Irish Chemical News

The Journal of the Institute of Chemistry of Ireland
Issue No. 3, July, 2016

Feature Articles:-

Polymorphic Transformations

Optical Micrograph of a metastable Form II piracetam.

Navigating the Challenges of Method Validation – with a little help from Eurachem.

Characterisation of Biopharmaceuticals

Irish Chemical News

The Institute of Chemistry of Ireland

PO Box 9322, Cardiff Lane, Dublin 2

www.instituteofchemistry.org

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The Professional Body for Chemists in Ireland

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Note: Opinions expressed in this Journal are those of the authors and not necessarily those of the Institute.

A Message from the President

Dear Fellows, Members, Graduates and Associates,

Congratulations to our Editor, Pat Hobbs, for publishing 3 issues of 'Irish Chemical News' in the space of six months! In his February Editorial, Pat wrote that he hoped to publish at least four issues in 2016. A quarterly publication seemed an ambitious goal at the time. However, there is now a real possibility that we may see five or six issues during the course of this year.

Since the April issue was published, a number of Institute events have been held. Professor Kieran Hodnett of UL gave the Boyle-Higgins award lecture on April 14th, at DIT Kevin Street. His lecture, entitled: 'Polymorphic Transformations in Pharmaceutical Compounds' was well received. Following the lecture, Professor Hodnett was presented with the Boyle-Higgins gold medal. To make its content more widely available, the Professor has kindly provided the text of his talk, which is published in the current issue of 'Irish Chemical News'.

On the same date, medals were presented to four candidates who tied for top place in the 2015 Leaving Certificate Honours Chemistry examination. They are Hayley Blair (High School Rathgar), Eimear Conroy (Loreto, Kilkenny), Sean Kavanagh (St. Vincent's, Castleknock) and Eleanor Windle (Dominican College, Muckross Park, Donybrook.) All four are now attending 3rd level colleges in Dublin, where they are taking science, or science related courses. We wish them well in their future careers. In fact, in 2015, a total of six candidates obtained highest marks in the L.C. Chemistry paper. The remaining two candidates, (Vera O'Riordan and Sinead McCarthy) are from Cork and are currently studying at UCC. They were presented with their medals at the close of the Irish Universities Chemistry Research Colloquium by Institute President Margaret Franklin at UCC on Friday, June 24th. After the medal presentations on April 14th, everyone enjoyed a wine and finger food reception, followed by the AGM. All of the current officers and ordinary members of Council were re-elected, including myself, so I am now into the second year of my term of office and look forward to working with Council during the coming months.

We are delighted that the Eurachem Analytical Measurement Competition is back again, after a lapse of one year. It took place on April 15th. Thanks and congratulations to Athlone Institute of Technology for organizing a very successful competition. Congratulation to the winning team from Limerick Institute of Technology, and to DCU and UCD who both took runner up prizes. We look forward to the judges' report, which will be published in due course.

The Annual Congress was held in Galway, on May 27th hosted by GMIT. The theme was 'Chemistry and Society' and included speakers from academia as well as industry who covered a varied range of topics, illustrating the importance of chemistry in our modern world. Thanks to GMIT for organizing the Congress and to our sponsors, Waters, Labplan and Mason for their generous support.

We hope to publish some of the Congress proceedings in future issues of ICN.

Margaret Franklin FICI, President. June 2016

Again we thank our Sponsors:-

Editorial

This is the 3rd Issue of ICN and I am well on the way to reach the target of 4 Issues in 2016 and I expect to exceed the target this year. There are 3 great papers. One from our Gold Medal Winner 2016 Prof. Kieran Hodnett at SSPC, UL on polymorphic transformations. The second big paper is on the characterisation of biopharmaceuticals, a most important topic given the shift in the pharmaceutical industry from small molecule APIs to complex large biomolecules. There is a report on another important topic "Navigating the Challenges of Method Validation" from Eurachem of relevance to anyone involved in analytical chemistry. Finally the Obituary for Professor Richard Butler NUI Galway whom I had the pleasure of meet on a couple of occasions.

There are two very important Conferences happening over the summer and I urge you to support one or both. The first is the 31st International Symposium on Chromatography at UCC in late August. The second is 6th EuCheMS Chemistry Congress in the ancient Moorish city of Seville, Spain, in Mid-September. This is where all the chemical societies in Europe and further afield meet to present high level chemistry topics.

The EuCheMS Congress is a biannual event and the next one will be in Liverpool in 2018. EuCheMS have invited member societies to submit a bid to host the 2020 Congress. The Institute of Chemistry of Ireland, I am happy to say are in the final stages of preparing a bid document to submit by July 8th for the 2020 Congress. This will be a major achievement if we win as it will attract 2500 – 3000 chemists to Dublin. The venue will be the Convention Centre on Dublin's River Liffey which is ideal for this size of conference. We are bidding against 6 other countries. The Chair of the Local Organising Committee is Professor Thorri Gunnlaugsson PhD, FTCD, MRIA, TCD and Chair of the Scientific Committee is Professor David A Leigh FRS FRSE FRSC MAE, University of Manchester. This bid is supported by state institutions and the biopharmachemical industry and state grants are available to us. A copy of the bid document will be available for members to download after it is completed.

Have a great summer and enjoy any chemistry conferences or symposiums you attend. I would like to receive reports on these events for publication. Papers or articles on chemistry topics are most welcome.

You can send these to The Editor at:-

mailto:info@instituteofchemistry.org

Patrick Hobbs MSc, FICI, CChem, CSci, MRSC.

Editor ICN,

Immediate Past President.

Boyle Higgins Lecture 2016

Polymorphic Transformations in Pharmaceutical Compounds

B K Hodnett

Synthesis and Solid State Pharmaceutical Centre
Department of Chemical and Environmental Sciences
Materials and Surface Science Institute
University of Limerick

Professor Kieran Hodnett joined the University of Limerick (NIHE) in 1984 and has been Professor of Physical Chemistry since 1996. A focus of his group's work in the last number of years has been on understanding the mechanism at the molecular level whereby solution mediated polymorphic transformations occur. This work includes investigations of dissolution, nucleation and growth of pharmaceutical solids and their interactions with solvent molecules. By investigating a number of these transformations, by defining the precise thermodynamic and kinetic factors which trigger the transformations and by combining in situ methods with a molecular modelling approach, his group has arrived at an understanding of dissolution of a host crystal and the nucleation of a new polymorph in close contact and sometimes in epitaxial relationship with a host polymorph. Professor Hodnett has published over 120 Journal articles and one book. He has supervised the PhD work of over 30 students, his work has been cited over 3000 times and he has a h-index of 32. Significant leadership roles have include Director of the Solid State Pharmaceuticals Cluster (2007-2013) and the Synthesis and Solid State Pharmaceutical Centre (2013-2019), Dean Faculty of Science and Engineering, University of Limerick (2007-2013), Leader of the Bernal Project, University of Limerick (2009-2013), Leader of the Rusal Aughinish Alumina- University of Limerick Research Collaboration (2002-2007) and Founding Director of the Materials and Surface Science Institute, University of Limerick (1998-2004) Professor Hodnett has led research projects which have attracted over 100 million Euro in competitive funding since 2000 and was awarded the Boyle Higgins Medal of the Institute of Chemistry of Ireland in 2016.

Abstract

This paper is an extract from the Boyle Higgins Lecture 2016, presented at the Dublin Institute of Technology on April 14th. The structural origin of polymorphism in molecular solids is introduced, including the role of hydrogen bonding and van der Waal's interactions. The general features of solution mediated polymorphic transformations is described and a rational explanation offered for the emphatically observed Ostwald's Rule of Stages based on the interplay of kinetic and thermodynamic factors. The mechanism of solution mediated polymorphic transformations in Piracetam, Glutamic Acid, Carbamazepine and Sulphathiazole is described. In each case, the polymorphic transformation is facilitated through nucleation of the stable polymorph on the dissolving surface of the metastable polymorph. In one example, a clear epitaxy could be identified between a specific cleavage plane on the metastable polymorph and a plane of identical structure on the nucleating stable phase.

Introduction.

Polymorphism is a well-recognized phenomenon whereby a pure chemical compound may exist in two or more structural orientations in the solid state, each polymorph displaying different physical characteristics. Polymorphs display individual physical properties such as density, melting point and solubility. Polymorph control is vitally important to the manufacture of chemicals, in particular, pharmaceuticals. Production of an unwanted polymorph will give a product that most likely will not satisfy the intended purpose or processing characteristics required [1-5].

The structural origin of polymorphism is molecular crystals.

The thermodynamic driving forces for polymorph formation is often associated with the development of a network of weak interactions between molecules. Two types of polymorphism are identified in molecular solids (as opposed to ionic solids). These are conformational polymorphism which arise due to differing conformers of the same molecule and packing polymorphism which arise from differing packing of the same conformer inside a unit cell. Generally in molecular solids, polymorphs are based on intermolecular hydrogen bonding and van der Waals interactions. The molecular structure of Stavudine is shown in Figure 1. This molecule can dimerize through a number of different hydrogen bonding motifs, as shown in Figure 2. Each dimer is developed through a different synthon and can lead to different packing polymorphs in the solid state [6]. Nomenclature in this area is not well regulated; sometimes we refer to alpha and beta, sometimes to Form A or B but most often as Form I, II, III, IV, etc.

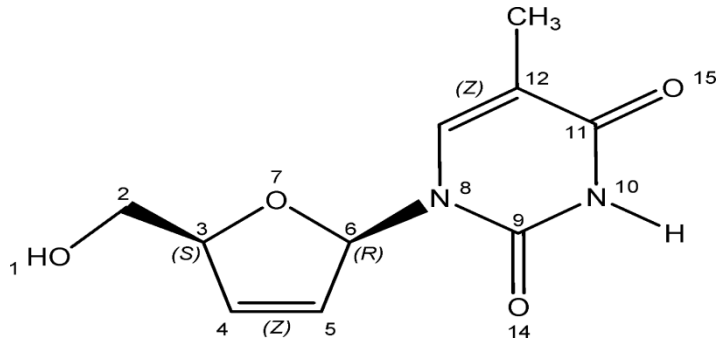


Figure 1. The molecular structure of Stavudine

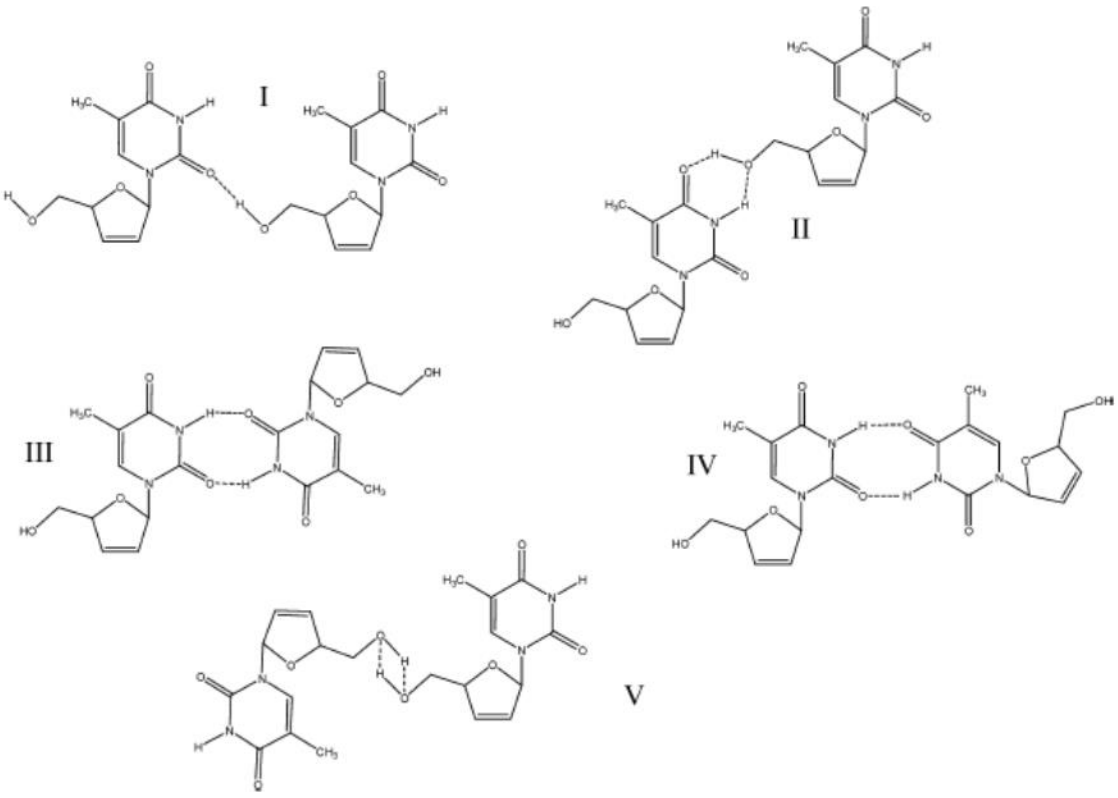


Figure 2. The origin of polymorphism in Stavudine

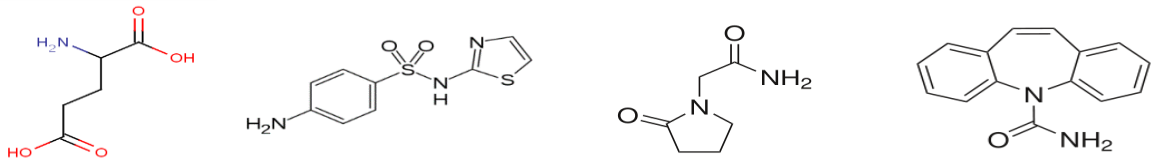
Knowledge of the possible transformations in a polymorphic system is essential when designing a process to isolate a desired polymorphic form. Isolation of a metastable polymorph will require operating conditions which prevent its transformation into the thermodynamically most stable form, whereas isolation of the stable form will require operating conditions which ensure the transformation has gone to completion [7-10]. The

ability of individual polymorphs to undergo polymorphic transformations and interchange polymorphic form is a vast area which is very much dependent on the nature of the compound under study.

Generally there are two types of polymorphic transformations:

1. Solid state transformations wherein the crystals of a metastable forms change into the stable form with the entire process occurring in the solid state [11] and
2. Solution mediated polymorphic transformations (SMPT) whereby the metastable form dissolves and, simultaneously, the stable form grows from solution [12].

This paper will examine SMPTs in Glutamic Acid, Sulphathiazole, Piracetam and Carbamazepine, whose molecular structures are shown in Scheme 1.



Glutamic Acid Sulphathiazole Piracetam Carbamazepine

Scheme 1.

Case Studies in Solution Mediated Polymorphic Transformations: Piracetam.

There are five known polymorphs of Piracetam, as summarised in Table 1.

Table 1. Properties of the five polymorphs of Piracetam[13]

New Name proposed	FI	FII	FIII	FIV	FV
Polymorph	I	II	III	IV	V
space group	$P2_1/n$	$P\bar{1}$	$P2_1/n$	$P2_1/c$	$P\bar{1}$
a (Å)	6.747	6.403	6.525	8.9537	6.3903
b (Å)	13.418	6.618	6.440	5.4541	6.2932
c (Å)	8.090	8.556	16.463	13.610	8.6450
Beta (°)	99.01	102.39	92.19	104.93	113.680
Z	4	2	4	4	2
Cell Volume ($Å^3$)	723.361	348.508	691.286	642.199	314.262
Symmetry Cell Setting	Monoclinic	Triclinic	Monoclinic	Monoclinic	n/a
D ($g.cm^{-3}$)	1.306	1.351	1.371	1.47	1.502
Pressure	Ambient	Ambient	Ambient	0.4 GPa	0.9 GPa
CCDC Ref Code [5]	BISMEV03	BISMEV	BISMEV01	BISMEV04	BISMEV08

The SEM images in Figure 3 show two of the polymorphs of Piracetam, namely the metastable FII and the thermodynamically stable FIII. Differences in particle shape do not always occur in polymorphs, but when it does, it is a useful method of identifying the individual forms.

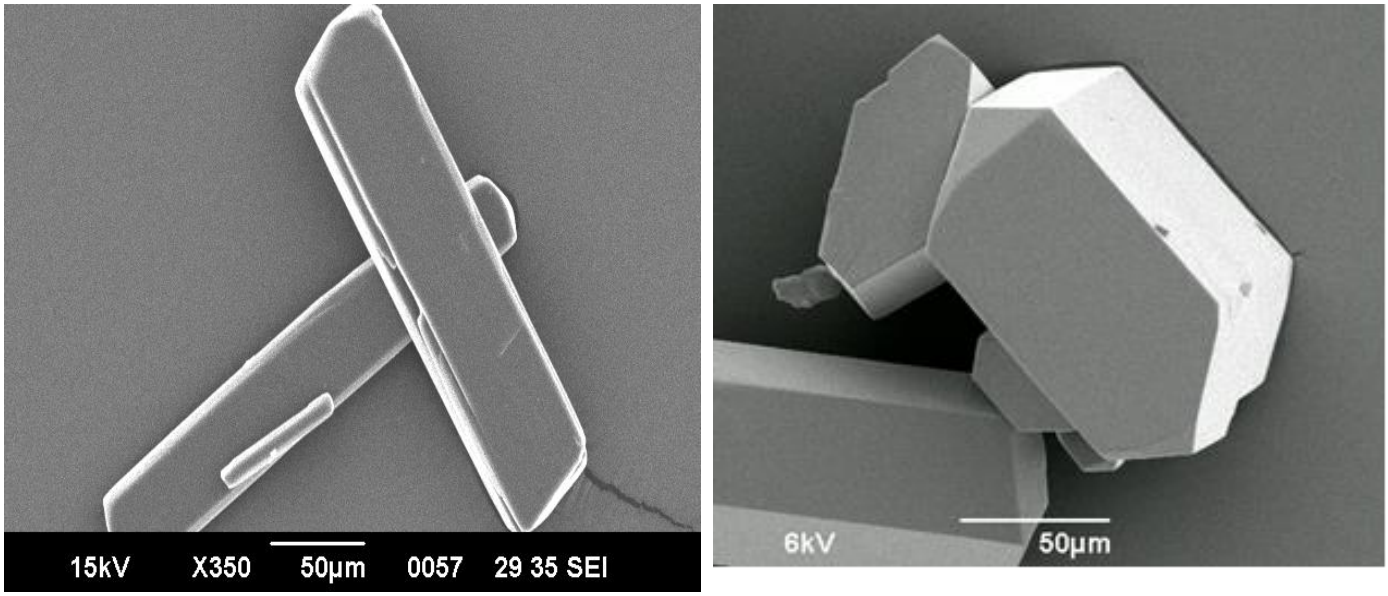


Figure 3. Metastable FII Piracetam (left) and the Stable FIII (right)

These two polymorphs are highlighted because they occur most often during crystallization from solution at and close to room temperature. Central to our understanding of crystallization and polymorphic transformations is the role of solubility and supersaturation. Figure 4 presents the equilibrium solubilites of FII and FIII piracetam. The more stable polymorph displays the lowest solubility in all solvents, and in this case, FIII is the more stable polymorph over the entire range of temperatures explored. This system is said to be monotropic as opposed to enantiotropic, which is characterized by solubility curves which cross over [14].

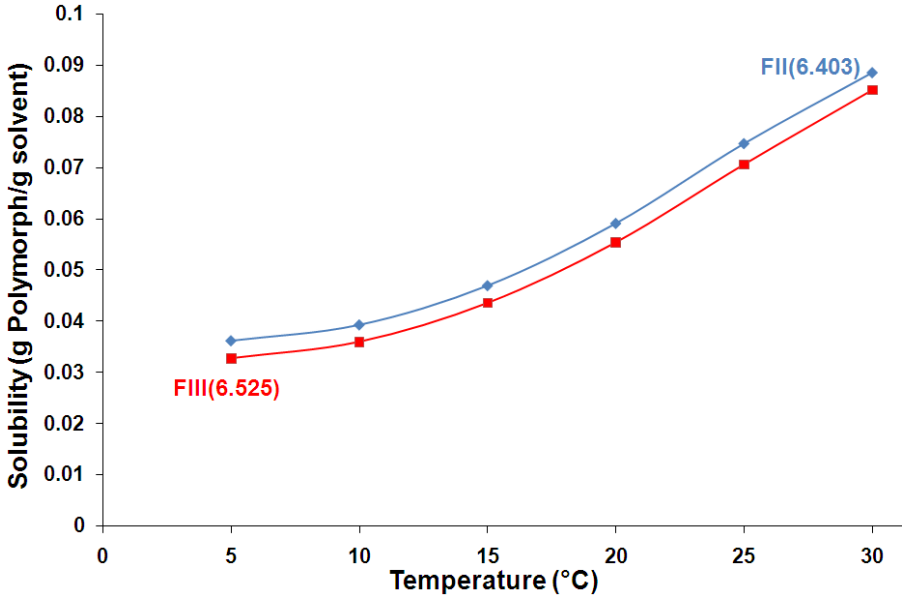


Figure 4. Solubility of FII and FIII Piracetam in Isobutanol:Water 95:5 (v/v) [15,16]

During SMPT in Piracetam, FII changes into FIII and the change in crystalline form is accompanied by a reduction in the measured solubility from the equilibrium value for FII to the equilibrium value for FIII. (Figure 5) [9].

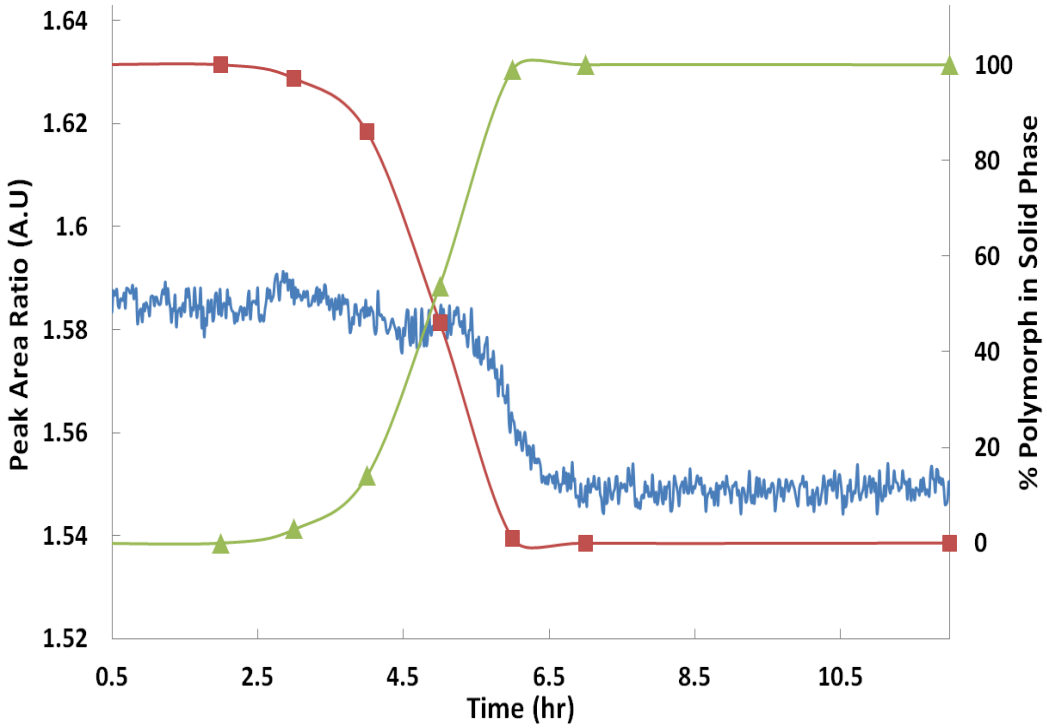


Figure 5. Concentration-Time profile and polymorphic composition during the transformation of FII Piracetam to FIII at 50 ˚C in ethanol: Blue Concentration of Piracetam in Solution; Percentage FII (■); Percentage FIII (▲).

Figure 6 presents an image of a Piracetam cluster in mid-transformation. The large roughened crystal is a dissolving metastable FII crystal (generally dissolving crystals roughen) whereas the small smooth faceted crystal on the surface of the dissolving crystal with nicely defined inter-facet angles (growing crystals tend to appear very smooth) is the developing FIII crystal.

Figure 6. Optical Micrograph of a metastable Form II piracetam (rough dissolving crystal) undergoing a solution mediated polymorphic transformation to Form III (smooth growing crystal) in methanol at 25 ˚C.

Continued ….p14

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METTLER TOLEDO

Compounds displaying polymorphism exhibit a stability hierarchy whereby one polymorph is the preferred, or stable, polymorph for a given set of conditions. The stable polymorph represents the lowest possible free energy state for the system and will always be the polymorph with the lowest solubility in any solvent. Other polymorphs are termed metastable and will attempt to transform to the stable form, if possible. This was first systematically described by Ostwald in his 1897 "Rule of Stages" [17]. Our understanding is that Ostwald's Rule is a manifestation of the interplay of kinetic and thermodynamic factors during polymorphic transformations.

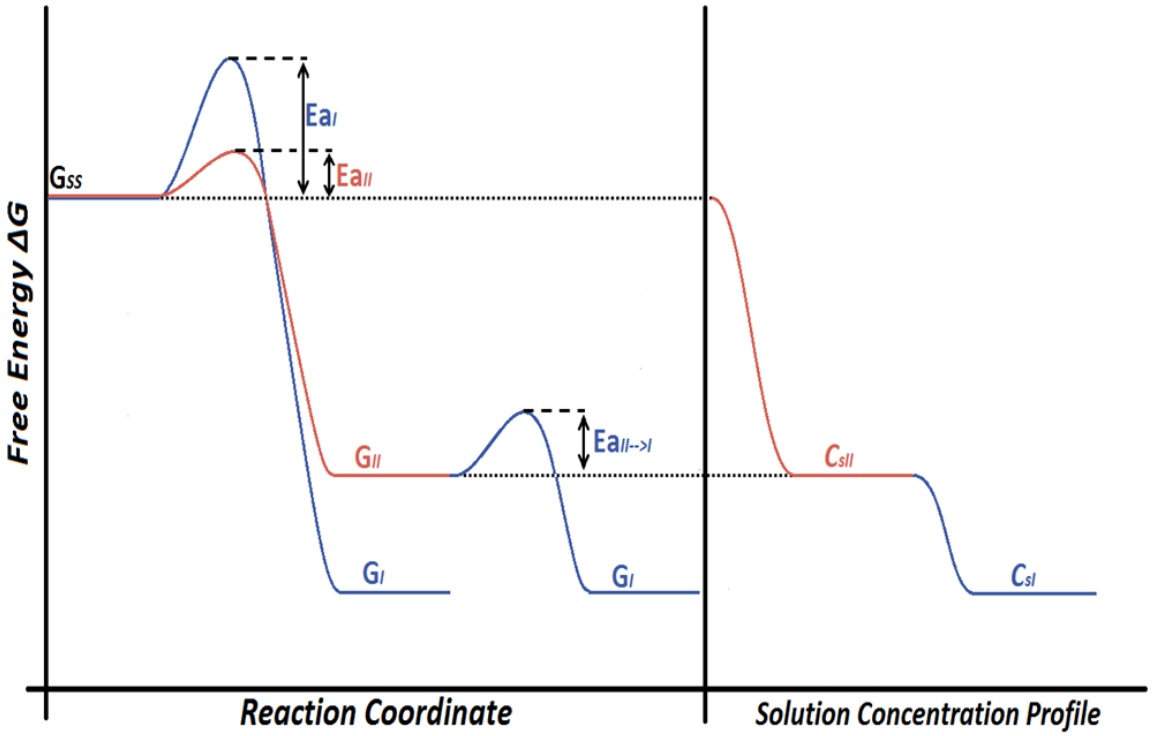


Figure 7 Interplay of thermodynamic and kinetics factors which underlie Ostwald's Rule of Stages

The Gibbs Free Energy (G) of the supersaturated state is represented by any point above the solubility curves shown in Figure 7. The higher the supersaturation the higher the Free Energy. This is an unstable state because the thermodynamics will drive the system to its lowest possible Gibbs Free Energy. Consider a state where the starting Gibbs Free Energy of the supersaturated state is represented as the highest energy state on the left hand side of Figure 7 and is plotted on the same scale as solubility on the right hand side. During a polymorphic crystallization starting with a state which is supersaturated with respect to two polymorphs, the system can transform into either of the two polymorphs. The polymorph which appears first will be determined by the activation energy associated with crystallization into each form. The metastable form crystallizes only in circumstances where its crystallization has a lower activation energy than crystallization directly into the stable form. In the scheme shown, the red route is followed leading to the metastable form. The blue route essentially disappears in a kinetic sense. The system then relaxes to the Gibbs Free Energy associated with the metastable form characterized by the equilibrium solubility of the metastable form. At this stage, the solution is still supersaturated with respect to the stable form. This is the driving force for the SMPT and can occur over any timescale depending on the polymorphs in question.

An important aspect of polymorphic transformation is the timescale involved. Essentially, this is a measure of the timescale during which the metastable form can survive in contact with the solution before it starts to be converted into the stable form. In the case of Piracetam, solution concentration was found to be an important factor, as shown in Figure 8. Solution concentrations can be varied by solvent or temperature choice. Clearly, the higher the solution concentration the faster the transformation from the metastable to the stable form [18].

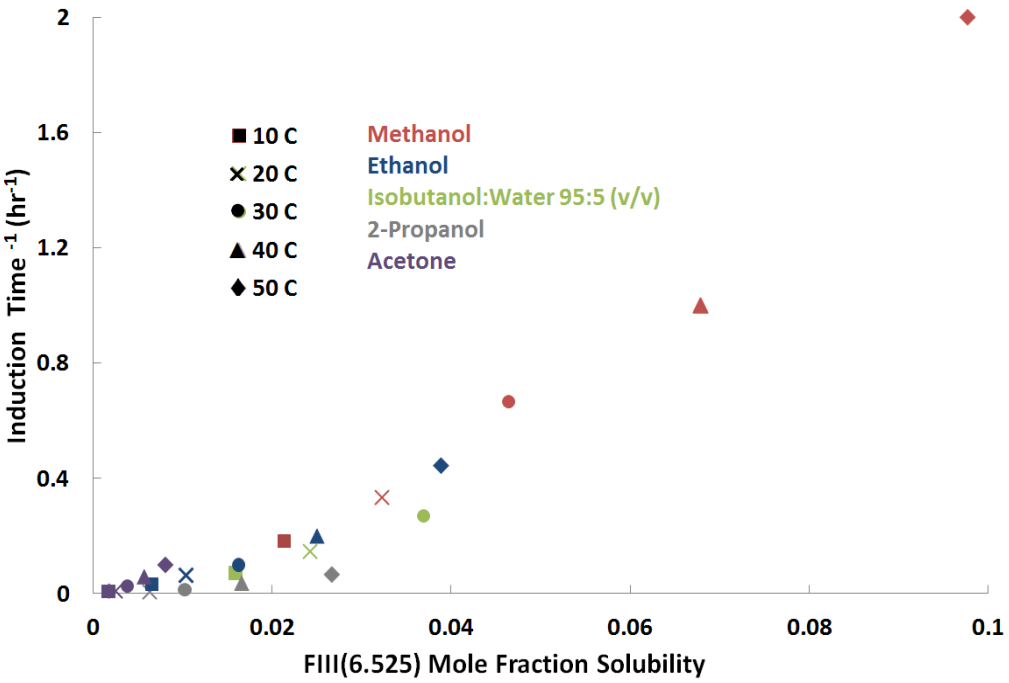


Figure 8. A plot of the inverse of the induction time for the transformation against the solubility of FIII Piracetam in five solvents and five temperatures indicated [18].

The Mechanism of Solution Mediated Polymorphic Transformations

A general finding of our research into the mechanism of SMPTs is that the metastable polymorph starts to dissolve, and in so doing, it starts to roughen. The next step involves nucleation of the stable polymorph on the roughened surface of the metastable phase. Some images of these transformations are shown below. Figure 9 shows the stable beta polymorph of Glutamic acid (the platelet like particles) developing from metastable alpha polymorph [19, 20]. A further example is the nucleation of Form III carbamazepine on the surface of needle-like FI particles (Figure 10) [13, 21]. The stable polymorph first nucleates as tiny features on the faceted surface of the needle-like metastable polymorph and then grown. The order of polymorph stability has little to do with particle shape.

The exact mechanism of interaction, especially for the nucleation step, can sometimes be related to the roughness of the meta stable form, perhaps a zone at the surface in which supersaturation can build up atypically and induce nucleation, sometimes to a function group interaction, namely a favourable interaction between a functional group on the surface of the metastable polymorph and the nucleating stable form and very occasionally an epitaxial interaction between the metastable and the stable polymorphs [22-25]. Figure 11 shows the development FII sulphathiazole from the metastable FV. Clearly, there is no evidence for epitaxy in this case. FII sulphathiazole is more typically prepared by crystallization from ethanol. When the resulting crystals are allowed in contact with the alcohol solution the middle part of each crystal roughens; an indication of dissolution. Raman point analysis indicated that this middle layer was FIV sulphathiazole, whereas the non-dissolving outer layers were FII. In this case, we postulated that the middle layer (Form IV) develops first as a fairly thin platelet. The stable FII then nucleates and grows on both basal planes of the platelet. The shape of the FII crystals which form are determined by the shape of the basal planes of Form IV, which acts as a kind of template. At first sight, the crystals appear to be FII, but the central layer is revealed when it starts to slowly dissolve on prolonged contact with the solution. Modelling of the two polymorphs revealed that there is a perfect match between the (100) face of FII and the (10-1) face of Form IV, as shown in Figure [24, 25].

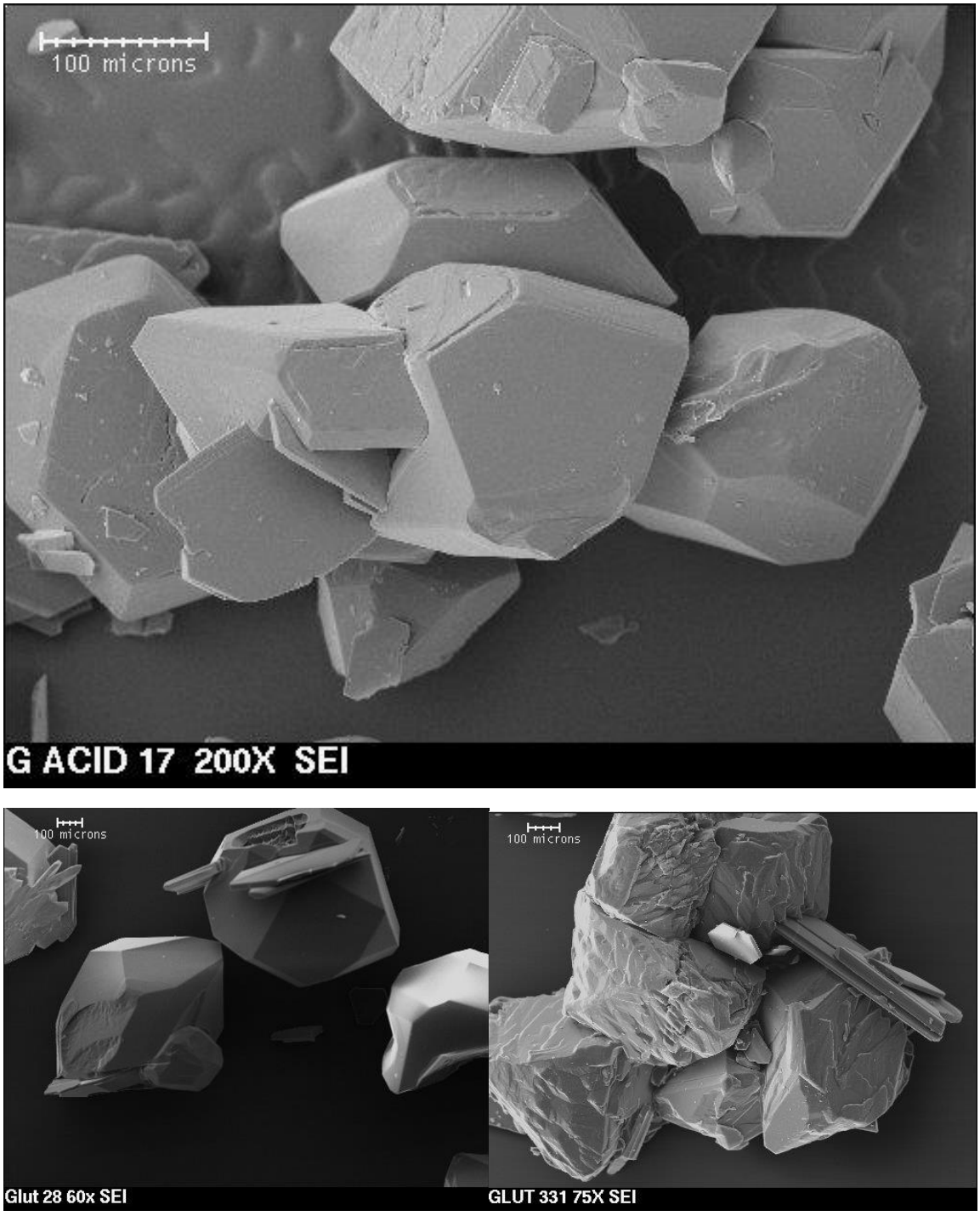


Figure 9. Polymorphic Transformations in Glutamic Acid: Electron micrographs of β-crystals growing *out of* the surface of α-L-Glutamic acid [19].

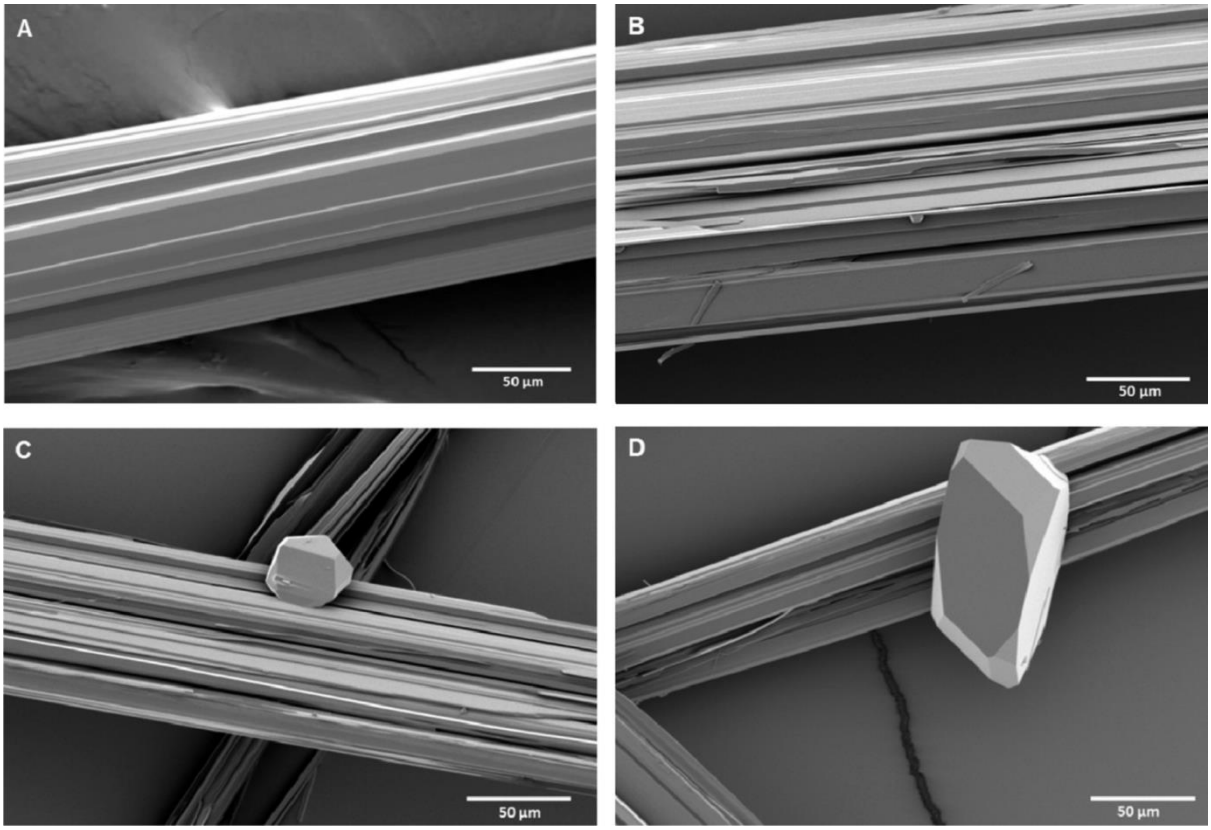


Figure 10: Polymorphic Transformation in Carbamazepine: SEM images of the typical surfaces of FI crystals imaged before the transformation (A) and the surface of samples of FI crystals taken during the transformation (B−D). The nucleation and growth of the blocky FIII crystals is evident on the dissolved surface of the FI crystals [13,21].

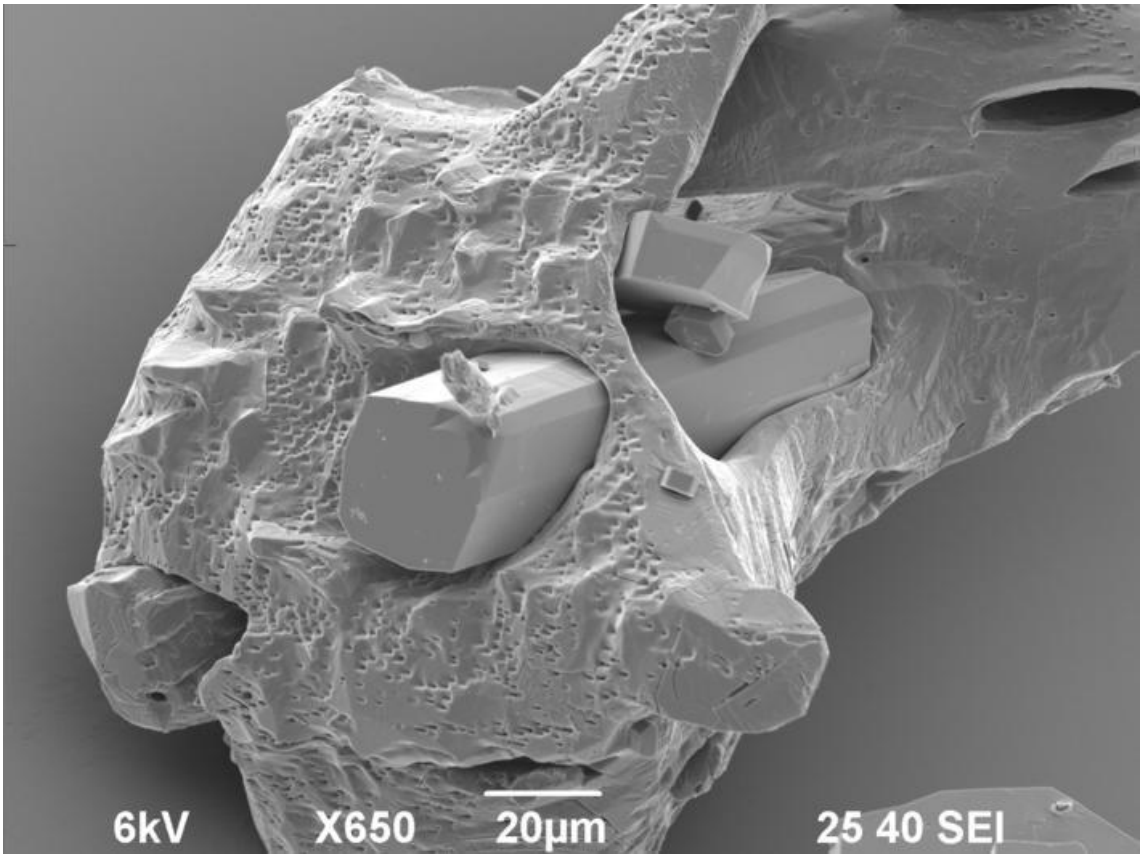


Figure 11. Polymorphic Transformation in Sulphathiazole: SEM images of the transformation of FV in ethanol to FII and FIV at 10 °C showing evidence for the growth into the metastable FV and multiple orientation effects. [22,23].

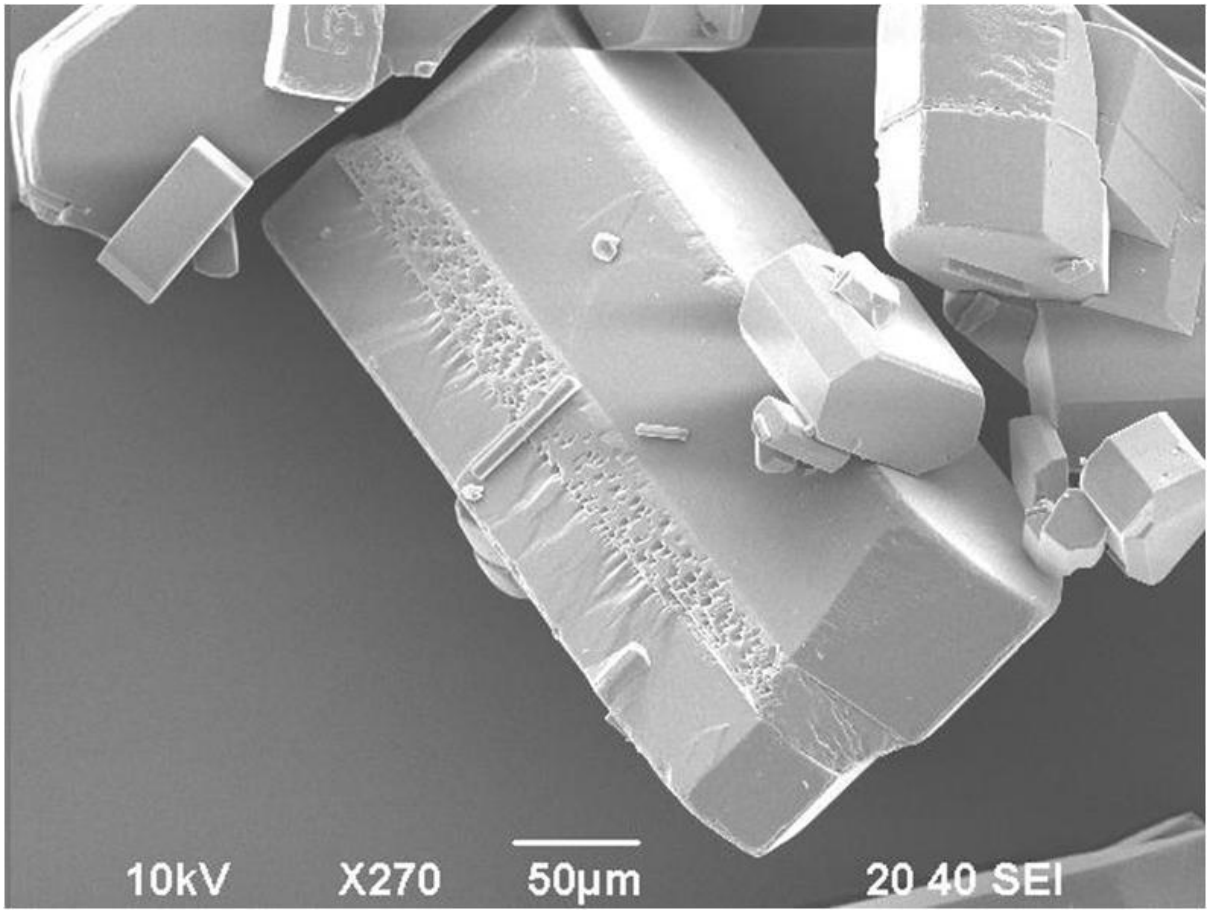


Figure 12. Polymorphic Transformation in Sulphathiazole: Scanning Electron Micrographs of sulfathiazole crystals exhibiting the characteristic middle layer of dissolving FIV crystal onto which has grown epitaxially layers of FII polymorph. [23,24].

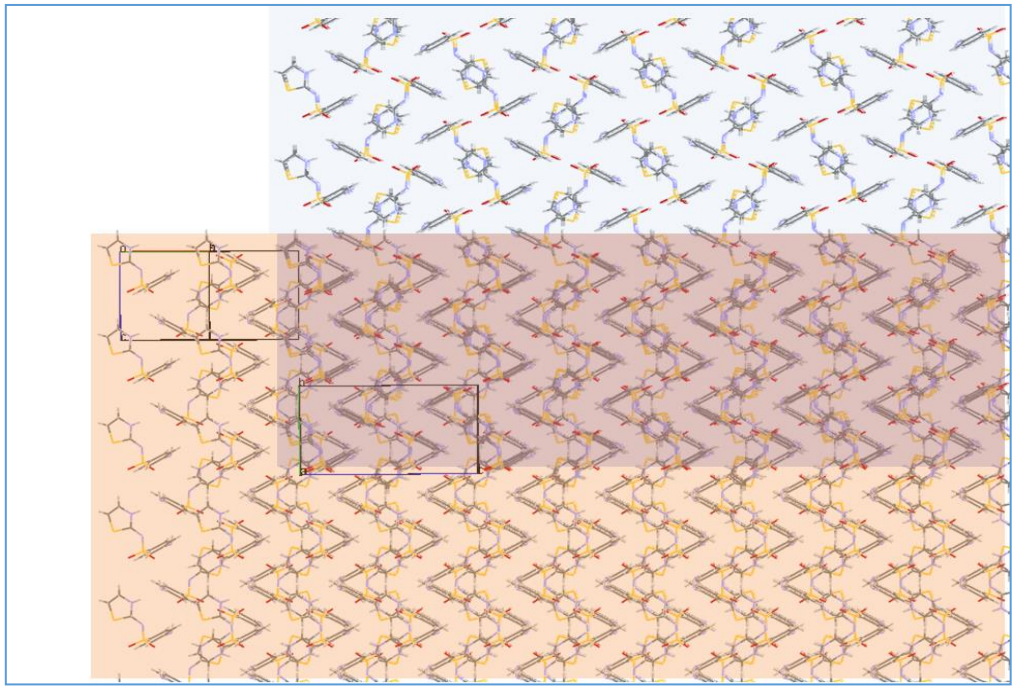


Figure 13. Representation of the molecular packing in FII and FIV Sulphathiazole viewed from the proposed faces in contact showing how the structures align. The (100) face in FII is highlighted in red and (1-10) face in FIV is highlighted in blue. The overlapping zone is highlighted in purple [24, 25].

Conclusions

The mechanism of solution mediated polymorphic transformations in Piracetam, Glutamic Acid, Carbamazepine and Sulphathiazole is described. In each case, the polymorphic transformation is facilitated through nucleation of the stable polymorph on the dissolving surface of the metastable polymorph. In one example, a clear epitaxy could be identified between a specific cleavage plane on the metastable polymorph and a plane of identical structure on the nucleating stable phase. The role of surfaces in facilitating solution mediated polymorphic transformations can be viewed in terms of a role for surface interactions reducing the activation energy for transformation from the metastable to the stable polymorph. It has not always been possible to identify the exact mechanism whereby surface nucleation proceeds, except in the case of epitaxy. A knowledge of nucleation times (induction times), which determine the point at which transformation starts, and transformation times, the point at which the transformation is complete, is important in terms of our ability to manufacture pharmaceutical compounds as pure polymorphs.

Acknowledgements: This work has been possible through successive awards from Science Foundation Ireland to the Solid State Pharmaceutical Cluster (2007-2013, Grant no 07/SRC/B1158) and to the Synthesis and Solid State Pharmaceutical Centre (2013- present Grant no 12RC/2275). Additional funding to students was awarded through the Irish Research Council for Science, Engineering and Technology, IRCSET.

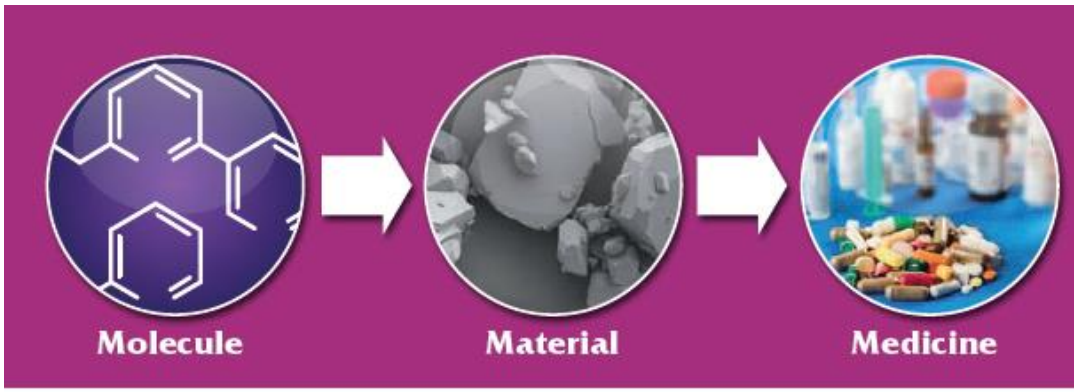
Overview of the Synthesis and Solid State Pharmaceutical Centre

The Synthesis and Solid State Pharmaceutical Centre (SSPC), a Global Hub of Pharmaceutical Process Innovation and Advanced Manufacturing, funded by Science Foundation Ireland (SFI) and industry, is a unique collaboration between 24 industry partners, 9 research performing organisations, and 12 international academic collaborators

The SSPC is a €42 million state-industry investment, which supports a globally-leading research team of 38 investigators, 38 post-doctoral researchers and 57 PhD candidates. The SSPC transcends company and academic boundaries and is the largest research collaboration in Ireland, and one of the largest globally within the pharmaceutical area.
The role of the SSPC is to link experienced scientists and engineers in academia and the pharmaceutical industry, to address critical research challenges, and to deliver industry relevant solutions, which result in job growth and retention within the pharmaceutical industry.

From the Molecule to the Medicine

The SSPC leads the way for next generation medicines manufacture. The SSPC research programme spans the entire pharmaceutical production chain from synthesis of the molecule, to the isolation of the material, and the formulation of the medicine.



The SSPC's globally-leading research programme is organised into three interconnecting strands (Molecule, Material, Medicine), which actively reflect the three distinct steps in the manufacture of modern medicines.

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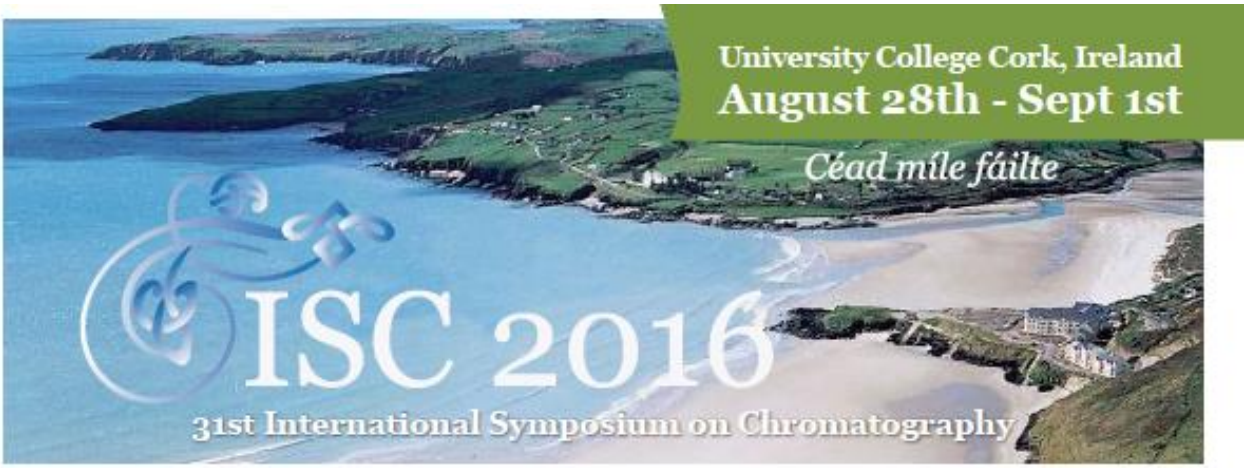

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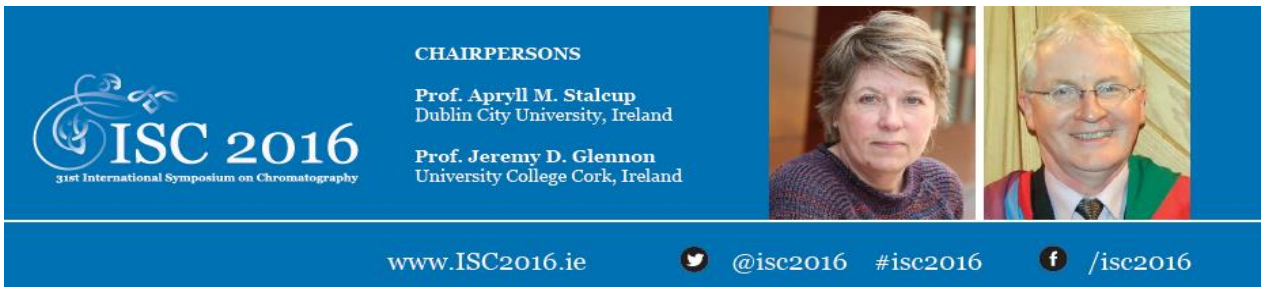
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Navigating the Challenges of Method Validation – with a little help from Eurachem

Validation in Analytical Science – Current Practices and Future Challenges

Eurachem Workshop 9-10 May 2016, Gent, Belgium

Dr. Helen Cantwell, The State Laboratory

As an analytical scientist working in a regulatory laboratory, method validation is a part of my daily working life. In my field of veterinary drug residue analysis, all methods used must be accredited to ISO17025. There is also constant change. New drugs are licensed, permitted limits change or are newly introduced, new matrices must be analysed and new analytical technology is developed. Also, method validation is not a one-off thing. We cannot demonstrate that a newly developed method is fit for purpose, introduce it into routine use and then sit back happy in the knowledge that our work is done. A significant part of method validation involves demonstrating that our method remains fit for purpose while in routine use. Method validation is therefore an ongoing process which requires significant time and resources. It is important then that we, as analytical scientists, ensure that we have the knowledge and expertise to effectively carry out validation studies and to keep abreast of new techniques which may maximise the information we gather from validation experiments while minimising the experiments themselves. It was with this in mind that I became interested in a Eurachem workshop run in May of 2016 entitled Validation in Analytical Science – Current Practices and Future Challenges.

Eurachem is a network of organisations in Europe with members from 32 countries. It aims to establish a system of international traceability for chemical measurements and promotes good quality practices. Technical activity is carried out by a number of working groups which publish the highly respected Eurachem guides which are freely available on the website www.eurachem.org. The working groups also organise international workshops. In May of 2016, the Method Validation Working Group ran a workshop which took place over two days in Gent in Belgium. The workshop consisted of a programme of lectures from international speakers, poster presentations and working group sessions where the workshop participants could discuss features of method validation, challenges or issues faced and ways in which these challenges may be overcome. The Safefood Travelling and Mobility Programme kindly agreed to sponsor my attendance at this workshop so, in May of 2016, I and two of my colleagues headed off to Gent.

Waiting for the presentations to start

The first thing I have to mention is that the facilities were wonderful. The conference room itself and the catering were all top class. The content also was highly applicable. It started with an overview of international guidance in the area of method validation. This presentation, which outlined the development of method validation requirements over the years, was given by Lorens Sibbesen, chair of the Method Validation Working Group. I was surprised to find that the concept of method validation in analytical chemistry, which is now so endemic, is a relatively recent one. Laboratories seeking accreditation before the advent of ISO 17025 worked to EN 45001 *General criteria for the operation of testing laboratories* (1989). This standard stated that, where it was necessary to employ test methods and procedures which are not-standard, that these shall be fully documented. Documented only. It wasn't until the 90s that the term validation started to emerge, initially in a set of harmonised guidelines published by the pharmaceutical industry, the ICH guidelines from the *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*. This also introduced the idea that there were characteristics of an analytical method that a validation procedure should monitor.

Since then the concept of method validation in analytical chemistry has taken a firm hold - and rightly so. ISO 17025 the international standard documenting *General requirements for the competence of testing and calibration laboratories* incorporates a section on validation of methods and requires that the validation demonstrates that analytical methods are fit for purpose. Legislation and guidelines pertaining to method validation in different fields of analysis – food safety, pesticides, pharmaceuticals, water, environment, forensics etc., have been published. While very important for ensuring the quality of analytical results reported, lack of harmonisation in these guidelines and legislation has yielded further challenges which became obvious during some of the working group discussions. The Eurachem Guide *The Fitness for Purpose of Analytical Methods* (available free to download from the website www.Eurachem.org) attempts to combat this by documenting a generic approach to method validation with some references to sector specific guidelines. Lorens finished his presentation by highlighting the ongoing work of the Eurachem Method Validation Working Group. The group will be addressing challenges remaining in the field of method validation. These include validation of multi-parameter and multi-matrix methods, verification of test kits, instrument qualification and setting performance requirements.

Bertil Magnusson from the SP Technical Research Institute of Sweden addressed the topic of setting requirements for a method. Method validation involves demonstrating that our method is fit for purpose. We can only do this if we are clear on what our method is required to do. Our first task, therefore, is to discuss with our client and determine their needs. We then need to study relevant legislation and guidelines. When this is complete we can document our method requirements. These will include: 1) Measurement range – can

our instrumentation cover the entire measurement range? Is there adequate sensitivity and linearity? May samples have to be diluted to bring them within the working range of the instrument? 2) Precision – how will precision be determined? Is a single laboratory validation sufficient or will an inter-laboratory study have to be carried out, for example if a method is being developed to be used as a standard method? 3) Trueness – is there a maximum bias that is acceptable? 4) Measurement uncertainty – is there a maximum uncertainty that is acceptable? When the requirements of the method have been determined and have been clearly documented, then we can assess whether our analytical method is fit for purpose.

With my colleague Paula after a full day at the workshop.

Stephen Ellison of LGC in the UK continued with the theme of planning validation studies and addressed some frequently asked questions: How many performance characteristics should be studied? How many samples and replicates are needed? How can the necessary information be gathered with the minimum amount of work? Unfortunately, there is no single answer to these questions. The question of which performance characteristics to study revolves again around fitness for purpose. Validation of a method which aims to identify an analyte only may require the determination of selectivity/specificity whereas validation of quantitative methods must always incorporate a determination of trueness, precision and linearity. An assessment of detection capability is usually required while ruggedness testing, although usually carried out, is often incorporated into the method development process with only some fields insisting it must be addressed as part of the method validation procedure. The number of experiments, or observations, necessary to determine these characteristics differs in different sectors and the best way to determine this is to study the relevant legislation and guidelines. A statistician's approach - that of using test power, is being documented in the IUPAC guidance document, *Experiments for Single Laboratory Validation of Methods of Analysis: Harmonized Guidelines*, which is currently in draft. The test power is defined as the probability of rejecting the null hypothesis when it is false. The approximate precision, the size of bias required and the confidence

level must all be known or assumed. The number of observations necessary can then be calculated and these observations carried out in whatever combination of experiments and levels is deemed appropriate. However, because of the number of assumptions which must be made, use of test power must be approached cautiously. It is useful for comparing different validation designs.

Efficient experimental design is the way to maximise the information gathered from the minimum amount of work. Firstly, it is possible to determine more than one performance characteristic from a single set of experiments. Legislation published in the area of veterinary drug residue analysis, 2002/657/EC describes a set of three experiments with 7 observations at each of three concentration levels in each experiment. From this single set of experiments precision at three concentration levels, bias at three concentration levels, an assessment of linearity and determination of detection capability can be calculated. Similarly, a factorial design can be used to minimise the number of experiments required to determine the ruggedness of a method.

Sarah de Saeger of the University of Ghent runs an accredited laboratory in an academic environment. Accreditation was initially sought as Sarah's laboratory also carries out drug residue and contaminant testing in food and animal feed for which accreditation is required. The extent of method validation, including proficiency testing, required of an accredited lab may often cause academics to balk due to the high cost in both time and money. What benefit then, if any, does implementing a quality system with stringent validation protocols have in a university laboratory? A primary function of a university is to teach. Carrying out research in such a laboratory will ensure the production of highly trained graduates who are perfectly placed to integrate into the workplace. Another function of a university is to carry out research. The presence of a quality system means that the research will generate reliable results and high quality data. The benefits seen in Sarah's laboratory include an increase in the number of projects funded and publication in journals with high impact factors.

Method validation in accreditation was discussed in a very interesting talk given by a representative of BELAC, the Belgian equivalent of our own Irish National Accreditation Board. ISO 17025 *General requirements for the competence of testing and calibration laboratories* is currently being updated. It is due to come into effect in 2017 and some significant changes are proposed. The concept of verification is being introduced; this will align it with ISO 15189 *Medical laboratories – requirements for quality and competence*. Requirements for sampling will be increased and the role of the laboratory as a conformity assessment body will be addressed. We therefore need to be prepared for changes, and, quite possibly the need to update our method validation procedures.

Workshop attendees – 125 people from 25 different countries.

Along with a poster session and other presentations covering validation in clinical chemistry and of microbiological methods, inter-laboratory validation and the use of both Quality by Design and chemometrics, the workshop included two working group sessions where participants discussed topics relating to method validation. I attended a working group session on the determination of trueness/bias and one on challenges in validation of multi-parameter methods. From participation in these working groups it became clear to me that one of the main challenges in method validation is the lack of harmonisation across sectors. The attendees at the working group sessions were from many areas of analytical science. When determining trueness/bias we follow different guidelines, are bound by different legislation, use differing numbers of observations and have different criteria for acceptance and rejection. In the working group session on validation of multi-parameter methods it was obvious that even basic terminology was different in different fields. The official language of the conference was English and I am a native speaker and yet I found myself having to translate before speaking. I was translating from those terms I, working in veterinary drug residue analysis, use everyday such as maximum residue limit and CCalpha values to those the other participants would understand – permitted limit and level at which a sample is considered non-compliant. We had to clarify our terminology before we could discuss anything relating to multi-parameter methods. Once we did we agreed that the challenges faced included a lack of certified reference materials, a lack of proficiency testing, validation in multiple matrices and the cost and time involved.

Method validation is an essential part of analytical chemistry and will continue to constitute a large part of the workload of the analytical chemist. Development of new technologies, movement towards multi-analyte and multi-matrix methods and changes in legislative and accreditation requirements mean that procedures for method validation must constantly evolve. The Eurachem Method Validation Working Group is an important component of this evolution, functioning as a centre of expertise and assembling and promoting best practice. I have decided to become involved and have recently joined the working group as one of the Irish representatives. I would be happy to raise any concerns or queries Irish scientists may have at the working group meetings so please feel free to contact me.

All of the presentations, posters and reports from the working group sessions are available on the Eurachem website www.eurachem.org.

I gratefully acknowledge the financial sponsorship of SafeFood in attending this workshop.

Helen Cantwell is a chemist working in the Veterinary Toxicology Section of the State Laboratory. Her areas of interest are method development, validation, mass spectrometry and quality management. She is a member of the Eurachem Ireland secretariat and one of the Irish representatives on the Eurachem Method Validation Working Group.

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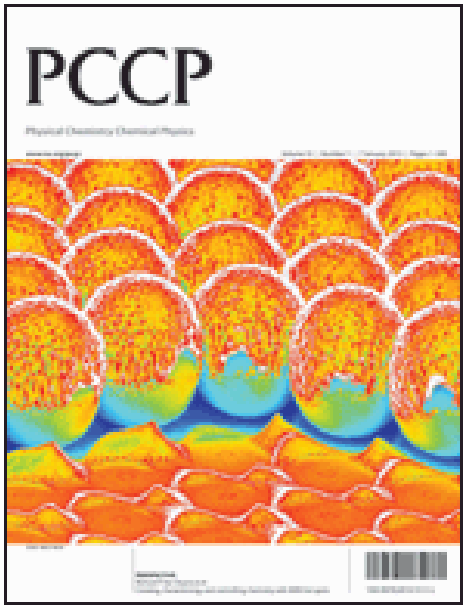

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Obituary: Professor Richard Butler NUI Galway, February 10th, 2016

Richard (Dick) Noel Butler (Emeritus Professor of Chemistry, NUI Galway), who died aged 73 in February of this year, was a leading figure in chemistry in Ireland and had an international reputation for his research.

Dick Butler was born in Dunmanway, in Co Cork, where his family operated the local pharmacy (Medical Hall). He was a boarder at Farranferris College, Cork and played hurling there and also with the Dohenys Club in Dunmanway. He went on to study at University College Cork and obtained his BSc (with first class honours) in chemistry in 1964. He obtained a PhD in Chemistry in 1967 under the direction of the late Professor F. L. Scott, where he developed his interests in mechanistic and heterocyclic chemistry. He won an 1851 Royal Exhibition and used this to go to the University of Leicester, where he studied applications of magnetic resonance spectroscopy (1967-1969) under the direction of the late Professor M. Symons. While in Leicester he attended the 1851 scholars annual reunion in London in 1986 and spent a very long period speaking with Prince Philip about Ireland and the incipient troubles in the North. Prince Philip, who was the patron of the 1851 Exhibitions, had an immense interest in Ireland and its problems. Prof. Butler returned to Ireland in 1970 as the Principal of the new Sligo Regional Technical College and then moved to University College Galway (now NUI Galway) as a lecturer in 1971.

In his teaching in both Irish and English he inspired his first year students and encouraged them to pursue a career in chemistry. His enthusiasm for chemistry was infectious and many were undoubtedly inspired by him. He liked impromptu discussions about particular chemical problems. Those who were struggling were encouraged when he told them ‘that he had not done chemistry at all in school’. He personally gave late evening tutorials for those students several times a week. More than 350 post-graduates passed through the Department during his watch gaining Ph.D.s and M.Sc.s. He had personally overseen the studies of 45 of them. Galway graduates made major contributions to the establishment of the pharmaceutical and chemical industries in Ireland and some diversified into other areas.

He had begun to make his mark as a research chemist publishing original contributions in top ranked journals. These led him to obtaining his higher doctorate (D.Sc.) from the NUI in 1976 and he was subsequently elected to the Royal Irish Academy in 1979, the highest honour that can be given to an academic in Ireland. He was later appointed as the Professor of Chemistry and Head of the Chemistry Department at NUI Galway in 1981, posts which he held until his retirement in 2008.

Under his direction the Department grew considerably both in teaching and in research. He oversaw the purchase and installation of new instrumentation which was a great help to in the research being actively

pursued by the Department staff and their students. He served on many University committees and bodies and was a member of UCGs governing body for two terms.

He was known Worldwide for his contributions in heterocyclic chemistry, especially tetrazoles and latterly the elusive pentazoles and other areas. His versatility was displayed in 2015 when he published his work on the effect of water on organic reactions. In all he had over 200 scientific papers published. He had a lifelong interest in astronomy and was a keen observer of the skies and he regularly lectured on topics such as 'Is there life on Mars? ' Before these lectures he used to modestly introduce himself as not being an expert in astronomy. His most recent lecture on astronomy was on January 4th last.

He was a fellow of both the Institute of Chemistry of Ireland and the Royal Society of Chemistry (UK) and he had served as President of the former for two years. During his tenure of the presidency he worked hard on behalf of the Institute especially in recruiting new members. He was passionate about this and it was said that at various interview boards he always asked the candidates if they were members! The Institute honoured him with their Boyle-Higgins Gold medal in 1998 for his contributions to Chemistry.

He had a great interest in hurling especially Cork hurling and often surprised people with his detailed knowledge of teams and events on and off the pitch. He told one inter-county player who was also studying chemistry at UCG that as long as they knocked out Tipp and Clare that would be great but knocking out Cork would be a different matter!

Dick was a warm and kind person and was always very good to all the chemistry students and staff who passed through the Department during his watch.

He is sadly missed by his loving wife, Jean, sons, Noel, Eoin and Richard, daughters, Eimear, Deirdre, and Tríona and his grandchildren. His passing is deeply regretted by his sisters, Mary and Rosarie, brothers-in-law, sisters-in-law, daughters-in-law, sons-in-law, relatives, neighbours, friends and academic colleagues.

Richard (Dick) Noel Butler: born November 24th, 1942; died February 10th, 2016.

Provided by WJS

Congress 2016

Chemistry and Society

The theme 'Chemistry & Society' was very broad and this was reflected in the great variety of the presentations, not all of which were given by chemists.

The first session provided an insight into some of the research that has been carried out by GMIT staff.

Dr Lisa Ryan, of GMIT, is a dietician and spoke about the benefits of certain phytochemicals in the diet, particularly polyphenols and anthocyanins from brightly-coloured fruit and vegetables.

This was followed by a presentation of some good detective work by Dr Philip White who analysed samples from remote parts of Mayo, in an attempt to find the cause of the decline in the Eel population & found dioxins in the most unlikely places!

Philip has recently moved to GMIT and the work he described was part of a project he had been involved in while employed at the Marine institute.

Then Dr Florian Stefanov (who apologised for the fact that he is a Biomedical Engineer, rather than a chemist) described some of the research being carried out at the GMedTech research centre at GMIT and illustrated how chemistry had helped in solving some of the challenges they faced, for example by providing low Melting Point alloys (e.g. Bismuth/Tin/Indium) and flexible polymers (ABS, or acrylonitrile/butadiene/styrene) for fabricating the models they are using to simulate the cardio-vascular system.

Dr Yvonne Lang, of Sligo IT, described the use of diatoms which she described as 'the jewels of the sea' in environmental monitoring and cleanup.

Dr Andrea Erxleben discussed the importance of being able to control crystallisation in the the production of active pharmaceutical ingredients, to ensure that the desired polymorph is obtained.

The afternoon session consisted of two very different presentations from chemists working in industry. Dr Brendan Feeney of Mylan described the various stages in the development of a pharmaceutical product from the laboratory to the patient.

The final speaker was Dr Hugh Fay from Henkel, who gave an overview of the many different adhesives which are produced by his company and went on to show how society had influenced Henkel to make changes in their processes and products to ensure sustainability and environmental protection.

We are grateful to our sponsors, Labplan, Mason and Waters for their generous financial assistance.

DEVELOPMENT OF SEED COATING AGENT WITH ι-CARRAGEENAN HYDROGEL FOR SUSTAINABLE AGRICULTURE

Maya Hotta, James Kennedy, Clement Higginbotham and Noreen Morris*

**Corresponding author: Department of Life and Physical Sciences, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland. E-mail: nmorris@ait.ie*

Feeding 9.6 Billion People…

- United Nations[1] estimated human population to reach **9.6 billion by the year 2050**
- World food production must increase even more to meet **food demand**
- **Pollution & environmental damage** caused by agriculture is currently a major concern
- Climate change – **drought** also becoming a threat for field crop cultivation
- **Sustainable agriculture** is essential to protect the environment while increasing food production
- **Natural hydrogels**[2,3] are key materials that can promote plant growth in critical conditions while keeping environmentally-safe

Project Aim

This project is based on the concept of applying hydrogel technology for agricultural/horticultural use.

The aim is to film coat seeds (**Fig. 1**) with environmentally friendly hydrogels (**Fig. 2**) made of natural polymers such as ι-carrageenan (**Fig. 3**) that are biodegradable and non-toxic.

Potential benefits include:

- Faster and higher seed germination rates (**Fig. 4**)
- Promote water and soil conservation
- Provide protection of seeds against pests
- Low material cost[4] and safe handling

Fig. 1: Uncoated (A) and film coated (B) durum wheat seeds with natural hydrogel.

Fig. 2: Dry hydrogel (right) swells up (left) by absorbing water.

Fig. 3: ι-Carrageenan (left) extracted from red seaweed (right).

Fig. 4: Grass growing better in the presence of ι-carrageenan hydrogel (right), but less in the pot without the hydrogel (right).

Preparation of Hydrogels and Analytical Tests

1. **Hydrogels were formulated by blending natural polymers**
   - Agar/ι-carrageenan blend (**AC**) hydrogel
2. **Hydrogels were characterised by swelling studies (Fig. 5) in:**
   - 0.1 M NaCl and $CaCl_2$ solutions
   - pH 4, 7 and 10 buffer solutions
   - The weights of dry and swollen gels

   were recorded in set time intervals for up to 168 hrs

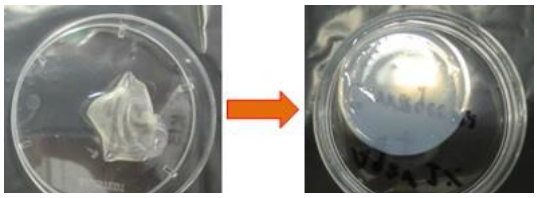

Fig. 5: Example of dry **AC** hydrogel (left) swelling in water (right) after 24 hrs.

Results

Swelling studies of AC hydrogels in Saline solutions

From this study it was found that the natural agar/ι-carrageenan (**AC**) hydrogel was more sensitive to $CaCl_2$ solution than NaCl solution. It was also noticed that in both saline solutions, the **AC** hydrogels showed higher resistance to disintegration compared to the hydrogels kept in distilled water shown by the large error bar (**Fig. 6**). In summary, the swelling % of the **AC** hydrogel and its life-span can change by the difference in the types of salt present in the water.

Swelling studies of AC hydrogels in pH buffer solutions

From this study it was found that the swelling capacity of natural agar/ι-carrageenan (**AC**) hydrogel under pH 4 and 7 were similar but tended to reduce in pH 10 buffer solution (**Fig. 7**). Particularly at times between 0.5 to 72 hrs seemed to swell less in pH 10 than the other two buffer solutions. The swelling % then gradually increased until it reached closer to those of the hydrogels in pH 4 and 7. The **AC** hydrogel swelling rate changed according to the change in the pH of the buffer solution.

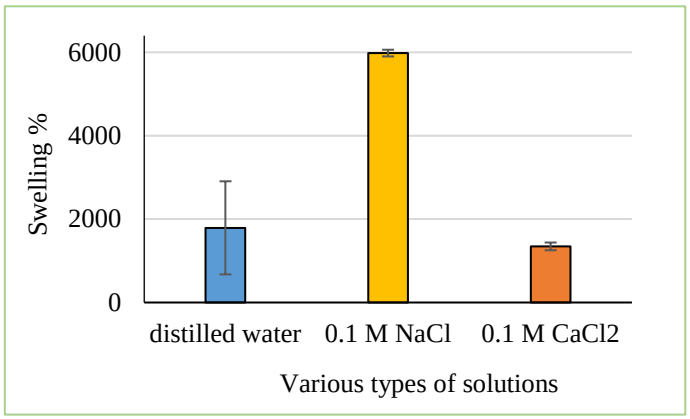

Fig. 6: The swelling % of agar/ι-carrageenan (**AC**) hydrogel in distilled water (blue bar), 0.1M NaCl solution (yellow bar) and 0.1M $CaCl_2$ solution (orange bar) measured at 22±2°C after 168 hrs.

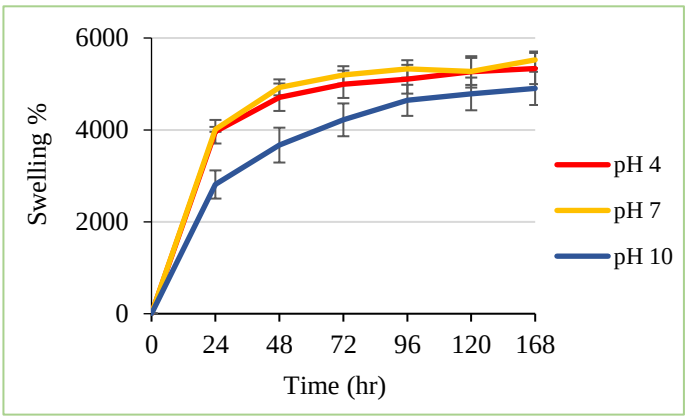

Fig. 7: The swelling % of agar/ι-carrageenan (**AC**) hydrogel in pH 4, 7 and 10 buffer solutions measured at 22±2°C for 168

Summary and Future Work

The novel agar/ι-carrageenan blended hydrogel coating showed some interesting swelling characteristics under different types of saline solutions and pH levels. The hydrogels acted as smart-gels[5,6] where it changes its swelling behaviour depending on the change in stimulation by the surrounding environment, typically ions, pH levels, or temperatures for example.

Applying the concept of smart-gels which acts as a controlled drug releasing device, incorporation of 'green pesticides' and or 'beneficial microbes' into the hydrogel is suggested for future work in this study, in order to provide protection of seeds and further growth promoting effect. The controlled release of these active ingredients can greatly promote sustainable pesticide or nutrient management which could contribute to the reduction of pollution into the environment without reducing crop yield.

The preliminary germination study gave an insight to the use of our novel natural hydrogel as seed coatings to speeding up germination for wheat seeds[7]. It is anticipated that this natural seed coating can be applied to different variety of seeds and contribute to future food sustainability.

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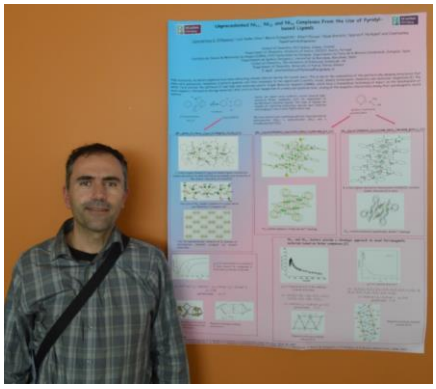

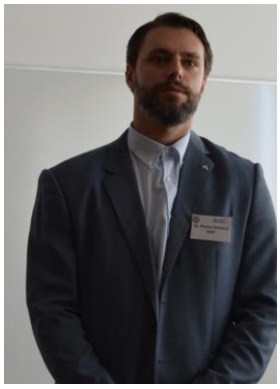

Research Review

Characterisation of Biopharmaceuticals

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Introduction

Biopharmaceuticals are drugs which are primarily (glyco)protein in nature, that are produced in living cells using recombinant DNA technology – the combination of genetic material from multiple sources, creating sequences that would not otherwise be found in the genome. They have become an essential component of modern pharmacotherapy, and are the only effective treatment option available for many severe, often life-threatening, diseases. Biopharmaceutical products have seen unprecedented sales growth in the last decade, particularly when compared with the conventional drug market. Most of the world's top-selling drugs in 2015 were biopharmaceuticals, accounting for over 70% of the sales revenue for the top ten drug products in that year – see Table 1. The global biopharmaceuticals market currently enjoys a compound annual growth rate of approximately 9%, and is expected to reach an estimated value of over US$250 billion by 2017.[1]

Table 1: Global sales revenue of the top 10 pharmaceutical products in 2015

Rank	Product	Active Ingredient	Company	Sales - US$ million
1	Humira	Adalimumab	AbbVie	14,012
2	Harvoni	Ledipasvir and Sofosbuvir	Gilead Sciences	13,864
3	Enbrel	Etanercept	Amgen / Pfizer	8,697
4	Remicade	Infliximab	Johnson & Johnson / Merck	8,355
5	MabThera/Rituxan	Rituximab	Roche	7,115
6	Lantus	Insulin Glargine	Sanofi	7,029
7	Avastin	Bevacizumab	Roche	6,751
8	Herceptin	Trastuzumab	Roche	6,603
9	Revlimid	Lenalidomide	Celgene Corporation	5,801
10	Sovaldi	Sofosbuvir	Gilead Sciences	5,276
			Total revenue for top 10 drugs of 2015	***83,503***

	US$ million	Percentage of total
Biopharmaceuticals	58,562	70.1
Small molecule drugs	24,941	29.9

Data source: http://www.pharmacompass.com/pharma-news/top-drugs-by-sales-revenue-in-2015-who-sold-the-biggest-blockbuster-drugs. Accessed 18/05/2016

Biosimilars represent a distinct class of biopharmaceuticals that are essentially 'copy-versions' of innovator biologics that emerge upon patent expiry of the innovator drug. By the end of 2015, important innovator biologics with a combined global annual revenue in excess of US$50 billion, including Herceptin®, Rituxan® and Remicade® had lost patent protection, and many more key patents are set to expire in the

period up to 2020 (frequently termed the 'patent cliff'). This creates an attractive opportunity for big pharmaceutical companies to develop biosimilars, enabling them to diversify their thinning pipelines.

Due to the highly complex nature of biologics, and their dependence on biological processes for production, biosimilars cannot be considered to be identical to innovator drugs. Therefore, legislation put forward for licensure of generics (such as the Drug Price Competition and Patent Term Restoration Act of 1984 in the United States of America (USA), often referred to as the Hatch-Waxman Act) is inadequate for biosimilars. Unlike biologics, generic versions of conventional drugs contain active substances whose safety and efficacy profiles are well-established. The FDA definition of a generic is that it should be comparable to the reference product in dosage, strength, route of administration, quality, performance characteristics, and intended use.[2] Generics' developers only need to prove average bioequivalence in order to obtain approval. However, considerably more data is required for biosimilars, as slight differences between biosimilar and innovator may have significant consequences, such as eliciting a potentially dangerous immune response, when administered to patients. Therefore, specific legislation for biosimilar approvals was required.

In the European Union (EU), the "Guideline on similar biological medicinal products"[3] was published in 2005, which introduced the concept of a biosimilar medicine, and describes the approach for demonstrating biosimilarity of a proposed biosimilar. The first biosimilar was licensed in EU in 2006 (Sandoz Inc.'s Omnitrope®; a recombinant somatropin), and more than 20 biosimilars have since gained authorisation. As such, considerable experience has been gained on biosimilars in the EU, not only from a conceptual perspective, but also from available data.

While biosimilar licensing legislation was pioneered in the EU, the US has significantly lagged behind in developing its own legislation. Indeed, some products that have been registered as biosimilars in the EU, such as Omnitrope®, have gained approval in the US via the full 'Biological Licensing Application' pathway before US biosimilar regulations were even created. However, in recent years, there has been a surge of regulatory activity in the US laying out the route for approval of biosimilars. A major step forward in this process was the issuing by the FDA of three draft guidance documents in 2012 which cover quality considerations, scientific considerations, and FAQ's regarding the implementation of the legislation relating to biosimilars.[4-6] In an exciting development in March 2015, the FDA announced a first biosimilar approval in the US – Sandoz's Zarxio®, a biosimilar to Amgen's Neupogen® (filgrastim), used to combat chemotherapy-induced neutropaenia in cancer patients. More recently in April 2016, the FDA approved Hospira's Inflectra® (infliximab-dyyb), a biosimilar to Janssen Biotech, Inc.'s Remicade® (infliximab), which is used to treat a range of autoimmune disorders. These recent approvals indicate that the licensure pathway for biosimilars in the US is well-established at this point.

Biosimilar development can take advantage of these abbreviated licensure pathways based upon characterisation programs that demonstrate sufficient similarity between the biosimilar and innovator product. This can offer enormous time and cost savings when compared to development of a new molecular entity (NME) as there is less dependence on lengthy and costly pre-clinical and clinical studies. According to the Tuft's Centre for the Study of Drug Development (an highly-reputable independent, non-profit organisation dedicated to researching drug development), the cost of taking an NME from concept to market can exceed US$2.6 billion.[7] By contrast, a biosimilar development programme typically costs in the region of US$100 million – 250 million.

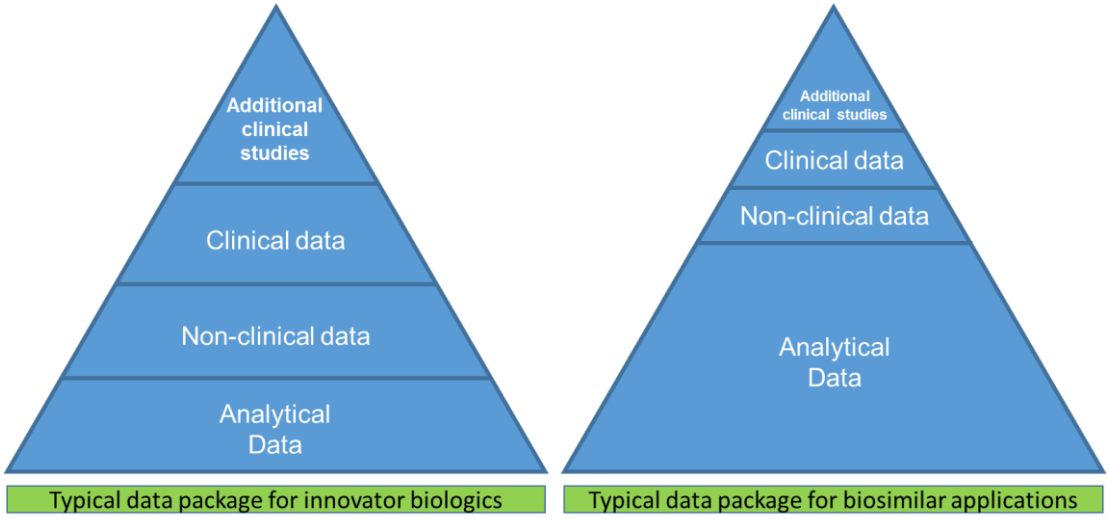


Figure 1: Data requirements for innovator biologics and biosimilar licensing applications

The importance of having analytical methods that can fully characterise biologics lies in the fact that even minor differences between a biosimilar and its reference product can have significant patient safety implications. Indeed, even slight changes in production processes may introduce subtle differences between batches of commercial biological product, and these differences may have potential for a clinical impact. Therefore, the analytical technologies developed for characterisation and release testing of biopharmaceuticals should be capable of detecting differences in product structure where they occur. Guidance from the International Council for Harmonisation on the characterisation of biopharmaceuticals states that new technologies (and improvements or modifications of existing technologies) are continually being developed and that these should be used if they can provide additional information or discriminating power when characterising biologics.

Our interests lie in the exploration and development highly discriminating analytical strategies for characterising biologics, in order to support biosimilar licensing applications. The research is being conducted through a joint industrial/academic setting, in a collaborative programme involving BioClin Research Laboratories and the BioSciences Research Institute at Athlone Institute of Technology (AIT), both of which are in close proximity to one another in Co. Westmeath, Ireland. The research is part-funded by a scholarship award from the Irish Research Council under the 'Employment-Based Postgraduate Programme". An impressive suite of *state-of-the-art* analytical technologies exist between BioClin and the Research Hub at AIT, and are at our disposal for performing this research. Undertaking this research at BioClin has enabled the company to diversify service offerings to include biopharmaceutical characterisation programs, which ensures they remain current and competitive as an analytical service provider in a rapidly-changing pharmaceutical market.

Analytical strategies for characterisation of biologics

Marketing authorisation for a new biopharmaceutical or a biosimilar, requires that all characteristics of the proposed drug that may have an impact on safety or efficacy are fully evaluated. Proteins may exhibit a high degree of heterogeneity due to the biosynthetic processes that living cells use to produce them. Owing to this heterogeneity, and the diverse and complex structure of biopharmaceuticals, there is no 'one-size-fits-all' analytical strategy for characterising them; therefore, each biologic requires a tailored approach. The goal for the analytical laboratory is to develop methods and technologies that can characterise all attributes of a biological drug. The International Council for Harmonization (ICH) Topic Q6B – "Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products",[8] which was adopted in

1999, provides guidance on tests and specifications that are appropriate for characterising biologics. The guidance indicates that applications for licensing of biologics should be supported by a comprehensive analytical package which characterises all the critical quality attributes (CQA's) of the drug. It provides direction on the setting of specifications and acceptance criteria that will ultimately serve as release test specifications, which will be a condition of approval for the drug. The document details that the reduction in dependence on clinical data permitted for a biosimilar application depends on the 'weight-of-evidence' from analytical studies that no functionally important differences exist between biosimilar and the reference drug. The following sections highlight the range of strategies typically applied in line with ICH Q6B guidelines, and comments on the structural elements that can be explored and the limitations of each strategy.

Confirmation of primary structure

Amino acid compositional analysis

The relative amount of each amino acid in a protein provides a characteristic profile for each biopharmaceutical, and can therefore confirm identification and support structural elucidation. Results from quantitative amino acid determination can be used for a precise determination of protein quantity in a sample (without the need for a reference standard), and this information can be further used to determine the extinction co-efficient – an important characteristic of a protein. Furthermore, amino acid analysis results can help to evaluate digestion strategies for peptide mapping and aid in identifying the presence of atypical amino acids that may have been incorporated into the protein. The test is often used to demonstrate comparability and consistency between batches for lot release of finished products.

The analysis involves the hydrolytic degradation of the protein into its constituent amino acids, followed by separation and quantitation of the free amino acids. Prior purification is essential, as buffer components can interfere with hydrolysis. Additionally, high-purity materials are required, as some reagents may be contaminated with low levels of amino acids that can distort the results – e.g. general reagent-grade hydrochloric acid (HCl) frequently has relatively high levels of amino acids such as glycine present.[9] Glassware and other consumables used for analysis must be free from contaminants – e.g. depyrogenised by baking at 500ºC for 4 hours, or certified pyrogen-free disposable glassware should be procured.

Hydrolysis is typically performed by heating to 110ºC for 24-72 hours in the presence of 6 M HCl (constant boiling), during which peptide bonds are hydrolysed, releasing the free amino acids. The wide range of treatment durations is due to the fact that some peptide bonds (such as those between isoleucine and valine) are more difficult to break than others. As such, hydrolysis duration is dependent upon amino acid content and sequence, and should be determined empirically for each unique protein. Hydrolysis can be performed in either the liquid phase (in which the protein is dissolved in HCl), or the vapour phase (in which only HCl vapours come into contact with the sample) – the former approach can reduce sample contamination from low-grade HCl. Accelerated methods involving higher temperatures for a shorter duration, or the use of microwave energy can significantly reduce hydrolysis times – in part, this research is evaluating rapid hydrolysis methods based on the latter.

One of the drawbacks of acid hydrolysis is that amino acids vary considerably in their stability to such treatment. Tryptophan is completely destroyed, and asparagine and glutamine are both deamidated to aspartic acid and glutamic acid, respectively. The complete loss of these three therefore limits analysis to 17 of the 20 common amino acids. Also, serine and threonine are partially destroyed; some amino acids are prone to oxidation, and others, such as tyrosine, can become halogenated. Certain treatments can address many of these concerns (e.g. addition of phenol can prevent halogenation of tyrosine, and removal of oxygen from the headspace of the reaction tube can reduce oxidation). However, the addition of agents to

protect one amino acid from degradation or alteration can often be at the expense of another amino acid. Therefore, where the sequence is available for a test protein, it should be consulted in order to evaluate the best approach for hydrolysis, and a time-course study initiated to determine appropriate hydrolysis duration. As part of our research, we characterise the rate of degradation of all naturally occurring amino acids subject to a range of hydrolysis treatments. This information will prove useful when interpreting experimental results from subsequent test samples.

Following hydrolysis of proteins, the free amino acids must be separated from each other and detected, in order to allow the relative quantities of each to be determined. Ion-exchange or reverse-phase HPLC are the methods of choice for separating the amino acids, followed by ultra-violet or fluorometric detection. In order to increase sensitivity and enhance detection, amino acids are typically derivatised, either pre-column (with ninhydrin or *o*-phthalaldehyde) or post-column (with phenyl isothiocyanate, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbonate, or other agents). Based on available technologies, we perform this analysis using pre-column methods employing both of the derivatisation agents specified above. Phenyl isothiocyanate (PITC) reacts with free amino acids to form phenylthiocarbamoyl derivatives, which are then separated on a reverse-phase octadecylsilane column with detection at 254 nm. Reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate produces amino acid-urea derivatives that fluoresce strongly at 395 nm (excitation wavelength 250 nm) – a representative chromatogram from amino acid analysis is shown in Figure 2.

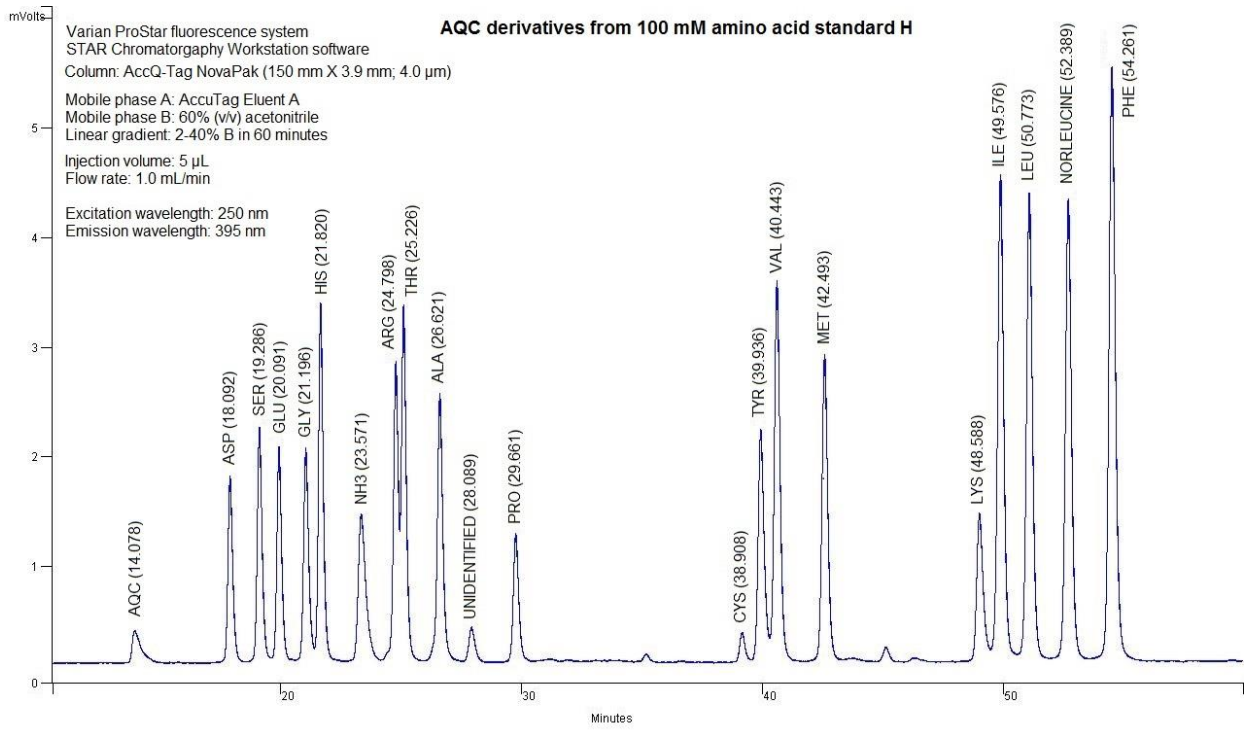


Figure 2: Representative chromatogram from amino acid analysis

Peptide mapping

Peptide mapping is an indispensable tool for characterising biologics. The technique is sensitive enough to detect even very minor changes to primary or secondary structure in a biopharmaceutical, and allows localisation of where this change has occurred. The technique can be performed using standard HPLC alone;

however, HPLC combined with mass spectrometry (LC-MS) releases enormous characterisation potential, as the mass information provided by mass spectrometry can reveal the nature of any change that is detected. Peptide mapping is one of the core techniques that is applied to release testing for biopharmaceuticals, and should be extensively used when comparing a biosimilar to the innovator drug. The technique is also used for assessing genetic stability of production cell lines, as mutations in the encoding gene often result in a change in the amino acid encoded – this change can be readily identified from peptide mapping data.

Peptide mapping makes use of commercially-available 'sequencing grade' proteolytic enzymes, such as trypsin (which cleaves at the *C*-terminal side of arginine and lysine residues) and endoproteinase Glu-C (which cleaves at the *C*-terminal side of hydrophobic amino acids). These enzymes (being protein in nature themselves) are often chemically treated in order to eliminate or reduce autolytic activity (where the enzyme cleaves neighbouring enzyme molecules). Chemical fragmentation strategies are also possible, such as treatment with cyanogen bromide, which cleaves at the *C*-terminal side of methionine residues. Table 2 details the commonly used enzymatic and chemical cleavage agents, and their respective specificities.

Table 2: Cleavage agents commonly used for peptide mapping

Type	Agent	Specificity
Enzymatic	Trypsin	*C*-terminal side of arginine and lysine residues
	Chymotrypsin	*C*-terminal side of leucine, methionine, alanine, tyrosine and tryptophan
	Pepsin	Non-specific digest
	Lysyl endopeptidase	*C*-terminal side of lysine
	Endoproteinase Glu-C	*C*-terminal side of glutamic acid and aspartic acid
	Endoproteinase Asp-N	*N*-terminal side of aspartic acid
	Endoproteinase Arg-C	*C*-terminal side of arginine
Chemical	Cyanogen bromide	*C*-terminal side of methionine
	2-Nitro-*thio*-cyanobenzoic acid	*N*-terminal side of cysteine
	O-Iodosobenzoic acid	*C*-terminal side of tyrosine and tryptophan
	Dilute acid (<0.1 M HCl)	Aspartic acid and proline
	3-Bromo-3-methyl-2-(2-nitrophenylthio)-3*H*-indole (BPNS-skatole)	Tryptophan

These agents cleave the biopharmaceutical at specific sites along the protein backbone with very high regularity. Treatment therefore results in the protein being broken down into a number of fragments dependent upon the sequence of amino acid residues of the parent protein. As all proteins have a unique amino acid residue sequence, treatment of different proteins with the same enzyme will give rise to distinctive peptide maps. An example of the mechanism of action of enzymatic cleavage of a peptide bond is illustrated in Figure 3, for the serine protease, chymotrypsin.

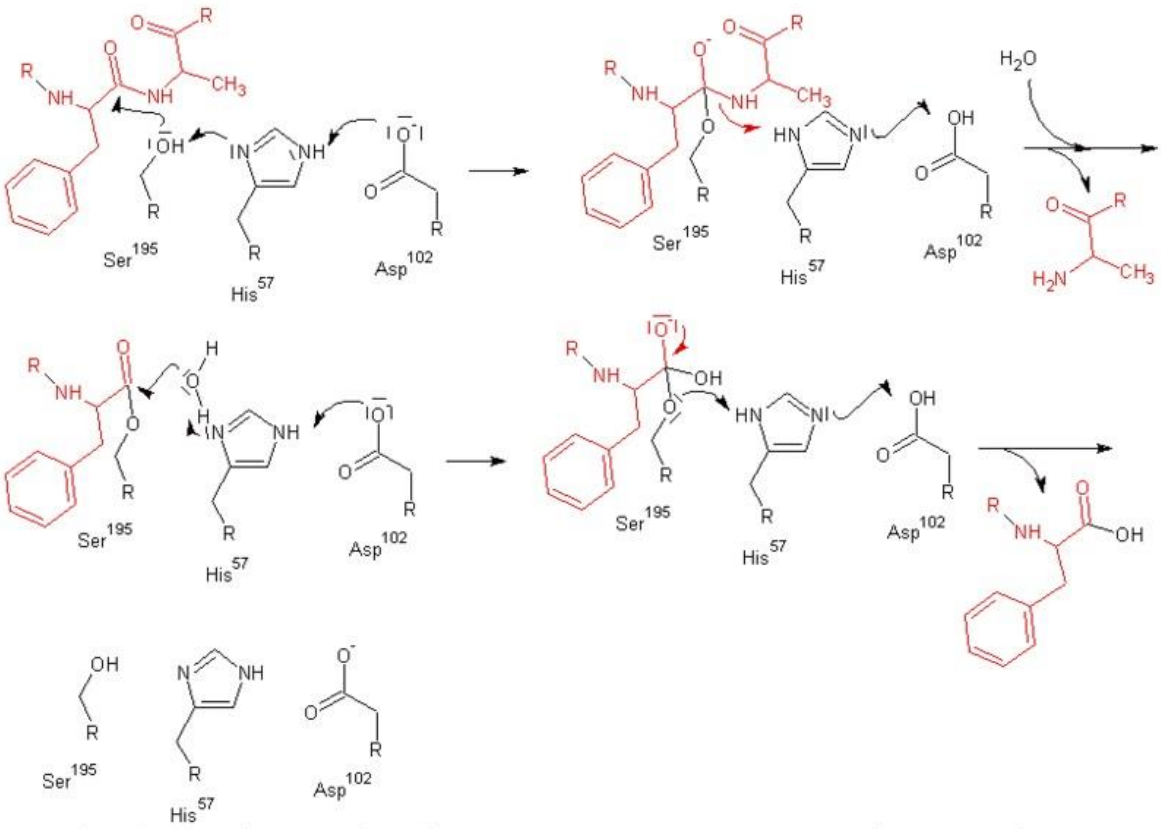


Figure 3: Mechanism of action of chymotrypsin.
Image credit: Felix Plasser CC-BY-SA 3.0.

Prior to digestion with an enzyme, it is often necessary to break disulphide bonds within the protein in order to ensure the proteolytic enzymes have access to all cleavage sites. This is typically accomplished by treating the protein with an agent that reduces disulphide bonds, followed by alkylation of the free thiol groups to prevent the disulphide bonds from reforming. Dithiothreitol or *β*-mercaptoethanol are commonly used reducing agents and iodoacetamide is a frequently used alkylating agent for this purpose. It is important to remember that these treatments increase the mass of peptide fragments – e.g. alkylation with iodoacetamide increases the mass of each fragment by approximately 58 Da for each cysteine present.

When the amino acid residue sequence of a protein is known (which is usually the case for biopharmaceuticals) the fragments that should be generated from treatment with a given cleavage agent can be predicted using *in silico* digestion. Many software applications and online resources are available for performing this task, such as the freely-accessible 'PeptideMass' from the website http://www.expasy.org.[10] The experimentally obtained peptide map results can then be compared to those predicted from the *in silico* digestion.

When HPLC alone is used for analysing results from peptide mapping, differences (between a biosimilar and its reference biologic, for example) are revealed by shifts in retention time of proteolytic fragments. When the nature and precise location of differences is to be determined, LC-MS is used instead of HPLC. With this method of analysis, each fragment is detected with a characteristic retention time, but the mass of

each eluting fragment is also obtained. With this additional information, the analyst can determine which peak corresponds to each fragment from the *in silico* digestion. If any changes in mass are observed, there will be a shift in the mass of the fragment that corresponds with the nature of the change (likely also accompanied by a shift in retention time). For example, where a single amino acid residue in a peptide fragment is oxidised, the overall mass of the fragment will increase by approximately 18 *Da*, or where a lysine has been substituted for an alanine, there will be a mass decrease of approximately 85 *Da* due to the difference in mass between these two amino acid residues. This information allows the analyst to pinpoint with high accuracy the location of any observed modification. Subsequently, the exact nature of the modification can be further explored by using tandem mass spectrometry (LC-MS/MS). Figure 4 illustrates a typical BioClin workflow for peptide mapping using LC-MS, while Figure 5 shows experimental peptide mapping data for asparaginase following digestion using trypsin. Asparaginase is an enzyme (Enzyme Commission (EC) Number: 3.5.1.1.) which is used to treat acute lymphoblastic leukaemia (ALL) in children. Asparaginase exploits the observation that acute lymphoblastic leukaemia cells cannot synthesise asparagine, and therefore depend on it being supplied in the bloodstream. Asparaginase catalyses the conversion of circulating asparagine to aspartic acid and ammonia, which deprives leukemic cells, leading to cell death. Table 3 shows the predicted peptide fragments from *in silico* digestion of asparaginase.

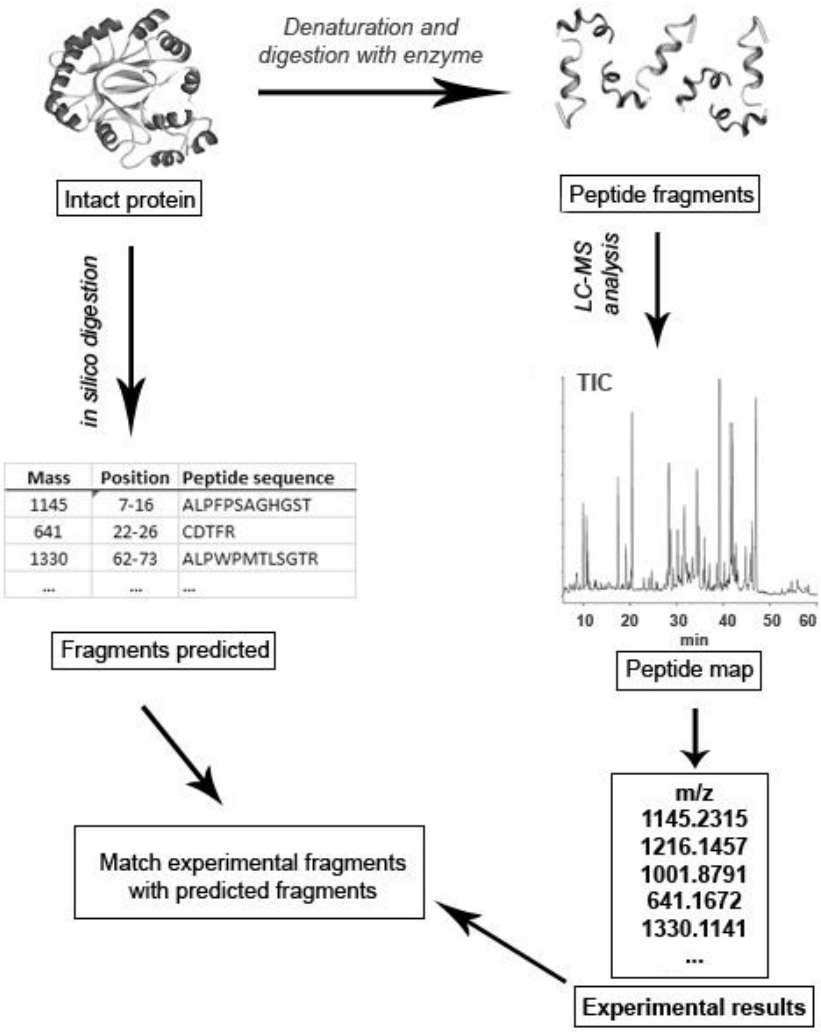
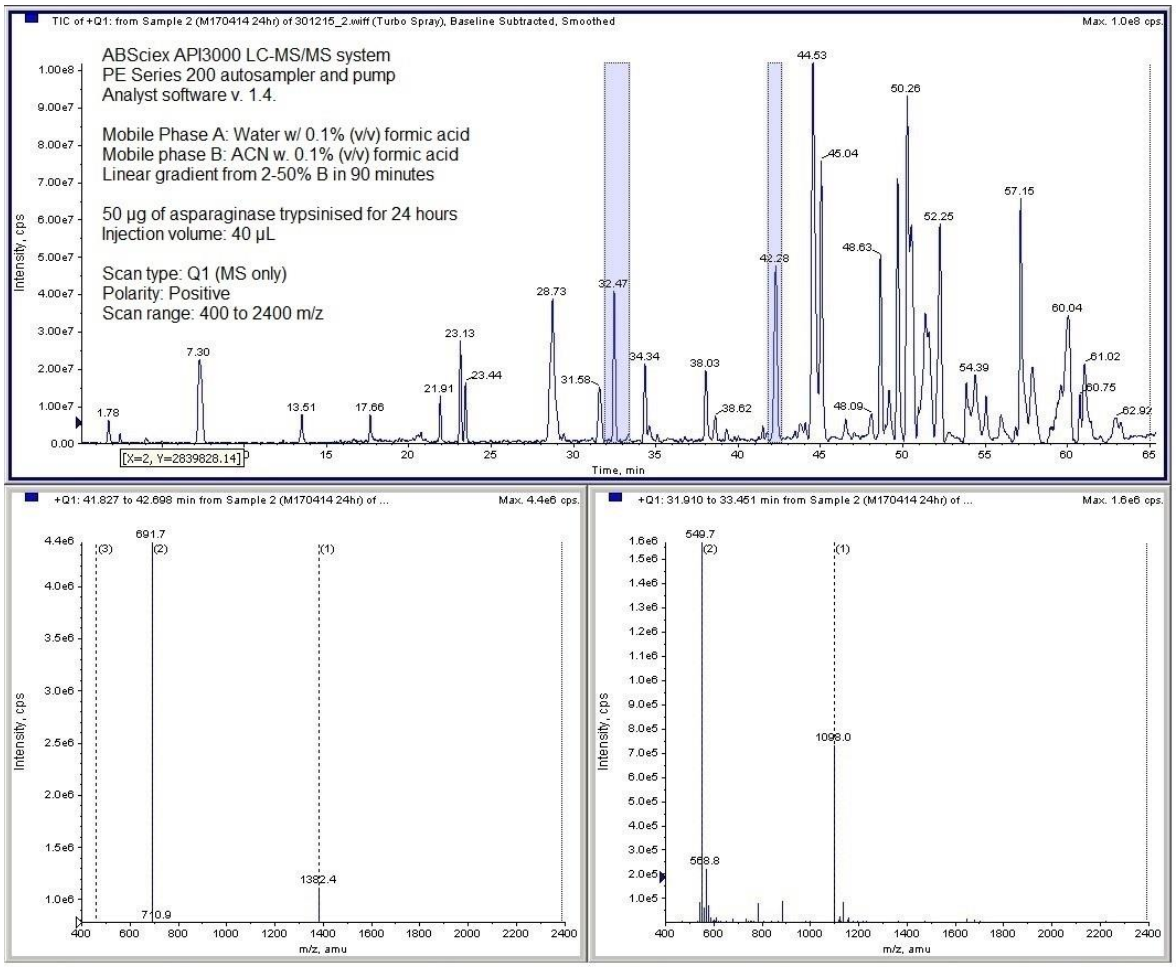


Figure 4: Peptide mapping using LC-MS

Table 3: *In silico* digestion of asparaginase using trypsin

Mass - [M+H]+	Position	Sequence
3642.7	127-161	CDKPVVMVGAMRPSTSMSADGPFNLYNAVVTAADK
3472.9	7-44	TALAALVMGFSGAALALPNITILATGGTIAGGGDSATK
2804.3	102-126	TDGFVITHGTDTMEETAYFLDLTVK
2431.2	72-93	GEQVVNIGSQDMNDNVWLTLAK
2153.1	252-273	ALVDAGYDGIVSAGVGNGNLYK
1694.9	236-251	VGIVYNYANASDLPAK
1617.8	295-310	VPTGATTQDAEVDDAK
1521.7	337-348	DPQQIQQIFNQY
1518.8	195-208	SVNYGPLGYIHNGK
1487.8	167-180	GVLVVMNDTVLDGR
1479.9	52-65	VGVENLVNAVPQLK
1381.7	***311-323***	***YGFVASGTLNPQK***
1233.6	219-229	HTSDTPFDVSK
1227.8	326-336	VLLQLALTQTK
1123.6	274-284	SVFDTLATAAK
1097.5	***185-194***	***TNTTDVATFK***

Data in bold italics correspond to peaks that are highlighted in Figure 5 below



Peptide mapping results for asparaginase from *E. coli*. The top pane shows the total ion current (TIC) from the mass spectrometer, while the bottom panes show the respective peptide masses for two well-resolved peaks in the TIC - 1382.4 and 1098.0, both of which are predicted from the *in silico* digestion (See Table 3). The slight differences in masses reported are related to the resolution and calibration of the mass detector used.

Figure 5: Peptide mapping results of asparaginase

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Amino acid sequencing

The sequence of a protein refers to the linear arrangement of amino acid residues in that protein. Due to the *in vivo* processing (and possible post-translational modification, explained later) associated with biopharmaceuticals, the *N*-terminal and *C*-terminal sequences of a protein are not always readily predictable from the gene sequence. As such, sequence analysis of a biopharmaceutical is typically limited to the *N*-terminal and *C*-terminal ends of the protein. *N*-terminal sequencing can reveal if truncation of the protein has occurred. Such truncations may result from the presence of trace levels of expression cell proteases that were not removed through downstream processing (involving a series of chromatographic steps which result in successively higher purity), and truncated protein may not exhibit the desired therapeutic effect. The sequence information obtained is used to confirm consistency between batches and for demonstrating similarity between biosimilars and reference drugs. There are two main approaches presently used by industry for the terminal amino acid sequence analysis of biopharmaceuticals – Edman degradation chemistry and mass spectrometry.

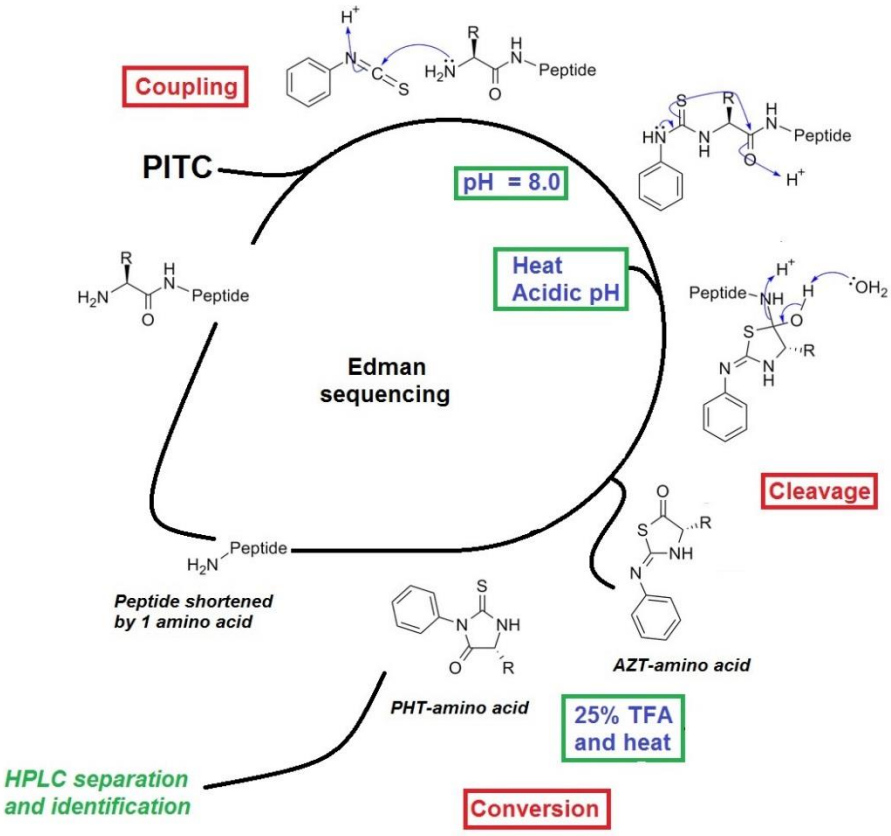


Figure 6: Edman degradation chemistry used for sequencing of proteins

Edman sequencing, illustrated in Figure 6 above, involves derivatisation and cleavage of one amino acid at a time from the *N*-terminus, followed by analysis and identification of the derivatised amino acid. The protein is first combined with a reagent that will selectively react with the *N*-terminal amino acid. PITC (which is also used for amino acid compositional analysis, described earlier) is combined with the protein sample under mildly alkaline conditions, where it selectively reacts with the uncharged terminal amino group to give a phenylthiocarbamoyl derivative. Subsequently, under acidic conditions, a thiazolinone derivative of the amino acid is liberated, leaving the remainder of the polypeptide chain intact, but shortened in length

from the *N*-terminus by one amino acid. The thiazolinone derivative is then extracted into an acidified organic solvent to give a more stable phenylthiohydantoin (PTH) amino acid derivative, which can subsequently be identified using HPLC. This procedure is continued until the entire length of the polypeptide chain has been sequenced.

Theoretically, it should be possible to sequence an entire protein using the Edman sequencing method, however, in practise this is not achievable. The efficiency of each cycle of the reaction is approximately 98%,[11] which means that only approximately 50 amino acid residues can be reliably sequenced using this method. In practise, no more than 30 residues should be sequenced by the Edman method in order to produce reliable and reproducible results. However, some automated sequencers claim 99% efficiency and manufacturers claim that they can reliably sequence 50-100 residues or more.[12]

This limitation of the Edman method can be overcome using a method developed by Frederick Sanger in 1955, for which he received the Nobel Prize in Chemistry in 1958.[13] Sanger's method involves selectively cleaving the original protein into smaller peptide fragments using trypsin, then separating these fragments using electrophoresis. Each fragment can then be sequenced using the Edman method. The order in which these fragment sequences are to be recombined is determined by cleaving the protein with a second proteolytic enzyme with a different specificity, such as chymotrypsin, followed by Edman sequencing of the fragments as previously described. Overlapping the partial sequences obtained from the two different digests allows the correct order of the fragments to be determined, thereby allowing reconstruction of the complete protein sequence.

Another important limitation of the Edman method is that it requires an unmodified amino group at the *N*-terminal of the molecule. Unlike prokaryotic cells, which are not thought to significantly post-translationally modify proteins, evidence suggests that up to 80% of intact proteins from eukaryotic organisms, such as Chinese hamster ovary (CHO) cells, have modified *N*-terminal amino groups.[14, 15] This has potential to result in significant complications for biologic characterisation, as the majority of biologics in present-day use are expressed in eukaryotic cells.[16] Several methods for unblocking these amino groups to facilitate Edman sequencing exist, but they require comparatively large amounts of protein, and don't produce consistent results, particularly when the nature of the blocking group is unknown. This limitation is less significant when enzymatic fragmentation of the protein is employed, as each of the internal polypeptide fragments would have unmodified N-terminal amino groups, and are therefore amenable to Edman sequencing.

Tandem mass spectrometry (MS/MS) is emerging as one of the most powerful methods of sequencing proteins.[17-22] It also offers the most reliable approach for C-terminal sequencing of proteins (analysing only the C-terminal peptide from the proteolytic digest of the protein). Using this approach, proteins are first cleaved into smaller fragments of approximately 20 amino acid residues or less (as for peptide mapping). Trypsin is the enzyme of choice for cleaving proteins prior to sequencing by MS/MS, as it typically gives rise to peptide fragments from 8-20 amino acid residues long, which is the ideal range for most mass spectrometers. The resulting series of peptide fragments can then be analysed via (MS/MS) to gain sequence information. In a typical sequencing workflow, proteolytic digests are infused directly to the mass spectrometer, and each predicted fragment (from the *in silico* digestion) is selected independently in the first quadrupole (see Figure 8). Selected peptides are then passed into the second quadrupole, where they are broken down through a process called collision induced dissociation (CID), and the resulting ions analysed in the third quadrupole. During CID, bond breakage most frequently occurs through the lowest energy pathways in the molecule, which for peptides are the amide bonds. Therefore, the major fragments generated differ from each other by a single amino acid. Roepstorff-Fohlmann-Biemann nomenclature is used to describe the ions that are produced in this process.[23, 24] Using this system, the fragments are called b-ions when the charge is retained by the amino terminal fragment, and y-ions if the charge is retained by the

carboxy terminal fragment, with ions being labelled consecutively from the original amino terminus – other fragments are also possible, albeit with a lower frequency - see Figure 7. As with the Edman method, reconstruction of the partial sequences to build up the overall sequence of the parent protein is accomplished by using a second enzyme, by overlapping both sets of results. Many software platforms and online resources are available for simplifying this task. Sequencing via mass spectrometry is illustrated in Figure 8.

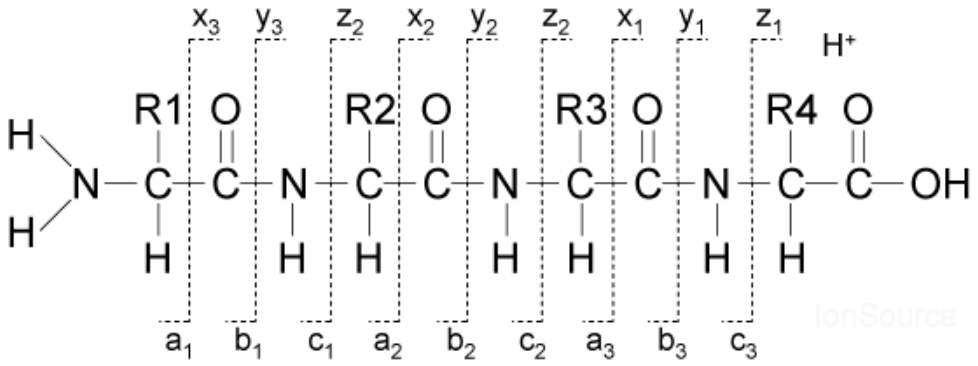


Figure 7: Roepstorff-Fohlmann-Biemann nomenclature.
Ionsource.com. Available at http://www.ionsource.com/tutorial/DeNovo/full_anno.htm.

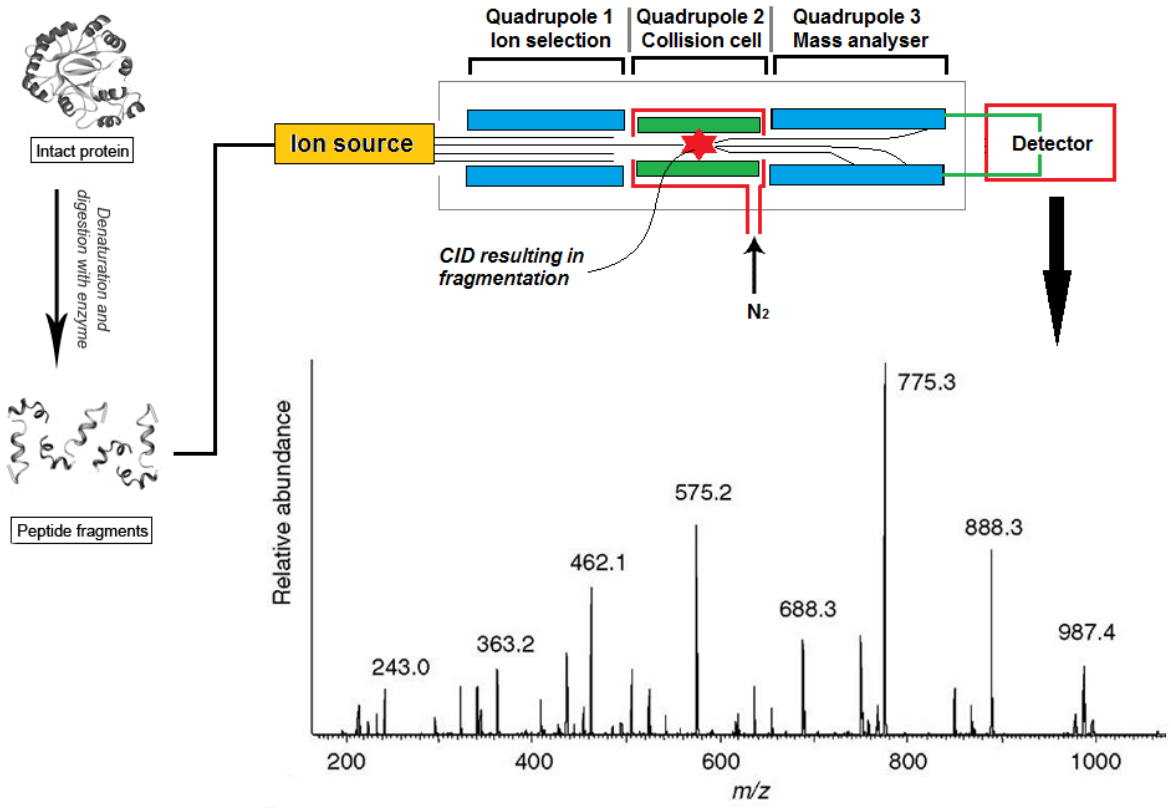


Figure 8: Workflow for protein sequencing via mass spectrometry

Sulphydryl groups and disulphide bonds

The side chains of cysteine residues contain thiol groups that can react with one another to form covalent disulphide bonds (denoted S-S) through a process called oxidative folding. An enzyme called 'protein disulphide reductase' (PDI) oxidises the thiol group of cysteine residues, thereby catalysing the formation of S-S bonds. Disulphide bonds can form between cysteines in the same polypeptide chain (intramolecular) or between cysteines from separate polypeptide chains (intermolecular). Intramolecular S-S bonds are responsible for stabilising the tertiary structure of a protein, while intermolecular S-S bonds are attributed to stabilising quaternary structure in complex proteins consisting of two or more subunits. While methionine also contains a sulphur atom, only cysteine residues can form S-S bonds. Reduced cysteines will react with each other if they are in close proximity, even if the protein isn't properly folded. Therefore, the greater the number of cysteines in a protein, the greater the potential for mismatched disulphide bonds. This is likely to result in a protein which does not act as intended when used as a biopharmaceutical. Therefore, in order to ensure drug safety and efficacy, it is important that the location of cysteine residues and disulphide bonds are determined, as this cannot be accurately predicted from the gene sequence.[25]

Conventional methods such as high-field NMR have been used to characterise disulphide bonds in proteins.[26] However, such methods typically require high concentrations of protein, which may not always be available at the early drug development stage. Mass spectrometry using soft ionisation techniques, such as electrospray ionisation, are gaining in popularity for disulphide bond analysis. Typical approaches involve performing peptide mapping via LC-MS under both reducing and non-reducing conditions. Where non-reduced protein is digested for peptide mapping, the disulphide linkages will keep fragments covalently combined, giving rise to larger molecular mass fragments. The reduced sample will result in more fragments, as fragments connected by disulphide bonds will be separated from one another when those bonds are broken. Data from these analyses can be combined and used to determine where disulphide bonds are present. This process is illustrated in Figure 9 below.

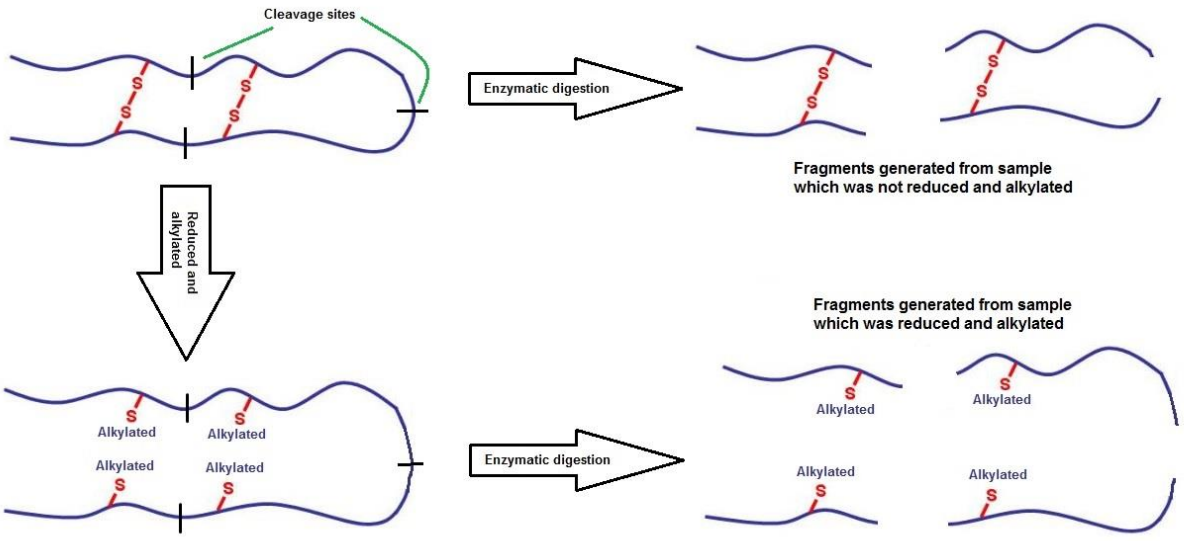


Figure 9: Disulphide bond analysis using reducing and non-reducing peptide mapping

Glycosylation analysis

Approximately two-thirds of the biopharmaceuticals in the current market are glycoprotein in nature. The carbohydrate content of proteins often plays a significant role in the function of the protein, having an impact on physiochemical properties and thermal stability, and helping to mediate effects such as circulating half-life and their reactivity towards target receptors (and therefore their pharmacological efficacy). Indeed, proteins with unanticipated glycan structure may promote a potentially-harmful immune response. Therefore, it is imperative that the constancy of carbohydrate moieties is maintained to ensure the safe and efficient use of glycosylated biopharmaceuticals. Glycosylation can occur at any number of sites on a protein molecule. *N*-glycosylation is the attachment of glycans to the carboxamido nitrogen on asparagine, and this is the most common type of glycosylation seen. *O*-glycosylation involves the attachment of glycans to serine or threonine residues. Other forms of glycosylation are less common. No universally applicable approach for glycan structural analysis of proteins is available, and a combination of methods is typically employed, many of which exploit various forms of mass spectrometry.[27] Glycosylation analysis presents a significant analytical challenge, as glycans typically form highly-branched structures (unlike the simpler linear arrangement of amino acids in peptides). As many of these variant arrangements of sugars will be isobaric (have the same molecular mass) it can be particularly challenging to tease out the actual glycan structure. However, three general approaches are typically employed, often in combination: (i) *characterization of glycans in intact glycoproteins*; analysis is by means of techniques including capillary isoelectric focussing, capillary zone electrophoresis, or mass spectrometry – results give partial characterisation of glycan profile and often serve as the starting point for glycan analysis; (ii) *characterization of glycopeptides derived from the protein*: proteins are digested and fragments analysed via capillary zone electrophoresis and tandem mass spectrometry – this provides information on glycosylation sites and some information on the nature of glycans present; (iii) *analysis of glycans that have been chemically or enzymatically removed from the protein*: acid hydrolysis of proteins or treatment with enzymes with specificity for various forms of glycosylation (such as PNGase F) releases the glycans which can be subsequently analysed by HPLC and other methods – this provides data on total glycan content.

Characterisation of physiochemical properties

Molecular weight or size

The molecular weight of a biopharmaceutical is often the first analysis applied during a characterisation programme. A molecular weight that differs from the predicted molecular weight immediately highlights a difference in the actual and expected structure. Many methods are used for this determination, ranging from size exclusion chromatography (SEC-HPLC), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and mass spectrometry. These techniques differ significantly in their resolution, and the type of technique chosen is often dependent on the equipment that is available at the test laboratory. Resolution is sacrificed with SDS-PAGE, where proteins can typically be estimated to within approximately 500 *Da*; however, this technique has the benefit that it allows early visual inspection for other protein-based contaminants (of different molecular weights) in the sample. Also, separation of proteins *via* SDS-PAGE effectively removes impurities and formulation excipients, so proteins can be excised from the gel for downstream analysis, such as peptide mapping. For determination of molecular mass, the highest resolution is obtained with mass spectrometry, particularly matrix-assisted laser desorption ionisation coupled with time-of-flight mass spectrometry (MALDI-TOF-MS), which can determine protein mass down to the sub-Dalton range.

Electrophoretic patterns

SDS-PAGE, native PAGE (in which the protein is not denatured prior to electrophoresis, but run in its native form), isoelectric focussing (IEF), capillary electrophoresis (CE), capillary isoelectric focussing (cIEF) and Western blotting are some of electrophoretic techniques commonly used to provide data on identity, homogeneity, and purity of a biopharmaceutical. These techniques are widely employed as part of a biopharmaceutical characterisation programme in support of new biologic or biosimilar licensing applications. IEF and SDS-PAGE are also included in the battery of specialised tests for release testing of biotherapeutics, according to USP General Chapter <1045>.

SDS-PAGE provides molecular mass information, while also revealing the presence of protein contaminants that may be present in a sample, which would appear as additional bands on the gels. SDS-PAGE has applications in forced degradation studies, where samples are extracted at various time-points during the degradation study and subject to SDS-PAGE analysis.[28] Sample degradation would be revealed through shifts in the location or changes in the appearance of the bands on the gel. For example, where a protein undergoes significant fragmentation during forced degradation, the band corresponding to the protein would be seen to decrease in intensity, and the presence of additional bands or smearing of the main band corresponding to the protein of interest may be observed. These observations provide insight into degradation pathways and products, which can serve as a basis for more detailed analysis using other techniques, such as mass spectrometry.

Aggregation refers to the non-covalent association of protein molecules in solution. Aggregation of proteins can be a significant problem during storage of finished product, as it can be intimately tied to protein folding. Physical factors (such as light exposure and temperature excursions) and chemical factors (including the impact of formulation excipients and pH shifts) may promote the development of aggregates,[29] and these may have an adverse impact on the biological activity of the protein, particularly where immunogenic responses to the protein are considered.[30] The presence of aggregates may be determined using native PAGE, as such aggregates would not dissociate during the relatively mild sample preparation steps employed in this analysis. The appearance of bands corresponding to ‘multiple times’ the mass of the protein of interest on native-PAGE gels would be indicative of aggregate formation.

IEF enables the detection of charge heterogeneity of a biopharmaceutical preparation. Charge variants have the potential to influence stability and biological activity of biopharmaceuticals, particularly those of the monoclonal antibody (mAb) class.[31] IEF can reveal the presence and ratio of charge variants due to post-translational modifications, as these differences can shift the isoelectric point of a protein considerably. The technique involves application of the sample to an immobilised pH gradient, then applying current to the system. Due to the variability in number of charged side groups in proteins, they generally have a charge at a given pH. Therefore, when an electrical current is applied to the IEF gel, they will migrate through the pH gradient towards either the anode or cathode, until they arrive at a pH at which the native charge is suppressed (i.e. where the net charge on the protein is zero) – refer to Figure 15. If the protein then diffuses away from this point in the immobilised pH gradient, it will gain a charge again, causing it to migrate back in the opposite direction under the influence of the electric current – see Figure 10. This has the effect of ‘focussing’ the sample to a very sharp band within the gradient, allowing high resolution detection of charge variants, which would be present as additional bands in the gel. IEF can be performed in gel format, on IPG strips (solid matrix with immobilised pH gradients), or in capillary format. The latter has a greater resolution than the first two techniques, allowing charge variants that differ by less than 0.1 pH units to be readily resolved. However, where information of the charge heterogeneity is available (e.g. from prior analysis) resolution in the gel format can be greatly improved by using narrower gradients (e.g. using a pH gradient from 7 to 9, instead of from pH 3 to 10).

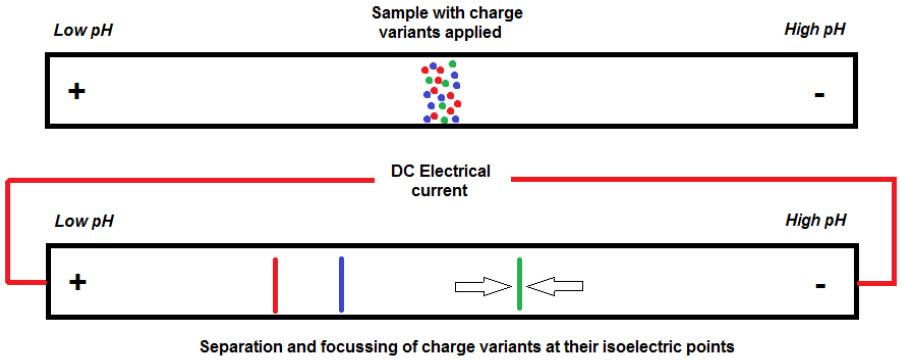


Figure 10: Isoelectric focussing for analysis of charge heterogeneity

Western blotting allows for unequivocal confirmation of protein identity. The technique is particularly useful for mixtures of complex proteins, as it allows detection of only the protein of interest in that mixture.[32] It can also reveal whether or not additional bands that are observed on a gel are related to the protein of interest (e.g. dimers or truncated protein) or if they are unrelated proteins that may be of host-cell origin, for example. Proteins are first separated using an electrophoretic method such as SDS-PAGE, with subsequent electrophoretic transfer to an inert membrane (such as PVDF or nitrocellulose), where they bind tightly to the membrane, becoming immobilised. The immobilised proteins can then be probed with a primary antibody ('anti-drug' IgG) that binds specifically to an epitope (the part the immobilised protein to which the antibody attaches itself) on the target protein. Following this, a secondary antibody (anti-IgG) (which has a 'reporter molecule' that generates a signal which can be detected) is added to the blot where it binds to bound primary antibody. A common example of a reporter molecule is the enzyme horseradish peroxidase – this catalyses the conversion of a chromogenic substrate to a derivative that can be detected via colorimetry, fluorimetry or through luminescence.

Chromatographic patterns

The identity and heterogeneity of formulated biotherapeutics should be thoroughly evaluated, with a number of important characteristics requiring consideration. Because of the high molecular complexity of biologics, they can be very sensitive to even minor changes in any detail of production. Examples include production process changes (either intentionally or inadvertently introduced), changes in batches of raw materials, or changes induced or promoted by formulation excipients. Other product changes may evolve slowly over time during storage, such as methionine oxidation; a primary degradation pathway for biologics.[33] Even minor changes (such as modification of a single susceptible amino acid), or more significant changes such as mismatched disulphide bonds, can result in significant peak shifts in chromatographic profiles. These retention time shifts allow rapid detection of variant forms that may be present in a biopharmaceutical preparation. For many biologics, particularly those based on monoclonal antibodies, a certain degree of heterogeneity is expected; however, this heterogeneity should be thoroughly characterised between production batches in order to ensure that it remains stable over time. Ion exchange (IEX) chromatography, size-exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) are orthogonal approaches, which offer excellent selectivity and resolution for separating charge variants, size variants and hydrophobicity variants, respectively, in biopharmaceutical preparations.

IEX separates proteins based on charge heterogeneity. Among the various different modes of IEX described in the literature, cation exchange chromatography is the most appropriate mode for biologics. A typical approach involves elution of the sample using a linear salt concentration gradient, with charge variants eluting in order of increasing binding charge. A modified approach (termed 'chromatofocusing') was introduced by Sluyterman *et al.* between 1977 and 1981.[34] This approach involves the use of a pH gradient that can be generated internally in an IEX column. The column is packed with beads of highly-cross-linked poly(styrene–divinylbenzene) (PS/DVB) – selected for its stability across a broad pH range (pH 2 to 12). This alternative approach allows for high resolution separation of isoforms with very minor differences in their isoelectric points.

Unlike many forms of separation, SEC offers a significant advantage in that the comparatively milder aqueous mobile phases used allow biologics to be characterised with minimal impact on their native conformation. The technique is widely employed for the qualitative and quantitative determination of protein aggregates in biopharmaceuticals. Proteins are separated based on their hydrodynamic radius, using a column packed with spherical, porous beads with strictly-controlled pore size. Larger proteins and aggregates cannot diffuse into the pores, and so pass through the column unimpeded, eluting first in the chromatographic run. Smaller proteins diffuse into the pores of the beads, and so take longer to elute. SEC can also be used for approximating protein size by plotting a standard curve of molecular mass (of a range of proteins of different masses) versus retention time.

HIC exploits the hydrophobicity of proteins, which enables their separation on the basis of hydrophobic interactions between the non-polar regions of proteins and immobilised hydrophobic ligands present on the column packing. The adsorption of protein on the column is greater with higher salt concentrations. Therefore, proteins are separated by decreasing the salt concentration of the mobile phase over time. Proteins are not significantly altered using this separation technique – indeed, the technique is often used to purify proteins which maintain biological activity from formulated biopharmaceuticals; such bioactive proteins can subsequently be used for specific activity bioassays.

Reversed-phase HPLC is gaining in popularity for separating variant forms of biotherapeutics, owing to its compatibility with mass spectrometry (LC-MS) and its high resolving power. Hydrophobic proteins adsorb onto a hydrophobic solid support in an aqueous (polar) mobile phase. Increasing the organic solvent in the mobile phase decreases its polarity, and this reduces the hydrophobic interaction between the proteins and the stationary phase, resulting in desorption. The more hydrophobic the protein, the higher the concentration of organic solvent that is required to promote desorption.

Developments and advancements in column and separation technology (such as UHPLC) are greatly accelerating the use of chromatographic techniques for characterising biologics. These techniques offer improvements over more classical techniques (such as electrophoresis) from the perspective of analysis time, precision, selectivity, resolution and a range of other considerations.[35] This is due to the fact that most testing laboratories would have HPLC systems available, and only need to acquire new specialist columns to enable them to perform biopharmaceutical characterisations. UHPLC is particularly attractive during the early development stage (when available sample can often be as low as microgram quantities) as it has extremely small sample requirements when compared to more conventional HPLC.

Spectroscopic profiles

The three-dimensional structure of a protein not only determines size and shape, but also dictates physiological behaviour and biological activity: for example, solubility of a protein depends on a predominance of polar variable groups on the outside of the protein, where they interact with the aqueous

environment, and biological activity is intrinsically linked to the shape of the active site of a protein. Indeed, incorrectly folded proteins may elicit potentially harmful immune reactions, or cause loss of efficacy of the drug as a consequence of antibody response.[36, 37] As such, the higher-order structural elements of biopharmaceuticals need to be thoroughly evaluated in order to ensure drug efficacy and safety. Protein higher-order structure is typically evaluated using a range of spectroscopic analyses, including circular dichroism (CD) spectroscopy, nuclear magnetic resonance (NMR), and infrared (IR) spectroscopy. Stabilisation of protein higher order structure so as to provide practical shelf lives is an ongoing challenge for formulation scientists.[38] Spectroscopic techniques represent the most frequently encountered approach for determining stability of higher order structure as part of product development programmes. Many excipients exhibit their own characteristic responses in CD and FT-IR spectroscopy. Software applications to 'subtract' these exist, but the best approach is to extract the target analyte (i.e. the therapeutic protein) from formulations using solid phase extraction, and perform analysis in the absence of excipients.

CD analysis (in the 'far UV' spectral region; 190-250 nm) can be used for estimating the secondary structural elements (such as the α-helix or β-sheet) of proteins in solution. Each secondary structural element gives rise to spectra of a characteristic profile, as shown in Figure 11.

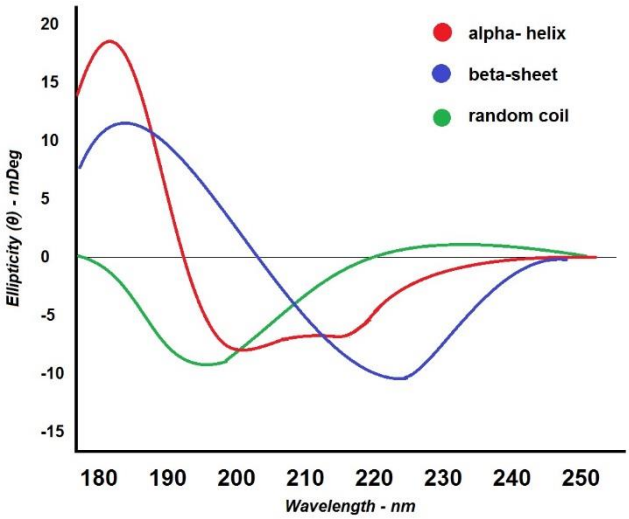
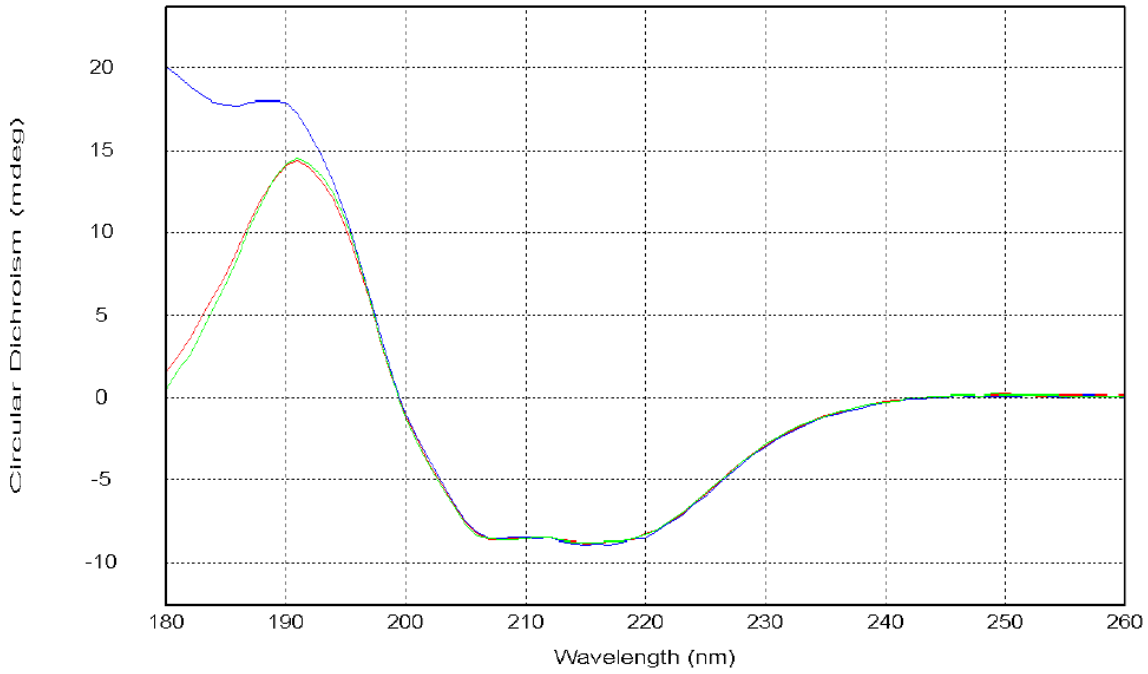


Figure 11: Typical spectra associated with secondary structural features in proteins

Since secondary structures in proteins are subject to denaturation upon exposure to physical or chemical stresses, CD analysis also offers a convenient method to determine thermal stability and formulation stability (including pH) of biopharmaceutical products. For instance, CD is frequently used to explore solvent conditions that increase the melting temperature (and reversibility of thermal unfolding) of proteins, which greatly facilitates development of formulations that prolong product shelf life. CD analysis has very minimal sample requirements (typically in the order of micrograms), and analysis can generally be completed in a matter of hours. The technique measures the difference in adsorption of left-handed and right-handed circularly polarised light by the asymmetric centres of chiral molecules. Ordered secondary structures within biomolecules result in a CD spectrum which can contain both positive and negative signals. Where no ordered structure is present, CD analysis results in a 'zero-intensity' signal.

A limitation of the CD technique is that it does not provide the residue-specific information that can be obtained with high-field NMR or X-ray crystallography analysis – the CD signal obtained for a protein sample represents the average signal for the entire population of molecular chiral centres. However, this

information is complimentary to information from other spectroscopic techniques, enabling detection of differences in a protein that may not be revealed through other spectroscopic analyses – see Figure 12, showing experimental data for three asparaginase preparations, highlighting a difference detected in the 180-200 nm region of the spectra. Therefore, CD analysis can provide information on the proportions of secondary structural elements (for instance, 50% α-helix), but it cannot reveal which specific residues are involved in those structures. CD in the far UV region may also be sensitive to certain elements of tertiary structure,[39, 40] providing information on whether denaturation occurs in a single step (i.e. with simultaneous loss of both tertiary and secondary structure), or if it occurs in a two-step process.



Analysis performed by Applied Photophysics, Leatherhead, UK on the Chirascan-plus qCD Spectrometer with 'Global 3 analysis' software

Figure 12: Normalised CD spectra of three assumed-identical preparations of asparaginase (Native, Recombinant, and Lyophilised).

Fourier transform infrared spectroscopy (FT-IR) is becoming increasingly recognised as a valuable tool for investigating protein structure. It can provide a wealth of information on folding, unfolding and misfolding of proteins. Information provided is complimentary to other methods of higher order structure analysis – e.g. some molecular features that produce a weak signal in CD analysis may produce a much stronger signal using FT-IR. Practically all biological molecules absorb infrared light, and the wavelength and magnitude of infrared light absorption by proteins produces characteristic spectra. The repeating units present in polypeptides and proteins give rise to nine highly-conserved IR absorption bands; amide bands A and B, and seven bands denoted by Roman numerals (I−VII). The amide I and II bands are the two most important vibrational bands in protein IR spectra.[41, 42] Other amide bands result from molecular force fields, the side chains present, and hydrogen bonding; these are often very complex and are of little practical use in the protein conformational studies.[43] FT-IR can be used to analyse proteins in either solution or solid-state, and is not significantly impacted by other sample components, such as buffer salts. Sample preparation, analysis and data interpretation can be completed relatively quickly (from our experience in as little as 15 minutes), and the presence of changes in protein secondary structure can be readily identified from IR spectra.

Pioneering work by Ernst and Wüthrich[44] led to NMR spectroscopy becoming one of only two techniques currently available that can provide structural data with resolution to the level of single atoms (X-ray crystallography also offers this resolution, but this technique is not dealt with in this article). Analysis involves sending radio signals across a range of frequencies through a sample contained in a powerful magnetic field, and measuring the absorption of the different frequencies by protons and isotopically-labelled atoms in the molecule. Absorption of these radio frequencies by atomic nuclei depends upon the local molecular environment, and on how atoms are covalently linked, arranged and move with respect to one another in three-dimensional space. Absorption signals may be perturbed by the presence of neighbouring nuclei, and these perturbations can provide an estimate of the distance between adjacent nuclei. These distances can be reconstructed to determine overall protein structure. Limitations of NMR include that proteins can only be analysed when in highly purified solution (and must not form aggregates when in solution); sample quantity requirements are much higher than for other spectroscopic techniques, and the upper practical limit of protein size is in the order of 50 kDa due to problems arising from overlapping spectra for larger proteins. Also, data collection times can often extend to days, and data processing and interpretation can be a very cumbersome process. More recent developments in NMR analysis of proteins detail solid-state analysis, where proteins are not required to be in solution for analysis,[45, 46] and adaptations of the technique to make it applicable to larger proteins[47] – however, these adaptations still require significant development, with multidimensional tools to the forefront in facilitating signal assignment.

A rationale for biosimilar characterisation

Conventional, small-molecule drugs (such as ibuprofen) depend entirely on easily-regulated chemical manufacturing methods, and production processes for these drugs readily delivers a consistent quality (identity, potency, purity and physical characteristics) of drug between production batches. Upon patent expiry of chemically-synthesised drugs, generics manufacturers can readily replicate production of the active ingredient, and can therefore produce a drug product that is identical to the innovator product. However, biopharmaceuticals are mostly protein in nature, and exhibit molecular complexity several orders of magnitude greater than that for small-molecule drugs. Production of biopharmaceuticals depends upon biological systems (e.g. animal cell cultures), where interplay between metabolomic processes and the environment of the producing cells can result in a high degree of variability in the finished product. This is particularly important when post-translational modifications (which some production cells may perform while others do not) are considered. Therefore, ‘copy-versions’ of biopharmaceuticals cannot be considered generic, as they are very unlikely to be identical to the innovator, and the more appropriate term ‘biosimilars’ has been widely adopted to describe this class of drugs. Since minor differences in structure may produce a biosimilar that is not comparable to the innovator in terms of its safety or efficacy profile, it is important that they are thoroughly structurally characterised and compared to the reference product prior to being granted authorisation. Licensing of biosimilars depends upon having technology available that allows for all the characteristics of a biologic drug to be closely examined. The technologies used should be sensitive enough to be able to detect even minute changes in structure between the biosimilar and the reference product. Therein lies a technical challenge in the biosimilar drug development sector.

Due to the enormous development and production costs for innovator biologics, they are often prohibitively priced, with some treatments costing tens of thousands of US dollars per patient per annum. This puts enormous financial burden on patients, national healthcare systems, and insurance providers. When a sufficient degree of similarity has been demonstrated using analytical characterisation strategies, biosimilars can take advantage of abbreviated licensure pathways, which have reduced dependence on costly and time-

consuming clinical studies. This results in biosimilars typically arriving on the market faster than innovators, and also with substantially reduced development costs. These cost savings can be passed on to the end users, and therefore reduce the cost burden on patients and payers. However, biosimilars still have comparatively large production costs when compared with generic small-molecule drugs, so the cost of a biosimilar still commands typically 80% that of the innovator. Nonetheless, this cost reduction still increases availability of these ground-breaking treatment to a wider patient group.

With patents for a large number of innovator biologics with combined global annual revenue of over US$50 billion set to expire in the period up to 2020, development and production of biosimilars is fast gaining ground. While licensing legislation for biosimilars has been slow to develop in the Unites States, two biosimilars have recently been approved there by the FDA, Sandoz's Zarxio® (filgrastim) and Hospira's Inflectra® (infliximab-dyyb), and these approvals are likely to be followed by many more. This will see the biosimilars market expand enormously over the coming decade, as the US is the primary market for biological drugs. These facts have resulted in a large increase in demand for specialist testing services (and experts) such as mass spectrometry and spectroscopic profiling. This has potential to release significant business opportunities for contract research organisations, such as BioClin Research Laboratories, as the biosimilars market becomes increasingly crowded.

Biotherapeutics compared to conventional drugs

Production methods for biologics compared to conventional drugs

The vast majority of biotherapeutics in common use today are proteins, which are extracted from living cells following growth of those cells in large-scale bioreactors. Many are produced through recombinant DNA technology, involving the insertion of a gene which encodes the protein of interest into the genome of the target cell. When these target cells are cultured under strictly controlled conditions in a bioreactor, they produce the protein encoded by the inserted gene, and this can then be extracted and purified using a range of downstream processing steps. Production of a biopharmaceutical is a highly dynamic process, with parameters such as temperature, pH, and dissolved oxygen continually changing under the influence of metabolising cells, and these parameters need to be monitored and controlled during production. For example, the application or removal of heat is used to control temperature, and the addition of acids or bases in order to maintain the pH within pre-defined limits. There is also the requirement that the specifications of the growth media components and other raw materials used in producing biopharmaceuticals are tightly controlled. A failure to adequately control can impact cell cultures even more than the fermentation process.[48] Since the production of recombinant proteins depends on biological substrates and biological processes, even the slightest alteration in production process parameters can lead to changes in the final product that can affect the identity, safety and/or efficacy profile of the drug. Biopharmaceuticals are almost exclusively intended for parenteral administration which means that they must be sterile. However, being protein in nature, they are often highly sensitive to extremes of temperature, pH and other harsh conditions commonly used to control or eliminate microbial contamination. Therefore, manufacture of biologics must be done under custom-designed 'clean-room' conditions, and using sterile equipment and processes throughout production.

With conventional drugs, production depends on purely chemical means, whereby drugs are synthesised in large reaction vessels, typically through a number of intermediates, using chemicals as raw materials. For example, the production of acetylsalicylic acid (the active ingredient in aspirin) involves esterification of

salicylic acid by acetic anhydride; illustrated in Figure 13, below. The identity of the drug produced through such processes is dependent upon of stringent control over the quality of starting materials, detailed understanding of the stoichiometry involved, and characterisation of any possible side reactions. Process parameters such as temperature and pH are usually easily regulated, and are not subject to unpredictable or erratic drift, such as may be observed in a bioreactor during biopharmaceutical production. Once a process for production of a conventional drug has been appropriately defined and regulated, production of a consistent quality of drug is readily achieved. Indeed, any manufacturer with details of the production process, and the specifications of the starting materials, can readily manufacture the same quality of drug at a site remote from the original manufacturers' site. Clean-room conditions are not usually required, as most chemically-derived drugs are not designed for parenteral administration, or can be sterilised post-filling using heat or other treatments if necessary, as the active ingredient and other constituents in the final formulation are typically stable during such treatments.

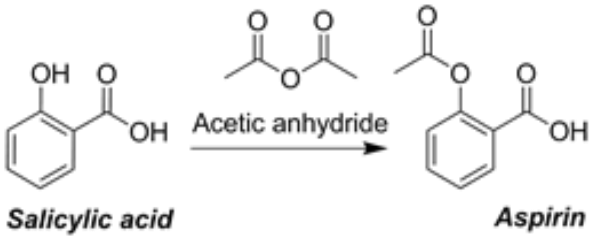


Figure 13: Synthesis of acetylsalicylic acid

Biopharmaceutical complexity

Biopharmaceuticals are much more complex than conventional drugs in many ways, the most obvious of which is the number of atoms of which they are composed and relative molecular mass, as illustrated between aspirin and a monoclonal antibody (a framework on which many biopharmaceuticals are based) in Figure 14, below. It is this complexity that makes biopharmaceuticals extremely difficult to characterise fully, when compared to the same task for small-molecule drugs. The following sections give a brief description of the structural levels of proteins.

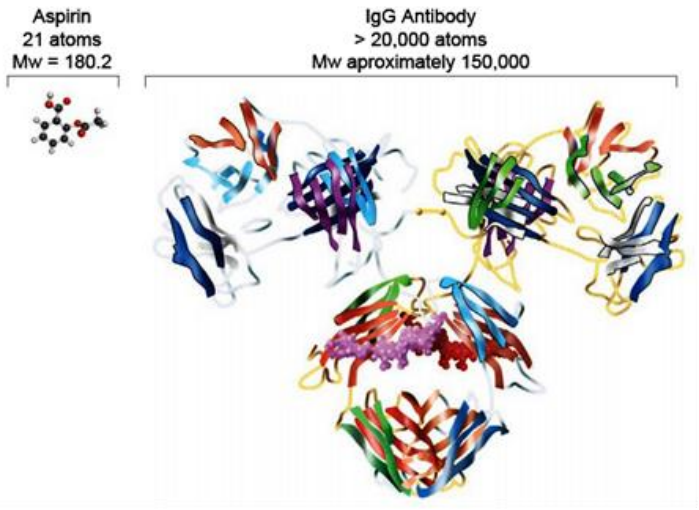


Figure 14: Comparison of size and complexity between conventional drugs and biologics. Source: http://www.amgenbiosimilars.com/the-basics/the-power-of-biologics/

Amino acids – the building blocks of all proteins

All amino acids (with the exception of proline) are primary amines, and all possess an asymmetric carbon (with the exception of glycine) and are therefore chiral. Of the naturally occurring amino acids, only L-amino acid enantiomers are naturally incorporated into proteins. All amino acids possess both a carboxylic acid group and an amine group, both of which are ionised at neutral pH in an aqueous environments (zwitterionic) with the carboxyl group having a net negative charge (COO^-), and the amino group having a net positive charge (NH_3^+) at physiological pH. This ionisation state varies as the pH of the solvent environment varies – in an acidic environment, ionisation of the carboxyl end is suppressed; in an alkaline environment, ionisation of the amino group is suppressed. Figure 15 illustrates the change in ionisation state of amino acids as pH changes in an aqueous environment.

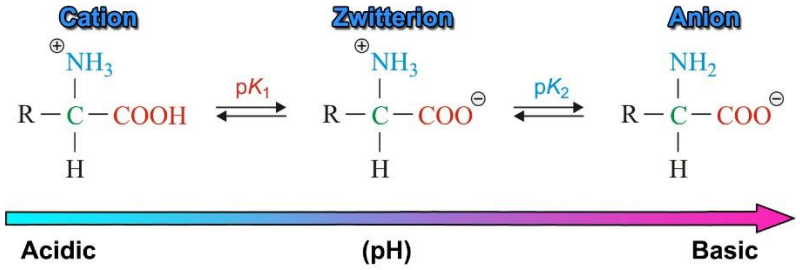
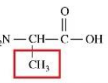
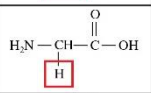
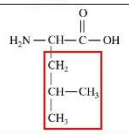
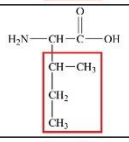
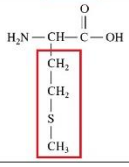
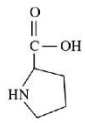
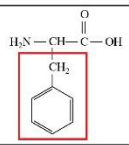
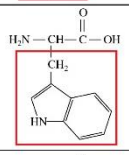
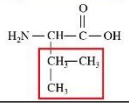


Figure 15: Ionisation state of amino acids in aqueous environments

Each amino acid is characterised by a variable side-group (or 'R'-group) which imparts a range of characteristics including size, charge, water solubility, and reactivity. Tables 4, 5 and 6 below group the amino acids into various categories, based on similar properties of their side groups, and also provides further information on important characteristics for each amino acid.

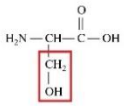
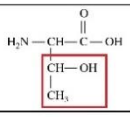
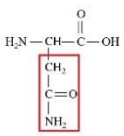
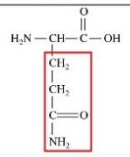
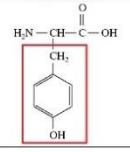
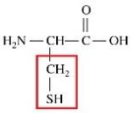
Table 4: Properties of amino acids containing non-polar side chains

Amino acids with non-polar side chains							
Amino Acid	**Abbreviation (3-letter, 1-letter)**	**Structure**	**Molecular mass - *Da***	**pKa1 (α-carboxyl)**	**pKa2 (α-amino)**	**pKa3 (side chain)**	**Properties**
Alanine	Ala, A		71.1	2.35	9.87	N/A	(2S)-2-aminopropanoic acid: one of the simplest amino acids. Its side chain is very non-reactive, and so it is rarely directly involved in protein function. This non-reactive nature also means that the side chain of alanine can be found both on the inside and outside of a protein molecule.
Glycine	Gly, G		57.1	2.35	9.78	N/A	2-aminoacetic acid: the only achiral amino acid and also the smallest and most flexible. Within proteins, it often acts as a structure breaker because of the entropy required to restrain its flexibility. Glycine can play a distinct functional role, such as using its side chain-less backbone to bind to phosphates.
Leucine	Leu, L		113.2	2.33	9.74	N/A	(2S)-2-amino-4-methylpentanoic acid: the most common amino acid. It is hydrophobic due to the presence of the isobutyl side chain.
Isoleucine	Ile, I		113.2	2.32	9.76	N/A	(2S,3S)-2-amino-3-methylpentanoic acid: an isomer of leucine which differs from valine by just one methyl group.
Methionine	Met, M		131.2	2.13	9.28	N/A	(2S)-2-amino-4-methylsulfanylbutanoic acid: one of two sulfur containing amino acids, meaning it can interact with atoms such as metals. However, as the sulfur in methionine is bound to a methyl group, it is far less reactive than the sulfur in cysteine (which is bound to a hydrogen atom). Primarily found in the hydrophobic cores of globular proteins.
Proline	Pro, P		97.1	1.95	10.64	N/A	(2S)-pyrrolidine-2-carboxylic acid: the only amino acid where the side chain is connected to the protein backbone twice, forming a five-membered nitrogen-containing ring, meaning it is more correctly referred to as an imino acid. This structure means that it cannot adopt many of the main chain conformations. Therefore, it can often be found in very tight turns in protein structures such as where the polypeptide chain must change direction. Because of this, it is commonly found on the outer surfaces of proteins, despite being both aliphatic and hydrophobic.
Phenylalanine	Phe, F		147.2	2.20	9.31	N/A	(2S)-2-amino-3-phenylpropanoic acid: one of the three aromatic amino acids along with tyrosine and tryptophan. It absorbs UV light, all though not as well as the latter two. Phenylalanine is converted to a different amino acid, tyrosine by hydroxylation, and is a precursor for the biosynthesis of dopamine and norepinephrine. Its side chain is fairly unreactive and so it is rarely involved directly in protein function, however, it is frequently involved in protein binding or substrate recognition.
Tryptophan	Trp, W		186.2	2.46	9.41	N/A	(2S)-2-amino-3-(1H-indol-3-yl)propanoic acid: the largest and least abundant amino acid. It is a precursor of serotonin, through the precursor 5-hydroxy-tryptophan. Along with the other aromatic amino acids, it is frequently involved in 'stacking' interactions within the cores of protein structures.
Valine	Val, V		99.1	2.29	9.74	N/A	(2S)-2-amino-3-methylbutanoic acid: found in many proteins, primarily in the interior of globular proteins helping to determine three-dimensional structure.

Note: molecular masses in column 4 refers to the residue mass when the amino acid is incorporated into a protein (or peptide)

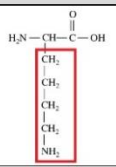
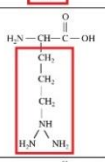
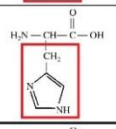
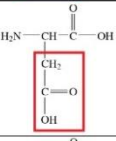
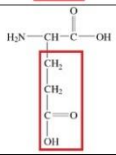
Amino acids are linked together by peptide bonds (peptide bond formation is described below). Two amino acid residues linked by a peptide bond is called a 'dipeptide', while three amino acid residues linked by peptide bonds is called a 'tripeptide'. When many amino acid residues are linked together by peptide bonds, the structure is called a polypeptide, and when a polypeptide is sufficiently long to exhibit higher levels of structure (detailed below), it is referred to as a protein. The possible diversity of proteins is enormous – for example, a simple octapaptide (peptide consisting of eight amino acid residues), there are over 2.5 billion (20^8) possible arrangements. Proteins are typically several hundred amino acid residues long with molecular weights of several tens of kilodaltons (k*Da*), and often, much greater. The correct functioning of a protein is dependent upon the polypeptide chain folding into the correct three-dimensional shape. This is dependent upon four levels of structure – denoted 'primary', 'secondary', 'tertiary', and 'quaternary'.

Table 5: Properties of amino acids containing uncharged polar side chains

Amino acids with uncharged polar side chains							
Amino Acid	**Abbreviation (3-letter, 1-letter)**	**Structure**	**Molecular mass - *Da***	**pKa1 (a-carboxyl)**	**pKa2 (a-amino)**	**pKa3 (side chain)**	**Properties**
Serine	Ser, S		87.1	2.19	9.21	N/A	(2S)-2-amino-3-hydroxypropanoic acid: can be synthesised from glycine or threonine, and is the only amino acid with a primary hydroxyl group. Acts as a nucleophile in the active site of many proteins including serine proteases such as trypsin and chymotrypsin. Can be a hydrogen bond donor or acceptor. Can undergo phosphorylation, and is the most commonly phosphorylated amino acid.
Threonine	Thr, T		101.1	2.09	9.10	N/A	(2S,3R)-2-amino-3-hydroxybutanoic acid: the only amino acid with a secondary hydroxyl group. Can be phosphorylated, though less frequently than Serine. Threonine is common in protein functional centres. The hydroxyl group is fairly reactive, and can readily form hydrogen bonds with many polar substrates.
Asparagine	Asn, N		114.1	2.14	8.72	N/A	(2S)-2,4-diamino-4-oxobutanoic acid: biosynthesized from aspartic acid and ammonia by the enzyme asparagine synthetase. Typically found interacting with the aqueous environment on the surface of proteins owing to its polar nature. Very frequently involved in binding sites as the polar side chain can interact with other polar groups on substrates. Can become hydrolysed to form aspartic acid. When identity is uncertain, it is often abbreviated to ASX to represent 'either aspartic acid or asparagine'
Glutamine	Gln, Q		128.1	2.17	9.13	N/A	(2S)-2,5-diamino-5-oxopentanoic acid: differing by only a methyl group, it has similar characteristics and functions to those of asparagine. Can become hydrolysed to form glutamic acid. When identity is uncertain, it is often abbreviated to GLX to represent 'either glutamic acid or glutamine'.
Tyrosine	Tyr, Y		163.2	2.20	9.21	10.46	(2S)-2-amino-3-(4-hydroxyphenyl)propanoic acid: synthesized from phenylalanine and is the precursor of epinephrine, thyroid hormones, and melanin. Typically found in protein hydrophobic cores. The aromatic side chain means that it is often involved in stacking interactions with other aromatic side-chains. Contains a reactive hydroxyl group, thus making it likely to be involved in interactions with non-protein ligands.
Cysteine	Cys, C		103.1	1.92	10.70	8.37	(2R)-2-amino-3-sulfanylpropanoic acid: the second of two common sulfur-containing amino acids. The role of cysteine in proteins depends on the cellular location of that protein. Within extracellular proteins, pairs of cysteines become oxidised to form disulfide bonds, which covalently link different parts of the polypeptide chain serving to stabilise protein structure. The reducing environment within cells makes the formation of disulfide bonds extremely unlikely, however, cysteines can still have an important structural function in this environment as the sulfhydryl side chain can effectively bind metals, which can be very important for enzyme functions. Cysteines are therefore prevalent in protein active and binding sites.

Note: molecular masses in column 4 refers to the residue mass when the amino acid is incorporated into a protein (or peptide)

Table 6: Properties of amino acids containing charged polar side groups

Amino acids with charged polar side chains							
Amino Acid	**Abbreviation (3-letter, 1-letter)**	**Structure**	**Molecular mass - *Da***	**pKa1 (a-carboxyl)**	**pKa2 (a-amino)**	**pKa3 (side chain)**	**Properties**
Lysine	Lys, K		128.2	2.16	9.06	10.54	(2S)-2,6-diaminohexanoic acid: most often found on the surface of proteins, lysines frequently play a part in determining protein structure - involved in formation of salt-bridges, where they pair with a negatively charged amino acid to create stabilising hydrogen bonds, that can be important for protein stability. Quite often found in protein binding sites.
Arginine	Arg, R		156.2	1.82	8.99	12.48	(2S)-2-amino-5-(diaminomethylideneamino)pentanoic acid: the most basic amino acid. Its positive charge is extensively delocalised meaning it can donate several H-bonds. It is frequently involved in determining protein structure, being frequently involved in forming salt bridges. The positive charge means it can interact with negatively charged non-protein atoms making it frequently found in protein active and binding sites.
Histidine	His, H		137.1	1.80	9.33	6.04	(2S)-2-amino-3-(1H-imidazol-5-yl)propanoic acid: the only amino acid whose pKa is in the physiological range, meaning it is relatively easy to move protons on and off the side chain. This means that it can be found in both the core and surface of proteins and is the most common amino acid involved in protein active centres.
Aspartic Acid	Asp, D		115.1	1.99	9.90	3.90	(2S)-2-aminobutanedioic acid: generally found on the surface of proteins exposed to an aqueous environment, although are often also found in hydrophobic cores involved in salt bridges where they pair with positively charged amino acids, stabilising protein structure. Often involved in active centres where they can bind positively charged non-protein atoms, such as zinc.
Glutamic Acid	Glu, E		129.1	2.10	9.47	4.07	(2S)-2-aminopentanedioic acid: similar characteristics and functions to those of aspartic acid above – indeed, these two amino acids can often be found substituted for one another in proteins with no measurable impact on protein function or specificity.

Note: molecular masses in column 4 refers to the residue mass when the amino acid is incorporated into a protein (or peptide)

Continues….. p67

Protein primary structure

The primary structure of a protein refers to the linear sequence of amino acid residues in the polypeptide chain(s) (which is determined by the encoding gene sequence). The positions of cysteine residues and disulphide bonds, which are covalent bonds between cysteine residues in the polypeptide chain, are also considered as part of primary structure. Amino acids form unbranched polymers (polypeptides) through nucleophilic attack of the electrophilic carbonyl group at the carboxyl end of a polypeptide, by the amino group of the amino acid being added to that polypeptide. For this to take place, the carboxyl group must first be activated by adenosine triphosphate (ATP) to provide a better leaving group than the hydroxyl. The link formed in this reaction is called a peptide bond – illustrated in Figure 16. A single water molecule is liberated in the formation of each peptide bond, which means that amino acids once incorporated into a growing polypeptide, are more correctly referred to as amino acid residues. The primary structure of a protein is always written as the sequence of amino acid residues from the amino terminal end (*N*-terminus) to the carboxy terminal end (*C*-terminus). Post-translational modifications (PTM's) of a protein, such as phosphorylation and glycosylation, are also considered to be part of the protein primary structure, however, information on these cannot be derived from the encoding gene.

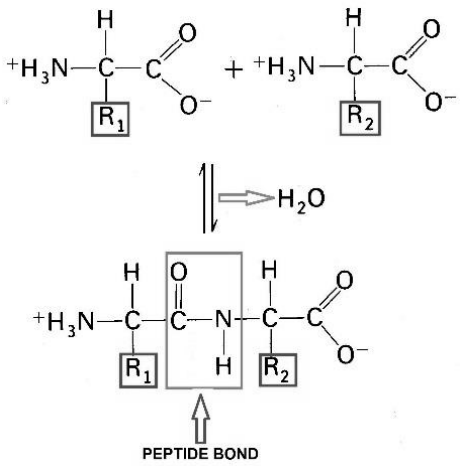


Figure 16: Peptide bond formation

Protein secondary structure

Secondary structure arises when the variable groups of amino acid residues interact locally with each other (and with their immediate environment) through hydrogen bonds and other non-covalent interactions. These interactions give rise to highly-stable structural motifs, the most prevalent of which include the alpha helix (α-helix) and the beta sheet (β-sheet). The α-helix forms between residues that are within close proximity to each other in the polypeptide chain, while β-sheets are formed between residues that are distant from each other. Figure 17 illustrates these structural elements.

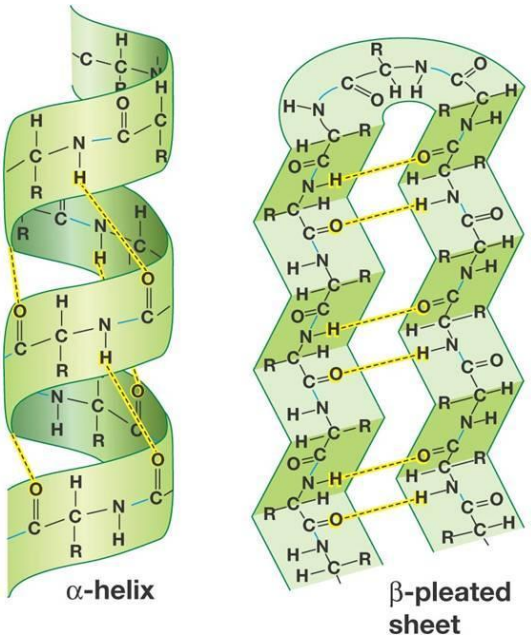


Figure 17: Common secondary structural elements of proteins

The α-helix, discovered in 1953,[49] is a 'right-handed' helix. The internal part of the helix is composed of the polypeptide backbone, with the R-groups of the amino acid residues projecting outwards on the helix. The structure is stabilised by hydrogen bonds between the carbonyl group of each amino acid residue with the amine group of the amino acid residue four resides away in the polypeptide chain. The length of α-helices in proteins can range from a few to several tens of residues, and the presence and number of α-helices in proteins can vary considerably. For example, globular proteins (which function as membrane receptors) tend to have greater α-helix content than other proteins.

The β-sheet structure which was elucidated in 1951,[50] is composed of two β-strands of polypeptide (either intermolecular or intramolecular). These strands are approximately five to ten residues long, and are associated with each other through hydrogen bonding between the carbonyl groups of one β-strand, and the amine groups of the adjacent β-strand. The alternate α-carbons between adjacent amino acid residues lie above and below the plane of the sheet, giving the structure a pleated configuration. The strands may both be aligned with their *N*-termini at the same end, in which case they are called parallel β-pleated sheets, or with the *N*-termini at opposite ends, in which case they are called antiparallel β-pleated sheets.

A number of well-ordered three-dimensional structural motifs are also commonly found in proteins, and these are collectively referred to as 'supersecondary structure'. These structural motifs facilitate correct folding of the protein. Examples of these include the 'helix-loop-helix' motif, which is composed of two α-helices joined by a loop; and the 'hairpin β-sheet motif', which contains two antiparallel β-sheets joined by a loop. Figure 18 illustrates some of the more commonly occurring supersecondary structural motifs.

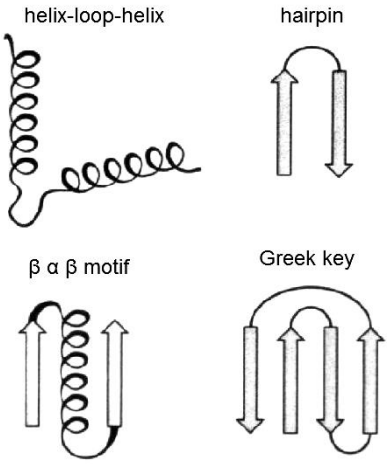


Figure 18: Protein supersecondary structural motifs

Torsion angles and the Ramachandran plot

The torsion angles in a polypeptide describe the rotation of the polypeptide backbone around two bonds – the bond between the α-carbon and nitrogen (called the phi, or φ), and the bond between the α-carbon and the carbonyl carbon (the psi, or ψ) – refer to Figure 19. These torsion angles are very important local structural parameters that control protein folding, because certain bond angles are restricted, as they would result in steric hindrance between atoms.

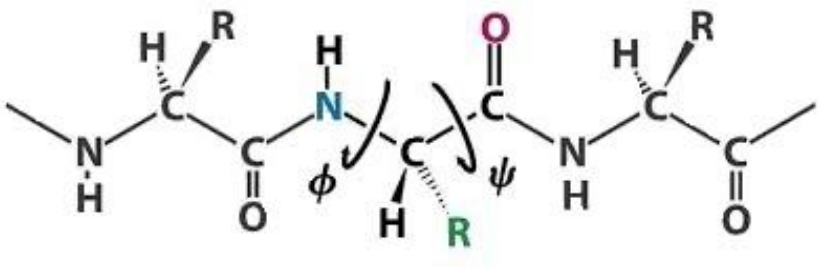


Figure 19: Torsion angles in polypeptide chains

The Ramachandran plot (illustrated in Figure 20), which was developed in 1963 by Ramachandran *et al*.,[51] is a way to visualise the distribution of all possible torsional angles in protein structure. It plots φ angles on the x-axis and the ψ angles on the y-axis which provides an overview of allowed and disallowed torsional angles. Due to steric hindrance, the allowed torsional angles are constrained within specific areas of the plot, particularly for secondary structures such as the α-helix or the β-sheet. In practical terms, the Ramachandran plot is a reliable method for predicting protein structure, but some proteins may include angles in the disfavoured regions – where this occurs, additional interactions will be present that help to stabilise the structure.[52] Ramachandran plots are often used to validate results obtained from structural analysis via X-ray crystallography or NMR analysis – problems with the experimentally-derived structures will be revealed where a large number of torsion angles are found in the disfavoured regions of the plot.

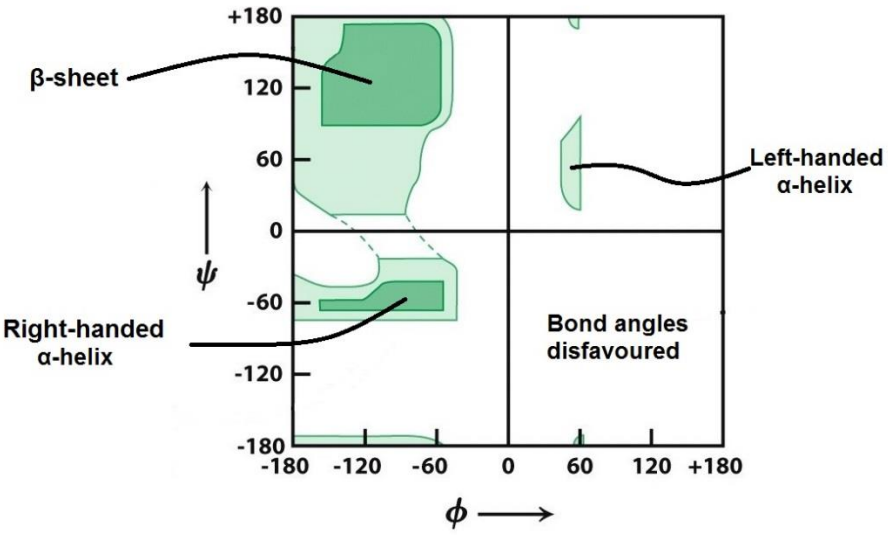


Figure 20: Ramachandran plot: dark green: low-energy regions where torsion angles are highly-favoured; light green: allowed regions; white: highly disfavoured regions.

Protein tertiary structure

Tertiary structure refers to the overall spatial arrangement of the polypeptide chain following the development of the secondary and supersecondary structural elements, to produce the compact globular shape of the protein. This conformation is determined by the combination of secondary structures to form protein 'domains'. It is generally accepted that the tertiary structure of a protein is the most thermodynamically stable arrangement. The tertiary structure of proteins is absolutely critical to their function, and for this reason is a critical characteristic of therapeutic protein that needs to be thoroughly characterised. Globular proteins generally have tertiary structures with hydrophobic residues at the core of the molecule, and a surface with hydrophilic residues exposed. This arrangement helps to stabilise the protein while also increasing its water solubility. Figure 21 shows a 'ribbon diagram', also known as a "Richardson diagram", which is a 3-D representation of protein tertiary structure in common use today.

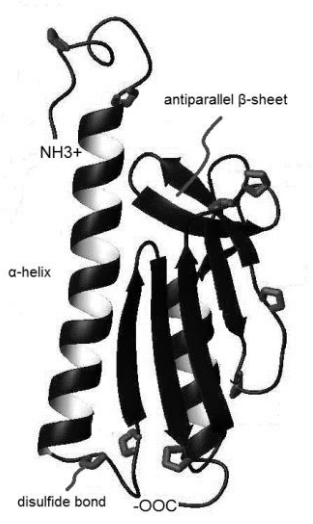


Figure 21: Richardson diagram illustrating protein tertiary structure

Protein quaternary structure

Quaternary structure refers to the covalent and/or noncovalent association of two or more protein subunits to form a functional protein. These subunits may have identical or different amino acid sequences and structures. Not all proteins exhibit quaternary structure (i.e. proteins composed of only one polypeptide chain). Monoclonal antibodies are examples of proteins that exhibit quaternary structure, being composed of four subunits; two identical 'heavy chains', and two identical 'light chains', which are linked together through a series of disulphide bonds and noncovalent interactions. In order to characterise proteins which exhibit quaternary structure, it is often necessary to first dissociate the subunits from one another to be characterised separately, then rebuilding the picture of the complete functional protein.

Post-translational modifications of proteins

Post-translational modifications (PTM's) of polypeptide chains can extend the functionality of proteins by covalently attaching chemical groups, or in some cases, cleaving chemical groups or signal peptides from the molecule. PTMs often play a critical role in the functionality of proteins,[53] and can also be important in regulating cellular functions – for example, many enzymes are activated through phosphorylation by kinases. PTMs are highly dependent on production cell lines. Prokaryotic organisms, such as *Escherichia coli*, (which has been used as an expression system for biopharmaceuticals for many years) do not significantly modify proteins following translation, however, eukaryotic cells often do modify proteins. For instance, recombinant asparaginase produced in *Escherichia coli* is non-glycosylated, whereas recombinant erythropoietin (used in treating anaemia) produced in Chinese hamster ovary (CHO) cells is heavily glycosylated, with carbohydrate accounting for up to 40% of the mass of the molecule.[54]

A large number of PTMs are commonly encountered including glycosylation, *S*-nitrosylation, methylation, *S*-palmitoylation, and many others. Glycosylation is acknowledged as one of the most significant PTMs, and can have an effect on protein secondary (and higher order) structure, function and stability.[53, 55, 56] Glycosylation involves the attachment of sugar moieties ranging from simple monosaccharides to highly complex branched polysaccharides. *S*-nitrosylation involves reaction of free cysteines with nitric oxide (NO) to form *S*-nitrothiols. This PTM has a major stabilising effect on proteins and also plays a part in regulating enzymes involved in gene expression.[57] Methylation involves the addition of methyl groups to nitrogen or oxygen (*N*- and *O*-methylation, respectively) or to amino acid R-groups, which increases the hydrophobicity of the protein, thereby enhancing cell membrane association.[58] *S*-palmitoylation attaches a C_{16} palmitoyl group to cysteine residues; this long hydrophobic group facilitates anchoring of the protein in the lipid membrane of cells.[59] Identifying, characterising and understanding the role that PTMs play in protein function is critical to the study of recombinant proteins as biopharmaceuticals.

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Derek Bradley *is a PhD candidate, having successfully secured a scholarship from the Irish Research Council's 'Employment-Based Postgraduate Programme'. Derek's research aims to develop methods to address a significant challenge in the biopharmaceutical industry: – in-depth characterisation of biopharmaceuticals using state-of-the-art analytical techniques. The research is based primarily in an industrial setting at BioClin Research Laboratories in Athlone, with the BioScience Research Institute (BRI) at Athlone Institute of Technology (AIT) providing the academic framework. Prior to this research project, Derek worked as an analytical scientist at BioClin for over two years.*

Mary Burke *has worked in the pharmaceutical industry for 32 years. After graduating from University College Galway with a BSc (Hons) in Biochemistry, she joined Elan Corp as a Bioanalytical scientist in 1984. She was Senior Director of Clinical Pharmacology/ Bioanalysis before she left Elan in 2002 to set up Bioclin Research Laboratories, where she holds the position of Managing Director. In addition to overseeing the strategic development of BioClin, she still maintains direct responsibility for the bioanalysis/mass spectrometry department.*

James J. Roche *worked for ten years in Elan Corp before transferring to Athlone Institute of Technology where he co-ordinates the BSc (Honours) in Pharmaceutical Sciences and lectures in analytical chemistry in the Faculty of Science & Health. Eligible for nomination as a Qualified Person for the pharmaceutical industry, Jim is a Chartered Chemist, a Member of the Royal Society of Chemistry and a Member of the Institute of Chemistry of Ireland. His research activities in follow-on biologics are informed by interests in trace analysis and in the evolution of the concept of patent sufficiency.*

This research is supported in part by a scholarship from the Irish Research Council under the Employment-Based Postgraduate Programme (EBPP), for which the authors extend their gratitude.

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EcoBalt 2016 is an international research conference that will be held on **9.–12. October 2016** in **Tartu, Estonia** (in Dorpat conference centre). The conference will focus on the most recent scientific and technological developments in the field of environmental analysis, environment and its protection. With the support by the EcoBalt conference series, EcoBalt 2016 is organized by the *University of Tartu* with *Tallinn University of Technology* and *Estonian Environmental Research Centre*.

!Great news! EcoBalt2016 has received grant for organisation of international events and conferences from Estonian Ministry of Economic Affairs and Culture in cooperation with the Enterprise Estonia tourism development centre. This means that a lot is included in the conference fee, most notably, conference dinner and excursions.

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Eurofin Lancaster announces 500 new jobs for Waterford

Posted on16 June 2016.

eurofins | Lancaster Laboratories

Eurofin Lancaster has announced that it will be creating 160 for Waterford. The jobs will be created over the next five years and will bring the companies employee total to over 500.

IDA Regional Manager Anne Marie Tierney La Roux says securing new investment like this to Dungarvan is significant. She says they have been working with Eurofin on this for some time.

Waterford's Minister for Training and Skills John Halligan TD has welcomed the creation of the new biopharma jobs in Dungarvan.

Eurofins Lancaster Laboratories, part of the global Eurofins Scientific that works with pharmaceutical, chemical, biotechnology, medical device and cosmetic clients, announced on Monday that the new jobs would be created over the next five years.

Fine Gael Waterford Senator Paudie Coffey has welcomed news and says that this announcement will be a major boost for Dungarvan and will take the total number of employees to over 500 in West Waterford.

"This is a hugely significant announcement for West Waterford and Dungarvan Town. Before the last general election I consistently called for more of a focus on West Waterford as many IDA jobs had been brought to the City and East of Waterford County. I am delighted that this announcement has been made today and it will have a massive impact on Dungarvan's local economy.

"There can be no doubt that the economy is recovering and it is imperative that we bring the recovery home; so that families right around Waterford feel its impact in a real and tangible way.

"I will continue to work with my Ministerial Colleagues and the IDA to ensure that investments like this are brought to Waterford. While it's not possible to bring every investment to Waterford, it is critical that we get our fair share."

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The Sustainable Energy Authority of Ireland Appoints New CEO

Posted on21 May 2016.

seai SUSTAINABLE ENERGY AUTHORITY OF IRELAND

The Board of the Sustainable Authority of Ireland (SEAI) is pleased to announce the appointment of Mr Jim Gannon as CEO of the Authority. The appointment takes place with effect from 23rd May 2016 and Jim will lead the development and delivery of the Authority's new five year strategy.

SEAI Chair, Ms Julie O'Neill said: *"Jim Gannon is an energetic and widely respected energy professional with a wealth of experience across all aspects of energy policy implementation. Working with the executive team, he will build on the successes of recent years and lead the organisation into the next phase which demands a rapid decarbonisation of our entire energy system. I wish Jim all the best in his new role and have every confidence the organisation will prosper under his leadership."*

Speaking on his appointment, Mr Gannon said: *"The new White Paper and recent commitments made at COP21 in Paris provide Ireland with both challenges and significant opportunities over the coming period. As a result, Ireland is likely to experience change at an infrastructural scale in addition to a technology-led democratisation of the energy system across electricity, transport and heat. I look forward to leading SEAI as it plays a central role in our transition to a more economically, socially and environmentally sustainable energy future."*

Mr. Gannon is an Engineering Graduate of NUI Galway, with a Masters in Environmental Assessment from the University of Wales Aberystwyth and MBA from the UCD Smurfit School of Business. He has worked within the energy sector throughout his career, delivering projects at a European, national and regional level for public and private sector organisations. This has included projects across conventional and renewable energy, transmission and distribution infrastructure, energy demand management and technology development. Most recently, he held the position of Director at RPS Group, leading the Energy, Environment and Health and Safety sector.

Portable benchtop NMR spectrometer from GPE for Fluorine, Proton or Dual NMR

The spectrometer offers spectroscopic resolution at a fraction of the size and maintenance of current NMR instrumentation. The design brings analytical performance and point-of-need utility to the benchtop, fume hood or glovebox due to the compact size of the machine.

The NMReady was the first 60 MHz spectrometer available on the benchtop NMR market. Given its small footprint (Dimensions: 9.5 x 11x 17 inches) and light weight nature (only 45 lbs), the spectrometer is ideal for incorporation directly into the laboratory. The NMReady is compatible with all standard consumable 5mm NMR tubes, also available from GPE Scientific, so sample preparation is simple and fast.

The machine offers good sensitivity and the high resolution allows spectra to be measured quickly. The data can be processed directly (even while wearing safety gloves) through the built-in resistive touchscreen without connecting an external computer.

Contact Information:

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E-mail: info@gpescientific.co.uk

Website: http://www.gpescientific.co.uk/products/chemistry/nanalysis-nmready-benchtop-spectrometer

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GPE Scientific Ltd was established in 1962 and is a leading distributor and manufacturer of laboratory equipment, glass blowing products and specialised glass components for the industrial, laboratory and research markets. There are many reasons to choose GPE Scientific above our competitors; we pride ourselves in stocking thousands of products from leading suppliers providing you with the best selection of laboratory equipment on the market. This includes being the exclusive distributors for Chemglass Life Sciences and Chemical Reactors, Norell NMR Tubes and Accessories and the portable Nanalysis NMReady Benchtop Spectrometer.

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130 jobs at risk in Tipperary pharmaceutical company

Posted on17 May 2016. Tags: Jobs Loss, manufacturing, pharmaceutical, Suir Pharma, tipperary

More than 130 jobs are at risk following the appointment by the High Court of a provisional liquidator to a Co Tipperary based pharmaceutical manufacturing company.

Suir Pharma Ireland Ltd, which is located in Clonmel and has been making generic medicines for approximately 40 years in the town, is insolvent and is to be wound up.

The company cited sustained and irreversible losses of €4.9m during the 15 months before March 2015, and a large drop in sales in the US market late last year, for its difficulties, At the High Court on Tuesday

It projects further losses in both 2016 and 2017.

The court heard its parent company, Saneca Pharmaceuticals, was not willing to fund the company and it was left with no other option than to apply to the court for the appointment of a provisional liquidator.

Mr Justice Michael Twomey said he was satisfied to appoint insolvency practitioner Michael McAteer of Grant Thornton as provisional liquidator of the company.

The court also granted Mr McAteer a number of powers, including the ability to allow the company continue to trade, and to take steps to secure the company's assets.

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Merck to create 70 jobs for Cork

Posted on22 April 2016. Tags: Cork, investment, Jobs, Merck

MERCK
Be well

70 new jobs are to be created at Merck in Co. Cork. The announcement comes after the medical science and Technology Company officially opened their new manufacturing and R&D facilities at their key strategic site in Carrigtwohill, Co Cork on Tuesday.

Martin McAuliffe, site leader, says the speciality membrane facility (SMF) and chromatography media facility (CMF) represents a € 55million investment in Merck's Cork operation, reinforcing the strategic importance of the Carrigtwohill site to the wider Merck Group.

"We are adding R&D capability to develop and scale up. A plant in the US is closing so the development of membranes will be done in Cork. We also do antibody development on-site. We will be adding 60 to 70 new positions initially with additional positions in the next 18 months."

Roles in general manufacturing, R&D, engineering, chemistry, microbiology and quality assurance are being created. This will add to the already 700 people employed at Merck's Cork operation, with 400 construction workers to be employed to build the two specialist centres.

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Shire to expand biotechnology manufacturing capacity in Ireland

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Shire plc (LSE: SHP, NASDAQ: SHPG) announces that it plans to expand its global biotechnology manufacturing capacity over the next four years by investing US$400 million in Ireland to meet the rapidly growing demand for its highly innovative products and robust pipeline.

Shire will create a new, state of the art biologics manufacturing campus, which it expects will lead to the creation of approximately 400 permanent jobs on a 120 acre site at Piercetown, County Meath. Construction of the new site will begin in mid-2016 with the site expected to be operational by mid-2019.

"Increasing our biologics manufacturing capability is fundamental to achieving our ambition of becoming the world's leading Biotechnology Company focused on rare diseases and other speciality conditions. The expansion will enable us to meet increasing product demands, support our pipeline and, ultimately, help more patients. We are delighted to be expanding our operations in Ireland, where we have strong, existing relationships. The new campus in County Meath will provide necessary geographic diversification and will help us serve our growing international business."*Shire's Chief Executive Officer, Flemming Ornskov, MD, MPH,*

Employing the latest bioprocessing techniques, the facility will employ flexible production strategies enabling it to supply both clinical and commercial scale products. The roles at Shire's new campus in County Meath will consist of highly skilled, full time jobs in the areas of R&D, operations, technical staff, engineering and construction.

Shire's Senior Vice President of Technical Operations, Tim Kelly, and commented "The expansion supports our manufacturing strategy Ireland is a strategically important location for Shire, providing both excellence in life science R&D and manufacturing. We already have a strong team on the ground in Ireland and believe that it is the right location for us to build a new state of the art facility which will complement our existing manufacturing operations in the US."

Mr. Richard Bruton TD, Irish Minister for Jobs, Enterprise and Innovation, said "Today's announcement is a huge boost for the Irish economy and for County Meath. The scale of the global investment being made by Shire and the creation of 400 full-time positions in Ireland, as well as over 700 additional jobs during construction, will have a major impact, and further validates Ireland's position as a leading global location for biotechnology manufacturing and R&D. Biotechnology is a key sector which we have targeted as part of our jobs plans, and Shire's decision to build on its very capable team already in Ireland with a $400million investment is a very welcome step-up in its presence here."