Development of an immuno-mass spectrometric assay for validation of protein C inhibitor (PCI) as a biomarker for prediction of biochemical recurrence in prostate cancer patients

by

Morteza Razavi
B.Sc., University of Victoria, 2008

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

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Supervisory Committee

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Abstract

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Biomarker validation remains one of the most important constraints to development of new clinical diagnostic assays. To address this challenge, an immuno-mass spectrometric assay known as SISCAPA has been developed for quantitation of protein biomarkers in human blood. The SISCAPA assay overcomes the sensitivity barrier facing most mass spectrometric approaches by utilizing high affinity antibodies for enrichment of specific surrogate peptide analytes from complex mixtures such as trypsin-digested human plasma. However, several technological barriers remain before the SISCAPA technology gains widespread use for biomarker validation. Improvements are required in areas such as selection of high affinity anti-peptide antibodies, peptide detection sensitivity and increasing sample throughput to allow biomarker validation on large sample sets. The work presented in this dissertation describes the development of new methods for antibody selection and for high-throughput application of SISCAPA technology to biomarker measurement in human plasma. Specifically, two technological developments are described: 1) an assay called MiSCREEN was developed, which
allows high-throughput screening of anti-peptide antibodies, enabling selection of high affinity reagents for *de novo* SISCAPA assays and 2) a liquid chromatography (LC)-free SISCAPA assay was developed that enables quantitation of surrogate peptides using both MALDI-TOF and RapidFire/MS platforms. Taken together, these technological advances provide a meaningful solution to the biomarker validation dilemma and allow a unified system for biomarker qualification, verification, validation and development of clinical assays for diagnosis and monitoring of a variety of diseases.

To demonstrate the utility of the unified SISCAPA system for biomarker measurement, an assay was developed for protein C inhibitor (PCI) as a marker for prediction of biochemical recurrence in prostate cancer patients. The PCI-specific analyte was shown to predict biochemical recurrence of prostate cancer after radiation/hormone treatment. Early stage detection of recurrence was achieved, when compared to the ‘gold standard’ marker for prostate cancer, prostate specific antigen (PSA). Two-dimensional gel electrophoresis studies on PCI, revealed unique protein spots in a serum sample from a biochemically recurrent patient. Studying such alterations at the protein level may enable understanding of the molecular mechanisms by which PCI is involved in prostate cancer progression.
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Chapter 1. General Introduction

1.1. The biomarker validation dilemma

A biomarker is defined as a measurable indicator of the risk of contraction, presence or stage of a disease (Rifai, Gillette & Carr, 2006). By searching the scientific literature, Polanski and Anderson (2006) identified more than 1200 candidate protein biomarkers that have been proposed for diagnosis or monitoring of different types of cancer. It is interesting that since the inception of the Food and Drug Administration (FDA) only 10 proteins have been approved for diagnosis and management of different cancers (Polanski & Anderson, 2006).

More than 20 putative protein biomarkers have been identified for detection and monitoring of prostate cancer (PCa) (Veltri, 2006, p.269). The best known biomarker for diagnosis and clinical management of prostate cancer, prostate-specific antigen (PSA), lacks specificity and sensitivity for most clinical conditions (Adhyam & Gupta, 2012) and is generally held as a poor diagnostic biomarker that has limited use for clinical management. Despite this fact, none of the other putative protein biomarkers for PCa have been validated for clinical use.

The minimum patient cohort size required to validate a biomarker depends on many factors including the type of disease, prevalence of the biomarker in healthy vs. diseased states, possible post-translational alterations of the biomarker etc. While no specific formula exists to calculate the minimum required cohort size, the consensus in the proteomics community is that to validate an analyte, it needs to be measured in a
significant (N>1000) number of relevant human specimens (N. L. Anderson, 2005). The ‘gold standard’ method for this purpose has been immunoassays, specifically enzyme linked immunosorbent assays (ELISA). Almost 80% of FDA-cleared protein biomarkers are measured by immunoassays (N. L. Anderson, 2009). Immunoassays are sensitive (Elshal & McCoy, 2006) and amenable to automation (Vessella, Noteboom, & Lange, 1992) making them ideal for analysing analytes in large numbers of samples. However, there is a growing concern about the specificity of many of these assays, especially in complex matrices such as human plasma and serum. Immunoassays can be compromised by autoantibodies and anti-reagent antibodies endogenously present in the patients’ blood (Hoofnagle & Wener, 2009). In addition, the reagent antibodies themselves need to be highly specific so that they only recognize the antigen of interest and minimize false detection of interfering substances. Such reagents need to be generated de novo for every biomarker and must be rigorously selected to ensure specific binding without interferences. This stringency requirement and the need for two antibodies for every protein analyte makes development of immunoassays prohibitively expensive and time consuming (J. R. Whiteaker et al., 2011). Thus developing immunoassays for validating the growing number of putative protein biomarkers is a daunting challenge.

1.2. An approach to biomarker validation using mass spectrometry

The emergence of protein mass spectrometry methods over the past decade has led many to believe that current biomarker validation challenges can be met using mass spectrometry. As mentioned previously, developing highly characterized antibody pairs for use in immunoassays is a major hurdle in biomarker validation efforts. Typically, one
of the two required antibodies is used for capturing the protein analyte while the other antibody allows its detection and measurement. A mass spectrometer can detect and measure many analytes without using antibodies at all: The identity of the protein analytes can be deduced from their unique peptide fragments (Baldwin, 2003) and their quantity can be determined either by label-free techniques (Old et al., 2005) or by using externally applied stable-isotope standards (Gevaert et al., 2008).

A quantitative, mass spectrometric approach that has gained significant traction in recent years is selected reaction monitoring (SRM) (Addona et al., 2009; Deutsch, Lam, & Aebersold, 2008; Picotti & Aebersold, 2012). Like most mass spectrometric assays, SRM assays take advantage of proteotypic peptides for protein identification and quantitation. A proteotypic peptide is a peptide that is unique to the protein of interest. Useful proteotypic peptides are usually selected as tryptic peptides that are suitably released from the target protein by digestion of human plasma or sera and that are effectively ionized for mass spectrometric detection and identification (Mallick et al., 2006). SRM assays thus usually require predetermined peptide analytes with known fragmentation patterns in trypsin digested human plasma or serum. The peptides are typically analysed using a triple quadrupole mass spectrometer (Picotti & Aebersold, 2012). Individual ion fragments from the parent peptides are referred to as ‘transitions’, which serve as unique identifiers for the protein of interest. If used for biomarker validation, such SRM assays provide a number of advantages over immunoassays. Four of the main benefits of SRM assays are: a) The problem of non-specific interferences due to autoantibodies and anti-reagent antibodies is avoided since the serum or plasma is trypsin digested prior to use and thus these interfering antibodies are eliminated.
(Hoofnagle & Wener, 2009), b) Developing antibody pairs for immunoassays is expensive and time consuming while with SRM assays no affinity reagents are required, c) The analytes measured using SRM assays can be unequivocally identified as proteotypic sequences using tandem mass spectrometry (MS/MS), in contrast to immunoassays where the sequence identity of the analyte is not directly determined and d) The databases of predetermined analytes and their fragmentation patterns (the main requirement for an SRM assay) are becoming exceedingly comprehensive (Picotti et al., 2009).

Unfortunately, despite all the efforts, there is not a single biomarker to date that has been validated through mass spectrometric approaches and cleared by the FDA. This is due to the fact that SRM assays, in particular, face challenging limitations, which have prevented their widespread utilization for biomarker validation. Notably, given the complexity of human plasma/serum, these assays suffer from lack of sensitivity. Reported sensitivities for SRM assays generally fall in the low microgram per milliliter and high nanogram per milliliter range (Picotti & Aebersold, 2012), while many of the clinically useful biomarkers are found at lower concentrations (N. L. Anderson & Anderson, 2002). The other major limitation of SRM assays is their throughput level; measuring specific peptide analytes in unfractionated serum, given the complexity of this matrix, is a time-consuming procedure. If specimens from more than 1000 patients are to be analyzed, sample analysis time on the order of seconds is required if biomarker validation using mass spectrometric (MS) assays is to become a reality.
1.3. Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA)

To overcome the challenges of SRM assays, Anderson et al. (2004) developed a method called SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies). In this method, unlike SRM assays, a high affinity anti-peptide antibody is used to enrich the surrogate peptide of interest from digested human serum or plasma prior to MS analysis. Although SISCAPA assays depend on an affinity reagent, the need for antibody pairs is eliminated because a single anti-peptide antibody captures the analyte and the mass spectrometer serves as an auxiliary secondary antibody for detection and quantitation purposes. Like the AQUA (Absolute Quantification of Proteins) technique (Gerber, 2003), SISCAPA assays use synthetic stable isotope standard (SIS or ‘heavy’) peptides to quantitate the endogenous analyte. In this approach, a SIS peptide is generated that is identical in sequence to the endogenous peptide of interest but it has heavy isotopes incorporated in selected amino acids (usually $^{13}$C and/or $^{15}$N isotopes are used). Because its sequence is identical to the endogenous peptide, the antibody capture process and subsequent ionization in the mass spectrometer are uniform for both the SIS peptide and the corresponding analyte. However, because of the presence of the heavy isotope, the mass to charge (m/z) ratio is different for the SIS peptide and thus it can be resolved from the endogenous analyte on a mass spectrometer. The ratio of the intensity (or peak area) of the endogenous peptide compared to the SIS peptide is then used to quantitate the analyte. Theoretically, given that the analyte is unique to the protein from which it is derived, the concentration of the protein can be calculated by inference.
Enriching the peptide analyte by a high affinity antibody dramatically improves the sensitivity compared to unfractionated SRM assays because the analyte is enriched from a relatively large volume of digested serum (usually 10-100 microliters in SISCAPA assays compared to nanoliter volumes in SRM assays). Moreover, the throughput is improved since enrichment of a specific analyte reduces the complexity of the matrix and hence the cycle time is reduced. In fact, SISCAPA assays have been shown to reach sensitivities of low nanogram per milliliter with cycle times of several minutes (Hoofnagle, Becker, Wener, & Heinecke, 2008; Miller, Pope, Razavi, Pearson, & Anderson, 2012). Using the SISCAPA assay, Whiteaker et al. (2010) demonstrated sensitivities in the low picogram per millilitre range using one milliliter of digested plasma. Although the first limitation of SRM assays (i.e. sensitivity) is largely alleviated using the SISCAPA approach, throughput remains a challenge.

One of the factors that affects throughout is cycle time. Cycle time is the product of the number of transitions and the time that is spent to acquire each transition (dwell time). Longer dwell times, therefore, improve the signal to noise ratio. The second, and more prominent factor that affects throughput is the liquid chromatography (LC) step upstream of MS analysis. LC is necessary to separate the target peptide(s) from other peptides present in the sample background. In SISCAPA assays, non-specific “background peptides” are usually derived from cleavage of high abundance proteins such as albumin. These peptides bind non-specifically to the protein G coated magnetic bead supports that are used to capture the antibodies. Background peptides can saturate the MS signal, thus to detect the analyte of interest, the target peptide must be separated from background peptides prior to MS analysis. Such separation requirements contribute
to increased sample cycle times (often several minutes per sample) which is suboptimal for validating biomarkers in large sample sets.

1.4. Peptide enrichment: The antibody debate

Over the past decade, methods have been developed for derivation and characterization of anti-peptide antibodies suitable for use in SISCAPA assays. Typically, such antibodies are generated in rabbits using a lengthy (>90 day) immunization that allows extensive antibody affinity maturation to occur, thus antibodies with higher affinities are generated. Rabbits are chosen as the animal of choice since they have a delayed affinity maturation system that yields antibodies of high affinity (Zhu and Yu, 2009). If monoclonal rabbit antibodies (RabMAbs) are sought, splenectomy is performed at the end of the immunization process and splenocytes are fused with the proprietary fusion partner 240E-W to form viable hybridomas (Zhu and Yu, 2009). The fusion and production process takes an additional 8-12 weeks, thus contributing to the total elapsed time of ~6 months for derivation and selection of appropriate monoclonal anti-peptide antibodies by this “conventional” cell fusion technique. Because of this long lead-time before antibodies (both polyclonal and monoclonal) are available for use in SISCAPA assays, many people are critical of this approach to making affinity reagents for peptide enrichment. Thus several groups have proposed generating antibodies through phage display technology (Barbas III, Kang, Lerner, & Benkovic, 1991; Hust & Dübel, 2004). Such reagents may be useful but the technologies are not widely practised and sufficient anti-peptide antibody selection strategies have not yet been applied using these methods.
The crucial point that needs to be highlighted is that the function of antibodies is an assay-dependant phenomenon. For example, an antibody might be an excellent reagent for use in immunoblotting yet it may not be useful for immunohistochemistry (Blow, 2007). Anti-peptide antibodies that are used in immunoproteomics assays (i.e. SISCAPA) must have affinities in the subnanomolar range (minimum half off-time of 10 minutes) to allow capture of peptides from complex peptide mixtures and to hold onto them during immunoadsorbent washing procedures. To my knowledge there are no reports that demonstrate a shorter path to antibody derivation that can systematically and reproducibly accelerate the affinity maturation process. There are recent efforts to produce proprietary adjuvants that speed up the immunization process (AnaSpec Inc., Fremont, CA) and to capture individual, specific B-lymphocytes from peripheral blood, a technology known as BLAST, which reduces the monoclonal antibody generation time compared to standard hybridoma-based procedures (Babcock, Leslie, Olsen, Salmon, & Schrader, 1996).

Other non-antibody approaches, for example using synthetic affinity reagents such as RNA aptamers (Brody et al., 1999; Eaton, Gold, & Zichi, 1995) or “click chemistry” (Agnew et al., 2009; Wahlberg, 2003), have not proven to be useful yet as there is less control of the specificity of these reagents, which becomes problematic in binding specific peptides in complex mixtures such as digested human plasma. Such non-antibody approaches may prove useful in the future but have not yet been shown to be robust and effective for use in immunoproteomics.

In many cases, polyclonal antibodies may be useful for peptide enrichment. For example, if the peptide target is abundant, lower affinity antibodies may be sufficient for
enrichment and polyclonal, affinity-purified antibodies can be used. As described above, production of polyclonal antibodies (pAbs) is quicker and less expensive compared to monoclonal antibody (mAb) production. However, mAbs are still the reagents of choice since unlike pAbs, they are homogenous as they are derived from a single hybridoma clone and are renewable since the parental clone can be cryopreserved and grown indefinitely (Blow, 2007). Moreover, unpublished studies in the Pearson lab have demonstrated that highly selected rabbit monoclonal anti-peptide antibodies (RabMAbs) can often have higher peptide retention time in solution compared to their polyclonal counterparts raised against the same target. Hence, SISCAPA assays generally tend to use monoclonal antibodies.

Aside from the cost and time that is required to generate this class of antibodies, selecting clones that produce mAbs with subnanomolar affinity is a challenge. Most investigators select positive clones based on the avidity (i.e. the strength of interactions between the antigen and multiple antigen-binding sites of the antibodies that are produced) and not their affinity (i.e. strength of interaction between the antigen and a single antigen-binding site). For this reason a novel surface plasmon resonance (SPR)-based technique was developed to select high affinity anti-peptide antibodies (Pope, Soste, Eyford, Anderson, & Pearson, 2009). In this method, goat anti-rabbit capture antibodies are immobilized on an SPR (Biacore) chip through amine coupling. The RabMAbs in hybridoma supernatants are then captured on the chip and the antigen is pumped through the system in solution. The kinetics of antigen-antibody interaction are then determined as the antigen is washed away over time. Using this strategy, Pope et al. (2009), were able to select antibodies with affinities in the subnanomolar range for use in
SISCAPA assays. The drawback of this method is that it is time-consuming (~1 hour per hybridoma supernatant), expensive and very different from the assay of ultimate use.

1.5. Scope of the dissertation

This research project has formed around the belief of my mentors and myself that SISCAPA technology has the prospect of mitigating the biomarker validation dilemma. When I became involved with the project, there were two rate-limiting steps within the SISCAPA procedure: First, antibody selection impeded the development of de novo SISCAPA assays and second, lengthy liquid chromatography step prior to MS analysis of SISCAPA eluates prohibited the analysis of patients’ specimens in a high-throughput fashion, which is a crucial requirement for biomarker validation. My project objectives therefore were: 1) To develop a high-throughput method for selecting high affinity anti-peptide antibodies directly from hybridoma supernatants, 2) To develop a method that would eliminate the need for time-consuming liquid chromatography in SISCAPA assays, thus shortening sample cycle times and 3) To develop a SISCAPA assay using the above methods to demonstrate their utility in high-throughput analysis of a biomarker in sera from human patients.

In Chapter 2, I describe the development of a method called MiSCREEN that allows selection of antibodies in an assay which is similar to the assay of ultimate use (binding of peptides from solution) and thus offers an improvement over existing antibody selection procedures. In Chapter 3, I describe the invention of liquid-chromatography-free SISCAPA methods that allow their use with high-throughput mass spectrometry platforms (MALDI-TOF/TOF and RapidFire/MS). In Chapter 4, I describe
the application of the technological advances outlined in Chapters 2 and 3 to
development of an assay for a putative cancer biomarker (protein C inhibitor; PCI). A
surrogate peptide from PCI was shown to be useful for monitoring the status of prostate
cancer progression in patients who receive radiation treatment with or without hormone
therapy. In Chapter 5, I describe biochemical work performed to examine the PCI protein
itself in the context of prostate cancer and discuss why it is important to study further the
relationship between PCI and prostate cancer progression. A schematic overview of the
Biomarker Validation Problem and the work performed to address it is shown in Figure
1.

Two concepts that are used in this dissertation in abundance and that need to be
differentiated from one another are ‘validation’ and ‘verification’. After the ‘discovery’
phase and before committing colossal amounts of resources to ‘validation’ of a biomarker
in large cohorts of patients for clinical use (N>1000), its potential value needs
‘verification’ in a smaller size cohort (N = 10s – 100s). In this dissertation I demonstrate
the utility of an LC-free SISCAPA assay for measuring a PCI peptide in clinical
specimens with clinically acceptable precision and verify its value as a predictor of
prostate cancer recurrence. A validation study on a large number of patients’ samples is
the next required step, and is beyond the scope of this thesis. However, validation of the
PCI peptide as a biomarker for predicting biochemical recurrence of prostate cancer after
radiotherapy will build upon the foundation that this thesis research provides.
Figure 1.1. The biomarker validation dilemma and scope of the dissertation
Chapter 2. MALDI immunoscreening (MiSCREEN): A method for selection of anti-peptide monoclonal antibodies for use in immunoproteomics

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All experiments presented in this chapter, except for the SPR analyses of antibody kinetics, were conducted by Morteza Razavi. The SPR analyses were performed by Matthew E. Pope.

2.1. Introduction

In the post-genomic era, more attention has been given to the ~22,000 proteins that are encoded in the human genome. Studying proteins can be as simple and direct as measuring their abundance in various bodily fluids or as convoluted as deducing their network of interactions or post-translational modifications. In either case, antibody reagents play a central role in the study of proteins. However, there is a severe shortage of antibody reagents against the many human proteins comprising the human proteome (Blow, 2007). To overcome this challenge, several ambitious projects are underway in both Europe (http://www.hupo.org/research/hai/; www.proteomebinders.org) and the United States (http://antibodies.cancer.gov) to make and characterize antibodies for use in immunohistochemical assays and for immuno-enrichment of proteins from complex mixtures. These anti-protein antibody reagents are also useful in “top-down” proteomics methods where protein targets are enriched prior to trypsinisation and subsequent peptide analysis by mass spectrometry (Nelson, Krone, Bieber, & Williams, 1995).
Much less thought and effort has been focused so far on the development of anti-peptide antibodies suitable for “bottom-up” proteomics approaches where a protein-specific proteotypic peptide is used to quantitate proteins by inference. These kinds of antibody reagents are used in quantitative assays such as immuno-Matrix-Assisted Laser Desorption/Ionization (iMALDI) (Jiang, Parker, Fuller, Kawula, & Borchers, 2007; Raska et al., 2003) or Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) (N. L. Anderson et al., 2004). To overcome the limitations that exist for anti-peptide antibodies, Anderson et al. (2009) proposed the human Proteome Detection and Quantitation (hPDQ) project. The goal of this project is to develop anti-peptide antibody reagents to quantitate surrogate peptides for all human proteins. This regenerative antibody resource will allow researchers to measure proteins in complex mixtures and will aid in biomarker validation efforts and ultimately in clinical assay developments.

Anti-peptide antibodies that are used in immunoproteomics assays, such as SISCAPA, need to have affinities (K_D) in the sub-nanomolar range. Essentially, such antibodies must be able to bind the target peptides from complex tryptic digests (typically from human plasma) and retain them through the washing steps prior to peptide elution and mass spectrometric analysis. With current SISCAPA assays, the retention time required for effective peptide enrichment is a minimum of 10 minutes during which time non-bound peptides are washed away. This unique requirement makes it a challenge to derive and select antibodies with the desired characteristics.

We previously developed a surface plasmon resonance (SPR) method that allows selection of monoclonal anti-peptide antibodies based on their affinities (not avidities)
and is useful for kinetic analysis (Pope et al., 2009). Antibodies selected by this method, specifically those with low off-rates ($k_d$) have been shown to work in SISCAPA assays. However, the method is expensive, cumbersome, time-consuming and importantly, very different from the assay of ultimate use. Therefore, it would be difficult to use this SPR-based method to screen antibodies for large-scale projects such as the hPDQ.

I sought to develop a method that would allow selection of high affinity anti-peptide monoclonal antibodies (mAbs) that are suitable for immunoproteomics applications in a more cost effective and high-throughput fashion. To do this I developed a method called MALDI-immunoscreening (MiSCREEN) for rapid screening of hybridoma supernatants. Importantly, the MiSCREEN workflow was designed to mimic the SISCAPA procedure so that antibodies are selected based on criteria that are very similar to the assay of ultimate use. The method allows the identification of antibodies that are able to bind specific peptides in solution phase from complex mixtures and that have low off-rates ($k_d$) suitable for use in immuno-MS assays.

2.2. Materials and Methods

2.2.1. Peptides

Synthetic tryptic peptides chosen as proteotypic surrogates of protein biomarkers were used throughout. Peptides that are uniquely encoded within the human genome and that yield multiple, strong selected reaction monitoring (SRM) transitions were selected based on previously described criteria (N. L. Anderson et al., 2004). Peptides were
synthesized by solid-phase methods by either the Chinese Peptide Company (Hangzhou, China) or by the UVic-Genome BC Proteomics Centre (Victoria, BC) and were tested by the vendors for the correct masses by MALDI-TOF mass spectrometry and for purity by high performance liquid chromatography (HPLC). Vendors were requested to supply peptides of greater than 80% purity and while this requirement was met for most of the peptides, in one case (peptide CPTAC-43c; from protein HE-4) the peptide contained significant impurities, thought mainly to be due to multiple cysteine modifications. The peptides were quantified by amino acid analysis (Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Ontario) and were stored at -20 °C in solution phase to prevent solubility problems that occur with some peptides after lyophilization. After thawing and/or just before use in MiSCREEN, all peptides were analysed by MALDI-TOF MS to determine their integrity and to assess the presence of altered forms.

Peptides were first synthesized with C-terminal cysteines to allow thiol-coupling to keyhole limpet hemocyanin (KLH) carriers for immunization (Pierce Chemical Co., St. Louis, MO). The same peptides synthesized without C-terminal cysteines were used in enzyme linked immunosorbent assays (ELISA; see peptide ELISA below) and in MiSCREEN and SPR assays for measuring antibody-peptide binding without interference from the linker cysteine. Although the peptides for this work were chosen as proteotypic surrogates of a variety of prospective biomarkers, any peptide of interest that can be bound by an antibody and detected by MALDI-TOF mass spectrometry can be used. The peptides used in this work are described in Table 2.1.
2.2.2. Anti-peptide antibodies

Rabbit monoclonal antibodies (RabMAbs) were produced by Epitomics Inc. (Burlingame, CA) using their proprietary, stabilized rabbit plasmacytoma cell line derived from the original parental myeloma 240E-W (Spieker-Polet, Sethupathi, Yam, & Knight, 1995) as the parental myeloma fusion partner. For each fusion, 4000 hybridoma supernatants were tested by peptide ELISA using the immunizing peptides (without carrier or added C-terminal cysteine). By using free-peptides in the ELISA assay we eliminated the possibility of selecting antibodies that recognize cysteine as part of the epitope (cysteine is used for coupling the hapten to the carrier but is not part of the endogenous analyte released by trypsin digestion of human plasma). It is important to note that the monoclonal antibodies selected by peptide ELISA screening may not be suitable for binding of peptides from solution phase since antibody avidity may allow selection of reagents with low affinities (single site interactions). For this reason, peptide ELISA is used only to select hybridoma clones that are secreting peptide-specific RabMAbs before they undergo further selection based on their association/dissociation rates (affinity measurements of peptide binding in solution). Positive rabbit hybridoma supernatants (usually obtained in volumes of 400 µL after the initial peptide ELISA) were used for MiSCREEN and SPR assays. All supernatants were stored at 4 °C before use to avoid freeze-thaw cycles and subsequent denaturation of antibodies.

We also used recombinant, purified, mouse monoclonal antibodies to demonstrate the utility of the assay for anti-peptide antibodies that have already passed the production stage and are offered commercially for purposes other than bottom-up proteomics. One of these, mAb 2A7 specific for peptide PPI-1b from LPS binding protein (see Table 2.1)
was produced by Immunoprecise Antibodies Ltd. (Victoria, BC) using ClonaCell-HY® single-step selection and cloning medium (Cat. No. 03800; StemCell Technologies Inc., Vancouver, BC) and the other, mAb BGN/KA/4H, specific for angiotensin I, was purchased from AbCAM (Cambridge, MA).

Table 2.1. Protein targets and their surrogate (proteotypic) peptides
(Table re-printed with permission; Elsevier: 3017341038317)

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Surrogate Peptide ID</th>
<th>Amino Acid Sequence</th>
<th>Mass (Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin Light Chain</td>
<td>CPTAC-14b</td>
<td>KPAEDEWGK</td>
<td>1059.14</td>
</tr>
<tr>
<td>Ferritin Light Chain</td>
<td>CPTAC-14d</td>
<td>LGGPEAGLGEYLFER</td>
<td>1607.78</td>
</tr>
<tr>
<td>Alpha-fetoprotein precursor</td>
<td>CPTAC-23a</td>
<td>GYQELLEK</td>
<td>979.10</td>
</tr>
<tr>
<td>Alpha-fetoprotein precursor</td>
<td>CPTAC-23c</td>
<td>YIQESQALAK</td>
<td>1150.30</td>
</tr>
<tr>
<td>Receptor tyrosine-protein kinase</td>
<td>CPTAC-36c</td>
<td>NNQLALTLIDTNR</td>
<td>1485.80</td>
</tr>
<tr>
<td>Receptor tyrosine-protein kinase</td>
<td>CPTAC-36d</td>
<td>AVTSANIQEFAGC*K</td>
<td>1495.72</td>
</tr>
<tr>
<td>Mucin-16 (CA-125)</td>
<td>CPTAC-38b</td>
<td>ELGYPYTLDR</td>
<td>1063.54</td>
</tr>
<tr>
<td>Mucin-16 (CA-125)</td>
<td>CPTAC-38c</td>
<td>VLQGGLGPPIFK</td>
<td>1184.74</td>
</tr>
<tr>
<td>Thyroglobulin precursor</td>
<td>CPTAC-39c</td>
<td>FSPDDSAGASALLR</td>
<td>1406.69</td>
</tr>
<tr>
<td>Thyroglobulin precursor</td>
<td>CPTAC-39d</td>
<td>VIFDANAPVAVR</td>
<td>1271.71</td>
</tr>
<tr>
<td>WAP four-disulfide core domain protein</td>
<td>CPTAC-43c</td>
<td>C<em>C</em>SAGC<em>ATFC</em>SLPNDK</td>
<td>1847.72</td>
</tr>
<tr>
<td>LPS Binding Protein</td>
<td>PPI-1b</td>
<td>ITLPDFTGDLR</td>
<td>1247.41</td>
</tr>
<tr>
<td>Protein C Inhibitor</td>
<td>PPI-4d</td>
<td>EDQHYLLDR</td>
<td>1351.44</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>Ag-I</td>
<td>DRVYIHPFHL</td>
<td>1296.49</td>
</tr>
</tbody>
</table>

1 The asterisk (*) denotes carbamidomethyl cysteine
2 Angiotensin I was chosen as a positive control peptide since it ionizes extremely well and is often used as a standard in MALDI-TOF mass spectrometry
2.2.3. Peptide ELISA

In ELISA, many different RabMAbs and mouse mAbs raised against peptide-KLH conjugates showed reactivity with unrelated peptide-carrier conjugates (i.e. different peptides coupled to a different carrier protein; unpublished observations). Such antibodies bind to the linker structure that is used for coupling the peptide hapten to the carrier. For this reason, we modified a standard indirect ELISA method (Tolson, Turco, Beecroft, & Pearson, 1989) to use unconjugated peptide antigens (i.e. not coupled to protein carriers) to coat polystyrene microtitre ELISA plates (Greiner Bio-One Microlon™ 600, Cat. No. 655081). In this specialized peptide ELISA, peptides were dissolved in distilled water to a final concentration of 0.1 to 5.0 µg/mL (each peptide was first titrated to select the optimum concentration to give good signal to noise ratios) and 100 µL of this solution were dried onto each well by overnight incubation at 37 °C in a dry incubator. The appropriate peptides used as immunogens (but not containing terminal cysteines) were used as antigens in peptide ELISA along with different (control) peptides for specificity analysis. Using this peptide ELISA, we were able to screen the large number of hybridoma clones obtained from each fusion (typically 4000 clones) for production of anti-peptide antibodies.

2.2.4. Measurement of solution-phase peptide binding by MiSCREEN assay

To identify high affinity anti-peptide antibodies first selected by peptide ELISA, mAbs in hybridoma supernatants were first captured by magnetic affinity beads (see below) followed by binding of specific peptides from solution. After the antigen-capture step the bead-antibody-antigen complex undergoes a carefully timed wash step. For all
MiSCREEN assays presented here, the wash step was approximately 10 minutes since the selected antibodies were to be used in SISCAPA assays, which require a half off-time of ~10 minutes. However, the time for the wash step can be modified based on the needs of the ultimate assay. To demonstrate this, we screened the RabMAbs for one of the targets (thyroglobulin FSP; CPTAC-39c) using a 4-minute wash cycle. The rationale behind the MiSCREEN approach is that antibodies with higher affinities retain more of the target antigen during the wash step, which is reflected in the intensity of the MALDI signal for that peptide.

As with the SISCAPA workflow, the MiSCREEN assay was designed to use a magnetic bead-handling robot (KingFisher 96, Thermo Fisher Scientific, MA, USA). The buffer used throughout this procedure was phosphate buffered saline (PBS)/0.03% (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (CHAPS) detergent. CHAPS is used to prevent non-specific adherence of the magnetic beads to the plastic surfaces. In MALDI-TOF MS, CHAPS appears as a single peak at 1229.7 Daltons and in triple quadrupole mass spectrometers it elutes as a single major hydrophobic peak late in the reverse phase chromatographic separation, after most peptides. It is thus more practical than other detergents, most of which are polymeric, yielding many peaks revealed by MS, which may interfere with peptide ionization and analysis of MS spectra.

The MiSCREEN workflow uses six 96-well polypropylene microplates (Cat. No. CA83007-596, Thermo KingFisher 96 KF plate, Thermo Scientific). The wells of the first plate (“Bead Wash”) contain 10 µL of sheep anti-rabbit IgG Dynabeads (Cat. No. 112.03D, Invitrogen, Oslo, Norway; the volume corresponding to the original concentration in the bottle) suspended in 200 µL of PBS/0.03% CHAPS. If mouse mAbs
were being screened, protein G coated Dynabeads (Cat. No. 100.04D) or goat anti-mouse IgG magnetic beads (Cat. No. 110.33) were used. After washing by agitation for 5 minutes, the beads were transferred by the Kingfisher magnet array into wells of the second plate (“Ab Capture”), each containing 50 µL of hybridoma supernatant. The beads were incubated in the hybridoma supernatant for 2 hours with constant shaking at room temperature. After the incubation the magnetic beads and captured antibodies were transferred to the third plate (“Ag Capture”), which contained 1 pmol of the target peptide in 100 µL of PBS/0.03% CHAPS. After another 2-hour incubation step, the magnetic bead-antibody-peptide complexes were transferred sequentially to three microplates for washing (in 200 µL of PBS/0.03% CHAPS). The washed beads were finally transferred to an elution plate (96-well Hard-Shell® PCR plate, Bio-Rad, Ca, USA), each well containing 25 µL of 5% acetic acid, and incubated for 5 minutes to release any bound target peptide. The eluted peptides from all 25 µL were desalted and concentrated using ZipTip C18 tips (Cat. No. ZTC18S960, Millipore, MA, USA) before spotting the entire sample in a 2 µL volume onto a MALDI plate. After drying, 1 µL of the matrix alpha-cyano-4 –hydroxycinnamic acid (CHCA) was added to each spot. A Voyager DE™ STR (Applied Biosystems, Foster City, CA) was used to analyze the eluted samples from the MiSCREEN experiments. The instrument was set to reflectron mode with laser intensity of 2800, accelerating voltage of 20 kV, delay time of 220 nsec and the mass range was set to 800-3000 Daltons. One hundred shots were accumulated per spectrum and 5 spectra were accumulated for each sample spot. All experiments were performed in duplicate.
Prior to MiSCREEN analysis, a dilution series of each peptide was made and known amounts were spotted onto MALDI plates to establish a standard curve. In this way, the signal intensity for each peptide could be gauged as a measure of peptide performance. Multiplexing of the MiSCREEN assay was tested by mixing supernatants from a number of different hybridomas (each secreting different peptide-specific RabMAbs) and measuring the enrichment of the relevant peptides from mixtures that also contained irrelevant peptides as specificity controls. In addition, multiplexing was tested using a different set of five peptides, two of which were specificity controls. Multiplexing experiments were performed with three replicates per sample.

2.2.5. Measurement of solution-phase peptide binding by SPR

Kinetic screening of anti-peptide RabMAbs was performed by SPR using a Biacore 3000 optical biosensor (Biacore, Uppsala, Sweden) according to a previously published method designed specifically for measuring affinities of monoclonal antibodies (Pope et al., 2009). Research-grade CM5 chips (Order Code BR-1003-99) were used for all experiments and were obtained from Biacore Life Sciences (Piscataway, NJ). The CM5 chips were coated with affinity-purified goat anti-rabbit IgG (Fc fragment specific; Jackson ImmunoResearch Laboratories, West Grove PA; Cat. No. 111-005-008) through an amine coupling method with 100 nM N-hydroxysuccinimide/390 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride as cross-linker. RabMAbs were captured by flowing hybridoma supernatant over the chip. Lastly, through the microfluidic networks, the peptide antigen was introduced to the flow-cell and captured by the solid-phase adsorbed RabMAb. The antigen-antibody interaction was then
modeled using a 1:1 Langmuir binding model, which determines the on- and off-rate constants \( (k_a \text{ and } k_d) \). The affinity \( (K_D) \) was then calculated based on the rate constants \( (K_D = k_d/k_a) \). The assumption behind this SPR method is that the on-rate is very similar for antibodies that are raised against the same target. Therefore, the off-rate has a more profound impact on the affinity. Thus the SPR method selects antibodies based on their off-rates.

### 2.3. Results

#### 2.3.1. Synthetic peptides

It was important that all peptides synthesized for this work be of high purity and that they be handled in a way that would preserve their integrity and prevent post synthesis modifications. Initially, peptide vendors were instructed that 80% purity was required and that both MALDI-TOF and HPLC traces be supplied. Further quality control was undertaken whereby after thawing and just before use of the peptides, they were examined by MALDI-TOF MS to ensure that the appropriate peptide mass was the predominant species. Examples of results obtained for two peptides, one highly pure the other not, are shown in Figure 2.1: A typical high-purity peptide, CPTAC-39d, is shown in Figure 2.1.a while a peptide that contains unacceptable impurities, CPTAC-43c, is shown in Figure 2.1.b. This peptide, derived from human epididymis protein 4 (HE-4; a putative biomarker for ovarian carcinoma), was an unusual peptide since it contained 4 cysteines that required special blocking by carbamidomethylation.
Figure 2.1. Peptide purity

A high-purity peptide (CPTAC-39d) (A) and a low-purity peptide (CPTAC-43c) (B) are shown. Figure reprinted with permission (Elsevier: 3017341038317).
2.3.2. Peptide titration curves

The peptides used in this work were originally chosen on the basis of their performance in electrospray ionization - triple quadrupole mass spectrometry. Therefore, it was important to test each peptide for its ability to ionize effectively by MALDI-TOF since not all peptides ionize equally well under different ionization conditions. Since peptides differ in the strengths of their MS signals, for each MiSCREEN analysis we performed a standard curve by directly spotting varying amounts of the appropriate peptide onto the MALDI target. To demonstrate this, the results obtained from three selected peptides are shown in Figure 2.2. While in all three cases we observed acceptable linearity (coefficient of determination \( R^2 > 0.95 \)), ionization of the peptides varied widely. Panel A shows the strong MS signals obtained with varying amounts of angiotensin I, a peptide that is often used as a standard in MALDI-TOF MS. Panel B shows the more typical MS signals obtained with tryptic peptides that we use in our immuno-MS assays. One peptide, (CPTAC-38b) ionized well whereas the other (CPTAC-23c) did not. This observation is consistent with previous reports that demonstrate MALDI-TOF instruments are biased towards tryptic peptides with a C-terminal arginine residue (Krause, Wenschuh, & Jungblut, 1999). In other words, peptides with a lysine C-terminal (e.g. CPTAC-23c) usually do not ionize well on these instruments. In the latter case, we would be sensitized to look carefully at low signal levels when assessing antibody capture in MiSCREEN analysis.
Figure 2.2. Peptide ionization

Excellent ionization was observed for angiotensin I (A) compared to typical proteotypic peptides (B). Tryptic peptides with C-terminal lysine (CPTAC-23c) usually ionize poorly by MALDI-TOF. Note: The signal for angiotensin I beyond 400 fmol is out of the linear range of the instrument. Figure re-printed with permission (Elsevier: 3017341038317).
2.3.3. Screening of hybridoma supernatants by MiSCREEN

To illustrate how high affinity anti-peptide mAbs were selected, the results of screening assays for selection of hybridoma supernatants containing high, medium and low affinity RabMAbs against a thyroglobulin specific peptide (CPTAC-39d) are shown in Figure 2.3. Panels A-C show the MiSCREEN results with Panel A representing a hybridoma clone that produces low affinity RabMAbs and Panel C representing the clone that produces the highest affinity antibodies amongst the clones from this fusion. Panels D-F show the respective SPR sensograms that were created using the same hybridoma supernatant used in the MiSCREEN assay. A good correlation was observed between the two assays. Moreover, the results indicate that by changing the duration of the wash step in the MiSCREEN assay, it is possible to select antibodies with varying off-rates depending on the requirements of the ultimate assay (Figure 2.4). Hybridoma supernatants were screened using either a 10-minute washing procedure (Figure 2.4.a) or a 4-minute washing procedure (Figure 2.4.b). In each case only high affinity antibodies that are able to retain the antigen during the washing step exhibit a high MALDI signal intensity.

More than 500 hybridoma supernatants producing RabMAbs against fifteen different tryptic peptides were screened for peptide binding in the MiSCREEN assay. A summary of the results is shown in Table 2.2. As expected, although all of the supernatants tested contained anti-peptide mAbs detected by peptide ELISA, only a fraction of them were positive by MiSCREEN, suggesting that the latter assay detected only high affinity antibodies and that with ELISA there are complicating avidity considerations.
Figure 2.3. Peptide enrichment by MiSCREEN and surface plasmon resonance

Panels A, B and C show MiSCREEN spectra for clones producing RabMAbs specific for CPTAC-39d, a thyroglobulin specific peptide, with low (Panel A) medium (Panel B) and high (Panel C) affinities. Antibodies with higher affinities retain more of the target peptide during the washing procedure, which is reflected in the MALDI signal intensity (y-axes). Panels D-F demonstrate the SPR sensograms for the same hybridoma supernatants. The half off-times were 8 min, 22 min and 174 min for the low affinity, medium affinity and high affinity RabMAbs, respectively. Figure re-printed with permission (Elsevier: 3017341038317).
Hybridoma supernatants containing antibodies specific for CPTAC-39d (thyroglobulin peptide) were screened by the MiSCREEN assay with a washing time of ~10 minutes (A). This wash time corresponds to a $k_d$ of approximately 1.7E-03 as determined by the SPR assay. Antibodies that had a high enough affinity to retain the target peptide during the wash steps (approximately 10 min; $k_d$ of ~1.7E-03) showed a high MALDI signal by MiSCREEN. Hybridoma supernatants containing RabMAbs specific for a different thyroglobulin peptide (CPTAC-39c) were screened by MiSCREEN using a wash time of approximately 4 minutes ($k_d$ of ~4.0E-03) as shown in panel (B). Once again, antibodies with off-rates below this threshold performed weakly in the MiSCREEN assay while high affinity RabMAbs capable of retaining peptides beyond the wash period yielded a strong MALDI signal. The vertical lines in each panel represent the half off-times (10 min in Panel A and 4 min in Panel B). Figure re-printed with permission (Elsevier: 3017341038317).

Figure 2.4. Screening hybridoma supernatant with varying wash times
Table 2.2. Summary of MiSCREEN vs. SPR selection of hybridoma clones producing high affinity RabMAbs

(Table re-printed with permission; Elsevier: 3017341038317)

<table>
<thead>
<tr>
<th>Surrogate Peptide ID</th>
<th>Selected Hybridoma</th>
<th>Accumulated MALDI Peak Intensity</th>
<th>SPR $K_D$ (nM)</th>
<th>SPR $k_a$ (1/Ms)</th>
<th>SPR $k_d$ (1/s)</th>
<th>SPR Half Off-time (min)</th>
<th>Antibody Selected (Yes/No)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPTAC-14b</td>
<td>10-10</td>
<td>465</td>
<td>172.4</td>
<td>4.75E5</td>
<td>8.19E-2</td>
<td>0.14</td>
<td>No</td>
</tr>
<tr>
<td>CPTAC-14d</td>
<td>10-58</td>
<td>8014</td>
<td>0.177</td>
<td>1.27E6</td>
<td>2.25E-4</td>
<td>51.3</td>
<td>Yes</td>
</tr>
<tr>
<td>CPTAC-23a</td>
<td>19-40</td>
<td>2012</td>
<td>9.60</td>
<td>9.01E4</td>
<td>8.65E-4</td>
<td>13.3</td>
<td>Yes</td>
</tr>
<tr>
<td>CPTAC-23c</td>
<td>19-44</td>
<td>Peptide ionizes poorly</td>
<td>3.99</td>
<td>2.71E5</td>
<td>1.08E-3</td>
<td>10.7</td>
<td>Yes</td>
</tr>
<tr>
<td>CPTAC-36c</td>
<td>None$^2$</td>
<td>No binding of peptide detected by SPR</td>
<td>No binding of peptide detected by SPR</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CPTAC-36d</td>
<td>32-27</td>
<td>3802</td>
<td>1.16</td>
<td>1.36E5</td>
<td>1.58E-4</td>
<td>73.1</td>
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<tr>
<td>CPTAC-38b</td>
<td>34-102</td>
<td>33774</td>
<td>0.22</td>
<td>1.23E5</td>
<td>2.71E-5</td>
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<td>CPTAC-38c</td>
<td>34-15</td>
<td>6174</td>
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<td>4.32E5</td>
<td>7.41E-4</td>
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<td>CPTAC-39c</td>
<td>35-31</td>
<td>2966</td>
<td>0.040</td>
<td>6.16E5</td>
<td>2.44E-5</td>
<td>473.5</td>
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<td>CPTAC-39d</td>
<td>35-41</td>
<td>2842</td>
<td>1.16</td>
<td>7.71E4</td>
<td>6.64E-5</td>
<td>174.0</td>
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<td>CPTAC-43c$^3$</td>
<td>38-12</td>
<td>2108</td>
<td>0.82</td>
<td>7.26E5</td>
<td>5.93E-5</td>
<td>194.8</td>
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<tr>
<td>PPI-1b</td>
<td>2A7$^4$</td>
<td>12300</td>
<td>ND$^7$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
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<tr>
<td>PPI-1c</td>
<td>109-4</td>
<td>10000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
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<tr>
<td>PPI-4d</td>
<td>58-4$^5$</td>
<td>13000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>PPI-6d</td>
<td>79-9</td>
<td>6739</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>Yes</td>
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<td>Angiotensin-I</td>
<td>BGN/KA/4H$^6$</td>
<td>3464</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
</tr>
</tbody>
</table>

$^1$ Only antibodies that demonstrated a half off-time of greater than 10 minutes were selected
$^2$ None of 12 hybridoma supernatants selected by ELISA showed any peaks detected by MiSCREEN
$^3$ Peptide impurities were present
$^4$ Mouse mAb (Immunoprecise Antibodies Ltd.)
$^5$ Recombinant rabbit mAb
$^6$ Mouse mAb (AbCAM)
$^7$ ND=Not Done (An anti-mouse capture reagent for SPR was not available at the time of experiment)
One notable weakness of the MiSCREEN method is illustrated by the results shown in Table 2.2. SPR analysis of mAb 19-44, specific for peptide CPTAC-23c, showed that this mAb had a half off-time of 10.7 minutes and is probably useful for immuno-MS assays. However, this was not detected by the MiSCREEN assay since the peptide itself does not ionize well by MALDI-TOF MS. On the other hand, we observed a number of unanticipated, positive attributes of the MiSCREEN method in our experiments. The first advantage of the MiSCREEN method is the unequivocal identification of the analyte. When screening the hybridoma supernatants against an impure peptide (e.g. CPTAC-43c; Figure 2.1.b) using SPR, the method is incapable of differentiating between antibodies that capture the ‘wrong’ peptide and the ones that bind to the ‘correct’ form of the peptide, whereas MiSCREEN analysis revealed that mAb 38-12 recognized the appropriate peptide mass (containing four S-carbamidomethyl cysteines) and is therefore likely a useful reagent for peptide enrichment.

Moreover, the MiSCREEN assay selects affinity reagents based on their performance in solution-phase in a format that is identical to the arrangement used by immunoproteomics assays. We observed that in some instances the SPR method was not able to acquire the necessary data for measuring binding kinetics since either the RabMAb capture was inadequate or inexplicable binding kinetics were observed. In these instances the MiSCREEN assay showed strong MALDI signal with the antibodies in question, indicating that these antibodies will be useful in immunoproteomics assays (Figure 2.5.a and b). As mentioned previously, the SPR method is focused on the off-rate for selection of affinity reagents since the on-rate is generally uniform for antibodies raised against the same target. However, in some instances the antibody shows excellent
on-rate characteristics while the off-rate is poor. In this instance, the antibody is still useful for use in immunoproteomics since although the antigen is released relatively easily from the antibody, because of the excellent on-rate the antibody is likely able to re-capture the antigen immediately after each release. An example of this phenomenon is shown in Figure 2.5 (c and d) where an acceptable MiSCREEN signal was observed using this antibody despite it showing a moderately fast off-rate.

Not surprisingly, we were also able to select high affinity recombinant RabMAbs (e.g. mAb 58-4 in Table 2.2) using the rabbit antibody capture system. In addition, high affinity murine mAbs (e.g. mAbs 2A7 and BGN/KA/4H, see Table 2.2) were detected when using either protein G Dynabeads or anti-mouse IgG Dynabeads as capture agents, illustrating the general utility of the MiSCREEN method.
Figure 2.5. Similarity of the MiSCREEN assay to the ultimate immunoproteomics assays allows for efficient selection of high affinity antibodies

A MiSCREEN spectrum is shown for a hybridoma supernatant against a proteotypic peptide specific for serum albumin. The strong MALDI signal demonstrate that this antibody is capable of retaining the antigen during the washing procedure. An SPR analysis could not be obtained for this sup (B). The peptide signal from a selected RabMAb by MiSCREEN specific for human IgM is demonstrated in (C). SPR analysis (D) demonstrates that this antibody has an excellent on-rate ($k_a = 1.14E+06 \text{ M}^{-1}\text{s}^{-1}$) but a poor off-rate ($k_d = 3.05E-02 \text{ s}^{-1}$; half off-time = 0.38 min) and as such is not considered to be a candidate for use in immunoproteomics. The fast on-rate presumably allows immediate re-capturing of the antigen and thus a strong MALDI signal is observed, indicating that this antibody will be useful for peptide capture in immuno-MS applications.
2.3.4. Multiplexed binding of peptides by MiSCREEN

Two sets of multiplexing experiments were performed, each using different combinations of RabMAbs and peptides. Representative results from one of these experiments are shown in Figure 2.6. In this case, MALDI-TOF analysis showed that all five peptides (four target peptides and one control) were resolved after directly spotting the mixture onto a MALDI plate (Panel A). A mixture of four hybridoma supernatants containing specific RabMAbs captured all four cognate peptides whereas the control peptide was not captured (Panel B). Indeed, the control peptide CPTAC-28d, which ionizes well, was not detected at all (Panel C) showing that specificity of each antibody was retained by the multiplexed hybridoma supernatants. Similar results were seen with all three replicates in this experiment. In addition, in a second set of multiplexing experiments also performed in triplicate, RabMAbs selectively enriched 3 (of 3) of their cognate peptides whereas the two control peptides were not captured. In this latter experiment we also used two control RabMabs (of “wrong” specificity) and none of the 5 peptides were bound.
Figure 2.6. Using MiSCREEN to screen for high affinity RabMAbs in a mixture of hybridoma supernatants

One picomole of 5 tryptic peptides was mixed and directly spotted onto a MALDI plate as a positive control (A). One of the peptides (CPTAC-28d) served as a specificity (negative) control. Forty microliters of hybridoma supernatants containing RabMAbs specific for each of the four remaining peptides were pooled and MiSCREEN was performed using this mixture. The spectrum shows that all four specific peptides were captured while the specificity control peptide was not (B). An expanded view of the spectrum shown in Panel B shows that the control peptide was not present, even at background levels (C). Figure re-printed with permission (Elsevier: 3017341038317).
2.4. Discussion

High affinity anti-peptide antibodies are required for several different immuno-MS assays used for measurement of protein biomarkers in complex peptide mixtures. ELISA assays are often used to screen for hybridoma clones that are producing antigen-specific antibodies. The antigen used in these assays is often an intact protein or a peptide surrogate of the protein linked to a carrier molecule different from the one used in immunization (for example if KLH-coupled peptide was used in immunization, BSA-coupled peptide will be used for screening). This strategy is sub-optimal because the possibility exists that the linker region is recognized as part of the epitope but the linker region will not be present in the naturally occurring analyte. We were able to avoid selecting such antibodies by using a “peptide ELISA” in which titrated amounts of ‘linker-free’ peptide antigens are dried onto the ELISA wells. These ELISAs, although not useful for measuring antibody affinity, were useful for initial selection of peptide-binding mAbs.

Screening anti-peptide mAbs using SPR has proven to be a useful technique for identification of high affinity anti-peptide antibodies (Pope et al., 2009). However, this SPR protocol, with a cycle time of approximately 30-45 minutes, is too slow and expensive for processing large numbers of hybridoma supernatants. Moreover, the SPR method is drastically different from the assay of ultimate use. For these reasons, we developed the MiSCREEN method that combines the same peptide enrichment procedures used in SISCAPA assays with robust MALDI-TOF MS for analysis of bound peptides. Using this assay we are now able to select affinity reagents for use in immuno-
proteomics in a time and cost effective manner. Moreover, the method is capable of
selecting antibodies in instances were the SPR method is not useful for measuring the
kinetics of antibody-antigen interactions. In MiSCREEN, both peptide enrichment and
MALDI-TOF MS analysis of the bound-eluted peptides can be automated so that high-
throughput is possible. In fact, after the publication of this study, and in line with the
objectives of the hPDQ proposal, we successfully used the MiSCREEN assay on a
relatively large scale to screen more than 5000 hybridomas raised against 50 different
targets.

For a chemically homogeneous mAb reagent, half the peptide dissociates in
$\frac{LN/2}{k_d}$. To retain 75% or more of the captured peptide and to avoid losing assay
sensitivity, a half off-time of greater than twice the washing period is desirable. Current
quantitative SISCAPA protocols involve serial washes over a total elapsed time of 8-12
minutes after removal of antibody-coated magnetic beads from the sample digest,
suggesting that antibodies with half off-times of at least 10 minutes and ideally 20-30
minutes are desirable. Antibodies of even higher affinity (and by extension, longer half
off-times) allow extended washing and removal of non-specifically bound peptides.
Hence the totalled elapsed washing time for MiSCREEN was selected to be 10 minutes.
However, we have demonstrated that this time can be altered as needed to select
antibodies with specific peptide antigen retention times.

In the recent literature, there has been some skepticism about the prospect of using
antibodies for proteomics biomarker discovery and use (Zichi, Eaton, Singer, & Gold,
2008). The argument has been made that antibodies do not have the requisite specificity,
that off-target proteins would interfere with detection and with multiplexing and that
autoantibodies present in many people confound the problem. Many of these criticisms will hold true for top down proteomics approaches where intact proteins are captured before processing for analysis by mass spectrometry. However, such criticisms do not hold true for anti-peptide antibodies used in bottom up proteomics since in this approach many interferences are eliminated through the process of sample digestion. We have shown that regardless of the species of origin, mAbs that bound peptides yielding high MALDI-TOF peak intensities (and low off-rates as determined by SPR) are clearly able to retain peptides long enough for use in immuno-MS methods such as SISCAPA under current configurations. In fact, selected antibodies presented here have been used in SISCAPA assays through the Clinical Proteomic Technology Assessment for Cancer (CPTAC) initiative to enrich specific target peptides from digested human plasma/serum with exquisite specificity and sensitivity (Kuhn et al., 2004; J. R. Whiteaker, Zhao, Anderson, & Paulovich, 2010).

A criticism that can be substantiated, however, is the fact that generating anti-peptide monoclonal antibodies through traditional hybridoma methods is an expensive and time-consuming process. Although solving this problem is beyond the scope of this dissertation, I believe that the multiplexing capabilities of the MiSCREEN method presented here can provide a feasible solution for selection of excellent anti-peptide capture antibodies. I believe that it should be possible to mix lymphocytes from different animals, each immunized with a different peptide, and perform a single fusion to produce mixtures of hybridomas. Each cloned hybridoma supernatant can then be screened for the presence of a different specific anti-peptide antibody using a single peptide mixture. This will decrease the cost and time required for mAb derivation and screening.
Chapter 3. Ultra-fast quantitation of peptides from human plasma digests using liquid chromatography-free SISCAPA assays

This chapter is published in the form of two separate manuscripts:


The experiments presented in this chapter were conducted by Morteza Razavi. Lauren E. Frick (Agilent Technologies) ran the peptide eluates on the RapidFire and LC/6490 QQQ platforms. Gary Kruppa (Bruker Daltonics) ran the peptide eluates on the microflex™ LT MALDI instrument.

3.1. Introduction

Separating chemical or biological analytes from complex mixtures is a routine laboratory practice. Amongst all fractionation techniques, chromatography is perhaps one of the most widely used techniques in biochemistry. Chromatography (from Greek *chroma* “color” and *graphein* “to write”) is a collective term that is used to refer to a variety of techniques that allow separation of analytes in complex mixtures based on their various characteristics such as molecular weight, charge and hydrophobicity. A special type of chromatography that gained a lot of attention during the 1960s was liquid-solid chromatography (L. R. Snyder, 1974; Soczewinski, 1977). The chromatography system in this case consists of a column that is packed with an adsorbent (usually silica or
alumina) called the solid phase. The analytes are introduced to this column in solution phase, which is generically called the mobile phase.

Adsorption of different analytes to the solid phase depends on the inherent characteristics of the analyte and the surface chemistry of the adsorbent thus allowing specific interactions with varying strengths for different analytes. In the original liquid-solid chromatography (now simply referred to as liquid chromatography or LC) experiments, the silica or alumina resins used in the solid phase were unmodified thus adsorbing hydrophilic molecules in the mobile phase and allowing the passage of hydrophobic molecules. After the analytes are separated on the solid phase, they are eluted from the column by altering the polarity of the mobile phase (Soczewinski, 1977). The elution step for multi-component mixtures is a delicate matter since analytes with similar characteristics will co-elute if the elution is isocratic (constant composition of the mobile phase) (L. R. Snyder, 1968). Therefore, researchers usually use ‘gradient elution’ where the composition of the mobile phase gradually changes over time, which improves the resolving power of the technique (Scott & Kucera, 1973).

A modified version of LC that is routinely used for separation of proteins and peptides is referred to as reversed-phase liquid chromatography (RPLC). Unlike the original LC, the solid phase (usually silica) in RPLC has hydrophobic characteristics (hence called ‘reversed’) because the surface is coated with alkyl chains of varying lengths (C$_4$, C$_8$, C$_{18}$) (Regnier & Gooding, 1980). The mobile phase in this case is aqueous and thus depending on their hydrophobicity, proteins and peptides in the mobile phase repel the aqueous solution and interact with the solid phase. The retention time for each analyte depends on the strength of the hydrophobic interactions and the polarity of
the mobile phase. Therefore, if the polarity of the mobile phase is gradually decreased (by adding organic solvents), a gradient elution is achieved allowing separation of proteins and peptides in complex mixtures.

In proteomics, two-dimensional gel electrophoresis is often used for separating proteins (O'Farrell, 1975); however, since the 1990s, RPLC coupled with mass spectrometry has become a formidable alternative for separation and detection of proteins and peptides (Mann, Hendrickson, & Pandey, 2001). The ‘miniaturized’ RPLC system used in mass spectrometry features small columns and slow (nL/min) flow rates. This configuration is essential to attain much needed sensitivity (Mitulovic & Mechtler, 2006). The challenging issue in the study of the human proteome is that the analytes of interest are often at low concentrations in the medium in which they are found. For example, the top 12 proteins in human plasma constitute 95% of the protein mass (Zhang et al., 2010) and peptides derived from these abundant proteins often saturate the MS signal and prevent identification of peptides derived from lower abundance proteins.

To improve the sensitivity of the LC/MS platforms, researchers have developed multi-dimensional LC systems (e.g. by combining ion-exchange chromatography with RPLC) especially for analysis of protein-specific tryptic peptides in digested human plasma by selected reaction monitoring (SRM). Briefly, after the LC separation step, selected peptides (based on mass) enter the collision-induced dissociation chamber of the mass spectrometer where they are fragmented. The specific fragment ions from a particular peptide form multiple ‘transitions’, which act as unique identifiers for that peptide. The sensitivity of the SRM assays has been improving exponentially in recent years mainly due to the extensive LC step prior to MS analysis. Typical SRM assays can
quantitate analytes in the high ng/mL range (Picotti & Aebersold, 2012) and there is a recent report of a highly specialized SRM assay called PRISM (high-pressure, high-resolution separations coupled with intelligent selection and multiplexing) that can reach sensitivity levels of pg/mL (Shia et al., 2012). However, there is a fine balance between sensitivity and throughput since with the PRISM assay only 10 individual samples can be analysed per day.

Immuno-MS assays such as SISCAPA offer an alternative for quantitation of low abundance analytes in human plasma. Instead of lengthy LC fractionation of peptides in plasma digests, the matrix can be simplified by enriching the peptide of interest using antibodies, thus decreasing the LC separation time. Using a nanoflow rate (~300 nL/min) the LC step for SISCAPA assays is usually 30 - 40 minutes (J. R. Whiteaker et al., 2010). Recently, with introduction of more sensitive mass spectrometers (for example, an Agilent 6490 mass spectrometer equipped with iFunnel technology) the LC step has been reduced to approximately 4 minutes using standard flow rates (~1.2 mL/min), while retaining equivalent sensitivity (Miller et al., 2012). While this is a respectable improvement over standard SRM assays it is interesting to consider that with a 4-minute sample analysis time, one would need to run a mass spectrometer non-stop for more than 10 years to validate all the putative cancer biomarkers that have been identified to date.

This chapter outlines my research and findings on eliminating the need for liquid chromatography in SISCAPA assays. By eliminating the LC step, sample analysis times of less than 10 seconds have been achieved. Using this strategy, the specimens required to validate all the putative cancer biomarkers (~1200 biomarkers; ~1,200,000 specimens to be analysed) could theoretically be analysed on a single mass spectrometer in less than
6 months. Moreover, eliminating the LC step allows us to combine the SISCAPA workflow with clinically friendly mass spectrometers (MALDI-TOF) and a new, high-throughput MS platform called the RapidFire/MS.

The RapidFire High-Throughput Mass Spectrometry System is an ultra-fast online solid-phase extraction (SPE) platform coupled to a triple quadrupole mass spectrometer that has been extensively employed for the MS analysis of high-throughput screening campaigns against large compound libraries and for absorption, distribution, metabolism and excretion (ADME) studies of drug candidates (Highkin et al., 2011; Hutchinson et al., 2012). The instrument aspirates a portion of each sample (directly from 96- or 384-well assay plates) onto a small SPE column chosen to retain the analyte(s) of interest. After a washing step, the sample is reverse eluted from the SPE column and sprayed directly into a mass spectrometer for detection. The entire cycle time between aspiration of one sample and the next is ~7 seconds. This system has not been previously tested for quantitation of peptides in complex mixtures such as trypsin digested human plasma.

3.2. Materials and Methods

3.2.1. Peptides

All proteotypic peptides for the proteins of interest were selected based on previously published guidelines (N. L. Anderson et al., 2004). All peptides were produced by JPT Peptide Technologies, Berlin, Germany and were of greater than 90% purity as determined by HPLC. Upon receipt from the vendors, peptide stocks were adjusted to approximately 10 nmol/µL (based on dry weight) in 30% acetonitrile (ACN)/0.1% formic
acid (FA). However, stock concentrations determined by peptide weight are inaccurate due to differing hygroscopic properties, hydrophobicity and solubility of peptides; thus aliquots of these initial stocks were sent for quantitation by amino acid analysis (AAA; Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Ontario). Using AAA data, the peptide stocks were then readjusted to 10 nmol/µL. Peptides were diluted immediately prior to use and were stored for short periods at 4 °C in solution phase. After thawing and/or just before use, all peptides were analyzed by MALDI-TOF MS to determine their integrity and to assess the presence of altered forms.

*a) Peptide for MALDI experiments*

We have recently demonstrated the general utility of MALDI-TOF instruments for quantitation of tryptic peptides in buffer (N. L. Anderson et al., 2012). However, the utility of this instrument for LC-free SISCAPA assays in complex mixtures such as digested human plasma has not been demonstrated. For this purpose, I have chosen to work with a proteotypic surrogate peptide derived from protein C inhibitor to develop a model system. The peptide sequence is EDQ YHY LLD R. The corresponding stable isotope standard (SIS) peptide was produced with a labeled arginine residue at the C-terminus (EDQ YHY LLD R*). The labeled residue causes a mass shift of +10 amu (for singly-charged ions) compared to the endogenous analyte and thus allows quantitation using mass spectrometry.

*b) Peptides for RapidFire experiments*

The utility of the RapidFire platform for analysis of tryptic peptides has not been previously demonstrated. For this purpose I chose 5 tryptic peptides representing proteins of varying abundance in human plasma: Protein C inhibitor, thyroglobulin (2
peptides), LPS binding protein and mesothelin. The stable isotope standard (SIS) version of each peptide was produced by synthesizing peptides with either C-terminal arginine (+10 amu) or lysine (+8 amu), providing mass shifts of \( m/z = +5 \) or +4 for typical doubly-charged peptide ions.

### 3.2.2. Anti-peptide monoclonal antibodies

All rabbit monoclonal antibodies (RabMAbs) were derived and produced by Epitomics Inc. (Burlingame, CA) and were selected by screening for high affinity using the MiSCREEN/SPR assays as discussed in Chapter 2.

### 3.2.3. Human plasma

Pooled human plasma was purchased from BioReclamation Inc. (Westbury, NY) and PCI-deficient human plasma was purchased from Affinity Biologicals Inc. (Ancaster, ON).

### 3.2.4. Selecting the beads

The key to eliminating the need for an LC step is to minimize non-specific background binding of peptides from high abundance proteins such as serum albumin. The most abundant background peptide in the current SISCAPA workflow was found to have the sequence RHP DYS VVL LLR with a mass of 1467.73 Da, which is derived from human serum albumin. Background peptides were observed to bind non-specifically to the magnetic bead support that is used to capture antibodies in SISCAPA assays. For
this reason, I studied the background binding to magnetic beads manufactured by several companies to find the beads that provided the best signal to noise ratios.

3.2.5. Selecting optimum washing procedure

The dominant background peak from serum albumin contains residues that contribute to both hydrophilic (RHPD) and hydrophobic (YSVVLLL) characteristics at neutral pH. This amphipathic characteristic contributes to the ‘stickiness’ of this peptide. To address this observation, I experimented with using the organic solvent acetonitrile as part of the washing procedure in order to reduce the non-specific background binding especially from the albumin peptide.

3.2.6. Digestion

A trypsin digestion protocol was originally developed by a previous member of the Pearson lab, Angela Jackson (currently at the UVic-Genome BC Proteomics Center). This “addition only” method enables facile, efficient and reproducible tryptic digestion of plasma samples and has been tested using plasma volumes of 10-1000 µL. First, urea, tris(2-carboxyethyl)phosphine (TCEP) and Tris buffer (pH 8.1) were mixed and lyophilized to yield a final concentration of 9 M, 0.05 M and 0.2 M, respectively. Neat plasma/serum was then added to this denaturation mix followed by 30 min incubation at room temperature. To prevent reformation of disulfide bonds, iodoacetamide was added at a 1.5 molar excess over cysteine residues and the reducing agent TCEP, and the mixture was incubated for 30 minutes in the dark. The reaction mixture was then diluted to a final urea concentration of 1M before adding trypsin (Worthington Cat. No.
LS003740) at a 1:20 ratio of enzyme to substrate. The sample was incubated 16 hours at 37 °C before adding a 2-fold excess of tosyl-L-lysine chloromethyl ketone (TLCK) to stop the tryptic cleavage. The digested sample was then purified using 150 mg solid phase extraction (SPE) columns (Oasis® HLB 6 cc cartridges; Waters, Milford, MA). For SPE purification, peptides were acidified using 0.2 M formic acid and were desalted by passing the solution through the SPE cartridge and washing with 5 mL of 0.1% formic acid. Purified peptides were eluted in 3 mL of 50% ACN/0.1% FA and lyophilized. The tryptic peptides were reconstituted to the original plasma volume with PBS and brought to pH 7.0 using 5 N NaOH.

I contributed to improving and testing this protocol in two ways: 1) by devising the washing procedure explained in 3.2.4. I eliminated the need for SPE purification of peptides from trypsin-digested plasma and 2) I applied the optimized protocol to digestion of proteins in dried whole blood/serum spots (Appendix I).

3.2.7. SISCAPA assay protocol

The SISCAPA assay described here used trypsin digest from 10 µL of plasma for antibody capture of peptides. All procedures were performed using a KingFisher 96 bead-handling robot (Thermo Electron Corporation, Vantaa, Finland). A schematic diagram of the generic SISCAPA peptide enrichment and assay procedure is shown in Figure 3.1. For the RapidFire work reported here, KingFisher 96-deepwell microplates were used for antibody and peptide capture whereas KingFisher 96-well standard microplates were used for MALDI experiments. A series of KingFisher plates were prepared as follows:
Plate 1: A Bead Wash Plate containing 1.43 µL/well of MyOne Protein G Dynabeads (Invitrogen-Dynal; custom made, 1.0 micron diameter; low peptide binding) brought up to 200 µL/well in PBS/0.03% CHAPS. It is important to note that with the LC-free system used here, the CHAPS was removed from the sample (by extensive bead washing prior to peptide elution from the magnetic bead immunosorbent) before the peptides were loaded onto the MALDI or RapidFire platforms.

Plate 2: An Antibody Capture Plate containing 1.0 µg/well of desired antibody brought up to a final volume of 100 µL/well in PBS/0.03% CHAPS.

Plate 3: A Peptide Capture Plate containing the trypsin digest of 10 µL plasma. The corresponding Stable Isotope Standard (SIS) peptides were added at this stage at 500 fmol/well. Here the samples can be prepared as a “master mix” before addition to the plate as long as the final concentration of the digest and the amount of SIS peptide per well remain unchanged.

Plates 4 and 5: Two Wash Plates (#1 and #2) containing 200 µL/well of PBS/0.03% CHAPS.

Plate 6: A Wash Plate (#3) containing 350 µL/well of 75% acetonitrile in PBS/0.03% CHAPS.

Plate 7: An Elution Plate containing 13 µL/well of 0.1% formic acid (25 µL/well for RapidFire experiments).

Briefly, the washed beads were transferred through the antibody plate where they were allowed to capture 1 µg of the relevant antibodies. The bead-antibody complex was then transferred to the plasma digest plate to capture the target peptides. The non-specifically bound peptides were washed away by the optimized wash buffer in the Wash
Plates and finally the captured peptides were eluted in the elution buffer.
Figure 3.1. Schematic representation of the SISCAPA workflow
3.2.8. SISCAPA-MALDI studies

a) Linearity study

A combination curve strategy was used to examine the linearity of PCI measurement by SISCAPA-MALDI and also to determine the percent coefficient of variation (CV) as a function of the dynamic range. Briefly, synthetic light (unlabeled) and heavy (labeled) PCI peptides were used to create two peptide solutions (using 0.1% FA as buffer) one with a L:H ratio of 10:1 and the other with a L:H ratio of 1:10. These two solutions represent the two ends of an 11-point combination curve with a 100-fold dynamic range. The remaining points on the curve were then generated by mixing equal volumes of consecutive solutions; for example, when equal volumes of the 10:1 and 1:10 solutions were mixed, a third solution was created with a L:H ratio of 1:1 representing the center of the curve etc. Using this strategy the total peptide concentration remains constant at 660 fmol/µL in every sample and pipetting errors are reduced. Two microliters of each sample was then spiked into 10 µL of digested PCI-deficient plasma and the SISCAPA capture procedure was performed. As a control, the peptide solutions were directly spotted onto a MALDI plate without subjecting the analyte to antibody capture. Four technical replicates of this experiment were conducted to obtain CVs corresponding to each point of the combination curve.

b) Precision and reproducibility studies

To determine within run (well-to-well) precision, 10 human plasma samples from 10 different individuals (BioReclamation) were digested and the levels of the PCI analyte was measured over 10 replicates per individual. To examine between run (day-to-day)
variation, pooled human plasma (BioReclamation) was digested on three separate occasions and the peptide analyte was measured in 5 replicates for each digest.

c) Peptide recovery study

The recovery of PCI peptide was investigated by spiking two levels (250 femtomole/well and 50 femtomole/well) of the synthetic light peptide in PCI-deficient plasma. The heavy peptide was spiked into the elution plate at 500 femtomole/well. The experimental ratio of L:H compared to the theoretical ratio was used to calculate percent recovery.

d) Limit of detection/quantitation study

To determine the limit of detection (LOD) and limit of quantitation (LOQ), two types of standard curves were generated in digested pooled plasma. The first type, called a forward curve, was generated by spiking constant amounts of the heavy peptide (500 fmol/well) and varying amounts of the synthetic light peptide to generate a 12-point curve with the light peptide being titrated in 2-fold dilutions from 1000 fmol to 0 fmol. The forward curve plateaus at a level that is representative of the endogenous level of the analyte in the digested plasma sample. The second standard curve, called a reverse curve, was generated by spiking constant levels of the synthetic light peptide (500 fmol/well) and titrating the heavy peptide (2-fold dilution from 1000 fmol – 0.5 fmol). Since no heavy peptide is expected to be endogenously present in the digest, the reverse curve will not plateau. By combining the forward and reverse curves, the endogenous level of the analyte (forward curve) with respect to the sensitivity of the assay (reverse curve) is determined. This experiment was repeated three times.
e) MALDI analysis of eluates

A 4800 MALDI-TOF/TOF Analyzer with 4000 series Explorer v3.5 software (Applied Biosystems/MDS Sciex, Framingham, MA) was used for all experiments. I also used a ‘clinically-friendly’, linear-mode only instrument called the microflex™ LT (Bruker Daltonics, Billerica, MA) to repeat a technical replicate of all experiments (except for reproducibility/recovery studies; when I had no further access to the instrument).

In both cases, 6 µL of the eluate were dried on the MALDI plate targets followed by placing of 1 µL of the CHCA matrix (5 mg/mL CHCA and 1 mg/mL ammonium citrate dibasic in 70% ACN/0.1% FA) on top of the sample. On the 4800 MALDI instrument, the acquisition of the data was automated in the 800-4000 Da mass range and in the positive-ion reflector mode with a laser intensity of 3400 at 1000 shots/spectrum. For MS/MS analysis of the specific peptide precursors, the collision energy was set to 2 kV and the relative precursor mass window at 300 (FWHM). The MS/MS spectra were collected with collision-induced dissociation (CID) turned on and by accumulating 1250 shots/spectrum. To verify the sequence identity of the peptides, the MS/MS spectra were manually examined using MS-Product tool from the ProteinProspector software v5.5.0 (University of California, San Francisco, CA). On the microflex™ LT instrument, the automated acquisition was performed in the linear mode. The parameters were optimized to give at least partially resolved isotopic envelopes up to m/z 1600 and 500 to 800 laser shots were averaged per sample.
3.2.9. **SISCAPA-RapidFire studies**

*a) Determination and selection of optimum transitions*

SRM transitions for each surrogate peptide were first determined by direct infusion into an Agilent 6490 QQQ mass spectrometer. The transition list was used to examine each peptide after Agilent RapidFire 300 direct injection and transitions that gave robust signals with no interferences were selected for our subsequent SISCAPA-RapidFire/MS experiments. The optimum collision energies for these SRM transitions were also determined on an Agilent 6490 triple quadrupole mass spectrometer using ultra high-pressure chromatographic (UHPLC) separation.

*b) Linearity study*

As with the SISCAPA-MALDI studies, peptide curves were generated using a ‘combination strategy’ to generate samples containing both light and heavy peptides (L+H). In the case of the RapidFire experiments, however, the 11-point curve spanned a dynamic range of 2500-fold and the final peptide concentration in each sample was 510 fmol/µL. Five different tryptic peptides were used in a 5-plex format and the curves were generated in triplicate.

*c) Limit of detection/quantitation study*

As with the SISCAPA-MALDI studies, forward and reverse curves were used to determine the LOD and LOQ of the SISCAPA-RapidFire assay. However, a low abundance biomarker (mesothelin) was used to test the sensitivity of the system.
*d) Mass spectrometric peptide detection (RapidFire/6490 QQQ)*

The predetermined optimum SRM transitions for antibody-enriched mesothelin peptide were analyzed on an Agilent RapidFire 300 High-Throughput Mass Spectrometry System using an Agilent 6490 QQQ mass spectrometer fitted with an electrospray ionization source and running in positive SRM mode. With the RapidFire instrument the eluted samples were aspirated directly from the 96-well elution plate and loaded onto a micro-scale solid-phase extraction (SPE) cartridge. The purpose of the SPE cartridge is to desalt, and to some extent purify, the sample before injection into the mass spectrometer. More specifically, the instrument aspirated aliquots of each sample sequentially, removing sample until the sip sensor determined that the 10-µL loop was full (usually about 200 ms). The contents of the loop were then applied to an SPE cartridge containing C₄ packing material and washed with ultrapure H₂O supplemented with 0.1% formic acid for 3000 ms. The purified sample was reverse-eluted using 90% ultrapure acetonitrile supplemented with 0.1% formic acid in a 3000 ms step and sent to the mass spectrometer, which was already monitoring the mass transitions of interest for that well. A re-equilibration of 500 ms brought the total cycle time to approximately 7 seconds. Data analysis was performed using RapidFire Integrator v3.4 software, which generated an output file of integrated peak areas for each SRM transition for each well of a sequence.

*e) Mass spectrometric peptide detection (LC/6490 QQQ)*

As a control, half of the eluted samples for the mesothelin forward and reverse curves were also analyzed with a system consisting of a 6490 triple quadrupole mass spectrometer with upstream liquid chromatography. A 20 µL aliquot of each sample was
separated on a 2.1 x 50 mm Zorbax 300 SB-C18 column with a flow rate of 1.2 mL/min. The target peptides were separated using a 3-minute gradient with 0.1% formic acid in water as solvent A and 90% acetonitrile/0.1% formic acid in water as solvent B. The gradient increased to 70% over 1.95 minutes and then dropped to 10% for column re-equilibration. Ions were isolated in Q1 using 1.2 FWHM resolution and in Q3 using 0.7 FWHM resolution.

\section*{f) Detecting a thyroglobulin-specific peptide using SISCAPA-RapidFire}

A surrogate peptide from thyroglobulin with sequence FSP DDS AGA SAL LR was analysed using both the RapidFire/6490 QQQ and the LC/6490 QQQ platforms. Forward and reverse curves of the peptide were prepared using 250 µL of human plasma and was analysed using the LC/6490 system. The SISCAPA capture of the analyte was independently repeated using 250, 500, 750 and 1000 µL of human plasma and peptide eluates were analysed using the RapidFire/6490 platform.

\section*{3.3. Results}

\subsection*{3.3.1. Reducing non-specific background peptides}

Eliminating the need for LC is directly contingent on the purity of the final eluted sample. To significantly reduce non-specific background peptides, a three-point strategy was used. First, magnetic beads providing the best signal to noise ratio were selected. Of all beads tested, Sepharose-based beads (GE Healthcare, UK) had the lowest background, although the best signal to noise ratio was achieved using protein G coated magnetic beads from Invitrogen (Oslo, Norway). Therefore, for all subsequent experiments,
protein G coated, custom-made, 1-micron diameter beads from this company were used. These beads exhibit low background binding and their small diameter (increased surface area/volume ratio) increases antibody binding capacity, making them more suitable for multiplexed analyses where a number of antibodies (at 0.1-1 µg each) can be used in a single SISCAPA capture reaction. Second, a washing procedure was designed where the first two washes contained a polar solution (PBS/0.03% CHAPS) while the third wash was a 75% (v/v) mixture of an organic solvent (acetonitrile) in PBS/0.03% CHAPS. This washing regimen addresses the amphipathic characteristics of the background peptides. Third, the robotic mixing during the washing steps was maximized. It is important to highlight that such an extensive washing procedure is only possible because the antibodies had subnanomolar affinities for their target peptides, thus the effort that was put into selecting high affinity antibodies (Chapter 2) is arguably the most important aspect of the method used in experiments presented henceforth. The effect of this optimized protocol on peptide enrichment is shown in Figure 3.2.
Figure 3.2. MALDI-TOF mass spectra showing low non-specific peptide background

(A) A MALDI-TOF spectrum showing poor specific peptide enrichment from trypsin-digested human plasma due to the presence of non-specific background peptides, mainly derived from serum albumin. (B) A MALDI-TOF spectrum showing excellent peptide enrichment from the same sample described above using the optimized affinity bead washing procedure. The target peptides are specific to protein C inhibitor (1351.6 Da) and soluble transferrin receptor (1672.8 Da).
3.3.2. SISCAPA-MALDI studies

a) Linearity of L:H peptide ratios

To examine the linearity of peptide detection in digested human plasma, a combination curve strategy was used. The points on the curve represent 11 solutions with varying ratios of the PCI light and heavy peptides. The L:H ratios varied from 10:1 to 1:10 representing a 100-fold dynamic range, which is the typical range for MALDI instruments. Two microliters of each solution were spiked into digested PCI-deficient plasma, which was subjected to SISCAPA capture procedure. The experimental L:H peptide ratios were plotted against theoretical values (Figure 3.3.a and b). The power law fit was determined for the curve, which had a slope of 1.033 and a coefficient of determination \((R^2)\) of 0.999. Four technical replicates of this experiment were repeated to examine the percent coefficient of variation (CV) for each data point across the 100-fold dynamic range. As a positive control, 11 peptide mixtures were directly spotted onto a MALDI plate without subjecting the analytes to the SISCAPA capture procedure. As with our previous report (N. L. Anderson et al., 2012) when the peptide solutions in buffer were directly spotted onto the MALDI plate target, the CVs ranged from 2-8\% with the best CVs towards the center of the dynamic range (i.e. close to L:H ratio of 1:1). Here I report that a similar trend was observed when the analyte was spiked into trypsin digested human plasma and subjected to antibody capture (Figure 3.3.c). The CVs in this case ranged from 4-10\% depending on the L:H ratio.

This experiment was repeated on the ‘clinically friendly’ microflex\textsuperscript{TM} LT MALDI instrument. The slope of the power law in this case was determined to be 0.947 with an
R² of 0.999. The CVs ranged from 3-17% with the CVs at the center of the curve (near L:H of 1:1) at approximately 5% (Figure 3.3.d-f).

b) Precision and reproducibility studies

To determine within run (well-to-well) precision, human plasma from 10 individuals (BioReclamation) were separately digested and the levels of the PCI-specific peptide were measured in 10 replicates per individual. The heavy peptide was spiked at 500 fmol/well so that the endogenous:heavy ratio was close to 1:1, which should provide the best CVs according to the previous results. CVs ranging from 1.2-7.8% were observed in different individuals with the average CV for all individuals at 3.2%. Between run (day-to-day) precision was determined by digesting pooled human plasma on three separate occasions and measuring the PCI analyte in 5 replicates for each digest. The PCI peptide was measured in the three digests to be 17.4 ± 1.7 pmol/mL, 18.5 ± 1.4 pmol/mL and 17.3 ± 2.6 pmol/mL, respectively. A one-way analysis of variance (ANOVA) for the data revealed a p-value of 0.42 with a significance level of 0.05, indicating that the mean values of PCI measurements for different digests were not significantly different.
Figure 3.3. SISCAPA-MALDI linearity studies

Combination curves with L:H peptide ratios of 10:1 to 1:10 were generated in PCI-deficient trypsin-digested human plasma. The error bars represent the standard deviation over 4 technical replicates. A power law fit was used to determine the slope and coefficient of determination as shown on each graph. The data are plotted both on a log-log scale (A and D) and on a linear scale (B and E). The percent coefficient of variation is shown as a function of the dynamic range (C and F). Panels A, B and C were analysed by AB 4800 MALDI-TOF/TOF MS while panels D, E and F represent a replicate of the same experiments on the clinically-friendly microflex™ LT instrument (Bruker).
c) Recovery of the PCI peptide

To determine the analytical recovery of the PCI peptide from digested plasma, synthetic light peptide was spiked into digested PCI-deficient plasma at both a high level (250 fmol) and a low level (50 fmol). The heavy peptide was spiked into the elution buffer at 500 fmol/well. The theoretical light:heavy peptide ratio was compared to the recovered amount of the light peptide to calculate the percent recovery. The recovery was 82% for both spike levels (Figure 3.4.), which is consistent with the recovery for other SISCAPA assays when a high affinity (sub-nanomolar) antibody is used (Schoenherr et al., 2010).

d) Determining the LOD and LOQ

Two types of standard addition curves were used to determine the endogenous level of the PCI analyte (forward curve) with respect to the sensitivity of the assay (reverse curve). The lower limit of detection was reached at 1 fmol of the heavy peptide, which was approximately 150-fold lower than the endogenous level of the analyte in 10 µL of pooled human plasma. Consistent with our previous results, the CV at the endogenous level was 3.8%. The precision of measurement was disrupted at 15 fmol level, where the CV rose to 30% (Figure 3.5). Therefore, the limit of quantitation of the PCI analyte is approximately 15 fmol.

The experiment was repeated on the microflex™ LT instrument. The endogenous level was calculated to be 16.0 ± 1.3 pmol/mL, which is consistent with the results on the more sophisticated AB 4800 instrument (16.5 ± 0.6 pmol/mL). The limit of quantitation, however, was 60 fmol on the microflex instrument compared to 15 fmol on the AB 4800 instrument (Figure 3.5.a vs. b).
Figure 3.4. Analytical recovery of the PCI-specific peptide

Synthetic light PCI peptide (1351.6 Da) was spiked into PCI-deficient digested plasma at 250 fmol (A) and 50 fmol (B). Corresponding SIS peptide (1361.6 Da) was spiked into the elution buffer at 500 fmol/well in both cases. Overlay spectra representing the three technical replicates of the experiment are shown. The ratio of the experimental L:H ratio was compared to the theoretical ratios of 1:2 for spectrum (A) and 1:10 for spectrum B to calculate the percent recovery which was 82% in both cases.
Figure 3.5. Limits of detection and quantitation

Forward and reverse curves were generated by titrating the light peptide while the SIS spike was constant at 500 fmol/well (forward curve) or by titrating the SIS peptide and maintaining a constant level of the light peptide at 500 fmol/well (reverse curve). The concentration of the endogenous peptide was calculated to be 16.5 ± 0.6 pmol/mL on the AB 4800 instrument (A) and 16.0 ± 1.3 pmol/mL on the microflex LT instrument (B). The limit of quantitation was approximately 15 fmol (in 10 µL of digested plasma) on the AB 4800 platform where the CVs fluctuated by 30%. The limit of detection on this instrument was 1 fmol. The limit of quantitation was approximately 60 fmol on the microflex platform with a CV of 12%. No SIS peptide was detected beyond this point and thus the LOD and LOQ are roughly the same on this instrument.
3.3.3. SISCAPA-RapidFire studies

a) Peptide analysis by RapidFire/MS

The utility of RapidFire/MS for ultra-fast throughput SRM analysis of proteotypic peptides has not been investigated before, primarily because the RapidFire/MS system, unlike standard LC/MS platforms used for SRM analysis, does not separate peptides chromatographically prior to injection into the mass spectrometer. With RapidFire, all molecules in each sample are injected as a single peak approximately 2 seconds wide (Figure 3.6.a). The linearity of response and instrument reproducibility was investigated using a range of concentrations of five pairs of pure synthetic tryptic peptides. The light and heavy peptides were combined in varying L:H ratios as previously described for SISCAPA-MALDI assays. For RapidFire experiments a dynamic range of 2500-fold was examined. For all peptides a linear response was observed with \( R^2 \) values of > 0.99 for four of the peptides and > 0.97 for the surrogate peptide from LPS Binding Protein (LBP) (Figure 3.6.b). As an example, RapidFire spectra representing the combination curve for mesothelin surrogate peptide is shown in Figure 3.6.c. The CVs ranged from 2-16% at different points over the 2500-fold dynamic range (Figure 3.7.d). The average percent coefficient of variation (within-run) over the 2500-fold dynamic range for the 5 peptides ranged from 6.0-8.3%.

b) SISCAPA-RapidFire/MS quantitation of mesothelin

To demonstrate that the SISCAPA-RapidFire/MS system can be used for measuring low abundance analytes in trypsin-digested human plasma, I performed replicate SISCAPA peptide response curves for the low-abundance cancer biomarker mesothelin, which is found at 1-10 ng/mL in healthy individuals (Hassan, Remaley, & Sampson,
As a control, the eluate at the end of the SISCAPA run was spilt: Half of the sample was analysed using RapidFire/Agilent 6490 QQQ MS platform with a 7 second cycle time and the other half using LC/Agilent 6490 QQQ MS platform with a 4 minute cycle time. Unfractionated, trypsin-digested human plasma was used in all experiments. The quantitated amount for mesothelin was identical between the two MS platforms (Figure 3.7.a-d) and the average CV of peak area ratios for endogenous mesothelin peptide (0 fmol spike level of the light peptide on the forward curve) was 3.9% on the RapidFire/MS platform and 1.1% on the LC/MS platform. The LOD on both platforms was 2.5 to 3 fold lower than the endogenous level. The CV at LOD was 7.4% on the RapidFire/MS and 6.5% on the LC/MS. It is important to note that three different transitions for the L and H mesothelin peptides were measured to ensure the identity of the analyte.

c) SISCAPA-RapidFire/MS detection of thyroglobulin

Detecting surrogate peptides from thyroglobulin using mass spectrometry is a challenging task and its measurement in human plasma has been a goal of the biomarker community for several years. Like mesothelin, thyroglobulin is a low abundance analyte that is endogenously present at ~1 ng/mL; however, the molar abundance is 20 fold lower for thyroglobulin since its mass is 20 times greater than that of mesothelin (660 kDa compared to 32 kDa). I attempted to detect thyroglobulin by measuring a surrogate peptide using both the LC/6490 and the RapidFire/6490 platforms developed as part of my thesis research. Because of the normally low levels of thyroglobulin, peptide enrichment was performed using larger than usual volumes of plasma (up to 1000 µL vs 10 µL used in standard SISCAPA captures).
the endogenous peptide was detected when 250 µL of plasma were used and the eluate was analysed using the LC/MS platform (Figure 3.8.a). The thyroglobulin peptide could not be unequivocally identified (even using different transitions) in 250 µL of plasma when the RapidFire/MS method was used (Figure 3.8.b). However, the peptide was detected successfully when plasma volumes of 750 µL or more were used.
Figure 3.6. SISCAPA-RapidFire linearity studies

All peptide analytes co-elute as a 2-second wide chromatographic peak using the RapidFire/MS platform (A) and thus highly purified eluents are required to use this platform for quantitating peptides in complex mixtures. L:H peptide ratios for 5 tryptic peptides prepared in a 5-plex format in buffer show a linear response on this platform with $R^2 > 0.99$ for four of the peptides and $R^2 > 0.97$ for the LBP peptide (B). RapidFire spectra representing the combination curve for the mesothelin peptide are shown where 11 samples are analysed in less than 2 minutes (C). The percent CV as a function of dynamic range is shown for each peptide at every point on the combination curve (D).
Figure 3.7. SISCAPA-RapidFire quantitation of a mesothelin-specific peptide

Forward and reverse curves are shown for the mesothelin peptide analysed on the RapidFire/6490 QQQ (A) and LC/6490 QQQ (B). Error bars represent the standard deviation over three technical replicates. Three transitions for light and heavy peptides were used to ensure the identity of the analyte. The plasma levels of the mesothelin peptide was measured as 0.297 ± 0.011 pmol/mL using the RapidFire platform and 0.305 ± 0.003 pmol/mL when LC was used upstream of the MS analysis. The spectra for one of the replicates of the forward curve are shown for the RapidFire (C) and LC (D) analyses.
Figure 3.8. Detection of a thyroglobulin-specific peptide on two different platforms: SISCAPA-LC/6490 QQQ and SISCAPA-RapidFire/6490 QQQ

Forward and reverse curves (peptides enriched from 250 µL of plasma) are shown for the SISCAPA-enriched thyroglobulin peptide analysed on the LC/6490 QQQ (A). The endogenous peptide was identified using three separate transitions at a level that was 4-fold higher than the LOD of the assay. Detection of the peptide using the RapidFire platform is shown in panels (B) and (C) for enrichment out of various volumes of plasma. Panel (B) shows the light peptide signals while the corresponding heavy peptide signals are shown in (C). Two transitions were monitored for each of the light and heavy peptides.
3.4. Discussion

It was hypothesized that the high purity of peptides in the eluates obtained by immunocapture in the optimized LC-free SISCAPA workflow presented here would allow a wide variety of peptides to be quantitated by the SISCAPA-MALDI and SISCAPA-RapidFire platforms in a high-throughput fashion. Using the SISCAPA-MALDI approach, it was shown that the peptide response was linear in digested human plasma over a 100 fold dynamic range. Peptide quantitation using these instruments can be precise not only when the peptides are measured in buffer (N. L. Anderson et al., 2012) but also when they are analysed in the context of a complex mixture such as digested human plasma. Average within-run CVs of 3.2% were observed for quantitation of the PCI-specific analyte in the plasma of 10 different individuals. It was also demonstrated that a clinically friendly MALDI-TOF instrument, the Bruker microflex, can be used to quantitate peptides in digested human plasma with great accuracy. The utility of this device for analysing true clinical specimens is discussed in Chapter 4.

It is important to note that average CV changes as a function of the dynamic range with the best precision achieved at endogenous:heavy ratios close to one. Another factor that affects precision is signal count: The higher the signal to noise ratio the more accurate the quantitation will be. Ideally, the MALDI-TOF instruments should be set to an automated mode such that the laser strikes the peptide spots long enough to reach 10,000 counts for the desired analyte. However, in reality this approach will be very costly to implement because it dramatically reduces the life span of the laser (personal communication with Gary Kruppa, Bruker Daltonics).
The limit of detection (LOD) and limit of quantitation (LOQ) were determined for the PCI-specific analyte in digested human plasma using forward and reverse standard curves. It was demonstrated that the LOQ for the PCI-specific peptide was 15 fmol, which is approximately 10 times lower than the average endogenous level for this analyte in 10 µL of pooled human plasma (~160 fmol). The SISCAPA-MALDI method has been applied to a number of different peptides with various abundance levels in plasma (experiments not performed as part of this dissertation) and as a rough estimate, analytes with concentrations greater than 100 ng/mL can be analysed with current configurations. However, many biomarkers will have plasma concentrations lower than 100 ng/mL. To apply the LC-free SISCAPA approach to these low abundance analytes, SISCAPA peptide enrichment was coupled to the RapidFire/MS platform that employs a triple quadrupole instrument for peptide detection.

As described earlier, the RapidFire platform is a high-throughput, microfluidic sample preparation system that is compatible with triple quadrupole mass spectrometers. The instrument aspirates samples into a micro solid-phase extraction cartridge to remove the salts and injects the washed analyte into the mass spectrometer. The elapsed time between consecutive injections is approximately 7 seconds. Given that there is no liquid chromatography step involved, all the analytes in the sample are eluted as one, 2-second wide chromatographic peak. Therefore, the RapidFire platform can analyse a limited number of analytes and heavy multiplexing is not currently supported.

However, using five tryptic proteotypic peptides, we demonstrated that despite the lack of LC separation, we were able to monitor 10 SRM transitions from the 10 analyte peptides (5 L + 5 H) that eluted together in a single 2-second-wide peak (Figure 3.6.a).
This allows quantitation of 5 protein analytes if only 1 transition per surrogate peptide is monitored (both L and H peptides must be measured for each analyte). Thus the RapidFire method described here currently allows 5-plex detection of peptide analytes with a 7-second cycle time, although with careful selection of peptide transitions and modification of binding and elution conditions, a higher level of multiplexing should be attainable. Linear responses were observed for all peptides and the average CVs ranged from 6-8%. It is worth highlighting that other SRM assays with upstream LC might outperform the SISCAPA-RapidFire platform on any one parameter (e.g. better precision, higher sensitivity, higher number of plexes etc.). However, the collective properties of the SISCAPA-RapidFire platform (i.e. reproducibility, linear range, specificity, sensitivity, multiplexing and most importantly, throughput) have not been matched by any other MS platform.

The RapidFire/6490 QQQ platform was used to measure the plasma levels of a peptide analyte specific for mesothelin (protein levels in plasma: 1-10 ng/mL). As a control, peptides in half of the final eluate in this experiment were analysed using an LC/6490 QQQ MS platform. Given that the same eluate and the same mass spectrometer were used in both cases, this allowed direct examination of how the presence or absence of a liquid chromatography step affected quantitation and sensitivity. The data showed that quantitation of the mesothelin analyte was identical with both LC/MS and RapidFire/MS (LC-free) platforms and that the sensitivity (reverse curve) was not significantly different between the two methods. However, when the sensitivity threshold was extended to detect a surrogate peptide from thyroglobulin, the LC/MS platform showed 3-fold better sensitivity than the RapidFire/MS method. Nevertheless,
the thyroglobulin peptide could be detected after enrichment from 750 µL of plasma using the (potentially) high-throughput RapidFire/MS platform. Unfortunately the KingFisher robot is not capable of handling the large volumes of plasma digest required for the thyroglobulin assay and thus performing proper quantitation experiments for thyroglobulin is contingent on implementing the SISCAPA peptide capture workflow on more sophisticated robotic instruments such as the Bravo (Velocity 11, Agilent Technologies), an endeavour that is well underway.

I believe that the results of the RapidFire experiments are especially encouraging for biomarker validation purposes: Using the RapidFire platform it is theoretically possible to analyse 1,000 samples in less than 3 hours. By combining the automated SISCAPA workflow to this high-throughput MS platform, its utility for biomarker validation will be almost certainly demonstrated in the near future.
Chapter 4. Verification of a specific peptide from protein C inhibitor as a biomarker for prediction of biochemical recurrence in prostate cancer patients

This chapter has been submitted for publication as:


All the experiments presented in this chapter were conducted by Morteza Razavi. I thank Alex Camenzind for his technical help with running the AB 4800 MALDI instrument and Lisa Johnson, Nancy Nesslinger and Nav Chima for their help in organising the clinical data. Gary Kruppa (Bruker Daltonics) analysed the peptide eluates on the microflex™ LT MALDI instrument.

4.1. Introduction

The path from biomarker discovery to validation is an uncertain one. In an attempt to systematize this path, Rifai, Gillette and Carr (2006) present four distinct phases that ultimately lead to biomarker validation. The process starts with the discovery of the biomarker, which should ideally happen in the context of human specimens. The next step, the authors argue, is to ‘qualify’ the biomarker using SRM assays in digested human plasma to determine its differential abundance in specimens drawn from ‘healthy’ and ‘diseased’ individuals. The third step is called ‘verification’, which tests the biomarker in ‘population-derived’ human plasma. For this step, too, the authors argue that LC/MS technology is the most appropriate option. The final stage is ‘validation’, which is an expansion of the verification step in a large cohort. Given the low throughput of SRM assays (Picotti & Aebersold, 2012; Shia et al., 2012), and that 1000s of
specimens need to be analysed (N. L. Anderson, 2005) the authors assert that the validation step needs to be done using immunoassays.

In chapter 3, an LC-free SISCAPA assay was discussed that reaches the throughput of average immunoassays with a sample analysis time of less than 10 seconds. This refined method allows for the same assay to be used for biomarker qualification, verification, validation and even biomarker measurement in a clinical setting. We believe that such a unified approach eliminates inevitable variations that are introduced when different methods are used at different stages of the biomarker validation path.

This chapter focuses on qualification and verification of a proteotypic peptide of protein C inhibitor (PCI) as a biomarker for prediction of biochemical recurrence of prostate cancer after radiation/hormone therapy. The SISCAPA-MALDI assay described in the previous chapter was used for this work. For the qualification step, the analyte was analysed in plasma obtained from 10 ‘healthy’ men and 5 ‘prostate cancer patients’ (BioReclamation, NY). In order to verify the predictive value of the PCI-specific analyte, sera derived from a population of 51 men who received treatment at the BC Cancer Agency in Victoria, BC from 2004-2008 were used.

Protein C inhibitor belongs to the superfamily of serine protease inhibitors and it is best known for its role in inhibiting activated protein C, an anticoagulant that is also involved in regulating anti-apoptotic and anti-inflammatory pathways (Flight, 2010). Recent studies have identified new biological functions for PCI (Geiger, 2007; Malmström et al., 2009; Suzuki, 2010). For instance, it has been demonstrated that PCI inhibits cathepsin L, a protein involved in blood vessel remodeling, cardiovascular diseases and different types of cancer (Fortenberry, Brandal, Bialas, & Church, 2010). It
has also been demonstrated that PCI interacts with glandular human kallikrein 2 and prostate specific antigen (PSA) in seminal fluid (Bourgeois et al., 1997; Christensson & Lilja, 1994), and its expression in high-grade prostate tumour cells is decreased (Cao et al., 2003). These findings, along with other similar studies, have led researchers to hypothesize about possible role of PCI as a suppressor of prostate cancer progression. Interestingly, an independent biomarker discovery effort has recently demonstrated that serum levels of an N-terminal fragment of PCI is lower in prostate cancer patients who had a biochemical recurrence of the disease compared to the patients who received the same treatment (radical prostatectomy) but who did not have a recurrence (Rosenzweig et al., 2009). The precise mechanism by which a decline in serum levels of PCI might be related to prostate cancer progression is not yet understood.

The current ‘gold standard’ method for monitoring prostate cancer involves measurement of prostate specific antigen (PSA) in patients’ sera. The PSA tests can be categorized into four main groups: 1) total PSA measurement 2) PSA density measurement (total PSA divided by the volume of the prostate gland) 3) PSA velocity (rate of change in concentration) and 4) ratio of free PSA to bound PSA (Adhyam & Gupta, 2012; Bostwick et al., 2000). There are more than 30 different commercial immunoassays that have been developed for PSA testing, the results of which are not always comparable (Kuriyama et al., 1999). Moreover, there is no consensus amongst practitioners regarding the cut-off values for the different PSA tests (Bostwick et al., 2000). Nevertheless, PSA tests are effective in determining “biochemical failure” in patients who receive radical prostatectomy since the prostate gland is completely removed in these patients and detectable PSA levels in the serum is indicative of residual
prostatic tissue. Detecting biochemical failure is a much more difficult task for patients who receive an alternative treatment (i.e. radiation with or without hormone therapy) since for these patients the prostate gland is not removed and thus a cut-off for PSA levels cannot be defined due to varying sizes of the gland in different individuals (Adhyam & Gupta, 2012).

The American Society for Therapeutic Radiology and Oncology (ASTRO) held a conference in Phoenix in 2005 to define biochemical recurrence in patients who receive radiation with or without hormone therapy. According to the ASTRO-Phoenix definition, a patient is considered to have a biochemical recurrence if a spike of more than 2 ng/mL is observed after the nadir PSA levels (Roach et al., 2006). Despite such efforts to reach a consensus definition, predicting biochemical recurrence in these patients remains a challenge since radiation patients often suffer from a condition known as the “PSA Bounce”, which corresponds to a rise in PSA levels due to inflammation of the gland. This rise in PSA levels is not due to disease recurrence (McGrath et al., 2010; Mitchell et al., 2008). For this reason, I chose to examine the potential value of PCI in predicting biochemical recurrence in prostate cancer patients who received radiation (with or without hormone therapy) as opposed to prostatectomy. It is important to mention that biochemical failure is correlated with clinical failure due to prostate cancer (Freedland et al., 2005) thus predicting it at an early stage will have clinical advantages.
4.2. Materials and Methods

4.2.1. Anti-peptide monoclonal antibodies

Anti-peptide rabbit monoclonal antibodies (RabMAbs) were produced against the PCI-specific and sTfR-specific peptides by Epitomics Inc. (Burlingame, CA) and high affinity (sub-nanomolar) clones were selected as described in Chapter 2. The mAb specific for the PCI peptide had an affinity of 0.09 nM (half off-time of 68 min) and the mAb specific for the sTfR peptide had an affinity of 1.8 nM (half off-time of 54 min).

4.2.2. Qualification study

The PCI-specific analyte was measured in digested plasma from 10 ‘healthy’ individuals and 5 ‘prostate cancer’ patients (Bioreclamation; Westbury, NY). The healthy individuals’ specimens consisted of 4 African Americans, 4 Hispanics and 2 Caucasians whose age ranged from 22 to 63 years old. The prostate cancer samples were obtained from patients with an age range of 68 to 87 years old. Details of the treatment, Gleason scores, PSA levels and tumour stages were not known for these patients. Each sample was measured in triplicate. As a negative control, the PCI-deficient plasma digest was used in assays to demonstrate antibody specificity.

4.2.3. Verification study

a) Control peptides

In the verification study, two control peptides were simultaneously measured along with the PCI analyte. A digestion control peptide, flanked at both ends with 4 amino acid residues, was produced to control for digestion abnormalities. This peptide
contains two tryptic sites and after digestion releases a peptide that is identical in sequence to the endogenous PCI peptide but with different isotopic configuration to cause a mass shift of +24 amu with respect to the endogenous peptide and +14 amu with respect to the SIS peptide (MMSR EDQ YHY L*L*D R* NLSC $\rightarrow$ EDQ YHY L*L*D R*). A second control peptide was used to ensure consistency in sample collection over time. This peptide (GFV EPD HYV VVG AQR) is specific to soluble transferrin receptor (sTfR), which has serum concentrations similar to PCI (Allen et al., 1998; Hortin, Sviridov, & Anderson, 2008). The synthetic endogenous peptide (light or L) and the SIS peptide (heavy or H) with a labeled arginine residue (+ 10 amu) were produced as described in Chapter 2.

b) Sera from cancer-free controls and prostate cancer patients

One hundred and fifty-nine sera from prostate cancer patients and ten sera from cancer-free, age-matched controls were obtained from the repository at the BC Cancer Agency in Victoria, BC. The average age of the cancer-free controls was 72.6 ± 6.5 years and the average age of the prostate cancer patients was 73.5 ± 7.5 years as of September 2012. These specimens were mainly collected between 2004 and 2008 from prostate cancer patients who received external beam radiation with neoadjuvant androgen deprivation or brachytherapy with or without neoadjuvant androgen deprivation for prostate cancer. Usually, specimen(s) were collected before any treatment (pre-treatment), 1-18 months after treatment (post-treatment < 18m) and beyond 18 months post treatment (post-treatment > 18m). The number of specimens collected for different patients ranged from two to seven. This variance was due to a lack of staff or the
unwillingness of patients to donate blood on every visit. Importantly, the outcome of treatment was not known at the time of sample collection.

c) Experimental design for measuring the PCI peptide in prostate cancer patients

This study was conducted in a double-blinded format. Sera collected at the BC Cancer Agency were randomized and coded by a third party before they were presented to us. The PCI and sTfR levels were measured in triplicate for all patients. After the measurements were complete, the key was revealed to us and the data were assembled in the presence of Dr. Lisa Johnson of the BC Cancer Agency. Patients in this study are still being monitored for the status of the disease at the agency and any changes in the status of the disease (i.e. if the patients develop biochemical recurrence) are provided to us by the agency. The outcome data used in this manuscript are up to date as of September 2012. Of the 51 patients, we excluded 8 from our analysis: Two of the patients developed secondary cancers (Patients #6 and #12) and three had known outcome at the time of sample collection (Patients #4 and #16 and #17). For three patients (Patients #1 and #31 and #38) we did not have long-term follow up data (i.e. 5-year biochemical recurrence unknown).

Although these patients were excluded from the box plot (Figure 4.5) and receiver operating characteristic (ROC) curve (Figure 4.6) analyses, the PCI/sTfR measurements and the clinical data for these patients are presented in Appendix II. The ROC curve was generated using XLSTAT (Addinsoft, NY), which aside from generating the curves, calculated the best cut-off value according to the data and the corresponding sensitivity/specificity levels. To determine biochemical recurrence, the BC Cancer Agency uses the ASTRO-Phoenix definition (Roach et al., 2006). Based on this
definition, a patient is considered to have a biochemical recurrence if he experiences a spike of greater than 2.0 ng/mL in PSA levels compared to the nadir level (the nadir level refers to the lowest PSA measurement obtained for the patient after the treatment).

4.2.4. Reproducibility of peptide measurements

The within-run precision was analysed using the triplicate runs of the samples. To examine between run (day-to-day) variation the PCI analyte was measured (in triplicate) in sera from 25 prostate cancer patients on two occasions, 12 months apart, to show the reproducibility of measurements in clinical samples.

4.3. Results

4.3.1. Digestion control peptide

Pooled human plasma was trypsin digested with and without the ‘winged’ peptide to examine the release of the control peptide as a consequence of the digestion (Figure 4.1). Possible digestion abnormalities were monitored using this peptide when digesting individual samples. The ratio of the digestion control peptide to the SIS peptide was very consistent in every batch that was digested with a CV of 2.31%. This observation is consistent with the between-run precision results (Chapter 3) where no significant differences were observed between different digestions of pooled human plasma, demonstrating the uniformity of the digestion protocol.
Figure 4.1. Digestion control peptide

One hundred microliters of pooled human plasma were digested with (A) and without (B) the ‘winged’ peptide. Five picomoles of the winged peptide were spiked into the plasma sample before the digestion to a final concentration of 50 fmol/µL. The SIS peptide was spiked at 500 fmol/well level and the SISCAPA capture process was performed using 1 µg of the PCI-specific antibody. When the digestion control was added (A) a peptide was observed at 1375.6 Da, which is +24 amu from the endogenous PCI peptide and +14 amu from the SIS peptide. The ratio of this control peptide to the SIS peptide was measured for every sample of the verification study to ensure the integrity of the digestion. The average CV of the digestion control peptide:SIS peptide ratio was 2.31% and no abnormal digestion was detected.
4.3.2. Qualification study

The PCI analyte was measured in triplicate in plasma of 10 ‘healthy’ men and 5 ‘prostate cancer patients’ (BioReclamation). The average CV for all measurements was 3.2%. The p-value of a two-tailed t-test was 6.11E-05 with a significance level of 0.05, which is indicative of a significant difference in the levels of the PCI analyte between the two groups (Figure 4.2). The data for ‘healthy’ individuals were divided into two groups of 5 based on age (above 45 years and below 45 years). No significant differences were observed based on age (p-value = 0.55).

Figure 4.2. Results of the PCI peptide qualification study

The boxplots were generated with a confidence interval of 0.999 (z-value = [3]). A two-tailed t-test showed a p-value of 6.11E-05 between the two groups. No endogenous peptide was detected in the PCI-deficient plasma.
4.3.3. Verification study

In a 2-plex format, I measured the PCI peptide analyte and the sTfR peptide analyte in 159 sera collected from 51 prostate cancer patients and 10 sera from 10 cancer-free, age matched controls in triplicate. The average CV for PCI peptide measurements (within-run) was 4.7% and for sTfR peptide was 7.6%. The sequence identity of the two peptides was confirmed by MS/MS analysis (Figure 4.3). The measurements were repeated on both a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) and a microflex™ LT instrument (Bruker Daltonics). Between the two instruments, an $R^2$ of 0.972 was observed for the PCI peptide measurements and an $R^2$ of 0.878 was observed for the sTfR analyte (Figure 4.4). The average CV (within-run) on the microflex instrument was 5.7% for PCI and 8.2% for the sTfR analyte.

Since different specimens for each patient were collected over a long period of time (months to years), the sTfR analyte was used as a control to determine whether sample collection caused adverse effects on the specimens. The sTfR protein was chosen because its serum levels are roughly the same as PCI (Allen et al., 1998; Hortin et al., 2008). The variation in the sTfR peptide was 8 times less compared to the PCI analyte (Figure 4.5.a), suggesting that the variation observed in the levels of the PCI peptide were less likely to be due to sample collection biases. The levels of the PCI peptide were analysed in 43 prostate cancer patients receiving external radiotherapy or brachytherapy (with or without hormone therapy). Ten of the patients have had a biochemical recurrence to date. The specimens were divided into three groups: 1) Collected before the treatment 2) Collected less than 18 months after treatment(s) and 3) Collected more
than 18 months after treatment(s). A significant reduction (p-value = 0.006) in the levels of the PCI analyte was observed in recurrent patients within the first 18 months after the treatment(s) compared to non-recurrent patients who received the same treatment (Figure 4.5.b). No significant difference was observed between the two groups for the sTfR analyte (Figure 4.5.c).

A receiver operating characteristic (ROC) analysis was conducted on the PCI and PSA data for the post-treatment (<18 months) time point. PSA values for these patients had been independently obtained by staff at the BC Cancer Agency and were supplied to me accordingly. Based on the ROC analysis, the area under the curve (AUC) for the PCI analyte was 0.774 compared to 0.506 for PSA (Figure 4.6.a). A sensitivity/specificity plot demonstrated that at a cut-off value of 12.77 pmol/mL, the sensitivity of the PCI assay was 0.778 and its specificity was 0.846 (Figure 4.6.b). The same analysis for the PSA assay showed a sensitivity of 0.556 and specificity of 0.538 at a 0.51 ng/mL cut-off value (Figure 4.6.c). In the published literature, the sensitivity and specificity of the PSA assay has been reported to be 0.240 and 0.930 respectively using a cut-off value of about 4 ng/mL (Lilja, Ulmert, & Vickers, 2008). Using a cut-off of 4.2 ng/mL, I observed a sensitivity of 0.222 and specificity of 0.923 for the PSA assay.

### 4.3.4. Reproducibility of PCI peptide measurements

Between run (day-to-day) precision was determined by measuring the PCI-specific peptide in plasma from 25 of the prostate cancer patients (in triplicate) on two occasions, 12 months apart. An $R^2$ of 0.965 was observed between the two measurements (Figure 4.7).
Figure 4.3. MS/MS analysis of PCI and sTfR peptides enriched from the serum of one patient

The identity of the endogenous PCI (A) and sTfR (B) peptides were confirmed in a specimen from one of the patients selected at random.
Figure 4.4. Measuring the PCI and sTfR peptides using two different MALDI-TOF mass spectrometers

Measurement of endogenous PCI (A) and sTfR (B) peptides using two different MALDI-TOF mass spectrometers is shown. Aliquots of the same samples were tested on an Applied Biosystems 4800 MALDI-TOF/TOF instrument and on a Bruker microflex™ LT instrument. Measurement of the PCI peptide analyte showed $R^2 = 0.971$ and the sTfR peptide analyte an $R^2 = 0.878$ (degrees of freedom = 149).
Figure 4.5. Results of the PCI biomarker verification study

Variations in the serum levels of the PCI-specific analyte are shown relative to the serum levels of the control peptide (sTfR) in 159 sera collected from 51 prostate cancer patients (A). The variance for the sTfR analyte was 8 times less than the variance for the PCI peptide. The levels of the PCI analyte in 10 cancer-free controls, 33 patients without a biochemical recurrence and 10 patients with a biochemical recurrence are shown in (B). The boxplots were constructed with a confidence interval of 0.999 (z-value = 3); outliers are shown with a “*”. The values for patients who experienced a biochemical recurrence are shown (circles) relative to the recurrence-free patients (boxplots). To determine whether there were significant differences in PCI values between recurrent and non-recurrent patients, a two-tailed t-test was used (α = 0.05) to calculate the p-value at each time point. The p-values for the pre-treatment, post-treatment (<18 m) and post-treatment (>18 m) were 0.935, 0.006 and 0.274, respectively. The same analysis was performed for the sTfR analyte (C). The p-values for the pre-treatment, post-treatment (<18 m) and post-treatment (>18 m) were 0.787, 0.339 and 0.102, respectively.
Figure 4.6. Receiver operating characteristic (ROC) curve analysis of PCI-specific peptide and PSA data

Receiver operating characteristic (ROC) curves for PCI and PSA are shown (A). The curves were constructed using the PCI and PSA data from sera drawn within the first 18 months after treatment (35 patients had samples collected during this period, 9 of whom experienced a biochemical recurrence). The sensitivity and specificity of the PCI analyte for predicting biochemical recurrence as a function of varying cut-off values is shown in (B). At the cut-off value of 12.77 pmol/mL, the sensitivity was 0.778 and the specificity was 0.846. A similar analysis of PSA data is shown in (C) where the sensitivity was 0.556 and specificity was 0.538 at a 0.51 ng/mL cut-off value.
Figure 4.7. Reproducibility of peptide measurements

The PCI analyte was measured in triplicate in sera from 25 prostate cancer patients with varying levels of the PCI peptide. Measurements were repeated 12 months later using the same samples from the same patients. Comparison of the results obtained 12 months apart revealed $R^2 = 0.965$. 
4.4. Discussion

Biomarker validation is nominally divided into a 4-step process: Discovery, qualification, verification and validation. Due to the low throughput nature of most protein or peptide mass spectrometry-based assays, they are most suited for qualification and verification studies but not for validation purposes where immunoassays are the method of choice (Rifai, Gillette, & Carr, 2006). In Chapter 3 it was demonstrated that high-throughput analysis of peptide analytes in digested human plasma using mass spectrometry is possible using an LC-free SISCAPA approach. In particular, high-throughput is achieved by the use of MALDI-TOF instruments (generally for moderately abundant biomarkers) or the RapidFire/MS (for low abundance analytes), depending on the performance of the particular peptide. To demonstrate the utility of this LC-free approach in clinical specimens, I conducted qualification and verification studies using a surrogate peptide specific to protein C inhibitor (PCI).

Rosenzweig et al. (2009) demonstrated that PCI is a putative biomarker with a potential value in predicting recurrence in prostate cancer patients who were treated by radical prostatectomy. The results of our verification study are consistent with the report of the Rosenzweig’s group. In a randomized, double-blinded study, we measured a peptide analyte of PCI in patients receiving radiation (either external radiation or brachytherapy with or without hormone therapy). We observed that the serum levels of the PCI-specific analyte were abnormally low (p-value = 0.006) in the specimens collected within the first 18 months of the treatment from patients who experienced biochemical recurrence. Interestingly, the PSA values measured in these patients at the same time points did not have a predictive value. It is important to note that PSA levels
eventually spiked months to years after this time point, which was the basis for determining biochemical recurrence; however, in the 18-month period after treatment, PSA levels were not predictive of a recurrence. The levels of the control sTfR peptide, were relatively constant compared to the PCI analyte, which suggests that the variations in the levels of the PCI analyte were less likely to be due to aberrations caused by sample collection, handling and storage.

The verification study was also analysed on the microflex™ LT MALDI-TOF instrument (Bruker). This is important because the same assay that was used to qualify, verify and validate a biomarker should ideally be also amenable to use in the clinic. Using mass spectrometers for protein and peptide analysis in clinical settings is challenging since they are usually complex, labour-intensive machines. The microflex™ LT uses the same internal technology as the Bruker BioTyper, which is a mass spectrometer already installed in more than 600 clinical laboratories worldwide. The instrument is currently used to provide microbial identification, although the successful use of this instrument in the PCI assay indicates that SISCAPA assays can be performed on the widely held machines.

The outcome of the radiation/hormone treatments was not known at the time of sample collection and the process was randomized in the sense that patients were not divided into subgroups based on baseline factors such as Gleason score, tumour stage etc. However, it is important to note that this study did not follow a randomized controlled trial (RCT) design where the subjects are randomly assigned a treatment and baseline equality is unequivocally assured (Ransohoff & Gourlay, 2009). Adapting such an RCT design, however, is difficult for this project because satisfying the ethical needs of such a
study, given that the subjects are patients who already have cancer and need a certain course of treatment, might be difficult. We thereby propose a study with a large cohort of patients (N>1000) receiving radiation/hormone therapy to validate PCI as marker for predicting cancer recurrence.

**Future Work: Validation of PCI**

The results of the qualification and verification studies presented here justify studying PCI in the context of a large population of prostate cancer patients. However, during the verification study we gained insight into the importance of designing a comprehensive sample set for the validation stage. The specimens analysed for the verification study had two important shortcomings: 1) different number of samples were collected for different individuals and 2) only a few serum samples (1 or 2) were collected from each patient within the first 18 months of the treatment. The first shortcoming is important because the availability of equal numbers of specimens for each individual will allow a more comprehensive analysis of differences between them. It can now be hypothesized that patients showing biochemical recurrence of their cancers experience the most significant drop in the levels of the PCI analyte within the first 18 months after treatment. Therefore, it is important to obtain as many specimens as possible within this period (ideally 1 draw per month over 18 months) to unequivocally test this hypothesis.

The verification study was internally controlled because the outcome of treatment was not known at the time of sample collection. Thus the patients who developed biochemical recurrence of their cancers could be compared to the patients who received
the same treatment but who did not develop a biochemical recurrence. Though this is an effective control, it would be ideal to include an external control to monitor the PCI levels only with respect to progression of the disease. This is important in order to eliminate the possibility of PCI fluctuations due to factors such as possible variations in treatment, etc. For this purpose, a class of patients called ‘weight watchers’ can be used. These patients choose not to receive any treatment for their prostate cancer and thus monitoring them could demonstrate how the levels of the PCI-specific analyte change with respect to the aggressiveness of the disease.

Currently, serum samples are being collected from prostate cancer patients and external controls with the above mentioned criteria. Given that limited numbers of prostate cancer specimens are available in British Columbia, obtaining samples from a broad range of cancer agencies, researchers and pharmaceutical companies in North America is necessary. The proposed validation study will follow a triple-blinded format where the samples are randomized and coded when presented to us, then after completion of our analysis the clinical data and the SISCAPA measurements will be sent to a third party (a bio-statistician) for analysis. We believe that despite the outcome of the study (i.e. whether or not the PCI analyte is validated for clinical use) this will be a valuable study as it will demonstrate, for the first time, how a single mass spectrometric assay can be used to qualify, verify and ultimately validate a biomarker in a reasonable amount of time.
Chapter 5. Studying the molecular forms of PCI protein may help elucidate its role in prostate cancer progression

All the experiments presented in this chapter were conducted by Morteza Razavi with technical help from Melissa Fowler and Richard Yip who aided in running the ISO-DALT 2-dimensional gel electrophoresis apparatus.

5.1. Introduction

The verification study described in Chapter 4 demonstrated that a significant drop occurs in the serum levels of the PCI-specific peptide in prostate cancer patients who ultimately experience a biochemical recurrence. Throughout this dissertation I have been mindful of differentiating between the serum concentrations of the ‘PCI-specific peptide’ and that of the PCI protein. In theory, when there is a 1:1 stoichiometry between the surrogate peptide and the protein, the concentration of the protein can be inferred from the concentration of the peptide. For example, in Chapter 3 the plasma levels of a peptide specific to mesothelin can be calculated from the forward curve in the SISCAPA assay to be approximately 9.5 ng/mL, while the concentration of mesothelin at the protein level has been reported independently by immunoassay to be approximately 9.0 ng/mL (Hassan et al., 2006). Similarly if we consider the sTfR peptide (control peptide in the verification study) its concentrations in cancer-free controls ranged from approximately 0.5 µg/mL – 1.2 µg/mL, which is in good correlation with the reported values of 1.3 µg/mL – 2.0 µg/mL at the protein level (Allen et al., 1998). However, if we look at the concentrations of the PCI peptide in cancer-free controls (or in pooled plasma samples) it
ranges from 0.7 µg/mL – 1.2 µg/mL, which is approximately 5-fold lower than the reported concentrations at the protein level (Hortin et al., 2008).

This discrepancy between peptide levels and protein levels for PCI is not a novel issue in proteomics and is especially common for proteins that are known to form complexes with other proteins in the serum or have alternate post-translational modifications. In either case, trypsin digestion of such proteins may not liberate all the possible tryptic peptides with an acceptable efficiency. For instance, the levels of a surrogate peptide of C-reactive protein, a protein that complexes with phosphocholine in response to an inflammation, have been shown to be much less than the protein concentrations as measured by immunoassays, likely due to inefficiency of releasing the peptide by trypsinization due to the complex formation at the protein level (Kuhn et al., 2004). It is important to note that utilizing a peptide as a clinical biomarker is independent of whether or not its concentrations match that of the parent protein; it is the peptide itself that is the biomarker.

However, from a biological standpoint, this discrepancy in the levels of the PCI peptide and literature values for the PCI protein intrigued me to explore PCI at the protein level in the context of prostate cancer. More specifically, I wanted to analyze the PCI protein itself at the time that the peptide levels drop (in a patient who experiences biochemical recurrence) compared to when the peptide levels remain normal (in a recurrence-free patient). Understanding biochemical changes at the protein level may improve our understanding of the biological role of PCI in prostate cancer patients and also why the serum levels of the peptide decreases in patients prior to biochemical recurrence of their cancers.
5.2. Materials and Methods

5.2.1. Measuring PCI protein in ‘healthy’ individuals and ‘prostate cancer patients’ using Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA kit for measuring PCI was purchased from US Biological (Massachusetts, USA). The plasma samples used in the qualification study described in Chapter 4 were used for the ELISA assay. Two technical replicates of the assay were conducted according to the manufacturer’s instructions. Briefly, the capture antibody was diluted 1:100 in 50 mM carbonate buffer (pH 9.6) and 100 µL/well of this solution were added to an ELISA plate followed by a 2-hour incubation at room temperature. Next, 150 µL of a blocking buffer (PBS/1.25% BSA (w/v)) were added to every well followed by a 1-hour incubation step at room temperature. The plate was washed 3 times with PBS/0.05% Tween-20 (v/v). The washing procedure was repeated 2 more times with a 5-minute incubation per wash. The plasma samples were diluted 1:100 in HBS-BSA-Tween-20 and added in 100 µL volume to each well. After a 1-hour incubation at room temperature the washing procedure was repeated as before. The horseradish peroxidase (HRP)-conjugated secondary antibody supplied in the kit was diluted 1:100 in HBS-BSA-Tween-20 and 100 µL were added to each well followed by a 90-minute incubation at room temperature. The washing procedure was repeated as before and 150 µL of the 3,3’,5,5’-tetramethylbenzidine (TMB) substrate were added per well. The reaction was allowed to develop for approximately 10 minutes and 50 µL of 1N HCL were added to stop the enzyme activity. An ELISA plate-reader (Bio-Tek, Germany) was used to measure color development at a wavelength of 490 nm.
Unfortunately, standard samples were not provided in the ELISA kit, thus PCI protein concentrations could not be calculated. The results represent the relative absorbance of the ‘healthy’ versus ‘prostate cancer’ samples from which the relative abundance of the PCI protein in the two groups could be inferred. As a negative control, PCI-deficient plasma was used at the same concentration as the test samples.

5.2.2. Analysing PCI protein in sera from patients with biochemically recurrent and non-recurrent prostate cancer using two-dimensional gel electrophoresis and immunoblotting

Pooled human plasma (BioReclamation) and sera collected after hormone therapy from a patient with biochemical recurrence of the cancer (patient # 13) and from a patient who did not show biochemical recurrence (patient # 3) were used in two-dimensional gel electrophoresis/immunoblotting experiments. The two patients were both 86 years old, both had a cancer at the T2a stage and both received hormone therapy before external beam radiation. Patient # 13 has had a biochemical recurrence while patient # 3 is still recurrence-free as of September 2012. By SISCAPA-MALDI assay, the concentration of the PCI peptide was 16.47 ± 0.63 pmol/mL in the pooled human plasma, 24.31 ± 0.17 pmol/mL in the serum sample from patient # 3 and 3.07 ± 0.51 pmol/mL in the serum sample for patient # 13.

Two-dimensional gel electrophoresis was conducted using the ISO-DALT system (Tollaksen, Anderson, & Anderson, 1984). Briefly, the isoelectric focusing gels (ISO) were prepared by mixing 8.25 g of urea, 2 mL of 30% acrylamide/1.8% N,N’ methylenebisacrylamide and 0.75 mL of pH 3.0 – 10.0 ampholines in 6 mL of distilled water. After degassing, 2.0 mL of 15% (v/v) NP-40, 70 µL of 10% ammonium persulphate and 10 µL of tetramethylethylenediamine (TEMED) were added to the
mixture and the mixture was used to fill the ISO-tubes by capillary action. The gels were solidified over a 1-hour period and pre-focused for 1 hour at 200 Volts. Ten microliters of each sample were diluted in twenty microliters of ‘SDS mix’ (50 mM N-Cyclohexyl-2-aminoethanesulfonic acid at pH 9.5, 2% sodium dodecyl sulphate, 1% dithioerythritol, 10% glycerol and 5 mM phenylmethanesulfonylfluoride brought to a final volume of 100 mL in distilled water). The samples were heated to 90 °C and 25 µL were loaded onto the ISO gels. The proteins were brought to their isoelectric point by applying 10,000 Volt-hours of electric potential across the ISO gels.

The second dimension, or ‘DALT’, gels were prepared by mixing ‘heavy’ (15%) and ‘light’ (10%) acrylamide solutions. The gels were solidified overnight. The ISO gels and a standard protein ladder (Fermentas Inc., ON) were then placed on top of the DALT gels. The proteins were electrophoresed through the DALT gels using 1 Amp of current, over approximately 4 hours. After the completion of the run, the gel-separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane in a 125 mM Tris base solution. The membrane was blocked in PBS/0.1% Tween-20/5% skim milk for 1 hour with mixing at room temperature. PBS/0.1% Tween-20 was used to wash the membrane for 30 minutes with regular changing of the washing solution. The capture antibody that was provided in the ELISA kit was used as the primary antibody for the immunoblot analysis. The primary antibody was diluted 1:1000 in the blocking buffer and 10 mL were incubated with the membrane overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated donkey anti-goat secondary antibody (Abcam Inc., ON) was used at a 1:25,000 dilution (in blocking buffer) and was applied in a 10 mL volume to the membrane. After a 1-hour incubation at room temperature, SuperSignal HRP substrate
(Thermo Scientific, IL, USA) was applied according to the manufacturer’s instructions. The blot was placed in an airtight cassette for approximately 10 minutes before developing the film.

5.3. Results

5.3.1. Examining the amino acid sequence of the PCI protein

A literature review of glycosylation sites in the PCI amino acid sequence revealed that the asparagine residue that follows the PCI-specific peptide is a site for N-glycosylation (Saito, 2011; Sun et al., 2010). The presence, absence or the complexity of glycosylation at this site can affect the efficiency of liberating the PCI-specific peptide by trypsin (Figure 5.1).

```
1 hrhhpremkk rvedlhvgat vapssrrdft fdlyralasa apsqniffsp vsismslaml
61 slgagssstkm qileqlglnl qkssekelhr gffqllqeln qprdfqqlsl gnalftdlvv
121 dlqdtfysam ktlyladtfp tnfrdsagam qkindyvakq tkqkivdilk nldsnvvim
181 vnyiffkakw etsfnhkgtq egdfyvtset vvrvmmsre dqgyhylldrN lscrvgvpy
241 ggNatafllil psekgmqvqe nglsektlrk wlkmfkkrql elylpkfsie gsyqlekvlp
301 slgisnvfts hadlsgisNn sniqvsemvh kavvevedsg traatatgti ftfrsarlns
361 qrlvfnrpfl mfvdnnilf 1gkvnrp
```

Figure 5.1. Amino acid sequence of PCI

The PCI-specific peptide is underlined. Potential N-glycosylation sites (Asn\textsuperscript{230}, Asn\textsuperscript{243} and Asn\textsuperscript{319}) are capitalized and italicised. The amino acid sequence was retrieved from the NCBI database on October 9, 2012; (Hayashi & Suzuki, 1993).

5.3.2. Examining the three-dimensional structure of the PCI protein

Analysis of the three-dimensional structure of the PCI protein revealed that the PCI-specific peptide is located in close spatial proximity to the reactive center loop
(RCL) (Figure 5.2). The RCL is the interactive portion of the protein that forms complexes with other proteins through the catalytic triad (Phe$^{353}$-Arg$^{354}$-Ser$^{355}$) (Phillips et al., 1994). The specificity of the RCL interactions is determined by the presence or absence of heparin, which forms a bridge between PCI and some of its substrates (Li, Adams, Nangalia, Esmon, & Huntington, 2008). Complex formation at this site is another factor that might affect the efficiency of liberating the PCI-specific peptide by trypsin.

Figure 5.2. The three-dimensional structure of PCI

The three-dimensional structure of PCI (light blue) is shown when bound to one of its many substrates, prothrombin (green). The catalytic triad (purple) is located at the tip of the reactive center loop (RCL). The surrogate PCI peptide, the potential biomarker peptide discussed in this thesis is shown in red. The image is from protein structure 3B9F (Li et al., 2008) and was created with Protein Workshop (Moreland, Gramada, Buzko, Zhang, & Bourne, 2005).
5.3.3. Analysis of PCI protein by ELISA

The plasma samples that were used for the qualification study (10 ‘healthy’ and 5 ‘prostate cancer’ plasma samples) were used in the ELISA assay to measure the PCI protein. The levels of the PCI were significantly higher in prostate cancer patients compared to cancer-free controls (p-value = 2.2E-06), which is in stark contrast with the levels of the PCI specific peptide measured in the same patients (Figure 5.3). As a negative control, the PCI-deficient plasma was used in the ELISA assay, which as expected shows absorbance at background levels.

![Figure 5.3. Measuring PCI protein in ‘healthy’ and ‘PCa’ specimens by ELISA](image)

The results of the qualification study (measuring the PCI-specific peptide) are shown in (A). The same specimens were used in the ELISA assay (B). A significant increase (p-value = 2.2E-06) in protein levels was observed in ‘prostate cancer patients’. This is in contrast to the results obtained for the PCI peptide where a significant decrease (p-value = 6.11E-05) was observed in the same specimens. No peptide was observed in the PCI-deficient plasma and ELISA absorbance for this sample was at the background level as expected.
5.3.4. Analysis of PCI protein by 2-dimensional gel electrophoresis and immunoblotting

To demonstrate the reproducibility of the 2-D gel electrophoresis and immunoblotting protocol, the pooled human plasma sample from BioReclamation was analysed 3 times, twice with a secondary antibody dilution of 1:50,000 and once with a dilution of 1:25,000. The results of this experiment established that the assay was reproducible and that a secondary antibody dilution of 1:25,000 produced a more detailed protein trace. The PCI isoforms ran between the 40 kDa and 55 kDa bands of the standard protein ladder and had isoelectric points (pI) ranging from approximately pH 4.5 to 6.0. The observed patterns of the isoforms, the molecular weight and the pI range were consistent with the results of a recent study where purified PCI was analysed using 2-dimensional gel electrophoresis (Saito, 2011). When comparing the immunoblot from patient # 13 (recurrent patient) with either the pooled human plasma sample or the serum from the non-recurrent patient, a new set of protein isoforms was observed (Figure 5.3.c). Appearance of this new set of protein isoforms coincides with a sharp decline in the serum concentrations of the PCI-specific peptide (3.07 ± 0.51 pmol/mL).
Figure 5.4. Two-dimensional gel electrophoresis and immunoblotting analysis of PCI protein in sera from recurrent and non-recurrent patients

PCI detection in pooled human plasma (A), a serum sample from a non-recurrent patient #3 (B) and a serum sample from a recurrent patient #13 (C) are shown. In these same samples, the PCI-specific peptide was 16.47 ± 0.63 pmol/mL in the pooled human plasma, 24.31 ± 0.17 pmol/mL in the serum sample from patient #3 and 3.07 ± 0.51 pmol/mL in the serum sample from patient #13. The unique protein spots in the sample from the recurrent patient #13 are shown with an arrow (C).
5.4. Discussion

In bottom up proteomics approaches, the assumption is that the stoichiometry between a surrogate peptide and the parent protein will be retained through the digestion procedure. However, there are well-documented cases where this principle does not apply. In some cases, protein structure can restrict accessibility to trypsin cleavage sites (Kuhn et al., 2004; Proc et al., 2010). In such cases some tryptic peptides will not be released from the parent protein efficiently and thus the concentration of the parent protein cannot be inferred based on its stoichiometric relationship to the surrogate peptide. A possible example of this phenomenon was observed in this study of a PCI-specific peptide where the peptide concentration in normal controls was approximately 5-fold lower than the published concentrations for the parent protein (Geiger, 2007; Hortin et al., 2008). This discrepancy may possibly be explained by examining the amino acid sequence and the three-dimensional structure of the PCI protein. Adjacent to the PCI-specific peptide used in this study, there is an N-glycosylation site (Asn\textsuperscript{230}), which has been shown to be glycosylated in normal humans (Sun et al., 2008). Such a configuration may restrict access to the trypsin cleavage site, which prevents effective release of the peptide analyte. Moreover, the peptide is located in close proximity to the reactive center loop (RCL), which is known to interact with molecules such as heparin during the complex formation events. Such complex formations can also restrict access to trypsin-cleavage sites of the PCI-specific peptide and thus absolute protein concentration in a given sample cannot be inferred by quantitating the PCI peptide.
To further examine this matter, the samples from the qualification study were used to measure the relative abundance of the PCI protein in healthy individuals versus prostate cancer patients. While at the peptide level a significant decline was observed for prostate cancer patients, at the protein level a significant increase was detected compared to the healthy individuals. Therefore, it follows that relative changes in peptide levels between samples is not necessarily indicative of similar changes at the protein level. This is an important observation since it strengthens the possibility that individuals who experience abnormally low levels of the PCI peptide may have unique post-translational modifications, perhaps differential glycosylation at the Asn$^{230}$ N-glycosylation site, which could inhibit release of the peptide by trypsin. Alternatively, unique protein-protein interactions could similarly interfere with tryptic digestion and release of the PCI peptide.

To further investigate the possibility that unique post-translational modifications/interactions of PCI protein occur in recurrent prostate cancer patients, 2-dimensional gel electrophoresis was used to examine PCI isoforms. Pooled human plasma was used as a control and sera from a recurrent and a non-recurrent patient were used as test samples. The patients were selected because they were both of the same age, had stage two prostate cancer, received the same treatment regimen and their samples were drawn in the same time period after hormone therapy. The PCI isoforms observed in the control sample were consistent with previously published 2-D gel spot patterns of this protein in terms of molecular weight, pl and isoform configurations (Saito, 2011). Unique PCI isoforms were observed in the serum sample from the patient with recurrent cancer when compared to either the control sample or the sample from the patient who
did not show cancer recurrence. Appearance of these unique isoforms in this sample corresponded with a significant drop in the concentration of the PCI peptide in the serum of the patient with recurrent cancer.

Based on the results of the ELISA assay and especially the 2-D gel analysis of PCI isoforms, I hypothesize that patients who experience a biochemical recurrence may at some point develop unique PCI isoforms likely due to unique post-translational modifications. These modifications are likely to be in an area of the protein that prevents release of the PCI-specific peptide and thus a decline in the levels of this peptide is observed in these patients as discussed in Chapter 4. One possibility is that the structure of the N-glycan at Asn^{230} is different in patients with recurrent cancer, which could severely inhibit trypsin activity at this site, thus the peptide concentration declines. Alternatively, it is possible that the PCI protein engages in unusual interactions with other biomolecules in these patients and because of the proximity of the PCI peptide to the reactive center loop (RCL) the tryptic release of the peptide becomes especially inefficient and a drop in the levels of the peptide is observed.

*Future Work*

The results of the work presented in this thesis suggest that a drop in the PCI peptide level is not reflected at the PCI protein level. This observation is likely due to the fact that tryptic release of the target peptide is prohibited by post-translational modifications adjacent to the peptide or by complex formations with other biomolecules at the RCL site, which is in close proximity to the peptide. Identifying these PCI isoforms/interactions in the context of prostate cancer may lead to unravelling yet another biological role for this diverse protein. Purification of larger amounts of PCI from
different individuals will be required in order to identify the biochemical changes leading to different PCI isoforms or to deduce interactions with other biomolecules. All published purification protocols for PCI have used in excess of 50 mL of plasma/serum for the purification step (Saito, 2011; Suzuki, Nishioka, & Hashimoto, 1983). In the work presented here we had access to limited amount of sera from each patient (~100 µL), thus we were prohibited from identifying specific isoforms/interactions at the protein level. It may be fruitful to obtain larger amounts of plasma/sera to allow biochemical analysis of the PCI isoforms from different patients.
Chapter 6. General Discussion

6.1. The biomarker validation dilemma: A fresh perspective

Validating biomarkers using immunoassays is problematic since these assays require two antibodies, one for antigen capture and the other for detection. Such antibody pairs must be specific for the antigen and not subject to interferences, a major problem that contributes to the great expense in developing immunoassays. In the past decade a number of mass spectrometric approaches have emerged with the promise of providing an alternative to often-flawed immunoassays. These can be developed with less expense and theoretically with fewer problems from interferences (Hoofnagle & Wener, 2009). Thus, such methods, if made to be robust and with high-throughput capability, should alleviate the biomarker validation issue. Two of the main approaches under investigation for biomarker validation are selected reaction monitoring (SRM) assays and an immuno-MS assay called SISCAPA. Both assays involve quantitation of protein-specific peptide analytes as surrogates of the parent proteins. SRM assays are particularly advantageous over immunoassays in the sense that they do not require affinity reagents (i.e. antibodies) and thus lengthy, expensive antibody production and selection process is avoided. However, the sensitivity of typical SRM assays is not sufficient to quantitate analytes in the low nanogram per millilitre range. As an alternative, SISCAPA assays have found the middle ground between sensitivity and the use of affinity reagents: These assays require only one antibody and replace the second antibody with a mass spectrometer.
SISCAPA assays have been shown to reach sensitivities of low nanogram per milliliter when ten microliters of human plasma are used.

However, both SRM and SISCAPA assays have suffered from low throughput since these assays require a lengthy fractionation step (usually by liquid chromatography) prior to mass spec analysis. The LC step is on the order 30-40 minutes for typical SRM assays and on the order of 3-5 minutes for typical SISCAPA assays, both too time-consuming when large sample numbers (likely more than 1000) must be analyzed in the validation process. The work outlined in this dissertation showed that non-specific background peptides in SISCAPA assays could be reduced, thus eliminating the need for a liquid chromatography step prior to MS analysis.

The key to reducing non-specific backgrounds is a thorough affinity bead washing procedure. However, to retain sensitivity during this washing procedure, antibodies with sub-nanomolar affinities must be used. Selecting such high affinity antibodies has been previously demonstrated using a surface plasmon resonance (SPR)-based method. In my thesis research, I developed an alternative assay called MiSCREEN that can be used to select high affinity antibodies in a rapid and cost efficient manner. Most importantly, the assay selects the antibodies under conditions that closely resemble their ultimate use in SISCAPA peptide enrichment from solution phase. MiSCREEN has subsequently been used to select high affinity monoclonal antibodies for more than 50 different peptide targets by screening more than 5000 hybridoma supernatants. Thus the MiSCREEN assay is of use for addressing the main premise of the human Proteome Detection and Quantitation (hPDQ) project (Anderson et al., 2009), which proposes producing anti-peptide antibodies against the primary proteins encoded by the human genome.
In addition to the use of high affinity antibodies, optimization of conditions for antibody capture and enrichment of peptides was required to obtain analytes of sufficient purity for LC-free MS analysis. Empirically, it was determined that of several affinity bead supports tested, protein G coated magnetic beads from Invitrogen (Oslo, Norway) provided the highest signal to noise ratio. In addition, based on the characteristics of the background peptides, a washing regimen was designed that included both an organic solvent (acetonitrile) and a solution containing a high salt concentration (PBS) to reduce background peptides. This allowed the implementation of SISCAPA assays using two LC-free mass spectrometric platforms, MALDI-TOF MS and RapidFire/MS. With these platforms it was clearly shown that peptide analytes can be quantitated accurately in digested human plasma/serum in a high-throughput fashion. This will allow biomarker validation that requires analysis of large numbers of samples.

Whether or not mass spectrometric assays will be able to replace most clinical immunoassays is still not clear. Despite the fact that immunoassays have serious shortcomings, they are still the ‘gold standard’ assays for many diagnostic tests. However, mass spectrometric assays offer several advantages, including the unequivocal identification of the analyte being measured and the ability to ‘multiplex’ the measurement of analytes (i.e. measure multiple analytes simultaneously). It is clear that some problematic immunoassays (such as thyroglobulin immunoassays) will be replaced by MS-based assays and that many new biomarker panels will require MS-based assays for their implementation.
6.2. Towards validating a protein C inhibitor (PCI)-specific peptide as a biomarker for predicting biochemical recurrence in prostate cancer patients

To demonstrate the utility of the LC-free SISCAPA assay for quantification of analytes in clinical specimens, a surrogate peptide from PCI was measured in sera from prostate cancer patients. The results indicated that the PCI specific analyte could be measured using the SISCAPA-MALDI approach with consistency and precision. In addition, the analyte could be measured accurately using a user-friendly mass spectrometer (Bruker microflex™ LT), which bodes well for adoption of SISCAPA assays in clinical settings.

In a randomized, double-blinded study, longitudinal samples collected from 51 prostate cancer patients were analysed for levels of the PCI peptide. Serum levels of the peptide analyte declined in prostate cancer patients who eventually experienced biochemical recurrence of the disease. More specifically, a significant decline in the levels of the PCI peptide was seen within the first 18 months after treatment in patients who eventually experienced a biochemical recurrence. This decline was not seen in patients who received the same treatment but who did not have a biochemical recurrence of their cancers. The patients’ PSA and PCI data for this specific time-point were plotted using a receiver operating characteristic (ROC) curve: While the PSA data showed no predictive value, the PCI data showed an area under the curve (AUC) of 0.774 and predicted biochemical recurrence in this cohort with a sensitivity of 78% and specificity of 85% using a 12.77 pmol/mL cut-off value.

The work presented here sets the stage for a validation study to be conducted in a large cohort of prostate cancer patients and controls. In addition, another serine protease
inhibitor (pigment epithelium-derived factor; PEDF) will be measured along with the PCI peptide as a separate (potential) biomarker. This protein will be included because it too is a serine protease inhibitor whose serum concentrations have been shown to decline with respect to progression of prostate cancer (Qingyi et al., 2009). By combining the PEDF and PCI analytes, it is possible that an assay with a high degree of clinical sensitivity and specificity for predicting prostate cancer recurrence can be developed.

6.3. **Studying PCI at the protein level may lead to unravelling its biological role in prostate cancer**

During the verification study, it was realized that the concentration of the PCI-specific analyte did not correlate with the literature-reported concentrations for the PCI protein. Consistent with previous mass spec-based studies on other proteins, this discrepancy may be due to inefficient release of the target peptide by trypsin, either because of unique or aberrant glycosylation at asparagine 230, which is adjacent to the target peptide, or because of protein complex formation through the reactive center loop (RCL), which is in close proximity to the peptide.

PCI protein was measured by ELISA using plasma samples from healthy people and prostate cancer patients. Despite low levels of the peptide analyte in samples from prostate cancer patients, the levels of the PCI protein were not reduced in any of these plasma samples. Hence, to understand this, 2-dimensional gel electrophoresis and immunoblot analysis was performed on pooled human plasma and serum samples from one patient with recurrent cancer and one patient who did not have a biochemical recurrence. The patients were selected because they were of the same age, had the same cancer stage and received the same treatment. Unique PCI protein spots were clearly
observed in the sample from the patient with recurrent cancer (who had a low PCI peptide level) compared to the sample from the patient without cancer recurrence and the control pooled human plasma. I hypothesize that the different protein spot patterns represent modifications of the PCI protein and that such modifications severely restrict the tryptic release of the target peptide, hence the low PCI peptide levels in the serum of this patient.

As a way to test this hypothesis, I propose that PCI should be purified from serum or plasma samples obtained from several patients with and without recurrent prostate cancer; the purified PCI can then be used for biochemical analysis of the post-translational modifications and possible PCI protein complexes. The presence of any unique modifications or protein-protein interactions might lead to the discovery of yet another biological role for this diverse protein. Understanding the protein interaction network(s) of PCI within the context of prostate cancer may provide invaluable information regarding the mechanism by which this disease progresses.
Bibliography


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Figure Appendix I. Digestion of dried blood and serum spots

SISCAPA enrichment of the PCI and sTfR analytes from a digested dried whole blood spot (A) and a digested dried serum spot (B) are shown. A 1/4 inch punch of the dried whole blood sample provides equivalent of 10 µL liquid plasma while the same punch out of the dried serum spot provides equivalent of 20 µL of liquid plasma. For this reason the concentration of the analytes is roughly twice as much in the dried serum spot compared to the dried whole blood spot.
## Appendix II

### Table Appendix II. Patients' data for the verification study

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