8b) + CC stretch (14)</td>
</tr>
<tr>
<td>nTyr'6</td>
<td>1510/3010</td>
<td>1507/3014</td>
<td>CC stretch (8b)/CC stretch (8b)</td>
</tr>
<tr>
<td>nTyr'7</td>
<td>1540/2865</td>
<td>1575/2879</td>
<td>CO stretch/CO stretch + CC stretch (14)</td>
</tr>
<tr>
<td>nTyr'8</td>
<td>1545/3085</td>
<td>1575/3150</td>
<td>CO stretch/2[CO stretch]</td>
</tr>
<tr>
<td>nTyr'9</td>
<td>1625/2965</td>
<td>1626/2930</td>
<td>CC stretch (8a)/CC stretch (8a) + CC stretch (14)</td>
</tr>
<tr>
<td>nTyr'10</td>
<td>1625/3160</td>
<td>1626/3201</td>
<td>CC stretch (8a)/CC stretch (8a) + CO stretch</td>
</tr>
</tbody>
</table>

* Value inside bracket corresponds to the aromatic mode assignment in Varsanyi notation [98].

decrease the likelihood of encountering issues with spectral congestion. Thus, analysis of proteins should preferably be carried out at basic pH in order to isolate the charged nTyr species.

The theoretical observation of a 3 order of magnitude enhancement in the signal for the charged nTyr residues was not observed in the peptide data, thus suggesting more accurate approximations are required.
4.3 BSA as a model of protein-tyrosine nitration

EVV 2DIR spectroscopy was then tested on its ability to identify Tyr nitration in a large and structurally complex protein-model. Bovine serum albumin (BSA) has been chosen as a model of protein nitration for various reasons. Firstly, nitrated BSA (nBSA) protein standards are widely available commercially. Secondly, it was very important to choose a protein with abundant Tyr residues in order to maximise our ability to detect nitration.

BSA is a globular-protein of approximately 66 kDa which, given its stability and lack of interference with biological reactions, is widely used as a protein standard. It can be abundantly produced at high purity levels and at low cost. nBSA standards are produced via two main nitration reactions: one using tetranitromethane (TNM) and other using peroxinitrite anion (ONOO−). While nitration of Tyr residues happen via peroxinitrite reactions in vivo, it is important to highlight that peroxinitrite is a very strong oxidant that favours oxidations of cysteine and methionine residues - at their thiol groups, and of Tyr into 3-hydroxytyrosine. Thus, it was suspected that BSA nitrated through addition of ONOO− would also contain several oxidised forms of other amino acids. Given TNM is a much more selective nitrating agent, despite it not being biologically abundant, it is often used in research and was expected to serve as a better nitrating agent for this study.

Samples of BSA, nBSA(TNM) and nBSA(ONOO−) were prepared in milliQ water without addition of any buffer. A comparison between quality of commercially available nBSA standards was carried out via MS analysis and is provided here.

Dr. K. Tveen Jensen3 has kindly analysed the BSA samples through LC-MS, followed by a MASCOT search for oxPTMs and verification by de novo sequencing (more detail may be found in the Section 2.5).

---

3Mass spectrometry data were acquired at Dr A. R. Pitt’s group at the School of Life and Health Sciences, Aston University. This collaboration was supported by the Proxomics project, through the Engineering and Physical Sciences Research Council grant EP/I017887/1.
Table 4.4: Nitrated peptides detected in nBSA(ONO$^-$). Modified Tyr are underlined in the sequence, with dotted lines indicating where it was not possible to determine the exact site of modification.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Peptide (charge state)</th>
<th>MASCOT ion score</th>
<th>Modification abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLYEIA R</td>
<td>486.7440 (2)</td>
<td>42</td>
<td>0.2</td>
</tr>
<tr>
<td>LGFYGFQNALIVR</td>
<td>762.8942 (2)</td>
<td>99</td>
<td>0.33</td>
</tr>
<tr>
<td>HPFYAPELLLYANKYNGVFQECQAE DK</td>
<td>938.8931 (4)</td>
<td>42</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Using the MASCOT search engine, a sequence coverage of 74% was identified for the nBSA(ONO$^-$) protein sample and 82% for the untreated BSA. In both cases, 15 out of 21 Tyr residues in the sequence were covered. Three nitrated peptides were detected in the nBSA(ONO$^-$) sample and none were detected in the untreated BSA sample. Also, a range of peptides with oxidation of methionine were detected in both samples.

When oxidation of Tyr and cysteine sulfonic acid were included in the variable modification search, these modifications were found in both BSA and nBSA samples, but with significantly higher levels in the latter. In both BSA and nBSA(ONO$^-$) samples, a range of peptides with containing sulphur oxidations and alternative Tyr oxidations were also detected, including cysteine sulfonic acid and hydroxytyrosine, with oxidation being significantly more prevalent in the nBSA(ONO$^-$) protein. The data was also searched for nitrosylcysteine and nitrotryptophan, but these could not be detected with statistical significance.

The relative percentage modification of the nitrated peptides was estimated via the generation of ion chromatograms at the masses of the nitrated peptides from the raw data using a window of 0.05 Da. In all cases, the abundance of the nitrated peptides was found to be less than 1% (Table 4.4).

The sequence of the modified peptides was verified by de novo sequencing of the raw data. The sequence of the peptide of M/Z value of 938.8931 could not be verified by de novo sequencing and no nitrotyrosine immonium ion could be identified, suggesting that this identification is probably a false positive.
The nBSA(TNM) sample was analysed in the same way as the other two samples and similar sequence coverage was obtained. However, the nBSA(TNM) protein was much more heavily nitrated than the nBSA(ONO-O−). Also, significantly less oxidation of sulfur was observed. Some Tyr oxidation and cysteine sulphonyl acid modifications were identified, but with lower levels than in the native BSA sample. No nitrosylcysteine or nitrotryptophan were detected with statistically significant ion scores.

The nitrated peptides identified are shown in table 4.5, along with the relative abundance of each modification found through LC-MS. It also shows the presence of methionine oxidation in a few of such peptides.

In summary, the MS data confirms the expected preference for oxidation over nitration of the nBSA(ONO-O−) samples. Although levels of nitration in nBSA(TNM) were significantly higher, certain nitrated peptides still showed relatively low abundances. Clearly, while it is easy to ensure nitration in the model peptides discussed in the previous section (which is due to the nature of their synthesis process), the same does not apply to the protein samples, which are nitrated via incubation with nitrating agents. In such cases, Tyr residues buried within the structure of the protein may provide difficult to become nitrated.

Given the very poor levels of nitration found in the nBSA(ONO-O−) sample, no further analysis was performed on it and nBSA(TNM) was then chosen as the most appropriate sample. To assess the required concentration for the nBSA(TNM) sample, a simple estimation was used:

- **Firstly the level of nitration of peptides and BSA was assessed:** given BSA has 3.6% Tyr to total number of residues, while the hepta-peptides utilised as earlier models have 14.3%, for the same concentration of peptide to BSA, the former would have four times more Tyr residues than the latter.

- **Then the homodyne detection scheme utilised in the EVV 2DIR spectroscopy experiments is accounted for:** a homodyne detection leads to signal
Table 4.5: Nitrated peptides detected in nBSA(TNM). Modified Tyr are underlined in the sequence. Peptides containing additional oxidative modifications are presented in bold.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Peptide (charge state)</th>
<th>m/z</th>
<th>MASCOT ion score</th>
<th>Modification abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLYEIAR</td>
<td></td>
<td>486.7405 (2)</td>
<td>45</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>509.2361 (2)</td>
<td>33</td>
<td>83</td>
</tr>
<tr>
<td>LGEYGFQNALIVR</td>
<td></td>
<td>762.2555 (2)</td>
<td>59</td>
<td>32</td>
</tr>
<tr>
<td>GLVLIASFQYLQCPFDHEVK</td>
<td></td>
<td>846.4125 (3)</td>
<td>106</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>635.0641 (4)</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>ETYGDMADCCEK</td>
<td></td>
<td>508.5042 (3)</td>
<td>21</td>
<td>33</td>
</tr>
<tr>
<td>ETYGDMADCCEK</td>
<td></td>
<td>762.2555 (2)</td>
<td>59</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>770.2526 (2)</td>
<td>84</td>
<td>23</td>
</tr>
<tr>
<td>RHPYFYAPELLYYANK</td>
<td></td>
<td>697.3413 (2)</td>
<td>61</td>
<td>69</td>
</tr>
<tr>
<td>RHPYFYAPELLYYANK</td>
<td></td>
<td>1068.005 (2)</td>
<td>67</td>
<td>-</td>
</tr>
<tr>
<td>RHPYFYAPELLYYANK</td>
<td></td>
<td>727.3267 (3)</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>RHPYFYAPELLYYANK</td>
<td></td>
<td>545.7471 (4)</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>556.9950 (4)</td>
<td>52</td>
<td>95</td>
</tr>
<tr>
<td>HPYFYAPELLYYANK</td>
<td></td>
<td>645.3109 (3)</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>HPYFYAPELLYYANK</td>
<td></td>
<td>989.9509 (2)</td>
<td>57</td>
<td>50</td>
</tr>
<tr>
<td>HPYFYAPELLYYANK</td>
<td></td>
<td>1012.4384 (2)</td>
<td>52</td>
<td>41</td>
</tr>
<tr>
<td>HPYFYAPELLYYANK</td>
<td></td>
<td>1034.9321 (2)</td>
<td>49</td>
<td>43</td>
</tr>
<tr>
<td>YNGVFQECQCQADK</td>
<td></td>
<td>896.8419 (2)</td>
<td>82</td>
<td>92</td>
</tr>
<tr>
<td>YICDNQDTISSK</td>
<td></td>
<td>744.8140 (2)</td>
<td>74</td>
<td>15</td>
</tr>
<tr>
<td>DAFLGSFLYEYSR</td>
<td></td>
<td>538.2441 (3)</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>DAFLGSFLYEYSR</td>
<td></td>
<td>829.3559 (2)</td>
<td>78</td>
<td>74</td>
</tr>
<tr>
<td>RHPEYAVSVLLR</td>
<td></td>
<td>742.9010 (2)</td>
<td>71</td>
<td>70</td>
</tr>
<tr>
<td>HPEYAVSVLLR</td>
<td></td>
<td>664.8513 (2)</td>
<td>49</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>443.6595 (3)</td>
<td>44</td>
<td>72</td>
</tr>
<tr>
<td>EYEATLLEECAK</td>
<td></td>
<td>774.3015 (2)</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>DDPHACYSTVFDK</td>
<td></td>
<td>800.3221 (2)</td>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>533.0844 (3)</td>
<td>49</td>
<td>14</td>
</tr>
<tr>
<td>LGEGYGQNALIVR</td>
<td></td>
<td>762.2909 (2)</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>508.9300 (3)</td>
<td>72</td>
<td>99</td>
</tr>
<tr>
<td>MPCTEDYLSLILNR</td>
<td></td>
<td>885.4042 (2)</td>
<td>68</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>590.6117 (3)</td>
<td>45</td>
<td>99</td>
</tr>
<tr>
<td>MPCTEDYLSLILNR</td>
<td></td>
<td>893.4063 (2)</td>
<td>118</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>595.9404 (3)</td>
<td>48</td>
<td>98</td>
</tr>
<tr>
<td>RPCFSALTLPDETXYVK</td>
<td></td>
<td>482.2297 (4)</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>963.4562 (2)</td>
<td>67</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>642.6382 (3)</td>
<td>45</td>
<td>61</td>
</tr>
</tbody>
</table>
Figure 4.9: Spectra of BSA and nBSA at pH 9. Labels represent Tyr2 and Tyr4 cross-peaks, as assigned earlier in this section. Circled cross-peak is only present for the nBSA. Enlarged spectral features are provided in (b) to assist on discussion.

- Find a concentration of peptide which lead to measurable signatures and apply the proportionality correction based on the above numbers. So, based on a 10 mM peptide solution, one would expect to require at least \( (4^2 \times 10) = 160 \) mM concentration of BSA in order to achieve the same signal.

It is worth mentioning that this estimation assumes identical oscillator strengths between modes in the peptide and protein Tyr residues, as well as 100% yield of Tyr nitration in the BSA samples and no decrease in vibrational life-times in larger molecules. Thus, it is likely that much higher concentrations would be required, which unfortunately are extremely high for proteins, especially given that the pH-adjustment step requires a minimum of 60 \( \mu \)L of solution in order to ensure accurate performance by the pH meter.

EVV 2DIR spectra have been collected for 225mM BSA and nBSA(TNM). Figure 4.9 shows data for a spectral range of interest, which was selected after thorough inspection of a wider spectral range. A shorter range was used to allow for an increase of signal averaging, thus preventing weak cross-peak signals from being buried in the signal noise.
Spectra in Figure 4.9 show Tyr2 and Tyr4 cross-peaks for both BSA and nBSA(TNM). This is in line with the LC-MS analysis which showed levels of nitration varied significantly between each Tyr residue in the nBSA(TNM). The second observation highlighted in Figure 4.9 is that both Tyr2 and Tyr4 cross-peak intensities are considerably lower in the nBSA spectrum. This confirms that, indeed, a number of Tyr residues have been modified. Note in Figure 4.9.a that another previously identified (however, not labelled) Tyr cross-peak at 1515/3000 cm\(^{-1}\) also has its intensity dropped significantly on the nBSA sample.

Ideally, however, identification of the presence of a chemical modification would be best done through the observation of a new cross-peak arising from the modification itself, rather than the decrease/disappearance of a cross-peak related to the unmodified amino-acid. One of such cross-peaks can be found at 1510/3165 cm\(^{-1}\) (circled in Figure 4.9). The frequency 1510 cm\(^{-1}\) has indeed been observed on earlier models of nTyr\(^{-}\) and has been assigned to the CC stretch (8b) mode. However, the observed cross-peak would require a combination band between the 1510 cm\(^{-1}\) mode and a mode at 1655 cm\(^{-1}\). The second frequency component of this combination band appears higher than earlier observations have suggested and may arise from couplings involving peptide backbone modes.

Upon further inspection, it was possible to observe a very weak cross-peak at a similar frequency (1520/3175 cm\(^{-1}\)) for the capped SPSnYSPS peptides. Data provided in the following chapter shows that not all peptide models of nitration have such cross-peak, which may indicate this vibrational coupling may be particularly sensitive to the environment of the nTyr moiety.

While identification of characteristic cross-peaks of nitration have been successfully car-
ried out for side-chain analogues and peptides, EVV 2DIR spectroscopy's sensitivity to the local environment of the chemical modification means that a bottom-up approach to understanding vibrational signatures may not hold when transferred to complex molecules. Instead, it appears that utilising a top-down approach for studying EVV 2DIR spectra might be preferable. This may be due to a variety of reasons which will require further study. To name a few, this bottom-up approach to identifying oxPTM assumed identical oscillator strengths between modes in the peptide and protein models. Also, vibrational life-times may decrease for larger molecules. Therefore, it has become clear that, unless a small model is carefully chosen to represent the local environment of the chemical group of interest of a larger molecule, there is a decreased chance that the characteristic cross-peaks would remain throughout the spectra of different molecules.

4.4 Conclusion

The identification of Tyr nitration via EVV 2DIR spectroscopy has been discussed for a variety of models in this chapter. The following research questions have been addressed:

1. Can EVV 2DIR spectra be used to identify oxPTMs?

2. Does the current model utilised in the simulation of EVV 2DIR spectra provide a good tool to pre-assess the ability of EVV 2DIR spectroscopy to experimentally probe vibrational modes arising from oxPTMs?

The research question 1 above was first investigated through the use of side-chain analogues. The relative ease in sample preparation of solutions containing 4MP and 4MN2P was a particularly favourable aspect of this experiment, even though such a simplistic model could only partially address this question.

Side-chain analogues confirmed the theoretical predictions that EVV 2DIR spectroscopy would be able to probe characteristic cross-peaks of nitrated phenolic structures. By
extending this study to include the identification of Tyr nitration of more biologically relevant models, such as peptides and proteins, it was possible to fully assess research question 1.

For the first time, EVV 2DIR spectroscopy was used to identify nitration of Tyr peptides. Also, taking into consideration the pKa of the hydroxyl group in nTyr is very close to physiological pH, spectra were produced for both the neutral and charged nTyr species.

nBSA samples produced through two chemical routes were analysed via LC-MS. Nitrilation via tetranitromethane was identified as leading to much higher yields. There was no statistically significant evidence of nitrotryptophan or nitrophenylanalanine in either sample, which suggests the nitration we are seeing is fairly selective. Unfortunately, the EVV 2DIR spectra for nBSA did not contain any of the expected characteristic cross-peaks for nTyr.

A weak cross-peak at 1515/3175 cm\(^{-1}\), which had not initially been considered as spectral marker of nTyr, was observed in both SPSnYSPS peptide and nBSA samples and is thought to arise from the coupling of the CC stretch (8b) mode at 1510 cm\(^{-1}\) and a backbone mode at 1655 cm\(^{-1}\).

Since local charges affect the transition dipole moment of the molecule and, therefore, will have an effect on the EVV 2DIR signal arising from EVV-IR\(_a\) pathway, it is important to appreciate that the intensity and frequency of vibrational modes, as well as their couplings, may be altered significantly by the local environment of the chemical group of interest. Therefore, it appears that future studies should give preference to a top-down approach for EVV 2DIR analysis.

Research question 2 was directly addressed in this section and it was shown that good prediction accuracy for vibrational mode frequencies could be achieved by utilising a 0.98 scaling factor to the results of the Gaussian calculations. However, a large increase in signal was observed in simulated spectra of the nitrated tyrosine side-chain analogue molecules, which was not observed experimentally. Therefore, the methods employed in
the calculations of relative cross-peak intensities may require further refinement.

Apart from answering the two research questions above, additional information was also gained via analysis of the 2DIR spectra. For example, experimental spectra suggest that the peptide conformation and aggregation, as well as the frequencies and life-times of the observed vibrational modes, are affected by nitration and sample pH. Clear differences were observed between the spectra from the nTyr species and particular attention should be paid to cross-peaks arising from asymmetrical NO₂ stretch, which experiences a large shift from ~1545 cm⁻¹ for the neutral nTyr to ~1420 cm⁻¹ for the deprotonated species.

In summary, while identification of nTyr in small peptides can be achieved by EVV 2DIR spectroscopy, it has not been possible to gather substantial evidence to confirm these findings in the chosen protein model. The nBSA(TNM) spectrum may suggest that either the number of nTyr residues was below the limit of detection of the apparatus, or, that nTyr residues in large proteins undergo a significant shift in vibrational frequencies and/or life-times, thus preventing its correlation with data acquired for simpler models. The local environment of the nTyr residue is highly likely to lead to changes in the EVV 2DIR spectra, given its sensitivity to intra- and intermolecular vibrational coupling.
In this chapter it is shown that EVV 2DIR spectroscopy has been successfully employed to detect 3-nitrotyrosine (nTyr) levels down to 5% in peptide mixtures, with an estimated detection limit of 1% nitration content.

A large portion of the work presented in this chapter has been previously published [80] (Appendix A.1). The contents of this chapter provide the reader with a deeper understanding of estimations of the limit of detection of nitration via EVV 2DIR spectroscopy.

Figure 5.1: Experimental EVV 2DIR spectrum of a mixture of peptides containing Tyr and nTyr. Reproduced from [80] under a Creative Commons Attribution (CC-BY) license.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Detection of nTyr in peptide mixtures</td>
<td>109</td>
</tr>
<tr>
<td>5.2 Construction of calibration curves</td>
<td>112</td>
</tr>
<tr>
<td>5.3 Quantification of mixtures containing low percentages of nTyr</td>
<td>113</td>
</tr>
<tr>
<td>5.4 The limit of detection of EVV 2DIR spectroscopy</td>
<td>115</td>
</tr>
<tr>
<td>5.5 Conclusion</td>
<td>116</td>
</tr>
</tbody>
</table>
Optical multidimensional spectroscopy offers the possibility of unambiguously quantifying biomarkers such as oxidative post-translational modifications (oxPTMs), based solely on the intrinsic properties of the sample. While mass-spectrometry is the gold standard for proteomic quantification, it relies on very extensive data analysis, the quality of which depends on the experience and knowledge of the user. Thus, this chapter assesses EVV 2DIR spectroscopy’s ability to quantify the nitration content of mixed peptide samples.

5.1 Detection of nTyr in peptide mixtures

Solutions of Ac-SPSYSPS-NH₂ peptide, containing varying ratios of Ac-SPSnYSPS-NH₂ peptide have been used. EVV 2DIR spectra were obtained for gel spots formed from solutions containing 100, 75, 50, 25, 10 and 5% Ac-SPSnYSPS-NH₂ at pH 5.6 and 9.1, respectively, in order to isolate the neutral and charged nTyr species.

In samples which are heterogeneous in height/surface geometry, such as the case of the coffee-ring gel spots, it has been found that the shape of the samples leads to various in the signal ratios of cross-peaks. Since these signal variations pose a problem for quantification, positioning of the different samples was carefully chosen by ensuring the signal ratio of CH₂-Amide I cross-peaks remained constant. EVV 2DIR spectra shown in this chapter have their intensity normalised against the CH₂ cross-peak intensity.

The EVV 2DIR spectrum for mixture containing 25% of nTyr pH 5.6 (Figure 5.2-d) shows characteristic nTyr cross-peaks at 1540/3080 and 1545/2900 cm⁻¹, which are labelled nTyr4 and nTyr6, respectively. The same applies to a variety of cross-peaks for the pH 9.1 mixtures. Since the spectrum for the mixture containing 25% nTyr peptide at both pH 5.6 and pH 9.1 contain easily identifiable nTyr cross-peaks, it suggests that the detection limit for tyrosine nitration is well below 25%.

Additionally, the spectrum for mixture containing 50% of nTyr pH 9.1 (Figure 5.3-c) shows that the peak nTyr8, at 1545/3085 cm⁻¹, is more intense than any Tyr cross-peak,
Figure 5.3: Experimental EVV 2DIR spectra of heptamer peptides mixtures at pH 9.1. Spectra are provided for samples containing: a) 0%, b) 25%, c) 50%, d) 75% and e) 100% nitrated peptide. The pH of the solution has been set so that the hydroxyl group of the nTyr side-chain is in its deprotonated form. The square root of the signal intensity of several cross-peaks has been plotted as a function of the ratio (f) in order to produce calibration curves for future quantification of unknown ratios. Adapted from [80] under a Creative Commons Attribution (CC-BY) license.
Table 5.1: Linear fit coefficients of calibration curves extracted from peptide mixtures in pH 5.6.

<table>
<thead>
<tr>
<th>Cross-peak Label</th>
<th>Freq. (cm⁻¹)</th>
<th>R²-value</th>
<th>Intercept Value</th>
<th>Std. Error</th>
<th>Slope Value</th>
<th>Stand Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr4</td>
<td>1600/3190</td>
<td>0.999</td>
<td>2.276</td>
<td>0.022</td>
<td>-0.019</td>
<td>0.000</td>
</tr>
<tr>
<td>nTyr4</td>
<td>1540/3080</td>
<td>0.994</td>
<td>0.219</td>
<td>0.026</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
<td>nTyr6</td>
<td>1545/2900</td>
<td>0.987</td>
<td>0.209</td>
<td>0.046</td>
<td>0.011</td>
<td>0.001</td>
</tr>
<tr>
<td>nTyr10</td>
<td>1635/2980</td>
<td>0.971</td>
<td>0.087</td>
<td>0.046</td>
<td>0.008</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 5.2: Linear fit coefficients of calibration curves extracted from peptide mixtures in pH 9.1.

<table>
<thead>
<tr>
<th>Cross-peak Label</th>
<th>Freq. (cm⁻¹)</th>
<th>R²-value</th>
<th>Intercept Value</th>
<th>Std. Error</th>
<th>Slope Value</th>
<th>Stand Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr4</td>
<td>1600/3190</td>
<td>0.952</td>
<td>1.647</td>
<td>0.119</td>
<td>-0.015</td>
<td>0.002</td>
</tr>
<tr>
<td>nTyr1</td>
<td>1365/2700</td>
<td>0.988</td>
<td>0.065</td>
<td>0.050</td>
<td>0.013</td>
<td>0.001</td>
</tr>
<tr>
<td>nTyr2</td>
<td>1375/2910</td>
<td>0.985</td>
<td>0.047</td>
<td>0.021</td>
<td>0.006</td>
<td>0.000</td>
</tr>
<tr>
<td>nTyr8</td>
<td>1545/3085</td>
<td>0.996</td>
<td>0.188</td>
<td>0.037</td>
<td>0.016</td>
<td>0.001</td>
</tr>
<tr>
<td>nTyr9</td>
<td>1625/2965</td>
<td>0.999</td>
<td>0.140</td>
<td>0.007</td>
<td>0.009</td>
<td>0.000</td>
</tr>
</tbody>
</table>

indicating that the signal from charged nTyr is larger than that from non-nitrated Tyr residues. Additionally, the relative intensities of the nTyr and Tyr cross-peaks in the 25% nTyr spectrum are significantly higher for the pH 9.1 mixtures (Figure 5.3-d) than for the pH 5.6 mixtures (Figure 5.2-d). This demonstrates that the detection sensitivity for Tyr nitration is expected to be higher for samples containing charged nTyr, i.e at basic pH. This increase was predicted theoretically and discussed in Chapter 4. While the observed increase in signal for the protonated nTyr residues does not match the predicted increase by 3 orders of magnitude, the experimentally observed 5-fold increase is still considerably favourable.

5.2 Construction of calibration curves

As EVV 2DIR spectroscopy uses a homodyne detection scheme, the measured signal is proportional to the square root of the polarisation $P_{NL} = N \mu F_{p_a}$, as shown in section 1.5.2. This equation clearly shows a linear relationship between the polarisation and the
number of molecules in the sample (N). Thus, the relationship between the signal and the number of molecules follow a square root dependence.

The square root of the signal was plotted against the known nTyr:Tyr ratios in each sample. The plots were linearly fitted to assess the linearity of the square of the measured signal, as well as to build calibration curves for future quantification.

As seen in Figures 5.2 and 5.3, given the abundance of coupling observed for the nTyr peptide, various cross-peaks have been used to build calibration curves. The plotted curves shown in Figures 5.2-f and 5.3-f displayed very good linearity with R²-values greater than 0.971, for the pH 5.6 mixtures, and greater than 0.952, for the pH 9.1 mixtures. The coefficients of the linear fits have been given in tables 5.1 and 5.2.

The good linearity of the calibration curves suggested that EVV 2DIR spectroscopy could lead to reliable relative quantification, as long as the nitration content of the sample is above the detection limit of the apparatus. To confirm the accuracy of quantification and to test the limit of detection, samples containing nTyr at ratios below or equal to 10% were analysed at pH 9.1.

5.3 Quantification of mixtures containing low percentages of nTyr

As previously mentioned, nitration of Tyr residues in vivo is a selective process which occurs at very low copy numbers. To test the ability of EVV 2DIR spectroscopy to detect low percentages of nitration, mixtures containing 10 and 5% nTyr-peptides were studied. Their spectra were analysed (Figure 5.4) and cross-peak signal intensities were used, in conjunction with the calibration curves discussed in the previous section, to estimate the percentage of nitration occurring in those samples.
Figure 5.4: Experimental EVV 2DIR spectra for peptide mixtures containing 10 and 5% of nTyr. Samples of capped SPSYSPS peptides were mixed with SPSnYSPS at pH 9.1 to form mixtures containing (a) 10 and (b) 5% of nTyr. A weak nTyr-8 cross-peak at 1545/3085 cm\(^{-1}\) is observed in both spectra and is indicated by a white circle. Line profiles with \(\omega_a/2\pi c\) frequency fixed at 1545 cm\(^{-1}\) for 10, 5, and 0% nTyr samples are shown in (c). Adapted from [80] under a Creative Commons Attribution (CC-BY) license.

The calibration curve for the nTyr-8 cross-peak at 1545/3085 cm\(^{-1}\) for the charged nTyr peptide was used (Figure 5.3-f). Line profiles were measured across EVV 2DIR spectral features using a fixed \(\omega_a/2\pi c\) frequency value of 1545 cm\(^{-1}\) for 10, 5, and 0% nTyr samples (Figure 5.4-c).

The normalised intensity of the 1545/3085 cm\(^{-1}\) cross-peak values for 5 and 10% mixtures were then used to extract a corresponding nTyr percentage from the calibration coefficients provided in Table 5.2. The results were found to be 6 and 11%, respectively.

In order to independently verify the levels of nitration in the above samples, LC-MS was used to characterise the peptide solutions by collaborators from Dr Andrew Pitt’s group at Aston University. Samples of 100% nTyr peptide and 100% Tyr peptide were used to obtain a correction factor for relative quantitative analysis of the mixtures. This is due to the fact that the addition of the nitro group to the Ac-SPSYSPS-NH\(_2\) peptide resulted in significantly different signal intensities in LC-MS (the nTyr peptide signal was observed to be approximately 2.61-fold more intense than that of the Tyr-containing peptide). These differences are mostly due to changes in ionization efficiencies, amongst other physicochemical properties.

The areas of the extracted ion chromatograms (Figure 5.5) were added for all observed signals for the \(1^+\) and \(2^+\) ions and their respective adducts with sodium. This has allowed
the quantification of these species. LC-MS calculation resulted in 5.4 and 12.1% for the 5 and 10% mixtures, respectively. This is in good agreement with the values extracted from the calibration curve from EVV 2DIR spectroscopy.

5.4 The limit of detection of EVV 2DIR spectroscopy

On the basis of the low noise level observed for the line profiles in Figure 5.4, as well as the overall good linearity observed on the square root of the cross-peak intensity as a function of nTyr ratios, it has been estimated that the limit of detection would be defined by the nTyr ratio leading to a signal which was distinguishable from the noise.

For the nTyr-8 cross-peak shown in Figure 5.4, the minimum distinguishable signal has been defined to be 0.2, which was then fitted on the regression equation containing the coefficients listed on Table 5.2. The resulting relative abundance of 0.75% was then rounded up in order to provide a conservative estimation of 1%.

It is important to recognise that quantification of nTyr can be performed via more conventional tools, such as UV-Vis spectroscopy, which relies on the shift of the electronic state resonant with 357 in neutral nTyr and 428 nm in charged nTyr. However, EVV 2DIR spectroscopy can be further used to gain important information on how a given modification, in this case nitration, can affect the molecule. As shown in Chapter 4, Amide I
band can provide information on folding/aggregation, and individual cross-peaks can be monitored to gain insight on intra- and inter-molecular interactions resulting from such modification.

Additionally, EVV 2DIR spectroscopy could be turned into a triply resonant technique by tuning the frequency of the third excitation beam to be in full resonance with nTyr’s electronic state. This is expected to lead to a great enhancement in 2DIR signal and potentially be used to significantly lower the limit of detection of the technique. Pairing this enhancement with the ability to produce chemical maps of samples, which is done by spatially mapping EVV 2DIR signal, one can, for example, potentially learn how the spatial distribution of nitration relates to different structures in a given tissue sample.

5.5 Conclusion

This chapter has addressed the following research question: Can quantification be performed to assess the levels of oxPTM within a sample? Peptide mixtures containing 100, 75, 50, 25 and 0% nTyr were analysed to create calibration curves for a wide range of cross-peak signals as a function of the known nTyr:Tyr ratios. Then, samples containing 10 and 5% nTyr were quantified by EVV 2DIR spectroscopy, as well as LC-MS. Results show that spectroscopic quantification led to similar results to those acquired via LC-MS analysis.

Interestingly, the observed cross-peak signal arising from deprotonated nTyr is higher than the protonated nTyr and unmodified Tyr, as predicted theoretically and discussed in Chapter 4. While the observed increase in signal does not match the predicted increase by 3 orders of magnitude, the experimentally observed 5-fold increase is still considerably favourable.

Limit of detection was estimated to 1% nTyr content in peptide mixtures at basic conditions, which is used to isolate the deprotonated nTyr species due to its higher signal
intensity. In order to further improve this limit of detection, EVV 2DIR spectroscopy should be utilised in triply-resonant mode. This can be done by introducing a resonant Raman probe step set at a wavelength that is resonant with an electronic excitation level in nTyr such that it selectively enhances the FWM signal arising from nTyr residues. This could increase the signal intensity by several orders of magnitude, as previously reported [77].

A useful application of EVV 2DIR spectroscopy-based quantification relates to chemically mapping oxPTMs across tissue samples to gain insight about their occurrence sites. This is discussed in the next chapter and poses a very exciting future development of EVV 2DIR spectroscopy.
Chapter 6

Conclusions and Outlook
## Contents

6.1 Experimental limitations .................................................. 121

6.2 Future work ................................................................. 122
   6.2.1 Study of oxidative markers in proteins ......................... 122
   6.2.2 Chemical mapping of oxidative markers ....................... 123

6.3 Work summary ............................................................. 125
6.1 Experimental limitations

Experimental limitations arising from a range of issues have been discussed. Firstly, current protocols utilise dried sample droplets, which generally result in the formation of coffee-ring structures. These, tend to be rather narrow and pose issues with the heterogeneity of the efficiency of four-wave mixing as a function of wavelength. Several methods have been tested with the goal to eliminate coffee-ring formation during the drying process and concentrate the sample in a small uniform area. The following techniques were used a) micropatterning of the substrate to make hydrophilic wells where the sample could be deposited; b) microarraying the samples using high-precision, software-controlled delivery via the use of fine needles and c) applying surface acoustic waves (SAW) to the substrate during the drying process.

While it was not possible to fully eliminate coffee-rings via the methods listed above, it was observed that, when subject to low frequency vibrations, droplets of sample were instead drying into a doughnut topology, which led to a flat region of material at the centre of the spot. This, while not optimum, due to the wasteful amount of sample which is still at the periphery of the coffee-ring, has facilitated the acquisition of spectra enormously.

Secondly, the current set-up experiences large beam walk-off, which is caused by wide tuning angles in the nonlinear crystal inside the optical parametric amplifiers. As a result, there are significant variations to the properties of the excitation beams as a function wavelength.

Finally, the current limit of detection is still reasonably high and may impede analysis of nitration levels in large molecules and at copy numbers comparable to those occurring in vivo. However, it is still possible for many peptide models to be studied through EVV 2DIR spectroscopy. The limit of detection may be dramatically improved by new developments on detection schemes. Furthermore, signal levels of characteristic cross-peaks of biomarkers, such as nTyr, could be amplified by utilising EVV 2DIR spectroscopy
as a triply-resonant technique. This can be done by tuning the wavelength of the third excitation beam to be in resonance with a known electronic transition of a molecule of interest.

While most research questions have been addressed in the current work, a wide range of experiments should be carried out to fully assess the capabilities of EVV 2DIR spectroscopy. The rest of this chapter will, hopefully, give an overview of future applications of the technique in the study of oxidation.

6.2 Future work

6.2.1 Study of oxidative markers in proteins

As already discussed, when compared to one-dimensional IR spectroscopy, considerable spectral decongestion is achieved through EVV 2DIR spectroscopy due to its ability to spread information across two dimensions and to probe only coupled vibrational modes. These features make it a valuable technique in the study of biological samples.

In Chapter 4, it was shown that when performing EVV 2DIR spectroscopy on nitrated BSA samples, it was not possible to detect any of the characteristic nTyr cross-peaks previously identified in model peptides and side-chains. 225 mM BSA solution was used. To put these numbers into perspective, BSA has 21 Tyr side chains, which corresponds to 3.6% of its total 583 amino acids. Proteins which have been reported in the literature to be affected by tyrosine nitration usually have much lower percentage of Tyr and only a few of their total number of Tyr are actually nitrated. Also, various studies report biological effects resulted from nitration of a single Tyr residue. Thus, copy numbers involved in in vivo detection are extremely low. Table 6.1 provides an overview of proteins which have been reported to be targets of tyrosine nitration.
Table 6.1: Example of proteins reported to be targets of tyrosine nitration.

<table>
<thead>
<tr>
<th>Protein</th>
<th>UniProt entry</th>
<th>Function</th>
<th>Number of Tyr</th>
<th>Tyr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSOD</td>
<td>P04179</td>
<td>Superoxide anion scavenger in the mitochondria.</td>
<td>10</td>
<td>4.50</td>
</tr>
<tr>
<td>Cytochrome-c</td>
<td>P08574</td>
<td>Electron transport chain in mitochondria.</td>
<td>14</td>
<td>4.31</td>
</tr>
<tr>
<td>α-synuclein</td>
<td>P37840</td>
<td>Dopamine regulator, tau protein fibrilization.</td>
<td>4</td>
<td>2.86</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>P02671</td>
<td>Major function in hemostasis due to role in clotting.</td>
<td>24</td>
<td>2.77</td>
</tr>
<tr>
<td>p53</td>
<td>P04637</td>
<td>Transcription factor, tumour suppressant, cell cycle regulator.</td>
<td>9</td>
<td>2.29</td>
</tr>
<tr>
<td>Tau</td>
<td>P10636</td>
<td>Promotes microtubule assembly and stability.</td>
<td>6</td>
<td>0.79</td>
</tr>
</tbody>
</table>

It is important to recognise that the limit of detection needs to be improved significantly in order for the technique to be useful in studies requiring nitrination levels of the same order of magnitude of the levels observed in vivo. Once those improvements are in place, proteins identified on Table 6.1 could be studied to enable important information to be gained on the structure and interactions involving them. Proteins involved in inflammatory processes are found in relatively large concentrations at inflammation sites. Amongst those, α-synuclein is particularly interesting given it is a soluble protein, thus facilitating sample preparation, and has been documented as displaying high levels of accumulation in Lewy bodies, which are protein aggregates known to be the pathological hallmark of Parkinson’s disease.

Finally, apart from proteins, a wide range of other molecules could be analysed, from DNA to lipids, both of which are well known to be targets of oxidative damage.

6.2.2 Chemical mapping of oxidative markers

Imaging methods based on IR and Raman spectroscopies have grown in popularity given their chemically sensitive label-free capabilities. ATR-FTIR has been successfully used to image arterial samples in contact with solutions containing drug molecules [99]. Spatial distribution of protein and lipid rich domains was also obtained. However, given FTIR spectroscopy/spectromicroscopy is a one-dimensional technique, ATR-FTIR imaging relies on the observation of amide I band (1680–1660 cm⁻¹) to track protein rich areas and of the carbonyl band at 1780–1720 cm⁻¹ to track lipid rich areas.
Another FTIR spectromicroscopy study has been able to analyse hippocampal tissue sections from Alzheimer disease mouse model [100]. Samples were mounted on IR reflective slides to allow imaging to be carried out in reflectance mode. Amyloid beta plaques have been identified by either taking the ratio of the area of the $\beta$-sheet amide I band envelope (1662-1652 cm$^{-1}$) to that of $\alpha$-helix amide I (1630-1620 cm$^{-1}$) or by processing for the area of the $\beta$-sheet amide I shoulder alone. The area of a sharp doublet (located between 1410-1384 cm$^{-1}$) was found to be adequate for detection of all creatine deposits. The latter is significantly inactivated by oxidation in Alzheimer’s disease, whereas amyloid beta plaques are well known to occur in sites with high levels of oxidation/nitrination. However, spectral congestion is still a problem intrinsic to such one-dimensional techniques. If a two-dimensional spectroscopy technique were to be used, then one could potentially differentiate between amino acids within proteins, allowing much richer analysis of the sample. Thus, we turn to EVV 2DIR spectroscopy.

It has been shown that EVV 2DIR spectroscopy is capable of producing chemical maps of tissue sections [77]. This is normally done by setting the IR frequencies to be in resonance with a known cross-peak and then translating the sample’s lateral and vertical axes to acquire signal levels across the entire sample. However, as learned from the work in this thesis, a good approach to this type of analysis is to select a few different regions of interest of the sample and acquire full spectra. By doing so, spectral features of interest can be selected from the 2DIR spectra. This can, for example, be particularly useful in the identification of spectral signatures that may aid in quantitatively grading tumours and, by doing so, we may gain a deeper understanding of the biochemical make up of specific diseased regions.

While EVV 2DIR chemical mapping has been previously performed, it has only so far been carried out on stained tissues. Thus, it would be particularly interesting to extend EVV 2DIR imaging towards label-free samples. Tissue microarrays (TMAs) are very good candidate samples. These are slides containing arrays of small tissue cores. They are very efficient in concentrating a large number of tissues on a minimal area by densely mounting the cores of interest, thus reducing costs, resources, and tissue usage.
TMAs are made from formalin-fixed paraffin embedded (FFPE) tissue sections, which are about 5 $\mu m$ thick and are fixed via paraffin embedding following three main steps: dehydration, where the water is replaced by alcohol; clearing, where the alcohol is replaced by xylene and impregnation, where xylene is replaced by paraffin. While histoimmunological practices require dewaxing in order to perform staining of features of interest, EVV 2DIR spectroscopy could potentially be performed in waxed samples.

Finally, chemical maps could be built at different polarisation of the IR beam, potentially allowing information regarding molecular conformation to be obtained.

### 6.3 Work summary

The work presented in this thesis proposes the use of EVV 2DIR spectroscopy as an analytical tool for the study of biomolecules under oxidative damage. Tyrosine nitration was used as a model of oxidative post-translational modification given the large number of studies which suggest it being responsible for protein deactivation.

Theoretical simulation and experimental data collected for side-chain analogues 4MP and 4M2NP, as well as peptides have been studied via EVV 2DIR spectroscopy. By using small molecules, it has been possible to show that EVV 2DIR spectroscopy can successfully detect couplings between vibrational modes of the native and nitrated tyrosine side chains.

The work was extended to peptides in order to model tyrosine nitration in biologically-relevant conditions. Sample pH was varied in order to separate the two species of nTyr, which are found at physiological pH: a species containing a neutral phenolic hydroxyl group and another containing its deprotonated counterpart. EVV 2DIR spectroscopy was able to identify, for the first time, characteristic spectral signatures for nitrated tyrosine (nTyr) residues in both protonated and deprotonated forms.

Bovine serum albumin protein has been also analysed via mass spectrometry and EVV
2DIR spectroscopy and, it was identified via mass spectrometry that the sample contained high percentages of nTyr modifications. Tyr2 and Tyr4 cross-peaks, which are the characteristic tyrosine cross-peaks, were successfully detected in BSA. They were found as well in nBSA but with considerably lower intensities, as expected given the nitration of the Tyr residues. Although no cross-peak similar to the prominent ones found in nitrated peptide models were detected, a specific cross-peak was detected at around 1510/3165 cm\(^{-1}\) in nBSA. Upon further investigation, a similar cross-peak was found in a specific model peptide and could be a result of coupling involving a backbone mode. This cross-peak was not systematically observed in other peptides and might be very sensitive to environment and molecular conformation, which is consistent with the hypothesised involvement of backbone vibrational modes. The inability to observe the expected cross-peaks suggests that potentially the absolute number of modifications was below the limit of detection of EVV 2DIR spectroscopy. Alternatively, the nitro group may be interacting with surrounding chemical groups and, as a result, its vibrational signature may have changed in terms of its frequency and/or its dephasing time.

Analysis of Amide I substructures for peptides containing Tyr, nTyr and deprotonated Tyr have provided information on the conformation of the sample with respect to the nitration status of the peptide. Data suggests that nitration of the nTyr residue and the solution pH leads to a significant change in the distribution of Amide I substructures.

It has also been shown that relative quantification of nitration levels can successfully be performed via EVV 2DIR spectroscopy, with results comparable to the ones from mass spectrometry, while not requiring any special sample preparation, apart from the deposition of a drop of solution onto a substrate for subsequent analysis.

Future applications of EVV 2DIR spectroscopy for the study of oxidatively modified proteins can be done via mapping of the spatial distribution of cross-peaks, in order to build chemical maps of samples such as tissue sections.
REFERENCES


APPENDICES

A Publications

A.1 L Rezende Valim et al 2014

Abstract:

Nitration of tyrosine in proteins and peptides is a post-translational modification that occurs under conditions of oxidative stress. It is implicated in a variety of medical conditions, including neurodegenerative and cardiovascular diseases. However, monitoring tyrosine nitration and understanding its role in modifying biological function remains a major challenge. In this work, we investigate the use of electron-vibration-vibration (EVV) two-dimensional infrared (2DIR) spectroscopy for the study of tyrosine nitration in model peptides. We demonstrate the ability of EVV 2DIR spectroscopy to differentiate between the neutral and deprotonated states of 3-nitrotyrosine, and we characterize their spectral signatures using information obtained from quantum chemistry calculations and simulated EVV 2DIR spectra. To test the sensitivity of the technique, we use mixed-peptide samples containing various levels of tyrosine nitration, and we use mass spectrometry to independently verify the level of nitration. We conclude that EVV 2DIR spectroscopy is able to provide detailed spectroscopic information on peptide side-chain modifications and to detect nitration levels down to 1%. We further propose that lower nitration levels could be detected by introducing a resonant Raman probe step to increase the detection sensitivity of EVV 2DIR spectroscopy.
Identification and Relative Quantification of Tyrosine Nitration in a Model Peptide Using Two-Dimensional Infrared Spectroscopy

Lays Rezende Valim†, Julia A. Davies†, Karina Tveen Jensen,‡ Rui Guo,†§ Keith R. Willison,†
Corinne M. Spickett,‡ Andrew R. Pitt,‡ and David R. Klug†,‡

†Department of Chemistry, Imperial College London, London SW7 2AZ, U.K.
‡School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.

Supporting Information

ABSTRACT: Nitration of tyrosine in proteins and peptides is a post-translational modification that occurs under conditions of oxidative stress. It is implicated in a variety of medical conditions, including neurodegenerative and cardiovascular diseases. However, monitoring tyrosine nitration and understanding its role in modifying biological function remains a major challenge. In this work, we investigate the use of electron-vibration-vibration (EVV) two-dimensional infrared (2DIR) spectroscopy for the study of tyrosine nitration in model peptides. We demonstrate the ability of EVV 2DIR spectroscopy to differentiate between the neutral and deprotonated states of 3-nitrotyrosine, and we characterize their spectral signatures using information obtained from quantum chemistry calculations and simulated EVV 2DIR spectra. To test the sensitivity of the technique, we use mixed-peptide samples containing various levels of tyrosine nitration, and we use mass spectrometry to independently verify the level of nitration. We conclude that EVV 2DIR spectroscopy is able to provide detailed spectroscopic information on peptide side-chain modifications and to detect nitration levels down to 1%. We further propose that lower nitration levels could be detected by introducing a resonant Raman probe step to increase the detection sensitivity of EVV 2DIR spectroscopy.

1. INTRODUCTION

Oxidative damage of proteins is involved in mechanisms such as insulin resistance, cell-cycle arrest, senescence, and apoptosis.† Reactive oxygen/nitrogen species (ROS/RNS) such as the superoxide radical (O_2^-) and nitric oxide (NO) can be converted to peroxynitrite (ONOOH/ONOO^-), which is a very strong oxidant. The principal targets of peroxynitrite in proteins are sulhydryl and tyrosyl side chains with nitration of tyrosine (Tyr) to 3-nitrotyrosine (nTyr) being one of the most extensively studied ROS/RNS-driven post-translational modifications (PTMs).†–§

Protein tyrosine nitration is thought to interfere with a range of biochemical processes§ and is known to prevent PTMs such as phosphorylation.♯–¶ Accumulation of nTyr is seen in neurodegenerative diseases,²–⁵ such as amyotrophic lateral sclerosis (ALS), Alzheimer’s disease, and Parkinson’s disease, as well as in chronic inflammation⁶ and cardiovascular disease.⁷–⁹ Conversely, at low NO concentrations, nitration of a specific Tyr residue (Tyr327) has been shown to have a positive regulatory effect through the activation of p53,¹⁰ a tumor-suppressant protein that acts as a transcription factor for various proteins involved in apoptosis and cell cycle arrest.

The main techniques currently employed for quantitative analysis of nTyr are immunooassays and mass spectrometry (MS) coupled to separation techniques, such as two-dimensional electrophoresis and liquid chromatography.⁴,⁵,¹⁴ Also, MS may be used in conjunction with chemical-labeling techniques to achieve higher selectivity and sensitivity.¹⁸ However, MS is a destructive technique that consumes the sample, and it relies on an interpretation of the patterns of ions generated upon fragmentation of the peptide, which can lead to false-positive identifications. Immunoassays depend highly on the quality of the antibodies involved and may suffer from selectivity issues.¹

The use of infrared spectroscopy to study proteins is beneficial because it relies on a sample’s intrinsic vibrational properties, which allow for label-free and nondestructive analysis. However, backbone vibrational modes of proteins lead to very congested one-dimensional IR spectra. Thus, we turn to electron-vibration-vibration (EVV) two-dimensional infrared (2DIR) spectroscopy, a two-dimensional frequency domain technique, which is capable of probing both infrared and Raman transitions.¹⁷ When compared to one-dimensional IR spectroscopy, considerable spectral decongestion is achieved through EVV 2DIR spectroscopy due to its ability to spread information across two dimensions and to probe only coupled vibrational modes. These features make it a valuable technique in the study of complex systems such as proteins.

EVV 2DIR spectroscopy uses one visible/near-IR and two mid-IR picosecond laser beams to induce a nonlinear third-order process in the sample. When the variable mid-IR frequencies are resonant with coupled vibrational modes, a
four-wave mixing (FWM) process results, and the emitted photons are detected via a homodyne scheme, which leads to signal intensities proportional to the square of the number of molecules. The picosecond laser pulse durations are shorter than the vibrational coherence dephasing times, allowing the pulse ordering to be used with variable delays to select specific coherence pathways. By choosing the pulse ordering to follow \( \omega_a \rightarrow \omega_p \rightarrow \omega_a \), we are able to select the nonparametric EVV-IR pathway (Figure 1). The \( \omega_p \) pulse is required to be resonant with an IR-active vibrational mode, \( a \), whereas \( \omega_a \) must be resonant with an IR-active combination band, \( c \), which is equal to \( a + b \) (or an overtone) where \( b \) is a Raman-active mode. The Raman probe step, which is initiated by \( \omega_a \), means that the FWM signal is dependent not only on the vibrational levels of the molecules studied but also on their electronic levels.

\[ \begin{align*}
\text{Figure 1. Schematic of an energy level diagram for the EVV-IR pathway depicting the generation of a signal beam through four-wave mixing of the } \omega_a, \omega_p, \text{ and } \omega_c \text{ laser beams. The time delay between successive laser pulses is typically chosen to be on the order of a few ps. The labels } a, b, \text{ and } c \text{ represent vibrational states, whereas } g \text{ and } e \text{ indicate the ground state and a virtual electronic state, respectively.} 
\end{align*} \]

The results shown in this paper follow a doubly resonant setup, where the IR beams are in resonance with coupled vibrational modes of the molecule, whereas the \( \omega_p \) beam is scattered from a nonresonant (virtual) electronic level. However, it is possible for EVV 2DIR spectroscopy to be used as a triply resonant technique, where an electronic resonance is excited by the \( \omega_a \) beam. This resonant Raman enhancement is known to increase the signal by several orders of magnitude and can greatly improve the detection of vibrational modes coming from low-abundance species in complex samples.

In this paper, we report on the ability of EVV 2DIR spectroscopy to identify tyrosine nitration in model peptides, to quantify nitration levels in peptide mixtures down to 1%, and to differentiate between nTyr residues containing a neutral versus deprotonated hydroxyl group. This proof of concept lays the groundwork for the application of EVV 2DIR spectroscopy to the study of protein tyrosine nitration in complex samples through future implementation of a triply enhanced setup.

2. METHODS

2.1. Peptide Sample Preparation. Lyophilized powders of Ac-SPSYERS-NH\(_2\) and its nitrated version Ac-SPSNsPSYERS-NH\(_2\) (Peptide Protein Research Ltd.) with greater than 98% purity were stored at \(-20^\circ\text{C}\) and allowed to thaw for 1 h before being dissolved in Milli-Q water to achieve a 10 mM concentration. NaOH solutions were used to adjust the pH to 5.6 and 9.1, which was measured using a double junction pH probe. For EVV 2DIR experiments, 1 \( \mu \text{l} \) droplets for each peptide solution were deposited onto glass coverslips and dried under ambient conditions to form gel-phase coffee-ring structures that allow the peptides to remain hydrated. The height of each coffee ring is \( \sim 4\ \mu\text{m} \), which was measured using a stylus profilometer. On the basis of a shift in frequency of the electronic transition of nTyr upon deprotonation, the pH of the solution can be monitored throughout the drying process (neutral nTyr leads to bright yellow solution whereas its charged counterpart is dark orange). Because the dried spots remained the same color as their initial solutions, we can confirm that they retained their protonation state.

2.2. EVV 2DIR Spectroscopy. The experimental setup of EVV 2DIR spectroscopy has been described in detail elsewhere. In brief, an ultrashort Ti:sapphire laser system (Newport Spectra-Physics) is used to generate a near-IR laser beam at 790 nm with a pulse duration of 1 ps and a repetition rate of 1 kHz. The generated beam is split into three beams, two of which are directed toward optical parametric amplifiers (Newport Spectra-Physics) to generate 1 ps pulses with tunable mid-IR frequencies calibrated using a 38 \( \mu\text{m} \) thick polystyrene calibration film traceable to NIST 1921b frequencies. All three beams are linearly polarized parallel to the plane of propagation (PPP), spatially overlapped, and focused at the sample. The pulse energy of the near-IR beam is set to 200 nJ at the sample, and the mid-IR beams display near-symmetric energy profiles across an \( \omega_p/2\pi \) range from 1300 to 1700 cm\(^{-1}\) (\( E_{\text{max}} = 800 \text{ nJ at 1500 cm}^{-1} \)) and an \( \omega_a/2\pi \) range from 2600 to 3400 cm\(^{-1}\) (\( E_{\text{max}} = 2.0 \text{ \mu J at 3000 cm}^{-1} \)). These pulse energies are measured through a 100 \( \mu\text{m} \) diameter pinhole because it closely matches the 1/e\(^2\) diameter of the near-IR beam, which defines the sample area from which the FWM signal is generated. Delay stages are used to adjust the variable delays between the laser pulses, thus controlling their arrival times to the order \( \omega_a \rightarrow \omega_p \rightarrow \omega_a \), which leads to the coherence pathway described in Figure 1. The emitted signal is monitored using a photo-multiplier detector (Hamamatsu H7422-50).

Prior to measuring spectra, the system is enclosed and purged with \( \text{N}_2 \) to reduce the humidity level to less than 3%. Unless otherwise stated, EVV 2DIR spectra were obtained using pulse time delays of \( \tau_{\text{ar}} = 1.75 \text{ ps } \) and \( \tau_{\text{rp}} = 1 \text{ ps } \), a mid-IR frequency step size of 5 cm\(^{-1}\), and an acquisition rate of 100 laser shots per data point. The FWM signal intensity was normalized to the methylene cross-peak at 1465/2925 cm\(^{-1}\) and smoothed using the shrinking/expanding algorithm in OriginPro (OriginLab Co.). The data were then plotted as a contour map with a logarithmic scale.

2.3. DFT Calculations and EVV 2DIR Simulations. As previously described, GAUSSIAN 03 is used to perform DFT calculations at B3LYP/6-311+G(\(\text{p,d}\)) level of theory, and a scaling factor of 0.98 is applied to the resulting vibrational frequencies. The theoretical EVV 2DIR signal intensity corresponding to the pathway shown in Figure 1 was numerically calculated as a function of the IR frequencies.
employing the method reported by Kwak et al. The resulting simulated EVV 2DIR spectra were produced by representing each cross-peak as a 2D Gaussian function with a full width at half maximum (fwhm) of 20 cm$^{-1}$ and a center frequency matching the harmonic frequencies of the bands involved.

2.4. Liquid Chromatography-Mass Spectrometry. The peptide mixtures were diluted to 4 µM and separated on an Ultimate 3000 liquid chromatography system (Dionex, Camberley, U.K.) interfaced with a 5600 TripleTOF mass spectrometer (ABSciex, Warrington, U.K.) via a New Objective
of 11 CH\textsubscript{2} groups: one for each Ser residue, three for each Pro residue, and one for each Tyr or nTyr residue. In previous work by our group, ratios of the side-chain cross-peak intensities with respect to an internal reference were determined in which the internal reference was either the CH\textsubscript{2} or CH\textsubscript{3} cross-peak intensity. Because the peptides used in the present work contain only a single CH\textsubscript{2} group, which is in the acetyl group of the N-terminal end cap, the CH\textsubscript{3} cross-peak intensity is used as the internal reference here.

The elongated diagonal feature centered at ~1640/3320 cm\textsuperscript{-1} is present in the spectra of all three peptides (circled in Figure 2a c) and can be assigned to the amide I band fundamental and its overtone. This band is predominantly associated with the backbone C=O stretch mode, rendering it sensitive to the peptide’s secondary structure and its environment. The amide I band has been extensively studied using conventional 2DIR spectroscopic techniques to obtain information on the conformation and aggregation of peptides and proteins. However, in the present work, the experimental conditions have been optimized to directly probe vibrational modes associated with the Tyr and nTyr side chains rather than the amide I band. This has resulted in an optical density of ~1 for the amide I band, which is too high for detailed analysis and interpretation of the band structure; thus, only a basic discussion of the substructure will be provided here. In Figure 2a c, component peaks are observed at approximately 1620/3240, 1635/3270, and 1660/3320 cm\textsuperscript{-1}, which arise from structural inhomogeneity in the sample. On the basis of extensive literature describing amide I substructures, we can assign the highest frequency component at 1660/3320 cm\textsuperscript{-1} to either a short \( \alpha \) helix or a disordered structure, whereas the component that appears at ~1635/3270 cm\textsuperscript{-1} can be assigned to \( \beta \) strands. The third component at 1620/3240 cm\textsuperscript{-1} is in close agreement with frequencies reported to arise from a red-shift of the \( \beta \)-strand component due to proline residues. The relative intensities of these three frequency components vary dramatically among the spectra shown in Figure 2a c, suggesting that the peptide conformation and/or aggregation changes upon tyrosine nitration and is also dependent on the protonation state. However, we refrain from drawing any further conclusions because of the high optical density for the amide I band, as discussed above.

A cross-peak at 1710/3120 cm\textsuperscript{-1} is observed in the spectra for the neutral and deprotonated states of the nTyr peptide (Figure 2b and c) and is assigned to the C=O stretch of the acetyl end cap on the N-terminus of each peptide. The deprotonated nTyr side chain also contains a C=O group; however, the quantum chemistry calculations discussed in section 3.2 and in the Supporting Information indicate that the frequency of this peak is much lower than 1710 cm\textsuperscript{-1}, possibly due to the negative charge that results from deprotonation. The intensity of the peak at 1710/3120 cm\textsuperscript{-1} is ~4 times weaker for the Tyr peptide and is not visible in Figure 2a due to the lower limit used for the intensity range, which has been chosen so that the strong features are more easily discernible. In our experience, the appearance of CH\textsubscript{2} amide I, and C=O stretch cross-peaks in 2DIR spectra is typical for peptides and proteins.

The main differences between the spectra shown in Figure 2a c are the presence or absence of cross-peaks associated with the side chains of Tyr (Figure 2a), neutral nTyr (Figure 2b), and deprotonated nTyr (Figure 2c). It is interesting to note that the spectral fingerprint is very different for each of the
three peptide samples. There are five obvious cross-peaks in Figure 2a that can be assigned to vibrational modes of the Tyr side chain. In order of decreasing intensity, the frequencies of these peaks are 1600/3190, 1520/3130, 1595/3025, 1520/3000, and 1615/2810 cm⁻¹. In previous work using EV2DIR spectroscopy, two Tyr cross-peaks were observed in the spectra of various peptides containing Tyr residues.12,13 A very weak peak was observed at 1470/3040 cm⁻¹, and a strong peak was observed at 1530/3130 cm⁻¹. The latter peak was assigned to ν15/(ν13 + ν16), where ν13 is an aromatic stretching mode, and ν16 is a CH₂ deformation mode on the Tyr side chain. Assignments of the remaining cross-peaks for Tyr and nTyr residues will be based on DFT calculations and simulated EV2DIR spectra.

3.2. Spectral Characterization. For the purpose of characterizing the experimental EV2DIR spectra presented in Figure 2a–c, EV2DIR spectra have been simulated for 4MP and 4M2NP, and the results are presented in Figure 2d–f. These molecules are side-chain analogues of Tyr and nTyr residues. Each simulated spectrum is placed directly below the corresponding experimental spectrum to aid comparison. The correlation between the relative intensities of the cross-peaks for each pair of spectra is not expected to be strong because the simulations do not consider the variable lifetimes of the coherences excited by the IR laser pulses. In addition, the incident laser intensity at the peptide sample varies with the tunable IR frequency for both the ωᵣ and ωₛ beams. However, the frequencies of the cross-peaks are expected to agree to within 50 cm⁻¹ in most cases.

Before comparing the cross-peak frequencies between the simulated and experimental spectra in more detail, we first discuss the differences observed for the three simulated spectra only. In this case, we can compare the intensities because the method used to perform the simulations is the same for all three spectra. From Figure 2d–f, it is clear that the maximum signal intensity calculated for these side-chain analogues varies greatly; the signals from the deprotonated and neutral states of 4M2NP are 14 and 229 times higher, respectively, than that for neutral 4MP. In other words, the simulated spectra predict that the experimental signal intensity from nTyr residues will be...
substantially higher than that from Tyr residues. Another observation from the simulated spectra is that there are substantially larger numbers of cross-peaks observed in both of the 4M2NP spectra than in the 4MP spectrum. The same observation is made in the experimental spectra (Figure 2a–c).

Tables 1, 2, and 3 list the assignments and vibrational frequencies for a selection of cross-peaks observed in the experimental EVV 2DIR spectra; there are five peaks for Tyr, ten peaks for neutral nTyr, and ten peaks for deprotonated nTyr, each of which is given a unique peak label for easy identification. Each cross-peak assignment consists of two parts; the first corresponds to the mode description for the fundamental band excited by the first mid-IR laser pulse, and the second gives an assignment for the combination band or overtone excited by the second mid-IR laser pulse. For aromatic modes, a descriptive assignment is given along with a numerical label in parentheses, which follows Varanyi notation. This notation has been chosen because it extends the Wilson vibrational mode numbering system for benzene to cover more than 700 benzene derivatives, and it provides general rules for the assignment of additional benzene derivatives based on the anticipated frequency and vibrational motion of each mode, thus removing some of the ambiguity often associated with mode assignment using alternative schemes. Details of the assignments for the fundamental modes are given in the Supporting Information, along with a comparison of the calculated and experimental frequencies for each mode (Tables S1, S2, and S3).

The correlation between the cross-peaks in the experimental and simulated spectra is indicated by the peak labels shown in Figure 2 and Tables 1–3. For the Tyr peptide, three of the five cross-peaks observed in the experimental spectrum (Figure 2a) correlated with cross-peaks in the simulated spectrum (Figure 2d); these peaks are labeled Tyr2, Tyr4, and Tyr5. Upon initial inspection of the spectra, one might presume that the simulated cross-peak at 1623/3011 cm⁻¹ correlates with the experimental cross-peak at 1585/3025 cm⁻¹ (Tyr3). However, the simulated cross-peak, which is assigned to CC stretch (8a)/CC stretch (8a) + C=O umbrella, is an artifact because the side-chain analogue contains a CH₃ group that is not present in the Tyr residue. This is also true for the series of four simulated cross-peaks observed between 1388/2776 and 1514/2902 cm⁻¹ in Figure 2d. The assignments for all five of the Tyr cross-peaks observed in the experimental spectrum are presented in Table 1.

A good understanding of the spectral signature for nTyr in both protonation states is indicated by the substantial number of cross-peaks that have been successfully correlated and assigned in Tables 2 and 3; these correlations are also indicated in Figure 2. For the protonated nTyr peptide, the two most intense cross-peaks observed in the experimental spectrum have frequencies of 1545/2900 and 1540/3080 cm⁻¹ and are labeled nTyr6 and nTyr7, respectively, in Figure 2b and Table 2. The first component of these cross-peaks (~1545 cm⁻¹) is assigned to the asymmetric NO₃ stretch vibration, indicating that these spectral features can provide information relating to the site of the peptide modification. Three less intense cross-peaks at 1430/2965, 1455/2795, and 1545/2970 cm⁻¹ (nTyr2, nTyr5, and nTyr7, respectively) also involve excitation of the asymmetric NO₃ stretch mode. In contrast, the cross-peaks at 1630/3205 and 1635/2980 cm⁻¹ (nTyr9 and nTyr10, respectively) are associated with a CC stretch (8a) aromatic mode, which is the mode responsible for the most intense peak in the unmodified Tyr peptide spectrum at 1600/3190 cm⁻¹ (Tyr4 in Table 1 and Figure 2a).

In the experimental spectrum for the deprotonated nTyr peptide, there are two cross-peaks at 1415/2745 and 1420/2845 cm⁻¹ that have fundamental frequencies assigned to the asymmetric NO₃ stretch mode, and these peaks are labeled nTyr3 and nTyr4, respectively, in Figure 2c and Table 3. However, the most intense cross-peak is at 1545/3085 cm⁻¹ (nTyr8) and is assigned to the fundamental and overtone of the CO stretch mode. As revealed in Table S3 in the Supporting Information, the CO stretch mode contains significant asymmetric NO₃ stretch motion as well as stretching motion along the CO bond. It is also interesting to note that half of the cross-peaks in Table 3 (nTyr'1, nTyr'3, nTyr'5, nTyr'7, and nTyr'9) have combination bands containing the CC stretch (14) mode, which can be explained by the very high Raman intensity calculated for this mode (Supporting Information). The detailed spectral characterization that we have achieved could potentially be used to interpret changes in EVV 2DIR spectra that result from an altered environment around nTyr residues when studied under complex biological conditions.

3.3. Experimental EVV 2DIR Spectra for Peptide Mixtures

To ascertain whether EVV 2DIR spectroscopy could be used to determine the percentage of tyrosine that is nitrated in a peptide or protein sample, it is first necessary to determine the relationship between the relative intensities of the Tyr and nTyr cross-peaks and the percentage of nitration. This characterization can be performed using samples containing known proportions of Tyr and nTyr residues; therefore, EVV 2DIR spectra have been measured for five different peptide mixtures containing 0, 25, 50, 75, and 100% of Tyr heptamer Ac-SPSPrYYS-YILy with the non-nitrated version constituting the remainder of the peptide mixture. The resulting spectra measured at pH 5.6 are presented in Figure 3a–e. The spectrum at 0% nTyr (Figure 3a) displays the spectral signature for Tyr residues and is similar to that shown in Figure 2a, which was measured for a Tyr peptide sample at pH 9.1. This similarity is expected because the Tyr side chain is neutral at both pHs. The 50% nTyr mixture contains equal amounts of the nTyr and Tyr peptides; therefore, this spectrum (Figure 3c) provides a simple indication of whether the signal is more intense from a Tyr or an nTyr residue. In this case, the Tyr peak at 1600/3190 cm⁻¹ is the most intense. This contradicts the results from the simulations, which incorrectly predicted that the signal from neutral nTyr would be ~14 times higher than that from Tyr. This discrepancy is most likely due to inaccuracies in the quantum chemistry calculations caused by the use of a simplified molecular model that neglects the effects of hydrogen bonding due to water solvation and peptide interactions.

As discussed in section 1, a homodyne-detected signal is produced by a FWM process in EVV 2DIR experiments; therefore, the measured signal intensity is proportional to the square of the number of peptides that contribute to the signal. Thus, for the mixed-peptide samples at pH 5.6, the square root of the signal intensity has been plotted against the percentage of Tyr peptides for various Tyr and nTyr cross-peaks, and the results are shown in Figure 3f. As expected, the signal intensity of the Tyr cross-peak at 1600/3190 cm⁻¹ (Tyr4) decreases as the percentage of nTyr peptide increases, whereas the nTyr cross-peaks at 1540/3080, 1545/2900, and 1635/2980 cm⁻¹ (nTyr6, nTyr9, and nTyr10, respectively) show an increase in
signal intensity. The solid lines represent linear regressions and indicate a good linear correlation between the square root of the signal intensity and the percentage of nTyr with $R^2$ values between 0.973 and 0.995. From these linear regressions, the ratio of intensities for a chosen nTyr peak with respect to a Tyr peak can be interpolated at any percentage of nTyr. This provides our calibration curve, such that the percentage of nitration in a wide variety of samples can be uniquely determined from a measurement of the relative intensities of a pair of nTyr and Tyr cross-peaks in the EVV 2DIR spectrum. In cases where the spectrum of a sample is particularly congested, measuring the relative intensities of more than two nTyr and Tyr peaks may be preferred.

For the 25% nTyr peptide mixture at pH 5.6 used in the present work, the EVV 2DIR spectrum (Figure 3b) reveals that two nTyr cross-peaks at 1540/2900 and 1540/3080 cm$^{-1}$ are easily observable, indicating that the detection limit for tyrosine nitration is well below 25%. A more accurate estimation can be determined from the plot in Figure 3f by taking the difference between the $y$-value for a data point and the $y$-value predicted by the linear regression for a chosen nTyr cross-peak for both the 0 and 25% nTyr peptide mixtures. The sum of these differences, which indicates the uncertainty in the cross-peak intensities, is converted to a percent nTyr value using the slope of the linear regression. An average value for all three nTyr peaks in Figure 3f provides a conservative estimate of ~12% as the detection threshold level for tyrosine nitration. Even though a sensitivity limit of ~12% may seem high, the potential for substantial improvement only requires using a resonant Raman probe step as mentioned in section 1. Furthermore, the advantage of this technique is that it does not require any background subtraction or deconvolution of the spectral features, making data analysis unambiguous and prompt.

Spectra measured for the peptide mixtures at pH 9.1 for the deprotonated state of nTyr are presented in Figure 4. The 50% nTyr spectrum (Figure 4c) reveals that the nTyr peak at 1545/3085 cm$^{-1}$ (nTyr*8) is more intense than the Tyr peak at 1600/3190 cm$^{-1}$ (Tyr4), indicating that the signal from deprotonated nTyr is stronger than that from non-nitrated Tyr. The relative intensities of the nTyr and Tyr cross-peaks in the 25% nTyr spectrum are significantly higher for the deprotonated sample (Figure 4b) than for the neutral sample (Figure 3b); therefore, the detection sensitivity for Tyr nitration is expected to be higher for the deprotonated nTyr sample also. Furthermore, the measured $R^2$ values of greater than 0.984 for all four of the nTyr cross-peaks (nTyr*1, nTyr*2, nTyr*8, and nTyr*9) indicate good linear correlation between the square root of the signal intensity and the percentage of
deprotonated nTyr. From the data presented in Figure 4f and using the analysis described above for the pH 5.6 sample, we initially estimate a lower limit of 3% tyrosine nitration for the deprotonated state.

Spectra have also been measured for mixed-peptide samples containing 10 and 5% of the deprotonated nTyr peptide at pH 9.1 and are presented in Figure 5a and b, respectively. Because the relative intensities of the Tyr and nTyr peaks are so large at such low percentages of nTyr, the data have been plotted over a small frequency range so as to exclude the most intense Tyr peaks. In the 9% nTyr spectrum, the nTyr peak at 1545/3085 cm\(^{-1}\) is clearly visible, but in the 5% spectrum, this peak is barely discernible above the signal from neighboring features. The nTyr spectral signature is more clearly observed in the line profiles shown in Figure 5c for the 10 and 5% nTyr samples; these have been measured with the \(\omega_p/2\zeta c\) frequency fixed at 1545 cm\(^{-1}\) on the center of the cross-peak, whereas the \(\omega_p/2\zeta c\) frequency is varied across the peak. The peaks centered at 3085 and 3132 cm\(^{-1}\) are assigned to nTyr and Tyr, respectively. The data presented in Figure 5c are the average of at least three line profiles, each obtained using longer data acquisition times per point than for the spectra. Furthermore, a step size of 2 cm\(^{-1}\) has been used for the profile measurements compared to 5 cm\(^{-1}\) for the spectra, and a 5-point smoothing function has been applied. The consequence of using this alternative method is that the nTyr\(^+8\) peak at 3085 cm\(^{-1}\) is clearly discernible above the underlying signal in the line profiles for both the 10 and 5% samples.

The nTyr peak intensity values measured at 3085 cm\(^{-1}\) in the line profiles shown in Figure 5c were compared to the linear regression in Figure 4f for the 1545/3085 cm\(^{-1}\) peak (nTyr\(^+8\)). This analysis yielded nTyr percentage values of 11 and 6%, which are in close agreement with the known values of 10 and 5% nTyr, respectively. On the basis of the low noise level observed for the line profiles in Figure 5c, and the clear separation observed between the profiles for the 5 and 0% samples, we estimate a lower detection limit of 1% for deprotonated nTyr, and we infer a lower detection limit of 4% for neutral nTyr.

An advantage of using EVV 2DIR spectroscopy is that it can also distinguish between the neutral and deprotonated states of nTyr. Therefore, this technique can potentially be used to
detect the level of tyrosine nitration in biological samples in their native states and to determine the relative abundances of the two protonation states, which will also provide an indication of the sample pH. Finally, potentially significant improvement in the sensitivity limit of EVV 2DIR can be had by introducing a resonant Raman probe step using a UV wavelength that is resonant with an electronic excitation level in nTyr such that it selectively enhances the signal level from nTyr residues. This could increase the signal level by several orders of magnitude, which is anticipated to improve the detection limit for nTyr to a value substantially lower than 1%.

3.4. Mass Spectrometry of Tyr/nTyr Peptide Mixtures. For comparison with the EVV 2DIR quantification of tyrosine nitration, relative quantification was also performed by liquid chromatography-mass spectrometry (LC-MS). Small changes in the peptide structures, such as replacement of Tyr with nTyr, can result in significantly different signal intensities in LC-MS depending on a number of physicochemical properties of the peptides, the most important being their ionization efficiencies. To allow for this, accurately prepared solutions of pure peptides were used to generate a correction factor for relative quantitative analysis of the mixtures. Signals for the 1+ and 2+ ions, and their respective adducts with sodium, were observed in the spectra, and the sum of the areas of the peaks in the extracted ion chromatograms (XICs) for all of these species was used in the quantification (Figure 6). The nTyr peptide signal was observed to be approximately 2.6-fold more intense than that of the Tyr-containing peptide, and this factor was used to correct for the relative intensities in the LC-MS profile of the peptide mixtures.

Quantitative mass spectrometric analysis of the peptide mixtures based on extracted ion chromatograms gave ratios of 5.4 and 12.1% for the 5 and 10% mixtures, respectively. This is in reasonably good agreement with the values of 6 and 11%, respectively, obtained using EVV 2DIR spectroscopy. It is interesting to note that the values obtained using both LC-MS and EVV 2DIR are consistently higher than the "known" nTyr values of 5 and 10%, which may indicate that these particular peptide samples have a higher proportion of nTyr than anticipated. This is highly plausible because of difficulties in handling small volumes of nTyr peptide solutions that are required for preparing low-percentage nTyr samples.

4. CONCLUSIONS

We have demonstrated the ability of EVV 2DIR spectroscopy to identify tyrosine nitration in short peptide models and to differentiate between the neutral and deprotonated states of nTyr. Detailed characterization of the experimental spectra has been carried out using the results of quantum chemistry calculations and by comparison with simulated EVV 2DIR spectra. Spectra have been measured for mixed-peptide samples containing levels of tyrosine nitration down to 5%. Following optimization of our data collection procedure, we were able to deduce nitration levels of 6 and 11% for selected mixed-peptide samples, which is in close agreement with the known values of 5 and 10%, respectively. Mass spectrometry was used to independently verify the level of nitration, yielding values of 5.4 and 12.1%. Interpolation of our EVV 2DIR line profiles indicates that the detection limits for tyrosine nitration are 1 and 4% for the deprotonated and neutral states of nTyr, respectively. We propose that this technique has the potential to detect low levels of protein tyrosine nitration in biological samples following the introduction of a resonant Raman probe step to increase the detection sensitivity. In summary, EVV 2DIR spectroscopy is able to provide detailed spectroscopic information on peptide side-chain modifications and to detect tyrosine nitration levels down to 1%.

ASSOCIATED CONTENT

Supporting Information

Results obtained from quantum chemistry calculations are provided for side-chain analogues of Tyr, neutral nTyr, and...
deprotonated nTyr. The calculated normal modes are assigned, and the vibrational frequencies are compared with experimental values determined from EVV 2DIR spectra of model peptides (Tables S1, S2, and S3). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION
Corresponding Author
E-mail: d.klug@imperial.ac.uk. Phone: (+44) 207 594 5806. Fax: (+44) 207 594 5880.

Present Address
R.G.: Department of Chemistry, University College London, 20 Gordon St., London WC1H 0AJ, U.K.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
This work has been supported by the Proxomics project funded by the U.K. Engineering and Physical Sciences Research Council (EPSRC) (Grant EP/I017857/1). L.R.V. acknowledges additional support from the U.K. EPSRC through a doctoral training grant studentship (EP/J500239/1).

■ REFERENCES
Supporting Information for:

Identification and Relative Quantification of Tyrosine Nitration in a Model Peptide Using Two-Dimensional Infrared Spectroscopy

Lays Rezende Valim, Julia A. Davies, Karina Tveen Jensen, Rui Guo, Keith R. Willison, Corinne M. Spickett, Andrew R. Pitt, David R. Klug

1Department of Chemistry, Imperial College London, London SW7 2AZ, UK.

2School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK.

† Currently at Department of Chemistry, University College London. London WC1H 0AJ.

* To whom correspondence should be addressed. Phone: (+44) 207 594 5806; fax: (+44) 207 594 5880; e-mail: d.klug@imperial.ac.uk.
Vibrational frequencies have been calculated for neutral 4-methylphenol (4MP), neutral 4-methyl-2-nitrophenol (4M2NP) and charged 4M2NP, where 4MP and 4M2NP are considered to be side-chain analogues for Tyr and nTyr residues in a model peptide. These calculations were carried out using Gaussian 03 at the B3LYP/6-311++G(d,p) level of theory and a scaling factor of 0.98 was applied. The calculated frequencies of fundamental vibrational modes that lie within the range from 1150 to 1750 cm\(^{-1}\) are presented in Tables S1, S2 and S3, along with the calculated infrared and Raman intensities, and vibrational assignments. A number of the calculated normal modes for 4M2NP exhibit substantial motion in different parts of the molecule such that two assignments are provided, where the primary assignment represents the dominant motion. For aromatic modes, a descriptive assignment is given along with a numerical label in parentheses that follows the Varsanyi notation for benzene derivatives, which is based on the Wilson numbering scheme for benzene.

Experimental frequencies have been obtained from electron-vibration-vibration two-dimensional infrared (EVV 2DIR) spectra of model peptides, Ac-SPSYSPS-NH\(_2\) and Ac-SPSnYSPS-NH\(_2\) at pH 5.6 and pH 9.1. The Tyr residue is neutral at both pHs, whereas the nTyr residue is neutral at pH 5.6 and deprotonated at pH 9.1. Cross-peaks in the EVV-2DIR spectra contain two frequency components; the first is resonant with a fundamental mode whilst the second is resonant with a combination band or overtone. Wherever possible the experimental frequency values given in Tables S1, S2 and S3 have been taken directly from the first component of the cross-peak specified in the right hand column of the table, otherwise they are taken from the frequency difference between the first and second components of the cross-peak. The errors in experimental frequency values are ±10 cm\(^{-1}\) for the former case and ±20 cm\(^{-1}\) for the latter due to the effects of anharmonicity.

When assigning the experimental frequencies to particular vibrational modes, it was necessary to not only select a mode that had a similar calculated frequency, but to also consider the correlation between cross-peak intensities in the experimental and simulated 2DIR spectra. For example, assignment of the 1600 cm\(^{-1}\) experimental frequency to the CC stretch (8a) mode leads to favorable
correlation between an intense experimental cross-peak at 1600/3190 cm\(^{-1}\) and an intense simulated cross-peak at 1623/3246 cm\(^{-1}\). In contrast, assignment of the 1600 cm\(^{-1}\) experimental frequency to the CC stretch (8b) mode would lead to poor correlation, i.e. an intense experimental cross-peak and an extremely weak simulated cross-peak. Experimental frequencies are not given for the CH\(_3\) modes listed in Tables S1-S3 because the CH\(_3\) group is a feature of the side-chain analogues only and is not present in the Tyr and nTyr sidechains. Good agreement is observed between the experimental and calculated vibrational frequency values listed in Tables S1, S2 and S3, and is typically within ±20 cm\(^{-1}\).
Table S1. Comparison of calculated vibrational frequencies for a tyrosine side-chain analogue (4-methylphenol) and experimental vibrational frequencies measured using EVV 2DIR spectroscopy for a heptamer peptide containing a Tyr residue.

<table>
<thead>
<tr>
<th>calculated frequency (cm(^{-1}))</th>
<th>calculated infrared intensity</th>
<th>calculated Raman intensity</th>
<th>experimental frequency (cm(^{-1}))</th>
<th>assignment(^a)</th>
<th>2DIR cross-peak frequency (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1163</td>
<td>164</td>
<td>8</td>
<td>1195(^b)</td>
<td>COH bend</td>
<td>1615/2810</td>
</tr>
<tr>
<td>1171</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>CH rock (9a)</td>
<td>-</td>
</tr>
<tr>
<td>1205</td>
<td>5</td>
<td>14</td>
<td>1215(^b)</td>
<td>CC stretch (13)</td>
<td>1520/2735</td>
</tr>
<tr>
<td>1250</td>
<td>98</td>
<td>15</td>
<td>1280(^b)</td>
<td>C-OH stretch (7a)</td>
<td>1520/2800</td>
</tr>
<tr>
<td>1305</td>
<td>3</td>
<td>2</td>
<td>1300(^b)</td>
<td>CC stretch (14)</td>
<td>1610/2910</td>
</tr>
<tr>
<td>1332</td>
<td>29</td>
<td>2</td>
<td>1325(^b)</td>
<td>CH rock (3)</td>
<td>1610/2935</td>
</tr>
<tr>
<td>1388</td>
<td>0</td>
<td>18</td>
<td>-</td>
<td>CH(_3) umbrella</td>
<td>-</td>
</tr>
<tr>
<td>1426</td>
<td>10</td>
<td>1</td>
<td>1445(^c)</td>
<td>CC stretch (19b)</td>
<td>1445/3045</td>
</tr>
<tr>
<td>1457</td>
<td>7</td>
<td>10</td>
<td>-</td>
<td>asymm CH(_3) bend</td>
<td>-</td>
</tr>
<tr>
<td>1472</td>
<td>11</td>
<td>8</td>
<td>-</td>
<td>asymm CH(_3) bend</td>
<td>-</td>
</tr>
<tr>
<td>1514</td>
<td>112</td>
<td>1</td>
<td>1520(^c)</td>
<td>CH rock (19a)</td>
<td>1520/3130</td>
</tr>
<tr>
<td>1599</td>
<td>17</td>
<td>6</td>
<td>1615(^c)</td>
<td>CC stretch (8b)</td>
<td>1615/2810</td>
</tr>
<tr>
<td>1623</td>
<td>30</td>
<td>28</td>
<td>1600(^c)</td>
<td>CC stretch (8a)</td>
<td>1600/3190</td>
</tr>
</tbody>
</table>

\(^a\) Numbers in parentheses are aromatic mode assignments in Varsanyi notation.

\(^b\) Determined from difference between frequency components for a 2DIR cross-peak.

\(^c\) Determined from first frequency component of a given 2DIR cross-peak.
Table S2. Comparison of calculated vibrational frequencies for a protonated nitrotyrosine side-chain analogue (4-methyl-2-nitropheno1) and experimental vibrational frequencies measured using EVV 2DIR spectroscopy for a heptamer peptide containing a nTyr residue at pH 5.6.

<table>
<thead>
<tr>
<th>calculated frequency (cm(^{-1}))</th>
<th>calculated infrared intensity</th>
<th>calculated Raman intensity</th>
<th>experimental frequency (cm(^{-1}))</th>
<th>primary assignment(^a)</th>
<th>additional assignment(^a)</th>
<th>2DIR cross-peak frequency (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1192</td>
<td>69</td>
<td>4</td>
<td>-</td>
<td>CH rock (9a)</td>
<td>COH bend</td>
<td>-</td>
</tr>
<tr>
<td>1220</td>
<td>131</td>
<td>120</td>
<td>1210(^b)</td>
<td>CC stretch (7a)</td>
<td>COH bend</td>
<td>1630/2840</td>
</tr>
<tr>
<td>1258</td>
<td>137</td>
<td>48</td>
<td>1250(^b)</td>
<td>symm NO(_2) stretch</td>
<td>CC stretch (13)</td>
<td>1545/2795</td>
</tr>
<tr>
<td>1290</td>
<td>221</td>
<td>103</td>
<td>-</td>
<td>COH bend</td>
<td>asymm CNO stretch</td>
<td>-</td>
</tr>
<tr>
<td>1331</td>
<td>94</td>
<td>1</td>
<td>1320(^b)</td>
<td>CH rock (3)</td>
<td>symm NO(_2) stretch</td>
<td>1540/2860</td>
</tr>
<tr>
<td>1381</td>
<td>29</td>
<td>56</td>
<td>1360(^c)</td>
<td>CC stretch (14)</td>
<td>COH bend</td>
<td>1360/2705</td>
</tr>
<tr>
<td>1392</td>
<td>2</td>
<td>15</td>
<td>-</td>
<td>CH(_3) umbrella</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1419</td>
<td>139</td>
<td>9</td>
<td>1430(^c)</td>
<td>CC stretch (19b)</td>
<td>COH bend</td>
<td>1430/2850</td>
</tr>
<tr>
<td>1457</td>
<td>8</td>
<td>10</td>
<td>-</td>
<td>asymm CH(_3) bend</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1469</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>asymm CH(_3) bend</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1494</td>
<td>121</td>
<td>8</td>
<td>1490(^c)</td>
<td>CH rock (19a)</td>
<td>-</td>
<td>1490/3070</td>
</tr>
<tr>
<td>1550</td>
<td>274</td>
<td>37</td>
<td>1545(^c)</td>
<td>asymm NO(_2) stretch</td>
<td>-</td>
<td>1545/2900</td>
</tr>
<tr>
<td>1584</td>
<td>42</td>
<td>33</td>
<td>1585(^c)</td>
<td>CC stretch (8b)</td>
<td>COH bend</td>
<td>1585/3165</td>
</tr>
<tr>
<td>1638</td>
<td>70</td>
<td>8</td>
<td>1635(^c)</td>
<td>CC stretch (8a)</td>
<td>-</td>
<td>1635/2980</td>
</tr>
</tbody>
</table>

\(^a\) Numbers in parentheses are aromatic mode assignments in Varsanyi notation

\(^b\) Determined from the difference between frequency components for a 2DIR cross-peak.

\(^c\) Determined from the first frequency value of the given 2DIR cross-peak.
Table S3. Comparison of calculated vibrational frequencies for a deprotonated nitrotyrosine side-chain analogue (4-methyl-2-nitrophenol) and experimental vibrational frequencies measured using EVV 2DIR spectroscopy for a heptamer peptide containing a nTyr residue at pH 9.1.

<table>
<thead>
<tr>
<th>calculated frequency (cm⁻¹)</th>
<th>calculated infrared intensity</th>
<th>calculated Raman intensity</th>
<th>experimental frequency (cm⁻¹)</th>
<th>primary assignment⁷</th>
<th>additional assignment⁷</th>
<th>2DIR cross-peak frequency (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1167</td>
<td>67</td>
<td>25</td>
<td>-</td>
<td>CH rock (9a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1225</td>
<td>1</td>
<td>11</td>
<td>-</td>
<td>CC stretch (13)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1242</td>
<td>873</td>
<td>46</td>
<td>1250ᵇ</td>
<td>symm NO₂ stretch</td>
<td>-</td>
<td>1415/2665</td>
</tr>
<tr>
<td>1304</td>
<td>166</td>
<td>154</td>
<td>1340ᶜ</td>
<td>CC stretch (14)</td>
<td>CN stretch</td>
<td>1340/2970</td>
</tr>
<tr>
<td>1361</td>
<td>10</td>
<td>17</td>
<td>1375ᶜ</td>
<td>CH rock (3)</td>
<td>-</td>
<td>1375/2910</td>
</tr>
<tr>
<td>1377</td>
<td>6</td>
<td>48</td>
<td>-</td>
<td>CH₃ umbrella</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1421</td>
<td>23</td>
<td>38</td>
<td>1445ᶜᵈ</td>
<td>CH rock (19a)</td>
<td>-</td>
<td>1445/2770</td>
</tr>
<tr>
<td>1446</td>
<td>83</td>
<td>19</td>
<td>1415ᶜᵈ</td>
<td>asymm NO₂ stretch</td>
<td>CC stretch (19b)</td>
<td>1415/2745</td>
</tr>
<tr>
<td>1451</td>
<td>4</td>
<td>15</td>
<td>-</td>
<td>asymm CH₃ bend</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1465</td>
<td>3</td>
<td>69</td>
<td>-</td>
<td>asymm CHI₃ bend</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1507</td>
<td>61</td>
<td>39</td>
<td>1510ᶜ</td>
<td>CC stretch (8b)</td>
<td>-</td>
<td>1510/2845</td>
</tr>
<tr>
<td>1575</td>
<td>437</td>
<td>42</td>
<td>1545ᶜ</td>
<td>CO stretch</td>
<td>asymm NO₂ stretch</td>
<td>1545/3085</td>
</tr>
<tr>
<td>1626</td>
<td>227</td>
<td>43</td>
<td>1625ᶜ</td>
<td>CC stretch (8a)</td>
<td>CO stretch</td>
<td>1625/2965</td>
</tr>
</tbody>
</table>

⁷ Numbers in parentheses are aromatic mode assignments in Varsanyi notation.

ᵇ Determined from the difference between frequency components for a 2DIR cross-peak.

ᶜ Determined from the first frequency value of the given 2DIR cross-peak.

ᵈ These have been assigned with reference to the simulated 2DIR spectra and are tentative.
A.2 Conference abstract

Society for Free Radical Research International 16th Biennial Meeting

Keywords: Mass spectrometry, Protein adduct, Phospholipase A2, Oxidised phospholipid
doi:10.1016/j.freeradbiomed.2012.08.447

[0596]

Study of protein-tyrosine nitration by two-dimensional infrared vibrational spectroscopy

Imperial College London, UK

ROS- and RNS-mediated mechanisms have been suggested to play an important role in health and disease of various organisms, including mammals. Oxidatively damaged proteins are known to become more resistant to proteolytic degradation and to impact mechanisms such as insulin-resistance, cell-cycle arrest, senescence and apoptosis [1,2]. Additionally, conformational changes arising from protein-tyrosine nitration could prevent certain post-translational modifications such as phosphorylation and nucleotidylation from taking place [3].

Unfortunately, a better understanding of the mechanisms involved in oxidative damage of proteins in vivo is hampered by difficulties in implementing quantitative analysis, which mainly arise from the low concentration of the resulting o xoforms. The development of new analytical methods is, therefore, of great importance to this field.

Electron-vibration-vibration (EVV) spectroscopy is a third-order nonlinear optical technique analogous to 2D NMR, which has been displaying the potential to become a powerful proteomics tool [4-7]. It has the ability to probe inter- and intramolecular vibrational couplings resulting from mechanical and electrical anharmonicities with remarkable spectral deconvolution.

We are currently investigating EVV spectroscopy as a tool to study nitration of samples ranging from isolated tyrosine residues to tyrosine-containing peptides and proteins. We will present theoretical and experimental data demonstrating the ability of EVV spectroscopy to obtain, with high specificity, characteristic cross-peaks of 4-methylphenol and 4-methyl-2-nitrophenol, the analogues of tyrosine and 3-nitro-tyrosine, respectively.


Keywords: tyrosine nitration, oxidative damage, ageing, 2DIR spectroscopy
doi:10.1016/j.freeradbiomed.2012.08.448

[0676]

Cytosolic Thioredoxin System Facilitates the Import of Mitochondrial Small Tim Proteins

R. Durigon, Q. Wang, E. Ceh Pavia, C.M. Grant, H. Lu*
Faculty of Life Science, The University of Manchester, UK

Thiol-disulphide redox regulation plays a key role during the biogenesis of mitochondrial intermembrane space (IMS) proteins. Only the Cys-reduced form of precursor proteins can be imported into mitochondria, which is followed by disulphide bond formation in the mitochondrial IMS. In contrast to the wealth of knowledge on the oxidation process inside mitochondria, little is known about how precursors are maintained in an import-competent form in the cytosol. Here we provide the first evidence that the cytosolic thioredoxin system is required to maintain the IMS small Tim proteins in reduced forms and facilitate their mitochondrial import during respiratory growth.

Keywords: mitochondria, thioredoxin, oxidation
doi:10.1016/j.freeradbiomed.2012.08.449

Posters - Oxidative Stress in Animals and Plants

[0994]

Eicosapentaenoic acid as an antidote against 2, 3, 7, 8- tetrachlorodibenzo-p-dioxin induced oxidative stress and inflammatory responses in hepg2 cells

P. Kalaiselvi*, V. Vijaya Padma
Bharathiar University, India

2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD), a member of the polychlorinated dibenzo-p-dioxin family is
The 8th International Conference on Coherent Multidimensional Spectroscopy CMDS 2016

**Preferential Solvation: Spectral Dynamic Slowdown of a Rhenium Photocatalyst**

**P58**

**Laura Kiefer, Kevin J. Kubarych**  
*University of Michigan, ANN ARBOR, MI, United States of America*

Equilibrium 2D IR spectroscopy was used to measure spectral diffusion of the photocatalyst Re(bpy)(CO)$_3$Cl in multiple TEOA/solvent mixtures. The slowest spectral diffusion time was observed in the 20%/80% TEOA/solvent (v/v) mixture, indicating occurrence of preferential solvation.

**2DIR Spectroscopy Study of Oxidation - From Biomarker Quantification to Spectral Imaging of Tissue Sections**

**P60**

**Lays Rezende Valim$^1$, Julia A. Davies, Keith R. Willison$^1$, David R. Klug$^1$**  
*$^1$Imperial College London, LONDON, United Kingdom*

Electron-Vibration-Vibration two-dimensional infrared spectroscopy is used here to study oxidation biomarkers, such as 3-nitrotyrosine. We aim to demonstrate how the spectral information obtained through this technique could be used to generate 2DIR images able to map the localisation of biomarkers across healthy and diseased human tissue sections.

**Can Solvent Vibrational Modes Generate Coherent Oscillation in Excited Organic Dye?**

**P62**

**Luca Bolzonello, Elisabetta Collini**  
*University of Padua, PADOVA, Italy*

The coherent excited state dynamics of a charged organic molecule in different solvents have been studied with 2DES. The data demonstrated an unexpected enhancement of a specific vibrational mode of the molecule promoted by the coupling with a resonant mode of the solvent.

**Background-Free Fourth-Order Optical Spectroscopy of Interfaces**

**P64**

**Michael Schleeger, Maksim Grechko, Mischa Bonn**  
*Max-Planck Institute for Polymer Research, MAINZ, Germany*

Recent development of the two-dimensional sum-frequency generation spectroscopy has enabled an insight into the molecular vibrational dynamics at interfaces. Its implementation, however, has so far remained limited to the pump-probe geometry, with its inherent restrictions. Here, we report proof-of-concept background-free measurements of the fourth-order susceptibility using non-collinear optical layout.
B  HPLC & mass spectrometry traces of peptides

B.1  Trimer peptides

---

Imperial College London
Customer report: 18207  Date: 11th September 2012

<table>
<thead>
<tr>
<th>Compound code</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ac-AY(3-NO2)A-NH2</td>
</tr>
<tr>
<td>Modifications</td>
<td>Ac = N terminal acetylation</td>
</tr>
<tr>
<td></td>
<td>Y(3-NO2) = 3-nitro tyrosine</td>
</tr>
<tr>
<td>Other / Comments</td>
<td>Molecular mass (Av.)</td>
</tr>
<tr>
<td>Amount</td>
<td>409.396</td>
</tr>
<tr>
<td>Batch No.</td>
<td>01</td>
</tr>
<tr>
<td>Purity (Determined by HPLC - see enc.)</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Physical properties</td>
<td>Lyophilised yellow powdered solid</td>
</tr>
</tbody>
</table>

Signed

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Date: 06/09/2012
Sample No: 18207P
File: C:\CHEM32\DATA\06092012\016-3601.D
Operator: PHIL

Column: Kinetex 2.6u XB-C18 100A
Method: C:\CHEM32\2\METHODS\10-90 FINIAL ANALYSIS.M
Instrument: Instrument 2
Flow Rate: 1.5mls/min
Injection Volume: 20ul
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 10% - 100% Acetonitrile, in 13 minutes. At 60°C.

<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.959</td>
<td>5672.700</td>
<td>100.000</td>
</tr>
</tbody>
</table>
**Imperial College London**

**Customer report: 18207**

**Date**: 24th September 2012

<table>
<thead>
<tr>
<th><strong>Compound code</strong></th>
<th><strong>Ac-AY(3-NO2)A-NH2</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence</strong></td>
<td><strong>Ac = N terminal acetylation</strong></td>
</tr>
<tr>
<td><strong>Modifications</strong></td>
<td><strong>Y(3-NO2) = 3-nitro tyrosine</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Other / Comments</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular mass (Av.)</strong></td>
<td>409.396</td>
</tr>
<tr>
<td><strong>Amount</strong></td>
<td>3x1mg</td>
</tr>
<tr>
<td><strong>Batch No.</strong></td>
<td>02</td>
</tr>
<tr>
<td><strong>Purity (Determined by HPLC - see enc.)</strong></td>
<td>&gt;98%</td>
</tr>
<tr>
<td><strong>Physical properties</strong></td>
<td><strong>Lyophilised yellow powdered solid</strong></td>
</tr>
</tbody>
</table>

**Signed**

Dr. Robert Broadbridge

**Enc. HPLC trace, mass spec. trace & safety data sheets.**
Sample No: 18207
File: C:\CHEM32\DATA\21092012\095-0201.D
Operator: Phil

Column: Kinetex 2.6µ C18 100A
Method: C:\CHEM32\METHODS\FINAL_ANALYSIS_80.M
Instrument: Instrument 1
Flow Rate: 1.5mL/min
Injection Volume: 60µL
Method Info: Analysis carried out using a 100A 4.6 x 50 mm column, gradient from 0% - 80% Acetonitrile, in 8 minutes.

<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.969</td>
<td>3748.865</td>
<td>100.000</td>
</tr>
</tbody>
</table>
Imperial College London
Customer report: 20790

<table>
<thead>
<tr>
<th>Compound code</th>
<th>AY(3-NO2)A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>Y(3-NO2) = 3-nitro tyrosine</td>
</tr>
<tr>
<td>Modifications</td>
<td></td>
</tr>
<tr>
<td>Other / Comments</td>
<td></td>
</tr>
<tr>
<td>Molecular mass (Av.)</td>
<td>369.157</td>
</tr>
<tr>
<td>Amount</td>
<td>5x1.0 mg</td>
</tr>
<tr>
<td>Batch No.</td>
<td>01</td>
</tr>
<tr>
<td>Purity (Determined by HPLC - see enc.)</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Physical properties</td>
<td>Lyophilised yellow powdered solid</td>
</tr>
</tbody>
</table>

Signed

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Analysis carried out using a 100A 4.6 x 50mm column, gradient from 2% - 80% Acetonitrile, in 8 minutes. At 60°C.
### B.2 Heptamer peptides

**Imperial College London**  
Customer report: 22243  
Date: 8th August 2013

<table>
<thead>
<tr>
<th>Compound code</th>
<th>Sequence</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ac-APAY(3-NO2)APA-NH2</td>
<td>Ac = N terminal acetylation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y(3-NO2) = 3-nitro tyrosine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other / Comments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular mass (Av.)</td>
<td>746.02</td>
<td></td>
</tr>
<tr>
<td>Amount</td>
<td>5 x 1.0 mg</td>
<td></td>
</tr>
<tr>
<td>Batch No.</td>
<td>01</td>
<td></td>
</tr>
<tr>
<td>Purity (Determined by HPLC - see enc.)</td>
<td>&gt;98%</td>
<td></td>
</tr>
<tr>
<td>Physical properties</td>
<td>Lyophilised yellow powdered solid</td>
<td></td>
</tr>
</tbody>
</table>

Signed

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Sample No: 22243PA
File: C:\CHEM32\1\DATA\08082013\083-0701.D
Operator: Phil

Column: Kinetex 2.6u C18 100A
Method: C1_FA0-80_60C.M
Instrument: Instrument 1
Flow Rate: 1.5mls/min
Injection Volume: 60ul
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 2% - 80% Acetonitrile, in 8 minutes. At 60°C.

<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.159</td>
<td>21.197</td>
<td>0.426</td>
</tr>
<tr>
<td>2</td>
<td>5.222</td>
<td>41.994</td>
<td>98.241</td>
</tr>
<tr>
<td>3</td>
<td>5.364</td>
<td>13.232</td>
<td>2.566</td>
</tr>
<tr>
<td>4</td>
<td>5.863</td>
<td>38.087</td>
<td>0.765</td>
</tr>
<tr>
<td>5</td>
<td>7.211</td>
<td>15.055</td>
<td>0.302</td>
</tr>
</tbody>
</table>
# Imperial College London

**Customer report: 22244**

Date: 8th August 2013

<table>
<thead>
<tr>
<th>Compound code</th>
<th>APAY(3-NO2)APA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>Modifications</td>
<td>Y(3-NO2) = 3-nitro tyrosine</td>
</tr>
<tr>
<td>Other / Comments</td>
<td></td>
</tr>
<tr>
<td>Molecular mass (Av.)</td>
<td>704.547</td>
</tr>
<tr>
<td>Amount</td>
<td>5 x 1.0 mg</td>
</tr>
<tr>
<td>Batch No.</td>
<td>01</td>
</tr>
<tr>
<td>Purity (Determined by HPLC - see enc.)</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Physical properties</td>
<td>Lyophilised yellow powdered solid</td>
</tr>
</tbody>
</table>

Signed

Dr. Robert Broadbridge

**Enc. HPLC trace, mass spec. trace & safety data sheets.**
Sample No: 22244PC
File: C:\CHEM32\1\DATA\05082013\007-0701.D
Operator: Phil
Column: Kinetex 2.6u C18 100A
Method: CI_PA0-80_60C.M
Instrument: instrument 1
Flow Rate: 1.5mL/min
Injection Volume: 60uL
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 2% - 80% Acetonitrile, in 8 minutes. At 60°C.

<table>
<thead>
<tr>
<th></th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.033</td>
<td>7577.694</td>
<td>99.846</td>
</tr>
<tr>
<td>2</td>
<td>7.339</td>
<td>11.685</td>
<td>0.154</td>
</tr>
</tbody>
</table>
Imperial College London  
Customer report: 25129  

<table>
<thead>
<tr>
<th>Compound code</th>
<th>Ac-PPPY(3-NO2)PPP-NH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>Ac = N terminal acetylation</td>
</tr>
<tr>
<td>Modifications</td>
<td>Y(3-NO2) = 3-Nitro Tyrosine</td>
</tr>
<tr>
<td>Other / Comments</td>
<td>Mass Spec. shows +Na on +1 peak</td>
</tr>
<tr>
<td>Molecular mass (Av.)</td>
<td>850.171</td>
</tr>
<tr>
<td>Amount</td>
<td>5 x 2.0 mg</td>
</tr>
<tr>
<td>Batch No.</td>
<td>01</td>
</tr>
<tr>
<td>Purity (Determined by HPLC - see enc.)</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Physical properties</td>
<td>Lyophilised yellow powdered solid</td>
</tr>
</tbody>
</table>

Signed

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Sample No: 25129PTGP
File: C:\CHEM32\1\DATA\31012014\094-2401.D

Column: Kinetex 2.6u C18 100A
Method: C1_FA0-80_60.M
Instrument: Instrument 2
Flow Rate: 1.5mls/min
Injection Volume: 60ul
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 0% - 80% Acetonitrile, in 8 minutes.

<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.848</td>
<td>8.818</td>
<td>0.066</td>
</tr>
<tr>
<td>2</td>
<td>5.442</td>
<td>22.242</td>
<td>0.168</td>
</tr>
<tr>
<td>3</td>
<td>5.556</td>
<td>132.8123</td>
<td>99.623</td>
</tr>
<tr>
<td>4</td>
<td>5.901</td>
<td>18.951</td>
<td>0.143</td>
</tr>
</tbody>
</table>
**Imperial College London**  
**Customer report: 25130**  

<table>
<thead>
<tr>
<th>Compound code</th>
<th>Sequence</th>
<th>Modifications</th>
<th>Other / Comments</th>
<th>Molecular mass (Av.)</th>
<th>Amount</th>
<th>Batch No.</th>
<th>Purity (Determined by HPLC - see enc.)</th>
<th>Physical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ac-PPYPPP-NH2</td>
<td>Ac = N terminal acetylation</td>
<td></td>
<td>804.926</td>
<td>5 x 2.0 mg</td>
<td>01</td>
<td>&gt;98%</td>
<td>Lyophilised off-white powdered solid</td>
</tr>
</tbody>
</table>

Signed

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Sample No: 25130PB  
File: C:\CHEM32\1\DATA\29012014\093-0801.D  

Column: Kinetex 2.6u C18 100A  
Method: CI_FA0-80_60_rF_M  
Instrument: Instrument 2  
Flow Rate: 1.5ml/min  
Injection Volume: 60uL  
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 0% - 80% Acetonitrile, in 8 minutes. At 60C.

---

<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.047</td>
<td>10815.828</td>
<td>100.000</td>
</tr>
</tbody>
</table>
# Imperial College London

**Customer report: 25133**

**Date:** 4th February 2014

<table>
<thead>
<tr>
<th>Compound code</th>
<th>Ac-SPSY(3-NO2)SPS-NH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>Ac = N terminal acetylation</td>
</tr>
<tr>
<td>Modifications</td>
<td>Y(3-NO2) = 3-Nitro Tyrosine</td>
</tr>
<tr>
<td>Other / Comments</td>
<td>Mass spec shows +Na on +1 peak</td>
</tr>
<tr>
<td>Molecular mass (Av.)</td>
<td>810.017</td>
</tr>
<tr>
<td>Amount</td>
<td>5 x 2.0 mg</td>
</tr>
<tr>
<td>Batch No.</td>
<td>01</td>
</tr>
<tr>
<td>Purity (Determined by HPLC - see enc.)</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Physical properties</td>
<td>Lyophilised yellow powdered solid</td>
</tr>
</tbody>
</table>

**Signed**

![Signature]

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Column: Kinetex 2.6u C18 100A
Method: C1_FA0-80_60.M
Instrument: Instrument 2
Flow Rate: 1.5mls/min
Injection Volume: 60ul
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 0% - 80% Acetonitrile, in 8 minutes.

```
<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.802</td>
<td>41.574</td>
<td>0.870</td>
</tr>
<tr>
<td>2</td>
<td>4.877</td>
<td>4725.607</td>
<td>98.828</td>
</tr>
<tr>
<td>3</td>
<td>5.909</td>
<td>11.079</td>
<td>0.232</td>
</tr>
</tbody>
</table>
```

DAD1 A, Sig=216.8 Ref=360.100 (31012014095-2501.D)
**Imperial College London**  
**Customer report: 25134**  

<table>
<thead>
<tr>
<th>Compound code</th>
</tr>
</thead>
</table>
| Sequence      | Ac-SFSYSPS-NH<sub>2</sub>  
| Modifications | Ac = N terminal acetylation  
| Other / Comments | Mass spec shows fragmentation  
| Molecular mass (Av.) | 764.772  
| Amount        | 5x2.0 mg  
| Batch No.     | 01  
| Purity (Determined by HPLC - see enc.) | >98%  
| Physical properties | Lyophilised off-white powdered solid  

Signed

Dr. Robert Broadbridge

Enc. HPLC trace, mass spec. trace & safety data sheets.
Sample No.: 25134UP  
File: C:\CHEM32\DATA\03022014\012-2001.D

Column: Kinetex 2.6u C18 100A  
Method: C1_FA0-80_60_LF.M  
Instrument: Instrument 2  
Flow Rate: 1.5mLs/min  
Injection Volume: 60ul  
Method Info: Analysis carried out using a 100A 4.6 x 50mm column, gradient from 0% - 80% Acetonitrile, in 8 minutes.

---

<table>
<thead>
<tr>
<th>#</th>
<th>Meas. Ret.</th>
<th>Area</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.235</td>
<td>13.782</td>
<td>0.177</td>
</tr>
<tr>
<td>2</td>
<td>4.392</td>
<td>7774.798</td>
<td>99.652</td>
</tr>
<tr>
<td>3</td>
<td>5.269</td>
<td>7.288</td>
<td>0.093</td>
</tr>
<tr>
<td>4</td>
<td>9.085</td>
<td>6.104</td>
<td>0.078</td>
</tr>
</tbody>
</table>

---