Mass spectrometric methods for analysis of therapeutic products and advanced proteomic analysis

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Abstract of Dissertation

The biopharmaceutical industry has played an important role in human lives in both academia and industry. Biopharmaceutical drugs have much higher development costs and complexity but with studies of basic science and biological, genomic research, there is continual progress in these important fields which have great potential to create commercial value in this market place. On the other hand, the basic sciences of manufacturing and analytics also are attracting many research efforts. Other key issues of concern include the manufacture of vaccines, and safe transportation of the drug product under refrigeration. It has become a global challenge to efficiently produce high quality drugs in remote areas or under emergency situations.

In this dissertation, the team headed up by MIT, designed a novel manufacturing platform and have applied novel yeast (*Pichia*) genomics to rapidly produce high quality biopharmaceutics which are more readily purified due to less host cell contamination. The InSCyT (Integrated and Scalable Cyto Technologies) for Flexible microbial manufacturing platform is both portable and convenient to produce biologics in remote locations.

In Chapter 2, we described the characterization of purified recombinant drugs from this novel system with full sequence coverage, ranging from the primary structure identification to possible post translational modifications by sensitive liquid chromatography coupled with tandem mass spectrometry (LC-MS). With the analysis of any product degradations that can occur during the fermentation or purification steps or during product storage, we observed that the manufactured biopharmaceuticals are at a high-quality level and comparable with the innovator drugs. The quality analysis was used to guide the upstream team to optimize their manufacturing protocol. We have built the resulting methodology for product identification into a robust approach for quality control and demonstrated that this final manufacturing platform is efficient, convenient and consistent in terms of product manufacture.
In Chapter 3, we provided the application of the InSCyT platform on to the production of the important biopharmaceutical, namely glargine an insulin analog. We also describe the characterization of the product with both bottom-up and top-down methods and the results from these various methods are comparable and consistent. The results of the analysis demonstrated that the products were prepared in a good quality in the initial upstream product. The characterization of glargine required a variety of techniques, including MALDI-TOF, Q-TOF, and LC-MS and we found that the products were secreted with full sequence coverage and with few modifications. In the future these analytical technologies will assist fermentation development and improve the subsequent production by a more robust system.

In Chapter 4, we demonstrated several analytical methods and techniques to characterize the protein therapeutics after incubation under stressed conditions. The resulting degradations from the accelerated stability study were monitored with LC-MS-MS. In addition, real time Raman spectroscopy techniques and electrokinetic measurements were utilized in this study and demonstrated a powerful ability to monitor degradation reactions, as well as determine the identity of a given biosimilar therapeutic product and any product variants. The study indicates these techniques are sensitive and robust and will provide an alternative method to conventional analytical methods for the monitoring of the manufacture of protein therapeutics. Such methods also have the potential to provide quality assessment of protein therapeutics manufactured in remote locations.

In Chapter 5, we described a proteomics study of breast cancer biomarkers with 1D gel and enzyme digestion coupled with LC-MS. The early detection of disease is always an important research area for the diagnosis and effective treatment of patients. In this study, we have characterized four cell lines including the secretome of cancer non-aggressive and aggressive cell lines using appropriate conditional medium to obtain information on potential biomarkers. We have applied HPLC tandem MS techniques and a proteomic strategy to characterize the cell lines. With a serial data analysis using information listed in data bases of
GeneCards VarElect, we have successfully curated a proteomic protein list. The evaluation of biomarker candidates derived from the searching of proteomic databases was then processed for bioactivity measurement, and as a promising lead we have found significant difference in expression of fibronectin in the aggressive cell lines. Future studies using RNA-sequence analysis, signal pathway analysis and bioactivity will be explored to confirm the disease associations of this biomarker.

In Chapter 6, we summarized and concluded our findings and provided a future view of the global health biopharmaceutical manufacturing and production studies.
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List of Acronyms

aa amino acid
ABC amnion bicarbonate
ACN acetonitrile
Asn asparagine
Asp aspartic acid
BRCA2 breast cancer type 2 susceptibility protein
CE capillary electrophoresis
CHO Chinese Hamster Ovary
C-HPP chromosome-centric human proteome project
CID collision induced dissociation
Cys cysteine
Da Dalton
DARPA Defense Advanced Research Projects Agency
DTT Dithiothreitol
EGFR epidermal growth factor receptor
ESI electrospray ionization
ETD electron transfer dissociation
FDA Food and Drug Administration
FT-ICR Fourier-transform ion cyclotron resonance
GCSF Granulocyte-colony stimulating factor
HEK human embryo kidney
hGH Human growth hormone
HPLC High Performance Liquid Chromatography
IAA Iodoacetic acid
IE ion exchange
IEF isoelectric focusing
IgG immunoglobulin G
**IsoAsp** isoapartic acid
**kDa** kilodalton
**LC** liquid chromatography
**LIT or LTQ** linear ion trap
**mAb** monoclonal antibody
**MALDI** matrix-assisted laser desorption/ionization
**Met** methionine
**mg** milligram
**MS** mass spectrometry
**MS/MS** tandem mass spectrometry
**m/z** mass to charge ratio
**PD** pharmacodynamics
**pI** isoelectric point
**PK** pharmacokinetics
**ppm** part-per million
**Pro** proline
**PTM** post-translational modification
**Q** quadrupole
**QE** Quadrupole Exactive
**Q-TOF** quadrupole time-of flight
**RP** reverse phase
**SDS-PAGE** sodium dodecyl sulfate-polyacrylamide gel electrophoresis
**SE** size exclusion
**SRM** selected reaction monitoring
**Tiam1** T-lymphoma invasion and metastasis-inducing protein 1
**TP53** DNA damage response/usually called tumor suppressor
**Tris** 2-amino-2-hydroxymethyl-propan-1,3-diol
**XCorr** cross-correlation
Chapter 1

Overview of the development and the analytical qualification of biopharmaceutical products
1.1 Abstract

The biopharmaceutical industry has played an important role in human lives in both academia and industry. Biopharmaceutical drugs have much higher development costs and complexity but with studies of basic science and biological, genomic research, there is continual progress in these important fields with potential to create commercial value in this market place. On the other hand, the basic sciences of manufacturing and analytics also are attracting many research efforts. Other key issues of concern include the manufacture of the vaccines, and safe transportation of the drug product under refrigeration.

This chapter describes the development of protein therapeutics from the cultivation and the quality control aspects and an overview of the background. Other important topics include the importance of global health and proteomics to characterize disease biomarkers. Also introduced is the significance of analytical developments in the biopharmaceutical arena and the overview introduces the nature of protein modifications, which are crucial for drug structure and functions. In the last part, this chapter briefly describes the techniques and applications of liquid chromatography coupled with mass spectrometry.

1.2 Global health and biosimilars

Global health is always a topic for scientists and socialists to explore due to the rapid development of medical and chemical technologies.\(^1\) Decades ago, scientists discussed the arrival of a new generation of health treatments especially from the 1990s.\(^2, 3\) Lancet has recently reported on health issues and opportunities in 1993 to 2035 from national and international viewpoints and from the financial viewpoint.\(^4\) Global burden of diseases was estimated. The mortality and burden of disease by region and cause were serious issues of WHO’s concern. The deaths causes from tuberculosis, malaria, and AIDS; that is the communicable
diseases, then maternal, perinatal and nutritional health; the deaths from non-communicable disease; and the
deaths caused by accident injury are all taken into consideration. For the baseline scenario, the trends in total
deaths from 2002 to 2030 are summarized in Fig.1.1 by WHO scientists.\textsuperscript{5} Global deaths are increasing in
the coming years, not only for the aging population reason, but the increase in aging related mortalities. The
biopharmaceutical area is now prevailing the whole world. China and India are remarkable new emerging
markets.\textsuperscript{6,7} The development of the biopharmaceutical area is regardless of the economic development of
a country, but has become a worldwide system for solving the health problem effectively.\textsuperscript{8} As far as we’ve
seen, with the dramatic gains in global health in these years and with an optimistic trend, scientific advances
will continue into the future if accompanied with a matched growth in technology and industry policies.

![Projected trends in total deaths for selected causes, baseline scenario, world, 2002-2030](image)

\textbf{Fig 1.1:} Projected trends in total deaths for selected causes, baseline scenario, world, 2002-2030

In 2015, the government has addressed the topic of Precision Medicine\textsuperscript{9} and encouraged research on pro-
teomics, metabolomics, and genomics to guide clinical practice. Oncology prevention, diagnosis and other effective treatments by precision medicine are expected to provide better understanding of the disease process. Therefore, many novel therapies will be expected to obtain benefits from this program. The biopharmaceutical industry is a growth market, with both high financial impact and with reduction of health issues.

From 1996, Bill and Melinda Gates Foundation has contributed millions of dollars to investigate the supply of medicines and health education worldwide which has resulted in the production of new vaccines, including monoclonal antibodies. From the year 1982, with the approval of the first biopharmaceutical, insulin, research including biology, chemistry, analytical sciences that are related to biosimilars has accumulated. The reduced development expense of biosimilars from both time and cost has appealed to the industry in recent decades.\textsuperscript{10,11} The biosimilar drug definition quoted from the FDA 351(k) is "A biosimilar product is a biological product that is approved based on showing that it is highly similar to an FDA-approved biological product, known as a reference product, and has no clinically meaningful differences in terms of safety and effectiveness from the reference product. Only minor differences in clinically inactive components are allowable in biosimilar products."\textsuperscript{12,13}

The definition of biosimilars has varied in different countries and regions,\textsuperscript{14,15} as indicated in Table 1.1. The drug analogs are referred to in different terms, which are described as similar biotherapeutic product (SBP) by WHO, subsequent-entry biologics by Canada, follow-on biologics (FOB) by US FDA, and only called biosimilars in Korea. Even the definition are not interpreted in a uniform manner, however, the important common disciplines are centered around the demonstration of safety and efficiency.
### Tab 1.1: The definition of biosimilar products in the world

<table>
<thead>
<tr>
<th>Term</th>
<th>By</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>WHO</td>
<td>A biotherapeutic product similar to an already licensed reference biotherapeutic product in terms of quality, safety and efficacy</td>
</tr>
<tr>
<td>SEB</td>
<td>Canada</td>
<td>A biologic drug that enters the market subsequent to a version previously authorized in Canada with demonstrated similarity to a reference biologic drug</td>
</tr>
<tr>
<td>FOB</td>
<td>US FDA</td>
<td>A product highly similar to the reference product without clinically meaningful differences in safety, purity and potency</td>
</tr>
<tr>
<td>Biosimilar</td>
<td>Korea</td>
<td>Biological products which demonstrated its equivalence to an already approved reference product with regard to quality, safety, and efficacy</td>
</tr>
</tbody>
</table>

The identification and quality control of the biotherapeutics requires a long and complex procedure. Generally speaking, as long as the amino acid sequence and 3D structure of active region of the biosimilar drug is same with the original patented medicine, the biosimilar would not require extensive clinical trials for different indications.\(^{16,17,18}\) However, the investigation process for a new drug is still a long and expensive way to achieve.\(^{19}\) For example, protein activity is often controlled by amino acid modifications which may effect structural stability and thus activity of the drug. Millions of dollars have been invested into the biopharmaceutical medical industry, but according to the safety, stability, toxicity, and other concerns, the approvals of innovative biopharmaceuticals are not very frequent. The FDAs Center for Drug Evaluation and Research (CDER), has approved a number of cancer related therapeutics in 2015 and recent years, as shown in Fig[1,2] and in fact oncology drugs have taken one third of the total approvals.
Among the past 15 years,\textsuperscript{20} FDA\textsuperscript{21} and EMA\textsuperscript{22} have granted 76 unique pharmaceuticals approval without the randomized control studies. The EMA has approved the first antibody in 2013,\textsuperscript{23} which has become a milestone in this area. To approve biopharmaceuticals, there are many controversial opinions around, especially focusing on clinical treatment studies. Randomized controlled trails are sometimes unnecessary for biosimilar pharmaceuticals due to a high degree of structural confidence in these drugs, however, the quality of the manufacturing process and the resulting of biopharmaceuticals still remain important issues. Hence, improvements in manufacturing and quality control testing are crucial to the field. The product safety is always the most important factor to consider when producing biosimilars.\textsuperscript{24, 25} As an example, the efficacy
and safety of many biosimilar and clinical trials have been studied, such as infliximab biosimilar. While the industry and market for biosimilars is a worldwide field the use of definitions and guidelines have differences in different regions. Wang has reviewed the regulatory approval procedures of biosimilar products by regions. The main guidelines are based on EMA and WHO, while at the same time scientists in Japan have reviewed their industry view of biosimilar development, and the regulation process in America is always important.

1.3 Biosimilar manufacture and delivery

The recombinant DNA technique that is used to produce a biosimilar was created in 1982, and during the past decades, the technologies have developed and advanced significantly. Based on special medical needs in emergency situations, DARPA has launched a program for medical treatments in remote areas especially focusing on the battlefield, which aims to provide a spontaneous cure based on the patients need. Although there are ways to produce therapeutics on small scale in the laboratories, the needs of a larger scale in short time with high quality are not easy as we can imagine. How the drug could be produced within one week or even overnight became a serious issue to save lives in the battlefield. There are many issues to manufacture vaccines and drugs in remote areas based on the traditional highly resourced pharmaceutical manufacturing process. Under stressed manufacturing conditions, how can one develop a process to efficiently produce a biosimilar by a suitable cell line and with high purity and safety and with suitable storage conditions that protect the drug? Clearly, there remain a lot of questions.

Besides the quality issues, the scale of the fermentation reactors is an important issue and portable small scale reactors are trending the research studies. For example, some institutes are making efforts on the development of portable devices to produce and evaluate biopharmaceuticals to more efficiently research
new medical treatments. Rao’s group\textsuperscript{33,34} has worked on innovating a portable fluorescence sensing bioreactor for many years with documentation in patents and publications, which focused on the measurement of amount and identification of biopharmaceuticals in relation to their bio-process techniques. However, while fluorescence is an effective technique it is not discriminating with large molecules such as proteins or antibodies. A number of novel techniques were applied in a related research program funded by DARPA, where the Love labs at MIT assembled a team to investigate a platform named InSCyT that focused on on-demand drug production in an efficient time period. The research aim of the team was to create a whole integrated platform to produce, purify, evaluate and release the biosimilar product for use at remote location. The DARPA mission is extremely challenging with the complexity of the biosimilar drug, the need for thorough characterization and the avoidance of safety issues.\textsuperscript{35} This challenging goal of assuring quality and safety of such new drugs, has been mitigated by the contributions of scientists for over a century, with novel technologies, clinical trial statistics, regulations, and other aspects. In our modern academic atmosphere and society, scientists already have contributed a lot.

The traditional manufacturing fermentation process for a recombinant DNA technique requires a long time to produce the final products where, the expression system mostly used mammalian (such as CHO) and \textit{E.coli} cell lines. These methods have been well studied and there are numerous researches on novel expression technologies traditional cell cultivation approaches. However, there are drawbacks with these techniques, for example with \textit{E.coli} production, in spite of an economical and timesaving fermentation process, the cellular secretory process may not be fully understand which can result in inefficient generation of a large protein.\textsuperscript{36} On the other hand, the most crucial aspect is a possibility of serious contamination with adventitious agents (endotoxins) and other host cell related impurities. With these considerations, the MIT team decided not explore more opportunities with the commonly used cell lines for innovations and development of the familiar upstream and downstream processes.
In recent decades, increasing studies have focused on exploring alternative expression systems, and the FDA has approved some novel products using alternative cultivation systems.\textsuperscript{37, 38, 39} The \textit{P. pastoris} produced Kalbitor has been approved by FDA because of the high tolerance in a condensed fermentation environment compared with the similar and traditional \textit{S. cerevisiae}. The yeast expression system has advantages over \textit{E.Coli} on purification and can result in isolation procedures, and the sufficient production in large proteins as well. With the InSCyT platform, research focused on using yeast production, \textit{Pichia}, which is easier to control in a fermenter, gave good yields of secreted bioactive product with minimal contamination of host cell protein. The Love lab produced different strains of \textit{Pichia} including \textit{K. pastoris}, \textit{K. phaffii} wildtype, and \textit{K. phaffii} GS115 and compared the proteins produced in the three strains.\textsuperscript{40, 41} During the manufacturing of protein drugs, the engineered modifications of the process such as changes in the amino acid sequence and undesired processing modifications such as chemical or physical degradations are all monitored in our quality analysis.

\section*{1.4 InSCyT platform}

The Love lab\textsuperscript{42} at MIT has developed an effective manufacturing platform named Integrated and Scalable Cyto-Technologies for flexible microbial manufacturing (InSCyT) for the Biologically-derived Medicines On Demand (BioMOD) program, aiming to produce high quality biopharmaceutics on demand in a short time period. The overview of the manufacturing process is shown in Fig\textsuperscript{1.3} containing upstream production and fermentation process, and downstream purification, polishing and ultrafiltration process. This platform also applied both on-line and off-line analytics to assure the quality. The cultivation fermentation only consumes less than 24 hours for one dose of therapeutic protein in 1.5L in volume. With further innovation, the team can now produce the therapeutic target in a fermentation system that contains three tanks to produce
within one day triplicates doses for emergency situations.

The InSCyT platform has innovated the manufacturing bioprocess with the use of yeast rather than mammalian CHO cell or plant cells productions. In this way, multiple drugs will be produced in the same time period by an easily programmed switch with chemical induction. For example, Tim Lu’s group has already developed and demonstrated the exposure of the production cells to methanol as an inducer to produce hGH or interferon.

This platform will generate selected pharmaceutical products with the use of controlled transcription factors. Another advantage of this system is the ability to use lyophilized yeast strain samples to minimize the cold
chain burden which is particularly advantageous in remote locations. The MIT team also innovated and developed a closed-loop process control with on-line techniques in order to monitor the fermentation process and if necessary adjust the production cycle. The platform utilized of novel affinity reagents, produced with a fusion tag system to efficiently capture and purify the products. Subsequently, product profiling utilized off-line mass spectrometry to discover product variants produced by fermentation issues in the development phase and then provide the evidence of a high level purity and low level degradations for products produced by the final platform. To monitor the quality of the products, several of analytical technologies and strategies have been utilized, including real-time monitoring Raman spectroscopy, UV detection, NMR, spectrofluorimetry, in addition to including off-line liquid chromatography with mass spectrometry. A fundamental need of the product testing stage is to determine the primary and secondary structure of the protein products. Then for measurement of the required biological properties, further testing with binding and bioassays were performed by the MIT team or contract testing laboratories but will not be further discussed in this report.

As discussed earlier the expiration of original pharmaceutical patents has allowed the development of biosimilar products and these were the focus of the MIT team. In this dissertation, we will demonstrate the characterization of the recombinant biopharmaceuticals produced from the InSCyT system, including human growth hormone, GCSF, glargine, interferon and antibodies. Aspects of the recombinant proteins such as the primary structures, stability, degradation variants and post-translational modifications will be discussed.

1.5 Proteomic studies

Proteomics is a challenging area of global study due to the complexity of the human set of proteins and much research must be performed before a targeted method for a disease specific biomarker can be developed.
High sensitivity, specificity and resolution are needed in the LC/MS analysis to well characterize the proteins or peptides in a complex samples such as human serum or plasma. To goal of such research is to identify biomarkers that can detect the early stage of a disease or to guide treatment of the patients. However, these aims will encounter the obstacles of protein post-translational modifications, degradations, and the presence of mutant variants, which can misdirect the analysis and the discovery of a biomarker suitable for the evaluation of the illness. If one looks at the history of medicine its important even urgent to investigate the advanced analytical methods and then differentiate the human proteins and their modifications precisely. The study of cancer biomarker proteins began in 1847 with discoveries by Henry Bence-Jones.\textsuperscript{45} The nowadays proteomic researchers have a huge advantage of the sequencing of the human genome and their studies rely on the derived amino acid sequence databases and predicted mass spectrometry data sets,\textsuperscript{46} however, not all potential sequences are expressed and determined. The identification of specific biomarkers from thousands of secreted proteins is still a challenge and termed the secretome.\textsuperscript{47}

Although predicted mass spectrometric datasets have been applied to many advanced proteomic studies with promising data results, the use of this tool has remained restricted by the complexity of clinical samples. The field continues to advance with innovative MS technologies which are well developed and updated in a fast pace. The high sensitivity, resolution and multi fragmentation functions of the latest mass spectrometers are designed and marketed by companies such as Thermo, Waters and Agilent. Mass spectrometry techniques, nowadays are more and more powerful than previous decades.\textsuperscript{48,49} Consequently, these novel instruments have been used in the research studies of biomarker discoveries and human diseases understandings. For these studies, the accuracy and tolerance of measurement of the protein ions are crucial to the identifications. From the other side, the identification of random mutations or post translational modifications must rely on sophisticated instrumentation, both in the liquid chromatography separation and an on-line mass analyzer. However, low abundance data sets are difficult to process and thus accurately diagnose the con-
stituent proteins or genes. MALDI-TOF\textsuperscript{50} has been applied in many research studies and aided the detection and evaluation of cancer biomarkers. While many advanced and sophisticated techniques have been applied in this field, however, to distinctly predict the biomarkers then directly help the patients in clinical trials there are still the need for continued progress in the analytical techniques. Research studies focusing on the molecular level of cancer cells and upregulated disease mechanisms are essential for scientists to comprehensively understand cancers. However, the barriers to identify the true biological indicators still exist and the field requires additional high resolution studies of cancer cells with different phenotypes.

1.6 Protein degradation and PTMs

the amino acid sequence of a protein is determined by the corresponding gene sequence, however, variants of expression may occur as a result of alternative splicing mechanisms. Then the biosynthetic process introduces post-translational modifications (PTMs) of the structure which may affect function by the inducing conformational changes in the 3D structure, altered cellular locations and modified protein-protein interactions. The diversity of PTMs is shown in Fig 1.4\textsuperscript{4} The PTMs described in this study include the most common ones, asparagine (Asn) deamidation, methionine (Met) oxidation, cysteine (Cys) disulfide bond linkages and glycosylation. The efficacy of a protein therapeutic may be influenced by dynamic changes of PTMs, which result in functional changes.\textsuperscript{52} Previous reports have monitored the existence of degradation reactions in samples corresponding to an innovator drug substance, biosimilar and counterfeit forms. In the reported study the approved biosimilar version contained similar level of the variants to the innovator product, however, a significantly higher level of variants existed in the unauthorized medication.\textsuperscript{53} The consequences of the variants on the drugs still remain to be studied in biological researches, but the quality control to accurately identify and characterize them are very necessary and can be accurately performed.\textsuperscript{54, 55, 56}
Aiming to detect the subtle differences of the biosimilar with the original product, a rigorous stability study is very necessary. The evaluation of protein stability will illustrate any changes in structure on storage that can effect protein dynamics and kinetics. These studies will also guide the development of appropriate formulations and storage conditions. Thus in order to well understand the function and efficacy of the biosimilar pharmaceuticals, stability test must be carefully designed, not only from a research view, but also in product development which is very important in both industry and clinical studies.

Fig 1.4: Diversity of post-translational modifications

There are many aspects of product quality to concern the development scientist. Beside the results of clinical trails, only from the analytical aspects, one determines the product degradations over time such as aggregations, oxidations under regular storage, which results in changes in the structures and functions of the protein, which could be crucial for predicting safety concerns for the patients. As for the complexity
of the protein drugs, since the biosimilar is not completely identical with the innovator product, a small differences could impact hugely in the stability of the final products. In some researches, scientists did stability examinations for a series of temperatures for 4 °C, 25°C, 37°C. In other studies, the shelf storage conditions were explored, and under freeze-thaw cycles. Moreover, stability evaluations have been taken under additional stressed conditions such as high oxygen, or extreme pH levels. These tests are compared with the innovator products and then the performance of biosimilar can be indicated. The reason for product instability can originate from many manufacture aspects, such as the upstream process with cell culturing, the cultivation environments, the level of gene product expression, also the downstream process including issues with purification or the polishing system that adds the final formulation buffer. Based on the theoretical three-dimensional conformation of a given proteins, the design of the stability study will include various conditions. For specific amino acid locations, the outside residues can be modified more easily than the buried core. In this study the stability detections will be focused on oxidation, deamidation, aggregation, as well as other modifications. As a consequence, the approval of a final product is not simply based on the presence of a correct sequence but will be evaluated from many other aspects to ensure the appropriate level of safety in the clinic.

1.6.1 Proteolysis

Proteolysis is a process used to enzymatically digest proteins into small peptides with proteases in order to effectively separated all protein fragments by HPLC and successfully analyze them by MS. Each enzyme has its best conditions for specificity and efficiency such as temperature, pH, duration of reaction, ratio of enzyme to substrate and other aspects. Peptide mapping also has limitations by the efficiency of proteolysis step. Enzyme selection is one of key parts of the experimental optimization process which is based on
both the structure of the protein of interest and the goal of the research. The enzymes can include specific and non-specific proteases, both of which have their unique limitations in the sequence mapping and their use will be applied based on known digestion specificity.\textsuperscript{59} Trypsin is the most commonly used specific protease that precisely cleaves at the carboxyl side of the amino acids arginine and lysine, with predictable peptides. Other well used enzymes in this study include LysC to cleave at the carboxyl side of lysine, GluC to cleave at the carboxyl side of glutamate or aspartate acids. On the other hand, non-specific enzymes such as pepsin aims to cleave at the carboxyl side of hydrophilic amino acids, and chymotrypsin to cleave at the aromatic amino acids. Additionally, multiple enzymes can be applied in one experiment to generate more cleavage sites to obtain peptides more appropriate for identification. For example, in a glycosylation study, PNaseF is a specific enzyme that releases N-linked oligosaccharides and is added after a trypsin digestion to identify the corresponding peptide backbones of glycopeptides.

1.6.2 Deamidation

Deamidation is one of the most common degradation reactions of proteins that occurs under high pH or temperature conditions in which the amide group is converted to a carboxylate group in the asparagine or rarely a glutamine residue. The mechanism\textsuperscript{60} is shown in Fig1. The symmetric succinimide intermediate results in two products from its hydrolysis, either forming an aspartate, or isoaspartate residue. Deamidation occurs readily if the Asn or Gln residue is followed by a glycine residue that provides minimal steric hindrance to the degradative reaction.\textsuperscript{7}
The strategy to characterize and quantitate the deamidation reaction has been developed in several ways even if there is only a 1 Da mass change, however, this small mass shift between asparagine and aspartate or isoaspartate can be detected and differentiated by a high resolution mass spectrometer. At the same time, the elution order of Asn, Asp and isoAsp are different according to their isoelectric point and to monitor the deamidation reaction, SDS-PAGE with IEF is commonly applied.

The MS-based method with either ECD or ETD fragmentation method have been studied and applied to differentiate between Asp and isoAsp residues which as isomers present unusual detection challenges. In this approach, the isomeric residues containing the respective side chain are cleaved with different fragmentation patterns and then generate c and z ions of different mass. After the fragmentation, isoAsp will generate c+57 and z-57 fragments which are unique from the Asp cleavage reactions.\textsuperscript{60, 61} Isotope labeling of oxygen is an alternative method that has been used to monitor and quantitate deamidation.\textsuperscript{62}
1.6.3 Oxidation

Oxidation may occur on the residues of methionine, tyrosine, tryptophan, and histidine, and the most common site is methionine. This residue in the protein sequence will be oxidized to methionine sulfoxide or even methionine sulfone and the chemical mechanism was shown in Fig 1.6. The sulfone is formed when a harsh oxidized condition occurs. The 3-D structure is the major determinant for the site of the degradations as steric accessibility often limits such reactions. With our previous experience, we expect that the amino acids that located on the outer region of the protein are easily exposed and degraded.

Fig 1.6: Mechanism of methionine oxidation

Since oxygen is everywhere, the oxidation reaction may occur in any stage of the procedures of protein therapeutic productions, such as fermentation, purification, formulation or transportation. Oxidation of residues of a protein can then promote aggregation processes, deactivation of function, and dynamic alteration of structure. The oxidation differences in original drug, biosimilars and counterfeit have been analyzed by Wu, which indicated the higher oxidation level in the counterfeit version. To monitor and evaluate the level of oxidation is essential to assure the quality and stability of a biopharmaceutical. In some cases, the
stability of the proteins under stressed condition will be investigated as well to further evaluate the oxidation level under extreme conditions and the drug capacity to resist degradation.

The oxidized peptide containing methionine and the normal peptide are easily differentiated by the large mass shift due to an additional oxygen which can be detected on a medium resolution mass spectrometer as well as elution order changes in the reversed phase LC separation as a result of the decrease in hydrophobicity of the peptide that contains the oxidized methionine. Under severe oxidized conditions, such as a hydrogen peroxide incubation, the sulfone moiety will formed as well. Of course, the structure and sequence of the protein decide the possibility of conformation changes. Based on previous studies, a methionine residue located on outer loop is more readily oxidized than a residue located in the inner loop of the protein structure.

1.6.4 Disulfide bond linkages

The covalent linkage between cysteine residues plays an important role in stabilizing protein structure. The formation of the disulfide linkage has been described in Fig 1.7 which is critical to maintain the structural composition of a protein. To characterize and identify a disulfide bond, mass spectrometry has become an essential technique, which determines not only the mass difference due to the linkage, but also the residue site and quantitation. For a MS analysis, ETD has the power to cleave the disulfide bond while CID cannot. For a small protein and simple structure, CID can be applied to the characterization of the peptide with cysteine residue based on the predicted sequence. ETD enables the breakage of the cysteine linkage and generates disulfide-dissociated species as well as charge-reduced species which can be determined with a high resolution and accuracy mass spectrometer.
1.6.5 Glycosylation

Glycosylation is an important PTM that occurs in the Golgi body and endoplasmic reticulum of a eukaryotic cell. As well studied, glycosylation has been separated into two major types, N- and O-glycosylation. While the motif that determines O-glycosylation if less predictable than for N-glycosylation the glycans are always attached to the residues of serine or threonine. For the N-glycosylation, there are many studies that have resulted in the determination of the NXS/T (the X is not proline) motif that allows the prediction of these structures. The research studies focusing on the functions of glycans and the safety concerns due to glycosylation changes, is summarized in the following review. 

In addition, compositional and linkage analysis are necessary to well characterize glycans. The pipeline of glycan qualification and evaluation always includes releasing glycans from the backbones and identification of glycopeptides with enzymatic methodologies. With the drawbacks of limited information of site-specificity of O-linked glycosylation, its complicated to identify the glycan structures with a straightforward method and thus multiple steps sample preparation are needed. The most widely used enzyme to
release N-linked glycans is PNGaseF.

Since the glycosylation structures are complex, the recognition and identification of the site location of individual structures is always determined by MS analysis of enzymatically generated glycopeptides (bottom-up techniques). The characterization of glycopeptides are basically estimated from the parent mass and abundance differences of the backbone peptides before and after the glycan release. Several ionization techniques could be applied to identify the structures, such as collision induced dissociation (CID), electron transfer dissociation (ETD) and high-energy collision dissociation (HCD).

In recent years, the bioinformatics technologies related to glycan analysis have undergone rapid exploration and put into service with huge databases. The data generated will assist the discovery of biomarkers and the structure predictions. The evaluation and monitoring of glycans are significant in clinical researches, in which the past has been an ignored field. Although the characterization of glycans is challenging, the utilization of online bioinformatic tools together with MS software are readily applied and effective.

There are several types of glycosylation, including N-linked glycosylation, O-linked glycosylation C-glycosylation and S-linked glycosylation (only found in bacteria). Aberrant patterns of N- and O-linked oligosaccharide modification in proteins are found in most of human cancers. This project will focus on N-linked glycosylation, and in order to fully understand the characterizations of glycans and the mutations in associated diseases, more research on this area needs to be achieved.

The source of the biological material used for analysis and structural detection provides different difficulties which can compromise results of the analysis. Blood or serum of the patients is the most commonly used, while tumor tissues, pleural and saliva samples also are used for diagnosis and validations. Glycosylation proteomics has become a new trend for cancer monitoring, since accumulating studies have indicated can-
cer cell shows a significantly different level in amount of glycans or mutated structures. Since the prediction of early stage cancers are not positive enough from characterization of these biomarkers, we must conclude that the detection of the disease stages is a challenge that will require better analysis tools. In recent years, although increasing attention has been focused on glycan biomarkers and glycomics, limitations still exist in most aspects, because of the complex nature of glycosylation modification and abnormal patterns in disease.

1.7 Analytical aspects

In today's research, many analytical techniques are used to identify chemical structures, such as fluorescence, UV-Vis, and Raman spectroscopy, etc. These techniques are effective in specific situations, however, they are not capable to determine the complex protein structural changes or quantitation of variants. Mass spectrometry is considered as one of the most powerful analytical techniques to identify and profile protein biomarkers. With the progress of methodology and techniques, the matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) methods have been commonly used in many research studies in the past decades. The sensitivity, efficiency and resolution of these two ionization techniques have had extensive use by scientists in this field.

MALDI was first mentioned/invented by Tanka, Hillenkamp and Karas in late 1980s and is a commonly applied ionization technique to analyze proteins and other biomolecules. MALDI ionization is usually coupled with time-of-flight (TOF) mass spectrometer and has the advantages of tolerances to moderate amounts of salts in millimolar concentrations, unlike ESI as well as sample preparation of complex mixtures with minimal steps. ESI offers a more gentle ionization procedure than MALDI, and the development of an online separation with LC is convenient. Desalting the sample is a crucial step needed to be considered before the MS analysis. A scheme of the mechanism of MALDI and ESI is shown in the Fig.
the convenience and effectiveness of this ionization method for both protein therapeutic characterization and clinical proteomics measurements, ESI has been selected in this project.

In most cases, MALDI will be applied with a time of flight mass spectrometers, named MALDI-TOF which used a laser pulse to desorb the samples from the matrix and then for ionization. The analyte was co-crystallized with an excess of the matrix such as α-Cyano-4-hydroxycinnamic acid (CHCA), and sinapinic acid (SA), etc. The matrix plays an essential role to vaporize the analytes by absorbing the laser energy. The laser pulse was applied to desorb and ionize the samples then the sample ions was accelerated by the high voltage in the vacuum system. The analyte ions pass through the evacuated tube arriving to the reflectron then to be analyzed.

The characterization of protein is a complex process and LC-MS is the most powerful way to accomplish that goal. This technique will lead the protein characterization into a well understood way. The proteins sequence, modifications, degradation variants are required to be determined, for the reason of the purity,
quality, efficacy, safety or toxicity of the biopharmaceutical. In the biological market and pharmaceutical industry, the analysis of bioproducts with LC-MS is very critical to success of the industry. In the recent decades, the analysis has majorly divided into top-down and bottom-up analysis, in other words, which the protocols are separated to intact protein and peptide fingerprint analysis, respectively. The two mass analysis tools have their own advantages and shortcomings to monitor the protein quality. In this section, the advanced LC-MS techniques for both top-down and bottom-up method will be described.

Fig 1.9: Characterization comparison of bottom-up and top-down methods; A, Bottom-up MS approach; B, Top-down MS approach
The bottom-up method is more commonly used to characterize the protein structure with digested peptide information derived from mass spectrometry analysis. With more detailed information from additional enzymatic digestions and repeated LC/MS analysis, this analysis can give a complete picture of protein structures but the preparation and post-analysis consumes significantly amounts of time and the digestion procedures can generate artifacts. Fig.1.9A indicates the process of the bottom-up approach.\textsuperscript{82} The proteins, or bioproducts will be digested into peptide fragments by a selected protease and then for analysis by a LC separation and MS analysis. The peptides will then be identified by the mass detector with advanced MS/MS determinations. The amino acids of the protein sequence will be specifically identified in this process. Although there are several limitations such as enzyme efficacy or resolution of instrument, peptides could still be precisely predicted and monitored because the analysis could be adjusted for a selected multi-enzymes digestion and development of an optimized LC-MS method for each digest. For a biotherapeutic characterization a 100% sequence coverage is required to ensure that there are no mutations in the sequence or PTMs. Proteins are much more complex to be measured than small molecules or short peptides due to the large structures and lower solubility, moreover, the modifications are also challenging to be easily detected by top-down techniques.

As shown in Fig.1.10, the peptide backbone will be cleaved in the indicated pattern.\textsuperscript{83} By mass spectrometry, in general, this will determine the amino-acid sequence according to the mass differences between the mass peaks. Peptide sequencing is a sensitive and robust way to identify the proteins. The peptide fragmentations are induced by the collisions and the amide bonds are the main breakage site due to the preferred lowest energy pathway.\textsuperscript{84} This pathway will provide b-ions by the amino-terminal fragment or y-ions by the carboxy terminal fragment.
Fig 1.10: MSMS peptide fragmentation

Top-down and bottom-up methodologies are both applied in modern proteomics researches. The top-down techniques are widely used\textsuperscript{85, 86, 87} for intact protein determination with high mass accuracy measurement. The proteins or bioproducts will be analyzed directly as the intact molecule without digestion which saves preparation time as well as artifacts generated by the digestion process. In our research, the bottom-up method is mainly applied to evaluate the quality of the proteins, and top-down instruments are used as well to accompany the digested analysis for small proteins drugs. At the level of a small protein level, intact protein analysis can reach full sequence coverage and successful determination usually can be achieved with a molecular weight less than 70kDa.\textsuperscript{88} However, the peptide fragmentation of the intact protein in the mass spectrometer at a large molecule level is less predictable than for analysis of an enzyme digested protein.

In summary top-down applications require a short time period from sample preparation to final analysis, however, the detailed information of post translational modifications may not be specifically obtained. For traditional methods, the FT-MS is more applicable for intact protein analysis based on its resolving power while so far, Orbitrap, TOF and more sophisticated instruments and software are under explored.\textsuperscript{89}

In this dissertation, the technology used is proposed to be optional to characterize the biological products in both crude and purified stages. The peptide mapping digests and the natural post-translational modifications in the proteins are required to be evaluated. In this analytical perspective, the intact protein analysis still remain challenging such as for variants identification, amino acid substitution, and quantitation, etc. The
intact analysis methodology may not provide an indicative strategy to the manufacturing process as the crude biologics may contain a high portion of buffer, which will give a large background signal that interferes with identification. Additionally, the remained host cell protein determination is another concern from the manufacturing process, which is challenge to a top-down analysis. Although intact protein analysis is a meaningful tool to rapidly evaluate the quality of a complex protein, our team decided to majorly apply the bottom-up analytical strategies in this dissertation.
References


[27] Dae Hyun Yoo, Nenad Prodanovic, Janusz Jaworski, Pedro Miranda, Edgar Ramitterre, Allan Lanzon,


[50] Haleem J Issaq, Timothy D Veenstra, Thomas P Conrads, and Donna Felschow. The seldi-tof ms


[85] Zachery R Gregorich and Ying Ge. Top-down proteomics in health and disease: Challenges and


Chapter 2

Advanced analysis of a series of recombinant therapeutics from the InSCyT platform by LC tandem mass spectrometry

Contributions:
Di Wu: concept contribution, experimental design, experiment procedures on recombinant hGH, GCSF, interferon, data analysis, manuscript writing and revision; Annie Y. Wang: Co-worker, experiment procedures on different recombinant proteins; MIT team: supply of samples from InSCyT platform; William Hancock: goal of the study, concept contribution, manuscript revision

Publications:

2.1 Abstract

Biosimilar therapeutics are marketed worldwide nowadays and stability assessment is necessary to validate the long term safety, purity and potency of the products. In addition, the manufacture of biopharmaceuticals are not easily controlled from either the upstream or downstream processes. In this study, we have applied yeast (Pichia) to produce biopharmaceutics instead of the traditional method using E.coli manufacture, to improve product stability with less host cell contamination. The InSCyT (Integrated and Scalable Cyto-Technologies) for Flexible microbial manufacturing platform is both portable and convenient to produce biologics. This generation platform is designed on a demand basis, so the quality obtained from a short time production is important. Our study has described the characterization of purified recombinant drugs from this novel system with coverage from the primary structure identification to possible post translational modifications by sensitive liquid chromatography coupled with tandem mass spectrometry (LC-MS). With the analysis of any product degradations, we observed that the manufactured biopharmaceuticals are at a high-quality level and comparable with the innovator drugs. The quality analysis was used to guide the upstream team to optimize their manufacturing protocol. We have built the resulting methodology for product identification and demonstrated that this final manufacture platform is efficient, convenient and consistent in terms of product manufacture.
2.2 Introduction

Biological therapeutics are a large market for both the academic arena and the clinical and financial field in modern society. However, the global health market for biopharmaceuticals is facing serious issues including drug innovations, methodology developments, quality control and transportation and storage conditions. To efficiently produce new clinical products to meet specific medical needs, however, traditional drug development requires long innovation times and huge financial support. In nowadays, biosimilar drug development has taken an important role in the market with short approval times with FDA review, minimizing cost as long as a highly similar structure and function is demonstrated with regard to the innovator therapeutics. To develop a stable formulation platform is important to produce high quality biopharmaceuticals and minimizing modifications. Recognizing the limitation of traditional manufacture, our goal aims to develop a small-scale platform to produce biological drugs efficiently and convenient for transportation to remote areas. The recombinant proteins are usually produced by E.coli, however, the DARPA team has focusing on minimizing host cell contamination, increasing cell density and expression accuracy, and thus have applied the production process to the yeast Pichia.

Our collaborators at the MIT lab have developed an effective manufacturing method named Integrated and Scalable Cyto Technologies for Flexible microbial manufacturing (InSCyT) for the Biologically-derived Medicines On Demand (BioMOD) program, aiming to produce high quality biopharmaceutics on demand in a short time. The recombinant DNA manufacturing process contains the upstream production and fermentation process, downstream purification, final polishing and an ultrafiltration process. This platform also applied both on-line and off-line analytics to assure the quality, however, traditional manufacturing of therapeutics require long cycle time of several days. The cultivation fermentation used by our team only consumed less than 24 hours for one dose. In fermentation development, the team evaluated different Pichia
strains to produce in high yield therapeutic proteins including, *K. pastoris*, *K. phaffii* wildtype, and *K. phaffii* GS115.\textsuperscript{5, 6}

Each step utilized during the production of recombinant proteins will possibly induce modifications and any PTMs and degradations reactions occurring during the cultivation are of concern and must be monitored. The safety, toxicity of resulting product analogs are required to be evaluated based on the structural characterization.\textsuperscript{7} As an example, the characterization of recombinant human growth hormone has been traced back to 1970s.\textsuperscript{8} The prediction of any post-translational modification and degradation reactions of the proteins target will depend both on the protein structure and manufacturing conditions.\textsuperscript{9} For example, in the sequence of human growth hormone, deamidation and oxidation are the major concern for the clinical efficacy and the safety of the therapeutic product.

In nowadays, the high resolution of modern analytical techniques as well as significant market opportunities are attracting more research studies. In this project, we have used liquid chromatography coupled with tandem mass spectrometry to evaluate the quality of yeast based recombinant protein therapeutics. From primary structure to modifications analysis to residual host cell proteins, the follow on products were compared with innovator marketed product samples. We observed that the new DARPA platform has a high level of reproducibility and stability to produce high quality biopharmaceuticals and that the platform could generate multiple protein products efficiently and with high purity. In this chapter, we will demonstrate result from the analysis of three purified protein drugs produced from InSCyT platform to show success of the system.
2.3 Experimental

2.3.1 Chemicals and materials

The chemicals including acetonitrile, hydrogen peroxide, ammonium carbonate sodium phosphate, dithiothreitol, and enzymes (trypsin, GluC and LysC) were analytical grade, obtained from Thermo (Thermo Fisher, USA). The reference standard was provided by commercial companies. The strain recombinant human growth hormone(hGH), GCSF, interferon α2b (IFN) samples were produced by InCSyT platform by our MIT collaborators. Stock samples were stored in -80°C.

2.3.2 SDS-PAGE

The strain hGH samples were standardized to 15 µg per loading based on the concentration provided by the collaborators in order to equalize the loading amount for comparison of proteins samples. The samples were diluted with deionized water and adding 5 µL sample buffer for non-reduced conditions. At the same time, the other batch of samples were prepared with additional 2 µL 1M DTT solution incubated at 90°C for 30 min for reduced condition. The protein mixtures were separated on SDS-PAGE at 160V for 45 min and stained with Coomassie blue. After the destaining process, the bands were monitored by BioRad Image system for further evaluation of the protein quality. In this experiment, an in-gel digestion was not performed.
2.3.3 In-solution digestion

Proteins were dialyzed via 10kD membrane Amicon centrifugal filter at 13,000 rpm for 15 minute and three times in ammonium carbonate sodium phosphate buffer (pH=7). The following in-solution digestion process with Trypsin for hGH and IFN samples, GluC and LysC for GCSF was kept overnight at 37°C. The digestion was terminated by addition of 20µL 5% formic acid. Proteins were then ready for LC-MS analysis, the remained materials were aliquot to 20 µL and stored in -80°C for further analysis.

2.3.4 Peptide assignment by LC-MS

LC-MS analysis used an Ultimate 3000 nano LC pump (Dionex, Mountain View, CA) and self-packed C18 column (Magic C18, 200Å pore and 5 µm particle size, 75µm internal diameter by 100 mm) connected to a coated 10µm internal diameter emitter (New Objective, Woburn, MA). LTQ-Orbitrap XL mass spectrometer was connected (Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source (New Objective, Woburn, MA). Mobile phase A was using 0.1% formic acid in HPLC grade water and mobile phase B was using 0.1% formic acid in acetonitrile. During sample injection, the flow rate was set 250 nL/min with 2% B for 25 min. The flow rate of the gradient was set at 200 nL/min, with mobile phase B, 0-60 min 2-40%, 60-70 min to 90% , 70-75 min 90% and 75-78 min 2%. The mass spectrometer was operated in a data dependent mode to switch between MS and CID-MS². Briefly, after a full-scan MS spectrum from m/z 400-2000 in the ion-trap, 8 CID-MS² activation steps were performed on the 8 most intense precursor ions from the full scan.

For peptide identification, raw data were searched against human Growth Hormone, GCSF, interferon α 2b sequence using in BioPharma Finder 2.0 software (Thermo Fisher Scientific). For peptide mapping,
searches were performed using a single-entry protein FASTA database with oxidation and deamidation set as variable modifications, 20 ppm mass accuracy, and a confidence level of 0.8 for MS/MS spectra. Final confirmation of the peptide identification was determined by manual inspection, extracting the base peak from the chromatogram and matching the MS² fragmentation data with theoretical prediction. The modification percentage was calculated by peptide peak area. Both non-degraded and degraded peptides were evaluated in the same LC-MS analysis with characteristic m/z differences and elution time shifts. The quantitation of the degradation could be calculated as the following: ratio of Degradation (such as oxidation, deamidation, etc) = peak area of [(degraded peptide )/(degraded peptide) + (Non-degraded peptide)] × 100%.

In this study, the biosimilar products from the advanced InSCyT platform were analyzed for not only the primary structures, as well as comparison of degradation products vs the standard to understand the purity profile to reach the FDA approval. For further stability studies, we developed an integrated stability method development and analytics which will be described in later Chapters. As the post-translational modifications are related with the efficacy,¹⁰,¹¹ stability or safety of the therapeutic drugs, the evaluation of the oxidation, deamidation and other variants are commonly measured.¹² As concerned, the N-terminal truncation of GCSF and IFN and the two-chain variants and oxidations of hGH are on our analysis priority.

### 2.4 Results for GCSF products

The first drug produced from InSCyT platform is the recombinant GCSF, which is a protein with 175 amino acids. The GCSF products were digested with GluC combined with LysC to improve the digestion efficiency of GluC.
2.4.1 Primary structure identification

The N-terminus of GCSF product has two significant modifications, the truncation resulting from gene expression in the host organism and oxidation of the first residue methionine. The HPLC comparison of each products and reference material are shown as Fig. 2.10. There protein samples are observed with full sequence coverage and comparable with the control. There are a total 12 cleaved peptides that were detected. The N-terminal peptide MTPLGPASSLPQSFLKCLE with a m/z 1067.07$^{(2+)}$ eluted at 29.64 min and was detected with the highest abundance in the reference sample, however, the G1 peptide (first peptide from GluC digestion) in GCSF products was not observed with the highest signal due to modifications occurring in the biosynthetic process. As an example of a less successful fermentation the product GCSF#2 was produced with a low concentration and its MS signal abundance was lower and with incomplete sequence coverage. The separated peptides were eluted based on their hydrophobicity, and with a consistent retention time in the four samples, and the results of peptide mapping has been summarized in the supplementary data Table 2.1. The products were produced at different times but the reproducibility of the manufacture platform was proven to be robust.
Fig 2.1: HPLC base peak of strain GCSF samples
2.4.2 Variants of GCSF products

2.4.2.1 Determination of oxidation

The sequence of GCSF starts from a N-terminal methionine residue that is located on the outer space of the structure. This N-terminal methionine is the easiest residue to be oxidized. There are three other methionine residues, Met122, Met127 and Met138 located at the inner side of the structure. The tendency of oxidation of these three methionines are various based on different buffer formulations and storage conditions and Met127 and Met138 may switch in the tendency for higher oxidation levels depending on the conditions. However, Met1 is the most likely oxidized residue, with the crucial first residue position, and thus the N-terminal peptide measurement was more complex than for other modified peptides.

![HPLC base peak of strain GCSF](image)

Fig 2.2: HPLC base peak of strain GCSF

As shown in Fig.2.2, the HPLC base peaks were shown for the N-terminal peptide. Not only the modification was demonstrated, but also the truncation of N-terminal residue was determined. The expected N-terminal
peptide with the mass of 893.98(2+) was eluted at 26.14 min, and the oxidized variant was eluted at 25.23 min, while the truncated N-terminal peptide with the m/z of 672.87(2+) was eluted at 22.90 min. The integrated peak area were calculated to give a 2.9% variant composition and used to guide the upstream team to optimize the generation process. The extracted fragment ion peaks were illustrated in Fig 2.3 and show peaks with a high confidence of identification.
Fig 2.3: N-terminal peptide characterization, MS2 identification of peptide MTPLGPASSLPQSFLLK
The missing residue which resulted in a protein variant with a lower pI is shown in Fig. 2.4 with the detection of significant additional bands. This variant band indicated lower pI of the product, illustrating the N-terminal truncation may induce the acidic variants or there are other impurities in this protein. From this point of view, the manufacturing team should be aware of the contamination in the fermentation process and to minimize the impurity level.

### 2.4.2.2 Determination of O-glycan variants

During the fermentation production, the reference GCSF sample corresponding to Lenograstim was derived from Chinese hamster ovary (CHO) cells and glycosylated and the sugar chain has been first studied by Ohdea.\(^\text{14}\) Two structures were obtained and analyzed by NMR. The glycosylation has been proved to be of limited clinical relevance.\(^\text{15}\) In our products from InSCyT platform, the existence of possible O-mannose
have been studied. The experimental data was shown in Fig. 2.5, which illustrated the residue T134 on peptide LGMAPALQPTQGAMPAPAFA which has potential glycosylation. The extracted ion peak for the non-glycosylated peptide with the m/z of 866.44(4+) and the detected glycosylated peptide with m/z 1048.49(4+) was eluted at 25.81 min, earlier than the non-glycosylated peptide eluting at 27.06 min, according to the relative polarity.

Fig 2.5: Analysis on O-glycosylation of strain GCSF products; XIC base peak and CID-MS2 of normal form and glycosylated form of peptide "LGMAPALQPTQGAMPAPAFA"

The figure shows a labeled fragmentation pattern for the fragment ions of the non-glycosylated peptide and the glycosylated peptide with two mannose residues. The structural heterogeneity of the peptide leads to a relatively low signal of the target peptide, however, we have detected this modification consistently in all
strain products except the #4 sample. Therefore, we could conclude that the potential glycosylation struc-
tures produced by the MIT platform contained two mannose residues in the GCSF products and for final
product characterization further analysis are needed for a precise structural confirmation and ratio of the
glycans. The existing glycan ratios in each product were varied, which will provide guidance to the manu-
facturing team to optimize the control conditions. All the modifications are summarized in supplementary
data Table.2.2. The T134 mannosylation was at a high level, present in above 50% in the strain samples.

### 2.5 Results for Interferon α2b products

The second product from the final test of the InSCyT platform is Interferon α2b (IFN). The purified samples
were concentrated and collected by centrifugal filters to obtain a suitable amount for HPLC analysis.

#### 2.5.1 Primary structure identification

Interferon α2b is a protein with 165 amino acids, including two pairs of disulfide bond, the Cys1-Cys98 and
Cys29-Cys138. The samples and the standard were treated under identical conditions as same with trypsin
in-solution digestion. The N-terminal sequence often contains the signal peptide of a protein and also know
as the leader peptide. In the biosynthetic process this short sequence will promote the cell to translocated
the newly synthesized protein to the cellular membrane and help secretion. In the strain interferon products,
residual leader peptide EEGVSLEK with a mass of 445.72 (2+) was identified. This leader peptide was
eluted at 12.72 min in the strain products, and was not detected in the reference material. The HPLC
separation chromatogram is shown in Fig.2.6 and the peptide mapping result is shown in supplementary
data Table.2.4.
Fig 2.6: HPLC base peak of strain Interferon samples
2.5.2 Variants of IFN products

2.5.2.1 Determination of leader peptide sequence

Fig 2.7: LC-MS analysis of leader peptide EEGVSLEK for the strain Interferon sample; Top figure: XIC of leader peptide of 446.72$^{2+}$; Bottom figure: CID-MS2 assignment of leader peptide

In our previous study, the leader peptide was identified as EEGVSLEKR, however, in this study trypsin was used to cleave the protein to prepare a digest for LCMS analysis and the terminal amino acid R was not detected. Therefore for a successful analysis we needed to select another enzyme to apply to a further study to specifically monitor the existence of an additional residue in the signal peptide. Even if the length of leader peptide has no effect of the function of a protein,\textsuperscript{16} the upstream manufacturing team for effective fermentation development needs to monitor any changes in N-terminal sequence. In Fig\textsuperscript{2.7} the extracted
ion chromatogram of the m/z 445.72(²⁺) ion is demonstrated, and the fragment ion peaks are shown. The y₁, y₂, y₄ and y₆ were detected, and the b₂, b₄, b₆ and b₇ were monitored as well. The leader peptide was measured with a level of 2.1% in strain interferon IFN#1, and raised up to 4.6% in IFN#4. This accumulation could be caused by an increase in the fermentation time to achieve an increased product yield.

![Image](image_url)

**Fig 2.8:** IEF gel image of strain Interferon samples

The variant of the leader peptide sequence resulted in a change of the pI of the protein and variant bands were detected in a gel analysis, shown in Fig 2.8. The additional N-terminal residue Glu (E) with an additional acidic carboxyl group lead to lower observed pI. The theoretical pI reported on Drugbank of interferon α 2b is 5.99. The major band of the biologic interferon products was observed with a pI at 6.0 and was comparable with the drug standard although migration values varied slightly for each lane. The amount of acidic bands observed from #1 to #4 was based on the darkness of the bands, and the observed result could be related to the date of each production batch and longer fermentation times could result in accumulated variants and thus be an issue in the future cultivation process. Besides, due to the lack of a potential deamidation residue.
in interferon sequence in the appearance of an additional acidic bands must be due to another mechanism such as an altered amino acid sequence.

2.5.2.2 Characterization of Oxidation

There are total of five methionine residues in the interferon sequence, with locating ranging from the surface to the hydrophobic core, and the entire sequence contains residue Met16, Met21, Met59, Met111 and Met148. The peptide T2 of interferon TLMLLAQMR contains two potential oxidized methionine residues, Met16 and Met21. The mass spectrometric data was used to differentiate the site of a specific oxidized residue and as shown in Fig.2.9 a peptide with the m/z 538.79(2+) was eluted at 23.31 min and two oxidized peptides with the same m/z 546.79(2+) were eluted at 20.50 min and 21.47 min. The assigned MS2 ion fragments confirm the presence of an oxidized residue. The Met16 oxidized peptide was not detected as the 2+ ion, however, the 1+ fragment was monitored. The detected b and y ion illustrated the modification site which allowed determination of the oxidized site and the amount.

The other potential oxidized residues have been characterized with the same data analysis method and the data are summarized in supplementary data Table 2.4. The residue Met111 is the least likely candidates to be modified. As a result of our analyses we have concluded that the purified products from InSCyT platform have comparable quality to the standard reference material.
Fig 2.9: LC-MS analysis of strain Interferon oxidation at Met16 and Met21: Base peak of non-oxidized peptide TLMLLAQMR and oxidized peptide; CID-MS2 peak assignments of non-oxidized and oxidized peptide.
2.6 Results for strain hGH products

The last protein product for evaluation of the platform is human growth hormone (hGH) which is essential for the treatment to children with growth hormone deficiency or hypopituitarism. hGH has been studied for a very long time for this manufacturing and production system. For the final product characterization, the MIT platform was able to manufacture hGH to achieve a product level of 5mg/mL that was the same unit level as with the commercial drugs. The products have been treated with tryptic digestion and without reduction of the disulfide linkages.

2.6.1 Primary structure identification

Recombinant human growth hormone (rhGH) was produced by yeast expression (*Pichia*) with an identical sequence for human growth hormone with 191 amino acids. The intra-disulfide linkages are Cys53-Cys156 and Cys182-Cys189 as the precise sequence and disulfide linkage are critical to the function and toxicity of biopharmaceuticals. In the analysis, the 191 amino acids sequence would be cleaved to 21 peptides by trypsin digestion.

We summarized the peptide mapping in Table 2.5 (supplementary data) for the primary structure identification. In the analysis all the expected peptide sequences may not be cleaved theoretically, and thus the missing residues are observed adjunceted with adjacent peptides, such as with peptides T3-T5, T17-T19 and need to be identified manually. In this section the hGH amino acids sequence is provided to show the connection of the intra disulfide bonds and potential modification sites.
Fig 2.10: HPLC base peak for the rhGH samples

The HPLC comparison of each product and reference material are shown as Fig 2.10. The peptides were eluted based on their size and polarity; the least RPLC retained peptides are T3 and T5, which only contain three amino acids and would not be retained on the reversed phase column. The peptides were eluted with a consistent retention time in the four samples, and the peptide mapping results have been summarized in Table 2.5. The N-terminal peptide FPTIPLSR which was observed with a m/z 465.77(2+) and eluted at 30.19 min was detected with the highest abundance. The manufacture platform was proven to be robust as the growth hormone products were produced with a high degree of reproducibility at different times of fermentation.
2.6.2 Variants of hGH products

For the concerns about the effects of degradation reactions on toxicity and efficacy for patient therapy it necessary to not only confirm primary structure but also measure degradative reactions such as oxidation.

2.6.2.1 Characterization of Oxidation

There are three methionine residues in the hGH sequence, which are the Met14, Met125 and Met170. Located at the surface area, the residue Met14 was the mostly easily oxidized methionine and the Met170 is located in the core area and resistant to oxidation and thus a crucial indicator of the protein structure.

The oxidized peptides will have additional mass of 15.994 Da for a 1+ oxygen ion or 7.997 Da for 2+ oxygen ion than the corresponding non-oxidized peptides. For the characterization method, the base peak from HPLC separation as well the extracted ion peaks for the oxidized species were analyzed. As shown in Fig 2.11 with the different polarity, the oxidized peptides have eluted earlier than non-oxidized peptides on reverse phase liquid chromatography, which could be differentiated by the base peak as well.
Fig 2.11: LC-MS analysis of the oxidized peptide (T2) from the tryptic digest rhGH
2.6.2.2  Characterization of Deamidation

Deamidation of the residue Asn149 was well characterized in our previous study\textsuperscript{20} and we have developed an optimized analytical method to determine the nature of the degradation reaction. The amount of deamidation monitored in this study based on the peak areas was low at 0.2% in the drug standard and lower than 1% in the products and corresponded to the retention time and mass observed in the previous studies (Table 2.6). However, the low levels of this modification resulted in the inability to observe the corresponding MS/MS fragmentation pattern. In addition, the corresponding IEF gel image (Fig 2.13) supported this conclusion with the observation of an additional minor band at a more acidic pI of 5.2.

![Fig 2.12: IEF gel image of strain hGH products](image)

Fig 2.12:  IEF gel image of strain hGH products
Fig 2.13: LC-NS analysis of the peptide T15 (FDTNSHNDALLK); Top: XIC of peptide with m/z 745.34$^{(2+)}$; Bottom: CID-MS2 peaks assignments

In this particular biological product sample set, we didn’t detect fragment ion peaks but the variant could be present at a trace amount and a lower level than the mass spectrometer detection setting range. Because the extracted base peak of the target peptide was detected based on previous experience, and the detection of CID MS2 assignment from previous study is shown in Fig.2.18 (supplementary data). The variant data was summarized in Table.2.6 (supplementary data). The amount of deamidation monitored in this study based on the peak area measurement was 0.2% in the drug standard and lower than 1% in the products. The IEF method was applied as well and the gel image was shown as Fig.2.13, the deamidation of asparagine will decrease the iso electric point of the protein. The additional faint band of variants at pI 5.2 were detected and reported by order of batch time.
In our previous study, the nondeamidated and deamidated peptides (T15) were identified through the accurate mass assignment by LC-MS. The deamidation mass shifts are the result of an increase of 0.9840 Da for 1+ ion and 0.4920 Da for 2+ ion as a result of the side chain changing of the deamidated peptide from asparagine to aspartic acid, NH$_2$ substituted by OH. The peptide LFDNAMLR was detected with the most abundance 2+ with 745.35, and the second high abundance 3+ with 490.25 and the deamidated peak was eluted later than the nondeamidated peptide. For specific hGH deamidation analysis, there are still ETD fragmentation techniques that could be applied in the future study.

2.6.2.3 Characterization of Disulfide linkage and two-chain cleavage

There are two disulfide linkages that exist in the hGH structure, that is the linkage of Cys53-Cys165 and Cys182-Cys189. To precisely characterize the connection, non-reduced conditions for enzyme digestion should be applied to assure the presence of the intact structure. In this research, CID fragmentation was used instead of ETD. The mass assignment of the strain samples was compared with the commercial standard.
Fig 2.14: LC-MS analysis of the disulfide linked peptide (Cys53-Cys165, T6-T16) from the tryptic digest rhGH

The connecting peptide with the m/z 941.71 for the 4+ ion, was eluted at 26.05 min. With the intra disulfide bond, the mass will be expected for the theoretical total mass minus 2 hydrogen atoms. The ion fragments were assigned to each backbone of the disulfide linkage in the CID fragmentation mode. In Fig 2.14, the major peaks of mass 671.22, 1031.18, 1545.84 are y6 and b17(3+) from T6 as the backbone. Fragments with the mass 897.81, 1067.78, and 1217.90 were detected as b8(4+), y4(2+) and y8(3+) from T16 as the backbone. Therefore, the observed fragment ions from the expected connecting peptides confirms the correct structure for the protein.
There are reported studies on the observation of two-chain variants of growth hormone and the effects this modification on protein function, safety or clinical efficacy. A scheme of hGH sequence and two-chain structure is compared shown in Fig. 2.15. The two-chain cleavage sites may not be reproducible by different cultivation methods due to the secretion of different proteases. In previous studies, the cleavage site was determined as residue T142, however, in this experiment with Pichia as the host organism, we detected the cleavage site is at residue Q141, which is reproducible in different fermentation samples, as shown in Fig. 2.17.
Fig 2.16: SDS-PAGE gel image of strain hGH products

The SDS-PAGE gel images of the strain hGH samples with reduced condition are shown in Fig.2.16. As the gel image indicated, the protein samples did not contain a significant level of covalent aggregates, but as the bands showed in the figures, there are fragments or two-chain variants observed. The identification of the variants and cleavages were described in detail in the following discussion. The gel images showed the major band with a molecular weight of approximately 22kDa with both standard and strain products. With the disulfide bonds cleaved by the reducing reagent, the major bands representing the intact protein sequence exhibit a slight increase in apparent MW due to an increase in hydrodynamic volume while the fragment bands at around 15kDa and 7kDa are observed as faint bands in the nonreduced gel, and get darker under the reduced conditions.
Fig 2.17: LC-MS analysis of the two-chain cleaved peptides from the tryptic digest strain hGH; XIC base peak of T13 with m/z 347.19(2+); T13+Q 411.22(2+); and the corresponding CID MS2 assignments

The precursor ion of T13 is 347.19(2+), eluted at 12.43 min; . The variant cleaved at Q134 peptide has the precursor ion with 411.22(2+) and elutes at 12.80 min. The fragmentation pattern of CID with b ion and y ion were labeled and indicated in the figure.

To characterize these variants, we needed to collect the peptide data of the predicted peptide with trypsin,
that is peptides T13 and T14, and the non-specific cleaved peptide. The specific protease site is the lysine (K133), on the peptide TGQIFK, however, the peptide of TGQIFKQ, which cleaved at residue Q134 has also been detected. From the CID-MS2 spectroscopy, the b ions and y ions of the peptides have been identified with high confidence level. The peak area of the variant and the amount of the variant are calculated with the assumption of a similar MS sensitivity.

2.7 Conclusions

Recombinant protein production and manufacture is a popular topic with many research studies. The manufacturing stage is a huge issue that needs the development of efficient techniques with high quality. In remote areas or at emergency situations, to requirement support the patient in their urgent need and to avoid fatal issues are very important. We have demonstrated the capability of the *Pichia* based production on the of InSCyT platform to operate with consistency and reproducibility. This bench size system is capable of generating multiple biopharmaceutics in a continuous production mode. Comparing with the drug quality level required for FDA approved, our products have been produced in a sufficient amount, full sequence coverage, and low level of host cell proteins. Although modifications such as oxidation were generated in the fermentation process, the quantity was within safety range and not clinically relevant. From the viewpoint of extended manufacturing, the amount of protein modifications observed in each sample lot was observed to accumulate from beginning to the end, which will suggest the need for continuous analytical monitoring of the manufacturing process to monitor the freshness of the system. For other properties of the drug such as toxicity, biofunction and pharmacokinetics, further studies will need to be performed to ensure comparability with the innovator product.
References


## 2.8 Supplementary Data

Tab 2.1: Peptide mapping of GCSF products

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Tab 2.3: peptide mapping of strain Interferon products

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<td>ITLYLKL</td>
<td>375.74^{2+}</td>
<td>19.07</td>
<td>19.04</td>
<td>19.04</td>
<td>19.00</td>
<td>19.03</td>
</tr>
<tr>
<td>T11 (132-134)</td>
<td>EKK</td>
<td>N/D</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>T13 (145-149)</td>
<td>AEIMR</td>
<td>310.16^{2+}</td>
<td>10.33</td>
<td>10.37</td>
<td>10.32</td>
<td>10.24</td>
<td>10.25</td>
</tr>
<tr>
<td>T14 (150-162)</td>
<td>SFSLSTNLQESLR</td>
<td>741.37^{2+}</td>
<td>22.33</td>
<td>22.36</td>
<td>22.37</td>
<td>22.36</td>
<td>22.34</td>
</tr>
<tr>
<td>T15 (163-165)</td>
<td>SKE</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
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<td>Std</td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
<td>#4</td>
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</tr>
<tr>
<td>Leader peptide (T0)</td>
<td>EEGVSLEK</td>
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<td>2.6%</td>
<td>3.7%</td>
<td>4.6%</td>
<td></td>
</tr>
<tr>
<td>Met16</td>
<td>TLMLLAQMR</td>
<td>0.3%</td>
<td>0.7%</td>
<td>1.5%</td>
<td>1.0%</td>
<td>1.0%</td>
<td></td>
</tr>
<tr>
<td>Met21</td>
<td>TLMLLAQMR</td>
<td>1.1%</td>
<td>0.8%</td>
<td>0.5%</td>
<td>0.2%</td>
<td>0.9%</td>
<td></td>
</tr>
<tr>
<td>Met59</td>
<td>AETIPVLHEMIQQIFNLFSTK</td>
<td>1.9%</td>
<td>1.1%</td>
<td>0.7%</td>
<td>0.9%</td>
<td>2.6%</td>
<td></td>
</tr>
<tr>
<td>Met111</td>
<td>FYTELYQQLNDLEACVIQGVGTETPLMK</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>Met148</td>
<td>AEIMR</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
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Tab 2.5: Peptide mapping of strain hGH products

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<th>Peptide</th>
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<th>m/z</th>
<th>Std</th>
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<th>#2</th>
<th>#3</th>
<th>#4</th>
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</thead>
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<tr>
<td>T1(1-8)</td>
<td>FPTIPLSR</td>
<td>465.77^{2+}</td>
<td>21.03</td>
<td>21.02</td>
<td>21.02</td>
<td>21.08</td>
<td>21.01</td>
</tr>
<tr>
<td>T2(9-16)</td>
<td>LFDNAMLR</td>
<td>490.25^{2+}</td>
<td>20.49</td>
<td>20.51</td>
<td>20.51</td>
<td>20.48</td>
<td>20.5</td>
</tr>
<tr>
<td>T2-T3</td>
<td>LFDNAMLRAHR</td>
<td>448.20^{3+}</td>
<td>12.83</td>
<td>12.95</td>
<td>12.93</td>
<td>12.89</td>
<td>12.93</td>
</tr>
<tr>
<td>T4(20-38)</td>
<td>LHQLAFDTRYQEFEE-EAYIPK</td>
<td>781.38^{3+}</td>
<td>24.47</td>
<td>24.49</td>
<td>24.47</td>
<td>24.56</td>
<td>24.89</td>
</tr>
<tr>
<td>T4-T5</td>
<td>LHQLAFDTRYQEFEEAYIPKEQK</td>
<td>682.83^{4+}</td>
<td>22.8</td>
<td>22.79</td>
<td>22.89</td>
<td>22.89</td>
<td>22.86</td>
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<tr>
<td>T6(42-64)</td>
<td>YSFLQNPCQPSLCEFSE-SIPTPSNR NYGLLYCFR</td>
<td>941.703^{4+}</td>
<td>26.05</td>
<td>26.05</td>
<td>26.02</td>
<td>26.01</td>
<td>26.07</td>
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<tr>
<td>T7(65-70)</td>
<td>EETQQK</td>
<td>762.36^{+}</td>
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<td>1.98</td>
<td>2.09</td>
<td>1.95</td>
<td>n/d</td>
</tr>
<tr>
<td>T8(71-77)</td>
<td>SNLELLR</td>
<td>422.74^{2+}</td>
<td>18.15</td>
<td>18.12</td>
<td>18.09</td>
<td>18.06</td>
<td>18.07</td>
</tr>
<tr>
<td>T7-T8</td>
<td>EETQQKSNLELLR</td>
<td>529.94^{3+}</td>
<td>15.65</td>
<td>15.72</td>
<td>15.7</td>
<td>15.7</td>
<td>15.8</td>
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<tr>
<td>T9(78-94)</td>
<td>ISLLLIQSWLEPVQFLR</td>
<td>1028.10^{2+}</td>
<td>31.74</td>
<td>31.8</td>
<td>31.8</td>
<td>31.84</td>
<td>31.88</td>
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<tr>
<td>T10(95-115)</td>
<td>SVFANSLVYGASDNS-VYDLLK</td>
<td>754.71^{3+}</td>
<td>27.24</td>
<td>27.14</td>
<td>27.21</td>
<td>27.18</td>
<td>27.1</td>
</tr>
<tr>
<td>T11(116-127)</td>
<td>DLEEGIQLTLMGR</td>
<td>681.34^{2+}</td>
<td>25.92</td>
<td>25.94</td>
<td>25.92</td>
<td>25.89</td>
<td>25.85</td>
</tr>
<tr>
<td>T12(128-134)</td>
<td>LEDGSPR</td>
<td>387.19^{2+}</td>
<td>8.32</td>
<td>8.35</td>
<td>8.4</td>
<td>8.34</td>
<td>8.45</td>
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<tr>
<td>T13(135-140)</td>
<td>TGGIFK</td>
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<td>12.54</td>
<td>12.53</td>
<td>12.51</td>
<td>12.55</td>
</tr>
<tr>
<td>T14(141-145)</td>
<td>QTYSK</td>
<td>626.31^{+}</td>
<td>2.79</td>
<td>2.85</td>
<td>2.82</td>
<td>2.83</td>
<td>2.85</td>
</tr>
<tr>
<td>T15(146-158)</td>
<td>FDTNSHNDALLK</td>
<td>497.23^{3+}</td>
<td>15.37</td>
<td>15.43</td>
<td>15.41</td>
<td>15.43</td>
<td>15.39</td>
</tr>
<tr>
<td>T19(173-178)</td>
<td>VETFLR</td>
<td>382.71^{2+}</td>
<td>16.52</td>
<td>16.45</td>
<td>16.54</td>
<td>16.53</td>
<td>16.51</td>
</tr>
<tr>
<td>T20(179-183)</td>
<td>IVQCR</td>
<td>701.82^{2+}</td>
<td>14.5</td>
<td>14.56</td>
<td>14.55</td>
<td>14.46</td>
<td>14.53</td>
</tr>
<tr>
<td>T21(184-191)</td>
<td>SVEGSCGF</td>
<td></td>
<td></td>
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</table>
Tab 2.6: Summary of observed modifications of strain hGH products

<table>
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<tr>
<th>Sequence Coverage</th>
<th>STD</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
</tr>
</thead>
<tbody>
<tr>
<td>M14 (oxidation)</td>
<td>LFDNAMLR</td>
<td>0.77%</td>
<td>0.67%</td>
<td>0.77%</td>
<td>1.55%</td>
</tr>
<tr>
<td>M125 (oxidation)</td>
<td>DLEEGIQTLMGR</td>
<td>1.89%</td>
<td>0.72%</td>
<td>0.48%</td>
<td>0.90%</td>
</tr>
<tr>
<td>M170 (oxidation)</td>
<td>KDMDKVETFLR</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>N149 (deamidation)</td>
<td>FDTNSHNDALLK</td>
<td>0.2%</td>
<td>0.6%</td>
<td>0.2%</td>
<td>0.9%</td>
</tr>
<tr>
<td>Q141 (Two-chain)</td>
<td>n/d</td>
<td>0.13%</td>
<td>1.29%</td>
<td>3.90%</td>
<td>3.30%</td>
</tr>
</tbody>
</table>
Fig 2.18: LC-MS analysis of the deamidated peptide (T15) from the tryptic digest of rhGH
Chapter 3

Combined top-down and bottom-up mass spectrometric approach to recombinant glargine product characterization approaches

Contribution:

Di Wu: concept contribution, experiment procedures on recombinant glargine, data analysis and interpretation, manuscript writing and revision;

Di Liu: MIT team collaborator, strain sample preparation

William Hancock: goal of the study, manuscript revision

This material is based upon work supported by the Defense Advanced Research Projects Agency (DARPA) and SPAWAR Systems Center Pacific (SSC Pacific) under Contract No. N66001-13-C-4025.
3.1 Abstract

The industry of biopharmaceuticals has been studied in great detail in humanity in recent decades. To efficiently produce the high quality drugs in remote areas or under emergency situations has become a global challenge. Insulin is very commonly required and when available used for the treatment of diabetes. To produce high quality biosimilar drug with a improved treatment profile and lower cost, the development of glargine would be significant for both the medical and biological field. The research funded by DARPA and developed by MIT has developed a small scale production technique, namely the InSCyT platform. We describe the characterization of the product with both bottom-up and top-down methods and the results from these various methods are comparable and consistent. The results of the analysis demonstrated that the products were prepared in a good quality even in the initial upstream product. The characterization of insulin glargine includes applying a variety of techniques, including MALDI-TOF, Q-TOF, and LCMS and we found that the products were secreted with full sequence coverage and with few modifications. These analytical technologies will assist fermentation development and improve the subsequent production by a more robust system in the future.
3.2 Introduction

Diabetes is one of the worst diseases globally, and due to co-morbidities patients with diabetes may induce cancer as well. There are many studies focusing on the chemical, biological or clinical views of diabetes and insulin therapy. With strong clinical evidence to indicate that insulin will significantly decrease the glucose level of patients with both type I and II diabetes. In the insulin pharmaceutical market, the original insulin patents will expire in the coming future with the promise of biosimilar versions. This is a potentially welcome development as the global market has increased rapidly and reached a high cost level of 50 millions.

As well known, insulin is essential for the treatment of diabetes type I and II. As the first time to produce biosimilar of insulin (Humulin) was in the 1980s, this industry has appealed accumulated number of studies. The therapeutic from the successful insulin production, the market is continuously expanding from the past decades. Both industrial companies or academic institutes are focusing on more opportunities to introduce and explore the development of high quality biosimilar insulins to the market. However, the approval of analogs are not easy in any countries, such as in the U.S and EU, the application and approval process are complicated and the failed follow-on applications will be detailed. The safety, quality, stability, manufacturing efficiency, efficacy and many other aspects must be evaluated to obtain an approval. Sequence modification between of the biosimilar (Lantus glargine, Novolin, Levemir, and etc.) with the innovator insulin may affect the pharmacokinetics and pharmacodynamics. To date, the modifications are designed to improve the functions for patients need to change the glucose level, such as rapid acting insulin analogues (Novolog, etc) and long duration drug (Lantus glargine, etc).

The traditional cultivation of recombinant proteins with E.coli expression induce contamination to the man-
ufacturing process, other bioactivities, or even inaccuracy on expression. To minimize these issues, our team applied yeast *Pichia* cultivation to enhance gene expression and improve product quality. This platform called Integrated and Scalable Cyto-Technologies for Flexible microbial manufacturing (InSCyT) was developed by our team for the Biologically-derived Medicines On Demand (BioMOD) program, aiming to efficiently produce high quality biopharmaceutics in remote locations. The platform and products have been introduced in Chapter II. The quality of the final purified biopharmaceuticals such as recombinant human growth hormone and GCSF were performed at a high analytical level. In fact, during the development of the upstream and downstream processes, there is long development cycle to achieve the required level of purified product. The fermentation, manufacture and progress of each production steps are important and in this context the crude protein drug is the beginning of the final success. It's hard to control the manufacture quality and reproducibility and difficult to determine the quality of the resulting crude proteins. In this chapter, we have not only introduced both top-down and bottom-up technologies on to characterize the reference materials we will also report on the analytical data of the crude protein product and illustrate the guidance and suggestions we gave to the manufacturing teams. In the future, the advanced purified drugs from the platform will be further studies by our techniques to provide a detailed analysis relative to the corresponding reference drug.

The bottom-up method is more commonly used to characterize the protein structure with digested peptide information from mass spectrometry analysis. With more detailed information from additional enzymatic digestions, the analysis would be more accurate but the preparation and post-analysis consumes significantly more time. The proteins, or bioproducts will be digested into small pieces by a selected protease and then for analysis by a LC separation and MS analysis. The peptides will be identified by the mass detector with advanced MS/MS determination. The amino acids of the protein sequence will be specifically identified in this process. Proteins are much more complex than small molecule or short peptides to be measured due
to the large structures and lower solubility, moreover, the modifications are also challenging to be easily detected by top-down techniques. Top-down and bottom-up methodologies are both applied in analytical researches. The top-down techniques are widely used\textsuperscript{17, 18, 19} for intact protein determination with high mass accuracy measurement. The proteins or bioproducts will be analyzed directly as the intact molecule without digestion which saves preparation time as well as artifacts generated by the digestion process. At the level of a small protein level, intact protein analysis can reach full sequence coverage and successful determination usually can be achieved with a molecular weight less than 70kDa.\textsuperscript{20} However, the peptide fragmentation of the intact protein in the mass spectrometer at a large molecule level is less predictable for analysis of an enzyme digested protein. In summary top-down applications require a short time period from sample preparation to final analysis, however, the detailed information of post translational modifications may not be specifically obtained. For traditional methods, the FT-MS is more applicable for intact protein analysis based on its resolving power while so far, Orbitrap, TOF and more sophisticated instruments and software are under explored.\textsuperscript{21}

In this chapter, we will introduce the characterization of recombinant glargine with both top-down and bottom-up approaches. We also applied the method to crude strain glargine protein samples.

3.3 Experimental

3.3.1 Chemicals and materials

The glargine standard was obtained from Lantus (refer as glargine), the compared insulin standard was obtained from Humulin (refer as insulin), and the strain glargine samples were produced by MIT laboratory. The chemicals including acetonitrile, Tris-HCl sodium phosphate, guanidinium chloride, dithiothreitol,
iodoacetamide, and trypsin and GluC were analytical grade, obtained from Thermo (Thermo Fisher, USA).

3.3.2 In-solution digestion

50µg glargine sample were dissolved in 6M guanidinium chloride, reduced by 10 mM dithiothreitol under 70°C for 30 minute and followed by alkylation with 55mM iodoacetamide under room temperature in dark condition. Proteins were dialyzed via 10kD membrane Amicon centrifugal filter at 13,000 rpm for 15 minute and three times in Tris-HCl buffer (pH=4.0). The following in-solution digestion process with Trypsin and GluC was kept overnight at room temperature to avoid artificial oxidation. Proteins were then ready for LC-MS analysis, the remained materials were aliquot to 20 µL and stored in -80°C for further analysis.

3.3.3 In-gel digestion

The strain samples were prepared to 15 µg as the concentration provided by the collaborators in order to equalize the loading amount of proteins for comparison. At the same time, the other batch of samples were prepared with additional 2 µL 1M DTT solution incubated at 90°C for 30 min for reduced conditions. The protein mixtures were separated on SDS-PAGE at 160V for 45 min and stained with Coomassie blue. After a destaining process, the bands were monitored for further evaluation of the protein quality.

The gel slices were washed with 500 µL ACN and 0.1 M NH₄CO₃ for 45 min shaking and then centrifuged, the supernatant were removed. Proteins were followed by a reducing process by adding 500 µL of 10 mM dithiothreitol in 0.1 M NH₄HCO₃ and incubated for 30 min at 56°C with centrifuging and removing supernatant before the alkylation process. Proteins were alkylated with 500 µL of 55 mM iodoacetamide in 0.1 M NH₄HCO₃ under room temperature and kept in dark for 60 min. All supernatant were removed after
spinning the gel pieces down and the following digestion process. Trypsin was added to protein solution and the concentration was based enzyme : protein ratio of 1:100 to 1:20 (w/w). The samples were covered by trypsin and GluC buffer and incubated overnight at 37°C to yield peptides. The digestion process was stopped with 50 µL 5% formic acid and extracted with acetonitrile. All supernatants were combined and stored for the following LC-MS analysis.

### 3.3.4 Isoelectric Focusing measurement

Insulin Humulin and Insulin glargine standard samples were prepared under stressed condition at pH 9, 37°C incubation for 3 days. This stability tests the variation of deamidation level with the protein sequence difference as the variants will be observed with different isoelectric focusing points relative to the test samples. After the stressed incubation, we prepared the humulin and glargine samples to 1 mg/ml total protein concentration and mixed 10 µL of the samples 1:1 with Novex IEF sample buffer pH 3-10 (2x (Thermo Fisher, USA)). After loading the humulin and glargine standard and the samples which has been with stressed under alkaline conditions, we set the program in the power supply at constant voltage of 100V for 1hr, 200V for 1h and 500V for 30min. When the program is complete, the gel was fixed with 12% trichloroacetic acid. The staining and destaining will terminate the whole process to evaluate the bands.

### 3.3.5 Measurement of HPLC mass spectrometry

LC-MS analysis was used an Ultimate 3000 nano LC pump (Dionex, Mountain View, CA) and a self-packed C18 column (Magic C18, 200Å pore and 5 µm particle size, 75µm internal diameter by 100 mm) connected to a coated 10µm internal diameter emitter (New Objective, Woburn, MA). A LTQ-Orbitrap XL mass spectrometer was connected (Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source.
(New Objective, Woburn, MA). Mobile phase A was using 0.1% formic acid in HPLC grade water and mobile phase B was using 0.1% formic acid in acetonitrile. During sample injection, the flow rate was set 250 nL/min with 2% B for 25 min. The flow rate of the gradient was set at 200 nL/min, with mobile phase B, 0-60 min 2-40%, 60-70 min to 90%, 70-75 min 90% and 75-78 min 2%. The mass spectrometer was operated in a data dependent mode to switch between MS and CID-MS$^2$. Briefly, after a full-scan MS spectrum from m/z 400-2000 in the ion-trap, 8 CID-MS$^2$ activation steps were performed on the 8 most intense precursor ions from the full scan. All control and variants samples were run in triplicate.

BioPharma Finder 2.0 software (Thermo Fisher Scientific) was used for analysis of all data acquired on the peptide and protein level. For peptide mapping, searches were performed using a single-entry protein FASTA database with oxidation and deamidation set as variable modifications, 20 ppm mass accuracy, and a confidence level of 0.8 for MS/MS spectra. Final confirmation of the peptide identification was determined by manual inspection, extracting the base peak from the chromatogram and matching the MS$^2$ fragmentation data with theoretical prediction. The modification percentage was calculated by peptide peak area.

### 3.3.6 Q-TOF measurement of intact protein analysis

Protein profiles were analyzed using reverse-phase liquid chromatography (RPLC) and a Xevo G2-S Q-ToF (Waters Corp, Milford, MA). Liquid chromatography was performed at 0.2 mL/min using an H-Class Acquity ultra-high pressure liquid chromatography system (UPLC) (Waters Corp, Milford, MA) on a BEH300-C4, 2.1 mm x 100 mm column, with pore size of 1.7 µm (Waters Corp, Milford, MA). Buffer A consisted of 0.1% formic acid (v/v) in HPLC grade water and buffer B consisted of 0.1% formic acid (v/v) in 100% HPLC grade acetonitrile (v/v). A 26 minute gradient was used: 0-5 min 5% B, 6-15 min 5-90% B, 16-20 min isocratic at 90% B and reducing to 5% B from 21 min to the end, samples were introduced via an
electrospray ion source in-line with the Xevo G2-S Q-ToF. External calibration of m/z scale was performed using sodium cesium iodide. The Q-ToF parameters were run in resolution mode, scanning m/z 400-4000, 3.00kV capillary voltage, 40V cone voltage, 120°C source temperature, 350°C de-solvation temperature, and 800L/h de-solvation gas. MS/MS data were collected at a scan speed of 0.1s. Data were manually interpreted using the UNIFI software package (Waters Corp, version 1.7.1).

3.3.7 MALDI measurement of intact protein analysis

Sinapic acid (Sigma Aldrich, 85429) at 10 mg/mL in ACN:wate (1:1) was mixed with sample solution in 40:1 ratio, and 0.5 mL of the mixture was deposited on MALDI plate and air-dried. The MALDI-TOF/TOF-MS was a model 5800 from AB-SCIEX (Framingham, MA) with a frequency-tripled Nd:YAG laser at 349 nm, and operated in the positive ion mode. The delay time for reflectron MS mode was 750 ns. TOF/TOF was in a positive 1 kV mode with air ($1 \times 10^{-6}$ Torr) as the collision gas, and a mass window of $\pm 5$ Da.

3.4 Results and discussion

Insulin, as we know, as a treatment for diabetes, is very important for adults to lower levels of glucose in the blood. The sequence of insulin and glargine has differences from the native human insulin by 3 amino acid residues, which lead changes in the stability of the protein and degradations pathways. In glargine vs. insulin the amino acids glycine in substituted in the place of asparagine at chain A site21, and the two arginines attached to the chain B at sites 31 and 32 as the C-terminal difference, see Fig[3.1] for the sequence of glargine and amino acid substitutions. The identification and characterization include intact protein identification with bottom-up identification with HPLC-MS/MS and the top-town strategy such as
As shown in Fig. 3.1, the sequence of glargine and differences of the amino acids. The sequence of insulin and glargine has differences with the native human insulin by 3 amino acids, which lead to the stability and degradations reactions changing.
3.4.1 Bottom-up analysis

3.4.1.1 Primary structure identification

Fig 3.2: Base peak ion chromatogram of the digested glargine

Tab 3.1: Peptide mapping of glargine standard

<table>
<thead>
<tr>
<th>Peptide</th>
<th>amino acid</th>
<th>sequence</th>
<th>m/z</th>
<th>t_R (min)</th>
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<td>P1</td>
<td>1-13</td>
<td>(-)FVNQHLCGSHLVE(A)</td>
<td>770.37 (2^+)</td>
<td>22.09</td>
</tr>
<tr>
<td>P2</td>
<td>14-21</td>
<td>(E)ALYLVCGE(R)</td>
<td>924.45 (1^+)</td>
<td>29.75</td>
</tr>
<tr>
<td>P2’</td>
<td>14-22</td>
<td>(E)ALYLVCGER(G)</td>
<td>540.78 (2^+)</td>
<td>25.14</td>
</tr>
<tr>
<td>P3</td>
<td>23-29</td>
<td>(R)GFFYTPK(T)</td>
<td>859.42 (2^+)</td>
<td>26.15</td>
</tr>
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<td>P4</td>
<td>30-32</td>
<td>(K)TRR(G)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>33-49</td>
<td>(R)GIVEQCCTSICSLYQLE(Q)</td>
<td>1030.46 (2^+)</td>
<td>39.11</td>
</tr>
<tr>
<td>P6</td>
<td>37-49</td>
<td>(E)QCCTSICSLYQLE(N)</td>
<td>831.35 (2^+)</td>
<td>24.81</td>
</tr>
<tr>
<td>P7</td>
<td>50-53</td>
<td>(E)NYCG(-)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>
With 53 amino acids, glargine was cleaved by trypsin combined GluC, the multi-enzyme strategy and the theoretical digested peptide mapping data are shown as below in Table 3.1 and the base peaks were shown in Fig 3.2. The multi enzyme strategy worked well in this experiment and the peptides were observed in high abundance. The protein was processed with reduction and alkylation so that the disulfide bonds were cleaved. The N-terminal peptide FVNQHLCGSHLVE was shown with the highest signal with m/z 770.37 (2+) was eluted at 22.09 min. With the connecting and cleaved residue K, R and E in the sequence, several peptides contained miscleaved residues such as P2’. The peptide P5 with a high m/z was not shown in a significantly high signal, but the extracted m/z has been identified. The residue difference of glargine and insulin in peptide P4 ”TRR” and P7”NYCG” were not determined in bottom-up experiment due to generation of small digested fragments. The intact mass difference was identified by top-down mass spectrometry and will be demonstrated in following sections.

### 3.4.2 Disulfide bond identification

For the linkage identification, with the non-reduced conditions, the protein was digested and remained as the intact disulfide bonds, so the linked peptides would be detected. The characterized fragmentation mass assignments were shown in Fig 3.3 and Fig. 3.4. In this project, CID was applied to identify the linkage with the whole connected peptide information. Since the chain A and chain B of glargine is connected the disulfide bonds, the proteolysis may not generate unique form peptides with two enzymes. The fragmentation has enabled the determination of the intra and inter disulfide linkage with the predicted structures and mass with six forms of linked peptides identified in the control samples. The demonstration of forms 2 and 3 are shown in detail with extracted ion peaks in Fig 3.5 and Fig 3.6.
Fig 3.3: Base peak of glargine standard disulfide bond assignment

Fig 3.4: Structures of glargine disulfide bond assignment
Fig 3.5: Disulfide bond assignment of glargine standard Cys53-Cys19

Fig 3.5 is an example of disulfide bond connected peptides, the linkage is between Cys53 and Cys19, the m/z is 661.77(2+), which was form 2 in Fig 3.4. The b ion and y ion from each peptide (P1 and P2) indicated in the figure were identified separately. Fig 3.6 illustrated another inter and intra disulfide bonds of Cys38-Cys43 and Cys7, the m/z is 1123.50(3+), indicated form 3. In Fig 3.5, the accurate mass matched the mass of the two enzymatically generated peptides linked together with 1 disulfide bond, and the corresponding MS2 spectra matched the fragment ions of the expected peptide sequence.
Fig 3.6: Di-sulfide bond assignment of glargine standard Cys38-Cys43, C39-C7

In Fig 3.6, the accurate mass matched the expected mass of the two enzymatic peptides linked together with 2 disulfide bonds were the peptide mass with the loss of 4H, and the corresponding MS2 spectra matched the fragment ions of the expected peptide sequence. The fragment ion patterns indirectly indicated the C38-C43 linkage even though no fragment ions was observed inside the ring as expected. Nevertheless, the exact Cys linkages can be confirmed by further ETD identification in future analyses.
3.4.2.1 Degradation evaluation

The changing of amino acids on human insulin to glargine can make the protein more soluble in an acidic environment but insoluble in neutral conditions. The carboxyl terminal of chain B changes leads to isoelectric point close to neutral and more inactive to degradation in a high pH environment. The glycine substitution of asparagine on chain A prevents deamidation of the acid-sensitive asparagine at acidic pH. As shown in Fig. 3.7, the deamidation of glargine has been studied under stressed conditions where the glutamine (Q37) in glargine is a potential deamidation site. While the Q deamidation is not common, we would like to observe if indeed any deamidation occurred to generate a glutamate residue.

Fig 3.7: IEF gel image of glargine samples

The glargine standard has been dialyzed into sodium borate at pH 9 condition, with the 37°C incubation for
three days. From the gel image, this gel image shows electrophoresis migration and broad bands, however, the isoelectric point of glargine standard and the stressed sample were obviously indicate around 7.0. As lane 5 and lane 6 shows, the stressed insulin had variants bands besides the major bands indicating pH around 5.1. Thus we concluded that glargine has more structural stability than insulin, due to the absence of asparagine residues and glutamine deamidation.

Besides IEF gel observation, a digested procedure has been performed by LC/MS and the resulting extracted ion peak shown in Fig [3.8] The potential glutamate residue is located in P1 FVNQHLCGSHLVE, while the +1 mass shift was not found. The detected m/z was 770.36(2+), and the +0.5 amu difference at charge 2 was not detected. This peptide under stressed condition has been found to be the same as with the normal conditions. Also the isotopic peak of this peptide has no signal shift at the second abundance, 770.86(2+) peak. From these different analyses we cannot conclude there is any deamidation that occurred in the glargine sample with our stressed conditions. Thus we conclude that the stability of glargine is better than insulin.

Fig 3.8: XIC peak of Q4 deamidation evaluation of glargine
3.4.3 Top-down analysis

Top-down analysis is a very efficient and convenient way to measure and evaluate the product at the first stage. This may guide the cultivation process in a correct direction although for the strain samples, further purification and polishing procedures are still necessary to achieve the analytic quality required for a therapeutic product. In our research, we have performed the intact protein analysis by several instruments including Thermo Orbitrap, Waters Q-TOF and Sciex MALDI.

![Fig 3.9: Base peak ion chromatogram of the intact glargine analysis](image)

As shown in Fig 3.9, the protein was analyzed by Orbitrap detector, eluted at 39.38 min. The peak shape was not smoothly shown due to the amount of protein and the column capacity. At this elution time, the observed m/z fragments included 758.862(8+), 867.127(7+), 1011.479(6+), and 1516.715(4+). This instrument has
been set the limitation mass range of m/z 2000, then the ion of $3^+$, $2^+$ or $1^+$ were not detected. The deconvoluted mass is 6062.889Da, which matched with the theoretical molecular average mass for Lantus Glargine 6063Da. Thus we concluded that the Orbitrap is a powerful mass detector to measure small protein.

Fig 3.10: QTOF result of glargine with non-reduced and reduced conditions

Using the QTOF technology to identify the intact protein in non-reduced and reduced condition. The drug samples were reduced with dithiothreitol and not alkylated prior to analysis. Thus the addition of hydrogen mass will be observed under reduction conditions. In the Fig 3.10, a has indicated the intact glargine elution on the LC column, as well as the reduced A chain and B chain have been separated and eluted.
by the order shown as Fig. 3.10B. The intact protein peak was eluted at 10.42 min, and for the reduced sample, chain A was eluted at 11.56 min while chain B was eluted earlier at 10.59 min. As the figure indicated, the m/z of intact glargine shown in Fig. 3.10A1 from charge 4+ ion of mass 1516.9179, to charge 8+ ion with mass 758.845, and the accurate mass has been deconvoluted at 6062.7Da. The fragments ion of chain A were shown in Fig. 3.10B2 with 2327.2869(1+), 1164.1478(2+) and 776.4466(3+). As the result of chain B, shown in Fig. 3.10B1, the ions were observed as 1872.2091(2+), 1248.4774(3+), 936.6144(4+), 749.4995(5+), 625.0945(6+) and 535.6510(7+). After deconvolution, the mass has been calculated to 2326.2869Da for chain A and 3742.2091Da for Chain B, which could be identified as the reduced products, comparable with theoretical molecular weight of 2326Da for chain A and 3742Da for chain B.
Fig 3.11: MALDI TOF/TOF data of glargine with non-reduced and reduced conditions; A: MS of Intact glargine; B: Reduced glargine MS/MS with zoomed in Chain B, from the asymmetric and symmetric cleavage of disulfide bonds.

In this study, MALDI-TOF\textsuperscript{23} has been applied to measure the intact protein molecular mass as well, and our data was demonstrated. Fig\textsuperscript{3.11}A, illustrated the 6061.8(1+) peak and the corresponding 3031.2 (2+) peak of the protein. As the Fig\textsuperscript{3.11}B indicating, the chain B with the quintet peak centring with 3740.9 Da with the difference of 32 amu, as a result of the asymmetric and symmetric cleavage of disulfide bonds and chain A and chain B connection\textsuperscript{24} However, the strain glargine sample didn’t provide distinctive peaks for structure determination by MALDI. Although MALDI is capable with relative high salt tolerance, the desalting and purification of crude protein samples are necessary prior to initial analysis.
3.4.4 Evaluation of strain proteins

Because the cultivation of glargine does not result in a strain product that is not mature enough for detailed analysis at the analytical level, we performed a bottom-up in-gel analysis on the crude recombinant protein sample for sequence and structure determination and top-down analysis on a QTOF for intact protein mass. The strain glargine samples were tested and compared with standard insulin and glargine by SDS-PAGE, and the gel image was shown in Fig. 3.12. Lane 2 and 3 were Lantus glargine standard with 10 ug and 2 µg loading. The lane 4 to 6 were the triplicates of strain glargine sample and. The strain glargine with a relative concentration around 0.05mg/mL, and the loading volume were adjusted to 25 µL. Due to the buffer components in the strain sample, the glargine bands exhibit a change in migration under high voltage, which is highlighted by a red box. The bands under the box were cut and combined into one vial and processed with an in-gel digestion with trypsin and GluC for primary structure identification.
Fig 3.12: SDS-PAGE gel image of strain glargine samples; Lane 1 and 8: marker; Lane 2, 10µg control; Lane 3, 2µg control; Lane 4-6, strain glargine with adjusted volume of 25 µL; Lane 7, another strain test sample.
At the pre-purified stage, the strain protein samples were examined with full sequence coverage with medium confidence. As shown in Fig. 3.13, the digested protein with reduction and without digestion result were shown as A and B. The N-terminal peptide of glargine FVNQHLCGSHLVE was detected in a relative high confidence and marked P1. The $1^+P6$ QCCTSICSLYQLE was observed. These peptides had an elution time shifting due to sample impurities such as salt. With non-reduced conditions, form 6 was detected in a high abundance, shown in Fig. 3.13B. With extraction of each expected peptide m/z, only form 4 and 6 were identified. Prior to the purification and polishing stage, the crude sample from the fermentation supernatant was in a good condition with sufficient concentration to allow us to determine accurate gene expression. However, the crude samples were not present at a purity level to enable the evaluation of in fermentation sample degradation or stability evaluation.
The crude glargine samples were analyzed on QTOF detector as well and the result compared with the standard and shown in Fig. 3.14. The buffer gave a high background signal shown as Fig. 3.14A, and the expected elution time 10.08 min with mass of 1011.376$^+$ was extracted and the chromatogram was shown in
Fig 3.14B compared with reference material elution time 9.67min. The deconvoluted mass peak of 6063Da of standard was shown in Fig 3.14C, and the crude sample was observed with 6044Da shown in Fig 3.14D. The mass shift could be as a result of dehydration (loss of H₂O), however, the purified samples may need further polishing procedures to obtain more conclusive analytical information.

### 3.5 Conclusion

As diabetes is one of the most serious disease in the world, scientists from clinical, pharmaceutical or biological areas are focusing on methods to improve the treatment of the rapidly increasing diabetic population. In our research, a series of efficient methods have been established to characterize the biopharmaceutical products from pre and post purified stage. The methodology is effective to evaluate the sequence structures and monitor the degradation. Different mass spectrometers were applied and compared, which will guide further analysis based on protein properties to select the best instrument. The crude protein drug produced from InSCyT system has been analyzed in this research and the data was demonstrated. Even the purification still need polishing by downstream teams, our data could illustrate the high capability for the production team and provide guidance to manufacture optimization.
References


Chapter 4

Stability analysis of biopharmaceutical products by LC tandem mass spectrometry

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Contributions: Northeastern University, Di Wu: LC-MS experiment procedures on protein drugs, data analysis and interpretation, manuscript writing and revision; William Hancock: goal of the study, concept contribution; MIT, William Herrington; Raman Spectroscopy experimental procedures and manuscript writing; MIT Wei Ouyang: Electrokinetic concentration binding assay experiment operating and manuscript writing

Wei Ouyang, Sung Hee Ko, Di Wu, Annie Yu Wang, Paul W. Barone, William S. Hancock, and Jongyoon Han Rapid Activity Assessment for Biologics using Electrokinetic Concentration Binding Assays with Charge-Modulated Receptors, Analytical Chemistry, 2016, 88 (19)

William F. Herrington, Gajendra Pratap Singh, Di Wu, Paul Barone, , William S. Hancock, Ram Rajeev Optical Detection of Degraded Therapeutic Proteins, Scientific Reports (Accepted)
4.1 Abstract

Biosimilar therapeutics are marketed worldwide nowadays and stability assessment is necessary to validate the long term safety, purity and potency of the products. In this study, a range of controlled stressed conditions, including temperature, humidity and pH, have been applied to a set of therapeutic proteins. The resulting degradations from the accelerated stability study are monitored with LC-MS-MS. With the high efficiency, accuracy, and sensitivity of mass spectrometry, deamidation and oxidation variants were detected. The differences in structural integrity and conformational stability profiles were monitored and potential degradations were evaluated by the MS analysis. In addition, real time Raman spectroscopy techniques were utilized in this study and demonstrated a powerful ability to monitor degradation reactions as well as determine the identity of a given biosimilar therapeutic products as well as to detect product variants. Data obtained from Raman spectrometry and electrokinetic measurement are comparable with mass spectrometry, which can differentiate modified protein from the normal form. The study indicates these techniques are sensitive and robust and will provide an alternative method to conventional analytical methods for the monitoring of the manufacture of protein therapeutics. Such methods have the potential to provide quality assessment of protein therapeutics manufactured in remote locations.
4.2 Introduction

Biopharmaceuticals have captured global attention and increasing market share in recent decades especially from 1980s when the first biosynthetic insulin has been produced and approved.\textsuperscript{1} The high sensitivity and specificity of protein therapeutics are important for safety and efficacy of these drugs in patients. After accurate diagnosis and effective treatment programs biologic drugs can be set up to treat difficult diseases. Although an increasing panel of such drugs have been discovered and studied, there are still many challenges in the biopharmaceutical market, such as the cost, time of treatment and targeting specificity and to assure the efficacy of biosimilars solutions are crucial.\textsuperscript{2,3} The safety and quality of a given biopharmaceutical may change significantly with the generation of subtle differences in the molecular species of the drug during the steps of production, transportation and storage conditions. In previous studies, we characterized the highly complex protein structures produced by the novel production system, InCSyT (Integrated and Scalable Cyto-Technologies), which is designed to produce high quality biologic medicine in the field. We have demonstrated that the biologics from this platform are with high quality produced under optimized conditions.\textsuperscript{4} However, the post-production quality will have additional quality concerns which are based on formulation and stability issues and thus we need to detect and evaluate further variations in the products. Stability is always a key feature of biological medicines. Furthermore the knowledge of potential toxicity caused by subtle changes in the therapeutic can be used to improve the production system. In this study, we have designed a set of experiments forcing the generation of stability degradations and then measured the resulting degradation products by different techniques.

Mass spectrometry is a powerful technique to characterize and quantitate biological products. Bottom-up analysis is commonly applied in many studies to identify and evaluate the structure, sequence or any mutations in the sequence of the protein product. To perform a bottom-up analysis we use enzymes with
high specificity and efficiency to digest the large protein molecule into small peptides. However, while a completed analysis can be achieved by mass spectrometry, the challenges of on-line samples preparation mean that timing of the sampling will be a challenge for real time monitoring of the fermentation. Therefore, for the InCyST program, other measurement techniques were considered for on-line measurement of the intact protein during the fermentation.

As we know, the drugs bioactivity was determined by the binding of the drugs to their physiological targets or receptors. Biologics are efficacious only when they are bound to their physiological targets (receptors). Both the binding equilibrium and binding kinetics, which correspond to the in vivo efficacy and duration of efficacy, are deemed as key metrics in drug discovery and pharmacokinetics. In this project, one of our collaborator teams has applied the molecular charge modulation (MCM) and electrokinetic concentration (EC) to assess the protein activity. They have obtained distinctive results of the correlation of degradation and bindings.

Another collaborator team working with Raman spectroscopy has also provided effective experimental data. Raman spectroscopy is a technology to verify the primary and secondary structure of proteins since 1950s. Previous work on proteins characterization by Raman spectroscopy has focused on high concentration in solution or in solid powder form. Our technique was with sensitive detection and to powerfully monitor the single molecule level biologics by Surface Enhanced Raman Scattering technique (SERS). If the biologics was not in control of its orientation, the absorbance will be differed by the sensitive SERS technology. Our Raman collaborator team has reported on the accurate identification and quantification of various therapeutic proteins by monitoring the SERS signal shifts according to the protein backbone or amino acids changes.
4.3 Experimental

4.3.1 Chemicals and materials

The recombinant human growth hormone was purchased as Humatrope (Lilly, USA). The recombinant GCSF was purchased from Neupogen (Amgen, USA). The chemicals including acetonitrile, hydrogen peroxide, Tris-HCl sodium phosphate, guanidinum chloride, dithiothreitol, iodoacetamide, and trypsin were analytical grade, obtained from Thermo (Thermo Fisher, USA).

4.3.2 Stressed conditions for oxidation

hGH was initially reconstituted in sample buffer (10mM sodium phosphate, pH=7), for a concentration of 2 mg/mL. The oxidation variants were prepared by addition of hydrogen peroxide to a final concentration of 0.5% (v/v), then incubated under 37°C overnight. After the incubation, the oxidized hGH were dialyzed back into sodium phosphate sample buffer, to avoid further oxidation.

G-CSF, initial concentration at 0.6 mg/mL was reconstituted into sample buffer (20mM glutamic acid with 5% sorbitol, w/v, pH=4.4). The oxidation variants were prepared by addition of hydrogen peroxide to a final concentration of 0.5% (v/v), then incubated at 37°C for 2 hour. The oxidized G-CSF was then dialyzed back into glutamic acid sample buffer and was executed by 200µL buffer at 13,000 rpm for 20 minutes, repeated three times in a 500µL 10kDa Amicon (EMD Millipore Corporation, Merck, Germany) centrifugal filter.

The test samples were produced by proportionally mixing of control and oxidized variants, with oxidation percentage at 10%, 40%, 70% and 100% (v/v). All test samples and control were lyophilized overnight and reconstituted again to give a final concentration 2 mg/mL of hGH, 0.6mg/mL of G-CSF, and stored at -80°C.
4.3.3 Stressed conditions of deamidation

100µL hGH was reconstituted into 0.1M sodium borate buffer with pH 8 and incubated under 37 degree for total four weeks. 20µL sample was collected at each time point for control, 1 week, 2 week, three week and four week. The samples were stored at -80°C after collection and all samples were performed in-solution digestion and analysis at same time.

4.3.4 In-solution digestion

hGH sample were dissolved in 6M guanidinium chloride, reduced with 10 mM dithiothreitol at 70°C for 30 minutes and followed by alkylation with 55mM iodoacetamide under room temperature in dark conditions. Proteins were dialyzed via a 10kD membrane Amicon centrifugal filter at 13,000 rpm for 15 minute and three times in Tris-HCl buffer (pH=6.8). The following in-solution digestion process was used trypsin and the solution was kept overnight at room temperature to avoid artificial oxidation. The digestion was terminated by addition of 20µL 5% formic acid.

GCSF samples were adjusted pH to 3 by HCl, and followed by digestion with pepsin at 37°C for 30 minutes. The digestion process was terminated by adjusting pH to 8 by 0.1M NH₄HCO₃. Protein samples were then ready for LC-MS analysis and the remained materials were aliquotted to 20 µL sample and stored at -80 °C for further analysis.
4.3.5 LC-MS measurement

LC-MS analysis used an Ultimate 3000 nano LC pump (Dionex, Mountain View, CA) and a self-packed C18 column (Magic C18, 200 Å pore and 5 µm particle size, 75 µm internal diameter by 100 mm) connected to a coated 10 µm internal diameter emitter (New Objective, Woburn, MA). LTQ-Orbitrap XL mass spectrometer was connected (Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source (New Objective, Woburn, MA). Mobile phase A used 0.1% formic acid in HPLC grade water and mobile phase B was using 0.1% formic acid in acetonitrile. During sample injection, the flow rate was set at 250 nL/min with 2% B for 25 min. The flow rate of the gradient was set at 200 nL/min, with mobile phase B, 0-60 min 2-40%, 60-70 min up to 90%, 70-75 min kept for 90% and 75-78 min 2%B. The mass spectrometer was operated in a data dependent mode to switch between MS and CID-MS². Briefly, after a full-scan MS spectrum from m/z 400-2000 in the ion-trap, 8 CID-MS² activation steps were performed on the 8 most intense precursor ions from the full scan. All control and variants samples were run in triplicate.

For peptide identifications, raw data were searched against human growth hormone and Granulocyte-colony stimulating factor sequence using BioPharma Finder 2.0 software (Thermo Fisher Scientific). For peptide mapping, searches were performed using a single-entry protein FASTA database with oxidation and deamidation set as variable modifications, 10 ppm mass accuracy, and a confidence level of 0.8 for MS/MS spectra. Final confirmation of the peptide identification was determined by manual inspection, extracting the base peak from the chromatogram and matching the MS² fragmentation data with theoretical prediction. The modification percentage was calculated by peptide peak area. Both non-degraded and degraded peptides were evaluated in the same LC-MS analysis with characteristic m/z differences and elution time shifts. The quantitation of the degradation could be calculated as the following: ratio of Degradation (such as oxidation, deamidation, etc) = peak area of [(degraded peptide)/(degraded peptide) + (Non-degraded peptide)] ×
4.3.6 Raman techniques

Raman spectra were collected using a purpose built system designed around the low volume sample holder illustrated in the Figure. The sample holder featured a 100m thick fused silica sampling window used for both excitation and collection of the Raman signal. A microscope objective was used to focus the excitation light and collect the Raman scattered light. A gold mirror at the back plane of the sample holder, positioned near the focal point of the microscope objective (NA=0.75, 40x magnification), increased the excitation energy by redirecting the excitation light back through the sample. Additionally, the mirror increased the systems Raman signal collection by redirecting forward scattered Raman light back towards the microscope objective for collection.

Raman signal levels increase with excitation intensity, so most of the Raman signal in the system was generated close to the focal point of the microscope objective. The liquid containing well of the sample holder was 4mm wide, 6mm long, and 0.8mm deep along the optical path for a total volume of approximately 19L. To use the holder a 20-25L drop of material was added to the well, over filling it slightly, and then the well was capped with the mirror. This prevented the formation of an air bubble between the sample and mirror. The sample holder was designed so that the focal point could be placed near the mirror for increased collection as described above, and the depth of the sample holder was chosen so that the excitation spot was far from the fused silica window to minimize the background signal from the window. The walls and mirror in the sample holder were metallic, so they added no background Raman signal. The inverted configuration ensured that neither the sample holder nor the microscope objective needed to move during sample loading or cleaning operations. This allowed the system to be used without re-aligning the optics each time a new
sample was presented to the system.

Fig 4.1: Scheme of Raman spectroscopy mechanism; A: Raman spectroscopy sample holder; B: Raman spectroscopy mechanism

A schematic diagram of our sample holder is shown in Fig 4.1A and the Raman system shown in Fig 4.1B. The total volume is kept low by recognizing that the volume of sample directly illuminated by the excitation light will produce the strongest Raman signal. The microscope objective used in this system is a Nikon Plan Fluorite, 40x 0.75NA objective.

4.3.7 Electrokinetic Concentration (EC) Binding Assays

Our partner group\(^7\) has previously reported on EC devices for simultaneous concentration and separation of charged species based on their mobility. Under the voltage configuration shown in Fig 4.2(a), a spatially decaying electric field is created in the vicinity of the cation-selective Nafion membrane due to the ion concentration polarization phenomenon, which imposes an electrophoretic force on charged species.
Meanwhile, all species are brought downstream by the electroosmotic flow. Therefore, negatively charged species are concentrated where electrophoresis and electroosmosis balance, with high mobility species being farther and low mobility species being closer to the nanojunction. It is also to be noted that positively charged species go downstream without being concentrated due to the cation-selectivity of the nanojunction. Fig 4.2(b-c) shows the design of the device, with five channels in parallel for testing five samples at the same time. EC can easily separate negative species with high mobility difference, such as aptamers and aptamer-IgE complex, at voltages < 100 V. However, as with other mobility-based separation technologies, it
is incapable of separating species with minimal mobility difference, including many proteins and protein-protein complexes. Fig 4.2 shows the positive and negative MCM for biologic-receptor binding assays.
4.4 Results and discussion

4.4.1 LC-MS results of hGH

4.4.1.1 Primary structure identification

Human growth hormone is essential for the treatment of children with growth hormone deficiency or hypopituitarism.\textsuperscript{11,12} The recombinant human growth hormone (rhGH) produced by the expression in the Pichia yeast strain had the identical sequence to natural growth hormone with 191 amino acids. The 191 amino acids sequence is cleaved to 21 peptides by proteolysis with the enzyme trypsin. Under non-reducing conditions, the intra disulfide linkages at Cys53-Cys156 and Cys182-Cys189 are maintained. The precise sequence and disulfide linkage are critical to the function and toxicity of biopharmaceuticals.\textsuperscript{13} Prior to any degradation analysis, peptide mapping is the fundamental procedure to assure a completed protein structure was detected. The tryptic peptide mapping was shown in Table 4.2.

The samples measured are all obtained by purchase of the corresponding FDA approved drugs with known primary sequences and primary structures were confirmed. The project aims were to design and control a reproducible set of stressed conditions and to subsequently evaluate the stability. At the same time, this experiment aims to generate specific degradation reactions and not denature the protein structure. The three dimensional structure of human growth hormone and the sequence information are shown in Fig. 4.3. The major degradation pathways of growth hormone were observed to be oxidation of methionine and deamidation of asparagine residues respectively. In this study, these variants were primarily evaluated by MS and correlated with Raman measurements.
The amino acid residues located in the flexible outer loop of hGH, the outer loop of growth hormone (residue M14) will be more easily oxidized, especially under stressed conditions. However, the methionine residue located in the hydrophobic inner core will not. A structural change induced by unfavourable manufacturing or storage conditions can therefore have a significant effect on the quality of a protein drug. It is important, therefore, to keep a consistent temperature, moisture, oxygen proportions or other conditions at remote supply areas or during the transportation of the product. The stability of the biopharmaceuticals will be thus crucial for the drug quality, then for the patient’s safety. Our work is to effectively evaluate the drug stability and measure any degradation reactions with our optimized methods.
The stressed oxidized condition was primarily targeted to the surface methionine residues instead of denaturing the protein and exposing the buried methionine residue at position 170. To ensure that each sample contained the correct sequence of amino acids, we used trypsin digestion for identification, although in another study a combination of trypsin and LysC were used and gave similar results. As Tab. 4.2 indicated, the T3, T5, T17, and T18 are small peptides and difficult to detect but were observed as the result of incomplete trypsin digestion, such as in the peptide T3-T5, T17-T19.
4.4.1.2 Oxidation determination of hGH

We observed that artificial oxidation of methionine residues occurred when the electrospray ion source is operated under high voltage. While a peptide with oxidized methionine would be expected to elute earlier in RPLC due to the greater polarity of the sulfoxide moiety, the artifactual oxidation that occurs after separation, would result in the oxidized peptide eluting at the same time as the non-oxidized peptides but with an increased m/z value. The artificial oxidation may not occur all the times, however, this calculation will be used to obtain an accurate oxidation ratio. In this study, we applied the coated tip for ESI to minimize any artifactual oxidation in the analysis so that the two types of oxidation variants will be differentiated by elution order differences, which shown in Fig 4.5. The voltage will approach the mobile phase solvent via the emitter that was equipped with conductive coating. The artifact oxidation induced by the high voltage will be differentiated from the real oxidation according to the elution time.

![Scheme of coated tips](image)

**Fig 4.5: Scheme of coated tips**

Considering the product stability and the type of formulation, previous researchers have indicated that higher oxidation levels may occur in lyophilized proteins than with the liquid products which usually contained a trace amount. The oxidation reaction could occur readily with oxygen existing in the product sample and is promoted by the presence of a less stable denatured structures either generated during the lyophilization process or any storage conditions. In this project, the samples were lyophilized to a stable form and then diluted to the required concentration for evaluation. All samples were prepared using the
same conditions, so the levels of observed oxidation in the reference standard could be used as a control.

Fig 4.6: Extracted ion peak of T2 and the oxidized T2 peptides from the tryptic digested rhGH test1 sample

Fig 4.7: LC-MS analysis of the T2 and the oxidized T2 peptides from the tryptic digested rhGH test1 sample
As shown in Fig 4.6, the elution order of oxidized peptide and non-oxidized peptide could be differentiated by the retention times due to differences in hydrophobicity. With oxidation, the peptide T2 LFDNAMLR, the oxidized methionine will bring a more hydrophilic property to this peptide, which will elute earlier than the normal peptide on the reversed phase separation.

Both non-oxidized and oxidized peptides were evaluated in the same LC-MS analysis with m/z difference and elution time shifts. Fig 4.7 presents one example of the extracted ion chromatogram (XIC) of non-oxidized T2 and oxidized T2. The methionine residue locates at position 6 in this peptide T2. By CID fragmentation, the b6, b7 ions and y3 to y7 ions will have a mass change due to the methionine oxidation. As shown in Fig 4.7, it is indicated here that y6 ion 719.31$^+$ has been oxidized to 735.31$^+$, with the change of amu as did the y7 ion from 866.37$^+$ to 882.33$^+$.

This base peak chromatogram was made up of the extracted ion peak from sample Test1, with a 20% mixing of control and harshly stressed oxidized sample Test4. The abundance shown in Fig 4.8 illustrates the oxidation amount, which indicates the trend of oxidation accumulation with an increased mixing ratio of oxidizing agent to sample. However, the residue Met14 in Test1 had a 36.3% oxidation, while in Test2 a value of 30.4%. Such minor variations are more likely caused by differences in sample handling. The data was summarized in Supplementary information.

The residue Met125 is not as easily oxidized as Met14 due to the conformation location difference. In this study, with overnight incubation, the residue Met125 was detected 96.20% oxidation in Test4, with a higher ratio than Met14. However, the oxidation on Met14 accelerated faster than on Met125 during the overall stressed conditions study. As the residue Met170 is located at the inner core of the protein it was resistant to oxidation, the data illustrated for the protein used in this study indicates a robust structure under stressed conditions, and that the developed method can effectively induce specific degradations of the biological
drug samples. Under very harsh oxidation conditions the reaction of residue Met170 indicated loss of native protein structure and thus the stability of this residue allows an analyst to monitor the structural stability of recombinant human growth hormone drugs. We conclude that the stressed oxidation design used in this study is effective for evaluating product stability. To evaluate other storage stability potentials, we would adjust temperature, humidity or incubation time in the future.

Fig 4.8: hGH oxidation result
4.4.1.3 Deamidation result of hGH

The generation of deamidation used incubation at high pH levels and higher temperature than normal drug storage to forced more degradation reactions. In this study, we incubated the protein for a total of one month and analyzed each week time point to monitor the tendency of deamidation in hGH.

Fig 4.9: Deamidation characterization; XIC of peptide FDTNSHNDDALLK of each test sample (745.35$^{2+}$ and 745.83$^{2+}$);

The peptide FDTNSHNDDALLK with the most labile residue Asn149 is the target peptide site. The extracted ion peak with m/z 745.33$^{2+}$ eluted at 29.30 min was shown as Fig 4.9 while the peak with m/z 745.83$^{2+}$ eluted 31.75 min contained the expected deamidation site. The changes of the structure resulted in a 1 Da mass change and a polarity difference which led the two minutes elution delay in the RPLC separation. The results showed the increasing level of deamidation of peptide T15 from the control (0 week) with 5.16% variant and accumulated to each week point 40.34% (1 week), 43.53% (2 weeks), 74.13% (3 weeks) and 82.00% (4 weeks). As mentioned before, this harsh condition may induce a large amount deamida-
tion, indicating other potential residues such as Asn99 and Asn152 deamidation or Asp130 isomerization to isoAsp. However, in this study we didn’t characterize these additional sites of deamidation or differentiate between Asp and isoAsp which were not the focus of this study.

4.4.2 Results for GCSF

4.4.2.1 Primary structure identification

The test samples to be used to evaluate of the Raman spectroscopy were produced by proportionally mixing of the control and oxidized variants, with a resulting oxidation percentage at 10%, 40%, 70% and 100% (v/v). With experimental condition limitations, the preparation of a GCSF oxidation standard focused on a harsh condition with the expectation of a fully oxidized sample for the subsequent preparation of a well controlled oxidation sample set.

Fig 4.10: 3-D structure of GCSF
We have illustrated the three-dimensional structure of GCSF in Fig. 4.10. The potential modification site amino acids have been highlighted. As similar with human growth hormone, the methionine residues are reported as being oxidized, but the tendency of reaction at each methionine positions are different. The sequence of GCSF starts from a N-terminal methionine residue that is located on the outer space of the structure. This N-terminal methionine is the easiest one to be oxidized. There are three other methionines, Met122, Met127 and Met138 located at the inner side of its structure. The tendency of oxidation of these three methionine residues are variable based on different formulation buffers and storage conditions and Met127 and Met138 may switch with the tendency for oxidation levels depending on the conditions.

![Base peak ion chromatogram of the pepsin map of GCSF test samples](image)

Fig 4.11: Base peak ion chromatogram of the pepsin map of GCSF test samples; Top: Control standard; Bottom: Test sample

As indicated, the elution and base peaks of GCSF test samples have been shown in Fig. 4.11. The upper one
is the elution base peaks of the reference material, and the lower one is the test sample. The peptides were labeled by the residue amino acids position numbers.

4.4.2.2 Oxidation of GCSF

The GCSF analyte was digested with pepsin under acidic conditions according to the optimal pH for this enzyme activity. The N-terminal peptide MTPLGPASSLPQSFL is the primary methionine oxidation peptide. Both non-oxidized and oxidized peptides were evaluated in the same LC-MS analysis with m/z difference and elution time shifts. Fig 4.12 presents one example of the extracted ion chromatogram (XIC) from the control sample of non-oxidized P1 and oxidized P1.
Fig 4.12: LC-MS analysis of the N-terminal and the oxidized N-terminal peptides from the pepsin digested GCSF sample

As shown in the figure, the diagnostic peptide was detected with m/z 773.39^{2+}, and the oxidized form 781.39^{2+} indicating that the N-terminal residue methionine was the most readily oxidized site. The CID fragmentation MS2 data was shown as Fig 4.12. All the b ions will add an oxygen mass in the oxidized status, while the y ions will keep the same mass since the residue methionine located the first position. The oxidation data of all four methionine residues in the control and test samples were shown in Fig 4.13, which illustrated the percentage of the oxidation level. The test sample has indicated the significant increasing of the oxidation of each methionine residue. In this case, our designed stressed method is effective. This
strategy is also unique to target only methionine oxidation instead of inducing other modifications, such as there were no increasing deamidation was detected in this test sample. The levels of the oxidized base peak area increased significantly over the harsh conditions. The data here indicated that the stressed sample Test4 has 92.5% oxidation and the control has only 19.30% on Met1. As previous studies described, the oxidation would occur M1 > M138 > M127 > M122, and our data shows comparable results with that reported study. The data was provided in the supplementary information Table 4.5.

Fig 4.13: Stability measurement of GCSF oxidation result
4.4.3 Raman measurement

The Raman spectra of a protein will be composed of peaks due to the content of amino acids, the secondary structure of the protein, and bonds such as the disulfide bond. The individual amino acids which make up the proteins have their own possible vibrational modes and contribution to the proteins Raman spectra, but in a protein the strongest amino acid Raman signals are seen in the amino acids which have an aromatic ring structure in their side-chain including Tyrosine, Phenylalanine, Tryptophan, and Histidine as well as sulfur containing side-chain.

![Fig 4.14: Raman spectra of control and oxidized hGH; a, Raman spectra shift from 400 to 1800 cm\(^{-1}\); b, zoomed in spectra Raman shift from 1020 to 1140 cm\(^{-1}\); control in red, test hGH in blue](image)

Fig 4.14: Raman spectra of control and oxidized hGH; a, Raman spectra shift from 400 to 1800 cm\(^{-1}\); b, zoomed in spectra Raman shift from 1020 to 1140 cm\(^{-1}\); control in red, test hGH in blue

The Raman peaks due to Tyrosine, Phenylalanine, and Tryptophan occur at a small enough Raman shift to be visible in the system which captures signal from 450 cm\(^{-1}\) to 1700 cm\(^{-1}\). The peaks associated with the aromatic side-chain in Histidine (around 3100cm-1) and the sulfhydryl bond in cysteine (2500-2600cm\(^{-1}\)) occur beyond the range of the system. The Raman signal from disulfide bridges appears at a shift around 500cm\(^{-1}\), which is near the lower limit of the systems range in a region impacted by the signal from the fused silica of the sampling window. The Amide I band due to the protein backbone produces a visible and relatively strong signal for all four proteins of interest in the region 1500-1700cm\(^{-1}\).
Raman spectra for reference and highly oxidized hGH Data is shown as normalized counts per second after background subtraction. Changes are seen in the 847 cm$^{-1}$ Tyrosine band, between 1100 and 1150 associated with the oxidized methionine residue, and in the Amide I band. The spectra shown in Fig 4.14b has indicated significant shift differences between the test hGH and the control. Our collaborator then applied Circular Dichroism Spectrometer (Aviv Model 202) to measure the same sample. They didn’t detect major changes in the circular dichroism spectrum in both control and stressed sample, which indicated the alpha-helix segment in hGH was still folded. As reported, $\alpha$-helix is about 45% of the whole structure of hGH.$^{24}$ Thus, the result from the Raman study indicated the shift changes may be induced by structural changes in the non-helix regions in addition to the oxidation of methionine residues during the stressed condition process.

Although Raman spectroscopy is not a technique for quantitation in our project, it can differentiate the oxidized sample from the standard material, which is powerful to be applied in the on-line QC measurements for InSCyT platform in the future. This will allow the scientists to monitor the product quality efficiently at the early stage after manufacture in a remote area with medical need.

### 4.4.4 Electrokinetic Concentration Binding Assay

The platform designed by our collaborator for the electrokinetic concentration binding assay enabled the application to separate the bound and unbound species with a mobility difference, so that to differentiate the inactive degraded biologics from the active control sample. We have demonstrated comparable results for our mass spectrometry methods with the distinctive measurement of the equilibrium and kinetic binding behavior of both hGH and GCSF degradation samples and the correlation between different levels of biologics analysis technologies has been shown in Fig 4.15.
Our collaborator has designed and applied three different assays to evaluate a biologic drug by this MCM-EC platform, which included direct assay, competitive assay and degradation determination assay. The degraded drug samples were assessed by the MCM-EC platform, which indicated the decreased product activity when oxidation increased which demonstrated the capability of this system. The MCM-EC platform applied in this project is efficient, economical and with easy-control.
4.5 Conclusions

In this chapter, we have introduced a series of characterizations analytics tools that can be used to monitor the stability of protein drugs. The products under stressed condition illustrate the stability of a given bio-therapeutic and the susceptibility of different residues to degradative reaction and provide an example of the evaluation of pre-commercial products. The results shown here indicated that the refinements made by our MIT collaborators have produced a sophisticated Raman technology which has potential to detect the modifications in protein therapeutics both in the manufacturing process and on storage.

Bottom-up LCMS techniques may require more offline times for sample preparation and analysis, however, by this way, there will be more detailed information on the exact structural changes in the biologic product, such as the level of change in specific amino acids. On the other hand, other techniques used in this study such as Raman spectrometry and electrokinetic concentration binding assays will differentiate the modifications directly on intact proteins in a short assay time period. These technologies are very efficient for a quick product evaluation or diagnosis of product quality.

We have introduced the design of stressed modifications to generate degradation products and the required characterization methodology. Our methods are effective in analyzing changes at specific amino acid residues, and the oxidation and deamidation harsh conditions did not interfere with the detection of the different set of variants. In addition, we detect that the observed modifications were specific to a set of forced degradation conditions. We are therefore confident that our designed methods could be applied in newly produced biosimilars and to monitor specific stability risks.
References


### Supplementary Data

Tab 4.1: Raman spectroscopy peak assignments of hGH samples

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<tr>
<th>Pixel Number</th>
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<td>515.8 to 576.2</td>
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<td>252</td>
<td>735.7</td>
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<td>417</td>
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<td>476</td>
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<td>T21(184-191)</td>
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### Tab 4.3: GCSF peptide mapping

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<th>( t_R ) (min)</th>
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### Tab 4.4: hGH oxidation result

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<td>Test 1</td>
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### Tab 4.5: GCSF oxidation result

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<td>64.80%</td>
<td>0.022</td>
<td>87.20%</td>
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Chapter 5

Proteomics analysis of the secretome of a cancer cell line (Tiam-1) regulated cell medium by HPLC-MS

Contribution: Northeastern University, Di Wu: LC-MS experiment procedures on cell medium, data analysis, William Hancock: goal of the study, concept contribution; Tufts Medical Center, Kun Xu: cell medium preparation, bioassay; Rachel Buchsbaum: concept contribution
5.1 Abstract

Breast cancer is a serious disease among the constellation of global health issues and the early detection is always an important research area for the correct disease diagnosis and effective treatment of these patients. In this study, we have characterized the secretome of four cell lines including non-aggressive and aggressive cancer cell lines using appropriate conditioned media to obtain information on potential biomarkers. We have applied HPLC tandem MS techniques and a proteomic strategy to study these cell lines. With a serial data analysis protocol using information listed in data bases of GeneCards VarElect, we have successfully curated a protein list derived from the proteomic study. The proteomic database was then evaluated with relevant bioactivity measurements, and as a promising lead we have found a significant difference in the expression of fibronectin relative to other gene products in the protein list. Future studies using RNA-sequence analysis, signal pathway analysis and bioactivity will be explored to confirm the disease associations of this biomarker.
5.2 Introduction

Cancer has become one of the most serious threats amongst all the diseases.\textsuperscript{1,2} In the past decades, knowledge, research, and health information focusing on cancer has accumulated tremendously, however, clinical detection of a patient status still has unknown issues.\textsuperscript{3} There are various topics on cancer biomarkers, as Sawyers\textsuperscript{4} has pointed out three types of biomarkers in his review which are prognostic, predictive and pharmacodynamic biomarkers. It’s not easy to choose the correct associated cell line since the first breast cancer cell line was established, BT-20 in 1958.\textsuperscript{5} A commonly used breast cancer cell lines, the SUM series, are derived at the University of Michigan and licensed and distributed on to the market.\textsuperscript{6} The SUM cell lines are known as aggressive cancer cell lines and have allowed scientists to learn many new aspects of breast cancer biology including early stage detection of the disease, biomarker identification, and drug discovery for treatment.\textsuperscript{7}

Cancer spreading from the primary site to other parts of the body is termed metastasis and is often fatal.\textsuperscript{8} The cancer cell can be spread to the whole body through the lymphatic system or the bloodstream when tumor cells break away from the primary tumor. There is urgent patient need for the discovery of cancer biomarkers for early detection of cancer and prognostic markers of those who may have risk of developing tumor metastases.\textsuperscript{9} The metastasis associated cells will have unregulated activities which been partially characterized cancer detection studies.\textsuperscript{10}

Mass spectrometry technology is important to proteomic research and a popular approach is to use a gel based digestion method followed by LC/MS analysis and protein database searching. The accuracy of measurement depends on the control of experimental conditions, which can be challenging, including resolution and sensitivity of the mass spectrometer, concentration of secretome sample from cell lines, contamination
of the preparation with components from the fermentation media, and so on. The bottom-up methodology with enzyme digestion and a MS-based strategy is commonly used nowadays in biomarker discovery. In this way, controlled identification and quantitation are very important to the validation process. The protein variants that exist naturally in the cell is also a challenge for identification and quantitation studies.

To identify well-curated focused set of potential target biomarkers from a long proteomic list is challenging. Data dependent acquisition (DDA), data independent acquisition (DIA) and selected reaction monitoring (SRM) are three main acquisition modes applied in the biomarker proteomic studies. We used the data dependent mode in our experiment and determined the peptides and proteins with confident MSMS measurements.

In this study we focused on the discovery of predictive biomarkers, whereas, in our previous studies, we have characterized several cell lines by the proteomic analysis to evaluate potential disease biomarker candidates and identified, differential gene mutations with resultant amino acid substitutions or different post-translational modifications, such as glycosylation than observed in the normal cell. In this study, we are comparing cancer aggressive cell lines to non-aggressive cell lines and will describe the differences between the cell lines and findings with disease associations. We assumed that the gene activities are different from the normal human cell line HMEC to pleural effusions MCF7 to the aggressive SUM cell line, to the primary breast cancer cell line SUM159 and to the mouse xenograft of the metastatic nodules cell line SUM1315. The ideal expectation is to discover the genes that have an unique existence in the cancer aggressive secretome as well as differences in gene product abundance in the aggressive cell lines. In this project, we will introduce the experimental design and characterization of the cell line by our mass spectrometry proteomic strategy and the comparative results will guide the resulting search of promising cancer biomarkers for the early detection of this disease.
5.3 Experimental section and methods

5.3.1 Cell medium and cell lysates

Cancer aggressive cell line secretome samples derived from the SUM159 and SUM1315 cell lines as well as the cancer non-aggressive cell lines HMEC (Human mammary epithelial cell) and MCF7 (Michigan Cancer Foundation-7) were prepared by our collaborators at the Tufts Medical Center (Boston, MA).

5.3.2 In-gel digestion

All four cell lines have been aliquotted to fifty microliters and kept in -80°C for further analysis. Twenty microliters of cells secretome sample were loaded onto a gel (SDS-PAGE, 4-12% gradient) to separate proteins by molecular weight. The gel was followed by the Coomassie blue staining process, and each gel lane was cut into five individual slices as shown in supporting information. The gel procedure has been duplicated, and the slices of same cell line with same position have been combined in order to get adequate concentration for a LC-MS validation.

Each slice was then minced into tiny pieces (approximately 1 mm$^2$), and transferred into a 1.5 mL microcentrifuge tube. The gel slices were washed with 500 µL ACN and 0.1 M NH$_4$CO$_3$ for 45 min shaking and then centrifuged, the supernatant were removed. Proteins were analyzed under a reduced process by adding 500 µL of 10 mM dithiothreitol in 0.1 M NH$_4$HCO$_3$ and incubated for 30 min at 56°C followed by centrifugation and removing supernatant before the alkylation process. Proteins were alkylated with 500 µL of 55 mM iodoacetamide in 0.1 M NH$_4$HCO$_3$ under room temperature and kept in dark for 60 min. All supernatant was removed after spinning gel pieces down and the following digestion process. Trypsin was
added in a protein solution and the concentration was based on an enzyme : protein ratio of 1:100 to 1:20 (w/w). The samples were covered by a trypsin buffer (200 µL 10 ng/mL of trypsin in 50 mM NH₄HCO₃, pH=8) and incubated 30 min at 4°C to saturate gel pieces. Then the gel pieces were covered with the solution of 50 mM NH₄HCO₃ and incubated overnight at 37°C to yield peptides. Digestion process was stopped with 50 µL 5% formic acid and extracted with ACN. All supernatant were combined and stored for the following LC-MS analysis.

5.3.3 LC-MS/MS methods

The in-gel digested peptides were analyzed by LC coupled to a Quadrupole Exactive (QE) plus mass spectrometer (Q Exactive Plus, Thermo Electron, San Jose, CA) with a Dionex nano-LC instrument (Utimate 3000, Sunnyvale, CA) and a 4.6 mm × 150 mm packed with stable bond C-18 column (Zorbax 300 Å pore size, SB C18) (Agilent Technologies, Santa Clara, CA). The analytical LC separation was carried out using a three step linear gradient, starting from 0.2% B to 40% B in 50 minute (A: water with 0.1% formic acid; B: ACN with 0.1% formic acid), increased to 80% in 5 minute, and kept for 5 minute. The flow rate was maintained at 200 nL/min. The following QE plus mass analysis was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. The mass analyzer provide full-scan MS spectra with two microscans (m/z 300-2000) with a resolution of 70 000.

5.3.4 Protein identification

Thermo Proteome Discover 1.4 was using to identify peptide sequences, with database Homo sapiens, full trypsin specificity and up to three internal missed cleavages. The static modification was carbamidomethylation for cysteine, and dynamic modifications were for deamidation of asparagine and oxidation of methio-
nine residues. The tolerance has been set at 20 ppm for precursor ions and 1.0 Da for product ions. Peptides were identified with high confidence level and Xcorr scores above the following thresholds: $\geq 3.8$ for $3^+$ and for higher charge state ions, $\geq 2.2$ for $2^+$ ions, and $\geq 1.9$ for $1^+$ ions. Several housekeeping proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and b-actin (ACTB) have been selected as internal standards for relative quantification to minimize variations in the amount of samples loaded on the gel, which provide consistent ratios among all four cell medium samples with the required criteria of high abundance.

5.4 Results and Discussion

5.4.1 Characterization of the secretome (cell line medium)

Fig 5.1: SDS-PAGE image of four secretome samples

![SDS-PAGE image of four secretome samples](image)

Fig 5.1: SDS-PAGE image of four secretome samples
The cell line secretome samples were analyzed by SDS-PAGE and followed by a gel based digestion. The gel image was shown as Fig. 5.1 which indicated the concentration and protein expression of the cell culture samples. The 1D gel method is effective to separate the proteins secreted from the cell lines and help to remove the contaminations from the media components used to enable cell growth.

As shown, the cell line SUM1315 was observed with a relative low protein concentration. As marked in the gel image, the gel segments for each cell line were separated into five segments for enzyme based digestion and LC/MS analysis and the proteomic/genomic data from each segment were then combined a proteome of a whole cell line. The replicate observation of high abundance proteins was eliminated from the whole protein list and the final proteogenomic lists were then analyzed by bioinformatic tools. The cell medium gel based analysis was repeated and consistent observations from both analysis were marked with high confidence for further analysis.

The preliminary study was performed on a linear ion-trap mass spectrometer and then the optimized study was performed in duplicate with the SDS-PAGE procedure on a more powerful instrument the Quadrupole Exactive (QE) plus ion trap mass spectrometer. The optimized study gave a better result with higher confidence and larger scale identifications due to the improved sensitivity and resolution of the instrument. The observed gene products that were observed consistently with cancer associations are shown in Table 5.1. For purposes of comparison the top 25 gene products from each cell line is shown in the supplementary data and it can be seen that some cancer related proteins such as fibronectin and thrombospondin-1 are also present at high levels.
Tab 5.1: Results of cancer-related proteins

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>Novoseek Hit</th>
<th>SUM159</th>
<th>SUM1315</th>
<th>MCF7</th>
<th>HMEC</th>
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<tbody>
<tr>
<td>AHSG</td>
<td>Alpha-2-HS-glycoprotein</td>
<td>$3^a$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C3</td>
<td>Complement C3</td>
<td>$4^a$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CLEC3B</td>
<td>Tetranectin</td>
<td>$17^a, 4^b$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin</td>
<td>$56^a, 6^c, 43^m$</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>$39^a$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>HSPG2</td>
<td>Basement membrane-specific heparan sulfate proteoglycan core protein</td>
<td>$14^a, 1^c$</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>Insulin-like growth factor-binding protein 4</td>
<td>$11^a, 22^b, 15^c$</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>LDHA</td>
<td>L-lactate dehydrogenase A chain</td>
<td>$15^a, 8^b$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>LDHB</td>
<td>L-lactate dehydrogenase B chain</td>
<td>$5^a, 4^b$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>LTF</td>
<td>Lactotransferrin (Fragment)</td>
<td>$3^c$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>NQO1</td>
<td>NAD(P)H dehydrogenase [quinone] 1</td>
<td>$92^a, 4^m$</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>THBS1</td>
<td>Thrombospondin-1</td>
<td>$143^a, 114^m$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>++</td>
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<td>TIMP1</td>
<td>Metalloproteinase inhibitor 1</td>
<td>$153^a, 115^b, 28^c, 241^m$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>TIMP2</td>
<td>Metalloproteinase inhibitor 2</td>
<td>$81^a, 37^b, 160^m$</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
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<tr>
<td>TPI1</td>
<td>Triosephosphate isomerase</td>
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<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>VIM</td>
<td>Vimentin</td>
<td>$87^m$</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

$^a$Cancer  
$^b$Breast cancer  
$^c$Colon cancer  
$^d$Metastasis

The total amount of protein candidate numbers observed in both preliminary study and the optimized study were compared, the gene observed was shown “+” and the nonexistence was shown as “−”. The number of total gene products with high confidence for each cell line in both studies are, in SUM159 are 101 and 167; in SUM1315 are 36 and 190; in HMEC are 155 and 268; in MCF7 are 207 and 471. Table 5.1 provides the comparable cancer-related protein results from the four cell secretome samples derived from two batches. The protein names are the symbols used in UniProt and GeneCards, which are listed alphabetical order.
Biomarker proteomics does not allow a straightforward analysis for biomarker discovery as numerous pro-
teins will be searched in a large data set, however, only a small set of proteins will be targeted for subsequent
clinical studies. Novoseek Hits from GeneCards indicated biomedical literature numbers in which both the
gene symbol and the protein product and disease associations appear. We have shown the relevance of the
observed proteins with general associations to breast, cancer, colon cancer and metastatic disease in this
table.

Tab 5.2: Gene data from search of VarElect GeneCards

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>GIFTs</th>
<th>Global Rank (of 10931)</th>
<th>Score</th>
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<tbody>
<tr>
<td>TIMP1</td>
<td>TIMP Metallopeptidase Inhibitor 1</td>
<td>55</td>
<td>210</td>
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<tr>
<td>TIMP2</td>
<td>TIMP Metallopeptidase Inhibitor 2</td>
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<td>249</td>
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<tr>
<td>VIM</td>
<td>Vimentin</td>
<td>61</td>
<td>347</td>
<td>34.72</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
<td>61</td>
<td>428</td>
<td>30.69</td>
</tr>
<tr>
<td>THBS1</td>
<td>Thrombospondin 1</td>
<td>55</td>
<td>524</td>
<td>26.25</td>
</tr>
<tr>
<td>LTF</td>
<td>Lactotransferrin</td>
<td>53</td>
<td>671</td>
<td>21.27</td>
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<td>LDHA</td>
<td>Lactate Dehydrogenase A</td>
<td>62</td>
<td>778</td>
<td>19.14</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>Insulin Like Growth Factor Binding Protein 4</td>
<td>52</td>
<td>1122</td>
<td>14.56</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
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<td>1172</td>
<td>14.05</td>
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<tr>
<td>HSPG2</td>
<td>Heparan Sulfate Proteoglycan 2</td>
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<td>1297</td>
<td>13.01</td>
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<tr>
<td>CLEC3B</td>
<td>C-Type Lectin Domain Family 3 Member B</td>
<td>49</td>
<td>1326</td>
<td>12.85</td>
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<td>C3</td>
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<td>LDHB</td>
<td>Lactate Dehydrogenase B</td>
<td>57</td>
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<td>7.85</td>
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<td>YWHAZ</td>
<td>14-3-3 protein Zeta</td>
<td>57</td>
<td>3052</td>
<td>7.04</td>
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<td>YWHAB</td>
<td>14-3-3 protein Beta</td>
<td>58</td>
<td>4189</td>
<td>5.37</td>
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<tr>
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<td>Ribosomal Protein Lateral Stalk Subunit P0</td>
<td>50</td>
<td>4693</td>
<td>4.79</td>
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</tbody>
</table>

The VarElect searching engine has functions to classify the identified gene products based on the gene data
and the queried phenotypes. The scores are usually in a range of 1-200 to illustrate the strength of the
connection. Table 5.2 shows the input of our gene list together with the additional inquiry of breast cancer, and the ranking of the strength score. The GIFT number, with the range 1 to 100, indicated the GeneCards Inferred Functionality Score uses the wealth of gene annotations within GeneCards to produce the degree of knowledge about the functionality of more than 169k human genes entries.\textsuperscript{18} The Global rank number has indicated the rank of a giving gene product out of the total 10931 genes related with breast cancer. In our result, the top five genes from the list are TIMP1 (TIMP Metallopeptidase Inhibitor 1), TIMP2 (TIMP Metallopeptidase Inhibitor 2), VIM (Vimentin), FN1 (Fibronectin 1) and THBS1 (Thrombospondin 1).
5.4.2 Pathway analysis of targeted proteins

Fig 5.2: Pathway Cell adhesion ECM remodeling

The candidate protein list with 16 genes was analyzed by Gene A La Cart tool on the GeneCards analysis suite. The relative pathways were calculated and the best matched one was the cell adhesion ECM remodeling signal pathway. The FN1 gene was shown in red and in the center of the pathway picture. EGFR was shown in this pathway as well. As we know, EGFR is an important gene of breast cancer signaling as well as a growth factor receptor. From the data collected so far, fibronectin has attracted our interest as a biomarker.
as it matched well our discovery criteria and will be the subject of a clinical study by our collaborator at Tufts Medical center.

## 5.5 Discussion

![Circular visualization of MCF7 genome](Image)

**Fig 5.3: The circular visualization of MCF7 genome**

It's a complicated process to discover and confirm a gene as a cancer biomarker, and then even more difficult to find a treatment for the resulting disease. The MCF7 cell line with estrogen, progesterone and glucocorticoid receptors is a human breast cancer cell line,\(^{19,20}\) which was derived from the pleural effusion in 1970s.\(^{21}\) MCF7 was recognized as a fundamental cell line serving as reference in many breast cancer and genomic research studies, according to its ability to generate RNA/DNA leads to support downstream
validation studies. For example a unique property of this cell line is associated with the estrogen receptor in the cell cytoplasm which made MCF7 a useful model for hormone responsive breast cancer research.\textsuperscript{22} As we show in Fig.\textsuperscript{5,3} the large set of data from these studies can be visualized by the software package Circos where the visual circle can be used to track genomic rearrangements and gene duplications.\textsuperscript{23, 24} In this manner the relationship of cancer tumor cell lines with the cell line MCF7 can be significantly validated by a comparison of the Circos plots of the cell lines.

From our gene list, our collaborator and us found several interesting genes that are reported association with breast cancer, such as LDHA (L-lactate dehydrogenase A chain), LDHB (L-lactate dehydrogenase B chain),\textsuperscript{25} CLEC3B (Tetranectin), IGFBP4 (Insulin-like growth factor binding protein 4),\textsuperscript{26} TIMP1 (Metalloproteinase inhibitor 1), TIMP2 (Metalloproteinase inhibitor 2),\textsuperscript{27, 28} THBS1 (Thrombospondin 1)\textsuperscript{29} and FN1 (Fibronectin).\textsuperscript{30} Based on our analysis and the curation by our collaborator of the potential biomarker list they have performed bioassays to validate promising gene candidates.

Our collaborator has selected FN1 and IGFBP4 from the list and monitored for activity associated with Tiam1 (T-Cell Lymphoma Invasion And Metastasis 1) regulation. The two genes were connected in the ECM (Extracellular matrix) pathway. The IGFBP4 had no effect on the fibroblast Tiam-osteopontin (OPN) pathway, however, with the increase of fibronectin secretion in aggressive breast cancer cell lines, our collaborators observed that exogenous fibronectin at physiological concentrations induces a decrease in Tiam1 expression and increased osteopontin levels in treated fibroblasts. As far back to 1980’s, there are findings on fibronectin with breast cancer linkages,\textsuperscript{31} where the scientists discovered the loss of fibronectin in cancer cells on comparing with normal cells and has cancer connections.\textsuperscript{32} Fibronectin is found in the extracellular matrix of all cells as linear and branched networks that surround and connect neighbouring cells.\textsuperscript{33} Fibronectin peptides can also mediate HMEC (Human Mammary Epithelial Cells) adhesion a to
porcine-derived extracellular matrix.\textsuperscript{34}

Another promising lead is that tumor lactate levels are associated with increasing metastasis and tumor recurrence. Thus the LDH proteins serve as indicators of lactate metabolism genes and were therefore targeted for cancer research. The inhibitors of LDHA have been shown to suppress tumor progression in prostate cancer.\textsuperscript{35} Although lactate was considered a byproduct from glycolysis, it has emerged as a critical regulator of cancer development and metastasis.

5.6 Conclusions

In this study, we have demonstrated that an initial proteomic analysis of the secretome (cell medium) characterized proteins of biological importance which can be correlated with breast cancer etiology. This study was based on advanced mass spectrometry technology, which gave a detailed view of the proteins secreted by the cell lines. The study of proteins that are related to metastasis correlated genes will yield insights into the biology of aggressive cancer cell lines. Subsequent studies can include the associated pathways, the metabolism of the gene products, RNA-sequence measurement to explore alternative splicing mechanisms, as well as other bioinformatic tools such as gene set enrichment and pathway analysis as well as targeted animal model studies using new genomic tools such as CRISPER. However, proteomic technology gives a unique view on the actual gene expression product with the additional complexity of post translational modifications that is produced and secreted by cancer cell and has a higher probability of generating a signal with a indicative prognosis of metastatic potential in an individual patient. With the development of the bioinformatics and biostatistics, the enriched genomic/proteomics data sets will be a promising approach to discover the cancer biomarkers for diagnosis and treatment in the future.
References


[14] Emma Yue Zhang, Massimo Cristofanilli, Fredika Robertson, James M Reuben, Zhaomei Mu,


## 5.7 Supplementary Data

Tab 5.3: Top 25 genes list from MCF7 cell line

<table>
<thead>
<tr>
<th>UniProt</th>
<th>Gene</th>
<th>Description</th>
<th>Xcorr Score</th>
<th>Cov %</th>
<th>Uniq. Pep.</th>
<th>PSMs #</th>
<th>AAs #</th>
<th>MW (kDa)</th>
</tr>
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<tbody>
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<td>P02751</td>
<td>FN1</td>
<td>Fibronectin</td>
<td>1848.08</td>
<td>40.70</td>
<td>54</td>
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<td>2386</td>
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<td>P60709</td>
<td>ACTB</td>
<td>Actin, cytoplasmic 1</td>
<td>1597.51</td>
<td>56.53</td>
<td>8</td>
<td>181</td>
<td>375</td>
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<td>P49327</td>
<td>FASN</td>
<td>Fatty acid synthase</td>
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<td>194</td>
<td>2511</td>
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<td>ENO1</td>
<td>Alpha-enolase</td>
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<td>Pyruvate kinase PKM</td>
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<td>Calsyntenin-1</td>
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Conclusions

Global health is always a topic for natural, health and social scientists to explore due to the rapid development of medical and chemical technologies. Recombinant protein production and manufacture is a popular topic with many researches. The requirement of low cost manufacturing is a huge issue that needs efficient techniques with high quality. In remote areas or emergency situations, to support the patient in their need and avoid fatal issues is very important. We have demonstrated the capability and consistency of *Pichia* based production and the reproducibility of InSCyT platform. This bench top system is capable to generate multiple biopharmaceutics with continuous running. Comparing with the quality level of FDA approved biotherapeutics, the InSCyT products have been produced in sufficient amount, full sequence coverage, and low levels of host cell proteins. Although modifications such as oxidation were generated in the fermentation process, the quantity of degradation products and process impurities was within the required safety range and not clinically relevant. We have developed protocols for the stability studies that generate degradation products as well as the required characterization methodology. Our methods are effective in analyzing changes at specific amino acid residues, and the harsh conditions developed to study oxidation and deamidation did not interfere with the detection of different sets of variants. In addition, we detected that the modifications were specific to a set of forced degradation conditions. We are therefore confident that our designed methods could be applied in newly produced biosimilars and to monitor specific stability
risks. During a fermentation run, the observed modification for each sample will indeed be continuously accumulated from beginning to the end, which suggests that the manufacturing process should be monitored continuously for product impurities. For other properties of the drug such as toxicity, biological function or in vivo stability, further studies will need to be performed.

In summary, the methodology we developed is effective to evaluate the sequence structures and monitor any degradation reactions. Different mass spectrometers were applied and compared, which will guide further analysis to select the best instrument based on the properties of the protein product. The crude protein drug produced from InSCyT system has been analyzed in this research and consistent data was demonstrated. Bottom-up LC-MS techniques may require more offline time for sample preparation and analysis, however, by this way, there will be more detailed information on the exact structural changes in the biologic product, down to the level of amino acids. On the other hand, the other techniques used in this study such as Raman spectrometry and electrokinetic concentration binding assays will differentiate the modifications directly on intact proteins in a short time period. These technologies are very efficient for a quick product evaluation or diagnosis of product quality.

For the cancer proteomic study, we performed a deep study on the genes and the corresponding proteins associated with metastasis and well as a comparison of aggressive vs non aggressive cancer cell lines. These results will help direct subsequent studies that can include associated pathways, the metabolism of the genes, RNA-sequence measurements, as well as other bioinformatic tools such as in depth gene pathway analysis and targeted animal model studies using new genomic tools such as CRISPER. However, proteomic technology gives a unique view on the actual expression product that is produced and secreted by cancer cell and has a higher probability of prognostic signals of metastatic potential in an individual patient. With the further development of the bioinformatics and biostatistics tools, the genomic data will be further explored
to discover informative cancer biomarkers for the diagnosis as well as treatment in the future.