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Structural and Functional Studies of a Tumour Killing Protein-Fatty Acid Complex

Louise Sullivan

A dissertation submitted to the University of Dublin
in candidature for the degree of Doctor of Philosophy

School of Biochemistry and Immunology
Trinity College
Dublin

and

Teagasc Food Research Centre
Moorepark
Fermoy
Cork

2013
Overview of thesis

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Summary

HAMLET (Human Alpha-lactalbumin Made LETHal to Tumour cells) is a complex between the milk protein \( \alpha \)-lactalbumin (\( \alpha \)-LA) and a fatty acid, oleic acid (OA), and displays remarkable properties of selectively killing tumour cell lines while leaving healthy differentiated cells relatively intact. HAMLET and BAMLET (its bovine equivalent) both exhibit toxic activity against a wide range of tumour and bacterial models, both in vitro and in vivo.

\( \alpha \)-LA is known to unfold under acidic conditions. Free oleic acid is naturally present in the stomach of infants due to gastric lipolysis. It was shown that when \( \alpha \)-LA unfolds during in vitro digestion it binds OA. Structural analysis by FTIR and NMR and biological activity of the complex against lymphoma cell line U937 indicates a BAMLET-like complex formed under the simulated infant stomach conditions.

A feeding study using nasogastric tubes inserted in the stomach of healthy adult volunteers was performed to determine potential BAMLET complex formation in vivo. Subjects drank specially formulated test drinks containing combinations of protein and fatty acid with sucrose present. Gastric aspirates were taken through the tube and their pH was recorded. Structural analysis of aspirates by intrinsic fluorescence confirmed the partial unfolding of \( \alpha \)-LA. No cytotoxic activity against U937 cells was observed.

An acidifier (D-gluconic acid \( \delta \)-lactone; GDL) was added to an \( \alpha \)-LA:OA solution and monitored in situ with NMR, its \(^1\)H spectra captured real-time to map protein unfolding. 2D-DOSY spectra were also acquired of the unfolded protein and the protein upon return to physiological conditions. Peak area decay as a function of signal strength showed differences in the diffusion patterns of the aromatic region of the protein and the olefinic carbon protons based on the pH of the complex.

The importance of the OA in the complex was highlighted through microfluidisation of the OA. The incubation of BAMLET with BSA resulted in the inactivation of BAMLET through the removal of OA. A BSA:OA complex was used as a comparison to determine the protonation state of the OA in the complex by \(^{13}\)C NMR. Regardless of whether the fatty acid starting material was protonated/deprotonated, the oleate form was found bound to the protein.
Acknowledgements

First and foremost I would like to thank my two supervisors, Dr. André Brodkorb of Teagasc, Moorepark and Dr. Ken Mok of Trinity College Dublin. Their infinite patience and wisdom, along with their help, support and guidance throughout my time in both Moorepark and Trinity College, was a source of inspiration for getting this thesis written. Particular thanks must be extended to André for (literally) putting his body on the line for the project. I am a better scientist for having had the pleasure and experience of working with you both and sharing your knowledge.

A massive thank you must go to Dr. Kamila Lišková for introducing me to all of the machines in the protein chemistry lab and of course for sharing all of your BAMLET related knowledge with me. Similarly thank you to Dr Joseph Kehoe for all of his assistance throughout the project and for providing great insight into all things protein related. Without you both there would be no thesis.

Thanks must go to Helen Slattery for her wealth of knowledge on all things chromatography and freeze drying related, Dr. John O’Brien for his technical skills in NMR, and to Dr. Manuel Ruether for introducing me to the world of Agilent and always being on hand to answer my questions.

To the past and present members of the protein chemistry lab in Moorepark: Kamila, Sinéad, Ian, Joe, Roman, Zhanmei, Solène – thank you for making science fun and sharing the laughs over beeping fume hoods, emails about boxes, infinite experiments with dry ice, lab Olympics and many, many sweets from trips abroad. Sharing a lab with all of you guys drove me crazy at times but mostly made me smile. Thanks guys.

To my lab members in Trinity: Soyoung, thank you for introducing me to the world of PC12’s and for always being so helpful; Yeon, thank you for helping with the initial sample preparation for NMR Nial, thank you for running cytotoxicity assays and for being a listening ear for all of my complaints; Yongjing, thank you for making BAMLET, always going above and beyond the call of duty in showing me how to do things and helping me whenever you could.
ACKNOWLEDGEMENTS

To all of the people who helped in anyway with the human trial from the people who were screened, the people to went on to have a nasogastric tube inserted, to Andrea Doolan, Prof. Fergus Shanahan, Prof. Eamonn Quiggley and Anne O’Neill of the APC, Lillian Barry and Dr. Martin Buckley of Mercy University hospital, to Ian, Solène, Noelle and Zhanmei for tirelessly measuring pH values and to Joe and Andre for taking the samples – that was some day for one day!

Thank you to our collaborators in Lund University, particularly to James Chin Ho for producing and providing HAMLET samples, and to Prof. Catherina Svanborg for exchanging ideas and providing advice and guidance.

To the members of the reading room in Trinity: Jenni, Ryan, (Dr.) Rob, Niki, Ewelina, Emily, Laura, Andy, Laura, Kate, Nial, Yongjing, Simon, Ray, Natalie, Roisin and Nick – thank you for making the thesis write up process a little easier with helpful advice, proof reading and copious amounts of (Earl Grey) tea, coffee (angel) and black Wednesday wine. Special mention to Andy for giving me food envy & amazing brownies.

To the people who made tea and lunchtime walks so much fun in Moorepark – Eoin, Noel, Pa, Sandra, Murray, Ian, Solène, Tony: lunchtime walks just won’t be the same anymore. I won’t ever think of snowball fights in the same way.

To the Lionesses and DULFC: rugby got me through my Ph.D. Thank you for giving me bruises and providing me with a stress relief. Lionesses thank you for letting me be your captain and helping me grow as a person throughout my time in Cork.

To my friends – Christine, Noirin (& Peter): thank you for proving me with a very comfortable bed on my trips to Cork but mainly thank you for being the most fantastic friends anybody could ask for. Trisha – thank you for the tea and letting me hide out in Farren – you have it on record now, I promise to come to Cahirciveen. Clare – same to you, I will finally visit you in Corfu to make new memories (seeing as we can’t remember the old ones!). Laura Collins – since
playschool you’ve had my back – thank you for always understanding what I’m trying to say. Holly: thank you for the coffee breaks and the chats. Rosie, what can I say other than cha cha cha, who said we couldn’t sing. Thank you for being a constant presence in my life and knowing me better than I know myself, and of course for letting me be a big child with Lauren and Dylan. Martina – thank you for reassuring me towards the end of the PhD – even if you are the other side of the Atlantic! Sinead – thanks for all the fun times in 2B (and not 2B!!) and for understanding the importance of a nice white shirt. To all of you: thank you for being understanding, particularly the past few months when I haven’t been around much. That will all change and ye’ll be sick of the sight of me.

And last, but by no means least, thank you to my family who have stood by me every step of the way since that first day in Corpus Christi. Thank you to my cousins Saoirse, Sam, Sadhbh and Lucy for keeping me on my toes, even if I am the undisputed champion of Monopoly Millionaire. A special thanks to the not-so-little man Cormac, you make me smile and you let me play science at home. Who knew mentos and diet coke could be so much fun. Although not strictly human, thanks to Misty for being the cutest thesis distraction ever by giving the best high 5’s.

To Gran – thank you for keeping me fed, watered and clothed. You are, without doubt the best Grandma in the world. To Brian, Eamon (& Niamh), Mandy (& Andy) and Auntie Marie – thanks for all of your support and being the best aunts and uncles I could have hoped for.

To Mum – there are not enough words. Thank you for being a constant support and investing so much time (and money!!) in my education. Turns out it may have been worth it after all. Who knew! Thank you for believing in me when I couldn’t believe in myself.

Now for the M. BA.!! ☺
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<th>Description</th>
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<tr>
<td>a-LA</td>
<td>α-Lactalbumin</td>
</tr>
<tr>
<td>β-LG</td>
<td>β-Lactoglobulin</td>
</tr>
<tr>
<td>ζ</td>
<td>Zeta</td>
</tr>
<tr>
<td>λmax</td>
<td>Wavelength at highest intensity occurs</td>
</tr>
<tr>
<td>Å</td>
<td>Angstroms</td>
</tr>
<tr>
<td>AA</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>All-Ala</td>
<td>α-Lactalbumin modified to have cysteine residues converted to alanine residues</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilino-1-naphthalenesulfonic acid ammonium salt</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
</tr>
<tr>
<td>BAMLET</td>
<td>Bovine Alpha-lactalbumin Made LEthal to Tumour Cells</td>
</tr>
<tr>
<td>BLA</td>
<td>Bovine Alpha-lactalbumin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BSSL</td>
<td>Bile Salt Stimulated Lipase</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
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<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>(k)Da</td>
<td>(kilo)Dalton</td>
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<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DOSY</td>
<td>Diffusion Ordered Spectroscopy</td>
</tr>
<tr>
<td>DQF-COSY</td>
<td>Double Quantum Filtered Correlated Spectroscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELOA</td>
<td>Equine Lysozyme in complex with Oleic Acid</td>
</tr>
<tr>
<td>EPI</td>
<td>Echo planar imaging</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GAMLET</td>
<td>Goat Alpha-Lactalbumin Made LEthal to Tumour Cells</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GDL</td>
<td>D-Gluconic acid δ-lactone</td>
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<td>GLA</td>
<td>Goat Alpha-Lactalbumin</td>
</tr>
<tr>
<td>GPLA</td>
<td>Guinea Pig Alpha-lactalbumin</td>
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<tr>
<td>Glu</td>
<td>Glutamine</td>
</tr>
<tr>
<td>HAMLET</td>
<td>Human Alpha-lactalbumin Made LEthal to Tumour Cells</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>Her-2</td>
<td>Herceptin positive breast cancer</td>
</tr>
<tr>
<td>HGL</td>
<td>Human Gastric Lipase</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
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<td>HLA</td>
<td>Human Alpha-lactalbumin</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focussing</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>LDV</td>
<td>Laser Doppler Velocimetry</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MAL</td>
<td>Multimeric Alpha-Lactalbumin</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MG</td>
<td>Molten Globule</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Magnesium</td>
</tr>
<tr>
<td>Min-Leu</td>
<td>α-Lactalbumin modified to have a minimum amount of hydrophobic residues</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>Manganese</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>Pen-Strep</td>
<td>Penicillin Streptomycin</td>
</tr>
<tr>
<td>PFG</td>
<td>Pulsed Field Gradient</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Photo-CIDNP</td>
<td>Photo Chemically Induced Dynamic Nuclear Polarisation</td>
</tr>
<tr>
<td>Ppm</td>
<td>Chemical shift (NMR)</td>
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<tr>
<td>PSI</td>
<td>Pressure per square inch</td>
</tr>
<tr>
<td>RH</td>
<td>Hydrodynamic radius</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small Angle X-ray Scattering</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatograph</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate (Sodium Laurel Sulphate)</td>
</tr>
<tr>
<td>SO</td>
<td>Sodium Oleate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N’N’N’N’- Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<tr>
<td>UF</td>
<td>Ultrafiltration</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
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<tr>
<td>ZP</td>
<td>Zeta potential</td>
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<tr>
<td>1H</td>
<td>Hydrogen Proton NMR</td>
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<tr>
<td>13C</td>
<td>Natural abundance carbon NMR</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
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Oral Presentations

40th Annual Conference on Food and Nutritional Sciences, UCC, Cork, April 2011 - Structural and functional studies of a bioactive protein complex produced during digestion

Irish Area Section of the Biochemical Society meeting, Maynooth, Kildare, November 2011 – Structural analysis of a bioactive protein

1st International Conference on Food Digestion, Cesena, 19th-21st March 2012 - Structural and functional studies of a bioactive protein complex produced during digestion: \textit{in vitro} and \textit{in vivo} studies

41st Annual Conference on Food and Nutritional Sciences, UCC, Cork, April 2012 - Structural and functional studies of a bioactive protein complex produced during digestion: \textit{in vitro} and \textit{in vivo} studies

Irish Area Section of the Biochemical Society meeting, Trinity College Dublin, November 2012 – The formation of an anti-cancer complex during \textit{in vitro} and \textit{in vivo} digestion of milk

Walsh Fellowship Seminar, RDS, November 2012 – The formation of an anti-cancer complex during \textit{in vitro} and \textit{in vivo} digestion of milk

2nd Meeting of the Irish NMR Society, Trinity College Dublin, April 2013 - Highly tumour-selective protein complexes: The importance of weak binding interactions
Poster Presentations

39th Annual Conference on Food and Nutritional Sciences, September 2009 - Structural changes of bovine $\alpha$-Lactalbumin in a simulated gastro-intestinal model

8th Annual EUROMAR Conference, UCD, 1st-5th July 2012 – Real-time NMR spectroscopy of the in situ partial unfolding of a protein: Glucono-delta-lactone-based conversion of $\alpha$-lactalbumin to its molten-globule form

3rd Conference on HAMLET and other tumour killing proteins, Lund, December 2012 – Real-time NMR spectroscopy of the in situ partial unfolding of a protein: Glucono-delta-lactone-based conversion of $\alpha$-lactalbumin to its molten-globule form

2nd Meeting of the Irish NMR Society, Trinity College Dublin, April 2013 - Real-time NMR spectroscopy of the in situ partial unfolding of a protein: Glucono-delta-lactone-based conversion of $\alpha$-lactalbumin to its molten-globule form

9th Annual EUROMAR Conference, Crete, Greece, 30th June – 5th July 2013 – Highly tumour-selective protein-fatty acid nanoparticles: NMR studies describing that its remarkable properties are due to conformational malleability and weak ligand-binding affinities
List of Publications

Scientific Publications


Popular Publications

Reactivating HAMLET Science Spin – Issue 56; Page 22-23 – January 2013
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Chapter 1

Introduction and Literature Review

This chapter contains work that has been published:
Section I. The structure and NMR of α-LA

1.1.1. α-Lactalbumin

Alpha-Lactalbumin (α-LA) is a small, globular whey protein found in the milk of all mammals. It has a molecular weight of 14.2 kDa and consists of 123 amino acid residues. α-LA is a metalloprotein, typically binding calcium as its co-factor. Due to its ability to bind metal ions it has been used as a model protein for calcium binding. Whilst α-LA has a specific calcium binding site, it also binds other metal cations. The binding site is formed by three Asp residues (Asp 82, Asp 87 and Asp 88) and two carbonyl groups of the peptide backbone (residues 79 and 84) to form a binding loop located between the two helical regions of the protein (Acharya et al., 1991). A second calcium binding site was discovered, along with zinc binding sites (Permyakov et al., 1991), however α-LA can bind other metal co-factors, including magnesium, manganese, sodium and potassium (Permyakov and Berliner 2000).

Table 1.1: Apparent binding constants of metal ions for bovine α-LA

<table>
<thead>
<tr>
<th>Cation</th>
<th>Association constants (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2 x 10⁷</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>211 ± 20; 46 ± 10</td>
</tr>
<tr>
<td>Na⁺</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>K⁺</td>
<td>6 ± 3</td>
</tr>
</tbody>
</table>

Taken from Permyakov and Berliner 2000.

α-LA consists of a large α-helical domain and a smaller β-sheet domain. The α-helical domain is composed largely of three α-helices at residues 5-11, 23-24 and 86-98, with two 3₁₀ helices at 18-20 and 115-118. The smaller β-sheet domain is a three-stranded antiparallel sheet at residues 41-44, 47-50 and 55-56. There is a cleft between the two domains and the domains are joined by two cysteine bridges at residues 73 and 91 (in the α-domain; responsible for the Ca²⁺ binding site) and
61-77, present in the β-domain. Two other cysteine bridges are present in α-LA at residues 6-120 and 28-111.

Acting with galactosyltransferase, α-LA is a vital component in lactose synthesis within the mammary gland. In a study by Brew et al., 1968, mice were genetically modified whereby the gene that specifies the substrate for synthesizing α-LA within the mammary gland was knocked down. The milk of the mice subsequently did not contain any lactose. A second important function of α-LA is that it is nutritionally significant to newborns – it provides all of the essential amino acids in their required levels (Jensen 1995). Recent research has shown that α-LA can be used within a potential breast cancer vaccine (Jaini et al., 2010, Tuohy and Jaini 2011). Interestingly, apo-α-LA can form a spherical supramolecular assembly with hen egg-white lysozyme, providing an alternative and intriguing model for protein self-assembly (Nigen et al., 2007). Finally, α-LA has been shown to be a vital component in the tumouricial molecule, HAMLET (Svanborg et al., 1999).

α-LA has three distinct molecular states: holo, apo and the molten globule state (of which the A-state is best known). The holo (native) form exists when the calcium is bound to the protein. The apo form exists when the protein is partially unfolded, and is induced through the removal of calcium through the use of a chelating agent such as EDTA. The A-state is a partially unfolded, molten globule-like state induced when the protein is under acidic conditions below its isoelectric point (Permyakov and Berliner 2000).

Species variations of α-LA also exist. Human α-LA is known to be the most predominant protein in human milk (Jensen 1995) and is present for its nutritional qualities. Bovine α-LA is also widely studied. The sequence of human α-LA was determined in 1972 by Findlay and Brew, showing a 72% sequence homology between the human and bovine variants. α-LA of origin of pig, sheep, goat (Schmidt & Ebner, 1972), rat (Qasba et al., 1977), guinea pig (Brew et al., 1972), mouse (Vonderha et al., 1973) and camel (Beg et al., 1985) have also been studied.
In terms of significantly advancing our understanding of the folding, two well characterised mutants of α-LA exist: Min Leu (Wu and Kim 1997) and All-Ala (Peng et al., 1995). For the Min Leu variant the Trp, Met, Phe, Ile, Leu, Tyr, Val and His hydrophobic residues within the sequence of the helical domain – of which its N-terminal (1-39) and C-terminal (81-123) segments were connected with a (Gly)₃ linker – were uniformly changed to leucine. The proline within the sequence was left unchanged as it was thought this proline residue has an influence in the structure of the protein through helix-capping effects rather than within the side chain. A tryptophan residue was also added to the sequence to aid protein concentration determination. The resulting variant was dubbed Min Leu, meaning the helical domain of α-LA was minimised with respect to amino-acid type. This variation was not prone to aggregation – all tested samples were monomeric. The Min Leu variant has a preference for the native like fold, this was confirmed by Min Leu adopting native disulfide pairings (Wu and Kim 1997). With regards to limited proteolysis performed on the Min Leu variant there were inconsistencies between the mutant and the native form with reference to the positioning of cleavage sites (Wu and Kim 1997).

A second α-LA mutant is that of All-Ala. All-Ala is a form of α-LA where all cysteines within the protein were replaced by alanines (Peng et al., 1995). The All-Ala variant is monomeric up to concentrations of 60 μM and the molecular weight was between 13.3 kDa and 15.3 kDa, which is within the expected range for α-LA (14.2 kDa). Another variant which contained the native 28-111 disulfide bond has a higher level of helix content over the All-Ala variant. The formation of a non-native disulfide bond involving Cys28 negatively affects the secondary structure suggesting that the 28-111 disulfide bond is important for the maintenance of secondary structure. The circular dichroism spectra for α-LA at pH 2 and All-Ala α-LA at pH 2 are similar suggesting that both have a native-like helical content but lack the aromatic side chain characteristics of native proteins.
Figure 1.1: The 3D crystal structure of bovine α-LA highlighting the large α-helical domain (pink) and the smaller anti-parallel β-sheet domain (blue). (Image created in PyMOL (PyMOL Schrödinger, 2009) using PDB 1HFZ (Pike et al., 1996).
Figure 1.2: Ribbon structure of α-LA with the calcium binding region highlighted. The calcium is depicted by a red sphere with the surrounding calcium binding residues depicted in ball and stick representation. Also shown: disulfide bonds in yellow. Image reproduced from Wijesinha-Bettoni et al., 2001.
1.1.2. NMR studies of the native structure of α-LA

Over the years, a wealth of significant studies on the different molecular states of α-LA using NMR have been performed (Alexandrescu et al., 1993; Permyakov and Berliner, 2000). In a study by Baum et al., 1989, guinea pig α-LA was characterised using NMR methods. Different proteins can exist in partially unfolded forms (Kuwajima 1977) and are classed as “molten globule” which are compact states existing with a large amount of secondary structure (Dolgikh et al., 1981). An early example is found in Baum et al., 1989, where different residues within molten globule states of α-LA were observed. Whereas lysozyme conforms to the classical two-state model for protein folding (Tanford et al., 1966; with the exception of equine lysozyme; Morozova-Roche, 2007), α-LA proteins do not.

Prior to the studies of Baum et al., there had been previous recordings of $^1$H spectra for α-LA where it was noted that the spectra are typical of what is expected of globular proteins (Berliner and Kaptein 1981). Spectra were recorded of native α-LA, α-LA at pH 2 and α-LA in 9 M urea at pH 2. In the case of the native protein, in the aromatic region there were interresidue interactions in the folded protein, which is characteristic of globular proteins. At the pH 2 A-state the spectra were markedly different from those of the native state and the pH 2, 9 M urea state, specifically in the aromatic chemical shift regions, with poor chemical shift dispersion and broad peaks. Two residues at 6.15 and 6.3 ppm were illustrated as an example. The sample was heated to 82°C and as the temperature increased there was a shift and sharpening of two peaks which revealed themselves as a pair of proton intensity doublets, which were assigned as the ring protons of a tyrosine residue. Overall the NMR spectrum of the A-state of α-LA exhibited different properties from both native and unfolded proteins (Baum et al. 1989).

NMR sequence assignments of α-LA have previously been a challenge: (i) the NMR signals of α-LA are quite broad; (ii) aggregation of the protein can occur around its isoelectric point (4.8) and optimum sequence assignments are at pH 4-5 (Wüthrich, 1987); (iii) the sequence of α-LA contains amino acid residues that are difficult to assign and have complex spin systems, such as isoleucine and leucine, and in the sequence of α-LA there are 12 isoleucines and 14 leucines resulting in
spectral crowding. The sequence differences between differing species of α-LA have been exploited. For example, based on aromatic-ring protons forming isolated spin systems, residues that impact the overall structure when the protein is in its molten globule state have been identified (Alexandrescu et al., 1992). Aromatic-ring side chain protons form isolated spin systems, and provide NOE-based connectivities with backbone and non-backbone protons. Combining NOE analysis with RELAY spectra and DQF-COSY spectra, the spin system for three of the four tryptophan residues, the four phenylalanine residues, the four tyrosine residues and the three histidine residues were identified. This allowed the aromatic spin systems of human α-LA and guinea-pig α-LA to be assigned, with the exception of the single Trp residue that could not be determined (Bruylants and Redfield, 2009). Comparisons of the spin systems of human, bovine and guinea-pig α-LA showed both similarities and differences. For example in bovine and guinea pig α-LA there is a similar chemical shift for a single tryptophan residue. This chemical shift is not seen in human α-LA, possibly due to the fact that human α-LA has one less Trp present. There is also a similarity between a Trp residue in HLA and BLA that is not seen in GPLA. This Trp residue was provisionally assigned as Trp60, which in the case of GPLA is a Phe residue. HLA has a unique Phe3 residue, which results in a characteristically unique spin system. Ultimately, using three-dimensional gradient-enhanced pulse sequences with the isotopically-labelled protein (^1H and ^15N), plus comparisons with crystal structures (Acharya et al., 1991) the ^1H NMR resonances of HLA and BLA could be assigned unambiguously (Forge et al., 1999).
1.1.3. The molten globule state of α-LA

A unique function of certain globular proteins is that they can be partially unfolded to yield a molten globule state, which is typically a state different from both the native form and the denatured form of the protein. For this reason the molten globule form of globular proteins is often referred to as a third, non-native phase of proteins – as it is different in structure and function to both the native and the denatured protein (Pande and Rokhsar 1998). The molten globule state can be induced in many proteins either through low pH conditions, high ionic strength conditions, or through the use of a chelating agent to remove compounds that are required for the protein to be in its folded state. Induction of the molten globule form of the protein can also be reversed through reversal of the conditions used to induce the state. Many proteins have the ability to form equilibrium molten globule forms and are outlined in Table 1.2.

The molten globule state is defined as “an equilibrium intermediate between the completely folded (‘native’) and completely unfolded (‘fully denatured’) states” (Ptitsyn, 1995) and was discovered to occur in carbonic anhydrase (Wong and Tanford 1973), β-lactamase (Robson and Pain, 1976), α-LA (Dolgikh, Gilmanshin et al., 1981) and horse cytochrome c (Ohsugi and Wada 1983; this paper was the first to mention the term “molten globule state”). These studies were significant in light of providing a potential solution to Levinthal’s paradox which states that “the search for a unique native conformation in a reasonable period of time would be practically impossible due to an astronomically large number of possible conformational states” (Levinthal, 1968; Levinthal 1969). Due to a much narrower scope of conformational ensembles offered by the presence of molten globule states, the acquisition of the native state would now be a feasible achievement.
Table 1.2: Proteins that can form equilibrium molten globule states.
(Accessed through the UniProt database, November 2013)

<table>
<thead>
<tr>
<th>Name</th>
<th>pI</th>
<th>Accession Number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta lactamase (penicillinase) S. Aureus</td>
<td>9.52</td>
<td>P00807</td>
<td>Robson B &amp; Pain RH Biochem J. 1976 May 1; 155(2):331-44</td>
</tr>
<tr>
<td>Diphtheria Toxin fragment A</td>
<td>5.06</td>
<td>P00588</td>
<td>Dumont ME &amp; Richards FM. J Biol Chem. 1988 Feb 5;263(4):2087-97</td>
</tr>
<tr>
<td>Human Brain-derived neurotrophic factor</td>
<td>9.59</td>
<td>P23560</td>
<td>Philo JS et al., Biochemistry. 1993 Oct 12;32(40):10812-8</td>
</tr>
<tr>
<td>E.coli (K12) Aspartate amino transferase</td>
<td>5.54</td>
<td>P00509</td>
<td>Herold M &amp; Kirschner K. Biochemistry. 1990 Feb 20;29(7):1907-13</td>
</tr>
<tr>
<td>Horse cytochrome C</td>
<td>9.59</td>
<td>P00004</td>
<td>Jeng MF et al., Biochemistry. 1990 Nov 20;29(46):10433-7</td>
</tr>
<tr>
<td>E.coli RNase H1</td>
<td>4.43</td>
<td>P0A7Y4</td>
<td>Dabora JM &amp; Marqusee S. Protein Sci. 1994 Sep; 3(9):1401-8</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>6.56</td>
<td>P62988</td>
<td>Harding MM et al., Biochemistry. 1991 Mar 26;30(12):3120-8</td>
</tr>
</tbody>
</table>

Table reproduced from Mollet, 2010
Studies into the molten globule form of proteins were initially performed using the acid-induced state of human and bovine $\alpha$-LA. Based on the outcomes of the preliminary studies using $\alpha$-LA a set of criteria were drawn up for the presence of a molten globule state in proteins: ‘compactness, the presence of a secondary structure, and the absence of a rigid tertiary structure’ (Ptitsyn 1992). Since its discovery many proteins have been found to enter the molten globule state (Table 1.2; Ptitsyn 2000; Arai and Kuwajima 2000). Due to rapid conformational exchange in MG proteins, analyses by NMR can be challenging in the first instance (Redfield 2004), and by definition, impossible by X-ray crystallography. However, a host of biophysical techniques, including stopped-flow circular dichroism (Matthews 1993), hydrogen-exchange labelling with 2D NMR (Baldwin 1993), chemical denaturant / heat titration with HSQC (Ramboarina and Redfield, 2003; Wijesinha-Bettoni et al., 2001), and pulse-labelled NMR spectroscopy (Mok et al., 2005) have been successfully used to probe this somewhat recalcitrant state to yield high informational content.

![Figure 1.3: Illustration of the molten-globule state: Pictorial depiction of the native state and molten globule state of proteins showing the preservation of the overall structure of the protein but with looser packing and higher motility (Ptitsyn, 1995).](image-url)
The use of NMR allows the determination of the hydrodynamic radius of a protein. Techniques such as diffusion NMR can use their gradient sequences to compare the different hydrodynamic radii of native and non-native states of proteins. An internal standard of which the decay rate is known is used in these calculations and is typically 1,4-dioxane.

The following equation is used to determine the $R_H$ of proteins:

$$R_{H}^{protein} = \frac{D_{ref}}{D_{protein}} \times R_{H}^{ref}$$

Where:

- $R_H$ is the hydrodynamic radius
- $D$ is the decay rate of the molecule
- $Ref$ is the values for 1,4-dioxane
- $Protein$ is the values for the protein samples

The decay rate is calculated through the use of diffusion NMR. A pulsed-field gradient (PFG) is applied to samples and a Gaussian decay curve is produced. The diffusion coefficient for the protein is calculated through the integration of the aromatic region and plotting it as a function of intensity of PFG applied (Jones et al., 1997).

A comprehensive overview of the differing $R_H$ values for $\alpha$-LA, $\alpha$-LA in its A-state at pH 2, the All-Ala variant and the All-Ala variant at pH 2 in 8 M urea (predicted as a fully unfolded state of $\alpha$-LA) exists (Redfield et al., 1999). Table 1.4 gives a comprehensive overview of the $R_H$ values for the different molecular states of $\alpha$-LA. The native state the calculated $R_H$ of 15.2 Å is in good agreement with the previously calculated value of 15.7 Å from x-ray scattering experiments (Kataoka et al., 1997). The value for native $\alpha$-LA at pH 2 (20.9 Å) is 6% greater than that of the native protein at physiological pH. The $R_H$ is further increased with the measurement of All-Ala at pH 2 (21.7 Å) however this value is still only 10% greater than that of the native state of the protein. The value obtained for All-Ala at pH 2 and in 8 M urea (33.3 Å) is in good agreement with predicted values of a fully unfolded protein of 124 AA residues (35.1 Å) (Redfield et al., 1999).
Table 1.3: Hydrodynamic radius values for α-LA

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$D_{\text{diox}}/D_{\alpha-LA}$</th>
<th>$R_g (\text{Å})^2$</th>
<th>$R_s (\text{Å})^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type α-LA</td>
<td>pH 6.2</td>
<td>9.12±0.03</td>
<td>15.2±0.1</td>
</tr>
<tr>
<td>Wild type α-LA</td>
<td>pH 2.0</td>
<td>9.69±0.10</td>
<td>16.2±0.2</td>
</tr>
<tr>
<td>All-Ala α-LA</td>
<td>pH 2.0</td>
<td>10.06±0.06</td>
<td>16.8±0.1</td>
</tr>
<tr>
<td>All-Ala α-LA</td>
<td>pH 2.0, 8 M urea</td>
<td>15.43±0.11</td>
<td>(25.8±0.2)$^2$</td>
</tr>
</tbody>
</table>

1 $D_{\text{diox}}/D_{\alpha-LA}$ is the ratio of the measures diffusion coefficients of 1,4-dioxane and α-LA.

2 $R_g$ is the radius of gyration of the protein. This is calculated as the product of $D_{\text{diox}}/D_{\alpha-LA}$ and the $R_g$ of dioxane (1.7 Å). $R_g$ is not a meaningful parameter for an unfolded protein; a value is given in parenthesis for comparison.

3 $R_s$ is the effective hydrodynamic radius defined as $R_s = (3/5)^{1/2} R_g$.

Table from Redfield et al., 1999.

α-LA is the best characterised of all the molten globule proteins (Kuwajima, 1996). It was shown by Hiraoka and Sugai 1985, that the apo form of α-LA could in fact assume a native-like structure at a low temperature, even though it was shown previously that the apo form of the protein could not maintain a native-like structure without calcium as a co-factor (Kronman et al., 1981). The stability of the native protein structure was found to be dependant on the salt content of the samples, and altering the salt content of the samples to up to 0.5 M NaCl was also found to increase the thermal stability of the protein (Hiraoka & Sugai, 1985). Hence in the studies of Wijesinha-Bettoni et al., 2001, the recombinant apo-α-LA protein was stabilised by using high salt contents. In samples of the recombinant apo protein with no salt there was a poorly resolved spectrum obtained but after the addition of 0.5 M NaCl the spectrum is well resolved and many native-like peaks can be seen such as the upfield methyl resonances. HSQC spectra for holo
and apo BLA have significant differences in the peak positions of Asp84 and Asp88, which are two residues involved in the binding of the calcium to the protein. The HSQC spectra for apo BLA in the presence and absence of NaCl are similar, thus supporting the hypothesis that NaCl stabilises the protein in its apo state. Also in HSQC spectra there are differences in the identification for Leu105 and Ala109 for the apo state compared to the holo state and this is due in part to the broadening of the peaks and thus they cannot be detected. The Asp87 which is implicated in the calcium binding loop is also undetectable with the protein in the apo form. It was expected that the NOE spectra for the apo state would be different for the C-helix region as there is a lack of calcium and two of the calcium binding residues are at the beginning of the C-helix, but no major differences were detected.

Slight, yet significant changes were shown to exist in the structures of BLA and HLA under 10 M urea at 50°C. A core region remains in HLA that is not seen in BLA under the same conditions, showing that, based on the $R_\text{h}$ values obtained for the proteins, BLA is less resistant to urea based unfolding than HLA. Hydrophobic residues exist in HLA that, due to sequence variation in BLA, are not present in the latter, thus giving rise to the core stability of HLA (Wijesinha-Bettoni et al., 2001).

The effect of temperature on the NMR spectrum of α-LA at pH 2 was investigated further through a series of HSQC experiments with a temperature range spanning from 20°C to 70°C (Ramboarina and Redfield, 2003). From circular dichroism experiments it was possible to determine that approximately 60% of the helical structure of the protein was retained at 70°C; with HSQC NMR 120 peaks were observed, suggesting that the peaks seen correspond to the structured regions of the molten globule rather than the unfolded regions. Increasing the temperature increases the number of visible peaks, however only 75% of the helical structure is retained, suggesting that the increase in temperature changes the dynamic properties of the molten globule. Similar trends were observed with the heating of α-LA at pH 2 as were observed in the study of Lassalle et al., 2003 on the All-Ala variant of α-LA. Under the molten globule conditions at pH 2 α-LA aggregated at
elevated temperatures thus in order to obtain good quality 3D data samples were only heated to 50°C.

The molten globule form of proteins are typically found at low pH conditions with high salt content (Ptitsyn 1991), however they can also be found in the presence of alcohol (Kamatari et al., 1998). In addition it is possible to induce further unfolding of the molten globule form of proteins, without the addition of a chemical denaturant, through the application of pressure. Lassalle et al., 2003 looked at the addition of pressure to All-Ala samples and investigated the effect on volumetric properties of the protein. The effect of both pressure and temperature were recorded through HSQC analysis of the protein. At low pressure (30 bar) and 20°C only 16 cross-peaks were visible in the aromatic region of the spectra with HN-15N HSQC. The loss of intensity of cross-peaks can be attributed to the restriction of mobility of the polypeptide chain within the molten globule (Baum et al., 1989). A reference of fully unfolded All-Ala in 8 M urea shows that cross-peaks for all are seen in the HSQC spectra for α-LA (Redfield et al., 1999). Increasing the pressure resulted in an increase in the visible cross-peaks. At 100 bar 32 cross-peaks were observed. Of these 32 cross-peaks, 26 were assigned to residues within the β-domain. At 1500 bar 42 cross-peaks were observed. This was an increase of 10 from 1000 bar, and these 10 cross-peaks were assigned to the β-domain. At 2000 bar a further 2 cross-peaks were visible and assigned to the β-domain. At 2000 bar and 20°C 90% of the cross-peaks for the β-domain were assigned compared to only 8.4% of the cross-peaks were assigned for the α-domain. Combining pressure treatment and heat resulted in 66 peaks being visible. This could be as a result of the unfolding of α-LA as a function of temperature. Heating at 36°C resulted in the identification of all cross peaks of the β-domain. Visualisation of 25 cross-peaks from the α-domain was also achieved; corresponding to 30% unfolding of the α-domain. The temperature induced unfolding is not dependant on the heating of α-LA; unfolding also occurs in the All-Ala variant when the temperature was decreased from 20°C to -18°C however this is thought to be as a result of cold denaturation of the protein (Ptitsyn, 1995b).
Unfolding of the molten globule form of α-LA, and a variant with just one disulfide bond in the presence of urea, showed that α-LA exhibit a similar unfolding trend, with just a small loss of stability in the α-LA variant. Two mutants were also studied – one with a proline substituted in the A-helix and one with a proline substituted in the C-terminal 3_{10}-helix. It was shown that the mutant with the substitution in the α-helix was unfolded without urea. The mutant with the substitution in the C-terminal 3_{10}-helix retained its structure. It was concluded that three of the four major α-helix regions of the protein can form a stable, compact core, and that molten globule α-LA has different structures within the different regions, which are all linked by long range interactions (Quezada et al., 2004).

Following on from studies of the molten globule form of α-LA at pH 2, the structure of the molten globule form was elucidated at pH 7 to compare differences between the two pH levels. Both the perpetually molten globule form, All-Ala, and the variant with a single disulfide bond [28-111] α-LA, exist in the molten globule form at pH 7 (Peng et al., 1995). With increasing urea concentrations the number of peaks observed increased, with peaks appearing as a function of protein conformation and pH: All-Ala had the most peaks appearing at lower urea concentrations, followed by [28-111] α-LA, and finally the native four disulfide bonded α-LA. From there the individual residues can be determined as all peaks were assigned (Rösner and Redfield 2009).
Section II. The HAMLET phenomenon

The field of protein folding has evolved to encompass research and development in protein folding (Dill and MacCallum, 2012), protein misfolding (Stefani, 2004) and its related associated diseases (Chiti and Dobson, 2006). Recently it has been shown that the study of partially unfolded states of proteins is also important, particularly in the case of HAMLET. HAMLET is an acronym for Human Alpha-Lactalbumin Made LEthal to Tumour cells and is a protein-fatty acid complex discovered by serendipity by Catherina Svanborg and her colleagues, who were screening human milk for its anti-infective properties. When applied to cancer cells the milk fraction displayed remarkable cytotoxic effects against the cancer, however had no effect on the healthy differentiated cells. Full analysis of this complex showed that it was composed of α-LA and fatty acid oleic acid (OA) (Håkansson et al., 1995).

Originally identified as MAL, multimeric α-lactalbumin, the complex was found in the casein fraction of human milk and was determined to contain α-LA and OA (Håkansson et al., 1995). In 1999 a method to produce this complex was developed whereby an ion-exchange chromatography column was preconditioned with a sonicated OA emulsion. α-LA in its apo form (Ca\(^{2+}\) removed with the addition of EDTA) was then applied to the column and the mixture was then allowed to equilibrate. Unbound protein was eluted from the column using 0.15 M NaCl and the bound fractions containing the bioactive compound were eluted using 1 M NaCl. These fractions then underwent extensive dialysis to remove any unbound OA and were lyophilized prior to analysis (Svensson et al., 1999).
To date HAMLET has been tested on over 40 cancer cell lines of different origins, including epithelial cells, leukaemia cells and glioblastomas of the brain, and has exhibited strong cytotoxic activity against them all. The action is also not specific to human cells and HAMLET has killed tumour cells originating from other species including bovine, murine and canine (Svanborg et al., 2003).

HAMLET has also shown activity against a range of bacterial cell lines including Streptococcus pneumonia and Haemophilus influenza (Håkansson et al., 2000). HAMLET has been shown to activate sodium dependant calcium influx in bacteria cells, thus activating ion channels (Clementi et al., 2012). Ion channel activation has also shown to be of importance in cancer cells (Storm et al., 2013). Co-treatment of Streptococcus pneumonia with HAMLET increased the toxicity of common antibiotics, thus increasing the sensitivity of bacterial cells to antibiotics. Co-treatment of antibiotic resistant bacteria strains Acinetobacter baumanii and Moraxella catarrhalis with HAMLET resulted in the loss of resistance to antibiotics (Marks et al., 2012). Recently, and perhaps most important, HAMLET has been shown to induce sensitivity to methicillin in methicillin resistant Staphylococcus aureus (MRSA) by killing biofilms, leading to an increase in antibiotic association with bacteria (Marks et al., 2013).

Despite sequence variation, HAMLET can also be formed from the milk of other animals, showing that the ability to form an anti-tumour complex is an inherent characteristic of the protein (Pettersson et al., 2006). Fragments of δ-LA...
complexed with OA (Tolin et al., 2010) or sodium oleate (SO) (Ho et al., 2013) have been shown to illicit the same cell death response as seen with the native protein in complex with the fatty acid.

After the discovery of HAMLET, other proteins have been investigated for their binding capabilities. It was reported by Wilhelm et al., (2009) that equine lysozyme is able to form a molten globule like state and bind OA in order to produce a cytotoxic complex, equine lysozyme with oleic acid (ELOA). NMR studies of the complex show that there is a higher amount of OA bound to ELOA than seen in HAMLET complexes. The whey protein β-lactoglobulin, β-LG, was also shown to produce a bioactive complex when heated in the presence of the salt form of oleic acid, sodium oleate (Lišková et al., 2011). The binding of hydrophobic compounds to proteins has also resulted in the alteration of the protein function (Le Maux et al., 2012).

Whilst the HAMLET phenomenon was thought to be specific to oleic acid or its analogue sodium oleate, it was recently shown that α-LA in complex with vaccinic acid, linoleic acid, palmitoleic acid, stearic acid and elaidic acid exhibited cytotoxic effects against HL60 cells. These effects were comparable to the effect seen when α-LA is in complex with OA. A dose response was also observed: increasing the FA content resulted in increasing toxicity of the complex (Brinkmann et al., 2013).
1.2.1. Methods of production of HAMLET and related complexes

The first published method for the production of HAMLET, the chromatographical method of Svensson *et al.*, 2000, is the most widely used method for production of the complex. However several other methods have been proposed for the formation of the complex of late.

Titration of α-LA with OA vesicles at pH 8.3 resulted in the formation of a complex with up to nine oleic acid molecules bound per molecule of protein (Knyazeva *et al.*, 2008). Heating α-LA in the presence of OA (Kamijima *et al.*, 2008) or in the presence of SO (Brodkorb & Lišková, 2009) resulted in the formation of bioactive complexes. In the case of heating in the presence of SO, it was possible to control the final molar ratio of fatty acid to protein. This method was also shown to work with β-LG (Lišková *et al.*, 2011), and in the production of complexes with other fatty acids bound (Brinkmann *et al.*, 2013).

Interestingly, the incubation of α-LA with OA under acidic conditions has been shown to produce a bioactive complex, however this method results in the reported formation of tetramers rather than monomers (Zhang *et al.*, 2009).

There are variations in the temperature at which the protein is heated in the presence of fatty acid. The incubation of α-LA with OA at 45°C at pH 12, dubbed by Brinkmann *et al.*, 2013 "the alkaline method", resulted in the formation of a bioactive complex. This complex was also formed with β-LG and parvalbumin (Permyakov *et al.*, 2011). Differing the temperature at which α-LA was heated in the presence of SO showed that there was no difference on the final SO content, and that pH is a parameter that should be regulated for complex formation (Kehoe and Brodkorb 2012). Heating α-LA in the presence of OA or linoleic acid at 60°C for 10 minutes resulted in a partially unfolded protein complex that exhibited cytotoxic activity against human prostate cancer cells (Atri *et al.*, 2011).

Variation of the pH was shown to affect the amount of OA it is possible to bind to the protein. Increasing the pH and heating at 50°C resulted in increased oleic acid content at elevated pH (Fang *et al.*, 2012). The reverse was seen in a similar study.
where decreasing the pH and heating allowed increased flexibility of the protein and an increase in hydrophobic residue exposure, increasing OA content at lower pH (Stanciuc et al., 2013).

Given that the acidic conditions under which MAL was initially discovered resembled those of a nursing infant, this prompted speculation that a HAMLET-like complex could be formed in the gastrointestinal tract of infants. To this end, a complex between bovine α-LA and OA was produced under simulated GI conditions, resulting in the induction of apoptosis in cancer cells (Sullivan et al., 2013).

1.2.2. The mechanism of action of HAMLET

Apoptosis is a form of programmed cell death that plays an essential role in the cell cycle. Several morphological events occur during apoptosis including cell shrinkage, blebbing, the retention of the cytoplasm and the lack of initiation of an immune response. Apoptosis is the preferential form of cell death over necrosis due to these factors (Shivapurkar et al., 2003). There are several characteristic events that occur during apoptosis, showing that it is a separate cell death entity. These events include caspase activation, DNA fragmentation and condensation of chromatin. Two apoptotic pathways exist: the extrinsic, or death-receptor pathway, and the intrinsic, or mitochondrial pathway. The extrinsic apoptosis pathway involves death receptors on the surface of cells and involves proteins, typically of the Tumour Necrotic Factor, TNF-α, family. Intrinsic apoptosis is activated within the cell as a result of an external stimulus and results in the activation of the B-cell lymphoma family, BCL-2 (Elmore, 2007).
Figure 1.5: Schematic of the pathways involved in apoptotic cell death showing the different cellular components activated by the intrinsic and extrinsic pathways. The mitochondrial pathway represents the intrinsic apoptotic pathway. The death-receptor pathway represents the extrinsic apoptotic pathway. Reproduced from Shivapurkar et al., 2003.

The mechanism with which HAMLET acts has been the subject of much investigation and at present, unlike many other apoptosis inducing agents, HAMLET appears to act on many different cellular targets. Given its broad range of activity and its differing mechanisms of action, the complex has been likened to the "Lernaeon Hydra" (Mok et al., 2007).

When treated with MAL, cells were found to have an activation of caspase-3 and caspase-6, and MAL was shown to interact with the mitochondria and release cytochrome c thus HAMLET was initially thought to induce an apoptosis-like mechanism of cell death due to caspase activation (Kohler et al., 1999). Similar experimentation with HAMLET indicated that a permeability transition pore was formed with subsequent release of cytochrome c potentially leading to the
activation of caspases (Kohler et al., 2001). However, although caspases were shown to be activated, upon inhibition of caspases cell death did still occur (Hallgreen et al., 2006), showing that HAMLET does have many cellular targets. Although apoptosis has an important role to play in cell death, it is not the main mechanism of cellular death.

For the case of secretory epithelial cells, HAMLET induces morphological changes in the cells that are in line with changes seen with apoptosis. These morphological changes are not seen when cells are incubated with α-LA (Baltzer et al., 2004).

HAMLET has been shown to interact with the nucleus and induce DNA fragmentation by binding to the histones (Düringer et al., 2003) and interacting with the chromatin, activating P-53 apoptotic response (Hallgren et al., 2006). Combining the treatment of cells with HAMLET and histone deacetylase inhibitors showed that there was an increased cell death response (Brest et al., 2007).

It has been shown that HAMLET interacts with the 20S proteasomes in vitro, and alteration of these 20S proteasomes inhibits their activity, triggering cell death (Gustafsson et al., 2009). This may shed some light on the ability of HAMLET to differentiate between healthy cells and tumour cells, however it was shown that inhibition of the proteasome was not the main mechanism of action of HAMLET. Surface Plasmon Resonance (SPR) experiments show there is a high affinity for HAMLET to immobilized lipid vesicles. HAMLET accumulates in the vesicles and changes the structure of the membranes, increasing membrane fluidity (Mossberg et al., 2010). α-LA with a 17 amino acid C-terminal extension can form a complex with OA and act similarly. Site specific labelling of this C-terminal extension, and subsequently HAMLET, with biotin, will allow identification of HAMLET within different cellular components (Mercer et al., 2011). It has also been shown that the c-Myc oncogene is important in HAMLET sensitivity, and that glucose starvation increased sensitivity to HAMLET, suggesting that oncogenes and the Warburg effect play a role in HAMLET activity (Storm et al., 2011).
HAMLET has been shown to induce macroautophagy in tumour cells (Aits et al., 2009). Autophagy was investigated further and it was recently shown that the autophagy protein p62/SQSTM1 is implicated in HAMLET activity by regulating apoptosis in cancer cells (Zhang et al., 2013).

HAMLET binding to α-actins promotes tumour cell detachment, resulting in cell death (Trulsson et al., 2011). HAMLET also binds to cell membranes, resulting in membrane leakage. HAMLET can form annular oligomers, which may be important for its activity. However OA is not a necessary factor for the formation of oligomers (Baumann et al., 2012).

Most recently the effect of HAMLET on ion channel activation has been assessed. HAMLET was shown to activate a cation current, and this activation was necessary for cell death by HAMLET to occur (Storm et al., 2013). A similar response was seen in bacterial cell death (Marks et al., 2012).

HAMLET exhibits preferential selectivity towards killing tumour cells than healthy, differentiated cells. This was extensively shown by the Svanborg’s group’s testing of more than 40 different cell lines (Svanborg et al. 2003). Other groups have shown that this selectivity does not necessarily hold, and that depending on the cell types, the LC50 values for primary cells may be as low as cancer cell lines (Brinkmann et al. 2011).
1.2.3. \textit{In vivo} activity of HAMLET

Unlike many other anti-cancer compounds that display cytotoxic activity \textit{in vitro}, HAMLET also exhibits remarkable cytotoxic activity \textit{in vivo}. HAMLET is active against bladder cancer, both in humans (Mossberg \textit{et al.}, 2007) and human tumour growth in the bladders of mice (Mossberg \textit{et al.}, 2010). In these studies treatment with instillations of HAMLET resulted in an increase in tumour cells shed in the urine and a subsequent decrease in tumour size. There was also no effect on the surrounding healthy tissue. Treatment of bladder cancer in mice provided more information and TUNEL staining of the tissue confirmed that there was cell death by apoptosis, that HAMLET entered the nucleus of the cell and that mice treated with HAMLET had a prolonged survival rate over their placebo treated
counterparts (Mossberg et al., 2010). A recent study showed that HAMLET was active against human bladder cancer models in rats, killing urothelial cell cancer (Xiao et al., 2013).

HAMLET displayed activity in human glioblastoma xenografts in mice. Glioblastoma tumours are the most difficult of all tumours to treat and after diagnosis there is a 90% mortality rate. Direct application of HAMLET to the tumour resulted in a decrease in tumour size, coinciding with a decrease in pressure related symptoms in the mice. Again, mice treated with HAMLET exhibited a prolonged survival rate and a better quality of life than those treated with the placebo (Fischer et al., 2004).

HAMLET has shown activity against the Human Papillomavirus (HPV). Patients with papillomas resistant to other treatments had HAMLET or a placebo applied daily for three weeks, with lesion size recorded. In the treated group there was a decrease in over 75% of the lesions, compared to less than 15% in the control group. In a second phase, HAMLET had activity against 82% of the lesions with a long term follow up reporting no return of lesions. No adverse effects were recorded (Gustafsson et al., 2004).

More recently HAMLET was used per-orally in the treatment of colon cancer in mice. HAMLET survived the gastrointestinal tract of the mice and acted selectively against the tumours. Not only did treatment with HAMLET act against the colon cancer polyps in the intestines of the mice, it also acted as a preventative treatment. The mice used in the study had a genetic predisposition to developing cancer and administration of HAMLET prophylactically resulted in a significantly lower number of tumours forming and prolonged survival (Puthia et al., 2013).

In the studies of the efficacy of HAMLET on the in vivo treatment of bacterial infections, HAMLET was shown to be active in vivo in mice models against Streptococcus pneumonia (Marks et al., 2012). Treatment of MRSA in the nasal cavity of mice was achieved by co-treatment of the biofilm with HAMLET and methicillin. This resulted in a significant decrease in bacteria levels that was not seen with treatment with methicillin alone (Marks et al., 2013).
1.2.4. Structural studies of HAMLET

Initial studies of HAMLET suggested that there was a 1:1 binding ratio of protein to oleic acid (Svensson et al., 2003). However NMR and other techniques have shown that this is not the case. Peak area integration of $^1$H spectra of HAMLET can give a detailed analysis of the fatty acid content within the fraction. Integrating the aromatic region of the protein (from 6 – 10 ppm) and comparing it to the olefinic binding region for the fatty acid (5 – 5.5 ppm) shows that in HAMLET prepared through the chromatographical method there is a 1:4-5 protein:OA ratio within the sample (Pettersson-Kastberg et al., 2009). This was confirmed by gas chromatography methods (Pettersson et al., 2004).

NMR also has the advantage that unlike other techniques such as FTIR, GC or chemical based fatty acid quantification kits, it can selectively distinguish between free OA and bound OA, both through the chemical shift of the fatty acid (5.4 ppm for free OA; 5.3 ppm for bound OA) and also more recently through the application of pulse field gradient diffusion NMR (Pettersson-Kastberg et al., 2009). Samples that have OA present in the sample and bound to the protein will have the same diffusion decay pattern as the protein, whereas free OA within the sample will either not decay, or possess a much different diffusion decay pattern. NMR was employed to differentiate between free and bound oleic acid for complexes produced with both wild-type and chemically modified α-LA (Xie et al., 2013). Changing or blocking the residues of the protein changed the protein's ability to bind OA. Sullivan et al., 2013 discussed the formation of a BAMLET-like complex between α-LA and OA during simulated gastric transit and through the use of NMR could distinguish between free and bound oleic acid, whilst simultaneously quantitating the amount of OA present.

The quantification of OA within the sample may seem a trivial issue. It has been shown by many (Brinkmann et al., 2011, Permyakov et al., 2011) that the OA within the sample is the active component – the protein may act as a mule for the delivery of the drug (Min et al., 2012). While OA, and its high pH counterpart sodium oleate (SO) do exhibit cytotoxic activity against cancer cell lines, much higher concentrations are needed to kill the cell. They also lack the specificity
seen by HAMLET and act with a necrotic rather than the preferential apoptotic form of cell death. In this instance apoptosis is preferential as it does not illicit an immune response thus no inflammation occurs. This is also seen with HAMLET (and BAMLET) samples with a large amount of OA present/bound to the protein thus the advantage of the use of NMR over conventional laboratory techniques cannot be overstated.

NMR has also been used to elucidate the structure of HAMLET and its related complexes, however much is still unknown on the structural aspects of the complex. The study of Fast et al., 2005 was the first detailed 2-dimensional homonuclear and heteronuclear NMR based study involving the protein and the fatty acid. Fast et al., 2005, determined that OA has a different chemical shift depending on its state within the sample: when OA is present only in solution and not bound to the protein it has a chemical shift of 5.4 ppm, whereas when OA is present and bound to the protein it has a chemical shift of 5.3 ppm. In the study of Sullivan et al., 2013, BAMLET produced through chromatography and studied by NMR has a small shoulder on the peak at 5.3 ppm, indicating that while most of the OA within the solution was bound to the protein, a small amount of OA remained unbound in solution.

Table 1.4: $^1$H and $^{13}$C chemical shifts for free and bound oleic acid

<table>
<thead>
<tr>
<th>Carbon no.</th>
<th>$\delta$ ($^1$H, free)</th>
<th>$\delta$ ($^1$H, bound)</th>
<th>$\delta$ ($^{13}$C, free)</th>
<th>$\delta$ ($^{13}$C, bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.29</td>
<td>2.24</td>
<td>38.01</td>
<td>39.72</td>
</tr>
<tr>
<td>3</td>
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<td>1.60</td>
<td>28.06</td>
<td>28.84</td>
</tr>
<tr>
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<td>2.09</td>
<td>2.02</td>
<td>30.08</td>
<td>29.62</td>
</tr>
<tr>
<td>9/10</td>
<td>5.43</td>
<td>5.33</td>
<td>132.38</td>
<td>132.88/131.97</td>
</tr>
<tr>
<td>18</td>
<td>0.96</td>
<td>0.90</td>
<td>16.87</td>
<td>16.72</td>
</tr>
<tr>
<td>4–7, 12–15</td>
<td>1.39</td>
<td>1.30</td>
<td>32.41</td>
<td>32.40</td>
</tr>
<tr>
<td>17</td>
<td>1.44$^b$</td>
<td>1.43</td>
<td>25.89$^b$</td>
<td>23.09</td>
</tr>
<tr>
<td>16</td>
<td>1.35</td>
<td>1.29</td>
<td>34.90</td>
<td>34.74</td>
</tr>
<tr>
<td>X</td>
<td>1.37</td>
<td>1.32</td>
<td>25.28</td>
<td>25.42</td>
</tr>
</tbody>
</table>

X, not assigned.

$^a$Protons are numbered according to their attached carbon, see Fig. 1(a).

$^b$Overlapped.

Table reproduced from Fast et al., 2005
Figure 1.7: Graphical representation of oleic acid with individual carbon numbers labelled; arrow indicating omega end of molecule (Neitzell, 2010)

A second important study using NMR in the analysis of HAMLET is that of Pettersson-Kastberg et al., 2009. Here, a structural variant of α-LA where the cysteines were replaced by alanine residues, All-Ala, was used to produce a HAMLET-like complex. In this study NMR was also used to quantify the amount of oleic acid present in the sample. Gas chromatography/mass spectrometry was used to determine the true OA content of the sample (1:5.4) and then a comparison of the $^1$H proton peak areas for the OA binding region and the aromatic region of the protein was performed and it was determined that the binding ratio of protein to OA was 1:5.1. NMR was also used to determine the hydrodynamic radius of the complexes and it was shown that the $R_h$ for both HAMLET and All-Ala -HAMLET was ~30 Å – an intermediate between native HLA (20.9 Å) and All-Ala α-LA (21.7 Å).

Broadening of the peaks was observed in HAMLET samples compared to native HLA, indicating that there is a structural change towards the MG state when the protein is bound to the fatty acid. Heating of HAMLET and HLA resulted in further broadening of the peaks at a temperature of 55°C. However the presence of the upfield valine and isoleucine peaks in HAMLET indicate that HAMLET retains a native structure with a well defined tertiary structure.
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Figure 1.8: $^1$H NMR for HAMLET and α-LA and its various spectra as a function of temperature (Pettersson-Kastberg et al., 2009).

Figure 1.9: Upfield $^1$H NMR spectra of HAMLET and α-LA and its variants at a) 30°C and b) 55°C. (Pettersson-Kastberg et al., 2009).

The molecular state of the fatty acid within the HAMLET sample dependent on the protonation state of the fatty acid during production was determined using natural abundance $^{13}$C NMR. Samples were prepared using the previously published chromatographic method however conditioning of the column was performed using both the protonated form, oleic acid, and the deprotonated form, sodium oleate. Controls of OA and SO at different pH levels were obtained and it was determined that at pH 7, there was a dual population of oleic acid and sodium oleate within the sample; both in the bound state. Residual EDTA was also
present, and detected, within the sample. This shows that the protonation state of the fatty acid is insignificant in the overall formation of the complex by chromatographical methods.

Figure 1.10: Natural abundance $^{13}$C NMR of HAMLET produced by chromatography with oleic acid (red) or sodium oleate (blue) showing the similarity of the complexes regardless of the starting material (Ho et al., 2013).

NMR has also been used to elucidate the structure and binding state of OA in the ELOA complex. The observations of Fast et al., 2005, the different chemical shift for oleic acid depending on the binding state of the fatty acid, can clearly be seen. As the amount of oleic acid present in the samples increased, there was a shift from unbound oleic acid, with a chemical shift of 5.4 ppm, to bound oleic acid with a sharp peak, at 5.3 ppm. 2D exchange NMR also showed the differences in the chemical shifts of the bound and free oleic acid.
Figure 1.11: $^1$H proton NMR spectra of the oleic acid binding region of ELOA produced with increasing concentrations of OA. Inset: 2D NMR of the OA binding region (Nielsen et al., 2010)

Whilst NMR has given lots of data on the structure of HAMLET and related complexes, due to poor chemical shift dispersion, broad peaks, and conformational exchange, few detailed 2-dimensional HSQC spectra of HAMLET exist. In a detailed NMR study, including PFG analysis and 2D HSQC, a structure for HAMLET, and the goat equivalent GAMLET, has been proposed.
Figure 1.12: 2D $^1$H-$^{15}$N HSQC analysis of HLA (A), GLA (C) in the molten globule state and HAMLET (B) and GAMLET (D), where B and D (red) are superimposed on the spectra for MG state (black) (Nakamura et al., 2013).

X-ray scattering experiments, small angle X-ray scattering (SAXS), have been completed on HAMLET. Whilst the structure is not complete, there is evidence to suggest that the region between residues L105 and L123 have an extended conformation in HAMLET and form a tail to the protein, indicating that this may be as a result of the binding of fatty acid and partial unfolding of the protein. The alpha domains of the protein were also shown to play a significant role in the activity of HAMLET (Ho et al., 2012).
Section III. Biological properties and functionality of milk

Cancer is the generic term given to a group of diseases which have common characteristics such as unregulated cell growth and proliferation, and the subsequent translocation of cells to other parts of the body through the process of metastasis (Vermeulen et al., 2003). Cancer cells, or tumours, evade typical cell death pathways, resulting in the unregulated growth. Treatment of cancer is a difficult process. The aim of cancer treatment is to induce cell death in cancer cells while having little/no detrimental effect on healthy cells, and apoptosis is the preferential method of cell death (Desch and Smith 1995).

Typical treatment of cancer will first involve surgery to remove tumours that have grown. A secondary treatment is undertaken to effectively eradicate any residual cancer cells. The most common therapeutic method of cancer treatment is chemotherapy. Chemotherapeutic agents are typically administered intravenously, however as chemotherapeutic agents can also have an effect on healthy, differentiated cells within the body there are many reported adverse effects with this treatment including suppression of the immune system, gastrointestinal disturbances and hair loss. Treatment with these agents over an extended period of time can also result in damage of specific organs. A second method used concurrently with surgery is radiotherapy, however as with chemotherapy it may also damage healthy cells. Radiation has also been linked with the formation of certain types of cancer including skin cancer (Vermeulen et al., 2003). Targeted therapies, including monoclonal antibody based therapeutics such as trastuzumab, act with a high specificity but are only active against a very narrow range of cancer cells. It is therefore clear that an alternate treatment which acts with a high specificity over a broad range of cancer types is needed (Smith et al., 2004).

HAMLET belongs to a group of recently discovered proteins with tumour killing capabilities. Like HAMLET, these other proteins possess the selectivity against healthy cells, showing little or no cytotoxic activity against healthy differentiated cells. Members of this family of proteins all induce an apoptosis-like, or
autophagic mechanism of cell death (Bruno et al., 2009). Many factors are necessary for cell growth and proliferation.

Apoptin is a protein isolated from chicken anaemia virus. Like HAMLET it localizes in the cytoplasm of healthy cells and in the nucleus of cancer cells. It activates apoptosis through intrinsic pathway activations, independent of p53. Apoptin has exhibited activity over a range of different cell lines, and has recently shown to be effective in the treatment of bladder cancer in mice (Wang et al., 2013).

TRAIL, a TNF family related ligand which induces apoptosis, has been widely studied but is also categorized as a tumour cell killing protein. It binds extracellularly to receptors on tumour cells and induces apoptosis through extrinsic pathways, independent of p53. TRAIL has recently been shown to suppress tumour growth in mice (Diao, Shi et al., 2013). MDA7 is an IL-10 member of the cytokine family that induces apoptosis in cancer cells by binding to extracellular receptors (similar to TRAIL). It also acts in vivo and a recent study looked at the stabilization of the protein for delivery to the colon for treatment of colon cancer (Xu, et al., 2013).

Other tumour killing proteins include adenovirus sourced E4orf4 (Lavoie, Nguyen et al., 1998) and parvovirus sourced NS1 (Lamana, et al., 2001).

The functional properties of milk have been a topic of research for many years. Milk is the natural source of nutrients to newborns after birth. However it can also be a vital source of nutrition for adults (Sheehy et al., 2009; Livney 2010; Thompson et al., 2009). The components contained within milk possesses many binding properties, such as the binding of Ca^{2+} ions to α-LA (Fox and McSweeney 2003); and the binding of hydrophobic molecules such as the binding of lipids to proteins (Raikos 2010), all of which can affect the function of milk. The bioactive properties of whey proteins have also been discussed extensively by Chatterton et al., 2006. Milk proteins have also been the subject of many health and dietary function studies. Bioactive peptides, such as the ACE inhibitory peptide, which has anti-hypertensive properties and functions in vivo (Murray and
FitzGerald 2007), have been produced and extracted from milk under physiological conditions. It is therefore helpful to screen milk digests at different stages of gastrointestinal digestion for the potential discovery of new functional peptides and bioactive complexes.

1.3.1. The composition of milk

The composition of human milk changes with respect to time after birth. Initially there is a very high protein concentration due to the high levels of functional proteins present in the colostrum milk, the first milk produced after birth, such as secretory immunoglobulin A (sIgA) and lactoferrin (LF). As a result of the high levels of these functional proteins, some proteins, such as β-casein, are not present in the colostrum. However the volume of milk produced is much lower than milk produced after the colostrum.

Caseins are a family of proteins made up of αS1-, αS2-, β-, and κ-caseins, and constitute the main protein constituent in cheese. Caseins are present in human milk in micellar form (Fox and Brodkorb, 2008). However, unlike in bovine milk, caseins are not the predominant protein constituent in human milk. Colostrum and early milk contains little or no casein, however casein concentration does increase over time (Kunz and Lonnerdal, 1992). The micellar form of casein is based on hydrophobic interactions and electrostatic binding between the charged parts of the casein subunits. Examples of these subunits include calcium and phosphates (Azuma et al., 1985), and nanoclusters of calcium phosphates (Holt, 2009). Casein is an important source of infant nutrition as it is easily digested and provides essential amino acids, calcium and phosphorus. Typically the ratio of casein:whey in human milk is 20:80, whereas in bovine milk it is 65:35, depending on the state of lactation (Fox and McSweeney, 2003).

The isoelectric point of casein is 4.6. At its pl, casein precipitates out of solution. The remaining proteins in solution – the soluble proteins – are called whey proteins. Human milk whey contains a variety of functional globular proteins. Whey was once deemed to be a waste product from cheese production, however recent research has shown that whey contains many bioactive protein components.
Milk globular proteins

Globular proteins, unlike their fibrous or unstructured counterparts, are soluble in water as their hydrophobic regions are sequestered within the molecule. Globular proteins extracted from milk can have a variety of different functions. The main function of the protein serum albumin is as a fatty acid transporter in vivo as it has many hydrophobic binding sites exposed on its surface (van der Vusse 2009). Lysozyme is another globular protein present in milk that functions as an antibacterial protein, by inhibiting bacterial growth. Like other functional proteins, lysozyme is present in higher quantities in the colostrum than in later milk (Gurr 1981). The predominant protein in human milk is α-lactalbumin, discussed previously. Other proteins of interest in human milk include serum albumin and lactoferrin.

β-Lactoglobulin

Although the predominant whey protein in bovine milk, β-lactoglobulin, β-LG, is not present in human milk. It is present in bovine milk at concentrations of 3.2 gL⁻¹. It has 160 amino acid residues and when present in monomeric form, has a molecular weight of 18 kDa. However β-LG is typically present in the dimeric form. Two genetic variants of β-LG exist: A and B. There are two disulfide bridges present in the protein, with one free Cys residue. To date, there is no known biological function of β-LG (Jensen, 1995; LeMaux et al., 2013).

Serum albumin

Serum albumin is the most abundant protein in blood. It is present in concentrations of 35 – 53 gL⁻¹ in blood. It is also present in milk. Its main function in the body is to maintain osmotic pressure within the circulatory system and regulate blood pH (Saguer et al., 2008). Serum albumin also acts as a transport protein. It has many different binding sites and can bind a variety of compounds including metals, hormones and fatty acids. Serum albumin binding can increase the half-life of compounds, however the binding of compounds to albumin can also alter the activity of various compounds in vivo (Hamilton et al., 1991). There have been studies completed by binding ¹³C labelled oleic acid to serum albumin in order to determine the binding sites on serum albumin (Sarver...
et al., 2005). Serum albumin has seven binding sites that are specific for fatty acid binding (van der Vusse 2009).

There is an ~80% homology between human serum albumin and bovine serum albumin. Serum albumin has an amino acid sequence that comprises of 582 amino acids which yields a molecular weight of 66.4 kDa. Serum albumin contains many highly charged amino acids including Asp, Glu, Lys and Arg, which may account for the large binding capacity of serum albumin. There are 17 disulphide bonds in albumin, and one free sulphydryl group.

Genetic variants of serum albumin exist, including mercapto and non mercapto forms (Sogami et al. 1984), glycated (Syrovy et al., 1994), and polymerized (Jordan et al., 1994) forms.

**Lactoferrin**

Lactoferrin is an iron binding protein found in many human secretions including tears, nasal secretions and saliva. It is also found in human milk at concentrations of ~3.7 g/L. It is a glycosylated protein with an amino acid sequence of ~700 AA and consequently a molecular weight of 80 kDa. It has a high affinity for binding iron however it has also been shown to bind other metals including copper, magnesium and zinc. Similarly to α-LA, lactoferrin can be found in holo, iron bound, or apo, iron free, forms. The binding of iron can induce structural changes in lactoferrin (Norris et al., 1991).

Lactoferrin has many functions. As previously mentioned it is an iron binding protein. It maintains iron levels within the body. Studies involving breast-fed infants and infant formula fed infants showed that there was a higher amount of iron absorbed by breast-fed infants, and this is due to the higher levels of lactoferrin present in breast-milk. It also has antimicrobial activity against a range of bacteria and also anti viral and anti fungal activity. Recent studies have shown that lactoferrin is a modulator in the immune system; and also that it possesses anticarcinogenic activity against human kidney cell carcinomas (Rascon-Cruz et al., 2009).
Enzymes in human milk

There are several enzymes that are indigenous to human milk that function in the infant’s body. Lipoprotein lipase is secreted in by the mammary gland and it functions by hydrolysing the lipids in lipoproteins. Bile salt stimulated lipase (BSSL) is also present in milk. It is activated in the presence of bile salts and although it has limited activity under acidic pH conditions, it is not inactivated by the acidic environment of the stomach; therefore it remains active in the intestines. Other enzymes that are present in human milk include antiproteases whose function is to protect the proteins from premature degradation. Protease inhibitors are also present. The function of these protease inhibitors is to assist in the delivery of digestive enzymes such as lipase and amylase in their active form to the infants and preventing them from being hydrolysed before reaching their active site (Gurr 1981).

Fatty acid content in human milk

The predominant fatty acid in human milk is oleic acid. Oleic acid is a monounsaturated omega-9 fatty acid and is present in the triglyceride form in human milk. It has a molecular weight of 282.46 gmol$^{-1}$ and its salt form, sodium oleate has a molecular weight of 302.46 gmol$^{-1}$. It is a C18:1 fatty acid in the cis formation with a single double bond on the C9/10 carbon (Nelson et al., 1998). Oleic acid is poorly soluble in water (Knyazeva et al., 2008), exhibiting a CMC value of 6 µM in solution at pH 7.4 (Davies et al., 2011). Sodium oleate has two CMC values and they are dependent on concentration. At 20°C the CMC values of SO are 1 mM and 2.3 mM (Hildebrand et al., 2004), and these CMCs are shown to have different shapes based on their diffusion NMR spectra (Mathieu et al., 1991). The reported pK$_a$'s of oleic acid/oleate vary across a wide range: from a pK$_a$ of 4.78 (Davies et al., 2011), 6.2 – 7.3 (Lieckfeldt et al. 1995), to 9.85 (Kanicky and Shah 2002).
1.3.2. Digestion

A key theme throughout this thesis is the digestion of α-LA and the subsequent formation of BAMLET-like complexes under both \textit{in vitro} and \textit{in vivo} conditions. Human milk has a pH of ~6.7, making it nearly neutral. It has a high protein content and thus has a high buffering capacity. Several studies have been performed whereby the pH experienced in the stomach of infants was monitored based on feeding times (Mitchell, \textit{et al.}, 2001). Nasogastric tubes equipped with pH probes were inserted into the stomach of infants and the pH was monitored. Based on the results observed in the study cited below, there is an elevated pH in the stomach for up to two hours after the beginning of the ingestion of milk. This is due both in part to the pH remaining high as feeding progresses – full ingestion does not occur for ~10 minutes, but also due to the low acid secretion levels observed in the stomach of infants. There was an eventual decrease to pH 2.5, which is likely to be a result of gastric emptying.

Due to the indigenous qualities of human milk such as the presence of BSSL, a particular emphasis has been placed on the gastric phase of infant digestion in this thesis. Gastric secretions occur from both parietal and non-parietal cells of the gastric mucosa of infants and are composed of water, gastric acid, minerals and importantly, enzymes (Geigy 1973). There are two main gastric enzymes of interest: lipolytic enzymes encompassing BSSL and human gastric lipase (HGL) and proteolytic enzymes, typically pepsin, within the stomach.

There is elevated gastric lipolysis within the stomach of breast fed infants. This is due to the levels of bile salt stimulated lipase, BSSL, in breast milk. The major gastric protease is pepsin, which is secreted early but only reaches approximately 20% of the activity of adults (Armand \textit{et al.}, 1996). Pepsin is activated from zymogen precursors, pepsinogen, by selective cleavage of a small peptide. There is low pepsin secretion (Agunod \textit{et al.}, 1969). Pepsin has a low pH activity optimum – 1-2.5, and is irreversibly denatured above pH 7.

With the higher/elevated gastric pH levels there is an overall lower level of proteolysis in the stomach of infants is found. This is followed through within the
intestines and proteins such as lactoferrin and immunoglobulin A can be found intact in the stools of breast fed infants. Intact proteins, namely lactoferrin and immunoglobulin A, but also α-LA, are also found in the blood of infants.

**In vivo validation**

Due to difficulties in obtaining human gastric and intestinal juices, there is a growing trend towards the use of *in vitro* models that have been validated with *in vivo* data. Furlund *et al.*, 2013, developed a method for the collection of human gastric and upper intestinal juices from healthy volunteers. For this study, three-lumen tubes were used for the aspiration of human gastric juice and human duodenal juices. The tubes were inserted through the noses of volunteers and a stimulatory solution was introduced to the GI tract through the tube. Gastric juices and duodenal juices were collected through the tube and fully characterised for proteolytic and amylase activity, along with bile acid concentrations.

Enzyme activity levels *in vivo* are vital parameters to consider for any work relating to digestion. The recognised standards for proteolytic digestion in a physiological context, includes a gastric phase of pepsin at pH 2, 37°C for 2 hours followed by the intestinal stage of trypsin, chymotrypsin and bile salts and pH 7 for 4 hours. Recent *in vivo* studies have shown that this is not the case and each model should be modified according to individual situations and conditions.
Figure 1.13: *In vivo* gastric pH monitoring of infants stomachs after feeding with A) 4 hourly, B) 3 hourly, C) 2 hourly feeding schedules (Mitchell *et al.*, 2001).
Section IV. Alternative protein folding

The initial concept of “one-gene one-protein one-function” was proposed by Beadle and Tatum 1941 after their discovery that specific genes code for specific proteins (Gerstein et al., 2007). The general consensus is that proteins may be functional only in their native, folded state, and almost always the protein will fold in such a way that it remains physiologically stable (Dobson et al., 1998). Typically protein unfolding, or protein misfolding, can lead to the development of certain pathological conditions (Dobson 2003). These conditions are developed as a result of a protein either folding incorrectly, or failing to remain folded correctly (Radford and Dobson 1999). Diseases such as cystic fibrosis (Thomas et al., 1995) and certain types of cancer (Bullock and Fersht 2001) are developed as a direct result of proteins not folding correctly and therefore are lacking in their intended biological functions. Other neurodegenerative conditions that are developed as a result of protein misfolding include both Alzheimer’s and Parkinson’s diseases, which are associated with the deposition of protein aggregates on the brain (Dobson 2001; Tan and Pepys 1994). These diseases are each linked to a particular protein and aggregates of the protein and are often only discovered later in life as they are as a result of a build-up of aggregates (Dobson 1999).

Partially unfolded and misfolded proteins can also form well-defined aggregates of different proteins (Chiti et al., 2003), called amyloid fibrils. The term amyloid was initially used to describe protein aggregates which appeared to have similar properties to those of starch, whose main constituent is amylose. Analysis of these amyloids showed they consisted of fibrous units (Dobson 1999). Typically fibrils are not formed by globular proteins as they remain folded under normal physiological conditions (Chiti et al., 2003). However the hydrophobic regions of globular proteins may become exposed as the protein partially unfolds under acidic conditions (Caughey and Lansbury 2003) or as a protein is fragmented through hydrolysis (Bitan et al., 2003). Under these conditions conversion to amyloid fibrils will be possible. A study was performed to show that under denaturing conditions, proteins not previously associated with amyloid formation,
could be used to form fibrils (Chiti et al., 2003). This suggests that amyloid formation is a generic property of all proteins, however the conditions under which amyloid fibrils were formed were not physiological therefore conversion in vivo is unlikely (Sanchez and Kiefhaber 2003). Several functional amyloids have been discovered including Pmel17 protein in melanosomes (Fowler et al., 2007) and Saccharomyces cerevisiae Sup35 prions (Shorter et al., 2005).

The unfolding of proteins in the cell typically results in both functional and destructive changes in cellular activity (Radford and Dobson 1999). A list of proteins that result in changes in biological functions are outlined in Table 1.7 (Chiti and Dobson 2006).

However in recent years, several functional properties of partially unfolded proteins have been discovered. Initial research into the beneficial effect of human breast milk led to the discovery of multimeric α-lactalbumin, MAL, which was found to induce apoptosis in cancer cells while having no detrimental effect on healthy, differentiated cells (Håkansson et al., 1995). Further investigations showed that unfolded α-lactalbumin could be converted into a protein complex named HAMLET (Håkansson et al., 1999, Svensson et al., 1999). This complex confirms the existence of partially unfolded proteins which bind co-factors and have a new biological function, in this case the HAMLET complex.
Table 1.7: Functional changes associated with protein mis-folding

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aggregating protein or peptide</th>
<th>Number of residues&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Native structure of protein or peptide&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurodegenerative diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s disease&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Amyloid β peptide</td>
<td>40 or 42&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Spongiform encephalopathy&lt;sup&gt;5, 6&lt;/sup&gt;</td>
<td>Prion protein or fragments thereof</td>
<td>255</td>
<td>Natively unfolded (residues 1–125) and α-helical (residues 125–230)</td>
</tr>
<tr>
<td>Parkinson’s disease&lt;sup&gt;2&lt;/sup&gt;</td>
<td>α-Synuclein</td>
<td>140</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Dementia with Lewy bodies&lt;sup&gt;2&lt;/sup&gt;</td>
<td>α-Synuclein</td>
<td>140</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Frontotemporal dementia with Parkinson’s&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Tau</td>
<td>332–441&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Superoxide dismutase I</td>
<td>153</td>
<td>All-β, Ig like</td>
</tr>
<tr>
<td>Spastic cerebellar ataxia&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Huntingtin with polyQ expansion</td>
<td>3144&lt;sup&gt;j&lt;/sup&gt;</td>
<td>Largely natively unfolded</td>
</tr>
<tr>
<td>Spastic cerebellar ataxia&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Ataxins with polyQ expansion</td>
<td>816&lt;sup&gt;k&lt;/sup&gt;</td>
<td>All-α, AXH domain (residues 526–694); the rest are unknown</td>
</tr>
<tr>
<td>Spastic cerebellar ataxia&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TATA-box binding protein with polyQ expansion</td>
<td>1390&lt;sup&gt;n&lt;/sup&gt;</td>
<td>All-α, TRB like (residues 159–199); unknown (residues 1–178)</td>
</tr>
<tr>
<td>Spinal and bulbar muscular atrophy&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Androgen receptor with polyQ expansion</td>
<td>910&lt;sup&gt;r&lt;/sup&gt;</td>
<td>All-α, nuclear receptor (ligand-binding domain (residues 699–919); the rest are unknown)</td>
</tr>
<tr>
<td>Hereditary dentatorubral-pallidoluysian atrophy&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Atrophin-1 with polyQ expansion</td>
<td>118&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Unknown</td>
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<td>Familial British dementia&lt;sup&gt;2&lt;/sup&gt;</td>
<td>All-β</td>
<td>23</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Familial Dutch dementia&lt;sup&gt;2&lt;/sup&gt;</td>
<td>AβDun</td>
<td>23</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Nonneuropathic systemic amyloidoses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL-amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Immunoglobulin light chains or fragments</td>
<td>~50&lt;sup&gt;t&lt;/sup&gt;</td>
<td>All-β, Ig like</td>
</tr>
<tr>
<td>AA-amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Fragments of serum amyloid A protein</td>
<td>76–104&lt;sup&gt;h&lt;/sup&gt;</td>
<td>All-α, unknown fold</td>
</tr>
<tr>
<td>Familial Mediterranean fever&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Fragments of serum amyloid A protein</td>
<td>76–104&lt;sup&gt;h&lt;/sup&gt;</td>
<td>All-α, unknown fold</td>
</tr>
<tr>
<td>Senile systemic amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Wild-type transthyretin</td>
<td>127</td>
<td>All-β, prealbumin like</td>
</tr>
<tr>
<td>Familial amyloidotic polyneuropathy&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Mutants of transthyretin</td>
<td>127</td>
<td>All-α, prealbumin like</td>
</tr>
<tr>
<td>Hemodialysis-related amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(β2-microglobulin</td>
<td>99</td>
<td>All-β, Ig like</td>
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<tr>
<td>ApoA1-amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>N-terminal fragments of apolipoprotein A1</td>
<td>80–99&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>ApoA1-amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>N-terminal fragment of apolipoprotein A2</td>
<td>98&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Unknown</td>
</tr>
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<td>ApoA1V-amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>N-terminal fragment of apolipoprotein A1V</td>
<td>~70&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Unknown</td>
</tr>
<tr>
<td>Finnish hereditary amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Fragments of globulin mutants</td>
<td>71</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Lysosome amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Mutants of lysosome α-chain</td>
<td>130</td>
<td>α-β, lysosome 6f</td>
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<tr>
<td>Fibrinogen amyloidosis&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Variants of fibrinogen α-chain</td>
<td>27–31&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Unknown</td>
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<td>Icelandic hereditary cerebral amyloid angiotrophy&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Mutant of cystatin C</td>
<td>120</td>
<td>α-β, cystatin like</td>
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<td>Nonneuropathic localized diseases</td>
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<td></td>
<td></td>
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<tr>
<td>Type II diabetes&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Amylin, also called insulin amyloid polypeptide (IAPP)</td>
<td>37</td>
<td>Natively unfolded</td>
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<td>Medullary carcinoma of the thyroid&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Calcitonin</td>
<td>32</td>
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<td>Atial amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Atial natriuretic factor</td>
<td>28</td>
<td>Natively unfolded</td>
</tr>
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<td>Hereditary cerebral haemorrhage with amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Mutants of amyloid β protein</td>
<td>40 or 42&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Pineaityri predementia&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Pradin</td>
<td>199</td>
<td>All-α, 4-helical cytokines</td>
</tr>
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<td>Injection-localized amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Insulin</td>
<td>21 ± 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>All-α, insulin like</td>
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<td>Aortic medial amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Medin</td>
<td>58</td>
<td>Unknown</td>
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<td>Hereditary lattice corneal dystrophy&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Mainly C-terminal fragments of kerato-epithelin</td>
<td>50–200&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cerebral amyloidosis associated with trichiar&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Lactoferrin</td>
<td>692</td>
<td>α-β, periplasmic-binding protein like II</td>
</tr>
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<td>Cataract&lt;sup&gt;6&lt;/sup&gt;</td>
<td>y-Crystallins</td>
<td>Variable</td>
<td>All-β, y-crystallin like</td>
</tr>
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<td>Calculifying epithelial odontogenic tumours&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>~46</td>
<td>Unknown</td>
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<td>Pulmonary alveolar proteinosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Lung surfactant protein C</td>
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<td>Unknown</td>
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<td>Inclusion body myositis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Amyloid β peptide</td>
<td>40 or 42&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Natively unfolded</td>
</tr>
<tr>
<td>Cataractous lichen amyloidosis&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Keratins</td>
<td>Variable</td>
<td>Unknown</td>
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Reproduced from (Chiti and Dobson 2006)
Section V. Objectives of the project

**Development of methods for the characterisation of H/BAMLET and other bioactive protein complexes**

In order to effectively determine if the HAMLET-like complex is formed under different conditions, including during *in vitro* and *in vivo* digestion studies, and during the production of the complex without the use of the lengthy chromatographic based method, it is initially necessary to fully characterise the complex HAMLET. Methods will be developed to assess structural changes experienced by the protein when it binds to the oleic acid. A full battery of biophysical techniques that will probe both the protein moiety (as well as the fatty acid) at the levels of secondary, tertiary, quaternary, and at the atomic levels, will be used (fluorescence, Fourier transform infra-red spectroscopy, zeta potential, light scattering, NMR spectroscopy, and others). Stoichiometric quantification of the fatty acid will be undertaken, and the samples subjected to bioactivity testing against both cancer cell lines and healthy, differentiated cell lines.

**Development of a GI digestion model and the formation of BAMLET under simulated digestion conditions**

A simulated human digestive model for the digestion of proteins and fats will be used by effectively mimicking the pH and enzymatic conditions experienced in the upper digestive tract of infants. This method will be used to digest mixtures of α-LA and oleic acid in levels that mimic those of human milk. Samples will be taken at regular intervals and analysed using a battery of biophysical techniques as outlined above. Polypeptide chain integrity will be determined using HPLC techniques, and the complexes tested for bioactivity against both cancer cell lines and healthy, differentiated cell lines to compare with the properties of H/BAMLET.
Determination of the potential formation of a BAMLET-like complex \textit{in vivo}
While \textit{in vitro} digestion methods have evolved over time and now sophisticated methods exist, a gap still remains between \textit{in vitro} digestion and \textit{in vivo} digestion. In order to determine whether a complex is formed in the stomach of adults, a feeding study using nasogastric tubes inserted in the stomach of healthy adult volunteers will be performed. Subjects will be given specially formulated test drinks containing combinations of protein and fatty acid with sucrose present. Gastric aspirates will then be taken through the tube and their pH recorded.

Characterisation of the fatty acid in complex with BAMLET
Much of the research within the study of HAMLET have focussed on the protein or the complex. Little is known about the role the fatty acid plays. Given the dose dependant response of increasing cell death with increasing fatty acid concentrations, further analysis will be performed on the fatty acid. The role of the protein in the delivery of the fatty acid to the cell will also be determined.
Chapter 2

Materials and Methods
2.1. Materials

2.1.1. \(\alpha\)-lactalbumin and other proteins

BioPURE-alpha-lactalbumin\textsuperscript{TM}, commercial, food grade \(\alpha\)-LA was purchased from Davisco Foods International Inc. (Minnesota, USA). The technical analysis for the protein showed that it is a minimum of 95% protein with a minimum of 90% \(\alpha\)-LA present. The remaining protein was \(\beta\)-LG with traces of BSA. This data was correlated with Kjeldahl analysis of total nitrogen content of the samples and native HPLC. Conformational analysis of the protein was performed to assess the molecular state of \(\alpha\)-LA using a combination of intrinsic fluorescence spectroscopy and atomic absorption spectroscopy. Results showed the protein was 70% in the apo form from the \(\text{Ca}^{2+}\) content and the protein was adjusted accordingly using EDTA or \(\text{CaCl}_2\) to produce the apo form or holo forms, respectively.

For certain cases calcium depleted \(\alpha\)-LA purchased from Sigma Aldrich (L6010) was utilised. Bovine Serum Albumin (A7030) was purchased from Sigma Aldrich at 98% purity.

BiPro Whey Protein Isolate, commercial, food grade, was purchased from Davisco Foods International Inc. (Minnesota, USA).

2.1.2. Oleic acid and its derivatives

Oleic acid used in all experiments was purchased from Sigma Aldrich, 99% purity, product code O1008 and sodium oleate with 95% purity was used, product code O3880, also Sigma Aldrich.

2.1.3. Reagents

Diethylaminoethyl (DEAE) Trisacryl ion exchange matrix was purchased from Pall Corp. (France); alamarBlue assay from Invitrogen (California, USA); Bio-Rad Protein Assay, ampholytes for Rotofor experiments, and molecular weight
markers for SDS-PAGE were from Bio-Rad Laboratories (Hertfordshire, UK). The apoptosis test kit (Cell Death Plus ELISA test) from Roche Diagnostics.

Unless otherwise stated all other chemicals and reagents were purchased from Sigma-Aldrich.

**Table 2.1:** List of chemicals and reagents used

<table>
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<th>Product</th>
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<td>Bovine Hemoglobin</td>
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<td>L-Histidine</td>
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2.2. Methods

2.2.1. Simulated digestion

A dynamic *in vitro* gastric model for mimicking infant gastric conditions was used. Simulated digestion experiments were performed at 37°C. Porcine pepsin, (determined to have an activity 653 U/mg protein using bovine haemoglobin as a substrate), was used at an E:S ratio of 1:100. The pH of the digest was controlled as per Figure 2.1 using a computer controlled Tiamo 842 pH monitoring and titration system (Metrohm, Germany) equipped with a pH probe for protein analysis. pH was maintained using 1 M HCl and 1 M NaOH. The pH gradient was used to effectively mimic the pH of the stomach of infants during and after feeding (Mitchell *et al.*, 2001). Homogeneity of the digest was maintained through the use of a submerged magnetic stirrer at a rate of 80 rpm. Digestions were monitored for 2 hours with samples taken at regular time points for analysis.

To formulate the test milk, α-LA was dissolved at a concentration of 700 μM. CaCl₂ was added at a concentration of 0.986 μM to ensure α-LA was in the holo conformation. OA dissolved in 1 mL ethanol to solubilise the fatty acid and the FA-ethanol emulsion was added to buffer A to give a final OA concentration of 10 mM. Samples were taken at time zero, T0; in the gastric phase at pH 2.5; and when the mixture entered the simulated intestinal phase at pH 7. Each sample was dialysed against distilled water at 4°C, lyophilised and stored at -20°C prior to analysis.
Figure 2.1: pH curve mimicking the pH conditions within the stomach of infants, outlining the important stages of digestion: (i) ingestion phase: pH of the stomach remains elevated as milk continually enters the stomach; (ii) gastric lipolysis occurs from gastric lipase enzymes, cleaving/hydrolysing free fatty acids from the triglyceride; (iii) α-LA unfolding occurs under the pI of the protein; iv) proteolysis occurs.

2.2.2. BAMLET production

Chromatographic method

A stock of BAMLET was produced using the previously published chromatographic method (Svensson et al., 2000; termed “conditioning of the column with fatty acid”).

Briefly, two buffers were produced: 10 mM Tris-HCl, pH 8.5 (buffer A) and 10 mM Tris-HCl, 1 M NaCl, pH 8.5 (buffer B). An oleic acid emulsion was produced by dissolving 112 μL OA to 1 mL ethanol. Mixing was achieved by vortexing. 40 mL of buffer A was added to the OA:ethanol mixture and the resulting solution was sonicated for 30 seconds, ten times, to give a total sonication time of 5 minutes. The fatty acid emulsion was added to the column under a low flow rate to achieve equilibration of FA throughout the column. The column was equilibrated with two column volumes of buffer A. Apo α-LA was
produced by adding EDTA to α-LA, which was dissolved in buffer A at a concentration of 700 μM. 40 mL of the protein was injected onto a DEAE-Trisacryl ion exchange column (column volume 180 mL) that was preconditioned with oleic acid. Again, the column was equilibrated with two column volumes of buffer A. The gradient changed to 15% B, 150 mM NaCl and equilibrated for 4 column volumes to remove any unbound protein. The salt concentration was increased to 100% buffer B, 1 M NaCl and the resulting protein fractions were collected, dialysed against distilled water, lyophilised and stored at -20°C before analysis.

Alternatively, the preparation was performed using a disposable HiTrap DEAE FF (GE Healthcare; 5 ml column volume) subjected to analogous conditions. 235 μL OA was dissolved in 500 buffer A and α-LA was dissolved in 3 mL buffer A at a concentration of 1 mM. (A dummy run of the eluent gradient was performed for the 5 mL column.)

**BAMLET complex generation by mixing**

BAMLET was prepared in solution at pH 7.4 using different molar excesses of sodium oleate. α-LA was dissolved in a two times concentrated solution of PBS at a concentration of 700 μM. Sodium oleate was dissolved in water at 5, 10, 15, 20, 25 and 30 times molar excess of the protein concentration and mixed in a 1:1 ratio to give a final volume of 50 mL with a 2.5, 5, 7.5, 10, 12.5 and 15 times molar excess of the protein concentration in a 1X PBS solution at pH 7.4. The mixture was heated to 60°C for 1 hour and cooled rapidly on ice prior to extensive dialysis and lyophilisation. Samples were stored at -20°C before use.
2.2.3. High-Performance Liquid Chromatography (HPLC)

All HPLC experiments were performed on a Waters 2695 Separations Module equipped with a dual wavelength detector set at 214 nm and 280 nm. For reversed phase experiments the column was kept at a constant temperature of 28°C in the column oven, which was chosen to preserve, as much as possible, the native properties of the protein or peptides. The sample chamber was set at 10°C. A sample volume of 20 μL of a 17 μM protein solution was injected onto the column.

Reversed-phase HPLC (RP-HPLC)

The two solvents used for RP experiments were 0.1% TFA in MilliQ™ (A) and 90% acetonitrile (MeCN), 0.1% TFA in MilliQ™ (B).

For native protein analysis RP-C4/C5 was performed using a Source RPC5 column (GE Healthcare, Buckinghamshire, UK). The starting conditions of solvents were 60:40 A:B with a run time of 34.1 minutes. The flow rate was 0.8 mL/min⁻¹.

For peptide analysis a μSymmetry C18 column was used (Phenomenex, UK). The starting conditions of solvents were 100:0 A:B, changing to 0:100 in a linear fashion over 25 minutes, with a 5 minute decrease back to 100:0. Overall there was a run time of 30 minutes and a flow rate of 1 mL/min⁻¹.

Figure 2.2: RP-C4/5 Acetonitrile gradient for native protein analysis
Size Exclusion Chromatography (SEC)

Digestion progression was monitored through SEC-HPLC using a TSK G2000 column (Tosoh Bioscience, Japan). The flow was isocratic and the solvent used was 30% MeCN with 0.1% TFA. There was a flow rate of 0.5 mL/min and a run time of 60 minutes.

2.2.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as per the modified method of Laemmli, 1970. Separating gels were prepared to a concentration of 15% (protein samples) to 20% (peptide samples) acrylamide. Stacking gels were prepared to a concentration of 4% acrylamide. Samples were dissolved at a concentration of 10 gL⁻¹ and diluted 1:8 with reducing SDS-PAGE buffer. Samples were heated at 95°C for 5 minutes and cooled at room temperature. Gels were prepared as outlined in Table 2.2. Gels were run at 140 V until the sample entered the separating gel, at which time the voltage was increased to 180 V. Molecular weight markers, (MW range 10 – 250 kDa; 161-0373, Bio Rad, UK) were used in each gel.

Table 2.2: Concentrations of each solution in SDS-PAGE for two 15% gels

<table>
<thead>
<tr>
<th></th>
<th>Separating Gel</th>
<th>Stacking Gel</th>
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<tr>
<td>30% Bis-Acrylamide</td>
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<td>0.65</td>
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<tr>
<td>1.5 M Tris HCl pH 8.5</td>
<td>2.5</td>
<td>-</td>
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<tr>
<td>0.5 M Tris HCl pH 6.5</td>
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<td>1.25</td>
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<td>10% SDS</td>
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<td>10% Ammonium Persulfate</td>
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<tr>
<td>TEMED</td>
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<tr>
<td>Distilled water</td>
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</tbody>
</table>
2.2.5. Zeta Potential Measurements

When a charged particle is in a liquid, ions contained within the liquid that are of an opposite charge to the particle will be attracted to the surface of the charged particle. The attraction of these ions forms an electrical double layer. This double layer consists of two parts – the Stern layer, which is the tightly bound oppositely charged ions, and the diffuse layer, which consists of more loosely bound ions. Within the diffuse layer there is a boundary, and inside this boundary, ions and particles are stable. When the particle moves, ions inside the boundary move with it, whereas ions outside of the boundary do not move. This boundary is known as the slipping plane, and the potential at this slipping plane is known as the zeta (ζ) potential.

![Diagram of Zeta Potential Measurements](image)

**Figure 2.3:** Schematic of measurements taken during the calculation of zeta potential. (Image taken from Malvern User Guide for Nanosizer, 2007)

The magnitude of the zeta potential indicates how stable the particle is. Typically the difference between stable and unstable particles is between ±30 mV, i.e. particles with a measured zeta potential of greater than ±30 mV tend to be more stable in solution than particles with a measured zeta potential of less than ±30 mV.
The most important factor in the measurement of zeta potential is the pH of the sample. A zeta potential vs. pH curve will be negative at a high pH and positive at a low pH. The point at which the particle has no charge – where the zeta potential is zero – is referred to as the isoelectric point.

Electrophoresis is one method used to measure the zeta potential. When an electric field is applied to a solution, the charged particles will migrate to either the anode or the cathode. The particles move at a constant velocity, which depends on the strength of the voltage applied, the dielectric constant of the solution, the viscosity of the solution and the zeta potential. The velocity is known as the electrophoretic mobility, and with the other known constants, it is possible to calculate the zeta potential using Henrys Equation.

\[
U_E = \frac{2\varepsilon zf(ka)}{3\eta}
\]

Where:

- \( U_E \) = Electrophoretic mobility
- \( z \) = Zeta Potential
- \( \varepsilon \) = Dielectric constant
- \( \eta \) = Viscosity of the solution
- \( f(ka) \) = Henrys Function

Zeta potential measurements were recorded using a Malvern Zetasizer Nanosizer (Malvern, United Kingdom). Protein and protein/fatty acid samples were prepared at a concentration of 5 mgmL\(^{-1}\). Fatty acid samples were diluted to 1 mM. Unless otherwise mentioned samples were measured at a recorded pH of 7. Measurements were made at 25°C with an applied voltage of 75 mV. Samples were measured 20 times in triplicate and an average of these 60 scan results was taken.
2.2.6. Dynamic Light Scattering

Assuming that the particle is spherical in shape and uniform in distribution, dynamic light scattering (DLS) offers a method of measuring the particle size of compounds in solution or suspension. A laser is focused through the sample and the scattering of the light beam measured by the detector. Small particles will have large angle scattering; larger particles will have small angle scattering.

Particle size measurements were recorded on a Malvern Zetasizer Nanosizer (Malvern, United Kingdom). Measurements were made at 25°C with the scan numbers determined by the optical clarity of the sample (a minimum of 8 and a maximum of 12). It is assumed that solutes have identical viscosity and refractive index as water. Samples were measured in plastic cuvettes with 1 mL of sample. The raw data of the distribution of particles within the solution as a function of particle size by volume was plotted to determine the z-average values.

2.2.7. Turbidity

Turbidity measurements were performed using a Cary UV-Visible Spectrophotometer (Varian). The optical density of the samples was measured at 600 nm.

2.2.8. Microfluidisation

Microfluidisation is a process used to reduce particle size and produce nano scale emulsions. In a Y type processing chamber there are two streams that meet in the interaction chamber at a right angle under pressure, which results in the formation of a high shear environment, disrupting the fat membrane, forcing the fat droplets to decrease in size and become a suspension.
Oleic acid and sodium oleate were dissolved at 1 mM at pH 2, pH 7 and pH 11. Samples were homogenized using an M110-EH Microfluidizer (Microfluidics International Corp., MA, USA) at 10,000 psi at room temperature (25°C) to produce fine suspensions. A 75 mm Y type single slotted ceramic interaction chamber was used. Samples went through the processing chamber 3 times.

2.2.9. Tissue culture

All tissue culture experiments were performed under sterile conditions within a laminar flow hood with correct aseptic technique. All samples were sterile filtered through a 0.1 μM syringe filter before bioactivity testing.

**Cell revival**

Cells were frozen in aliquots and stored in liquid nitrogen. For revival, the aliquot of cells was removed from the liquid nitrogen storage unit and left at room temperature for 1 minute. The aliquot was then transferred to a 37°C water bath for 2 minutes, or until nearly thawed. The cells were pipetted into a 25 mL T-flask and pre-warmed RPMI + 10% FBS was slowly added to the mixture of cells. The cells were then incubated at 37°C in 5% CO₂ for 24 hours and re-passaged.
**Human histiocytic lymphoma suspension cells: U937**

U937 cells are a suspension cell line extracted from a human diffuse histiocytic lymphoma. Cell were grown at a density of $1 \times 10^5$ in RPMI 1640 supplemented with 10% fetal bovine serum and incubated at 5% CO$_2$ at 37°C. For cytotoxicity assays cells were subcultured at a density of $2 \times 10^5$. Samples for cytotoxicity analysis were dissolved in RPMI 1640 and sterile filtered with a 0.1 μM syringe filtered and diluted to a concentration of 5 mgmL$^{-1}$. Samples were then diluted to desired concentrations between 0 and 2.5 gL$^{-1}$ with RPMI to give a final volume of 50 μL. Cells at a density of $2 \times 10^5$ were then added to the samples in the well to give a final volume of 100 μL. It should be noted that all of the cell viability assays performed in the Svanborg laboratory deplete serum when HAMLET samples are applied prior to incubation for 24 hours (Svensson et al., 2000). For this reason, cell viability assays with U937 cells were competed with final FBS concentration at 5% (instead of 10%). Plates were incubated for 24 hours prior to viability assays. For samples with digestive enzymes present, pepstatin, a potent pepsin inhibitor was used to inhibit enzyme activity during bioactivity testing.

**Human osteogenic sarcoma adherent cells: U2OS**

U2OS, derived from human osteogenic sarcoma, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FCS), 100 mg/mL of streptomycin, 100 mg/mL of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5% CO$_2$. Media was replenished every 4 days. When cells became sufficiently confluent, they were detached using 0.25% trypsin and 0.05% EDTA for 2 minutes. Aliquots of U2OS for cellular experiments were subcultured as required. Plates were incubated for 24 hours prior to cell viability assay. The cell viability assays with U2OS were performed with no depletion of FCS.

**Rat pheochromocytoma adrenal cells: PC-12**

Undifferentiated PC12 (PC12$^{undiff}$) cells were maintained in RPMI 1640 medium-GlutaMAX™-1 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C in a 95% air/5% CO$_2$ humidified environment. Culture medium was replaced every 3 days. For the purpose of cell viability assays, cells were plated at a density of $10^6$ cells/well in 96 well plates. For nerve
growth factor (NGF) - induced differentiation, PC12 cells were seeded onto collagen coated 96 well plates at a cell density of $15 \times 10^4$ cells/well in RPMI 1640 medium-GlutaMAX™-1. Wells were supplemented with 1% FCS, 1% penicillin/streptomycin and 0.01% NGF. Cells were maintained in a 95% air/5% CO$_2$ humidified atmosphere at 37°C. Culture media was replenished every 2 days for a period of 7 days. At this point PC12 cells exhibit a differentiated morphology characterised by extensive neurite growth (PC12$^{\text{diff}}$). Plates were incubated for 24 h at 37°C in 5% CO$_2$ humidified environment prior to treatment with AlamarBlue.

**AlamarBlue Cell Viability Assay**

AlamarBlue reagent (resazurin), 10 μL, was added to each well and the plate was incubated for 4 hours. The fluorescence intensities of the samples were measured with an excitation wavelength of 530 nm and emissions were recorded at 590 nm.

**Apoptosis test kit**

The type of cell death occurring after treatment with samples was analysed using a Cell Death Detection ELISA$^{\text{PLUS}}$ kit (Roche Applied Science). Cells were seeded and supplemented with different samples in the same manner as for cytotoxicity assay. After 4 h of incubation at 37 °C and 5% CO$_2$, the cell viability was determined with a trypan blue assay. Cells with 50% viability were used for the apoptosis assay. Apoptosis was determined by quantifying the enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm of the treated cells.
2.2.10. Fluorescence Spectroscopy

Fluorescence spectroscopy is a highly sensitive technique used to study the physicochemical properties of proteins, protein interactions and protein dynamics. We employ three types of fluorescence measurements in the study of proteins – intrinsic, and 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS)-bound, and ThioflavinT fluorescence.

Samples are excited at a particular wavelength, such as 280 nm, and then their emissions measured over a defined number of wavelengths, such as 300-420 nm (the emission spectra). Many proteins contain naturally fluorescent amino acid residues such as tyrosine and tryptophan. Human α-LA contains 3 tryptophan residues which are contained within the calcium binding loop, therefore when calcium is removed from the α-LA, the residues, which previously had a low intensity, experience a change in their environment, thus resulting in both a shift at which the maximum intensity is observed, and an increase in the intensity recorded. This is due to both the hydrophobic regions of the protein being exposed and also as the protein unfolds residues such as Asp and Glu, which quench the signal from Trp, change location with respect to Trp thus resulting in a red-shift. A comparison of the amino acid residues of human and bovine α-LA can be seen in Figure 2.5. Fluorescence measurements based on the naturally fluorescent AA in proteins is known as intrinsic fluorescence.
In addition, a dye that binds to hydrophobic residues can be used to probe unfolded proteins. The hydrophobic dyes bind to the protein, and unfolding can be monitored through this. This is known as extrinsic fluorescence. The most common form of extrinsic fluorescence is 8-anilino-1-naphthalene sulfonate (ANS). A study of human α-LA, a genetic variant, and HAMLET was performed using ANS fluorescence spectroscopy. It can be seen in Figure 2.6 that there is an increase in intensity from just α-LA to HAMLET. This is due to the dye binding to the hydrophobic regions in HAMLET that are produced with α-LA in its partially unfolded form, resulting in more of its hydrophobic core exposed.

A third form of fluorescence utilised is ThioflavinT (ThT) fluorescence. ThT is a dye that binds to amyloid fibrils. An increase in fluorescence intensity between untreated sample (native protein) and treated sample (protein:fatty acid complexes) is indicative of the production of amyloid fibrils (Nilsson, 2004).
Figure 2.6: ANS fluorescence analysis of HAMLET (continuous black line), a HAMLET like complex formed from a genetic variant (black dashed line), and human α-LA (dot-dash line). The spectra for ANS alone is represented by a dotted line. (Pettersson-Kastberg et al., 2009).

Fluorescence measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer (Varian) equipped with a multicell holder, Peltier unit and temperature controller. Protein concentrations were determined by the method of Bradford and the protein concentration of the samples was diluted to 5 μM in the appropriate buffer. Spectra were recorded at 25°C and at a scanning speed of 80 nm min⁻¹. The excitation and emission slits were set to 10 nm.

**Intrinsic fluorescence**

Samples were dissolved in 10 mM Tris.HCl buffer, pH 8.5. Samples were excited at 280 nm and emissions were recorded between 300 and 420 nm.

**ANS fluorescence**

Samples were dissolved in 10 mM Tris.HCl buffer, pH 8.5 and ANS dye was added to samples at a concentration of 10 μM and left to incubate at room
temperature for 30 minutes. The samples were excited at 390 nm and emissions were recorded between 410 and 600 nm.

**Thioflavin T (ThT) fluorescence**

ThT was dissolved in 10 mL phosphate buffer (10 mM Na₂HPO₄; 150 mM NaCl), filtered through a 0.2 μM syringe filter and stored in the dark. On the day of testing 1 mL of this stock was added to 50 mL phosphate buffer. The fluorescence of the working buffer was measured with an excitation wavelength of 440 nm and emissions were recorded between 470 and 550 nm. 15 μL of the native protein solution was added to the working buffer and the spectra were recorded. This procedure was repeated for protein complexes. An increase in intensity between the control and test sample is indicative of amyloid formation.

### 2.2.11. Fourier Transform Infra-Red Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) provides essential information on the secondary structure of proteins. Infrared spectroscopy was developed through the discovery of IR radiation by Herschel in 1800, who then developed prism-based techniques to measure spectra with IR light. This discovery led to the development of the interferometer by Michelson (1891 and 1892), which could be used to measure the wavelength of light. Computing the Fourier Transform of the pattern generated by the interferometer (an interference pattern) produces a spectrum. Subsequently, Cooley and Tukey developed the fast Fourier transform (FFT) methodology in 1965, which significantly reduced the computation time while also increasing the resolution of the spectra.

An interferometer is a device used to generate interference patterns. It contains an IR light source which emits light in the IR region, a beam splitter which splits the IR beam into two separate beams, and two mirrors – one fixed which is used as a reference, and one moving mirror. The mirrors are set up so that the two beams can be reflected and then recombine at the beam splitter. A combination of the distance between the two mirrors and the recombined beam passing through the samples produces an interference pattern with different intensities for different samples.
The IR source provides IR radiation based on its resistance to conduct a current when electricity is passed through the source. Typically the generation of an IR beam results in huge heat generation, therefore a cooling system is needed to maintain a constant temperature within the system. Too much heat generation with inadequate cooling can result in the production of noisy signal and inaccurate readings as the detector used in FTIR is extremely sensitive to temperature changes. There is also a laser source within FTIR systems, which emits light at 15,798 cm\(^{-1}\). This serves as both an internal standard for wavelength determination and it is also used to determine the position of the moving mirror.

FT-MIR is FTIR in the mid-IR range (4000 – 400 cm\(^{-1}\)). It measures changes in the protein based on movements and vibrations in molecules. The most common bands analysed for changes in the secondary structure of proteins are given in Figure 2.7. Examples of the changes in the Amide I bands of proteins can be seen in Figure 2.8. The most common movements experienced by molecules include stretching, scissoring and twisting. The two main types of sample measurement in FT-MIR are transmittance and reflectance. Transmittance involves passing IR light through the sample and measuring/detecting the beam on the other side. The most common form of reflectance measurement is attenuated total reflectance (ATR), which measures samples based on changes in refractive index between the sample and the crystal. Light travels from an area of high refractive index (a crystal) to an area of low refractive index (sample), light is reflected back based on total internal reflection. The spectra are obtained from the surface of the samples based on the reflection experienced by the beam.

FTIR is a simple, rapid, sensitive method of analysis with a very high sample throughput. It yields consistent, well-defined spectra. It cannot detect diatomic molecules such as nitrogen and oxygen, therefore no vacuum is needed for measurements. Nitrogen can also be used for a constant airflow throughout the instrument or wherever the light beam passes through i.e. chamber of interferometer, sample and detector.
FTIR measurements were performed using a Bruker Tensor 27 instrument equipped with a thermally controlled BioATR Cell™ II, equipped with a single bounce ZnSe crystal (Bruker Optik, Germany), which was designed for analysis of protein in aqueous solution. Measurements were performed at 20°C and an average of 180 scans at a resolution of 4 cm⁻¹ were recorded. Protein samples were diluted to a concentration of 10 gL⁻¹ and filtered through a 0.1 µM syringe filter. Data was processed by performing atmospheric compensation and then vector normalisation at 1600-1720 cm⁻¹ for the Amide I region, and 2800-2900 cm⁻¹ for the oleic acid region. Spectra capturing software Opus (version 5.5) was used for data processing. The molar ratios of oleic acid to α-LA were determined using a multivariate calibration method that was developed using OPUS/QUANT software (Lišková, 2011).

![Figure 2.7: Typical FTIR spectra for protein sample showing the different bands of interest measured (α-LA sample; 1 mM in distilled H₂O).](image-url)
Figure 2.8: Typical conformation of the amide I band based on structure of proteins (image taken from Bruker Tensor 27 manual, 2005).
2.2.12. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance is a vital tool that can be used to study the biomolecular structure, dynamics and function of a variety of molecules including proteins. It was originally shown by Wüthrich and Wagner, 1978, that the aromatic residues in globular proteins were able to flip 180° from their original orientation as a function of temperature, despite the fact that the protein was still tightly packed. Results obtained here provided information that could not be obtained with other biophysical analysis of proteins.

NMR can show changes in a protein’s motion to give a clearer understanding of both protein dynamics, and subsequently its effect on the bioactivity of the protein. Chemical shifts are used to determine any structural changes that may occur within the protein. For example, the $^1$H signals for the aromatic regions of proteins appear between 6 and 8 ppm (Shimizu et al., 1993). Some aromatic regions of $\alpha$-LA that are tightly packed in the native state may become partially unfolded when the conformation of the protein changes. NMR is able to provide further structural information for $\alpha$-LA as it progresses from its holo to apo form and as it unfolds under acidic conditions.

The techniques employed here to further probe the protein or protein and fatty acid complex include $^1$H NMR, diffusion NMR (including Diffusion Ordered Spectroscopy; DOSY (Brand et al., 2007)) and natural abundance $^{13}$C NMR.

$^1$H proton NMR detects proton signals. However as most solvents in protein NMR are water based the water peak can cause difficulties in obtaining spectra. Deuterated solvents lessen the water peak however for protein NMR, particularly involving the quantification of oleic acid within proteinaceous samples, fully deuterated solvents cannot be used as the exchangeable aromatic peaks would not be detectable. To circumvent water peaks different water suppression methods are employed including:

(i) Presaturation: selectively saturates a peak at a specific frequency, with a long low-power pulse, typically the water signal, increasing signal to noise ratio.
(ii) WATERGATE (Piotto et al., 1992): WATER suppression by GrAdient Tailored Excitation, is a pulse sequence applied to suppress the water signal in aqueous samples and is particularly useful in DOSY experiments. Watergate uses a pair of gradients and results in the inversion of all signals apart from the water peak.

\(^{13}\text{C}\) natural abundance NMR detects the \(^{13}\text{C}\) isotope of carbon as the \(^{12}\text{C}\) carbon is undetectable as its net spin is zero. Approximately 1\% of all carbon is \(^{13}\text{C}\), thus there are greater sensitivity issues with \(^{13}\text{C}\) NMR than observed with \(^{1}\text{H}\).

**Liquid State NMR**

\(^{1}\text{H}\) NMR spectra were obtained using an 800 MHz shielded NMR Spectrophotometer (18.8 Tesla magnetic field strength) with an indirect detection cold probe (Agilent Technology, USA). Samples were prepared to a concentration of 1 mM in 10\% D\(_2\)O. 1,4-Dioxane was used as an internal standard (referenced to chemical shift 3.75 ppm). Spectra were obtained at 25°C. The stoichiometry of the protein-fatty acid complexes was determined using integration of the peak areas for the aromatic region of the protein and the region where oleic acid binds.

\(^{13}\text{C}\) Natural abundance NMR spectra were obtained. 1,4-Dioxane was used as an internal reference with a chemical shift of 67.3. Spectra were obtained at 25°C with a relaxation delay of 9 s. A total of 8000 scans were recorded.

**Magic Angle Spinning Solid State (MAS-SS) NMR**

Solid state magic angle spinning, where the sample is angled at 54.7° towards the magnet to reduce nuclear dipole-dipole interaction and increase resolution, was performed on BAMLET and related complexes. Samples were packed into 3.2 mm rotors and spectra were obtained with a spinning rate of 12,000 kHz at 4°C with 8,000 scans.
Diffusion Ordered Spectroscopy (DOSY) NMR

DOSY experiments were performed using stimulated echo and bipolar gradient pulses on the samples (Brand et al., 2007). A pre-saturation step, presat, was employed to reduce the water signal. The gradient time was 150 ms with a diffusion delay of 2 s. The gradient ran in a linear fashion from 1300 to 32500 Gauss/cm with 32 increments. Processing of the FIDs was performed within the Agilent VnmrJ software where three regions were integrated for peak area: 6-10, 5-5.5 and 3.6-3.8 ppm representing the aromatic region, the olefinic region and the 1,4-dioxane peaks respectively. The decay in signal was plotted as a function of the gradient strength.

In situ, real-time acidification of L-Histidine

A solution of 10 mM L-histidine was prepared in 10% D$_2$O. α-Gluconic acid delta-lactone, GDL, was added to the mixture at a concentration of 98 mM in order to decrease the pH. $^1$H proton NMR spectra were captured of the mixture as a function of time, for a period of 45 minutes.

In situ, real-time unfolding of α-LA and partial refolding in the presence of OA

A solution of α-LA and OA was prepared in 10% D$_2$O as outlined in 2.2.1. α-Gluconic acid delta-lactone, GDL, was added to the mixture at a concentration of 2.5% (w/v) in order to decrease the pH and effectively simulate the pH gradient of gastro-intestinal digestion. $^1$H proton NMR spectra were captured of the mixture as a function of time. After completion of GDL hydrolysis (the point at which the pH did not decrease further), DOSY experiments were performed as described previously. The sample was removed from the NMR tube and the pH was returned to pH 7 using NaOH and the resulting mixture was lyophilised. The powder was resuspended in 10% D$_2$O and further $^1$H and DOSY experiments were performed.

In situ heating of α-LA in the presence of SO

α-LA and sodium oleate were mixed as outlined previously (BAMLET by mixing). Using temperature control within the NMR unit the temperature was increased from 25°C to 30°C and then in increments of 10°C until heated to 60°C.
The samples were heated at 60°C for 1 hour and removed from the NMR machine and placed into an ice-bath. Further $^1$H and diffusion NMR experiments were performed at each stage.

**Stoichiometry and quantification of oleic acid in BAMLET samples by NMR**

It is essential that the rates of exchange between $^1$H spins in proteins (mainly $^1$H$^N$ originating from the backbone or the side chains of Trp, Asn, and Gln) and those $^1$H spins in water be taken into consideration when enumerating the total number of protons. Such rates of exchange is pH-dependent, and especially at the pH which ensures that BAMLET/HAMLET stably holds the oleic acid moieties (= pH 7.4), the intrinsic backbone $^1$H$^N$ exchange rates are known to be rather high (Connelly *et al.*, 1993). Hence, using pre-saturation as the solvent suppression technique will invariably lead to saturation transfer to $^1$H$^N$ spins (particularly known to be deleterious above pH 7) (Cavanagh *et al.*, 2010). This was accounted for by a rigorous comparison of the fully deuterated BAMLET with BAMLET in 90%/10% H$_2$O/D$_2$O buffer. The integrated area between 6.0 – 12.0 ppm for the fully deuterated sample will reflect the non-exchangeable $^1$H spins originating from the aromatic ring amino acid side chains (Phe, Tyr, Trp, and His). The sum total for this is 62 non-exchangeable protons, detailed in Table 2.3. For the 90%/10% H$_2$O/D$_2$O buffer case, the exchangeable $^1$H spins will be additionally contributing, albeit reflecting any chemical exchange with the solvent. The sum total for this summation is 218 protons, detailed in Table 2.3.

There are two protons within the olefinic region of the spectra (5.0-5.5 ppm), representing the C$_9$-C$_{10}$ protons. With a known protein concentration (1 mM as determined by absorbance spectroscopy) it is possible to determine the OA concentration within the sample by comparing the integrated peak area of the two regions.
### Table 2.3: Exchangeable and non-exchangeable protons contained within the polypeptide chain of $\alpha$-LA: detailing the number of protons used for the summation of protons contributing to the peak area contained within the 6.0 – 12.0 ppm region of $^1$H NMR spectra.

<table>
<thead>
<tr>
<th>Source $^1$H</th>
<th>Number of sidechain $^1$H (non-exchangeable)</th>
<th>Number of sidechain $^1$H (exchangeable)</th>
<th>Number of residues (Identity)</th>
<th>Total number of $^1$H (non-exchangeable)</th>
<th>Total number of $^1$H (Exchangeable)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypeptide chain backbone NH</td>
<td>123 (i) Including protonated amino terminus (NH$_3^+$; pKa = 8.0); and (ii) Excluding two Pro residues.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>5</td>
<td>1 ($^1$H: pKa of ~ 12; Chemical shift approx. 10.1 ppm)</td>
<td>4 (Trp$^{26}$, Trp$^{60}$, Trp$^{118}$ and Trp$^{184}$)</td>
<td>20</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>Tyr</td>
<td>4</td>
<td>1 ($^1$H: pKa of ~ 10.9; Chemical shift approx. 8.18 ppm; Not included in the summation.)</td>
<td>4 (Tyr$^{18}$, Tyr$^{36}$, Tyr$^{50}$, and Tyr$^{103}$)</td>
<td>16</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Phe</td>
<td>5</td>
<td>0</td>
<td>4 (Phe$^{6}$, Phe$^{31}$, Phe$^{33}$, and Phe$^{60}$)</td>
<td>20</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
<td>1 ($^1$H: pKa of ~ 6.04; Chemical shift approx. 8.41 ppm; Not contributing to spectra because of the pH of the sample.)</td>
<td>3 (His$^{32}$, His$^{68}$, and His$^{107}$)</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Asn</td>
<td>0</td>
<td>2 ($^1$H$^{621}$ and $^1$H$^{622}$; pKa &gt; 10; Chemical shift approx. 6.91 and 7.59 ppm)</td>
<td>8 (Asn$^{44}$, Asn$^{45}$, Asn$^{56}$, Asn$^{77}$, Asn$^{66}$, Asn$^{71}$, Asn$^{24}$, and Asn$^{102}$)</td>
<td>0</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Gln</td>
<td>0</td>
<td>2 ($^1$H$^{621}$ and $^1$H$^{622}$; pKa &gt; 10; Chemical shift approx. 6.87 and 7.59 ppm)</td>
<td>6 (Gln$^{2}$, Gln$^{39}$, Gln$^{43}$, Gln$^{54}$, Gln$^{65}$, and Gln$^{117}$)</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td></td>
<td></td>
<td>62</td>
<td>156</td>
<td>218</td>
</tr>
</tbody>
</table>

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CHAPTER 2

MATERIALS AND METHODS

Figure 2.9: $^1$H spectra of BAMLET in 90%/10% H$_2$O/D$_2$O (red; 1.0 mM) and fully deuterated in D$_2$O (blue; 0.93 mM). As an internal reference with a $^1$H chemical shift of 3.75 ppm, 1,4-dioxane was used. Samples were dissolved in 50 mM phosphate buffer, pH 7.4. Data were recorded at 25°C with 64 scans. For the 90%/10% H$_2$O/D$_2$O sample, presaturation was used at a power of 7 db (109 Hz). The integrated peak areas used for integration were 6.0 – 12.0 ppm and 5.0 – 5.5 ppm for the exchangeable and non-exchangeable aromatic and –NH protons envelope, and the olefinic region of the spectra respectively.

Integration of the 6.0 – 12.0 ppm and 5.0 – 5.5 ppm regions for the 90%/10% H$_2$O/D$_2$O sample yielded peak areas of 131,785 and 9,160 respectively. Comparing these peak areas with the number of protons as 218 yields a molar excess value of 7.6 moles of oleic acid per mole of protein. This sample was dried and solubilised in D$_2$O to allow complete exchange of the exchangeable protons to ensure full deuteration of the protein. Subsequent integration of the 6.0 – 12.0 ppm and 5.0 – 5.5 ppm regions for the fully deuterated sample yielded peak areas of 27,081 and 6,011 respectively. Comparing these peak areas with the number of protons as 62 yields a molar excess of 6.9 moles of oleic acid per mole of protein. Integration of the 1,4-dioxane peak at 3.75 ppm in the 90%/10% H$_2$O/D$_2$O sample and the subsequent deuterated sample shows a decrease in the integrated peak area which is congruent with the decrease in both the protein concentration and oleic acid concentration between the 90%/10% H$_2$O/D$_2$O sample and the deuterated sample. This consistent decrease can be attributed to loss of sample during the drying and deuterating process.

Repeated trials have led us to prefer the stoichiometry be measured in 100% D$_2$O. This will allow the measurements to be made irrespective of the sample pH, since
the number of non-exchangeable \( ^1 \text{H} \)'s would be a constant quantity of 62. We wish to note that most of the quantitations performed in this thesis were done in 90%/10% H\(_2\)O/D\(_2\)O.

### 2.2.13. Rotofor Isoelectric Focussing

The Rotofor\textsuperscript{®} system (BioRad, UK), was used for the separation of protein mixtures and protein-fatty acid mixtures. The Rotofor is a lab scale preparatory system that separates mixtures based on isoelectric focussing (IEF). A concentrated buffer solution, ampholytes, is added to the protein fraction prior to the sample being added to the chamber. An electric charge is applied to the chamber and the compounds within the chamber migrate to their isoelectric point. The ampholytes act as individual buffers for the individual chambers. Fractions are then harvested for analysis.

![Schematic of the sample chamber used for IEF with the Rotofor system](image)

**Figure 2.10:** Schematic of the sample chamber used for IEF with the Rotofor system (BioRad Rotofor user guide, 2011)

Solutions were prepared at 10 mgmL\(^{-1}\) protein and high concentration ampholyte solutions (pH range 3 to 10) were added at a concentration of 2%. Sample was loaded into the sample well and 15 W of power was applied until the voltage stabilised. Fractions were harvested and each fraction was analysed for pH, protein and fatty acid content using FTIR and NMR.
2.2.14. Gas Chromatography

The OA content of the complexes were determined as described by Palmquist and Jenkins, 2003. An OA standard curve was prepared by adding 0, 10, 25 and 50 µL OA dissolved in dichloromethane (1 mg/mL) to separate tubes. As an internal standard 50 µL palmitic acid dissolved in dichloromethane (2 mg/mL) were added to each tube used for the standard curve and to the tubes used for test samples. After evaporation of dichloromethane in a fume cupboard for 30 min, 100 µL PBS were added to the OA tubes and 100 µL of the protein complexes (BAMLET and digested complex) were added to the test samples tubes. To prepare derivatives of OA and palmitic acid for gas chromatography (GC) methyl esters were formed by the addition of 1.5 mL 10% methanolic HCl (prepared by slowly adding 20 mL acetyl chloride to 100 mL of dry methanol while stirring), and 1 mL heptane to each tube followed by heating to 90°C for 2 hours. After cooling on ice, 1 mL heptane and 3 mL 10% K$_2$CO$_3$ were added to each tube followed by vortexing and centrifugation (500 x g, 5 min). The heptane phase (upper phase) containing the fatty acid methyl esters was transferred to GC vials. Samples were analysed on a HP 6890 GC-system (Hewlett Packard Co., Palo Alto, CA, USA) with a flame-ionisation detector and a Restek Rt 2560 column (100 m x 0.25 mm x 0.2 µM, Restek U.S., Bellefonte, PA, USA). The inlet temperature was 275°C with a split ratio 40:1, and the carrier gas helium with a constant flow of 1.5 mL per minute. The starting temperature of 140°C was held for 5 minutes and increased by 4°C per minute to an end temperature of 300°C. The detector temperature was 300°C. Results were displayed as mg oleic acid per mg of powder thus to quantitate the oleic acid content in the samples the mg protein per mg of powder was determined using the Bradford method and molarities were determined on this basis.
2.2.15. BSA and α-LA competitive binding

Contact Plate

BSA was immobilised onto a polystyrene plate by filling it with a solution of 4.4% BSA and incubating it at 37°C for 1 hour. The BSA solution was removed by pipetting and the well was gently washed with distilled water. A solution of 1% BAMLET was then put into the well and the plate was incubated at room temperature for varying times. The samples were freeze dried for further analysis through FTIR, electrophoresis and cytotoxicity.

Inactivation of BAMLET with BSA

BAMLET solutions were prepared at a concentration of 1 mM. Amounts of BSA were added to the solution at concentrations from 0.5 – 4.4% (up to physiological conditions of serum albumin in blood). Samples were incubated at 37°C for 24 hours. Samples underwent ultrafiltration through a 30 kDa membrane and the retentate and permeates were kept for analysis.

2.2.16. Ultrafiltration of samples

Whey was isolated from milk as follows. Fresh raw milk was obtained from the Dairy Production Research Centre, Moorepark. The milk was centrifuged at 30,000 g for 30 minutes at 4°C. The resulting fat layer was removed and the skimmed milk was filtered through glass wool to remove residual fat. The pH of the milk was adjusted to the pl of casein, 4.6, and centrifuged at 30,000 x g for 30 minutes at 4°C. The casein pellet was removed and the whey solution was vacuum filtered through a #1 Whatman filter. The whey was then ultra filtered. As the molecular weight of α-LA is 14.2 kDa the sample was first filtered through a 30 kDa ultrafiltration membrane. The permeate of this filtration was then filtered through a 10 kDa ultrafiltration membrane. The Millipore diafiltration system was used with a flow rate of ~1 mL min⁻¹ and the pressure/flow rate was controlled through nitrogen gas.
2.2.17. *In vivo* human study

In conjunction with the Alimentary Pharmabiotic Centre (UCC), Cork University Hospital and Mercy University Hospital, a feeding study to determine the potential formation of a BAMLET-like complex in the digestive tract of adult volunteers was performed in the Gastrointestinal Clinical Measurement facility in Mercy University Hospital under the supervision of Dr. Martin Buckley and clinical research technician Lillian Barry. In this study volunteers had a nasogastric tube inserted into their stomach, which allowed gastric content removal during ingestion of specially formulated beverages.

**Ethical approval**

Ethical approval was obtained from the Cork Research Ethics Committee. A protocol was drafted outlining all stages in the trial and sent for approval along with advertisements to be used for recruitment and application form. Subjects received €50 to cover expenses and inconvenience.

**Pre-Screening**

A pre-screening step was undertaken to ensure that volunteers were healthy and did not suffer from any pre-existing medical conditions that may be have been exasperated by participating in the trial. Volunteers were to be between the ages of 18-55 with no pre-existing medical conditions. Volunteers underwent a medical questionnaire and a bowel disease questionnaire. Female applicants of child-bearing age took a pregnancy test. Blood was taken by clinical research nurse Anne O’Neill from volunteers and screened in Cork University Hospital for pre-existing medical conditions.

**Feed**

The feeds were formulated using food or pharmaceutical grade products as outlined in Table 2.3. Feeds were made up in water and a volume of 250 mL was ingested for each feed.
Table 2.4: Composition of the test drinks

<table>
<thead>
<tr>
<th>Feed 1</th>
<th>Feed 2</th>
<th>Feed 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (α-LA)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>25 gL⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty Acid</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4 gL⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>50 gL⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Subjects:
Subjects (n = 10; 6 female, 4 male) participated in the study [median age 32.5 years (25-48)]. None of the subjects were required to take any daily medication. A pre-screening step was performed to ensure the subjects were healthy and showed no symptoms of gastrointestinal disease. Female subjects of childbearing age were required to take a pregnancy test prior to nasogastric tube insertion.

Procedure
After an overnight fast, subjects attended the Clinical Measurement Laboratory in Mercy University Hospital. A nasogastric tube, 10CH/FG ryles tube, 3.3 mm diameter (Pennine Healthcare) was inserted into the stomach of subjects (Thomsen et al., 2006). A 10 mL Luer syringe was used for removing samples from the stomach. An aliquot of gastric juice was obtained and the pH was measured to confirm the tube was in the stomach. The void volume of the tube was calculated to be 6.5 mL thus after each sample was taken through the tube, 7 mL of air was injected through the tube to return gastric content to the stomach. Subjects were then given 250 mL of a test drink to consume. Samples were taken every 3 minutes. Each sample was 5 mL and the pH was recorded prior to aliquoting of samples into 1 mL vials. Each aliquot was immediately placed on ice and after pH measurement and frozen within 2 hours of sampling.

Mirocam® Imaging Study
Capsule endoscopy (IntroMedic, Seoul, Korea), was used for intragastric imaging of α-LA during digestion. Eight individual electrodes were attached to the torso of
the volunteer. These electrodes allowed the transmission of 3 images per second to a workstation, allowing real-time imaging of the stomach and its contents. The camera was equipped with an LED, which transmitted light to allow correct imaging of the milk-like solution. A combination of the intragastric pH probe and the camera allowed real-time pH measurement and real-time imaging of the stomach thus allowing the correlation of gastric pH with gastric content. The camera was swallowed simultaneously with the feed.

**Figure 2.11:** Mirocam® Capsule endoscope with dimensions shown, showing the LED light and the range of vision of the camera (IntroMedic, 2011).

**Intragastric pH monitoring**

The intragastric pH levels were recorded using a nasogastric pH probe inserted through the nose into the stomach. The probe was connected to a recording unit that allowed real-time monitoring of the pH within the gastric environment.
Chapter 3

The formation of an anti-cancer complex under simulated gastrointestinal conditions of infants

This chapter contains work that has been published:
3.1. Introduction

HAMLET, Human Alpha-Lactalbumin Made LEthal to Tumour cells is a protein-fatty acid complex between partially unfolded α-LA and OA. HAMLET was discovered by serendipity in 1995 when screening human milk for its anti-infective properties (Håkansson et al., 1995). Since then the field of HAMLET has expanded greatly to include many structural (Gustafsson et al., 2005; Pettersson-Kastberg et al., 2009; Pettersson-Kastberg et al., 2009) and functional (Düringer et al., 2003; Hallgren et al., 2006; Aits et al., 2009) studies of the complex. To date it is known that HAMLET can kill a range of over 40 different cancer cell lines with some specificity, while having little or no detrimental effect on healthy, differentiated cells (Mok et al., 2007). HAMLET acts with an apoptosis-like mechanism. It induces microautophagy in cells (Aits et al., 2009) and activates caspases (Düringer et al., 2003). However it has also been shown that cell death is independent of caspase activation (Hallgren et al., 2006). HAMLET also has anti-microbial activity against Streptococcus pneumonia (Håkansson et al., 2000). The activity of HAMLET was tested in several in vivo clinical trials and it was observed that through application of HAMLET to bladder cancer (Mossberg et al., 2007; Mossberg et al., 2010), skin papillomas (Gustafsson et al., 2004) and also brain glioblastoma xenografts (Fischer et al., 2004), all of which a decrease in tumour size was observed with no effect on the healthy surrounding tissues.

HAMLET-like complexes have also been previously formed from the α-LA of milk from other mammals including caprine and ovine, with the most notable species variation being bovine α-LA, denoted BAMLET (Pettersson et al., 2006), which is used by a number of different research groups (Lišková et al., 2010; Rammer et al., 2010; Spolaore et al., 2010).

α-LA is a small (Mw 14,200 Da), acidic (pI ~4.8), globular protein found in the whey fraction of the milk of all mammals. α-LA contains four disulphide bridges
cross-linking the polypeptide chain. One of the disulphide bonds bridges the large α-helical domain and the smaller β-sheet domains thereby forming the calcium binding loop. The calcium is bound to the protein through aspartic acid (Asp) residues. However when the pH of the protein decreases below its isoelectric point these Asp residues start to become protonated and release the calcium thus leaving the protein in a partially unfolded state (Hiraoka et al., 1980). This unfolding also leaves the hydrophobic core exposed. This partially unfolded α-LA is structurally similar to the apo (calcium free form) and is dubbed the A-state or acid-state (Kuwajima, 1996).

The main biological function of α-LA is to specify the substrate for lactose synthesis in the mammary gland (Brew et al., 1968). A follow-up clinical trial with mice where the gene that expressed α-LA was removed, the mice did not produce any α-LA or lactose in their milk (Stinnakre et al., 1994). A second and equally important function of α-LA is that it is nutritionally significant for nourishment, growth and development, particularly in humans. The amino acid profile of α-LA delivers most of the essential amino acids in the required concentrations needed for neonatal development.

The protein composition of human milk is also of significant importance. α-LA is the predominant protein in human milk, present at concentrations of 2-3 gL⁻¹ (Lönnerdal 2003). Similarly oleic acid is the most abundant fatty acid (as glycerides) in human milk and accounts for 34% of all fat (Gibson and Kneebone, 1981). Indigenously present in human milk is bile salt stimulated lipase (BSSL), which has lipolytic activity in the stomach, increasing lipolysis levels in both the stomach and intestines of infants (Jensen, 1995).

Monitoring the pH profile in early infants stomachs shows that the pH remains elevated (Mitchell et al., 2001) and above the required pH levels for proteolysis with pepsin (Favilla et al., 1997), thus there is a relatively low rate of proteolysis in the stomach of infants. Low proteolysis levels result in polypeptide chain
stability and presence of significant amounts of native α-LA as milk passes through the stomach phase of gastric digestion.

The conditions under which HAMLET was initially discovered were similar to those in the stomach (Svensson et al., 2000; Barbana et al., 2006). Given that the composition of human milk may lend itself to the formation of HAMLET – high α-LA levels, high OA levels and high gastric lipolysis – there has been speculation that a HAMLET-like complex may be formed in the gastric tract of breast-fed infants.

The aim of this study was to determine if a BAMLET-like complex could be formed under simulated gastric conditions. Fractions were produced and analysed for structural and biological similarities to characterised BAMLET – monomeric with approximately 5 times molar excess of oleic acid, produced using the previously published chromatographical method as a reference.
3.2. Results

A simulated gastric model of infants was used for simulated digestion. *In vitro* models such as the US Pharmacopeia use static models with a set incubation time at pH 2. However *in vivo* data from neonates show a more gradual decrease in gastric pH (Mitchell *et al.*, 2001). Therefore a model which employed a pH curve was used in order to allow sufficient time for indigenous lipase to act, protein to unfold and a small amount of proteolysis to occur. Complexes were produced and analysed as outlined in Table 3.1.
Table 3.1: Outline of different stages before, during and after *in vitro* gastric digestion, with corresponding samples and abbreviations used throughout the study.

<table>
<thead>
<tr>
<th>Stage 1: Formulation of milk</th>
<th>Abbreviation</th>
<th>Symbol</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Lactalbumin</td>
<td>α-LA</td>
<td>⬤</td>
<td>Structural Analysis</td>
</tr>
<tr>
<td>Oleic acid dissolved in ethanol</td>
<td>OA</td>
<td>⬪</td>
<td>FTIR</td>
</tr>
<tr>
<td>Apo α-Lactalbumin</td>
<td>Apo</td>
<td>○</td>
<td>NMR</td>
</tr>
<tr>
<td>Stage 2: Gastric phase – pH 2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Addition of HCl to mimic pH conditions (as per figure 1A)</td>
<td>Gastric α-LA</td>
<td>□</td>
<td>OA Content</td>
</tr>
<tr>
<td>Addition of pepsin</td>
<td></td>
<td></td>
<td>FTIR</td>
</tr>
<tr>
<td>Stage 3: Post-gastric phase – pH 7</td>
<td></td>
<td></td>
<td>NMR</td>
</tr>
<tr>
<td>Addition of NaOH to bring pH to neutral conditions</td>
<td>Post-gastric α-LA</td>
<td>▼</td>
<td>GC</td>
</tr>
<tr>
<td>Stage 4: Ultrafiltration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration of digest through a 10 kDa membrane at pH 7</td>
<td>Retentate</td>
<td>△</td>
<td>Bioactivity</td>
</tr>
<tr>
<td></td>
<td>Permeate</td>
<td>—</td>
<td>Testing on U937 cells</td>
</tr>
</tbody>
</table>

Compared to BAMLET produced through chromatography (BAMLET ♦)
3.2.1. Simulated digestion

The pH of the stomach of infants was mimicked according to the pH gradient in Figure 3.1A, as per the study of Mitchell *et al.* This gradient represents several different studies where gastric pH levels were monitored during feeding. Pepsin has an optimum activity of between pH 1.8 and pH 2. While these pH conditions are not favourable for proteolytic hydrolysis they are ideal for gastric lipolysis. Gastric lipase enzymes are active between pH 4 and pH 6 thus high levels of lipolysis occurs in the stomach of breast-fed infants due to the presence of BSSL.

![Figure 3.1: A) pH gradient used during simulated gastric digestion with highlighted areas: I) the optimum pH range for gastric lipolysis; II) the point at which α-LA unfolding commences, III) the pH range of highest gastric proteolysis and IV) the pH for α-lactalbumin re-folding. B) SDS-PAGE of 1- α-LA 2- Post-gastric α-LA 3- UF Retentate 4- UF Permeate. MW – protein molecular weight markers 113 – 21 kDa.](image)
3.2.2. Structural analysis of the complex

Fluorescence Studies

It was previously shown by Svensson et al., 2003, that α-LA unfolding is a pre-requisite for the conversion of α-LA to its bioactive form. Typically Ca^{2+} chelators are used to induce the partially unfolded apo form of α-LA. However in this study the A-state of α-LA, a structurally similar form of α-LA, was induced under these acidic conditions. Unfolding was observed after acidification of α-LA during the simulated gastric phase below pH 4. This was confirmed through the comparison of the fluorescence spectra for holo α-LA and apo α-LA (Figure 3.2A). When the gastric pH model experienced a pH increase, this resulted in a reverse conformational change towards a partially re-folded structure (Figure 3.2B). It should be noted that intrinsic fluorescence is a reporter of global structural changes, and hence there may be different populations of conformers that contribute to that intermediate signal.

Using ANS it is possible to detect changes in the surface hydrophobicity of the protein (Pettersson-Kastberg et al., 2009). ANS binds to holo α-LA in limited amounts, however there is an increase in hydrophobicity as the molecular state of the protein changes from holo to apo, and a further increase in hydrophobicity between apo α-LA and α-LA in the A-state (Figure 3.2C). This suggests a higher level of hydrophobic residues exposed thus a marked increase in surface hydrophobicity.

ThioflavinT (ThT) fluorescence is a rapid screening method for the detection of extended β-sheets such as those seen in amyloid fibrils. Any treatment of proteins that changes their structure or functionality can also increase the risk of amyloid-like formation, which would be a negative characteristic of proteins due to the association of fibrils with degenerative diseases such as Alzheimer’s and dementia (Dobson 1999). For both the BAMLET produced through chromatography and the complex extracted from the simulated gastric conditions
there was no increase in intensity at 490 nm, which is indicative that OA does not promote amyloid fibril formation under these conditions (Figure 3.2D).

Through intrinsic fluorescence it was shown that BAMLET and α-LA:pH 7 have a similar tertiary structure. Their secondary structure was also deemed to be similar thus it is a fair assumption that their respective spectra for ANS fluorescence would be alike. However this was not the case. This would suggest that ANS not only binds to the hydrophobic regions of the protein, but also the hydrophobic fatty acid oleic acid. This is further shown when the oleic acid stoichiometries for the respective complexes are examined as this appears to be the sole difference in the two complexes. BAMLET has a higher molar ratio of OA bound (see OA quantification data, Figure 3.4, Figure 3.5) and also a higher ANS fluorescence intensity.
CHAPTER 3

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![Graph B](image1)

![Graph C](image2)
Figure 3.2: Fluorescence spectra of 5 μM α-LA in 10 mM Tris buffer at 25°C with a scanning speed of 80 nm/min⁻¹. All measurements were performed in triplicate on three individually produced samples with an average of nine spectra are displayed above:

A) Intrinsic fluorescence spectrum recorded with an excitation wavelength of 280 nm with spectra recorded between 300 and 420 nm of: Gastric α-LA (at pH 2.5) (green); for comparison, spectra of holo (blue) and apo (red) α-LA are also shown. The fluorescence maximum for Gastric α-LA was 349 nm, for holo α-LA 327 nm and for apo α-LA 349 nm.

B) Intrinsic fluorescence spectrum recorded with an excitation wavelength of 280 nm with spectra recorded between 300 and 420 nm of: Post-Gastric α-LA at pH 7 (purple); for comparison, spectra of holo α-LA (blue), and apo α-LA (red) and BAMLET (green) are also shown.

C) ANS fluorescence spectra recorded with an excitation wavelength of 390 nm with spectra recorded between 410 and 600 nm of: Gastric α-LA (cyan); Post-Gastric α-LA (green); for comparison, spectra of apo α-LA (red); BAMLET
(purple) and holo α-LA (blue) are also shown. Samples were incubated with a 2 times molar excess of ANS to protein for 30 minutes.

D) ThioflavinT fluorescence spectra recorded with an excitation wavelength of 440 nm with spectra recorded between 470 and 550 nm of: α-LA (blue); Post-Gastric α-LA (red) and BAMLET (green). Samples were incubated on a 1:100 volume basis with a 1 in 50 dilution of ThT in phosphate buffer.

FTIR

FTIR detects changes in the secondary structure of proteins by measuring changes in the Amide I band (1600-1720 cm⁻¹) which measure C=O stretching, giving information on the conformation of the protein. α-LA has a mainly α-helical structure thus has a peak at 1652 in the Amide I region of the FTIR spectra. Under acidic conditions there is a shift in the absorbance maximum of α-LA. A random coil formation has a maximum intensity at 1645 cm⁻¹. α-LA at pH 2.5 under simulated gastric conditions has a maximum intensity at 1647 cm⁻¹ suggesting that it is in the molten globule-like form, which is in agreement with both intrinsic and ANS fluorescence. These secondary structure results are in agreement with the tertiary structures measured with intrinsic fluorescence, and as with intrinsic fluorescence, the digested complex and BAMLET have a similar secondary structure. This corroborates the hypothesis that a BAMLET-like complex is formed under simulated gastric conditions (Figure 3.3).
Figure 3.3: Amide I region of vector-normalised with offset correction FTIR spectra of Gastric α-LA at pH 2.5 (red) and Post-Gastric α-la at pH 7 (green); native α-LA (blue); BAMLET (purple) and oleic acid (cyan) shown for comparison. All measurements were performed at 20°C with an average of 180 scans. Measurements were made in triplicate on three individually produced samples with an average of nine spectra are displayed above.

NMR

The aliphatic region of the spectra, from 0 to -1 ppm, corresponding to the δCH3 protons of Ile and the γCH3 protons of Val residues, provides information on the structure and folding of the protein (Wijesinha-Bettoni et al., 2001; Pettersson-Kastberg et al., 2009). Comparing spectra (a) from holo α-LA to α-LA in its molten globule form at pH 2.5 (d) shows that the molten globule form lacks any well-defined 3° structure, which is characterised by the smooth line, whereas the spectra for holo α-LA, which has a series of peaks corresponding to its defined tertiary structure. The BAMLET complex that was produced through chromatography exhibits native like-structure compared to the molten globule form, although it is possible that there exists a partitioning of the population of
protein molecules into native and molten globule states (Pettersson-Kastberg et al., 2009). It should be noted that column-BAMLET possesses cytotoxic activity, and the same was observed for the digested complex after the pH increase post-gastric phase of digestion, (Figure 3.7A). This would suggest that structurally, it is possible that the partially unfolded state of the protein may coexist in equilibrium with the native state of molecules, as shown by NMR, within the ensemble of the protein-fatty acid complex.

Figure 3.4: $^1$H-NMR spectra of 1 mM protein (dissolved in 10% D$_2$O, 50 mM phosphate buffer, pH 7.4) of (a) a-LA; (b) BAMLET produced through chromatography; (c) Post-gastric a-LA; (d) Gastric a-LA (at pH 2.5). (I) and (II) are magnified section of the spectra corresponding to the oleic acid and aliphatic protein region of the spectra, respectively. Spectra were recorded at 25°C with pre-saturation of the water peak. Proteins
3.2.3. Oleic acid content

FTIR

Analysis of the FTIR spectra for α-LA, OA and BAMLET shows that a band at wave number 2854 cm\(^{-1}\) which corresponds to OA is not present in α-LA but is present in BAMLET. Thus FTIR can detect the presence of oleic acid, and subsequently quantify the oleic acid content within the samples.

When α-LA is in its molten globule or apo form it is in the required conformation to bind to oleic acid and form a BAMLET-like complex. Under simulated gastric conditions at pH 2.5 minimal levels of oleic acid were present. This suggests that hydrophobic interactions alone are not enough to bind oleic acid to α-LA. When the pH of the digest increased, α-LA refolded (Figure 3.5A). FTIR data suggests that there is a higher quantity of oleic acid present. Quantification of this oleic acid peak gives an oleic acid stoichiometry of 4.4 moles of oleic acid per mole of α-LA. Quantification of the BAMLET sample produced through chromatography, performed as described in materials and methods, yields an oleic acid stoichiometry of 5.8, which is in line with the previously published literature of 5.4 with GC (Svensson et al., 2003) and 5.1 with NMR (Fast et al., 2005).

Evaluation of the ultrafiltration permeate and retentate showed that there was oleic acid present in fractions of the ultrafiltrate. This would suggest that oleic acid that is present in the retentate is bound to the protein as free oleic acid has been removed through the diafiltration step. The permeate contained the highest amount of oleic acid and also the low molecular weight fractions of the digest. (Figure 3.5B).
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A

B

Wavenumber (cm⁻¹)

Intensity (a.u.)

2900 2890 2880 2870 2860 2850 2840 2830 2820 2810 2800

2900 2890 2880 2870 2860 2850 2840 2830 2820 2810 2800

Wavenumber (cm⁻¹)

Intensity (a.u.)
Figure 3.5: Vector-normalised region of FTIR spectra corresponding to oleic acid during (A) simulated gastric digestion and (B) subsequent fractionation by ultra-filtration.

(A): Gastric $\alpha$-LA (red), Post-Gastric $\alpha$-LA (green); for comparison OA alone (cyan), $\alpha$-LA (blue) and BAMLET (purple) are also shown.

(B): OA spectra for the different stages during ultra-filtration after digestion showing the permeate with an increased OA content (red) and the retentate (purple), along with Post-Gastric $\alpha$-LA prior ultra-filtration (green), holo $\alpha$-LA (blue).

NMR

Nuclear Magnetic Resonance has previously been used to quantify oleic acid content in HAMLET and HAMLET-like complexes (Pettersson-Kastberg et al., 2009). By integrating the peak area of both the aromatic region of the protein (6-10 ppm) and the peak area of the olefinic oleic acid region (5-5.5 ppm) it is possible to quantify the amount of oleic acid present in samples. What distinguishes NMR from techniques such as GC and FTIR for OA quantification is that it can differentiate between bound and unbound oleic acid in samples rather than just determining if it is present in the samples. Oleic acid that is bound to protein has a chemical shift of 5.3 ppm whereas free, unbound oleic acid has a chemical shift of 5.4 ppm (Fast et al., 2005).

Analysis of both the column BAMLET and the digested complex show that the OA peak has a chemical shift of 5.3 ppm, verifying that the oleic acid is bound to the protein and not just in solution. Quantification of the oleic acid region of the digested complex shows that there is a 4.1 molar excess of oleic acid bound per mole of protein, as compared to 5.6 for BAMLET produced through chromatography.
3.2.4. Polypeptide chain composition of the complex

Ultrafiltration

In order to determine if BAMLET-like complexes can be formed with the peptides produced during *in vitro* simulated digestion an ultrafiltration step was employed using a 10 kDa UF membrane. Using size exclusion chromatography it was possible to determine that peptides were all of molecular weight less than 6.5 kDa, therefore the BAMLET-like complex (MW ~14,200 Da protein) is retained by the membrane while peptides and oleic acid were in the permeate (Figure 1B; Figure 5B). Analysis of the retentate of the UF sample of post-gastric α-LA shows that the OA present in the sample has a chemical shift of 5.3 which shows that oleic acid is bound to the protein.

HPLC

Reversed-phase chromatography was used for both native protein analysis (C4/5), and peptide analysis (C18). C4/5 chromatography detects the loss in native protein and analysis of the samples showed that the peak obtained for α-LA had a retention time of 11 minutes, which reduced slightly as digestion time progressed, suggesting that a slight decrease in hydrophobicity of the protein due to the loss of hydrophobic amino acids during hydrolysis. Quantification of the peak areas for α-LA before and after digestion shows that under the tested conditions ~60% of the native protein remains undigested after 30 minutes of digestion. (Figure 3.6A).

Size exclusion chromatography, SEC, showed that there was a breakdown of α-LA into smaller peptides. After the addition of the digestive enzyme, proteolysis began to occur. The intensity of the peak for native α-LA decreased as digestion time progressed, suggesting that there was still native protein present. There was an increase in the amount of breakdown products obtained during digestion (Figure 3.6B).
C18 chromatography was used for peptide analysis. There was a decrease in native protein content and a subsequent increase in peptide formation as digestion progressed. (Figure 3.6C).
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Figure 3.6: HPLC chromatographs of α-LA (blue) and post-gastric α-LA (pink) using (A) native protein-reversed phase; (B) size exclusion chromatography; (C) C18 reversed phase. In each case a decrease in native protein content can be observed, and a subsequent increase in peptide formation.

Electrophoresis

SDS-PAGE analysis (Figure 1B) confirmed that the digest was composed of protein and peptides (lane 2). UF with a 10 kDa membrane removed some peptide material (lane 3) however some remained in the retentate. The permeate consisted of peptides (lane 4).

3.2.5. Activity of the fractions

All fractions that were isolated throughout the process were tested against lymphoma cell line U937 for their cytotoxic activity and their activity was compared to the activity of BAMLET produced through chromatography. BAMLET had an LC₅₀ value of 0.13 ± 0.02 mg/mL (9.15 ± 1.4 μM). The digested complex had an LC₅₀ value of 0.2 ± 0.01 mg/mL (14.08 ± 0.7 μM) (Figure 3.7A). The difference in the activity of the two samples can be related back to the oleic acid content within the samples. Testing of the fractions showed an enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm of the treated cells, indicating that the cell death was as a result of apoptosis, with values of 17.18 ± 0.36 and 19.73 ± 0.85 for BAMLET produced through chromatography and the digested complex respectively.

The UF permeate which contained the low molecular weight fractions from the digest was shown to contain the highest amount of oleic acid (Figure 3.3B) however bioactivity testing showed that this complex was not toxic to the cells in the tested concentrations.
THE FORMATION OF AN ANTI-CANCER COMPLEX UNDER SIMULATED GASTROINTESTINAL CONDITIONS OF INFANTS

![Graph A](image1)

![Graph B](image2)
Figure 3.7: Cytotoxicity against U937 cells tested with alamarBlue® after a 24 hour incubation with A) the digested complex (red), BAMLET (green), and α-LA (blue); and B) the UF retentate (green), the UF permeate (purple), BAMLET (red) and α-LA (blue). Data are the means of three independent measurements with SDs represented by vertical bars.
3.3. Discussion

There has been speculation regarding the potential formation of HAMLET in the stomach of breast-fed infants (Svensson et al., 2003, Barbana et al., 2006). A recent study on the formation of BAMLET from denatured α-LA also alluded to the potential formation from α-LA from infant formula (Lišková et al., 2010). The data presented in this study indicates that a BAMLET-like complex, which is structurally and actively similar to BAMLET, can be formed under simulated gastric conditions.

Recently Tolin et al., 2010, published results that suggest that α-LA fragments can bind to oleic acid and induce apoptosis in cancer cells with comparative activity to BAMLET. These complexes are produced through mixing oleic acid with the fragments of α-LA to produce the complexes, suggesting that should α-LA undergo digestion it may bind oleic acid and be active. However these fragments are produced under limited proteolysis and thus may not be representative of fragments that may be produced through *in vitro* and subsequently *in vivo* digestion. Comparatively speaking, there were in excess of 20 peptides formed with the conditions tested in this study, which is considerably greater than the 3 fragments produced through limited proteolysis. Thus while bioactive complexes can be formed through α-LA fragments, this was not observed with the fragments produced in this study.

It was previously shown that unfolding is a pre-requisite for the conversion of α-LA to BAMLET; however unfolding alone is not enough to convert α-LA to its bioactive form (Svensson et al., 2003). The unfolding of α-LA results in an increase in its hydrophobicity (Pettersson-Kastberg et al., 2009) thus the current consensus is that the hydrophobic regions of α-LA act as binding sites for oleic acid, and that the formation of BAMLET is based on hydrophobic interactions (Tolin, et al., 2010). Sequencing of peptides derived from α-LA could therefore result in the identification of hydrophobic peptides, which may form HAMLET-
like complexes. Other forces, such as electrostatic charge, may also have an impact on the formation of complexes, thus the altered charge of apo α-LA and α-LA in its molten globule state impact the formation of the complex. The effect of the pH conditions on both the solubility and the charge of the oleic acid may also therefore have an affect on the formation of the complex. Hydrophobic interactions may be the initial cause of interaction, however this hydrophobic interaction alone is not enough to produce the complex as at pH 2.5 the oleic acid present was not enough to have a cytotoxic effect against the cells. A secondary effect is needed to partially refold the α-LA in order to convert it to its bioactive form. Thus either the structural change in the protein (partial refolding) or the change in the charge on the protein had an impact on the complex formation.

Another important factor to consider when determining the formation of the complex is the molecular state of oleic acid. Oleic acid has no charge below its pKₐ, and while the pKₐ of oleic acid is approximately pH 4.8, this can change depending on osmotic conditions. Thus should the electrostatic force between the protein and the fatty acid be a factor in the production of the complex, the oleic acid will not be in a suitable state to bind to the protein. Oleic acid is also pH dependant in that it can interchange between its acid state and its oleate (salt form) state. Therefore when looking at the complex formation it is necessary to not only look at the conformation of the protein, but also of the fatty acid state.

The structure of the complex remains a point of interest. There have been many structural studies on HAMLET and HAMLET-like complexes, the most recent by Pettersson-Kastberg et al in 2009 where they produced a perpetually molten-globule form of α-LA (rHLA\textsuperscript{Ala-Ala} -OA) and used it to produce a HAMLET-like complex. NMR spectroscopy of this complex and HAMLET produced through chromatography showed there were no chemical shifts in the upfield region of the spectra, suggesting a lack of tertiary structure within the protein. A similar structure has also previously been observed in other studies (Casbarra et al., 2004). However as shown in Figure 3.4, there are many peaks seen in the upfield
region of the spectra, which corresponds to a native-like structure. We speculate that the reason lies in the fact that the alpha-lactalbumin-fatty acid complex forms a continuum of various partially-unfolded states of varying degrees that make up the entire population (Pettersson-Kastberg et al., 2009).

The indigenous properties of human milk are also important for the formation of the complex. The presence of gastric lipase in the milk means that there will be elevated levels of lipolysis in breast-fed infants (Jensen 1995). The second important factor in the formation of the complex is the digestive tract of infants. The conditions are favourable for complex formation as the pH decreases over time (Mitchell et al., 2001), which are unfavourable for proteolysis, thus less proteolysis will occur. The pH decrease also promotes lipolysis prior to protein unfolding thus free fat will be present prior to protein refolding.

The resistance of α-LA to proteolysis in the presence of oleic acid (Casbarra et al., 2004) is another interesting factor. This study has shown that as α-LA is converted to its bioactive form, 60% of the native protein remains. This is a significant finding, as conversion to the bioactive form is not seen with α-LA peptide fragments. It is also an inherent characteristic of α-LA that it is more resistant to tryptic hydrolysis than other whey proteins (de Laureto, 1999). Thus once peptic hydrolysis is complete, α-LA will remain more stable in the GI tract.

The binding of OA to α-LA also results in the stabilisation of α-LA. α-LA in its apo and A-states are inherently unstable as it does not have a co-factor bound (Halskau et al., 2002). Thus the binding of OA to the protein may also stabilise it to intestinal digestion.

Activity analysis of the digested complex compared to BAMLET yielded interesting results. Samples were solubilised in RPMI media with regards to protein content. Thus their oleic acid content is different based on oleic acid molar ratio estimation. BAMLET produced through chromatography has a lower LC$_{50}$
value than the BAMLET-like complex produced through digestion. As the samples are structurally homologous it can be concluded that the difference in activity may be as a result of the difference in oleic acid levels, thus suggesting that OA is the active component of H/BAMLET and α-LA acts as a mule to deliver OA to the cells. This confirms previous results including Wilhelm et al., 2009, and Permyakov et al., 2011.

This study also raises the potential for the production of BAMLET without chromatography. Many methods have been suggested for the formation of H/BAMLET without the use of chromatography, including mixing at room temperature and titrating with OA to its critical micelle concentration (Knyazeva et al., 2008), mixing at elevated temperatures (Zhang et al., 2009), and simple mixing in solution (Spolaore et al., 2010; Brodkorb & Lišková, 2009).

Thus it can be concluded from this study that under the tested simulated gastric conditions, a complex that is structurally and actively homologous to that of BAMLET can be produced.
Chapter 4

The \textit{in vivo} gastric digestion of $\alpha$-Lactalbumin in adults

Manuscript prepared for submission to the \textit{British Journal of Nutrition}.
CHAPTER 4

THE IN VIVO GASTRIC DIGESTION OF α-LACTALBUMIN IN ADULTS

4.1. Introduction

Once deemed to be a waste commodity or by-product of cheese production, the whey fraction of milk has received much interest from scientists and industry of late. Known for its traditional functions, including that of nutrition (whereby the amino acid sequence of the protein provides all of the essential amino acids), it also possesses other functional properties. Whey has been used in gelation studies (Kehoe et al., 2007) including its use as a carrier for targeted delivery of probiotic bacteria to the intestines (Doherty et al., 2010) and emulsification (Bylaite et al., 2001).

Along with the known health benefits of components such as the iron binding protein lactoferrin and the immunological benefits of immunoglobulins, both components of whey, further research is currently on-going to determine other health benefits that can be attributed to the consumption of whey-containing products. Examples include the formation of ACE inhibitory peptides through the hydrolysis of the main protein in the whey of bovine milk, β-lactoglobulin, β-LG, (Murray and FitzGerald 2007) and the formation of anti-microbial peptides from α-lactalbumin, α-LA (Pellegrini et al., 1999), a protein found in the whey fraction of all mammals. Many other health promoting benefits have been reported through the consumption of whey proteins (Chatterton et al., 2006), including the supplementation of α-LA into infant formulae to further “humanize” infant formula (Rudloff and Lönnerdal 1992) and the potential use of α-LA in the treatment of stress through the increase of serotonin levels after consumption of tryptophan-rich α-LA (Markus et al., 2000).

Another function of whey proteins is the binding of hydrophobic compounds to affect the bioactivity of the proteins, including the binding of sodium linoleate to β-LA (Le Maux et al., 2012); and more extensively the binding of oleic acid to both α-LA (Svensson et al., 2000) and β-LG (Lišková et al., 2011) to produce tumouricial and antimicrobial (Håkansson et al., 2011) complexes.
\( \alpha \)-LA, is a small, globular protein found in the whey fraction of all mammals. It is composed of 123 amino acid residues with a resulting molecular weight of 14.2 kDa. It is a known component of lactose synthase in the mammary gland (Brew et al., 1968). It is also known to be nutritionally significant in that it provides all of the essential amino acids in their required concentrations (Jensen, 1995). Recently there have been reports that \( \alpha \)-LA is thought to protect somewhat against breast cancer and thus was the focus of a potential vaccine against breast cancer (Jaini et al., 2010).

Another function of \( \alpha \)-LA is its role in the production of the known anti-microbial and tumouricidal complex between \( \alpha \)-LA and milk fatty acid oleic acid (OA), dubbed HAMLET (Human \( \alpha \)-lactalbumin Made LEthal to Tumour cells) (Svensson et al., 2000). HAMLET is active against a range of over 40 different cancer cell lines, and exhibits certain selectivity and can differentiate between cancer cells and healthy cells (Brinkmann et al., 2011). It kills cancer cells with an apoptosis-like mechanism, activating caspases (Düringer et al., 2003) and induces microautophagy (Aits et al., 2009). HAMLET has been shown to be active in vivo in a range of clinical trials including bladder cancer (Mossberg et al., 2007; Mossberg et al., 2010), brain glioblastomas (Fischer et al., 2004), and colon cancer (Puthia et al., 2013). It was shown by Sullivan et al., 2013, that under simulated infant gastrointestinal conditions that a complex that was structurally and actively similar to BAMLET, the bovine analogue of HAMLET, was formed.

The initial concept of a HAMLET-like complex being formed in the gastrointestinal tract was described by Svensson et al., 2003. During its initial discovery, HAMLET, previously known as MAL, multimeric \( \alpha \)-LA, the complex was found in the acidic fraction of precipitated human milk. This led to speculation that the conditions within the stomach of nursing infants may be favourable for the conversion of \( \alpha \)-LA to its active form: there are high rates of gastric lipolysis occurring thus free OA is present (Roman et al., 2007); upon pH changing to below the isoelectric point of the protein it unfolds, potentially
allowing complex formation (Kuwajima 1996); and low rates of proteolysis occur due to the pH gradient experienced in the stomach of infants (Mitchell et al., 2001). This was again alluded to by Barbana et al., 2006. α-LA, and fragments of α-LA produced through limited proteolysis of the protein, were shown to bind the fatty acid and in turn possessed cytotoxic activity.

The aim of this study was to determine whether the required unfolding of the protein occurs \textit{in vivo} in the stomach of healthy adults, and then to determine whether enough native protein remains during gastric digestion for conversion of α-LA to its bioactive form and to test \textit{ex vivo} gastric samples against cancer cells.
4.2. Results

4.2.1. pH within the gastric environment

The fasting pH of the stomach of the subjects was measured prior to the ingestion of each test drink. Typically the pH of the stomach was acidic with average fasting pH levels of 2.31 ± 1.19, 2.22 ± 1.91, and 1.89 ± 1.80 prior to drinks 1-3 respectively. The pepsin activity within the stomach was determined prior to the ingestion of the protein-only feed and was 812 ± 257 U/mg protein (Table 4.1).

Table 4.1: Measured experimental values of baseline levels of pH and pepsin activity (range of values in parenthesis)

<table>
<thead>
<tr>
<th></th>
<th>Fasting levels</th>
<th>Before feed 2</th>
<th>Before feed 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>2.31 (± 4.05)</td>
<td>2.22 (± 2.85)</td>
<td>1.89 (± 3.25)</td>
</tr>
<tr>
<td></td>
<td>(1.8 - 6.3)</td>
<td>(1.2 - 4.5)</td>
<td>(0.8 - 5.7)</td>
</tr>
<tr>
<td><strong>Pepsin activity</strong></td>
<td>812 (± 257) U/mg</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aPepsin activity defined as units of pepsin per mg (U/mg) of protein using bovine haemoglobin as a substrate.

The pH of the aspirate was measured immediately after it was removed by suction through the previously inserted nasogastric tube. The pH of the stomach increased due to the pH of the protein only feed (pH 6.7) and the buffering capacity of the protein within the feed. A similar trend was seen with the protein and OA feed 2. No difference was observed between the curves for the protein and the protein-fatty acid drinks. Prior to the ingestion of the oleic acid only beverage the resting pH of the stomach was 1.89 ± 1.80 (0.8 - 5.7). After ingestion of this drink the pH of the stomach remained stable and no increase was observed. This was due to the acidic nature of the OA and the lack of a buffer present (Figure 4.1). If OA is present in the non-emulsified form it will not affect the pH as it is just an oily liquid and water mixture.

For the purposes of the current study gastric emptying was defined as when only air or gastric mucus was removed with the syringe via the nasogastric tube at the
location accessible to the tube, thus no sample was remaining. It is a fair assumption that this is close to actual gastric emptying however it would need to be confirmed by other methods such as MRI scanning or scintigraphy. Gastric emptying was estimated to have occurred at 30 minutes for each of the three feeds. The calorific value of the three feeds was calculated using standard values per 1 g of protein (4 kcal), sugar (4 kcal), ethanol (7 kcal) and fat (9 kcal). The respective values for feed 1 – 3 were 30 kcal, 46 kcal and 36 kcal respectively. Gastric emptying rates are influenced by the calorific value of the feed (Kwiatek et al., 2008, Kwiatek et al., 2009). However, differences in calorific values of the feeds can be assumed to be negligible and so the gastric emptying times were not affected by calorific content of the feeds. This was observed in the current study.

Figure 4.1: Gastric pH levels in healthy humans (n = 8) after ingestion of 250 mL of feeds: 2.5% α-LA (blue); 2.5% α-LA with oleic acid (red); oleic acid alone (green) showing an increase in pH for the two samples containing protein and a similar trend for the two samples, and the steady pH level of the oleic acid alone sample.
In addition, the pH of the gastric content was also measured using a nasogastric pH probe in one volunteer. The in vivo measurement of the pH of the stomach was effective and was comparable with the pH of the gastric aspirates measurements previously discussed (Figure 4.5a). There were small differences and several pH jumps observed in the sample measured in vivo. Although measures were taken to ensure the tube remained in the same position (subject remained lying down in a stationary position) movement of the stomach cannot be accounted for.

4.2.2. Structural changes within the protein

Using intrinsic fluorescence spectroscopy it was possible to monitor protein unfolding as a function of the decrease in pH within the stomach. The isoelectric point of α-LA (~4.8) is the point at which the protein unfolds. Upon protein unfolding there is a change in the environment of the tryptophan residues thus resulting in a change in the intrinsic fluorescence spectra of the protein. Upon unfolding there is a red shift in the wavelength at which the maximum intensity occurs and also an increase in the maximum intensity recorded. An indicative spectra of folded and unfolded protein is present in Figure 4.2a. This confirms a change in the tertiary structure of the protein upon changes in the pH environment.

Plotting the maximum fluorescence intensity at λ_{max}, recorded for the protein as a function of pH, shows two distinct regions with no discernable differences between the protein feed (Figure 4.2b) or the protein and oleic acid feed (Figure 4.2c) recorded. There are two distinct regions within the graph - folded and unfolded, as characterized by the clusters of points within the groups.
Figure 4.2: a) Typical intrinsic fluorescence spectroscopy spectra with $\lambda_{ex}=280$ nm of the test drink prior to ingestion (black line) and the intragastric sample of the protein below its isoelectric point (dashed line) showing a shift in both the maximum intensity and the wavelength at which the maximum intensity occurs, as a result of the acidification of the solution.  
Plot of the wavelength at which the maximum intensity was recorded as a function of pH in b) the protein alone feed (drink 1); and in c) the protein:OA feed (drink 2). Each symbol represents one individual. Outliers removed from Figure 4.1 are included here to show pH decrease did not occur in all individuals.

Fourier Transform Infrared spectroscopy (FTIR) evaluates secondary structure of proteins by measuring vibrational changes within the amide I region of the spectra, 1600 – 1720 cm$^{-1}$, allowing differentiation between $\alpha$-helices, $\beta$-sheets and random coil conformations. $\alpha$-LA is composed mainly of $\alpha$-helices, with only a small amount of $\beta$-sheets present. Typically there is a vibration at 1652 cm$^{-1}$ corresponding to $\alpha$-helical structure. In this study this was observed in the original protein feeds and within samples above the isoelectric point of the protein. Molten globule-like structures have vibrational shifts at 1645 cm$^{-1}$ which is indicative of a random coil structure which was seen in the samples below the isoelectric point.
confirming that there was an alteration of the secondary structure of the protein along with changes in the tertiary structure, however α-helix structure still remains (Figure 4.3) (Troullier \textit{et al.}, 2000).

![Figure 4.3: FTIR analysis of the amide I region of the protein and OA after ingestion (red) and after the protein was deemed to be unfolded by intrinsic fluorescence spectroscopy (blue).]

4.2.3. Polypeptide chain composition of α-LA during gastric transit

Molecular weight calibration of size exclusion HPLC allowed the identification and quantification of the α-LA peak at 23 minutes (Figure 4.4a). Undigested monomers of α-LA remained present in the stomach for each sample up to and including gastric emptying (Figure 4.4a). However the levels of native protein remained low – approximately 5% of the remaining protein content. Levels of proteolysis increased during gastric digestion, resulting in the production of lower molecular weight material. The pH rapidly decreased within 9 minutes of
ingestion (Figure 4.4b), reaching the optimum pH range for pepsin activity (pH 0.8 to 4) (DiPalma et al., 1991).

![Figure 4.4: a) Typical SEC-HPLC chromatographs for α-LA as it is digested in the stomach of healthy adults with retention times of molecular weight standards, the direction of the arrows show the progression of loss of native protein and](image-url)
increase in peptides post ingestion time (0 – 24 minutes); and b) integrated peak area percentages as a function of digestion time showing: greater than 10 kDa (blue), 5-10 kDa (red), 1-5 kDa (green), 500 Da – 1 kDa (purple) and less than 500 Da (orange), as a function of time.

Similar results were observed in SDS-PAGE (Figure 4.5). Large peptides were easily visible in the initial gastric aspirate, with increasing amounts of peptides appearing as digestion time progressed, coinciding with the decrease in band intensity of α-LA. Residual β-LG remained intact even after 30 min.

Figure 4.5: Typical SDS-PAGE gel for the protein drink as digestion progresses: lanes 1-7: 3, 6, 9, 12, 15, 18 and 21 minutes respectively, showing native α-LA, β-LG, BSA and peptides.
4.2.4. In vivo imaging

*In vivo* gastric imaging using capsule endoscopy was performed on one subject. Resulting images are displayed in Figure 4.6. The nasogastric pH probe can be seen clearly within the stomach (Figure 4.6a). The camera became lodged in the rugae of the stomach at the start of the fundus, allowing real-time imaging of the gastric content to occur prior to the camera entering the duodenum. The pH of the stomach decreased upon acid secretion within the stomach. Acid secretion could be visualized through the formation of white streaks within the gastric content and also along the lining of the stomach wall (Figure 4.6b). These white streaks are as a result of isoelectric precipitation of the protein whereby apo α-LA precipitates out of solution forming a white mixture. Real time measurement of the pH of the gastric content was correlated to the visual appearance of the protein solution. The pyloric sphincter is where the most shear within the stomach occurs and thus where the most mixing will occur. Based on the images (Figure 4.6c) and videos, the shear within the stomach was not adequate for the formation of a homogenous mixture similar to that prior to digestion. Gastric emptying time could also be determined with *in vivo* imaging for two consecutive protein feeds. Based on images and videos, the feeds had emptied from the stomach after approximately 30 minutes of gastric digestion. Gastric emptying occurs by 30 minutes via the pyloric sphincter opening allowing the gastric content to enter the small intestine. This is achieved through the opening of the pyloric sphincter, allowing the entire content of the pylorus enters the small intestine, and not done in a step-wise manner. Gastric emptying can be seen in Figure 4.6d.

A real time video was recorded, monitoring the fate of α-LA during gastric digestion. Two feeds at time 0 minutes and 97 minutes were ingested. The camera passed the pyloric sphincter at approximately 150 minutes, where the typical yellow colouration of bile can be seen. Slow but complete dissolution and digestion can be observed within the jejunum. Recording was stopped after 200 minutes. Using the video function on the software MiroView it was possible to see peristalsis occurring within the stomach. This took place in the form of waves of stomach muscle contractions of
the pyloric sphincter and was very visible when the stomach was empty around the pyloric sphincter.

*In vitro* digestion was also performed and mixed at 150 rpm, an internationally recognized value (Dupont *et al.*, 2011). Turbidity of the sample changed as a function of pH. The sample went from clear to cloudy with a white precipitation forming, resulting in the formation of a homogenous white solution, before finally a clear solution was formed when the mixture reached pH 3.5 (Figure 4.6). This was contrasting what was observed *in vivo* where a homogenous mixture was not obtained.

Selected video clips of the gastrointestinal digestion of α-LA are available in Appendix 2.
Figure 4.6: A) The intra-gastric pH as measured using a nasogastric pH probe showing the initial pH increase upon the secretion of gastric acid with the two feed times highlighted; and capsule endoscopy images of 25 gL-1 α-LA, 50 gL-1 sucrose within the stomach: B) A pixelated image of the video file showing gastric transit and each feed after ingestion. C) visualization of the pH probe from within the stomach prior to administration of the α-LA feed; D) 5 minutes after the administration of the α-LA feed (pH 5.2); E) 8 minutes after the administration of the α-LA feed (pH 4.3); and F) after gastric emptying occurred; and for comparison, examples where homogeneous mixing occur during in vitro digestion at G) pH 7; H) pH 4 and I) pH 2.5, are given.
4.2.5. Cytotoxicity measurements

Samples were tested for their cytotoxic activity against human lymphoma cell line U937 cells. From *in vitro* digestion work (Chapter 3) it was shown that protein folding and refolding occurred in order for complex formation. Samples containing native, undigested protein that was deemed to have undergone unfolding by monitoring the intrinsic fluorescence spectra were pH adjusted for cytotoxicity testing against the cancer cells and compared to the activity of BAMLET produced through chromatography (Sullivan *et al.*, 2013). The effect of pepstatin, a potent pepsin inhibitor on U937 cells was assessed and did not have a detrimental effect on the cells at the tested concentrations. BAMLET killed the cancer cells however the gastric aspirate samples did not affect the viability of the cells. Due to the overlapping of bands for sucrose and OA in their FTIR vibrations, and the same chemical shift in NMR, ANS fluorescence was used to estimate the presence of fatty acids. The data suggested that fatty acid was present in the samples, however it was not possible to determine if the OA present in the sample was bound to the protein and was therefore cytotoxic.

In order to determine whether the sucrose present may impede the cytotoxicity of the gastric aspirates, cell viability assay was performed against U2OS, a human osteosarcoma cell line that have displayed sensitivity to HAMLET and BAMLET (Xie *et al.*, 2011) using BAMLET with and without added sucrose. There was no difference in the activity of BAMLET after the addition of sucrose, indicating that the presence of sucrose within the samples did not affect the cytotoxicity of the samples and was confirmation that the complex was not formed under the tested *in vivo* conditions (Figure 4.7).
Figure 4.7: Cytotoxicity against U2OS cells tested with alamarBlue® after a 24 hour incubation of BAMLET with (red) and without 5% (w/v) sugar (blue). Data are the means of three independent measurements with SDs represented by vertical bars.
4.3. Discussion

Due to a rapid gastric emptying time (determined to be approximately 30 minutes) it was not possible to fully determine % native protein entering the pH neutral conditions of the duodenum. Although gastric emptying occurs in the form of “dumping” when the pyloric sphincter opens allowing all of the gastric content contained within the antrum to enter the duodenum. Given the nature of the feeds, liquid, there is a possibility that some liquid enters the duodenum thus more native protein may remain.

α-LA is the most abundant protein and OA is the most abundant fatty acid in human milk (Jensen, 1995), thus the potential for association and production of a HAMLET-like complex in vivo in the gastrointestinal tract of infants is of clinical interest to researchers. Under simulated infant gastric digestion, a BAMLET-like complex can be formed (Sullivan et al., 2013). However the current study shows that there is a higher rate of digestion in the stomach of healthy adults than seen in both in vitro (Rudloff and Lönnerdal 1992) and in vivo (Agunod et al., 1969) studies of digestion in infants. Different pH profiles also exist within the gastric environment of infants (Mitchell et al., 2001, Omari and Davidson 2003) to the gastric environment of adults (Troost et al., 2001). In all in vivo monitoring of gastric pH post feeding a pH gradient exists (Mitchell et al., 2001; Omari and Davidson, 2003) where the pH gradually decreases as a function of time, however within adults the pH decreases over a shorter time due to higher levels of acid secretion (Troost et al., 2001).

While enzyme activity levels are not too different in infants and adults, the optimum pH level for pepsin activity is pH 1.8 with no activity occurring above pH 4, (Agunod et al., 1969), thus as the pH of the stomach decreases faster in adults (Troost et al., 2001) there is a higher level of proteolysis occurring over a 30 minute period.

A recent study highlights both the prevention and treatment of colon cancer after ingestion of HAMLET in mice (Puthia et al., 2013). While this study is of important clinical significance, conclusions cannot be drawn that should HAMLET be ingested
that it would result in activity against colon cancer \textit{in vivo} in humans, mainly due to the differences in the anatomy of mice and humans. Although the presence of OA does reduce the digestibility of \(\alpha\)-LA (Casbarra \textit{et al.}, 2004), it is shown within the current study that the harsh gastric conditions within the stomach are not conducive to \(\alpha\)-LA polypeptide chain stability, thus the protein would not survive transit to its active site within the intestines. The relative resistance of HAMLET to gastric digestion can be attributed to changes of the structure of the protein.

Antimicrobial peptides (Pellegrini \textit{et al.}, 1999) and peptides with opioid activity (Chatterton \textit{et al.}, 2006) have been derived by hydrolysis from \(\alpha\)-LA. It is still unclear whether peptides with any biological activity can be derived from the \textit{in vivo} digestion of \(\alpha\)-LA. Currently few studies exist on the \textit{in vivo} digestion of food proteins. Studies exist on the digestion of lactoferrin, the iron binding and transport protein. A similar pH curve exists, with a similar gastric emptying rate. However one striking difference is the remaining native protein present after gastric digestion: in the case of lactoferrin, depending on the molecular state of the protein, between 60-80\% of native protein remains intact, significantly higher than in the case of \(\alpha\)-LA gastric digestion present in this study (Troost \textit{et al.}, 2002; Troost \textit{et al.}, 2003).

Gastric mixing and emptying rates have been discussed by (Marciani \textit{et al.}, 2003; Hoad \textit{et al.}, 2007; Marciani \textit{et al.}, 2009) whereby magnetic resonance imaging (MRI) and echo-planar imaging (EPI) were used to track gastric emptying. The gastric emptying rate is determined by a number of factors including the calorific value of the feed, the rheology of the samples, the buffering capacity of the feed and the stability of the emulsion. Given that no increase in gastric retention time was observed in the sample containing fatty acid it can be concluded that the solution was not an emulsion or emulsified within the stomach, further highlighting that no complex is formed within the tested conditions.
α-LA enters a molten globule like state under acidic conditions (Kuwajima 1996). This structural change may be a necessary prerequisite for the formation of the protein fatty acid complex (Svensson et al., 2003). It was shown in Chapter 3 that under simulated gastric conditions within the stomach of an infant that this structural change occurred irrespective of the presence of pepsin and other digestive enzymes.

However despite (i) protein unfolding occurring, (ii) the presence of oleic acid and (iii) the presence of native protein, the complex produced in vivo was not toxic to cancer cells. Different reasons have been hypothesized, mainly the importance of mixing within the stomach. Measuring the gastric forces is an invasive and laborious task, the cytotoxicity of the mixture can also be attributed to the mixing and visual aspects of the solution, thus it was decided that capsule endoscopy technology would be utilized to visualize the mixture in vivo.

To our knowledge this is the first use of capsule endoscopy in the visualization of protein digestion within the gastric environment. There are no previous publications in the literature that use capsule endoscopy in conjunction with the consumption of food – typically capsule endoscopies are used in the diagnosis of illnesses in the small intestine of people who present with abdominal pain or bleeding that cannot be diagnosed using either traditional endoscopy or colonoscopy techniques (Kurien et al., 2013).

Standardization of digestion within the pharmaceutical industry has been proposed, however it was discussed by (Hur et al., 2011) that no such standardization exists to date for food digestion. COST action FA1005 INFOGEST (Dupont et al., 2011) is a group recently set up whose aim it is to standardize food digestion across the discipline. One initial proposal of the action was the standardization of mixing rates within in vitro digestion and the mixing rate of 150 rpm was proposed. This mixing speed was employed in a previous study while mixing the in vitro digestion mixture and visually comparing this to the in vivo digestion mixture shows that the mixing within the stomach does not produce a homogenous mixture similar to that produced.
in vitro. One outcome of the current study is that the rate of mixing within the stomach is overestimated in many in vitro digestion studies (80 rpm: Sullivan et al., 2013; 95 rpm: Granado-Lorencio et al., 2009; 1000 rpm: Golding et al., 2011). However mixing is a common limitation of one vessel digestion even though commonly used in the pharmaceutical industry.
Chapter 5

Real-time monitoring of protein unfolding using D-gluconic acid delta-lactone
5.1. Introduction

α-Lactalbumin (α-LA) is a small globular protein obtained from milk whey (123 AA residues; MW 14.2 kDa) and functions as a key component in lactose synthesis within the mammary gland (Brew et al., 1968; Permyakov and Berliner 2000). Extensively studied as an archetypal example for protein folding, α-LA contains a single Ca\(^{2+}\) binding site, which when occupied increases the stability of the protein. Removal of the ion using chelators such as EDTA induces the apo state, and providing an acidic pH environment results in partial unfolding of the protein, or its “molten globule state” (Ptitsyn 1995; Arai and Kuwajima 2000; Permyakov and Berliner 2000). More recently α-LA has shown to be a constituent of the H/BAMLET (Human/Bovine α-lactalbumin Made LEthal to Tumour cells) complexes with the fatty acid, oleic acid (OA), exhibiting potent cytotoxic properties against tumour cell lines while leaving healthy, differentiated cells intact (Svensson et al., 2000; Min et al., 2012). In addition, the protein has been discussed as a potential vaccine against breast cancer (Jaini et al., 2010).

H/BAMLET are part of a range of recently discovered partially unfolded protein-fatty acid complexes involved in tumour cell death including equine lysozyme (Wilhelm et al., 2009) and β-lactoglobulin (Lišková et al., 2011). These form a “family” of proteins in a partially unfolded state that exhibit bioactivity beyond the initial and known biological functions of the proteins. Noteworthy is that despite possessing beneficial bioactivity, these proteins are in a non-native conformations unlike those non-native or partially folded forms correlated with pathological diseases such as cystic fibrosis (Thomas et al., 1995), cancer (Bullock and Fersht, 2001), and neurodegenerative diseases like Alzheimer’s and Parkinson’s disease (Tan and Pepys 1994; Dobson 2001). Exposure of the hydrophobic core of proteins under acidic conditions has been linked with the formation of amyloid fibrils (Caughey and Lansbury 2003), hence the study of these proteins under non-native conditions has gained widespread attention over the years.
Acidification of α-LA results in the formation of a partially-folded, molten globule-like form called the A-state (Ptitsyn, 1995; Aral and Kuwajima 2000), which is a distinct molecular state of proteins that shares properties of both native and denatured protein through its retention of secondary structure and loss of tight, tertiary packing. Atomic-level studies of the molten globule form of proteins are integral towards the understanding of the folding of proteins, and significant details have been uncovered especially with NMR spectroscopy (Redfield 2004). Provided that aggregation is minimized, it would be helpful to chart changes of structure from native conformation to the acidic pH form, or alternatively, the native, holo (calcium bound) conformation to the apo form.

The molten globule form of α-LA is interesting in the study of HAMLET as the conditions within the stomach of a nursing infant are similar to the conditions under which HAMLET was initially discovered (Svensson et al., 1999), and this research has prompted speculation that HAMLET may be formed in the digestive tract of breast-fed infants (Svensson et al., 2003; Barbana et al., 2006) due to the elevated pH levels in the stomach (Mitchell et al., 2001) and the low enzyme activity in the stomach (DiPalma et al., 1991). It was shown by Sullivan et al., 2013 that a BAMLET-like complex is formed under simulated gastric conditions. Because gastric pH conditions are known to be low, a question naturally arose into whether the molten globule state of α-LA generated from such acidic conditions would have the opportunity to expose its solvent-inaccessible core and allow hydrophobic interactions to occur between the protein and the free fatty acid in situ. Upon restoration to neutral pH conditions in the small intestine the protein would refold and form the complex with fatty acid.

D-Glucono-δ-lactone (GDL; molecular weight 178.14 g mol⁻¹) is a small, naturally occurring food substance/additive that is found in honey, fruit juices and wine. GDL can be used as an acidifier as it produces a slow, controllable rate of acidification in aqueous solutions (Pocker and Green 1973). For this reason GDL can be used to effectively modulate the changing pH conditions of the stomach of infants. The hypothesis of the current study is that GDL can be used to decrease
the pH *in situ* and induce structural changes within the protein. Other small molecules have previously been used to induce structural changes within α-LA, including a caged photo labile compound DM-nitrophen, which, upon exposure to laser light through photo-CIDNP, releases calcium, refolding the unfolded protein (Kühn and Schwalbe 2000; Wirmer *et al.*, 2001).

Diffusion Ordered Spectroscopy (DOSY) separates different components within a mixture based on their diffusion coefficients (Jones *et al.*, 1997; Wilkins *et al.*, 1999), determined using Gaussian curve fitting for each of the components, in the case of this study α-LA, OA and 1,4-dioxane. The diffusion coefficients for each individual component in the mixture can determine the state of binding of the OA to the protein. If they decay together and have similar diffusion coefficients they are bound together in solution.

The aim of this study was to first initiate the molten globule form of α-LA *in-situ* using GDL and capture $^1$H spectra as a function of time to map the protein partial unfolding in real-time, and then to use diffusion NMR methods to assess if the oleic acid that is present in the sample is bound to the protein. A control experiment to test the feasibility and sample homogeneity of this *in situ* NMR experiment was carried out prior through the monitoring of the C2 $^1$H chemical shift change of L-histidine. The α-LA-oleic acid sample was then compared to chromatographically produced BAMLET using a battery of optical and NMR spectroscopic techniques (Pettersson-Kastberg *et al.*, 2009).
5.2. Results

5.2.1. pH changes

An initial, proof-of-principle test was performed by monitoring the pH-jump of L-histidine, which undergoes a pH-induced acid-base equilibrium change through protonation/deprotonation of its imidazole group. In a previous study showing an *in situ* stopped-flow injection to alter the pH from pH 4.2 to pH 6.5, a chemical shift change of the C2 and C4 carbon $^1$H signals could be observed (from 8.57 ppm to 7.71 ppm), reflecting the course of an acid-base chemical reaction in real-time (Mok *et al.*, 2003). The direction of the chemical reaction is reversed in our case, with the initial pH starting at pH 7 and ending at approximately pH 4 after a time course of 45 min (Figure 5.1). There is a C2 $^1$H chemical shift change from 8.48 ppm towards 8.65 ppm, with significant broadening occurring with the early time periods. The likely reason for this is due to greater chemical exchange going on at the early stages – the pH being closer to the pK$_a$ of the imidazole group. This confirms that the acidification of proteinaceous material with GDL can occur.

For pH jump experiments involving the protein-fatty acid mixture, a gradual pH decrease was needed to mimic the conditions within the stomach of infants (Mitchell *et al.*, 2001). The pH decrease within the stomach is a slow process due to the low acid secretion levels for infants.

The pH of the solution was decreased through the addition of GDL. The amount of GDL needed to decrease the pH of the solution from neutral to acidic pH (approximately pH 7 to pH 3) over a set period of time was determined through the addition of varying amounts of GDL to a fixed volume of protein solution.
CH APTER 5

REAL-TIME MONITORING OF PROTEIN UNFOLDING USING GLUCONO-DELTA-LACTONE

Figure 5.1: Sequential $^1$H NMR spectra of the C2 $^1$H signal for 10 mM histidine undergoing acidification through incubation with 98 mM GDL. The time periods (and corresponding pH values) are given. The bottom trace (brown) reflects the conventional $^1$H NMR spectrum of L-His at approximately pH 4.

For the pH-jump of the protein-fatty acid mixture, values between 23 mM and 252 mM were chosen. 23 mM GDL did not decrease the pH past pH 6. Increasing the GDL concentration to 252 mM resulted in the rapid initial decrease of the pH from pH 6.7 to pH 3, and this was also the case with concentrations of 236 mM and 198 mM GDL. Therefore it was determined that 112 mM GDL provided the temporal course for the in vitro digestion pH conditions utilized (Figure 5.2).

Along with the pH change came an alteration in the turbidity of the mixture. There was an initial increase in the turbidity of the protein around the pI (~4.8) and fluorescence data (Figure 5.3) confirms that this is the point at which the
protein unfolds. Once the protein solution reached ~pH 4 the solution became clear again.

Figure 5.2: Change in solution pH as a function of concentration of GDL with 1 mM α-LA, and 5 mM OA. The different concentrations applied are as follows: Navy, 23 mM; Pink, 98 mM; Red, 112 mM; Yellow, 196 mM; Azure, 236 mM; Purple, 252 mM. The resulting pH gradients obtained show that 23 mM GDL does not decrease the pH below pH 6, whereas at 198 mM GDL and higher concentrations the pH decreases too rapidly to effectively mimic the conditions within the stomach of an infant. The temporally optimum pH decrease was determined to be 112 mM GDL.

Particle size was also determined as acidification occurred. Due to the mixed nature of the sample – protein and fatty acid – the particle size distribution was multi-modal and no significant differences were seen between samples at differing pH levels (Figure 5.3).
5.2.2. Structural changes within the protein

It was previously shown by Dolgikh et al., 1984, that α-LA unfolds under acidic conditions and enters a molten-globule like state. Therefore, as expected under the acidic conditions within the NMR tube after the addition of GDL, α-LA exhibited unfolding. The reaction of GDL being hydrolysed to gluconic acid is relatively slow, therefore effectively mimicking the gradual pH decrease experienced in the stomach of infants.

Intrinsic Fluorescence

Intrinsic fluorescence maps protein unfolding by monitoring the changes of the environment of intrinsically fluorescent amino acids such as tryptophan. At 280 nm the sample is excited and the emission spectra are recorded. Typically for holo α-LA there is a fluorescence intensity maximum at ~330 nm and for apo/A-state it
is at ~345 nm. This represents a change in the environment of the tryptophan residues, which ordinarily are not exposed when the protein is in its native form however they become exposed when the protein unfolds. This was experienced with these data, and can be seen in Figure 5.4. The initial protein sample had an intensity maximum at 330 nm, and this intensity shifted towards the apo state upon acidification.

![Graph showing fluorescence shift](image)

**Figure 5.4:** Intrinsic fluorescence spectroscopy of the 1 mM protein-fatty acid solution showing a shift in the wavelength at which the maximum intensity was recorded from 330 nm (cyan) to 340 nm (pink) and an increase in intensity at 340 nm.

**ANS Fluorescence**

ANS is a dye that binds to exposed hydrophobic regions of proteins and fluoresces upon excitation at 390 nm. Samples were taken from the *ex situ* protein-GDL mixture and the ANS fluorescence spectra were captured. After unfolding occurred, as determined by intrinsic fluorescence, there was an increase in intensity observed for samples that had been incubated in the presence of ANS.
While there was a small amount of ANS fluorescence in the folded protein, due to the easily accessible surface hydrophobic residues, upon the exposure of the hydrophobic core of the protein more ANS binds and thus results in a higher fluorescence intensity (Figure 5.5).

![Fluorescence spectra of ANS-bound 1 mM α-LA 5 mM OA solution with 112 mM GDL during in situ acidification showing the transition from folded (blue, red) to partially-unfolded (pink, navy) protein with pH decrease.](image)

**Figure 5.5:** Fluorescence spectra of ANS-bound 1 mM α-LA 5 mM OA solution with 112 mM GDL during *in situ* acidification showing the transition from folded (blue, red) to partially-unfolded (pink, navy) protein with pH decrease.

**NMR experimental work**

As a reference experiment, NMR spectra were captured of the native protein, the protein in complex with oleic acid and the A-state of the protein. This served a 3-fold purpose: (i) to determine the native-structure of α-LA as a reference point for a folded protein; (ii) to determine the molten globule structure of α-LA as a reference point for a partially unfolded protein; and (iii) to determine the presence
and bound state of oleic acid within the BAMLET sample produced through chromatography (Figure 5.6).

The native structure of the protein shows peaks within the aromatic region of the protein along with a lone tryptophan resonance at 12 ppm. There is also significant sharpness of the peaks in the upfield region of the spectra. The molten-globule form of the protein shows significant peak broadening and a decrease in the number of peaks in the upfield region of the protein and also within the aromatic region.

A study of oleic acid within HAMLET and its respective chemical shift based on the binding state to the protein was completed. It was reported that when OA is bound to the protein it has a chemical shift of 5.3 ppm and when it is just free in solution and not bound to the protein it has a chemical shift of 5.4 ppm (Fast et al., 2005). In the case of the column produced BAMLET in this study the OA peak resonates at 5.3 ppm thus was deemed bound to the protein. There is a small shoulder on the OA peak suggesting that there is a small amount of unbound OA present in the solution, however a DOSY experiment on the sample confirmed that all OA within the sample had the same rate of decay as the α-LA.

One interesting thing of note with the BAMLET produced in this study – it has a very native-like structure compared to previous NMR-based studies on the complex (such as Pettersson-Kastberg et al., 2009). Current research in the area of HAMLET is focusing on the role of the fatty acid and this data would suggest that the structure of the protein produced is not important in bioactivity once the oleic acid is present and bound to the protein.
Zeta potential measurements were taken every 5 minutes for 1 hour while the pH decreased from pH 6.7 to pH 3.5. Initial measurements began at -38 mV and increased to 25 mV after 1 hour. The changes in zeta potential were as expected – negative above the pI and positive below the pI (Figure 5.7).
5.2.4. GDL-induced changes in the mixture

Focusing on the upfield region of the spectra (-2.5-0 ppm), which reflects the native-packing of aliphatic side chains, we see a gradual loss in the native structure through a decrease in the sharpness of the peaks. This results in an eventual total loss of native-like structure, as a function of pH. pH is measured ex situ and identical conditions are employed in situ. Similarly to intrinsic fluorescence, where the point of unfolding of the protein was determined to be between pH 4 and pH 5, there was a distinct structural change in this pH range. However NMR is a more sensitive biophysical technique therefore a lot more information on the structure of the protein can be learned from unfolding experiments (Figure 5.8).
Figure 5.8: Progressive $^1$H spectra of the upfield region of 1 mM α-LA incubated with 112 mM GDL at 37°C. Upfield region, -2 – 0 ppm, region shown as a function of pH change induced by the hydrolysis of GDL.

Broadening of the peaks also occurred in the aromatic region of the protein which coincides with protein unfolding and the induction of the partially unfolded molten globule state of the protein.

Another area of interest to monitor protein unfolding using NMR is at 12 ppm where there is a single tryptophan resonance. This Trp resonance can be clearly seen in the native protein and is indicative of tertiary structure of the protein. Looking at the change in the spectra as the protein decreases in pH there is a distinct loss of signal at 12 ppm and this coincides with the pH decrease which ultimately leads to the loss in native-like structure (Figure 5.9).
Figure 5.9: Real-time $^1$H proton NMR spectra of the α-LA and OA mixture undergoing acidification. Note the change in the upfield aliphatic region of the spectra and the sequential broadening of the peaks, indicating the formation of the partially-unfolded protein.

Another thing of note is the impact of pH decrease on the broadness of the oleic acid peak. Upon acidification the OA peak became sharper, indicating that there is a change in the fatty acid along with a change in the protein. Carbon NMR analysis of the fatty acid at pH 2 indicates that the C1 carbonyl group becomes protonated (see chapter 6).

5.2.5. State of binding of oleic acid

DOSY-NMR was used to determine the rates of signal decay of different regions of the spectra with respect to the internal control 1,4-dioxane. Three regions were integrated as a function of the gradient of magnetic field strength applied to the samples: the aromatic region 6-10 ppm, the olefinic binding region 5-5.5 ppm, and the region for 1,4-dioxane 3.75 ppm. DOSY experiments were carried out on 3 occasions – before the addition of GDL, 90 minutes after the addition of GDL when it was estimated the protein was in its' molten globule form, and on a separate sample that the pH was increased to physiological conditions. Both the sample prior to the addition and the sample under acidic conditions had signal decay rates for the protein and the fatty acid that were not coincident, suggesting that while both were in solution, the fatty acid was not bound to the protein. After the pH was increased ex situ and there was a recovery to neutral pH conditions the rates of decay became in good agreement, suggesting that they are in solution
together and the oleic acid is bound to the protein. This would suggest that for the generation of a H/BAMLET-like complex, a return to a native-like environment is necessary, possibly to allow the refolding of the protein and the "encapsulation" of the oleic acid within the protein matrix. This also assists in allowing the closure of the exposed hydrophobic regions and assisting in the harbouring of the weakly-bound fatty acid moieties (Figure 5.10).

Figure 5.10: Comparison of the signal decays as a function of applied gradient field strength for the olefinic CH9-10 peak of oleic acid with the aromatic region envelope of the protein-oleic acid mixture:
(i) pH 2: aromatic envelope (red) and olefinic peaks (red dotted line)
(ii) pH 7 (upon return to physiological conditions): aromatic envelope (blue) and olefinic peaks (blue dotted line). The general agreement of the two curves for the *ex situ* and the *in vivo* cases after recovery to native state conditions indicate that the oleic acid is bound to the partially-unfolded protein.
5.2.6. OA quantification

Integration and comparison of the peak area for the OA region (5-5.5 ppm) and the aromatic region of the protein (6-10 ppm) for the BAMLET sample produced through chromatography indicates that the OA is bound to the protein, and that there is an estimated 5.6 moles of OA per mole of protein. The same analysis performed on the post-gastric α-LA/OA complex yielded results of 4.1 moles of OA per mole of OA. The variances in the OA content can account to differences in a) the structure and related biophysical analyses, and b) the bioactivity of the protein complex.
5.3. Discussion

In obtaining the bioactive protein-fatty acid complex, H/BAMLET, the binding of the fatty acid molecules to the protein moiety is key, as the partially-folded protein appears to act as a “mule” in delivering the fatty acid across the cellular membrane (Nielsen et al., 2010; Min et al., 2012). In this study the protein underwent partial unfolding through acidification by GDL. Real-time analysis of the spectra showed that the molten globule form of the protein was obtained at the conclusion of the reaction. These spectra were in line with other studies on the MG form of α-LA (Wijesinha-Bettoni et al., 2001; Pettersson-Kastberg et al., 2009), and display the utility of the addition of acidifier combined with real-time NMR monitoring of the reaction. However under the tested conditions the initiation of the MG form of the protein alone was not enough to produce the bioactive complex.

The structure of the complex produced during in situ acidification is different to the structure of HAMLET seen in the studies of (Pettersson-Kastberg et al., 2009) where the upfield region of the protein has less defined peaks in HAMLET than in the complex produced. The complex produced had a similar upfield structure to the BAMLET produced during chromatography, which was produced using the same starting material. Thus the native-like upfield structure is likely to be a deviation based on the material used.

The oleic acid loading of the samples is in line with what was seen with other studies (Fast et al., 2005; Pettersson-Kastberg et al., 2009). The complex created in situ, however, has a slightly lower oleic acid loading. The work of Brinkmann et al., 2011, Brinkmann et al., 2013, and Permyakov et al., 2011 shows that the oleic acid is the toxic component of the complex and suggests that α-LA exists in the complex to deliver the fatty acid to the cell. This would suggest that the complex created during the simulated gastrointestinal model is less toxic to cancer cells, due mainly to its lower oleic acid content.
This study monitors the real-time unfolding of proteins, conversely to the study of Hore et al., 1997, whereby photo-CIDNP was used to monitor the real-time refolding of proteins using a stopped-flow technique which monitors the changes in side-chain accessibility that occurs when a protein refolds. There have been many NMR-based studies performed on the refolding of proteins (Baum et al., 1989; Wirmer et al., 2006; Redfield, 2004) whereas few studies exist on the real-time unfolding of proteins.

The addition of small molecules to solutions to induce conformational changes within proteins is not unique to this study. Kuhn & Schwalbe, (2000), used a photo-labile ion chelator, i.e. a caged molecule, which, upon the addition of light (through photo-CIDNP) is released and induces conformational changes in the protein. This study was performed on a-LA, the calcium binding site of which is important for its tertiary structure. It was possible to measure refolding kinetics for proteins which had been unfolded using urea through monitoring the upfield region of the spectra. There was an increase in the native-like state of the protein with increasing amounts of calcium addition.

pH induced structural changes of proteins have also previously been studied (Mok et al., 2003) where a solution was manually injected into an NMR tube to produce a pH gradient for structural analysis. The use of GDL reduces the need for the injection of a second compound for pH jumps over time.
Chapter 6

Biophysical characterization of protein-fatty acid complexes and their constituents

This chapter contains work that has been published in part:
6.1. Introduction

Early studies of the HAMLET complex neglected the importance of oleic acid: (i) there was an assumption of a 1:1 binding ratio based on molarity (Svensson et al., 2003); and (ii) there was an assumption that OA alone was not cytotoxic to the cells (Håkansson et al., 2011). Recent studies have shown that using different methods including NMR and GC there is approximately a ~5:1 OA:α-LA molar ratio (Pettersson-Kastberg et al., 2009; Sullivan et al., 2013). Other studies have also shown that with increasing amounts of oleic acid bound to the protein, there is a decrease in the LC50 value i.e. there is a decrease in the amount of sample required to kill half of the population of cells (Brinkman et al., 2011; Permyakov et al., 2012). There are also publications showing that the protein alone is not of importance to the formation of the complex: a complex similar to BAMLET can be formed from bovine whey protein β-LG (Lišková et al., 2011) and, equine lysozyme, the only lysozyme protein known to have a partially unfolded molten globule like state (Wilhelm et al., 2009).

This raises the question of the importance of the fatty acid. Oleic acid is a monounsaturated omega 9 fatty acid that is found naturally in many plants and mammals. It is the main component in olive oil – triglyceride esters of oleic acid are present in quantities of up to 60% (Rana and Ahmed 1981). Oleic acid is the predominant fatty acid in human milk (Jensen, 1995). It is a C18 fatty acid with one double bond in the cis formation on the carbon chain at carbon 9. Oleic acid is not water-soluble, however, its salt form, sodium oleate (Ananthapadmanabhan and Somasundaran 1988) is. Oleic acid is known to have toxic effects against cells. It is used in sheep and rats to initiate lung damage in order to test the efficacy and toxicity of new drugs for the treatment of lung disease (Yeh et al., 2009).

Given the toxicity of OA and the lack of specificity with the protein used in the formation of the complex, biophysical characterization of the fatty acid under different conditions is essential to understanding the biological activity of the
complex. Complexes were produced, using the method of Brodkorb & Lišková, 2009, with varying amounts of sodium oleate and characterised to determine protein structure and fatty acid content compared to BAMLET. To further test the hypothesis of the role of the protein in the solubilising of the fatty acid, the fatty acid solution was solubilised to create finer emulsions in order to determine whether a smaller particle will be more toxic to cells, and whether the mechanism of cell death is different when the fatty acid is in complex rather than just the fatty acid alone. Oleic acid is known to induce necrosis in cells at high quantities (Zhu et al., 2005) and HAMLET kills cells by an apoptosis-like method of cell death.

In this study, a complex of oleic acid with BSA was generated for comparison with HAMLET and BAMLET. BSA was chosen because it is the bovine equivalent of HSA, the most abundant protein in human blood, (present at concentrations of 38 – 52 gL⁻¹), and because of its known binding capabilities with OA. If a complex between BSA and OA were to be cytotoxic, not only would this be a new mechanism of delivery via the blood system, but also corroborate the hypothesis that a complex can be formed with any protein that can bind OA. The stability of BAMLET in the presence of BSA was determined as BSA is a known fatty acid transporter in vivo.

Finally, many methods of production of HAMLET and BAMLET have been proposed. Different molar ratios of OA are reported, however these values are reported for a crude mixture of the complex. Within a solution containing BAMLET with a 5X molar excess, α-LA with 2X to 10X OA bound may be present within the mixture, thus the idea of microheterogeneity within the mixture was investigated by isoelectric focusing.
6.2. Results

6.2.1. Structural characterization of the complex and quantification of oleic acid within the complex

Quantification of oleic acid

Complexes were produced by heating α-LA in the presence of increasing molar excess of sodium oleate and dialysed extensively against distilled water and lyophilised. Zeta potential measurements of the complexes set at pH 7 showed a linear negative increase in surface charge as a function of increasing sodium oleate bound to the complexes (Figure 6.1). A similar trend was also seen during quantification of the OA peak in NMR, FTIR and GC (Table 6.1). \(^1\)H NMR analysis confirmed that the OA present within the samples had a chemical shift at 5.3 ppm and thus, bound to the protein. Zeta potential measurements of the α-LA:OA mixture prior to heating showed no significant differences from native α-LA thus it can be concluded that the surface charge of protein is only altered when the fatty acid is bound to the protein (Table 6.2).
Table 6.1: Stoichiometry of samples as quantified by FTIR, NMR and GC. Standard deviations are means of two individual preparations of complexes each measured in triplicate.

<table>
<thead>
<tr>
<th>Molar Excess During Preparation</th>
<th>FTIR (OA/Protein)</th>
<th>NMR (OA/Protein)</th>
<th>GC (OA/Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.02 ± 0.00</td>
<td>0.33 ± 0.12</td>
<td>0.21 ± 0.14</td>
</tr>
<tr>
<td>1.25</td>
<td>1.29 ± 0.23</td>
<td>0.43 ± 0.24</td>
<td>0.56 ± 0.27</td>
</tr>
<tr>
<td>2.5</td>
<td>2.41 ± 0.74</td>
<td>2.3 ± 0.21</td>
<td>2.4 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>3.74 ± 0.87</td>
<td>4.6 ± 0.54</td>
<td>5.6 ± 0.22</td>
</tr>
<tr>
<td>6.25</td>
<td>5.06 ± 1.03</td>
<td>5.7 ± 0.76</td>
<td>5.7 ± 0.43</td>
</tr>
<tr>
<td>7.5</td>
<td>6.21 ± 0.64</td>
<td>6.3 ± 0.82</td>
<td>6.1 ± 0.63</td>
</tr>
<tr>
<td>8.75</td>
<td>7.44 ± 0.98</td>
<td>7.2 ± 0.45</td>
<td>7.3 ± 0.58</td>
</tr>
<tr>
<td>10</td>
<td>8.4 ± 0.49</td>
<td>8.7 ± 0.53</td>
<td>8.9 ± 0.69</td>
</tr>
</tbody>
</table>
Zeta potential

![Zeta Potential curve of α-LA:SO complexes prepared by heating: 2.5X, 5X, 7.5X and 10X molar excess of sodium oleate present during preparation; zeta potential measured at pH 7.4 with a protein concentration of 7 mgmL⁻¹ and an applied voltage of 75 mV.](image)

**Figure 6.1:** Zeta Potential curve of α-LA:SO complexes prepared by heating: 2.5X, 5X, 7.5X and 10X molar excess of sodium oleate present during preparation; zeta potential measured at pH 7.4 with a protein concentration of 7 mgmL⁻¹ and an applied voltage of 75 mV.

Different methods were used to quantify the oleic acid within the samples. Whilst the "gold standard" for fat analysis is GC, this pertains to samples primarily consisting of lipids and fatty acids only. Table 6.1 represents values for quantification of the oleic acid content within the samples with a defined sodium oleate content. Here, the three methods are in a good agreement, and are also in agreement with zeta potential data. While GC analysis requires an extraction of the fatty acid from protein, FTIR and NMR have the advantage of measuring the sample intact. NMR has several advantages over other methods: (i) the ability to determine the molecular state of the OA (bound/unbound; protonated/deprotonated) through the \(^1\)H and \(^{13}\)C chemical shifts and also through PFG-NMR, and (ii) a standard curve is not needed to predict the OA content, unlike with GC, FTIR and zeta potential.
**Table 6.2:** Zeta Potential Values for α-LA prepared with 5X molar excess of sodium oleate during preparation by heating measured at pH 7.4 with a protein concentration of 7 mg/mL and an applied voltage of 75 mV.

<table>
<thead>
<tr>
<th>Stage of production</th>
<th>Measured Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Heating</td>
<td>-10.36 ± 0.81</td>
</tr>
<tr>
<td>After Heating</td>
<td>-25.87 ± 1.06</td>
</tr>
<tr>
<td>After Dialysis</td>
<td>-28.93 ± 0.27</td>
</tr>
<tr>
<td>After Freeze drying</td>
<td>-27.84 ± 0.98</td>
</tr>
</tbody>
</table>

In order to effectively compare samples produced through the heating method to BAMLET produced through chromatography, a sample with a similar amount of OA bound as in the column method was used. For the purpose of explanation within the text chromatographically-prepared BAMLET will be referred to as “BAMLET”, the complex produced through the heating method will be referred to as “α-LA:OA complex”. The 5X sample was deemed to be the most similar to BAMLET and was the sample used for analysis.

Comparing the zeta potential of the two complexes (BAMLET and α-LA:SO complex), measured against the native protein as a function of pH both complexes exhibit excellent agreement in surface charge across different pH levels. In strong contrast, the native protein sample, while negatively charged, displays a much lesser electrokinetic potential than the protein in complex with fatty acid. Hence, for non emulsion systems, the more homogeneous in structure the sample, the lesser the absolute value of the zeta potential (Figure 6.2).
Figure 6.2: Zeta potential of preparations of BAMLET: native α-LA (green), BAMLET (red) and the α-LA:OA complex produced with a 5X molar excess (blue) as a function of pH measured with a protein concentration of 7 mgmL$^{-1}$ and an applied voltage of 75 mV.

Fluorescence studies

ANS fluorescence was used to determine the surface hydrophobicity of the different complexes. There was an increase in surface hydrophobicity between native α-LA and the complexes (Figure 6.3). The change in the hydrophobicity can be attributed to (i) changes in the structure of the protein and (ii) an increase in the amount of hydrophobic material in the sample (for example, an increase in fatty acid may increase the hydrophobicity of the different samples). Previous experiments showed an increase in the hydrophobicity of the samples based on the amount of SO within the samples – samples had an increased ANS fluorescence intensity from 0X to 10X, in line with increasing amounts of SO present.
Figure 6.3: ANS Fluorescence of preparations of BAMLET: native α-LA (green), BAMLET (cyan) and the α-LA:OA complex produced with a 5X molar excess (blue) at a concentration of 5 μM, incubated with 10 μM ANS.

ThioflavinT (ThT) fluorescence is a rapid screening method for the detection of extended β-sheets such as those seen in amyloid fibrils. Any treatment of proteins that changes their structure or functionality can also increase the risk of amyloid-like formation, a negative characteristic of proteins associated with fibrils formation often seen with degenerative diseases such as Alzheimer’s and dementia (Dobson 1999). There was no increase in intensity at 490 nm in samples heated in the presence of SO, indicative that heating in the presence of SO does not promote amyloid fibril formation under these conditions (Figure 6.4).
Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis of the oleic acid region of the spectra, stretching of the O-H bonds at 2854 cm\(^{-1}\), indicated a difference in the oleic acid content of the BAMLET produced through chromatography and the \(\alpha\)-LA:OA complex (Figure 6.5). This was an expected result as they were prepared with differing amounts of oleic acid/sodium oleate. A similar trend was observed for the samples with increasing SO content within them – there was an increase in the size of the oleic acid peak relative to the SO content within the samples.
Figure 6.5: FTIR spectra of the OA region 2854 cm$^{-1}$ of different BAMLET preparations: BAMLET (green) and the $\alpha$-LA:OA complex produced with a 5X molar excess (purple) in comparison to the OA control (blue). Also shown for reference: $\alpha$-LA (red)

FTIR analysis of the amide I band (1600 – 1720 cm$^{-1}$) shows subtle changes in the secondary structure of the complexes compared to the native protein. The native protein has a spectrum indicative of that observed in an $\alpha$-helical protein – in agreement with the overall structure of native $\alpha$-LA. The two complexes are very similar in structure. There is a shift towards the random coil conformation, similar to that observed with the molten globule form of the protein, however native structure remains. The shift towards random coil formation occurs in conjunction with the loss of $\beta$-sheets, indicated by the decrease in signal at 1620 and 1680 cm$^{-1}$ (Figure 6.6).
Figure 6.6: FTIR spectra of the protein region – 1600 - 1720 cm$^{-1}$ – showing the protein secondary structure of BAMLET (green) and the α-LA:OA complex produced with a 5X molar excess (purple) in comparison to the native α-LA control (red).

**Nuclear Magnetic Resonance (NMR)**

NMR was used in a similar fashion to FTIR, observing the overall structure of the protein and also the OA portion of the complexes (Figure 6.6). Region I is indicative of the oleic acid content within the samples. There is an increase in peak size in the oleic acid region as a function of increasing sodium oleate content during preparation. The peak observed for the olefinic protons of C5/6 was at 5.3 ppm – indicating that the oleic acid was bound to the protein. An example of the presence of unbound OA within BAMLET samples has been previously discussed in Figure 3.4. Region II represents the upfield region of the protein where it assesses the overall structure of the protein. Unexpectedly, both complexes had a native-like structure. However this did not impact their cytotoxic capabilities (Figure 6.7).
Figure 6.7: NMR spectra of samples produced with increasing concentration of sodium oleate: a) native α-LA, b-h) increasing amounts of SO; i) BAMLET produced through chromatography and j) the molten globule form of α-LA. Region (I) scaled analysis of the oleic acid binding region of the protein showing the increase in the OA peak as a function of the increasing SO content. Region (II) scaled analysis of the upfield region of the protein showing the sharp peaks indicative of native structure of the protein and the peak broadening occurring in j), the molten globule form of the sample.
Bioactivity of the complexes

Quantification of oleic acid in BAMLET produced through chromatography by NMR (Chapter 3) shows that there is a 5.4 times molar excess of oleic acid bound to the protein. Quantification of the α-LA:OA complex yields 4.6 moles OA per mole of protein. Measuring cytotoxicity of the complexes shows a slight difference in cytotoxicity of the samples, with BAMLET exhibiting slightly more toxicity for the three cell lines tested. A similar trend was observed for both complexes between PC-12\textsuperscript{indiff} and PC-12\textsuperscript{diff} indicating that the heated complex has the same selectivity as BAMLET.

BAMLET cytotoxicity data yielded interesting results. Against the two cancer cell lines the two complexes had similar LC\textsubscript{50} values due, in part, to the higher levels of oleic acid within the sample. Previous experiments (Brodkorb & Lišková, 2009) indicated that there is a dose response to sodium oleate – the more sodium oleate present in the sample, the more toxic it becomes. There is, however, a change in the method of cell death based on oleic acid content within the complex. The higher value complexes give a cell death that is more representative of necrosis rather than apoptosis (Lišková, 2011).

The cell model used for testing the complexes were rat adrenal carcinoma cell line, PC-12 models, which are representative of a cancer cell line. Differentiation of these cells with a nerve growth factor (NGF) results in the formation of neurons and the cells become representative of a healthy cell model. Testing of the complexes against the healthy, differentiated cell model shows that at the concentrations tested for the cancer cells, there was no change in cell viability within the healthy model, in fact there is a 10 fold increase in both the BAMLET and the complex in the amount of sample needed to kill the cells (Lišková, 2011).
Table 6.3: LC₅₀ values for BAMLET produced through chromatography and the α-LA:OA complex with a 5X molar excess of OA during preparation produced by heating

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>BAMLET mg/mL (µM)</th>
<th>α-LA:OA Complex mg/mL (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937</td>
<td>Human Lymphoma</td>
<td>0.13 (9.15)</td>
<td>0.18 (12.67)</td>
</tr>
<tr>
<td>PC-12&lt;sup&gt;undiff&lt;/sup&gt;</td>
<td>Rat Adrenal Carcinoma</td>
<td>0.04 (2.81)</td>
<td>0.05 (3.52)</td>
</tr>
<tr>
<td>PC-12&lt;sup&gt;diff&lt;/sup&gt;</td>
<td>Healthy Cell Model</td>
<td>0.45 (31.69)</td>
<td>0.51 (35.92)</td>
</tr>
</tbody>
</table>

6.2.2. Real-time monitoring of structural changes within the protein during production of the complex by heating

NMR was also used to assess what structural changes occurred within the protein during the production by heating process (Figure 6.8). The sample was prepared within the NMR tube without heating and then heated in situ on a 400 MHz NMR. The machine was heated gradually in increments of 10°C, with ¹H spectra captured at each temperature. Once 60°C was reached the sample was held in the NMR machine for 1 hour. After 1 hour a ¹H spectra was captured and the sample was removed from the machine, rapidly cooled on ice and put back into the machine for further analysis. A ¹H spectra was captured at 25°C and the sample was moved to a 600 MHz NMR for PFG-NMR experiments. The in situ heating experiment showed that unfolding occurred within the protein as the sample was heated – peak broadening was observed both in the aromatic region and within the upfield region. Peak sharpening of the OA peak occurred during heating, however the peak sharpness disappeared after the rapid cooling. PFG-NMR analysis of this sample showed that the OA did not decay at the same rate as the protein. OA decayed at a slower rate, indicating that there was unbound OA present in the sample. This was expected as no dialysis step was performed prior to the PFG-NMR, thus residual OA would be present in an unbound state.
Figure 6.8: Preparation and characterization of a complex of α-LA and OA during heating in situ in a 400 mHz NMR unit. Sample was heated at temperatures indicated within the graph at pH 7.4. NMR allowed the monitoring of structural changes as a function of temperature increase.
6.2.3. Oleic Acid and Sodium Oleate: Structural and Activity Considerations

It was shown previously that OA is the toxic component in HAMLET and related complexes (Permyakov et al., 2011; Lišková et al., 2011). The cytotoxic effect of the neat fatty acid solubilised by simple mixing or ethanol was compared to the cytotoxic effect of the fatty acid post micro-fluidization. The fatty acid, OA, and the salt form, SO, were analysed and compared pre- and post-microfluidisation for their biophysical attributes and biological activity.

Physical properties of SO/OA

The turbidity of the samples was measured pre- and post-microfluidisation as a function of pH by measuring the absorbance at 600 nm (Table 6.4). Figure 6.9 shows the visible changes in the samples pre- and post-microfluidisation. Whilst at pH 11, there were no visible changes upon microfluidisation, a dramatic cloudiness could be observed for oleic acid samples in both pH 2 and pH 7. This is due to inherent solubility issues, where pre-microfluidisation there is a phase separation of oleic acid in the case of pH 2 and pH 7.

The particle size of the samples microfluidised at pH 7 was measured by dynamic light scattering and compared to the particle size of the unprocessed oleic acid/sodium oleate. This was measured in order to determine the effect of microfluidisation on the particle size of the different fatty acids. Prior to microfluidisation both oleic acid and sodium oleate had a particle size of roughly 1 μm. Both were monomodal. After microfluidisation there was a decrease in particle size from 1 μm to 600 nm (Figure 6.10).
Table 6.4: Turbidity of oleic acid and sodium oleate samples before and after microfluidisation; absorbance values measured at 600 nm

<table>
<thead>
<tr>
<th></th>
<th>pH 2</th>
<th></th>
<th>pH 7</th>
<th></th>
<th>pH 11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SO</td>
<td>OA</td>
<td>SO</td>
<td>OA</td>
<td>SO</td>
</tr>
<tr>
<td>Before</td>
<td>1.12±.09</td>
<td>0.05±0.00</td>
<td>1.50±0.01</td>
<td>0.68±0.00</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td>After</td>
<td>0.12±0.01</td>
<td>0.13±0.00</td>
<td>1.54±0.0218</td>
<td>1.68±0.0085</td>
<td>0.04±.00</td>
</tr>
</tbody>
</table>

Table 6.5: Zeta potential of oleic acid and sodium oleate samples before and after microfluidisation; measured at 75 mV

<table>
<thead>
<tr>
<th></th>
<th>pH 2</th>
<th></th>
<th>pH 7</th>
<th></th>
<th>pH 11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SO</td>
<td>OA</td>
<td>SO</td>
<td>OA</td>
<td>SO</td>
</tr>
<tr>
<td>Before</td>
<td>-32.46±1.37</td>
<td>-34.7±3.25</td>
<td>-94.93±3.85</td>
<td>-92.9±2.35</td>
<td>-79.1±4.16</td>
</tr>
<tr>
<td>After</td>
<td>-8.25±0.58</td>
<td>-6.08±.61</td>
<td>-65.03±4.52</td>
<td>-63.5±3.17</td>
<td>-78.03±2.87</td>
</tr>
</tbody>
</table>
**Figure 6.9:** Images of OA and SO at pH 2, 7 and 11 before and after microfluidisation at 10,000 psi, room temperature with three passes through a Y-chamber
Figure 6.10: Particle size data by dynamic light scattering for fatty acid samples: sodium oleate (red) and oleic acid (blue), before microfluidisation (♦) and after microfluidisation (●) all at pH 7. Samples were microfluidised at 10,000 psi using 3 passes on a Y-chamber.

Zeta potential is the measure of effective surface charge of a sample and is measured as a function of pH. At native pH, sodium oleate has net negative charge and oleic acid has net neutral charge (= zero). A negative zeta potential is indicative that a sample is below its isoelectric point. In the emulsions produced, and in the non-microfluidised samples, the charges on the surface of the suspended droplets were measured (Table 6.5). The results can be described as follows:

Firstly, the zeta potential values for both SO and OA within each pH were similar (for example, -32.5/-34.7 at pH 2; -94.9/-92.9 at pH 7; -79.1/-78.2 at pH 11, etc.), indicating that acid-base equilibria was achieved rapidly. This also suggests that in the preparation of the protein-fatty acid complex, theoretically, there would be no difference from using either OA or SO. (The particular conditions used during complex generation/production would determine which would be best, as there
would be marked differences (Brodkorb & Lišková, 2009)). Secondly, regardless of the starting sample, the differences pre- and post-microfluidisation were quite pronounced (for example, -32.5/-34.7 vs. -8.25/-6.08 at pH 2), indicating that producing smaller vesicles negatively impacts the stability of the emulsion. One prominent exception was at pH 11 where there were no significant differences in any measured zeta potential value – regardless of source sample and/or particle size (all showing values around -79). As the fatty acid is fully deprotonated at pH 11, this suggests that any emulsions/vesicles generated must commonly incorporate the amphipathic character present in all molecules - the negative charge of the polar group along with its hydrophobic tail. As such, the samples made at pH 11 (which would presumably be all oleate in chemical property) are less stable – emulsion-wise – than those produced with the protonated OA pre-microfluidisation. At pH 7 and pH 2 with microfluidisation, the zeta potential for the emulsion becomes successively less than at pH 11, indicating that phase separation upon protonation works against emulsion stability. These results support a working hypothesis that if the fatty acid molecules were to bind protein, the charged form (oleate) would be relatively more advantageous than the uncharged OA form. Indeed, the absolute values of the protein-fatty acid complexes (Fig 6.1) show that fatty acid bound to protein display very different emulsive properties, which may help in enhancing its media-solubility and ultimately biological activity compared to the fatty acid vesicles alone.

As shown previously, the presence of the fatty acid only becomes important when in complex with the protein. This data shows that the fatty acid alone likely possesses a charge when not bound to the protein, also supporting that the change in zeta potential when the protein is bound to the fatty acid may be as a result of the solubilisation effect of the protein.

**Diffusion NMR**

Diffusion NMR applies a magnetic field gradient of increasing magnetic strength to a sample. The rate of decay of the sample as a function of the strength of magnetic field applied can be used to determine different parameters about the sample, including the hydrodynamic radius of compounds (Jones *et al*., 1997;
Wilkins et al., 1999). Diffusion NMR can also be used to determine the state of samples in solution – if the peaks decay at the same rate relative to the control it is said that all of the sample is a unibody, or complexed. If the peaks do not decay at the same rate as the control it can be said that the samples are not associated in solution. Diffusion NMR has been used in the study of HAMLET to determine if all of the oleic acid is bound to the protein.

Oleic acid and sodium oleate at pH 2, pH 7 and pH 11 exhibited very similar diffusion patterns relative to each other. Interestingly, using this technique, it was possible to detect the presence of oleic acid/sodium oleate at pH 2, whereas observation of signal was somewhat difficult with the zeta potential experiments. Samples at pH 2 and at pH 7 had similar decay patterns. Based on their patterns, it would suggest that the fatty acid within the samples was not in solution. Samples at pH 11 exhibited a different decay pattern to samples at pH 2 and pH 7. The pattern was similar to that of the control (1,4-dioxane) thus it can be concluded that the samples were in solution, not just in suspension, at pH 11 (Figure 6.11).
Figure 6.11: Diffusion NMR spectra for measurements of % magnetic field strength as a function of signal decay for oleic acid and sodium oleate post-homogenisation at pH 2, pH 7 and pH 11 (representative of 1,4-dioxane (u) and olefinic protons (n))

Cytotoxicity of oleic acid and sodium oleate
The cytotoxicity of the microfluidised SO and OA samples at pH 7 were determined against U2OS, undifferentiated PC-12 cells and differentiated PC-12 cells, and their LC₅₀ values were compared to the non-microfluidised samples. Only samples at pH 7 were tested as the samples generated at pH 2 and pH 11 would not stay constant as soon as they were applied to the cell culture media. In this study it was shown that both processed and unprocessed SO and OA are toxic to cancer cells. Untreated SO and OA were toxic to the U2OS cells at concentrations of 100 ± 12 μM and 110 ± 11 μM respectively. After
microfluidisation the LC$_{50}$ values decreased to 50 ± 8 μM and 60 ± 7 μM respectively. A similar trend was observed in the UPC-12 cells where the decrease in the LC$_{50}$ value was of a similar order of magnitude to that seen in the U2OS cells. However there was a large difference in the LC$_{50}$ values for the differentiated cells. There was an almost 7-fold decrease in the LC$_{50}$ values for the OA samples from 400 ± 7 μM to 60 ± 6 μM, and over an 11-fold decrease for the SO samples, from 800 ± 5 μM to 70 ± 9 μM (Table 6.6).

Table 6.6: Cytotoxicity of Oleic Acid and Sodium Oleate samples before and after microfluidisation

<table>
<thead>
<tr>
<th></th>
<th>OA (μM)</th>
<th>SO (μM)</th>
<th>MF OA (μM)</th>
<th>MF SO (μM)</th>
<th>BAMLET mg/mL</th>
<th>BAMLET SO/OA Equivalent (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>110±11</td>
<td>100±12</td>
<td>60±7</td>
<td>50±8</td>
<td>0.048 (3.4)</td>
<td>18.36</td>
</tr>
<tr>
<td>PC-12$_{undiff}$</td>
<td>95±4</td>
<td>85±8</td>
<td>50±5</td>
<td>50±7</td>
<td>0.064 (4.5)</td>
<td>24.3</td>
</tr>
<tr>
<td>PC-12$_{diff}$</td>
<td>400±7</td>
<td>800±5</td>
<td>60±6</td>
<td>70±9</td>
<td>0.45 (32)</td>
<td>172.8</td>
</tr>
</tbody>
</table>

The mechanism of cell death was also determined. After treatment of the cells with the different samples for 4 hours it was determined that the cells were undergoing apoptotic cell death, with DNA fragmentation of the cells into apoptotic bodies occurring, as confirmed by the Roche Cell Death ELISA test kit. This was seen through the increase of the enrichment factor as a result of incubation with the different complexes.
Table 6.7: Enrichment factors of mononucleosomes and oligonucleosomes for bioactive complexes tested at the concentration of the LC50 for each component. Tested against U937 cells after 4 hours incubation with representative sample. Error bars are representative of three preparations in triplicate.

<table>
<thead>
<tr>
<th>Sample used</th>
<th>Enrichment Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-LA</td>
<td>0</td>
</tr>
<tr>
<td>BAMLET</td>
<td>18.72 ± 2.61</td>
</tr>
<tr>
<td>Sodium Oleate</td>
<td>21.12 ± 3.84</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>22.43 ± 1.06</td>
</tr>
<tr>
<td>Microfluidised Sodium Oleate</td>
<td>20.32 ± 4.11</td>
</tr>
<tr>
<td>Microfluidised Oleic Acid</td>
<td>19.83 ± 3.34</td>
</tr>
</tbody>
</table>

While processing of the SO and OA does indeed lead to smaller particles that are more toxic to the cancer cells, it is also significantly more toxic to the healthy cells. This would suggest that in the case of H/BAMLET that the role of the protein is not just in the assistance of the solubilisation of the fatty acid, but that it also has a protective effect on the healthy cells.

From the range of biophysical techniques employed to test the fatty acid it is clear that there are discernable differences between the fatty acid forms pre- and post-microfluidisation. However after the process of microfluidisation has been completed, at each pH the sodium oleate and oleic acid became equivalent in their physical characteristics regardless of the starting material used.
6.2.4. BAMLET and BSA: The issue of competitive binding - Inactivation of BAMLET through incubation with BSA

Direct addition of BSA

It was shown previously that the fatty acid is the important co-factor in the production of the complex, and that it is possible to produce a complex between β-lactoglobulin, parvalbumin, equine lysozyme and OA. A complex was produced between BSA, a known fatty acid transporter, and oleic acid. With increased levels of OA bound to both α-LA and β-LG there was a subsequent increase in toxicity. Thus, a similar response was expected when BSA was in complex with either OA or SO. However, when complexed with SO or OA BSA did not exhibit any cytotoxic activity against U937 cells.

Treatment of U937 cells with BAMLET that had been incubated with increasing amounts of serum albumin resulted in the inactivation of BAMLET – i.e. at the tested concentrations of BAMLET there was a decrease in activity of the complex with increasing concentrations of BSA. There was no detrimental effect of BSA on the action of the complex up to 1% BSA, however towards physiological concentrations of BSA, up to 4.4% (44 g/L), there was a decrease in activity, eventually resulting in the inactivation of BAMLET (Figure 6.12).
Figure 6.12: Cell death curves for BAMLET incubated with increasing amounts of BSA from 0% (♦ - orange), 0.5% (■ - green), 1% (▲ - blue), 2% (×), 3% (☆ - purple), and 4.4% (● - cyan) (physiological serum albumin concentration). Overall there was an increase in the LC50 value as a function of increasing serum concentration. Data are the means of three independent measurements with SDs represented by vertical bars.

Quantification of the oleic acid content remaining in complex with α-LA showed that with increasing amounts of BSA there was a subsequent decrease in the amount of oleic acid. Samples underwent ultracentrifugation through a 30 kDa UF membrane whereby the retentate contained BSA and the permeate contained BAMLET. Quantification of the OA was completed using integration of peak areas for known standards of OA content on FTIR. The loss of OA in the permeate can be attributed to the binding of OA to BSA (Table 6.8).
Table 6.8: Quantification by FTIR of oleic acid levels in BAMLET samples after incubation with BSA

<table>
<thead>
<tr>
<th>BSA concentration co-incubated with 1 mM BAMLET (%)</th>
<th>OA content in permeate (mole_{OA}/mole_{protein})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.05</td>
</tr>
<tr>
<td>0.5</td>
<td>5.71</td>
</tr>
<tr>
<td>1</td>
<td>4.32</td>
</tr>
<tr>
<td>2</td>
<td>4.74</td>
</tr>
<tr>
<td>3</td>
<td>1.48</td>
</tr>
<tr>
<td>4.4</td>
<td>0.81</td>
</tr>
</tbody>
</table>

**BSA contact plate**

Immobilization of BSA resulted in the same phenomena occurring. BSA was incubated in a 24 well plate at 37°C for one hour. Excess BSA was removed and the wells were washed three times with distilled water to remove unbound BSA. Plates were air dried before the addition of a 10 mg/mL solution of BAMLET to the wells. Samples were taken after 30 minutes, 1, 2, 3 and 4 hours for analysis.

The protein concentration in samples taken from the well remained at a constant level within the samples. Analysis of the protein content of the samples via SDS-PAGE showed that there was no detachment of BSA from the plate, or loss of α-LA to the plate. As there was no loss of α-LA, any loss of OA can be attributed to competitive binding with BSA (Figure 6.13).
Figure 6.13: SDS-PAGE of BAMLET (lane 1) after incubation on a BSA coated plate for 30 minutes (lane 2), 1 hour (lane 3), 2 hours (lane 4), 3 hours (lane 5), 4 hours (lane 6) and 24 hours (lane 7).

The oleic acid content of the samples was determined using FTIR. There was a decrease in intensity at 2,854 cm$^{-1}$ coinciding with incubation time on the BSA coated plate (Figure 6.14). This is indicative that oleic acid was removed from BAMLET through contact/interaction with BSA.
**Figure 6.14:** Oleic acid (blue) and protein content (red) as a function of incubation time on a BSA coated plate from 30 minutes to 4 hours. Data are the means of three independent measurements with SDs represented by vertical bars.

The cytotoxicity, as expected, was negatively impacted by the decrease in OA levels within the samples – there was an increase in the LC\textsubscript{50} value for BAMLET as a function of incubation time with BSA (Figure 6.15). This further confirms the importance of oleic acid in BAMLET activity, and that BAMLET can be inactivated by BSA.

**Figure 6.15:** Increasing LC\textsubscript{50} values (red) of 1 mM protein samples with decreasing oleic acid content (blue) present in samples as a function of incubation time on a BSA coated plate from 30 minutes to 4 hours. Data are the means of three independent measurements with SDs represented by vertical bars.
6.2.5. BSA:OA complexes as model proteins: NMR studies

Liquid state $^{13}$C NMR

Given the high affinity for OA, BSA is a perfect model protein for determining the bound state, and importantly the protonation state of oleic acid and sodium oleate when in complex with proteins. BSA is known to have six specific binding sites for fatty acid transport (Petipas et al., 2001). In order to ensure that all oleic acid or sodium oleate within the solution was bound to the protein a complex with a 5 times molar excess of the fat was produced with the protein. The complex was produced by overnight agitation. Natural abundance $^{13}$C NMR was performed on oleic acid alone and sodium oleate alone in order to determine the chemical shifts for the free states of the fatty acid. With BSA in complex with oleic acid, the mixture was maintained at pH 4 where it was known that any fatty acid present would be in the acid, or protonated form, and it was possible to decipher a difference between the carbonyl region for the protein and the fatty acid. At pH 12 the fatty acid present was in the oleate, or deprotonated form and thus the carbon 1 proton was deprotonated and had a different chemical shift than that observed for the OA/pH 4 sample (Table 6.9). At pH 7.4, the physiological pH in which BAMLET acts (in buffered cell culture media, in PBS for in vivo studies), there is a mixed population of bound sodium oleate and free sodium oleate – no oleic acid is present. Oleic acid alone, sonicated at pH 7.4 in PBS has the same chemical shift as free sodium oleate, confirming that oleic acid and sodium oleate are interchangeable based on pH changes (Figure 6.16).

Table 6.9: Chemical shift values for the carbonyl group of the fatty acid in liquid state NMR

<table>
<thead>
<tr>
<th></th>
<th>Oleic Acid</th>
<th>Sodium Oleate</th>
<th>HAMLET Oleic</th>
<th>HAMLET Oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAMLET</td>
<td>181.21</td>
<td>180.2</td>
<td>184.09</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>183</td>
<td>183.17</td>
</tr>
</tbody>
</table>
Using chromatographically produced HAMLET, produced by James Chin Ho in Lund University (Svanborg group), $^{13}$C NMR studies were performed. HAMLET was produced using both oleic acid and sodium oleate, $^{13}$C NMR of complexes gave some interesting results (Ho et al., 2013). The carbon 1 chemical shift of oleic acid in HAMLET produced with oleic acid has the same chemical shift of the sodium oleate in HAMLET produced with sodium oleate, showing that regardless of the starting material, the same overall complex is produced. Comparing these HAMLET complexes to BAMLET produced in this laboratory shows that the complexes exhibit remarkable similarities. In both HAMLET complexes there is a large peak at 171 ppm, this was confirmed as residual amounts of EDTA from the production of the apo form of $\alpha$-LA. This may have implications for the activity of the complex as EDTA is a known ion chelator and may exploit the ion channels within the cell, disrupting cell survival (Figure 6.17).
Figure 6.17: Liquid state natural abundance $^{13}$C NMR spectra of BSA in complex with oleic acid at pH 4, sodium oleate at pH 12, a mixed population at pH 7.4 and oleic acid alone at pH 7.4, in deuterated PBS at 25°C, relaxation delay of 9 seconds with 8,000 scans.
Solid state MAS $^{13}$C NMR

In order to fully confirm the chemical shifts for the native states of oleic acid and sodium oleate i.e. without altering the pH or solubilising the compound, magic angle spinning, MAS, solid state NMR was performed on the compounds and on BAMLET and the BSA complex. The chemical shifts followed the same overall trend as observed in solution state NMR thus it can be concluded that when oleic acid or sodium oleate are bound to a protein, the fatty acid is in the deprotonated state (Figure 6.18).

Figure 6.18: 3.2 mm Magic Angle Spinning Solid State natural abundance $^{13}$C NMR spectra of BAMLET produced through chromatography with oleic acid, oleic acid and sodium oleate, at 4°C and a spinning rate of 12,000 kHz.

Table 6.10: MAS-SS NMR carbonyl chemical shift values of the fatty acid component for BAMLET, oleic acid, sodium oleate and a BSA:SO complex produced with a 5X molar excess of fatty acid.

<table>
<thead>
<tr>
<th>BAMLET</th>
<th>Oleic Acid</th>
<th>Sodium Oleate</th>
<th>BSA:SO Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>Red</td>
<td>Blue</td>
<td>-</td>
</tr>
<tr>
<td>182.41</td>
<td>181.06</td>
<td>182.05</td>
<td>182.78</td>
</tr>
</tbody>
</table>
6.2.6. BSA:OA complexes as model proteins: Isoelectric focussing

The importance of weak binding was highlighted previously – the OA bound to BSA is not cytotoxic compared to α-LA and β-LG in complex with OA. Given the ability to produce complexes with differing amounts of OA/SO bound to the protein, it is possible that within one single preparation of BAMLET, different populations with varying levels of oleic acid bound exist. In order to determine whether this micro heterogeneity exists within individual preparations of BAMLET, liquid phase isoelectric focusing was used. The principle behind the use of IEF is that the overall isoelectric point of the complex will change based on the amount of oleic acid/sodium oleate that is bound to the protein. The isoelectric point of α-LA is ~4.8. With an increase in surface charge being observed, (as measured by zeta potential) with increasing amounts of oleic acid bound to the protein, it was expected that the distribution of the protein as a function of pH would change based on the amount of fatty acid bound.

Different preparations of BAMLET were used, along with a control of native α-LA to determine the correct protein distribution for the native protein. Ampholytes with a pH range of 3 – 10 were used in Rotofor experiments (Bio Rad Laboratories). Ampholytes contain compounds with both acidic and basic isoelectric points, and when an electric field is applied each compound within the ampholytes migrates to their individual isoelectric point. Ampholytes are present in high concentrations relative to the protein concentration and thus provide a stable pH level for proteins to migrate to their isoelectric point.

For native α-LA the distribution of protein was relatively tightly centred around its isoelectric point, with a peak at pH 4.14 – 5.53. For BAMLET produced through chromatography there was a maximum peak at pH 5.53 with two large peaks following at 6.03 and 6.17. For BSA:SO complex, the maximum peak is at 7.29 with sister peaks existing either side.

The pI for BSA is 4.7 (Girard et al., 1997), similar to α-LA. However due to the bound oleate molecules the pI of the complex increases to approximately 7.29.
This is expected as the pK_a of OA/SO is 9.5 (Kanicky and Shah, 2002), hence the pl of the complex increases accordingly. The relative tightness of the distribution (similar to that of native α-LA) reflects the strong binding of the fatty acid (dissociation constant on the order of ~ 8 x 10^8).

In contrast for chromatographically produced BAMLET distribution of peaks is relatively wide, suggesting that there is a distribution of bound oleates. The maximal peak shift towards higher pl is analogous to the case of BSA, the pK_a of the bound oleates are higher than the protein itself. It is, however, clear that even within one batch of chromatographically prepared BAMLET, a polydispersion of bound fatty acids is observed.

Figure 6.19B shows that the heating method yields a very similar profile of heterogeneous complexes with a different number of oleate molecules bound. We feel that, therefore, the heating/cooling method of complex formation may be superior in large scale production. The reason into why/how such wide ranges of microheterogeneous complexes are generated is currently being investigated.
Figure 6.19: Normalised protein concentration plotted against an average pH value for each Rotofor run with ampholytes giving a pH gradient of 3-10, for:

A) α-LA (red), BAMLET produced through chromatography (black), a BSA:SO complex (blue);

B) BAMLET produced through chromatography (black), BAMLET complex produced by heating with a 5X molar excess of SO (orange) and a 10X molar excess of SO (green).

Quantification of oleic acid was achieved by creating a standard curve of oleic acid concentrations and quantifying based on peak areas of the olefinic protons at 5-5.5 ppm. Samples of a known protein concentration and known volume were
dried and the resulting powder was dissolved in a deuterated methanol solution. In this case the protein component remained insoluble and the oleic acid entered the organic phase. $^1$H proton analysis of the ampholyte solution showed that there were no overlapping peaks in the 5-5.5 ppm region thus the presence of ampholytes is negligible for quantification of oleic acid.

Oleic acid content within the samples increased as a function of pH increase (Table 6.10). Samples also had a lower protein content and when normalized to protein concentration there is a higher molar excess of fatty acid per mole of protein, thus proving the hypothesis that within a homogenous, well prepared solution of BAMLET, micro-heterogeneity exists.

**Table 6.10:** Stoichiometries of oleic acid/sodium oleate content in select Rotofor fractions

<table>
<thead>
<tr>
<th>pH</th>
<th>$\alpha$-LA</th>
<th>BAMLET</th>
<th>BSA:SO</th>
<th>$\alpha$-LA:SO 5X</th>
<th>$\alpha$-LA:SO 10X</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.66</td>
<td>0.00</td>
<td>1.10</td>
<td>1.92</td>
<td>1.09</td>
<td>3.16</td>
</tr>
<tr>
<td>5.53</td>
<td>0.00</td>
<td>0.89</td>
<td>2.09</td>
<td>1.17</td>
<td>3.18</td>
</tr>
<tr>
<td>6.17</td>
<td>0.00</td>
<td>2.08</td>
<td>2.35</td>
<td>3.06</td>
<td>3.66</td>
</tr>
<tr>
<td>7.29</td>
<td>0.00</td>
<td>3.68</td>
<td>3.57</td>
<td>3.27</td>
<td>4.13</td>
</tr>
<tr>
<td>8.38</td>
<td>0.00</td>
<td>5.41</td>
<td>4.1</td>
<td>3.21</td>
<td>5.43</td>
</tr>
<tr>
<td>8.72</td>
<td>0.00</td>
<td>5.70</td>
<td>5.38</td>
<td>3.45</td>
<td>6.55</td>
</tr>
<tr>
<td>8.88</td>
<td>0.00</td>
<td>6.63</td>
<td>5.84</td>
<td>4.43</td>
<td>8.76</td>
</tr>
</tbody>
</table>

Overall the isoelectric focusing experiments show that microheterogeneity exists within a given preparation, regardless of the method of production. Within each fraction harvested for the different preparations, different stoichiometries exist. As the pH of the fraction increases the amount of fatty acid increases, indicating that the non- $\alpha$-LA binding component (= oleic acid/oleate) exhibits a pI higher than the protein itself.
6.3. Discussion

Overall it was shown that the complex produced by the method of Brodkorb & Lišková, 2009, is structurally and actively similar to BAMLET produced through chromatography. In the original publication of Svensson et al., 1999, oleic acid was solubilised in ethanol before addition to a Tris buffer at pH 8.5 and sonicated. This produced an oleic acid suspension, which within production of BAMLET for our own laboratory is a white, turbid solution. In most publications oleic acid is used, with the exception of the most recent publication from the Svanborg group where sodium oleate was used to produce HAMLET via the chromatographical route. HAMLET produced through oleic acid and sodium oleate were deemed to possess the same cytotoxic capabilities, and it was shown, and expanded further within this study, that the protonation state of the carbonyl group of the fatty acid is identical and independent of the starting material (Ho CS, J, et al., 2013). pH ranges for complex formation in all other studies bar the digestion study of Sullivan et al., 2013 where oleic acid was maintained in the acid form, the pH of the solutions were maintained above pH 7.4, sometimes reaching pH ~10 (Brinkmann et al., 2011). Within the current study it was shown that at the same pH levels, oleic acid and sodium oleate exhibit the same behaviour, and that there is a transition between the acid (protonated) and salt (deprotonated) forms around pH 7.4 where a mixed population of oleic acid and sodium oleate exist. However various values exist for the pKₐ of sodium oleate depending on the conditions of the experiment. Therefore it makes characterisation of the complex challenging.

Controlling the oleic acid content results in the ability to alter the protein structure of the complex. Differences exist in the upfield structure of BAMLET produced by heating compared to the upfield structure of HAMLET. Wijesinha-Bettoni et al 2001 report the upfield structure of α-LA and proteins as being broad when the protein is in its molten globule like form. Initial and recent NMR based studies of HAMLET indicate that HAMLET, and derivatives of HAMLET made with All-Ala α-LA, has a molten globule like tertiary structure (Pettersson-Kastberg et al., 2009). The same is observed in BAMLET produced via chromatography using α-LA from Sigma-Aldrich. However when the complex was produced using
commercially available α-LA the overall structure of the complex was more native like, irrespective of the oleic acid/sodium oleate loading.

When correlated to quantified molar excess content, there is a linear decrease in zeta potential, indicating that the complex is more negatively charged with increasing amounts of the fatty acid bound. The increase in charge is not seen by simple mixing of the protein with sodium oleate and only occurs after the heating/dialysis steps suggesting that the structural changes within the protein upon heating and the mixing/incubation time as a result of the dialysis allows the fatty acid to bind to the protein.

The results observed in the diffusion NMR studies are in contrast with the zeta potential data. Clearly at pH 11 the diffusion NMR results suggest that the SO/OA molecules are monomeric, in solution, and with a similar decay curve of that of a small molecule, in this case, 1,4-dioxane. The pH 11 data for the zeta potential exhibits an intermediate value between those of pH 2 and pH 7. It was, therefore, expected to see a similar trend in the diffusion NMR. We attribute this inconsistency to the different observables between the two techniques. Whilst diffusion NMR primarily looks at the radius of hydration, zeta potential is a complex measurement that incorporates surface charge, electrokinetic potential, electrophoretic mobility, and particle size, amongst others. As SO/OA at pH 7 did not follow a Gaussian decay, it was not possible to correlate this diffusion NMR data to the particle size data.

The proposed function of the protein initially discussed by Mok et al., 2007, is that HAMLET acts as a “biological Hydra” – it has many heads, as determined by its varying mechanisms of action (Mok et al., 2007). More recently it was proposed that HAMLET and related complexes work by acting as a mule delivering the fatty acid to the cell and aids solubilisation of the fatty acid (Min et al., 2012). Mechanically solubilising the fatty acid, while increasing its toxicity, still did not result in the formation of a compound more toxic than BAMLET. Microfluidised oleic acid and sodium oleate were also more toxic towards healthy cells, suggesting a protective role of the protein with the healthy cells. However
the nature of the protein is also of vital importance. Having too high an affinity to the fatty acid results in the fatty acid being retained by the protein, thus losing its effectiveness against cancer cells. The presence of serum albumin may also negatively impact the activity of the complex and at physiological conditions the complex may not be as active.

Mechanistically different preparations of HAMLET and BAMLET possess slight differences. However the $^{13}$C NMR data published by Ho CS, J, et al., 2013, shows that there is residual EDTA present in HAMLET samples. This EDTA is not present in BAMLET samples. EDTA is a chelator and will bind excess calcium, thus may leach intracellular calcium from cells, affecting their metabolic pathways, yielding different cell death pathways to samples free of EDTA. While EDTA alone is not toxic to cells at the concentrations tested, there is a decrease in cell viability.

It is important to note that oleic acid is the protonated form of sodium oleate. The $pK_a$ for sodium oleate, the aqueous form of the fatty acid, is pH 8 (Kanicky, 2003). Experimentally it was shown that as the pH of the fraction from IEF increased, the molar excess of oleic acid within the sample also increased. More fatty acid was present per mole of protein where protein was present, and at the extreme pH fractions no protein was present thus the composition of the samples was just sodium oleate. Hence the distribution of the different microheterogeneity populations reflects the bound stoichiometry well.

The cytotoxicity of the preparations of oleic acid and sodium oleate compared to the microfluidised samples yields interesting results. Before microfluidisation oleic acid and sodium oleate were toxic to U2OS cells and PC-12$^{undiff}$ cells, with PC-12$^{diff}$ cells showing more resistance to the compounds. After microfluidisation the particle size of the compounds is smaller and this influences the toxicity. For U2OS cells and PC-12$^{undiff}$ cells there is an increased toxicity and remarkably the cytotoxic effect was also observed in the PC-12$^{diff}$ cell line. The resistance that was exhibited in the differentiated cells has dramatically decreased and the LC$_{50}$ values are in line with those obtained with the undifferentiated cell line.
Comparing the LC_{50} values of BAMLET against the different cells the fatty acid content needed to have a cytotoxic effect is approximately half of that needed in the microfluidised samples. However what is important to note is that the resistance of the differentiated cells is retained when OA is in complex with the protein. This indicates that the solubilisation of the fatty acid is not the only important factor and that the protein acts as a protective, barrier layer for the healthy cells while also delivering the fatty acid to the cancer cells.
Chapter 7

General discussion
Background and studies to date

The discoverer of HAMLET, Prof Catharina Svanborg (Lund University, Sweden), is a world-renowned clinical mucosal immunologist and urinary tract infection specialist (Ragnarsdottir et al., 2003). The discovery of MAL, multimeric α-lactalbumin, has prompted her research group to embark on a new and different challenge (Håkansson et al., 1995). Whilst screening human milk for its anti-infective properties and effect on bacterial cell adherence, interesting changes were noted in cells when treated with the precipitated casein fractions. The complex was noted by its remarkable properties of killing cancer cells with a mechanism similar to apoptosis, and later was shown to be composed of α-LA and OA (Svensson et al., 1999; Svensson et al., 2000). As a result, the Svanborg laboratory presently works on these two themes - urinary tract infection and HAMLET - with equal intensity and purpose of mission.

Since this discovery, the field of HAMLET research has expanded in great measures of both breadth and depth. There have been many notable publications from the Svanborg group and others, including:

- Characterisation of the complex and demonstration that the protein moiety is partially-unfolded and in the molten globule state (Svensson et al., 1999)
- The method of production of HAMLET with an OA conditioned column (Svensson et al., 2000)
- NMR study to suggest OA is bound to the α-LA protein in a compact conformation (Fast et al., 2005)
- Confirmation that the phenomena was not specific to HLA but can be formed from BLA (Pettersson et al., 2006)
- Quantification of oleic acid within HAMLET showing a 1:5 ratio of protein:fatty acid (Pettersson et al., 2009)
- Evidence showing that the native structure of α-LA need not be recovered upon uptake into the tumour cell (Pettersson et al., 2009)
- NMR study to differentiate between OA in the bound and free state (Nielsen et al., 2010)
- Publications relating to the use of HAMLET in vivo as a therapeutic agent:
  - Bladder cancer (Mossberg et al., 2007, Mossberg et al., 2010)
Mechanistic studies continued and due to the range of tumour cells HAMLET has activity against, amongst the assortment of different mechanisms elucidated, HAMLET was shown to act with caspase activation (Hallgren et al., 2006) and with histone interactions (Düringer et al., 2003). HAMLET was likened to a “Lernaean hydra” – many different heads representing its multi targeted approach to cell death (Mok et al., 2007).

Since the current research began, publications within the field have exploded with each publication answering questions as well as creating more. In 2008, HAMLET was a unique entity in the field of tumouricidal protein fatty acid complexes. In 2010 it was shown that, through limited proteolysis of α-LA, a cancer killing complex can be produced from fragments of α-LA and OA (Tolin et al., 2010). In 2009 the field expanded further to include the complex between equine lysozyme and OA (dubbed ELOA), exhibiting the same cytotoxic activity of HAMLET (Wilhelm et al., 2009), opening up the field for the production of complexes with other proteins which can enter a molten-globule state. The year of 2009 also marked the discovery that the salt form of the fatty acid, sodium oleate, can be used in the production of an active complex with α-LA, along with the ability to control the amount of fatty acid present in the complex (Brodkorb and Lišková 2009). A follow up study in 2010 on the efficacy of HAMLET against human bladder tumours in mice showed that HAMLET exhibited remarkable cytotoxicity against the tumours. In 2011 it was again shown that the protein source is not important when a complex was formed between β-LG and SO (Lišková et al., 2011). This discovery opened up the possibility of binding other bioactive components to proteins to alter their biological function and in 2012 β-LG was used to deliver sodium linoleate to the intestines (Le Maux et al., 2012). This also marked the first in vivo study of the treatment of bacterial infections in vivo and it was shown that HAMLET kills Streptococcus pneumonia in mice models (Marks et al., 2012). Other fatty acids were shown to interact with α-LA to illicit the same cytotoxic response in vitro, including stearic acid and vaccinic.
acid (Brinkmann et al., 2013). Two further *in vivo* mouse trials were published: the prevention and treatment of colon cancer by oral administration of HAMLET (Puthia et al., 2013), and the treatment of MRSA by co-treatment of HAMLET with methicillin (Marks et al., 2013).

From a structural point of view, new methods were developed to quantify the amount of OA present in HAMLET and related complexes via FTIR (Tolin et al., 2010, Lišková 2011) and NMR (Pettersson et al., 2009). The first in-depth structural studies of HAMLET were completed, including a SAXS study (Ho et al., 2012) and the first HSQC-based structure of HAMLET (Makabe et al., 2013); both hypothesizing structure and potential binding sites.

**Current research hypothesis**

It was stated by Svensson et al., 2003 that "... the conditions under which HAMLET was formed resembled those in the stomach of a nursing child..." and this was further alluded to by Barbana et al., 2006 with "... the conditions existing in the gastrointestinal tract could provide an environment fulfilling the requirements necessary for the conversion of α-LA into the active apoptotic form...". Around this time, HAMLET began to gain interest from "mainstream" media and featured sporadically in popular publications under the premise that consuming breast milk will allow the body to naturally form HAMLET and fight off cancer (Rehmeyer, 2006; Huff, 2013). This prompted many emails from patients to research scientists within the field, begging the question "... do you think it might work for me to simply ingest bovine alpha-lactalbumin with an oil high in oleic acid to see if my body would naturally make BAMLET?"

Therefore the general working title/research question of the project was to assess if a HAMLET-like complex can be formed under simulated gastric conditions. However, as with all research, when one question is answered another is asked. The most pertinent question was: How do we know if we have produced HAMLET? The simplest method was to produce BAMLET through the published chromatographic method and analyze it with a battery of biophysical techniques, using these results as a reference for complexes produced.
With other research groups publishing their data, and through results obtained in our own research group, other questions were raised, including, but not limited to: the importance of mixing and temperature in the production of the complex, and if these subsequent complexes are different to column BAMLET; the importance of the fatty acid (studies have shown that OA is the toxic component); why interaction with BSA is an important factor in activity testing *in vitro* and *in vivo*; and finally, if SO is equivalent to OA at a given pH, is it always SO inadvertently used in all preparations of HAMLET and related complexes?

**How the current research answers these questions**

**Is BAMLET formed in the stomach of infants?**

Yes. Under the current tested *in vitro* conditions, a complex that is structurally similar to, and exhibits the same toxicity as BAMLET is formed. pH levels (Mitchell *et al.*, 2001), enzyme activity levels (DiPalma *et al.*, 1991), indigenous properties of milk (Jensen, 1995) and inherent structural changes in the protein below its isoelectric point (Kataoka *et al.*, 1997), lend themselves to complex formation. The hypothesis was therefore deemed correct in this work.

Limitations do exist to the current study. The model used was an *in vitro* model requiring *in vivo* validation. A simple gastric model was employed and did not include the small intestine where further proteolysis may occur (Moreno *et al.*, 2005). Other assumptions made include that of gastric lipolysis occurring (Hamosh *et al.*, 1981) resulting in the formation of free fatty acids, and free oleic acid was used in experiments; and as it was a liquid based feed mastication and amylase enzymes were deemed negligible.

Drawing comparisons between this study and other publications is difficult: no other studies factor in the digestion aspect of complex formation. Studies exist whereby fragments of α-LA are produced through limited proteolysis and bound to OA, and these complexes possess activity (Tolin *et al.*, 2010). The same was observed with SO (Ho *et al.*, 2013). A complex was not formed with peptides in this study.
After success with *in vitro* digestion experiments, the natural progression was towards an *in vivo* study, with a slight change in the hypothesis: Is BAMLET formed in the stomach of adults? Unlike with *in vitro* experimentation, the results were less positive. However the results were in line with previous *in vivo* digestion studies of proteins. A similar pH curve was seen (Troost et al., 2001; Troost et al., 2002), and thus resulted in the generation of vital data in an otherwise rarely studied field of *in vivo* validation of *in vitro* models. It is envisaged that the data gathered could be used to generate a pH profile and enzyme activity model for studying milk/liquid protein digestion in adult humans. Comparing the pH data to previous studies on gastric pH monitoring in infants (Mitchell et al., 2001, Omari and Davidson 2003) shows that there is a higher level of acid secretion in adults than in infants, and comparing pepsin levels (Newton et al., 2004) shows a higher level of proteolysis in adults than in infants.

One major deliverable from this study is that of the *in vivo* visualization of gastric mixing. Whilst digestion cannot be tracked on a molecular level, it is possible to use capsule endoscopy technology to visualize changes in the appearance of food in the stomach. Co-measurement of pH allowed correlation of pH to sample appearance, and adequate comparison to *in vitro* visualization.

One oversight in the *in vivo* study was the addition of sugar to the feed in order to make it more palatable to the subjects. The chemical structure of sugar is such that it absorbs in the same range as OA in FTIR, and displays the approximately same chemical shifts with many of the key \(^1\)H signals of OA in NMR. This made quantification of the fatty acid difficult through the usual FTIR and 1-dimensional NMR analyses. We foresee the use of fast NMR techniques such as non-uniform sampling 2D \(^1\)H-\(^{13}\)C HSQC NMR to overcome this difficulty, as in the second dimension, the \(^1\)H signals do not overlap – chemical shifts are 129.8 and 94.8 ppm for OA and sucrose respectively. A second oversight was not using triple bore nasogastric tubes that are normally used in the collection of gastric and duodenal enzymes (Furlund et al., 2013). The use of these tubes would allow direct duodenal sampling, however these tubes are more invasive (larger diameter) and require an x-ray to confirm correct placement.
In 2013, Puthia et al published their work on the use of HAMLET in the treatment and prevention of colon cancer. Whilst relevant to this study, it is difficult to draw comparisons. Firstly the anatomy of a mouse varies greatly to that of a human – mice do not have a small intestine. Secondly, this study did not require the conversion of HAMLET in vivo – HAMLET was administered orally to the mice. However given the efficacy of HAMLET it may be potentially beneficial to investigate the instillation of HAMLET rectally for the treatment of colon cancer, thus bypassing gastric and intestinal proteolysis.

Through the addition of GDL it was possible to monitor protein folding real time as a result of acidification. Using this method the changes in the OA could be visualized simultaneously. This allowed us to see what exactly happens during acidification, particularly in relation to changes in the surface charge of the protein, changes in the surface hydrophobicity of the protein, and real time measurement of structural changes. Structural/chemical shift changes in histidine upon initiating the pH jump were also confirmed to occur using GDL, providing good agreement to previous real-time NMR studies (Mok et al., 2003).

**Why are the cellular cytotoxicity assays performed in the absence of serum?**

It was previously shown that BSA can lower the activity of BAMLET however a reason for this was not proposed (Brinkmann et al., 2011). Within Chapter 6 it was shown that increasing the amount of BSA in solution, or simply incubating BAMLET in the presence of BAMLET, results in the eventual inactivation of the complex. This was shown to be due to the high affinity of the protein for OA.

**Is there a difference between using OA from SO when preparing H/BAMLET?**

In all previously proposed methods for the production of HAMLET, whilst OA was the starting material, all bar one study (Zhang et al., 2009) inadvertently used the sodium oleate form, or at the very least, a mixed population of SO and OA. It is known that electrostatic interactions play a part in complex formation (Xie et al., 2013) and this further corroborates this study. The charge on the oleic acid form of the FA is much lower than that of the deprotonated form.
Variations within results or mechanisms of analysis also exist. By zeta potential analysis, emulsions produced at pH 7 are more stable than those produced at pH 11, however diffusion NMR shows that at pH 7, the OA signal does not decay in concert with the protein moiety. Diffusion NMR data recently published contradicts data obtained within this study (Makabe et al., 2013), however it is our opinion that this is due to differences in interpretation of the spectra. Structural analysis of all BAMLET samples produced within the remit of this study show a distinct presence of upfield structure showing that while in complex, α-LA maintains a native-like structure (Pettersson-Kastberg et al., 2009).

The general hypothesis of FA toxicity is they induce necrosis in cells (Zhu et al., 2005), and that in BAMLET with increasing sodium oleate levels there was a trend towards necrosis over apoptosis (Lišková 2011). However within the current study, at the concentrations tested even fatty acid alone induced apoptosis over necrosis.

**Future Considerations**

In order for the field to continue to expand, standardization of methods of production of HAMLET needs to occur. The chromatographic method is well regarded and well investigated thus should feature in publications as a reference complex if needed. However much variation exists in heating parameters – there should be a well-defined protocol outlining optimum temperature, incubation time, pH of reaction and concentrations.

Concluding the general hypothesis, answers were given to the questions posed by Svensson et al., 2003, and Barbana et al., 2006, – a complex is formed in vitro, however under the tested in vivo conditions, no bioactivity was found.

Irish comedian and science enthusiast, Dara O'Briain is famously quoted as saying:

"People complain that science doesn’t know everything. Science knows it doesn’t know everything; otherwise it’d stop".
Hence, from the current study the following questions have been asked:

- Can bioactive peptides be formed during the \textit{in vivo} gastric digestion of \( \alpha \)-LA?
- How significant is the finding of the presence of EDTA in some HAMLET preparations, and what implications does this have to the ion channel activation in cancer/bacterial cells by HAMLET?
- Does the importance of the stability of HAMLET in the presence of BSA merit further study?
- In light of its potent sensitising agent for antibacterial activity, does HAMLET exhibit the same selectivity against health beneficial bacteria?

Advances have been made within the field with the current research. A definitive answer to the question “does the starting form of the fatty acid affect complex production” was provided. \textit{In vivo} data was obtained for the digestion of proteins, along with the first recorded use of capsule endoscopy to visualize digestion of protein. New techniques have been introduced into the study, such as zeta potential, and methods that were already used extensively, such as NMR, have been shown to have new applications in the study of HAMLET and related complexes.
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Appendices
Appendix 1

List of HAMLET-related publications

A comprehensive overview of publications relating to HAMLET

Alphabeticised by corresponding author

Including subsection of publications relating to \textit{in vivo} clinical studies of HAMLET
Professor Catherina Svanborg and Associates


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**Fast, J and Linse, S**


Halskau, O


Hofman, P


Jaggi, R


Marino, G

Mok, K. H.


Zhivotovsky, B


Bahrim, G


Brinkmann, C


Brodkorb, A


De Laureto, P


Demura, M

Ding, W


Fontana, A


Håkansson, A


Holmsen, H


Jaattela, M


Kanamaru, Y


Kehoe, J

APPENDICES

Kuwajima, K


Liang, Y


Moore, R

Morozova-Roche, L


Niasari-Naslaji, A


Otzen, D

Permyakov, E


Petersen, J


Qasba, P


Ren, F


Wehbi, Z

*In vivo* studies incorporating HAMLET and related complexes

**Bladder Cancer**


**Glioblastoma**


**Human Papillomavirus**

Colon Cancer


Bacterial Strains

Appendix 2

CD containing video files of the \textit{in vivo} digestion of $\alpha$-LA referenced in Chapter 4
Video clip 1
Entire video clip of recording from ingestion of camera to gastric transit. Feed 1 enters the stomach with the camera. The video condensed into 40 minutes; actual gastric transit / recording time was 4 hours. A time of 1 minute in the video corresponds to approximately 6 minutes of transit.

Moments of note:
- 10 seconds: gastric acid secretion from gastric mucosa (characterised by precipitation of protein upon acidification)
- 3 min, 30 seconds: gastric mucosa mised with protein stuck on viewport of camera
- 7 min: pH probe with protein material caught in gastric mucosa
- 8 min: water consumed and camera moves location to antrum of stomach
- 8 min, 30 seconds: waves of peristasis seen
- 13 min, 30 seconds: reflux of bile into stomach
- 14 min: feed two enters the stomach
- 20 min: camera passes pyloric sphincter and duodenal contents can be seen

Video clip 2
External peristalisis imaging

Video clip 3
Gastric acid secretion from the wall of the stomach inducing a colour change in the mixture as characterised by white streaks in the solution

Video clip 4
Feed entering stomach and intragastric mixing

Video clip 5
Visualisation of the pH probe

Video clip 6
Peristalisis occuring in the stomach
Video clip 7
Second drink entering stomach and precipitation occurring

Video clip 8
Camera enters small intestine
Appendix 3

Publications (incl. Supplementary Information):

