Paper-based smart sampling for bottom-up protein analysis

Thesis for the degree Philosophiae Doctor

by

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Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 2240

ISSN 1501-7710

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

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Acknowledgments

The work presented in this thesis has been carried out at the Section for Pharmaceutical Chemistry, Department of Pharmacy, University of Oslo in the period August 2016 to December 2019. First and foremost, I would like to thank my supervisors Trine and Léon for an excellent collaboration. I would also thank mamma, pappa og brodern, for all the support!

Øystein Skjærvø

Oslo, December 2019

Research is to see what everybody else has seen,

and to think what nobody else has thought

- Albert Szent-Györgyi

Publications

This thesis is based on the following papers. The papers are further referred to by their respective roman number:

I. **Instant on-paper protein digestion during blood spot sampling.**
Øystein Skjærvø, Cecilie Rosting, Trine Grønhaug Halvorsen and Léon Reubsaet. *Analyst*, 142(20) p3837-3847, DOI: 0.1039/c7an01075c

II. **Smart blood spots for whole blood protein analysis.**
Øystein Skjærvø**,** Trine Grønhaug Halvorsen and Léon Reubsaet. *Analyst*, 143(13) p3184-3190, DOI: 10.1039/c8an00317c

III. **Pre-lab proteolysis for dried serum spots**
- An intelligent sampler targeting low abundant biomarkers
Øystein Skjærvø**,** Trine Grønhaug Halvorsen and Léon Reubsaet. *Analytical Methods*, DOI: 10.1039/c9ay01976f

IV. **Paper-based immunocapture for targeted protein analysis.**
Øystein Skjærvø**,** Eirik Solbakk**,** Trine Grønhaug Halvorsen and Léon Reubsaet. *Talanta: The International Journal of Pure and Applied Analytical Chemistry*, 195 p764-770, DOI: 10.1016/j.talanta.2018.12.013

V. **All-in-one paper-based sampling chip for targeted protein analysis.**
Øystein Skjærvø**,** Trine Grønhaug Halvorsen and Léon Reubsaet, *Analytica Chimica Acta, 1089 p56-65,* DOI: 10.1016/j.aca.2019.08.043.

Publication(s) not included in the dissertation:

SI. **Exploring bioimpedance instrumentation for the characterization of open tubular liquid chromatography columns.**

Øystein Skjærvø, Ole Kristian Brandtzaeg, Kristian Lausund Blindheim, Oliver Pabst, Ørjan Grøttem, Elsa Lundanes and Steven Ray Haakon Wilson, *Journal of Chromatography A*, 1534 p195-200, DOI: 10.1039/c7an01075c

SII. **Matrix-assisted ionization mass spectrometry in targeted protein analysis – an initial evaluation.**

Øystein Skjærvø, Sarah Trimpin and Trine Grønhaug Halvorsen, *Rapid Communications in Mass Spectrometry*, Special Issue Research Article 2019 p1-9, DOI: 10.1002/rcm.8437

SIII. **Microfluidic electromembrane extraction under stagnant conditions.**

Magnus Saed Restan, Øystein Skjærvø, Ørjan Grøttem Martinsen and Stig Pedersen-Bjergaard. Recently accepted, January 2020, *Analytica Chimica Acta,* DOI: 10.1016/j.aca.2020.01.058

Abbreviations

ABC	*Ammonium bicarbonate*
C18	*Octadecyl (chromatographic stationary material)*
DBS	*Dried Blood Spots*
DDA	*Data-dependent analysis*
DIA	*Data-independent analysis*
DMS	*Dried Matrix Spots*
DTT	*Dithiothreitol*
ELISA	*Enzyme-Linked Immunosorbent Assay*
ESI	*Electro Spray Ionization*
FDA	*Food and Drug Administration*
hCG	*Human Chorionic Gonadotropin*
HEMA	*2-Hydroxyethyl methacrylate*
IAC	*Iodoacetic acid*
ID	*Internal Diameter*
IS	*Internal Standard*
IT	*Ion-Trap*
LC	*Liquid Chromatography*
LCMS	*Liquid Chromatography-Mass Spectrometry*
LOD	*Limit Of Detection*
LOQ	*Limit Of Quantification*
m/z	*Mass-to-Charge ratio*
MIP	*Molecular Imprinted Polymer*
MS	*Mass Spectrometry*
MS/MS	*Tandem Mass Spectrometry*
OT	*Orbitrap*
pELISA	*Paper-based Enzyme-Linked Immunosorbent Assay*
pHEMA-TsCl	*2-Hydroxyethyl methacrylate-4-toluenesulfonyl chloride*
pHEMA-VDM	*2-Hydroxyethyl methacrylate-co-2-vinyl-4,4-dimethyl azlactone*
POC	*Point-of-Care*
proGRP	*Pro-Gastrin Releasing Peptide*
PTM	*Post-Translational Modification*
QqQ	*Triple Quadrupole*
RIA	*Radioimmunoassay*

RP	*Reversed-Phase (chromatographic separation principle)*
S/N	*Signal-to-Noise ratio*
SISCAPA	*Stable Isotope Standards and Capture by Anti-Peptide Antibodies*
SPE	*Solid Phase Extraction*
SRM	*Selected Reaction Monitoring*
TCEP	*Tris-2(-carboxyethyl)-phosphine*
TsCl	*4-Toluenesulfonyl chloride*
WADA	*World Anti-doping Agency*

Abstract

Paper-based sampling has been implemented in the determination of small molecules in biological samples for many decades. However, the use of paper-based sampling in combination with mass spectrometric analysis of proteins has been limited. Promising efforts have previously been demonstrated with blood and serum, sampled on paper materials. However, common for the methodologies is the time-consuming sample preparation and difficulties of reaching sufficient sensitivity and general performance for clinical applications. The latter methodologies also require a high degree of operator skills in order to obtain reliable data.

In this thesis, paper-based sampling is integrated with key elements (such as protein hydrolysis and immunoaffinity) required to perform qualitative protein determinations, as well as quantitative determinations of low abundant biomarkers. The presented concept is aimed towards the point-of-care strategy for consumer-based sampling with simple execution and high analytical performance.

In **paper I,** it was demonstrated that sampling combined with tryptic protein digestion *on-paper* was readily comparable to a conventional dried blood spot (DBS) procedure with respect to peptide generation. The DBS procedure was carried out with over-night protein digestion. The sampling concept was demonstrated with wax printing to hinder sample spread in the paper and polymer beads covalently immobilized with trypsin. The polymer beads was applied on top of the sampling surface for instant protein digestion during the sampling stage. The demonstration was carried out in a non-targeted proteomic investigation. The feasibility of protein reduction and alkylation after enzymatic digestion was demonstrated. Furthermore, the sampling stability was investigated. The sampling material could be stored both with and without sample for several weeks without a significant decrease in performance.

In **paper II**, the sampling concept was improved by coating the sampling paper with a hydrophilic polymer with reactive groups for covalent immobilization of trypsin, directly to the sampling material. Immobilization conditions (duration, temperature and trypsin amount), as well as characterization of filter paper structure (thickness, porosity, and weight) were evaluated and optimized. The evaluation was based on missed cleavage sites, the number of unique peptides and unique protein groups obtained in a non-targeted analysis of whole blood. The improved sampling device was investigated and compared alongside with protein analyses with conventional DBS sampling, similar to **paper I**. The polymer-coated sampling device showed promising performance with twice as many identified protein groups compared to our initial demonstration, and conventional DBS.

In **paper III**, the optimized fabrication procedure and immobilization conditions from **paper II**, alongside a new sampling cartridge developed for **paper V**, were utilized in a targeted analysis of the low abundant serum biomarker proGRP. The sampling concept of *in-device protein digestion* was used in combination with proteolytic epitope peptide immunoaffinity capture. Fast sample preparation (under 2.5 hours from sampling stage to LCMS analysis) with a high degree of performance (low detection limits and high correlation in the relevant concentration range) was demonstrated. Furthermore, the concept proved that *in-device protein digestion* is comparable to conventional digestion protocols. LOD and LOQ were 150 and 500 pg/mL in serum, respectively. A correlation of R^2= 0.99 was obtained between 0.5 – 1000 ng/mL (five points) and all RSDs were below 26 %.

In **paper IV**, the considerations regarding fabrication of the polymer layer (from **paper II**) as well as testing other polymers for *in-device immobilization of monoclonal antibodies* (mAb) was investigated. Determination of the most suitable polymer layer and immobilization conditions for mAB E27 (targeting hCG) were optimized. The sampling strategy was tested with the wax printed design from **paper I,** and *in-device immunocapture* was demonstrated in hCG spiked serum. The sampling spots allowed fast and easy sampling and preparation and enabled a detection limit of 1 ng/mL. The detection limit was demonstrated to be two times lower compared to conventional DBS sampling. A satisfactory correlation was obtained by a five-point concentration curve from 1 ng/mL to 20 ng/mL (R^2 =0.97) and 100 ng/mL to 2000 ng/mL (R^2=0.99), respectively. Intraday precision was within 16 % and 23 % for concentration range 1 ng/mL to 20 ng/mL and 100 ng/mL to 2000 ng/mL, respectively. Interday precision was within 20 %. Accuracy was determined to 10 % and 11 % for 2.5 ng/mL and 20 ng/mL, respectively. The sampling concept was further demonstrated in a realistic setting where serum samples from two confirmed patients with testicular germ cell cancer were analyzed and quantified with high precision.

In **paper V**, the optimized fabrication and immobilization conditions from **paper IV** were used to make a fully integrated sampling concept capable of performing *immunoaffinity, protein reduction, alkylation,* and *protein hydrolysis* all *in-device*. The sampling device was designed in a paper-based chip format aimed at the point-of-care sampling strategy. *In-device protein reduction* and *alkylation* were optimized with regards to reagent concentration and reaction pH. *In-device protein hydrolysis* was optimized with respect to the amount of trypsin and reaction time. The concept was evaluated by quantification of hCG spiked to serum and freshly collected whole blood. The sampling concept showed a high degree of performance between 10 and 1000 ng/mL (five points, R^2=0.99). The LOD (100 pg/mL) was up to ten times lower, with more than six times faster sample preparation compared to what has previously been reported for on-paper sampling of hCG in human serum samples.

1 Introduction

1.1 Proteomics

According to Nature[1], proteomics is described as the *study of proteomes* (the entire set of proteins expressed by a genome, cell tissue or organism at a given time), but the term is also used to describe techniques to determine entire sets of proteins of an organism or system, such as purification and analysis of specific proteins. This definition will often also include the function of proteins [2] and aims towards the study of changes in protein expression and modifications. The latter to obtain information of the cellular pathway and to improve the understanding of the biological pathways in living organisms[3]. The interest in proteomics, or protein analysis, has had a linear increase during the past few decades (**Fig. 1**). However, proteins and their role in biological systems has had a major focus in analytical chemistry and modern medicine for far longer and could be traced back to 1827[4]. Nevertheless, they have been difficult to analyze and to characterize. This, because the protein expression in biological systems is dynamic i.e. the protein content or set of proteins in an organism is always changing with time[5, 6], but also with external and internal changes such as aging, disease condition[7], medication[8], and stress[9]. Proteins are composed by a combination of 20 different amino acids (human, but depending on the organism). This makes for a vast variety in size, type, and functionality, all controlled by the amino acid composition and post-translational modifications (PTM). Compared the characterization of other biological building blocks, such as DNA, which is only composed of 3 elements (a phosphate group, a sugar group and one of three nitrogen bases), proteins are of high complexity. Proteins do also have a broad dynamic range in biological samples, and the concentration spans from pg/mL-mg/mL. The majority of the proteins related to biological changes lay in the lower concentration range. Therefore, with all into consideration, a complete characterization of the proteome is not yet accomplished. Nevertheless, with sensitive and high resolving separation, purification, and detection techniques much progress has been accomplished during the past two decades. Therefore, the increase in publications could be seen in correspondence to the technological advancements in analytical instrumentation as well as the

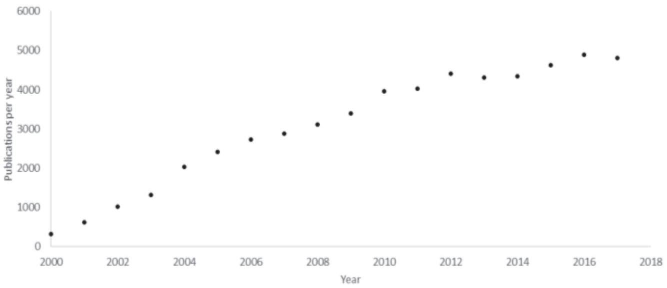

Figure 1 Number of publications per year according to a search with the key word "proteomics" in the web of knowledge database. Search performed September 2019

commercialization of electro spray ionization (ESI) for mass spectrometry (MS) (introduced in the late 1980's[10]) enabling MS detection of liquid samples with larger biomolecules.

1.1.1 Clinical proteomics

In contrast to the general description of proteomics as introduced in the previous section, clinical proteomics has been described with several different definitions throughout the last few decades and seems to be continuously changing[11]. Early definitions of the field were broad and was most often referred to as "*comprehensive studies of qualitative and quantitative profiling of proteins (and peptides) present in clinical specimens such as body fluids and tissues"* [12]. In later years Andrew N. Hoofnagle had an excellent summary for the most common definitions in a Clinical Chemistry Q and A[2] with sharpened, but still broad definitions that are frequently used in the literature today 1) t*he quantitative measurement of multiple proteins in a biological sample that is related to human disease*. 2*) The identification and relative quantification of proteins in a biological sample that is related to human disease* and 3*) the quantitative measurement of proteins in human samples that will directly lead to an improvement in the care of human patients*. To summarize the terms as one, clinical proteomics describes proteomic discoveries as well as applications related to diagnostic purposes and treatment of patients. When a protein is associated with a biological malfunction or mutation it is referred to as a biomarker, and as previously mentioned, biomarkers were described early and have sustained clinical interest. However, the use of proteomic tools for the discovery of new biomarkers and clinically approved methods is slow and according to the US Food and Drug Administration (FDA), only tests for 109 proteins in plasma and serum were approved by 2008[13]. Furthermore, only 23 protein tumor markers were FDA approved by 2013 for use in clinical practice[14]. It must, however, be stressed that there are several more biomarkers known that are related to cancer other than what FDA has approved. One of these is human chorionic gonadotropin (hCG). Though, obtaining approval for new biomarker candidates into routine clinical application varies from country to country and is generally a demanding process. In order to implement new biomarkers in routine use one has to prove evidence of technical stability (robustness), significant improvements over consisting method, cost per analysis and ease of use[15]. These requirements may be difficult to convey from a research facility to a clinical laboratory. Therefore, the gold standard of clinical test for biomarkers is still considered immunoassays, where biomarkers (antigens) are enriched by antibodies such as in enzyme-linked immunosorbent assay (ELISA). This, even though many people in the research community anticipated a shift in the analytical methodology from the demonstration of MS applicability to analyze and distinguish complex serum proteins in the early 2000s[4]. Ever since then, with MS-based protein mapping and the increased interest in the human proteome, it was believed that many new biomarkers were to be characterized and implemented into routine settings[4]. However, this has not been the case even though the sensitivity and applicability (ease of use) of the mass spectrometers have significantly increased during the later years. Thus, the newer MS-based methodologies with its benefits (further discussed in *section 1.3*) in clinical protein analysis have not yet become the gold standard, but it is expected to become so[16].

1.2 Immunoassays

For targeted protein analysis immunoassays have been, and are still preferred in clinical settings due to their specificity, detection limits and ease of use. Immunoassays, which employ antibodies to enrich and detect an analyte (antigen), have sustained superior performance for targeted protein determination ever since its introduction in the early 1960s[17] by Yalow and Berson (radioimmunoassay (RIA)). The assays are now used in various applications ranging from virus[18, 19] to larger biomolecules e.g. proteins[20] and peptides[21]. For in-depth characterization of proteins western blot (also referred to as immunoblot) is often used due to excellent specificity and as a compliment/confirmation of other screening methods. However, ELISA is considered the gold standard for clinical screening and protein determination due to its simplicity, sensitivity, automation potential, and good reproducibility[22, 23]. In addition, most of the reagents used in these methodologies particularly ELISA has been standardized and pre-fabricated kits are readily available from various manufacturers (e.g. Thermo Fischer Scientific, Sigma Aldrich, and Merck Millipore). Standard operating procedures and practical guides for immunoassay method validation of bio-fluids are also available making them straight forward to incorporate into clinical and routine applications[24].

1.2.1 ELISA

After the introduction of the first RIA for endogenous insulin levels in plasma by Yalow and Berson, which was an immunoassay for measuring insulin (by using radioactive-labeled antibodies as reporter label for concentration measurements) the concept of ELISA started to develop. The detection principle of Yalow and Berson's RIA was carried out by reacting to human insulin and beef insulin-I^{131}, with insulin-binding antibodies from guinea pigs serum (the test subjects had multiple injections with beef insulin prior to serum sampling). The tracer, insulin-I^{131}, was used due to the lack of crystalline preparation of human insulin and therefore the tracer would not be naturally occurring in the samples. The concentration measurements were carried out by separating radioactively labeled insulin with electrophoretic paper chromatography with subsequent radioactive measurement of insulin-I^{131} degradation[25]. From the introduction, RIA gained praise due to its performance of measuring protein concentration, but also much concern due to the use of radioactive labeling. However, the interest in the fundamental concept sustained and was applied to different applications the following years with different detection principles. This later evolved into ELISA[26-28]. From the introduced RIA application (with radioactive labeling), ELISA was later introduced simultaneously by two different research groups in 1971[26, 27]. Differentiating from the original RIA method, both groups replaced the radioactively labeled tracer antibody with enzymes. Detection was performed with a spectrophotometer for color intensity measurement by reacting the enzymes with a reactant (enzyme + reactant = conjugated product). Even though the detection principles evolved over time, the basic principle of ELISA remained the same (**Fig.2**). The antigen is captured by a primary antibody immobilized to a solid support such as tubes or microplates (e.g. 96-well plate). The matrix is then removed and a tracer antibody (with label) is added to bind to a different part of the antigen. Depending on the tracer antibodies label, a reactant is added and a product (between the reactant and the tracer antibody label) is measured e.g. by fluorescent, spectrophotometer or colorimetric detection.

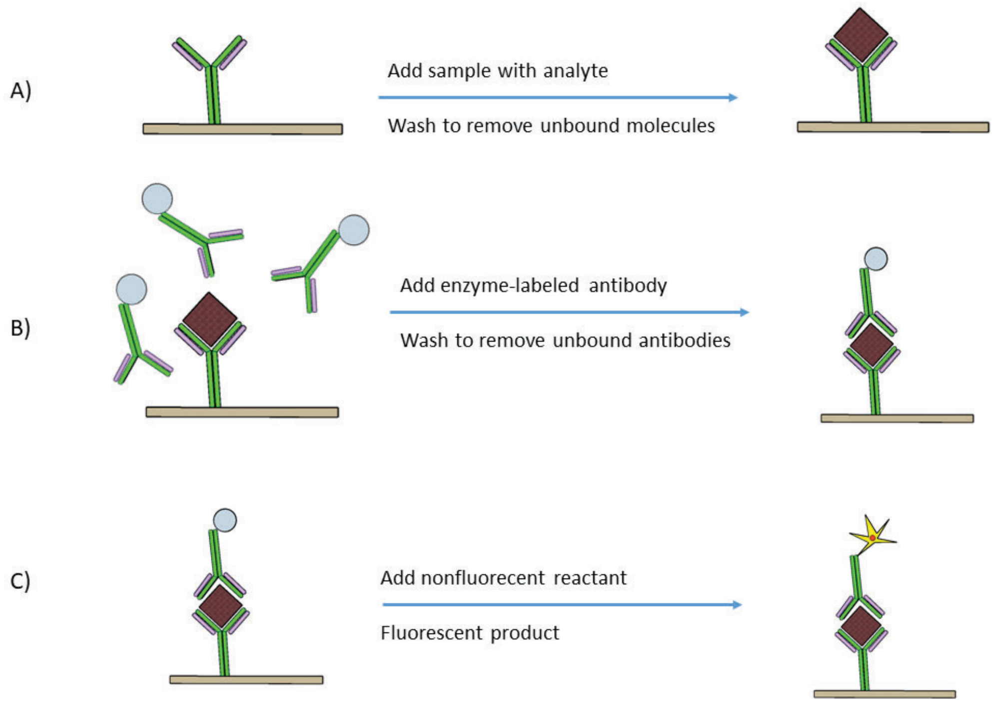

Figure 2 Schematic illustration of the ELISA principle (sandwich configuration) with fluorescent detection. A) sample is added to an ELISA plate with antibodies immobilized to a solid support, all unbound molecules are subsequently washed away prior to B) addition of antibody 2 (enzyme labeled) which recognize a different site of the bound molecule. The final step C) is adding a non-fluorescent reactant that yields fluorescent when reacted with the enzyme attached to antibody 2.

1.2.1.1 Types of ELISA

There are many configurations of ELISA, however, there are two distinct categories; homogenous assays and heterogeneous assays. Homogenous assays are based on enzymes becoming inactive when bound to an antibody. They are usually used to measure small quantities (e.g. drugs or viruses) and detected by colorimetric responses such as color change, intensity or appearance[29-33]. This technique is relatively easy to use with minimal actions involved, but holds lower selectivity due to lack of matrix removal and has historically been expensive to perform assay[23]. Heterogeneous assays involve immobilization/fixation of the antibodies to a solid support in such way that the antibody-antigen complex could go through a washing procedure to remove molecules from the matrix that could interfere with the signal response. Thus, the heterogeneous assays yield a higher sensitivity compared to their counterparts and are therefore more commonly used[23]. This washing step is also necessary to remove free antigen from the samples prior to adding a labeled antibody to avoid false reporting. In addition to the two main categories of ELISA, there are also four sub-types namely; direct-, indirect-, sandwich- and competitive ELISA.

Direct ELISA is carried out by fixating the antigen directly to a solid support. An enzyme-labeled antibody is then added to directly bind to the fixated antigen. For analyte measurement, a substrate is added which reacts with the enzyme creating a colored product that could be measured based on intensity.

Indirect ELISA[34] is more or less like direct ELISA, however, a secondary antibody with conjugated-enzyme labeling is added to bind to the primary antibody. The technique is mostly performed to detect antibodies in biological fluids. Compared to the direct method, the indirect method is marginally more sensitive and holds higher flexibility since more primary antibodies (binding directly to the antigen) could be used. However, common for both is that the antigen has to be fixated to a solid support and this procedure is not specific (in terms of fixation). This, in turn, can lead to high background signals.

The principle of *Sandwich ELISA*[35] differs slightly from the prior method because primary antibodies are fixated to a solid support. Secondary antibodies with conjugated enzymatic-labeling are then added after the antigen has been captured to the immobilized antibody. In between each step, washing procedures to remove unbound material and unbound antibodies are necessary. This method holds higher specificity and up to five times high sensitivity compared to the prior methods mainly due to the ability to include multiple washing steps to remove interferences, however, more reagents and steps are required[23].

The last ELISA principle used is *competitive ELISA*[36]. Here, primary antibodies (without a label) are fixated to a solid support prior to further addition of primary antibody and reference antigen simultaneously (both could be labeled). The antigen from the sample competes with the reference antigen for binding to the fixated antibodies. With a higher amount of antigen in the sample, less reference antibody will be able to bind to the antigen hence the nomenclature. After the washing procedure, a secondary antibody with enzyme labeling is added, followed by a reacting substrate to yield a measurable product e.g. coloration.

1.2.1.2 ELISA in the clinics

In accordance with what Van Weemen and Schuurs[27] suggested when first introducing the ELISA in 1971, the technique has indeed had high importance for clinical applications. Not only for hCG as Van Weemen and Schuurs used as model protein, but also for 109 other FDA approved (by 2008) protein biomarkers and 62 additional tests for peptides, PTMs and more, as previously discussed. According to the target analyte, multiple different types of ELISA kits are used. An example is commercially available ELISA kits for HIV screening, which are available as both indirect[37, 38] and sandwich[38] ELISA. For biomarkers, it seems that most manufacturers offer sandwich ELISA kits due to the sensitivity and assay range needed for low abundant biomarkers. As an example, the Booster ELISA kit for hCG (product code: EK7062) promise sensitivity of 2.0 IU/L with an assay range of 8-240 IU/L in serum and urine. Similarly, Abcam plc. hCG ELISA kit (ab100533) offers a sensitivity sub 50 pg/mL and an assay range of 54.87-40000 pg/mL with recovery above 90 percent. With this in consideration, the performance of commercial ELISA kits is adequate and with the ease of use and its rapid reporting, it is with a good reason why this technique is regarded as the gold standard in the clinical settings. However, there are also several drawbacks of commercially available ELISA kits. One of which is the variable kit performance between the manufacturers. Different manufacturers use different antibodies and several studies have shown that there could be significant differences between the commercially available ELISA kits, especially with regard to precision and recovery[39-41] and in some cases false reportings[42].

1.2.2 Western blot

Western blot (WB) is another technique for identifying specific proteins from biological matrices and is commonly used to confirm findings from ELISA analyses. Compared to the latter, WB is carried out mainly by three stages where the analyte is 1) separated by size, 2) transferred to a solid support such as a nylon membrane and 3) undergone similar treatment to ELISA with primary and labeled antibodies for reporting. Separation of the protein is carried out by e.g. gel electrophoresis before blotting the (selected or entire) gel piece onto a membrane prior to incubation and addition with labeled antibodies. Unbound antibodies are further removed. The remaining antigen-antibody complexes could be detected by e.g. color intensity. Given the separation prior to addition of antibodies WB show higher specificity as well as sensitivity than ELISA[43], however, WB involves several more steps and is a delicate as well as a time-consuming process making it less preferable in clinical settings compared to ELISA (if either could be used) as a primary measurement technique. Nevertheless, WB solves potential pitfalls related to other immunometric techniques such as antibody cross-reactivity and specificity issues (discussed in the next section). WB could also be used for quantitative determinations[44]. Furthermore, in light of more recent miniaturization of the technique such as WB in chip format, the technique could sustain importance for future application[45] even though most blotting investigations are anticipated to be replaced by MS.

1.2.3 Drawbacks of the Immunological platforms

Due to the above-mentioned attributes making ELISA a popular analytical platform, ELISA and other immunological methods suffers from significant challenges that could give rise to incorrect reporting and ultimately false diagnosis if used to screen for a disease[46]. Incorrect reporting could be due to several reasons such as auto-analyte antibodies, heterophile antibodies or human anti-reagent antibodies present in the sample. Cross-reactivity (antibody reactivity to other proteins than the target analyte)[47] and high dose hook effect[48] has also been comprehensively discussed as severe issues of ELISA in the literature. In particular, high dose hook effect, which is a measurement of significantly lower levels of analyte than the actual sample concentration in the sample. Ideally, the signal in ELISA should be linear with concentration. However, a signal plateau and eventually a decrease (particularly for sandwich assays without reagent washing steps) in signal will be reached due to saturation of the immobilized primary antibodies. If the analyte signal is plotted with respect to increasing concentration the plot will resemble a fishing hook, hence the name. This effect could also be seen if the antigen is in such high concentration that it binds to all possible binding sites of both the primary and the secondary antibody (with the reporting tag) as well as adsorption to the solid support, thus preventing the sandwich-formation. The secondary antibodies now connected to the antigen will then be washed away during the matrix removal step and the signal will be reported as untrue[49]. To overcome the hook effect, some laboratories dilute serum samples for repetitive measurements to correct for the untrue signal. Nevertheless, for some analytes such as prostate-specific antigen (PSA), these effects have shown troublesome to correct due to the high analyte concentration[50, 51]. Finally, the methodology also lacks the ability to differentiate between multiple isoforms, isovariants or subtyping tied to biomarkers and therefore multiplexing is not possible within a single analysis.

1.3 Mass spectrometry

Mass spectrometry is a separation and detection technique for ionized molecules in gaseous phase, where the signal is generated by counting ions with their respective mass-to-charge (*m/z*) ratio. The analyzers in MS come in multiple configurations depending on application, however, they all share common traits like having a separation step prior to detection. This separation could be carried out by separating ions based on flight time (TOF), separation ions based on a stable trajectory through a variable electrical field (Quadrupole, Q) or ion isolation in an electrical field (ion trap, IT). Other mass analyzers that differ from the above mentioned have also been commonly applied to proteomic investigations like fourier-transform ion cyclotron resonance (FT-ICR) and orbitrap (OT). The latter instrumentation differs from others because they do not have a detector and thus, do not count ions. However, the signal is generated by measuring image current of ions with stabile orbital trajectory and by applying a fourier-transformation of the recorded image current with respect to time, a mass spectrum is generated.

Common for MS is the ability to fragment the molecules prior to detection (MS^n). This is particularly powerful for characterization as molecules since most fragment in unique patterns. This is a feature that allows MS to be a superior technique for quantification and avoiding false-positive results. For protein analysis fragmentation is exclusively used and could be set continuously (QqQ) for e.g. clinical applications of a known target or altering (only portions of the sample is fragmented) for non-targeted approaches.

1.4 Mass spectrometry in protein analysis

As previously mentioned MS has gained increasing popularity during the last few decades for proteomic investigations as a quantification and characterization technique for proteins. In contrast to the immunometric techniques, MS is capable of multiplexing as well as separating matrix components prior to the detection of proteins, especially if coupled to an LC[52]. The recent evolution of the MS instrumentation has also enabled high sensitivity and low detection limits and the technique is now able to quantify femtograms of proteins from e.g. cell-[53] or exosome extracts[54] and picograms of proteins in serum and whole blood samples[55]. MS has also vastly contributed to the ongoing characterizing the human proteome and are slowly being incorporated into clinical applications for biomarkers. The main advantage over immunometric methods, besides multiplexing, is the ability to avoid false reporting. Patel et al.[42] demonstrated this with an analytical method comparison of analysis of signal-regulatory protein alpha (SIRPA) from patient samples undergoing open-heart surgery by ELISA, WB, and MS. In this study, it was demonstrated that commercially available ELISA kits gave false positive results and WB failed to identify recombinant SIRPA in the calibrators. MS analysis of the same sample set did, on the other hand, identify several inflammatory markers (and albumin from the calibrators), but no SIRPA. The authors further stated that caution should be used when utilizing ELISA kits marked “for research purpose only” since the market is over-flooded with kits from different manufacturers that has not necessarily gone through thorough validation. The latter statement might be true, nevertheless, assay kits approved for clinical use is undertaken extensive validation prior to use in real cases and should therefore not show as significant variations in signal reporting. Even though not for proteomic purposes, another historical example on speculations regarding the liability of immunoassays for clinical applications followed the Nimitz accident in 1981 where subsequent immunoassay test reported positive for marijuana metabolites in urine. Based on this accident, a lack of trust in immunoassays (due to false positive results) and previous years with a high number of positive drug test amongst the military employees, GC-MS (gas chromatography MS) was introduced as a mandatory technique in 1982 to confirm the immunoassays[56]. During the following years, several milestones for MS in the clinics (for analytes other than proteins) were achieved such as tandem MS for newborn screening in 1990. The clinics may have been embracing the MS methodology slowly as discussed elsewhere[57] compared to the research community, which is now, more than ever, relying on MS data prior to concluding (several journals even require MS data to publish results for certain analytes[56]). Even though several analytical platforms have previously been used for protein analyses, now the proteomic investigations are mostly carried out with MS and are divided into two main categories, targeted and non-targeted analysis. Additionally, analysis of proteins is mainly carried out in two different strategies, top-down (analysis of intact proteins) and bottom-up (indirect analysis of proteins by their respective peptides).

1.4.1 Mass spectrometry in clinical protein analysis

Mass spectrometry is a relatively new technique and is not widely applied in laboratories for routine clinical protein analyses. This, because it is not (yet) a routine technique it requires specialized skills from the operators and implementation of MS-based strategies instead of the already and high performing ELISA techniques may see many more years to come. Compared to ELISA, MS-based methodologies is also laborious and often require complex sample preparation (such as protein modification) that could introduce more harm than good (if sufficient training lacks), in addition to time-consuming instrument maintenance and method development. This issue could be limited if MS applications could be more robust and automated in the future. Nevertheless, several MS applications have already been introduced and validated for clinical determinations[58-61]. However, by 2014 no MS applications were FDA approved as the main analytical technique for clinical determinations of biomarkers[62]. This may resolve from the regulatory requirements from different governmental bodies, lack of standardized quality controls and the high cost associated with implementation and substitution of the already existing clinical methods[63, 64].

1.4.2 Non-targeted analysis by mass spectrometry

Non-targeted, or discovery analysis, is one of the fundamental MS-strategies for protein mapping and characterization of the human proteome. The strategy is based upon determining all proteins in a sample and uses the information of protein content to couple to a hypothesis. This hypothesis could for instance be, how is the protein expression changing caused by a disease state or organ failure?[65] or how is age contributing to the protein variety in biological systems?[66]. Past non-targeted approaches has been carried out with blotting techniques, however, MS has during the last twenty years become the gold standard for such analyses because it could deliver more information per sample run compared to other techniques. Additionally, high-performance instrumentation are becoming more accessible for laboratories. The main mass analyzer used in the current non-targeted approaches is by far OT-MS due to its high resolving power (separation of similar masses), acquisition speed, accuracy (trueness of *m/z*) and sensitivity. The cost of this instrument has also been reduced over the last decade as well as increasing capabilities (higher resolving power, sensitivity and acquisition speed) making the OT-MS widely applied. FT-ICR, on the other hand, is not as frequently used as it sits on high running costs (due to superconductors), large foot-print and a high purchasing cost. The instrument also has a slow acquisition speed, however, it has superior resolving power, mass accuracy and the capability of analyzing higher mass molecules compared to other MS-platforms[67]. Nevertheless, OT-MS may be the most applied instrument in research laboratories for non-targeted protein applications due to its flexibility. The operation/acquisition in OT-MS is carried out in two different modes *data-dependent* and *data-independent acquisition*. Both acquisition modes are capable of delivering extensive structure information of proteins in biological (or non-biological) samples by fragmentation with subsequent spectral interpretation by available database-based software such as Mascot or Proteome Discover.

Data-dependent acquisition (DDA) on OT-MS has been the dominant acquisition technique since the introduction of the instrument in the early 2000s. DDA is based on collecting MS^n spectra of the most abundant *m/z (singular or plural)* prior to setting the fragmented *m/z* of an exclusion list meaning it would not be fragmented again for x amount of scan cycles (typically for 15-30 seconds)[68]. Therefore, it will only select a specific part of the sample for fragmentation and eventual identification and large portions of the sample will not undergo any fragmentation. This could be troublesome if many peptides co-elute because the MS will then focus on a selected few *m/z* and exclude the other peptides for fragmentation i.e. excludes sample information. Another factor is the presence of too many high abundant proteins. This because a high number of very abundant proteins such as albumin, which might not be of interest, could potentially dominate the MS^n spectrums. Additionally, given the fragmentation in DDA, each peptide species will only be measured once or twice per run. Thus, absolute quantification is difficult[69]. In spite of the latter "flaws" DDA still remains as a flexible technique with broad usability and technical simplicity and might be the favored method for general proteomic investigations.

In contrast to DDA, data-independent acquisition (DIA) does not have an exclusion list and is capable of fragmenting the larger part of the sample. The acquisition in DIA is operated by fragmenting defined *m/z* segments covering the whole *m/z* window (e.g. first segment *m/z* 50 – *m/z* 150, second segment *m/z* 150– *m/z* 250, etc.). In this way, peptides are not excluded based on their signal intensity and each peptide could be measured several times making for reliable quantification. The concept of DIA has been around for a decade but has been more and more incorporated as a staple technique in the later years due to the advancements in instruments with a faster acquisition rate. Nevertheless, there are still challenges regarded with DIA as the spectrums are highly complex and often need deconvolution by advance software for spectral interpretation. DIA also requires more skill from the operator and may therefore not be considered as a common approach yet, compared to DDA[69, 70].

1.4.3 Targeted protein analysis by mass spectrometry

Quantification of protein samples could be performed by several methods. Quantification by DDA or DIA is often carried out as label-free methods (spectral counting) or label entire sets of proteins (Stable Isotope Labeling with Amino acids in Cell culture, SILAC). These methods could be targeted to specific proteins, and are mostly used to measure up- or down regulation proteins in a sample set. However, targeted protein analysis by MS is by most considered as the approach where you filtrate certain *m/z* for specific measurements. For these applications, faster instrumentation is more commonly used such as triple quadrupole (QqQ), IT, TOF or hybrid Q-instruments. The most common MS instrumentation for targeted protein application is the QqQ. Quantification with QqQ is performed with three steps; Q_1 filtrates out most of the sample ions except one or more with a specific *m/z* (e.g. only *m/z* 500 ±0.1). These *m/z* are then subjected to fragmentation (by collision with an inert gas) in the q-cell before a secondary *m/z* selection is performed in Q_3. This operation is referred to as multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) and may be considered as the most used targeted MS method. Because of the *m/z* selectivity prior to detection, these instruments have high sensitivity and acquisition speed suited for routine analyses or determinations of one or more known analytes. Therefore, when compared to other quantitative techniques such as ELISA, targeted MS is most often the comparison.

1.4.3.1 Quantification

For quantification of proteins by MS, a correction for variance in signal intensity related to ESI spray stability, matrix effects and loss of analyte during sample preparation is needed. This correction is, for the most part, carried out by internal standards (IS) added to the sample during or subsequently after sampling. The IS is a labeled analyte that is added to the sample in a known concentration and quantification could thus be performed by measuring the signal ratio between the IS and the unknown (concentration) analyte. The labeling of the IS is most often by isotopes e.g. ^{13}C or ^{15}N) (preferably by two or more labeling sites to avoid confusion with naturally occurring isotopes), but could also be by deuterium[71]. For protein determinations, isotope labeling may be the preferred method since heavy labeling with ^{2}H could face hydrogen exchange during storage resulting in degradation of the IS and thus, give inaccurate results[72]. Furthermore, retention shifts during LC separation is also a potential drawback of ^{2}H IS. Regardless of IS labeling, absolute quantification of protein could be troublesome due to the required sample preparation prior to analysis and complexity of the molecules. Intact protein analysis is very demanding regarding instrumentation because of molecular size and several charge states (often more than 10) but also charge dilution effects[73]. Higher energy fragmentation cells are also necessary for intact protein analysis. This calls for high-resolution instruments which, in a routine application may not be the ones with the fastest acquisition rate (suited for high throughput). In contrast, bottom-up analyses are more suited for MS determinations especially combined with trypsin as the proteolytic enzyme (further discussed in *section 1.4.5*). This because trypsin produces peptides of approximately 10 amino acids, thus the size of each molecule is MS favorable of ~1000 Da. However, the enzymatic step is difficult to correct for since it relies on enzymatic activity and other kinetic variables such as temperature and time. Therefore, IS for quantitative bottom-up protein determinations, is available in several configurations based upon the correction needed during the sample preparing stage. Most frequently used is stable-isotope labeled (SIL) peptides. SIL peptides is regarded as the gold standard, however, they do not correct for the enzymatic digestion. As an alternative, SIL peptides with cleavage sites and additional amino acids has been introduced (wSIL). Additionally, stable-isotope labeled proteins (PSAQ) is available[74]. Both wSIL and PSAQ could correct for the entire sample preparation including enzymatic treatment. However, in a recent study by Oeckl et al.[75], no significant differences between the different IS approaches were found under controlled conditions. Others have demonstrated that PSAQ to have advantages[76, 77]. However, if used for frequent applications PSAQ is both laborious and expensive[78].

1.4.4 Sample preparation for targeted bottom-up analysis

As elaborated earlier, MS analyses often demand extensive sample preparation prior to analysis. This mainly resolves from the limited dynamic range of the mass spectrometer combined with the large span in protein concentrations in biological samples e.g. plasma (over 12 orders of magnitude)[79]. Additionally, salts naturally present in biological fluids as well as co-eluting matrix components could contribute to ion suppression (masking of analyte MS response)[80]. If MS is combined with LC, column clogging is also an additional issue were larger proteins, lipids and fat are troublesome. All the above-mentioned results in lowered sensitivity and ultimately deterioration of instrument performance. With exception of urine[81] (could be applied by the dilute and shoot method), most biological matrices required several sample-preparing steps such as desalting (solid-phase extraction, SPE), protein precipitation (organic solvent) and/or fractionation and filtration according to molecular size. All the latter has to be performed either prior to, or after protein modification (*elaborated in section 1.4.5*). However, even if many of the above-mentioned sample preparing strategies were combined, the sample would still be highly complex and not necessarily “pure” enough for reliable quantification of low abundant proteins (avoiding masking of signal and LC clogging). This because the dynamic protein range would still be too large even though salts and other non-relevant matrix components are removed. Nevertheless, there are possibilities for increasing the specificity of the above-mentioned methods e.g. selective protein precipitation[82], centrifugal filter cut-off value[83] or the use of specific stationary phases for SPE[84]. Despite the possibility for enhancing selectivity, these methods are considered general and are in most cases not solely good enough for quantification of low abundant proteins. Immobilized metal affinity chromatography (IMAC) could, however, be combined in order to gain selectivity by using a suitable transition metal ion for the target, analyte. However, IMAC is not frequently used since the analyte protein often needs to be tagged (e.g. polyhistidine) and therefore is a time-consuming sample preparation[85]. In contrast to the latter methods, approaches that are more specific have been introduced during the last few decades. Sample preparation involving protein or peptide enrichment via immunoaffinity[86-88] or molecular imprinted polymers (MIP)[89] have shown superior performance compared to generic techniques and are capable of enriching a target (or multiple) analyte(s) directly in a serum sample followed by a rapid and thorough matrix removal step. Additionally, immunoaffinity[90] and MIP[91] have been demonstrated in on-line LCMS systems making for automated and highly selective sample preparation. Other platforms have also been introduced such as chip-based MIP[92] and immunoaffinity[93], immunoaffinity pipette tips (MSIA)[94], beads[89, 95-97] and 96-well plates[98].

1.4.5 Protein modification for bottom-up protein determinations

In the bottom-up strategy, quantification (or discovery) is carried out on the indirect determination of the proteins by their respective peptides. Determination of peptides, rather than proteins is beneficial due to several reasons such as higher mass sensitivity[99], less complex spectra, and the fact that peptides are more compatible with MS due to their smaller size[100]. Intact proteins are also prone to solubility issues which could be troublesome to overcome without the use of non-MS-compatible reagents[99, 101]. However, in order to cut the protein into smaller peptides, the protein is most often hydrolyzed by enzymes (enzymatic digestion). A variety of enzymes could be used for the purpose and each has their respective cleavage sites. However, in order to access the cleavage sites the protein 3D configuration (tertiary structure) must be unfolded. This is mostly carried out by reducing and alkylating the disulfide bridges formed by cysteines in the protein backbone, and/or denaturation.

1.4.5.1 Reduction and alkylation

Disulfide bridges stabilize the proteins 3D structure and are thus, important for proteins' biological functions. However, in the proteomic workflows, these intra-molecular bonds could hinder enzymatic access to cleavage sites causing poor peptide generation. Breaking these bonds can be accomplished by chemical reduction. Different chemicals could be used for protein reduction e.g. tris-2(-carboxyethyl)-phosphine (TCEP)[102], however, dithiothreitol (DTT) remains as the most popular reducing agent[103]. DTT is a small-molecule redox reagent. DTT has a low redox potential and is thus, capable of reducing most biological disulfides[104, 105]. Furthermore, DTT is capable of reducing all disulfides in a protein (with low concentration of DTT) if exposed, which could be accomplished in the presence of denaturing surfactants such as sodium dodecyl sulfate or sodium deoxycholate. However, most surfactants are not MS compatible and are now seldom used in proteomic investigations. The in-depth chemistry of protein reduction by DTT is reviewed elsewhere[104], in short: proteins are left with free SH-groups after the reaction. To avoid re-formation of a tertiary structure after the reduction, alkylation is frequently reported. Alkylation is dominantly carried out by iodoacetamide (IAA) or iodoacetic acid (IAC). These reagents alkylate (transfers alkyl groups) by substitution reactions (SN_2) to the free SH-groups, preventing reformation of the tertiary structure exposing the cleavage sites for enzymatic hydrolysis[106].

1.4.5.2 Tryptic hydrolysis

For the enzymatic digestion, a variety of proteases could be utilized, however, trypsin is mostly applied due to its specificity (two cleavage sites) and low cost. Trypsin exclusively hydrolyzes the peptide bonds on the carboxy-terminal side of arginine (R) and lysine (K), with the exception of either followed by proline. The peak activity of trypsin is reported to be between pH 7.5 and 8.5[107] making this protease readily compatible with biological samples such as serum and whole blood. Additionally, digesting proteins with trypsin, MS fragmentation by collision induced dissociation (CID) could readily be used. CID dominantly produce y- and b-ions making for relatively simple MS spectra interpretation. The conventional tryptic procedure relies on the addition of lyophilized trypsin into the matrix and is left to react for 8-24 hours in order to achieve complete digestion of the sample proteins[108, 109].

1.4.6 Immunoaffinity for mass spectrometric applications

Antibodies have been shown to be readily immobilized to magnetic beads or other particle shapes suitable for either column packing[110], precipitation beads[111] or magnetic applications[112, 113]. Having antibodies attached to solid supports, moreover, immobilized beads (**Fig. 3**) make the immunoaffinity-based MS strategies highly flexible towards a variety of sample preparing methods. For quantitative protein determinations, magnetic beads have shown rapid sample preparation with high performance (linearity, LOD, LOQ, precision and reproducibility) for complex samples such as serum[86, 89, 97]. These beads, could directly be added to a biological samples prior to agitation and subsequent matrix removal, followed by enzymatic digestion. The enzymatic digestion has also been demonstrated to be efficient both while the protein is bound to the antibody (immobilized to beads) or after protein elution off the antibodies (on-beads and off-beads digestion, respectively)[114]. As previously mentioned, this form of immunoaffinity purification (or enrichment) has proven high flexibility. Not only because the beads could be added directly to most sample matrices, but also due to the vast majority of antibodies[86, 113-117] that could be immobilized with generic and non-extensive coupling procedures via the magnetic beads functional groups.

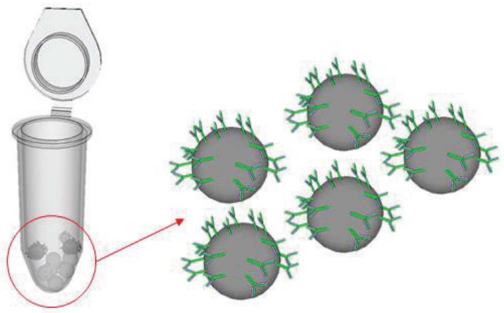

Figure 3 Magnetic beads immobilized with monoclonal antibodies for specific extraction of proteins or peptides in-solution. The illustration is not to scale

1.4.6.1 Immunocapture of intact proteins

For most protocols, intact protein capture (**Fig. 4A**) and enrichment are performed directly in the biological sample. This opens up for significant enrichment and ultimately the possibility of quantifying proteins in the low pg/mL range. Rossetti et al.[89] demonstrated that pro-Gastrin releasing peptide (proGRP) could be quantified at the reference limit (8 pM) by a 20 fold enrichment using immunoextraction of 1000µL serum (final volume 50 µL). The extraction of 1000 µL serum gave a LOD of 1 pM (RSD 7 %). For comparison, the same sampling method and considerations were applied with an initial sample volume of 50 µL serum. LOD was obtained at 14 pM (RSD 16 %). Immunocapture was further compared to protein precipitation (with cold ACN). Protein precipitation had 160 times higher LOD with an RSD of 57 %. From the investigation, Rossetti et al. demonstrated the power of protein capture by immunoaffinity and that enrichment is crucial for protein quantification (near the proposed reference limits). However, for other biomarkers enrichment has shown to be of less importance in order to obtain low detection and quantification limits if extracted by immunoafinity with subsequent MS determination. Zhang et al.[117] demonstrated a lower LOQ of 1 pM for free IP-10 (protein target for treatment of Crohn's disease) in monkey serum. This, from an initial 100 µL sample without enrichment during the sample preparation. However, even though the capture of a specific protein simplifies the sample matrix and opens up for clean extracts and sensitive analyses, the capture of large proteins still produces complex spectra. This, because larger proteins will turn into a high number of peptides (signature (protein-specific) or not) in addition to trypsin autolysis products (trypsin digest itself) as well as unspecific binding of unwanted proteins. The latter could still interfere with the analyte peptide of interest due to the abundance of trypsin applied to the sample. A solution to further simplify the matrix prior to analysis is to capture peptides rather than intact proteins by anti-protein or anti-peptide antibodies.

1.4.6.2 Immunocapture of peptides

Capturing, or enriching peptides (**Fig. 4B**) instead of intact proteins holds several benefits over the counterpart. Peptide samples are easier to handle because there is less chance for degradation, unfolding and solubility issues as seen for proteins[118]. Peptide capturing is also less likely to be inflicted by auto-antibodies since the proteolytic digestion is carried out prior to the affinity step and thus, the auto-antibodies will likely be degraded by the digestion[46]. Peptide capturing /enrichment is also expected to give cleaner sample extracts and therefore, potentially lower detection and quantification limits due to the lack of other tryptic peptides (from the target protein) and trypsin autolysis products present when performing intact protein capture (**Fig. 4A**)[118]. Furthermore, since the proteolytic step is carried out prior, the antibodies could be re-used since they are not susceptible to trypsin in low concentrations (or other proteases) and therefore costs related to the assays could be reduced. Nevertheless, there has been limited alternatives to perform peptide capturing during the last few decades. The first commercially available antibodies specifically targeting peptides were introduced in 2011 by SISCAPA Assay Technologies, with the nomenclature Stable isotope standards and capture by anti-peptide antibodies (SISCAPA^{TM}). This introduction followed the proof-of-principle study by Anderson et al.[119] in 2004. SISCAPA has over the last decade proven performance[88, 120-122], however, the assay remains expensive and developments for more biomarkers need to be made[123] (by 2019

SISCAPA^{TM} offers anti-peptide antibodies for 45 different target proteins in their catalog). As an alternative to SISCAPA, Schoenherr et al.[124] demonstrated that commercially available antibodies targeting intact proteins could be used for peptide enrichment. This theory was investigated by testing of 105 commercial monoclonal antibodies (mAb) targeting proteins associated with DNA damage response network. Levernæs et al.[86] later showed a similar approach. The latter works showcased the applicability of antibodies targeting intact protein capturing tryptic epitope peptides (from the same protein). The study by Levernæs et al. investigated three different antibodies targeting pro-Gastrin releasing peptide (ProGRP). Both studies mentioned showed that it was readily achievable. However, a criterion was shown to that the epitope peptide needed to be linear and be within cleavage sites of trypsin, and thus might not work with the majority of proteins. Also, since the enzymatic digestion is performed prior to the affinity capture, excess trypsin (e.g. digest the antibodies), reducing and alkylating reagents is troublesome since these could modify the antibody and ultimately hamper the immunoaffinity. Therefore, immobilized trypsin (alternatively addition of a trypsin inhibitor, granted the trypsin concentration is low) is advisable for peptide capture by anti-protein antibody workflows.

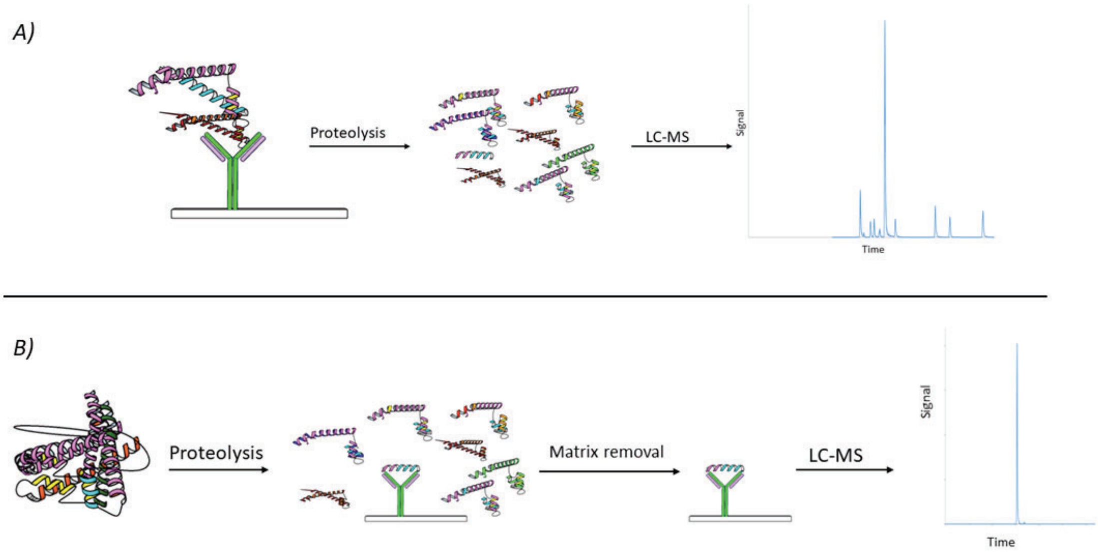

***Figure 4** Immunocapture mass spectrometry where A) intact protein is captured prior to proteolysis and B) signature epitope peptide is captured after proteolysis and analysis by LCMS is carried out after matrix removal*

1.4.7 Immobilized trypsin for accelerated proteolysis

As previously mentioned, enzymatic digestion is a key factor in the bottom-up methodology whether it is in targeted or non-targeted analyses. However, tryptic protein digestion is rather slow and most generic protocols report a time consumption for complete protein digestion in between 8-24 hours *in-solution*. Furthermore, tryptic digestion is shown to be a first-order kinetic reaction[125] and digestion of proteins of low concentration (e.g. biomarkers) is slow in the presence of high abundant proteins such as albumin. This is one of the reasons why tryptic hydrolysis often is carried out for an extensive amount of time. However, as previously mentioned, trypsin is prone to autolysis (trypsin self-hydrolysis, a second-order kinetic reaction[126]) which, in turn, generates trypsin products (often in abundance) and other artifacts[127] that could cause ion suppression due to co-elution with sample components. Therefore, increasing the trypsin concentration in the sample to shorten the reaction time could cause more harm than good. To minimize autolysis, as well as sustaining the enzymes catalytic activity, chemical fixation by covalent attachment of trypsin to solid supports have been proposed[128]. Other than reducing excessive trypsin self-hydrolysis, other benefits of covalent immobilization have been suggested such as a higher enzyme-to-protein ration, re-usability, and rapid reaction times[129, 130]. Additionally, trypsin fixated to a solid support, opens up for the possibility of peptide-capture by immunoaffinity with pre-affinity protein hydrolysis. The latter, because trypsin could be easily removed prior to addition of antibodies (to hinder trypsin cleaving the antibodies). Trypsin immobilization has been reported to many different platforms such as chromatographic columns[129, 131], micro-chips[132, 133], membranes[134, 135], and beads[136].

1.4.8 Antibody coupling to solid supports

Antibodies have been coupled to numerous solid supports during the last decades. Thus, different ways of attaching antibodies have been described in the literature. The main approach has been covalent attachment via leaving groups (such as sulfonyl-groups), amino groups or aldehyde moieties[137]. Chemical immobilization (covalent coupling) holds a stable and mechanically strong binding (between the antibody and substrate), suited for applications such as beads and chromatographic columns where large external forces are introduced. However, covalently coupled antibodies face the possibility of being randomly oriented (**Fig. 5A**) resulting in low availability for antibody-antigen interaction. This is especially true for immobilization via amino groups[137]. To ensure the orientation of covalently bound antibodies (**Fig. 5B**). Conradie et al.[138] demonstrated the applicability of antibody exposure to acidic conditions during coupling. Pei et al.[139] also proved the importance of pH during the immobilization of antibodies to ensure correct orientation. In the latter study, it was demonstrated that a successful immobilization was highly dependent on the surface pKa, antibody p*I*, and pH of the immobilization buffer. For most covalent coupling procedures, several solutions need to be introduced and although they might not be complex procedures, to perform they remain time-consuming. Thus, for simple analytical platforms such as devices with colorimetric detection, many have attached the antibodies via a simple adsorption (drip and dry)[140, 141]. Adsorption is a by far the easiest method of attachment of antibodies to a solid surface and is mostly caused by hydrophilic or hydrophobic interactions towards the substrate. These substrates are frequently reported as cellulose, nylon or metals. However, with antibody fixation by adsorption, antibody detachment, as well as incorrect and random orientation (**Fig. 5C**) remains an issue[137]. Butler et al.[142] demonstrated the latter by adsorption with optimal conditions yielded <10 % active/available antibodies in their ELISA assay. Additionally, antibody orientation has been shown to be highly dependent on the adsorbing surface composition. This has been characterized by different surfaces covered with calixarene[143, 144]. However, most of the sampling surfaces included with antibodies through adsorption uses less complex detection techniques e.g. fluorescence[143], blotting[144] or colorimetric[140] and are not widely reported in combination with MS determinations.

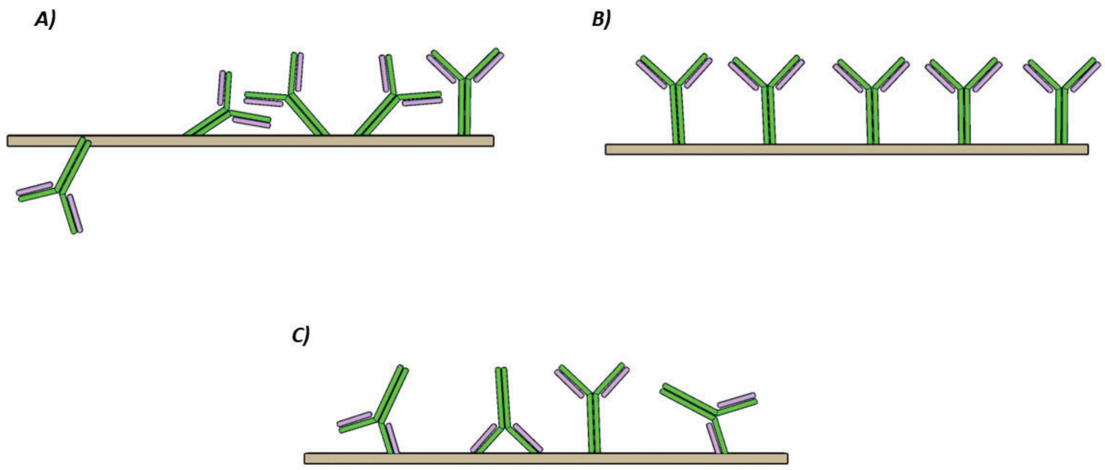

Figure 5 Coupling strategies of antibodies to a solid support. A) adsorption (drip and dry), B) chemical immobilization of antibodies with configuration and C) chemical immobilization without configuration of the mAb

1.5 Paper-based sampling of biological matrices

Paper has played an important role in analytical chemistry with applications such as paper-chromatography, electrophoresis, filtration and sample collection. During the last few decades, simple paper-based analytical devices have emerged, with the recent interest in the point-of-care (POC) strategy. These devices are often easily fabricated on commercially available filter paper and are combined with rapid reporting such as coloration or color intensity. Paper-based analytical devices are now more than ever considered to be an attractive alternative for miniaturized and portable analytical determinations due to excellent wicking capabilities (solvent transportation) due to its porous structure of fibers, low cost and, availability[145]. Paper is also bio-degradable and thus opens up for noninvasive disposal. Several studies have shown that cellulose (base component of filter paper) has a high degree of biocompatibility[145-148] and could readily be functionalized with different reagents such as anti-microbial agents[149], antibodies[140] and proteins[150] due to the cellulose hydroxyl groups. For bioanalytical applications, paper-based devices are often fabricated with means of channeling to guide the sample flow (or wicking) in a certain direction through the paper. These channels could be obtained by several methods such as photolithography[151], silanization (masking)[152], laser treatment[153], or wax printing[154]. Common for all the latter methods is to create hydrophobic barriers/channels to prevent sample spread and guide a hydrophilic sample though the device.

1.5.1 Wax printing

Wax printing may be the simplest method of fabricating microfluidic channels in filter paper since the only components involved are filter paper, a printer, wax, and a heat source to melt the wax into the paper structure. This technology remains fairly inexpensive and the limitations regarding channeling design are few. Fabrication could also be accomplished within a few minutes[154]. The wax itself has been demonstrated to be compatible with aqueous solvents of various pH, however, are not compatible with organic solvents such as methanol or acetonitrile. The wax will also be unaffected by strong acids or bases, though, the paper itself may dissolve from these reagents. Wax printing has widely been demonstrated with different designs and applications during the last few years such as multi-zone plates[155], lateral-flow devices[151], 3D testing devices[156], colorimetric assays[152], and immunoassays[157].

1.5.2 Paper-based assays

Paper-based assays has not only gained popularity within the research community but also been commercialized for applications such as glucose monitoring (urine), pregnancy tests, and E.coli.[158] The latter tests were introduced early to the public market with lateral-flow dip sticks for glucose monitoring in 1960, and later pregnancy test for hCG in 1977[158]. Now, many of the former, in addition to new microfluidic tests, remain on the market, however, with more clever designs catered by plastic housings, in-device sample cleanup (such as membranes) and branding. An example is a lateral flow assay by Merck Millipore Corporation where the device is incorporated with a sample port, conjugate pad, test zone, control line, an absorbent pad, all integrated in a plastic housing separating the in-device chemistry form the ambient surroundings[158]. In addition to single analyte tests such as the lateral–flow dipstick for hCG, multiplexed tests have also seen commercialized e.g. RAID^{TM} for the determination of biological threat agents[159]. Despite the commercialization of specific tests, microfluidic test devices have sustained the interest research community for the development of new applications. This, mainly due to the low fabrication costs, simplicity, and wide application areas.

1.5.3 Paper-based ELISA

As an alternative to commercially available ELISA kits, paper-based ELISA (pELISA) has frequently been reported in the literature as less expensive alternatives with sensitive detection[160, 161]. Both direct-[162], indirect[160, 163], and sandwich-ELISA[140, 164] configurations have been described. Common for these assays is the use of hydrophobic barriers applied to commercially available filter paper and adsorption of antibodies to non-treated cellulose filter paper. Additionally, modification of filter paper has been carried out for covalent antibody immobilization[164]. Covalent immobilization of antibodies has in similar fashion to immobilization of other reactants (enzymes) been demonstrated to be superior with regards to assay sensitivity[164, 165]. To reduce non-specific binding of proteins to the paper substrate and thus further increase sensitivity, many utilize proteins like bovine serum albumin (BSA) as a blocking agent[160, 164]. Zhu et al.[164] demonstrated a pELISA (with covalent immobilization of antibodies to modified cellulose) with similar results to a commercially available ELISA kit with adequate recoveries of α-fetoprotein in serum samples. The assay was demonstrated with a linear range from 0.1 ng/mL to 11.2 ng/mL and a detection limit of 0.04 ng/mL. A similar comparison to a commercially available ELISA kit was demonstrated by Cheng et al.[162], however, with antibodies adsorbed to non-treated filter paper. In the latter study, sensitivity was reported to 54 fmol and 4 fmol for pELISA and ELISA, respectively. Even though the analytical performance was slightly worse for the pELISA, the cost related to the fabrication and analysis as well as sample preparation time was significantly lower. In a future perspective, low cost analytical devices such as pELISA holds great potential for applications related to POC. However, they still suffer from the same drawbacks as conventional ELISA. Furthermore, paperbased analytical devices integrated with antibodies has not widely been reported with MS detection. If so, the paperbased methodology could greatly improve its specificity, sensitivity, and drawbacks such as false reporting, however, a sacrifice will be the simplicity of the methodology as MS instrumentation is, as previously discussed, demanding.

1.5.4 Dried matrix spots

In contrast to the latter discussed paper-based methodologies, dried matrix spots (DMS) has frequently been combined with LCMS[166]. DMS is a sampling technique introduced in the 1960s were a biological sample is spotted and subsequently dried on a paper sampling card[167]. The sample is further extracted out from the sampling material by aqueous solvent, prepared and analyzed. The benefits of collecting biological samples this way are many such as, less invasive sampling, sample stability when dried, simple sample collection and lowered HSE risk since many biomolecules are inactive when dried[168]. Sample collection is performed by either a finger or heel prick and sampling volumes typically range from 10-50 µL[169]. With regard to the low sampling volumes, this technique has been appreciated for newborn screening[170, 171] and also clinical trials in order to reduce invasive sampling of test rodents. However, the methodology suffers from some distinct drawbacks. One issue is the variability in sample viscosity often related to whole blood (dried blood spots, DBS) and hematocrit values. This is predominantly an issue in clinical trials since only a fixed area (3.00 mm – 6.00 mm diameter) of the sampling card is utilized for the analysis. Thus, one could risk excluding parts of the whole sample during the sample preparation, particularly if the target analyte is not homogeneously distributed within the dried sample spot. However, this issue could be avoided if hydrophobic barriers were implied in the sampling card e.g. wax printing (**Fig. 6**). Furthermore, given the low sampling volumes, the enrichment of target analyte remains difficult and low quantification limits could thus be difficult to obtain. During the course of DMS, low weight molecules such as drugs[169, 172, 173], or trace elements[170, 174] have dominantly been the target analytes and the use of this sampling method has predominantly been for pharmacokinetic and toxicology applications[175, 176]. However, recent efforts do suggest the applicability towards larger biomolecules[177, 178]. Some investigations has also utilized DBS and DMS for proteomic investigations as concept studies[87, 179-182]. Nevertheless, DMS and DBS are not extensively reported for analysis of low abundance biomarkers.

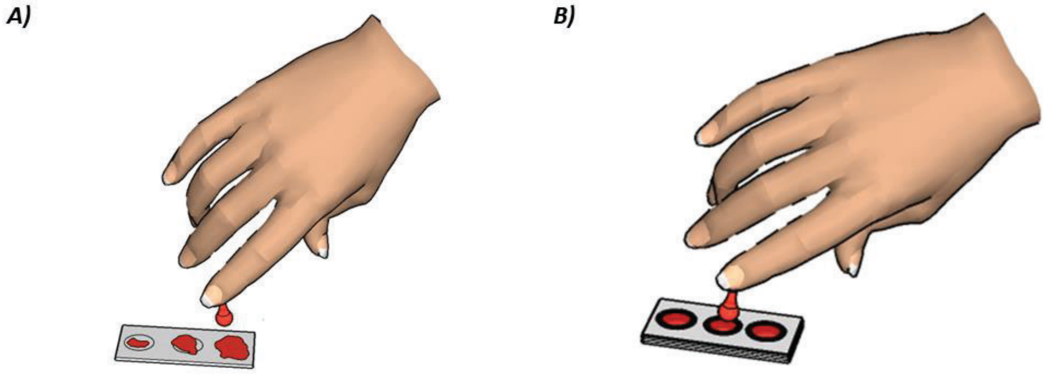

Figure 6 Sample application of whole blood from a finger prick directly to A) non-treated paper such as DBS cards and B) wax printed sampling device with hydrophilic barriers

1.5.5 Dried matrix spots in clinical protein analysis

Most of the clinical protein determinations are by far carried out by liquid specimens/samples and the use of DMS is limited[183]. This may resolve from the fact that many of the clinical assays used today, requires double the sample volume of what DBS could deliver. Additionally, since the sampling volume is low, the enrichment of target proteins could be troublesome with regard to simplicity and low-cost[183]. Furthermore, the correlation between plasma-concentration and DBS-plasma concentration has been demonstrated to be lacking for some analytes[184], which is essential for clinical assays. Additionally, the absence of DMS in clinical protein determination may not solely resolve from the sampling methodology, but rather the general time-consuming sample preparation needed for protein determinations in combination with the implementation of MS (as discussed earlier). If further technological advancements of the protein-analytical strategy are developed, DMS or paper-based sampling combined with MS might see a future for clinical protein applications.

2 Aim of the study

The aim of the study was to investigate the use of functionalized paper-based materials, to simplify the sampling methodology for protein determinations by LCMS. The focus is on the POC strategy allowing sampling outside the laboratory with subsequent, and non-laborious sample extraction prior to analysis. The sampling concept was designed around *in-device protein hydrolysis* (**paper I – III**) as well as *in-device immunocapture* (**paper IV-V**) of low abundant biomarkers in serum and whole blood. All this to simplify the analytical workflow upon arrival of the sample in the laboratory. The sampling strategy was evaluated with non-targeted (**paper I and II**) and targeted analyses (**paper III-V**). The concepts performance was assessed based on qualitative proteomic performance (Identified protein groups, peptides, and missed cleavage sites) and targeted quantitative performance (linearity, precision, accuracy, and repeatability).

The main objectives were:

- Prove the applicability of *in-device protein hydrolysis* and *in-device immunoaffinity* for complex matrices
- Solving fundamental challenges related to paper-based sampling with integrated sample preparing steps
- Performance comparisons to conventional DMS (among this DBS) in non-targeted protein analysis and targeted analyses of low abundant biomarkers

3 Main results and discussion

*This thesis is based on five papers from proof-of-concept to analysis of patient samples. The results and discussion consist of four main parts where **section 3.1** introduces the model proteins used for targeted demonstrations. **Section 3.2** elaborates on the architectural considerations for concept development. **Section 3.3** covers the performance of instant in-device proteolysis, both in discovery and targeted analysis. **Section 3.4** and **section 3.5** discusses the performance in targeted analysis with in-device immunocapture.*

3.1 Model proteins for the targeted approaches

To demonstrate the applicability to realistic settings, two low abundant biomarkers (proGRP and hCG) were chosen as model proteins for the targeted analyses. Both proteins have been investigated extensively in our group and by others, and validated immunoassays and LCMS methods are readily available with different sample preparation methodologies. Even though the proteins chosen for the demonstrations are in fact well-known biomarkers, it must be stressed that all the investigations related to this work are concept developments and complete method validation was not within the scope of this thesis.

3.1.1 Pro-Gastrin releasing peptide

Pro-Gastrin releasing peptide (ProGRP) has been extensively studied over the past few decades as a serum marker for small-cell lung cancer[185-187]. ProGRP is a neuropeptide and has three isoforms at the mRNA level that are linked to cancer development. Isoform 1 and 3 are more abundant (in serum samples) and thus, mostly used for quantification by LCMS methodologies. For determination, without isoform distinction, the signature peptide NLLGLIEAK (**Fig. 7**) is common for all isoforms, whereas the isotopes could be distinguished or multiplexed by measuring other isotope-specific signature peptide sequences such as LSAPGSQR (isoform 1) or DLVDSLLQVLNVK (isoform 3)[188]. A disease state of small cell lung cancer (SCLC) is regarded with proGRP concentration >100 pg/mL in serum[185, 189]. However, the proposed cut-off value (upper normal limit of circulation) for ProGRP in serum has been established around 50-60 pg/mL[186, 190, 191] and therefore a sample preparing method providing clean extracts prior to MS detection is crucial due to the low abundance in serum. The protein was chosen as a model due to elaborate work on the protein in our group[89, 90, 97, 192, 193] and the analytical simplicities due to the lack of disulfide bridges. The protein also introduces some significant challenges by less favorable digestion in complex matrices (unpublished observations) compared to larger proteins with more cleavage sites and thus, a high performing tryptic digestion was needed for determinations. The model protein was in the presented work used for instant proteolysis during on-paper sampling of serum combined with MS detection. By initiating the sample preparation with protein digestion the conventional sample preparation (staring with immunoaffinity of intact protein), needs to be reversed and thus alternative enrichment methods prior to LCMS injection are called for. Levernæs et al.[86] recently demonstrated enrichment of proteolytic epitope extraction of proGRP by using mAb targeting intact protein for post proteolytic immunocapture. This was possible since the enriched proteolytic epitope (ALGNQQPSWDSEDSSNFK, signature peptide for all isoforms) was a linear part of the intact protein (**Fig. 7**). Other means of peptide capture (or enrichments) could also be possible post proteolysis such as SISCAPA[119] or MIP as demonstrated for NLLGLIEAK[91, 194]. For the demonstration of the proteolytic epitope extraction, the mAb M18 (designed for intact protein capture) was chosen for the immunoaffinity antibody (**paper III**), based on the higher performance with respect to MIPs and the lower cost per analysis compared to SISCAPA. The results are further discussed in *section 3.3.3.*

ProGRP isoform 1

VPLPAGGGTVLTKMYPRGNHWAVGHLMGKKSTGESSSVSERGSLKQQLREYIRWEEAARNLLGLIEAK

ENRNHQPPQPKALGNQQPSWDSEDSSNFKDVGSKGKVGRLSAPGSQREGRNPQLNQQ

Figure 7 Amino acid sequence of proGRP isoform 1. The signature peptide shared by all isoforms is color-coded red and the proteotypic epitope peptide targeted by the mAb M18 is color-coded green. The green colored sequence is also common for all isoforms

3.1.2 Human chorionic gonadotropin

The second model protein used in the presented work is human chorionic gonadotropin (hCG). hCG is measured for tumor development for malignant trophoblastic disease and testicular and ovarian germ cell cancer[195-197]. hCG is also on the World Anti-Doping Agency (WADA) list of prohibited substances for male athletes (related to the use of anabolic steroids)[198]. Similar to proGRP, this protein has been extensively studied in our group[115, 199, 200] and there are several published papers describing sample preparing methods and quantification of hCG sampled on paper by DBS[199] and DMS[201]. Unlike proGRP, hCG contains several cysteines and is mostly present as a dimer (stabilized by three disulfide bonds), meaning that hCG is composed of two units (α and ß) were the α-unit is common for all glycoproteins and is not specific to hCG[202]. The ß-unit is, however, specific and could be used to quantify the total hCG content in serum and whole blood. In previous work from our group, the mAb E27 has dominantly been used for immuno-extractions prior to LCMS determinations. Therefore E27 was further used to explore on-paper instant immunocapture in this work, as discussed further in *section 3.4* and *section 3.5*. This antibody binds to a non-linear part of the ß-unit (**Fig. 8**) resulting in the need to reduce, alkylate and perform tryptic digestion after immunocapture, in contrary to what was performed for proGRP. For all quantifications and method developments regarding hCG, the signature peptide sequence for the ß-unit VLQGVLPALPQVVCNYR was measured (**Fig. 8**)

α-chain

APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQKNVTSESTC

C VAKSYNRVTVMGGFKVENHTACHCSTCYYHKS

ß-chain

SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNYR

DVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSS

S SKAPPPSLPSPSRLPGPSDTPILPQ

Figure 8** **Amino acid sequence for the hCG alpha and beta chain. The signature peptide sequence for the beta chain is color-coded green. The non-linear epitope recognized by the mAb E27 is color-coded red

3.2 Design of concept – *an initial assessment*

*In the following section, architectural and fundamental challenges are discussed regarding the development of the sampling device capable of delivering instant in-device immunocapture and instant in-device proteolysis. It must be stressed that these two sampling concepts do not lay in the same device and are used in ordinary and reversed sample preparation for bottom-up protein determinations, respectively. Ordinary sample preparation is here described as immunocapture of intact protein prior to necessary sample preparation e.g. proteolysis. Reversed sample preparation is here described as proteolysis prior to immunocapture of peptides. However, even though two different sampling strategies are presented, the fundamental chemistry and architectural consideration of the sampling device prior to immobilization remain the same. The presentation in this chapter is not chronological with respect to section **3.3** and **3.4**.*

3.2.1 On-paper protein interactions

Reaction time is often a necessity that has to be taken into consideration in most parts of sample preparation, whether it is interaction with a sorbent, antibody or protease. Thus, lies the challenge to incorporate different sample preparing strategies into the on-paper sampling methodology where a sample is rapidly drawn into a paper structure and subsequently dried. In a conventional paper-based sampling approach e.g. DBS or DMS, the sample will be absorbed into the paper structure and dried relatively fast. Also, since there are no wicking restrictions preventing sample spread, the degree of wetness throughout the sampling spot will be variable. This means that some parts of the applied sample will dry faster than others (especially in the middle of the spot). This, in turn, will results in variable reaction-time throughout the sample spot with minimal reaction time along the edges due to rapid evaporation of solvent, if reagents such as trypsin is directly added to the sampling paper (i.e. applying trypsin solution to the paper prior to the sample). Therefore a hydrophobic structure that confined the sample and kept the sample from wicking was created by wax printing. Circular patterns were printed and melted down in conventional laboratory grade filter paper. This resulted in sample encasement within the wax print, where the sample would lie on top as well as hanging underneath the sampling spot surrounded by wax (**Fig. 9A**).

Instant on-paper proteolysis

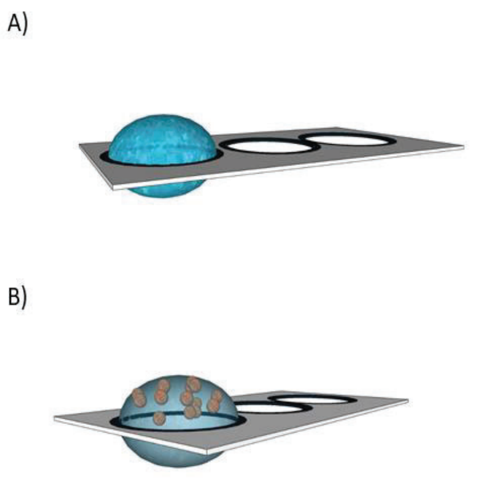

Figure 9 Filter paper with a circular wax print enclosing a drop of water (A) and wax printed filter paper enclosing a sample with applied polymer beads on top of the sampling spot (B). Illustration is not to scale

As mentioned earlier, the bottleneck in the bottom-up strategy is undoubtedly the proteolysis. Protein digestion with trypsin is mostly carried out over-night and relies on a system containing aqueous solvent with correct pH. Acquiring a successful proteolysis directly on the sampling paper was, therefore challenging in initial efforts with conventional DBS/DMS sampling cards. This, because both the applied trypsin and sample would spread randomly and evaporate fast (no wicking restrictions were used). In addition, the use of excessive amounts of trypsin directly applied to the sampling cards to accelerate the reaction, only suppressed the MS signal by autolysis products and did not yield sufficient digestion. Therefore, wax printing was incorporated with conventional filter paper to prevent sample wicking and prolong the drying time (reaction time). In order to avoid using excessive amounts of lyophilized trypsin powder (susceptible to autolysis) trypsin immobilized to a solid support to increase the enzyme-to-protein ratio, while still minimizing the trypsin autolysis products had to be utilized. As a consequence, in **paper I** on-paper proteolysis was assessed by using wax printing (6.0 mm ID per circle) with deployed polymer beads immobilized with trypsin in the sampling area (within the wax print) (**Fig. 9B**). The trypsin beads as introduced by Freije et al.[136] had shown to improve digestion efficiency and claimed capable of sufficiently digest a complex sample within a few hours *in-solution*[203]. To investigate whether or not these beads could be used to digest a protein sample directly on the sampling paper, the beads were applied to the sampling spot (and let dry off the application solution) prior to sampling with a protein standard or spiked whole blood. The combination of wax printing and immobilized beads was carried out to explore if proteolysis directly on the sampling material is possible allowing instant and viable protein digestion. This, in turn, opened up for extraction of peptides rather than intact proteins which could increase extraction recoveries given that peptides are smaller in size and the “stickiness” to cellulose could potentially be reduced compared to intact proteins. Additionally, utilizing the wax printed circles the sample was confined to perfectly fit a conventional DBS/DMS punching tool in such a way that the entire sample was going forward in the sample preparing process. The latter was considered important since variations in blood hematocrit levels lead to different viscosities i.e. different spread patterns if sampled on conventional paper or DBS/DMS sampling cards. This variability in viscosity, and thus the sample spread, is a potential problem in conventional paper-based sampling since parts of the sample could be excluded when only a fixed area of the sampling card is punched out and further processed. Wax printing solved this issue. However, from the initial experiments in **paper I**, the sample did create a droplet formation on both sides of the sampling area (the over-side and the under-side). The under-side droplet represents half of the sample assuming a homogeneous protein distribution in the sample droplet and the proteins' ability to penetrate and travel through the paper structure. Furthermore, since the trypsin beads lay on the top surface, the remaining underside would not go through with enzyme-protein interactions. This was considered troublesome. Additionally, during the testing of the concept, it was apparent that having the loose beads on top the sampling paper did create issues regarding handling. Nevertheless, the initial concept was further tested and compared to generic DBS with over-night proteolysis and proved promising results. These will be further discussed in *section 3.3*.

3.2.2 Polymeric supports for covalent immobilization of trypsin

From the introduction of the *on-paper* proteolysis concept in **paper I**, it was evident that relying on polymeric beads to perform the tryptic digestion would account for difficulties if the concept were to be used in a POC setting where transportation is needed. In addition, since more interaction between the enzyme and proteins was desirable (in such a way that the interaction would occur in the entire sampling spot, rather solely on top as previously discussed) a polymeric backbone was introduced in **paper II** for covalent attachment of trypsin directly to the sampling spot. The polymer was coated around each paper fiber to hold anchoring sites for direct immobilization of enzymes and therefore no loose parts (e.g. beads) were involved in the device, making performance (in terms of proteolysis capabilities) unaffected by handling. The polymer used, 2-hydroxyethyl methacrylate-co-2-vinyl-4,4-dimethyl azlactone (pHEMA-VDM), is hydrophilic enough in such a way that biological samples could be wicked into the sampling device and not being repelled on the surface. Compared to the previous use of beads, the pHEMA-VDM layer was believed to have a higher capacity with regards to immobilized enzymes and would serve as a proteolytic platform through the entire device instead of solely relying on proteins being on top of the sampling paper were the beads was deployed. The proteolytic process would also endure the entire drying process and not only when the sample lied as a droplet within the wax printed area. The testing of the polymeric sampling devices was carried out in a similar fashion (non-targeted analysis with a strict reporting rate during the data analysis) in **paper II** to what was done in **paper I**, however, without the wax print (i.e. the volume holding capacity was solely due to absorption of the polymeric sampling material). The pHEMA-VDM layer was further used in **paper II** – **paper V**.

3.2.2.1 Fabrication of the pHEMA-VDM polymer layer

Preparation of this polymer layer was carried out on commercially available filter paper (Whatman® grade 1, 4, 41, 114) and DMPK-C sampling cards. The preparation of the polymer was carried out similarly to what has been done by others[131]. The preparation step of the polymeric devices was started by punching out circles (6 mm ID) and further using a silanization reagent (3-(trimethoxysilyl)propyl methacrylate, TMSPM) to form anchor sites for the subsequent co-polymerization of HEMA and VDM. This particular polymer proved to suit our concept since it has been used and proven for a variety of enzymatic platforms[204-206]. A schematic illustration of the silanization and polymerization steps is presented in **Fig. 10**.

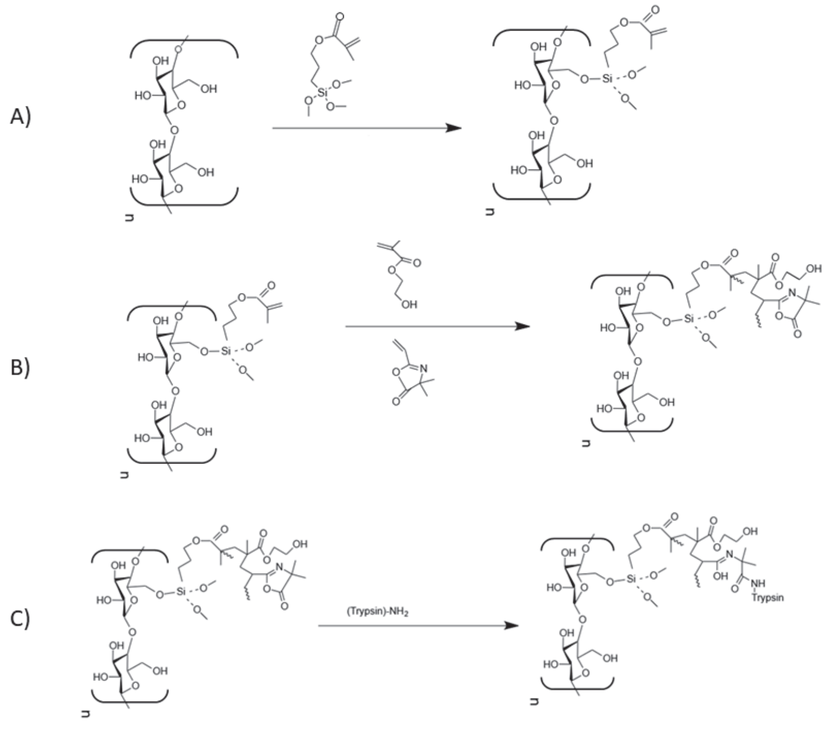

Figure 10 Reaction scheme of fabrication of the polymer layer where A) is silanization of filter paper, B) is polymerization of silanized filter paper and C) immobilization of trypsin to polymerized pHEMA-VDM filter paper

Initially, the idea of having a polymer layer to anchor trypsin seemed readily achievable since other research groups had already silanized filter paper by immersion[207-209] and vapor phase deposition[209, 210]. However, most other publications had solely silanized the paper and not gone through with further polymerization. Prior to the final preparation procedure (later elaborated), a variety of methods described in the literature for modifying cellulose was tested. Vapor-phase deposition did not turn out successfully for TMSPM and neither did immersion with subsequent drying. Also, using paper masks both wet and dry as described elsewhere[207] did not provide the modification needed. This, because the subsequent polymerization step would coat the masking filter papers (wet and dried) and not the target filter paper in between the sandwich configuration. With these introductory methods, coating/polymerizing the sampling filter paper was challenging. Especially, the silanizing reagent (TMSPM) that has been shown to give poor reaction yield if processed in the presence of water[211]. This could explain why our preliminary polymerization procedures failed. If no TMSPM anchoring sites were formed prior to the subsequent copolymerization, the polymer would have a hard time coating the fiber specimens. All preliminary attempts were open systems wetted (with water) or dried, but exposed to humidity. Other means were also investigated where paper was immersed in the polymerization solution, sealed and subsequently baked in a vessel (various designs). From the immersion attempts, it was difficult to acquire a thin coating around the fibers without over-polymerizing the sampling paper (creating a non-pours layer covering the paper fibers resulting in a non-porous device). Furthermore, polymerizing the pre-punched paper disc directly in a container with polymerization solvent formed a solid block (these attempts were not published). Thus lied one of the main issues of preparing the polymer layer, acquiring a thin polymer film around the fibers without intra fiber specimens. This problem was solved by fabricating an airtight vessel by removing the center (8 mm) of GC injection port septum. The removed center was in a diameter slightly bigger than the sampling paper (6 mm) to hinder contact between the rubber and the paper. The rubber septum was further pierced with glass capillaries and sealed between two glass plates (**Fig. 11**). The fabrication vessel was therefore airtight and polymerization process could be carried out without contact with the rubber septum and the glass plates. This fabrication method was introduced in **paper II** and further used in **paper III** - **paper V**.

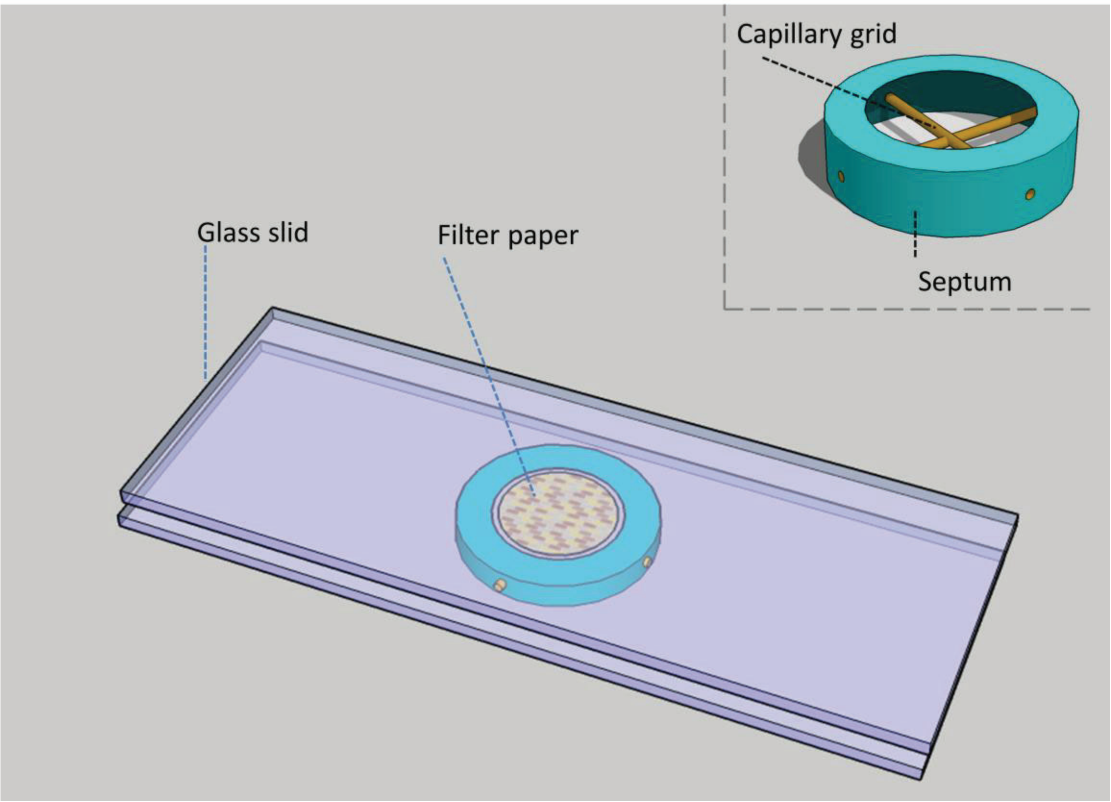

Figure 11 In-house made heating container for silanization and polymerization. Reproduced with permission from Fig. 1 paper II. Copyright © 2018, Rights managed by the Royal Society of Chemistry

3.2.2.2 Impact of filter paper morphology and immobilization conditions - for in-device proteolysis

In **paper I,** Whatman® grade 1 filter paper was used for all the investigations alongside DMPK-C for conventional DBS analyses. Since the filter paper acted as rebars for the polymer layer, the initial structure of the filter paper with respect to thickness, density, and porosity. The latter parameters defined not only the sample capacity but also how many anchor sites (for further immobilization of enzymes and antibodies) that could be formed. Therefore, in **paper II** we explored the significance of filter paper structure by polymerizing different types of filter paper (cellulose material and manufacturer was the same). All the filter paper types investigated (**Table 1**) were coated and immobilized with trypsin (at the same time and under the same conditions) prior to subsequent sampling with freshly collected whole blood. The analyses was performed with DDA non-targeted analyses. The performance characterization was based on the number of identified protein groups per filter paper type. From the investigation, filter paper weight (density) in combination with bigger pores proved to be the most prominent feature to increase the performance, where the denser and more porous filter papers (grade 4 and 41) had significantly better performance compared to their counterpart. This could resolve from thicker cellulose fibers with more clearance within the substrate and therefore leading to a more macro-porous end-product. This, in turn, was believed to deliver higher availability for trypsin to interact with the immobilization sites and later the sample. In addition to the filter papers, 6 mm discs of DMPK-C was also investigated alongside the others. DMPK-C proved the highest performance of all with respect to identified protein groups, however, not significantly (two-sided t-test, p>>0.05) more than grade 4.

Table 1 Commercially available laboratory filter paper tested for the fabrication of smart blood spots. All filter papers are branded under GE Healthcare Whatman®. All specifications are collected from the manufacturer

	Grade 1	**Grade 4**	**Grade 41**	**Grade 114**
Thickness	180 µm	205 µm	220 µm	190 µm
Pore size	11 µm	20-25 µm	20-25 µm	25 µm
Weight	87 g/m^2	92 g/m^2	85 g/m^2	77 g/m^2
Filtration speed	150 sec/100 mL	37 sec/100 mL	54 sec/100 mL	38 sec/100 mL

Trypsin immobilization on pHEMA-VDM coated filter paper

The immobilization procedure of trypsin on the pHEMA-VDM samplers used in **paper II** was predominantly adapted from what has been described for NHS activated beads by Freije et al.[136]. However, 20 mg/mL trypsin as used in the latter procedure seemed excessive (for immobilizing a single spot) and therefore the amount of trypsin was investigated regarding immobilization conditions on the two best performing filter paper types (grade 4 and DMPK-C). The best performance was obtained at 1.25 mg/mL trypsin per spot. The immobilization temperature and reaction time was also investigated and the peak performance was obtained at room temperature (21°C) and 1.25 mg/mL trypsin per sampling disc. At optimal conditions, no significant differences on digestion efficiency (identified protein groups) for the two paper substrates was observed. However, DMPK-C was chosen for the later studies in **paper III** due to a higher sample capacity.

3.2.2.3 pHEMA-TsCl polymer layer for in-device immobilization of antibodies

Immobilization of antibodies directly to the sampling spot holds many benefits compared to sampling on conventional paper material and subsequently performing in-solution immunoaffinity extraction on a single (or multiple) protein(s). Even though the previously introduced polymer with anchoring sites has been shown to successfully immobilize a variety of proteins via the ring-opening of azlactone [212-214], polymeric surfaces co-synthesized with toluene-sulfonyl chloride (Ts-Cl) may hold a bigger portion in the literature for immobilization of antibodies[113, 215-218]. Tosyl chloride is an excellent leaving group (nucleophilic substitution reaction) and immunoaffinity beads with toluene-sulfonyl groups have routinely been immobilized in our group for the past decades[89, 97, 188, 192, 216, 217]. Therefore, in **paper IV** it was of interest to synthesize Ts-Cl directly to the sampling material for direct immobilization of antibodies. In preliminary efforts, tosylation of cellulose fibers (non-coated filter paper) was tested according to procedures by others[219, 220], however with limited success (these attempts were not published). This was mainly due to the dissolution of the pre-punched cellulose disc in such a way that the sampling spot was not intact after the synthesis, leaving only tosylated cellulose fiber sediments. This was a result of the processes being carried out in basic aqueous solvent (NaOH) with high pH. The dissolution of cellulose in aqueous solutions has also been described and elaborated by others (for different purposes)[221]. From the latter observation, a more rugged and mechanical stable material to perform the synthesis on was needed. Therefore, a homopolymeric layer of pHEMA was created to capsulate the cellulose fibers. This polymer was chosen since HEMA contains several -OH groups (for the synthesis of TsCl) and has been shown to work well with protein samples in the previous work. Tosylation of the pHEMA layer was initially investigated in NaOH to maintain a low HSE risk during fabrication, however, with limited results. The final synthesis procedure was carried out in pyridine (**Fig. 12**). Pyridine is demonstrated to be an excellent organic solvent for the tosyl synthesis since it neutralizes the HCl formed under the coupling, but also contribute to swelling of the polymer layer in such way that the polymer opens up and becomes more exposed for modification. The swelling of the polymer was visually apparent as the polymeric discs approximately doubled in thickness and turned transparent while in contact with the solvent. For the tosylation procedure, a ratio of 1:4 (TsCl: pyridine) was used in compliance with others[220]. The HEMA-TsCl layer was only used in **paper IV** for immobilization of mAb E27 targeting hCG as later discussed. The pHEMA-TsCl polymer layer could, however, be coupled with other reagents grated compatible with basic coupling conditions.

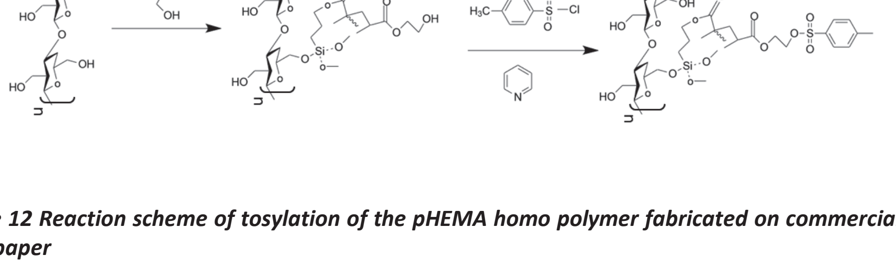

Figure 12 Reaction scheme of tosylation of the pHEMA homo polymer fabricated on commercially available filter paper

3.2.3 Fabrication of wax printed sampling chips

As previously mentioned, most sample preparation steps in protein analysis of biological samples require actions in liquid systems e.g. protein modification and hydrolysis. In preliminary work (**paper I** for instant protein digestion and **IV** for instant immunocapture), this requirement was kept by utilizing wax printed circles to confine the sample droplet and prevent vertical wicking. However, from the observations in the first paper, it was considered troublesome that half of the sample was wicking throughout the sampling material and left hanging on the underside of the sampling area. This made for cumbersome handling since the device could not lay on a surface prior to being completely dry. In **paper IV** wax printing was used to enclose and hold polymerized sampling material immobilized with mAb targeting hCG, however with subsequent protein modification and hydrolysis in solution after the sampling stage. Thus, the only sample interaction in the sampling material was protein capture by the antibodies. This study was similar to the first paper with respect to the aim, to prove the concept and to demonstrate the capabilities of instant protein capture directly in the sampling material. Later, in **paper V,** a full *lab-on-paper* methodology (also described as all-in-one sampling) was demonstrated for a protein that required both reduction and alkylation as well as tryptic digest prior to analysis. This sampling concept was fabricated to hold a chip like design where the sampling device could lay on a flat surface while still being able to perform all actions necessary for a bottom-up workflow. To prevent sample from hanging on the under-side of the sampling material, the holder was constructed with a solid panel of wax as a base-layer combined with four layers of wax printed circles glued on top to create a well-like structure. The polymeric sampling disc (now immobilized with mAb) was further placed inside the well so that the sample could wick neither vertical nor horizontal (**Fig. 13**). The volume holding capacities was optimized for the addition of additional solutions after sampling (reduction, alkylation, and trypsin). The volume holding capabilities could be increased by utilizing more wax prints to make deeper wells e.g. 4-ply, 5-ply or 6-ply. This sample holder was further used in **paper III** with instant tryptic digestion for targeted protein determinations.

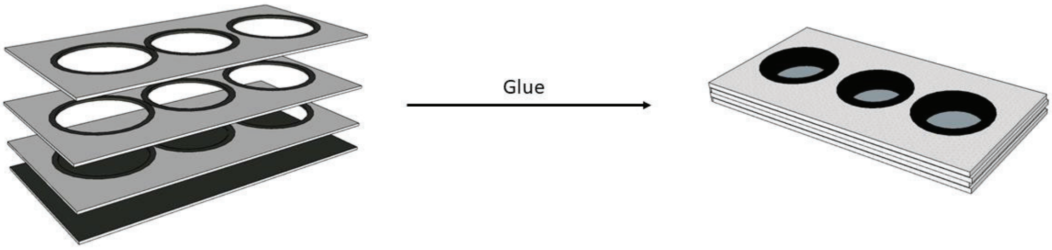

Figure 13 Schematic illustration of a 4 layer wax printed sampling cartridges. Three papers with a 6 mm ID wax print and one solid piece of wax print was glued in a sandwich construction to form sampling wells designated to polymerized and functionalized sampling material

3.2.4 Protein reduction and alkylation post protein hydrolysis

As discussed in *section 1.4.5*, determining proteins by the bottom-up approach reduction and alkylation of the proteins, more specifically cysteines S-S binding, prior to enzymatic digestion is needed. This is especially true for proteins containing cysteines/disulfide bonds, but could also be to avoid protein-peptide complexes during later sample preparation for separation and detection. With instant *on-paper*, or *in-device,* sampling this was a significant challenge since it is not possible to reduce and alkylate the proteins prior to sampling if the device is to be used in a realistic setting like DBS. Therefore, in **paper I** post proteolysis reduction and alkylation of a six-protein buffered sample, and later whole blood was investigated and compared to the conventional strategy of treating the proteins prior to enzymatic digestion. From the non-targeted investigations, it was shown that the placement of the reducing and alkylating steps did not have a significant impact on the number of identified protein groups. The *on-paper* sampling with reduction and alkylation post proteolysis was also compared to a conventional in-solution approach (no paper sampling and reduction and alkylation prior to proteolysis were performed in-solution). Minor differences between the two sampling strategies were observed (10 % less protein sequence coverage, 29 % fewer high confidence peptides and 19 % fewer high confidence peptides with zero missed cleavages for on-paper digestion). The *on-paper* protein digestion sampling strategy with on-paper proteolysis followed by reduction and alkylation was further compared to conventional DBS (with reduction and alkylation prior to over-night tryptic digestion). From this comparison, major differences in identified protein groups and peptides were not found with 75±25 and 267±72 compared to 76±5 and 335±11 high confidence protein groups and unique peptides for *on-paper* digestion and DBS, respectively. Though, it must be stressed that reversing of the protein modification strategy may not work for in-solution approaches given that the sample with its respective peptides dry into the sampling material and thus, lay with minimal interactions (in terms of reformation of the cysteine bonds) when dried prior to being extracted in a solution containing the reducing agent. Given the results, post digestion reduction and alkylation was further applied in **paper II** as elaborated later.

3.2.5 Storage stability

Paper-based sampling has many benefits compared to liquid sample handling e.g. capillary or venipuncture as mentioned earlier. However, the most important might be the high degree of sample stability in dried form (exceptions apply). Since the presented concept holds the potential to be used in a future POC setting where a sample is collected away from the lab e.g. in-field or at-home there is a requirement of the stability of the sampling devices. In **paper I**, storage stability was investigated both stored with (six weeks) and without protein sample (twelve weeks). The experiment was carried out with wax printed filter paper applied with trypsin immobilized polymer beads and a buffered sample of cytochrome C (cyt-C). The sampled and un-sampled wax prints applied with trypsin immobilized beads were during this time period frequently moved in order to simulate a realistic setting of a postal shipment. The wax printed filter papers stored without sample, but with beads, for twelve weeks were sampled with a buffered cyt-C sample in the same concentration as the control sample, which was freshly prepared wax prints applied with beads. The sampling devices stored with beads and applied protein-sample for six weeks showed a reduction of five percent in protein sequence coverage (total amino acid sequence), 33 % high confidence peptides and one less peptide with zero missed cleavage sites compared to the control samples. For the reactors stored for twelve weeks, only a decrease in 11 % in protein sequence coverage, four less high confidence peptides and two less high confidence peptides with zero missed cleavage sites were observed. The signal drop for both storage variants may have been a result of the handling of devices causing the beads deployed on top to fall off, which for conventional paper sampling would not be an issue, resulting in lower overall performance. Nevertheless, with the latter into consideration the storage stability of the concept was considered adequate and the stability assumptions were further applied in **paper II – V**.

3.2.6 Protein adsorption to sampling materials

The strategy of sampling small volumes of biological samples on-paper offers many benefits, but could also be troublesome for determination of lower abundant proteins due to rapid adsorption of proteins to cellulose[222]. This adsorption, often un-specific, could also be further increased by the use of surfactants during extraction or further sample treatment ultimately leading to loss or absence of MS signal. In **paper IV** it was therefore of interest to characterize how proteins bind to un-treated filter paper (cellulose) and filter paper coated with pHEMA-VDM. The characterization was performed by brilliant blue staining as described by McCann et al.[223], with increasing concentration of bovine serum albumin (BSA) in phosphate buffer as adsorbent. Since the sampling concept was designed to hold immobilized antibodies for selective protein capture, no protein adsorption to the sampling material was desired. For the characterization, non-treated filter paper, as well as pHEMA-VDM coated filter paper, was used. Blank samples was also included (i.e. sampling spots without fixated BSA) to show that the brilliant blue solution did not interact with the sampling materials. From the characterization (**Fig. 14**) increased color intensity was observed in correlation with a higher concentration of BSA. The blank sampling materials were unaffected by the staining solution. Interestingly, the non-treated filter paper showed a non-uniform distribution of the proteins with a dominant concentration-effect along the edges. This may be due to evaporation and migration of the proteins towards edges of droplets as described by the terminology "coffee ring effect"[224]. In contrast to the untreated filter paper, the pHEMA-VDM coated paper showed a homogenous distribution of the proteins. The color intensity did as well correlate to the increased protein concentration. However, the polymerized reactors were not immobilized with antibodies and it could, therefore, be assumed that BSA was immobilized to VDM`s functional groups since this polymer has been demonstrated to be covalently immobilized with BSA by others[212]. In retrospective, this experiment could be carried out including antibodies (not targeting BSA) immobilized pHEMA-VDM sampling substrate to further conclude whether or not BSA indeed was immobilized or adsorbed. However, a tentative conclusion was made that the polymer layer was indeed capable of covalently immobilize proteins as later shown and that the non-specific adsorption effects of proteins to non-treated cellulose was extensive.

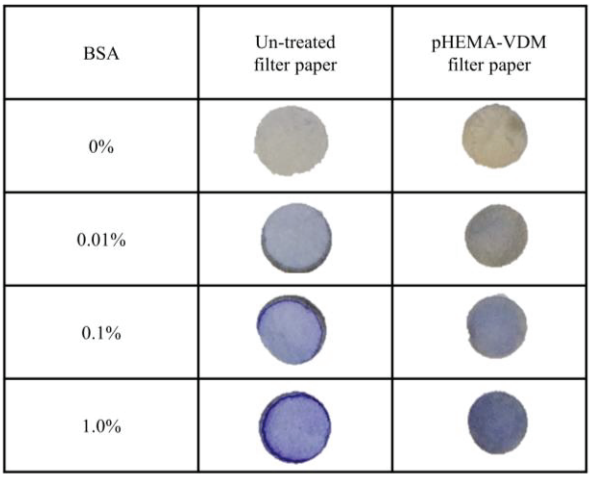

Figure 14 Brilliant blue staining of BSA immobilized to un-treated- and pHEMA-VDM coated Whatman® Grade 1 filter paper. Reproduced with permission from Fig. 1 paper IV. Copyright © 2018, Rights managed by Elsevier

3.2.7 Coupling strategies and choice of immobilization support of antibodies

A variety of coupling strategies of antibodies and enzymes to solid supports has previously described in the literature. For paper-based supports, this has mostly been through adsorption[140, 162, 225, 226]. Therefore, in **paper IV** we explored the coupling of antibodies directly to the sampling paper for instant *in-device /on-paper* immunoaffinity for MS determination of biomarkers. Antibodies have frequently been reported immobilized to filter paper in the literature as paper-based ELISAs (sandwich and direct). Most of these procedures involve a simple adsorption step of the antibodies to the paper support. Therefore, in initial experiments in **paper IV** adsorption of antibodies to the sampling filter paper was investigated. For the adsorption/ fixation of the antibodies, we adapted a previously described procedure by Lei et al.[140] however, with minor modifications since we were intended to use MS instead of colorimetric detection. Conventional filter paper (Whatman® grade 1) was used for the experiments since it was assumed that extended interaction time between the protein and antibodies was not needed for the antibody-based approaches (contradicting the instant digestion strategy). To evaluate the performance (in targeted mode) of the adsorption strategy buffered hCG was sampled on mAb-adsorbed filter paper alongside with filter paper without adsorbed antibodies. From this investigation, no significant difference ($p \gg 0.05$) in hCG ß-T5 proteolytic peptide was observed (between the filter paper with and without adsorbed antibodies. The results was believed to be a cause from non-specific adsorption of protein to the cellulose material. Especially given the lower concentrations (highest concentration investigated 2 µg/mL in buffered solution) used in the experiments. The lack of distinction between the variants ultimately pointed out no use of the antibody affinity as proteins have shown to be inherently sticky to cellulose materials. In turn, no difference was seen between the filter paper applied with antibodies, as hCG might have adsorbed to the structure and eventually released during the acidic extraction. To make the concept function, different immobilization strategies were consequently explored. Therefore, we looked at other structures and means where antibody immobilization has successfully been incorporated in. For chromatographic-, sample preparing columns, and other sample preparing means such as immunoaffinity beads covalent fixation of antibodies has mostly been carried out. There are many ways to chemically and permanently attach enzymes and antibodies to a solid support by reactive groups e.g. amino-, carboxylic acid-, and hydroxyl groups[227]. As previously discussed, Ts-Cl activated beads have routinely been utilized for immobilization of various antibodies for in-solution sample preparing procedures. Therefore, the polymer layer pHEMA-TsCl (*section 3.2.2.3*) was investigated alongside with pHEMA-VDM for covalent attachment of the antibodies to the sampling spot. To immobilize the polymer layers with antibodies (here E27 targeting hCG), the same strategy for immobilization as for trypsin was applied. The first strategy was carried out in phosphate buffer for three hours at room temperature, the second strategy was carried out in the same buffer, however for eighteen hours at 4 °C. These two coupling strategies did not give adequate MS signal when later sampled with hCG (20 µg/mL in phosphate buffer). Therefore, a third coupling method was investigated according to a more thorough procedure described by Paus and Nustad[113]. The third and final method included orientation correction of the antibodies by acidic conditions with HCl. This orientation step has previously been shown by Conradie et al.[138] to be of significance. This immobilization strategy was superior over the others (**Fig. 15**) and was further investigated in SRM analysis of hCG (buffered solution) between 100 and 2000 ng/mL (five concentration levels). Satisfactory linearity (n = 3, $R^2 = 0.996$) was obtained. This immobilization strategy was further applied in **paper V**.

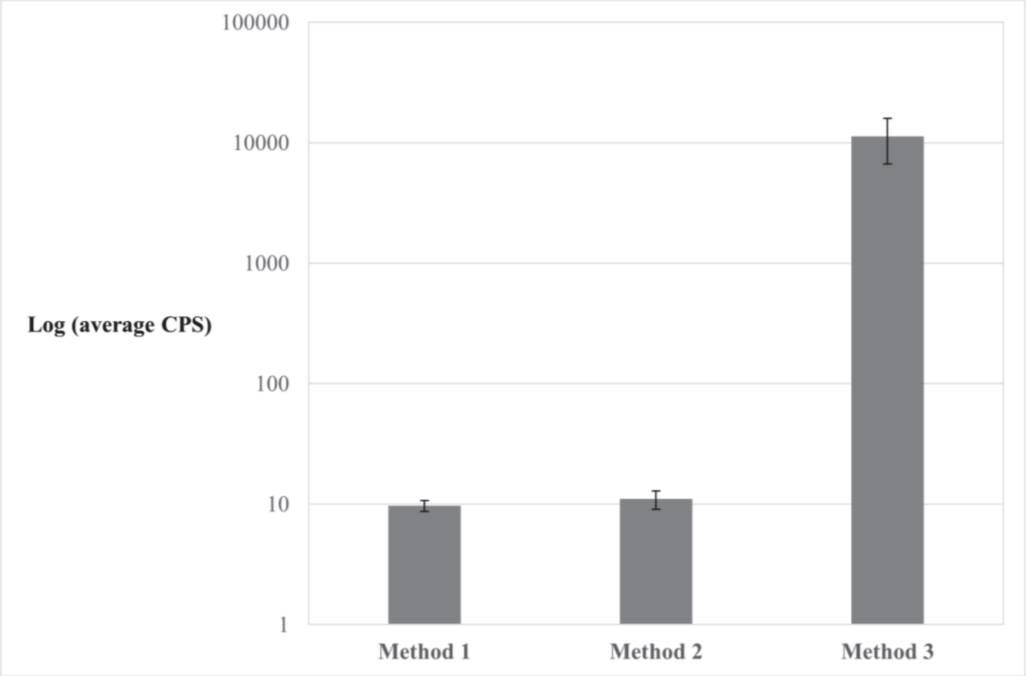

Figure 15 hCG (βT5) MS intensity for the three different immobilization strategies investigated in paper IV. The strategies was tested om pHEMA-VDM polymerized Whatman® grade 1 filter paper and subsequently sampled with 20 µg/mL hCG (from Ovitrelle®) in phosphate buffer. Reproduced with permission from Fig. 2 paper II. Copyright © 2018, Rights managed by Elsevier

3.3 Smart sampling with *in-device proteolysis*

*As previously discussed, in-device and instant proteolysis (on-paper in **paper I** and in-device in **paper II**) has been investigated and compared to generic DBS. In **paper I** the concept of instant on-paper digestion of proteins in whole blood was introduced as a proof-of-principle investigation. The concept and its considerations concerning reduction, alkylation and stability were further polished and reintroduced in **paper II** with a polymeric backbone for immobilization of trypsin directly in the sampling device. Both papers sampled freshly collected whole blood. The analyses were performed in a DDA non-targeted analysis aiming to discover as many proteins as possible. Contradicting to **paper I,** no wax printing was used in **paper II**. The fundamental principles investigated such as reduction and alkylation, storage stability and aim of usage was, however, further considered. In **paper I** the principle of operation was in focus whereas in **paper II** fabrication and design of the device was the main objective. The overall results from these two papers could be seen as a whole. The findings in these two papers and the consideration that had to be taken into account for in-device proteolysis was further applied in a targeted methodology for the biomarker proGRP in **paper III.***

3.3.1 On-paper digest compared to in-solution digest of buffered protein

To compare *on-paper digestion* to conventional *in-solution* digestion, a brief preliminary experiment was performed in **paper I** with a single buffered model protein (Cyt-C). The protein was not reduced and alkylated prior to digestion since it does not contain any disulfide bonds. After applying five µL of protein sample to the wax printed filter paper with trypsin immobilized beads, the *on-paper* digested sample was analyzed. The analysis was carried out subsequently to extraction and dilution to the exact same concentrations as in the *in-solution* digest. The total mass injected was 10 ng digested protein on column. From the analysis, 13 and 15 unique peptides (from a total of 16 and 20 peptides) were found for *on-paper digestion* and the *in-solution* approach, respectively. The two methods also showed 8 peptides with zero missed cleavage sites and similarly 80 % of protein coverage. The signal intensity for the three most frequent peptides (MIFAGIK, TGQAPGFTYTDANK, and EDLIYLK) were also compared. However, with exception of TGQAPGFTYTDANK, the peptide signal intensities did not show significant differences (two-tailed t-test, $p>>0.05$) (**Fig. 16**). Therefore, it was concluded that it was readily achievable to perform rapid protein digestion directly in the sampling paper with short reaction time; one hour compared to conventional 18 hours of over-night *in-solution* protein digestion.

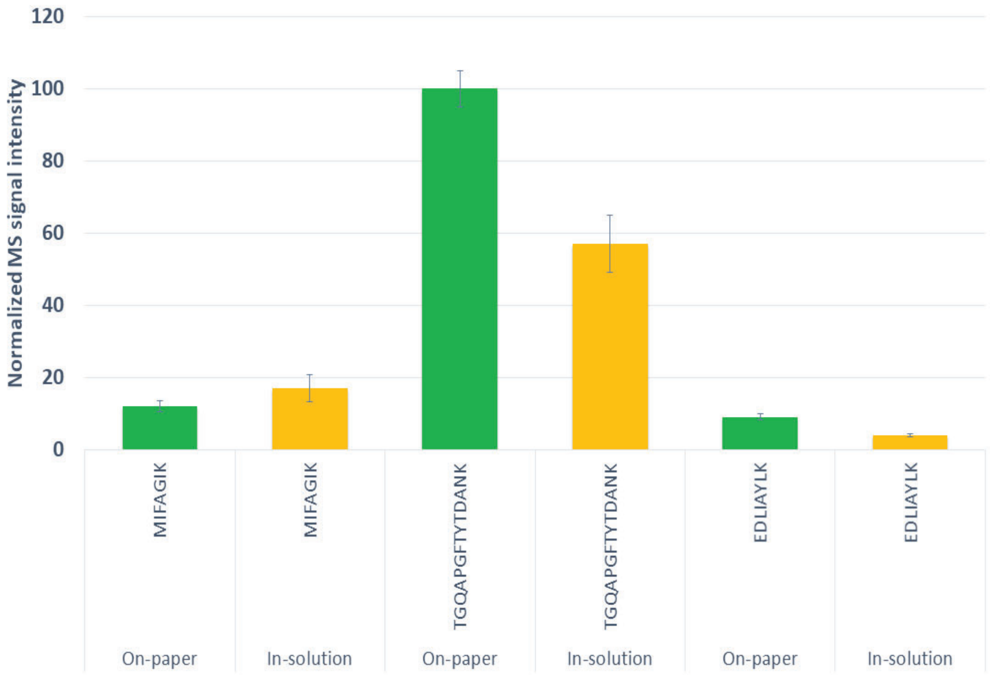

***Figure 16** Normalized MS signal intensity for the three most frequent tryptic peptides from Cyt-C. Cyt-C (buffered) was digested on-paper and in-solution and analyzed with DDA*

3.3.2 Non-targeted analysis of proteins in whole blood

To demonstrate that it was feasible to digest proteins in complex matrices directly in the sampling spot, non-targeted (DDA) analysis was used for measuring the digestion efficiency of sampled whole blood. Digestion efficiency was defined as the number of identified protein groups, number of unique peptides and in some cases protein sequence coverage. Both **paper I** and **paper II** utilized a previously published sample preparing procedure by Chambers et al.[180] with minor modifications regarding when the hydrolysis step was performed. For performance comparison, a reference was made by performing conventional DBS sampling exactly according to the non-targeted procedure described by Chambers et al. The reference sample set was performed in **paper I** with conventional over-night and in-solution protein digestion with whole blood from the same stock as used for the wax prints with instant digestion. The same LCMS settings and analytical conditions, as well as sample preparing procedure, were utilized for both sampling variants in **paper I** and **paper II**. From the reference set (DBS) 76±25 protein groups were determined as well as 335±11 unique proteins (**Fig. 17**). Compared to what Chambers et al.[179] reported in a later study using the same approach (average 253 protein groups from spotted whole blood) our findings were significantly lower. This may be due to MS limitations since the data interpreting software (Proteome Discover) and strict reporting criterion were the same. In short: precursor tolerance of 10 ppm, fragment mass tolerance of 0.6 Da, a maximum number of three missed cleavages and a strict false discovery rate (0.01). Compared to the DBS reference results our initial concept of instant *on-paper* digestion (**paper I**) showed to have comparable results by 75±25 and 267±72 protein groups and the number of unique peptides, respectively. In **paper II**, 134±14 and 124±7 protein groups were found from freeze thawed and freshly collected whole blood, respectively. Additionally, for the *in-device* digestion of the freshly collected whole blood 49 %, 31 % and 14 % of the unique peptides (252 in total) had zero missed cleavage sites, one missed cleavage site and two missed cleavage sites, respectively. All in all, the polymer coated filter paper immobilized with trypsin showed superior performance compared to our findings in **paper I** with filter paper deployed with immobilized trypsin beads. The performance was also significantly better (two-sided t-test; $p << 0.05$) than what was achieved with generic DBS.

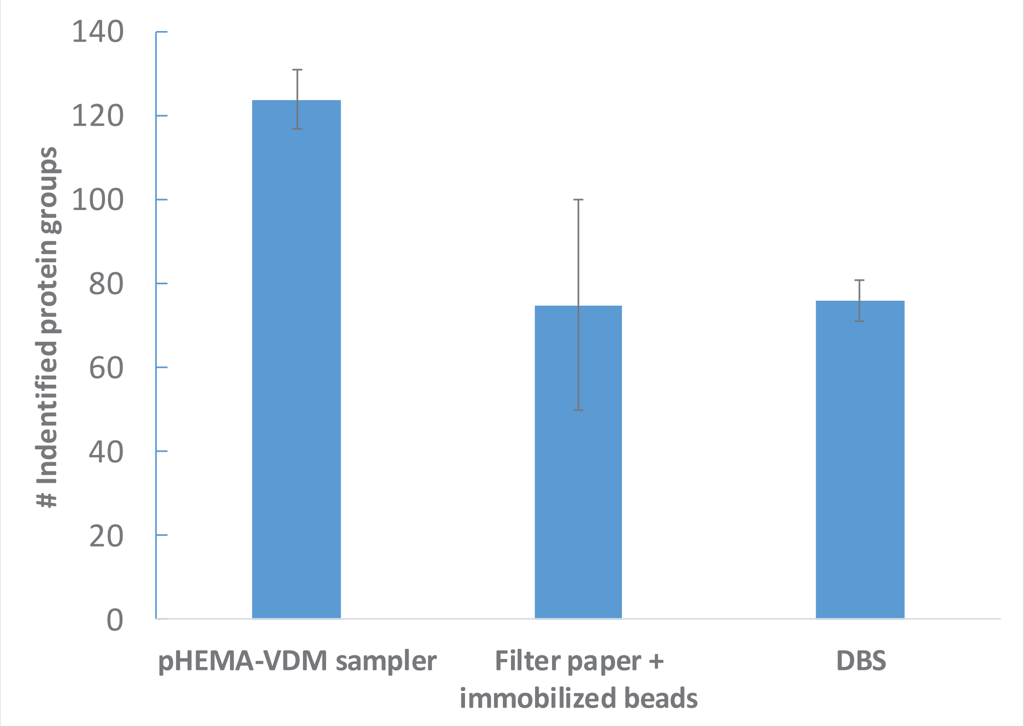

***Figure 17** Identified high confidence protein groups from whole blood sampled on A) instant on-paper proteolysis with trypsin immobilized pHEMA-VDM devices, B) wax printed filter paper with trypsin immobilized beads on top and C) conventional DBS procedure with over-night in-solution proteolysis. The figure is combined and adapted from paper I and paper II with permission. Copyright © 2018, Rights managed by the Royal Society of Chemistry*

3.3.3 Targeted analysis of proGRP

In the development of the smart-sampling concept with immobilized trypsin for instant protein digestion during sampling, all analyses were performed using a non-targeted approach. This way of collecting data was important to understand the differences in types and quantity of peptides generated compared to more conventional sampling and sample preparing techniques (in-solution, DBS, etc.). The non-targeted strategy was also efficient with respect to the general understanding of the concept, and its mechanism for further improvements, such as optimization of the polymer layer and the subsequent immobilization of trypsin, needed in order to digest complex matrices. However, this sampling concept was not designed and intended for discovery analyses. This, because it could not compete with sampling and fractionation of large volume samples combined with hyphenated analytical techniques generally required for extensive proteomic datasets. While one could combine *on-paper* sampling with more heavy analytical techniques, it would not hold too high of significance to shorten the sample preparation time and possibly sacrifice peptide generation when combined with cumbersome and time-consuming techniques such as fractionation and long LC separation gradients. Even though the initial investigations were carried out in a proteomic approach, the ultimate goal was to create a simple and robust, yet high performing sampling concept that could serve as a POC device for targeted analyses of lower abundant biomarkers. This was demonstrated in **paper III** were sampling with integrated and instant tryptic digestion was combined with tryptic peptide immunoaffinity extraction for quantitation of the low abundant biomarker proGRP. The proposed sampling concept and its subsequent sample preparation required minimal sample preparing time. It also showed similar or higher performance compared to what previously had been accomplished in our group, regardless of the sampling method. Moreover, little has been carried out with paper sampling and immunocapture other than what Rosting et al.[87] demonstrated with DMS sampling and immunocapture of extracted and intact hCG. The method Rosting et al. demonstrated had adequate performance, however, the sample preparing time was long (>22 hours). Even though the workflow in **paper III** is not directly comparable to what Rosting et al. demonstrated, it proved that smart sampling with integrated proteolysis is a promising concept for future MS-based clinical applications where high performance and fast sample preparation is required.

3.3.3.1 Sample preparation

In order to obtain low detection and quantification limits, *in-device* proteolysis was combined with peptide immunocapture (by conventional mAb, targeting intact protein) of the proteotypic signature peptide ALGNQQPSWDSEDSSNFK (A-peptide). Sample preparation with immunoaffinity was in this work necessary to measure the low concentrations relevant for proGRP. This considering the low sampling volume (15 µL) making no possibilities to perform enrichments. The capture of the tryptic peptide could be achieved by other methods previously demonstrated in our group such as MIP[89], however, the peptide capture by anti-protein antibody methodology had previously been proven to work efficiently by Levernæs et al.[86] for the target analyte. To incorporate immunoaffinity extraction with our chip-based sampling concept, antibodies were immobilized to magnetic beads and subsequently added to the extraction solvent. It must be stressed that this approach would not be efficient if trypsin were not immobilized to the sampling material. This because trypsin would digest the antibodies and thus, ruin the peptide-antibody interaction. If so, MIP must have been used instead. Furthermore, since the enzymes were chemically fixated to the sampling material, flexibility in terms of when to perform immunocapture and combining this step with extraction was possible. mAb-beads can be added during the extraction of the tryptic peptides (from the sampling device), rather than extracting peptides from the sampling material prior to subsequent immunocapture. This possibility was investigated in order to eliminate the conventional one-hour immunocapture (of peptides or proteins) that has routinely been carried out in our group[86, 89, 97, 188]. From the investigation, the addition of antibodies simultaneously with extraction proved significant increases (two-tailed t-test, $p<<0.05$) in MS signal intensities (**Fig. 18**). In addition, the presence of the non-ionic surfactant Tween 20® was also investigated in the extracting and capturing solution to potentially reduce non-specific binding of proteins towards the magnetic beads. This was examined to further minimize the sampling steps related to matrix removal. However, no improvement was observed between extracting and capturing of the peptide with Tween 20® present (data not shown).

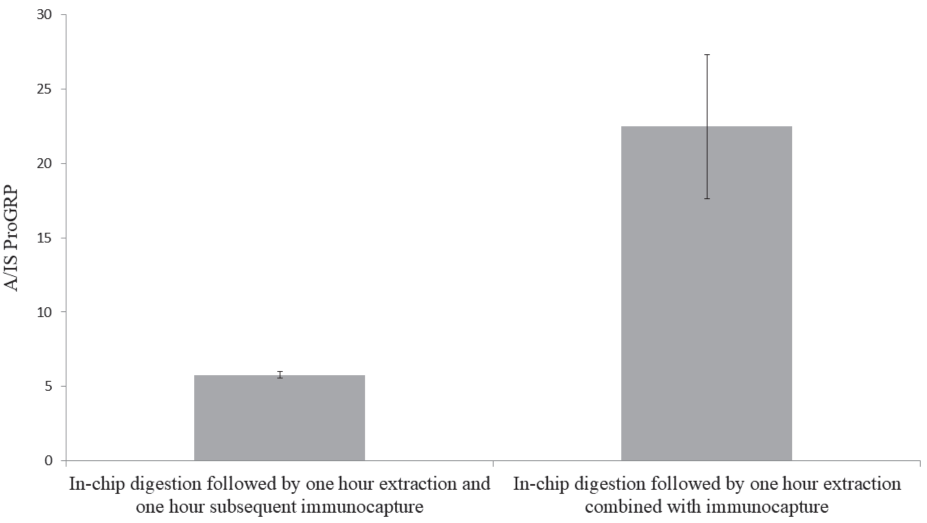

Figure 18 In-chip digestion of proGRP spiked serum (2 µg/mL) with A) subsequent immunocapture after 1 hour of extraction in PBS and B) one hour extraction combined with immunocapture. The y-axis is the ratio between the signal intensity of the A-peptide and the signal intensity of the IS. Reproduced with permission from Fig. 1 paper III. Copyright © 2020, Rights managed by the Royal Society of Chemistry

3.3.3.2 Extended tryptic digestion for targeted analysis of proGRP

As previously mentioned, tryptic digestion is a first-order kinetic reaction and in the presence of high abundant proteins, the lower abundant ones such as proGRP, would face a slower digestion time. This, in particular in the lower concentration levels investigated. With the latter in mind, it was explored whether or not the tryptic digestion needed to be extended. The experiments were performed in spiked serum and the additional buffer was added to the sampling material either at the time of sampling (to increase the volume and thus, drying and reaction time) or after the serum sample had dried i.e. performing a second digestion step. However, no increase in signal intensities or improved reproducibility was obtained. Extending the reaction time as well as increasing the enzyme-to-protein ratio was also investigated by applying trypsin immobilized beads (similar to what was done in **paper I**) in volumes 5 µL, 10 µL and 15 µL to the sampling spots. These efforts did not promote a higher degree of protein digestion with regards to the target peptide's MS signal intensity. Therefore, it was concluded that the sampling concept performed adequately in complex matrices without extended reaction times.

3.3.3.3 Performance considerations

The methodology presented in **paper III** showed a significantly lower time consumption from sampling-to-data treatment at under 2.5 hours. The LOD and LOQ were obtained at 150 pg/mL and 500 pg/mL in freshly collected whole blood, respectively. It also proved a high degree of linearity (R^2=0.99) between 500 – 1000 ng/mL with adequate precision. Compared to efforts by others the concept showed similar and in some cases higher performance. Torsetnes et al.[97] obtained a LOQ of 300 pg/mL by enriching *1.0 mL* of serum by the same type magnetic beads coupled with mAb 146 (in **paper III** mAb M18 was used). Correspondingly to Torsetnes et al.[97], Rossetti et al.[89] later demonstrated measurements of clinically relevant concentrations of proGRP in serum (< 60 pg/mL) with the immunoaffinity beads. The importance of the selective sample preparation was also demonstrated and compared to MIP and protein precipitation. For protein precipitation, a high sampling volume was used (1.0 mL) and in both studies (Torsetnes et al. and Rossetti et al.) intact protein was captured followed by over-night tryptic digestion resulting in sample preparation procedures longer than 20 hours. In a more recent study by Levernæs et al.[86] LOQ was estimated to be 162 pg/mL from 50 µL serum. This was demonstrated with peptide capture after 3-hour protein digestion (in-solution) by trypsin immobilized beads, rather than intact protein capture. In the latter work, it was shown that the capture of peptides holds benefits over an intact protein such as lower MS background signal (cleaner extracts) that could contribute to more sensitive analyses. Nevertheless, all the above-mentioned publications share a technical sample preparing procedure that requires high competence of the operator and therefore could be troublesome to incorporate in clinical routine analyses. Thus, regardless of performance numbers and their comparisons the principal benefit of the concept presented in **paper III** lies in the fast and semi-automated sample preparation making the sampling device simple to use with its integrated and semi-automated sample preparation. This holds benefits for use out of the lab e.g. in-field or at home as a POC device. If the sampling was performed outside the lab, minimal sample preparation would be necessary after the sampling device arrives at the lab, making for a more streamlined and cost effective alternative to conventional bottom-up approaches if used in a clinical setting.

3.3.4 *In-device proteolysis*, a perspective

The findings in **paper I** – **paper III** encircled the concept of protein digestion incorporated with paper-based sampling of biological matrices for bottom-up analyses. From its initial considerations (**paper I**), architectural design (**paper II**) and demonstration of applicability (**paper III**), smart sampling on paper has proved as a promising platform for quantitative protein determinations. Many of the challenges regarding protein digestion performed as the first step in the sample preparing workflow have been addressed. This, especially concerning protein reduction and alkylation, which is normally carried out prior to protein digestion. Protein reduction and alkylation was demonstrated to be applied after protein hydrolysis with minimal differences in terms of peptide yield compared to conventional in-solution digestion. Furthermore, similar peptides were found in DDA discovery analyses when compared to a conventional DBS strategy. The findings imply that if protein digestion is performed in-device, dried and subsequently reduced and alkylated, the risk of re-establishment of disulfide bonds is small. *In-device* (or *on-paper*) sampling has also been demonstrated to perform equally or better in terms of tryptic digestion compared to DBS with over-night and in-solution proteolysis. It has also been proven a promising alternative for targeted protein determinations of low abundant biomarkers with a very low sample preparing time. Still, there are fundamental challenges that have to be addressed if the concept was further developed and eventually reach the clinics. In terms of architectural design, the concept could readily be transferred over to other materials such as plastic holders instead of wax printing to prevent sample movement during sampling. It is also possible to solely rely on the polymer as sampling material or change the paper with more rigid materials (as rebars) for polymer coating such as metal or plastic making for a more streamlined production with a higher success rate with respect to fabrication (filter paper could be troublesome if not handled carefully prior to coating). If no rebar were used, a sampler shape similar to the Mitra® microsampling device (Neoteryx) could be fabricated (unpublished results). All in all, in-device protein digestion has proven good results and has proven similar (or better) performance to the more generic sampling approaches. For future optimization, it is likely that polymer composition, immobilization conditions, and sampler dimensions could further increase the concepts' performance.

3.4 Smart sampling for selective protein capture

The sampling concept with integrated tryptic digestion previously discussed in section 3.3 proved promising performance with minimal sample preparing time for targeted analyses. However, a reversed (protein hydrolysis prior to immunocapture) bottom-up strategy was used and thus, may not be suitable for the majority of biomarkers. Also, since the sampling devices immobilized with trypsin showed high performance, integration of mAb into a similar device seemed to be readily achievable. Therefore, a more conventional approach with integrated and instant immunoaffinity prior to enzymatic treatment was explored. Consequently, in ***paper IV*** *and* ***paper V*** *smart sampling with immobilized mAb was investigated for in-device immunocapture of the biomarker hCG. Both papers are based on the polymeric sampling device previously introduced along with the general considerations regarding fabrication, stability, and applicability towards POC. Additionally, other polymers were investigated.* ***Paper IV*** *could be regarded as the proof-of-principle study because only the immunocapture was performed in-device (or on-paper). However, most of the developments required for the concept such as immobilization conditions, characterizations, and choice of polymer layer was here explored. In* ***paper V*** *the sampling concept was transferred to a chip-format for an all-in-one analytical platform. This second iteration incorporated all steps necessary for a targeted bottom-up workflow in-device.* ***Paper V*** *is a demonstration of applicability towards POC applications.*

3.4.1 Paper-based immunocapture

The idea of incorporating mAb directly to the sampling material came about the inclusion of the time-consuming sample preparing step, associated with immunoaffinity prior to MS determination of low abundant proteins (**paper IV**). This, in an easy-to-perform paper-based sampler. Even though, the use of mAb-immobilized beads directly added to serum has proven to be high performing with relatively fast sample preparation[89, 97]. The latter in combination with paper sampling could be a time-consuming process since extraction of the sample (from the paper) has to be included prior to the immunocapture step and subsequently protein modification and enzymatic digestion[87]. Furthermore, the inclusion of instant immunocapture during sampling introduces a streamlined sample preparation that only calls for reduction and alkylation (performed in the extracting solution) with subsequent tryptic digestion prior to MS analysis. The initial goal for this particular concept was not to develop the fastest sample preparing method, but rather demonstrate a streamlined sample preparation with a high degree of performance and ease of use. Nevertheless, in **paper V**, further development of the sampling device from **paper IV** resulted in just that, with fast and minimal manual sample preparation. The second iteration of the sampling concept (**paper V**) was introduced in chip format aimed towards POC applications. To our knowledge incorporation of antibodies, on-paper /in-device in combination with MS analyses has prior to this work not been demonstrated.

3.4.1.1 Pilot experiments for the choice of polymer layer

One of the main challenges for the proposed concept was the fixation of the mAb. As previously discussed in *section 3.2.7* different strategies of fixating antibodies directly to the sampling material were investigated in **paper IV**. These strategies were mainly adsorption by different conditions and stronger chemical fixation by covalent attachment. From the investigations, covalent immobilization of mAb E27 (targeting hCG) showed superior performance when compared to adsorption methods. Additionally, the pHEMA polymer layer was also investigated with different functional groups in order to explore potential gains related to immobilization. To determine which polymer composition worked best for the concept, and also demonstrate the necessity of a polymer layer; the optimized immobilization strategy was used to immobilize antibodies on pHEMA-VDM filter paper and pHEMA-TsCl filter paper. Untreated filter paper was also immobilized (according to the same conditions as the polymer immobilization). A standard curve of hCG in buffered BSA was analyzed by the three variants. To verify that the mAb played a significant role in the sampling device, pHEMA-VDM and pHEMA-TsCl coated filter paper were also analyzed *without immobilized mAb*, however with the same spiked samples. From the analysis, the pHEMA-TsCl sampling spots produced higher MS signal intensities compared to pHEMA-VDM. However, the pHEMA-TsCl devices showed a significantly higher (two-sided; *t*-test $p \ll 0.05$) background signal compared to the pHEMA-VDM variant (signal for the variants without antibodies). For the untreated filter paper, no difference was observed between the immobilized and non-immobilized variants. The experiment did prove that the use of the polymer layers was superior to untreated filter paper. Nevertheless, for the polymerized versions without immobilized mAb, the functional groups on the polymers may have contributed to signal bias regarding the sampler-sets without immobilized mAb. This because these functional groups could couple the target analyte in addition to adsorption of both matrix protein (BSA) and target protein (hCG). With regards to nonspecific protein binding, the same experiment was performed in spiked serum. From the serum analyses, no significant (two-tailed t-test $p >> 0.05$) differences in terms of MS signal intensities were observed between the polymer layers (**Fig. 19**).

The pHEMA-VDM polymer was from this pilot experiment considered the most suitable coating for further developments. The coating showed a high degree of flexibility (regarding immobilization of other proteins), in such way that the sampling material could be immobilized with either trypsin (as described in **paper I-III**) or mAb making for convenience. It also involved less HSE hazardous chemicals under fabrication and required an easier fabrication procedure with lower time consumption compared to the counterpart. The pHEMA-VDM coating was further used throughout **paper IV** and **paper V.**

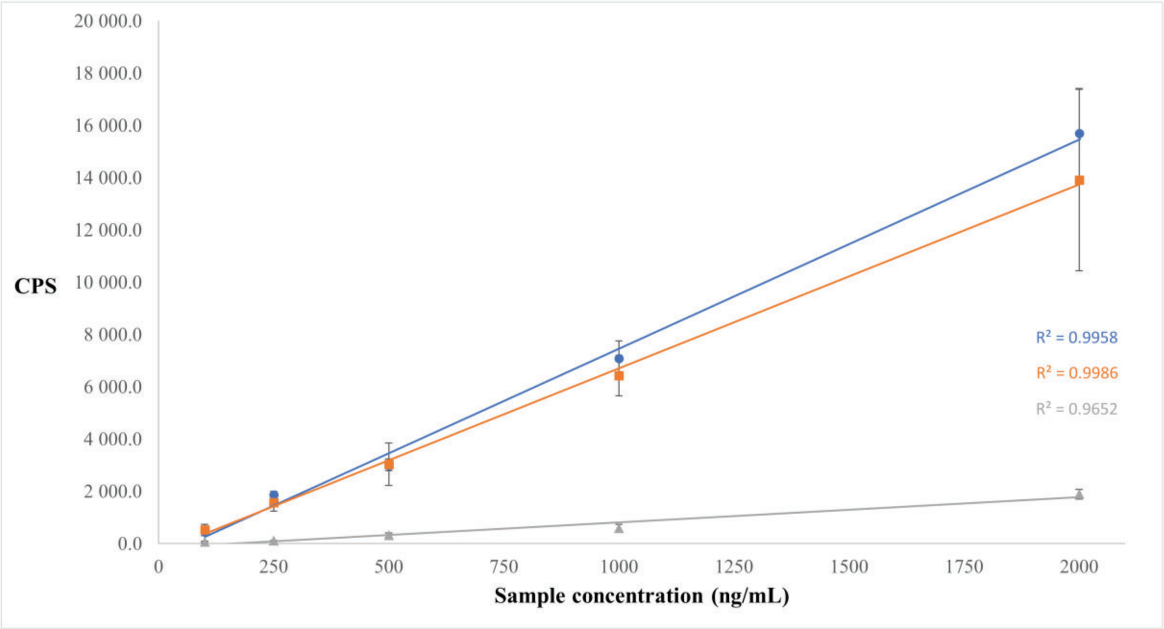

Figure 19 Ananlysis of hCG spiked serum in concentration 100 - 2000 ng/mL (n=5). The sample set was applied to E27 immobilized pHEMA-VDM, pHEMA-TsCl and un-treated filter paper. Reproduced with permission from Fig. 3 paper IV. Copyright © 2018, Rights managed by Elsevier

3.4.1.2 Performance considerations of in-device immunocapture

In order to evaluate the quantitative performance of the mAb immobilized sampling materials in **paper IV**, intraday and interday evaluations were conducted. The interday linearity (100-2000 ng/mL, (2.6-26 nM)) showed a satisfactory correlation, R^2=0.99. Intraday linearity (1-20 ng/mL, (26.4 pM – 528 pM)) was obtained with a correlation of R^2=0.97. Compared to earlier demonstrations of hCG determination with DMS followed by immunoaffinity by Rosting et al.[87] (R^2=0.93, concentration range 28-2900 pM (2 – 100 ng/mL)) improvements were obtained. However, Rosting and colleagues performed the linearity study in a broader concentration range and thus, a direct comparison may not be accurate. Furthermore, Rosting and colleagues used an additional SPE enrichment step and it could be assumed that this step contributes to some loss of analyte and variability. The RSDs reported was also higher compared to what were obtained in **paper IV**. LOD was obtained at 1 ng/mL (26.4 pM which was similar to what Rosting et al.[87] demonstrated with a conventional DMS sampling followed by immunoaffinity). Additional comparisons to paper-based microfluidic devices targeting hCG[228, 229], indicated that the quantitative numbers are similar, however, MS detection opens up for broader use. The latter due to multiplexing and distinction between isoforms as discussed earlier. This is particularly true for doping analysis were hCG is quantified for abuse of performance-enhancing substances or analyses capable of distinguishing cancer from pregnancy[230].

3.4.1.3 *Application to patient samples*

To demonstrate that the sampling concept with in-device immunoaffinity would work in a realistic setting, patient serum samples were sampled on the device and subsequently analyzed according to the optimized conditions. The demonstration was based on two different sample sets were both came from patients diagnosed with testicular germ-cell cancer. The quantified samples were determined to 418.5±4.2 ng/mL and 21±4.2 ng/mL (n=3 each). Compared to analysis of patient serum containing hCG by Rosting et al.[87] with conventional DMS sampling, the accuracy was better (here: ±1 % for the high concentration compared to ±10 % (580±65 ng/mL) with the DMS methodology). The results were considered satisfactory and showed promising potential for the concept to be used in a future clinical setting.

3.5 Lab-on-paper – *an all-in-one sampling device*

From the demonstrations of paper-based sampling with *in-device* immunoaffinity investigated in **paper IV**, the second iteration was presented in **paper V** with fast and semi-automated sample preparation more suited for POC testing**.** The first concept showed a high degree of performance compared to conventional DMS sampling for MS-based protein determinations, however, the sample preparation remained time-consuming. The latter mostly due to over-night tryptic digestion, but also due to additional sample preparing steps in-solution such as reduction and alkylation. As a result, the concept would not cater to a simple POC application. Therefore, the sampling material and immobilization conditions previously explored were applied to a wax printed holder (casing) for the polymerized sampling material tailored to perform all steps necessary for the same workflow, however, *in-device*.

3.5.1 Development of wax printed sampling cartridges

As elaborated in *section 3.2.3*, the wax printed cartridges were prepared by gluing several layers of wax printed circles to a solid underlayer of wax. Each sample cartridge (**Fig. 20**) held three sampling wells designed for polymerized filter paper (6 mm in diameter) and measured to 40x20x1 mm (LxWxD). Machined Delrin® plastic (acetal homopolymer) was also investigated for the purpose of hindering sample-spread in the polymerized sampling material. However, with the plastic cartridges, more fabrication and tools were required and thus, the wax printed filter paper was used for the experiments. Other fabrication techniques such as 3D-printing could be utelized[231]. However, with 3D-printing one should choose the 3D-printer and the respective plastic carefully since many of the plastics commonly used could leak into the sample and ultimately interfere with the MS analysis[232]. Nevertheless, 3-D fabrication of sampling cartridges would readily suit the concept (with the criterion for biocompatible and inert material for printing) in order to make it ready for the consumer market. Nevertheless, no performance differences were observed between the two materials (wax printing and Delrin® plastic).

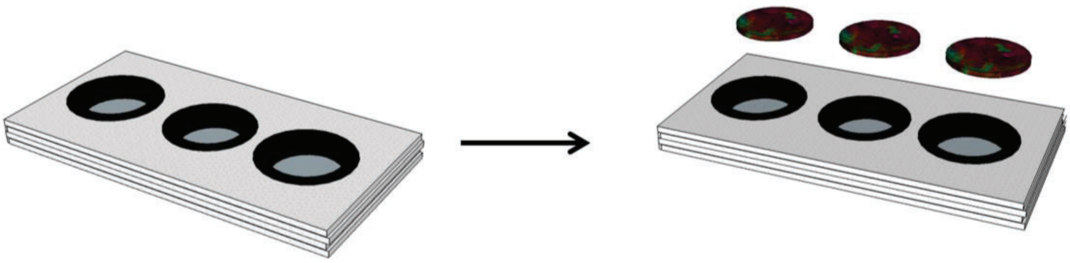

***Figure 20** Wax printed sampling holders design to perfectly fit a 6.00 mm polymerized sampling disk. The holder is glued together with 3-ply of wax printed circles and a solid wax printed panel to prevent droplet formation on the underside. Reproduced with permission from Fig. 2 paper V. Copyright © 2019, Rights managed by Elsevier*

3.5.2 In-spot sample preparation

Other than incorporating immunoaffinity into the sampling material alongside fabricating the cartridge, the main challenge with the *all-in-one sampling concept* was the execution of *in-device protein reduction*, *alkylation*, and *protein hydrolysis* (with trypsin). This, because these actions would be difficult to fully integrate simultaneously into the device without affecting the already immobilized antibodies. A similar concept of chip-based all-in-one sampling was recently explored by Leipert et al.[233] for non-targeted applications. In this work protein digestion, reduction and alkylation were also performed in-device. However, Leipert et al. demonstrated the concept with a cell lysate and would therefore not be directly comparable to efforts with whole blood and serum. Additionally, the procedure was performed with immobilized trypsin beads in a similar fashion to **paper I**. Furthermore, the reducing and alkylating reagents were introduced to their chip-system by solvent additions (pipetting droplets onto the chip). The tryptic digestion was also carried out for eight hours in a humidified incubator. Similar to what Leipert et al. demonstrated, other efforts with full integration of the actions needed for a bottom-up strategy[234, 235], as well as intact protein determination[236], has been described. Nevertheless, common for the latter procedures is a time-consuming sample preparation despite the cleaver sampling design. These devices would also face difficulties for the quantification of low abundant biomarkers due to the low sampling volume (~100 nL).

3.5.2.1 Reduction and alkylation

Conventional reduction and alkylation are normally carried out in solution, and an adequate reaction of the latter steps are often required in order to achieve successful tryptic digestion. Since the workflow in **paper V** aimed towards incorporating these steps into the sampling device, a high reaction rate was necessary since low reagent volumes, no agitation, and no temperature control were used. In similarities to Leipert et al.[233], the reagents had to be sequentially added with a respective drying step in-between. This did introduce a prolonged sampling time of ~20 – 30 min. per reagent, however, opened up for the use of different solution pH which for the most part is not achievable in conventional approaches in-solution. In addition to solution pH, different reducing agents alongside with concentrations of the reducing agent were investigated.

Reduction

From the investigation of in-device protein reduction, the major contributor for the reducing step was solution pH. DTT yielded the best performance of the reducing agents. The experiment was performed with a subsequent alkylation and tryptic digestion *in-solution*, to exclude bias from the latter steps according to the sampling procedure such as alkylation and tryptic digestion. In accordance with what Singh et al. [103] demonstrated for a rapid reduction of disulfide bonds, peak performance of the reducing agent was obtained with a pH of the reducing solution approximately matching the pK_a of the reducing agents' thiol group. This to maximize the thiol-disulfide interchange. The mechanisms of the latter has also been thoroughly elaborated by Péter Nagy[237]. For DTT (pK_a 9.2[103]) this was found between pH 9.2 and 10.1. Maintaining sufficiently high pH during the reduction was, however, a challenge since buffered solution could not be utilized due to potential ion suppression and the need for drastically changing the pH in-between reduction, alkylation, and protein hydrolysis. Therefore the reducing agent was dissolved in ammonia water with a pH on the higher side of the reported optimal conditions (pH 10.1). With a pH above 10, it was believed that the evaporation of ammonia did not alter the pH to sub 9 during the complete evaporation of the solvent. However, no significant differences were observed between pH 9.2 and 10.2 and therefore evaporation of ammonia was not considered troublesome (**Fig. 21a**).

Alkylation

In-device alkylation was investigated in similar fashion as the reducing step. The digestion step was performed in-solution in order to avoid bias and focus the results directly to the in-device reactions. Since the ammonia water was evaporated to dryness during the reduction step, alkylation could be performed at a different pH. The alkylating reagent, iodoacetic acid (IAC), has been suggested to give a maximum reaction yield if alkylation is carried out between pH 7-8[238]. However, it is also suggested that if the reaction is carried out between 7.0 and 7.5 over-alkylation could be troublesome and the reaction has to be carefully monitored[238]. Therefore, the *in-device* alkylation was carried out in ammonia bicarbonate (ABC) buffer (pH 7.8) with increasing concentrations of IAC to determine the threshold for over-alkylation. The concentration for maximum alkylation efficiency was in this experiment determined to be the concentration prior to where the MS signal dropped potentially due to over-alkylation or offsite-alkylation[106, 239] (alkylation of other amino acid residues than cysteine) ultimately resulting in different *m/z* values for the target peptide. The best results were obtained at 200 mM IAC (**Fig. 21b**).

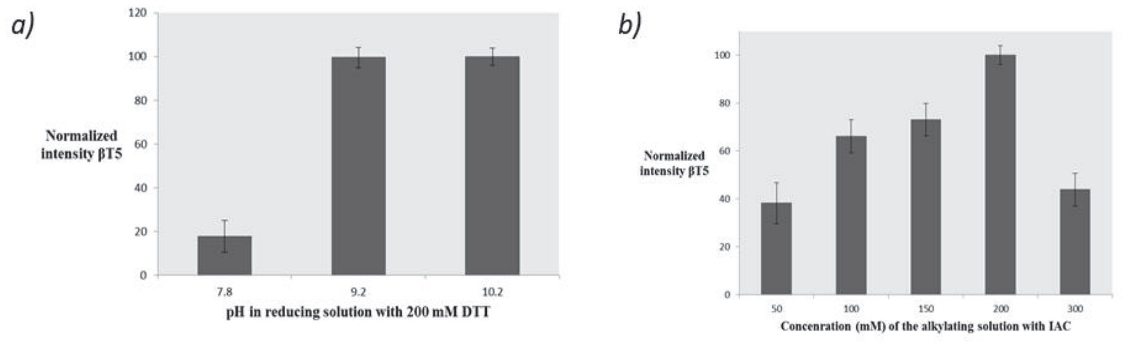

Figure 21 a) In-device protein reduction with DTT as reducing agent (20 µL) at varying pH in the reducing solution followed by in-spot alkylation (20 µL) by 200 mM IAC and b) In-spot protein reduction (20 µL) by 200 mM DTT at pH 10.2 followed by in-spot protein alkylation by IAC at varying concentrations (20 µL). The error bars represent the standard deviation, all experiments was carried out with n=4. Reproduced with permission from Fig. 3 paper V. Copyright © 2019, Rights managed by Elsevier

3.5.2.2 Protein digestion

As previously discussed in *section 1.4.7* applying high amounts of trypsin to a protein sample could be troublesome due to trypsin autolysis, secondary digestion kinetics and ultimately ion suppression despite the interesting results by Egeland et al.[240] on increased trypsin-to-protein ratio. At the same time, deploying trypsin immobilized polymer beads on top of the sampling paper (with a sample already dried into the paper structure) did not result in adequate digestion yield as discussed in *section 3.3.3.2*. Therefore, trypsin dissolved in ABC and subsequently applied to the sampling wells was carefully examined with respect to concentration, amount, and volume of solvent applied (to increase the reaction time). The pH in the digestion buffer was set to 7.8 in accordance with what has been proposed for the maximum proteolytic activity by others[241]. As initially expected, and in contrast to Egeland et al., increasing the trypsin concentration did inflict the digestion poorly (**Fig. 22a**). This could due to the autolysis products (which could compete with ionization in the ESI), however, since the analyses were run in SRM, autolysis was not further investigated. In order to confirm the theory, the same experiments should be performed in either DIA or DDA analysis in a future study. A discovery analysis would also be beneficial in order to determine the proteolytic reaction rate as well as characterizing potential modification of peptides exposed in the digestion buffer for an extended time (also causing signal decrease)[242]. Consequently, reaction time was investigated. This was carried out by applying various volumes of digestion buffer, however, with the same total amount of trypsin. From the latter experiment (**Fig. 22b**), no significant difference (two-sided t-test p>>0.05) was observed with regards to analyte signal intensity. These findings indicate that the protein digestion is performed within a short time, and additional reaction time is not necessary. This could be due to the low sample volume and thus, a low total amount of trypsin inhibitors to reduce the proteolytic activity naturally abundant in serum and whole blood[242, 243].

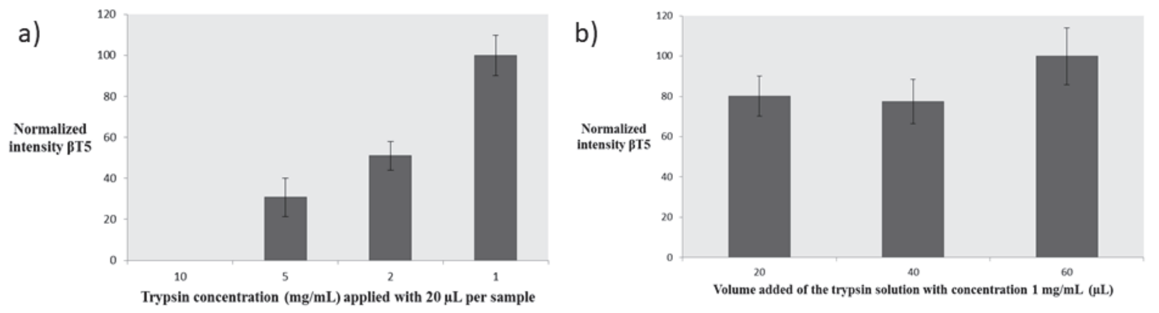

Figure 22 Normalized signal intensity for tryptic peptide βT5 digested in-device at a) different concentration of trypsin (V = 20 µL). The signal is normalized to trypsin concentration 1 mg/mL with respective average signal 2.7 * 10^3 MS counts per second. b) Different applied volumes with a fixed measure of 20 µg trypsin applied per sample. The signal is normalized to reaction volume 60 µL with respective average signal 3.4 * 10^3 MS counts per second. The error bars represent the standard deviation, all experiments was carried out with n=4. Reproduced with permission from Fig. 4 paper V. Copyright © 2019, Rights managed by Elsevier

3.5.3 Performance evaluation in human matrices

To prove that the all-in-one sampling concept (**paper V**) would be feasible to use in a realistic setting, spiked serum and freshly collected whole blood were sampled, prepared and analyzed with the optimized sample preparation conditions. Freshly collected whole blood was included in the experiments due to the concepts' potential use in POC. Additionally, since serum was exclusively used in **paper IV** performance comparisons were dominantly made to this matrix. Others have also previously stated that hCG (more specifically free ß-hCG) has improved stability by DMS sampling and therefore allows for comparisons to more general analytical methodologies[244].

3.5.3.1 hCG spiked serum

To compare the optimized sampling procedure to the previous work in **paper IV**, hCG spiked serum was analyzed from 10 ng/mL to 1000 ng/mL (five points). The concept was also sampled with 20 µL and 40 µL to characterize whether sampling volume would affect the optimized parameters with respect to the generation of the tryptic signature peptide ßT5. The concentration range for the experiments in **paper V** was also extended, compared to our initial efforts in **paper IV**. This allowed for a direct comparison in addition to more similarities to previous method developments on hCG sampled on DMS by others[87]. For the analysis sampled with 20 µL, thinner filter paper (Whatman® grade 1) was used to facilitate a direct comparison to **paper IV**. However, pHEMA-VDM coated DMPK-C cards were used when sampled with the higher volume since this paper substrate was proven more capable (*section 3.2.2.2*), at least for enzymatic digestion**.** Additionally, no signals were observed for blank samples (serum without external addition of hCG). From the experiments, a higher degree of correlation was observed when sampled by 40 µL (R^2=0.99) compared to 20 µL (R^2=0.98). Lower RSDs were also obtained when sampled with a higher sample volume. The RSD was under 26 % for all concentration levels and comparable to what Rosting et al.[87] demonstrated with hCG spiked serum sampled on conventional DMS. Overall, the implementation of wax printed cartridges (holding the sampling material) combined with less manual sample preparation steps, proved a significant increase in performance compared to our initial demonstration (**paper IV**).

LOD and LOQ

To assess the LOD and LOQ, 20 µL hCG spiked serum was applied to pHEMA-VDM coated (and subsequently immobilized) Whatman® grade 1 filter paper. The sampling volume and paper substrate was chosen to allow for a direct comparison to our findings in **paper IV**. From the investigation, the LOD (S/N=3) was experimentally demonstrated to be 10 times lower (0.9 IU/L, 100 pg/mL). Furthermore, it was also lower compared to what has previously been accomplished by DMS (7.8 IU/L) combined with immunocapture and LCMS[87]. Additionally, the concept demonstrated better detection limits compared to a fully validated methodology by Lund et al. [216] with large sample volumes (1.0 mL).

The LOQ (S/N=10) was from the LOD calculated to 3.0 IU/L (333 pg/mL). With comparisons to other paper-based immunoassays, the performance of the concept was similar, even though most of these methods have not demonstrated LOD in serum samples[228, 245]. Though, it must be stressed that the achieved LOD is still three times higher than the proposed reference limit (<0.3 IU/L) for healthy adults with regards to tumor development[246]. However, the sensitivity of the method would readily comply with specifications from the WADA controls (<5.0 IU/L)[245, 247]. It must be stressed that WADA is only measuring intact hCG, while E27 capture both the free and intact ß-hCG. This requirement could be solved by immobilizing a different antibody.

3.5.3.2 hCG spiked whole blood

The *all-in-one sampling chip* showed promising performance in serum samples for fast and targeted sample preparation of the low abundant biomarker hCG. However, if the concept were to be applied in a POC setting, the performance needed to be similar in freshly collected whole blood. The latter because the POC strategy would rely on finger- or heel pricks. To assess the performance in whole blood, freshly collected whole blood was spiked with hCG from 10 – 1000 ng/mL (five concentration points), sampled and analyzed accordingly. The results (**Fig. 23**) showed a high correlation (R^2=0.99) with satisfactory RSD values. LOD and LOQ were obtained at 630 pg/mL and 2.10 ng/mL, respectively. No signal was observed by analysis of blank samples (whole blood without external addition of hCG). From the demonstration, the sampling concept showed promising performance, however, adoptions had to be made compared to the sampling of serum. Whole blood faced encrustation of the sample droplet hindering the sample of a smooth drying rate. Additionally, this crust could potentially cause problems if the target analyte were trapped in the solid part. This would ultimately lead to reduced interaction between the mAb and proteins and thus, untrue low reporting. It may also have contributed to the lower sensitivity compared to serum. However, this issue could potentially be solved if the sampling cartridges were fabricated with deeper wells facilitating additions of solvent for decreasing the sample viscosity during the drying/interaction period. Nevertheless, compared to previous efforts on hCG determination by MS with DBS, the sensitivity was similar[87]. However, with significantly less time consuming and laborious methodology.

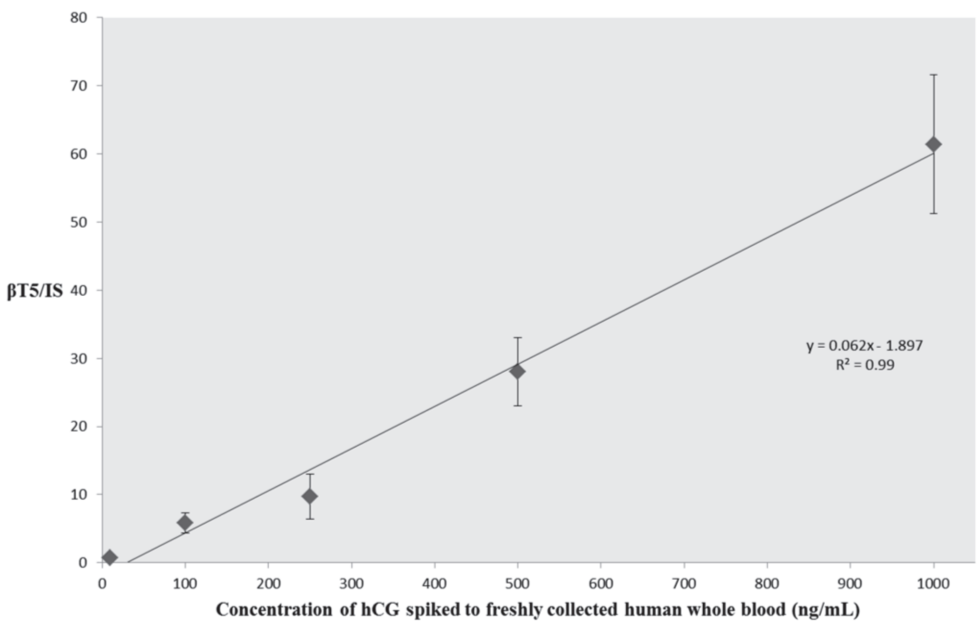

Figure 23 Five-point concentration curve from 10 to 1000 ng/mL of hCG spiked to human whole blood. Thirty micro liter spiked whole blood (freshly collected) applied to pHEMA-VDM modified and mAb E27 immobilized Whatman® DMPK-C cards. Reproduced with permission from Fig. 6 paper V. Copyright © 2019, Rights managed by Elsevier

3.5.4 *In-device immunocapture*, a perspective

In **paper IV** and **paper V**, it was demonstrated that in-device immunocapture of low abundant proteins was readily achievable. The concept utilized the polymer coating introduced in **paper II** for covalent immobilization of mAb directly to the sampling material. The fundamental investigations regarding the choice, and the need, of a polymer layer, was demonstrated in **paper IV** alongside with the immobilization strategy. The applicability of the sampling strategy was assessed by analyzing hCG spiked serum. The second iteration of the sampling concept was introduced in **paper V** with all steps required to perform bottom-up protein determination facilitated in the sampling device. These steps were immunocapture, protein reduction, alkylation, and tryptic digestion. The second iteration introduced a simplified sample preparation with low time consumption suited for a POC strategy. The results in **paper V** were comparable, or better than what has previously been achieved on targeted analysis of low abundant biomarkers sampled on paper. However, with a significantly lower time consumption and ease of use. The sensitivity was also demonstrated to be comparable, or better, compared to commercially available ELISA kits. Nevertheless, in compliance with other efforts on fully automated sampling devices, there are limitations. These limitations include the difficulty to immobilize multiple different reagents into the device without external additions as mAb are not readily compatible with proteases, reducing and alkylating reagents. This remains a challenge for future efforts. Regardless, the all-in-one sampling concept showed the potential to accelerate the current methodology of setting a diagnosis. If combined with a surface desorption technique such as MALDI[248], MAI[249], DESI[250] or paper spray[141], the concept could also be used with high throughput eliminating the need for chromatography. In a future perspective, the concept of integrating mAb into sampling materials to accelerate protein analytic workflow seems to be feasible if more commercial packaging is introduced, as discussed in *section 3.3.4*.

4 Concluding remarks and future perspective

In this thesis, the development, fabrication, and application of paper-based smart sampling for the bottom-up proteomic strategy has been demonstrated. The paper-based sampling format was integrated with key elements (such as protein hydrolysis and immunoaffinity) required to perform qualitative (non-targeted) as well as quantitative (targeted) determinations of low abundant biomarkers in a simple sampling format. The presented concept was aimed towards the point-of-care strategy for consumer-based sampling with simple execution and high analytical performance.

In **paper I-III**, immobilized trypsin was explored to enable instant protein digestion during the sampling stage, eliminating over-night in-solution protein digestion. These papers proved that it was readily possible to reverse the conventional proteomic workflow, i.e. performing protein hydrolysis prior to protein modification. In **paper I**, the fundamental challenges with regards to performing the proteolysis (with immobilized polymer beads deployed on the sampling material) prior to protein modification was addressed. From the exploration, protein reduction and alkylation could readily be performed post hydrolysis in contrast to prior hydrolysis. The stability of storing peptides, rather than intact proteins *on-paper*, was also proven satisfactory. The sampling concept was further polished in **paper II**, where the sampling paper was coated with a polymeric layer for covalent immobilization of trypsin directly to the sampling material. The performance of the sampling strategies in **paper I** and **paper II** was demonstrated in non-targeted (DDA) analysis of whole blood and compared to a conventional dried blood spot procedure. The initial concept in **paper I** showed similar performance (the number of unique tryptic peptides and protein groups) to conventional DBS. However, the performance was near doubled with the polymer-coated sampling device in **paper II**. The optimized sampling material was ultimately proven in a targeted analysis of the low abundant serum biomarker proGRP in **paper III**. **Paper III** combined the developments in **paper II** with peptide immunoaffinity for fast and selective sample cleanup. The sampling concept combined with peptide immunoaffinity extraction showed promising performance for future clinical applications.

In **paper IV** and **V**, smart sampling of proteins *on-paper* was demonstrated in the conventional workflow i.e. sample clean-up of intact protein prior to protein hydrolysis. By immobilizing the polymeric sampling material from **paper II** with monoclonal antibody E27 (targeting hCG), the sampling concept enabled *in-device immunocapture*. **Paper IV** was a proof-of-principle study and showed a high degree of performance as well as applicability to patient samples. Intraday and interday precision, as well as accuracy, was proven satisfactory. **Paper V** demonstrated an all-in-one paper-based sampling format. The optimized fabrication and immobilization principles from **paper IV** was applied. The sampling format in **paper V** allowed for *in-device immunoaffinity* followed by *in-device protein reduction*, *alkylation*, and *tryptic digestion*. The sampling concept was demonstrated with up to ten times lower detection limits. The sampling device also showed more than six times faster sample preparation compared to what has previously been reported for analysis of hCG in human serum sampled *on-paper*.

Future perspective

In this thesis, it has been demonstrated that paper-based sampling materials immobilized with trypsin or mAb could simplify the current bottom-up strategy for low abundant biomarkers. By utilizing smart and affordable sampling concepts, MS could readily be incorporated into the POC strategy. It could therefore be expected that future optimization of immobilized sampling devices have the potential to streamline the current methodology of protein analyses. Especially, if combined with direct ionization techniques such as MALDI. In a short-term aspect, optimization regarding the polymer composition should be investigated alongside the fundamental kinetics of the immobilization strategies. The latter will be important to characterize the amount of trypsin and mAb immobilized and their respective activity. It would also be of interest to further investigate wheatear or not autolysis is present when the sampling concepts are used for targeted analyses. The sampling strategy should also be investigated with respect to validation. This in order to further prove the capabilities of the sampling strategy. Finally, other materials than paper should be investigated in order to be closer to a commercial product form. Absorption of a fixed volume similar to VAMS by Neoteryx, would also be relevant for the concept since many POC applications do not rely on fixed volume applications. Initial demonstrations of the latter has been explored by modifying existing technologies such as VAMS by Neoteryx (unpublished work), in addition to conventional Q-tips compatible with swab sampling (unpublished work).

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Talanta 195 (2019) 764–770

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Paper-based immunocapture for targeted protein analysis

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ARTICLE INFO

Keywords:
Smart-sampling
On-paper immunocapture
Mass spectrometry
Bottom-up protein analysis
Dried matrix spots

ABSTRACT

A novel sampling concept for mass spectrometric bottom-up targeted protein analysis is here demonstrated with polymeric sampling spots integrated with instant immunocapture for analysis of dried matrix spots. The polymers 2-hydroxyethyl methacrylate-*co*-2-vinyl-4,4-dimethyl azlactone (pHEMA-VDM) and pHEMA-Tosyl for covalent attachment of antibodies where investigated alongside with adsorption on non-treated filter paper. From performance characterization, the pHEMA-VDM had the best performance. The sampling spots demonstrated fast and easy sampling and preparation of human serum spiked with the biomarker human chorionic gonadotropin. The sampling spots enabled a detection limit of 1 ng/mL (26.4 pM) within a five point concentration curve from 1 ng/mL to 20 ng/mL ($R^2 = 0.97$). The detection limit was demonstrated to be two times lower than previously demonstrated with standard DMPK-C sampling cards. A five point concentration curve from 100 ng/mL to 2000 ng/mL was also investigated ($R^2 = 0.998$). Intra day precision was within 16% and 23% for concentration range 1 ng/mL to 20 ng/mL and 100 ng/mL to 2000 ng/mL, respectively. Inter day precision was within 20%. Accuracy was determined to 10% and 11% for 2.5 ng/mL and 20 ng/mL, respectively. The sampling spots were also demonstrated in a realistic setting where serum samples from two confirmed patients with testicular germ cell cancer were analyzed. These analyses confirmed an elevated hCG content in the sera of 418.5 ± 4.2 ng/mL and 21 ± 0.02 ng/mL hCG for patient one and two respectively.

1. Introduction

During the past few decades paper-based immunoassays have rapidly gained popularity for a wide array of sample matrices and analytes e.g. neuropeptides [1], virus [2] and tumor markers [3]. These immunoassays, often fabricated by antibodies adsorbed to filter paper, have proven to be fast, simple and cost effective alternatives to conventional ELISA and are most often combined with colorimetric detection [2,4] for on-site test results. Other means of detection have also been reported such as electrochemical detection [5] and electrochemiluminescence [6,7]. The development of paper-based immunoassays and other microfluidics related to the interest in *point-of-care* diagnostics have the potential to simplify and accelerate the current workflow of setting a diagnosis. However, immunoassays with colorimetric detection do have a risk of reporting both false positive and false negative [8,9]. This is a challenge that mass spectrometry (MS) could resolve [10]. We have previously developed smart sampling spots where tryptic digestion is incorporated with paper sampling for fast and easy sampling of whole blood with instant proteolysis *on-paper* [11]. The polymer backbone of the proteolysis reactors holds a potential for immobilization of antibodies covalent for instant immunocapture. Incorporation of covalently bound antibodies to the sampling devices allows to streamline the current workflow of biological sample collection and preparation prior to MS analysis of low abundant proteins. Furthermore, it holds great potential for more cost effective shipping, sample preparation (significantly reduction in on-bench laboratory time), accessibility to sampling in remote areas (sample stability when dried [12]) and reduction in animal bleeding in e.g. preclinical studies [13]. Efforts in combining immunocapture directly to MS detection using paper have previous been published [14,15]. Chen et al. [15] reported indirect determination of protein biomarkers through a sandwich assay containing a cleavable probe for MS-detection. Although quantitative data were obtained, this set-up will suffer of the same susceptibility to false results as conventional immunoassays [16]. Zhang et al. [14] uses an antibody based cartridge system for immunocapture prior to paper spray MS for intact protein detection from diluted plasma and demonstrated detection limits in the sub µg/mL-µg/mL (aqueous standard solutions). The advantage of our proposed set-up is the combination of paper-based immunocapture with bottom-up analysis. By not relying on a tracer antibody the number of false results is greatly reduced, in addition the bottom-up workflow generally provides better sensitivity than when detecting intact

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https://doi.org/10.1016/j.talanta.2018.12.013
Received 8 October 2018; Received in revised form 5 December 2018; Accepted 7 December 2018

proteins. The aim of the study described in this paper is to develop a simple, high performing and cost effective sampling concept where selective protein capture by immunoaffinity is combined with paper sampling of human serum in a "lab-on-paper format" followed by bottom-up mass spectrometric protein determination. This proposed set-up reduces false results compared to sandwich assays and improves sensitivity compared to MS based intact protein determination. Different antibody immobilization strategies were evaluated using the biomarker human chorionic gonadotropin (hCG) as the model protein. The sampling formats investigated where un-treated filter paper and two polymer coated filter papers for adsorption and covalent attachment, respectively. The smart sampling spots were evaluated with respect to efficiency of antibody immobilization, non-specific binding of target analyte and capture efficiency. Finally, the quantitative performance (linearity, precision, accuracy and quantification and detection limits) was evaluated for the best performing sampling variant. The results were confirmed by analysis of serum form patients previously diagnosed with testicular tumor (contains elevated levels of hCG). To our knowledge, this is the first report of paper-based immunocapture as "smart sampling" combined with bottom-up mass spectrometric detection.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (99.9%, (ACN)), pyridine (Ph. Eur. grade), boric acid (99.8%), Sodium phosphate dibasic dehydrate (99–102%), Sodium chloride (≥ 99.5%), hydrochloric acid (37%), iso-propyl (HPLC grade), Methanol (HPLC grade) and di-sodium hydrogen phosphate anhydrous (99–102%) were purchased from Merck (Darmstadt, Germany). Formic acid (≥ 98%, (FA)), iodoacetic acid (≥ 98%, (IAA)), DL-dithreitol (≥ 99.5%, (DTT)), N,N-dimethylformamide anhydrous (DMF), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS, 98%), 1-heptanol, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 2-hydroxyethyl methacrylate (HEMA, 97%, containing 200–220 ppm monomethyl ether hydroquinone) and initiator 2,2´azobis(2-methylpropinonitrile), 2,2′-azobis (2-methylpropionitrile) (98%, (AIBN)), brilliant blue R (250, for microscopy), ammonium bicarbonate (ABC, ≥ 99.5%), tween 20®, bovine serum albumin (BSA, ≥96%) and trypsin from bovine pancreas TPCK treated (≥ 10,000 BAEE units /mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glacial acidic acid (≥ 99.7%), ethanolamine (≥ 99%), borax anhydrous (99.5%), trizma® base (≥ 99.9%), 2-Vinyl-4,4-dimethylazaltone (VDM) was purchased from Polysciences Inc. (Warrington, PA, USA). Water used in this experiment was filtrated through a Merck Millipore Milli-Q intergral 3 water dispenser (resistivity: 18.2 MΩ cm^{-1}). Monoclonal antibody E27 was donated by the Department of Medical Biochemistry, Oslo University Hospital (Oslo, Norway). Internal standard AQUA™ peptide (amino acid sequence: (VLQGVLPALPQVVCNY[$R\_^{13}C_{6}\_^{15}N_4$]) was purchased from Sigma-Aldrich. Ovitrelle® (Merck, London, UK) was used as source for hCG and was purchased from the local pharmacy. Human serum was obtained from healthy volunteers from Oslo University Hospital Ullevål (Oslo, Norway). Serum samples from patients diagnosed with testicular cancer were donated with patient acknowledgement from the Department of Medical Biochemistry, Oslo University Hospital.

2.2. Fabrication of sampling spots

2.2.1. Wax printing

Preparation of the wax printed filter papers was carried out as previously reported by Skjærvø et al.[12] In short: GE Healthcare Life Sciences (Buckinghamshire, UK) Whatman® Grade 1 filter paper was cut to A4- sheets and printed with a Xerox (Norwalk, CT, USA) Color Qube 8580 printer and Color Qube 8570 wax. The printed sheets were heated at 110 °C for 15 min and subsequently cooled down prior to use.

2.2.2. Preparation of pHEMA-VDM sampling spots

Fabrication of pHEMA-VDM sampling spots were carried out as previously described by Skjærvø et al. [11] on Whatman® grade 1 filter paper. In short: filter paper circles (6.0 mm ID) were punched out with a Philip Harris (Birmingham, UK) Unicore 6.0 mm and subjected to pretreatment in 1.0 M NaOH for 15 min at 600 rpm and ambient temperature. After pre-treatment silanization and polymerization was carried out at 80 °C and 100 °C for two and five hours respectively. The sampling spots were washed with ACN and dried prior to immobilization.

2.2.3. Synthesis of pHEMA-Tosyl sampling spots

Fabrication were initiated by silanizing Whatman® grade 1 filter paper with a subsequent homopolymerization of HEMA with the same work flow as previously described for the pHEMA-VDM polymer. The polymerization solution consisted of 300 mg HEMA, 0.005 g AIBN and 300 mg 1-heptanol. Tosylation was carried out as previously described by Albayrak et al. [17] for three hours. The mixture was stirred in a glass beaker at ambient temperature with 1:4 parts of tosyl-chloride in pyridine. Excess amounts of pyridine in the polymer sampling spots were removed by 5 mM HCl. The sampling spots were stored cooled in the wash solution prior to immobilization.

2.2.4. Antibody immobilization

Immobilization of antibody E27 was carried out as previously described by Paus and Nustad [18]. In short: non-treated filter paper or polymeric sampling spots (pHEMA-VDM or pHEMA-TsCl) were placed in individual protein LoBind tubes with 100 µL 0.1 M borate buffer (pH 9.6) containing 0.5 mg/mL monoclonal antibody E27. The tubes were mixed at 37 °C and 800 rpm for 20 h. The solution was replaced with 100 µL 1.0 M ethanolamine with 0.1% Tween 20® (pH 9.5) and mixed at 25 °C and 800 rpm for two hours. The solution was further replaced by 100 µL 0.1 M Tris-hydrochloride buffer (Tris-HCl) (pH 7.0) containing 0.1% Tween 20® (v/v) and 0.1% BSA (W/v) (pH 7.0) and mixed at 25 °C and 800 rpm for 30 min. The sampling spots were finally washed with 15 µL of 0.1 M phosphate buffer (pH 7.5) and dried in room temperature prior to use. The ready to use sampling spot was placed back in the wax circle. The wax circle confined the sample within the sampling spot.

2.3. Characterization

2.3.1. Characterization of polymer coating

Un-treated filter paper and polymeric filter paper was mixed separately in 1.0 M NaOH for 30, 60 and 90 min before subsequent removal of liquid. Characterization was carried out using a Leica (Wetzlar, Germany) Zoom 2000 optical microscope for visual evaluation of differences.

2.3.2. Adsorption by brilliant blue staining

Characterization of protein absorbance was carried out as previously described by McCann et al. [19] using BSA as adsorbant. In short: Three six mm ID filter paper circles were incubated with BSA for 30 min at 800 rpm at ambient temperature before a subsequent blotting on filter paper to remove excess solution. The circles were applied with 15 µL 50 mM ABC to wick out the unattached BSA before incubation at 80 °C for ten minutes. Thereafter, the circles where soaked in 0.1% (w/v) brilliant blue solution (50/40/10:MeOH /Milli-Q water/glacial acetic acid (v/v/v)) for ten minutes. The excess solution where removed with 10 mL (50/40/10:MeOH /Milli-Q water/glacial acetic acid (v/v/v)) agitated for one hour at 800 rpm with a removal and subsequent addition of new solution after 30 min. The circles were dried at room temperature before characterization.

2.4. Sample preparation

Sample application was performed by pipetting 20 µL sample (hCG spiked to 0.1 M phosphate buffer, 0.1 M phosphate buffer w/ 0.01% BSA, or human serum) and subsequently dried at ambient temperature. The polymeric sampling spots (and un-treated filter paper if specified) were washed with 500 µL phosphate buffered saline (PBS) with 0.05% Tween 20®. The wash solution was removed and replaced in the following order by: 500 µL PBS, 400 µL 10 mM Tris-HCl and 300 µL 50 mM ABC. During each washing step the sampling spots were vigorously mixed. After the final washing step 100 µL 50 mM ABC was added to the paper and subsequently reduction, alkylation and tryptic digestion were performed (without removing the sampling spot from the Eppendorf tube).

2.4.1. Reduction, alkylation and tryptic hydrolysis

Reduction was carried out by adding 10 µL 100 mM DTT in 50 mM ABC to each extract and mix at 37 °C and 800 rpm for 15 min. Ten microliter 200 mM IAA in 50 mM ABC was added and agitated a 25 °C and 800 rpm in the dark for 15 additional minutes. Tryptic hydrolysis was performed by addition of 10 µL trypsin (1.0 mg/mL in 50 mM ABC) and agitated at 37 °C and 1150 rpm for 16 h. If specified, 10 µL (50 pmol/mL) internal standard (VLQGVLPALPQVVcNY[R_^{13}C$_6$_^{15}N$_4$) AQUA™ peptide was added after tryptic digestion. The AQUA™ peptide was reduced and alkylated prior to addition as described by Lund et al. [10].

2.5. Analysis of patient samples

Serum samples from two patients previously diagnosed with testicular cancer were used in this work. All samples were stored at – 30 °C prior to analysis. Professor Elisabeth Paus at the Norwegian Radium Hospital (Oslo University Hospital, Oslo, Norway) is acknowledged for providing the patient samples and the monoclonal antibody E27 used for capturing of hCG. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for research involving human participants. Informed consent has been obtained from the participants involved. For analysis: twenty microliters of serum were spotted to E27 immobilized pHEMA-VDM sampling spots. Sample preparation and analysis was carried out as described elsewhere.

2.6. LC-MS analysis

2.6.1. Micro LC-MS analysis

The chromatographic separation was carried out with a Dionex (Sunnyvale, CA, USA) Ultimate 3000 pump module coupled to a Thermo Fischer (Waltham, MA, USA) BioBasic C8 (ID: 1.0 mm, L: 50 mm, d_p: 5.0 µm). The chromatographic separation was performed on 10 µL injected sample at 50 µL/min over 13 min from 0% to 90% mobile phase B (ACN/water/FA:95/5/0.1) followed by seven minute wash and a subsequent reconditioning for 10 min with mobile phase A (ACN/water/FA:5/95/0.1). Detection was performed with a Thermo Fischer TSQ Quantum Access operated in SRM with positive mode ESI. Applied voltage was set to 4 kV, heated capillary: 270 °C and CID with 0.5 scan per second scan interval. The system was operated by Dionex Chromeleon Xpress and Thermo Fischer Xcalibur 2.2 (version: SP 1.48). Micro LC-MS was used for initial experiments according to evaluation of antibody adsorption experiments, covalent immobilization conditions and evaluation of polymer layers.

2.6.2. Nano LC-MS analysis

The sample loading and chromatographic separation was performed with a Dionex Ultimate 3000 RSLC nano pump module. Injected sample (1 µL) was loaded onto a Thermo Fischer Acclaim™ PepMap™ C18 (ID: 300 µm, L: 5 mm, d_p: 5.0 µm) trap column over three minutes at 30 µL/min with 2% ACN and 0.07% FA. The sample was subsequently back flushed onto the separation column. Separation was carried on a Thermo Fischer Acclaim™ PepMap™ C18 (ID: 75.0 µm, L: 150 mm, d_p: 3.0 µm) over 25 min from 5% to 95% MP B (ACN/water/FA:95/5/0.07) with one minute hold at 95% MP B prior to subsequent reconditioning for 13 min with MP A (ACN/water/FA:5/95/0.07). Detection was performed with a Thermo Fischer TSQ Quantiva operated in SRM positive mode nano ESI with 2.25 kV applied voltage. The MS parameters were set as followed: 350 °C ion transfer tube, 30 V collision energy, 1.5 mTorr CID gas (Ar), 1 s scan cycle and Q1 and Q3 resolution at 0.7 and 0.7 at FWHM, respectively. This instrumental set-up was used for all quantitative experiments.

2.6.3. SRM transitions

Quantification was carried out by measuring fragment ions m/z 1036.3 (y_8^+) and m/z 1317.8 (y_{11}^+) for signature peptide ßT5 (VLQGVLPALPQVVcNYR – precursor ion m/z 964.2^{2+}) and fragment ions m/z 1046.3 (y_8^+) and m/z 1327.8 (y_{11}^+) for the internal standard (VLQGVLPALPQVVcNY[R_^{13}C$_6$_^{15}N$_4$] – precursor ion m/z 969.3^{2+}). All transitions were carried out with 30 V of collision energy.

3. Results and discussion

3.1. Initial experiments

3.1.1. Adsorption

Initial experiments evaluated immobilization of antibodies based on adsorption to un-treated paper. A method for on-paper immunoassay integrated with colometric detection [2] was slightly modified and tested with respect to mass spectrometric applications. The procedure for preparation of these filter papers and the conditions for subsequent application and analysis of the model protein hCG is described in Supplementary material (S1).

From the initial analysis it was apparent that hCG was captured *on-paper*. However, by comparison with sampling spots without applied antibodies the MS response from the proteotypic peptide, ßT5 did not give any significant difference in signal intensities (two-sided; *t*-test $p > > 0.05$ between the sampling spots with and without adsorbed antibody)(data not shown). To reduce non-specific binding the washing procedure was altered (tenfold increase in wash solution) and amount of BSA in blocking buffer (0%, 0.01% and 0.1%) was evaluated, none of which resulted in a reduction of non-specific binding. In addition, the use of increased volume of washing buffer seemed to affect the capture of the model protein negatively (data not shown).

The effect of BSA for blocking purposes was shortly evaluated. Without BSA in the blocking buffer a 20% signal reduction for ßT5 compared to use of a buffer with 0.01% BSA was observed. By increasing the BSA amount from 0.01% to 0.1% the signal intensities was additionally 10% lower. Therefore, a blocking buffer with 0.01% BSA was most likely the ideal composition. Consequently, a conclusion was made that the adsorbed antibodies did not have sufficient performance.

3.1.2. Evaluation of antibody attachment to un-treated paper and polymeric sampling spots

Based on our initial study with adsorption of antibodies to wax printed sampling spots, a stronger binding of the antibodies was desirable. Therefore, polymeric sampling spots previously used for covalent attachment of trypsin [11] were investigated. The protein-polymer interaction was assessed using a previously described staining procedure [19]. The attachment of proteins to polymeric sampling spots and un-treated Whatman® grade 1 filter paper was visually inspected after staining with brilliant blue. In this assessment, BSA was used to model the protein binding since BSA has been shown covalent attachment to pHEMA-VDM polymer layers [20]. From the procedure it was apparent that different mechanisms of protein attachment occurred to un-treated and polymeric sampling spots (Fig. 1). Un-treated filter papers showed

BSA	Un-treated filter paper	pHEMA-VDM filter paper
0%		
0.01%		
0.1%		
1.0%		

Fig. 1. Brilliant blue staining of BSA immobilized to un-treated- and pHEMA-VDM coated Whatman® Grade 1 filter paper.

a less uniform attachment of protein with the "coffee ring effect" [21] clearly visible indicating weak attachment. This effect was not visible on the pHEMA-VDM treated filter paper. The pHEMA-VDM sampling spot showed a more uniform distribution of protein throughout the sampling spot. Different levels of proteins were tested and the color intensity of both variants was increasing with respect to higher protein content. A blank sample was also included to prove that brilliant blue did not interact with surfaces without immobilized protein. All in all, the polymeric coating showed a uniform immobilization of protein and thus, it could be assumed that the attachment mechanism was covalent, and would also be covalent for the antibodies. Therefore, further development was carried out with the polymeric sampling material.

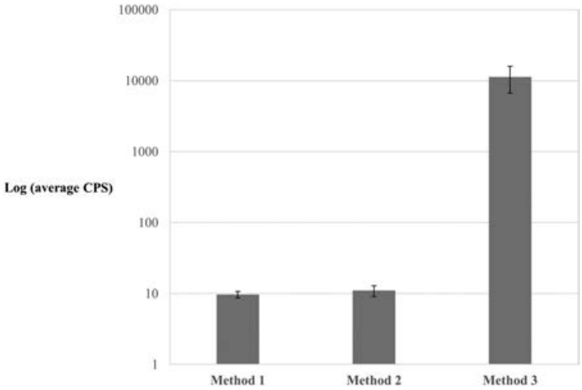

Fig. 2. hCG intensity by means of βT5 signal intensities for the three immobilization methods of mAb E27 to pHEMA-VDM polymerized Whatman® grade 1 filter paper. The signal intensity is presented with counts per second (CPS). Error bars are presented as standard deviation.

3.1.3. Evaluation of antibody immobilization methods for pHEMA-VDM sampling spots

To determine whether or not antibodies could be adequately immobilized onto the pHEMA-VDM sampling spots, hCG targeting monoclonal antibody E27 was immobilized by three different methods prior to sample application. Immobilization was initially carried out by agitation at 800 rpm for three hours at 25 °C. The temperature was chosen from previous work by others [22,23]. Sampling was carried out by application hCG (20 µg/mL) in phosphate buffer. The washing steps after sample application were performed as described earlier followed by a subsequent reduction, alkylation and tryptic digestion. This method is referred to as method 1. The method showed viable signal intensity (Fig. 2). However, limit of detection was poor. A different immobilization temperature and time (method 2) was therefore investigated and the process was carried out at 4 °C for 18 h. All other parameters were kept constant. Method 2 performed similar to method 1. Given the staining characterization, it is reasonable to assume that the antibody indeed was immobilized to the pHEMA-VDM sampling spots. However, the antibodies could have been attached in a less defined orientation, yielding lower performance and thus, not interacting sufficiently with the target protein [24]. The orientation of the antibodies to the sampling spot was considered important since the sampling spots have a static surface area similar to other means such as columns [25,26], however, with a static sample layer. Thus, the antibodies needed to be arranged in a way that the analyte could easily bind instantly as the sample wick throughout the spot. According to the latter immobilization conditions a third immobilization method (method 3) proven by Nustad and Paus [18] was investigated as previously demonstrated on TsCl activated magnetic beads. Included in this method was orientation of antibodies by acidic conditions with HCl as demonstrated by Lund et al. [10]. Fig. 2 showed that immobilization method 3 was superior regarding signal intensity (even though the concentration of hCG in the sample was very high). Consequently, the correlation regarding signal and concentration was investigated between 100 and 2000 ng/mL (five concentration levels). A satisfactory linearity (n = 3, R^2 = 0.996) (S2) was obtained. Therefore, immobilization method 3 was chosen for further experiments.

3.2. Evaluation of sampling polymers

Besides antibody immobilization through adsorption to un-treated filter paper and covalent binding to pHEMA-VDM, tosyl synthesized pHEMA sampling spots was developed. The new polymer composition was investigated since tosyl chloride has previously been demonstrated as an excellent leaving group for immobilization of enzymes, proteins and antibodies to materials such as beads [10] and cellulose [17]. These three antibody immobilization strategies were compared in order to evaluate if the new immobilization procedure had positive effect on non-specific binding and capture efficiency of the model protein. To determine performance differences for the different materials, a five point concentration curve from 100 to 2000 ng/mL spiked hCG in phosphate buffer containing 0.1% BSA was analyzed (n = 3). Additionally, the same sample-set was analyzed on sampling materials without immobilized antibodies (blank material). From the analysis, the pHEMA-TsCl sampling spots produced higher signal intensities than the pHEMA-VDM spots. However, a significant increase in signal intensities (two-sided; *t*-test $p \ll 0.05$) was observed compared to the pHEMA-VDM material for the analysis carried out on blank material (i.e. no immobilized antibodies). Thus, a higher degree of non-specific binding of hCG to the pHEMA-TsCl was observed. For the non-treated filter paper the signal intensities were low and no major differences in signal intensities were observed for the immobilized and blank sample spots. These results, however, might not represent a”real sample scenario” since serum and other biological matrices naturally contain high concentration of proteins that would mask / compete with potential non-specific binding sites. Therefore, spiked serum samples (n = 3) from 100 ng/mL to 2000 ng/mL (five concentration levels) were analyzed in order to further evaluate the most suitable sampling material. The evaluation of the various polymer types and non-treated filter paper was determined based on the following factors: signal intensity of proteotypic peptide (ßT5), the amount of non-specific interactions to the polymer layer and ease of fabrication. From the analyses (Fig. 3) no significant difference (two-sided; *t*-test $p > > 0.05$) was observed for the pHEMA-VDM and pHEMA-TsCl materials. Blank sampling spots (sampling spots without immobilized antibodies) were also analyzed (n = 3) with spiked serum in the same concentration range to determine the degree of non-specific interaction. The degree of non-specific binding in these blank sampling spots types did not show any significant differences between the ßT5 signal intensities at concentration levels below 2000 ng/mL (two-sided; *t*-test $p > > 0.05$). In fact no defined peak (containing both fragments) for ßT5 was observed in any of the concentration levels for the pHEMA-VDM sampling spot (w/o antibody). However, a signal for ßT5 was observed for the highest concentration level when sampled on the pHEMA-TsCl polymer w/o antibody (Supplementary S3). This could possibly been due to protein interaction with the leaving group since this group was left un-interacted i.e. not immobilized at the time of sampling. The pHEMA-VDM polymer was chosen for further experiments due to the less time invasive fabrication and lower HSE risk under fabrication.

3.3. Evaluation of quantitative performance

To demonstrate the quantitative performance of the sampling spots an inter day verification of linearity and inter day precision was carried out using a nano LC-MS system. The verification was carried out with three sample sets (n = 3) in serum, sampled and prepared at three different days with internal standard added after tryptic digestion. Both medium to high concentration levels were investigated (100–2000 ng/mL) as well as investigation of intra day performance in the low concentration range (1–20 ng/mL). The medium to high concentration range was studied in order to get an impression on the performance of the sampling spots at high target protein load. Additionally, since most protein biomarkers are expressed in a wide concentration range (< 5 ng/mL to 5 μg/mL for hCG depending of disease [27]) a five point concentration curve was also investigated in the lower range (1–20 ng/mL hCG spiked in human serum). The low concentration range covered the more realistic hCG levels.

The inter day linearity in the medium to high concentration level (100–2000 ng/mL, 5 concentration levels) was reported with a correlation factor of 0.998 (Fig. 4). Inter day precision was reported to 20%, 3%, 11%, 13% and 16% for 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 2000 ng/mL, respectively.

From the analysis of the samples in the low concentration range a correlation factor of 0.97 was obtained (Fig. 5). Intraday precision was reported to 16%, 15%, 14%, 11% and 7% for 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL and 20 ng/mL, respectively. Intraday precision was all within 23% in the medium to high concentration range. Accuracy (independently prepared samples) was tested at selected concentration levels and reported to 10% and 11% for 2.5 ng/mL and 20 ng/mL and 2% and 14% for 250 ng/mL and 1000 ng/mL, respectively. Limit of detection (3 S/N) was experimentally obtained at 1 ng/mL (26.4 pM) (S4), two times lower than what has previously been reported from our group with DMPK-C as sampling material for hCG spiked serum [28]. The LOD concentrations were included in the intraday linearity since their signal intensities showed to have a CV < 20%.

Considering the fact that this work is a conceptual study, the intraday linearity, inter day linearity (repeatability), inter day precision and accuracy was evaluated to be acceptable.

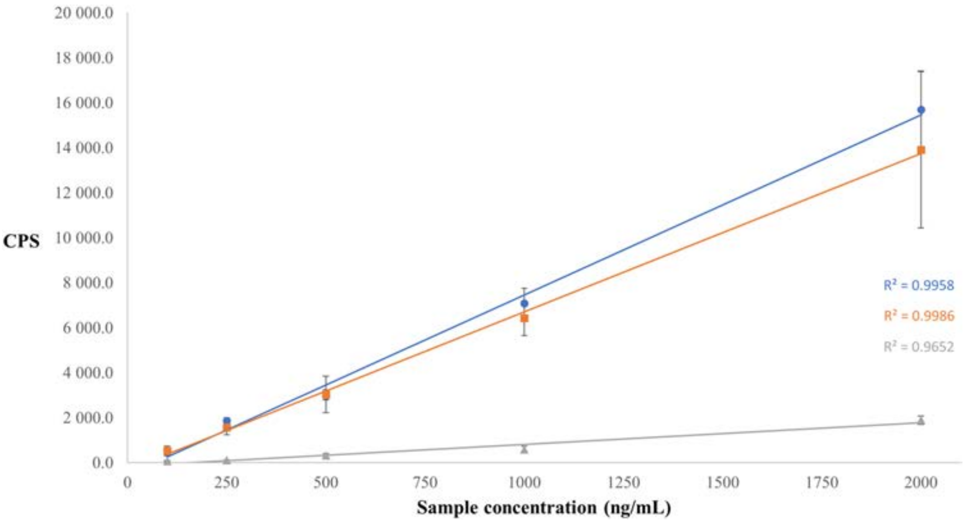

Fig. 3. Analysis of spiked serum in concentration 100, 250, 500, 1000 and 2000 ng/mL hCG on antibody immobilized pHEMA-VDM (Blue) and pHEMA-TsCl (Orange) reactors. The grey data points represent antibodies adsorbed to non-treated filter paper. Error bars are presented as standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

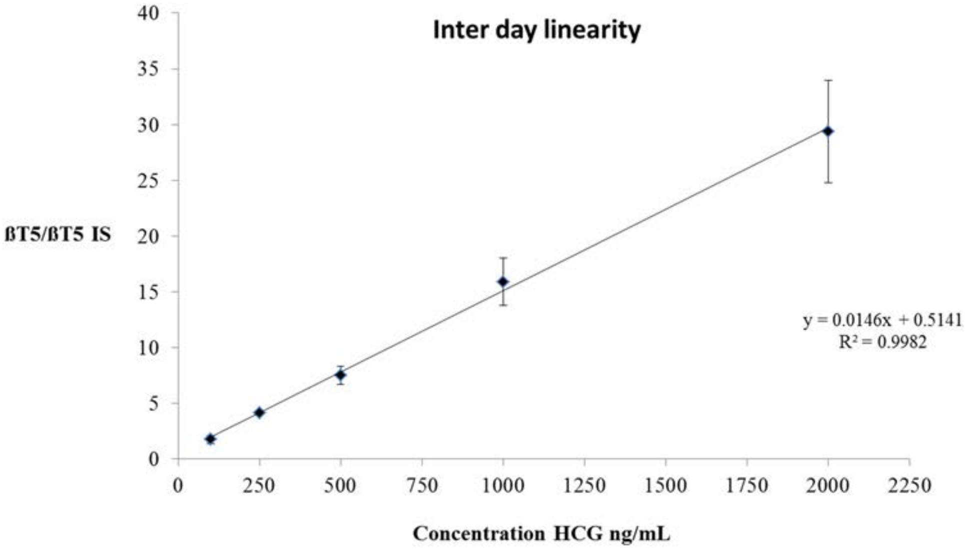

Fig. 4. Inter day analysis (N = 3, n = 3) of hCG spiked serum to pHEMA-VDM sampling spots immobilized with monoclonal antibody E27. Error bars are presented as standard deviation.

3.4. Applicability to patient samples

The sampling spots have proven capability to be used in both high and low concentrations range. To further assess the applicability, serum from two patients previously diagnosed with testicular germ cell tumor was analyzed (n = 3). The patient samples were selected based on previous estimates to suit the selected concentration ranges. However, the true concentration is not known for either of the samples as they have been stored and freeze thawed for several circles prior to sample application onto the E27 immobilized pHEMA-VDM sampling spots. Nevertheless, both sample sets were relevant to the concentration curves for quantification. From the analyses, the patient samples were determined to 418.5 ± 4.2 ng/mL and 21 ± 0.02 ng/mL for sample set 1 and sample set 2, respectively (S5). All in all, these results demonstrate that the polymeric sampling spots holds promising potential for a clinical application in a *lab-on-paper* format within the interest of *point-of-care* devices. As for the current state of this concept it must be stressed that it is by no means designed to compete with the current methodologies used in the clinics. The LOD obtained at 1 ng/mL (9.1 IU/L) is significantly higher than the recently proposed reference limit for healthy adults at < 0.3 IU/L [29]. It is expected that this reference limit is achievable with further optimization. That being said, no in-depth optimization regarding sample capacity (i.e. sample spot thickness) and other aspects related to sample preparation were examined in the presented study. This needs to be carried out in future works. Nevertheless, the concept shows that *on-paper* sampling hold performance for advanced and sensitive applications related to disease state protein analyses.

4. Conclusion

We have demonstrated a paper-based sampling concept for mass spectrometric bottom-up protein analysis in a novel *lab-on-paper* format. By immobilizing antibodies under acidic conditions on pHEMA-VDM, polymeric sampling spots proved a high degree of performance within 1 ng/mL and 2000 ng/mL by *on-paper* immunocapture of hCG in human serum. The concept showed promising intraday and inter day precision with adequate accuracy and was tested successful on donated patient samples.

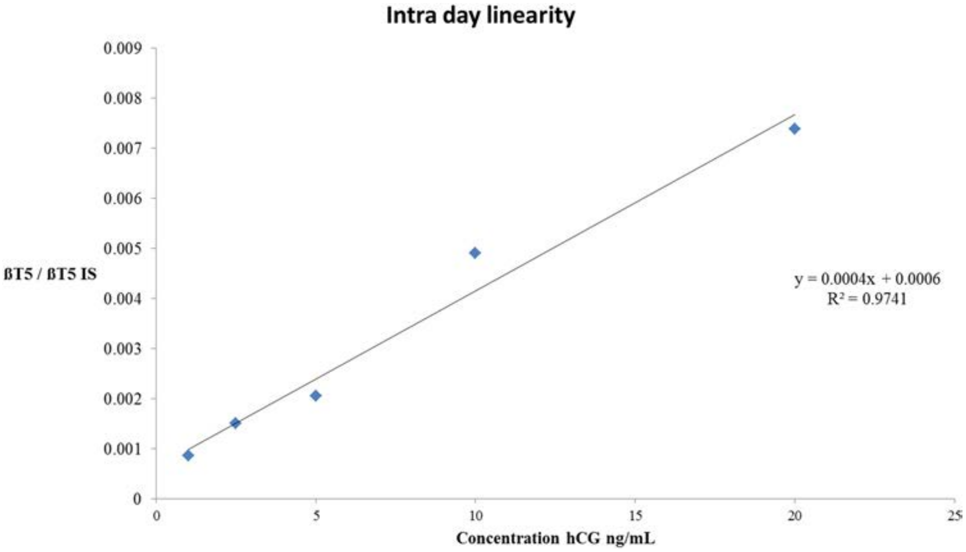

Fig. 5. Five point concentration curve of hCG spiked human serum from 1 ng/mL to 20 ng/mL sampled on E27 immobilized pHEMA-VDM sampling spots (n = 3). The RSD of the signal ratio at each concentration level are all below 16%. The reason for the intercept on the y-axis is not known, it is not expected that this is caused by the contribution of endogenous hCG.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.12.013.

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Supplementary material to:

Paper based immunocapture for targeted protein analysis

by

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Table of content

S1. Procedure for preparation and initial testing of reactors based on adsorption of antibody

On wax printed filter paper 20 µL 0.05 mg/mL antibody E27 (in 50 mM ABC) was applied and left to dry at room temperature in the dark. The sampling spots where then washed by wicking 10 µL 50 mM ABC to an underlying absorbent and left to dry. Ten microliters of 0.1 % BSA in 50 mM ABC (for blocking of non-specific intereactions) were added and dried with an additional wash of 20 µL 50 mM ABC. To the washed and dried sampling spots 20 µL hCG (Ovitrelle® diluted to 10 µg /mL or 2 µg /mL in 50 mM ABC) was applied. After the sample spots were dried, they were washed five times with 20 µL 50 mM ABC by wicking to an underlying absorbent. The spots were dried in room temperature after the final washing step with ABC. The dried spots were punched out alongside the inner wall of the wax print and transferred to 2.0 mL Eppendorf (Hamburg, Germany) protein LoBind tubes. The eppendorf tubes where further added 100 µL 50 mM ABC and agitated at 800 rpm and room temperature for one hour. Reduction was carried out by 10 µL 50 mM DTT. After 15 min at 60 °C and 800 rpm, 10 µL 100 mM IAA was added with a subsequent agitation at 800 rpm at 27 °C. The extract was transferred to new Eppendorf Protein LoBind tubes and subjected to 16 hour tryptic digest by addition of 10 µL 1 mg/mL trypsin in 50 mM ABC. A simplified schematic overview of the workflow can be seen in **Figure S1**.

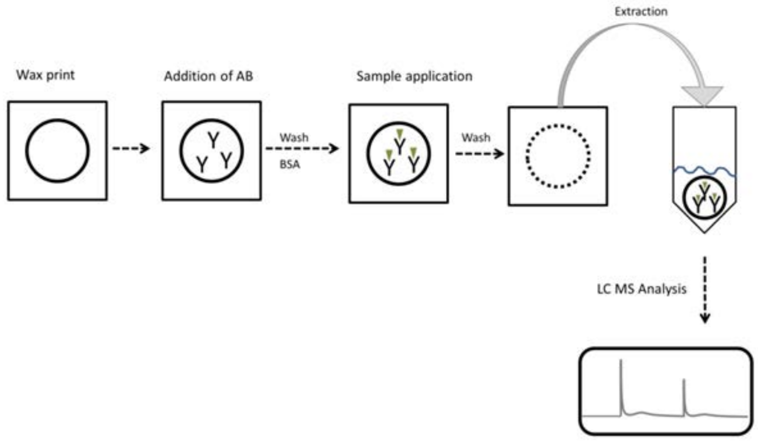

Figure S1 Schematic illustration of immune capture and subsequent analysis of hCG using antibody E27 adsorbed to wax printed filter paper.

S2 Concentration range for hCG in phosphate buffer sampled with pHEMA-VDM sampling spots

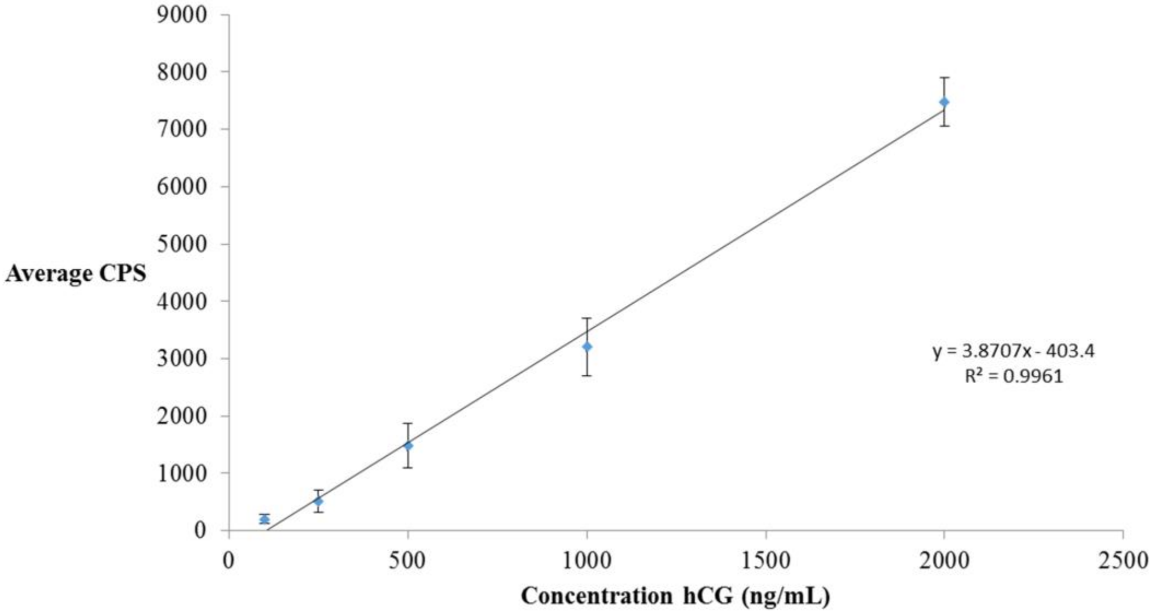

Figure S2 Concentration curve of hCG spiked 0.1M phosphate buffer with 0.1 % BSA from 100 ng/mL to 2000 ng/mL sampled on E27 immobilized pHEMA-VDM sampling spots. All concentration levels (n=3) are displayed with standards deviation. The signal is reported by counts per second (CPS)

S3. Chromatograms related to blank reactors

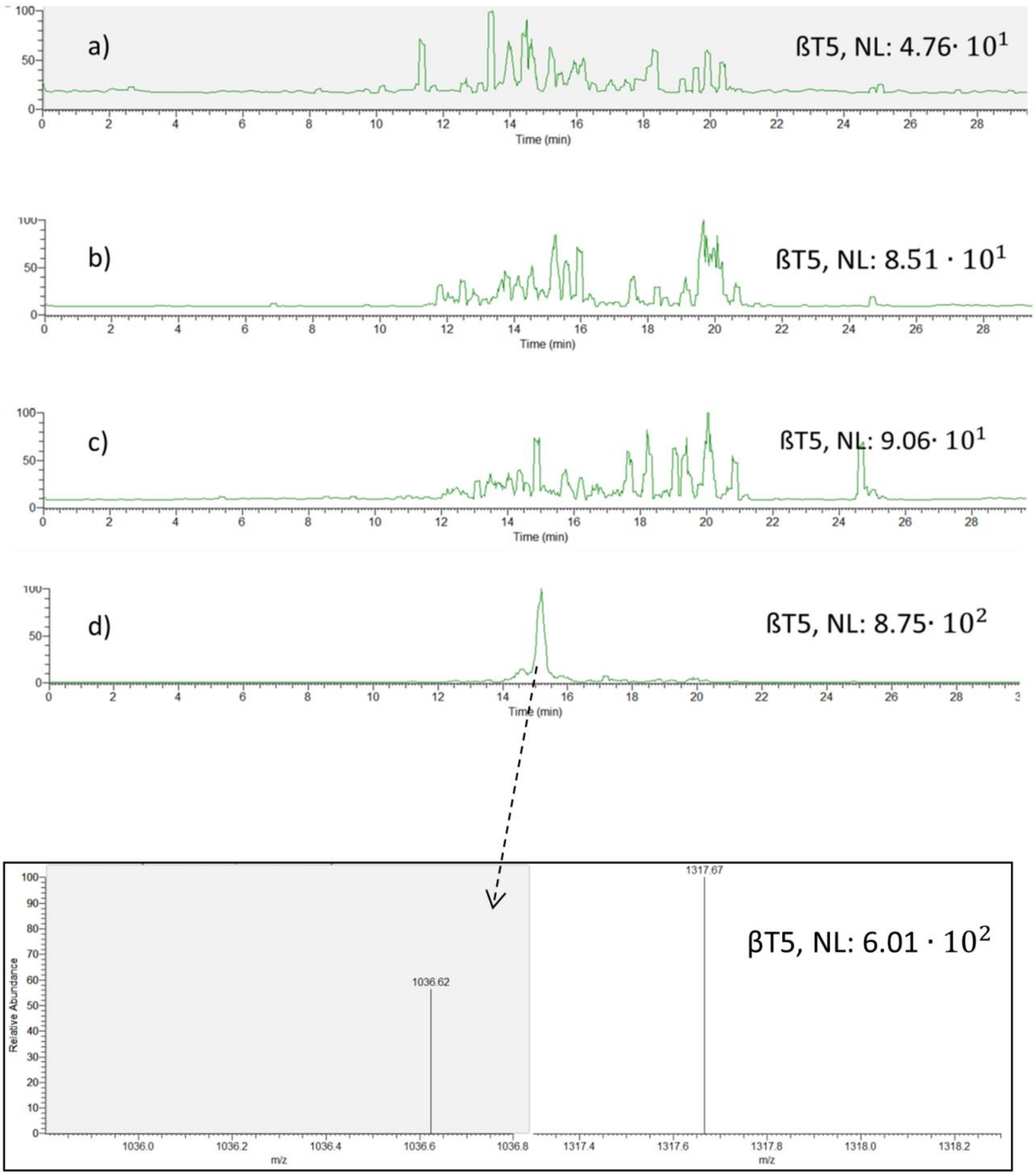

Figure S3 Analysis of hCG spiked human serum sampled on pHEMA-VDM and pHEMA-TsCl sampling spots without immobilization of monoclonal antibody E27. From the figure: **a**) 100 ng/mL hCG sampled on pHEMA-VDM sampling spot, **b**) 2000 ng/mL hCG sampled on pHEMA-VDM sampling spot, **c**) 100 ng/mL hCG sampled on pHEMA-TsCl sampling spot and **d**) 2000 ng/mL hCG sampled on pHEMA-TsCl sampling spot

S4. Limit of detection

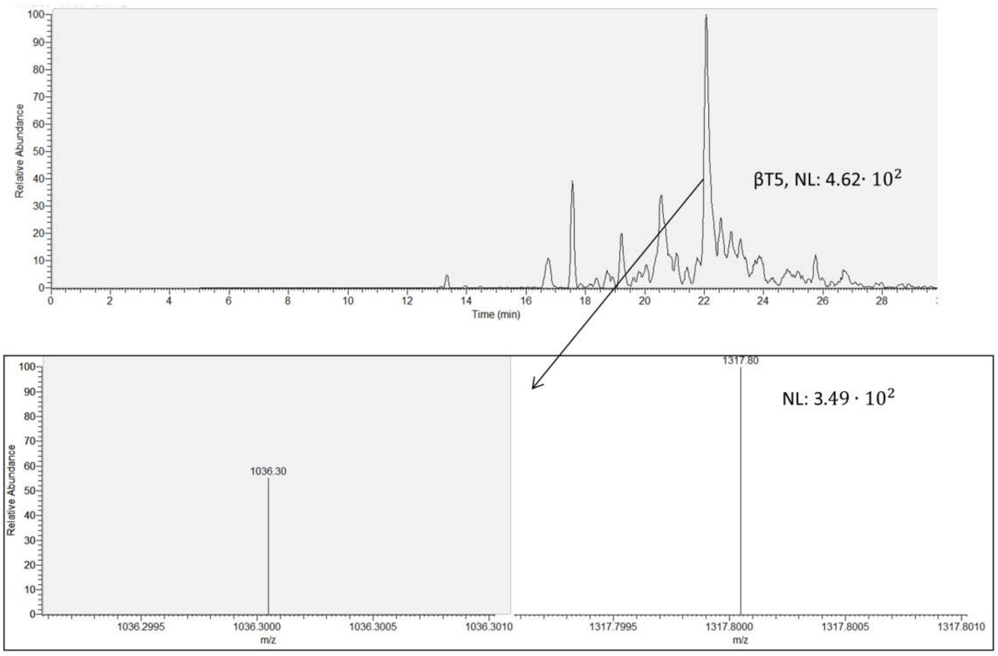

Figure S4 Analysis of hCG spiked to 1 ng/mL in human serum. The sample was sampled on pHEMA-VDM reactors immobilized with monoclonal antibody E27

S5 Chromatogram related to realistic samples

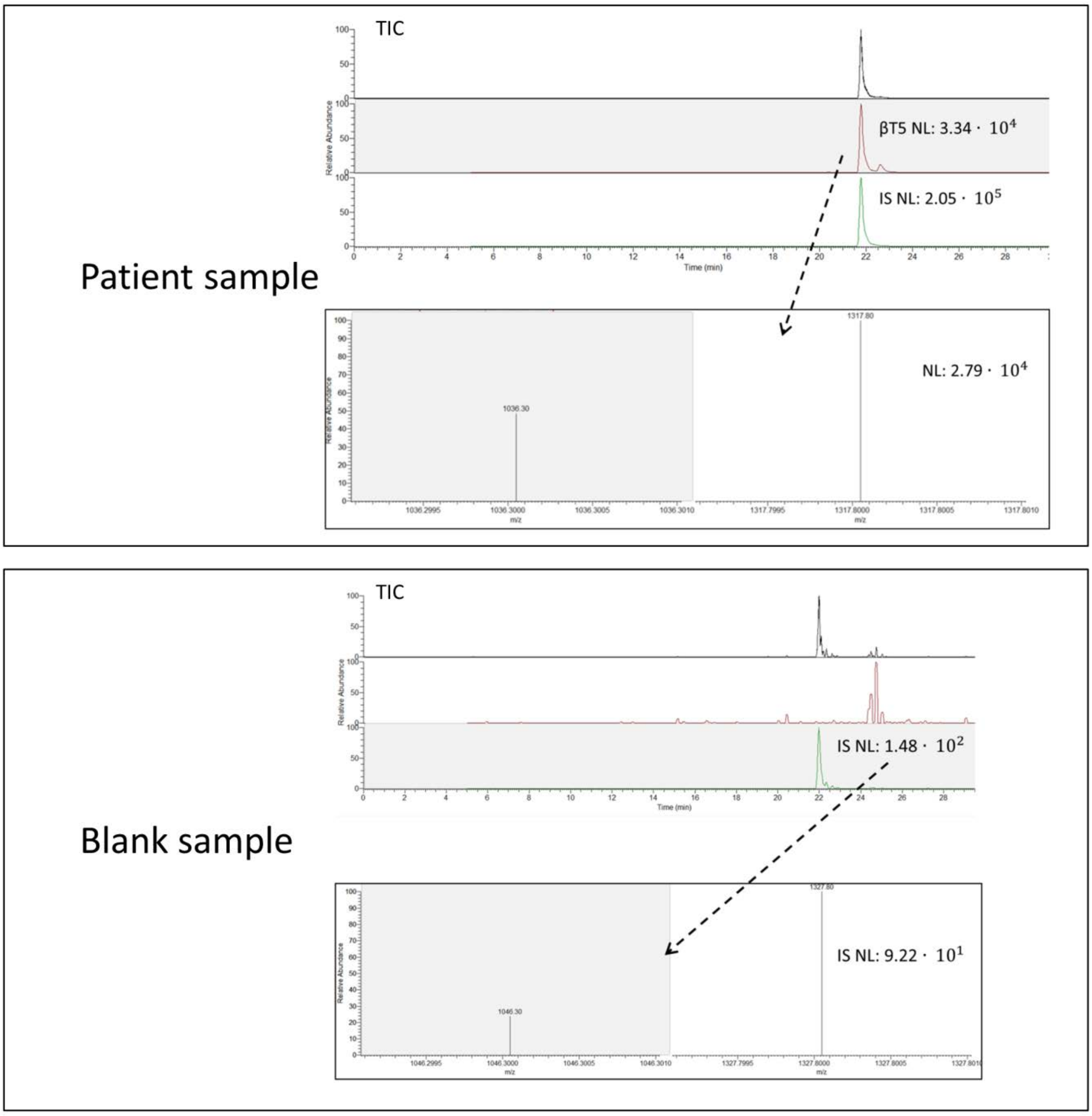

Figure S5 Chromatograms for patient sample 1. Twenty microliter serum was sampled on pHEMA-VDM sampling spot (immobilized with monoclonal antibody E27) spiked with 0.5 pmol (reduced and alkylated) internal standard after tryptic digestion and blank sample. Some carry over could be seen from the internal standard

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Analytica Chimica Acta 1089 (2019) 56–65

Contents lists available at ScienceDirect

Analytica Chimica Acta

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All-in-one paper-based sampling chip for targeted protein analysis

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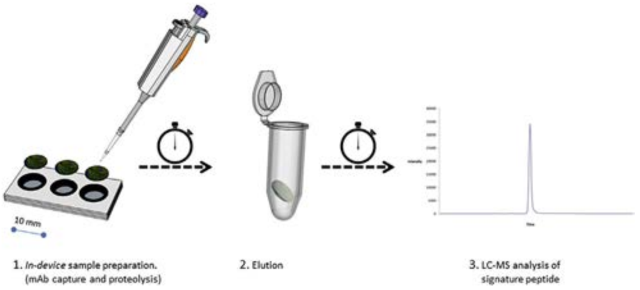
HIGHLIGHTS

- All-in-one paper-based sampling concept in chip format for mass spectrometric bottom-up protein analysis.
- A simple sampling concept for sensitive analysis of lower abundant proteins related to disease state protein applications.
- Paper-based sampling chip integrated with mAb E27 and all steps necessary for analysis of proteins in biological matrices.
- A high degree of performance sampled with human serum and freshly collected whole blood samples.
- Demonstrated with the biomarker human chorionic gonadotropin.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 14 June 2019
Received in revised form
13 August 2019
Accepted 19 August 2019
Available online 20 August 2019

Keywords:
Smart sampling
Protein analysis
Whole blood analysis
LC-MS
Paper-based chip sampling
Dried blood spots

ABSTRACT

A novel *all-in-one* paper-based sampling concept for mass spectrometric bottom-up protein analysis is here demonstrated in a chip format integrating instant immunocapture, protein reduction, - alkylation and tryptic digestion all *in-device*. Conventional laboratory grade filter paper was coated with the polymer 2-hydroxyethyl methacrylate-co-2-vinyl-4,4-dimethyl azlactone (pHEMA-VDM) with a subsequent covalent immobilization of the monoclonal antibody E27 targeting the biomarker human chorionic gonadotropin (hCG). *In-device* protein reduction and alkylation was optimized with regards to reagent concentration and reaction pH. The sampling concept showed a high degree of performance between 10 and 1000 ng/mL ($R^2 > 0.99$) by a five-point calibration curve sampled with hCG spiked to human serum samples and freshly collected whole blood samples, respectively. LOD (experimentally obtained at 100 pg/mL (2.64 pM/0.9 IU/L)) was demonstrated to be up to ten times lower with more than six times faster sample preparation than what has previously been reported for on-paper analysis of hCG in human serum samples.

1. Introduction

Paper-based sampling of biological matrices has been around for decades after the introduction of dried blood spots (DBS) in the 70's as a sampling technique aimed towards newborn screening [1]. DBS

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https://doi.org/10.1016/j.aca.2019.08.043

is a technique were a small volume of whole blood, typically 10–20 μL, is collected and stored on a paper card prior to extraction and analysis. The technique has proven many clinically relevant applications. In the decades following its introduction, DBS has successfully been implemented as a sampling technique of biological matrices targeting viruses, drugs [2,3] and other smaller molecules [4,5] both for research and clinical purposes [6]. The benefits are vast by utilizing paper based blood sampling rather than performing venipuncture e.g. lower bio-hazard when the sample is dry, low transportation costs, non-invasive sampling, reduction in animal bleeding while performing pre-clinical studies [7] and the possibility to sample at remote locations out of reach for a high quality testing facility. Reports on DBS sampling combined with mass spectrometric (MS) detection of proteins are however limited. DBS has suffered from not meeting the low detection limits and performance (in the lower concentration range) often required for analysis of low abundant proteins. This may resolve from strong adsorption of protein to cellulose [8], insufficient extraction [9] and low sampling volumes (eliminating the possibility for enrichment). Nevertheless, some efforts has been made with promising results both with non-targeted [10] and targeted [11–13] approaches. One of the biggest drawbacks of the DBS methodology for protein analysis is the extensive time consumption with regards to the sample preparation needed to perform sensitive analysis of low abundance proteins. This methodology most often consists of an extraction step (from the collecting device), protein reduction, alkylation, protein hydrolysis, protein precipitation and solid phase extraction prior to liquid chromatographic (LC) sample injection. The whole process demand in-between 20 and 30 h from the start of sample preparation to LC-MS injection. Many of these steps can thus, be eliminated if DBS is combined with immunocapture, however, high extraction efficiency and protein reduction and alkylation and protein hydrolysis for bottom-up approaches is still needed for the immunocapture techniques because the sample (in whole) needs to be extracted from the DBS card prior to sample preparation [14]. Apart from antibody based methods combined with MS, immunoaffinity techniques such as ELISA has previously been demonstrated in combination with DBS for larger molecules such as drugs [15] and biomolecules [16,17]. Nevertheless, MS detection offers several advantages over ELISA such as quantification of isoforms and eliminating the risk for false positive or negative response as elaborated by Lund et al. [18]. At our group we have recently demonstrated that paper-based sampling of proteins in biological matrices is readily performed with *in-device* tryptic hydrolysis [19,20] and *in-device* immunocapture [8]. These two concepts allowed a significantly shorter sample preparation time (Integrated tryptic hydrolysis: ~3 h and integrated immunocapture:~19 h, sample drying time not included) compared to conventional MS-based bottom-up protein determinations using DBS or dried matrix spots (DMS) as sampling technique (~22–30 h, sample drying time not included). This since the protein capture or tryptic digestion of the proteins was integrated in the sampling device. The instant immunocapture sampling proved to have a high degree of performance within 1–2000 ng/mL hCG spiked to human serum. This demonstrated to be competitive to conventional DBS/DMS (with respect to limit of detection (LOD)), however with a significantly lower sample preparing time. Nevertheless, the sampling device still called for some manual sample preparation such as protein reduction and alkylation and conventional overnight tryptic digestion. The aim of this study is to develop a simple, high performing, cost effective and *true* all-in-one sampling chip combining the necessary steps for bottom-up targeted analysis of proteins in serum and whole blood. The instant immunocapture sampling device described previously [8] was used as a starting point. In order to enable a *true* all-in-one chip the additional steps in the procedure were optimized for *in-device* performance i.e. *in-device* protein reduction and alkylation as well as *in-device* tryptic digestion. Two different kinds of paper quality were evaluated, and the combined sampling and sample preparation time estimated. The concept's quantitative performance was further assessed regarding linearity and, LOD/LOQ of hCG spiked to both human serum and same day collected whole blood samples. To our knowledge, this is the first paper-based *all-in-one* sampling concept for proteins in combination with LC-MS bottom-up protein analysis.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (99.9%, (ACN)), boric acid (99.8%), ammonia solution (25%) and ethanolamine (≥99%) were purchased from Merck (Darmstadt, Germany). Formic acid (≥98%, (FA)), ammonium formate (≥98%, (AF)) iodoacetic acid (≥98%, (IAC)), DL-dithiothreitol (≥99.5%, (DTT)), tris(2-carboxyethyl)phosphine (≥98% (TCEP)), *N*,*N*-Dimethylformamide anhydrous (DMF), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS, 98%), 1-heptanol, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 2-hydroxyethyl methacrylate (HEMA, 97%, containing 200–220 ppm monomethyl ether hydroquinone), initiator 2,2′azobis(2-methylpropinonitrile) (98%, (AIBN)), trypsin from bovine pancreas TPCK treated (≥10 000 BAEE units/mg protein), bovine serum albumin (≥96.0%, (BSA)), sodium phosphate dodecahydrate (99–102%), sodium phosphate monobasic (99–102%), Trizma® base (≥99.9%), tris(2-carboxyethyl) phosphine hydrochloride (≥98.0%), hydrochloric acid (37.0%), sodium chloride (≥99.5%), tween 20® and borax® (99.5%) were purchased from Sigma-Aldrich (St Louis, MO, USA). 2-Vinyl-4,4-dimethylazaltone (VDM) was purchased from Polysciences Inc. (Warrington, PA, USA). Ammonium bicarbonate (ABC, BioUltra, ≥99.5%) was purchased from Fluka (Milwuakee, WI, USA). Sodium hydroxide was purchased from VWR international (Leuven, Belgium). Water used in this experiment was filtrated through a Merck Millipore Milli-Q intergral 3 water dispenser (resistivity: 18.2 MΩ cm^{-1}). The monoclonal antibody (mAb) E27 was donated by the Department of Medical Biochemistry, Oslo University Hospital (Oslo, Norway). Internal standard AQUA™ peptide (amino acid sequence: (VLQGVLPALPQVVCNY[R_$^{13}C_6$_$^{15}N_4$]) was purchased from Sigma Aldrich. Ovitrelle® (Merck, London, UK) was used as source for hCG and was purchased from the local pharmacy. Human serum was obtained from healthy volunteers from Oslo University Hospital Ullevål (Oslo, Norway). Fresh whole blood was collected by certified personnel at the Department of Pharmacy, University of Oslo. Whatman® filter paper and DMPK-C cards was purchased from GE healthcare Life Sciences (Buckinghamshire, UK).

2.2. Ethics

Same day collected whole blood from one healthy volunteer was collected in BD (Franklin Lakes, NJ, USA) vacutainer® K2 EDTA tubes. Freeze thawed human serum samples from healthy subjects (stored at −30 °C) were obtained from Oslo University Hospital, Ullevål (Oslo, Norway). The authors state that they have obtained appropriate institutional review board approval and have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. For investigation involving human subjects, informed consent has been obtained from the participants involved.

2.3. Preparation of sampling spots and wax printed cartridges

2.3.1. Preparation of wax printed cartridges

Whatman® grade 1 filter paper was cut to A4 sized sheets and printed with 45 circles (6.00 mm ID and 10.0 mm OD) and three solid oblong rectangles (1.3 × 24.9 cm) in Microsoft Power Point (version14.0.7177.5000). The printing was carried out by a Xerox (Norwalk, CT, USA) Color Qube 8580 printer with Color Qube 8570 wax. After printing, the wax was melted for 10 min at 110 °C. The inner circle was punched out by a Philip Harris (Birmingham, UK) Uni-core 6.00 mm puncher and set aside for polymerization. The wax printed cartridge was fabricated by gluing three layers of pre-punched wax prints (with inner circle removed) and one oblong panel of solid wax in sandwich. The glue used was Pritt (Düsseldorf, Germany) original. The wax print template is attached in supplementary S1.

2.3.2. Polymerization

Polymerization of the sampling spots was carried out on Whatman® grade 1 and Whatman® DMPK-C cards as previously described by Skjærvø et al. [8]. The polymer coating used was pHEMA-VDM. In short; filter paper circles was punched out (6.00 mm diameter) and subjected to silanization. The silanization mixture was composed of 5 mg DPPH, 660 mg DMF and 313 mg TMSPM. Ten microliter solution was applied to each spot and baked in an in-house made reaction chamber for 2 h at 100 °C. The silanized spots were further rinsed with ACN and dried. The spots were subsequently polymerized by applying 10 µL polymerization mixture composed of 37 mg AIBN, 300 mg HEMA, 300 mg 1-decanol and 100 mg VDM and baked for two and 3 h at 80 and 100 °C, respectively. The polymerized sampling spots were rinsed with ACN and dried prior to immobilization of antibody.

2.3.3. Immobilization of sampling spots

Immobilization on the pHEMA-VDM sampling spots was carried out as previously described by Skjærvø et al. [8] and Paus and Nustad [21]. In short; pHEMA-VDM polymerized filter paper were placed in individual Eppendorf (Hamburg, Germany) protein LoBind tubes with 100 µL 0.1 M borate buffer (pH 9.6) containing 0.5 mg/mL mAb E27. The tubes were agitated for 20 h at 37 °C and 800 rpm by an Eppendorf comfort thermo mixer. The solution was replaced with 100 µL 1.0 M ethanolamine containing 0.1% Tween 20® (pH 9.5) and mixed at 25 °C and 800 rpm for 2 h. The solution was further replaced by 100 µL tris-hydrochloride buffer (Tris-HCl) containing 0.1% Tween 20® and 0.1% BSA (pH 7.0) and mixed at 25 °C and 800 rpm for 30 min. The reactors were finally washed with 15 µL 0.1 M phosphate buffer (pH 7.5).

2.4. Sample preparation

2.4.1. Sample application and on-device sample treatment

After immobilization of the mAb E27 on the pHEMA-VDM polymerized filter paper, the sampling spot was placed into a wax printed cartridge. Sample application was performed by pipetting 20 µL (if not stated otherwise) sample (hCG spiked to 50 mM ABC buffer, human serum or freshly collected human whole blood) to the center of each sampling spot. The sample was subsequently dried at ambient temperatures prior to sample clean-up. Sample clean-up was carried out by transferring the sampling spot to a 2.0 mL Eppendorf (Hamburg, Germany) Protein LoBind tube and added 500 µL phosphate buffered saline pH 7.4 (PBS) with 0.05% (w/v)Tween 20® and mixed vigorously. The solution was removed and replaced by 500 µL PBS, 400 µL Tris-HCl (pH 7.6) and 300 µL 50 mM ABC respectively. All solutions were removed (by pipetting out the volume, also through the sampling spot to remove the stagnant liquid) prior to addition of a new solution. The wash volumes were applied to all samples if not stated otherwise. After removal of the last washing solution the sampling spots were placed briefly on dry filter paper to remove excess solution prior to being placed back in to the wax printed cartridge and dried.

2.4.2. Reduction, alkylation and tryptic hydrolysis

Protein reduction was performed dark and at ambient temperatures by adding 20 µL of 200 mM DL-dithiothreitol (DTT) in ammonia water (pH 10.2). When all solvent was evaporated 20 µL of 200 mM iodoacetic acid (IAC) in 50 mM ABC (pH 7.8, room temperature). After evaporation, tryptic hydrolysis was performed by applying 60 µL 0.33 mg/mL trypsin (in 50 mM ABC) to each sampling spot. The reaction was carried out dark at room temperature. Reduction, alkylation and tryptic digestion was performed with the sampling spots in the wax printed cartridge. The spot was not removed during any of these reactions. Extraction of generated peptides from the sampling spot was carried out at 800 rpm for 30 min by transferring the protein digested sampling spot into 2.0 mL Eppendorf LoBind tubes with 90 µL 5% ACN and 0.1% FA and 10 µL internal standard (VLQGVLPALPQVVcNY[R_^{13}C$_6$_^{15}N$_4$], 50 pmol/mL, in 20 mM FA). After extraction the samples were centrifuged with an Eppendorf 5804 centrifuge equipped with a 30 place fixed angle rotor (F45-30-11) and transferred to HPLC-vials. A flow chart of the entire sample preparation is presented in supplementary S2.

2.5. LC-MS analysis

The sample loading and chromatographic separation was performed with a Dionex (Sunnyvale, CA, USA) Ultimate 3000 RSLC nano pump module. Twenty micro liter sample was loaded onto a Thermo Fischer (Waltham, MA, USA) Acclaim™ PepMap™ C18 (ID: 300 µm, L: 5 mm, dp: 5.0 µm) trap column over 3 min at 30 µL/min with 2% ACN and 0.1% FA and 10 mM AF. The sample was subsequently back-flushed onto a Thermo Fischer Acclaim™ PepMap™ C18 (ID: 75.0 µm, L: 150 mm, dp: 3.0 µm) analytical column. The separation was performed at 300 nl/min over 25 min from 5% to 95% MP B (95% ACN/5% water containing 0.1%FA and 10 mM AF) with 1 min hold at 95% MP B prior to subsequent reconditioning for 13 min with MP A (5% ACN/95% water containing 0.1%FA and 10 mM AF).

2.5.1. Quantitation

The analysis was performed with a Thermo Fischer TSQ Quantiva operated in SRM positive mode nano ESI with 2.20 kV applied voltage. Quantitation was carried out by measuring signature peptide βT5 fragment ions *m/z* 1036.3 (y_8^+) and *m/z* 1317.8 (y_{11}^+) for (VLQGVLPALPQVVcNYR– precursor ion *m/z* 964.22$^+$) and fragment ions *m/z* 1046.3 (y8$^+$) and *m/z* 1327.8 (y_{11}^+) for the internal standard (VLQGVLPALPQVVcNY[R_^{13}C$_6$_^{15}N$_4$] – precursor ion *m/z* 969.32$^+$). All transitions were carried out with 30 V of collision energy. The following MS parameters were used: 350 °C ion transfer tube, 1.5 mTorr CID gas (Ar), 1 s scan cycle and Q1 and Q3 resolution both at 0.7 FWHM.

3. Results and discussion

During the initial parts of this study (3.1 through 3.3) untreated filter paper was used to investigate the parameters influencing reduction, alkylation and tryptic hydrolysis *in-device*. These steps were investigated individually (to avoid variation/bias from other sample preparation steps such as extraction) prior to being incorporated in full for performance evaluation in human serum and freshly collected whole blood (Part 3.4). From part 3.4 and further, pHEMA-VDM polymerized paper immobilized with mAb E27 is used. A schematic presentation of the manuscripts work flow is presented in Fig. 1.

3.1. Fabrication of wax printed sample cartridge

In a bottom-up proteomic workflow, sample preparation steps like reduction, and alkylation are in most cases necessary in addition to the hydrolysis. These preparation steps can only be accomplished in solution. In order to meet this criterion a cartridge that could hold a static liquid layer was fabricated similarly to what has previously been described by Skjærvø et al. [19]. The cartridge, composed by a three dimensional wax print was made by gluing three circular wax prints (with the center piece removed) to a solid oblong panel of wax (as an under layer) with solvent free glue stick. These well-like holders (Fig. 2) were tailored to hold separate sampling spots (polymerized and immobilized with monoclonal antibodies as described by Skjærvø et al. [8]). The individual wells were capable of holding volumes surpassing 60 µL. A stack of three of wax prints was utilized to increase the volume holding capacities since the wells needed to exceed the thickness of the polymerized sampling spot. Solvent free glue stick was chosen as the binding agent between the paper sheets to minimize potential hydrophobic components to be introduced later in the sample preparing procedure. The wax printed cartridge could be replaced by other designs and solvent restricting materials such as machined Delrin® plastic or potentially 96 well plates. However, wax printing is a low cost, low weight (for potential transportation via regular mail) and flexible method and was therefore chosen for this concept. The wax printed cartridge combined with the polymerized and mAb immobilized sampling spots is further referred to as the sampling chip and when actions is performed it is discussed as *in-device* (stand-alone polymerized paper is further discussed as *in-spot*). The assembled chip dimensions with three sampling wells measured 40 × 20 × 1.0 mm (LxWxD).

3.2. In-device reduction and alkylation

3.2.1. In-device protein reduction

In order to evaluate the *in-spot* protein reduction, an aqueous hCG sample was prepared (2 µg/mL hCG in 50 mM ABC). Twenty microliters was applied to untreated wax printed filter paper. When the sample was dried, 20 µl of the reducing agent (100 mM DTT or tris(2-carboxyethyl)phosphine (TCEP) dissolved in 50 mM ABC) was applied and let to react (dark) until dry. The alkylating reagent (200 mM IAC dissolved in 50 mM ABC) was then subsequently added of the same volume. Based on previous results [8,19,20] the IAC concentration was for these initial analyses set to 200 mM. However, with an increased volume (20 µL instead to10 µL) and thus, amount of alkylating reagent compared to protein content to extend the reaction time (i.e. drying time) and potentially increase reaction efficiency and product formation. The wax print was then punched out, extracted and digested (*in-solution*) in 180 µL 50 mM ABC and 20 µL trypsin (1 mg/mL in 50 mM ABC) for 18 h at 1050 rpm and 37 °C. For these initial experiments, reduction and alkylation was carried out both *sequential* (in separate steps) and *combined* (reducing and alkylating reagent dissolved in conjunction) both at 25 °C and 60 °C. The reagents were dissolved in 50 mM ABC (pH 7.8) for both the reduction and alkylation in compliance to previous work [8]. Simultaneous addition of the reducing and alkylating reagents was carried out since TCEP and IAC has been demonstrated to work well as a mixture [22] and protein reduction with TCEP is mostly carried out in conjunction with the alkylating reagent [23].

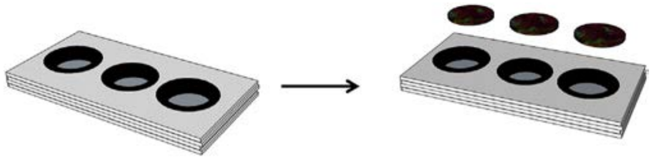

Fig. 2. Schematic illustration of the wax printed sample cartridge with antibody immobilized polymer sampling spots. A picture of the device and its assembly is presented in supplementary S3.

Using the above experimental conditions, low signals where obtained for the βT5 peptide when using DTT as reducing agent. For TCEP no signal was observed by any combination (separate additions or in conjunction) of reducing and alkylating with TCEP either at half (50 mM) and twice (200 mM) the concentration. Therefore, DTT was chosen as the reducing reagent for further experiments. In a next set of experiments the impact of DTT concentration was investigated by adding 200 mM or 500 mM of DTT while keeping

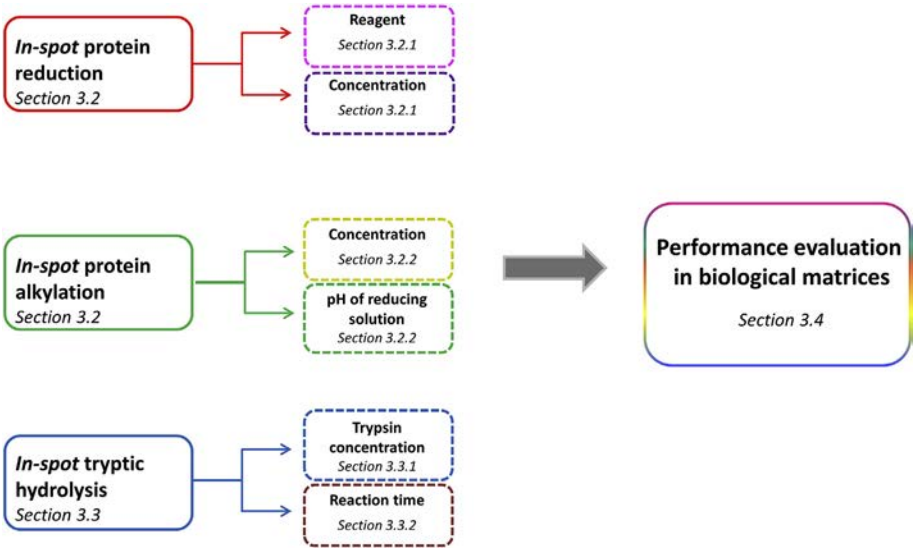

Fig. 1. Schematic presentation of parameters optimized (dotted boxes) in the manuscript.

the IAC concentration at 200 mM. From these investigations a significant difference between the DTT concentrations (200 mM and 500 mM) in terms of signal intensity was observed ($p < 0.05$). The lower concentration (200 mM) showed high signal intensities, but the standard deviation for this concentration level was significantly higher ($p > 0.05$) than what was observed for 500 mM DTT. Nevertheless, the chromatographic peak obtained at 500 mM DTT was of low symmetry and combined with low signal intensities, this indicated that either the reduction or alkylation in paper may not have been performed under optimal conditions. Therefore, 200 mM DTT was chosen for further experiments and investigated with respect to the pH in the reducing solution. The pH for reduction was investigated at pH 7.8 (ABC), pH 9.2 and pH 10.2 (the two latter with pH adjusted 25% ammonia water). These conditions were used to match the pH of the solution to the pK_a of the reducing agents thiol group (9.2 and 10.1 for DTT) to maximize the thiol-disulfide interchange as discussed by Singh et al. [24]. From the results it was apparent that increasing the pH in the reducing solution was beneficial both in terms of signal intensity (Fig. 3a) and chromatographic peak symmetry. No significant distinction was observed between pH 9.2 and pH 10.2. Nonetheless, pH 10.2 was used for further experiments to ensure basic conditions since ammonia probably evaporates during drying thus reduce the pH.

3.2.2. In-device protein alkylation

To optimize *in-spot* protein alkylation, wax printed filter paper sampled with hCG (2 μg/mL in 50 mM ABC) was reduced (*in-spot*) using the optimized conditions (200 mM DTT at pH 10.2), and subjected to alkylation (*in-spot*) in 50 mM ABC (pH 7.8) at varying concentrations of IAC. The reaction was performed dark and at ambient temperature. The reaction was carried out at the given pH because the efficiency of IAC may be at its maximum between pH 7–8 [25], however, to avoid over-alkylation it was kept at pH 7.8 since over-alkylation could readily occur if the reaction is carried out over an extended period of time between pH 7.0 and 7.5 as suggested by Boja and Fales [25]. The concentrations investigated (50, 100, 150, 200 and 300 mM IAC) were selected to be higher than what is normally performed in *in-solution* approaches to ensure

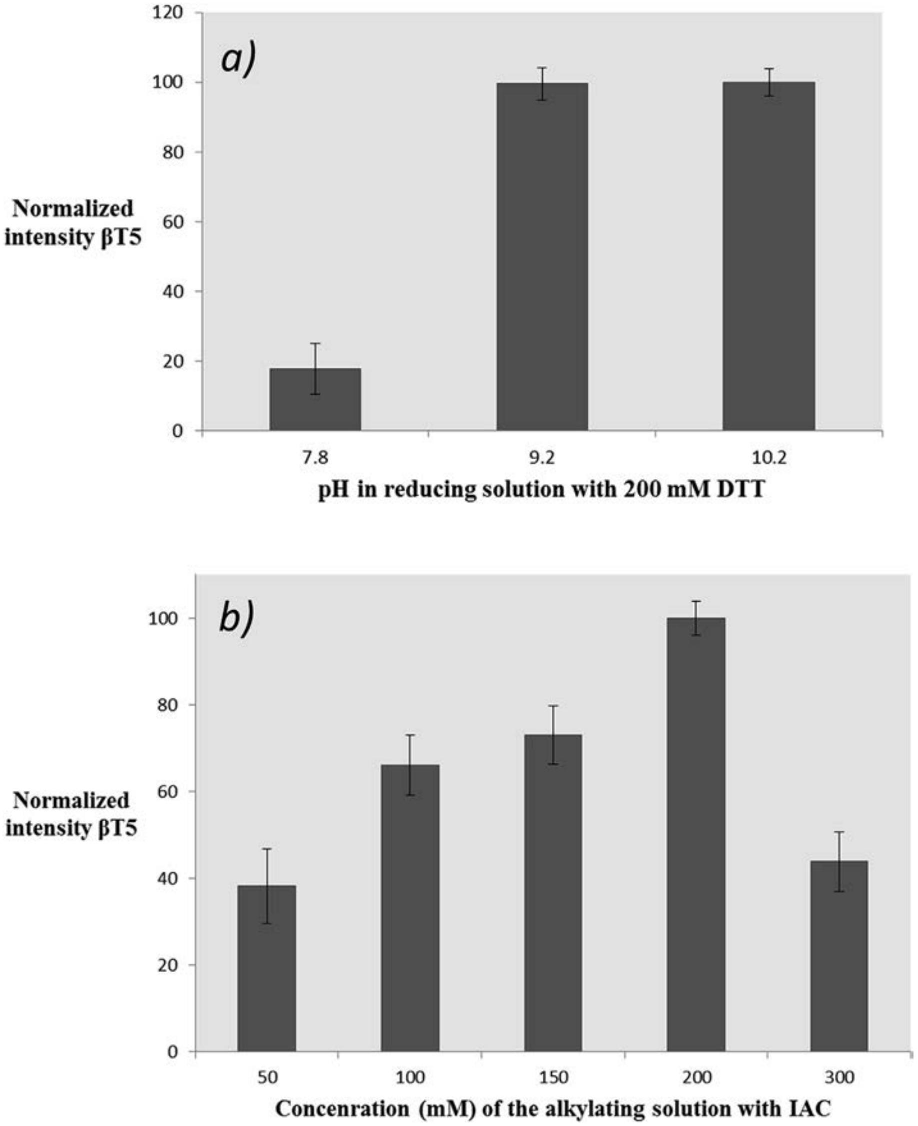

Fig. 3. *a)* In-device protein reduction with DTT as reducing agent at varying pH in the reducing solution followed by in-device alkylation (20 μL) by 200 mM IAC and *b)* In-device protein reduction (20 μL) by 200 mM DTT at pH 10.2 followed by in-spot protein alkylation by IAC at varying concentrations (20 μL). The error bars represent the standard deviation, all experiments was carried out with n = 4.

sufficient reaction time (which is similar or highly correlates to the drying time, approximately 30 min). From the results (Fig. 3b) it was shown that it is beneficial to increase the IAC concentration; however, not surpassing 200 mM IAC as then the signal intensity of the βT5 peptide significantly decreased. This decrease in signal intensity could be a result of over-alkylation and/or offsite alkylation (e.g. over-alkylation or alkylation at other amino acid residues than cysteine) as described by Müller and Winter [23]. This was, however, not further investigated. Nevertheless, the lowest RSD (4%) was obtained at 200 mM IAC.

3.3. In-device tryptic hydrolysis

Previous efforts performed in our group on tryptic digestion of proteins sampled on paper with conventional DMPK-C cards has showed to be challenging due to insufficient interaction time (due to water evaporation and water wicking) between the proteins and enzyme without volume restricting barriers. On the other hand, our group recently demonstrated that *on-paper* protein digestion was feasible if a hydrophobic wax print was utilized to hold the aqueous drop with trypsin solution static [19]. This concept was carried out by applying immobilized trypsin beads on the paper surface (for *in-spot* tryptic hydrolysis of proteins in a shot-gun approach). Immobilized trypsin was in this concept utilized to hinder excessive trypsin autolysis products. From that demonstration, *on-paper* digestion was readily comparable to conventional *in-solution* protein digestion when sampled on conventional DMPK-C cards. These trypsin beads was, however, not used in this experiment since the concept described here has a different workflow: extraction of the target protein needed to be performed before digestion was carried out. Thus the beads may be too big in size to actually interact with the proteins within the sampling spot structure. Therefore, a more conventional approach was investigated where trypsin was dissolved in 50 mM ABC and applied directly to the sampling spot. Since the sampling spots here presented lay in a wax printed well the interaction time between the protein and trypsin could readily be extended by increasing the reagent volume (trypsin solution).

3.3.1. Concentration of trypsin

The enzyme-to-protein ratio could be increased to potentially accelerate the hydrolysis reaction and provide fast and sufficient peptides [20]. Usually that can be achieved by immobilizing the enzyme to a solid support to reduce autolysis products while still maintaining a high enzyme-to-protein ratio. However, as mentioned earlier, it is not possible to use support to which trypsin is immobilized. That means that hydrolysis with dissolved trypsin in increased concentrations may lead to a significantly increase in autolysis products. This in its turn can lead to chromatographic complexity, ion suppression and low signal intensities. Nevertheless, Egeland et al. [26] reported that it may be beneficial to increase the enzyme-to-protein ratio for targeted applications even *in-solution* without sacrificing peptide reproducibility. They showed that high digestion efficiency under shortened hydrolysis time could readily be achieved in serum samples (here spiked with hCG) by using high trypsin concentrations (in a targeted approach). However, this may not be the case when performing the digestion directly *in-spot* (or *on-paper*) as there will be a high degree of physical hindrance between the enzyme and protein due to the sampling spot structure (coated fibers) especially in the absence of agitation. Therefore, an evaluation of different trypsin concentrations was carried out with respect to *in-spot* tryptic digestion. The pH for the experiments was set to 7.8 which is within the reported optimal proteolytic activity range for trypsin (pH 7–9) [27]. To determine the threshold of where the highest signal intensity for hCG βT5 was obtained combined with the lowest relative standard deviations; previously reduced and alkylated (*in-solution*) hCG standards (2 μg/mL) were applied to un-treated wax printed filter paper and allowed to dry. This to avoid variation during the prior sample preparing steps (drying, washing, reduction and alkylation). Twenty microliter of trypsin with a varying concentration (1, 2, 5 and 10 mg/mL trypsin) dissolved in 50 mM ABC was then applied and left to dry dark in ambient temperature. From the results (Fig. 4a) it was apparent that increasing the trypsin concentration resulted in lower signal intensities. No signal was observed for the highest trypsin concentration (10 mg/mL). These results may be misleading with regards to our previous efforts by covalently immobilizing trypsin directly to the polymer coating for *in-spot* hydrolysis (were a higher amount of trypsin could be used to accelerate the digestion without gaining higher concentration of autolysis products) [20], however the results do verify the general understanding of the autolysis of trypsin and were assumed to be reasonable. Additionally, it must be stressed that these analyses are performed in a targeted mode (on a QqQ system) thus information regarding trypsin autolysis products was not acquired.

3.3.2. Reaction time

As mentioned earlier it may not be beneficial to apply the same consideration when trypsin is *not* immobilized directly within the sampling spot as with the immobilized trypsin (i.e. apply a higher concentration of trypsin without altering the reaction time in order to accelerate the hydrolysis process) [20]. Therefore, to provide a longer reaction time while still keeping the amount of applied trypsin constant, varying volumes of applied trypsin solution (per sampling spot) were investigated. The amount of trypsin was kept constant at 20 μg regardless of volume ensuring that the only variable was drying time of the applied liquid and thus, reaction time. The drying time was observed to be approximately 30 min per 20 μL applied (i.e. 30 min, 60 min and 90 min for 20 μL, 40 μL and 60 μL, respectively). This volume-to-drying time was observed to be inflicted by humidity and temperature, thus, the drying time could potentially be a limiting factor for reproducible results. However, with an increased volume the evaporation rate seemed to be less inflicted by external variations such as humidity. From the experiments (Fig. 4b) it was shown to be beneficial to extend the reaction time. The gain from extending the reaction time by a threefold contributed to 20% higher signal intensity. However, it should be noted that this gain in signal intensity may not be of high importance if a high throughput sample preparing method is desirable. The relative standard deviation regarding signal intensity between the different volumes did not show significant variance ($p > 0.05$) and thus, the method showed similar performance regardless of trypsin digestion time. Nevertheless, 60 μL was chosen for further experiments due to potential gain for more complex samples such as serum and whole blood.

3.4. Quantitative evaluation of the all-in-one sampling concept

3.4.1. Effect of paper quality on signal reproducibility

As previously discussed, we have demonstrated that proteins sampled *on-paper* can readily be reduced, alkylated and digested by trypsin directly in the sampling device. However, all these experiments have been conducted with protein spiked to a buffered solution. In order to prove the applicability towards realistic complex samples the concept was evaluated by a five point standard curve ($n = 3$) from 10 to 1000 ng/mL with hCG spiked to human serum for an *all-in-one* sample preparation (i.e. immunocapture, protein reduction, protein alkylation and tryptic digestion) directly in the device with a subsequent extraction prior to LC-MS analysis. The investigation was performed with mAb E27 immobilized pHEMA-VDM Whatman® grade 1 filter paper (sampled with 20 μL serum)

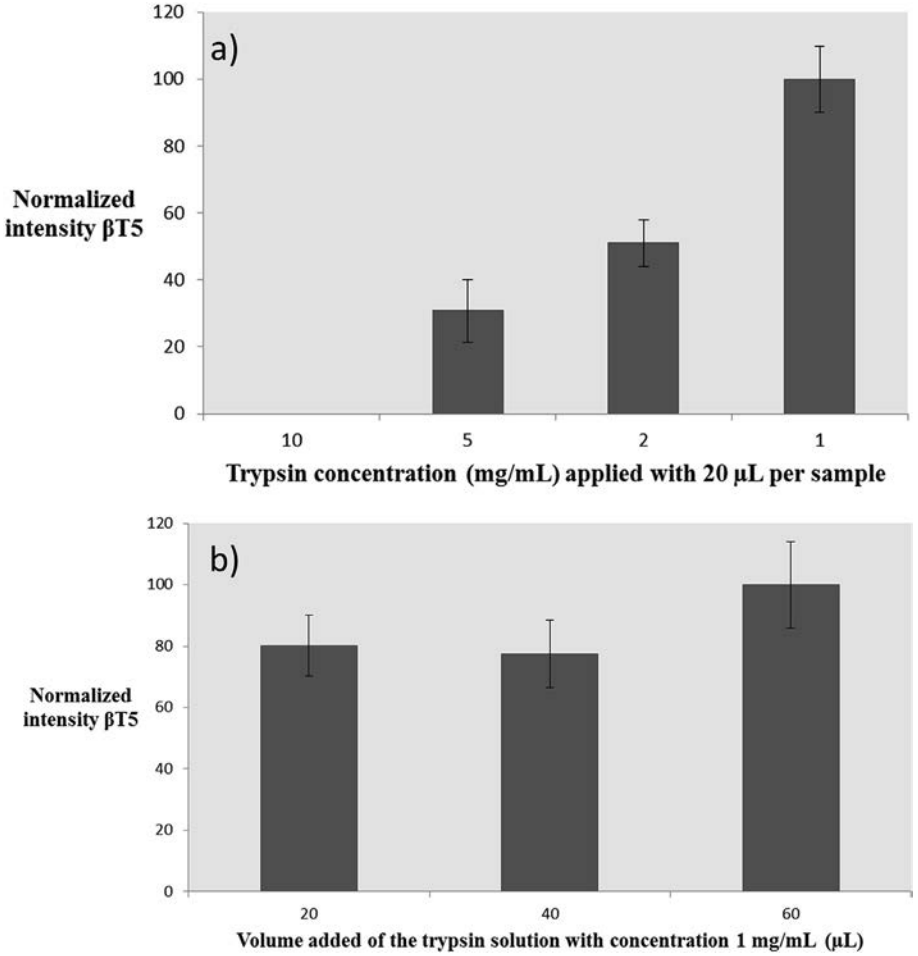

Fig. 4. Normalized signal intensity for tryptic peptide βT5 digested in-device at *a*) different concentration of trypsin (V = 20 µL) (Normalized to trypsin concentration 1 mg/mL with respective average signal 2.7 × 10^3 MS counts per second) and *b*) different applied volumes with a fixed measure of 20 µg trypsin applied per sample (Normalized reaction volume 60 µL with respective average signal 3.4 × 10^3 MS counts per second). The error bars represent the standard deviation, all experiments was carried out with n = 4.

for a direct comparison to our previous efforts [8]. The entire sample preparation was carried out in within 3 h (sample drying time not included). The concept showed satisfactory correlation of $R^2 = 0.98$ (Fig. 5a) and no signal was observed for the analysis of blank serum samples (**S4**). However, the relative standard deviation was high for the upper concentration level (1000 ng/mL). This variation may be a result of inhomogeneous design of these paper circles or capacity in terms of available antibodies immobilized. The thinner paper such as Whatman® grade 1 is prone to slight geometry changes (visual observations) under the polymerization and immobilization steps since these steps involve large aqueous volumes that can potentially dissolve/tear the cellulose backbone to some degree along the sampling spot edges if there is exposed cellulose such as cracks or holes (in the polymer coating along in the device edges). Also, as previously described [20] the thinner paper types could potentially face over-polymerizing (i.e. polymer layer between the individual cellulose fibers creating a less "porous" device with lower sample capacity). This alteration of the paper backbone is not as heavily present when polymerizing a thicker paper substrate such as Whatman® DMPK-C cards since these paper circles are more tolerant towards physical handling during the polymerization process (most likely due to a more dense network of fibers within the paper). It was also observed that sharpness of the punching device was of high importance for the highest reproducibility of the polymer coating process. All taken into consideration, the preparation process of the polymerized sampling spots and in combination with a higher sample capacity, DMPK-C cards were polymerized, immobilized and sampled with 40 µL hCG spiked serum. From these analyses a better correlation ($R^2 = 0.99$) with over all lower standard deviations were obtained (Fig. 5b). Compared to previous work on hCG spiked to serum sampled on conventional DMPK-C material (immunocapture sample clean-up combined with SPE) by Rosting et al. better correlation was seen, though it should be mentioned that the concentration range in the here presented study is slightly different (here: 10–1000 ng/mL, $R^2 = 0.99$ compared to 2 ng/mL – 100 ng/mL, $R^2 = 0.93$). The RSD was in-between 26% and 13% for the lowest (10 ng/mL) and highest (1000 ng/mL) concentration level n = 4, respectively. These RSD values is comparable to previous work by Rosting et al. [28] were the RSD (n = 5) was 13% for 2 ng/mL and 29% for 100 ng/mL (in the presented study the RSD for 100 ng/mL is 18%). Nevertheless, from both the experiments it indicated that it is beneficial to fabricate a thicker sampling spot. A thicker sampling spot (such as DMPK-C) could not only tolerate a higher sample capacity, but also allow for immobilization of potentially more antibodies (from the higher sampling spot surface area and thus, potentially more functional groups for immobilization). Furthermore, as elaborated in previous efforts [20] the thicker papers have been shown to be easier to fabricate in terms of geometry of the polymer layer.

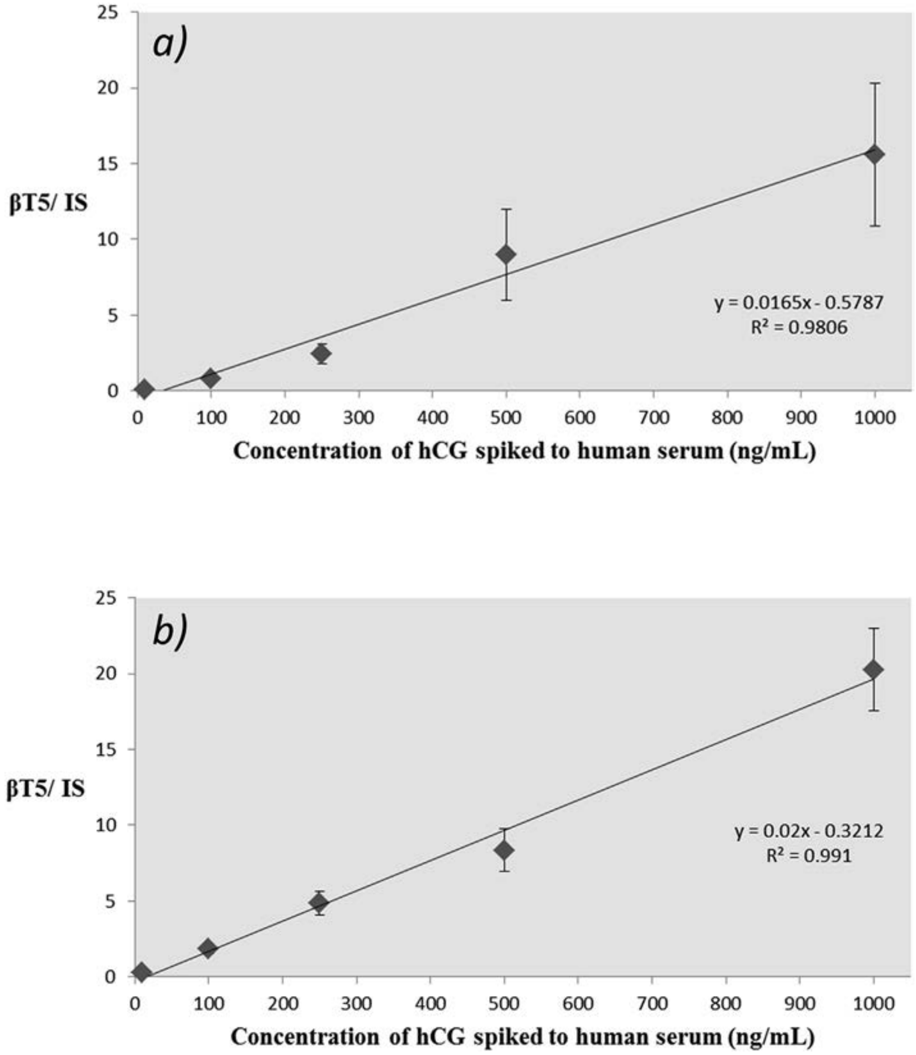

Fig. 5. Five-point concentration curve from 10 to 1000 ng/mL of hCG spiked to human serum. The signal was quantified by the respective βT5/IS peak area. Twenty micro liter sample was applied to pHEMA-VDM Whatman® grade 1 filter paper immobilized with monoclonal antibody E27 (*a*). Forty micro liter sample was applied to pHEMA-VDM Whatman® DMPK-C cards immobilized with monoclonal antibody E27 (*b*). The error bars represent the standard deviation, all experiments was carried out with n = 4.

3.4.2. *Evaluation of limit of detection in serum*

To assess the experimental limit of detection for serum Whatman® grade 1 filter paper was polymerized and immobilized prior to sampling of 20 μL human serum spiked with hCG. This filter paper type and sampling volume was chosen to allow for a direct comparison to our previous efforts on *on-paper* immunocapture [8] and to demonstrate the significance of the optimized sample preparation parameters and significant increase in performance. The limit of detection (S/N = 3) was experimentally obtained at 100 pg/mL (2.64 pM/0.9 IU/L) (**S5**). LOQ (S/N = 10) was theoretically calculated to be 333 pg/mL (3.0 IU/L). The LOD was ten times lower than what has previously been obtained for immunocapture *on-paper (*1 ng/mL *(9.1 IU/L))* [8], but also lower than what has previously been obtained by conventional DBS with DMPK-C cards (7.8 IU/L) and water soluble carboxymethyl cellulose (3.0 IU/L) with serum as sample matrix [28]. Compared to a validated method with more traditional sampling and analysis of hCG by Lund et al. [29] (antibody based sample clean-up) the concept presented here allows for a significant lower detection limit (0.9 IU/L compared to 2.0 IU/L). This, even though the sampling volume is much lower (20 μL compared to 1 mL). Nevertheless, the LOD is still three times higher than the proposed reference limit for healthy adults (˂0.3 IU/L) [30]. This reference limit is expected to be readily achievable by further optimization e.g. amount of antibodies immobilized, sampling spot thickness etc.

3.4.3. *Whole blood analysis*

To demonstrate that the concept also would work for more complex and “in-field” collected samples (i.e. samples collected outside the laboratory), freshly collected human whole blood was included as matrix. Same day collected whole blood was spiked with hCG (in a five point concentration range 10 ng/mL to 1000 ng/mL, n = 4) and subsequently sampled using 30 μL on E27 immobilized pHEMA-VDM polymerized DMPK-C sampling spots. The sampling volume was chosen regarding an experimental suggestion from Peck et al. [31] that the ideal blood volume would be 30 μL to ensure 1 mm clearance to the 6.00 mm ID punch perimeters (given that the blood has a low viscosity/hematocrit). These volumetric suggestions were in compliance with our experimental observations from blood distribution in the sampling spots. This volume could be increased due to the wax printed barriers (in the wax printed cartridge) in compliance with what has been described earlier [19]. However, whole blood possesses higher viscosity accompanied by clothing factors [32] and other components that creates a blood crust relatively fast. Compared to serum (which has a higher water content), a higher volume would be less beneficial since whole blood will wick slower into the sampling spot structure and the sample will lay as a bubble on top of the sampling spot for an extended time. Therefore, an even evaporation rate during the drying cycle (from time of sampling to completely dry) does not take place since a thin film is formed on the interface between the sample droplet and the air. This film slows down the evaporation rate and ultimately creates a crust on the blood surface (resulting in a part of the sample will not wick into the sampling spot. The result of this is; whatever analyte trapped in this crust would likely not have interaction with the immobilized antibodies and will be washed away during the sample preparation stages (assuming a homogenous content of analyte through the blood sample). Additionally, to dissolve the dried blood in a way that the matrix could be readily washed away (hCG is still attached to the mAb) the wash volumes for the whole blood samples was increased to 1000 μL (for all solutions) and extended to 15 min per wash cycle in order to completely dissolve the whole blood crust/matrix absorption in the sampling spot. After the washing procedure, the sampling spot was placed back into the wax printed cartridge and subjected to reduction (at pH 10.2), alkylation, and digestion as described above. From the analysis (Fig. 6) a high degree of correlation between sample concentration and signal intensity was observed ($R^2 = 0.99$). Blank samples (freshly collected whole blood without hCG spike) were also analyzed and did not contribute to any signal (**S6**). The LOD (S/N = 3) and LOQ (S/N = 10) in the freshly collected whole blood samples was theoretically calculated to be 630 pg/mL and 2.10 ng/mL, respectively (on pHEMA-VDM Whatman® DMPK-C cards).

3.4.4. Novelty compared to previous efforts

The presented concept has proven performance as an *all-in-one* sampling device for fast and effortless analysis of disease state proteins in human serum and freshly collected whole blood. The introduction of wax printed cartridges with optimization and integration of the individual sample preparing steps, proved a significantly lower detection and quantification limit (ten times lower) with a total sample preparing time of 3 h compared to 19 h in previous efforts. This may resolve from the possibility to especially perform protein reduction in-device and therefore more optimal reduction conditions could be utilized without affecting later sample preparing steps such as alkylation and hydrolysis in basis of a high pH. The sampling chip offers a potential to be incorporated with its small packaging and accelerated sample preparation for “in-filed” sampling in clinical studies. Nevertheless, the presented sampling device is still at a conceptual stage and therefore a full validation was not within the scope of this work. With further in-depth optimization of the workflow and design of the cartridge (3D printed plastic as an example), the concept holds potential to be validated and readily included in real applications. These are not limited to analysis of diagnostic proteins, but also applicable in environmental, forensic and anti-doping analyses.

4. Conclusion

We have here demonstrated a novel all-in-one sampling concept for bottom-up protein analysis in a chip design. The device performed satisfactory protein reduction, -alkylation and -hydrolysis all within the sampling device *within 3 h* from the sampling stage to time of LC-MS injection. By polymerizing and immobilizing conventional laboratory grade filter paper and DBS sampling cards the concept showed a high degree of performance between 10 and 1000 ng/mL by sampling of hCG spiked to human serum and freshly collected whole blood samples (the latter was only evaluated using polymerized and mAb immobilized DMPK-C cards). LOD (experimentally obtained) and LOQ (calculated) was shown to be ten times lower than previously obtained in human serum samples by conventional DMS sampling. The concept has shown potential for sensitive analysis of lower abundant proteins related towards disease state protein applications by its performance in analysis of serum and freshly collected whole blood samples.

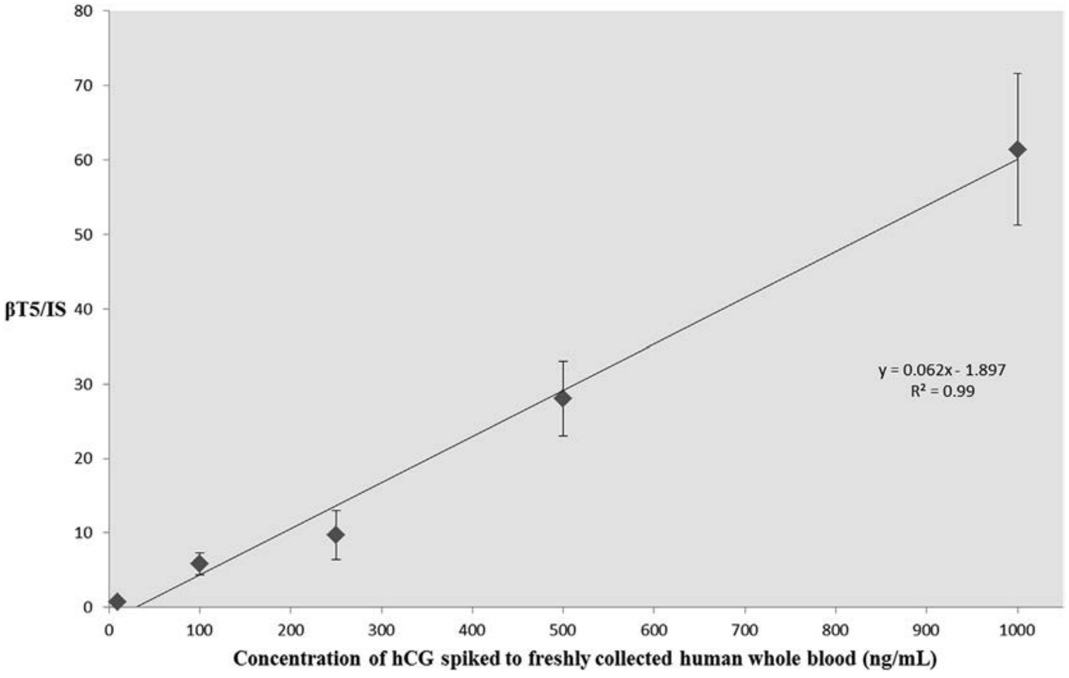

Fig. 6. Five-point concentration curve from 10 to 1000 ng/mL of hCG spiked to human whole blood. The signal was quantified by the respective βT5 and βT5 IS peak intensity. Thirty micro liter spiked whole blood (freshly collected) was applied to pHEMA-VDM Whatman® DMPK-C cards immobilized with monoclonal antibody E27. For all experiments, n = 4.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.08.043.

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Supplementary material to:

All-in-one paper-based sampling chip for targeted protein analysis

by

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Contents

S1 Wax print template

The sampling device holder was fabricated by gluing one solid oblong rectangle of wax with three sets of wax printed circles in a sandwich configuration as shown in Figure 2 (in the journal article). The wax printed template for the fabrication is presented in **Figure S1**.

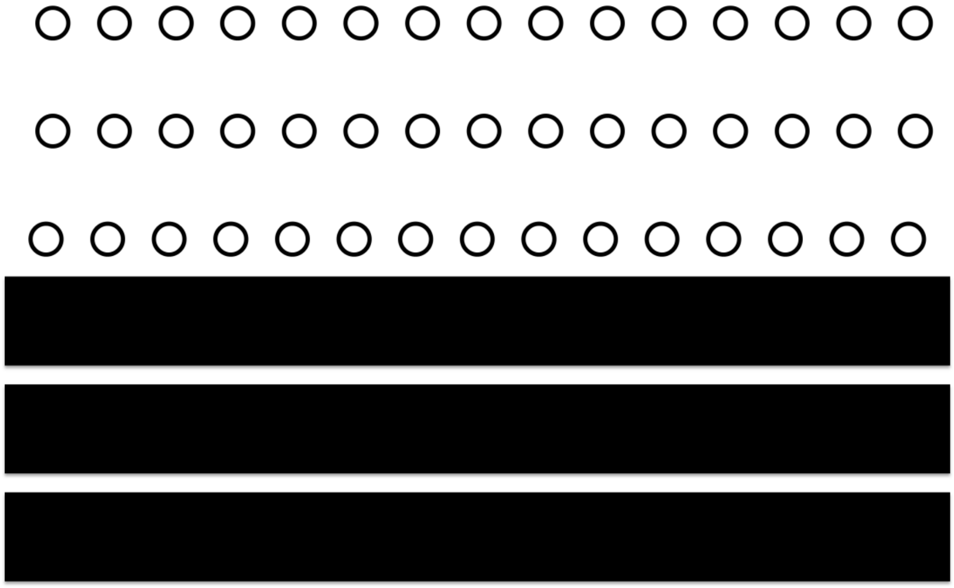

Figure S1 PowerPoint template for wax printing of the all-in-one sampling device cartridge

S2 Work flow diagram

The sample preparation workflow for the sampling chip is illustrated in **Figure S2**. The sample preparation was performed in short: hCG spiked sample was spotted and let to dry. Then, the sampling spots (the polymerized center) was removed and washed with phosphate buffered saline pH 7.4 (PBS) with 0.05% (w/v) Tween 20® and mixed vigorously. The solution was removed and replaced by 500 µL PBS, Tris-HCl (pH 7.6) and 50 mM ABC respectively prior to reinstallation into the wax printed framework. The sample was further reduced and alkylated by of 200 mM DTT in ammonia water (pH 10.2) and of 200 mM IAC in 50 mM ABC (pH 7.8, room temperature) prior to tryptic hydrolysis. The sampling spot was further transferred to a clean

Eppendorf tube and hCG was eluted by acidified water prior to HPLC-vial transfer and HPLC-MS analysis.

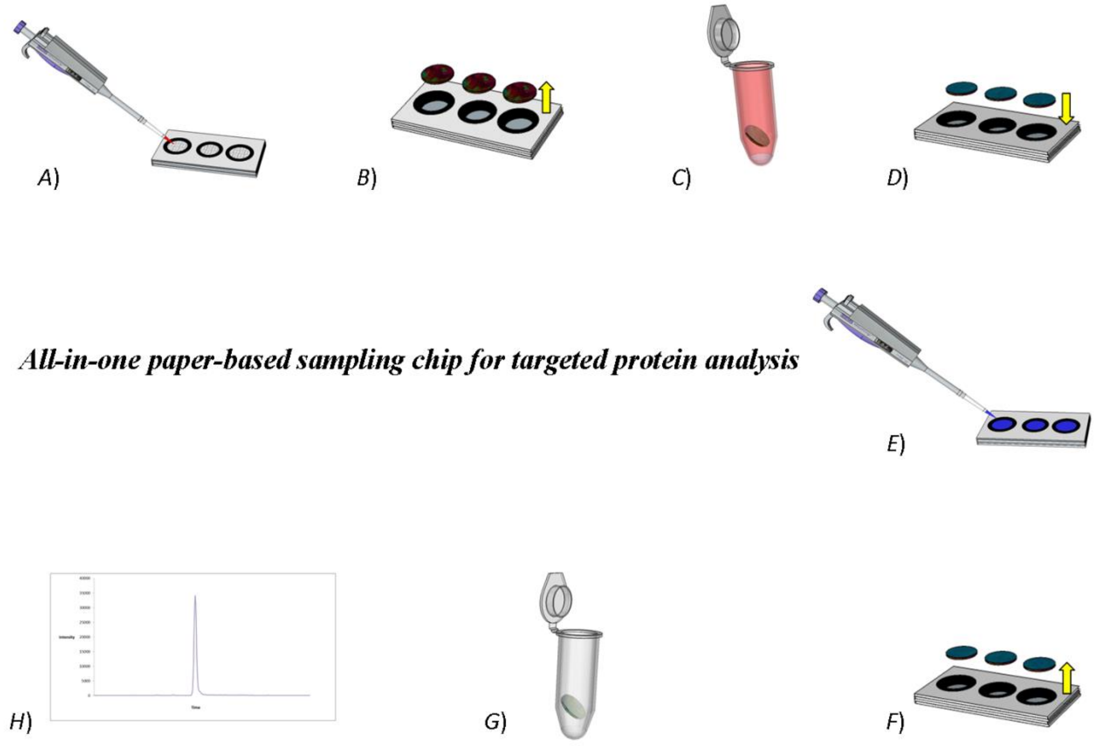

Figure S2 Work flow diagram for the All-in-one sampling chip immobilized with mAb E27 for targeted analysis of hCG. The schematics illustrates A) sampling B) removal of dried sampling spots with a subsequent transfer to a clean Eppendorf tube C) wash of the sampling spot D) re-installation of washed and dried sampling spot to the wax printed chip (could also be dried post installation) E) protein reduction, -alkylation and tryptic hydrolysis F) removal of dried sampling spot G) extraction with 2 % FA H) LC-MS analysis.

S3 Device assembly

The presented wax printed cartridge (**Figure S2**) was assembled by gluing one oblong panel of wax and three wax printed circles (6.0 mm ID) with the center removed (schematically illustrated in Figure 2 in the journal article). For rigidity the device could be glued to an underlying piece of cardboard. A top lid of solid wax could also be included for esthetic purposes. However, this top lid does not introduce increased performance.

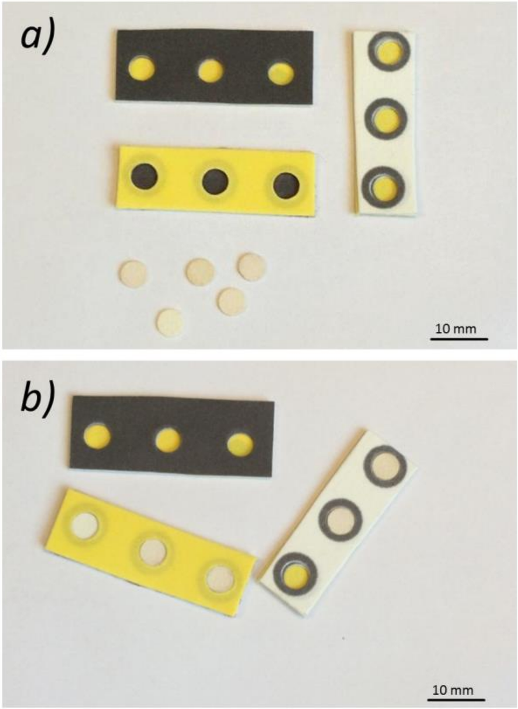

Figure S3 Photograph of the wax printed device a) without installed p-HEMA-VDM polymerized sampling spots and b) with p-HEMA-VDM sampling spots.

S4 Analysis of blank serum

Analysis of blank serum samples is presented in **Figure S4**. The retention time of hCG βT5 was 19.80 min (illustrated with a green area in the chromatogram). For a positive confirmation of the hCG βT5 peptide the ratio between the two fragment ions *m/z* 1327.8 and *m/z* 1036.3 should be 10:4, respectively. For the analysis of the blank serum samples, there was no chromatographic peak at the specified retention time; however one peak was observed at 20.0 min. This peak did not have the correct ratio between the fragment ions. Moreover, for these samples the did not contain the fragment ion *m/z* 1036.3 and thus, could not be identified as hCG βT5.

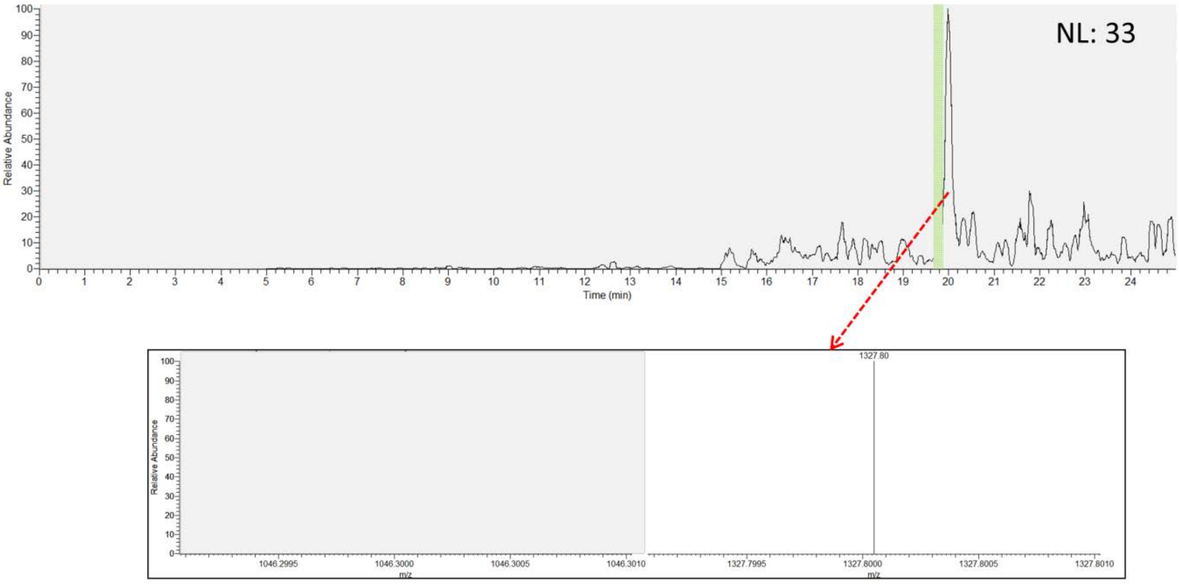

Figure S4 Analysis of blank serum sampled on a E27 immobilized pHEMA-VDM sampling device. The upper figure displays the chromatogram (normalized intensity; 33 cps). The lower box present the ions found in the marked peak (red arrow). The fragment ion ratio does not match the fragment ion ratio of hCG βT5. The green area in the chromatogram represent the retention time of hCG βT5.

S5 Limit of detection

The limit of detection for spiked serum samples was assessed on Whatman® grade 1 filter paper polymerized and subsequently immobilized with mAb E27 prior to sampling. Sampling was carried out with 20 µL human serum spiked with hCG. The limit of detection (S/N = 3) was experimentally obtained at 100 pg/mL (2.64 pM / 0.9 IU/L). The chromatogram for the LOD investigations is presented in **Figure S5**.

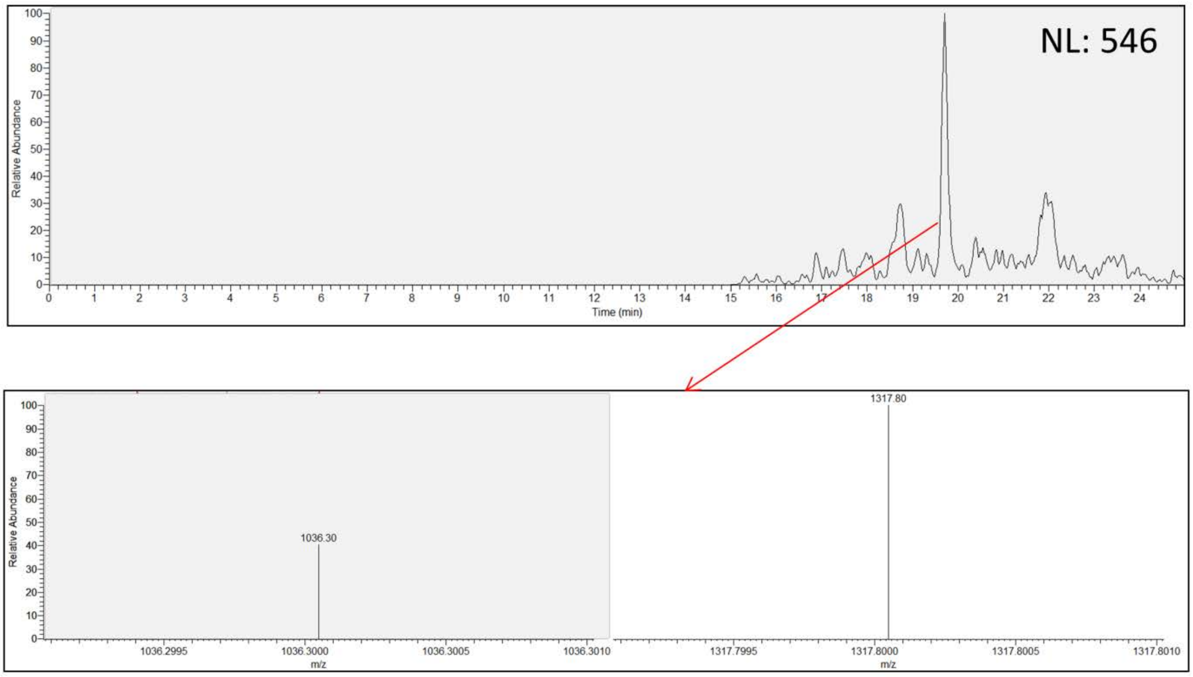

Figure S5 Experimentally obtained LOD at 100 pg/mL of hCG spiked to human serum and sampled on a mAb E27 immobiliized pHEMA-VDM sampling device fabricated from Whatman grade 1 filter paper. Normalized intensity of the hCG βT5 peak is 546 cps

S6 Analysis of blank whole blood

Analysis of blank whole blood samples is presented in **Figure S6**. The retention time of hCG βT5 was 19.80 min (illustrated with a green area in the chromatogram). No peak was observed at the hCG βT5 retention time. The fragment ion pattern at the given retention time did not match hCG βT5 either.

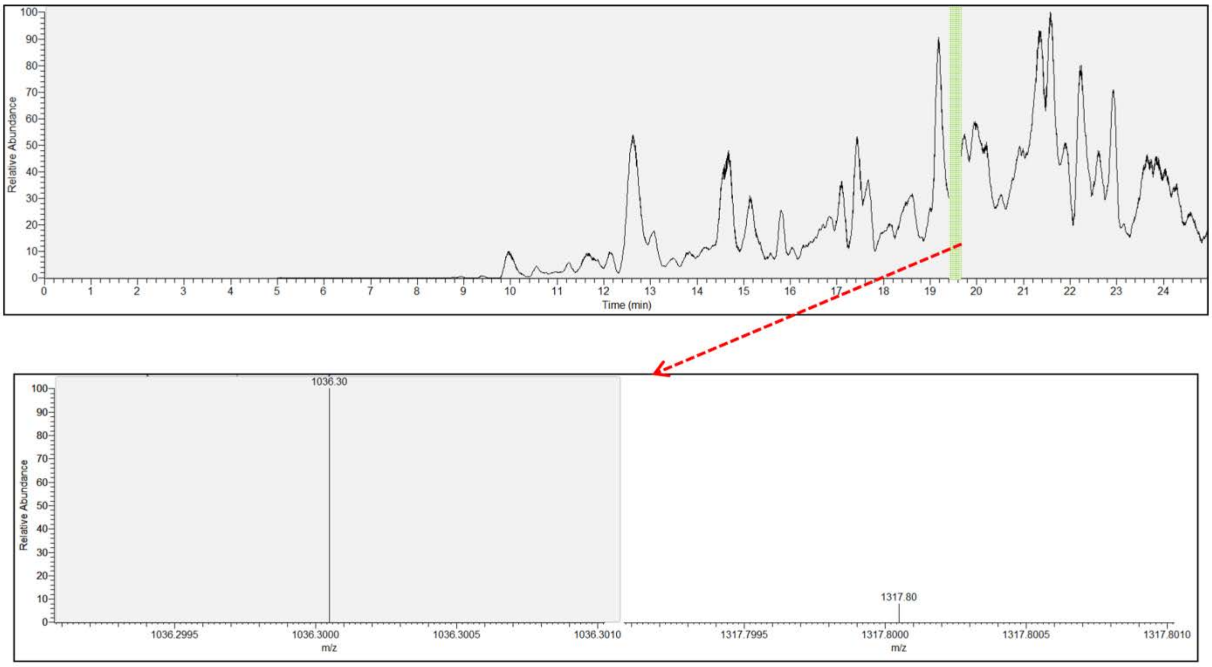

Figure S6 Analysis of blank whole blood sampled on a mAb E27 immobilized pHEMA-VDM sampling device. The upper figure displays the chromatogram (normalized intensity; 415 cps). The lower box present the ions found in the marked peak (red arrow). The fragment ion ratio does not match the fragment ion ratio of hCG βT5. The green area in the chromatogram represent the retention time of hCG βT5.