QUALITY BY DESIGN FOR THE DOWNSTREAM PROCESSING OF BIOPHARMACEUTICALS

Phillip Wayne Elliott

Bachelor of Biotechnology (Hons)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

THE UNIVERSITY of ADELAIDE

School of Chemical Engineering

University of Adelaide, Australia

February 2015

Thesis Abstract

This thesis explores the concept of "Quality by Design" (QbD) as proposed by the FDA and demonstrates how this concept can be used and applied to the manufacture of biopharmaceutical proteins. QbD principles and approaches have been adopted in carrying out the development of unit operations that are typical for most biopharmaceutical products and to carry out studies that are required for the successful filing of a biopharmaceutical application. The introduction defines Quality by Design and discusses why this is an important topic. To better understand and define QbD, the history of the concept is reviewed with emphasis on how it came to be applied in the area of Pharmaceutical development, how it should be defined in practice and how it has been applied to the manufacture of biopharmaceuticals.

Protein A chromatography is the most commonly used technique for the manufacture of monoclonal antibodies today. To make the results of this work widely applicable a QbD approach is developed to compare the suitability of different Protein A resins for the manufacture of a monoclonal antibody product with the aim of selecting an optimal or preferred Protein A resin for the purification of a monoclonal antibody at industrial scale. Resins identified for closer scrutiny are used to purify a biopharmaceutical monoclonal antibody and the product of the resins is compared to identify a Protein A resin to act as the basis of a manufacturing platform. Experimental work is also directed at determining how well the standard models used to explain the equilibrium binding of Protein A to an IgG molecule represent the binding behaviour seen in practice using an industrially-relevant IgG feedstock. As a result of this research a preferred equilibrium model for use with Protein A and IgG-conditioned medium is identified.

Isothermal titration calorimetry (ITC) is used to determine the mechanistic reasons why one Protein A resin (Mab Select SuRe™) was superior to other protein A resins in its performance. It is shown that Mab Select SuRe™ has a markedly higher K_a compared to

native Protein A and the ability to bind the monoclonal antibody under pH conditions much lower than native Protein A. Further, the affinity of native Protein A for IgG is shown to be increased by increasing salt concentration whereas Mab Select SuRe™ is shown to be insensitive to the salt concentration during binding.

QbD concepts and techniques are then applied to a chromatography resin lifetime study, a unique and specific requirement for biopharmaceutical manufacture, to improve the efficiency of the study and gain more knowledge from limited experimentation. A resin lifetime study is carried out using a model biopharmaceutical protein feedstock and the data analysed using statistical quality control techniques to understand how these techniques, an important part of QbD, can be used to improve understanding and control of the manufacturing system.

While chromatography is the most commonly used technology for downstream purification of biopharmaceuticals, tangential flow filtration (TFF) is probably the second most common unit operation in the manufacture of these products. To determine how QbD can be applied to TFF a case study is developed in applying QbD techniques and mechanistic understanding to the development of a TFF unit operation for a biopharmaceutical protein. Multiple membrane types and operating modes are compared for use with the protein. QbD techniques are used to optimize the manufacturing settings using the most preferred filter membrane to produce a robust and reliable manufacturing process at commercial manufacturing scale. The results of scaling up and using the developed process at full commercial manufacturing scale are also reported and compared to the laboratory scale development to confirm the effectiveness of the QbD development approach.

1 Table of Contents

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give consent to this copy of my thesis when deposited in the University library after the embargo period expires in February 2017, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library search and also through web search engines after February 2017.

Phillip Elliott

Acknowledgements

I would like to acknowledge Ben Page for assistance in carrying out TFF experiments, Irina Mitina for assistance in preparing a comparison of available Protein A resins and Dr Bulliraju Edupuganti for assistance in carrying out the resin lifetime study on CFT resin. I would also like to acknowledge the guidance and forebearance of my supervisors, Dr Jingxiu Bi, Dr Hu Zhang and Dr Sam Billingham and thank them for the review of drafts of various chapters.

I would also like to thank Hospira Ltd for supporting me through this work and giving me a lot to learn.

To my wife and son

Who make everything fun

Table of Figures

List of Tables

1 Thesis Introduction

The overall aim of the thesis is to explore the recent concept of "Quality by Design" (QbD), as proposed by the United States Food and Drug Administration (FDA), and define how this concept can be used and applied to the manufacture of biopharmaceutical proteins.

1.1 The problems of regulation and the pharmaceutical industry

There have been many recent drug shortages due to FDA warning letters or actions against drug manufacturers. In general these have arisen because the FDA felt that the manufacturers were not carrying out their production processes in a manner that assured quality or were sufficiently safe. The number of prescription drug shortages in the United States nearly tripled between 2005 and 2010, and shortages are becoming more severe as well as more frequent. These drug shortages have affected consumers, as they do not have access to important medicines, as well as tarnishing the reputations of both the FDA and drug companies and encouraging distrust and acrimony between these two groups [1]. This has been serious enough to result in an Executive Order from the US President about the matter [2]. In general this demonstrates a mismatch between the expectations of regulatory agencies as to what is acceptable and what manufacturers deem to be acceptable and profitable. In addition the FDA and others have criticized the low rate of innovation and high cost of pharmaceuticals[3].

1.2 Proposed benefits of QbD

A new approach to the development of pharmaceutical products and their subsequent manufacture has been advocated by the FDA and the International Conference on Harmonization (ICH). This approach has been termed "Quality by Design" (QbD) and is defined as " a systematic approach to development that begins with pre-defined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [4]. Quality by Design has been proposed as a solution

to the mismatch problem mentioned above, though it is yet to be seen if this will be successful. According to the concept of QbD, when designing and developing a product, a company needs to define the desired product performance. On the basis of this design, the company then designs the product formulation and process to meet those product attributes. Ideally this leads to understanding the impact of raw material attributes and process parameters on the pharmaceutical product and identification and control of sources of variability. As a result of all this knowledge, a company can continually monitor and update its manufacturing process to assure consistent product quality [5]. The QbD concept promotes industry's understanding of the product and manufacturing process starting with product development, with the aim of building quality in from the start rather than trying to test quality into the product during manufacture.

There is much enthusiastic literature on the proposed benefits and cost reduction that the use of QbD approach will achieve [6, 7]. The proposed benefits include ensuring better design of products and manufacturing process by thorough understanding of active components, excipients, and process; allowing implementation of new technology without regulatory scrutiny; and reducing the number of manufacturing supplements for post-approval changes.

1.3 Reception by Industry and initial outcomes of QbD

It should be noted that drug regulatory authorities generally base their decisions on three criteria – quality, efficacy and safety. However, they take no account of the actual or potential cost of the product to the company. Therefore in addition to the need to be compliant with regulatory requirements it is important for a company to examine the value for money it obtains from process development, acquisition of knowledge and QbD in general, and deploy these approaches in the most cost effective manner [8]. A 2012 survey of industry participants found that approximately 20 % of respondents thought lack of cost effectiveness was the biggest hindrance to adoption of QbD in biologics, with a similar number citing fears

of regulatory delays as the biggest hindrance [9]. This further emphasises the importance of the study of QbD as applied to biopharmaceuticals.

At a recent conference on QbD, Roger Nossal, Vice President and Head of Pfizer Global Chemistry, Manufacturing and Controls, spoke about some of the difficulties that have been encountered with implementing QbD in the biopharmaceutical industry. It has been Pfizer's experience that when regulatory filings to the FDA or the EMA are made using a QbD approach, much more scrutiny has been applied by the regulatory bodies than seen with more traditional filings with less information. He stated that Pfizer have prepared and shared case studies as "we cannot do this alone, we need other companies and regulators to help share the load". He also added that for regulatory bodies, change management is a big concern – whether or not companies will make scientifically sound decisions in moving within a "design space" if approved and whether the safety of a biopharmaceutical product will be assured by the change management process within a company. That is, whether companies could be trusted to move safely within an approved design space [10].

At the same conference Lynne Krummen, a representative of Genentech (Roche) also spoke about that company's experience with QbD submissions and the challenges and successes that had been encountered. Genentech (Roche) have participated in the FDA pilot program for QbD submissions, putting two molecules into the program as QbD submissions and working closely with the FDA and the European Medicines Agency (EMA) on development of the submission. Surprisingly, considering the close collaboration with the regulatory authorities, only one of the design spaces was accepted at filing and one of the submissions was rejected. This reflects just how difficult QbD is to interpret and implement, even when carried out by professionals in the field in close collaboration with the relevant authorities [11].

The European Federation of Pharmaceutical Industries and the EMA have stated that that each pharmaceutical product is unique - developing any product will require a "bespoke" approach and therefore there is no standard blueprint for applying QbD [7].

1.4 Purpose of this thesis

Due to the desire of governments to protect public health and safety, the Pharmaceutical industry is one of the most highly regulated industries. The pharmaceutical industry lies at the nexus of high technology, legal and regulatory requirements and QbD is a regulatory change designed or intended to spur technological innovation in the area or at least reduce some of the negative effects of regulation on development. It is through the QbD initiative that regulatory agencies are hoping to encourage innovation while also assuring the safety of citizens – the ultimate users of the products of the pharmaceutical industry.

Quality by Design is an important topic in the study of pharmaceutical science as this concept is being actively promoted by the FDA and the EMA, probably the most important pharmaceutical regulatory agencies worldwide. Due to the regulations and laws that apply to pharmaceutical development and approval these laws and regulations are very important considerations when developing any pharmaceutical product. To a large extent, the regulations determine what sort of development is required, the extent of development required and therefore the amount and type of resources required for new pharmaceutical products to be developed. The QbD concept was developed in response to perceived problems, delays and cost blowouts in the development of new pharmaceutical products. In an attempt to alleviate these problems QbD is now being actively promoted by the US FDA as the preferred method of development. The intent of QbD is to encourage pharmaceutical companies to demonstrate sufficient understanding of their products and manufacturing processes to the regulatory agencies in exchange for a more flexible regulatory approach. For example, if a pharmaceutical product is approved for sale on the basis of a QbD application, it may be possible to make improvements to the manufacturing process after the product has been approved for sale, without the need to go through an expensive "post-approval change process" with the pharmaceutical regulator. In this way it is hoped that the QbD approach will improve product quality and reduce regulatory compliance costs for pharmaceutical

manufacturers. QbD is an important topic of study as the QbD concept is currently being used to shape the regulatory landscape for pharmaceuticals and biopharmaceuticals worldwide, determining how they are developed and the techniques and sciences that will be required for future development and studies.

1.4.1 Summary of knowledge gap

While the principles of QbD have been used for the production of Drug Product (the final administered form of a pharmaceutical compound) there is a lack of successful case studies in the literature on applying these principles to the manufacturing process of biopharmaceutical proteins. This work will attempt to use the principles of QbD when developing manufacturing processes for biopharmaceutical products in order to help fill this gap and hopefully provide a useful resource in implementing the QbD concept.

1.4.2 Aims of thesis

Given the noted problems with innovation and regulation in the Pharmaceutical industry and the persistent Quality problems already noted it is the purpose of this thesis to determine whether QbD techniques can be effectively applied to the development of a biopharmaceutical manufacturing process. In doing so it is also reasonable to enquire into whether the QbD initiative as currently recommended and implemented is likely to achieve its intended results – whether it will reduce the cost of medicines and simplify or reduce the regulatory burden in this field. While there has been some very positive literature on the intended outcomes of QbD there has been a sceptical response from industry [12] with some suggesting that it would likely increase costs and possibly increase the regulatory burden.

With all these difficulties in mind, the purpose of this thesis is to better understand the QbD concept as applied to biopharmaceutical manufacture. In the first chapter, a literature review of QbD for biopharmaceuticals, an attempt has been made to better define the QbD concept by examining the historical context of the QbD initiative and surveying the literature to

identify examples in which QbD has been effectively implemented and the potential problems or pitfalls of the QbD approach. In later chapters experimental case studies have been produced to help fill the literature gap identified above - more case studies with actual biopharmaceutical products need to be made available so that both industry participants and regulators can educate themselves in the concepts and adopt best practices. This is an important part of implementing the QbD concept and achieving improvements in the biopharmaceutical industry. In this thesis, QbD principles and approaches have been adopted in carrying out the development of unit operations that are typical for most biopharmaceutical products or for mandatory studies that are required for the successful filing of a biopharmaceutical application.

In the first case study (Chapter 3), QbD principles have been applied to the selection of a preferred Protein A resin for the production of a monoclonal antibody product. The relevant properties of commercially available resins have been compared and experimental work has also been directed at determining how well the standard models used to explain the equilibrium binding of Protein A to an IgG molecule represent the binding behaviour seen in practice using an industrially-relevant IgG feedstock. In the following chapter (Chapter 4) further experimentation has been undertaken to determine the mechanistic reasons why one resin was found to perform better than the other resins examined using isothermal titration calorimetry (ITC).

A standard requirement in marketing applications for biopharmaceutical products is that prospective studies have been directed at determining the useful lifetime of a chromatography resin so that the manufacturing resin is replaced before this lifetime has been exceeded. These types of studies are referred to as “resin lifetime studies” and are a common part of the development of most biopharmaceutical products. In the second case study (Chapter 5) a resin lifetime study for a biopharmaceutical product has been developed following QbD principles. Additional experimentation has also been directed at measuring the fundamental physical

properties of the chromatography resin to determine the mechanism of degradation with continued use. In addition, statistical techniques have been used to analyse the data produced during the study to demonstrate techniques that can be used to extract more information to obtain the "enhanced knowledge" that is a goal of QbD[4].

The final case study (Chapter 6) uses Tangential flow filtration (TFF) as this unit operation is typical of many biopharmaceutical manufacturing processes. A case study has been carried out on the development of a TFF unit operation following QbD principles with a biopharmaceutical protein to illustrate how the QbD concepts can be used to speed development and identify development priorities, with the developed process finally being scaled up to full production scale and the results of that scale compared to the results of development work in the laboratory.

1.4.3 List of Specific Aims

1. Better define the QbD concept and identify potential shortcomings this approach.
2. Determine whether QbD techniques can be effectively applied to the development of a biopharmaceutical manufacturing process.
3. Identify the best commercially available resin for the production of a monoclonal antibody and identify the mechanistic reasons for its superiority.
4. Produce a resin lifetime study for a chromatography unit operation incorporating QbD principles and mechanistic analysis and understanding of the resin.
5. Develop a TFF unit operation for a biopharmaceutical protein following QbD principles.

1. USFDA. *Drug Shortages*. 2014; Available from: http://www.fda.gov/Drugs/DrugSafety/DrugShortages/default.htm.
2. Whitehouse, T. *Executive Order reducing prescription drug shortage*. 2011; Available from: http://www.whitehouse.gov/the-press-office/2011/10/31/executive-order-reducing-prescription-drug-shortages.

3. Abboud, L. and S. Hensley, *Drug Manufacturing, Out of Date for Years, Gets a Shot in the Arm --- U.S.'s FDA Prods Industry to Adopt Innovations, Raise Quality Standards.* The Wall Street Journal Europe, 2003(03 September).
4. ICH. *Pharmaceutical Development Q8(R2)*. 2009; Available from: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.
5. Rathore, A.S. and H. Winkle, *Quality by design for biopharmaceuticals.* Nat Biotechnol, 2009. **27**(1): p. 26-34.
6. Stevenson, D. and T. Cochrane, *Implementation of QbD Part 1 - Setting Product specifications.* Regulatory Rapporteur, 2011. **8**(2): p. 3.
7. Stevenson, D. and T. Cochrane, *Implementation of QbD Part 2 - Organisational Implications.* Regulatory Rapporteur, 2011. **8**(3).
8. Schmitt, S., *The Roadmap to QbD*, in *Quality By Design: Putting Theory into Practice*, S. Schmitt, Editor. 2011, Davis Healthcare Internation Publishing: Bethesda, MD. p. 31 -54.
9. BioProcessTechnologyConsultants. *Quality by Design: Just a Buzz Word?* 2012 30OCT2012]; Survey results]. Available from: http://www.bptc.com/sites/default/files/biopulse_reports/qbd_results_2012-05-15_final.pdf.
10. ISPE. *The State of the Art in Quality by Design: Criticality Assessment, Design Space Implementation and Control*. in *The State of the Art in Quality by Design: Criticality Assessment, Design Space Implementation and Control*. 2013. Sheraton Fisherman's Wharf, San Francisco: ISPE.
11. CMCBiotechWorkingGroup. *A-mAb: a case study in Bioprocess Development*. 2009 30OCT2012 [cited 2.1; Available from: www.ispe.org/pqli/a-mab-case-study-version-2.1.
12. Schmitt, S., *The regulatory framework*, in *Quality by design: putting theory into practice*, S. S, Editor. 2011, Davis Healthcare International Publishing: Bethesda, MD.

Statement of Authorship

Title of Paper	Quality by design for biopharmaceuticals: a historical review and guide for implementation
Publication Status	⊙ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Elliott, P., Billingham, S., Bi, J., Zhang, Hu. Quality by design for biopharmaceuticals: a historical review and guide for implementation (2013). Pharm. Bioprocess. (2013) 1 (1), 105–122

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Phillip Elliott		
Contribution to the Paper	The review was largely planned and written by Phillip Elliott.		
Signature	[redacted]	Date	19FEB2015

Name of Co-Author	Dr Jingxiu Bi		
Contribution to the Paper	Dr Bi completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20/02/2015

Name of Co-Author	Dr Hu Zhang		
Contribution to the Paper	Dr Zhang completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20FEB2015.

Name of Co-Author	Dr Sam Billingham		
Contribution to the Paper	Dr Billingham completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	23 Feb 2015

Review

Pharmaceutical BIOPROCESSING

For reprint orders, please contact reprints@future-science.com

Pharm. Bioprocess.
(2013) 1(1), 105–122

Quality by design for biopharmaceuticals: a historical review and guide for implementation

This article reviews the history of quality-by-design (QbD), how this concept has been applied to biopharmaceuticals, and what can be expected from implementation of QbD. Although QbD may lead to better design of products and manufacturing processes, and offers the potential for reduced regulatory compliance costs, it will likely increase development costs. Process developers will require additional skills and knowledge in the 'quality disciplines', which are not normally part of the training of those in biopharmaceutical process development. A model for implementing QbD in biopharmaceutical manufacture is proposed. The reader will gain an understanding of how QbD principles have been applied to the development of biopharmaceuticals, as well as learning of the potential drawbacks of applying QbD tools indiscriminately. Excellent examples of QbD applied to biopharmaceuticals in the literature will be highlighted and suggested as the direction for future development in this area.

Phillip Elliott*[1], Sam Billingham[1], Jingxiu Bi[2] & Hu Zhang[2]

[1]Hospira Adelaide, 8 Dalgleish Street, Thebarton, South Australia, 5031, Australia

[2]School of Chemical Engineering, The University of Adelaide, North Terrace, SA 5005, Australia

*Author for correspondence:
E-mail: phillip.elliott@hospira.com

A more modern approach to the development of pharmaceutical products and their subsequent manufacture has been advocated by the US FDA and the International Conference on Harmonization (ICH). This approach has been termed 'quality-by-design' (QbD) and is defined as *"a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management"* [101]. There is much literature promoting the proposed benefits of QbD [1,2]; the benefits claimed for QbD include [3]:

- Better design of product;
- Fewer problems in manufacturing;
- A reduction in the number of supplements required for post-market changes;
- Understanding and mitigation of risk;
- Allowing for the implementation of new manufacturing technology without regulatory scrutiny;
- A reduction in overall cost of manufacturing;
- Less waste;
- Faster regulatory approval;
- Enabling continuous improvement;
- Providing a better understanding of processes and a better business model.

The key tools of QbD are incorporation of prior knowledge, the use of statistically designed experiments, risk analysis and knowledge management. The intent of QbD is to encourage pharmaceutical companies to develop sufficient understanding of their products and manufacturing processes; ensure that their processes are robust; and, demonstrate this enhanced understanding to the pharmaceutical regulatory agencies. Regulatory agencies have in turn suggested that demonstration of this 'enhanced knowledge' could allow for a more flexible regulatory approach [101]. For example, if a pharmaceutical product is

FUTURE SCIENCE

10.4155/PBP.13.6 © 2013 Future Science Ltd ISSN 2048-9145

2 Quality by design for biopharmaceuticals: A historical review and guide for implementation

2.1 Abstract

This article will review the history of Quality by Design (QbD), how this concept has been applied to biopharmaceuticals and what can be expected from implementation of QbD. Although QbD may lead to better design of products and manufacturing processes and offers the potential for reduced regulatory compliance costs, it will likely increase development costs. Process developers will require additional skills and knowledge in the "Quality disciplines" which are not normally part of the training of those in biopharmaceutical process development. A model for implementing QbD in a biopharmaceutical manufacture is proposed.

The reader will gain an understanding of how QbD principles have been applied to the development of biopharmaceuticals, as well as learning of the potential drawbacks of applying QbD tools indiscriminately. Excellent examples of QbD applied to biopharmaceuticals in the literature will be highlighted and suggested as the direction for future development in this area.

2.2 Introduction

A more modern approach to the development of pharmaceutical products and their subsequent manufacture has been advocated by the US Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH). This approach has been termed "Quality by Design" (QbD) and is defined as "a systematic approach to development that begins with pre-defined objectives and emphasizes product and process understanding and process

control, based on sound science and quality risk management"[101]. There is much literature promoting the proposed benefits of QbD [6, 7]. The benefits claimed for QbD include [13]:

- Better design of product
- Fewer problems in manufacturing
- A reduction in the number of supplements required for post-market changes
- Understanding and mitigation of risk
- Allowing for the implementation of new manufacturing technology without regulatory scrutiny
- A reduction in overall cost of manufacturing
- Less waste
- Faster regulatory approval
- Enabling continuous improvement
- Providing a better understanding of processes and a better business model

The key tools of QbD are incorporation of prior knowledge, the use of statistically designed experiments, risk analysis and knowledge management. The intent of QbD is to encourage pharmaceutical companies to demonstrate sufficient understanding of their products and manufacturing processes to the pharmaceutical regulatory agencies in exchange for a more flexible regulatory approach. For example, if a pharmaceutical product is approved for sale on the basis of a QbD application, it may be possible to make improvements to the manufacturing process after the product has been approved for sale, without the need to go through an expensive "post-approval change process" with the pharmaceutical regulator. However, this level of regulatory flexibility is yet to be realised. It is hoped that the QbD approach will improve product quality and reduce regulatory compliance costs for pharmaceutical manufacturers. The concept promotes industry's understanding of the product

and manufacturing process starting with product development, with the aim of building quality in from the start rather than trying to test quality into the product during manufacture.

Under the concept of QbD, when designing and developing a product, a company needs to define the desired product performance and identify critical quality attributes (CQAs - see Table 2 for useful definitions in this area and the acronym guide in Table 1). On the basis of this information, the company then designs the product formulation and manufacturing process to meet those product attributes. Ideally it is hoped this will lead to understanding the impact of raw material and equipment attributes and manufacturing process parameters on the CQAs and identification and control of sources of variability. As a result of all of this knowledge, a company can continually monitor and update its manufacturing process to assure consistent product quality [5].

Table 1: Acronym guide

Acronym	Definition
ANDA	Abbreviated New Drug Application (see also NDA)
BLA	Biologics Licence Application
CMC	Chemistry, Manufacturing and Controls
CQA	Critical Quality Attribute
DoE	Design of Experiments
EMA	European Medicines Agency
FDA	Food and Drug Administration
NDA	New Drug Application (see also ANDA)
PAT	Process Analytical Technology
QbD	Quality by Design
QTPP	Quality Target Product Profile
TPP	Target Product Profile

Table 2: Defined key terms

	Definition
Critical Quality Attributes (CQA)	A physical, chemical, biological or microbiological property or characteristic of the product that should be within an appropriate limit, range or distribution to ensure the desired product quality.
Design of Experiments (DoE)	The use of statistically designed experimental arrays to determine the effect of multiple variables on an experimental system which take into account experimental variation and are able to determine both the effects of each variable alone and the combined effect (interaction) of multiple variables.
Design Space	The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality.
Drug Product	A pharmaceutical product type that contains a drug substance, generally in association with excipients. Also referred to as the Dosage Form or Finished Product.
Drug Substance	The active pharmaceutical agent which is subsequently formulated with excipients to produce the "Drug Product"
Knowledge management	A systematic approach to collecting, analysing, storing, and disseminating information related to products, processes and components
Process Analytical Technology (PAT)	A system for designing, analysing, and controlling manufacturing through timely measurements (i. e. , during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality.
Risk Analysis	The estimation of the risk associated with identified hazards. In a pharmaceutical context this term is often used interchangeably with Risk Evaluation - the comparison of the estimated risk to given risk criteria using a quantitative or qualitative scale to determine the significance of the risk.
Robustness	The ability of a process to reliably produce a product of the intended quality over a variety of operating conditions, at different scales or with different equipment.
Target Product Profile (TPP)	A summary of the quality characteristics of a drug product that ideally will be achieved to ensure that the desired quality, and thus the safety and efficacy, of a drug product is realised. Also referred to as the QTPP or Quality Target Product Profile

The first task of QbD is identifying the Target Product Profile (TPP) of the intended drug product. The TPP is defined as a "summary of the quality characteristics of a drug product that ideally will be achieved to ensure that the desired quality, and thus the safety and efficacy, of a drug product is realised"[101]. Once the TPP has been identified, the next step is to identify the Critical Quality Attributes (CQAs) of the intended product, with CQAs defined as "a physical, chemical, biological or microbiological property or characteristic of the product that should be within an appropriate limit, range or distribution to ensure the desired product quality"[101]. From the CQAs identified, the *product* design space can be determined, that is, specifications for in-process, drug substance and drug product attributes [5]. The sum of acceptable variability in each of these attributes defines the overall *product* design space . The *process* design space can then be determined in three stages. First, risk analysis is performed to identify important parameters for process characterization. Second, studies are formulated using DoE (Design of Experiment) techniques to define the process design space. Finally, these studies are carried out and the results analysed to determine the importance of the parameters as well as their role in establishing the design space and any interactions that may occur between parameters.

It should be noted that drug regulatory authorities generally base their decisions on three criteria – quality, efficacy and safety. However, they take no account of the actual or potential cost of the product to the company. Therefore in addition to the need to be compliant with regulatory requirements it is important for a company to examine the value for money it obtains from process development, acquisition of knowledge and QbD in general, and deploy these approaches in the most cost effective manner [8]. A 2012 survey of industry participants found that approximately 20 % of respondents thought lack of cost effectiveness was the biggest hindrance to adoption of QbD in biologics manufacturing, with a similar number citing fears of regulatory delays as the biggest hindrance [102].

In this paper, the historical background of QbD is first reviewed, and then the implementation of QbD in biopharmaceutical manufacturing unit operations is examined. Finally, an approach to implementation of QbD is suggested and QbD is critiqued.

2.2.1 Historical background

In the 1990s the FDA's focus shifted from regulating individual products to regulating the biotechnology industry as a whole [14]. The 1997 Food and Drug Administration Modernization Act (FDAMA) established a new approach to reporting manufacturing changes, with the intent of minimizing the differences between applications for biologics and for drugs approval, this act was later transposed into guidance documents [103-105]. The changes added more and more requirements for industry, resulting in increased review times. By the year 2000 the FDA realised that there were undesirable consequences of the regulatory review process [5] as manufacturers had become wary of implementing new technologies as it was unknown how regulators would perceive such innovation. This in turn led to higher costs for pharmaceutical manufacture due to the maintenance of wasteful and inefficient manufacturing processes. In many cases the FDA attributed these high costs to low manufacturing efficiencies and the difficulty of implementing manufacturing changes [5]. In addition, the pharmaceutical industry had been accused of under-performing in manufacturing innovation by the business community: “Even as is it invents futuristic new drugs, its manufacturing techniques lag behind those of potato-chip and laundry-soap makers”[3].

Due to concerns over the state of manufacturing, FDA oversight of firms increased. One result of this more stringent regulatory oversight was a dramatic increase in the number of manufacturing supplements to applications (these are approvals required from the FDA for a manufacturer to vary its process from that contained in the documentation already filed with the FDA). In 2007, the FDA received a total of 5000 supplements for new drug applications (NDAs), biological licence applications (BLAs) and abbreviated new drug applications

(ANDAs)[5]. Considering that each of these supplements costs approximately US$250,000 in direct costs alone [8], this increased regulatory oversight caused significant costs for the pharmaceutical industry (approximately US$1.25 billion in 2007 alone based on these estimates).

2.2.2 cGMPs for the 21st century

A 2-year initiative, "Pharmaceutical cGMPs for the 21st Century: A Risk-Based Approach" was launched by the FDA in August 2002. This initiative was intended to modernize the FDA's regulation of pharmaceutical quality for veterinary and human drugs and selected human biological products such as vaccines. The FDA acknowledged that the new strategy was required to alleviate concern among manufacturers that innovation in manufacturing and quality assurance would result in "regulatory impasse" [106] – effectively, the cost of gaining approval for innovations became so high that innovation in manufacturing was almost completely discouraged. As part of this initiative, both the pharmaceutical, as well as the chemistry, manufacturing, and controls (CMC) regulatory programs were evaluated [107].

The final report of this initiative was released in September of 2004 [107]. In this report the FDA stated that the guiding principles of its efforts to modernise the regulation of pharmaceutical manufacturing were:

Risk-based orientation

Science-based policies and standards

Integrated quality systems orientation

International cooperation; and

Strong public health protection

Importantly, in this report the FDA acknowledged that its primary focus remained the same - to minimize the risks to public health associated with pharmaceutical product manufacturing.

The FDA stated, perhaps hopefully, that pharmaceutical manufacturing was evolving from an art to a science- and engineering-based activity. It was hoped that application of this enhanced science and engineering knowledge in regulatory decision-making, establishment of specifications, and evaluation of manufacturing processes would improve the efficiency and effectiveness of both manufacturing and regulatory decision-making. In this report the FDA identified a "risk-based orientation" as one of the driving principles of the cGMP initiative with efficient risk management as the primary way to make the most effective use of FDA resources. A further guidance on Process Analytical Technology (PAT) was released as part of the "cGMPs for the 21st century" initiative which hoped to encourage the adoption of more modern and flexible manufacturing technology in the pharmaceutical industry [106].

2.2.2.1 ICH Q8 - Pharmaceutical Development

Further codification of the QbD concept came with the release of the ICH Q8 guideline "Pharmaceutical Development" [101] in November 2004. This guideline reached "step 4", recommendation for adoption by the regulatory agencies party to the ICH, in November 2005. A further annex to the guideline, intended to clarify the concepts in the original guideline was released for public consultation in November 2007 and reached step 4 in November 2008 (the original guideline and the annex have subsequently been combined into a single document).

It is in the ICH Q8 annex that the term Quality by Design or QbD is explicitly defined as, "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."[101]

Two other important terms for discussing QbD were also defined in ICH Q8; Design Space and Process Analytical Technology. Design space is defined as "the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality". According to ICH

Q8, working within the design space is not considered as a change as it has been demonstrated to have no impact on quality. Movement out of the design space would be considered to be a change and would normally initiate a regulatory post approval change process. Based on this guideline, design space was to be proposed by the applicant and would be subject to regulatory assessment and approval. Process Analytical Technology (PAT) was also defined in ICH Q8 as "a system for designing, analysing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality".

It should be emphasised that the ICH Q8 guideline provides guidance on the suggested contents of Pharmaceutical Development section of the Common Technical Document (CTD). This section of regulatory submissions relates to the manufacture of the "Drug Product" - this is a very specific term relating to the product that will actually be administered to the patient. This is in contrast to "Drug Substance" or "Bulk Material" which is the term usually given to the active pharmaceutical agent that is subsequently formulated with excipients to produce the "Drug Product"[108]. This difference between Drug Product and Drug Substance is important when considering how and to what extent the original guidance was intended to apply QbD concepts and controls to pharmaceutical and biopharmaceutical manufacture. This original guideline did not relate to the manufacture of "Drug Substance" - the active pharmaceutical ingredient (API) before it is formulated for administration to the patient. The complexity of unit operations for "Drug Product" is generally less than that for "Drug Substance" and it is appropriate that more control should be demonstrated for the Drug Product which will actually be administered to humans.

The ICH Q8 guideline indicated areas where the demonstration of greater understanding of pharmaceutical and manufacturing sciences could create a basis for flexible regulatory approaches. The guideline emphasised that more flexible regulatory approaches could be

achieved if the applicant could demonstrate an "enhanced knowledge" of product performance over a range of material attributes, manufacturing process options and process parameters. The methods suggested to achieve this enhanced knowledge were formal experimental designs or DoE studies, PAT and prior knowledge. Also suggested was the use of quality risk management principles to prioritise additional studies to collect such knowledge. ICH Q8 emphasized that it is the level of knowledge gained and not the volume of data generated which would lead to more favourable consideration by the regulatory bodies. A further suggestion was that applicant companies could assess the robustness of the manufacturing process, the ability of the process to reliably produce a product of the intended quality over a variety of operating conditions, at different scales or with different equipment, to support future manufacturing change and process improvement. The guideline suggests that changes during development should be looked upon as opportunities to gain additional knowledge and further support establishment of the design space [101].

2.2.2.2 ICH Q9 - Quality Risk Management

ICH Q9 "Quality Risk Management" [109] was released at approximately the same time as ICH Q8 and ICH Q10 and needs to be considered as part of the overarching QbD guidance released by regulatory agencies. The purpose of ICH Q9 was to offer a systematic approach to quality risk management. It provided guidance on the principles and some of the tools of quality risk management for use by both regulators and industry in managing Drug Substances and Drug Products. Importantly, it noted that use of quality risk management can "facilitate but does not obviate industry's obligation to comply with regulatory requirements and does not replace appropriate communications between industry and regulators."[109]

Two important principles were outlined in this document for the use of Quality Risk Management:

The evaluation of the risk to quality should be based on scientific knowledge and ultimately link to the protection of the patient; and

The level of effort, formality and documentation of the quality risk management process should be commensurate with the level of risk.

These are important caveats that should be remembered as risk assessment is a process that can easily be overused and lead to large amounts of unnecessary documentation.

In Annex 1 to ICH Q9 the following tools are suggested for risk management in the pharmaceutical industry:

Flow Charts

Check Sheets

Process Mapping

Cause and Effect Diagrams

Failure Mode Effects Analysis (FMEA)

Failure Mode Effects and Criticality Analysis (FMECA)

Fault Tree Analysis

Hazard Analysis and Critical Control Points (HACCP)

Hazard Operability Analysis (HAZOP)

Preliminary Hazard Analysis (PHA)

Risk Ranking and Filtering

Various Statistical Tools

Acceptance control charts

Design of Experiments (DoE)

Histograms

Pareto charts

Process capability analysis

While acknowledging that the selection of quality risk management tools is dependent on specific facts and circumstances, Annex 2 to ICH Q9 suggested areas to which quality risk management tools could be applied by a pharmaceutical company, ranging across all operational areas from Quality Management to Facilities maintenance and even final packaging and labelling. Of particular relevance to this review were the potential applications to the development phase of pharmaceuticals suggested by the ICH. Specifically, application of quality risk management techniques was suggested to assess the critical attributes of raw materials, active pharmaceutical ingredients (APIs), excipients and packaging materials as well as to determine the critical process parameters for a manufacturing process. Other areas suggested in development were to assess the need for additional studies (e.g., bioequivalence, stability) in technology transfer and scale-up and to the reduction of variability in quality attributes.

2.2.2.3 ICH Q10 - Pharmaceutical Quality System

ICH Q10 reached "step 4" in 2008 and described a model of an effective quality system for a pharmaceutical company. This model was intended to complement ICH Q8 and Q9 [110] and defines the ICH expectations for management responsibilities in a pharmaceutical company. The pharmaceutical quality system described had four key elements:

A process performance and product quality monitoring system;

A corrective action and preventive action (CAPA) system;

A change management system;

Management review of process performance and product quality.

Importantly, the guideline emphasized that these elements should be applied in a manner "proportionate and appropriate" for each of the lifecycle stages. That is, the same level of rigour is not appropriate for products in the development stage as in the commercial or discontinuation phases of a products lifecycle. It was the regulators' hope that adoption of ICH Q10 should "facilitate innovation and continual improvement and strengthen the link between pharmaceutical development and manufacturing activities." Knowledge management and Quality Risk Management were cited as "enablers" of this innovation and continual improvement. While movement within a registered "design space" would not require regulatory approval, the change should still be evaluated and documented by the company's change management system.

2.2.2.4 ICH Q11 - Development and Manufacture of Drug Substances

Dealing with the manufacture of Drug Substances, ICH Q11 "Development and manufacture of Drug Substances (Chemical and Biotechnological/Biological entities)" was released for public consultation in May 2011 and was adopted internationally in May 2012 [111]. Importantly for biological and biotechnological products this guideline stated that most of the CQAs of a biologically derived Drug Product are associated with the Drug Substance and thus are a direct result of the design of the Drug Substance or its manufacturing process. ICH Q11 re-iterates the commitment to QbD principles in ICH Q8 and provides examples of how this process can be applied to Drug Substance manufacture. It then goes on to suggest where the data produced by QbD studies and risk assessments can be located in the Common Technical Document (CTD) format.

While most of ICH Q11 is concerned with identifying what data should be presented in each section of the CTD, the appendices give some useful examples of the use of DoE experiments to establish the design space for different unit operations, both for small molecules (chemical entities) and biological products.

Of note is the fact that in these examples, when more than one CQA is affected by or dependent on a unit operation, the effective design space in which acceptable product is produced becomes smaller (see Figure 1). This is likely to be a general result, especially so when more than one variable controls the output of a unit operation and the corresponding design space therefore has more than one dimension. For example, the design space shown in Figure 1, has two operating dimensions – conductivity and pH, but other unit operations may have additional operating dimensions such as linear flow velocity and product load which may also affect the CQAs of the product. In addition, the example given in ICH Q11 should be regarded as highly simplified due to the linear relationship shown between the variables and the product CQAs which results in square or rectangular design spaces. In reality, these design spaces are generally complex curved surfaces for each CQA with statistical confidence intervals for each surface. Interpretation of such design spaces is more complex than for the example shown in ICH Q11.

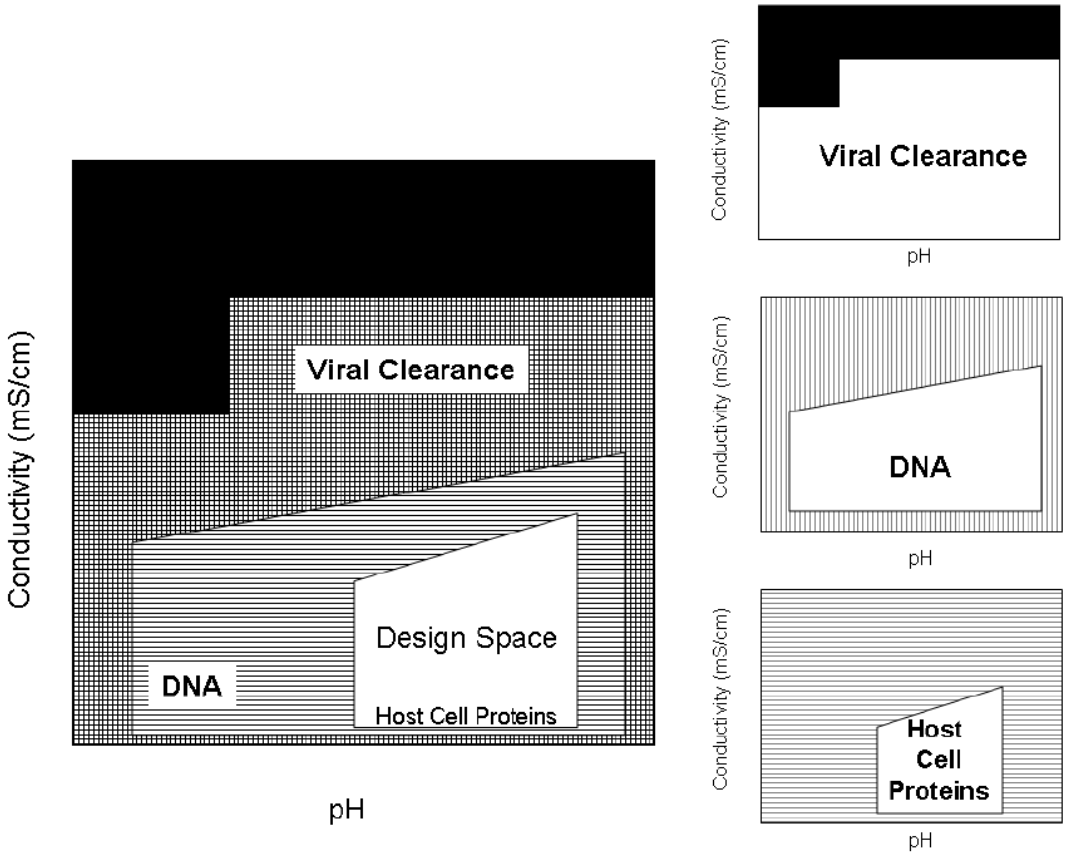


Figure 1: Design space for an anion exchange chromatography unit operation.

Figure demonstrates the reduction in the design space when more than one CQA is dependent on a unit operation (reproduced from [111]). The clear area in the small diagrams on the right illustrate the safe design space for each of the CQAs individually. Clear area in the large diagram on the left indicates the safe design space for all CQAs affected by the unit operation.

2.2.3 Review of QbD in Manufacturing Biological Products

The European Federation of Pharmaceutical Industries and the EMA have recognised that each product is unique - developing any product will require a "bespoke" approach and therefore there is no standard blueprint for applying QbD [8]. Various methods of implementing QbD for entire processes have been suggested in the literature. One of the more useful and easy to follow is shown in Figure 2 [5]. Initially, the TPP of the Drug Product is identified. Once the TPP has been identified the relevant CQAs are identified and prioritised through the process of risk assessment. Based on the CQAs, the product design space is proposed – this is the sum of specifications for in-process, drug substance and drug product attributes. These proposed specifications are ideally based upon non-clinical studies, previous clinical experience with similar products, published literature and known process capability. Risk analysis is then performed to identify and prioritise parameters for process characterization. DoE studies are formulated to examine the process design space, the studies are then carried out and the results are analysed to determine the importance of the parameters examined. If necessary, the design space is then refined, based on the results of these initial studies. The control strategy for the unit operation is then defined through the process of risk assessment and based on the importance of the CQAs affected by the unit operation and the capability of the unit operation. As the biopharmaceutical product progresses toward regulatory filing, the unit operation is validated and routine monitoring of the process is carried out.

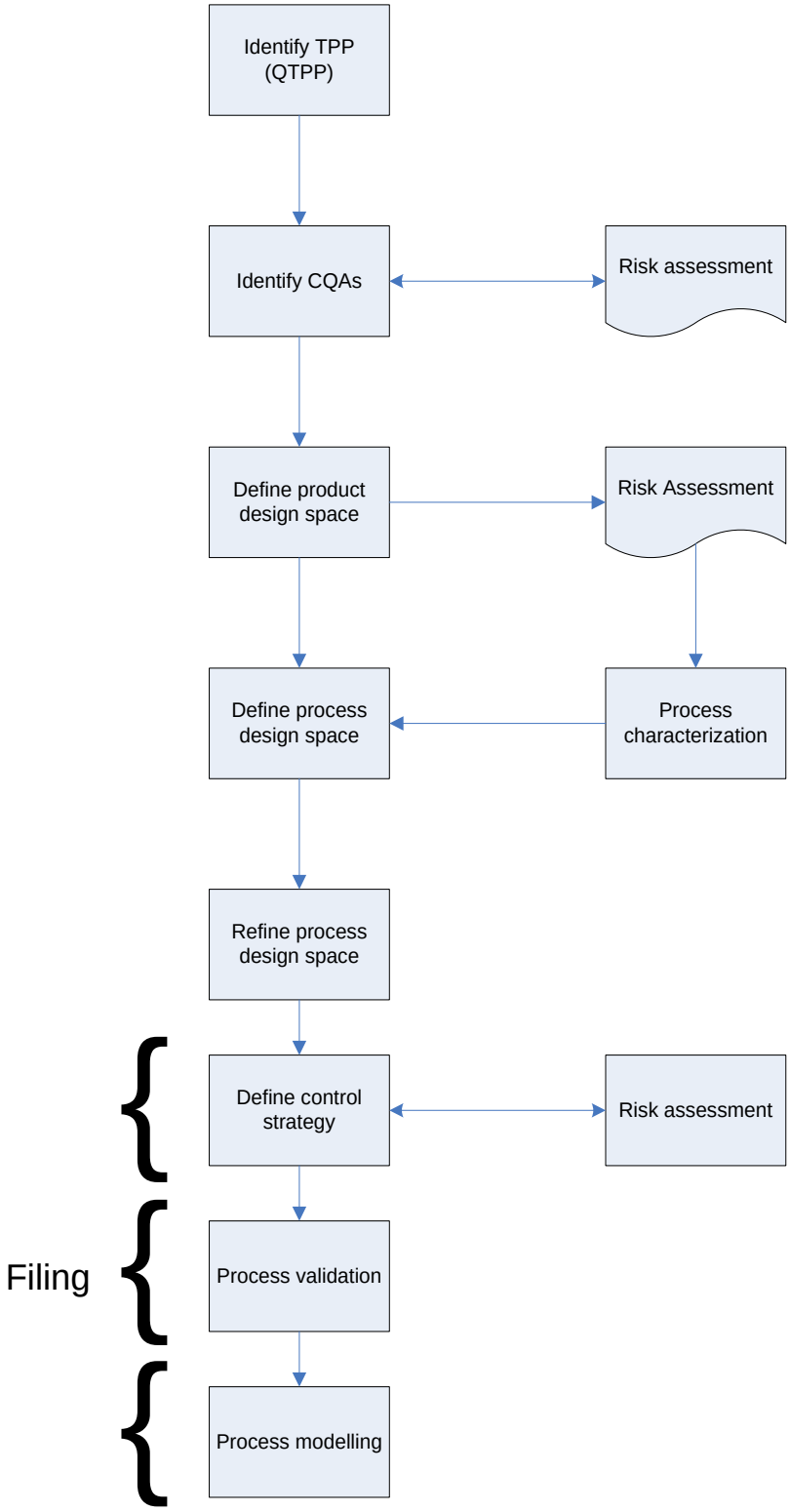


Figure 2: A suggested implementation plan for QbD (reproduced from [5])

A more general introduction to using FMEA to classify and prioritise the parameters in a biopharmaceutical manufacturing process for further study has been presented in [15].

A QbD case study on the production of a model monoclonal antibody (A-mAb) has been developed by the ISPE (International Society for Pharmaceutical Engineering) [112]. This example includes sections on Upstream (mammalian cell culture), Downstream and Drug Product unit operations. It also includes sections on anticipated post-launch process changes – what is described as movement within the design space.

The case study noted that the overall control strategy for a manufacturing process is based on the design spaces of the individual unit operations – it is the sum of the individual control strategies that represent the overall control strategy.

The case study gives examples of constructing the Target Product Profile (TPP) for the A-mAb molecule, followed by identification and risk assessment of quality attributes, leading to a rationale for selecting the quality attributes (CQAs) for the case study. Three types of tools for assessing the criticality of quality attributes were used (risk ranking; preliminary hazard analysis and safety assessment decision tree) and examples given of how these tools are applied to the assessment of different quality attributes of the product (aggregation, glycosylation, deamidation, oxidation etc.).

Various publications have reported the application of QbD techniques to many of the specific areas and unit operations of biopharmaceutical manufacture. The following section is a review of this literature as a reference for process developers to illustrate the different approaches that have been taken and the differences between individual areas and unit operations which make it necessary to modify the way in which QbD is implemented. In reviewing QbD literature and preparing DoE studies, careful attention should be paid to the definitions that will be used to classify each of the variables and the ranges within which each of the parameters can be controlled. A recognised scheme is to classify each operating variable as

either “critical”, “key” or “non-key” (Table 3) using the classification system produced by the Parenteral Drug Association [16]. The selection of variables to study and the definitions used to classify the criticality of those variables are pivotal in risk analysis and subsequent interpretation of DoE studies. This is because the criticality definitions include aspects of both how well the variable can be controlled within the defined range and how much of an effect that variable has on a CQA within that defined range. Many, if not all variables examined will affect the process to some degree, but the decision on whether to treat that variable as ”critical” depends on how it affects the final product administered to the patient and how easily the variable can be maintained within the desired range. Insufficient attention to the definitions used for variable classification can lead to haphazard overuse of both DoE and FMEA techniques.

Table 3: Parameter classification definitions

All input parameters outside this definition are non-critical.

Classification	Definition
Critical	An adjustable parameter (variable) of the process that should be maintained within a narrow range in order to not affect a product critical quality attribute
Key	An adjustable parameter (variable) of the process that, when maintained within a narrow range, ensures operational flexibility
Non-key	An adjustable parameter (variable) of the process that has been demonstrated to be well-controlled within a wide range, although at extremes could have an impact on process performance.

2.2.3.1 Cell Culture

Failure Modes and Effects Analysis (FMEA) has been used to identify and prioritise the parameters for process characterization in the fermentation of *Pichia pastoris* [17] and *E.coli* [18] and in mammalian cell culture producing monoclonal antibody products in [19, 20]. All of these examples start with the use of FMEA to classify and prioritise variables for experimentation, then move to DoE studies and development of the design space for the fermentations. The initial low resolution screening experiments using the selected variables (parameters) are followed by more targeted and thorough response surface designs exploring the active variables determined in the screening design. A recent study has also used quality risk management procedures to choose between alternative cell culture technologies for the production of a monoclonal antibody product [21].

In a fermentation process using *P.pastoris* [17] and methanol induction to express a soluble product , no critical parameters were identified in the fermentation, as none of the variables examined affected final product quality within the experimental ranges examined and the process was able to be well controlled to remain within these ranges. The fermentation was therefore found to have a wide design space. Key parameters (those which affect process performance) identified in the study were temperature, pH and dissolved oxygen, all of which affected cell growth and titre. A "worst-case" fermentation was then performed to verify the design space, in which all of the fermentation parameters were set to their most disadvantageous level in the design space. Although cell growth was affected, product yield and quality after purification were acceptable, verifying the robustness of the fermentation.

In comparison, an *E.coli* fermentation [18] in which the product was deposited as insoluble inclusion bodies, temperature, feed rate, pH and dissolved oxygen (and interactions between these variables) were shown to affect product quality. A "worst-case" combination of

parameters was then used to produce inclusion bodies. As the inclusion bodies produced in this "worst-case" could still be purified to produce acceptable product using the established downstream process it was concluded that there were no critical operating parameters within the ranges tested as none affected the final product. Monte Carlo simulation was then applied to parameters and ranges selected for the design space to set acceptance criteria for process validation.

In mammalian cell culture expressing monoclonal antibody products, the proportion of acidic variants has been found to be particularly sensitive to culture conditions [19, 20]. Temperature, pH and initial viable cell density were confirmed to affect the glycosylation profile of the antibody product[19], however, as the downstream purification process was still able to produce an acceptable product from "worst-case" culture conditions, no critical variables were detected in the cell culture conditions. Initial screening DoE and follow-up response-surface experimentation was used to produce predictive models that allowed optimization of the cell culture process and could be used to monitor the success of scale-up and later commercial operations [19].

2.2.3.2 Downstream processing

2.2.3.2.1 Chromatography

Examples of the use of FMEA applied to parameters of a chromatography unit operation are given in the appendix of ICH Q11 [111] and in the A-Mab case study [112].

Cecchini gives an example of the application of QbD techniques to the unit operations of cell harvest and product capture, Protein A capture chromatography, hydrophobic interaction chromatography and anion exchange chromatography [22]. As with the approach taken in applying QbD to cell culture, all of these case studies follow the sequence of 1) determining acceptable ranges for product quality attributes, 2) risk analysis to identify the most important unit operations to examine further to improve control of the overall process 3) parameter

screening – identification of key and critical parameters for modelling DoE through the use of Resolution III or IV screening DoE, 4) modelling DoE – Resolution V or response surface designs to determine important interactions between variables and establish an empirical model for the important outputs and 5) Scale-down model verification – comparison of modelling DoE to manufacturing scale runs.

A more mechanistic approach to ion exchange chromatography has been taken in [23, 24]. Both of these examples were built from the extensive theoretical knowledge of ion-exchange chromatography available in the literature to develop the design space for an ion-exchange unit operation and reduce the experimental and analytical requirements during process characterization. Kaltenbrunner *et al* [24] also compared the use of fractional factorial experiments to the theoretical model to validate the modelling approach. Both approaches identified pH, ligand density and ionic strength at the start of the gradient as the dominant variables, providing assurance that the assumptions made to allow the modelling were valid. A similar treatment by Mollerup *et al* [23] was extended to provide a simulation of the resulting chromatogram generated from fundamental protein parameters and the ionic strength and pH of the running buffers. This simulation was verified against small-scale and pilot-scale chromatograms and was able to confirm the root cause for a manufacturing problem which occurred at pilot scale.

2.2.3.2.2 Tangential Flow Filtration

Quality by Design principles have been combined with a mechanistic understanding of tangential flow filtration operations and applied to the development of a TFF unit operation in [25]. Due to this approach, extensive DoE experiments were unnecessary, but more targeted experiments, guided by the mechanistic model, were able to quickly generate a robust method of operation and examine the economic implications of different operating modes. This work addressed issues of membrane selection, TFF design objectives, operating parameter design and operating mode design. It especially emphasised the need to determine the effect of

temperature on the product, as the forces involved with TFF will generate heat - heat increases flux through the filtration membrane, but biopharmaceutical products can be especially sensitive to denaturation due to increased temperature. This article is important in that it begins from a mechanistic model of the technique of tangential flow filtration, while also considering the applied aspects of the unit operation that will be important and unique to each individual application. This approach is distinguished from more "naïve" DoE approaches which progress on a purely empirical basis and vary operating parameters without reference to mechanistic understanding of the unit operation.

This study does not consider the effect of electrostatic interactions during ultrafiltration of biopharmaceuticals since it deals primarily with a Drug Substance process. Electrostatic interactions should be taken into consideration in Drug Product TFF unit operations as protein biopharmaceuticals are highly purified and become the predominant charged species in solution. It is also important in the final Drug Product that exact concentrations of excipients are known and a target pH value is reached in the final solution. It has been shown that in the highly purified conditions of Drug Product TFF operations, electrostatic interactions between the biopharmaceutical, the membrane and charged excipients can interact to significantly alter the final pH and excipient concentrations through the Donnan effect [26-29]. Special attention is required during the development of Drug Product TFF unit operations or any TFF unit operation in which the resultant buffer concentration and pH needs to be controlled within a narrowly specified range.

2.2.3.2.3 Viral and Sterile Filtration

The A-Mab [112] case study provides a worked example of applying QbD principles to viral filtration and sterile filtration of drug product in which the combined use of risk assessment, prior knowledge from similar product experience and the development of a control strategy are illustrated. For virus filtration two parameters, volumetric load and filtration pressure, were found to be important controls on virus removal and were classified as well controlled

critical process parameters. For sterile filtration 5 parameters were found to be critical, though all were easily controlled. Two of these parameters, pre-run flush volume with Drug Product and WFI flush volume involved the preparation of the filter prior to use. The other three parameters identified as critical were the level of bioburden before filtration, the flow rate per unit of membrane area and the filter area.

2.2.3.3 Drug Product

Both the A-mAb case study and ICH Q8 provide examples of the use of QbD techniques to develop Drug Product unit operations such as lyophilisation, sterile filtration, filling, stoppering and capping. While these unit operations are, by definition, high risk due to their proximity to the patient they are generally relatively simple and well characterized. The examples provided by the ICH guidelines have been rightfully criticised [12] as they provide examples of a one or two parameter design space only, whereas the design space for many unit operations can have many more than two parameters or dimensions. In designing these unit operations, care should be taken to ensure that the design space is as simple as possible to enable the resultant design space to be correspondingly simple. Compared with most of the Active Pharmaceutical Ingredient (API) processing operations, linkage is not as complex in Drug Product processing primarily because the ranges are narrow and composition does not change as these are not purification steps. Therefore there are as many critical process parameters (CPPs) [30].

Martin-Moe *et al.*, have focussed on drug product unit operations for monoclonal antibodies as these are amenable to platform development and have a well-established clinical history [30]. This work used a risk ranking and filtering tool, rather than FMEA, to identify the CQAs affected by Drug Product unit operations. The output of this tool was used to determine which CQAs should be analysed after each unit operation. A DoE study was then performed for formulation characterization with a risk ranking tool used to support selection of ranges and the type of study proposed to establish the design space (multivariate vs univariate).

This work emphasised establishment of scale-down models to allow multivariate experimentation without excessive cost, and the importance of establishing the link between the large-scale process and the scale-down model. An alternative presented is to use at-scale surrogate models which are not as costly as use of the actual product itself, but it is similarly important to establish the link between the surrogate model and the actual unit operation with product. For this reason, several companies now only produce biologics for Phase III trials in the same equipment as commercial product as they cannot otherwise assure comparability.

As discussed above, TFF unit operations at the Drug Product stage of processing suffer from the extra complexity introduced by the interaction of the charged biopharmaceutical, charged excipients and residual charge on the TFF membrane (Donnan effect) [26-29]. For this reason, particular attention should be given to the pKa's of the excipients, buffers and product and the extent of diafiltration when developing TFF unit operations for Drug Product.

2.2.3.4 Raw Materials

Lanan makes the very important point that raw materials have several distinguishing features from other aspects of biopharmaceutical manufacturing and this affects how they need to be handled in a QbD approach [31]. Unlike the operating parameters of a process, raw materials are not under the direct control of the biopharmaceutical manufacturer – they are the products of others suppliers and their processes. In addition, complex or naturally derived raw materials can vary over much longer timescales than are required for process development. Therefore, even when efforts are made to use a variety of raw material lots or batches in process development, the total variation of the raw material may not be captured during process development due to the much shorter timeframe. Furthermore, for operating parameters and conditions less attention is given to detecting variables once a process has been transferred to manufacturing. Raw materials may require the constant detection of variables even after the process has been transferred to manufacturing or commercial scale. This is because lot-to-lot, time-dependent changes to raw materials can occur during the

manufacturing stage. QbD for raw materials may need to provide strategies to detect and manage changes in raw materials that may happen for the first time during commercial manufacturing. This is more in line with what has been described as a PAT or chemometric approach [32] .

Due to its complexity, special consideration needs to be given to cell culture media as a raw material. Cell culture media can contain more than 40 compounds [31]. Reactions can occur between these compounds, generating even more chemical complexity. The metabolism of the cells also alters these compounds, further increasing the complexity, making the whole system prone to variability from run-to-run. For some older mammalian cell-culture products, complex natural mixtures such as serum or plant hydrolysates are used in the media. These are complex mixtures and the analytical platforms required to characterize significant components of these mixtures are only now being developed [33]. Literature reports extensive use of many complex laboratory techniques (^{1}H-NMR, LC-MS, ICP-MS, LC-DAD) coupled with sophisticated statistical analysis (PCA – Principal component analysis, PLS – partial least squares analysis, MVA multi-variate analysis, multi-linear regression) to attempt to determine the root cause of variation in cell culture arising from these complex raw materials [31]. Some of these reports, even with such extensive efforts and sophisticated techniques, are not entirely successful in explaining the variation seen or completely determining root cause for out of trend results. For this reason it is probably prudent to avoid the use of complex or naturally derived components in cell culture as much as possible. Completely chemically defined media is likely to be more economical and reliable over the long term, even if it is not capable of producing the same yield of product in cell culture. It is fortunate that, while cell culture is often the most variable stage of biopharmaceutical production, it is also the most distant from the patient and variation at this stage is less likely to present risk to the patient. Experimental efforts to remove or replace naturally derived materials as far as possible, or explain, control and provide acceptance specifications for

naturally occurring materials that must be retained in the process are probably well spent and will contribute to overall control and QbD of cell culture. An examination of the history of quality improvement in the beer industry (the oldest biotechnology industry based on cell culture) shows the extensive efforts that have been exerted in controlling and reducing the variability of naturally derived raw materials in order to improve productivity and consistency [34]. Biopharmaceutical quality is already following the same pattern of development and the same approach to controlling raw materials is likely to be fruitful in improving the quality of the product.

Rathore and Low provide a useful scheme for classifying raw materials into Critical, Key and Non-key categories [35, 36]. They also provide a useful matrix or checklist of the categories of risk arising from raw materials and how these can be handled, though most of these are more traditional methods of handling raw material risk than being uniquely different for QbD. The second part of their paper gives examples of 4 different risk assessment tools and demonstrates the application of FMEA to upstream and downstream materials along with an explanation of the logic for assigning different values for severity, occurrence and detection. In general, processes that are closer to the patient, especially Drug Product processes and the associated raw materials, should be considered higher risk than earlier processes. The risk assessment team should begin with the most critical unit operations near the end of the process and work backwards through the process when assessing raw materials. At the end of the risk assessment process the team should benchmark their scoring against the outcome of previous assessments for similar products and materials to ensure a consistent view of risk.

2.2.3.5 Other

The use of risk assessment procedure to determine the critical quality attributes of monoclonal antibody products has been described in [37]. This example illustrated the combined use of risk assessment procedures with prior knowledge and literature review. A recent study has tried to link experience in human clinical trials with monoclonal antibodies to physiochemical

measures of the protein to further improve criticality determinations for this class of proteins [38].

The use of risk assessment as an adjunct to validation of an analytical assay is presented in [39]. The FDA has also indicated that it has accepted some NDA applications in which analytical methodologies are developed using a QbD approach and supplied a recommended approach for developing analytical tests in this manner [117].

Low pH viral inactivation, a common procedure for biopharmaceuticals expressed in mammalian cell culture is developed from a QbD perspective in the A-mAb case study [112].

2.2.4 Implementation of QbD

The concept of quality by design can be traced to Joseph Juran [40, 41] and it is useful to return to some of his original works for the clarity with which they describe the concept of Quality by Design. Juran's method for implementing quality by design is shown in Figure 3 and was referred to by Juran as the "Quality planning roadmap" [42]. The virtue of this model is the ease with which it can be reduced to a step-by-step guide - Juran was quite conscious of the fact that deciding at which point to begin planning partly came down to a judgement call and decided on this series of steps to avoid repeated looping through the planning cycle [40]. The "Roadmap" has 9 important steps: identifying customers, determining the needs of those customers, translating those needs into company language, developing a product that can respond to those needs, optimising the product's features to meet both company needs and customer needs, developing a process that is capable of producing the product, optimizing the process, proving that the process can produce the product under operating conditions, and transferring the process to operations [43]. Notably absent from the work of Juran is the use of risk assessment, as included in the ICH and FDA guidances. When considering use of the "Quality planning roadmap" in the biopharmaceutical industry it is useful to remember that some of the customers (indeed before

marketing approval, the dominant customers) are the regulatory authorities involved, so many of the requirements that need to be sought from the customer should be sourced from the regulatory authorities and the relevant guidance and regulations. This is a very important point to remember in order to produce manufacturing processes and products that are both compliant with regulation, while also producing a product with the required attributes.

Also pertinent is that Juran further sub-classified customer needs into:- stated needs, real needs, perceived needs, cultural needs and needs traceable to unintended use. This classification recognised the fact that the needs stated by customers are only a part of the whole needs - the customer has many other needs which are not consciously identified [40]. For example, regulatory agencies require that resin and membrane lifetime studies should be performed before a drug is approved for sale - this is a stated need. However, the real need is for a manufacturing process that performs consistently and reproducibly over the lifetime of the product and this real need should be considered for the process as a whole in addition to the stated needs of resin and membrane lifetime studies.

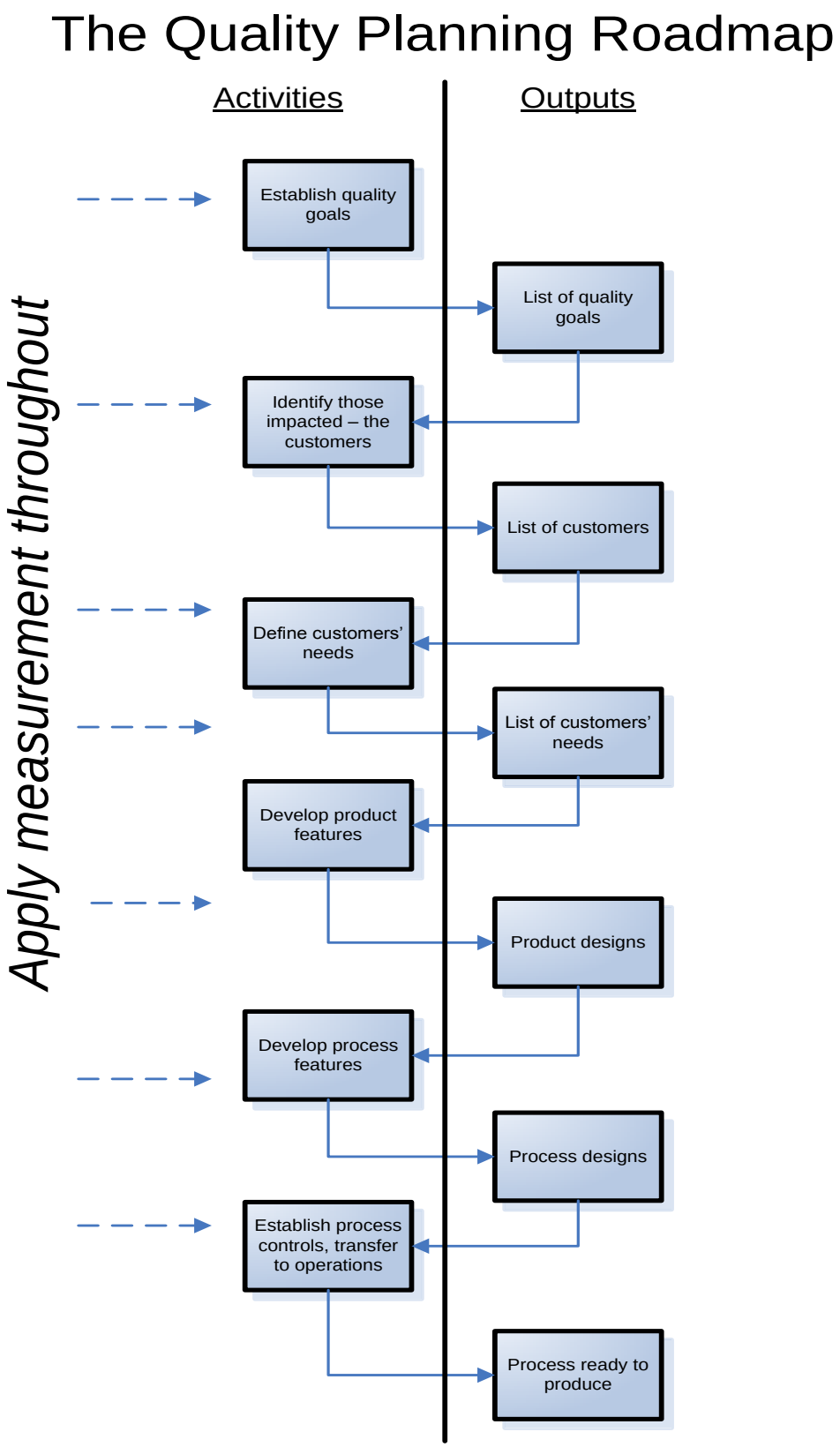


Figure 3: Juran's Quality Planning Roadmap (from [42])

Developing a manufacturing process for a biopharmaceutical using QbD principles corresponds to the last 3 steps of the Quality Planning Roadmap (Figure 3) - "Develop process features", "Process designs", "Establish process controls and transfer to operations". In order to carry out these steps it necessary for the previous 7 steps of the Quality Planning Roadmap to have been completed and documented for reference. These 7 prior steps are often not carried out by process development groups, they are generally the functions of different groups or Senior Management in the company so it is important to ensure the outputs of these previous steps are thoroughly communicated and recorded. It is also necessary for the process development group to remember that it has customers - the manufacturing or operations group to whom the process will be transferred for example - and the needs of these customers should be correctly identified.

A flowchart for implementing the "Quality Planning Roadmap" in biopharmaceutical process development is proposed by the authors and is shown in Figure 4. This approach is intended to identify distinct outputs during QbD efforts, outputs which are necessary for communication and collaboration amongst the various groups inside a pharmaceutical company that must coordinate their efforts before, during and after product development. Before QbD can be implemented in a manufacturing process it is necessary to have a rudimentary initial process. Once this has been established it is necessary to move backwards through the unit operations of the process, beginning with the last unit operation in the process train. It is important to progress in this manner as the output of each unit operation in a manufacturing process becomes the input for the next unit operation. Beginning at the start of the manufacturing process can lead to the initial unit operations being optimised, to the detriment of the subsequent unit operations and the overall manufacturing train. Progressing in this manner is also in itself a risk assessment or prioritisation of work, with the level of risk of a unit operation generally thought to increase with proximity to the patient. This aligns

with the fact that regulatory agencies will use risk assessments to determine which points in the process and which manufacturers and unit operations are most likely to cause risk to the final patient and therefore require increased scrutiny [107, 113, 114]. The general rule of thumb of moving from the last unit operation back through to the initial unit operation could be replaced by a more formal risk assessment to identify areas of the process more likely to cause harm if they become out of control (developing a priority list of unit operations as shown in Figure 4). Determining the safe design space for the highest risk unit operations should be placed as the highest priority.

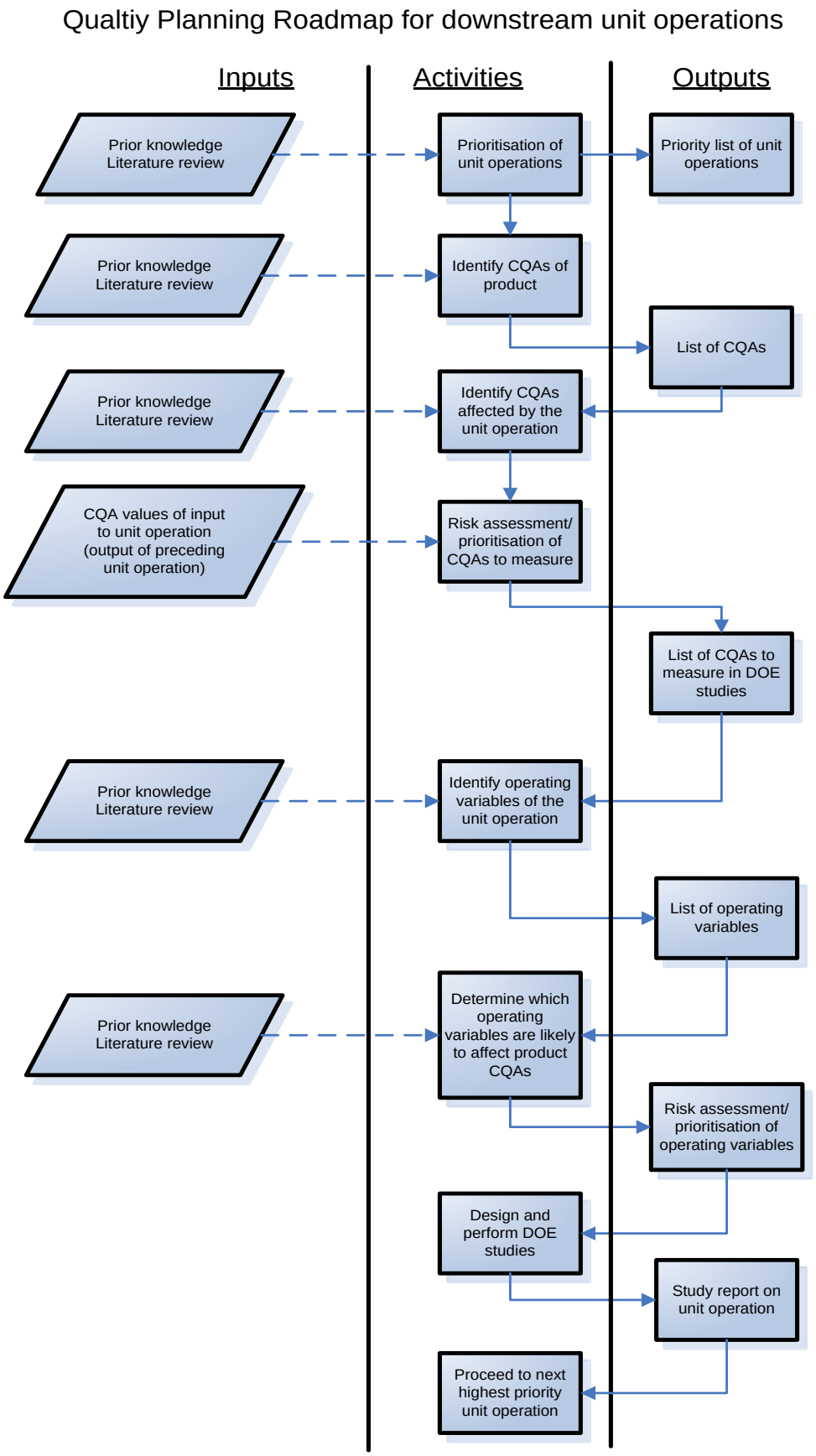


Figure 4: Suggested activities for implementing QbD in downstream unit operations

The next step in Figure 4 is to identify the CQAs of the product, then go on to identify which CQAs are thought to be affected by the specific unit operation - this can be done using a combination of literature reviews, prior knowledge and risk assessment. Following this, the operating variables of the unit operation can be identified. A risk assessment can be performed on these variables - this should include a literature review and other prior knowledge. This risk assessment can be used to determine which of these variables *will* affect products CQAs, which *may* affect product CQAs and which are unlikely to affect product CQAs. The next step in the process is to identify the values for the CQAs in the feed stock for the unit operation under consideration. This is effectively the output of the preceding unit operation. Having these values will allow determination of the effect of the unit operation on the CQAs. On the basis of the risk assessment and the values of the CQAs in the feed stock, the variables and outputs to measure in DoE studies can be determined and justified. Multivariate experimentation can then be carried out and the output levels of each of the CQAs compared to the inputs. This confirms whether each CQA is affected and whether the process variables chosen are robust within the ranges selected.

It may be useful to go through this exercise with a variety of processing technology alternatives for each unit operation in order to maintain flexibility for future improvements in technology. Demonstrating that acceptable CQAs are achieved over a multivariate range of parameters and a range of processing alternatives demonstrates the robustness of the manufacturing process. ICH Q8 identified that a more flexible regulatory approaches could be achieved if the applicant could demonstrate an "enhanced knowledge" of product performance over a range of " material attributes, manufacturing process options and process parameters". For unit operations that are intended to produce a specific CQA value or limit, it is useful to determine what maximum value can be used to challenge that unit operation i.e. if a chromatography unit operation is designed to reduce deamidation variants to a specification level it may be useful to challenge the unit operation in order to determine the maximum

amount of deamidation variants that can be brought within the final specification. A study report on each unit operation should then be produced which identifies operating variables affecting the product and the safe operating ranges for those variables, along with identifying operating variables that do not affect the product in the unit operation[16]. Classifying in this manner is necessary to enable this information to be used in regulatory submissions which require disclosure of the critical steps and operating parameters in the manufacturing process.

After this exercise is completed the same exercise can be repeated with the next highest priority unit operation until the whole manufacturing process train is complete or the highest priority unit operations have been addressed.

2.2.5 Pitfalls of QbD

Risk management, statistical tools and knowledge management are all useful tools when implemented in the appropriate manner. However these are all very broad concepts and, due to the large number of variables involved in any pharmaceutical manufacturing operation, the amount of experimentation and documentation required to thoroughly carry these out can become very large. In a recent marketing application from Genentech, who has worked closely with the FDA on QbD, the Chemistry, Manufacturing and Controls (CMC) section was approximately 1500 pages in length due to the inclusion of all the backup data, risk assessments and justifications [115].

Similarly, design of experiments studies are a very effective tool and in many published papers QbD and DoE have almost become synonymous. However, the large number of variables that can be considered in a manufacturing process, especially a Drug Substance process, make the number of possible experimental runs very large. For example, the typical process chromatography unit operation can have 50 - 100 operating parameters that can affect its performance [44]. Examining all of these parameters would require a prohibitively large number of experimental runs. As a result of the increased experimentation and

documentation required, a recent estimate has suggested that adoption of QbD approaches is likely to increase development costs by up to $US1 million [12]. Due to the provenance of this estimate (from a report commissioned by the FDA and used to promote the QbD concept) it should be considered a conservative estimate likely to err on the low side. Evidence from the FDA that the generics industry, in which low development and production costs are competitive advantages, is much less keen on adoption of QbD would appear to support this (cited in [12]). Both European Medicines Agency (EMA) and FDA representatives have stated that they expect a more costly assessment process for QbD applications and EMA representatives also expect more difficult and costly inspections for QbD-based processes [12].

An additional concern is that the output of statistically designed experiments and the resulting design space can be a large and complex mathematical equation. Interpreting and applying this equation takes considerable mathematical and statistical knowledge on behalf of both the pharmaceutical company and the regulatory agency reviewing the design space. A potential problem for companies is that the regulators, who would be required to approve the design space, may not have the mathematical knowledge required to correctly interpret the design space presented. At a recent conference of industry and representatives from various regulatory agencies, most attendees were unfamiliar with the concept of "n-dimensional space" - a space having more than 1, 2 or 3 dimensions (n-dimensions). This concept is essential for interpreting complex design spaces as many reactions or processes can have more than 2 variables which control the output. EMA representatives admitted that EMA staffs were "challenged" in statistical knowledge and the FDA had not yet evaluated whether its staff had the requisite knowledge [12]. Elsewhere, representatives of the Canadian regulator Health Canada have stated that they are struggling to deal with QbD applications [8]. A survey of industry participants in 2012 indicated that many people in the

pharmaceutical industry felt that the level of QbD understanding by FDA regulators was variable from individual to individual [115].

Not surprisingly, there appears to be widespread scepticism of the QbD concept, with a survey taken in 2008 showing that 58 % of companies had QbD either in the “ideas and vision” or “not started” stage of implementation [45]. As the industry and regulators are not in a position to publish examples of QbD applications (due to confidentiality and intellectual property concerns) it has not been possible to fully allay these concerns. Up to mid-2010 a total of only five BLAs and four post-approval supplements had been received for the biologics QbD trials [12].

2.2.6 Recent Developments

2.2.6.1 EMA-FDA Pilot Program

The FDA and EMA began a joint pilot program in March, 2011 for the parallel evaluation of quality-by-design applications. The purpose of this program was to share knowledge, ensure consistent adherence to international guidelines related to QbD and promote the availability of pharmaceutical products of consistent quality throughout the European Union and the U.S. With the agreement of the companies presenting applications, experts from the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) could also participate as observers. Through this program the EMA and FDA reached agreement on a number of QbD elements. One marketing authorisation and several requests for scientific advice were assessed by the participating agencies in the first three years of the pilot program and three question-and-answer documents have been published as a result [46-48]. In April 2014, this program was extended for a further two years and further publications on continuous processing and other QbD elements are planned.

In the first document question and answer document, specific guidance was provided on how CQAs and CPPs should be classified and included in the application documents. Also some guidance was provided on the use of Analytical Target Profiles (ATP) and Method Operational Design Ranges (MODR) for the application of QbD to analytical methods but "There is currently no international

consensus on the definition of ATP and MODR. Until this is achieved, any application that includes such proposals will be evaluated on a case-by-case basis."[46]

The second report provides information on how to verify the design space of a QbD submission at large scale, the relationship of validation to design space verification and what to do if unexpected results occur during verification. Both agencies agree that design space verification is not necessarily complete at the time of submission and can continue over the life cycle of the product. There is also agreement that when companies can demonstrate that the design space is independent of scale, additional risk mitigation steps are not necessary in the verification. The report contains two appendices covering areas on which the EMA and FDA differ. The European agency states that there should be no difference between the principles used for design space verification at commercial scale between chemical and biological pharmaceuticals and that a plan for design space verification should be included in the marketing application. The FDA states that verification plans do not need to be in the application and should be managed through the companies Quality system, though the FDA considers it "beneficial" to the application to include a high level overview of the plans for verification. EMA also explains in some detail the difference between validation and verification [48].

The third document was released on 19DEC2014 and covers the level of detail to include in regulatory submissions containing QbD elements. Specifically mentioned are the level of detail required for design spaces, risk assessments on process and product design and Design of Experiments (DOEs). For all of these areas the level of detail to provide should be commensurate with the impact any of these exercises had on the commercial product or process. In general the agencies have seen risk assessments on the selection of formulation variables, the delineation of the impact of process parameters and selection of in-process controls. A significant point in this document is the very detailed information required when submitting a DOE to define an important unit operation. Also significant is that it is not necessary to provide a mathematical description of a design space unless the design space being requested is quite complex e.g. is a response surface which includes quadratic terms. [47]

2.2.7 Conclusions

QbD is not a unified system as such, but is more correctly described as an *ethos* that "quality cannot be tested into products; it should be built in by design" [114]. As such it is difficult to prescribe a single solution for QbD - developing any product will require a "bespoke" approach and therefore there is no standard blueprint for applying QbD [8].

QbD is also an armoury of techniques or tools which should be used for development of good quality products and processes. These tools include: DoE, Risk assessment, Statistical Quality control techniques (control charts etc.) and mechanistic models and understanding of processes and products.

In order to be able to implement QbD, biopharmaceutical companies should ensure they have these capabilities. Development staff and company scientists should be conversant with these tools and techniques. Joseph Juran, to whom the origins of Quality by Design can be traced [41] stated that "Product development requires not only functional expertise; it also requires the use of a body of quality-related know how". He decried quality planning performed by 'amateurs'- people who have not been trained in the "quality disciplines" - as one of the main causes of poor quality processes and products [40]. Training those who develop manufacturing processes (development scientists and engineers) in these quality disciplines is the only way to address this fundamental problem. Development scientists need a thorough understanding of these quality disciplines as their data and experiments form the basis of the processes and methods used during commercial manufacture [41].

It has been remarked that in order to develop the design space for a process, well designed DoE experiments are required, but most process developers lack the statistical knowledge to effectively design these experiments [12]. For example, it is very easy with the use of DoE software to design a large, empirical study on a filtration or chromatography process. However, very detailed mechanistic models and understanding of these unit operations are already available in the chemical engineering literature. It is therefore not necessary to retreat

to an entirely empirical level of understanding of these unit operations. Bringing the insights of mechanistic models to bear on specific applications in biopharmaceutical manufacturing requires very highly trained development scientists who can reduce the models to practice and make the knowledge contained in the models available to technical staff who do not have the same level of scientific knowledge. Further, development scientists need to be able to determine how to use the insights of such models on specific pieces of manufacturing equipment. Thus a very important way for the quality of developmental products to be improved, and some of the hopes for the QbD initiative to be realised, is to ensure that development scientists are trained outside their original specialty disciplines in some (ideally all) of the tools of QbD. Similarly, as the QbD concept is being promoted by the pharmaceutical regulatory authorities it is imperative for those authorities to have staff that are familiar with and understand the limits and caveats of these tools. This is necessary in order for the authorities to be able to understand applications which present this information and be aware of when the information presented is insufficient.

Industry appears to have been restrained in the adoption of QbD, and considering the complexities involved and the apparent lack of understanding in the regulatory agencies on how to deal with applications of this type, such caution is understandable [12].

Hopes for greatly reduced costs of pharmaceuticals and reduced regulatory burden due to the QbD initiative should be limited. Much of the high cost of pharmaceuticals is due to economic, political and regulatory factors [49] which are unaffected by the science behind the original development work. None of these factors are likely to change due to the QbD or PAT initiatives; indeed adoption of QbD approaches is almost certain to increase development costs [12].

The QbD concept is being used in the literature by development scientists to publish and share effective methods for process development and characterisation. This is a welcome and useful development of the QbD concept and should help good practices become widely

embraced throughout the industry and aid process development scientists in becoming cross-trained in quality disciplines. Some excellent examples in the literature have begun from mechanistic models of unit operations and shown how these models can be used to develop robust and economical unit operations that are well characterized without having to assume only the empirical level of understanding implied in a "naïve" DoE approach to the development of unit operations [23-25, 44]. However, the way unit operations interact with a given product is less likely to be deducible from mechanistic models due to the complexity of biopharmaceutical products. How unit operations interact with the impurities in the product which are in fact a wide variety of individual species (i.e. host cell protein, host cell DNA, viruses) is likely to resist mechanistic understanding as each individual host cell protein (for example) will have its own specific properties and clearance from the Drug Substance. For this reason there will always be a level of "empirical-only" knowledge required in the development of a biopharmaceutical and the need for traditional validation will remain.

2.2.8 Future perspectives

The FDA has announced that it expects the pharmaceutical development (Drug Product) section of all ANDAs for small molecule generic drugs to be in a QbD format from January 1, 2013 [116]. This change was achieved by updating the ANDA submission checklist to include QbD elements as requirements. This is likely to indicate that QbD will also become a requirement for biopharmaceuticals in the next 5 - 10 years. Close attention should therefore be paid to how QbD is applied to generics so that the lessons learned can be applied to more complex biopharmaceuticals in the future. Close attention should also be paid to how the QbD process has been implemented in the semiconductor and microelectronics industries over the previous decades, as these industries have made significant improvements through the use of the quality disciplines and tools during development.

The majority of biopharmaceuticals under development are produced by small biotechnology companies who are under funding constraints and pressure to bring their products to market

[8, 12]. The increased funding requirements due to QbD may be most challenging to these companies and the development of literature and industry guidance documents from regulators that can assist in addressing QbD requirements in a cost-effective manner will be necessary for these smaller companies to thrive in the QbD regulatory environment and thus maintain a vital element of new product development.

2.2.9 References

1. Stevenson D, Cochrane T. Implementation of QbD Part 1 - Setting Product specifications. *Regulatory Rapporteur*, 8(2), 3 (2011).
2. Stevenson D, Cochrane T. Implementation of QbD Part 2 - Organisational Implications. *Regulatory Rapporteur*, 8(3) (2011).
3. Kozlowski S, Swann P. Current and future issues in the manufacturing and development of monoclonal antibodies. *Adv Drug Deliv Rev*, 58(5-6), 707-722 (2006).
4. Rathore AS, Winkle H. Quality by design for biopharmaceuticals. *Nat Biotechnol*, 27(1), 26-34 (2009).
5. Schmitt S. The Roadmap to QbD. In: *Quality By Design: Putting Theory into Practice.* Schmitt, S (Ed. (Davis Healthcare Internation Publishing, Bethesda, MD, 2011) 31 -54.
6. Kozlowski S. Protein therapeutics and the regulation of quality: a brief history from an OBP perspective: as the biotechnology industry has matured through various stages of growth, regulatory agencies have evolved in response to the need to define quality standards *Biopharm International*, 20(10) (2007).
7. Abboud L, Hensley S. Drug Manufacturing, Out of Date for Years, Gets a Shot in the Arm --- U.S.'s FDA Prods Industry to Adopt Innovations, Raise Quality Standards. *The Wall Street Journal Europe*, (03 September) (2003).
8. Seely R, Haury J. Applications of Failure Modes Effect Analysis to Biotechnology Manufacturing Processes. In: *Process validation in manufacturing of Biopharmaceuticals: Guidelines, Current Practices and Industrial Case Studies.* Rathore, AS, Sofer, G (Eds.) (CRC Press, Taylor & Francis Group, Boca Raton, FL, 2005) 13 - 31.
9. Brandreth EJ, Bussineau C, Butler M *et al.* Validation of Column-based Chromatography Processes fro the Purification of Proteins Technical Report No. 14 Revised 2008. *PDA Journal of Pharmaceutical Science and Technology*, 62(S-3) (2008).
10. Harms J, Wang X, Kim T, Yang X, Rathore AS. Defining process design space for biotech products: case study of Pichia pastoris fermentation. *Biotechnol Prog*, 24(3), 655-662 (2008).
11. van Hoek P, Harms J, Wang X, Rathore AS. Case study on definition of process design space for a microbial fermentation step. In: *Quality by Design for Biopharmaceuticals: Principles and Case studies.* Rathore, AS, Mhatre, R (Eds.) (John Wiley & Sons, Inc., Hoboken, New Jersey, 2009) 85 - 108.
12. Abu-Absi SF, Yang L, Thompson P *et al.* Defining process design space for monoclonal antibody cell culture. *Biotechnol Bioeng*, 106(6), 894-905 (2010).
13. Horvath B, Mun M, Laird MW. Characterization of a monoclonal antibody cell culture production process using a quality by design approach. *Mol Biotechnol*, 45(3), 203-206 (2010).
14. Mila L, Valdes R, Tamayo A, Padilla S, Ferro W. Application of a risk analysis method to different technologies for producing a monoclonal antibody employed in hepatitis B vaccine manufacturing. *Biologicals*, 40(2), 118-128 (2012).
15. Cecchini D. Applications of Design Space for Biopharmaceutical Purification Processes. In: *Quality by Design for Biopharmaceuticals.* Rathore, AS, Mhatre, R (Eds.) (John Wiley & Sons, Hoboken, New Jersey, 2009) 127 - 141.
16. Mollerup JM, Hansen TB, Kidal S, Staby A. Quality by design--thermodynamic modelling of chromatographic separation of proteins. *J Chromatogr A*, 1177(2), 200-206 (2008).
17. Kaltenbrunner O, Giaverini O, Woehle D, Asenjo JA. Application of chromatographic theory for process characterization towards validation of an ion-exchange operation. *Biotechnol Bioeng*, 98(1), 201-210 (2007).
18. Watler P, Rozembersky J. Application of QbD principles to tangential flow filtration operations. In: *Quality by Design for Biopharmaceuticals: Principles and case studies.* Rathore, AS, Mhatre, R (Eds.) (John Wiley & Sons, Inc., Hobokwn, New Jersey, 2009) 111 - 125.
19. Bolton GR, Boesch AW, Basha J, Lacasse DP, Kelley BD, Acharya H. Effect of Protein And solution properties on the Donnan effect during the ultrafiltration of proteins. *Biotechnol Prog*, 27(1), 140-152 (2011).
20. Noordman T, Ketelaar T, Donkers F, Wesselingh J. Concentration and desalination of protein solutions by ultrafiltration. *Chemical Engineering Sciences*, 57(693 - 703) (2002).

21. Rohani MM, Zydney AL. Role of electrostatic interactions during protein ultrafiltration. *Adv Colloid Interface Sci*, 160(1-2), 40-48 (2010).
22. Stoner MR, Fischer N, Nixon L *et al.* Protein-solute interactions affect the outcome of ultrafiltration/diafiltration operations. *J Pharm Sci*, 93(9), 2332-2342 (2004).
23. Schmitt S. The regulatory framework. In: *Quality by design: putting theory into practice.* S, S (Ed. (Davis Healthcare International Publishing, Bethesda, MD, 2011)
24. Martin-Moe S, Lim FJ, Wong RL, Sreedhara A, Sundaram J, Sane SU. A new roadmap for biopharmaceutical drug product development: Integrating development, validation, and quality by design. *J Pharm Sci*, 100(8), 3031-3043 (2011).
25. Lanan M. QbD for Raw materials. In: *Quality by Design for Biopharmaceuticals.* Rathore, AS, Mhatre, R (Eds.) (John Wiley & Sons, Hoboken, New Jersey, 2009) 193 - 208.
26. Pomerantsev A, Rodionova O. Process analytical technology: a critical view of the chemometricians. *Journal of Chemometrics*, 26, 11 (2012).
27. Luo Y, Chen G. Combined approach of NMR and chemometrics for screening peptones used in the cell culture medium for the production of a recombinant therapeutic protein. *Biotechnol Bioeng*, 97(6), 1654-1659 (2007).
28. Curinova L, Dudek F. Quality Management in Nineteenth-Century Czech Beer and Sugar Production. In: *A history of managing for quality: the evolution, trends, and future directions of managing for quality.* Juran, JM (Ed. (ASQC Quality Press, Milwaukee, Wisconsin, 1995) 657.
29. Rathore AS, Low D. Managing Raw Material in the QbD paradigm, Part 1: Understanding risks. *BioPharm International*, 23(11), 8 (2010).
30. Rathore AS, Low D. Managing Raw Materials in the QbD paradigm Part 2: Risk Assessment and Communication. *BioPharm International*, 23(12), 5 (2010).
31. Schenerman M, Axley M, Oliver C, Ram K, Wasserman G. Using a Risk assessment process to determine criticality of product quality attributes. In: *Quality by Design for Biopharmaceuticals: Principles and Case studies.* Rathore, AS, Mhatre, R (Eds.) (John Wiley & Sons, Hoboken, New Jersey, 2009) 53 - 82.
32. Goetze AM, Schenauer MR, Flynn GC. Assessing monoclonal antibody product quality attribute criticality through clinical studies. *MAbs*, 2(5), 500-507 (2010).
33. van Leeuwen JF, Nauta MJ, de Kaste D *et al.* Risk analysis by FMEA as an element of analytical validation. *J Pharm Biomed Anal*, 50(5), 1085-1087 (2009).
34. Juran JMJM. *Quality by design Juran on quality by design : the new steps for planning quality into goods and services* (Free Press ; Maxwell Macmillan Canada ; Maxwell Macmillan International,, New York : Toronto : New York :, 1992).
35. McConnell J, Nunnally BK, McGarvey B. The forgotten origins of Quality by Design. *Journal of Validation Technology*, 16(3), 5 (2010).
36. Juran JM. A call to action: the summit: Carlson School of Management, University of Minnesota-Minneapolis, Minnesota 26 June 2002. *Measuring Business Excellence*, 6(3), 4 (2002).
37. Bicheno J. *The Quality 60: A guide for service and manufacturing.* (PICSIE Books, Buckingham, UK, 1998).
38. Kaltenbrunner O, McCue JT, Engel P, Mollerup JM, Rathore AS. Modeling of Biopharmaceutical Processes. Part 2: Process chromatography unit operations. *BioPharm International*, 21(8), 13 (2008).
39. Neway J. How to make the business case for Quality by Design. *BioPharm International*, 21(12) (2008).
40. FDA-EMA. EMA-FDA pilot program for parallel assessment of Quality-by-Design applications: lessons learnt and Q&A resulting from the first parallel assessment. (Ed.^(Eds) (2013)
41. FDA-EMA. Questions and answers on level of detail in the regulatory submissions (Ed.^(Eds) (2014)
42. FDA-EMA. Questions and Answers on Design Space Verification. (Ed.^(Eds) (2013)
43. Friedman M. How to cure Health Care. *Hoover Digest*, 3(July 30) (2001).

2.2.10 Web References

101. ICH. Pharmaceutical Development Q8(R2). 2009; Available from: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.
102. BioProcessTechnologyConsultants. Quality by Design: Just a Buzz Word? 2012 30OCT2012]; Survey results]. Available from: http://www.bptc.com/sites/default/files/biopulse_reports/qbd_results_2012-05-15_final.pdf.
103. USFDA. Guidance for Industry: Changes to an Approved Application for Specified Biotechnology and Specified Synthetic Biological Products. 1997; Available from: www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM124805.pdf
104. USFDA. Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-derived Products. 1996 [cited 2012 23SEP]; Available from: as adopted internationally
105. USFDA. Revised guidance for industry: providing regulatory submissions to the Center for Biologics Evaluation and Research (CBER) in electronic format – biologics marketing applications [Biologics License Application (BLA), ProductLicense Application (PLA)/ Establishment LicenseApplication (ELA) and New Drug Application (NDA)]. 1999; Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072349.pdf.
106. USFDA. Guidance for Industry: PAT — A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance. 2004; Available from: www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070305.pdf
107. USFDA. PHARMACEUTICAL CGMPS FOR THE 21ST CENTURY — A RISK-BASED APPROACH: FINAL REPORT. 2004; Available from: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/Manufacturing/QuestionsandAnswersonCurrentGoodManufacturingPracticescGMPforDrugs/ucm137175.htm.
108. ICH. SPECIFICATIONS: TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS Q6B. 1999; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf.
109. ICH. Quality Risk Management Q9. 2005; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf.
110. ICH. Pharmaceutical Quality System Q10. 2008; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q10/Step4/Q10_Guideline.pdf.
111. ICH. DEVELOPMENT AND MANUFACTURE OF DRUG SUBSTANCES (CHEMICAL ENTITIES AND BIOTECHNOLOGICAL/BIOLOGICAL ENTITIES) Q11. 2012 May; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q11/Q11_Step_4.pdf.
112. CMCBiotechWorkingGroup. A-mAb: a case study in Bioprocess Development. 2009 30OCT2012 [cited 2.1; Available from: www.ispe.org/pqli/a-mab-case-study-version-2.1.
113. USFDA. Pharmaceutical cGMPS for the 21st Century — A Risk-Based Approach: Second Progress Report and Implementation Plan. 2003 [cited 2012 23SEP2012]; Available from: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/Manufacturing/QuestionsandAnswersonCurrentGoodManufacturingPracticescGMPforDrugs/ucm071836.htm.
114. USFDA. Innovation and Continuous Improvement in Pharmaceutical Manufacturing Pharmaceutical CGMPs for the 21st Century. 2004; Available from: http://www.fda.gov/ohrms/dockets/ac/04/briefing/2004-4080b1_01_manufSciWP.pdf.
115. Taylor, N. FDA mixed messages create QbD confusion; consultant. in-Pharma Technologist.com 2012; Available from: http://www.in-pharmatechnologist.com/Regulatory-Safety/FDA-mixed-messages-create-QbD-confusion-consultant.

116. Thomas, P. Is QbD a mandate for generics? FDA responds to confusion. pharma QbD 2012 [cited 2013 30JAN]; Available from: www.pharmaqbd.com/qbd_mandate_generics_fda/.
117. Tang, Y. Quality by Design Approaches to Analytical Methods - FDA perspective. 2011 [cited 2013 30JAN]; The FDAs intention for QbD for analytics]. Available from: http://www.fda.gov/downloads/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/UCM301056.pdf.

Statement of Authorship

Title of Paper	A QbD approach to selection of a Protein A resin for manufacturing use
Publication Status	O Published, O Accepted for Publication, O Submitted for Publication, ⊙ Publication style
Publication Details	Text in manuscript

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Phillip Elliott		
Contribution to the Paper	The work was largely planned and written by Phillip Elliott.		
Signature	[redacted]	Date	19FEB2015.

Name of Co-Author	Dr Jingxiu Bi		
Contribution to the Paper	Dr Bi contributed to the planning and analysis of the ITC, SBC and DBC sections. Dr Bi completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20/02/2015

Name of Co-Author	Dr Hu Zhang		
Contribution to the Paper	Dr Zhang contributed to the planning of the ITC section. Dr Zhang completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20FEB2015

Name of Co-Author	Dr Sam Billingham		
Contribution to the Paper	Dr Billingham completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	23 Feb 2015

Statement of Authorship

Title of Paper	A QbD approach to selection of a Protein A resin for manufacturing use
Publication Status	○ Published, ○ Accepted for Publication, ○ Submitted for Publication, ◉ Publication style
Publication Details	Text in manuscript

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Phillip Elliott		
Contribution to the Paper	The work was largely planned and written by Phillip Elliott.		
Signature	[redacted]	Date	20 FEB 2015.

Name of Co-Author	Yuzhe Tang		
Contribution to the Paper	Yuzhe contributed to the design and analysis of the ITC section results. Yuzhe largely carried out the experimental work with the ITC instrument.		
Signature	[redacted]	Date	19 Feb 2015

Name of Co-Author	D-Yee Lin		
Contribution to the Paper	D-Yee contributed to the design and analysis of the SBC and DBC section and carried out the experimental work in these sections.		
Signature	[redacted]	Date	20 Feb 2015

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

3 A QbD approach to selection of a Protein A resin for manufacturing use

3.1 Abstract

Three different types of modern Protein A chromatography resins (MabSelect SuRe, POROS MabCapture A and ProSep-vA Ultra) were used to purify a monoclonal antibody under manufacturing conditions to determine a "best" resin for manufacturing use. The resins examined differed in base matrix, ligand type (native versus engineered recombinant) and bead size.

MabSelect SuRe™ was found to be the best performing resin in terms of binding capacity (under both static and dynamic conditions) and residual Protein A levels.

In comparing the chromatography resins, different equations were also compared for their utility in analysing the static binding capacity data produced when challenged with a manufacturing feedstock. In this analysis the Langmuir-Freundlich isotherm was found to be superior to the individual Langmuir and Freundlich isotherms that have generally been used in the literature for this purpose and this equation is recommended for use in future comparisons of Protein A and other affinity-based resins.

To determine how much of the difference in performance could be attributed to the difference in ligand binding the interaction between the purified monoclonal antibody and the native Protein A ligand and the engineered MabSelect SuRe™ ligand was explored using isothermal calorimetry. The MabSelect SuRe™ ligand was found to have a significantly higher affinity constant than the native Protein A. A significantly higher ΔS value was also obtained with the MabSelect SuRe™ ligand, indicating a more significant structural re-arrangement was induced in the IgG upon binding. This may indicate a potential downside of the MabSelect SuRe™ ligand and associated resin in that some structural re-arrangements could lead to

denaturation or inactivation of the target IgG molecule. It is recommended that this risk be considered and examined in pilot experiments when using this resin with a new target molecule for the first time.

3.2 Introduction

Protein A is a polypeptide derived from the cell wall of *Staphylococcus aureus* which has a very high affinity for the fragment-crystallisable (Fc) region of IgG antibodies. The full length Protein A molecule has a C-terminus that begins with a cell wall/membrane-associated region, proceeding into a linear series of five homologous antibody-binding domains. These domains are designated as E,D,A, B and C (in order from the N-terminus; see Figure 5) and share 65 % - 90 % sequence homology [50]. The molecular weight of the complete Protein A molecule is 54 kDa, while that of a derivative with the cell wall/membrane-associated region deleted and used for attaching to chromatography resins is 42 kDa. Each of the antibody-binding domains has a molecular weight of approximately 6.6 kDa and consists of 3 helices. The antibody binding domains of Protein A interact with antibodies at their fragment crystallisable (Fc) region, specifically at the region between the CH2 and CH3 domains known as the consensus binding site [50].

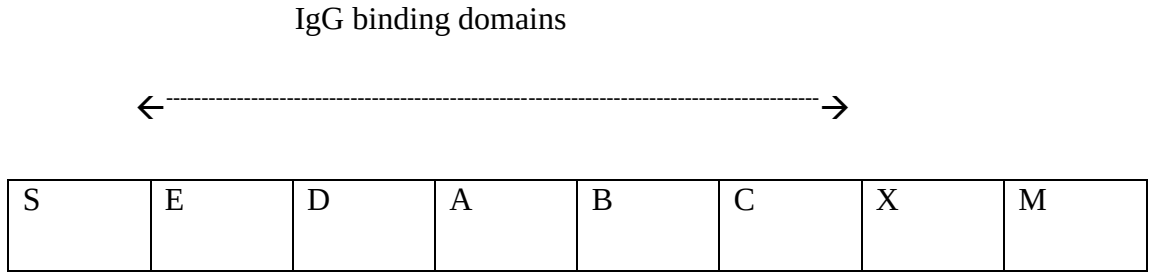


Figure 5: Schematic of the domain structure of Protein A [51]

Most industrial schemes for the purification of monoclonal antibodies employ Protein A affinity chromatography as the capture step due to its specificity for antibodies, physicochemical stability and the ease and simplicity of process development [52]. Protein A sorbent consists of Protein A linked to a chromatographic support such as agarose beads and is available from several commercial suppliers. These sorbents can vary with respect to the source of Protein A ligand (natural wild-type vs. recombinant), immobilization chemistries and the characteristics of the chromatographic support. The large purification factor obtained from Protein A chromatography simplifies downstream purification as, in general, only trace contaminants such as high molecular weight aggregates, residual host cell protein (HCP) and leached Protein A remain to be removed after this unit operation. The large purification factor achieved by Protein A chromatography enables the development of platform manufacturing processes, in which one downstream process can be developed and used for multiple antibody products with a minimum amount of tailoring and adjustment to account for the relatively minor differences between antibody products [53, 54].

In the growing field of biopharmaceuticals, monoclonal antibodies represent the largest category of products. Thirty monoclonal antibodies (mAbs) are currently licensed by the FDA and in 2007 mAbs accounted for $US27 billion in sales, occupying 8 out of the 20 highest selling drug positions [55]. Increasing cell culture titres have resulted in a shift of production bottlenecks away from bioreactors to the purification of mAbs and the use of Protein A chromatography. In response to the growing need for more efficient Protein A media, a number of novel resins have been developed. Amongst them are MabSelect SuRe™, ProSep vA Ultra and POROS MabCapture A. This work sought to compare these resins systematically in their performance when challenged with a model monoclonal antibody feedstock to provide an aid to decision making when choosing a resin for a platform manufacturing process.

Conventional Protein A ligands are not alkaline stable, so Protein A resin is commonly regenerated using high concentrations of chaotropes such as urea or guanidine hydrochloride. A relatively recent development in Protein A chromatography has been the use of recombinant Protein A ligands that have been altered to enable better resistance to the harsh cleaning and sanitisation procedures common in biopharmaceutical processes. MabSelect SuRe™ is one such resin that has been created by protein engineering of the B domain of native Protein A to remove amino acids sensitive to alkali (predominantly asparagine) and create a more stable ligand [52, 56]. The resulting engineered domain has been termed a Z domain, with the final MabSelect SuRe™ construct being a tetramer of Z domains, with a C-terminal cysteine residue added to enable single-point attachment to a bead matrix (Figure 6). Due to the engineering of the ligand, MabSelect SuRe™ resin is stable to the use of 0.1 – 0.5 M sodium hydroxide for regeneration.

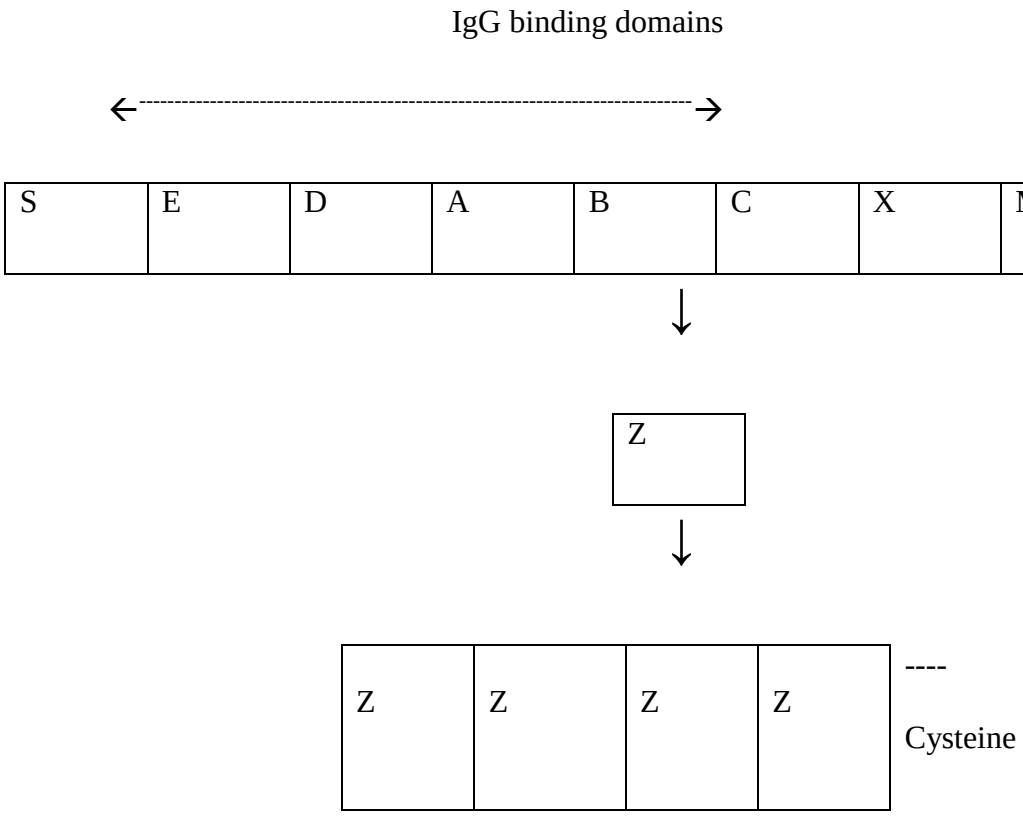


Figure 6: Schematic of the derivation of the recombinant Protein A ligand MabSelect SuRe™[51]

Various comparisons of the performance of available Protein A resins have been published in the literature [57-63]. Generally these studies have used polyclonal antibodies dissolved in buffer solutions to compare the binding properties of the various resins. This is not an accurate representation of the actual manufacturing conditions under which monoclonal antibodies are produced and may not give an accurate comparison of the resins tested. Under manufacturing conditions all of the IgG molecules are of one type (monoclonal rather than polyclonal) and the molecules are present in a solution of spent conditioned medium, with a high conductivity and a much larger amount of competing proteins and other contaminants than a standard buffer solution. Further, published studies that have tried to characterise the mass transfer properties and model the behaviour of resins have generally used the Langmuir isotherm to fit the interfacial adsorption equilibrium (the transfer of protein from liquid to solid phase) with no justification for this selection. The Langmuir isotherm is a homogenous model based on the theoretical principle that only one type of functional group on a surface interacts with one type of solute [64]. While it has generally been accepted that the primary region of binding between Protein A and IgG molecules is through binding to the Fc region of the IgG, many authors have shown that interactions between the Protein A molecule and the variable region of some antibodies can also occur and in some cases be the strongest determinant of binding between the two molecules [65]. This is especially so for IgG molecules with variable regions generated from the VH3 gene family. Therefore the Langmuir isotherm is unlikely to be a valid model of the binding of monoclonal antibodies to Protein A ligand. Complicating this analysis is the fact that engineered Protein A resins such as MabSelect SuRe™ are likely to demonstrate much more homogenous adsorption behaviour than resins derived from native Protein A due to the presence of only one type of Protein A domain and the fact that this resin has been shown to exhibit much less interaction with the variable domain of IgG molecules [65]. In evaluating and comparing Protein A resins in this

work, homogenous and non-homogenous models were used to model the adsorption of monoclonal antibody to the various Protein A resins and the accuracy of the models compared. To further confirm whether any differences in resin performance could be attributed to differences in the ligand, the binding between purified Mab Select SuRe™ ligand and a purified monoclonal antibody and that between native Protein A and the monoclonal antibody were compared using isothermal titration calorimetry.

Three resins from three chromatography manufacturers were compared in this study: MabSelect SuRe media (GE Healthcare), POROS MabCapture A media (Applied Biosystems) and ProSep-vA Ultra (Millipore). The relevant resin properties are further discussed and briefly summarised in Table 4. Various other resins were also selected for initial consideration, but were disregarded from further experimental examination on the basis of a literature review revealing properties undesirable for manufacturing-scale use. All three resins have very similar dynamic binding capacities, recoveries and final product purities reported in the literature however POROS MabCapture A and ProSep-vA Ultra are able to be packed to higher bed heights, operated at higher linear velocities and are capable of withstanding much higher back pressures than MabSelect SuRe™.

Table 4: Summary of the Protein A Resins selected for evaluation

Property	**MabSelect SuRe**	**POROS MabCapture A**	**ProSep-vA Ultra**
Manufacturer	GE Healthcare	Applied Biosystems	Millipore
Base Matrix	Rigid, highly cross-linked agarose	Cross-linked poly(styrene-divinylbenzene)	Controlled pore glass
Particle Size	85 μm	45 μm	75-125 μm
Ligand	Alkali-tolerant, recombinant Protein A	Recombinant Protein A	Native vProtein A (vegan origin)
Dynamic Binding Capacity	35 mg/mL at 2.4 min residence time at 10% breakthrough [66]	45 mg/mL at 2 min residence time at 5% breakthrough[67, 68]	35 mg/mL at 2.4 min residence time at 10% breakthrough[69, 70]
Recovery	94-104%[60]	>95%[71]	93-107%[60, 72]
Linear Velocity	up to 500 cm/hr	up to 2000 cm/hr	up to 1500 cm/hr
Bed Height	10 to 25 cm	5 to 40 cm	up to 45 cm
Maximum Operating pressure	3 bar	100 bar	200 bar
Residual HCP	0-756 ppm[60]	2300-2700 ppm[71]	5-5211 ppm[71]
Column Lifetime	200+ cycles	300+ cycles	300+ cycles[73]
Purity	>95%[51]	>95%[71]	99%[60, 72]
pH of elution	3 to 4	2 to 3	2 to 3
Operating pH range	3 to 12	2 to 10	1 to 9
Protein A leaching	5-20 ppm [66] 1-3 ppm[60]	< 50 ppm[67, 68] 4-5 ppm[71]	1-122 ppm [6, 7] (average = 40 ppm)[60]
Cost – Capital (per L resin)	$26,606	$18,030	$19,850
Regeneration conditions	PBS pH 7-8	100 mM phosphate pH 7.5	PBS
Cleaning conditions	0.1-0.5 M NaOH	1 M acetic acid in 20 % ethanol or 0.1 M NaOH	Phosphoric acid or HCl pH 1.5 or 6 M guanidine
Storage conditions	20% ethanol at 4-8 °C	100 mM phosphate, pH 7.5 in 20% ethanol	PBS + preservative 2-8 °C
Regulatory Support File	Available. No materials of animal origin used.	Available. No materials of animal origin used.	Available. No materials of animal origin used.

3.3 Materials and Methods

3.3.1 Materials

The natural Staphylococcus Protein A (catalog no. P6031-20mg) was obtained from Sigma, and purified MabSelect SuRe™ Ligand from GE Healthcare (catalog no. 28-4018-60). MabSelect SuRe™ resin was also obtained from GE Healthcare. ProSep-vA Ultra was obtained from Millipore and POROS MabCapture A from Applied Biosystems. Monoclonal antibody reference standard was purchased from Genentech/Roche.

3.3.2 Monoclonal antibody

Monoclonal antibody was produced by Hospira Adelaide in CHO cells using standard techniques. The cell culture harvest was clarified by filtration at concentrations exceeding 2 mg/ml and the antibody concentration of each batch was accurately determined using Protein A high performance liquid chromatography (HPLC).

3.3.3 HCP Assay

Host cell protein remaining in the monoclonal antibody preparations was determined using a commercially available ELISA kit (catalog # CM015) from Cygnus technologies, according to the manufacturer's instructions.

3.3.4 Protein A assay

Residual Protein A remaining in the monoclonal antibody preparations was determined using a commercially available ELISA kit (catalog # F400) from Cygnus technologies, according to the manufacturer's instructions. According to the manufacturer, this kit has been shown to be equally sensitive to the MabSelect SuRe™ ligand as well as unmodified Protein A ligand. Spike recovery tests were applied to each sample, in which standard Protein A is added to the test material, and found to give acceptable recovery, indicating no significant matrix interference.

3.3.5 SEC analysis

Size-related impurities were estimated using a BioSep S3000 300 x 7.8 mm column (Phenomenex) running on a Waters Alliance 2695 HPLC system with 2487 detector module. The column was equilibrated with a buffer containing 50 mM sodium phosphate and 250 mM Na_2SO_4 pH 6.5 at 0.5 ml/min. The column eluate was monitored by A280 and the column was eluted for 30 minutes per run. Samples were diluted to 1 mg/ml using the running buffer and 40 µl injections were used for each analysis.

3.3.6 Recovery

Recovery of the monoclonal antibody by each resin was determined by comparing the amount of monoclonal antibody eluted (estimated by UV spectrophotometry) to the amount of monoclonal antibody in the load solution as estimated by Protein A HPLC prior to purification.

3.3.7 UV analysis

UV spectrophotometry was performed using a Cary 50 UV spectrophotometer (Varian Instruments) to determine the concentration of the purified protein. An extinction co-efficient $\varepsilon^{0.1\%}$ 0.1% at 280 nm of 0.613 $ml.mg^{-1}cm^{-1}$ was used.

3.3.8 Protein A chromatography

Protein A chromatography was performed in the same manner for each resin tested. Using an ÅKTAExplorer FPLC system (GE Healthcare) the Protein A resin was equilibrated in a solution of 50 mM Tris, 100 mM Na_2SO_4, 5 mM EDTA pH 7.0 for 10 column volumes (CV) at 165 cm/hr. The conditioned medium was then loaded onto the resin at 165 cm/hr to provide a residence time of 4 min for the load solution. Once loading was complete the column was washed with the equilibration solution for a further 4 CV at 230 cm/hr. The column was then washed with the secondary wash solution of 15 mM sodium citrate, 100 mM Na_2SO_4 pH 5.7 for 4 CV at a linear velocity of 230 cm/hr. The monoclonal antibody was then eluted from the

column using a solution of 15 mM sodium citrate, 100 mM Na_2SO_4 pH 3.2 at a linear velocity of 75 cm/hr. The protein peak eluting from the column with this solution was collected and prepared for further processing.

3.3.9 Viral inactivation and filtration

The eluate pool from Protein A chromatography was adjusted to pH 3.4 by the addition of 1 M citric acid and left to stir for 45 minutes. The solution was then filtered through a 1.2 μm Supor membrane (Pall) before being further filtered through a 0.2 μm membrane of the same type.

3.3.10 Cation exchange chromatography

After viral inactivation and filtration the monoclonal antibody was further purified on the cation exchange resin Fractogel EMD SO_3^- (M) using an ÅKTAExplorer FPLC system (GE Healthcare). The column was equilibrated in a solution of 300 mM sodium acetate pH 6.0 for 2 CV, followed by 4 CV in a solution of 80 mM sodium acetate pH 5.0, with both washed performed at a linear velocity of 150 cm/hr. The antibody pool was then loaded onto the column at 150 cm/hr, to a maximum loading density of 34 mg/ml. The column was then washed with a solution of 50 mM sodium acetate pH 5.0 for 4 CV at 500 cm/hr. A secondary wash using a solution of 80 mM sodium acetate pH 6.0 at a linear velocity of 500 cm/hr was then performed for 4 CV. The column was then eluted with a linear gradient to 300 mM sodium acetate pH 6.0 over 34 CV at a linear velocity of 500 cm/hr, with the eluting protein collected for further processing on anion exchange chromatography.

3.3.11 Anion exchange chromatography

Q-sepharose FF resin was used for anion exchange chromatography using an ÅKTAExplorer FPLC system (GE Healthcare). The resin was equilibrated in a solution of 50 mM Tris, 50 mM NaCl pH 7.5 at a linear velocity of 600 cm/hr. The protein pool from cation exchange was adjusted to pH 7.5 by the addition of 1 M Tris base, then loaded onto the column at

600 cm/hr. The monoclonal antibody does not bind under these conditions, so the non-binding pool was collected as it eluted from the column. To wash the protein pool though the column the equilibration solution was used at a linear velocity of 600 cm/hr.

3.3.12 Maximum and Dynamic binding capacity

Maximum binding capacity of any resin is highly dependent on running conditions and sample preparation. Binding capacity studies were conducted with conditioned media containing the monoclonal antibody. A residence time of 4 minutes was selected for the determination of the dynamic binding capacity. Each resin was equilibrated in 50mM Tris, 100 mM Na_2SO_4, 5 mM EDTA pH 7.0 before being challenged with the protein load solution of conditioned media containing 2 g/L monoclonal antibody (mAb). Loading was continued until the UV trace of the eluate indicated significant protein breakthrough was occurring (the maximum binding capacity of the resin had been reached). The resin was then eluted with a solution of 15 mM sodium citrate, 100 mM Na_2SO_4 pH 3.2, the eluate collected and quantitated by UV spectrophotometry to determine the maximum binding capacity of the resin.

To determine dynamic binding capacity packed columns of the three different chromatography resins were challenged with conditioned media containing the recombinant monoclonal antibody at 2 mg/ml using a linear flow rate of 300 cm/hr. The non-binding eluate of the column was sampled at intervals and the amount of unbound antibody determined using Protein A HPLC. The derived data was expressed as the fraction of the bound compared to the initial load concentration (C/C_0). The Dynamic binding capacity (DBC) curves produced by the resins were fit to the sigmoidal dose response (Hill-Slope) curve (Y = Bottom + (Top - Bottom)*XH/(EC50H+XH) using GraphPad Prism 6 for Windows (GraphPad Software Inc.). EC50 indicates the resin loading density at 50 % breakthrough, H the slope of the Hill-Slope curve, X the total amount of ligand (mAb) loaded onto the column and Y the concentration of

free ligand divided by the concentration of ligand in the initial load solution. "Bottom" denotes the lower asymptote of the C/C_0 curve and "Top" the upper asymptote.

3.3.13 Static Binding Capacity

Static binding data was obtained by creating a 50% slurry in equilibration buffer (20 mM Phosphate 200mM NaCl pH 7.4 for MabSelect™ Sure and 100 mM PBS pH 7.4 for the other two resins) and transferring varying quantities of resin such that the settled volume of media ranged from 20 - 400µl. The amount of media for MabSelect™ Sure and POROS Mab Capture A was determined from the volume of slurry. In contrast the amount of media for ProSep-vA Ultra was determined by weight of the settled media with excess liquid removed. This was due to difficulty in pipetting the fast settling glass based ProSep-vA Ultra resin.

Clarified cell culture supernatant (1mL) was added to the resin. The clarified culture and resin was allowed to equilibrate overnight on a shaker at room temperature. After equilibration the bulk fluid was removed and mAb concentration (C) determined by Protein A HPLC. Bound antibody concentration (q) was determined by mass balance using Eq. 1.

$$V_a C_a = V_l C + V_m q \quad \text{(Eq. 1)}$$

Where V_a is the volume of culture added, C_a is the initial concentration of the culture, V_l is the volume of liquid occupied by the unbound antibody and V_m represents the volume of chromatography media added. V_l was obtained by Eq. 2.

$$V_l = V_a + \varepsilon V_m \quad \text{(Eq. 2)}$$

Where ε represents the bulk porosity. Adsorption data was fit to the Langmuir, Freundlich and Langmuir-Freundlich equations using GraphPad Prism 6.0 (GraphPad Software Inc.).

3.3.14 Protein A HPLC

Protein A HPLC was undertaken on a Waters Alliance 2695 HPLC system utilising Empower software and 2487 detector module. The column was an Applied Biosystems PA ImmunoDetection Sensor Cartridge. A standard of purified mAb was created at an approximate concentration of 25 mg/ml, the accurate concentration being determined using a Cary 50 spectrophotometer. Loads of 40 and 100 μL were used to construct a standard curve. Column temperature was set to 25 °C and runs were performed using equilibration buffer (0.15M NaCl, 7.2mM Na_2HPO_4, 2.8mM NaH_2PO_4) and elution buffer (0.15M NaCl, 10mM NaH_2PO_4, 12mM HCl).

3.3.15 Isothermal titration calorimetry

The Isothermal Titration Calorimetry was performed on the VP-ITC micro-calorimeter from Microcal ^{TM}, INC (Northampton, MA). The 1.4mL sample cell was filled with mAb diluted to the desired concentration (1.5 μM) and the injection syringe was filled with 250 μL of 7.5 μM native Protein A or MabSelect SuRe™ ligand solution.

Both the mAb and ligand solutions were dialyzed into a buffer of pH 7, 1 M NaCl before analysis. All samples were degassed for 10 minutes prior to reaction to remove any air bubbles. The initial delay was 60 seconds and the stirring speed was 307 RPM to ensure continuous mixing. Titrations consisted of 25 injections of 10.0 μL in 30 seconds with a spacing time of 300 seconds to ensure a complete reaction of each injection and time for the temperature to re-equilibrate.

3.4 Theory

The majority of Protein A chromatography experiments utilise the Langmuir isotherm (Eq. 3) to characterise Protein A adsorption, including work by Jiang et al.[62], Ghose et al.[63], Hahn et al.[59] and McCue et al.[57]. Throughout all articles using the Langmuir isotherm, very little is done to justify the applicability of Langmuir to Protein A-mAb adsorption.

$$q = \frac{q_m K_l C}{1+K_l C} \quad \text{(Eq. 3)}$$

Where q is the mAb concentration bound to media, q_m is the adsorption capacity of the media, K_l is the Langmuir equilibrium constant and C represents the concentration of unbound mAb in the bulk fluid.

The Freundlich isotherm (Eq. 4) is often used to empirically fit adsorption data but requires an independent determination of maximum binding capacity.

$$q = K_f C^m \quad \text{(Eq. 4)}$$

Where K_f and m represent the two Freundlich parameters.

The Langmuir-Freundlich isotherm (Eq. 5) is a heterogeneous model of binding that has been shown to accurately model other affinity systems.

$$q = \frac{q_m K C^n}{1+K C^n} \quad \text{(Eq. 5)}$$

Where K is the Langmuir-Freundlich association constant and n represents the heterogeneity index, n=1 being a perfectly homogeneous system and decreasing values of n indicating increasing heterogeneity.

The Langmuir-Freundlich isotherm was utilised by Umpleby et al.[74] to characterise the adsorption of molecularly imprinted polymers (MIPs). Wang et al.[64] explored the adsorption of recombinant human collagen in immobilized metal affinity chromatography. Both of these affinity systems display heterogeneity that was modelled successfully with the Langmuir-Freundlich isotherm over a wide range of concentrations.

3.5 Results

3.5.1 Performance comparison

To compare the performance of the three Protein A resins, each resin was used to purify a conditioned medium containing a recombinant mAb using identical conditions. The yield and purity of the product obtained was then determined to compare the resin performance under manufacturing conditions with the quantity of monoclonal antibody eluted determined using UV spectrophotometry (Table 5). Recovery and SEC purity were approximately the same for all resins, with the recovery values greater than 100 % obtained with two of the resins attributed to underestimation of the feedstock concentration by HPLC assay of the crude feedstock.

Table 5: Yield and purity of the mAb produced by the three different resins

Resin	Recovery (%)	SEC purity (%)	Maximum binding capacity (mg/mL)	HCP (ppm)	Protein A (ppm)
MabSelect SuRe™	114	98.0	44	14	1
POROS MabCapture A	111	97.7	31	8	36
ProSep-vA Ultra	94	96.4	37	8	14

MabSelect SuRe™ was found to have the highest binding capacity of 44 mg/mL of resin at 4 min residence time. POROS MabCapture A had the lowest binding capacity of approximately 31 mg/mL of resin, almost 30% lower than the dynamic binding capacity of MabSelect SuRe™ under the same conditions. ProSep-vA Ultra was found to have a maximum binding capacity of approximately 37 mg/mL of resin, approximately 15% lower than the DBC of MabSelect SuRe™ under the same running conditions. The concentration of HCP in the

eluted mAb was approximately the same for all resins. Although there are some differences in the HCP levels found, given that the results for each of the tested resins are so close to the limit of quantitation of the assay (4 ppm), there is little functional difference in results. Protein A chromatography is the first capture step of the downstream purification process, and there are two more orthogonal purification steps generally remaining in a standard purification which would be expected to remove HCP, so HCP of <20 ppm is expected to be acceptable in most purification trains.

To demonstrate that the levels of residual HCP and Protein A present in the eluates could be brought under control with further processing the POROS MabCapture A eluate was purified using cation and anion exchange chromatography (Table 6). The residual HCP and Protein A was brought to the same level as the commercially available reference standard by further purification when measured with the same assays, indicating that none of the HCP or Protein A levels detected in the Protein A eluates were high enough to cause significant difficulty in producing a sufficiently pure final product.

Table 6: HCP and Protein A removal in downstream purification

Chromatography step	**HCP (ppm)**	**Protein A (ppm)**
Affinity	6	43
Cation Exchange	<4	0.8
Anion Exchange	<4	0.6
Reference Standard	<4	1

MabSelect SuRe™ was found to have the highest ligand stability of the three tested media. An average Protein A ligand leakage of 1 ppm was found with this resin. The Protein A content of a reference standard of the monoclonal antibody was also measured at 1 ppm using this ELISA. POROS MabCapture A was found to have much higher Protein A leakage of approximately 36 ppm, similar to ProSep-vA Ultra (14 ppm).

3.5.2 Static binding capacity

Equilibrium adsorption data for the three media was fitted to the Langmuir, Freundlich and Langmuir-Freundlich isotherms to compare the suitability of the various isotherms and the performance of the various resins (Table 7, Table 8 and Table 9). The curve fits produced for the three resins are also displayed Figure 7, Figure 8 and Figure 9, respectively.

MabSelect SuRe™ displayed a static binding capacity (q_m) of 42 mg/mL using the Langmuir isotherm (Table 7). A significantly different result was achieved using the Langmuir-Freundlich isotherm which yielded a q_m of 75 mg/ml. The Freundlich isotherm does not produce a value for q_m. A high degree of binding heterogeneity was also indicated by the Langmuir-Freundlich isotherm (n=0.4654; Table 8). The slow rise of the equilibrium curve indicates a high degree of heterogeneity (Figure 7). Consequently the Freundlich and Langmuir-Freundlich isotherms were both better able to model the adsorption behaviour than the Langmuir isotherm on this resin as these are better able to model non-homogenous binding behaviour. The Freundlich and Langmuir-Freundlich isotherms produce significantly lower variance when fit to the data produced by MabSelect SuRe™, compared to the Langmuir.

ProSep vA Ultra displayed a q_m of 18 mg/ml when the data were analysed using the Langmuir isotherm. The Langmuir-Freundlich isotherm produced a much higher q_m (63mg/ml). With this resin the equilibrium curve also displayed a high level of heterogeneity (n=0.3154) that could not be accurately modelled with the Langmuir equation ($R^2 = 0.80$) and the R^2 values for the other two equations were consequently much higher ($R^2 = 0.96$; Table 7 to Table 9**Error! Reference source not found.**).

POROS MabCapture A was found to have a q_m of 36 mg/ml using both the Langmuir and Langmuir-Freundlich isotherms, as the resin displayed entirely homogenous binding behaviour (n = 1). Under these conditions the Langmuir-Freundlich equation is equivalent to the Langmuir.

The Freundlich isotherm does not provide a value for static binding capacity, but the R^2 value produced by this isotherm was the lowest of all three equations (R^2 = 0.83) for POROS MabCapture A.

Table 7: Langmuir Isotherm Parameters

Media	**K_l**	**q_m**	**R^2**
MabSelect SuRe™	10.26	41.77	0.8769
ProSep vA Ultra	20.56	18.01	0.7951
POROS MabCapture A	118.9	36.24	0.9609

Table 8: Langmuir-Freundlich Isotherm Parameters

Media	**K**	**q_m**	**n**	**R^2**
MabSelect SuRe™	1.033	75.49	0.4654	0.9747
ProSep vA Ultra	0.384	63.31	0.3154	0.9644
POROS MabCapture A	118.9	36.24	1.000	0.9609

Table 9: Freundlich Isotherm Parameters

Media	**Kf**	**n**	**R^2**
MabSelect SuRe™	38.11	0.3121	0.9762
ProSep vA Ultra	17.57	0.2599	0.9640
POROS MabCapture A	36.64	0.1893	0.8257

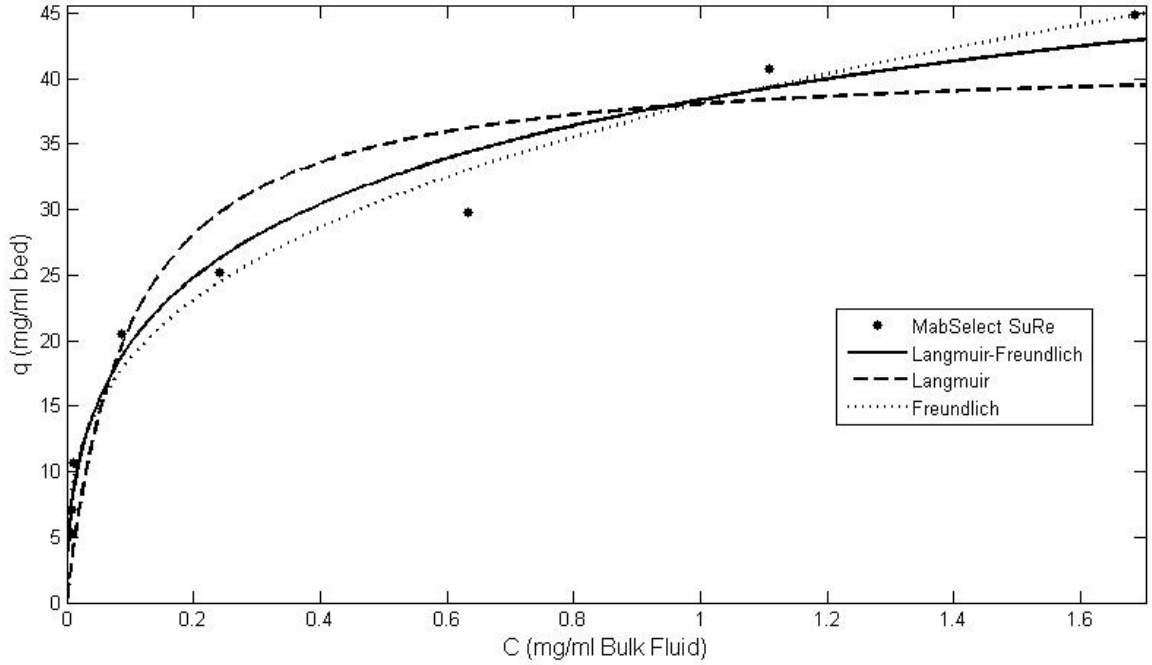


Figure 7: Equilibrium adsorption data for MabSelect SuRe™ using monoclonal IgG (2 mg/ml) in Dulbecco's modified Eagles Medium (DMEM).

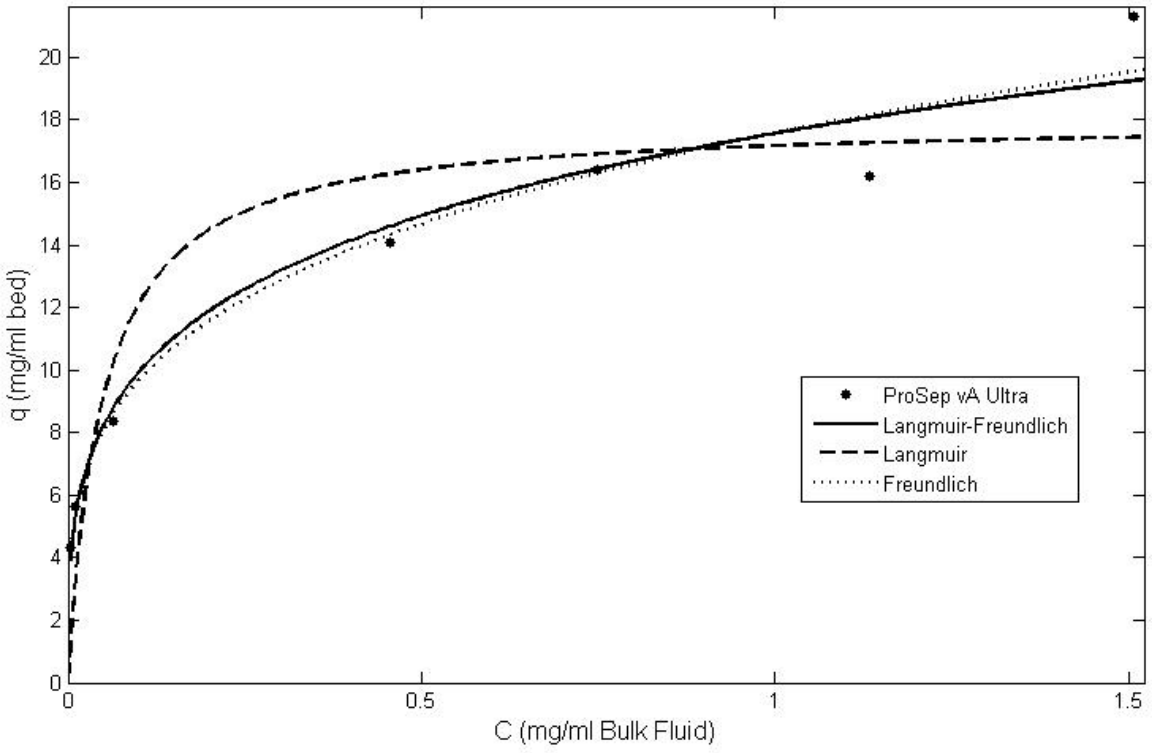


Figure 8: Equilibrium adsorption for ProSep vA Ultra using monoclonal IgG (2 mg/ml) in Dulbecco's modified Eagles Medium (DMEM).

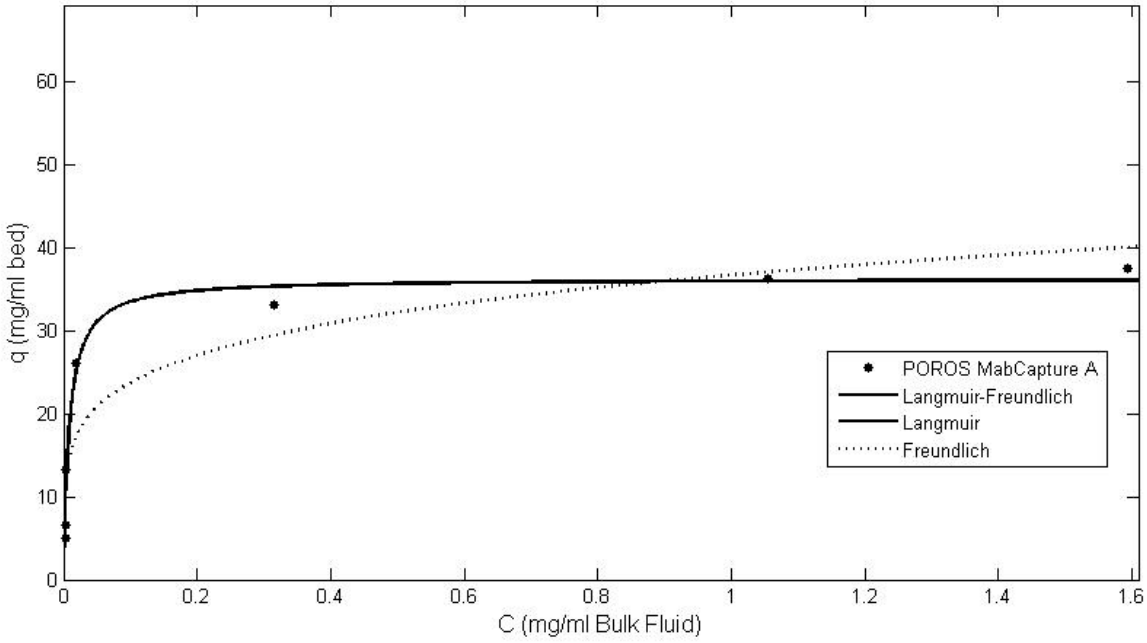


Figure 9: Equilibrium adsorption for POROS MabCapture A using monoclonal IgG (2 mg/ml) in Dulbecco's modified Eagles Medium (DMEM).

3.5.3 Dynamic binding capacity

Dynamic binding capacity experiments conducted with the various Protein A resins using a conditioned medium feedstock were broadly in agreement with the results obtained for maximum binding capacity and static binding capacity (Figure 10). Mab Select SuRe™ was found to have the highest DBC under the conditions studied and POROS MabCapture A the lowest (Table 10). All three resins produced noticeably different DBC curves indicating significant differences in their mass transfer and flow properties (Figure 10). Under the conditions studied, ProSep vA and Mab Select SuRe™ did not achieve complete saturation binding, whereas the POROS resin was clearly saturated well before the end of loading. The markedly different slope values obtained for the DBC curves illustrate the large differences in binding behaviour (Table 10). Pore diffusion and film mass transfer govern the behaviour and appearance of DBC curves as they govern the rate of adsorption of the product to the ligand

[75]. As the rate of adsorption to the ligand is so much faster than the pore diffusion and film mass transfer rates the contribution of the rate of adsorption to the DBC curve is negligible in packed bed experiments of this sort. The much more rapid saturation of the POROS resin is reflective of the smaller bead size (45 μm), larger pore diameter (50 – 1000 nm) and flowthrough pore structure of this resin as mass transfer resistance increases with the square of the particle diameter [58]. In this data set the slope of the binding curve appears significantly steeper for the ProSep resin than the POROS resin, however this is probably exaggerated as only the POROS resin reached saturation under the conditions used.

Effective pore diffusion rates for MSS and ProSep have previously been estimated and found to be very similar [58] so the much steeper slope for ProSep compared with MSS in this experiment reflects a more rapid film mass transfer coefficient for this resin despite it having a large average particle diameter than the MSS resin (100 versus 85 μm, respectively). Previous work has also indicated a steeper slope in DBC curves for the ProSep resin versus MSS [58].

Table 10: Dynamic binding capacities of the three resins

Resin	DBC (mg/ml)	Slope (H)
Mab Select SuRe™	60.6	3.7
ProSep vA ultra	51.4	15.2
POROS Mab Capture A	33.4	9.3

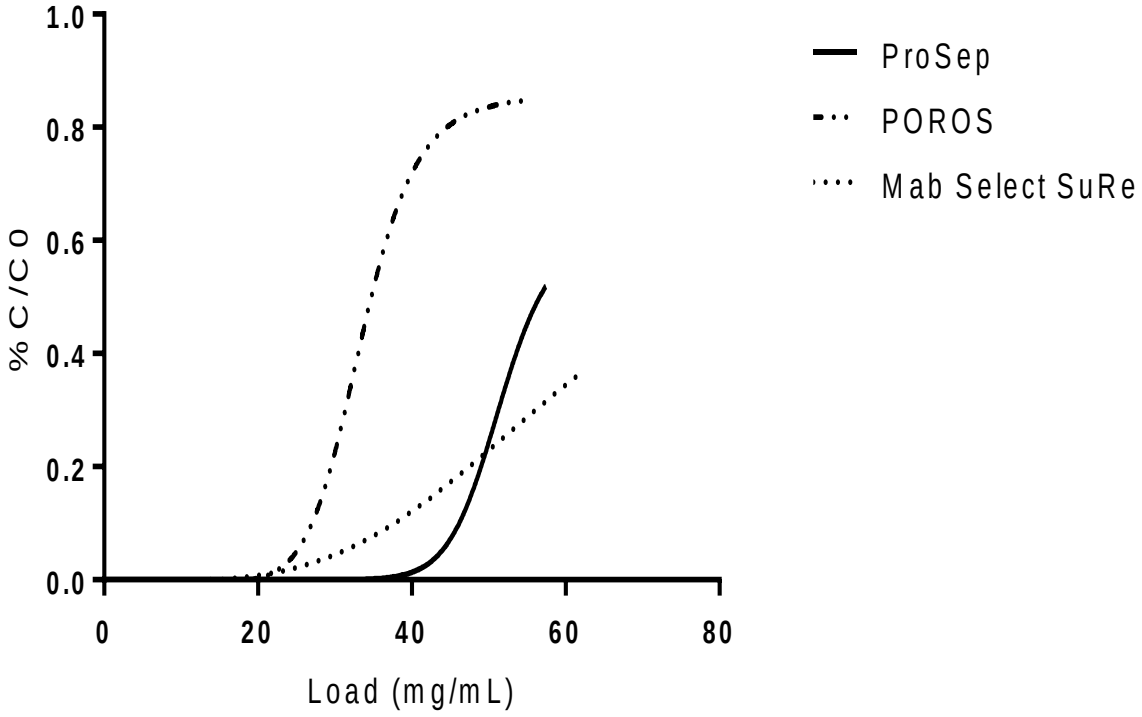


Figure 10: Dynamic binding capacity curves for the three resins.

Each resin was equilibrated in 50 mM Tris, 100 mM Na_2SO_4, 5 mM EDTA pH 7.0 before being loaded with the conditioned medium containing IgG at 2 mg/ml at a linear flow rate of 300 cm/hr.

3.5.4 Isothermal titration calorimetry

The MabSelect SuRe™ resin is composed of a modified Protein A ligand that has been optimised to improve tolerance to alkaline conditions attached to an agarose base matrix. The other two Protein A resins use the native Protein A molecule as ligand attached to cross-linked polystyrene-divinylbenzene (POROS MabCapture A) and controlled pore glass

(ProSep vA Ultra). As native Protein A is composed of 5 homologous antibody binding domains the variation in affinity between the five binding domains could contribute to the heterogeneity of Protein A-mAb systems when using native Protein A. This variance in domains is eliminated in MabSelect SuRe™ which contains 4 identical Z domains capable of withstanding alkali conditions [76]. To determine how much of the differing behaviours of the Protein A resins could be explained by the different Protein A ligands the binding of the purified ligand to the target monoclonal antibody was compared using isothermal titration calorimetry.

The purified monoclonal antibody was placed into conditions mimicking the solution conditions of spent cell culture (pH 7, 1 M NaCl, 25 °C) and the interaction analysed. The results are shown in Table 11. Of note is the dramatically higher affinity binding constant obtained with the modified ligand and the much higher ΔH and $T\Delta S$ values.

Table 11: ITC data from titrating native Protein A and Mab Select SuRe™ ligand into mAb solution

	Ka (M^{-1}) [x 10^8]	ΔG ($kJ.mol^{-1}$)	ΔH ($kJ.mol^{-1}$)	N	TΔS ($kJ.mol^{-1}$)
Native Protein A	1.7 ± 0.33	-46.9 ± 2.28	-189 ± 2.28	0.59	-142
MabSelect SuRe™	11.3 ± 1.96	-54.4 ± 3.81	-573 ± 3.81	0.41	-519
Recombinant Protein A (Arouri *et al* 2007)	3.5 ± 0.75	-48.8 ± 0.50	-272 ± 2.0	0.60	-223

3.6 Discussion

All three resins were found to perform well with model mAb conditioned medium and produced a highly purified eluate appropriate for further processing. Despite having a lower maximum flow rate, the MabSelect SuRe™ resin was found to have a slightly higher binding capacity than the other two resins when measured under flow and static binding conditions. When challenged with a manufacturing feedstock, all resins produced an eluate with very low levels of residual HCP. The stability of the Protein A ligand proved to be far better with the MabSelect SuRe™ resin as the eluate from this resin contained only 1 ppm residual Protein A, while all other impurities were roughly equal from all resins. This additional stability is important for an industrial Protein A resin as the resin would be expected to degrade with the repeated use that occurs in biopharmaceutical manufacturing and the level of leached Protein A would be expected to increase over time. The higher binding capacity, lower HCP and leached Protein A levels, when combined with the simpler and more economical chemicals required for sanitization and storage of this resin make it the leading choice for industrial applications.

The lower residual Protein A and higher binding capacity for MabSelect SuRe™ in this study was supported by the literature reported in this area. Literature also suggests that this resin requires less acidic elution conditions than the other two resins, making it advantageous for use with products that are more susceptible to acid denaturation. The narrower range of elution pHs for a range of antibodies [77] makes it suitable for use in a platform process designed for use with a variety of antibodies.

MabSelect SuRe™ and ProSep vA Ultra resins displayed highly heterogeneous binding behaviour in static binding capacity tests with clarified supernatant. The Freundlich equation was able to fit this data but failed to model the homogeneous system of POROS MabCapture

A. Further, the parameters obtained with the Freundlich equation did not allow for ease of interpretation and comparison. In contrast the Langmuir isotherm was only able to accurately model data that was highly homogeneous as displayed by POROS MabCapture A. With this resin the Langmuir-Freundlich isotherm reduced to the Langmuir equation (Figure 9) as the homogeneity index was equal to 1.

The Langmuir-Freundlich isotherm was demonstrated to provide a good fit for all three media of varying heterogeneity, whilst allowing for comparison of the media binding capacity, association constant and heterogeneity. That cell culture supernatant produced highly heterogeneous isotherms when compared to studies utilising purified IgG is important when considering industrial applications. Implementation of Quality by Design should account for the likely increase in heterogeneity due to impurities typically found at this purification stage. The Langmuir-Freundlich isotherm is the recommended equation for modeling the binding of monoclonal antibodies to Protein A chromatography resins in industrial settings as it is able to model both heterogonous and homogenous behaviour and allows simple comparison of chromatography resins which differ greatly in their binding behaviour.

It would be expected that the 5 identical domains of MabSelect SuRe™ would produce a high degree of binding homogeneity. This was not the case, which suggests that other factors are dominant in determining the heterogeneity of binding. An explanation of the heterogeneous character of the Protein A media:IgG system is offered in [53] - suggesting that accessibility of IgG to the binding site is an important factor. These authors suggest accessibility could be affected by factors such as a small pore size not allowing formation of the IgG-ligand complex and partial deactivation of the ligand during coupling. Further support for the idea that accessibility is important in this respect is provided in this study by the homogeneity determined for POROS MabCapture A. This resin has a rigid structure and flow through particles with pore size designed for rapid mass transport [67, 68] and correspondingly exhibited entirely homogenous binding behaviour. Problems of accessibility and hence

binding heterogeneity would be further compounded in a situation where contaminants are present and competing for binding sites as they are during the industrial purification of monoclonal antibodies, adding further support for the use of the Langmuir –Freundlich isotherm in industrial comparisons.

When the interaction between the model mAb and purified Protein A ligands was compared using isothermal titration calorimetry, much higher values for the affinity constant (K_a), enthalpy (ΔH) and entropy ($T\Delta S$) were obtained for the MabSelect SuRe™ ligand. However, approximately the same free energy change (ΔG) was measured with both ligands. The thermodynamic parameters measured for native Protein A were very similar to those reported previously for the interaction of recombinant Protein A with an IgG1 protein [78] under similar conditions (pH 7.2, 25 °C, 132 mM NaCl - Table 11).

The higher enthalpy value indicates that association between MabSelect SuRe™ ligand and the monoclonal antibody is much more favourable in terms of energy release than that between the antibody and native Protein A. This is also reflected in the higher affinity constant.

The ITC binding co-efficient (N) measures the concentration required for 50 % saturation of binding. It has previously been observed that although each IgG1 molecule has two binding sites for Protein A in the Fc region, a stoichiometry factor of 0.6 is observed in ITC measurements [78]. This is most likely due to the way in which the experiments were performed, with Protein A titrated into an excess of IgG molecule. and when the reverse experiment is performed (IgG titrated into an excess of Protein A) a stoichiometry factor of N = 1 is obtained [79]. The binding co-efficient value of N = 0.4 for MabSelect SuRe™, compared with 0.6 for the native Protein A in this study, possibly indicates some steric hindrance comes into play with the ligand due to the repeated structure of identical domains, a hindrance that does not affect the native or recombinant ligand as severely.

The significantly higher entropy change found with MabSelect SuRe™ could indicate a possible risk of the modified ligand. One of the reasons Protein A is so specific in its interaction with IgG is that its interaction with the antibody molecule involves an induced fit [80-82] which is achieved by destabilization of the $C\gamma_2$ domain of the IgG. The much higher entropy change obtained with the modified ligand indicates a much more significant re-arrangement of the monoclonal antibody molecule and ligand upon binding. This raises the possibility that partial denaturation of the antibody is more of a risk when using the MabSelect SuRe™ ligand compared with the native ligand. This observation, and the higher K_a value obtained for MabSelect SuRe™, appear in contrast to the observation of [60] that in general the interaction between MabSelect SuRe™ and IgG is weaker than with native Protein A, as milder elution conditions were generally required when examining the use of a range of antibodies with this resin.

3.7 Conclusions

MabSelect SuRe™ resin was found to have a slightly higher binding capacity than either ProSep-vA Ultra or POROS MabCapture A when measured under flow and static binding conditions with a conditioned medium containing recombinant monoclonal antibody. The stability of the Protein A ligand proved to be far better with MabSelect SuRe™ as the eluate from this resin contained only 1 ppm residual Protein A, while all other impurities were roughly equal from all resins. MabSelect SuRe™ is therefore the preferred choice as the basis for a platform manufacturing process. When the interaction between the model mAb and purified Protein A ligands was compared using isothermal titration calorimetry, much higher values for the affinity constant (K_a), enthalpy (ΔH) and entropy ($T\Delta S$) were obtained for the MabSelect SuRe™ ligand. The significantly higher entropy change found with MabSelect SuRe™ could indicate a possible risk of the modified ligand as it indicates a much more significant re-arrangement of the monoclonal antibody molecule and ligand upon binding.

This raises the possibility that partial denaturation of the antibody is more of a risk when using the MabSelect SuRe™ ligand compared with the native ligand.

The binding co-efficient value of N = 0.4 determined for MabSelect SuRe™ using ITC, compared to 0.6 for the native Protein A in this study, indicates some steric hindrance comes into play with this ligand due to the repeated structure of identical domains - a hindrance that does not affect the native or recombinant ligand as severely. The MabSelect SuRe™ ligand could be further improved by reducing this hindrance.

In using multiple models to analyse the binding of the multiple protein A resins to the monoclonal antibody feedstock it was found that the Langmuir-Freundlich isotherm is the recommended equation for modelling the binding of monoclonal antibodies to Protein A as it is able to model both heterogonous and homogenous behaviour and allows simple comparison of chromatography resins which differ greatly in their binding behaviour.

QbD requires consideration of all the parameters relevant to the consistent manufacturing performance of a process and this comprehensive approach has been adopted in this work comparing protein A resins. In addition, for a QbD approach to produce a truly fair comparison of the performance of resins it is also necessary to ensure that appropriate mathematical models are being used to analyse their performance. This work demonstrates that for modelling and comparing the equilibrium binding behaviour of protein A to a manufacturing feedstock containing monoclonal antibody the Langmuir-Freundlich isotherm is more appropriate and flexible than other models frequently used in the literature.

3.8 References

1. Gottschalk, U., ed. *Process Scale Purification of Antibodies*. 1st ed. 2009, John Wiley & Sons, Inc.: Hoboken, New Jersey. xvii - xx.
2. Vunnum, S., G. Vedantham, and B. Hubbard, *Protein A-Based Affinity Chromatography*, in *Process Scale Purification of Antibodies*, U. Gottschalk, Editor. 2009, John Wiley & Sons, Inc.: Hoboken, New Jersey. p. 79 - 102.
3. Perez-Almodovar, E.X. and G. Carta, *IgG adsorption on a new Protein A adsorbent based on macroporous hydrophilic polymers. I. Adsorption equilibrium and kinetics.* J Chromatogr A, 2009. **1216**(47): p. 8339-47.

4. Perez-Almodovar, E.X. and G. Carta, *IgG adsorption on a new Protein A adsorbent based on macroporous hydrophilic polymers II. Pressure-flow curves and optimization for capture.* J Chromatogr A, 2009. **1216**(47): p. 8348-54.
5. Scolnik, P., *mAbs: A business perspective.* MAbs, 2009. **1**(2): p. 179-84.
6. Braisted, A. and J. Wells, *Minimizing a binding domain from Protein A.* Proc Natl Acad Sci U S A, 1996. **93**: p. 5688 - 5692.
7. McCue, J.T., et al., *Evaluation of protein-A chromatography media.* J Chromatogr A, 2003. **989**(1): p. 139-53.
8. Hahn, R., et al., *Comparison of Protein A affinity sorbents II. Mass transfer properties.* J Chromatogr A, 2005. **1093**(1-2): p. 98-110.
9. Hahn, R., R. Schlegel, and A. Jungbauer, *Comparison of Protein A affinity sorbents.* J Chromatogr B Analyt Technol Biomed Life Sci, 2003. **790**(1-2): p. 35-51.
10. Hahn, R., et al., *Comparison of Protein A affinity sorbents III. Life time study.* J Chromatogr A, 2006. **1102**(1-2): p. 224-31.
11. Wang, C. and F. Mann, *Increasing MAb Capture Productivity.* BioProcess International, 2009. **7**(5): p. 56 - 61.
12. Jiang, C., et al., *A mechanistic study of Protein A chromatography resin lifetime.* J Chromatogr A, 2009. **1216**(31): p. 5849-55.
13. Ghose, S., B. Hubbard, and S.M. Cramer, *Binding capacity differences for antibodies and Fc-fusion proteins on Protein A chromatographic materials.* Biotechnology and Bioengineering, 2007. **96**(4): p. 768-779.
14. Wang, X.J., D.D. Fan, and Y.E. Luo, *Breakthrough model of recombinant human-like collagen in immobilized metal affinity chromatography.* Appl Biochem Biotechnol, 2009. **158**(2): p. 262-76.
15. Ghose, S., et al., *Antibody variable region interactions with Protein A: implications for the development of generic purification processes.* Biotechnol Bioeng, 2005. **92**(6): p. 665-73.
16. GEHealthcare, *MabSelect SuRe - studies on ligand toxicity, leakage, removal of leached ligand, and santization, GE Healthcare application note 11-0011-65 AB.* 2004.
17. AppliedBiosystems, *POROS® MabCapture™A Perfusion Chromatography® Media 4383704 Rev. A.* 2007.
18. AppliedBiosystems, *POROS® Perfusion Chromatography® Media 102BR05-02*. 2009.
19. Millipore, *ProSep®-vA Ultra Chromatography Media Lit. No. DS4241EN00*. 2004.
20. Millipore, *Affinity Chromatography Media Operating Instructions OIBP1131118 rev C*. 2005.
21. Fahrner, R.L., et al., *Performance comparison of Protein A affinity-chromatography sorbents for purifying recombinant monoclonal antibodies.* Biotechnol Appl Biochem, 1999. **30 (Pt 2)**: p. 121-8.
22. Swinnen, K., et al., *Performance comparison of Protein A affinity resins for the purification of monoclonal antibodies.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 97-107.
23. Millipore, *ProSep-vA Ultra Media 300 Cycle Lifetime Study*. 2005.
24. GEHealthcare, *Mab Select SuRe Instructions 11-0026-01 AD*. 2004.
25. Umpleby, R.J., 2nd, et al., *Characterization of molecularly imprinted polymers with the Langmuir-Freundlich isotherm.* Anal Chem, 2001. **73**(19): p. 4584-91.
26. Millipore. *ProSep-vA Ultra Chromatography media Data Sheet*. Available from: http://www.millipore.com/publications.nsf/a73664f9f981af8c852569b9005b4eee/e91495c1c046eb6f85256e90004eba49/$FILE/DS4241EN00.pdf.
27. Gottschalk, U., *Process scale purification of antibodies*. 2009, Hoboken, N.J.: John Wiley & Sons. xxvi, 430 p.
28. Shukla, A.A., et al., *Downstream processing of monoclonal antibodies--application of platform approaches.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 28-39.
29. Arouri, A., et al., *Hydrophobic interactions are the driving force for the binding of peptide mimotopes and Staphylococcal Protein A to recombinant human IgG1.* Eur Biophys J, 2007. **36**(6): p. 647-60.
30. Langone, J.J., et al., *Complexes prepared from Protein A and human serum, IgG, or Fc gamma fragments: characterization by immunochemical analysis of ultracentrifugation fractions and studies on their interconversion.* Mol Cell Biochem, 1985. **65**(2): p. 159-70.

31. Diesenhofer, J., *Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of Protein A from Staphylococcus aureus at 2.9 and 1.8 Å resolution.* Biochemistry, 1981. **20**: p. 2361 - 2370.
32. Dima, S., et al., *Effect of Protein A and its fragment B on the catabolic and Fc receptor sites of IgG.* Eur J Immunol, 1983. **13**(8): p. 605-14.
33. Ghose, S., B. Hubbard, and S.M. Cramer, *Protein interactions in hydrophobic charge induction chromatography (HCIC).* Biotechnol Prog, 2005. **21**(2): p. 498-508.

Statement of Authorship

Title of Paper	Isothermal titration calorimety comparison of the interaction between a model monoclonal antibody and native Protein A or the engineered mimic Mab Select SuRe
Publication Status	O Published, O Accepted for Publication, O Submitted for Publication, ⊙ Publication style
Publication Details	Manuscript in text

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Phillip Elliott		
Contribution to the Paper	Phillip contributed to the design and analysis of the project. Phillip contributed equally with Yuzhe in drafting the manuscript and was responsible for supply of experimental materials i.e. protein A and variants as well as the purified recombinant monoclonal antibody.		
Signature	[redacted]	Date	19FEB2015.

Name of Co-Author	Yuzhe Tang		
Contribution to the Paper	Yuzhe contributed to the design and analysis of the ITC experiments. Yuzhe also completed half of the drafting of the manuscript and largely carried out the experimental work.		
Signature	[redacted]	Date	19 Feb 2015

Name of Co-Author	Dr Jingxiu Bi		
Contribution to the Paper	Dr Bi contributed to the design and analysis of the ITC experiments. Dr Bi completed multiple reviews of the manuscript and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow as well as contributing to the design of the project.		
Signature	[redacted]	Date	20/02/2015

Name of Co-Author	Dr Hu Zhang		
Contribution to the Paper	Dr Zhang completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow as well as contributing to the design of the project.		
Signature	[redacted]	Date	20FEB2015.

4 Isothermal titration calorimetry comparison of the interaction between a model monoclonal antibody and native Protein A or the engineered mimic Mab Select SuRe™

4.1 Abstract

Three different types of Protein A resin have previously been used to produce a monoclonal antibody under manufacturing conditions. These resins differed in base matrix, ligand type (native versus engineered recombinant) and bead size. One resin, Mab Select SuRe™ (MSS) proved to be superior in terms of dynamic and static binding capacity, residual HCP and Protein A leachate [Chapter 3 of this thesis].

We hypothesize that the difference in separation performance of resins could be attributed to ligand types attached to the different resins. To test the hypothesis, the interaction between the purified monoclonal antibody and native Protein A ligand or the MSS ligand was explored using isothermal titration calorimetry (ITC). A range of conditions of pH, salt concentration and temperature were used to identify any differences in interaction between the two types of ligand and the monoclonal antibody.

ITC revealed a number of differences between the native and modified ligand. The MSS ligand was found to have an association constant 10 - 20 fold higher than the native ligand under all conditions of temperature, pH and salt concentration. Both ligands were relatively unaffected by temperature changes between 15 - 25 °C. The modified ligand also produced a $T\Delta S$ value 2 - 3 fold higher than the native ligand under all conditions studied, indicating that structural rearrangement of the antibody upon binding was much more significant than seen with the native ligand. No binding between native Protein A and antibody could be detected at

pH 4, whereas the MSS ligand retained some binding affinity for the antibody at this pH, though greatly reduced from the maximum value seen at pH 7.

4.2 Introduction

Protein A affinity chromatography is widely used as the initial capture operation in the purification of monoclonal antibodies [83-85]. The main purpose of this unit operation is the removal of host cell protein (HCP), DNA and a reduction in the total volume of the product solution [85, 86]. The native Protein A ligand specifically interacts with the Fc region of most immunoglobulins (IgGs) and forms the basis of this chromatography [87, 88]. Mab Select SuRe™ (MSS) is an engineered version of Protein A that is replacing native Protein A in a number of industrial processes for the manufacture of monoclonal antibody products. This engineered alternative to native Protein A has been designed to have greater alkaline resistance, allowing more stringent cleaning regimes and longer production life for the resin in industrial use [89, 90]. While native Protein A is composed of 5 highly homologous domains, the MSS ligand consists of five identical domains, referred to as Z-domains. These Z-domains are modified versions of the B-domain of native Protein A. Data presented earlier in this thesis indicates that resin using Mab Select SuRe™ ligand has some advantages over resin that uses native Protein A as the ligand. Specifically, Mab Select SuRe™ resin produced an eluate with lower levels of HCP impurities while having a higher static and dynamic binding capacity. As well as having different ligands, the resins compared also had different stationary phase properties i.e. the beads on which the ligands were grafted differed in their chemistry, size and porosity.

This work sought to determine whether the advantages noted were due to the different properties of the ligands on the resin. Isothermal titration calorimetry (ITC) is an excellent method of studying the interaction of two macromolecules as it allows the quantification of the complete set of thermodynamic parameters, providing insight into the stability, specificity and stoichiometry of the interaction. ITC also provides insight into the contribution of

conformational changes to a biomolecular interaction by direct measurement of the role of entropy changes in a binding event [78, 91].

In solution phase, the binding of a model monoclonal antibody to the native Protein A or engineered Mab Select SuRe™ ligand was measured using ITC. Binding of the antibody to the respective ligands was compared over a range of conditions of pH, conductivity and temperature to obtain a global comparison of the performance of the ligands. This was performed to enable a determination of the robustness of the interaction between the antibody and the Protein A ligand and it was hoped this would give some indication of the solution parameters that could be manipulated to promote specific binding of the antibody to the Protein A. This is relevant to the industrial manufacture of monoclonal antibodies as capture of antibodies from solution generally occurs in spent cell medium which can vary in pH, conductivity and temperature depending on the type and course of the cell culture prior to harvest.

4.3 Materials and Methods

A model recombinant monoclonal antibody (IgG) was provided by Hospira Adelaide. The native Protein A was obtained from Sigma. Purified MabSelect SuRe Ligand was from GE Healthcare.

The Isothermal Titration Calorimetry was performed on the VP-ITC micro-calorimeter from Microcal™, Inc. (Northampton, MA). The 1.4mL sample cell was filled with IgG diluted to 1.5 μM and the injection syringe was filled with 250 μL of 7.5 μM native Protein A or MabSelect SuRe ligand solution. Each ligand titration was carried out under the conditions of temperatures, salt concentration and pH as shown in Table 12. The buffer used was 50 mM sodium phosphate for all measurements. All experimental runs were performed in triplicate. Binding isotherms were analysed with non-linear regression analysis using the manufacturer's software to calculate binding parameters.

Table 12: Experimental design

Experimental Run No.	Temperature	Solvent pH	Salt concentration (mM NaCl)
1	15°C	7	100
2	20°C	7	100
3	25°C	7	100
4	25°C	4	100
5	25°C	5	100
6	25°C	6	100
7	25°C	7	100
8	25°C	7	100
9	25°C	7	500
10	25°C	7	1000

Both the IgG and ligand solution were dialyzed into the same buffer condition prior to ITC measurement. All samples were degassed under vacuum for 10 minutes to remove any air bubbles which could interfere with the measurement. The initial delay after titration was 60 seconds and the stirring speed was 307 RPM throughout to ensure a thoroughly mixed solution. Each titration was performed with 25 injections of 10.0 μL occurring over 30 seconds. Time between injections was 300 seconds to ensure a completed reaction prior to the next injection and allow sufficient time for the cell temperature to re-equilibrate.

4.4 Theory

When titration starts, injection of the ligand results in the evolution of heat within the sample cell causing a negative change in the differential power (DP) signal. Normally the signal reaches its highest value in the first few titrations due to complete reaction between antibody and ligand. With subsequent injections the IgG becomes saturated with ligand so the DP

signal diminishes until only the background heat of dilution is observed. The thermodynamic parameters of binding are obtained from the titration curve produced by successive injections. As shown in Figure 11, the enthalpy change (ΔH) is the difference between the initial and saturated state. The association constant (K_a) is the slope of the curve and the binding coefficient is the molar ratio of ligands at 50 % saturation.

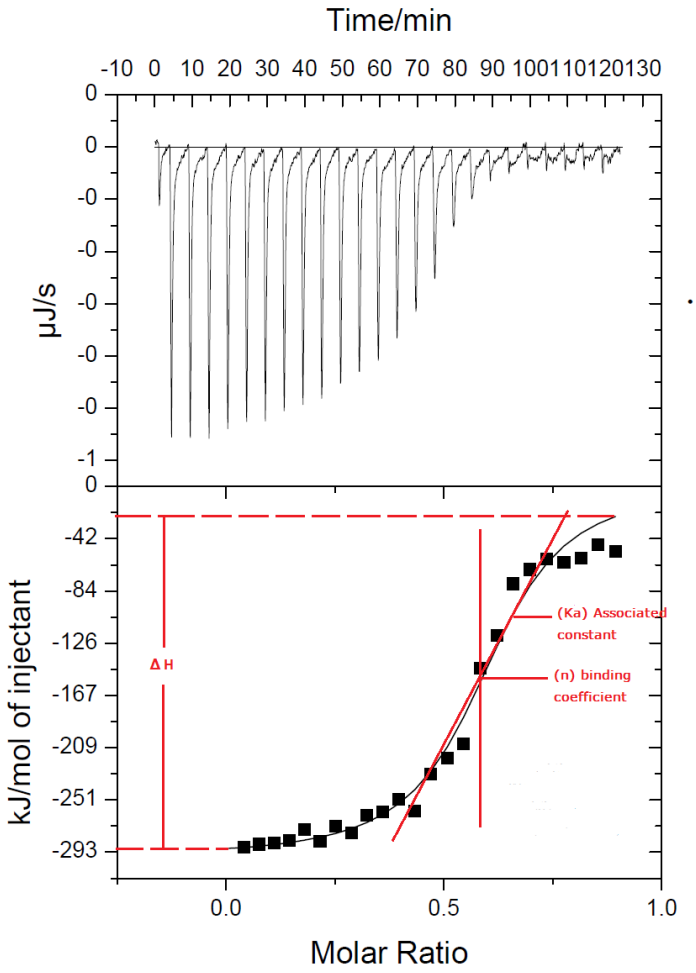


Figure 11: Thermogram (top) and binding isotherm (bottom) for the interaction between native Protein A and model IgG.

The concentration of Protein A and IgG were 7.5 and 1.5 µM, respectively. The experiment was performed at 25 °C in 50mM sodium phosphate, 100mM NaCl, pH 7.

For this work, a one-site binding model was used where K_a is given by the expression:

$$Ka = \frac{\Theta}{(1 - \Theta)[X]}$$

Where Θ is the fraction of sites occupied by the ligand and X is the concentration of free ligand.

The parameters ΔG (change in free energy) and ΔS (change in entropy) are calculated from the Van't Hoff equation:

$$\ln Ka = \frac{\Delta H^0}{RT} - \frac{\Delta S}{R} = \frac{\Delta G}{RT}$$

Where R is the gas constant and T is the temperature in degrees Kelvin.

4.5 Results

4.5.1 The effect of temperature

The temperature of the reaction cell was varied from 15 to 25 °C, while all other conditions were maintained at pH 7 and 100 mM NaCl. The association constant (K_a) between native Protein A and IgG increased as the temperature was decreased from 25 to 15 °C. In contrast, the association constant for MSS was unaffected by temperature in this range (Figure 12). The K_a for MabSelect SuRe was approximately 10 – 20 fold higher than native Protein A at all temperatures.

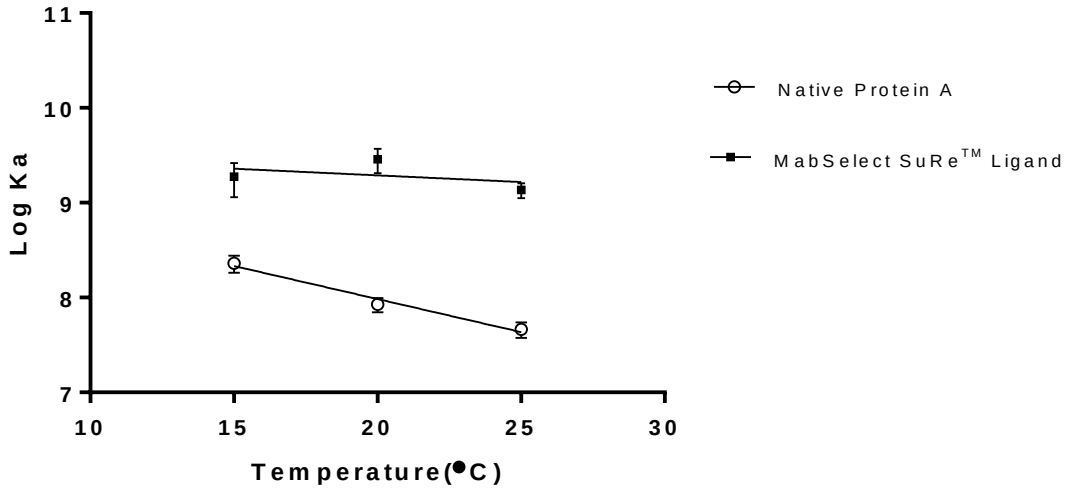


Figure 12: Ka changes with temperature for MSS and native Protein A at pH 7 in 50 mM sodium phosphate, 100 mM NaCl.

For both ligands ΔG was not significantly affected by the change in temperature (Figure 13). As expected, the binding interaction between the ligand and antibody was highly favourable, as indicated by the strongly negative ΔG value.

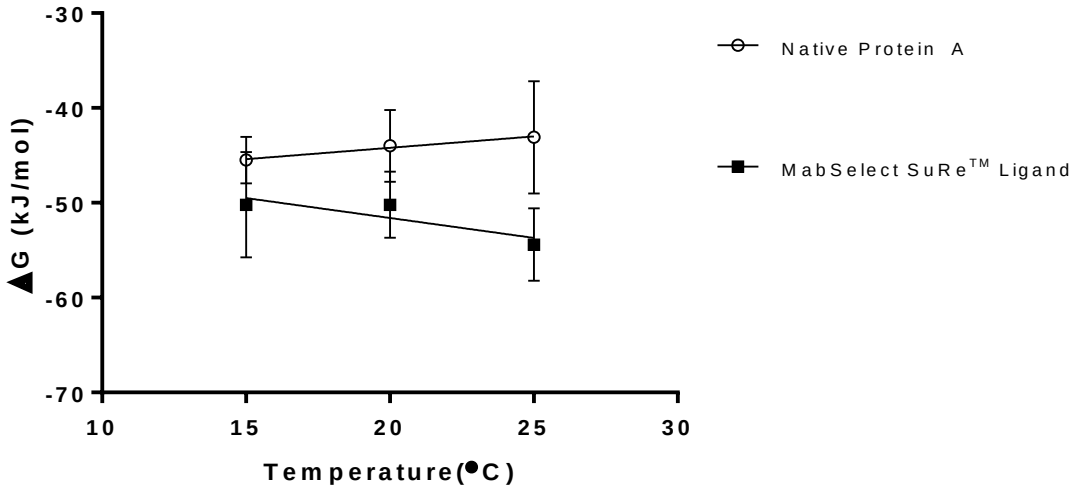


Figure 13: Free energy changes determined for both ligands with varying reaction temperature at pH 7 in a solution of 50 mM sodium phosphate, 100 mM NaCl.

Both enthalpy (ΔH - Figure 14) and entropy (TΔS - Figure 15) decreased with increasing temperature for the reaction with both ligands. As seen with the comparative K_a values, Mab

Select SuRe™ produced much higher enthalpy and entropy changes than the native Protein A at all temperatures examined.

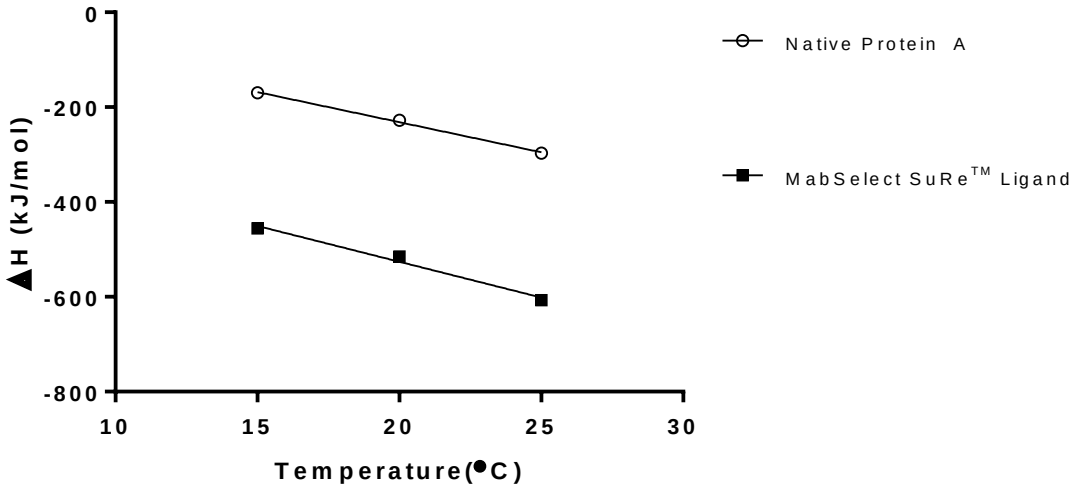


Figure 14: Measured enthalpy changes with varying temperature at pH 7 in a solution of 50 mM sodium phosphate, 100 mM NaCl.

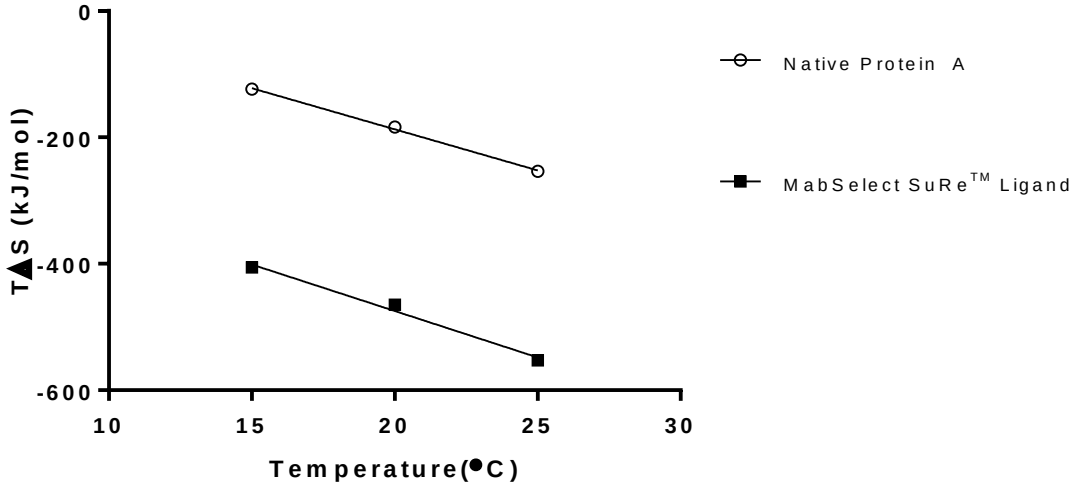


Figure 15: Measured entropy changes with varying reaction temperature at pH 7 in a solution of 50 mM sodium phosphate, 100 mM NaCl.

4.5.2 The effect of salt concentration

In determining the effect of increasing salt concentration on the reaction the NaCl concentration of the reaction was varied from 100 to 1000 mM NaCl, while the pH was maintained at 7 and the temperature at 25 °C. The affinity constant for Mab Select SuRe™ was significantly higher than native Protein A at all salt concentrations examined and was unaffected by increasing the salt concentration. In contrast, the K_a value for native Protein A increased significantly over this range of salt concentrations (Figure 16).

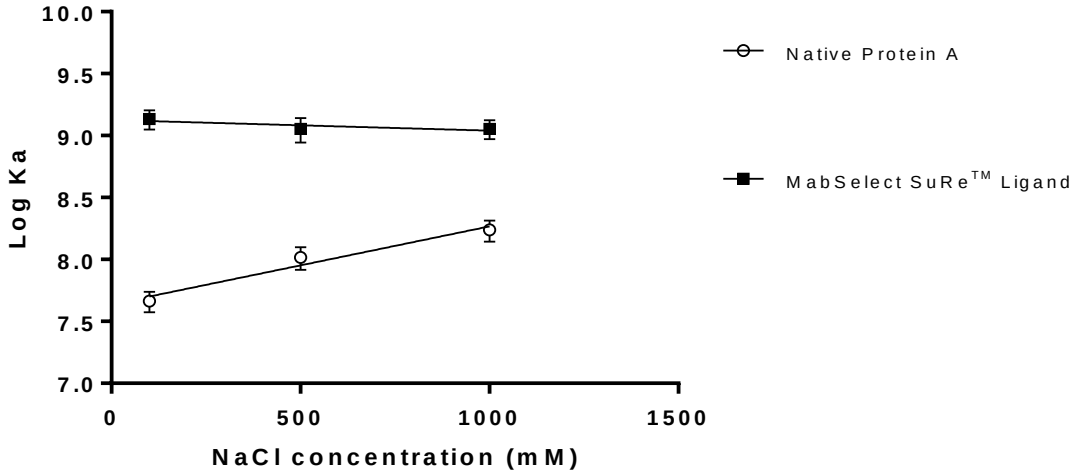


Figure 16: Ka values with increasing salt concentration in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

The free energy change upon association was unaffected by increasing the salt concentration for both ligands. However, the MSS ligand produced a noticeably higher value at all salt concentrations studied (Figure 17).

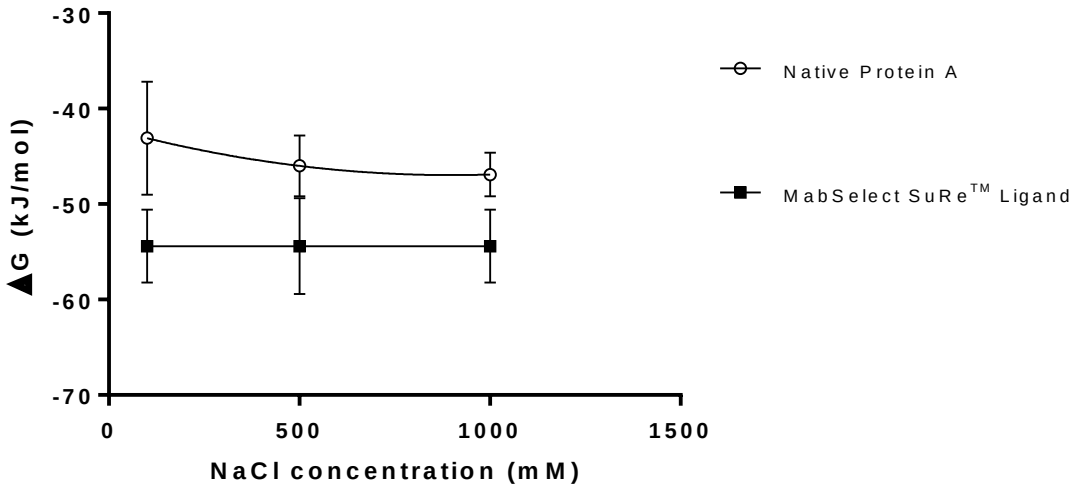


Figure 17: Free energy values with increasing salt concentration in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

Similarly, enthalpy was not significantly altered for either ligand by increasing the salt concentration and MSS was found to have a significantly higher enthalpy change upon binding compared with the native ligand (Figure 18).

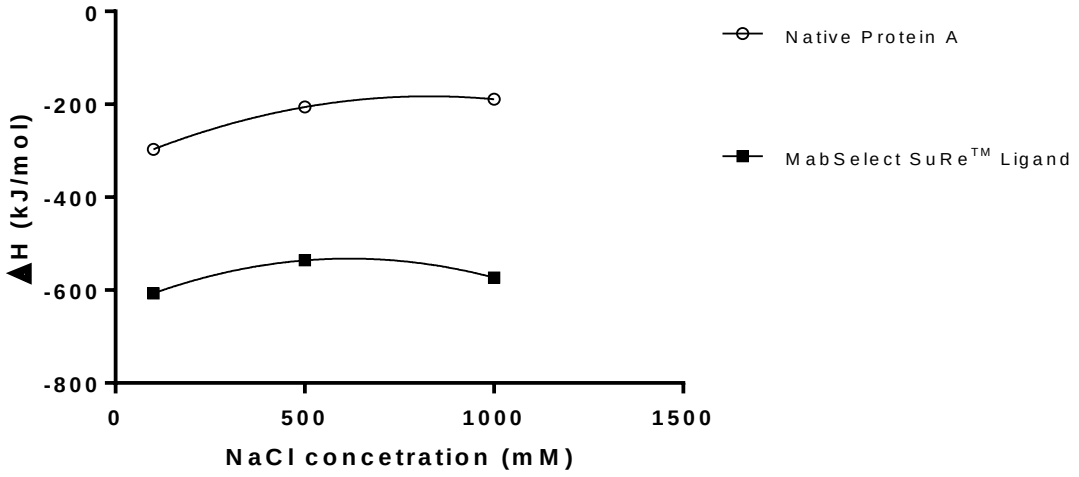


Figure 18: Enthalpy values with increasing salt concentration in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

Entropy changes with salt concentration followed a very similar pattern to enthalpy, with both ligands remaining relatively unaffected by changes in the salt concentration (Figure 19).

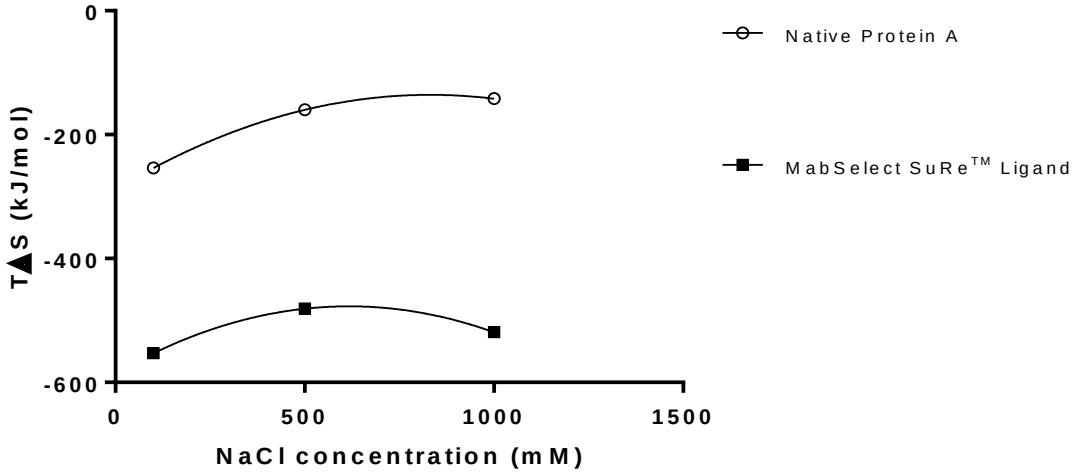


Figure 19: Entropy values with increasing salt concentration in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

4.5.3 The effect of pH

In estimating the effect of pH on the reaction between IgG and the respective ligands the salt concentration was maintained at 100 mM NaCl and the temperature at 25 °C while the pH was varied from 4 to 7 (Figure 20). The K_a for MSS reached a maximum value at pH 7, whereas native protein reached a maximum at pH 6 before declining slightly at pH 7.

No detectable signal was able to be obtained from the native ligand reaction at pH 4, whereas MSS continued to record significant reaction with the IgG at this pH.

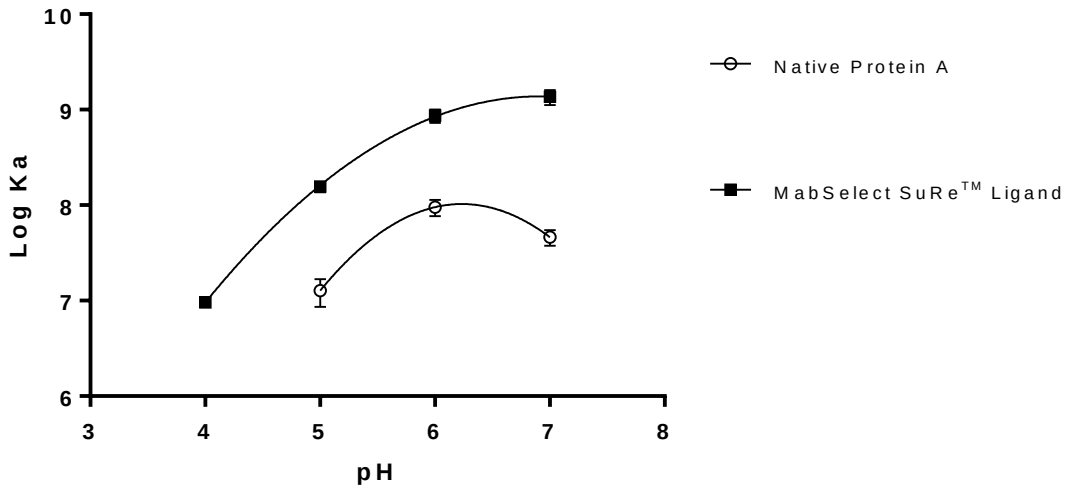


Figure 20: Ka values at reaction pH values from 4 – 7 in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

The free energy change of the reaction decreased with the decrease in pH for both ligands, with MSS generally recording a large ΔG at all pH values (Figure 21). Similarly, the enthalpy and entropy changes at all pH values were significantly higher with MSS than the native ligand at all pH values (Figure 22 Figure 23).

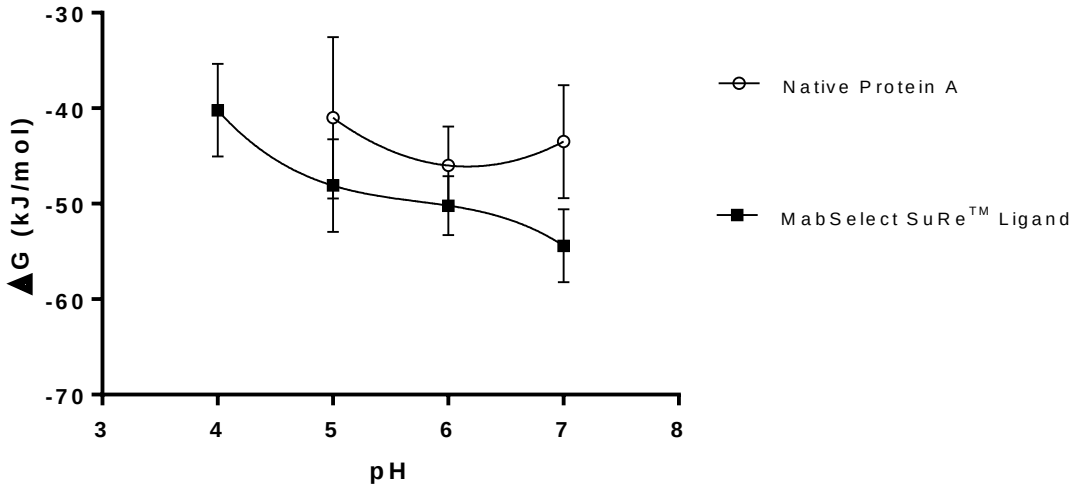


Figure 21: Free energy values measured at reaction pH 4 – 7 in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

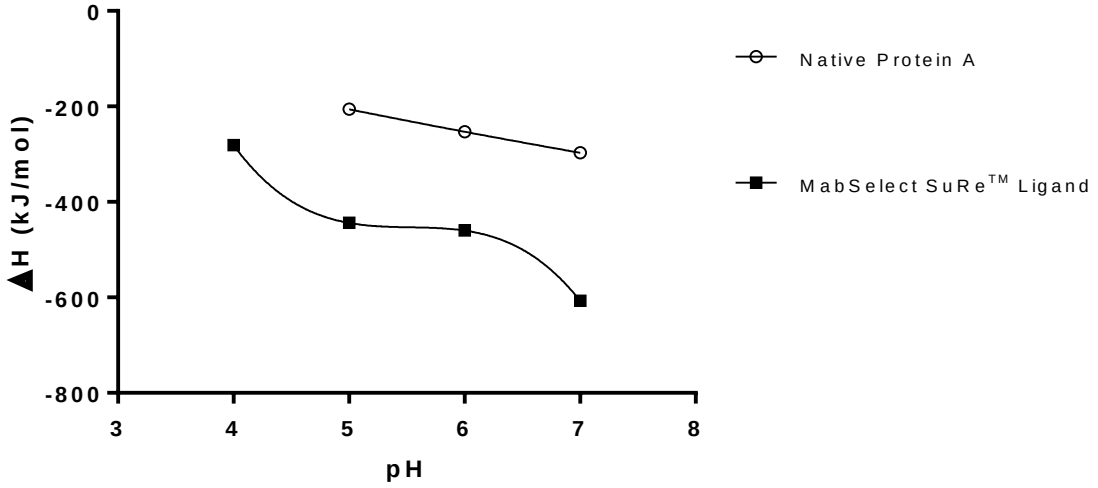


Figure 22: Enthalpy values of the reactions at pH 4 – 7 in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

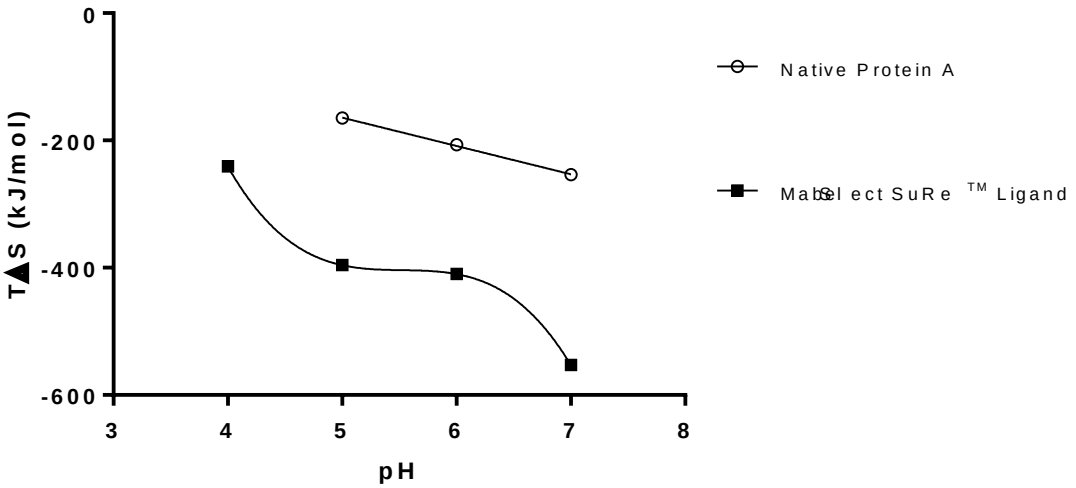


Figure 23: Entropy values measured at pH 4 – 7 in solution conditions of 50 mM sodium phosphate, 100 mM NaCl and 25 °C

4.6 Discussion

In varying the pH of the reaction of MSS and IgG it was found that the free energy of the reaction declined with declining pH from 7 to 4. This was accompanied by decreases in both the enthalpy and entropy changes associated with the interaction. A similar pattern was observed with the native Protein A ligand, with the exception that no detectable interaction occurred between IgG and native Protein A at pH 4. Coupled with the fact that MSS ligand was shown to be relatively insensitive to differing salt concentrations in the reaction this finding indicates that chromatography resins based on the MSS ligand are likely to be effective over a wider range of conditions than resins based on native Protein A or recombinant Protein A with the same domain structure as native Protein A.

The fact that MSS maintains significant interaction with IgG at pH 4 means that use of this resin allows the chromatographer an additional means of tailoring purification conditions. Specifically, the pH of the purification can be varied from pH 4 to 7 in order to increase specificity or decrease non-specific binding of contaminants. For resins based on native Protein A it may be possible to tailor the specificity of purification by manipulating the salt concentration of the binding condition. High salt washes are found in some of the published protocols for Protein A purification ([59, 92]) and this is probably due to the increased K_a between the two species at higher salt concentrations found in this work. Resins based on the MSS ligand would be expected to be unaffected by the salt concentration of the binding solution, based on our ITC observations.

Previous publications have found that the interaction of Protein A and IgG was accompanied by large negative changes in enthalpy, entropy and free energy [78]. This earlier work used a recombinant version of Protein A binding to an IgG but found very similar values to those obtained in this study with the K_a at 15 °C being approximately 3 x 10^8 M^{-1}, ΔH = -225 $kJ.mol^{-1}$, ΔS= -619 $JmolK^{-1}$and ΔG varying from -44 to -51 $kJmol^{-1}$ over a temperature range of 5 to 60 °C. The interaction of Protein A and IgG was found to be mainly driven by

hydrophobic effects and was associated with a large change in the water accessible surface area of the interacting species.

The significantly higher entropy change associated with the MSS ligand could indicate a possible drawback of the modified ligand. One of the reasons Protein A is so specific in its interaction with IgG is that its interaction with the antibody molecule involves an induced fit [65, 80, 81]. The induced fit is achieved by destabilization of the $C\delta_2$ domain of the IgG. The much higher entropy change obtained with the modified ligand indicates a much more significant re-arrangement of the monoclonal antibody molecule upon binding. This raises the possibility that denaturation of the antibody is more of a risk when using the MabSelect SuRe™ ligand compared with the native ligand. Similarly, the finding that an appreciable interaction remains between the ligand and IgG even at pH 4 suggests that even lower pH conditions may be required for elution. Lower pH values would be accompanied by an increased risk of acid denaturation of the target IgG. In summary, the MSS ligand has some unique properties and advantages over native Protein A in addition to its increase resistance to alkaline cleaning conditions. However, these unique properties are accompanied by increased risk of structural destabilization of the target antibody due to the very high binding affinity of MSS and the possibility that much more acidic elution conditions may be required, which can in some instances lead to denaturation of the target product.

The MSS ligand was created by protein engineering of the B domain of native protein A aimed at removing the asparagine-glycine dipeptide sequences and methionine residues to create a ligand resistant to treatment with hydroxylamine and cyanogen bromide [93]. The resulting recombinant protein A domain is referred to as a Z domain, having had 8 asparagine residues replaced per domain. MSS ligand is composed of a tetramer of these Z domains, each of which binds to the Fc region of IgG. In contrast, native protein A is composed of 5 domains – E, A, D, B and C – which, though highly homologous, are not identical. As a result native protein A has a theoretical pI of 5.1 and molecular weight of approximately 42 kDa

while the MSS ligand has a theoretical pI of 4.9 and a molecular weight of approximately 27 kDa.

Arouri *et al* [78] have previously shown that the interaction of IgG with protein A is mainly driven by hydrophobic effects and is associated with the large change in water accessible area that occurs for both molecules upon binding. This observation, along with the fact that native protein A and MSS differ little in pI, indicates that charge effects do not play a large role in the differences in the thermodynamics of binding seen between the two ligands. The lack of influence of charge effects on binding is also supported by the observations in this work using different salt concentrations. In these experiments the slope of the curve for ΔG, ΔH and ΔT against increasing salt concentration is almost identical for the two ligands indicating they both share the same sensitivity to increasing salt concentration despite starting at different baseline values. The dominant role of hydrophobic interactions is reinforced by observation that, for native protein A, the Ka increased with decreasing temperature as would be expected considering that the hydrophobic force increases with a decrease in temperature. The observation that the Ka of the MSS ligand was almost unaffected by changes in the reaction temperature suggests that the hydrophobic interactions are close to the maximum value achievable for the two interacting species at 25 °C. The finding that the binding energy is higher for MSS compared to native protein A is surprising as MSS ligand has a significantly lower molecular weight and hence lower surface area to result in a reduction in water accessible area upon binding between ligand and IgG to drive the hydrophobic interaction. Therefore the hydrophobic interactions between amino acid side chains of the Z domain and protein A Fc region must be significantly higher in the modified MSS ligand than the parent protein A.

Starovasnik *et al* [94], using ITC to examine the interaction between single ‘E’ and ‘Z’ domain repeats with the Fc region of a monoclonal antibody found that the isolated E domain had a slightly higher binding constant than the isolated Z domain, while the ΔH values for the

two ligands were almost identical. Our work indicates that this finding does not hold true for the multimers that make up the complete native protein A and MSS ligands.

4.7 Conclusions

The higher static and dynamic binding capacity of MSS resin compared to chromatography resins based on native or recombinant protein A resins is largely due to the much higher (10 – 20-fold) affinity constant of the MSS ligand compared to the other ligands.

Chromatography resins based on the MSS ligand are likely to be effective over a wider range of conditions than resins based on native or recombinant Protein A with the same domain structure as native Protein A. Resins based on the MSS ligand would be expected to be relatively unaffected by the salt concentration of the binding solution, whereas resins based on native or recombinant protein A of native sequence are significantly affected by the salt concentration of the binding solution.

An appreciable interaction remains between the MSS ligand and IgG even at pH 4 suggesting that lower pH conditions may be required for elution compared with unmodified ligands. Lower pH values would be accompanied by an increased risk of acid denaturation of the target IgG.

The MSS ligand has some unique properties and advantages over native Protein A in addition to its increase resistance to alkaline cleaning conditions. These unique properties are accompanied by increased risk of structural destabilization of the target antibody due to the very high binding affinity of MSS and the possibility that much more acidic elution conditions may be required, which could in some instances lead to denaturation of the target product.

4.8 References

1. Hober, S., K. Nord, and M. Linhult, *Protein A chromatography for antibody purification.* Journal of Chromatography B, 2007. **848**(1): p. 40-47.
2. Schwartz, W., et al., *Comparison of hydrophobic charge induction chromatography with affinity chromatography on Protein A for harvest and purification of antibodies.* Journal of Chromatography A, 2001. **908**(1–2): p. 251-263.
3. Sisodiya, V.N., et al., *Studying host cell protein interactions with monoclonal antibodies using high throughput Protein A chromatography.* Biotechnology Journal, 2012. **7**(10): p. 1233-1241.
4. Biosciences, A., *Protein Purification: Handbook*. 2001: Amersham Pharmacia Biotech.
5. Salvalaglio, M., et al., *Molecular modeling of Protein A affinity chromatography.* Journal of Chromatography A, 2009. **1216**(50): p. 8678-8686.
6. Xia, H.-F., et al., *Molecular Modification of Protein A to Improve the Elution pH and Alkali Resistance in Affinity Chromatography.* Applied Biochemistry and Biotechnology, 2014: p. 1-11.
7. Hahn, R., et al., *Comparison of Protein A affinity sorbents III. Life time study.* Journal of Chromatography A, 2006. **1102**(1–2): p. 224-231.
8. Ishihara, T., N. Nakajima, and T. Kadoya, *Evaluation of new affinity chromatography resins for polyclonal, oligoclonal and monoclonal antibody pharmaceuticals.* Journal of Chromatography B, 2010. **878**(23): p. 2141-2144.
9. Arouri, A., et al., *Hydrophobic interactions are the driving force for the binding of peptide mimotopes and Staphylococcal Protein A to recombinant human IgG1.* Eur Biophys J, 2007. **36**(6): p. 647-60.
10. Pierce, M.M., C.S. Raman, and B.T. Nall, *Isothermal titration calorimetry of protein-protein interactions.* Methods, 1999. **19**(2): p. 213-21.
11. Aldington, S. and J. Bonnerjea, *Scale-up of monoclonal antibody purification processes.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 64-78.
12. Hahn, R., R. Schlegel, and A. Jungbauer, *Comparison of Protein A affinity sorbents.* J Chromatogr B Analyt Technol Biomed Life Sci, 2003. **790**(1-2): p. 35-51.
13. Diesenhofer, J., *Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of Protein A from Staphylococcus aureus at 2.9 and 1.8 Å resolution.* Biochemistry, 1981. **20**: p. 2361 - 2370.
14. Dima, S., et al., *Effect of Protein A and its fragment B on the catabolic and Fc receptor sites of IgG.* Eur J Immunol, 1983. **13**(8): p. 605-14.
15. Ghose, S., et al., *Antibody variable region interactions with Protein A: implications for the development of generic purification processes.* Biotechnol Bioeng, 2005. **92**(6): p. 665-73.

Statement of Authorship

Title of Paper	QbD for resin lifetime studies
Publication Status	O Published, O Accepted for Publication, O Submitted for Publication, ⊙ Publication style
Publication Details	Text in manuscript

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Phillip Elliott		
Contribution to the Paper	This work was largely planned and written by Phillip Elliott.		
Signature	[redacted]	Date	19FEB2015.

Name of Co-Author	Dr Jingxiu Bi		
Contribution to the Paper	Dr Bi completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20/02/2015

Name of Co-Author	Dr Hu Zhang		
Contribution to the Paper	Dr Zhang completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20FEB2015

Name of Co-Author	Dr Sam Billingham		
Contribution to the Paper	Dr Billingham completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	23 Feb 2015

5 QbD for resin lifetime studies

5.1 Abstract

A resin lifetime study was performed on a ceramic fluoroapatite (CFT) chromatography unit operation used for the production of a recombinant Protein at industrial scale. In order to produce the enhanced knowledge and understanding of the unit operation required to enable Quality by Design (QbD), additional techniques and mechanistic determinations were performed.

The resin was found to remain functional over a period of 30 re-uses, with no change detected for the level of purification achieved by the chromatography over this period. The primary function of the CFT unit operation is to remove an enzymatic activity that had previously been shown to degrade an excipient in the final drug product. The resin lifetime study demonstrated that this enzymatic activity continued to be removed over 30 cycles of re-use. Furthermore, column cleaning was found to be effective as no product and/or impurity carryover was observed from one run to the next. Despite the acidic conditions under which the resin was used (pH 5.4), no degradation in the static or dynamic binding capacity was detected over the resin lifetime study. In addition, measurements of the inter- and intra-particle porosity of the packed column demonstrated that there was no attrition of the base media over the 30 cycles of re-use. This demonstrates the enhanced stability of the CFT resin under acidic conditions compared to the parent resin of ceramic hydroxyapatite, which has previously been shown to undergo significant attrition when used under mildly acidic conditions.

In-line with QbD, statistical control charts were used to analyse the resin lifetime data, demonstrating how this quality control technique can be applied to lifetime data in order to

quickly identify whether the unit operation is stable and demonstrate the level of control and underlying variability of the process. Static and dynamic binding capacity measurements and determination of the various diffusion parameters of the resin before and after multiple re-uses were also performed to demonstrate how measurement of these fundamental resin properties can elucidate the mechanism of any degradation in performance detected during the resin lifetime study.

5.2 Introduction

A more modern approach to the development of pharmaceutical products and their subsequent manufacture has been advocated by the US Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH). This approach has been termed "Quality by Design" (QbD) and is defined as "a systematic approach to development that begins with pre-defined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [4]. The key tools of QbD are incorporation of prior knowledge, the use of statistically designed experiments, risk analysis and knowledge management. The intent of QbD is to encourage pharmaceutical companies to demonstrate sufficient understanding of their products and manufacturing processes to the pharmaceutical regulatory agencies in exchange for a more flexible regulatory approach [95]. The concept promotes industry's understanding of the product and manufacturing process starting with product development, with the aim of building quality in from the start rather than trying to test quality into the product during manufacture. Under the concept of QbD, when designing and developing a product, a company needs to define the desired product performance and identify critical quality attributes (CQAs). On the basis of this information, the company then designs the product formulation and manufacturing process to meet those product attributes. Ideally it is hoped this will lead to understanding the impact of raw material and equipment attributes and manufacturing process parameters on the product critical quality attributes (CQAs), leading to identification and control of sources of variability. It is a goal of QbD that as a result of all of this knowledge a company can continually monitor and update its manufacturing process to assure consistent product quality [5].

In the production of a biopharmaceutical the cost of the chromatography and filtration media can often be the largest raw material cost in the downstream process [96]. Determining the useful lifetime of these re-usable media is necessary to ensure that the process will continue to

produce intermediates and final products that meet the defined quality and safety attributes. These studies are time-consuming and expensive but have become a regulatory requirement. The FDA's Therapeutic Compliance Program Guide, which serves as a guide for investigators, states, "There should be an estimated life span for each column type, i.e. number of cycles. Laboratory studies are useful even necessary to establish life span of columns." [97]. Other health authority guidance documents also mandate the prospective establishment of limits on the number of times a chromatography resin can be used in manufacturing [98]. The concern is that the performance of a chromatography resin could change with use and storage and thus impact the safety and efficacy of the final product. Regulators have stated that validation of the purification process should also include justification of the working conditions such as column loading capacity, column regeneration and sanitization, and length of use of the columns [99]. International guidelines on the development and manufacture of biotechnologically-derived drug substances allow for the lifetime of chromatography columns to be estimated using experimental studies carried out in small-scale models, as long as these studies are confirmed during commercial-scale production [100].

The extent of large-scale manufacturing experience is typically limited at the time of filing for approval of a new biotherapeutic product. It is rare to have taken any of the chromatographic resins out to the limit of their resin lifetime. Hence, a scale-down model is employed in which the resin is loaded and cleaned many times and the product is analysed to determine any deterioration in column performance. These characterisation studies are typically performed to establish a recommended lifetime in terms of the maximum number of re-uses [101, 102]. Scale-down studies also form a useful scale to optimise column regeneration and storage conditions and are also often used to generate aged chromatographic resin to assess viral clearance capability in production processes which use a mammalian cell line.

The FDA has previously stated in the "cGMPs for the 21st century" initiative [103], the forerunner of the QbD initiative, that one of the features of its desired state for the pharmaceutical industry was one in which product and process specifications are based on a mechanistic understanding of how formulation and process factors affect product performance. That is, it is the goal of the FDA for pharmaceutical companies to have a mechanistic understanding of their production processes and how they affect the final pharmaceutical product. In this sense, a mechanistic understanding or model of unit operations, their performance and effects on product is generally superior to the purely empirical level of understanding generally gained using a scale-down model– the effect of the variable "number of uses" on the output "product quality" is determined empirically. The empirical approach does not provide an understanding of the mechanism of resin degradation that is encountered during the resin lifetime study. Understanding the mechanism of resin degradation can enhance knowledge of the production process and feedstock properties and possibly lead to improvements in the process which further extend the lifetime of the resin and the robustness of the overall process. This is necessary in the QbD paradigm of development as it is the extent of knowledge which is derived, rather than the amount of data that is derived, which is the important factor [4].

Considering that resin lifetime studies are of such importance in the production of most biopharmaceuticals it is surprising that there are currently no published studies attempting to tackle the area using a QbD approach. One study has attempted a multifactorial or DOE approach to resin lifetime studies in which the effect of multiple parameters on the percent yield and various impurities was studied [104]. The parameters varied to challenge the model were various fermentation lots, elution and equilibration pH values and collection start points. This was achieved via a DOE (design of experiment) consisting of 42 experiments, and it was concluded that the media could be reused for 42 cycles. Other authors have felt that it is infeasible to study resin lifetime in a multivariate manner because of the number of

parameters involved and the associated complexity [105]. These opposing viewpoints emphasize that a QbD approach is required that focuses on the mechanism of action of the resin and the mechanism of any performance degradation in order to gain understanding of resin performance as opposed to simply compiling evidence that the resin remains functional over a certain period.

Jiang *et al*. have reported a resin lifetime study on Protein A resin in which further studies were carried out to determine the mechanism of resin degradation [62]. In this study, instability of the protein ligand during resin sanitization was shown to be the limiting factor in the resin lifetime. Subsequent investigations were able to discover stabilizing excipients which protected the protein ligand during sanitization and hence improved the overall lifetime of the resin. The important point here is that understanding the mechanism of resin degradation allowed effective action to be taken to improve the resin lifetime and hence the overall robustness of the entire downstream process. The larger point is that a mechanistic understanding of resin degradation, over and above the purely empirical understanding of the number of times the resin could be re-used, lead to effective action that achieved continuous improvement in the downstream process. This level of "enhanced knowledge" is necessary for a thorough understanding of a chromatography unit operation and is more in-line with the intent of the QbD initiative [4]. The study initially measured yield of the unit operation versus chromatography cycle, as is standard for a typical "empirical" resin lifetime study. Three additional performance characteristics of unused and aged resins were also evaluated to determine the cause of the decline in product yield with repeated use including adsorption isotherms, dynamic binding capacity breakthrough curves and the height equivalent to a theoretical plate (HETP) versus flow-rate.

This mechanistic study of the fundamental properties of ceramic fluoroapatite (CFT) resins was based on earlier work which characterised cation exchange resins [106]. CFT [$(Ca_5(PO_4)_3F)_2$] is an inorganic calcium phosphate used in the chromatographic separation of

biomolecules that has been available from Bio-Rad since 2006 [107]. CFT has separation characteristics similar to ceramic hydroxyapatite (CHT - $(Ca_5(PO_4)_3OH)_2$) from which it is derived, but can be used under chromatographic conditions as low as pH 5 to separate acidic proteins [108]. It is a composite of fluoroapatite and hydroxyapatite prepared by chemically converting hydroxyapatite nanocrystals with a fluorine reagent. The functional groups on CFT resin are the same as make up the backbone of the resin itself - Ca^{2+} PO_4^{3-} and F^- . As with CHT resin, binding of macromolecules can occur through the mechanisms of anion exchange and metal affinity (due to the Ca^{2+} residues) as well as cation exchange due to the PO_4^{3-} and F^- residues. Previously published resin lifetime studies have demonstrated the significant resin instability that can occur with CHT in large-scale manufacturing operations when the solution conditions are not ideal for the stability of the resin [109], demonstrating the need to establish resin lifetime information for CHT and derivative resins early in the development cycle to avoid costly and time consuming remediation later. According to the supplier, it is possible to re-use CFT resin for up to 100 cycles in pH solutions as low as pH 5.5 [107] with no noticeable loss of resin material, but there are currently no published studies on the usable lifetime of CFT resin. CFT is becoming increasingly important in the manufacture of biopharmaceuticals as it possess some unique mixed-mode properties and some advantages over current techniques. One study has reported the development of a one-step purification method for monoclonal antibodies, based on CFT resin. In addition, CFT and CHT may be preferred over the standard Protein A resin for many antibody products as these resins do not have the antibody sub-type restrictions of Protein A resin. For example, Protein A does not bind to the human IgG3 sub-class, most IgA and IgM molecule, nor does it bind to antibody fragments lacking the Fc region such as single-chain antibodies or Fab fragments. CFT (and CHT) can be used to purify IgA and IgM molecules whereas most single-mode chromatography methods have severe drawbacks [11, 19, 21, 25]. CFT (and CHT) may actually be the first choice for the efficient preparation of pharmaceutical grade plasmid DNA

(pDNA). With the expected rise of DNA medicine (gene therapy, DNA vaccination) the importance of CFT resin is likely to increase as apatite-based resins have distinct advantages in DNA purification, being able to differentiate between pDNA and most important impurities [33, 70–72]. In this context the factors affecting the ability of this resin to be re-used and continually produce acceptable product are an important area of study.

In the unit operation examined in this work the CFT resin is used to remove HCP, product dimer and a specific HCP enzyme that has previously been shown to cause excipient degradation in the final drug product. The target protein product does not bind to the resin in this unit operation, passing through unbound while the impurities are removed. The resin is then cleaned with a sodium phosphate solution and sanitized with sodium hydroxide. This study carried out multiple re-uses of the resin, including sanitization and re-equilibration procedures, then additional measurements of the interstitial porosity and particle porosity for estimating the mass-transfer properties of the resin were carried out to determine if any of these fundamental parameters had been affected by the conditions of use.

5.3 Materials and Methods

5.3.1 Column Chromatography

All CFT chromatography was performed using an ÅKTAExplorer FPLC system (GE Healthcare). A 1.1 cm diameter column was packed to a bed height of 5.4 cm with CFT resins (CFT ceramic fluoroapatite 40 μm, Type II Bio-Rad catalogue no. 158-5200). Each load was performed at a target nominal loading density of 100 g of filgrastim per L of resin. Before loading the column was sanitized for 1.5 column volumes (CV) with 1 M NaOH, and then equilibrated for use with 3 CV of a 10 mM sodium phosphate pH 5.4 buffer. The column was then loaded with the protein solution at 4.2 mg/ml pH 5.4 using a linear velocity of 225 cm/hr (all other flow rates used were 300 cm/hr, unless specified). The load solution contained 5.6 % RP-HPLC impurities and 2.4 % SEC impurities. After loading the column was washed with equilibration buffer for 3 CV. The eluting protein contained in the non-binding eluate was collected when the OD280 absorbance of the eluate on the column outlet exceeded 16 mAu. Protein collection was ended when the absorbance on the outlet receded below 40 mAu. After protein collection the column was washed with 400 mM sodium phosphate pH 5.4 for 3 CV, followed by 400 mM sodium phosphate pH 6.8 for 3 CV before being sanitized with 1 M NaOH for 3 CV and 1 hr incubation time. The column was then put through a storage cycle of 0.1 M NaOH/10 mM sodium phosphate for 5 CV before being cycled through to the start of the chromatography process for the next column load.

To measure the efficiency of resin packing the column was equilibrated in a solution of 200 mM sodium phosphate, 150 mM NaCl at a linear velocity of 90 cm/hr. A solution of 1.5 M NaCl, 200 mM sodium phosphate was injected as a tracer to check the packing using an injection volume equal to 1 % of the total column volume. The HETP and asymmetry of the column was then determined from the conductivity trace produced at the column eluate.

5.3.2 Experimental Design

Operational parameters other than the loading density and bed height were performed at the centre point of range values in Table 13 and the product from the first five cycles and subsequently every fifth cycle were analysed. Blanks runs using a load solution of buffer only (25 mM ammonium acetate pH 5.4) were conducted after every fifth cycle of product runs to check for product carry-over. All the pools from blank runs were analysed for protein concentration, hydrolase activity, TOC, HCP and SDS-PAGE. The CFT resin used for this study had already been used for five cycles of purification prior to the commencement of the resin lifetime study. This CFT media was then used for a further 25 cycles to examine the effects of resin ageing on the unit operation.

Table 13: CFT operational parameters

Operational Parameter	**Specification**
Column Diameter (cm)	1.1
Bed Height (cm)	5 ± 1
Loading Density (g of protein / L of resin)	Target 100g/L with each cycle
Linear Flow rate (cm/hr)	300
Sanitization (CV)	1.5
Contact Time	NLT 60 minutes
Resin Stripping (CV)	2.0
Equilibration (CV)	NLT 3.0
Load (mL)	As needed
Linear Flow Rate (cm/hr)	225 for Loading and post-load Washing only
Post-Load Wash (CV)	3.0
Elution (CV)	3.0
Final Wash (CV)	3.0
NBE Collection Criteria	Peak Start 16mAu to Peak End 40mAu
Sanitization (CV)	3.0
Contact Time	NLT 60 minutes
Storage (CV)	5.0

5.3.3 SDS-PAGE

SDS-PAGE was performed for the supernatants produced by the blank runs. The SDS-PAGE gel (NuPage 4-20% gel with MES running buffer [Invitrogen]) was stained with Oriole™ Fluorescent stain (Bio-Rad). 100 ng Controls and 10 ng of target protein were included on each gel to ensure the gel was sufficiently developed along with molecular weight markers. The presence and molecular weight of any contaminating bands present in the lanes was noted.

5.3.4 Determination of size-related Impurities

Size-related impurities of the product were estimated using a Superose 12 10/300 L column (GE Healthcare) run at 0.5 ml/min in a buffer of 50 mM borate, 100 mM NaCl pH 9.1 for 50 minutes on a Waters Alliance 2695 HPLC system. All analyses were performed at ambient temperature (22 °C) and a sample application of 100 μg was applied to each separation run. Samples were prepared by centrifugation at 10 000 g for 5 minutes before application to the column and the column effluent was monitored at 280 nm.

5.3.5 RP-HPLC Analysis of Products

RP-HPLC impurities were estimated using a version of the RP-HPLC assay described in [110]. Briefly, a Jupiter C4 5μm 300Å, 4.60 x 250 mm, HPLC Column (Phenomenex) was run at 0.6 ml/min and 60 °C using running buffers of 0.1% TFA/10 % acetonitrile and 0.1 % TFA/80 % acetonitrile using a Waters Alliance 2695 HPLC. Separation was performed using a gradient of 0.17 % acetonitrile per minute over 35 minutes using a target sample application of 10 μg for all runs. The column effluent was monitored at 215 nm.

5.3.6 Visible Spectrophotometry

Spectrophotometry in the visible range (380 – 770 nm) was performed on the supernatants produced by the blank and sample runs as per USP<1061>[111] to scan for any coloured species eluting from the resin during the chromatography run.

5.3.7 Total Organic Carbon

Total organic carbon in the eluate of blank runs was measured using a Sievers 900 total organic carbon analyser (GE analytical instruments) calibrated using a Sievers 900 Multi-Point Calibration Standard Set.

5.3.8 Static binding capacity

The Freundlich adsorption isotherm parameters, m and K, were calculated from static binding capacity studies. Used CFT media and New CFT media were equilibrated in 10mM Phosphate Buffer pH 6.8 prior to the addition of protein sample. Static binding capacity was performed in 4 sets (each set is in triplicate) with chicken egg white lysozyme solution (3X crystallised; Sigma Cat. No. L6876). Lysozyme was spiked into a fixed volume of resin slurry (400 µL) at various concentrations (theoretical calculation 25 to 55 mg/mL). The resulting volume of every experimental set up was 1mL; which included 0.4mL of CFT media. The protein-resin slurry mixture was incubated overnight on a rotating mixer at room temperature. The amount of lysozyme remaining in the supernatant was determined by UV spectrophotometry using a Cary 50 UV spectrophotometer (Varian Instruments). Each concentration point (c) was measured in triplicate. The amount of lysozyme bound to media was determined by mass balance. C and q values correspond to the concentration of lysozyme in supernatant and stationary phase respectively. GraphPad Prism was used to determine K_f and m values by best fit. Static binding capacity data was fit to the Freundlich isotherm. The parameters obtained (K_f and m) were compared between the used and unused resin to determine whether any deterioration in the resin ligand had occurred [62].

5.3.9 Dynamic binding capacity

To assess effects on dynamic binding capacity (DBC) of the resin a model compound lysozyme and conditions were used. The column was equilibrated in a solution of 10 mM sodium phosphate pH6.8 and a solution of 2 mg/ml chicken egg white lysozyme in binding buffer was injected at 150 cm/hr until the OD280nm absorbance of the eluate reached a plateau value. The concentration of protein in the eluate was measured at various points before the plateau and expressed as a percentage of the initial concentration of protein (C0). The percentage of C/C0 was plotted against the total protein loaded onto the column. These conditions were used to assess the DBC of used and unused resin to look for any degradation of the binding capacity. The DBC curves produced by used and unused resin were fit to the sigmoidal dose response (Hill-Slope) curve (Y = Bottom + (Top - bottom)*XH/(EC50H+XH) using GraphPad Prism 6 for Windows (GraphPad Software Inc.), with EC50 used to indicate the resin loading density at 50 % breakthrough of lysozyme and H the slope of the Hill-Slope curve.

5.3.10 HETP versus Linear Velocity Curve

HETP versus linear velocity experiments were performed with new and used resin packed into small columns. A solution of the target protein was adjusted to 400 mM $NaHPO_4$ pH5.4 to provide non-binding conditions on the CFT resin. The CFT column was then equilibrated in a solution of 400 mM $NaHPO_4$ pH 5.4. Injections of target protein (1 % of column volume) were then performed at linear flow velocities ranging from 60 to 300 cm/hr. The curve of HETP versus linear velocity was then plotted to determine the pore diffusion properties of the resin [62, 106].

5.3.11 Porosity measurements

Pulse injections of 1 % of column volume of 2 M NaCl were performed to determine total volume (Vt) of the resin. The total porosity of the resin was then be obtained from the equation $\varepsilon t = Vt/Vc$ [112], where Vc = total column volume. Pulse injections of 1 % of

column volume of 2 mg/ml Dextran blue 2000 were performed to determine V_0. This allowed determination of the interparticle (interstitial) porosity (ε_i) of the resin using the equation $\varepsilon i = V_0/V_c$.

Comparison of the intra-particle porosity (ε_p), interparticle porosity (ε_i) and total porosity (ε_t) of the new and used resin was then performed to determine whether any of the pores on the resin had been blocked with particulates due to lack of load filtration or insufficient cleaning [62].

5.3.12 Host Cell Protein (HCP)

HCP was estimated using the *E.coli* HCP ELISA assay available from Cygnus Technologies (catalog number F410) according to the manufacturer's instructions. Briefly, samples were reacted with antibody against *E.coli* host cell protein labeled with the HRP (horse radish peroxidase) enzyme. The sample and antibody mixture was then placed in micro titre strips coated with an antibody to E. coli HCP that has been affinity purified (the capture antibody) and incubated for 90 minutes at 25 °C. The reaction results in the formation of a sandwich complex of solid phase capture antibody-HCP-enzyme labelled antibody. The strips were then washed to remove any unbound reactants and TMB (3,3',5,5' Tetra methyl benzidene) was added. After 30 minutes of incubation at 25 °C the reaction was stopped with 0.5N H_2SO_4 and the absorbance of the resulting mixture measured at 450 nm. By comparing the absorbance of the sample wells to the absorbance of a standard curve, prepared with a standard curve of HCP from 1 – 100 ng/ml in concentration the amount of HCP present in the samples could be estimated. The limit of quantitation for the method was shown to be 1 ng/ml.

Zymography

Zymography with the substrate 4-Methylumbelliferyl heptanoate (4-MUH) was used for detection of the enzymatic activity removed by the CFT resin. After separation of the samples

by SDS-PAGE, the gel was incubated in 50 ml of 2.5 % Triton X-100 for 60 minutes with shaking. The gel was then transferred to 50 ml of 200 mM sodium phosphate pH 7 and 120 µl of a 25 mM 4-MUH solution in DMSO added. The gel was then incubated in the darkness for 10 minutes with shaking before being illuminated on a UV transilluminator and photographed. A bright band appears on the gel where the 4-MUH substrate has been converted to a fluorescent product by the action of the HCP enzyme. Lighter and more diffuse bands appear on the gel due to the intrinsic fluorescence of the product protein under UV illumination. The identity of these more diffuse bands was confirmed by comparison with control samples of the purified product protein.

5.4 Theory

5.4.1 HETP vs linear velocity

HETP (Height equivalent to a theoretical plate) is defined as:-

$$H = \frac{L}{5.54}\left(\frac{tw,0.5}{tr}\right)^2 \quad \text{(Eq. 1)}$$

Where L is the column linear length (cm), H is the height equivalent to a theoretical plate (cm), tw,0.5 is the width at half-height (min) and tr is the retention of the peak maximum (min).

Contributions to HETP are additive. The contribution from film and pore mass transfer can be estimated under unretained conditions by using the methodology described in [113]. The HETP under unretained conditions is expressed as:

$$H = 2\zeta + \frac{2(1-\varepsilon_i)\varepsilon_p u}{\left[\varepsilon_i + (1-\varepsilon_i)\varepsilon_p\right]^2}\left[\frac{R}{3k_f} + \frac{R^2}{15D_p}\right] \quad \text{(Eq. 2)}$$

Where H is HETP (m), ζ is axial dispersion parameter (m), ε_i is intraparticle porosity, ε_p is interparticle porosity, u is linear flow velocity (m/s), R is average particle radius (m), k_f is film mass transport coefficient (m/s), and D_p is pore mass transport coefficient (m^2/s).

The HETP vs u plot can be fitted to determine the intercept and slope to yield ζ and D_p once k_f is known [62]. The film mass transfer coefficient (k_f) is calculated from standard correlations [106].

$$Sh = 2 + 1.45Re^{1/2}Sc^{1/3} \quad \text{(Eq. 3)}$$

Where Sh (Sherwood number) is equal to $k_f d_p/D_m$, Sc (Schmidt number) is equal to η/D_m and Re (Reynolds number) is equal to ud_p/η. The symbol d_p is the particle diameter (cm), η is kinematic viscosity (cm^2/s) and D_m is molecular diffusivity (cm^2/s).

5.4.2 Porosity measurements

Pulse injections of the solutes were made at various flow rates with the column off-line to estimate extra column contributions from the chromatography system itself. The first and second moments of the resultant peaks were computed, and the porosity was calculated by:

$$\mu_1 = \frac{L}{u}(\varepsilon_i + (1 - \varepsilon_i)\varepsilon_p b_0) \quad \text{(Eq. 3)}$$

where $\mu 1$ is the first moment of the elution peak, u is superficial velocity (cm /s), b_0 is a parameter reflecting retention factor; under unretained conditions b_0 is equal to 1, ε_i is interstitial porosity, ε_p is particle porosity, and ε_t is total porosity. According to IUPAC definition, $\varepsilon_t = Vt/Vc$

The interstitial and particle porosities were obtained from unretained data. Pulse injections of solutes were made at high PO4- concentrations. Under these conditions, the proteins used in this study were unretained in the stationary phase. Under unretained conditions, the value of b0 is 1. Pulse injections of blue dextran (average MW 2,000,000) were made under unretained conditions. It was assumed that these bulky solutes could only access the interstitial volumes and were excluded from the pores. Thus, the interstitial porosities were estimated from the first moments of their peaks. Having determined the interstitial porosity, the particle

porosities of the various solutes employed in this study were estimated from the first moments of their unretained peaks using the equation above [106].

5.4.3 Static binding capacity

An adsorption isotherm is a measure of the relationship between the equilibrium concentrations of bound and free guest molecule over a certain concentration and is generated from equilibrium batch rebinding studies or chromatographic frontal-zone analysis [74]. Binding properties can be calculated from the binding isotherm by fitting the adsorption isotherm to specific binding models. The Freundlich isotherm is

$q = K_f C^m$ (Eq. 4)

where: q is stationary phase protein concentration [bound guest] (mg/mL), C is mobile phase protein concentration [free guest] (mg/mL), and K_f is the Freundlich association constant. K_f is related to the median binding affinity (K_0) as $K_0 = K_f^{1/m}$, and m is a heterogeneity index (where a homogenous binding surface has m = 1 and m < 1 is a heterogonous binding surface).

5.4.4 Statistical control charts

Additional quality tools, control chart techniques cited as important supporting statistical tools for quality risk management in ICH Q9, are briefly explained below.

5.4.4.1 I –charts

The individuals (I) chart assesses whether the process centre is in control. The I-chart consists of plotted points for each individual observation, a centre line (green), which is the estimate of the process average (average of all individual observations) and control limits (red), which are set at a distance of 3σ (σ is the standard deviation) above and below the centre line and provide a visual display for the amount of variation expected in the individual sample values. Minitab conducts up to eight tests for special causes for the I-chart, which detect points beyond the control limits and specific patterns in the data. Points that fail are marked with a

red symbol and the number of the failed test. A failed point indicates that there is a nonrandom pattern in the data that may be the result of special-cause variation. These points should be investigated [114].

5.4.4.2 I-MR charts

The I-MR chart is a combined chart consisting of: an I-chart, and a moving range (MR) chart, which plots the range calculated from artificial subgroups created from successive observations, and provides a means to assess process variation.

An I-MR chart is used to draw a combined control chart for assessing whether process center and variation are in control when the data are individual observations. An in-control process exhibits only random variation within the control limits. An out-of-control process exhibits unusual variation, which may be due to the presence of special causes. The MR-chart must be in control before I-chart can be interpreted because the I-chart control limits are calculated considering both process variation and centre. When the MR-chart is out of control, the control limits on the I- chart may be inaccurate and may not correctly signal an out-of-control condition. In this case, the lack of control will be due to unstable variation rather than actual changes in the process center. When the MR-chart is in control, it is possible to be sure that an out-of-control I-chart is due to changes in the process centre [114].

5.5 Results

5.5.1 CQAs for CFT resin life time study

For successful implementation of QbD, it is necessary to define the intended product performance and identify CQAs. Based on this information, the product manufacturing process can be designed. Therefore, CQAs for the life time study of CFT resin were firstly identified. The biopharmaceutical protein being purified in this application is a cytokine expressed recombinantly in *E.coli*. The CFT chromatography is used to remove general HCP, product dimer, RP-HPLC impurities and a specific HCP which had previously been shown to degrade one of the excipients in the final drug product formulation [data not shown]. A table displaying the relationship between the various unit operations in the downstream process and the CQAs of the final product is shown in Table 14. This table was used to help assess the criticality of the CFT unit operation as part of the overall downstream process and to determine which quality attributes of the final product would be useful to measure as part of the lifetime study of CFT chromatography. The table illustrates that there is considerable redundancy in the downstream process, with all of the CQAs affected by CFT also affected by later unit operations. For this reason the CFT step, while providing a useful role in removing many impurities, was not considered to be a critical unit operation, apart from its previously identified role in removing a residual host cell protein. For these reasons the CQAs measured during the CFT lifetime study were HPLC impurities, SEC impurities, residual HCP and removal of the excipient-degrading enzyme. Endotoxin and HC-DNA were not measured during the lifetime study as the levels of these impurities are generally negligible or undetectable by this stage in the downstream process and subsequent unit operations are able to remove these impurities.

Table 14: CQAs affected by the unit operations in the overall downstream process

CQAs of final product	**Unit operation**						
	Dissolution and Refolding	SP-BB	CFT	P/S-HP	SP-HP	UF/DF	Filter & Fill
RP-HPLC impurities	X	X	X	X	X		
SEC Impurities	X	X	X	X	X		
Final pH						X	
Final protein conc.						X	X
Residual HCP		X	X	X	X		
Endotoxin		X	X	X	X		
CE-HPLC impurities		X		X	X		
Residual HC-DNA		X	X	X	X		
Biological activity	X						
Bioburden							X
Excipient final concentration			X			X	X

Based on the CQAs identified using Table 14, the product quality attributes and process parameters were then determined for the specific life time study of CFT resins. The HPLC impurities, SEC impurities, residual HCP and the specific enzyme in the purified protein were tracked as the resin aged to determine whether any deterioration of functional performance was occurring. In addition, the standard performance attributes relevant to any chromatography unit operation – yield or recovery, column packing parameters and column pressure were also tracked with repeated use. In addition, spectrophotometry in the visible range of the spectrum was performed on the eluates of product and blank runs to check for any coloured contaminants eluting from the resin. This was because many product feed streams contain coloured substances that bind strongly to chromatographic media, with this being a noticeable phenomenon with *E. coli* feed streams from inclusion bodies and in plasma fractionation [96]. Total organic carbon was also measured in the eluates of blank cycles as an indication of whether the cleaning conditions were adequately removing organic components.

This is only possible in this specific application as the running buffers used for CFT are entirely inorganic. SDS-PAGE and HCP were also measured in the blank cycles to determine whether the resin was being adequately cleaned.

5.5.2 RP-HPLC, SEC and HCP Impurities

The data on RP-HPLC impurities remaining in the pool after CFT chromatography are summarised in an I-chart in Figure 24. The first 5 cycles of the unit operation were performed using manual operation of the chromatography system, after which point the system was automated and repeated runs were performed using the automated method. The I-chart shown in Figure 24 was able to detect the change in technique that occurred between these two methods of operation and indicates that three of the first 4 chromatography runs produce values more than 3 standard deviations (3σ) outside the mean established by the subsequent chromatography runs. The load solution contained a total of 5.58 % RP-HPLC impurities, with the RP-HPLC technique having a variation on repeat measurement of 0.4 %, which indicates that the CFT unit operation had relatively little effect on the total RP-HPLC impurities for the product. Further, the outlier points indicated in red by the statistical software should be treated with caution due to the low number of data points used (n = 9) and the fact that the level of variation detected is quite close to the inherent variation of the analytical assay (RP-HPLC).

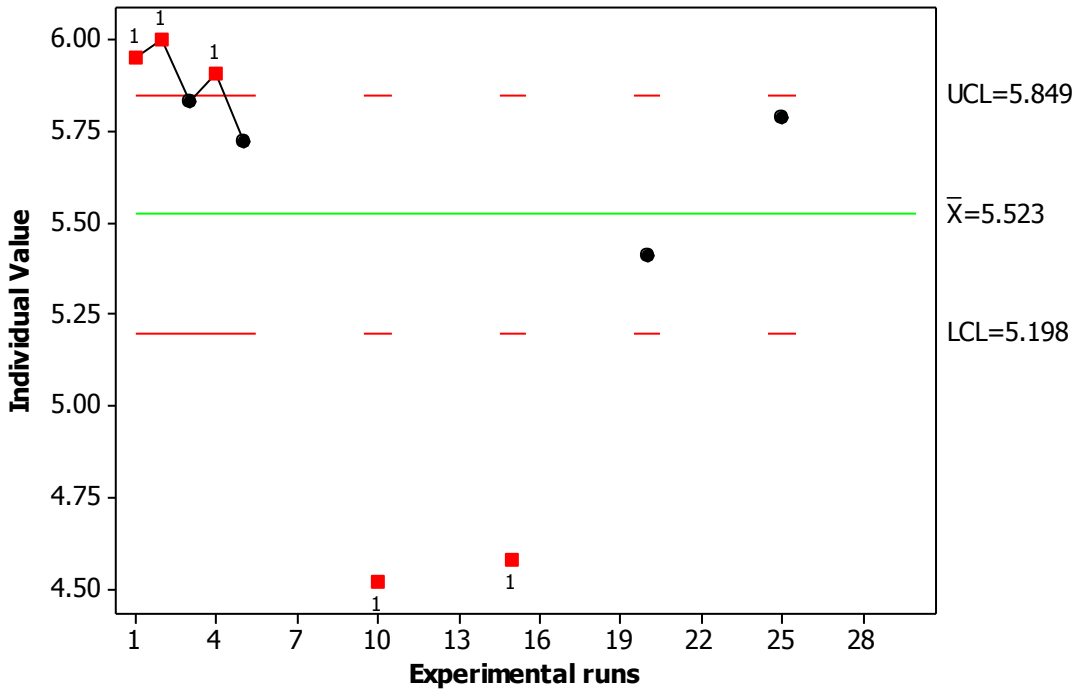


Figure 24: I-chart of HPLC impurities.

$\bar{X}$ denotes the mean of the measured values, UCL (upper control limit) the mean plus three standard deviations (3σ) and LCL (lower control limit) the mean minus 3σ.

Figure 25 shows the level of SEC impurities in the CFT eluate, with the data displayed as an I-chart. As discussed above, the first 5 cycles of the unit operation were performed manually and subsequent operations were performed using an automatic method. The increased level of variation is visible in the I-chart, though it does not result in any of the points measured exceeding the 3σ control limits. The amount of SEC impurities remaining in the pool after CFT chromatography was assessed for the first 5 product cycles, then every 5th product cycle after that. SEC impurities in the CFT load solution were 2.41 %, with the SEC assay itself having an inter-assay variability on repeat measurement of 0.1 %. In this context, Figure 25 indicates that the CFT unit operation provides robust removal of SEC impurities over the lifetime of operation measured in this process. This chart indicates that the performance of the CFT resin in removing SEC impurities did not change throughout the resin lifetime study as the amount of SEC impurities remained within the ranges established by the initial 5 chromatography runs on the resin. The I-chart indicates that the removal of SEC impurities is firmly in control throughout the resin lifetime.

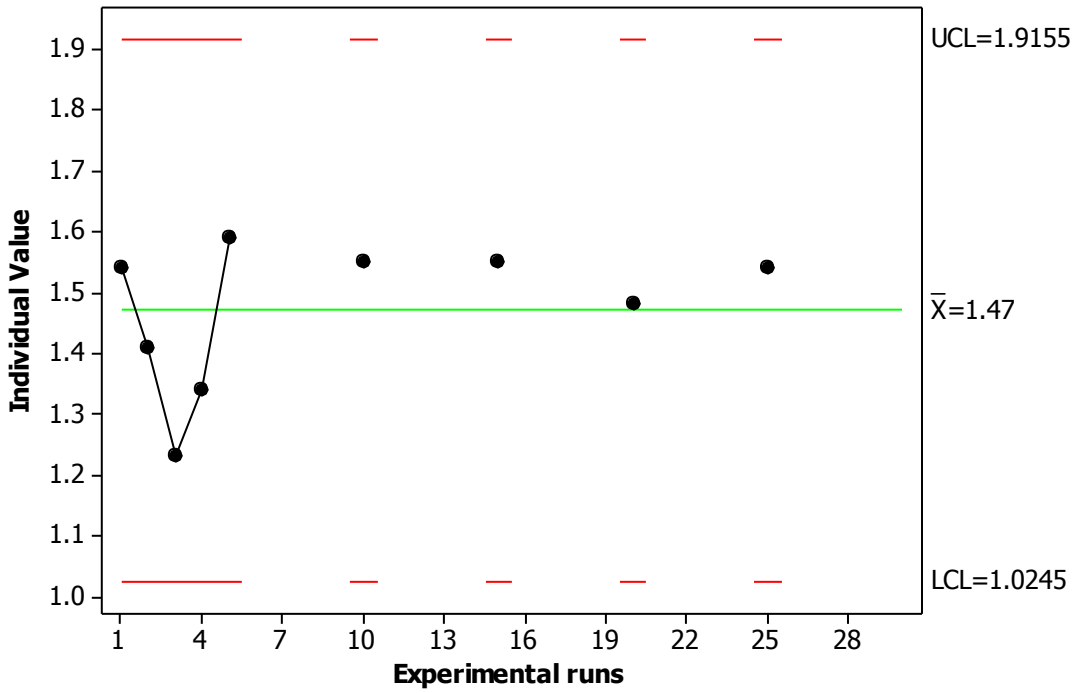


Figure 25: I chart of SEC impurities

Figure 26 shows the level of HCP impurities present in the CFT eluate throughout the lifetime study, with the data presented as an I-chart. The level of HCP impurities in the load solution was approximately 600 ppm. As with the other variables measured, the amount of HCP remaining in the product pool was assessed for each of the first 5 product cycles, then every 5th product cycle after that over the course of the lifetime study. The I-chart shows that the removal of HCP impurities is firmly in control throughout the resin lifetime study. Removal of HCP by the CFT unit operation was robust to repeated use and cleaning of the chromatography resin. No degradation of the ability of the CFT resin to remove impurities was discovered over the resin lifetime, with the amount of HCP in the product pool remaining within the ranges established in the first 5 cycles.

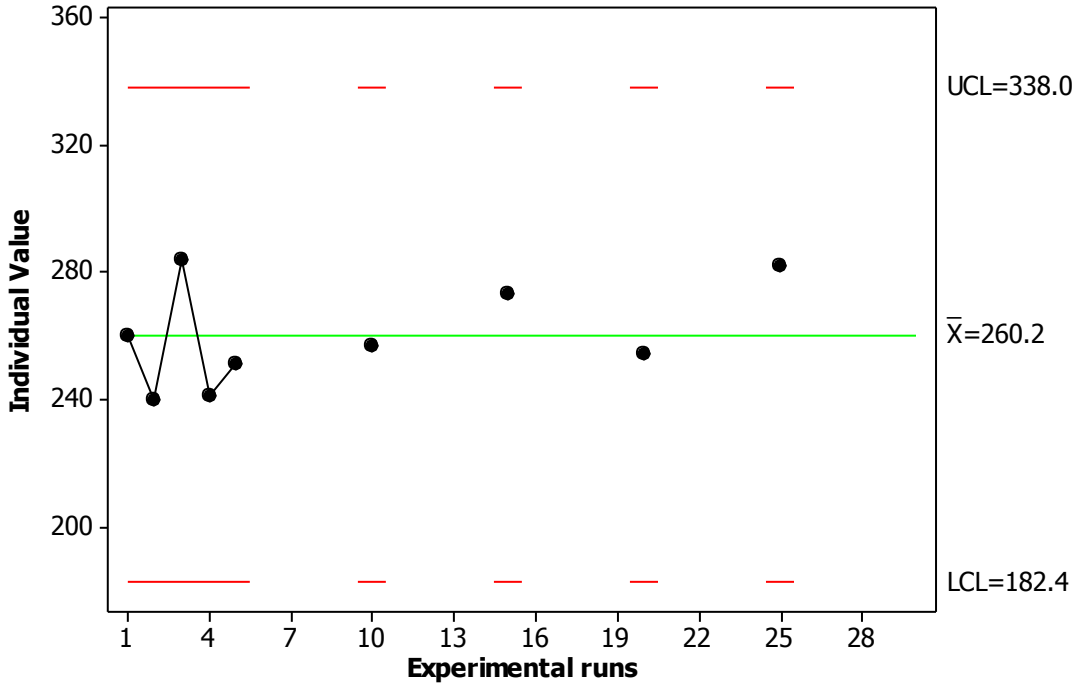


Figure 26: I Chart of HCP impurities

5.5.3 Recovery

Figure 27 displays the total protein recovery data collected throughout the resin lifetime study, displayed as an I-MR chart. The I-MR chart indicates that the protein recovery remained within control throughout the study. The only unusual point was cycle 31 which had an unusually low recovery of 84 %, followed by a more normal recovery of 95 % in the following cycle. This resulted in the very large moving range between these two runs, detected on the MR chart. This data probably indicates that recovery of cycle 31 was underestimated due to measurement error, as there is no discernible change in the process centre or the moving range between runs throughout the lifetime study and the process returned to more normal values immediately after cycle 31. The gaps in the I and MR chart are due to the fact that every fifth cycles of operation was a “blank” run which used a buffer solution as a mock load, so calculation of the % recovery for these cycles was not relevant.

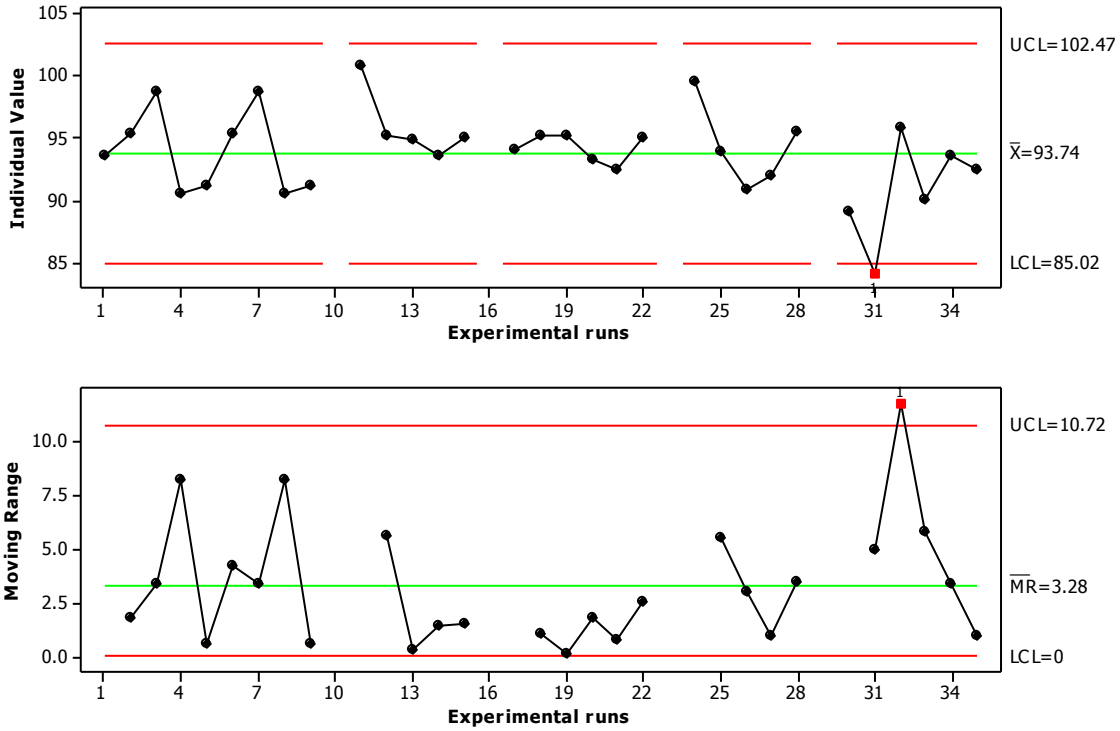


Figure 27: I-MR (interval and moving range) chart of total protein recovery (%)

Figure 28 displays the backpressure on the resin at the end of the load cycle for each run as an I-MR chart. Backpressure at the end of load is an important parameter to measure as an increase in pressure may indicate a build-up of solid material on the resin and possible clogging of the interstitial spaces or resin pores. The fifth and sixth cycle of operation are detected as out of the control limits by the I-chart, with the range between the sixth and seventh cycles being detected as out of the control limits by the MR-chart. After the seventh cycle the unit operation settles down and remains firmly in control for the remainder of the resin lifetime study. The outliers in these early operations may indicate that some settling in of the resin was occurring inside the packed bed, which later stabilized through repeat operation of the column. Included in this chart are the results from the blank runs were performed as the first cycle and then after every 5 product cycles. The fact that there was no discernible difference in backpressure between the product and blank cycles indicates that the

protein resin is not causing any build-up on the resin and has been appropriately prepared for loading onto the chromatography column.

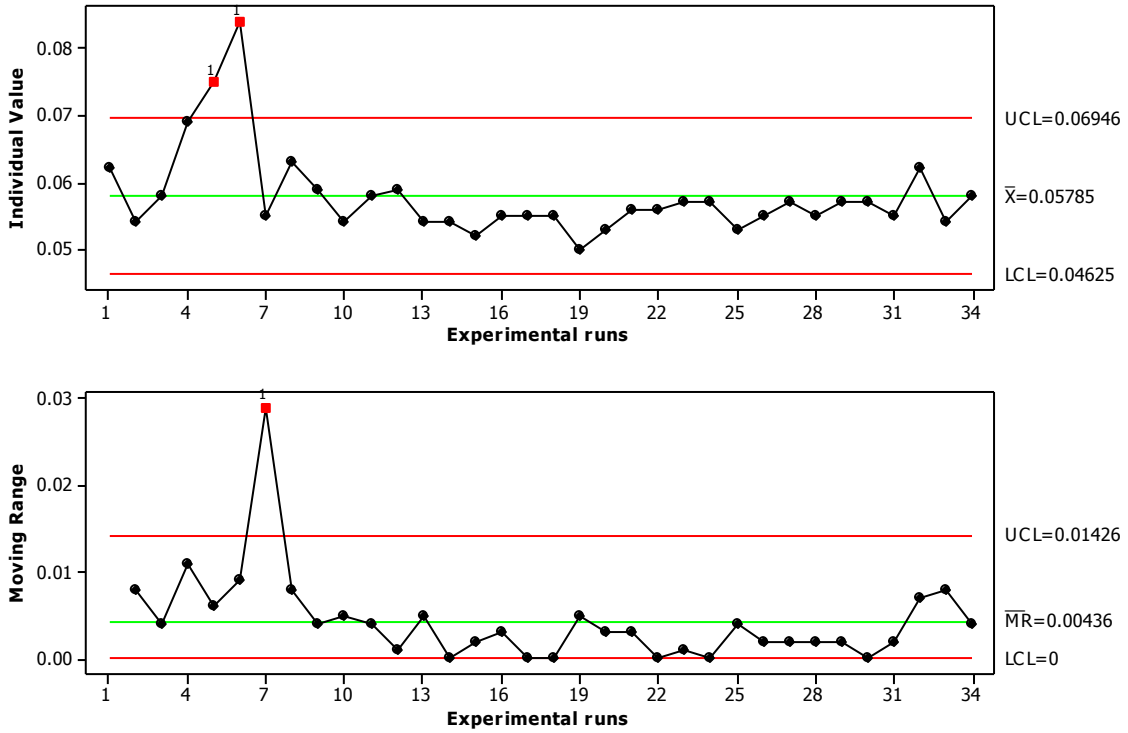


Figure 28: I-MR chart of backpressure

5.5.4 Column Packing and Qualification

The column was packed and qualified based on the qualification criteria used at manufacturing scale and the column efficiency was evaluated at the start of every cycle. The column qualification procedure involved equilibrating the column with 10 mM sodium phosphate with 150 mM NaCl and then injecting a salt spike of 1 M NaCl equal in volume to 1% of the column volume. Resin bed height remained constant throughout the study.

Figure 29 displays the HETP of the packed column as an I-MR chart, with HETP measured prior to each chromatography run. This chart indicates that the chromatography column resin packing was indeed changing throughout the resin lifetime study, an observation which is further supported by Figure 30, an I-MR chart of the column asymmetry throughout the resin lifetime study. If these charts were of measurements taken from a production-scale column in routine operation they would be cause for concern as the acceptance criteria for the resin, based on established best practices, would be for an HETP value between 0.008 – 0.02 and an asymmetry value of between 0.8 – 1.5. At manufacturing scale the figure obtained (an average HETP of 0.055 and average asymmetry of 2.5) would trigger a re-pack of the column to ensure the manufacturing column was meeting pre-determined acceptance criteria. As this data is derived from a scale-down model run, when coupled with quality attribute data obtained earlier in the study they provide assurance that the resin can achieve the required levels of product purity even when the chromatography bed is poorly packed. This provides added assurance of the robustness of the unit operation as a well-packed chromatography resin used at manufacturing scale would be expected to perform at least as well as the scale-down model examined in this study. Unlike most other chromatography resins based on agarose or other organic polymers, CFT is a ceramic, virtually incompressible and settles rapidly under gravity when poured into the chromatography housing. For these reasons it is more difficult to pack, especially at small-scale when it is less possible to exert force on the packed bed to compress the bed to obtain acceptable HETP and asymmetry values.

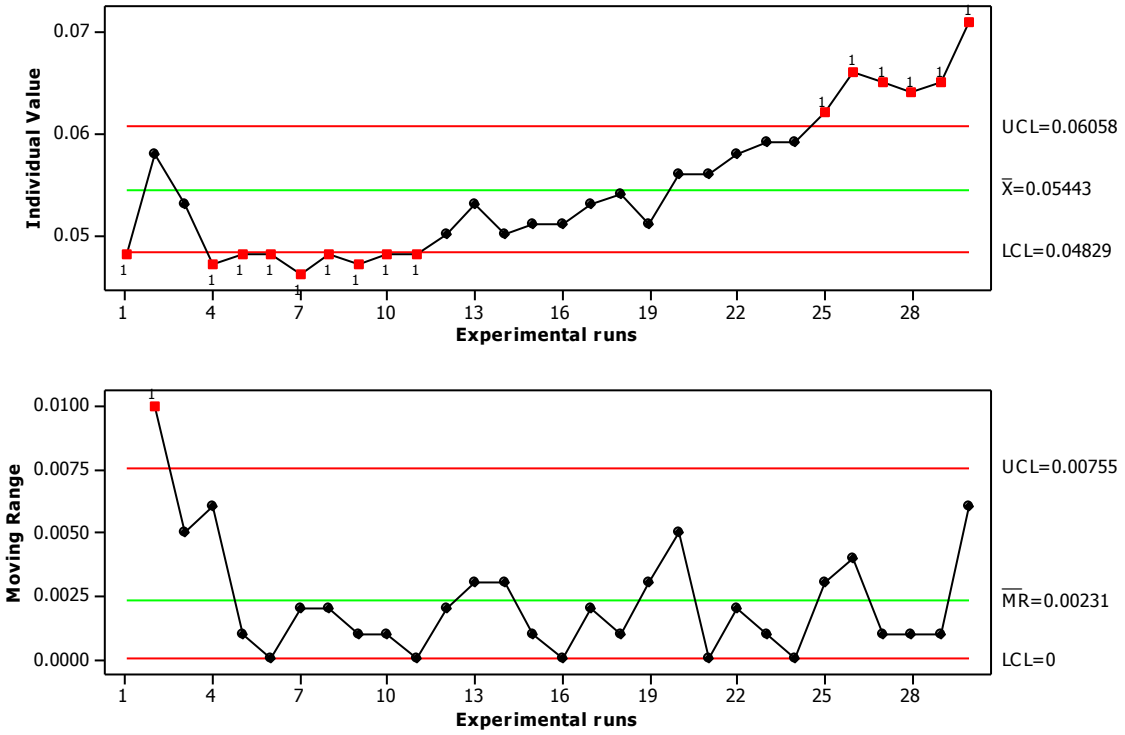


Figure 29: I-MR chart of resin HETP

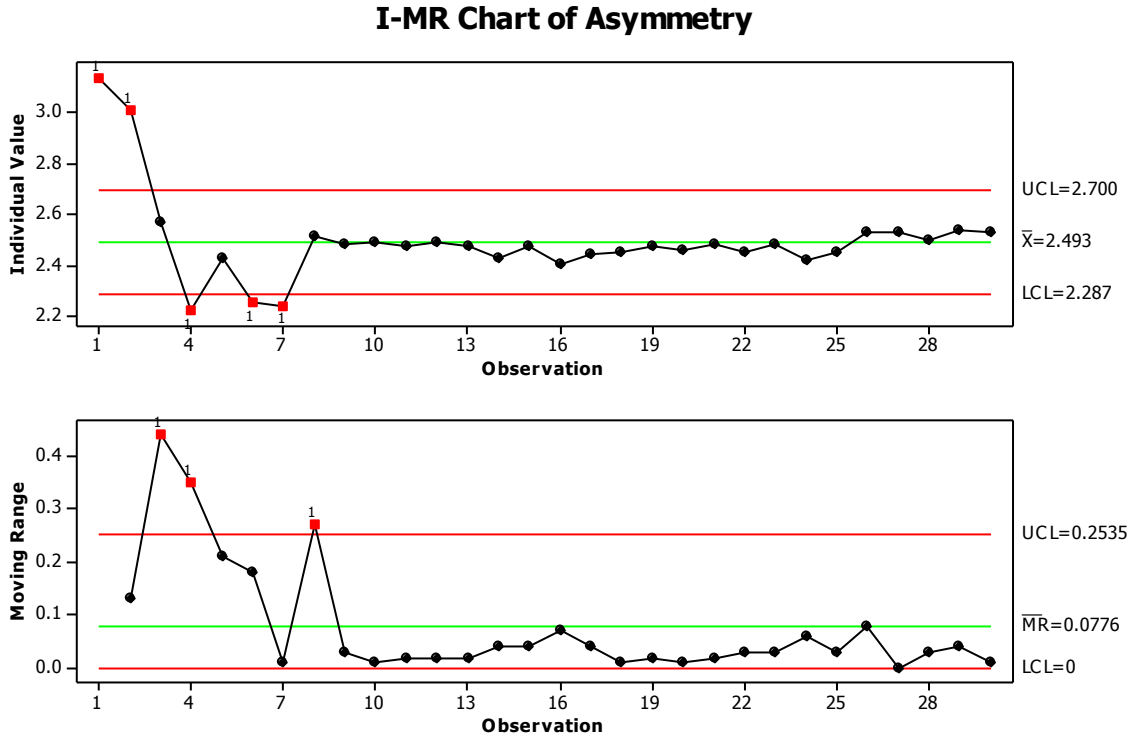


Figure 30: I-MR chart of resin Asymmetry

5.5.5 SDS-PAGE

SDS-PAGE analysis was performed on the CFT non-binding eluate from the blank runs to test for carryover of the protein. 100 ng of control and 10 ng of the target product protein were included on each gel to ensure the gels were sufficiently developed. The presence of any contaminating bands in tested lanes was noted, reported and compared to the negative control lanes (unused resin). No build-up of product was seen on the column throughout use (Figure 35; Appendix 1).

5.5.6 Visible Spectrophotometry

Visible Spectrophotometry was carried out on the eluate of each of the blank and product runs to check for the build-up of coloured contaminants on the resin which may elute during product runs and contaminate the product. No significant absorbance was observed in the eluate samples of blank or product runs (Table 17; Appendix 1), ruling out the build-up of any coloured contaminants on the resin.

5.5.7 Total Organic Carbon

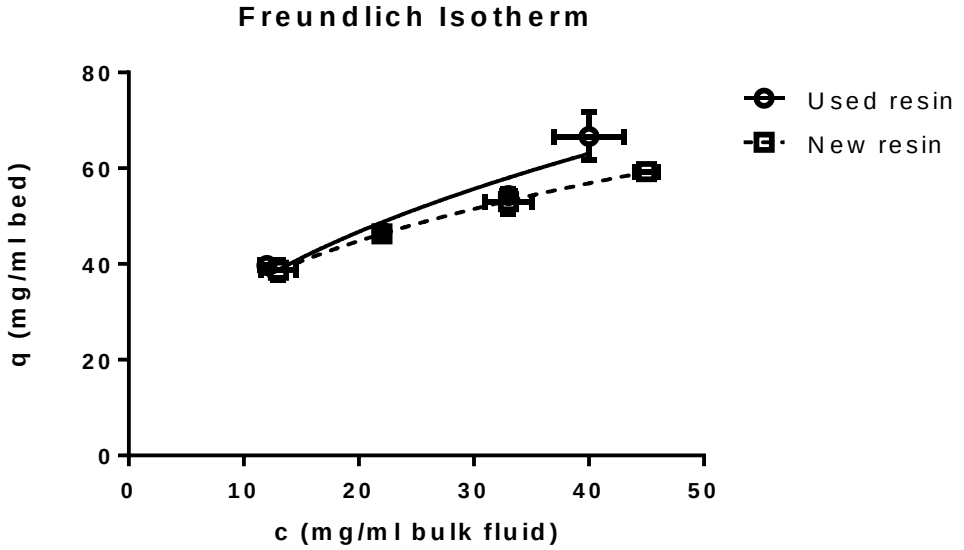
TOC analysis was performed on the eluate of blank runs 1 – 6 to check for organic content, which would indicate build-up of protein or other organic contaminants on the resin during repeated usage. The results of TOC analysis are shown in Table 15 and show that there was no build-up of organic carbon on the resin throughout the study.

Table 15: TOC level measured in the eluate of the blank cycles
Values are reported as the difference from the TOC value of the blank elution buffer

Blank cycle no.	TOC (ppm)
1	2.81
2	1.49
3	5.53
4	2.17
5	-2.82
6	-0.97

5.5.8 Static Binding Capacity

The static binding capacity of unused resin and the same resin after 30 cycles of use was determined using the model compound lysozyme as the CFT resin does not function to bind the product under the operating conditions but rather operates in flow through mode. In this mode the contaminants bind to the resin while the product flows through in the non-binding eluate. The static binding capacity of the used and unused resin is shown in Figure 31 along with the derived association constant and heterogeneity index (K_f and m). The 95 % confidence intervals obtained for both parameters are overlapping so there is no indication in degradation of the resin association constant or heterogeneity index with repeated use and cleaning of the resin. This result indicates that the current cleaning and use conditions are compatible with the resin and no significant degradation of the ligand was occurring over multiple re-uses.



	Used resin	New resin
Best-fit values		
Kf	12.79	15.99
m	0.4324	0.3440

	Used resin	New resin
95% Confidence Intervals		
Kf	7.406 to 18.18	13.35 to 18.62
m	0.3076 to 0.5572	0.2955 to 0.3924

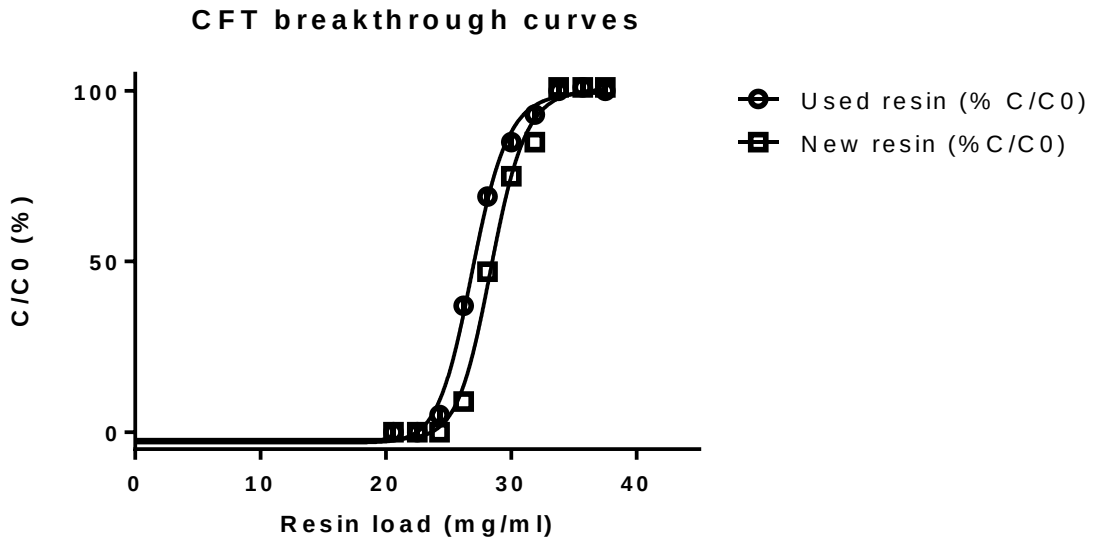
Figure 31: Static binding capacity of new and used resin using lysozyme.

Solution conditions of 10 mM phosphate buffered saline pH 6.8 containing chicken egg white lysozyme at varying concentrations from 25 to 55 mg/ml. Data were fit to the Freundlich isotherm to obtain the displayed parameters.

5.5.9 Dynamic Binding Capacity

The dynamic binding capacity study for the CFT resin was performed to determine the binding efficiency of the resin before and after 30 cycles of use, sanitization and regeneration and therefore to check for any resin degradation. The product does not bind to the CFT resin during normal use so the model protein chicken egg white lysozyme was used to determine the dynamic binding capacity.

The results of the dynamic binding capacity tests are shown in Figure 32, along with the parameters obtained from fitting of the breakthrough curve to the Hill-Slope equation. When comparing the used and unused resin the sigmoidal dose-response curve produced very similar values and overlapping confidence intervals for all curve parameters apart from the EC50 or the value at 50 % breakthrough. This indicates that there was a slight reduction in the binding capacity of the CFT resin after 30 re-uses of approximately 1 mg lysozyme per ml of resin or approximately or approximately 4 % of total capacity. The fact that there was no detectable difference in the slope (H) of the binding curve between new and used resin indicates that there was little change in the kinetics of binding of the model compound after the resin had been used multiple times.



	Used resin (% C/C0)	New resin (%C/C0)
H	19.12	20.66
EC50	26.96	28.45

	Used resin (% C/C0)	New resin (%C/C0)
95% Confidence Intervals		
Bottom	-9.226 to 3.095	-9.171 to 4.744
Top	95.33 to 104.0	93.79 to 107.5
H	14.27 to 23.97	13.41 to 27.91
EC50	26.56 to 27.35	27.92 to 28.98

Figure 32: Dynamic binding capacity curves for new and used resin using chicken egg white lysozyme.

Lysozyme at 2 mg/ml and all solution conditions 10 mM sodium phosphate pH 6.8; linear flow rate of 150 cm/hr. H = slope of the linear portion of the curve; EC50 = resin load at 50 % binding capacity (C/C0).

5.5.10 HETPs and Porosities

Comparing intra-particle porosity (ε_p), interparticle porosity (ε_i) and total porosity (ε_t) of the new and used resin allowed determination of whether any of the pores on the resin had been blocked with particulates due to lack of load filtration or insufficient cleaning. These porosities were estimated from HETP vs linear velocity plots and experimentally measured. HETP versus linear velocity experiments were performed with new and used media. CFT eluate (containing the product protein in 10mM Phosphate at pH 5.4) was adjusted to 400mM

$NaHPO_4$ to provide non-binding conditions on the CFT resin. The CFT column was equilibrated in 400 mM $NaHPO_4$, pH 5.4. An injection of filgrastim (1 % of column volume) was performed at linear flow velocities ranging from 100 to 300 cm/hr.

The curve of HETP versus linear velocity was plotted and is shown in Figure 13. The figure shows that there were no significant changes to the slope of the plots generated based on new and used media. No change in the slope indicated that pore diffusion properties are likely to be similar for new and used CFT media. The curve of HETP versus linear velocity was fit to the one phase decay equation using GraphPad Prism. A good fit (R^2>0.99) was produced by this curve with both used and new media having overlapping confidence intervals for all parameters analysed, indicating no detectable degradation of resin was produced by repeated use and sanitization of the resin.

No detectable changes in the interparticle, intraparticle, and total porosity were further confirmed from experimental measurements (Table 16). The parity between the porosity values of the new and old resin demonstrates that no fouling occurred during the process.

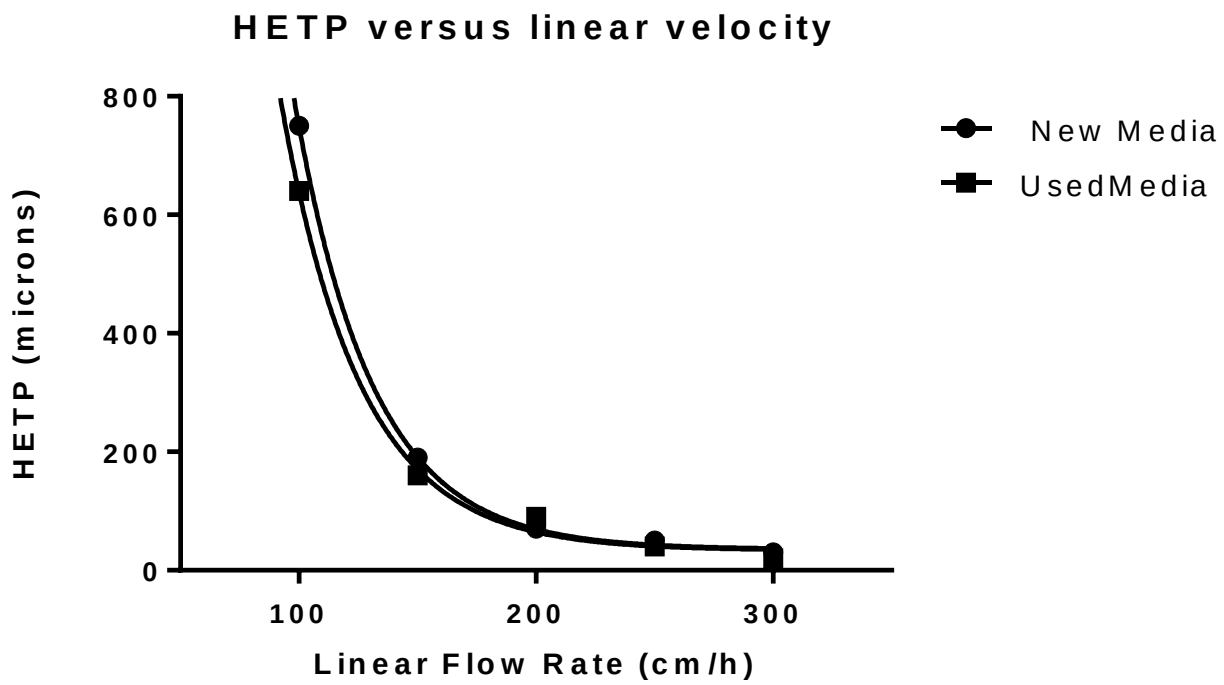


Figure 33: HETP versus linear velocity curves for new and used resin.

Running buffer of 400 mM NaHPO4 pH 5.4 (non-binding conditions) and an injection volume of 1 % CV of the target protein (filgrastim) at 4.2 mg/ml as measured by UV spectrophotometry.

Table 16: Results of Total Porosity studies with New and Old CFT media

	New Media	Old Media
Total porosity (εt)	0.85	0.80
Interparticle porosity (εi)	0.48	0.67
Particle porosity(εp)	0.77	0.64

5.5.11 Enzymatic Activity

Zymography was used to confirm that the contaminating enzymatic activity had been removed by the CFT resin. An example zymogram is shown in Figure 34. In all zymogram gels, a prominent band was observed at 28 kDa (Lane 2) in the positive control which was produced using a sample of the product load before CFT chromatography to demonstrate the presence of the enzymatic activity in the load sample. Also visible in the zymogram is the intrinsic fluorescence of the product protein, indicated by a band at 18 kDa on the gel. Removal of the contaminating enzyme was indicated by removal of the 28 kDa band. No enzymatic activity was observed in the CFT eluates from any of the process runs tested demonstrating that the resin continued to remove the enzymatic activity across the entire lifetime study. Furthermore, no enzymatic activity was detected in the blank cycle eluates produced throughout the lifetime cycle (data not shown).

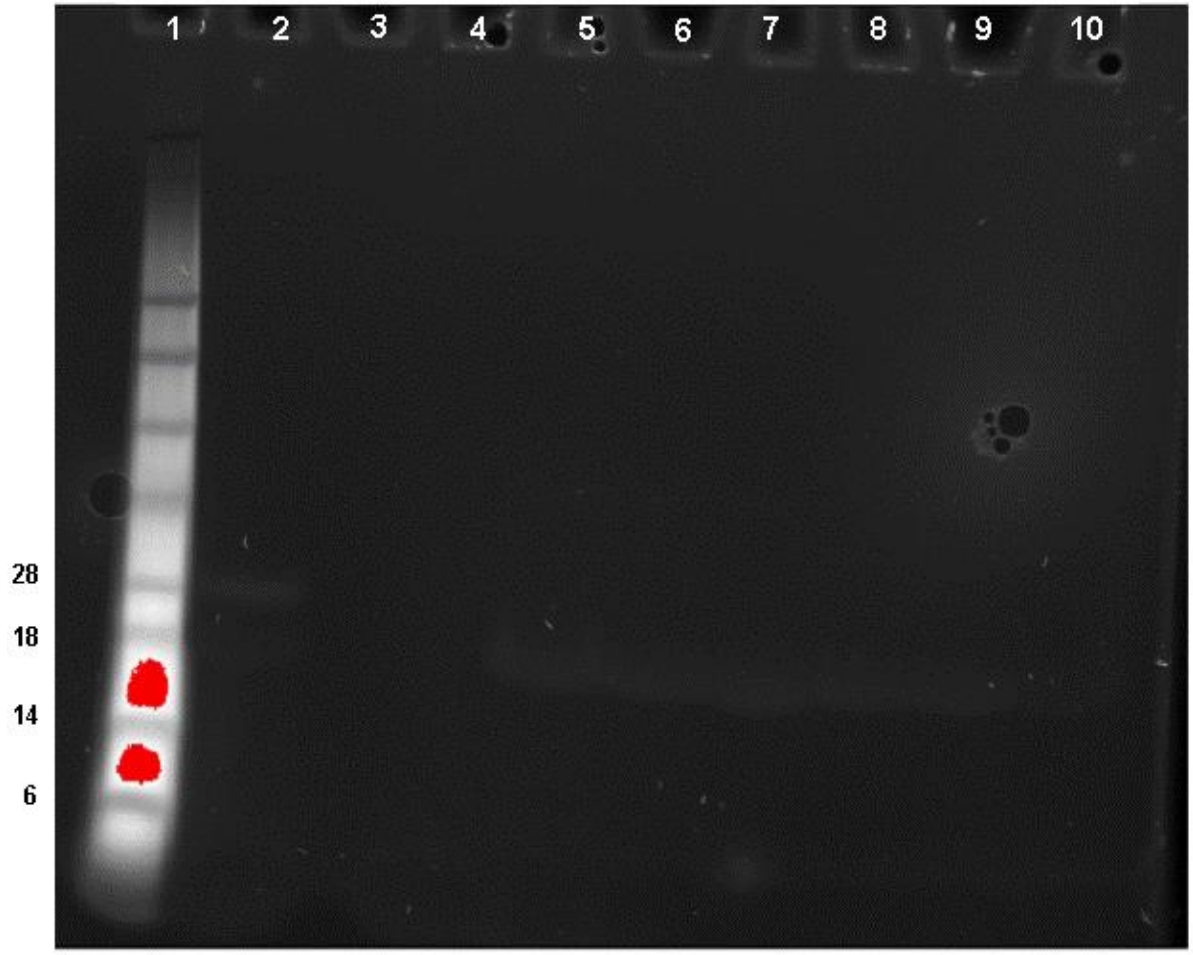


Figure 34: Enzymatic zymogram of CFT eluates

Lane 1: Molecular weight markers, Lane 2: Positive control (CFT load solution) showing the enzymatic activity band at 28 kDa and the intrinsic fluorescence of the product Protein At 18 kDa. Lane 3: Empty. Lane 4: Blank cycle 1 eluate. Lane 5: Process run 1 eluate. Lane 6: Process run 2 eluate. Lane 7: Process run 3 eluate. Lane 8: Process run 4 eluate. Lane 9: Process run 5 eluate. Lane 10: Negative control – product sample without enzymatic activity.

5.6 Discussion

This study was performed to determine whether the running conditions of a CFT chromatography unit operation were compatible with the resin over a lifetime of 30 re-uses including conditions of loading, cleaning and sanitization. Based on analysis of the CFT output, it was observed that step yield and quality of the product remained within the acceptable range over 30 runs (Yield > 90%, RP-HPLC impurity < 8%, SEC impurity < 1.7%). The primary function of the CFT unit operation is to remove an enzymatic activity that can degrade an excipient in the final drug product. This resin lifetime study demonstrated that this enzymatic activity continued to be removed over 30 cycles of use. Furthermore, column cleaning was found to be effective as no product impurity carryover was observed from one run to the next. Results indicate that the CFT resin can be used for a minimum of 30 cycles in commercial manufacturing.

Establishing the acceptable lifetime of the resin in a purely empirical manner as performed here is necessary to provide the information required by pharmaceutical regulatory authorities to demonstrate the robustness of a biopharmaceutical manufacturing process - that it will continue to produce acceptable product over repeated production cycles. In addition to providing empirical information, this study was performed to demonstrate the additional techniques that can be used to produce a Quality by Design resin lifetime study. A standard tool in many manufacturing industries is the use of control charts to monitor repeat manufacturing runs. This tool can provide early warning of when unit operations are starting to drift out of control, with the moving range chart being able to determine whether the underlying process is in-control and therefore whether variation seen in the outputs is due to the natural variation of the data or a “special cause” (i.e. an uncontrolled change or unknown variable) is responsible for some of the experimental variability. Use of this quality tool is recommended for resin lifetime data, which is really a form of time series data, in order to make more efficient use of limited data and to enable more conclusions to be drawn than the

purely empirical observation that the resin continues to function adequately. International guidelines on quality risk management for pharmaceuticals [115] recommend the use of acceptance control charts as part of a company's overall quality risk management strategy. This study demonstrates how control charts in particular can be used to gain further understanding from a standard resin lifetime study and to confirm the robustness of the underlying process.

In addition to using control charts to extract more information from a resin lifetime study, various tests were applied to the resin before and after it had been subjected to multiple re-uses to determine whether there were any changes to the underlying mechanisms of action of the resin. The use of these tests was based on earlier work used to examine Protein A and cation exchange resins [62, 106]. With the use of various tracer techniques it was possible to demonstrate that no blocking of the resin pores was occurring over the lifetime of use, demonstrating that the feedstock is adequately filtered and prepared for use and that no precipitation was occurring on the resin during use. Using static binding capacity tests it was demonstrated that no detectable loss of ligand had occurred during use of the resin as the equilibrium binding capacity of the resin remained unaltered. Dynamic binding capacity studies indicated that no changes to the mass transfer properties of the resin had occurred under the conditions of use and preparation over multiple cycles. As previously shown [62] the more detailed information these mechanistic studies provide can determine what aspect of the chromatography is degrading with multiple re-uses and therefore enable intelligent decisions to be made about remedial action to enhance resin lifetime. For example, if these additional tests demonstrate that the resin pores are becoming blocked with repeated use the utility of improved filtration of the load should be examined and the conditions of use examined for any potential to lead to on-column precipitation. These are different courses of action to those that would be considered if the conditions of use were shown to lead to ligand instability by the loss of equilibrium binding capacity or changes to the mass transfer

properties were indicated by changes in the dynamic binding capacity. In one of the seminal QbD documents the FDA has stated that having a mechanistic basis for understanding failure modes and variability should be one of the objectives of state-of-the-art pharmaceutical science [116]. This level of "enhanced knowledge" is recommended as part of a QbD approach and demonstrates the superiority of a mechanistic approach to resin lifetime studies, and QbD studies in general, over a purely empirical approach. Mechanistic studies can achieve a more fundamental understanding of the function of the unit operation and can allow earlier and more economical detection of whether improvements or changes need to be made to resin handling conditions, saving time, effort and expensive equipment.

The effect of the operating conditions on the resin itself is especially important with resins based on hydroxyapatite. Other researchers have shown that hydroxyapatite resin can undergo significant loss or gain of resin mass depending on running conditions and that these mass changes are associated with column failure during production [109]. The work reported in this paper demonstrates both how such degradation can be detected early in development and the superiority of CFT over CHT resin in its reusability and resistance to acidic conditions.

5.7 Conclusions

CFT resin was found to be very stable during use with a recombinant protein product over 30 cycles in operating conditions of pH 5.4. Control chart (I-charts and I-MR charts) were used to demonstrate the level of normal variation that could be expected in data produced by repeated use of the resin. Use of these charts demonstrated how this Quality technique can be applied to resin lifetime studies – with the control charts shown to be sensitive enough to detect when the individual chromatography runs switched from being run manually to being fully automated using the ÅKTA FPLC system.

Studies of the static and dynamic binding capacity of the resin before and after 30 manufacturing cycles coupled with determination of the intra-particle porosity (ε_p),

interparticle porosity (ε_i) and total porosity (ε_t) of the resin were able to demonstrate that the running and cleaning conditions and product feedstock were completely compatible with the resin, providing justification for their continued use at manufacturing scale. This study also demonstrated the uniquely stable nature of ceramic fluoroapatite resin in a manufacturing application compared to previous reports of the sensitivity of ceramic hydroxyapatite resin to operating conditions.

5.8 References

1. ICH. *Pharmaceutical Development Q8(R2)*. 2009; Available from: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.
2. Elliott, P., et al., *Quality by Design for biopharmaceuticals: a historical review and guide for implementation.* Pharmaceutical Bioprocessing, 2013. **1**(1): p. 105 - 122.
3. Rathore, A.S. and H. Winkle, *Quality by design for biopharmaceuticals.* Nat Biotechnol, 2009. **27**(1): p. 26-34.
4. Rathore, A. and G. Sofer, *Life span studies for Chromatography and Filtration media*, in *Process validation in manufacturing of Biopharmaceuticals: Guidelines, Current Practices and Industrial Case Studies.*, A. Rathore and G. Sofer, Editors. 2005, CRC Press, Taylor & Francis Group: Boca Raton, FL. p. 169 - 203.
5. USFDA, *Compliance Program, Chapter 41, Inspection of Licensed Therapeutic Products.* 1999.
6. USFDA, *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use.* 1997. **February**.
7. EuropeanCommission, *Production and Quality Control of medicinal products derived by recombinant DNA technology*, in *III/3477/92*, E. Commission, Editor. 1994. p. 10.
8. ICH. *DEVELOPMENT AND MANUFACTURE OF DRUG SUBSTANCES (CHEMICAL ENTITIES AND BIOTECHNOLOGICAL/BIOLOGICAL ENTITIES) Q11*. 2012 May; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q11/Q11_Step_4.pdf.
9. Bussineau, C., et al., *PDA Technical Report #42.* 2005.
10. O'Leary, R., et al., *Determining the useful lifetime of chromatography resins.* BioPharm International, 2001. **14**(9): p. 10.
11. USFDA. *Pharmaceutical cGMPS for the 21st Century — A Risk-Based Approach: Second Progress Report and Implementation Plan*. 2003 [cited 2012 23SEP2012]; Available from: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/Manufacturing/QuestionsandAnswersonCurrentGoodManufacturingPracticescGMPforDrugs/ucm071836.htm.
12. Breece, T., E. Gilkerson, and C. Schmelzer, *Validation of Large-Scale Chromatographic Processes, Part 2 Results from the Case Study of Neuleze Capture on Macroprep High-S.* BioPharm 2002. **July**: p. 35 - 42.
13. Bannerjee, A., *Designing in Quality: Approaches to Defining the Design Space for a monoclonal antibody process.* BioPharm, 2010(May).
14. Jiang, C., et al., *A mechanistic study of Protein A chromatography resin lifetime.* J Chromatogr A, 2009. **1216**(31): p. 5849-55.
15. Natarajan, V. and S. Cramer, *A methodology for the characterization of ion-exchange resins.* Separation Science and Technology, 2000. **35**(11): p. 1719-1742.
16. Hilbrig, F. and R. Freitag, *Isolation and purification of recombinant proteins, antibodies and plasmid DNA with hydroxyapatite chromatography.* Biotechnol J, 2012. **7**(1): p. 90-102.
17. BioRad, *CFT Ceramic Fluoroapatite Instruction Manual*. 2000.

18. McCue, J.T., et al., *Use of an alternative scale-down approach to predict and extend hydroxyapatite column lifetimes.* J Chromatogr A, 2007. **1165**(1-2): p. 78-85.
19. Council_of_Europe, *European Pharmacopeia. Filgrastim Concentrated Solution <2206>*. Vol. 7. 2013, Strasbourgh: Council of Europe.
20. USP, *Color - instrumental measurement*, in *USP29*. 2006, United States Pharmacopeia. p. 2896-2898.
21. IUPAC, *Nomenclature for Chromatography.* Pure and Applied Chemistry, 1993. **65**(4): p. 819-872.
22. Ghose, S. and S.M. Cramer, *Characterization and modeling of monolithic stationary phases: application to preparative chromatography.* J Chromatogr A, 2001. **928**(1): p. 13-23.
23. Umpleby, R.J., 2nd, et al., *Characterization of molecularly imprinted polymers with the Langmuir-Freundlich isotherm.* Anal Chem, 2001. **73**(19): p. 4584-91.
24. Minitab, *Minitab Stat guide*. 2005, Minitab.
25. ICH. *Quality Risk Management Q9*. 2005; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf.
26. USFDA. *Innovation and Continuous Improvement in Pharmaceutical Manufacturing Pharmaceutical CGMPs for the 21st Century*. 2004; Available from: http://www.fda.gov/ohrms/dockets/ac/04/briefing/2004-4080b1_01_manufSciWP.pdf.

5.9 Appendix 1

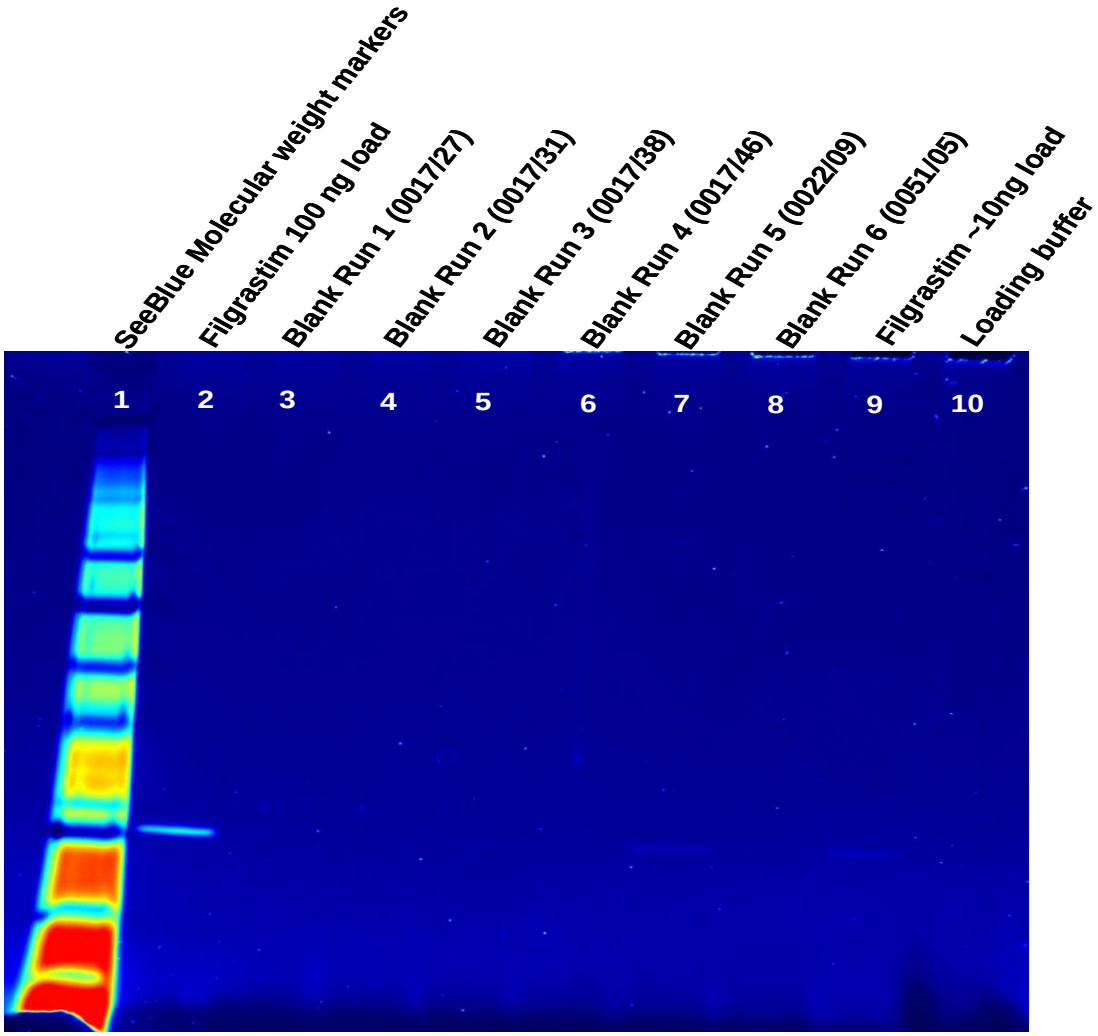


Figure 35: SDS-PAGE analysis of the blank runs performed throughout the resin lifetime study.

Positive control lanes containing 100 and 10 ng of filgrastim (lanes 1 and 9) were included to confirm the gel had been sufficiently developed.

Table 17: Visible spectrophotometry measurements of the eluate from blank and product runs throughout the resin lifetime study.

Sample	Results
Blank Run - 1	No absorbance
Product Run - 1	Max. ~0.008 at 380 nm
Product Run - 2	Max. ~0.008 at 380 nm
Product Run - 3	Max. ~0.008 at 380 nm
Product Run - 4	Max. ~0.008 at 380 nm
Product Run - 5	Max. ~0.008 at 380 nm
Blank Run - 2	No absorbance
Product Run 10	Max. ~0.016 at 380 nm
Blank Run - 3	No absorbance
Product Run - 15	Max. ~0.015 at 380 nm
Blank Run - 4	No absorbance
Product Run - 20	Max. ~0.021 at 380 nm
Blank Run - 5	No absorbance
Product Run - 25	Max. ~0.014 at 380 nm
Blank Run - 6	Max. ~0.001 at 380 nm

Statement of Authorship

Title of Paper	Use of Quality by Design for development of tangential flow filtration for a biopharmaceutical where standard TFF optimization techniques were problematic
Publication Status	○ Published, ○ Accepted for Publication, ○ Submitted for Publication, ◉ Publication style
Publication Details	Text in manuscript

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Phillip Elliott		
Contribution to the Paper	This work was largely planned and written by Phillip Elliott.		
Signature	[redacted]	Date	19FEB2015

Name of Co-Author	Dr Jingxiu Bi		
Contribution to the Paper	Dr Bi completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	24/02/2015

Name of Co-Author	Dr Hu Zhang		
Contribution to the Paper	Dr Zhang completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	20FEB2015

Name of Co-Author	Dr Sam Billingham		
Contribution to the Paper	Dr Billingham completed multiple reviews and made suggestions for increased focus in certain sections and suggestions for improvements of writing to improve clarity and flow.		
Signature	[redacted]	Date	23 Feb 2015

6 Use of Quality by design for development of tangential flow filtration for a biopharmaceutical where standard TFF optimization techniques were problematic

6.1 Abstract

A tangential flow filtration (TFF) procedure for the formulation of filgrastim (G-CSF) was developed using a Quality by Design (QbD) approach to ensure a robust and well understood manufacturing operation was produced. Four different TFF membranes, comprising two different molecular weight cut-off values (5 kDa and 10 kDa) and two different membrane types (polyethersulfone and regenerated cellulose) , were compared in their performance for formulating filgrastim. A design space comprising a safe range of transmembrane pressure (TMP), cross-flow and membrane loading rates was developed allowing for flexibility at future manufacturing scale and adjustment to future manufacturing equipment. Most of the previous work on TFF optimization has used model proteins to establish the principles due to the large volume of protein required, the high cost of biopharmaceutical Protein and the resulting high cost of the final experimentation. This work sought to apply the standard TFF principles to use with a biopharmaceutical protein to produce a case study of TFF development that is relevant to the current industry.

Excessive product loss to the permeate using 10 kDa membranes (both PES and RC) eliminated the use of these membranes. A design space of 4 – 8 L/m^2/min cross flow and 0 – 2.5 bar TMP (transmembrane pressure) was developed for formulation of filgrastim from 3.6 g/L to 10 g/L using 5kDa membranes of RC and PES at a filter loading of 50 g/m^2. Response curves for the relationship between flux and TMP were produced within this design space to provide information about processing times and yields that could be expected at

manufacturing scale. For PES membranes it was found necessary to use a cleaning solution comprising 0.5 M NaOH and 600 ppm NaOCl in order to adequately recover NWP (an indicator of effective cleaning) after use with the filgrastim solution. In contrast, use of 0.5 M NaOH alone provided effective cleaning for the RC membranes.

Application of all the standard principles for TFF unit operation such as gel layer concentration (C_{gel}) determination and determination of optimal diafiltration concentration (C_D) were found to be inappropriate for this Protein as aggregation of the filgrastim protein occurred below the optimal C_D determined under the conditions. Furthermore, an unusual breakthrough phenomenon was encountered with the regenerated cellulose membrane when the optimised conditions were implemented in trial production runs. This breakthrough coincided with the change of buffer condition during diafiltration, indicating a change to the protein conformation, allowing additional passage of the filgrastim protein. Different charge effects between the two membranes meant that the Donnan effect did not alter the final pH of the formulated protein when it was prepared using a regenerated cellulose membrane, but a significant change in the pH occurred with the use of PES membranes.

A 5 kDa PES membrane was selected for further use and scale-up due to its higher yield (i.e. > 95%), lower processing times and ability to be re-used up to at least 20 times. This work illustrates that while the principles for robust development of a TFF unit operation have been identified for industrial proteins and other products, these principles and experimental approaches need to be modified to account for specific attributes of particular biopharmaceutical proteins and formulations. This is certainly the case where the product is particularly susceptible to aggregation and/or multimer formation and when a specific final pH is required in the product to ensure stability.

After development of the design space at R&D scale the unit operation was scaled-up to commercial manufacturing scale, a scale factor of greater than 30-fold. The extensive knowledge gained during design space development allowed for relatively trouble free scale-

up and the cause of two scale-related changes was quickly identified because of the knowledge accumulated during the development process.

6.2 Introduction

Filgrastim is a recombinant version of human G-CSF which is a non-glycosylated 18.8 kDa protein of 175 amino acid residues in length produced in *Escherichia coli*. This molecule, as a cytokine, belongs to a distinct structural class of growth factors and folds into a four-helical bundle with a left handed twist with overall dimensions of 45Å×26Å×26Å [117]. The structure of filgrastim is stabilized by two disulfide bonds, both of which are required for activity. The molecule also contains one free cysteine residue [118, 119]. Filgrastim is particularly sensitive to aggregation and reversible self-association, so much so that it has been used as an experimental model protein in many aggregation studies. When dissolved only in water, recombinant human G-CSF produced both in mammalian cells and *E. Coli* is highly unstable [120], with the molecule being extremely sensitive to agitation, pH, ionic strength, temperature, light and freezing [121]. Due to these inherent features of the molecule careful development is required when formulating the protein to prevent aggregation.

Tangential flow filtration (TFF) is a unit operation used in the production of many biopharmaceutical products to both concentrate the product and change the solution conditions in a process similar to dialysis. The aim of this work was to develop an industrial-scale tangential flow filtration unit operation to transfer filgrastim to a stable formulation that could be frozen and transported without damage to the protein. Agitation, pH and ionic strength changes are inherent features of tangential flow filtration operations, therefore the process needed to be well designed to ensure successful and stable operation while maintaining the integrity of the protein. The protein feedstock intended for this TFF operation was in a buffer containing approximately 150 mM NaCl at pH 5.4. Tangential flow filtration under these conditions presents a number of risks to the protein. The first risk is that the pH of the protein solution is very close to the isoelectric point (pI) of filgrastim at 5.6, so the protein

is at high risk of aggregation and subsequent precipitation. Another risk is associated with the presence of a significant salt concentration. Prior literature on filgrastim indicates that increasing the ionic strength by the addition of 150mM NaCl induces aggregation even at pH 3.5 [122, 123], a notably stable pH for this protein. Proper design of the TFF unit operation is essential to reduce the risks to the Protein And to develop an efficient operation amenable to large-scale production.

An improperly designed and scaled TFF operation will be plagued with membrane fouling, low product recovery, long processing times, excessive buffer usage, frequent membrane replacement and inconsistent operation. Such problems can be avoided by applying Quality by Design (QbD) principles to understand and specify the TFF unit operation[25]. Quality by Design is a modern approach to the development of pharmaceutical products and their subsequent manufacture which has been advocated by the US FDA and the International Conference on Harmonization (ICH). This approach is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [103]. The intent of QbD is to encourage pharmaceutical companies to develop sufficient understanding of their products and manufacturing processes to ensure that their processes are robust and to demonstrate this enhanced understanding to the pharmaceutical regulatory agencies.

The application of QbD principles to TFF unit operations has been previously described in general terms [25] and this work sought to apply these principles to the development of a manufacturing process for filgrastim. Tangential flow filtration is a highly dynamic process which never operates in a steady state, and as a result a precise understanding of the operating parameters is critical to robust and consistent operation.

Major design objectives for this particular application were to obtain high product recovery, identify conditions that would allow the TFF membranes to be re-used, optimise the flux rate

in order to minimise overall process time, obtain high product purity and produce a formulation of the desired concentration and pH at the end of the unit operation. The intent was to develop a stable design space for the unit operation by determining the optimum membrane MWCO and membrane type, obtaining the relevant mechanistic parameters with the "best" membrane identified and to examine the re-use capabilities of the chosen membrane to establish cleaning conditions compatible with a biopharmaceutical manufacturing environment.

While the principles of QbD have been used for the production of Drug Product (the final administered form of a pharmaceutical compound) there is a lack of successful case studies in the literature on applying these principles to the manufacturing process of biopharmaceutical proteins. This work applied the principles of QbD when developing a manufacturing process for a biopharmaceutical product in order to help fill this gap and hopefully provide a useful resource in implementing the QbD concept with biopharmaceuticals.

6.3 Materials and Methods

6.3.1 Overall experimental approach

All four membranes were compared in their performance with a G-CSF solution initially using nominal conditions that had previously been identified. After this initial screening, the membranes which generated the highest yield and purity were employed to develop a design space of membrane load, cross-flow and TMP. Once this design space was developed, the optimal conditions were used to perform TFF formulation under expected manufacturing load rates (g/m^2) with the membranes selected to evaluate the membrane performance for the protein formulation. The best membrane and operational conditions were then used for further manufacturing development. A schematic of the overall approach to the TFF development is shown in Figure 36.

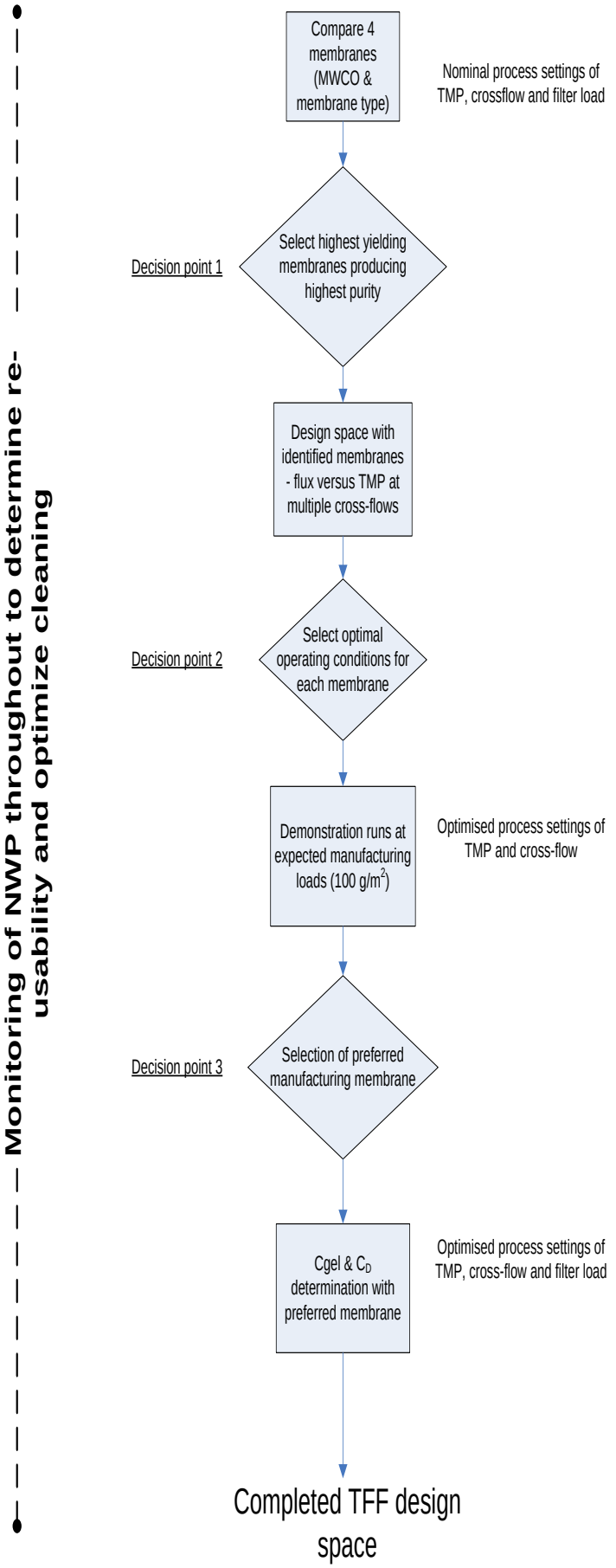


Figure 36: Schematic of the QbD approach to TFF development

6.3.2 Samples and Chemicals

All TFF experiments were performed on an AKTA™ cross-flow system (GE Healthcare, Sweden). Sodium acetate, sodium chloride and Sorbitol chemicals and Polysorbate 80 were from Merck (Australia). Polyethylene terephthalate glycol (PETG) containers were from Thermo Scientific (USA).

Recombinant human met-G-CCSF was produced in *E.coli* and purified by Hospira Adelaide. The protein preparation used as the feedstock for the TFF unit operation contained the Protein At a concentration of approximately 3.6 mg/ml in a solution of 25 mM sodium acetate, 150 mM sodium chloride at pH 5.4, which was the eluate from the previous chromatography purification step. Neupogen®, a recombinant version of filgrastim expressed in *E.coli* was purchased from Amgen Incorporated to act as a reference standard in some of the analytical assays.

Four different TFF membranes were evaluated all supplied from Merck-Millipore. TFF filters of 5 and 10 kDa MWCO composed of polyethersulfone and regenerated cellulose were used, with all filters having a total membrane area of 0.1 m^2. Manufacturer catalogue numbers were P2B010V01 (10 kDa, PES); P2C010V01 (10 kDa, regenerated cellulose); P2B005V01 (5 kDa, PES) and P2C005V01 (5 kDa, regenerated cellulose).

6.3.3 Initial Screening

Initial screening conditions for the TFF concentration phase used a target final protein concentration of 6.5 mg/ml and a filter load of 50 g/m^2. The protein concentration of the load solution before diafiltration was 3.6 mg/ml in a solution of 25 mM sodium acetate, 150 mM NaCl pH 5.4. Triplicate TFF runs using various membranes (PES and RC) of different MWCO (5 kDa and 10 kDa) were performed at a crossflow of 480 $l/m^2/h$ and a TMP of 120 kPa, with a starting protein solution volume of 1400 ml. After concentrating the protein

solution to 6.5 mg/ml the retentate was diafiltered against 10 diavolumes of 25 mM sodium acetate, 5 % sorbitol at pH 3.7. At the end of the diafiltration the solution was recovered from the retentate vessel. A flush solution (100 ml) of 10 mM sodium acetate, 5 % sorbitol at pH 4.0 was recirculated past the membrane for 5 minutes at a crossflow of 60 l/m^2/h with no applied backpressure to remove all the protein from the membrane. This flush solution was then also recovered from the retentate vessel and pooled with the recovered retentate.

Placebo/buffer runs were performed with each membrane to identify the effect of the protein presence on the performance of the system and pH and conductivity profiles produced during operation. The pH and conductivity of the permeate solution produced by each TFF run was recorded in real-time by the AKTA™ Crossflow instrument.

6.3.4 Design space for TFF operations

Once the two best performing membranes were identified these membranes were used to determine the response curve for flux and TMP at both initial (3.6 mg/ml) and final (10 mg/ml) protein concentrations. Solution conditions of 25 mM sodium acetate, 150 mM NaCl at pH 5.4 were used for all experiments as they were considered to be the worst case for protein stability. Three different cross-flow rates (4, 6 and 8 L/m^2/min or LMM) and TMP (0.3 – 2.5 bar) were used at each protein concentration to fully establish the design space.

The performance of both 5 kDa RC and PES membranes was then determined using a TMP of 2 bar, a crossflow of 480 L/m2/h (LMH) and a filter load of 100 g/m2 to simulate probable manufacturing conditions. The yield and purity of the protein produced and the conductivity and pH profiles of the individual runs were then examined and compared, along with the pH of the final retentate produced. In this set of experiments, 7 diavolumes of the diafiltration buffer (25 mM sodium acetate, 5 % sorbitol pH 3.6) were used and the protein was taken to a final target concentration of 10 mg/ml.

6.3.5 Product Recovery

After the protein was concentrated and diafiltered, the protein was recovered from the reservoir. The filter was then flushed with a set volume (50 mL) of buffer, which was recirculated for five minutes to ensure any protein remaining in the system hold-up volume or adhered to the membrane was recovered. The membranes were flushed with 100 ml solution of a 10 mM sodium acetate and 5 % sorbitol at pH 4.0 recirculated at 60 $l/m^2/h$ for 5 minutes.

6.3.6 UV spectrophotometry

UV spectrophotometry was performed using a Cary 50 UV spectrophotometer (Varian Instruments) to determine the concentration of the purified protein. An extinction co-efficient $\varepsilon^{0.1\%}$ at 280 nm of 0.862 $ml.mg^{-1}.cm^{-1}$ was used.

6.3.7 RP-HPLC

RP-HPLC impurities were estimated using the RP-HPLC assay described in [110]. Briefly, a Jupiter C_4 5µm 300Å, 4.60 x 250 mm, HPLC Column (Phenomenex) was run at 0.6 ml/min and 60 °C using running buffers of 0.1% TFA/10 % acetonitrile and 0.1 % TFA/80 % acetonitrile. Separation was performed using a gradient of 0.17 % acetonitrile per minute over 35 minutes using a target sample application of 10 µg for all runs. The column effluent was monitored at 215 nm. Microsoft excel was used to perform t-test and identify any significant differences in the amounts of impurities produced by different membranes with different MWCO values.

6.3.8 Size-exclusion chromatography (SEC)

Size-related impurities of the product were estimated using a Superose 12 10/300 L column (GE Healthcare) run at 0.5 ml/min in a buffer of 50 mM borate, 100 mM NaCl pH 9.1 for 50 minutes. All analyses were performed at ambient temperature (22 °C) and a sample application of 100 µg was applied to each separation run. Samples were prepared by

centrifugation at 10 000 g for 5 minutes before application to the column and the column effluent was monitored at 280 nm.

6.3.9 Turbidity

Measurements were performed using 2100Q Portable turbidimeter from HACH (Australia). G-CSF is noted for its ability to self-associate and aggregate under the conditions used in this study [119, 124]. Turbidity of the retentate solution was measured to provide a measure of large aggregates of the protein which had become too large to be measured by the SEC technique employed as they would be removed by the sample preparation techniques used i.e. centrifugation.

6.3.10 Aggregation point

Aggregation point measurements were performed using Zetasizer Nano ZS (Malvern Instruments, United Kingdom). Individual standard operating procedures (SOPs) for formulated filgrastim, unformulated filgrastim and Neupogen® were created. Measurements were performed by aliquoting 150 µL of solution into disposable low volume glass cuvettes. A 3 ^{o}C temperature ramp and a 2 minute equilibration time was used for each measurement. Dispersant parameters used for the aggregation point measurement are detailed in Table 18.

Table 18: Dispersant parameters for aggregation point measurement.

Name of dispersant	Refractive Index (RI)	Viscosity (cP)
Water	1.333	0.887
Sorbitol	1.337	1.230

6.3.11 Zeta Potential

Zeta Potential was determined using a Malvern Zetasizer Nano ZS (Malvern CO. UK). Approximately 800 µL of solution was placed in the clear disposable zeta cell. Electrophoretic mobility of the particles in the feedstock solution (25 mM sodium acetate, 150

mM NaCl pH 5.4), after formulation and in the Neupogen® reference solution was determined by using the M3-PALS technique which is a combination of Laser Doppler Velocimetry and Phase Analysis Light Scattering (PALS).

The background solution (dispersant) was considered to be Milli-Q water for the protein standard and unformulated samples. Sorbitol buffer (5 %) was considered as the background solution for the formulated samples. The measurement parameters were entered are detailed in Table 19.

Table 19: Dispersant parameters for zeta potential measurement.

Name of dispersant	Refractive Index (RI)	Viscosity (cP)	Dielectric constant
Sorbitol	1.333	1.230	33.5

6.3.12 pH measurement

The pH of the protein solution after TFF formulation was measured using a Mettler-Toledo SevenEasy pH meter at a sample temperature of 20 °C. During the diafiltration operation the pH of the permeate solution was measured using the in-line probes that are part of the ÅKTACrossflow instrument.

6.3.13 Normalised water permeability (NWP)

After sanitization, the cleanliness of the membrane was assessed using normalized water permeability tests. This was performed using a pre-programmed method on the ATKA Crossflow which controlled the cross flow to produce a stable TMP of 1 bar using a solution of purified water as sample. Once a stable TMP was achieved the flux through the membrane was measured to determine the normalised water permeability, which was adjusted for the temperature-induced changes in the density of water to normalise the flux to that expected with water at 25 °C. NWP is expressed in units of $Lm^{-2}h^{-1}bar^{-1}$.

6.3.14 Gel Concentration Determination

A C_G (C_{gel}) determination experiment was performed by measuring flux rates at increasing protein concentrations from 10 to 50 mg/mL using the 5 kDa PES membrane. The protein feedstock solution was present in 25 mM sodium acetate, 150 mM NaCl pH 5.4 at an initial protein concentration of 3.6 mg/ml. The TFF system was operated at 2 bar TMP using a cross-flow of 8 L/m^2/min and a filter load of 100 g/m^2. During the experiment flux through the membrane was measured and plotted against the calculated protein concentration remaining in the system.

6.3.15 Scale-up to manufacturing scale

The TFF operation was scaled up to full manufacturing scale using a AKTA Uniflux system (GE Healthcare) and a total of 2.5 m^2 of 5 kDa MWCO PES membrane (Millipore). During the course of technology transfer and process development seven TFF runs were performed at manufacturing scale, with total filter loads of between 94 - 137 g/m^2. The upper end of this filter load range was greater than highest load tested at small scale (100 g/m^2) but this was not expected to present any difficulties. Cross-flow and TMP were carried out at the same scale-independent values determined to be optimum during design space development - 8 LMM crossflow and 2 bar TMP.

6.4 Results

6.4.1 Initial Screening of Membranes

Selection of the appropriate membrane in a TFF unit operation is important to ensure a robust and successful TFF process [25]. In the current study, two different membrane types, PES and RC, were tested with the Filgrastim solution. Each of these membrane types were tested at two MWCO values (5 kDa and 10 kDa) to determine the best combination of membrane type and MWCO. Formulation with these membranes was performed and after the formulation process product yield and quality attributes were assessed. In addition, to ensure an economically viable unit operation, flux with the different membranes was determined along with the ability of the membranes to recover NWP after cleaning and be re-used multiple times.

6.4.2 Online monitoring profiles

The conductivity, pH and UV absorbance of filter permeate was measured during each run. Examples of the conductivity and UV profiles produced by 5 kDa and 10 kDa PES membranes are presented in Figure 37 and Figure 38, respectively. Similar profiles were observed for runs using 5 kDa and 10 kDa RC membranes. A constant UV absorbance level on the permeate line during the initial and final stages of diafiltration for the 5kDa PES membrane can be seen in Figure 37, which indicates no detectable protein breakthrough. However, a different UV profile was observed for the 10 kDa membrane runs as exemplified in Figure 38, with a significant increase in the UV absorbance of the permeate during the initial concentration phase and then again during the diafiltration phase indicating loss of the target protein to the permeate during the run. In both profiles the conductivity and pH profiles can be seen to fall in a smooth curve before reaching an equilibrium value as the diafiltration factor increases, indicating successful buffer exchange and formulation of the protein.

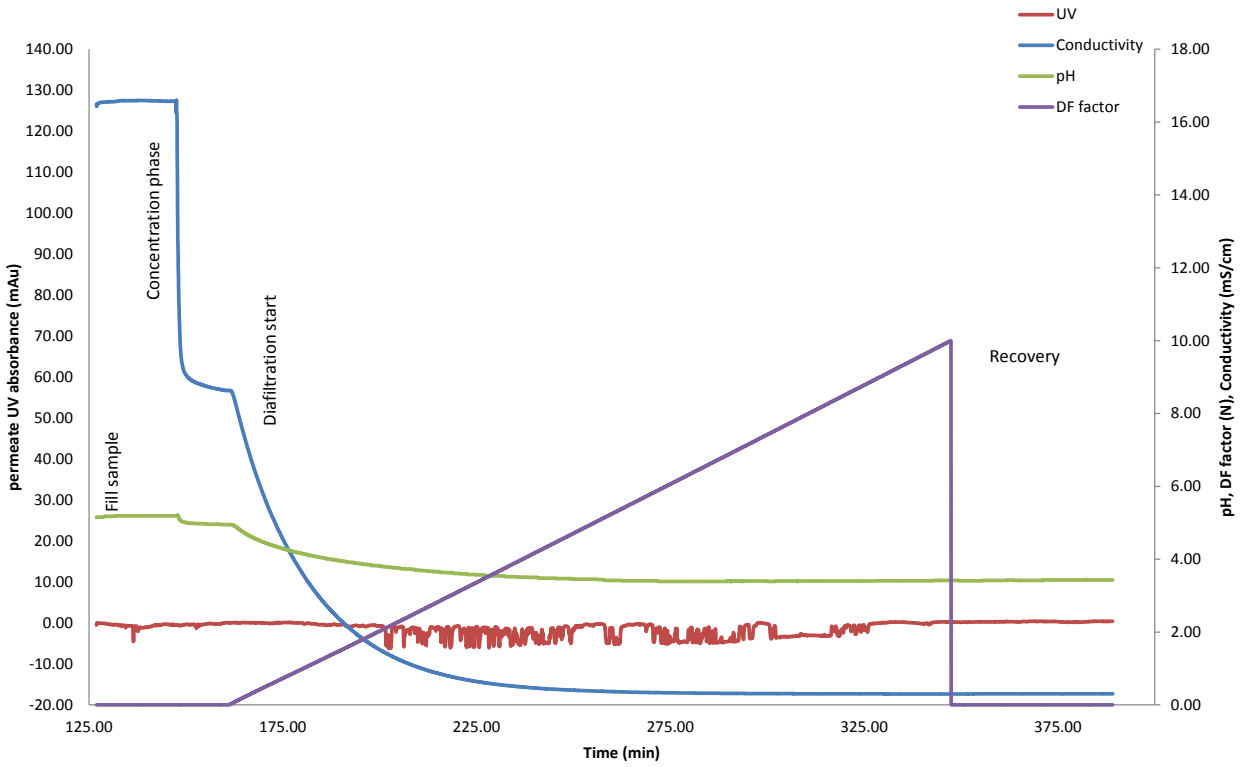


Figure 37: TFF profile of initial development run using a 5 kDa PES membrane. Starting conditions were filgrastim at 3.6 mg/ml in 25 mM sodium acetate, 150 mM NaCl pH 5.4 and TFF conditions were a crossflow of 480 L/m^2/h and TMP of 120 kPa to concentrate the protein to a final concentration of 6.5 mg/ml before diafiltration against 10 diavolumes of 25 mM sodium acetate, 5 % sorbitol at pH 3.7.

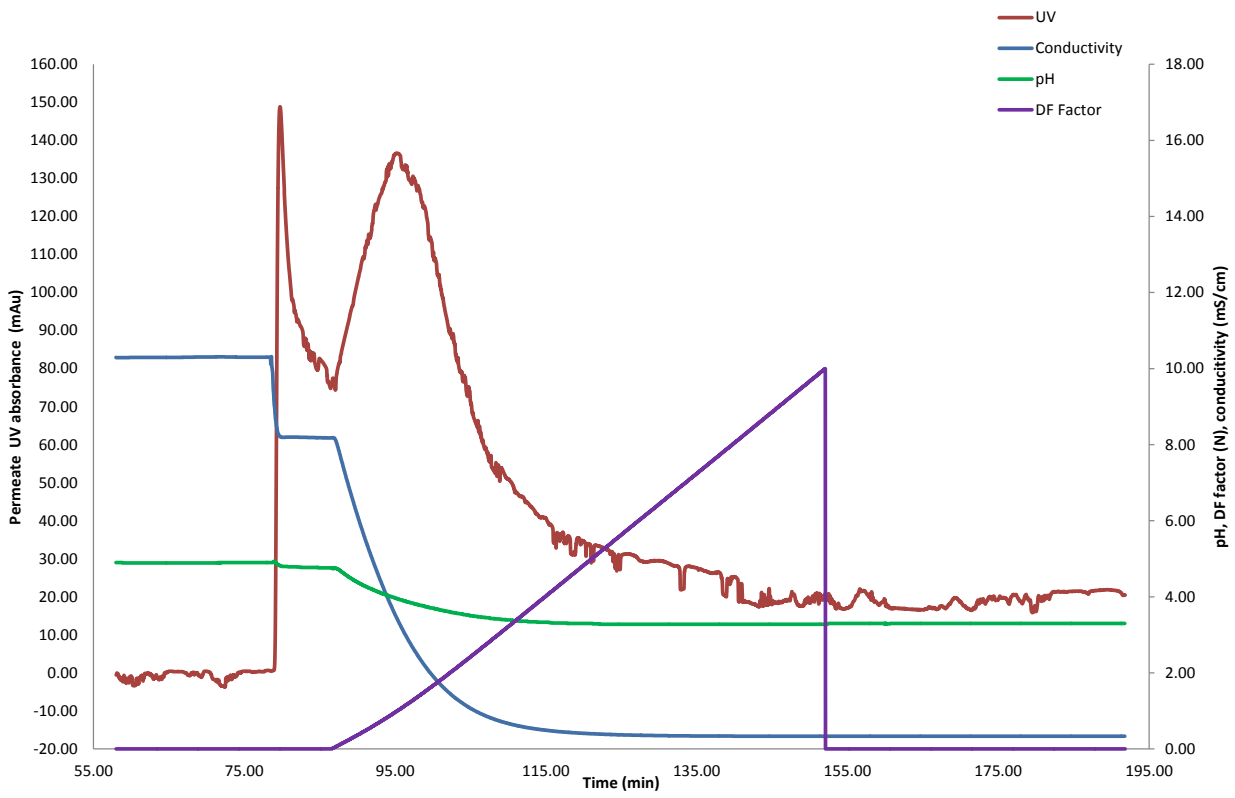


Figure 38: TFF profile of initial development run using a 10 kDa PES membrane.

Starting conditions were filgrastim at 3.6 mg/ml in 25 mM sodium acetate, 150 mM NaCl pH 5.4 and TFF conditions were a crossflow of 480 L/m^2/h and TMP of 120 kPa to concentrate the protein to a final concentration of 6.5 mg/ml before diafiltration against 10 diavolumes of 25 mM sodium acetate, 5 % sorbitol at pH 3.7.

6.4.3 TFF operational evaluation

An overview of the performance of the different membranes is shown in Table 20, all results are presented as averages of three replicates runs ± their standard deviation. Initial experiments were performed on both membrane types at a filter loading of 50 g/m^2 to a target final concentration of 6.5 mg/mL.

The 5 kDa membranes of both PES and RC produced much lower flux values than the 10 kDa membranes resulting in longer processing times. This effect was expected, as lower MWCO membranes provide more resistance to the flow of solution through the membrane. Of the 5 kDa membranes, the flux with the PES membrane was consistently higher than the RC membrane when run at the same cross-flow and TMP.

Both membrane types (PES and RC) with 5 kDa MWCO resulted in product yields ≥ 95%. The yields of slightly greater than 100 % seen with the RC membranes in Table 20 are most likely due to slight errors in the volume estimations of the starting and ending material. This high yield from 5 kDa membranes is in agreement with the UV absorbance profile in Figure 37 in comparison with 10 kDa membranes in Figure 38. Due to the significantly lower yield of the two 10 kDa membranes, further development with the 10 kDa membranes was discontinued.

Turbidity measurements were performed to check for grossly precipitated protein in the retentate which would not be expected to be measured by the size-exclusion chromatography technique. The turbidity of the unformulated filgrastim protein before TFF formulation was 11.7 NTU. The final retentate produced by the 5 kDa RC membrane had a turbidity of 12 ± 5 NTU compared with 7 ± 2 NTU produced by the corresponding PES membrane. The lower turbidity after formulation with the PES membrane is likely a consequence of the fact that the protein has been transferred to a pH 4.0 solution, which is known to be the optimally stable

pH for filgrastim [118]. The higher turbidity produced by the 5 kDa RC membrane suggests that some additional aggregation and precipitation was occurring to the protein in the presence of this membrane. This was most likely due to the formation of large aggregates of filgrastim which is known to have a strong propensity for formation of large, multimeric aggregates [119, 124]. This result suggests that RC membrane under these conditions caused a small amount of denaturation and precipitation of the filgrastim molecules. The aggregation could be produced by a different surface interaction between the filgrastim and regenerated cellulose material as compared to the PES membrane material.

Table 20: Performance parameters of various membrane types and MWCO values

Membrane type	**Nominal MWCO** (kDa)	**Average** Flux ($L/M^2/H$)	**Run time** (min)	**Yield** (%)	**Turbidity of final retentate** (NTU)
PES	5	20 ± 4	246 ± 41	96 ± 2	7 ± 2
RC	5	13 ± 0	395 ± 3	103 ± 5	12 ± 5
PES	10	63 ± 5	80 ± 6	58 ± 7	5 ± 1
RC	10	61 ± 3	81 ± 3	15 ± 1	6 ± 2

6.4.4 Product Evaluation

Purity of the formulated filgrastim protein was analysed by RP-HPLC chromatography and compared with the unformulated filgrastim and Neupogen® samples. The percentage of related proteins (impurities) in the formulated filgrastim, unformulated filgrastim and Neupogen® are presented in Table 21. There was no significant difference between the RP-HPLC impurities after formulation using either 5 kDa (PES or RC) and 10 kDa (PES or RC) membranes ($p>0.05$ for all membranes).

Size exclusion analysis of the formulated filgrastim protein was carried out to determine the amount of higher molecular weight impurities present. The percentage of higher molecular weight impurities in the formulated filgrastim intermediate is presented in Table 21. Analysis

of the samples from various membrane runs showed the amounts of high molecular weight (HMW) impurities are lower than the limit of quantitation (LOQ). No detectable aggregate or multimers were found in the product after formulation with any of the TFF membranes. This validates the decision to use turbidity as an alternative method for monitoring aggregate formation as some of the aggregates formed were too large to enter the SEC column and were therefore not detected by the assay, being either removed by the sample preparation procedures before analysis (i.e. centrifugation and filtration) or adhering to the inlet of the analytical column. This result also indicates that the TFF unit operation was compatible with the molecule as, other than the slightly increased turbidity of the retentate produced by the 5 kDa RC membrane, the molecule remained monomeric and intact.

Measurement of aggregation point (indicative of thermal stability) is important in the total characterization of the target protein, particularly if the protein is destined for integration into a pharmaceutical product or formulation. The aggregation point of filgrastim solution which had been obtained from the different TFF membranes was measured in order to determine whether any structural damage or rearrangement had occurred in the protein that may make it more susceptible to future aggregation or degradation upon storage. The average aggregation point values of the formulated filgrastim from triplicate runs are presented in Table 21. These values are compared with the value of 55 °C obtained with formulated Neupogen® and with the aggregation point value obtained for unformulated filgrastim protein. The values for filgrastim formulated using different membranes were not significantly different to the value obtained with formulated Neupogen®. This indicates that the formulation procedure had not damaged or altered the protein structure in any way to make it more susceptible to aggregation or heat-induced denaturation.

However, the aggregation point for filgrastim in the feed solution prior to formulation (25 mM acetate, 150 mM sodium chloride, pH 5.4) was significantly lower than for Neupogen® or formulated filgrastim. This is mainly attributed to difference in pH (pH 5.4 compared to

4.0). At pH 5.4 the protein is close to its isoelectric point (pI) of 5.6. As a result, there is minimal repulsive forces between the protein molecules due to charge and hydrophobic interactions between the molecules can dominate, favouring aggregation of the filgrastim molecules. In contrast, filgrastim formulated at pH 4.0 is strongly positively charged, resulting in significant repulsion between the protein molecules sharing the same charge and therefore disfavouring aggregation due to hydrophobic interactions. This is illustrated by the direct measurement of zeta potential in the various formulations.

Table 21: Analytical data for each membrane type at different MWCO values.

Note: < 0.2 is below the limit of quantitation (LOQ)

Membrane type	**MWCO** (kDa)	**Final Protein Concentration** (mg/mL)	**HPLC impurities** (% related proteins)	**SEC impurities** (%)	**Aggregation Point (°C)**	**Zeta Potential (mV)**	**Final pH**
Feedstock	-	3.60	3.4 ± 0.40	<0.1	46	0.7	5.4
PES	5	9.46	3.67 ± 0.35	<0.1	55 ± 3	54.6 ± 9.4	4.1
RC	5	6.81	3.70 ± 0.36	<0.1	58 ± 3	64.1 ± 11.7	4.0
PES	10	3.64	3.53 ± 0.51	<0.1	58 ± 3	57.1 ± 16.6	4.0
RC	10	0.96	3.47 ± 0.15	<0.1	55 ± 3	61.0 ± 10.7	4.0
Neupogen®	-	-	4.3 ± 0.45	<0.1	55 ± 3	27.7 ± 5.0	4.0

Zeta Potential is a measure of the magnitude of the electrostatic charge on a particle and is one of the fundamentals parameters known to affect stability of formulated protein solutions. Zeta potential measurements are shown in Table 21. The zeta (ζ) potential of formulated Neupogen® was measured at approximately 28 mV. This is significantly higher than the unformulated filgrastim intermediate, which was virtually uncharged in the pH 5.4 formulation containing salt. As identified above, this is most likely due to the protein being very close to its pI (5.6) at the pH of 5.4. Formulated filgrastim produced by Hospira had a high zeta potential in all samples tested (50 – 60 mV). The reason for the difference with

Neupogen® is unclear, but may be related to the fact that the Hospira filgrastim had been produced more recently than the Neupogen®, which had been stored for several years and passed its expiry date and the absence of polysorbate 80 in the Hospira produced filgrastim solution.

After formulation, the pH of filgrastim protein solution was measured and the values are presented in Table 21 Following extended diafiltration and formulation, the pH increased to a final value of 4.0 with all four membranes assessed, except PES 5 kDa in which a slightly higher final pH of 4.1 was obtained.

6.4.5 Membrane Evaluation

NWP experiments were used to determine the flux of clean water through the processed membrane under standard pressure and temperature conditions. The NWP should return to greater than 70% of the initial NWP value for the membrane to be considered sufficiently clean for reuse. This ensures that the cleaning process is effective and also helps to determine the lifetime of the filter. Investigations were carried out on both the PES and RC membranes to assess the NWP recovery after sanitization and the values are presented in Table 22. During the screening experiments (i.e. from runs 4-6), the NWP of the PES membrane declined with use as shown in Figure 39. This suggested that the cleaning solution in use (0.5 M NaOH) was insufficient to clean the membrane. Upon realisation that the 0.5 M NaOH was not sufficiently cleaning the PES membrane, NaOCl (600 ppm) was added to the cleaning solution (runs 7-12) for this membrane. Using this new sanitization procedure, the NWP flux values returned to greater than 90% suggesting restoration of membrane to its original condition. This membrane continued to return acceptable NWP values when used for up to 20 runs Figure 39. The NWP of the RC membrane was stable Figure 40 with repeated use for almost 15 runs, indicating the use of 0.5 M NaOH alone in the cleaning solution provided adequate cleaning with this membrane.

Table 22: Normalized water permeability after sanitization for different membrane types

Membrane type	**Nominal MWCO** (kDa)	**Average flux** ($L/M^2/H$)	**Post NWP** (%)
PES	5	20 ± 4	50 ± 10
RC	5	13 ± 0	100 ± 0
PES	10	63 ± 5	75 ± 5
RC	10	61 ± 3	100 ± 0

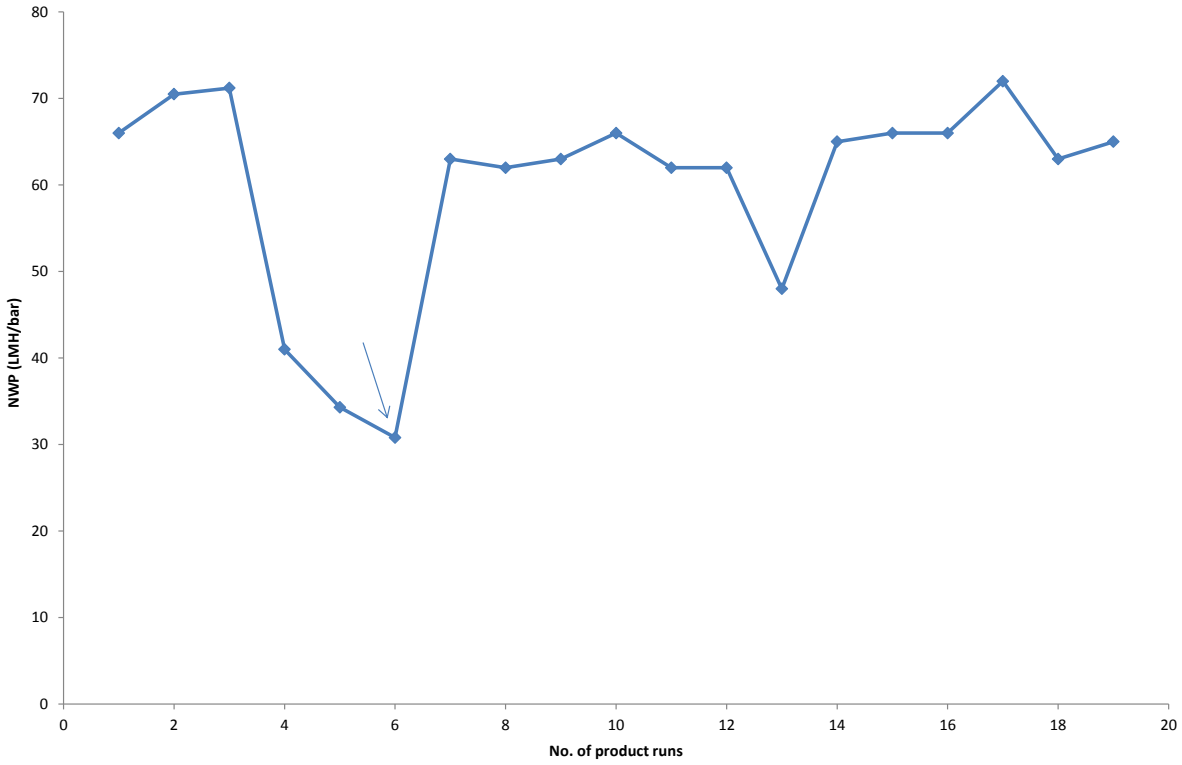


Figure 39: NWP recovery over 20 cycles of use and cleaning with a 5 kDa PES membrane.

A solution of 0.5 M NaOH (cycles 1 - 6) or a solution containing 0.5 M NaOH and 600 ppm NaOCl was used for cleaning of the membrane. The addition of NaOCl to the cleaning solution occurred after the 6^{th} product run (indicated by the arrow). The poor recovery seen in cycle 13 was attributed to an incorrectly executed cleaning procedure after investigation.

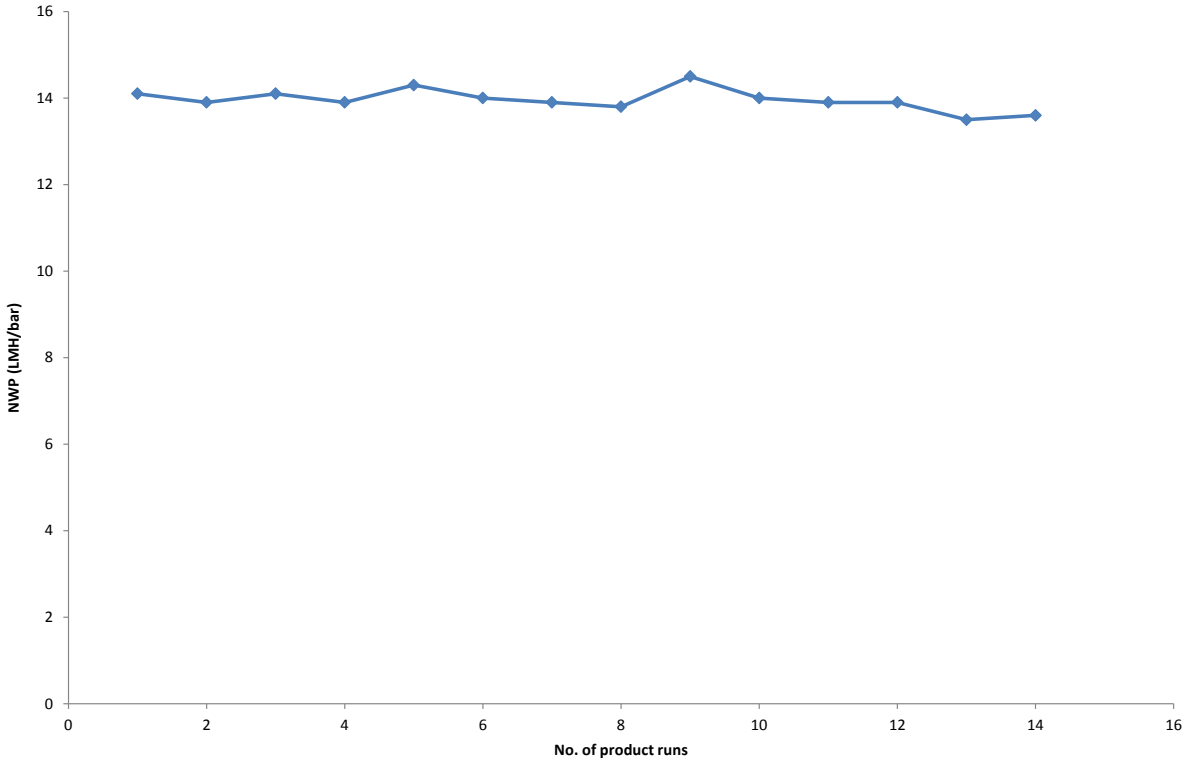


Figure 40: NWP recovery over 14 cycles of use and cleaning using a 5 kDa RC membrane and a cleaning solution of 0.5 M NaOH for all cycles.

6.4.6 Development of operating design space

The design space of any unit operation is that combination of operating parameters which affect the outcome of the given operation [100]. For a TFF unit operation this is the combination of membrane type, cross-flow rate, product concentration and TMP used during the operation. With this in mind, the two best performing membranes in the initial screening experiments (the 5 kDa PES and RC membranes) were further examined to determine the optimum TMP values at various cross-flow rates. The flux obtained through the membrane was measured at each combination of TMP and cross-flow to identify the optimal setting of TMP to use to minimise cycle time and the membrane area required for large-scale operation. These experiments are generally referred to as flux versus TMP profiles and are used to predict process cycle times. The experiments were carried out at both the initial and target final protein concentrations (3.6 and 10.0 mg/mL) using cross flows of 4, 6 and 8 L/m^2/min

and TMPs of 0 – 2.5 bar. The flux versus TMP profiles obtained for 5kDa PES and RC membrane are shown in Figure 41 and Figure 42, respectively. From both graphs, three distinct regions exist for both membranes, where the TMP and cross-flow rates influence permeate flux to varying degrees. At lower TMP, flux is influenced both by membrane and gel layer (product) resistance and has a linear relationship to TMP. In this region, flux is membrane controlled and pressure dependent. At higher TMP the development of a significant gel layer occurs. Flux through this layer becomes the limiting factor in overall flux through the system and the flux is not increased by further increases in TMP. This region is referred to as the gel-layer controlled region or pressure-independent region. The point between the pressure dependent and pressure-independent regions is referred to as the transition region and is generally considered to be the optimal operating condition as it optimizes flux while minimising TMP [25].

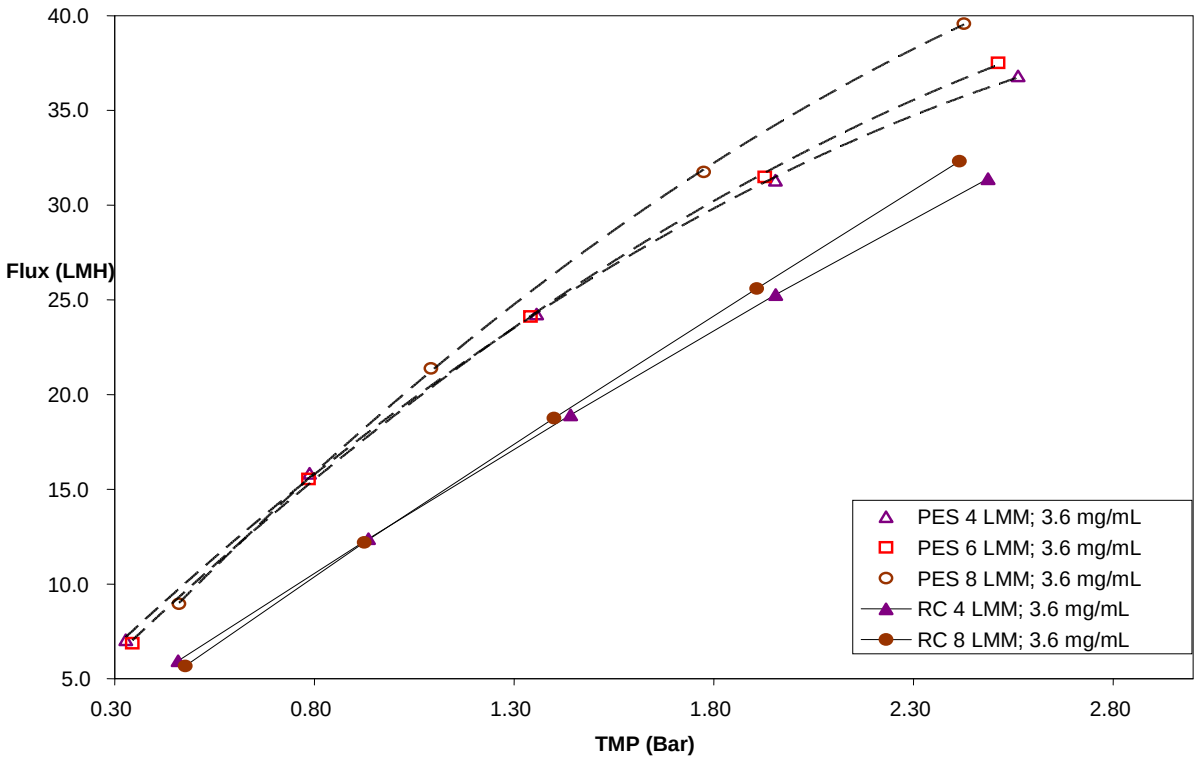


Figure 41: Flux and TMP relationship for the 5 kDa PES and RC membranes at the initial protein concentration of 3.6 mg/m in 25 mM sodium acetate pH 5.4.

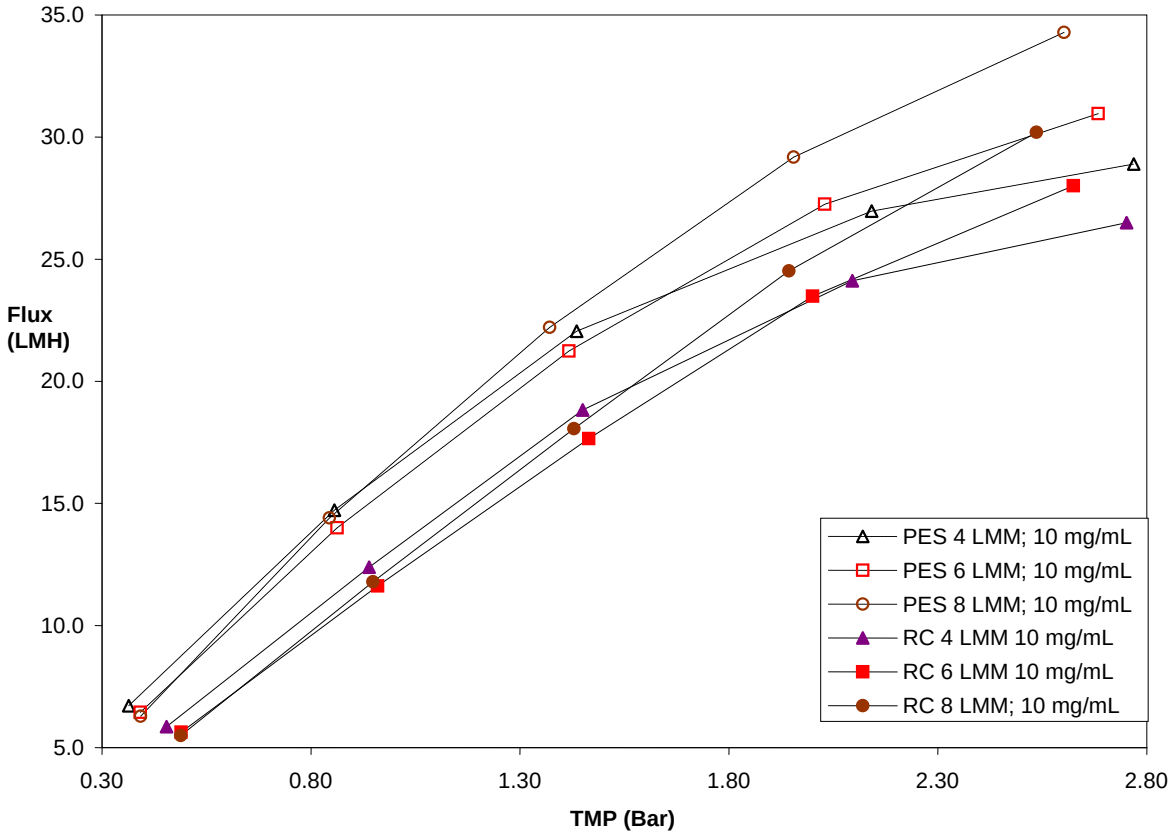


Figure 42: Flux and TMP relationship for the 5 kDa PES and RC membranes at the final protein concentration of 10.0 mg/mL in 25 mM sodium acetate pH 5.4.

The RC membranes produced lower flux than the PES membrane at equivalent TMP, crossflow and protein concentrations. Transition to the pressure-independent region occurred at lower TMP values with the 10 mg/ml final concentration than the initial concentration of 3.6 mg/ml. Within membrane types, there was relatively little difference between the flux values obtained at 4, 6 and 8 L/m2/min. The lowest TMP value for the transition region between pressure-dependent and independent operation occurred at 4 L/m2/min with the 10 mg/ml protein solution. From the results a TMP of 2.0 bar was identified as optimum for operation as this pressure either lay in the transition to pressure-independent operation or was just prior to the transition under all combinations of operating parameters examined. The flux profiles identified an operating design space of 4 – 8 L/m2/min cross-flow, protein concentration of 3.6 – 10 mg/ml and TMP of 0 – 2.0 bar TMP with TFF membranes composed of both PES and RC.

6.4.7 Demonstration Runs at 100 g/m^2 Filter load

After determining the operating design space for both filters, demonstration runs were carried out using a membrane load of 100 g/m^2 as this load would allow TFF operation of the process at a large scale without requiring large additional capital investment and would minimise the amount of membrane area required for the manufacturing scale operation. Both membranes were tested using a TMP of 2.0 bar, crossflow of 8 L/m^2/min to a target final concentration of 10 mg/mL. The profiles of permeate conductivity, pH and UV absorbance during the runs are shown in Figure 43 and Figure 44 for PES and RC membrane respectively. The operational results of each run are tabulated in Table 23.

Table 23: Results of example TFF runs carried out at 100 g/m^2 filter loading

Membrane	Retentate Final turbidity (NTU)	Final pH	Protein Yield (%)	Retentate final protein concentration (mg/mL)
PES	8	4.25	98.6	9.46
RC	7	4.01	68.1	6.81

The PES membrane produced a product yield of 98.6 % whereas the yield from the RC membrane proved to be only 68.1 %. Examination of the TFF record indicated significant protein breakthrough during the diafiltration stage using the RC membrane as shown in Figure 44 by a large increase in the UV absorbance of the permeate after diafiltration had commenced which continued throughout the diafiltration. The filter integrity was measured after use and the membrane was found to be integral so the protein loss was not due to a rupture of the membrane during use. The yield reduction indicates that the protein may interact with the membrane surface and undergo some reduction in hydrodynamic volume with the pH change in formulation from 5.4 to 4.0, allowing it to permeate the membrane. Based on these results the 5 kDa PES membrane was selected for future scale-up and use in manufacturing operations.

Of note also was the observation that the pH of the retentate had drifted significantly from the target value of 4.0 using the PES membrane to 4.25 but this drift did not occur with the RC membrane. It has previously been shown that in the highly purified conditions of drug product TFF operations, electrostatic interactions between the protein, the membrane and charged excipients can interact to significantly alter the final pH and excipient concentrations through the Donnan effect [19–22]. The Donnan effect results from the fact that during ultrafiltration, electroneutrality must be maintained on both sides of the membrane while at the same time maintaining identical chemical potentials for each species on the permeate and retentate side of the membrane. In order to simultaneously satisfy both these criteria, diffusible ions with

charge opposite that of the protein must be present in higher concentration on the retentate side of the membrane, while diffusible ions with charges similar to that of the protein's must be present in lower concentration on the retentate side of the membrane. This is known as the Donnan effect [29]. In this present application, acetate is the most important diffusible ion as it has both a charge opposite that of the Protein At the operating pH and intrinsic buffering capacity. In this application, the rise in acetate concentration that accompanied the rise protein concentration resulted in an increase in the retentate pH due to the pKa of acetate being 4.8. Use of the charge-state model to predict the pH of the final retentate predicts a difference of only 0.05 pH unit based on the differences in the protein concentration of the retentates produced with the two different membranes (RC and PES) [29]. Together with the observation that the PES membrane required use of NaOCl in addition to NaOH for adequate cleaning this reinforces the need to evaluate different material types and attributes during design space studies as subtle effects such as these can result in significantly different outputs for similar operations at large scale.

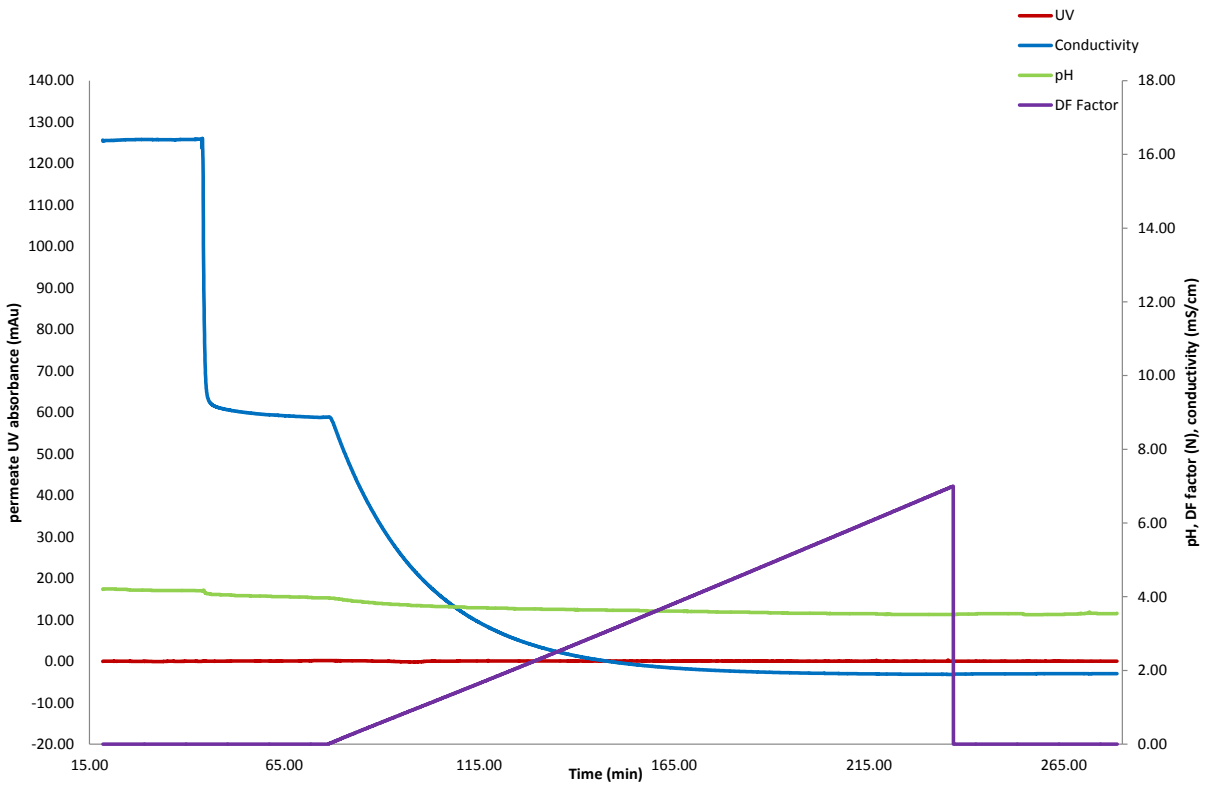


Figure 43: TFF profile produced during the demonstration run using a 5 kDa PES membrane at 100 g/m^2 loading.

Experimental TMP of 2.0 bar, crossflow of 8 L/m^2/h, initial protein concentration of 3.6 mg/ml in 25 mM sodium acetate pH 5.4 and final protein concentration of 10 mg/ml.

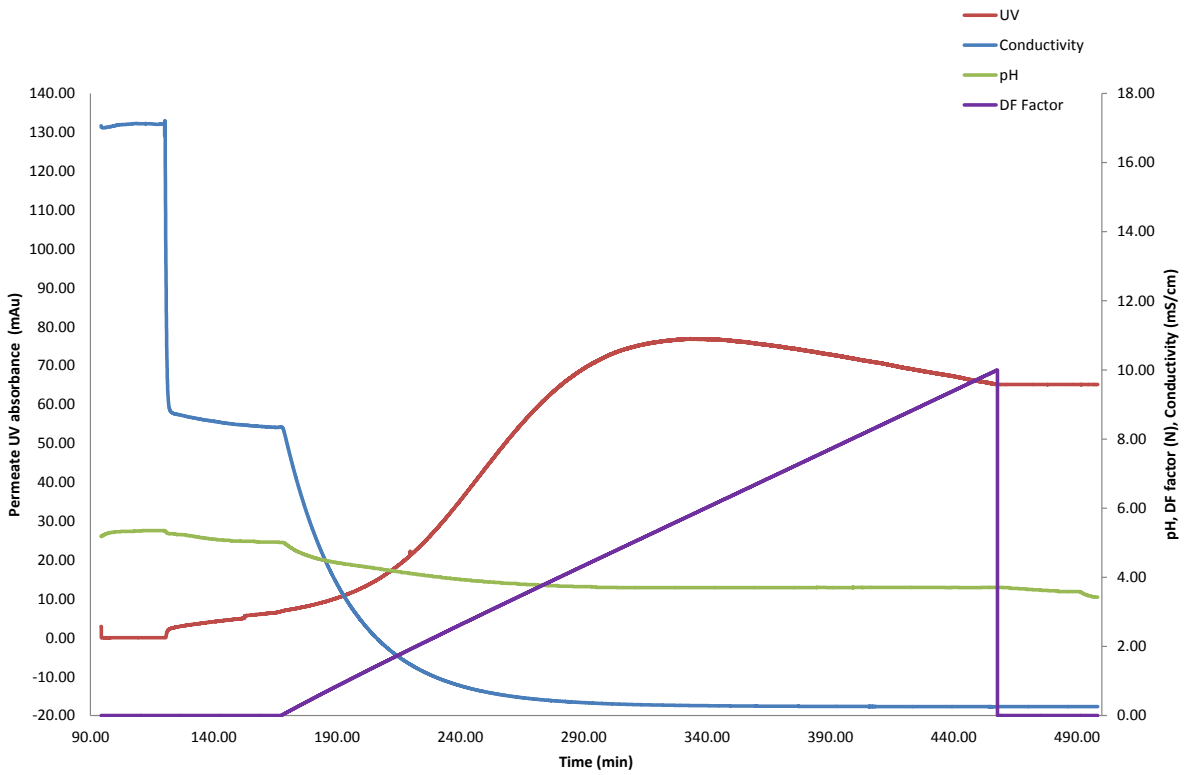


Figure 44: TFF profile produced during the demonstration run using a 5 kDa RC membrane at 100 g/m^2 loading.

Experimental TMP of 2.0 bar, crossflow of 8 L/m^2/h, initial protein concentration of 3.6 mg/ml in 25 mM sodium acetate pH 5.4 and final protein concentration of 10 mg/ml.

6.4.8 Gel concentration determination and operating mode design

Another standard parameter used to characterize and optimise TFF unit operations is the determination of the gel layer concentration (C_G) and optimal diafiltration concentration (C_D). Based on the flux-concentration relationship

$$J = k \ln \frac{c_G - c_P}{c_F - c_P}$$

Where J is the flux, k is the mass transfer co-efficient for the system, C_F is the concentration of the feedstock and C_P is the permeate concentration. For a completely rejected system (e.g. the 5 kDa PES membrane), C_P is negligible. Therefore, C_G can be determined by measuring the flux through the membrane while concentrating the product and plotting the results. Using the 5 kDa membrane the flux-concentration semi-log plot is shown in Figure 45, and the theoretical gel layer concentration (C_G) was determined as 440 mg/mL. Using film theory the optimal concentration for diafiltration (C_D) is calculated where $C_D = C_G/e$ [25]. The theoretical optimum protein concentration for diafiltration (C_D) was calculated as 163 mg/mL. Of note was the fact that significant levels of turbidity formed in the retentate during these experiments indicating aggregation and precipitation of the Protein At the concentrations used (up to 50 mg/ml) which were significantly below the value calculated for C_D. Furthermore, when concentrating the protein from 3.6 mg/mL to 163 mg/mL, the change in volume required would be more than 45-fold, placing the retentate volume below the minimum working volume for most manufacturing equipment. For both of these reasons the experimentally determined C_D value was not used in further development work as it was felt to place the product at risk and was most probably unworkable at manufacturing scale. The operating mode was therefore designed to concentrate the protein to the final target concentration of 10 mg/ml and then perform diafiltration at this protein concentration to

minimise buffer usage and process time as much as possible without compromising the stability of the product.

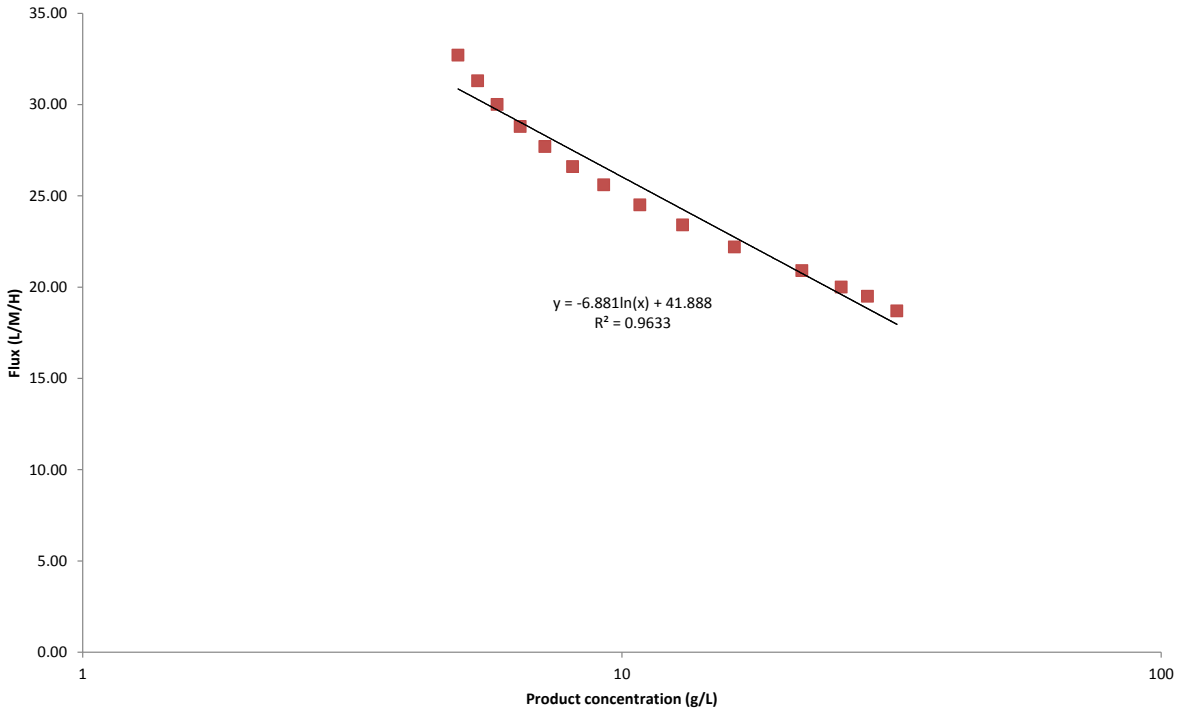


Figure 45: Filgrastim C_{gel} determination using the 5 kDa PES membrane.

Solution conditions of 25 mM sodium acetate, 150 mM NaCl pH 5.4 and an initial protein concentration of 3.6 mg/ml at a crossflow of 8 L/m^2/h and filter load of 100 g/m^2 and TMP of 2.0 bar.

6.4.9 Manufacturing scale performance

The results of the TFF runs carried out at manufacturing scale are shown in Table 24. The significant improvements in RP-HPLC impurities notable in the table were a result of upstream changes to fermentation and purification conditions and were not a product of the scale-up. The target concentration at the end of diafiltration was increased to ≥12 g/L from the ≥10 g/L used during development to ensure that a final product of 10 g/L could be produced after removing the product from the retentate vessel and formulating for use. The

yield produced in the manufacturing scale batches was as expected from the development scale, as was the final product pH demonstrating an effective scale-up of approximately 30-fold.

Problems with control of the TMP were noted during the operation of the first manufacturing scale batch, resulting in large swings in pressure during the initial concentration phase. These pressure instabilities were rectified during the second batch by refinement of the TMP PID controller settings. Significant pressure instabilities in first batch were more easily localised to an equipment problem due to the high level of specification set around the other process outcomes during development.

Table 24: Results of TFF performed at manufacturing scale.

Batch No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Load (g)	266	308	342	272	330	318	236
Product (g)	253	293	341	268	316	315	219
Yield (%)	95	95	100	99	96	99	93
Final protein conc. (g/L)	13.7	13.4	12.4	13.0	13.6	13.6	12.5
RP-HPLC impurities (%)	n.d.	1.6	1.8	1.4	0.8	1.5	1.0
SEC impurities (%)	n.d.	0.5	0.4	0.4	0.5	0.5	0.5
Final retentate pH	4.1	4.1	4.1	4.0	4.1	4.0	4.0

n.d. = analysis not performed

Size related impurities increased over the TFF operation from ≤0.2 % in the feedstock to an average of 0.5% in the TFF retentate. Frothing was observed during the diafiltration phase due to the height from which solutions were pumped into the reservoir - through a sealed port approximately 0.6 m above the bottom of the tank. The increase in size related impurities is likely related to the frothing and may be resolved by modifying the retentate inlet to reduce its height above solution level. Despite the increase in SEC impurities, the TFF retentate met target criteria for the manufacturing scale batches and the final product met all criteria of the target product profile.

6.5 Discussion

A TFF unit operation was developed for the production of the model biopharmaceutical protein filgrastim following QbD principles. Using ultrafiltration membranes of both

regenerated cellulose and polyethersulfone a design space consisting of a cross flow of 4 – 8 $L/m^2/min$, TMP of 0 – 2.5 bar and a membrane load of up to 100 g/m^2 was developed. Differences in membrane interactions with the product resulted in the 5 kDa PES membrane being chosen for future manufacturing work as this membrane resulted in the highest yield of product and produced a lower turbidity in the final retentate than the regenerated cellulose.

When the design space was implemented in mock production runs, subtle differences emerged in the performance of the membranes, emphasizing the need to evaluate a range of material properties and types during early development. This work illustrated that not all of the standard techniques for developing TFF operations are appropriate or applicable for biopharmaceutical applications. Normal theory would suggest that the optimal diafiltration concentration for this protein was 160 g/L but due to the sensitivity of this particular product to aggregation and the formation of higher molecular weight species, coupled with the significant safety concerns that accompany the formation of aggregate species in biopharmaceutical proteins, adoption of this standard practice is not appropriate. A significant observation is that the standard procedures for developing TFF unit operations need to be modified according to the properties of the protein and solution being produced to allow for the unique properties of each molecule.

When using the chosen membrane (PES) a significant pH difference developed between the target product pH and the final pH. Some of this can be attributed to the Donnan effect [29] as in the highly purified conditions of drug product TFF operations, electrostatic interactions between the biopharmaceuticals and charged excipients can significantly alter the final pH and excipient concentrations [19–22]. However, this effect could not account for the magnitude of difference in pH which appeared between the regenerated cellulose and polyethersulfone membranes at the end of diafiltration. Different authors have measured the zeta potential in solution of PES and RC membranes. In general PES membranes have been shown to possess a zeta potential of – 7 to – 15 mV [125, 126], while RC membranes have

been shown to be have a much lower charge of approximately -1 to -4 mV, depending on the manufacturers membrane used [127, 128]. The particular RC membrane used in this study (30 kDa Ultracel) has been reported as having a zeta potential of -1.6 mV in solution [28]. It appears likely that the significant difference in final pH emerged through the interaction between the charged acetate ions and protein species, as suggested by Donnan equilibrium considerations, in addition to interaction with the charged PES membrane under these conditions. This appears validated by the observation that the PES membrane was more prone to fouling and required the addition of hypochlorite to the cleaning solution in order to regain permeability to acceptable levels after use. Previous authors have correlated surface membrane charge with the degree of fouling undergone by a membrane with the greatest fouling occurring when the membrane and protein solute had opposite charge, as was the case in this application. That different surface interactions occurred between the PES membrane and filgrastim as compared to those between the RC membrane and filgrastim was also indicated by the higher turbidity produced in the final retentate by the RC membranes. Future work examining the nature and type of these protein-membrane interactions with a view to improving the properties of membranes would appear to be a promising area for future.

During scale-up to manufacturing scale it was noted that the TFF unit operation caused a slight increase in HMWS from an average of 0.2 % before to an average of 0.5 % after TFF. This increase was not observed in the R&D scale development runs and was attributed to the geometry of the TFF skid causing slight foaming in the protein solution. Filgrastim is known to be capable of forming reversible multimer species in this manner due to agitation. The increase in HMWS was ameliorated by the subsequent addition of formulation components (data not shown) however the observation goes some way to demonstrating the value of using the QbD approach. Firstly, a target product profile (TPP) had already been established with an acceptable level of HMWS ($\leq$ 2%) and despite the increase the product contained less than this limit. Secondly, the extensive knowledge gained from R&D development in using the

QbD approach allowed the cause of the HMWS increase to be identified quickly so the unexpected observation did not result in lengthy re-development of the process and accompanying delays in the project timeline. In a similar manner, the extensive knowledge gained at development scale with regards to the expected pressure and flow profiles allowed rapid identification of pressure instabilities during the first manufacturing scale batch and localisation of the problem to the PID setting of the manufacturing scale TFF skid.

Due to the large number of techniques involved in QbD there is the risk of applying some of the techniques poorly and thereby generating large amounts of documentation while gaining little additional understanding. To avoid these pitfalls a method for implementing QbD has previously been proposed and forms part of the introduction to this thesis [95]. This implementation sought to ensure that unit operations were prioritized before further study. A standard method of prioritization recommended was to begin working on the last unit operation until that is under control, before progressing to the preceding unit operation to ensure the highest priority unit operations receive the most attention. The way in which QbD was implemented for the development of the TFF unit operation using this scheme is illustrated in Figure 46 and Figure 47. A target product profile and CQAs for the product had already been identified; prior knowledge of the product and unit operation was used to inform which CQAs to monitor during experimentation and determine how to go about the development work most efficiently. Multiple process designs were examined in the use of alternative membranes of different contact materials, molecular weight cut-off values and operating regimes before the process controls were established and the unit operation was transferred to the commercial operating scale. The results of initial manufacturing runs were then compared to the data obtained during development to determine whether the scale-up had been successful.

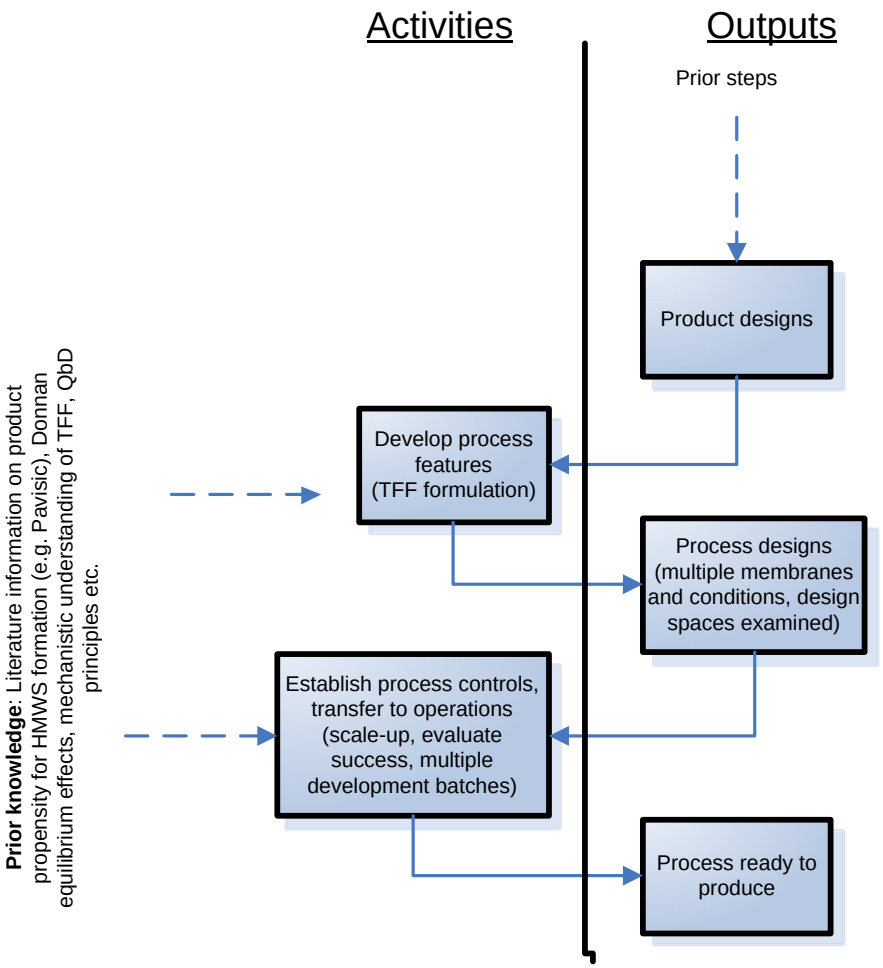


Figure 46: Implementation of the Quality planning roadmap with TFF development

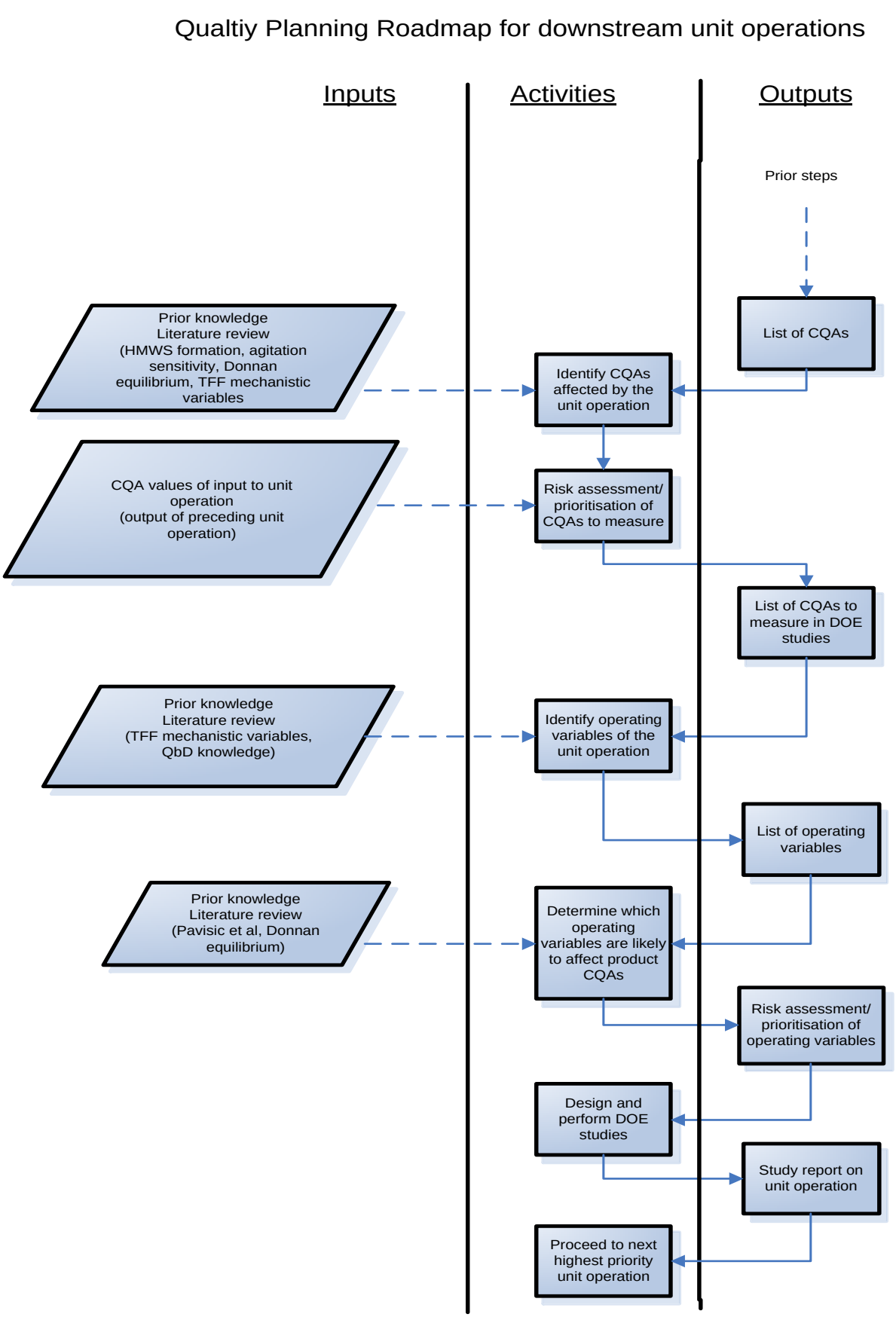


Figure 47: Quality planning roadmap for the development of the TFF unit operation

6.6 Conclusions

Using a QbD approach to the development of a TFF unit operation for filgrastim was found to be effective as relatively simple and reliable scale-up of the process to manufacturing scale was achieved. In developing the design space for this unit operation PES filter membrane of 5 kDa MWCO was found to be preferable to RC membrane of the same MWCO value despite the PES membrane causing a drift in the pH of the retentate solution during operation. This demonstrates the utility of examining a number of material types in development of a design space for a manufacturing unit operation, while also examining the range of operating variables such as TMP and cross-flow rate. In standard theory for the development of a TFF operation the optimal diafiltration concentration (C_D) is derived from the gel-layer concentration (C_G). Use of the C_D value obtained in this way was found to be inappropriate for this application as the derived concentration was high enough to cause aggregation and precipitation in the protein, both of which are serious drawbacks in a biopharmaceutical product.

6.7 References

1. Hill CP, Osslund TD, Eisenberg D. The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. *Proc Natl Acad Sci U S A*, 90(11), 5167-5171 (1993).

2. Ricci MS, Sarkar CA, Fallon EM, Lauffenburger DA, Brems DN. pH Dependence of structural stability of interleukin-2 and granulocyte colony-stimulating factor. *Protein Sci*, 12(5), 1030-1038 (2003).

3. Pavisic R, Hock K, Mijic I *et al.* Recombinant human granulocyte colony stimulating factor pre-screening and screening of stabilizing carbohydrates and polyols. *Int J Pharm*, 387(1-2), 110-119 (2010).

4. Piedmonte DM, Treuheit MJ. Formulation of Neulasta (pegfilgrastim). *Adv Drug Deliv Rev*, 60(1), 50-58 (2008).

5. Herman A, Boone T, Lu H. Characterization, formulation and stability of Neupogen (filgrastim), a recombinant human granulocyte colony stimulating factor. In: *Formulation, Characterizationand Stability of Protein Drugs.* Pearlman, R, Wang, Y (Eds.) (Plenum Press, New York, 1996) 303 - 328.

6. Chi EY, Krishnan S, Kendrick BS, Chang BS, Carpenter JF, Randolph TW. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor. *Protein Sci*, 12(5), 903-913 (2003).

7. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative Protein Aggregation. *Pharm Res*, 20(9), 1325-1336 (2003).

8. Watler P, Rozembersky J. Application of QbD principles to tangential flow filtration operations. In: *Quality by Design for Biopharmaceuticals: Principles and case studies.* Rathore, AS, Mhatre, R (Eds.) (John Wiley & Sons, Inc., Hobokwn, New Jersey, 2009) 111 - 125.

9. USFDA. Pharmaceutical cGMPS for the 21st Century — A Risk-Based Approach: Second Progress Report and Implementation Plan. (Ed.^(Eds) (2003)

10. Council_of_Europe. *European Pharmacopeia. Filgrastim Concentrated Solution <2206>* (Council of Europe, Strasbourgh, 2013).

11. Pavisic R, Dodig I, Horvatic A *et al.* Differences between reversible (self-association) and irreversible aggregation of rHuG-CSF in carbohydrate and polyol formulations. *Eur J Pharm Biopharm*, 76(3), 357-365 (2010).

12. ICH. DEVELOPMENT AND MANUFACTURE OF DRUG SUBSTANCES (CHEMICAL ENTITIES AND BIOTECHNOLOGICAL/BIOLOGICAL ENTITIES) Q11. (Ed.^(Eds) (2012)

13. Stoner MR, Fischer N, Nixon L *et al.* Protein-solute interactions affect the outcome of ultrafiltration/diafiltration operations. *J Pharm Sci*, 93(9), 2332-2342 (2004).

14. Burns D, Zydney AL. Buffer effects on the zeta potential of ultrafiltration membranes. *Journal of Membrane Science*, 172, 39 - 48 (2000).

15. Gasch J, Leopold C, Knoth H. Positively charge polyethersulfone membranes: The influence of Furosemide on the zeta potential. *Journal of Membrane Science and Technology*, 3(1), 1 - 5 (2013).

16. Suzuki Y, Kanamori T, Sakai K. Zeta potential of hollow fiber dialysis membranes and its effects on hydrogen phosphate ion permeability. *ASAIO Journal*, 39(3) (1993).

17. Kim K, Fane AG, Nystrom M, Pihlajamaki A, Bowen WR, Mukhtar H. Evaluation of electroosmosis and streaming potential for measurement of electric charges of polymeric membranes. *Journal of Membrane Science*, 116, 149 -159 (1996).

18. Rohani MM, Zydney AL. Role of electrostatic interactions during protein ultrafiltration. *Adv Colloid Interface Sci*, 160(1-2), 40-48 (2010).

7 Overall Thesis Conclusion

7.1 The application of QbD to Biopharmaceutical development

The purpose of this research was to determine how the concept of Quality by Design could be applied to the development of biopharmaceuticals in practice. This was both to assist practitioners in the field in applying QbD concepts and to determine whether QbD is likely to fulfil the stated aims of the initiative. In order to answer these questions the literature was consulted and surveyed to identify what constitutes QbD development of biopharmaceuticals, as opposed to traditional or more established development methodologies. An obvious, related question for those in the field of biopharmaceutical development is whether QbD is worth the effort of engaging in – to determine whether there is sufficient benefit to outweigh the likely cost, whether it is here to stay or a passing fad and whether there is sufficient substance to the concept to warrant the investment in learning required.

To attempt to answer all of these related questions a QbD development methodology was applied to three typical development activities that would be required by almost all biopharmaceutical products. A QbD approach was taken to the selection of a Protein A resin for the manufacture of a monoclonal antibody, for resin lifetime studies in a chromatography unit operation and for the development of a tangential flow filtration unit operation. In preparing these chapters and reviewing the state-of-the-art of QbD it became clear that the fundamentals of QbD development are deciding on a target product profile and defining the operating parameters of a unit operation. Once these fundamentals are established, Design of experiments and risk analysis are then tools for linking and prioritizing further study. This seems obvious, that final objectives should be considered and defined before starting - but the fact that it is a system being promoted by regulatory agencies and essentially being mandated by guidelines shows that in practice many developers omit these important steps. Often, understanding of the manufacturing process and the analytical assays is not built during

development as the large payoff in biopharmaceutical development occurs with market entry. In most industries, marginal process improvements are the basis of continuous improvement. In the biopharmaceutical industry, marginal process improvements can incur a very large cost if they require approval from the regulatory agencies before they are implemented. This acts as a significant disincentive to innovation. Therefore these marginal improvements are often neglected in biopharmaceutical development and knowledge that is built during the development process is less easily implemented.

7.2 Cost-benefit analysis of QbD

In promoting the concept of QbD the FDA hoped that this method of development would reduce overall development costs and reduce the time required for development. Early publications from industry tended to suggest this was unlikely [12] and that the use of many of the QbD techniques was likely to increase both development costs and times. More recent joint guidance from the FDA and EMA have conceded this point, indicating that significant additional information and documentation is required when submitting applications for a design space [48]. In submitting a design space a company must be prepared to undertake the validation of the manufacturing process, as essential for a "traditional" development route, in addition to verification of the design space at manufacturing scale [46]. Validation of a manufacturing process at the commercial production scale is generally regarded as a costly and time-consuming exercise in itself, so the addition of verification activities at commercial scale can only be expected to be of similar magnitude in cost. Despite these additional costs, examples provided in this thesis show that use of a QbD methodology is justifiable on the balance of costs and benefits. This was exemplified in the development of a TFF unit operation. Thorough process development and understanding of the TFF unit operation before scale-up allowed the rapid identification of problems and their resolution without additional development time or costs. It is in the prevention of lost time and additional costs to these types of problems that the real benefits of QbD are accrued. Similarly there are

literature examples such as the development of analytics and chromatography scale-up in which this has also proven to be the case [23, 129, 130].

7.3 Achieving the aims of QbD

Among the aims of the QbD initiative is to reduce the costs of medicine. On balance, the information produced in this work suggests that the additional effort of QbD is worth the cost in saved time in development and scale up. However, the additional work required means that more upfront investment is required in development. In effect this will translate to higher entry costs for developing new products. Economists generally believe that higher entry costs favour large market participants over smaller as large companies are more easily able to mobilise the required capital and resources [131]. Therefore the concern that QbD will disadvantage smaller players in bringing products to market is valid. For this reason QbD is not likely to lower costs of medicines and regulatory agencies are yet to grapple with the issue that this change in regulation is likely to disadvantage smaller companies, which are generally the source of most innovation in the biopharmaceutical industry [8].

7.4 QbD is here to stay

Recent developments have indicated that the QbD development track is here to stay and is not a passing fad. The submission guidelines for generic small molecules have been amended to require certain aspects of QbD to have been implemented before a generic molecule can be registered [132]. It is highly likely that, once established for this class of drugs, similar changes in requirements for biopharmaceuticals will take place, ultimately requiring all development to incorporate at least some QbD elements. Recent publications on the outcome of FDA-EMA pilot program illustrate how QbD information and the verification of design space at large scale are to be additional to validation information. This indicates that QbD requirements will be in addition to all of the current requirements for registration of a new drug.

One of the very positive outcomes of the QbD initiative is that it has become a recognised academic field that has allowed practitioners a vehicle for publishing ideas on how to go about process development in a comprehensive and economical manner. Examples such as the paper of [23] in which a mechanistic model of a chromatographic unit operation was used to determine how the unit operation will behave at large scale are the sort of application of QbD that is likely to raise development standards in the industry. In this example, the model was able to pinpoint the cause of a problem at large-scale easily, greatly reducing the time and effort usually necessary to solve problems of this kind. Development and publication of mechanistic models in this way allows continuous improvement or at least continuous monitoring in ways that are not achieved by the traditional "validation" approach to pharmaceutical development. These go some way to reducing the reliance on traditional "Quality by Testing" of the final product and in implementing some aspects of process analytical technology, as real-time data from the operation can be used to confirm that the process is in control or rapidly detect when the process is outside of its intended operating range. This is similar to the TFF development chapter in this thesis in which the development of expected pressure, concentration and impurity profiles from development data allowed rapid identification of changes upon scale-up and a solution to the problems identified. In allowing publication and sharing of these best practice models of process development, QbD is contributing to raising standards in the pharmaceutical industry and ensuring that processes are being designed for manufacturing from the outset.

In the QbD paradigm the process must be designed to manufacture product consistently over the lifetime of the pharmaceutical product. Reliability and re-usability of manufacturing platforms is therefore a very important part of the QbD development. The reusability of the manufacturing platform was examined in the resin lifetime, Protein A selection and TFF development sections in this thesis.

7.5 Significant new knowledge produced

As stated earlier the intention of this research at the outset was to identify how QbD could be applied to development of biopharmaceuticals and the likely benefits of performing development in this manner. Due to the collaboration with a pharmaceutical company it was possible to undertake this development with biopharmaceutical proteins rather than the model proteins generally examined in the literature due to cost constraints. In this way, relevant case studies could be built up to provide information and learning for industry. In the course of research several other pieces of knowledge were produced. In comparing the performance of some of the leading Protein A resins available it became clear that there was confusion in the literature as to what isotherm is best to use to compare the static binding capacity of these resins and additional work was performed to confirm that the Langmuir-Freundlich isotherm is the more correct model to use for this purpose. This was in addition to the original purpose of determining which of the Protein A resins available gave the best performance when tested with a model monoclonal antibody under manufacturing conditions. Due to the cost involved, many of the published comparisons of these resins have tended to use polyclonal antisera or other models of monoclonal antibody which do not give a completely accurate representation of the performance to expect in manufacturing conditions.

Once it was determined that the resin based on the MSS ligand performed better than resin based on the native ligand experiments were undertaken with the purified ligands were undertaken to determine how much of this difference could be attributed to the ligand itself. An examination of the thermodynamics of the reaction between modified Protein A (MSS) and native Protein A indicated that the MSS was able to maintain significant interaction with an IgG at pH 4, a pH value at which no detectable interaction occurred between native Protein A and the IgG. The affinity constant for the MSS ligand was also shown to be 10 – 20 fold greater than the native ligand under almost all conditions examined. This additional mechanistic understanding of the Protein A purification process yielded the insight that

tailoring of the pH value of purification may be a fruitful field of study when attempting to improve purification using MSS. The ITC results also indicated that tailoring the salt concentration of the binding solution when using resins based on native Protein A can be used increase the specificity of the interaction. The point here is not to re-hash the conclusions of the ITC section but to emphasise the additional knowledge and understanding gained by extending QbD studies to gain mechanistic understanding of the underlying processes involved.

In this course of this research a worked example of applying QbD to design, development and scale-up to manufacturing scale of a TFF unit operation was produced using an actual biopharmaceutical product. This development and scale-up was based on a mechanistic understanding of the TFF unit operation and the inherent properties and potential degradation pathways of the particular protein. The problems encountered during scale-up of the TFF unit operation - additional foaming leading to increased levels of HMWS due to the geometry of the system - provides new knowledge and knowledge relevant to the industry. This is also relevant to the field of QbD as the additional knowledge gained by establishing the design space in a mechanistic manner enabled rapid identification of the existence of a problem upon scale-up and concomitant rapid resolution.

7.6 Relation to the QbD field

This thesis illustrates the rapidly changing nature of QbD field - even during final write-up in December 2014 the FDA-EMA were issuing new Q&A guidance documents that answered some the questions previously identified by industry as concerns in the "Pitfalls of QbD" section of the literature review [47]. Furthermore, publications from the FDA in late 2014 admitted that there was some confusion in the industry regarding QbD and went on to give very detailed and highly specific definitions in order to help simplify matters and ease the confusion [133]. The approach defined in this recent publication broadly agrees with the process for QbD implementation developed in the review paper, especially with regards to

having a rudimentary process established and working backwards through the unit operations or using some other more formal means of prioritising development studies.

As identified there is a need in the QbD field for worked case studies to demonstrate how these principles can be actually applied and how they can have benefits. This need has been answered by the studies in this thesis.

In the QbD paradigm the process must be designed to manufacture product consistently over the lifetime of the pharmaceutical product. Reliability and re-usability of manufacturing platforms is therefore a very important part of the QbD development. The reusability of the manufacturing platform was examined in the resin lifetime, Protein A selection and TFF development sections in this thesis.

7.7 Future Work to advance QbD in biopharmaceuticals

For QbD to be realised there must be more use of quality related tools and the "Quality disciplines" by scientists in process development. One way in which this can be implemented was exhibited by use of control charts in the lifetime study carried out with CFT resin to extract more information from the data set. A fundamental understanding of a unit operation and an understanding of the reliability of the unit operation was therefore produced simultaneously. The combination of mechanistic studies of the resin before and after use with control charts is an ideal way to implement this Quality tool and also produce information that can lead to understanding of the underlying process involved. More efforts in this area will improve the quality of publications and lead to more reliable and better understood manufacturing processes.

Further development of the ITC technique to enable use of the actual derivatized resin rather than the free ligand is an area of experimentation that could be explored as, if developed, this technique could be applied to achieve rapid and detailed comparison and understanding of different chromatography resins with various products under a variety of conditions.

Some excellent examples in the literature have begun from mechanistic models of unit operations and shown how these models can be used to develop robust and economical unit operations that are well characterized without having to assume only the empirical level of understanding implied in a "naïve" DoE approach [23-25, 44]. The TFF study developed in this thesis was an attempt to take the same approach for tangential flow filtration, as the examples cited had focused on chromatography operations. As discussed earlier, recent joint guidance from the FDA and EMA has indicated that in submitting a design space a company must be prepared to undertake the validation of the manufacturing process, as essential for a "traditional" development route, in addition to verification of the design space at manufacturing scale [46]. Published, successful examples of design space verification at full production scale would greatly assist the field and regulators in assessing the costs and benefits of the QbD approach and increasing understanding of the additional flexibility allowed. For example, the TFF study reported here could be expanded by carrying out full production scale runs using the range of TMPs and cross-flows examined during development and analysing the product to confirm that all quality attributes remained within acceptable ranges. Due to the requirements for large quantities of recombinant product and for the work to be carried out at full production scale, this kind of experimentation cannot be carried out without the involvement of industry partners but is necessary to prove the utility of the QbD approach to development.

7.8 References

1. USFDA. *Drug Shortages*. 2014; Available from: http://www.fda.gov/Drugs/DrugSafety/DrugShortages/default.htm.
2. Whitehouse, T. *Executive Order reducing prescription drug shortage*. 2011; Available from: http://www.whitehouse.gov/the-press-office/2011/10/31/executive-order-reducing-prescription-drug-shortages.
3. Abboud, L. and S. Hensley, *Drug Manufacturing, Out of Date for Years, Gets a Shot in the Arm --- U.S.'s FDA Prods Industry to Adopt Innovations, Raise Quality Standards.* The Wall Street Journal Europe, 2003(03 September).
4. ICH. *Pharmaceutical Development Q8(R2)*. 2009; Available from: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.
5. Rathore, A.S. and H. Winkle, *Quality by design for biopharmaceuticals.* Nat Biotechnol, 2009. **27**(1): p. 26-34.
6. Stevenson, D. and T. Cochrane, *Implementation of QbD Part 1 - Setting Product specifications.* Regulatory Rapporteur, 2011. **8**(2): p. 3.
7. Stevenson, D. and T. Cochrane, *Implementation of QbD Part 2 - Organisational Implications.* Regulatory Rapporteur, 2011. **8**(3).
8. Schmitt, S., *The Roadmap to QbD*, in *Quality By Design: Putting Theory into Practice*, S. Schmitt, Editor. 2011, Davis Healthcare Internation Publishing: Bethesda, MD. p. 31 -54.
9. BioProcessTechnologyConsultants. *Quality by Design: Just a Buzz Word?* 2012 30OCT2012]; Survey results]. Available from: http://www.bptc.com/sites/default/files/biopulse_reports/qbd_results_2012-05-15_final.pdf.
10. ISPE. *The State of the Art in Quality by Design: Criticality Assessment, Design Space Implementation and Control*. in *The State of the Art in Quality by Design: Criticality Assessment, Design Space Implementation and Control*. 2013. Sheraton Fisherman's Wharf, San Francisco: ISPE.
11. CMCBiotechWorkingGroup. *A-mAb: a case study in Bioprocess Development*. 2009 30OCT2012 [cited 2.1; Available from: www.ispe.org/pqli/a-mab-case-study-version-2.1.
12. Schmitt, S., *The regulatory framework*, in *Quality by design: putting theory into practice*, S. S, Editor. 2011, Davis Healthcare International Publishing: Bethesda, MD.
13. Kozlowski, S. and P. Swann, *Current and future issues in the manufacturing and development of monoclonal antibodies.* Adv Drug Deliv Rev, 2006. **58**(5-6): p. 707-22.
14. Kozlowski, S., *Protein therapeutics and the regulation of quality: a brief history from an OBP perspective: as the biotechnology industry has matured through various stages of growth, regulatory agencies have evolved in response to the need to define quality standards* Biopharm International, 2007. **20**(10).
15. Seely, R. and J. Haury, *Applications of Failure Modes Effect Analysis to Biotechnology Manufacturing Processes*, in *Process validation in manufacturing of Biopharmaceuticals: Guidelines, Current Practices and Industrial Case Studies.*, A.S. Rathore and G. Sofer, Editors. 2005, CRC Press, Taylor & Francis Group: Boca Raton, FL. p. 13 - 31.
16. Brandreth, E.J., et al., *Validation of Column-based Chromatography Processes fro the Purification of Proteins Technical Report No. 14 Revised 2008.* PDA Journal of Pharmaceutical Science and Technology, 2008. **62**(S-3).
17. Harms, J., et al., *Defining process design space for biotech products: case study of Pichia pastoris fermentation.* Biotechnol Prog, 2008. **24**(3): p. 655-62.
18. van Hoek, P., et al., *Case study on definition of process design space for a microbial fermentation step*, in *Quality by Design for Biopharmaceuticals: Principles and Case studies*, A.S. Rathore and R. Mhatre, Editors. 2009, John Wiley & Sons, Inc.: Hoboken, New Jersey. p. 85 - 108.
19. Abu-Absi, S.F., et al., *Defining process design space for monoclonal antibody cell culture.* Biotechnol Bioeng, 2010. **106**(6): p. 894-905.
20. Horvath, B., M. Mun, and M.W. Laird, *Characterization of a monoclonal antibody cell culture production process using a quality by design approach.* Mol Biotechnol, 2010. **45**(3): p. 203-6.

21. Mila, L., et al., *Application of a risk analysis method to different technologies for producing a monoclonal antibody employed in hepatitis B vaccine manufacturing.* Biologicals, 2012. **40**(2): p. 118-28.
22. Cecchini, D., *Applications of Design Space for Biopharmaceutical Purification Processes*, in *Quality by Design for Biopharmaceuticals*, A.S. Rathore and R. Mhatre, Editors. 2009, John Wiley & Sons: Hoboken, New Jersey. p. 127 - 141.
23. Mollerup, J.M., et al., *Quality by design--thermodynamic modelling of chromatographic separation of proteins.* J Chromatogr A, 2008. **1177**(2): p. 200-6.
24. Kaltenbrunner, O., et al., *Application of chromatographic theory for process characterization towards validation of an ion-exchange operation.* Biotechnol Bioeng, 2007. **98**(1): p. 201-10.
25. Watler, P. and J. Rozembersky, *Application of QbD principles to tangential flow filtration operations*, in *Quality by Design for Biopharmaceuticals: Principles and case studies*, A.S. Rathore and R. Mhatre, Editors. 2009, John Wiley & Sons, Inc.: Hobokwn, New Jersey. p. 111 - 125.
26. Bolton, G.R., et al., *Effect of protein and solution properties on the Donnan effect during the ultrafiltration of proteins.* Biotechnol Prog, 2011. **27**(1): p. 140-52.
27. Noordman, T., et al., *Concentration and desalination of protein solutions by ultrafiltration.* Chemical Engineering Sciences, 2002. **57**(693 - 703).
28. Rohani, M.M. and A.L. Zydney, *Role of electrostatic interactions during protein ultrafiltration.* Adv Colloid Interface Sci, 2010. **160**(1-2): p. 40-8.
29. Stoner, M.R., et al., *Protein-solute interactions affect the outcome of ultrafiltration/diafiltration operations.* J Pharm Sci, 2004. **93**(9): p. 2332-42.
30. Martin-Moe, S., et al., *A new roadmap for biopharmaceutical drug product development: Integrating development, validation, and quality by design.* J Pharm Sci, 2011. **100**(8): p. 3031-43.
31. Lanan, M., *QbD for Raw materials*, in *Quality by Design for Biopharmaceuticals*, A.S. Rathore and R. Mhatre, Editors. 2009, John Wiley & Sons: Hoboken, New Jersey. p. 193 - 208.
32. Pomerantsev, A. and O. Rodionova, *Process analytical technology: a critical view of the chemometricians.* Journal of Chemometrics, 2012. **26**: p. 11.
33. Luo, Y. and G. Chen, *Combined approach of NMR and chemometrics for screening peptones used in the cell culture medium for the production of a recombinant therapeutic protein.* Biotechnol Bioeng, 2007. **97**(6): p. 1654-9.
34. Curinova, L. and F. Dudek, *Quality Management in Nineteenth-Century Czech Beer and Sugar Production*, in *A history of managing for quality: the evolution, trends, and future directions of managing for quality*, J.M. Juran, Editor. 1995, ASQC Quality Press: Milwaukee, Wisconsin. p. 657.
35. Rathore, A.S. and D. Low, *Managing Raw Material in the QbD paradigm, Part 1: Understanding risks.* BioPharm International, 2010. **23**(11): p. 8.
36. Rathore, A.S. and D. Low, *Managing Raw Materials in the QbD paradigm Part 2: Risk Assessment and Communication.* BioPharm International, 2010. **23**(12): p. 5.
37. Schenerman, M., et al., *Using a Risk assessment process to determine criticality of product quality attributes*, in *Quality by Design for Biopharmaceuticals: Principles and Case studies*, A.S. Rathore and R. Mhatre, Editors. 2009, John Wiley & Sons: Hoboken, New Jersey. p. 53 - 82.
38. Goetze, A.M., M.R. Schenauer, and G.C. Flynn, *Assessing monoclonal antibody product quality attribute criticality through clinical studies.* MAbs, 2010. **2**(5): p. 500-7.
39. van Leeuwen, J.F., et al., *Risk analysis by FMEA as an element of analytical validation.* J Pharm Biomed Anal, 2009. **50**(5): p. 1085-7.
40. Juran, J.M.J.M., *Quality by design Juran on quality by design : the new steps for planning quality into goods and services*. 1992, New York : Toronto : New York :: Free Press ; Maxwell Macmillan Canada ; Maxwell Macmillan International,.
41. McConnell, J., B.K. Nunnally, and B. McGarvey, *The forgotten origins of Quality by Design.* Journal of Validation Technology, 2010. **16**(3): p. 5.

42. Juran, J.M., *A call to action: the summit: Carlson School of Management, University of Minnesota-Minneapolis, Minnesota 26 June 2002.* Measuring Business Excellence, 2002. **6**(3): p. 4.
43. Bicheno, J., *The Quality 60: A guide for service and manufacturing.* 1998, Buckingham, UK: PICSIE Books.
44. Kaltenbrunner, O., et al., *Modeling of Biopharmaceutical Processes. Part 2: Process chromatography unit operations.* BioPharm International, 2008. **21**(8): p. 13.
45. Neway, J., *How to make the business case for Quality by Design.* BioPharm International, 2008. **21**(12).
46. FDA-EMA, *EMA-FDA pilot program for parallel assessment of Quality-by-Design applications: lessons learnt and Q&A resulting from the first parallel assessment*. 2013.
47. FDA-EMA. *Questions and answers on level of detail in the regulatory submissions* 2014 10DEC2014]; Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2014/12/WC500179391.pdf.
48. FDA-EMA. *Questions and Answers on Design Space Verification*. 2013 24 October 2013].
49. Friedman, M., *How to cure Health Care.* Hoover Digest, 2001. **3**(July 30).
50. Gottschalk, U., ed. *Process Scale Purification of Antibodies*. 1st ed. 2009, John Wiley & Sons, Inc.: Hoboken, New Jersey. xvii - xx.
51. GEHealthcare, *Mab Select SuRe Instructions 11-0026-01 AD*. 2004.
52. Vunnum, S., G. Vedantham, and B. Hubbard, *Protein A-Based Affinity Chromatography*, in *Process Scale Purification of Antibodies*, U. Gottschalk, Editor. 2009, John Wiley & Sons, Inc.: Hoboken, New Jersey. p. 79 - 102.
53. Perez-Almodovar, E.X. and G. Carta, *IgG adsorption on a new protein A adsorbent based on macroporous hydrophilic polymers. I. Adsorption equilibrium and kinetics.* J Chromatogr A, 2009. **1216**(47): p. 8339-47.
54. Perez-Almodovar, E.X. and G. Carta, *IgG adsorption on a new protein A adsorbent based on macroporous hydrophilic polymers II. Pressure-flow curves and optimization for capture.* J Chromatogr A, 2009. **1216**(47): p. 8348-54.
55. Scolnik, P., *mAbs: A business perspective*. MAbs, 2009. **1**(2): p. 179-84.
56. Braisted, A. and J. Wells, *Minimizing a binding domain from Protein A.* Proc Natl Acad Sci U S A, 1996. **93**: p. 5688 - 5692.
57. McCue, J.T., et al., *Evaluation of protein-A chromatography media.* J Chromatogr A, 2003. **989**(1): p. 139-53.
58. Hahn, R., et al., *Comparison of protein A affinity sorbents II. Mass transfer properties.* J Chromatogr A, 2005. **1093**(1-2): p. 98-110.
59. Hahn, R., R. Schlegel, and A. Jungbauer, *Comparison of protein A affinity sorbents.* J Chromatogr B Analyt Technol Biomed Life Sci, 2003. **790**(1-2): p. 35-51.
60. Hahn, R., et al., *Comparison of protein A affinity sorbents III. Life time study*. J Chromatogr A, 2006. **1102**(1-2): p. 224-31.
61. Wang, C. and F. Mann, *Increasing MAb Capture Productivity.* BioProcess International, 2009. **7**(5): p. 56 - 61.
62. Jiang, C., et al., *A mechanistic study of Protein A chromatography resin lifetime.* J Chromatogr A, 2009. **1216**(31): p. 5849-55.
63. Ghose, S., B. Hubbard, and S.M. Cramer, *Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials.* Biotechnology and Bioengineering, 2007. **96**(4): p. 768-779.
64. Wang, X.J., D.D. Fan, and Y.E. Luo, *Breakthrough model of recombinant human-like collagen in immobilized metal affinity chromatography.* Appl Biochem Biotechnol, 2009. **158**(2): p. 262-76.
65. Ghose, S., et al., *Antibody variable region interactions with Protein A: implications for the development of generic purification processes.* Biotechnol Bioeng, 2005. **92**(6): p. 665-73.
66. GEHealthcare, *MabSelect SuRe - studies on ligand toxicity, leakage, removal of leached ligand, and santization, GE Healthcare application note 11-0011-65 AB*. 2004.
67. AppliedBiosystems, *POROS® MabCapture™A Perfusion Chromatography® Media 4383704 Rev. A.* 2007.
68. AppliedBiosystems, *POROS® Perfusion Chromatography® Media 102BR05-02*. 2009.
69. Millipore, *ProSep®-vA Ultra Chromatography Media Lit. No. DS4241EN00*. 2004.

70. Millipore, *Affinity Chromatography Media Operating Instructions OIBP1131118 rev C*. 2005.
71. Fahrner, R.L., et al., *Performance comparison of protein A affinity-chromatography sorbents for purifying recombinant monoclonal antibodies.* Biotechnol Appl Biochem, 1999. **30 (Pt 2)**: p. 121-8.
72. Swinnen, K., et al., *Performance comparison of protein A affinity resins for the purification of monoclonal antibodies.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 97-107.
73. Millipore, *ProSep-vA Ultra Media 300 Cycle Lifetime Study*. 2005.
74. Umpleby, R.J., 2nd, et al., *Characterization of molecularly imprinted polymers with the Langmuir-Freundlich isotherm.* Anal Chem, 2001. **73**(19): p. 4584-91.
75. Horstmann, B.C.H., *Modelling the affinity adsorption of immunoglobulin G to Protein A immobilised to agarose matrices.* Chem Eng Res Des, 1989. **67**(May).
76. Gottschalk, U., *Process scale purification of antibodies*. 2009, Hoboken, N.J.: John Wiley & Sons. xxvi, 430 p.
77. Shukla, A.A., et al., *Downstream processing of monoclonal antibodies--application of platform approaches.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 28-39.
78. Arouri, A., et al., *Hydrophobic interactions are the driving force for the binding of peptide mimotopes and Staphylococcal protein A to recombinant human IgG1.* Eur Biophys J, 2007. **36**(6): p. 647-60.
79. Langone, J.J., et al., *Complexes prepared from protein A and human serum, IgG, or Fc gamma fragments: characterization by immunochemical analysis of ultracentrifugation fractions and studies on their interconversion.* Mol Cell Biochem, 1985. **65**(2): p. 159-70.
80. Diesenhofer, J., *Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9 and 1.8 Å resolution.* Biochemistry, 1981. **20**: p. 2361 - 2370.
81. Dima, S., et al., *Effect of protein A and its fragment B on the catabolic and Fc receptor sites of IgG.* Eur J Immunol, 1983. **13**(8): p. 605-14.
82. Ghose, S., B. Hubbard, and S.M. Cramer, *Protein interactions in hydrophobic charge induction chromatography (HCIC).* Biotechnol Prog, 2005. **21**(2): p. 498-508.
83. Hober, S., K. Nord, and M. Linhult, *Protein A chromatography for antibody purification.* Journal of Chromatography B, 2007. **848**(1): p. 40-47.
84. Schwartz, W., et al., *Comparison of hydrophobic charge induction chromatography with affinity chromatography on protein A for harvest and purification of antibodies.* Journal of Chromatography A, 2001. **908**(1–2): p. 251-263.
85. Sisodiya, V.N., et al., *Studying host cell protein interactions with monoclonal antibodies using high throughput protein A chromatography.* Biotechnology Journal, 2012. **7**(10): p. 1233-1241.
86. Biosciences, A., *Protein Purification: Handbook*. 2001: Amersham Pharmacia Biotech.
87. Salvalaglio, M., et al., *Molecular modeling of Protein A affinity chromatography.* Journal of Chromatography A, 2009. **1216**(50): p. 8678-8686.
88. Xia, H.-F., et al., *Molecular Modification of Protein A to Improve the Elution pH and Alkali Resistance in Affinity Chromatography.* Applied Biochemistry and Biotechnology, 2014: p. 1-11.
89. Hahn, R., et al., *Comparison of protein A affinity sorbents III. Life time study.* Journal of Chromatography A, 2006. **1102**(1–2): p. 224-231.
90. Ishihara, T., N. Nakajima, and T. Kadoya, *Evaluation of new affinity chromatography resins for polyclonal, oligoclonal and monoclonal antibody pharmaceuticals.* Journal of Chromatography B, 2010. **878**(23): p. 2141-2144.
91. Pierce, M.M., C.S. Raman, and B.T. Nall, *Isothermal titration calorimetry of protein-protein interactions.* Methods, 1999. **19**(2): p. 213-21.
92. Aldington, S. and J. Bonnerjea, *Scale-up of monoclonal antibody purification processes.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 64-78.
93. Nilsson, B., et al., *A synthetic IgG-binding domain based on staphylococcal protein A.* Protein Eng, 1987. **1**(2): p. 107-13.

94. Starovasnik, M.A., et al., *Antibody variable region binding by Staphylococcal protein A: thermodynamic analysis and location of the Fv binding site on E-domain.* Protein Sci, 1999. **8**(7): p. 1423-31.
95. Elliott, P., et al., *Quality by Design for biopharmaceuticals: a historical review and guide for implementation.* Pharmaceutical Bioprocessing, 2013. **1**(1): p. 105 - 122.
96. Rathore, A. and G. Sofer, *Life span studies for Chromatography and Filtration media*, in *Process validation in manufacturing of Biopharmaceuticals: Guidelines, Current Practices and Industrial Case Studies.*, A. Rathore and G. Sofer, Editors. 2005, CRC Press, Taylor & Francis Group: Boca Raton, FL. p. 169 - 203.
97. USFDA, *Compliance Program, Chapter 41, Inspection of Licensed Therapeutic Products.* 1999.
98. USFDA, *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use.* 1997. **February**.
99. EuropeanCommission, *Production and Quality Control of medicinal products derived by recombinant DNA technology*, in *III/3477/92*, E. Commission, Editor. 1994. p. 10.
100. ICH. *DEVELOPMENT AND MANUFACTURE OF DRUG SUBSTANCES (CHEMICAL ENTITIES AND BIOTECHNOLOGICAL/BIOLOGICAL ENTITIES) Q11*. 2012 May; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q11/Q11_Step_4.pdf.
101. Bussineau, C., et al., *PDA Technical Report #42.* 2005.
102. O'Leary, R., et al., *Determining the useful lifetime of chromatography resins.* BioPharm International, 2001. **14**(9): p. 10.
103. USFDA. *Pharmaceutical cGMPS for the 21st Century — A Risk-Based Approach: Second Progress Report and Implementation Plan*. 2003 [cited 2012 23SEP2012]; Available from: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/Manufacturing/QuestionsandAnswersonCurrentGoodManufacturingPracticescGMPforDrugs/ucm071836.htm.
104. Breece, T., E. Gilkerson, and C. Schmelzer, *Validation of Large-Scale Chromatographic Processes, Part 2 Results from the Case Study of Neuleze Capture on Macroprep High-S.* BioPharm 2002. **July**: p. 35 - 42.
105. Bannerjee, A., *Designing in Quality: Approaches to Defining the Design Space for a monoclonal antibody process.* BioPharm, 2010(May).
106. Natarajan, V. and S. Cramer, *A methodology for the characterization of ion-exchange resins.* Separation Science and Technology, 2000. **35**(11): p. 1719-1742.
107. Hilbrig, F. and R. Freitag, *Isolation and purification of recombinant proteins, antibodies and plasmid DNA with hydroxyapatite chromatography.* Biotechnol J, 2012. **7**(1): p. 90-102.
108. BioRad, *CFT Ceramic Fluoroapatite Instruction Manual*. 2000.
109. McCue, J.T., et al., *Use of an alternative scale-down approach to predict and extend hydroxyapatite column lifetimes.* J Chromatogr A, 2007. **1165**(1-2): p. 78-85.
110. Council_of_Europe, *European Pharmacopeia. Filgrastim Concentrated Solution <2206>*. Vol. 7. 2013, Strasbourgh: Council of Europe.
111. USP, *Color - instrumental measurement*, in *USP29*. 2006, United States Pharmacopeia. p. 2896-2898.
112. IUPAC, *Nomenclature for Chromatography.* Pure and Applied Chemistry, 1993. **65**(4): p. 819-872.
113. Ghose, S. and S.M. Cramer, *Characterization and modeling of monolithic stationary phases: application to preparative chromatography.* J Chromatogr A, 2001. **928**(1): p. 13-23.
114. Minitab, *Minitab Stat guide*. 2005, Minitab.
115. ICH. *Quality Risk Management Q9*. 2005; Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf.
116. USFDA. *Innovation and Continuous Improvement in Pharmaceutical Manufacturing Pharmaceutical CGMPs for the 21st Century*. 2004; Available from: http://www.fda.gov/ohrms/dockets/ac/04/briefing/2004-4080b1_01_manufSciWP.pdf.
117. Hill, C.P., T.D. Osslund, and D. Eisenberg, *The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors.* Proc Natl Acad Sci U S A, 1993. **90**(11): p. 5167-71.

118. Ricci, M.S., et al., *pH Dependence of structural stability of interleukin-2 and granulocyte colony-stimulating factor.* Protein Sci, 2003. **12**(5): p. 1030-8.
119. Pavisic, R., et al., *Recombinant human granulocyte colony stimulating factor pre-screening and screening of stabilizing carbohydrates and polyols.* Int J Pharm, 2010. **387**(1-2): p. 110-9.
120. Piedmonte, D.M. and M.J. Treuheit, *Formulation of Neulasta (pegfilgrastim).* Adv Drug Deliv Rev, 2008. **60**(1): p. 50-8.
121. Herman, A., T. Boone, and H. Lu, *Characterization, formulation and stability of Neupogen (filgrastim), a recombinant human granulocyte colony stimulating factor*, in *Formulation, Characterizationand Stability of Protein Drugs*, R. Pearlman and Y. Wang, Editors. 1996, Plenum Press: New York. p. 303 - 328.
122. Chi, E.Y., et al., *Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor.* Protein Sci, 2003. **12**(5): p. 903-13.
123. Chi, E.Y., et al., *Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation.* Pharm Res, 2003. **20**(9): p. 1325-36.
124. Pavisic, R., et al., *Differences between reversible (self-association) and irreversible aggregation of rHuG-CSF in carbohydrate and polyol formulations.* Eur J Pharm Biopharm, 2010. **76**(3): p. 357-65.
125. Burns, D. and A.L. Zydney, *Buffer effects on the zeta potential of ultrafiltration membranes.* Journal of Membrane Science, 2000. **172**: p. 39 - 48.
126. Gasch, J., C. Leopold, and H. Knoth, *Positively charge polyethersulfone membranes: The influence of Furosemide on the zeta potential.* Journal of Membrane Science and Technology, 2013. **3**(1): p. 1 - 5.
127. Suzuki, Y., T. Kanamori, and K. Sakai, *Zeta potential of hollow fiber dialysis membranes and its effects on hydrogen phosphate ion permeability.* ASAIO Journal, 1993. **39**(3).
128. Kim, K., et al., *Evaluation of electroosmosis and streaming potential for measurement of electric charges of polymeric membranes.* Journal of Membrane Science, 1996. **116**: p. 149 - 159.
129. Hubert, C., et al., *Improvement of a stability-indicating method by Quality-by-Design versus Quality-by-Testing: a case of a learning process.* J Pharm Biomed Anal, 2014. **88**: p. 401-9.
130. Orlandini, S., S. Pinzauti, and S. Furlanetto, *Application of quality by design to the development of analytical separation methods.* Anal Bioanal Chem, 2013. **405**(2-3): p. 443-50.
131. McAfee RF, M.H., Williams MA, *What is a barrier to entry?* The American Economic REview, 2004. **94**(2): p. 461 -465.
132. Thomas, P. *Is QbD a mandate for generics? FDA responds to confusion*. pharma QbD 2012 [cited 2013 30JAN]; Available from: www.pharmaqbd.com/qbd_mandate_generics_fda/.
133. Yu, L.X., et al., *Understanding pharmaceutical quality by design.* AAPS J, 2014. **16**(4): p. 771-83.