Biomarker assay systems for onsite monitoring of fatigue

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**BIOMARKER ASSAY SYSTEMS FOR ONSITE MONITORING
OF FATIGUE**

TSYRULNEVA IUNA

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2019

BIOMARKER ASSAY SYSTEMS FOR ONSITE MONITORING OF FATIGUE

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Tsyrulneva Iuna

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7 August, 2019

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Date

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Bo Liedberg

Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journal in which I am listed as an author.

Chapter 5 is published as I. Tsyulneva, P. Alagappan, B. Liedberg. Colorimetric Detection of Salivary α -Amylase Using Maltose as a Noncompetitive Inhibitor for Polysaccharide Cleavage. *ACS Sensors*, 4, **4**, 865-873 (2019). DOI: 10.1021/acssensors.8b01343

The contributions of the co-authors are as follows:

- I prepared the manuscript drafts. The manuscript was revised by Dr Alagappan Palaniappan.
- Prof Bo Liedberg edited the manuscript drafts.
- I co-designed the study with Prof Bo Liedberg and performed all the laboratory work at the School of Materials Science and Engineering. I also analyzed the data.

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Abstract

Overtraining is one of the most underestimated physiological states of athletes. Being recognized as a serious complication for high performance, overtraining is diagnosed based only on verbal complaint of a sportsman, making it highly subjective, or on exhibiting underperformance. Besides, numerous diseases and psychological disorders have the same symptoms. No specific biomarkers have been proposed for detection of overtraining, neither in blood or urine, nor in saliva. Various physiological, behavioral and biochemical markers are constantly examined. Recently, metabolic and hormonal biomarkers demonstrated their potential in assessing overtraining. The variations in concentration levels of salivary cortisol, testosterone and α -amylase are currently being used to evaluate the state of a sportsman. However, these changes can be caused by a variety of other disorders that greatly complicates the work of sport doctors. Presumably, constant monitoring of biomarkers' response to the influence of external and internal factors can provide tools for timely detection of early signs of overtraining development. In this work, saliva has been chosen as a biological fluid that can be tested onsite, being non-invasive and rapidly accessible object.

Recently, the ratio of two salivary heptapeptides was reported to be indicative of overtraining condition. However, saliva as a complex matrix needs to be cleaned up in order to remove high weight proteins, debris and bacteria that otherwise would interfere with the detection of peptides. Few different extraction protocols were explored with the aim to extract the peptides from saliva. High Performance Liquid Chromatography with UV detection (HPLC/UV) and Matrix-Assisted Laser Desorption Ionization-Time of Flight Spectroscopy (MALDI/ToF) were used to identify peptides in extract. Quartz Crystal Microbalance (QCM) platform was developed with the aim to confirm the presence of peptides in salivary extract. A series of antibodies raised against each of peptide were tested in order to distinguish the one with the highest affinity towards a peptide. The surface chemistry was optimized to achieve the best binding of antibody to an immobilized peptide. QCM platform was utilized to identify the peptides of interest in salivary extract.

This work also describes an assay for naked eye detection of salivary α -amylase for enabling assessment of overtraining. The ability of α -amylase to cleave α -bonds of polysaccharides is utilized for developing a colorimetric assay. In the proposed approach, 2-chloro-4-nitrophenyl- α -D-maltotrioside (G3-CNP) as substrate releases a colored by-product upon cleavage by salivary α -amylase. Introduction of maltose as product inhibitor yielded desirable linear responses in the range 20-500 $\mu\text{g/mL}$ and a limit of detection (LOD) of 8 $\mu\text{g/mL}$ (in aqueous solution). The concentration of substrate and product inhibitor were subsequently optimized for naked eye detection of salivary α -amylase. Finally a facile paper-based assay is proposed for analysis of human saliva samples with minimum interference from saliva components. The proposed paper-based assay is rapid, specific and easy-to-implement for onsite testing of saliva samples within 20-500 $\mu\text{g/mL}$ at a LOD of 11 $\mu\text{g/mL}$ (upon visual detection). The proposed assay is benchmarked against state of art method for spectrophotometric detection of α -amylase.

Another biomarker of overtraining that demonstrates distinct response to the intensity of exercise and represents the level of stress in organism is cortisol. To design a strip test where concentration of cortisol in saliva sample correlates significantly with exhibited color, molecularly imprinted polymers (MIPs) were chosen as recognition entities based on their stability to harsh environment, robustness, easy matching with different scaffolds and recognition based on both chemical and structural affinity. Being biodegradable, accessible, disposable, stable and easily modified, paper is a good choice for developing an assay. An indirect competitive assay based on the competition between free cortisol in solution and immobilized glucose oxidase (GOx)-cortisol-21-hemisuccinate (cortisol-21-HS) conjugate in grafted MIP layer at the paper disk was elaborated. The specificity of o-dianisidine dye to develop color gradually due to its sensitivity toward amount of hydrogen peroxide that is produced after enzymatic reaction between GOx and β -glucose was utilized for quantification of cortisol. Achieved LOD for detection of cortisol is 0.3 ng/mL in the dynamic range 0.5 – 20 ng/mL. Developed assay was benchmarked by HPLC/MS.

The combination of reported methodologies on one platform can serve as a potential diagnostic tool for assessment overtraining development in sportsmen.

Lay Summary

Overtraining is one of the most underestimated physiological states of athletes. Being recognized as a serious complication for high performance, overtraining is diagnosed based only on verbal complaint of a sportsman, making it highly subjective, or on exhibiting underperformance. Besides, numerous diseases and psychological disorders have the same symptoms. Imagine the situation when a sportsman visits a sports doctor and complains to a bunch of physiological problems as poor sleep, absence of appetite, anxiety, loss of libido, constant stress etc. A sports doctor after a careful interview diagnoses a sportsman with “depression”, since all symptoms correlate well with this mental condition. This is what happened in the past. Currently, with the science and technologies having done a huge leap forward, overtraining is diagnosed based on the set of various markers that include heart rate, weight, variation in levels of hormones or enzymes etc. There is no ultimate biomarker in human body that correlates with overtraining which means that overtraining cannot be established based on the appearance of a certain compound in blood or saliva. However, physical exercises leave a trace on multiple systems in human body that follow certain patterns in reaction to the amount of exercises. If a way to monitor these changes is found, it will become possible to predict the development of overtraining and thus, to enhance athletes’ performance. Saliva has been chosen for the analysis as a biological fluid that can be tested onsite, being non-invasive and rapidly accessible object.

Recently, the ratio of two salivary heptapeptides was reported to be indicative of overtraining condition. However, saliva is rich with peptides that have similar structure to the target ones and thus, needs to be cleaned up. Several clean up protocols were tested with the aim to remove interfering effect of salivary compounds. High Performance Liquid Chromatography with UV detection (HPLC/UV) and Matrix-Assisted Laser Desorption Ionization-Time of Flight Spectroscopy (MALDI/ToF) were used to identify peptides in extract. Platform that is based on the property of quartz crystal to change frequency of its oscillation upon recognition of antibodies specific for target peptides was developed with the aim to confirm the presence of peptides in salivary extract. However, the detection of mentioned peptides in saliva collected from sportsmen with satisfactory evidence was not possible.

With the aim to detect another overtraining biomarker – α -amylase - assay for naked eye colorimetric detection was developed. In the proposed approach, a substrate releases a colored by-product upon cleavage by salivary α -amylase. First, the system was developed in aqueous solution and it enabled detection of as low as 8 $\mu\text{g/mL}$. Subsequently, the assay was transferred to a paper membrane and optimized with the aim to provide good visual recognition of differences in color intensities of paper membrane that correlate with α -amylase concentration. The proposed paper-based assay is rapid, specific and easy-to-implement for onsite testing of saliva samples within 20-500 $\mu\text{g/mL}$ at a limit of detection (LOD) of 11 $\mu\text{g/mL}$ (upon visual detection). The assay has been tested in saliva samples collected from volunteers. The accuracy of developed assay was confirmed by commercial kit for detection of α -amylase.

Another biomarker of overtraining that demonstrates distinct response to the intensity of exercise and represents the level of stress in organism is cortisol. To design a test strip that doesn't require any equipment and the concentration of cortisol correlates significantly with exhibited color was primary aim. To achieve it, “artificial antibodies” – polymers that have cavities specific in shape and chemical properties to cortisol – were chosen. The cavities of polymer were saturated with a conjugate between cortisol and glucose oxidase (GOx) – an enzyme that turns β -glucose to hydrogen peroxide. When cortisol solution is added, molecules of cortisol that have higher affinity to cavities replace conjugate. Subsequently, conjugate molecules move towards opposite end of the strip. Along the flow they react with β -glucose placed on the strip and produce hydrogen peroxide which upon reaction with peroxidase (POx) and a colorless dye develops red color of different intensity that is commensurate with the amount added cortisol.

Combination of developed assays on one paper platform would enable simultaneous analysis of several biomarkers and can be potentially used as the tool to detect development of overtraining early. Moreover, this approach allows monitoring of concentration fluctuation of other biomarkers, namely testosterone, with the aim to control course of physical or mental condition.

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Figure 6.5.1 Structure of dimeric GOx with carbohydrates on the surface [40]. The active site where glucose binds is in a deep pocket.

Figure 6.5.2 SDS-PAGE of 20 $\mu\text{g/mL}$ GOx (first column) and GOx-cortisol-21-HS conjugate (second column) versus molecular-weight size marker (third column).

Figure 6.5.3 Frequency shift and dissipation upon removal of cortisol from synthesized MIP@SiO₂, addition of GOx-cortisol-21-HS conjugate to MIP synthesized on surface of SiO₂ chip, and its replacement by cortisol molecules. I – washing by buffer; II – washing by mixture of methanol and AcA (9:1); III – washing by buffer; IV – addition of 0.5 g/L cortisol; V – washing by mixture of methanol and AcA (9:1); VI - washing by buffer; VII - addition of 0.1 g/L GOx-cortisol-21-HS; VIII - washing by buffer; IX - addition of 0.5 g/L solution of cortisol; X - washing by buffer.

Figure 6.5.4 Frequency shift and dissipation upon removal of cortisol from synthesized MIP@SiO₂ and addition of GOx to MIP synthesized on surface of SiO₂ chip. I – washing by buffer; II – washing by mixture of methanol and AcA (9:1); III – washing by buffer; IV – addition of 0.5 g/L cortisol; V – washing by mixture of methanol and AcA (9:1); VI - washing by buffer; VII - addition of 0.1 g/L GOx; VIII - washing by buffer.

Figure 6.6.1 Kinetic curve of enzymatic reaction of GOx-cortisol-21-HS that was removed from non-modified paper (red curve) or from MIP@paper (blue curve) compared to enzymatic reaction of GOx-cortisol-21-HS in solution (without being in contact with paper or MIP@paper) (black curve). C (GOx-cortisol-21-HS) = 0.1 g/L. Sample size for MIP@paper and non-modified paper = 10 mm x 10 mm and $\lambda = 405$ nm.

Figure 6.6.2 Kinetic curve of enzymatic reaction of GOx that was removed from non-modified paper (red curve) or from MIP@paper (blue curve) compared to enzymatic reaction of GOx in solution (black curve). C (GOx) = 0.1 g/L. Sample size MIP@paper and non-modified paper = 10 mm x 10 mm and $\lambda = 405$ nm.

Figure 6.7.1 Kinetic curves of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS, 0.1 M β -glucose and 0.1 g/L POx for varying concentrations of o-dianisidine (0.1, 1 and 10 g/L).

Figure 6.7.2 Kinetic curves of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS, 1 g/L o-dianisidine and 0.1 g/L POx for varying concentrations of β -glucose (0.01, 0.05, 0.1 and 0.5 M) as a substrate for GOx.

Figure 6.7.3 Kinetic curves of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS, 1 g/L o-dianisidine, 0.1 M β -glucose and varying concentrations of POx (0.01, 0.05, 0.1 and 0.5 g/L) as a catalyst for the reaction of oxidation of o-dianisidine by H_2O_2 .

Figure 6.8.1 Cortisol homologues that were chosen to be conjugated with GOx.

Figure 6.8.2 Kinetic curve of enzymatic reaction of GOx-CS-HS, GOx-T-HS or GOx-TO that was removed from MIP@paper (navy, green and red curves, respectively) compared to enzymatic reaction of GOx-CS-HS, GOx-T-HS or GOx-TO in solution (pinkish, black and blue curves, respectively). C (conjugate) = 0.1 g/L. Sample size MIP@paper and non-modified paper = 10 mm x 10 mm and $\lambda = 405$ nm.

Figure 6.9.1 Scheme of a lateral flow displacement assay for detection of cortisol that comprises of following zones (from left to right): 1) loading zone, 2) flow equalizer, 3) displacement zone (MIP@paper), 4) flow equalizer, 5) reaction zone, and 6) detection zone. Overall length of a strip is 4 cm.

Figure 6.9.2 Optimized lateral flow displacement strip for detection of cortisol. Membrane components are as following: 1 – cellulose, 2 – glass fiber, 3 – MIP@paper saturated with GOx-cortisol-21-HS, 4 – glass fiber impregnated with 0.1 M β -glucose, 5 – glass fiber impregnated with 0.1 g/L POx, 6 – cellulose fiber with 1 g/L o-dianisidine deposited via vertical flow.

Figure 6.10.1 Colorimetric response of lateral flow displacement strip after addition of 0.1, 0.5, 1.0, 5.0, 10.0 and 20 ng/mL of cortisol, from left to right. Images are taken after 2 min of reaction. For RGB analysis detection pad marked by red box was resized to 75x150 pixels.

Figure 6.10.2 Colorimetric response of lateral flow displacement strip with 0.5 M β -glucose impregnated in glass fiber instead of 0.1 M, after addition of 0.1, 0.5, 1.0, 5.0, 10.0 and 20 ng/mL of cortisol, from left to right. Image is taken after 5 min of reaction. For RGB analysis detection pad marked by red box was resized to 75x150 pixels.

Figure 6.11.1 Estimation of expected color difference of two membranes for visual cortisol detection assays that include 0.15 mM (black box) or 0.3 mM (white box) H_2O_2 , pre-deposited on detection pad, with reaction time 2 min.

Figure 6.11.2 RGB analysis of detection pad with pre-deposited 0.15 mM (a) and 0.3 mM (b) H_2O_2 in response to cortisol with increasing concentration.

Figure 6.11.3 Analysis of RGB ratios as a variable of colorimetric response of evaluated assays that consist of 0.15 mM (a) and 0.3 mM (b) H_2O_2 pre-deposited in detection pad.

Figure 6.15.1 Correlation among the results obtained from quantitative estimation of cortisol level (by RGB analysis) and from semi-quantitative visual estimation versus results obtained by HPLC/MS taken as a standard for detection of cortisol.

Figure 7.3.1 Schematic representation of a onsite testing device for simultaneous colorimetric detection of α -amylase and cortisol in saliva.

Figure 7.3.2 An example of gamut of standard membranes (1) and real images of standard membranes (2) as a response to increasing concentration of α -amylase (from 20 to 500 $\mu\text{g/mL}$).

Figure 7.3.3 Color gamut for lateral flow displacement assay for detection of cortisol (0.1-20 ng/mL).

Figure 7.3.4 Mass-spectra of saliva collected from 6 sportsmen (a) before and (b) after training in a range of m/z 600-800.

Abbreviations

AA	Acrylamide
Ab	Antibody
AcA	Acetic Acid
ACN	Acetonitrile
ADH	Adipic Acid Dihydrazide
ADHD	Attention Deficit Hyperactivity Disorder
AIBN	Azobisisobutyronitrile
ANS	Autonomous Nervous System
AT	Anaerobic Threshold
BCAA	Branched-Chain Amino Acids
BSA	Bovine Serum Albumin
CNP	Chloronitrophenol
CNS	Central Nerve System
Cortisol-21-HS	Cortisol-21-Hemisuccinate
CS	Corticosterone
CS-HS	Cortisocosterone Hemisuccinate
DHB	2,5-Dihydroxybenzoic Acid
DHEA	Dehydroepiandrosterone
DI	Deionized
DMF	Dimethylformamide
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide
EGDMA	Ethylene glycol dimethylacrylate
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Formic Acid
FAD	Flavin Adenine Dinucleotide
FESEM	Field Emission Scanning Electron Microscopy
G3-CNP	2-chloro-4-nitrophenyl- α -D-maltotrioside
Gal-G2-CNP	2-chloro-4-nitrophenyl-4-O-b-D-galactopyranosylmaltoside
GC	Gas chromatography

GOx	Glucose Oxidase
GOx-cortisol-21-HS	Glucose Oxidase Conjugate with Cortisol-21-Hemisuccinate
HPA	Hypothalamic-pituitary-adrenal Axis
HPLC/MS	High Performance Liquid Chromatography with Mass Spectrometry
HPLC/UV	High Performance Liquid Chromatography with UV detection
IG	Immunoglobulin
LOD	Limit of Detection
MALDI/ToF	Matrix-Assisted Laser Desorption Ionization-Time of Flight Spectroscopy
MES	2-(N-morpholino)ethanesulfonic acid
MIP	Molecularly Imprinted Polymers
MIP@paper	Molecularly Imprinted Polymers At Paper Membrane
MS/MS	Tandem Mass Spectrometry
NHS	N-hydroxysulfosuccinimide
NIP	Non-Imprinted Polymers
NIP@paper	Non Imprinted Polymer at Paper Membrane
PBS	Phosphate-Buffered Saline
POx	Peroxidase
PVDF	Polyvinylidene Difluoride
QCM-D	Quartz crystal microbalance with dissipation
RGB	Red Green Blue
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SH-C15-COOH	16-Mercaptohexadecanoic Acid
SH-C18	1-Octadecanethiol
SNS	Sympathetic Nervous System
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
T	Testosterone
T-HS	Testosterone Hemisuccinate

TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TO	Testosterone 3-(O-carboxymethyl)oxime
Tsa	Salivary Threshold
UV-Vis	Ultraviolet-Visible

Chapter 1

Introduction

With a lot of effort being put into assessment of overtraining and mechanisms of its development, it still remains one of the most underestimated physiological states of athletes, although it is known as a serious complication for high performance. Till recent time, overtraining was diagnosed based on exhibiting underperformance or on verbal complaint of a sportsman, compromising the objectivity of diagnosis. Besides, numerous diseases and psychological disorders have the same symptoms. For the present, no specific biomarkers have been proposed for detection of overtraining, neither in blood or urine, nor in saliva. It is hypothesized that constant monitoring of biomarkers' response to the influence of external and internal factors is a viable strategy for timely detection of early signs of overtraining development. Selection of biomarkers that have specific response to the exercises, evaluating methods of their fast, non-invasive, low cost and facile detection and developing a platform that facilitates their application onsite are the main focuses of this thesis.

1.1 Background and problems statement

Investigation of biomarkers in biological fluids and of related disorders in human body engrosses minds of researchers. Identifying the sole biomarker that indicates the presence or onset of a health problem is a challenging task since plenty of unknown factors can affect its appearance. Overtraining syndrome that previously was ascertained from athletes' subjective judgments is characterized by various symptoms and changes in physiological parameters. Nowadays, multiple biomarkers for identification of overtraining are proposed, but they require constant monitoring of an athlete which puts a big burden on sports doctors and requires continuous control to establish the influence of other disorders on their concentration alteration. Moreover, methods that are currently proposed for detection of biomarkers are laboratory based, time and labor consuming that complicates routine assessment of biomarkers in field settings. Developing a simple, cheap and intuitively clear platform to enable simultaneous quantitative or semi-quantitative estimation of biomarkers' amount in human fluids within minutes could benefit sportsmen in terms of early detection of likelihood to develop overtraining. It would also have a positive impact on their overall performance and could potentially reduce the workload of doctors.

1.1.1 Overtraining

Significant efforts have been devoted to the improvement of sportsmen performance, which include both strength and endurance. Although the intensity of physical activities is generally known to improve the performance, intense training periods should be alternated with periods of rest and recovery. A persistent exposure to high levels of intense training load leads to overreaching [1]. Equally, when a sportsman is exposed to extra stressors while overreaching, it may lead to the overtraining syndrome [2]. The main symptoms include fatigue, heavy muscles, persistent soreness, inability to complete routine training which negatively affects the sportsman performance, mood disturbance and anxiety that provoke depression, physiological changes such as weight loss or gain, excessive sweating, constant minor infections and slow return of heart rate to normal after exercise. The explanation of overtraining is still debatable. In some works it is defined as just

unexplained underperformance syndrome [3]. Studies indicate that overtraining syndrome happens quite rarely. Non-functional overreaching, which is usually confused with overtraining, has the same symptoms as overtraining, but as mentioned before, it can be overcome after prolonged rest. Thus, high performance can be reached by adopting a balanced alternation of load and rest. Rest is important in terms of compensation of applied stress during physical exercises through an adaptation mechanism that involves enhanced heart performance, strengthening of capillaries in muscles, restoration of glycogen stores and mitochondrial enzymes. Therefore, if the recovery period is not long enough, the physical performance will gradually decline. Besides, lack of rest during recovery periods can result in increasing exhaustion that is difficult to recover from. Overtraining syndrome also results in increasing levels of cortisol and muscular breakdown products as well as decreasing levels of testosterone. Ultimately, the human body might lose its ability to fix the associated health problems during rest. Reduced performance of a sportsman who trains hard can arise from two reasons: constant recurrence of the same activity results in flat response due to the adjustment of the central nerve system (CNS) to the absence of stimulus [4] and the lack of time for sufficient rest and self-healing [5].

1.1.2 Importance of predicting overtraining development

Overtraining is one of the most underestimated physiological states of athletes. Whyte et al [6] claim that every tenth elite sportsman and every tenth American college swimmer experiences overtraining. Sports researchers predict that up to 70% of high-level endurance athletes are susceptible to facing overtraining during their career [7]. Being recognized as a serious complication for high performance, overtraining is diagnosed based only on verbal complaint of a sportsman or on exhibiting underperformance. It means that overtraining can be diagnosed only ex post, when general fatigue and stress already have negatively affected athlete's health. During training season, the overtraining may result from excessive load of exercises, both high volume (multiple sets to repeatedly target a specific muscle group in an attempt to breakdown muscle tissue and increase tension time as a means to illicit growth) and high intensity (exercises performed with a high level of effort which is thought to stimulate the body to produce an increase in muscular strength

and size). Although high load of exercises generally leads to increase of performance [8], there is a thin line between maximum adaptation to the loads and a condition when recovery time is not sufficient for full adaptation. Moreover, an athlete can be affected by his dietary and routine habits in the same amount as by training. Development of overtraining does not happen overnight – it is a continuous exposure to high loads that is not alternated with proper rest and that provides sports doctors with an avenue of predicting overtraining and preventing sportsmen from going under extreme loads [9].

Currently, full inspection of various body systems and functions is used to confirm overtraining. It includes interviewing an athlete that involves subjective evaluation of personal state, estimating physiological and behavioral biomarkers (weight control [10], sleep quality, heart rate [11-13], electrocardiography [14], echography [15] etc) non-invasively, biochemical markers (blood analysis [16-17], ionnogram [18-19], BCAAs [20-21], glutamine, non-organic constituents [22-24] etc), hormones and immunoglobulins as metabolic regulators [25-30] via blood or urine analysis.

Saliva is an ideal biological fluid for fatigue and overtraining assessment because of minimal invasiveness of sample collection process, and as it offers a route to conduct real-time physiological monitoring in a field setting (e.g. at a sports camp). Besides, saliva contains only free, biologically active, fraction of hormones that facilitates analysis. So far, salivary cortisol, testosterone and salivary α -amylase have been proposed to control overtraining condition [31]. However, the levels of these markers vary within an individual, which requires long term monitoring of its baseline level and other physiological parameters, as well as knowledge about the athlete's history of health disorders [32]. Recently, a ratio of two heptapeptides was proposed as an objective biomarker responsible for overtraining and fatigue in sportsmen [33]. However, this ratio was used to establish fatigue level in nine cyclists with no future studies for other sportsmen performed.

Collecting all parameters of an individual can be time consuming and stressful. Moreover, the methods that are currently used to detect biochemical markers are laborious, bulky, require professional equipment and personnel that drastically decreases the efficiency of

overtraining diagnosis. Although they enable specific and robust analysis, they cannot be implemented onsite, in field settings. Here the demand in onsite testing devices arises as the way to target main drawback of instrumental analysis and to ensure absence of special equipment, facile analysis of result and fast readout. Another advantage of onsite testing devices lies in enabling a sportsman to conduct analysis by himself and report about the results to the doctor.

Given that suggested biomarkers are always present in human biological fluids and have individual response to various external and internal factors, it is critical to establish a baseline for each sportsmen. Thus, sports doctors need to monitor the response of physiological functions to external factors in order to establish the deviations where onsite testing devices can become of great use.

1.2 Hypothesis

This thesis examines the hypothesis that fingerprint of two salivary heptapeptides along with α -amylase and cortisol has the perspective to be used for confirmation of fatigue and overtraining in sportsmen. Constant monitoring of these biomarkers can serve as a tool for investigating the mechanisms of overtraining development. Complex salivary matrix chosen as analysis object compels to optimize extraction protocol in order to avoid interference. Difference in chemical structure and properties of analytes enables investigation of various extraction procedures.

This thesis tests the premise that visual paper-based tests would demonstrate a viable route for detection of overtraining biomarkers in terms of sensitivity, speed, disposability, robustness and ease of use for onsite detection. It also opens up for cost effective biochemical analysis where highly qualified personnel and bulky/expensive equipment become redundant.

This thesis supports the assumption that paper-based assay for visual detection of α -amylase based on its specific property to cut α -bonds of polysaccharides coupled to a dye

and to release a dye would be characterized by high specificity and sensitivity. It also validates that assay comprised of a glucose oxidase – cortisol conjugate and molecularly imprinted polymers as recognition sites would perform the best for detection of cortisol. Visual detection in both cases will be done by intensity of colored pad in less than 15 minutes.

Besides, this thesis outlines continuous monitoring of salivary α -amylase and cortisol as a good approach for detecting overtraining.

1.3 Specific objectives

In this thesis, the initial focus would be on analyzing literature for the most relevant and representative salivary biomarkers of overtraining. Then protocols for efficient extraction of target peptides as overtraining biomarkers from saliva followed by the elaboration of a piezoelectric platform capable of recognizing and quantifying the peptide biomarkers will be examined. The next part of this research work will include the studies of kinetics of enzymatic reaction between α -amylase and a polysaccharide coupled to a dye, optimization of homogenous assay, its transfer to a paper membrane with subsequent visual (semi-quantitative) and instrumental (quantitative) analysis. The developed assay will be tested in saliva samples collected from a pool of volunteers. The last part of the work will focus on the synthesis of MIPs, characterized by high specificity towards cortisol and on the development of displacement test strip.

The objectives are as follows:

- 1) Evaluate biomarkers of overtraining proposed in the literature, and explore their potential. Choose a few biomarkers and validate their performance;
- 2) Examine available extraction protocols and propose one that offers the highest recovery degree of target biomarkers from biological fluid;
- 3) Optimize conjugation and surface chemistry for stable and efficient immobilization of target analytes, on the surface of the sensor chip with the aim to develop an assay that does

not require labeling of target analyte. Develop sensing platforms for recognition and detection of biomarkers and assay optimization;

4) Investigate and optimize kinetics of enzymatic reaction between α -amylase and 2-chloro-4-nitrophenyl- α -D-maltotriose (G3-CNP) as its substrate that releases a colored by-product (chloronitrophenol) upon cleavage by α -amylase. Analyze colorimetric response to variation in concentration of substrate and inhibitor as well as with time.

5) Benchmark developed assay versus gold standard in the analysis of salivary α -amylase.

6) Evaluate various protocols of synthesis of MIP@paper and synthesis of GOx – cortisol conjugate; explore the specificity of synthesized cavities towards conjugate and cortisol.

7) Design a lateral-flow assay for fast detection of cortisol; apply designed assay to the analysis of cortisol in saliva.

1.4 Novelty of this study

A plethora of research studies has been devoted to the study of overtraining, mechanisms of its development and methods of its confirmation. The response of physiological, behavioral, biochemical and metabolic biomarkers to the development of overtraining is under close attention of sports scientists. The methods for detection of biomarkers have been already elaborated and successfully applied. However, the methods currently utilized are mostly laboratory based and require qualified personnel for data acquisition and interpretation. Moreover, delay in obtaining results can lead to the missing of time point when a sportsman inevitably develops overtraining.

The existing concerns can be addressed by employment of onsite testing devices - simple devices that can be used by patient in home or field setting without a need to get the results explained by a trained professional. Currently onsite testing devices are embracing healthcare market through reducing costs and increased efficiency of diagnostic and monitoring. A simple paper based platform with several detection systems employed could potentially enable fast estimation of target analytes' level.

On the other hand, constant monitoring of essential biomarkers with fixation of a response to various external and internal factors can provide a doctor with a baseline of a healthy person's individual reactions to stimuli. When abnormality in reaction to same factors is observed, decision about disrupted signal exchange between body systems can be taken and sportsman can be asked to refrain from further exercises until results are confirmed. Fast reaction can help a sportsman prevent overtraining development. Currently, diagnosing of overtraining occurs post factum when sportsman's performance significantly drops. To attend to the issue that is widespread in sports, this thesis describes a paper based test that enables simultaneous analysis of two biomarkers from low volume of saliva that is collected by passive drool.

Based on the evaluation of literature, there were no previous reports that describe all-encompassing solution to a problem.

1.5 Dissertation overview

This paper consists of 7 Chapters. Chapter 1 presents an introduction to a current problem of overtraining diagnosing, states the biomarkers that are currently utilized for this purpose and methods used for their detection, advocates the necessity of a mobile device. Chapter 2 is literature review on development of overtraining syndrome, structure, properties and methods of detection of heptapeptides, α -amylase and cortisol in saliva. Chapter 3 covers description of extraction procedures of analytes from saliva, protocols for elaboration of sensing platforms, characterization techniques used for determination of analytes' concentration and methods used for the validation of developed approaches. Chapter 4 describes steps to improve peptides extraction from saliva samples, development of piezoelectric platform with antibodies as recognition molecules and optimization of surface chemistry with the aim to be utilized as a reference method for detection of heptapeptides. Chapter 5 depicts development of colorimetric method for detection of α -amylase with its subsequent transfer to a paper membrane. Chapter 6 describes synthesis and characterization of MIP@paper for detection of cortisol and illustrates the elaboration of

displacement assay on a paper-based strip test. Chapter 7 presents conclusions of this thesis, outlines directions for future research and mentions several reconnaissance studies.

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Chapter 2

Literature Review

Diagnosis of overtraining is a subject of careful investigation of symptoms and collecting background information about an athlete's health. In order not to confuse general underperformance with overtraining, continuous monitoring of physiological responses to stimuli with the aim to create a baseline for each athlete and recording inadequate non-characteristic deviations in reaction should be performed. Salivary cortisol and α -amylase proved to demonstrate distinct variation in concentrations in the response to exercises which makes them ideal biomarkers of overtraining. Current methods used for their detection are laboratory based, require expensive equipment and trained personnel that complicates analysis on-the-go. Elaboration of paper based platform that facilitates simultaneous visual (semi-quantitative) detection of cortisol and α -amylase in their natural range within minute amount of saliva and doesn't require bulky equipment could potentially benefit athletes and their doctors and could expedite more efficient predicting of overtraining development.

2.1 Overtraining versus overreaching

Significant efforts have been devoted to the improvement of sportsmen performance, which include both strength and endurance. Although the intensity of physical activities is generally known to improve the performance, intense training periods should be alternated with periods of rest and recovery. A persistent exposure to high levels of intense training load leads to **overreaching** [1]. Being recognized as the condition of the body when the loads accumulate and disappear after a period of rest and recovery, overreaching can enhance physical performance. Equally, when a sportsman is exposed to extra stressors while overreaching, it may lead to the **overtraining** syndrome [2, 3], the main symptoms of which include fatigue, heavy muscles, persistent soreness, inability to complete routine training which negatively affects the sportsman performance, mood disturbance and anxiety that provoke depression, physiological changes such as weight loss or gain, excessive sweating, constant minor infections and slow return of heart rate to normal after exercise. The explanation of overtraining is still debatable. In some works it is defined as just unexplained underperformance syndrome [4]. Studies indicate that overtraining syndrome is diagnosed quite rarely at the same time Morgan et al [5] state that overtraining is likely to develop in 70% of high level endurance sportsmen during their sports career. Whyte et al [6] claim that every tenth elite sportsman and every tenth American college swimmer currently experiences overtraining. The reason for ambiguity in estimation can lie in the fact that overtraining sometimes is confused with non-functional overreaching as it has the same symptoms, however, it can be overcome after prolonged rest.

Overtraining is characterized by exhibiting underperformance due to fatigue, heavy muscles, persistent soreness and inability to complete routine training; by mood disturbance (depression, loss of appetite, increased fluid intake, loss of libido, anxiety etc); and by physiological changes: weight loss/gain, swollen lymph glands, excessive sweating, gastrointestinal disturbances, constant minor infections, reduced VO_2 max, slow return of heart rate to normal after exercise and insomnia [1, 7, 8]. The symptoms of physiological conditions of athletes are shown in Figure 2.1.1.

Figure 2.1.1 Demonstration of difference between overreaching and overtraining

High performance can be reached by adopting a balanced alternation of load and rest. Rest is important in terms of compensation of applied stress during physical exercises through an adaptation mechanism that involves enhanced heart performance, strengthening of capillaries in muscles, restoration of glycogen stores and mitochondrial enzymes [9]. Therefore, if the recovery period is not long enough, the physical performance will gradually decline. Besides, lack of rest during recovery periods can result in increasing exhaustion that is difficult to recover from. Overtraining syndrome also results in increasing levels of cortisol and muscular breakdown products as well as decreasing levels of testosterone [10]. Ultimately, the human body might lose its ability to fix the associated health problems during rest.

Two different types of overtraining are recognized [11]. The *sympathetic* form of overtraining occurs after training of high intensity/activity, while the *parasympathetic* form can happen after exercises at high volume/loads. The symptoms of overtraining are different for both forms but they end up with fatigue and reduced performance. Reduced performance of a sportsman who trains hard can arise from two reasons: constant recurrence of the same activity results in flat response due to the adjustment of the central

nerve system (CNS) to the absence of stimulus [12] and the lack of time for sufficient rest and self-healing.

The sole mechanism of overtraining has not been established yet. Petibois et al [13] proposed that metabolic adaptation occurs in several places in an organism: liver, kidney and skeletal muscle cells. Changes of myocyte levels, for example, are believed to be caused by physical breach of proteins and by oxidation of subcellular components. Due to great leap in utilization of oxygen during exercises, the formation of highly reactive oxygen species takes place [14]. They can affect the membrane permeability by oxidizing lipids which leads to the increased level of certain substances, as myoglobin creatine phosphokinase etc, in blood. Estimation of level of released substances can predict the development of overtraining.

The Glutamine Hypothesis of overtraining development considers depletion of glutamine that is produced by skeletal muscles as a way to overtraining [15]. Level of glutamine typically returns to the baseline within several hours, however, inability of human body to replenish glutamine can be the evidence of overtraining development. However, glutamine, either in blood or in saliva, raises complications for its analysis because of complex nature that causes its release. Moreover, recent results obtained from a different group of athletes do not support this theory [16] as the depletion of glutamine is frequently occurring in overtrained sportsmen with immune insufficiency [17].

It is widely known that any type of exercises causes depletion of glycogen. Poor glycolysis might lead to formation of toxic compounds such as hypoxanthine and xanthine that preferentially accumulate in muscles cells that affects energy provision of muscles. Although high depletion of glycogen can be easily overcome before the training by glycogen refill but long term depletion can have an impact on branched-chain amino acids (BCAAs) by oxidizing them [18]. In turn, these BCAAs compete with tryptophan in binding to albumin. While free tryptophan increases and the level of BCAAs decreases sufficient adaptation of a human body to an activity is observed. Once this ratio of

tryptophan to BCAAs decreases, overtraining kicks in. Therefore, the assessment of BCAA level in blood can be an indicator of the development of fatigue.

It is generally recognized that endurance exercises trigger metabolic processes within muscles that can be linked to tissue inflammation. One of the widely used indexes to assess fatigue is a ratio of testosterone (T) to cortisol (C) that represents anabolic-catabolic processes in the organism and is a general estimation of protein uptake. Unlike other markers, hormones are released to the saliva that enables their fast and non-invasive assessment. Numerous studies on the high intensity resistance exercises did not demonstrate a good correlation between T/C ratio and overtraining [19, 20], especially immediately after training. However, protein metabolism might be not the primary mechanism to overtraining development but rather an energy metabolism that utilizes energy from carbohydrates and lipids.

Other symptoms such as increasing/decreasing heart rate might not be representative indications of overtraining. For example, during rest period the heart rate of a sprinter is increased while heart rate of a weightlifter is decreased [21]. Importantly, none of proposed mechanisms is unopposed. Overtraining can be caused by all of the above-mentioned factors and combinations thereof that greatly complicate definition and reasons of overtraining.

2.2 Saliva as an ideal object for estimation of overtraining syndrome

Biomarkers of overtraining can be found in blood, serum, saliva and sweat. Saliva was chosen as an ideal biological fluid for fatigue and overtraining assessment because of minimal invasiveness of sample collection process, and as it offers a route to conduct real-time physiological monitoring in a field setting (e.g. at a sports camp). Saliva is a complex fluid that basically consists of water (>99%). It has slightly less components as compared to serum. The abundance of sodium, potassium and calcium cations as well as chloride and hydrocarbonate anions plays an important role in salivary osmosis. Saliva contains a large number of different proteins, however, their concentration is much less than in serum,

blood or urine. The organic constituents of saliva comprise of lactates, urea, maltase, albumin, creatinine, mucin, immunoglobulins, ascorbic acid and small quantities of hormones. The proteins and enzymes are also present in saliva with highest quantity of salivary α -amylase and immunoglobulins [22]. Salivary amylase is the only protein in saliva that is synthesized by the salivary glands and its concentration significantly surpasses the levels of α -amylase found in other biological fluids.

Hormones that respond to physical exercises are also present in saliva, e.g. cortisol, testosterone and dehydroepiandrosterone (DHEA) [22]. Moreover, only free fraction of hormones is available in saliva [23]. In human fluids, most of hormones are bound to a carrier protein (albumin) and appear to form large complexes that make the hormone inactive. Thus, only free, unbound, hormones have biological activity and pose interest for investigation. Their levels fall behind the levels in blood (approximately in 10 times). Therefore, more sensitive methods are required for analysis than those currently used for detection of hormones in blood. Moreover, being threshold compounds, all constituents of saliva require constant monitoring of variation in biomarkers' concentrations as only alteration in response to external or internal stimuli as compared to baseline can be considered a sign of overtraining.

2.3 Biomarkers of overtraining in saliva

Unlike other biomarkers associated with fatigue, fluctuations in salivary α -amylase levels are directly caused by autonomous nervous system (ANS) response to parasympathetic and sympathetic stimulation [24]. For example, parasympathetic stimulation of salivary glands leads to a secretion of watery saliva, which is poor in organic and inorganic constituents. On the contrary, sympathetic stimulation is followed by production of saliva that is rich in proteins, especially α -amylase. Thus, increase/decrease in the amount of salivary amylase can be a marker of sympathetic nerve activity. Fluctuations of α -amylase are indicative of many physiological processes including fatigue and overtraining. Moreover, level of α -amylase is strongly associated with depression [25], stress [26, 27], panic disorders [28], ADHD [29] etc. With fatigue development being a consequence of inappropriate response

of ANS to physiological processes [24, 30], constant monitoring of α -amylase enables assessment of fatigue and could possibly be used to predict onset of fatigue.

Salivary α -amylase is a prevailing enzyme in saliva. Its main function is to degrade starch and glycogen at α -bond by forming glucose and maltose. The escalation of plasma catecholamines produced after exercises (that is an evidence of adrenergic effect) increases the amount of salivary proteins including α -amylase. According to Rohleder [31], salivary α -amylase is considered to be a more sensitive biomarker of overtraining-related stress than cortisol. This advantage relates to the production of α -amylase directly in salivary glands whereas cortisol is transferred from blood to saliva. At the same time, the levels of salivary α -amylase can be influenced by other conditions as insomnia, stomatopathy, and stresses induced by other factors than physical training. α -Amylase behaves contradictorily towards chronic influence of sympathomimetic agonists. For instance, Johnson et al. [32] reported diminishing of levels of α -amylase concentration whilst Amsterdam et al. [33] reported about increasing levels.

Besides following diurnal patterns of variations [34] with lowest concentration in the morning and gradual increase during the day, the level of α -amylase also fluctuates during physical exercises [35]. Figure 2.3.1 shows that, during an incremental exercise test to exhaustion, α -amylase levels decrease and then, at an exercise intensity (salivary threshold or Tsa) coinciding with the anaerobic threshold (AT), start to rise toward baseline levels, indicative of the on-set of anaerobic training and activation of the sympathetic adrenal system. The identification of AT was confirmed also by control of blood lactate, the level of which dramatically increases after its achieving.

Thus, in order to carefully control the training conditions of a sportsman and to avoid overtraining it is important to evaluate the concentration of salivary α -amylase at every time point of training. An increase of α -amylase secretion was observed after trainings of either short or long duration. Low flow of saliva rich in α -amylase during training can be associated with hyperventilation and dehydration. It is also believed that presence of catecholamines can induce secretion of α -amylase [36].

Figure 2.3.1 Determination of both the anaerobic (AT) and saliva thresholds (Tsa) of an individual participating in an incremental treadmill exercise test via observing fluctuations of α -amylase level in saliva and lactate in blood, respectively [35]

α -Amylase is 50 kDa enzyme that has 3 main building domains (Fig. 2.3.2) [37]. The active site of α -amylase is located in the largest unit (domain A) and comprises of two aspartate residues and one glutamate residue. A chloride ion is also present at domain A, thus, making activation of amylase by chlorides quite favorable [38]. The active site is situated close to domain B that also surrounds a calcium ion. Calcium is believed to play an important role in preserving amylase conformation near the active site. Domain C, being less related to domains A and B, does not express any influence on the catalytic activity [39].

Figure 2.3.2 Structure of α -amylase. Domain A is depicted in white/grey color, domain B - in blue, domain C – in red. Green ball is chloride ion, and yellow ball – calcium ion [37]

The catalytic action of α -amylase can be explained by involvement of ping-pong mechanism where carboxyl groups of amino acids at the catalytic site act as acid/base agents and as nucleophilic reagent with subsequent formation of covalent intermediate. The environment around catalytic site may influence the protonation process of carboxylic groups. For example, chloride ion has an impact on protonation of carboxyl groups by activating them through transferring of a formal charge of one dissociating group to another at the same active site [40]. Thus, acting as an activator that induces the possibility of substrate binding to enzyme, chloride ion must be present in reaction system.

The main function of α -amylase is to cleave polysaccharides with subsequent release of short chain by-products - maltose and maltotriose [41]. Gilles et al. [42] proposed that the predisposed center vulnerable to catalytic attack is located between third and fourth units in domain A (Fig. 2.3.3).

Figure 2.3.3 Schematic structure of domain A comprising active site between units 3 and 4 [42]

The physiological range of α -amylase in saliva is typically high and varies from 50 to 500 $\mu\text{g/mL}$, depending on saliva flow, collection method and the measurements based on the total amount of protein in saliva and direct measurements [43]. Although the numbers vary greatly in different works it was established that high values refer to the amount of total protein in saliva, whilst the measurements made by enzyme assay indicate one order lower concentration of amylase. Taking this into account we can assume that the level of overtraining can be controlled by simultaneous detection of both cortisol and salivary α -amylase in order to regulate the physical exercise and recovery cycles in sportsmen.

Another effective overtraining biomarker is cortisol which is commonly recognized as a “stress hormone” [44]. It belongs to glucocorticoid class and is secreted by adrenal cortex

in the adrenal gland. It plays an important role in the uptake of fats, proteins and sugars, in the inhibition of immune system by preventing inflammatory compounds from inflammation releasing [45]. High spurt of cortisol is a usual response to physical exercises followed by decreased glucose concentration in blood that in turn causes stress. When an athlete increases the level of exercises, the concentration of salivary cortisol is directly proportional to the amount of blood lactate [46]. Port et al [46] explained this by assuming lactate deposition in muscles that can trigger chemoreceptors and excite the hypothalamic-pituitary axis (HPA). Moreover, cortisol level is subjected to diurnal variation with incremental decline during the day. The level of cortisol increases gradually during training. It slowly augments till the training switches to anaerobic training and human body – to catabolic metabolism, after which a leap in the concentration is observed [47] (Figure 2.3.4). This switch, also called as ‘threshold intensity’, occurs after half an hour of training with more than 60% of oxygen use [19].

Figure 2.3.4 Serum and salivary cortisol responses to incremental exercise testing [46]

As far as cortisol in saliva mostly appears in the unbound, free state, it exhibits a significant response to corticotrophin-releasing hormone that can be administered via extracorporeal procedures. Crewther et al. [48] demonstrated that changes in the salivary cortisol level as a function of high-intensity training are a better marker for exercises compared to fluctuations of cortisol level in blood. Furthermore, independency of the cortisol amount on the salivary flow rate is a big advantage of assessing this hormone as an overtraining biomarker. The elevated amount of cortisol caused by training load represents the hormonal

adaptation to exercise [49]. The constant augmented level of hormone cortisol in overtrained sportsmen contributes to the prevalence of the catabolic mode over the anabolic. If the cortisol level doesn't decrease during rest, it can be regarded as a sign of fatigue [50]. At the same time, some overtrained sportsmen demonstrated exhaustion of the HPA axis during training that appears in parallel with decreasing levels of cortisol [51]. However, the levels of cortisol vary within an individual, which requires long term monitoring of its level and other physiological parameters as well as knowledge about the athlete's history of health disorders.

Another hormone that can be found in saliva is testosterone, which belongs to a family of androgens and is formed in hypothalamic–pituitary–gonadal axis [52]. In contrast to cortisol, testosterone contributes to anabolic processes in our body by improving protein synthesis and inhibiting protein degradation that in turn has a positive effect of the muscle strength. That's why a raise of testosterone is observed after high-strength exercises [53]. Anabolic activity of testosterone also influences the production of growth hormone. As for cortisol, only a small portion of testosterone is unbound and biologically active in blood. On the contrary, salivary testosterone is commensurable with the fraction of free testosterone [54, 55]. Crewther et al [48] showed that the assessment of salivary testosterone is more reliable than blood testosterone as salivary testosterone has a better response to the training load. Testosterone amount is also subjected to a circadian rhythm, with elevated concentration levels around 8 am and reduced levels around 8 pm.

Continuous field monitoring of testosterone and cortisol levels in a response to training load for individual athletes is crucial for improving training conditions that lead to enhanced training capacity and performance. Aldercreutz et al. [56] proposed that the ratio of free testosterone/free cortisol was a relevant indicator of overtraining which furthermore represents the activity of anabolic/catabolic processes. When the ratio falls below 30%, athletes are recommended to rest to avoid overtraining. This ratio was shown to be useful for detecting overtraining in athletes after rugby match [57], kickboxing [58] and wrestling [59]. Thus, continuous assessment of cortisol and testosterone may be used as an indicator

of alteration in physical activities. However, they cannot be recommended as a pair of definitive fatigue biomarkers.

Saliva is also rich in salivary immunoglobulins (IGs), biomarkers of mucosal immunity. However, the data about IGs in literature is not coherent. IGs is generally decreased for the sportsmen after training, coincidently with augmentation of cortisol level which is believed to play a direct role in suppression of mucosal protection [55].

Recently, the ratio of two heptapeptides was proposed as an objective biomarker responsible for overtraining and fatigue in sportsmen. Michael et al. [60] collected saliva samples of 9 sportsmen-cyclists before, during and after their training program, analyzed them by HPLC-MS and found that only 2 peptides (GGHPPPP and ESPSLIA) followed certain pattern during training. The ratio of these heptapeptides, derived from acidic and basic proline-rich proteins (aPRP and bRP), was identified as an objective biomarker for the evaluation of fatigue. The ratio of these peptides was used by Michael et al. [60] for evaluation of the fatigue level in sportsmen. These peptides are suggested to be good biomarkers of overtraining.

2.4 Detection methods of biomarkers of overtraining

This section describes recently employed instrumental methods for detection of cortisol and salivary α -amylase that together are considered to be the best biomarker combination to assess overtraining.

Current developments in the elaboration of enzymatic assays for the α -amylase detection include electrochemical (potentiometry [61, 62], amperometry [63, 64], cyclic voltammetry [65]), piezoelectric methods [66] and immunoassays [67]. The ability of amylase to cleave α -bonds of polysaccharides was widely utilized for elaboration of colorimetric detection methods [68-77]. A summary of methods can be found in table 2.4.1. Enzymatic assays typically require trained personnel and instrumentation for accurate analysis of salivary α -amylase. One of the commercially available assays for detection of

α -amylase activity is the Phadebas test that comprises water-insoluble starch-based granules incorporated with a blue dye, which slowly releases into solution upon cleavage of α -bonds by α -amylase [78]. Although this method is reliable and sensitive, heterogeneous structure of starch comprising different portions of amylopectin and amylose could influence the responses obtained within different batches. Besides, this method requires high volumes of saliva and a spectrophotometer to record and analyze the obtained responses. Thus, it is essential to develop simple and rapid assay platforms that enable continuous or frequent monitoring of α -amylase concentration levels in saliva, especially for correlating changes in salivary α -amylase concentration to any cognitive or physical activity.

Table 2.4.1 Current methods for detection of α -amylase in saliva

Chemical system employed	Analytical parameters of the method	Remarks	Ref.
Starch-triiodide complex on Pt electrode	LOD 0.03 mKNU for in the range of 0–9.6 mKNU (0–80 μ g)	Developed test is not intended for detection of high levels of α -amylase as in saliva	[61]
Two enzymatic membranes containing maltose phosphorylase and glucose oxidase and peroxidase were immobilized on the same planar surface.	0–190 kU/l α -amylase activity with R^2 of 0.97 was demonstrated with a sample volume of 50 μ l.	High dynamic range, low amount of saliva required for analysis	[62]
Amperometric biosensor fabricated by immobilizing glucose oxidase–gelatin via glutaraldehyde on the Au electrode surface	Dynamic range 0.819 – 13.11 U/mL Response time 15 min	Low Stability (15 days)	[63]
Indirect determination of α -amylase in a sequence of two chemical reactions. The first reaction is the hydrolysis of starch by α -amylase to produce maltose. Then, the generated reducing sugar promotes the conversion of $[\text{Fe}(\text{CN})_6]^{3-}$ into $[\text{Fe}(\text{CN})_6]^{4-}$ in a second reaction	LOD = 1.1 U/mL; Dynamic range between 182.8 and 1117.1 U/mL and the accuracy is between 90 and 97%	High interference from reducing compounds present in saliva	[64]

Cyclic voltammetry. The method is based on the hydrolysis of maltopentaose, immobilized on the hydrazide-derivatized surface of a gold electrode, and the induced charge changes of the substrate-modified electrode. The determination of α -amylase activity is achieved by analyzing the electrochemical signal obtained from the electroactive molecule $[\text{Ru}(\text{NH}_3)_5\text{Cl}]^{2+}$ during the hydrolysis of maltopentaose.	LOD = 0.022 U/mL Dynamic range 0.03 to 3 U/mL	Demonstrated assaying in blood serum. Sensitive to low concentrations. The biosensor exhibits a rapid response, good stability and anti-interference ability.	[65]
Oligosaccharides (glycogen and amylopectin) spin-coated onto gold coated quartz crystals with a base frequency of 10 MHz. The films were subsequently cross-linked with hexamethylene diisocyanate. Film degradation was monitored	Dynamic range 0.08–8 U/ml	Requires laboratory equipment and special storage conditions	[66]
QCM with immobilized Abs via Photochemical Immobilization Technique	LOD 0.1 $\mu\text{g/mL}$	Influence of viscosity effects	[67]
The variation in the intensity of the color with the concentration of α -amylase was estimated in three stages: (i) the paper-surface was illuminated with a light emitting diode, (ii) the transmitted (reflected) rays emitted through (from) the paper were collected on a photoresistor, and (iii) the variations in the electrical resistance of the photoresistor were correlated with the α -amylase concentration in analyte.	10–110 U/L	High concentrations lead to distortion of responses Requires pre-incubation	[68]
Disposable test strip and a hand-held reader with a digital display. The test strip integrates a collector pad at its tip and a reagent paper infused with 2-chloro-4-nitrophenyl-4- <i>O</i> - β -D-galactopyranosyl-maltoside (Gal-G2-CNP) and a chromogenic substrate for salivary α -amylase. When placed under the tongue, the	10–230 U/ml. Coefficient of variation < 9%	Rapid non-invasive sample acquisition (10 s). Lack of sample preparation steps and rapid reporting (within 30 s). Requires additional portable equipment	[69]

collector pad quickly saturates with fixed microliter quantities of saliva. The strip is inserted into the reader and the lever is raised to activate the reader and transfer the collected saliva onto the reagent paper.		
Involves maltopentaoside as a substrate for α -amylase and α -glucosidase, hexokinase and G-6-P dehydrogenase as additional enzymes to cleave by-products	LOD = 112 U/L. Assay time of 6 min Spectrophotometry as a method of detection	Multicomponent system depending on many factors. Not suitable for high concentrations of α -amylase [70]
2-chloro-4-nitrophenyl-4- <i>O</i> - β -D-galactopyranosylmaltoside (GalG2CNP) as the substrate; this assay was used with anti-human salivary α -amylase monoclonal antibodies for specific determination of the pancreatic isoenzyme.	Dynamic range 0-1100 U/L for serum. LOD = 1.95 U/L.	Serum and urine Requires special equipment [71]
GalG2CNP as the substrate. Amount of KSCN, CaCl ₂ examined	Dynamic range = 0–200 kU/L Assaying time = 150 s. Requires only 5 μ l of the whole saliva	Requires portable equipment Real application [72, 74]
Evaluation of various substrates for α -amylase and constituents that affect their efficiency.	The activities of α -amylase with good response from substrate were in between 5 to 1600 U/L	2-chloro-4-nitrophenyl α -maltotrioside is used for detection of α -amylase only in serum and urine [73, 75, 76]
Water-insoluble starch polymers are covalently bound to blue dye molecules, which are liberated in the presence of α -amylase. For Phadebas paper, starch-dye complexes are immobilized onto filter paper and visualization of α -amylase positive areas on membranes occurs when the blue dye molecules diffuse through the pores of paper.	2000 U/L in the 40-min examination of a stain. 189 U/L for saliva	Additional coloration of membrane due to presence of other biological fluids. Does not account for environmental effect [77]

Nowadays, most assays aim at detection of total amount of cortisol in biological fluids. However, only the free fraction of cortisol is biologically active and requires assessment that is beneficial for saliva as object of analysis, since it comprises only free fraction. The amount of free fraction in cortisol in saliva is at ng/mL/(nM) level and varies between 0.5 to 20 ng/mL [79, 80]. Possessing a molecular structure similar to other hormones, cortisol is difficult to detect, especially without proper sample preparation. Current methods that are being used are mostly chromatography [81-84] and immunoassays based on surface plasmon resonance (SPR) [85-94]. They are time and labor consuming, costly and not ideal for field application. However, high sensitivity and specificity of these methods provide accurate and robust detection of cortisol. The list of state-of-art methods for instrumental detection of cortisol is illustrated in Table 2.4.2.

Table 2.4.2 Current methods for detection of cortisol in saliva

Method details		Analytical parameters of the method	Remarks	Ref.
HPLC with MS/MS detection		LODs 0.07–0.11 nmol/L Low interference from drugs and antibodies, which are frequent problems in immunoassays	Saliva sample require prior clean up (solid-phase extraction and protein precipitation). All analysis is done in laboratory setting.	[81-84]
ELISA based immunoassays		Detection range 0.4–5.8 nmol/L, LOD 0.1 nmol/L	High cross-reactivity from other steroids. Low stability of Abs. Time consuming. Lab-based analysis	[85-89]
SPR biosensor based on competitive detection of Cortisol in saliva was detected across the physiologically relevant range.		Pre-treatment of saliva not required. LOD 49 pg/mL. Coefficient of variation (CV) of 13.5% and recoveries close to 100%. Stable responses for more than 140 binding and regeneration cycles, enabling re-use.	Ultrasensitive and rapid detection of salivary cortisol. The system can handle saliva matrix effects by use of chemical treatment during the assay to reduce non-specific binding to sensor surfaces. This sensor system provides an automated, high sensitivity analytical tool capable of yielding results in approximately 15 min. Requires special equipment	[90]
SPR based biosensor on		LOD of 46 pg/mL	Sophisticated equipment, long time of analysis	[90, 91]

competitive immunoassays.			
Miniature SPR system with gold sensor chips modified with BSA or colloidal gold	The system sensitivity and noise were tested through detecting different concentration of glycerol solutions with a group of refractive indices. Has automatic sample injection device which is able to work 24 hours unattended.	Indirect detection of cortisol with wide dynamic range. High sensitivity but not sufficient enough specificity. Cross reactivity with other steroids.	[92]
Electrochemical impedance spectroscopy	0.36 pg/ml to 3.6 ng/ml cortisol in saliva with a 40 min incubation time	Label-free immunosensor with sufficient sensitivity. Requires incubation. Doesn't cover dynamic range of saliva	[93]

As it can be seen from the Table 2.4.2, all methods require lab based equipment and trained personnel to operate it. Thus, one of the main shortcomings of lab-based techniques is that they do not ensure continuous analysis of cortisol levels but instead provide a snapshot, the results of which might be not relevant at the time when they are obtained. Therefore, there is a great demand in developing highly integrated system for cortisol detection that offers continuous monitoring using a portable device.

Unlike cortisol and α -amylase reported in Table 2.4.1 and 2.4.2, peptide biomarkers can be identified only by HPLC/GC coupled to MS, tandem MS or MALDI. The excessive amount of peptides of similar length and structure in saliva constitutes is the main complication of their detection. Thus, with the aim to avoid bulky cumbersome analysis and laborious experiments, preliminary pre-concentration and extraction of peptides from saliva is required. The necessity of saliva pre-treatment with the aim to detect peptides limits fast, reagent-free and online analysis.

Elaboration of simple methods that do not require special equipment for result readout and can be used onsite – in field setting – could potentially facilitate prediction of overtraining development and its diagnosis. Moreover, an opportunity of constant control of athlete's

physiological parameters (as change in cortisol and α -amylase levels) would provide a huge avenue for sports doctors in terms of creating a database of typical individual reactions to external and internal stressors.

2.5 Bridging gap between existing problem and demand (Importance of onsite testing devices)

With the increasing pace of life and stressful situations, development of sensors capable of detection of biomarkers and collection of health data after continuous monitoring of α -amylase and cortisol would benefit patient, and would provide the avenue for analysis of the effect of various factors on their levels. Onsite testing devices are biosensors that do not require sophisticated equipment, can be easily applied onsite, are disposable, environmentally and user-friendly and cheap. These requirements imply the use of cheap stable materials and highly reliable chemical systems employed on the surface that would withstand oxidation and humidification. One of the widely utilized platforms for onsite testing devices is paper. Paper as a membrane has been successfully employed in many biosensors that are widely used nowadays for visual detection of human chorionic gonadotropin [94] (based on lateral flow), metabolic or infectious species [95] etc. The requirement to the development of a paper strip is a clear and intensive colored signal that correlates with the amount of target analyte and can be read out visually, without any instrument.

Recognition molecules employed in paper-based test strips are antibodies raised against target analyte. While sensitivity of the measurement is sufficient enough, these strips provide non-threshold analysis and do not enable semi-quantitative estimation of a biomarker level in body fluids [96]. Moreover, they are highly susceptible to interference from both sample and analytical system. Thus, exploring different reporting systems and recognition molecules could potentially benefit their stability and robustness of analysis.

For example, the ability of α -amylase to cleave α -bonds of polysaccharides has been widely explored for colorimetric assaying. Oligosaccharides were coupled to a small dye, which

turned from white to yellow upon cleavage by α -amylase. Maltopentaosides were initially utilized as substrates for spectrophotometric and electrochemical detection of α -amylase [62, 70]. However, cleavage of α -bond between saccharide and dye required additional enzymes that complicated the assay and prolonged the assay time. Subsequently, maltotrioxide was proposed as a substrate for detection of α -amylase enzymatic activity [75, 76], without requiring additional enzymes. Although the assay yielded linear responses for low concentrations of α -amylase, the obtained dynamic range was beyond the physiological range of salivary α -amylase. Increasing the substrate concentration could offer an avenue to increase the dynamic range. However, the reaction may be very rapid, compromising detection of α -amylase at higher concentrations. Introduction of competitive inhibitor (a substance that is structurally similar to a substrate, but not recognized by enzyme as a substrate, e.g. maltose), that slows down reaction velocity would enable more accurate measurements of α -amylase at higher concentrations, and would potentially expand the dynamic range of the assay. Previously reported protocols utilizing maltotrioxide required sophisticated instrumentation and are therefore not ideal for onsite applications. A substrate consisting of two glucose and one galactopyranose residue was recently proposed, which disabled transglycosylation and hydrolysis that are inherent processes for other substrates [71, 97]. This substrate was successfully employed for testing paper strips to evaluate development of fatigue within individuals [72, 73]. However, the detection required additional instrumentation for reading the signal intensity, thereby limiting its usage for onsite applications.

Potentially, if it becomes possible to correlate the intensity of released in enzymatic reaction colored by-product with amount of α -amylase, it will be beneficial for customers to control level of salivary α -amylase by facile and easy-to-implement paper test. Moreover, marginal interferences are expected from salivary components because of the highly specific enzymatic reaction.

The development of onsite testing devices for detection of small molecules as cortisol is adjacent with search of recognition sites that enable fast and robust visual response that correlates to the amount of cortisol. Recognition sites that can be potentially used for

cortisol detection are aptamers, antibodies or molecularly imprinted polymers. Onsite testing devices available in market actively utilize antibodies and offer electrochemical molecular detection with gold nanowires coupled with cortisol Abs [98], GOx conjugated with cortisol molecule and attached to self-assembling monolayers [99], and modified interdigitated μ -electrodes [100].

Current assays for the visual detection of cortisol utilize anti-cortisol antibodies and labeled cortisol-glucose oxidase conjugates. For example, Yamaguchi [99] proposed a lateral flow device with immobilized anti-cortisol antibodies that is based on competitive replacement of conjugate between glucose oxidase and cortisol by cortisol. Visual evaluation of color intensity was performed with bench top reflectance reader with linear detection range was 1-10 ng/mL. However, no information about time of analysis, interference and application of an assay in real samples was provided. Visual methods are not widespread for the detection of low amounts of cortisol given slow and uneven development of color, low sensitivity and lack of specificity for analysis of complex matrices. Moreover, visual assessment of cortisol amount usually requires utilization of special equipment for precise read out. However, given the aim to study the individual behavior towards specific stressors it might be not necessary to control cortisol concentration quantitatively with high precision, but instead, to analyze the pattern of cortisol response to various factors and in future to predict development of fatigue.

Design of a test strip that doesn't require any equipment and the concentration of cortisol correlates significantly with exhibited color is one of the goals of this thesis. "Artificial antibodies" - MIPs - based on their stability to harsh environment, robustness, easy matching with different scaffolds and recognition based on both chemical and structural affinity can be a good choice for recognition system. MIPs have been widely incorporated into cellulose fibers as a platform for small molecules detection [101-103]. MIP layer grafted on the surface of cellulose fiber demonstrates enhanced surface area and shortened rebinding time [101]. Being biodegradable, accessible, disposable, stable and easily modified, paper is a good choice for developing an assay. For the moment, no MIP@paper for colorimetric detection of cortisol has been reported so far. An indirect competitive assay

based on the competition between free cortisol in solution and immobilized GOx-cortisol conjugate in grafted MIP layer at the paper disk can potentially enable fast and facile assessment of low amounts of cortisol in saliva. The exceptional properties of o-dianisidine as a bifunctional dye [104] to develop gradual change in color (from colorless through blue to red) based on its sensitivity to the amount of H_2O_2 that is produced after enzymatic reaction between glucose oxidase and β -glucose are utilized. The elaboration of a colorimetric membrane-based test strip for lateral flow analysis of salivary cortisol could potentially solve the problem of its onsite detection.

Integrating several detection systems within one paper platform is expected to facilitate simultaneous analysis of α -amylase and cortisol in saliva and to empower an athlete with a strong tool to control response of his markers to various parameters and therefore, to predict development of overtraining in the early stages.

2.6 Conclusions

According to analyzed literature, the mechanism of overtraining is not established yet and appears to be a fusion of complementary processes. Moreover, strong dependence of type of physical load, diet, sleep preferences and overall psychological and physiological health on the development of overtraining requires continuous monitoring of sportsmen's health and fixation of the response of certain biomarkers to external and internal factors. I hypothesize that prolonged monitoring of the metabolic response to the type of exercise can potentially help to predict overtraining and assist sportsman in customization of his training in order to ensure high performance.

Biomarkers that have been proposed for detection of overtraining if assessed separately do not represent the whole picture of formation of overtraining syndrome. Therefore, it is necessary to monitor several biomarkers that demonstrate the best response to physical activities and the alteration of which can be representative of overtraining development.

Saliva was chosen as object of analysis because of non-invasiveness of its assessment, non-stressfulness, applicability on site and easiness of operation. At the same time, saliva is a complex fluid with plenty of compounds that can interfere with the determination of target analytes by decreasing selectivity, which requires preliminary sample matrix clean-up or extraction of target analytes.

Methods that are currently used for detection of peptides, cortisol and α -amylase are bulky, expensive and laboratory-based and they usually require trained personnel to conduct the analysis. The demand for onsite testing devices that can be applied remotely or at patients' houses drives the burst in their development. Paper-based tests with visual detection represent a better option for determination of analytes in terms of facile utilization, disposability, low cost and instrument-free. Specific property of amylase to cut bonds of oligosaccharides with release of a colored product can be potentially employed for its detection. Grafting MIPs on paper that are complimentary in size and shape to cortisol and designing of a displacement assay at paper membrane seem prospective for elaboration of lateral flow assay for cortisol detection.

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Chapter 3

Experimental Methodology

This chapter provides information about protocols and methods that were used during development of assays for detection of overtraining biomarkers. Procedures for extraction and detection of heptapeptides in saliva are described in the chapter. Techniques investigated to develop colorimetric enzymatic assay for detection of α -amylase in saliva with utilization of maltose as non-competitive inhibitor are also depicted. Protocols on synthesis of MIP@paper as a recognition site for cortisol, evaluation of conjugation mechanism between GOx and cortisol, optimization of design of a lateral flow displacement strip and of saliva pre-treatment are discussed in detail.

3.1 Extraction and detection of heptapeptides in saliva

3.1.1 Materials

Acetonitrile (ACN) (Merck, USA), HPLC grade, was used as a mobile phase for HPLC/UV. Trifluoroacetic acid (TFA) (Merck, USA) was added to a mobile phase with the aim to dissolve hydrophobic compounds. Centrifugal filter units made of regenerated cellulose, 3 kDa, (Merck Millipore, Ireland) were used for ultrafiltration. Deionized (DI) water was used in all cases and is named water hereafter.

Heptapeptides GGHPPPP and ESPSLIA (SBS Peptide Core, Singapore) were used after lyophilization and dissolved in water to obtain required concentrations.

Different matrices were tested for MALDI/ToF in order to establish the one that offers the high ionization of the components of saliva sample. α -Cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) or 2,5-dihydroxybenzoic acid (DHB) (all Sigma, USA) were dissolved in absolute ethanol to obtain concentration 10 mg/mL.

Hydrogen peroxide, ammonium hydroxide (all Sigma, Singapore) were used for cleaning gold crystals used for QCM-D. The protocol of cleaning involved: cleaning with ozone-UV for 15 min, immersing into 75 °C solution of 8 mL water, 1.4 mL 30% H₂O₂ and 38% NH₄OH for 5 min, rinsing with water and drying in N₂ stream.

Ethanol (Sigma, Singapore), absolute, was used for dissolving the reagents. 16-mercaptohexadecanoic acid (SH-C15-COOH) and 1-octadecanethiol (SH-C18) (all Sigma, USA) were dissolved in ethanol to obtain concentration of 1 mM. mPEG7-COOH thiol and mPEG7-OH thiol (both Polypure, Norway) were dissolved in water to obtain concentration of 50 μ M. 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) (ThermoFisher Scientific, USA) was dissolved in ethanol in 10 fold excess to a linker. NHS was dissolved in ethanol in 5 fold excess to a linker.

The saliva investigated in this work was collected from 5 participants (3 males and 2 females) who are exposed to an intense training program at least 4 times per week, based on their verbal consent agreement. The saliva was collected by passive drooling before training, after rinsing mouth with distilled water, and immediately after training (both high volume and high intensity training regimens) when the participants were asked to train to the fullest extent. The participants did not consume any food and drinks for 2 hours prior to collection. Saliva samples were stored in the fridge at -20 °C. Before analysis the samples were defrosted and shaken.

3.1.2 Extraction of heptapeptides from saliva

Saliva as a complex matrix requires preliminary sample preparation with the aim to extract, pre-concentrate or separate the biomarkers and to avoid interferences from the matrix. No universal protocol has been proposed yet for extraction of peptides. Every sample preparation protocol is a compromise of different techniques and materials/chemicals. Extraction of short peptides from saliva samples is a challenging task due to the large amount of different components such as proteins, peptides with similar structures, electrolytes, hormones and enzymes that might interfere with the detection of target biomarkers [1]. By varying different steps of sample preparation, it is possible to reach high level of extraction of the target. Amongst various approaches for isolation of peptides liquid-liquid and solid-phase extraction procedures including membrane-based systems play important roles. Other common techniques applied for extraction and isolation of peptides include centrifugation, ultrafiltration and precipitation by acids, salts, chaotropic molecules and organic solvents (see Fig. 3.1.2.1).

Centrifugation is usually the first step for sample preparation of saliva, which aims to remove cell and organic debris, sediments, bacteria and other large molecules. Centrifugation always results in waste of the peptides that are tightly bound to glycoprotein or carrier proteins that can precipitate.

Figure 3.1.2.1 Protocols of extraction peptides from saliva

Liquid-liquid extraction by organic solvents demonstrates contradictory results. For example, ACN and acetone allow extraction of peptides while being the reason for aggregation and subsequent precipitation of high weight molecules (>20 kDa). The disruption of hydrogen bonds between protein-protein complexes is usually driven by chaotropic agents [2] such as guanidine, ethanol and SDS. In this case, a higher amount of low molecular weight compounds can be found in the solution. However, the co-elution of molecules with weight 10-50 kDa can be observed that can complicate the detection of peptide of interest. On the other hand, the use of ammonium sulphate that acts as an antichaotropic agent can cause the increment of compounds' hydrophobicity in the solution. According to Vitorino et al [1], extraction by ammonium sulphate results in low amount of recovered peptides, but is very selective towards peptides with molecular weight 1200-1500 Da.

Formic, acetic and trifluoroacetic acids (FA, AcA and TFA) are commonly used organic acids for separation of low and high weight molecules that exhibit different behavior towards the constituents of saliva. It is possible to achieve high recoveries of amphipathic and slightly hydrophobic peptides by careful selection of the solvents. Extraction with AcA

yields an increasing number of extracted proteins compared to TFA that is highly electronegative while possessing less oxidative properties than most of oxidizers has an impact on the solvation potential of the solvent. The solubility of proteins decreases on addition of TFA by affecting the repulsive electrostatic forces. Besides, organic acids demonstrate high recovery of hydrophobic peptides, with hydrophathy index higher than zero. The hydrophathy index represents hydrophobicity or hydrophilicity of the side chain of an amino acid – that is, a hydrophathy index below 0 is characteristic for hydrophilic peptide, and a hydrophathy index above 0 - for hydrophobic peptide [3].

Ultrafiltration is a process of mechanical separation of compounds through a membrane forced by the pressure. It is an ideal method for separation of peptides that can be found in filtrate from proteins that are retained on the membrane. Membranes differ by molecular weight cut-off. Although the controlled separation makes ultrafiltration an attractive technique, but the requirement of specific equipment (centrifuge) constraints its onsite use.

Based on applied approaches for the extraction of peptides Vitorino et al [1] came up with the list of all peptides (2497 sequences) that could be recovered by different extraction protocols from salivary samples of healthy rested individuals. The largest part of peptides belongs to aPRP, bPRP2, histatin 1 and SMR3B families. It is a noteworthy observation that target peptides (GGHPPPP and ESPSLIA), which are believed to be biomarkers for overtraining, are not found in saliva. This can be explained by the fact that saliva was collected from volunteers that were not involved in any kind of sports activities. Else, short peptide sequences were not monitored, with majority of peptides having molecular weight within 1-3 kDa. The prevalence of hydrophilic peptides from aPRP, bPRP1, bPRP2 and histatin 1 protein families was observed in samples without centrifugation [1] that affects the decision of preferable extraction protocol.

The apparent disadvantages of liquid-liquid extraction are in part the necessity of using of toxic organic solvents that can induce emulsification of solution. The procedure is also tedious, time- and labor-consuming. Although solid-phase extraction can be more selective but it demands several steps (conditioning, loading, washing and elution) that increases the

total time of analysis. One of the interesting approaches for extraction of short peptides that currently is being developed is the application of supported liquid membranes that include organic solvent with low dielectric constant allocated in the pores of a lipophilic porous membrane [4]. Applying an external potential leads to electrokinetic migration of the analytes [5]. Electromembrane extraction is achieved because of a potential and analyte concentration difference leading to the reactant transport. As peptides are readily ionizable compounds, the variation of the environmental acidity of both donor and acceptor solutions could be used. The mass transfer of peptides strongly relates to the nature of donor and acceptor solutions and type of membrane. Although the recovery of peptides from biological matrices was greatly affected by proteins and salts, the addition of metal-chelating and ion-pairing reagents enabled extraction of low weight peptides.

Ionic liquids (salts with great number of ions and short-lived ion pairs) offer another approach for selective extraction of peptides since they are very sensitive to the nature of peptides [6]. It was established that the substitution of amino acid in the peptide greatly affects its solubility on ionic liquid. The mechanism is the average of three processes that happen in the solution: the formation of supramolecular complexes which facilitates the transport through border ionic liquid – organic phase; fractional blending of the solvents near boundary due to distorted borderline; molecular diffusion as the way of partitioning.

3.1.3 Detection methods

UV-Vis spectroscopy (Lambda 35, Perkin Elmer) was used to record adsorption spectra of heptapeptides in order to choose the wavelength for HPLC detection where intensity of adsorbed by molecule light is the highest.

HPLC/UV (1260 Infinity II LC, Agilent) was used for separation of peptides from other constituents of saliva. All compounds interact in a different way with immobile phase (adsorbent material) based on difference in chemical properties and polarities. Target peptides have different values of hydrophobicity, assuring good separation. The separation of saliva components was performed using a C18 column (Zorbax 300SB-C18, 4.6 mm ID

x 150 mm, 5 μ m) which was specifically designed for the separation of peptides and small proteins. It provides a good addition to high sensitive detection method which requires low background such as mass-spectrometry. Chromatographic separation was performed at flow rate of 1 mL/min, with gradient elution of peptides from 0 to 10 min change from 5 to 100% ACN+TFA (5 v. %), from 10 to 20 min – from 100 to 5% ACN+TFA (5 v.%). Increasing the amount of organic solvent typically results in reduction of retention time of a sample. TFA was added to solubilize hydrophobic peptides. Detection of peptides was conducted at 220 nm, the wavelength where they are characterized with the maximum absorbance. Given the big amount of peptides in saliva, even after extraction, it was decided to use a reference wavelength (280 nm) where target peptides do not absorb which would allow to eliminate their false positive detection.

The fractions with eluted peptide were collected for subsequent analysis by MALDI/ToF (MALDI Shimadzu Performance). MALDI/ToF is a type of soft ionization of molecules that makes it highly desirable in analysis of proteins, peptides, DNA, polymers and other large biomolecules. While other types of ionization are expected to fragment target analytes, MALDI does not break the molecules. ToF mass-spectrometer is commonly used for the enhancement of resolution. Due to the reflection of ions from an ion mirror the flight pass increases twice which enhances the selectivity. Selection of matrix plays an important role for MALDI/ToF analysis. Matrix must be characterized by high extinction coefficient, presence of a chromophore and polar groups, high acidity and fast vaporization. A set of matrices has been studied with the aim to obtain high ionization of all constituents of saliva.

With the aim to detect the presence of peptides in saliva and saliva spiked with peptides in samples, that didn't undergo ultrafiltration, extraction with ethanol or acetonitrile was employed. Equal volumes of saliva and a solvent were mixed and analyzed. This approach aimed to reduce saliva viscosity and to augment ionization of salivary components.

Quartz crystal microbalance with dissipation (QCM-D) monitoring is the method of choice for initial evaluation of the binding between the raised antibodies and the peptide biomarkers. QCM-D is a relatively new technique that is used for surface characterization

and investigation the mechanisms of adsorption on a modified crystal [7, 8]. It enables the real-time monitoring of adsorption of different compounds onto a modified surface of various chemical nature. The principle of the technique lies in the change of oscillation frequency of quartz crystal caused by adsorbed compound with predefined mass. Dissipation in turn offers information about the viscoelastic properties of the deposited layer and provides the knowledge about hydration/swelling of molecule/polymer coatings. QCM-D can also be used for quantitative examination of adsorbed amount and for thickness calculation. The principle of formation of dissipation is depicted in Fig. 3.1.3.1. QCM-D is characterized by sensitivity of 1 ng/cm^2 [9].

Figure 3.1.3.1 Formation of dissipation when adsorbing to soft or rigid deposited layers [9]

Choice of piezoelectric quartz crystal plays the crucial role in QCM-D analysis. While variety of crystals has been proposed by different manufacturers, gold coated crystals are usually chosen for its stability and a big range of possible modifications. Q-Sense set up (Biolin Scientific) connected to a pump (Ismatec) was used for the experiments.

3.1.4 Employment of standard addition method for detection of peptides in saliva

One of the simplest methods to determine target analyte in complex object is to apply method of standard additions that allows to relate the analytical signals from both unknown sample and standard solutions with the help of calibration curve [10]. This method found its application in atomic absorbance spectroscopy and gas and liquid chromatography. Standard addition method stands for spiking the same volume of an unknown sample by known amounts of analyte standard solution, diluting the sample with solvent to the same

volume and measuring detector signals as a response to every added amount. Analytical signal reflecting increase of spiked concentrations is linearized and extrapolated to x-axis where $y=0$. The absolute value of intercept, obtained with the negative sign, indicates the amount of target compound in the sample.

Saliva samples collected from volunteers were spiked with increasing concentrations of peptides. The ultrafiltration was performed at centrifugal filter units 3 kDa made of regenerated cellulose (Merck Millipore, Ireland). HPLC/UV (Agilent, US) analysis was carried out to confirm the presence of peptides.

3.1.5 Development of a QCM platform

The modification of the QCM gold chip involved several steps (Fig. 3.1.5.1): I) formation a monolayer from a 1 mM solution of SH-C15-COOH (the hydrophobic chain linker for further modification) in absolute ethanol, or 50 μ M of mPEG7-COOH thiol (hydrophilic chain linker to prevent non-specific binding) in water; II) activation of the carboxylic groups of the linker by EDC/NHS; III) coupling of a peptide (1.4 mM) followed by washing with water and drying in a stream of nitrogen gas. All antibodies that were raised against the peptides were diluted in water to final concentration 100 nM. The QCM measurements started by injecting water in the sample chamber to establish a flat baseline. When antibodies were injected, a frequency drop was observed.

Figure 3.1.5.1 Immobilization of peptide on a surface of gold chip, covered with a monolayer of a linker

In order to fill the holes (plain gold) that potentially could be formed due to incomplete coverage of the linker and to prevent non-specific binding of antibody to a surface of gold

chip, a mixture of linker and blocker (1:1 and 4:1), where the blocker did not possess reactive COOH-group was introduced into the system. The blockers used are SH-C18 and mPEG7-OH thiol as hydrophobic and hydrophilic blockers, respectively. Immobilization procedure and measurements were conducted as described before. The scheme of immobilization is depicted in Fig. 3.1.5.2.

Figure 3.1.5.2 Immobilization of a peptide on a surface of gold chip, covered with a monolayer of linkers and blockers

The same procedures were repeated with the aim to immobilize target peptide on gold surface via hydrophilic linkers or blockers with concentration 100 μM .

3.1.6 Validation of developed QCM platform

In order to validate the developed QCM platform, two subsequent experiments were performed. In the first one, 0.1 μM of GGHPPPP or ESPSLIA were mixed with 0.6 μM of Ab 9 or Ab 5, respectively, as the Abs that demonstrated the highest affinity. The mixture was incubated for 15 min at room temperature. Excessive amount of Ab is expected to bind all peptide molecules, while its leftover could bind to the immobilized on gold chip peptide and result in a frequency shift. In the second experiment, 1 μM of GGHPPPP or ESPSLIA were mixed with 0.6 μM of Ab 9 or Ab 5, respectively. The mixture was incubated for 15 min. Excess of peptides is expected to bind to all Ab molecules in the solution, thus, demonstrating no frequency shift upon reaction with gold chip.

Saliva collected from one volunteer (female, 29 yo) spiked with GGHPPPP or ESPSLIA solutions to obtain final concentration of peptides equal to 0.1 μM and 10 μM . The samples

were ultrafiltrated at membranes with 3 kDa cutoff. Supernatants were collected and incubated with antibody solution (0.6 μ M) for 15 minutes. The resultant mixture was run through modified gold chip without any further treatment.

3.2 Colorimetric Detection of Salivary α -Amylase using Maltose as Inhibitor for Polysaccharide Cleavage

3.2.1 Materials

All the materials and reagents were purchased from Sigma-Aldrich (Singapore) and used without further purification. Maltose (Sigma, USA) was dissolved in water. Deionized (DI) water was used in all experiments. Qualitative filter paper (Toyo Roshi Kaisha, Japan) was used to prepare circular test paper membranes with a diameter of 10 mm. Spectrophotometric analysis for Phadebas test was carried out using Lambda 35 (Perkin Elmer, USA). Studies of kinetics and effect of non-competitive inhibitor utilized a plate reader Infinite M200 Pro (Tecan, Switzerland).

MES buffer was prepared at room temperature. MES was dissolved in water to obtain a concentration of 50 mmol. The pH was adjusted to 6.85 by 5 M NaOH. MES buffer was used for dissolving 2-chloro-4-nitrophenyl- α -D-maltotrioxide (G3-CNP) to obtain a concentration of 20 mg/mL and subsequently diluted to the final concentration. MES buffer that contained 6.7 mmol CaCl_2 and 400 mmol NaCl was used for dissolving α -amylase to obtain required concentrations. Buffer pK_a is recognized to change minimally with the temperature. MES buffer is very stable indicated by no visual changes in coloration, aggregation or precipitation during 5 months of storage.

Solutions of α -amylase were prepared by dissolving α -amylase from human saliva, type IX-A (Sigma, USA), in MES buffer to obtain a concentration of 5 mg/ml was aliquoted and stored at -20°C . Before use, the aliquot was thawed and fresh solutions with required concentrations were prepared in MES buffer.

3.2.2 Study of kinetics and evaluation of effect of non-competitive inhibitor

The solutions for kinetics study were prepared by mixing increasing concentration of G3-CNP (0.02 – 5 mg/mL) with constant amount of α -amylase (200 μ g/mL) in order to establish the amount of substrate for which reaction rate is independent of substrate concentration. The absorbance of released dye was recorded during 20 min. In order to evaluate the dynamic range of the assay, amount of substrate was kept constant (5 mg/mL) while amount of α -amylase was gradually increased (from 5 μ g/mL to 500 μ g/mL). Maltose (1 mM, 2 mM and 5 mM) was added to the solution along with varying concentrations of α -amylase and the absorbance of released product was recorded during 20 min.

3.2.3 RGB analysis of paper based assay

Test paper membranes were prepared by dropping required amount of G3-CNP and maltose in MES buffer. After drying under air stream, paper membranes were utilized for testing the α -amylase activity. For digital image analysis, tested paper membranes were placed on a white background and the images were taken with a digital camera ILCE 7R (Sony) fixed at a constant distance and angle from the samples. Subsequently, the images were transferred to a computer. Central part of the test paper membranes (75x75 pixels) was analyzed by ImageJ software for quantification of obtained colorimetric responses. The illumination for RGB analysis was kept constant (500-550 lum). The measurement of light intensity was performed by Android phone apps Luxmeter and Lightmeter in the environments with different lightning condition. The images were taken at three different illuminations: 540, 110 and 42 lum. The results from both apps were averaged.

3.2.4 Preparation of standard paper membranes

Standard paper membranes were prepared by dropping 10 μ L of a mixture of 20 mg/mL G3-CNP and 2 mM maltose and dried in air stream. 5 μ L of α -amylase (20, 50, 75, 100, 200, 300, 400 and 500 μ g/mL) were then dropped on the prepared membranes. After 15 min, 5 μ L of 1 M NaOH were added to stop the reaction. Obtained color intensities of the

membranes were used as standards (standard membranes) to obtain semi-quantitative estimate of α -amylase concentrations in saliva samples. Membranes were prepared freshly before each experiment that required estimation of α -amylase in saliva samples. Several membranes were also stored in dark for up to 70 days for stability analysis.

3.2.5 Collection of saliva samples and exposure to membranes

Saliva samples were collected from volunteers in different physiological conditions in order to cover a wide range of salivary α -amylase concentration levels. The samples were collected upon their verbal consent. Volunteers were asked to avoid consumption of food at least for 1 h (unless stated different) and to rinse their mouths with water prior to saliva collection.

In order to obtain saliva samples with low, average and high amount of α -amylase, volunteers were asked to provide their saliva during certain physiological conditions that are well-known to influence level of α -amylase. Hunger and satiety, rest and high load exercises, sleep and keeping awake are generally recognized to be characterized by fluctuations in α -amylase levels. The list of physiological conditions of volunteers is as below: 1 – Female, 31 yo, upon awakening; 2 – Female, 25 yo, in the middle of day; 3 – Male, 26 yo, before lunch; 4 – Female, 31 yo, before lunch; 5 - Female, 31 yo, after lunch; 6 – Male, 33 yo, before training; 7 – Male, 33 yo, after high load training. The collected saliva samples were then used (within 30 min) as is for evaluation of α -amylase response without any further pretreatment. Five μ L of saliva was added to membranes deposited with 20 mg/mL G3-CNP and 2 mM maltose. In order to estimate α -amylase level semi-quantitatively, after 15 min of enzymatic reaction, the resulting color was compared to the standard membranes. Saliva from a volunteer was stored for different periods of time in room temperature in order to explore the effect of storage on α -amylase activity.

3.2.6 Benchmarking of developed assay against Phadebas test kit

α -Amylase concentration in saliva samples was evaluated using Phadebas test kit. 200 μ L of freshly collected saliva samples were transferred into falcon tubes and diluted with 4

mL of water. Tubes were pre-incubated at 37 °C water bath for 5 min. One tablet of the Phadebas test kit was added into each tube, vortexed for 10 s and incubated in a well stirred water bath at 37 °C for 15 min. The reaction was stopped by adding of 1 mL of 0.5 M NaOH. Tubes were centrifuged at 1500 g for 5 min. Obtained blue supernatant was transferred into a cuvette. Spectroscopic measurements were carried out at 620 nm. All procedures were repeated with a blank solution that had all constituents except saliva. The actual amount of α -amylase was retrieved from the calibration table provided in the Phadebas test kit.

The developed assay was benchmarked against Phadebas test kit. Five μ L of saliva was added to membranes deposited with 20 mg/mL G3-CNP and 2 mM maltose. The concentration of salivary α -amylase is then estimated based on the obtained color intensities of the test membranes with reference to color intensities of standard membranes. The estimated salivary α -amylase concentrations are subsequently validated using responses from the Phadebas tests. Quantitative estimation of α -amylase concentrations obtained from the RGB analysis also was benchmarked against the Phadebas test.

3.2.7 Interference of saliva components

The experiments were designed to study the effect of varying salivary components that differ between volunteers and the interferences from salivary components that could potentially hydrolyze the α -bonds of the polysaccharide substrate.

Buffer solutions with varying concentrations of α -amylase (estimated using Phadebas test) that match the levels obtained in the saliva samples from 7 volunteers were prepared. The amount of α -amylase was tested semi-quantitatively (visually), quantitatively (by RGB analysis) and verified by Phadebas. Potential interference from salivary components could also be studied by maintaining the concentration of salivary components (in order to preserve the interferences, if any) and by gradually increasing the concentration of α -amylase. In case of positive interference from salivary components on α -amylase activity, the assay would yield erroneous estimation of α -amylase levels. Saliva sample that was

collected from a volunteer in the morning contained α -amylase in concentration of 81 $\mu\text{g/mL}$ as determined by Phadebas. Whole saliva was aliquoted and 5 μL of α -amylase solution of different concentrations (0.5, 2.6, 4.7 and 6.8 mg/mL) were added to 100 μL of saliva to achieve total concentration of α -amylase 100, 200, 300 and 400 $\mu\text{g/mL}$, respectively.

For the next experiment saliva was diluted with MES buffer and α -amylase was added to obtain the initial concentration of α -amylase in saliva. The saliva was collected from one volunteer in the middle of the day. Then, the same saliva was aliquoted by 100 μL , 50 μL and 20 μL . 100 μL of whole saliva were immediately used for determination of α -amylase concentration by Phadebas and developed assay (it contained 103 $\mu\text{g/mL}$ of α -amylase). 50 μL and 20 μL of saliva were diluted by MES buffer 2x and 5x times, respectively. Concentration of α -amylase was controlled by Phadebas and developed assay. Then, 5 μL of α -amylase of different concentration (as in Table 3.2.7.1) were added to achieve total concentration of α -amylase in aliquots 103 and 200 $\mu\text{g/mL}$.

Table 3.2.7.1 Protocol of preparation of saliva samples for subsequent colorimetric and spectrophotometric analysis

Dilution of saliva	Volume of saliva or diluted saliva, μL	Volume of α -amylase aliquot, μL	C(α -amylase), mg/mL	Final concentration of α -amylase in sample, $\mu\text{g/mL}$
Whole saliva	100	-	-	-
Whole saliva	100	5	2.1	200
Saliva diluted 2x times by MES buffer	100	-	-	-
Saliva diluted 2x times by MES buffer	100	5	1.1	103
Saliva diluted 2x times by MES buffer	100	5	3.1	200
Saliva diluted 5x times by MES buffer	100	-	-	-

Saliva diluted 5x times by MES buffer	100	5	1.7	103
Saliva diluted 5x times by MES buffer	100	5	3.8	200

3.2.8 Storage stability of membranes and saliva

Different concentrations of α -amylase (20, 100, 400 $\mu\text{g/mL}$) were added to membranes and stored in dark for 1 (freshly prepared), 42 and 70 days at room temperature. Storage stability of prepared assay membranes was verified by comparing the colorimetric responses of membranes stored for 42 and 70 days with that of responses obtained using freshly prepared membranes.

To test the activity of α -amylase one saliva sample was stored for different periods of time at room temperature and was analyzed immediately, after 30 min, 1 h, 5 h and 12 h after collection with no prior pre-treatment.

3.2.9 Effect of temperature on α -amylase detection in saliva sample

Experiment for the detection of α -amylase at room temperature 24 °C and at 31 °C was performed. The membranes were kept at mentioned temperatures 15 min before the start of experiment and during the assaying (15 min).

3.3 Development of lateral flow displacement assay using MIP@paper as recognition site for detection of cortisol

All reagents were purchased from Sigma (Singapore) unless stated different. o-Dianisidine was diluted in water with 0.1 M HCl (10%, v/v). Deionized (DI) water was used in all experiments.

3.3.1 Synthesis of MIP@paper

Prior to in-situ polymerization of MIPs, bare paper membranes (qualitative filter paper (Toyo Roshi Kaisha, Japan)) were treated with 3-(methacryloxypropyl)-trimethoxysilane with the aim to introduce C=C bonds via reaction of surface silanization due to abundant OH groups of cellulose. 95 μ L of 3-(methacryloxypropyl)-trimethoxysilane were hydrolyzed in 10 mL mixture of ethanol and water (80% ethanol, v/v) to form reactive silanol groups. Then paper strips were immersed into resulting solution and were incubated for 1 h at room temperature. Excess of reagent was removed by thorough rinsing with ethanol, 3 times, and paper membranes were dried in air stream.

Next, polymerization mixture that contained 50 mM template molecule (cortisol), 20 mM monomer (acrylamide (AA)), 20 mM crosslinker (EGDMA) and 0.8 mM initiator (AIBN) in 40 mL of ethanol and water (80% ethanol, v/v) was sonicated for 5 min, placed on ice, and bubbled with N₂ stream for 3 min. Activated papers were placed into the resulting solution and the polymerization was carried out at 60 °C in water bath for 2 h, with the aim to form MIP layer on cellulose surface. The obtained papers were thoroughly washed by ethanol multiple times in order to remove excess of reagents as well as poorly bound to cellulose MIP particles.

The same procedures were performed to synthesize MIP@paper in DMF as a polymerization solvent.

Lastly, with the aim to remove cortisol molecules from the specifically designed cavities washing with methanol and glacial acetic acid (AcA) was performed (90% methanol, v/v).

In the same manner, but without addition of cortisol to polymerization mixture, non-imprinted polymers at paper (NIP@paper) were synthesized.

3.3.2 Evaluation of the optimal solvent for MIP polymerization

MIP@paper (10 x 10 mm²) synthesized in mixture of ethanol and water or in DMF was immersed into 0.5 mL solution of 0.5 mg/mL cortisol and incubated for 5 min. MIP@paper was removed from the solution and leftover concentration of cortisol was determined by HPLC/MS. Paper membrane was rinsed by 0.5 mL of water and the resultant solution was collected for subsequent HPLC/MS analysis.

3.3.3 Confirmation of formation of MIP layer at the surface of paper

SEM is an ideal tool to identify size and position of nanoparticles on surface of fibrous material due to its ability to generate high-resolution images [11, 12]. SEM's principle lies in producing images of a sample by scanning the surface with a focused beam of high-energy electrons, whereby electrons lose energy by repeated random scattering and absorption within a teardrop-shaped volume of the specimen known as the interaction volume, which extends from less than 100 nm to approximately 5 μ m into the surface [13]. SEM has been widely utilized to observe formation of layers and to study the morphology of specimens [14-16]. Schematic structure of SEM is shown in Fig. 3.3.3.1.

Formation of MIP layer at paper was observed by FESEM 6340F in high vacuum. In order to enhance conductivity bare paper and MIP@paper were sputtered with a thin layer of gold. Prepared membranes were scanned by FESEM and images were captured with different magnification with the view to evaluate general topography of a surface and to estimate size of particles formed after polymerization.

Figure 3.3.3.1 Structure of SEM [11] (BSE – backscattered electrons, SE – secondary electrons, SC – specimen current, EBIC – electron-beam-induced current, X – X-rays, CRT – cathode-ray tube)

3.3.4 Evaluation of cortisol rebinding to the MIP@paper

MIP@paper, NIP@paper and bare paper membranes ($10 \times 10 \text{ mm}^2$) were immersed into 0.5 mL solution of 0.5 mg/mL cortisol and incubated for 5 min. All paper membranes were washed by 0.5 mL of water, methanol, AcA or mixture of methanol and AcA (90% methanol, v/v). Each washing was preceded by rinsing in water. All fractions were collected for subsequent HPLC/MS analysis to establish the amount of cortisol removed from the paper. To estimate the concentration of unbound or removed cortisol in experiments with studies of rebinding process HPLC/MS (LC1260 MS6120, Agilent) was used. Calibration curve for detection of cortisol was prepared in the range from 50 ng/mL to 0.5 mg/mL. The parent ion (363) and fragment ion (239) were used for confirmation of cortisol presence in SIM acquisition mode. Analysis was performed at C18 column (Zorbax

300SB-C18, 4.6 mm ID x 150 mm, 5 μ m). Chromatographic separation was performed at flow rate of 1 mL/min, with gradient elution of cortisol from 0 to 5 min change from 5 to 50% ACN+FA (5 v. %), from 5 to 10 min – from 50 to 95% ACN+FA (5 v.%). Increasing the amount of organic solvent typically results in reduction of retention time of a sample. FA was added to facilitate solubilization of slightly hydrophobic cortisol. The chromatograms were also recorded for cortisol in methanol and in solution of methanol with AcA (90%). Solutions that included glacial AcA and contained removed cortisol were evaporated in N₂ stream and dissolved in 0.5 mL of water to perform HPLC/MS. Chromatogram of cortisol in water as an example is shown in Fig. 3.3.4.1.

Figure 3.3.4.1 Chromatogram of 50 ng/mL cortisol in water with employed acquisition mode m/z 363

Retention time of cortisol was calculated 2.67 min as observed by SIM 363 in water and 2.24 min in methanol.

3.3.5 Synthesis of GOx-cortisol-21-hemisuccinate (GOx-cortisol-21-HS) conjugate

5 mg of cortisol-21-HS were mixed with 10 mg of NHS and 20 mg of EDC in 200 μ L DMF, 200 μ L of 1,4-dioxane and 100 μ L water. The mixture was incubated at 4 °C overnight. Modification of mannose on the surface of GOx occurred upon mixing 10 mg of GOx with 10 μ L NaIO₄ in 1 mL of water. The solution was incubated for 30 min at room temperature. Sephadex G-25 column was equilibrated with 10 mM ammonia carbonate (pH 9.3), and the solution was filtered through it to remove low weight compounds. Resulting yellow fraction was collected into a vial. Next, 1 mL of yellow fraction was mixed with 100 mg ADH and incubated at 4 °C overnight. Then, 10 μ L of 5 M NaBH₃CN in 1 M NaOH were added to a solution, incubated for 3 h at 4 °C and filtered through Sephadex G-25,

equilibrated with 10 mM phosphate buffer. Conjugation between GOx-ADH and activated cortisol-21-HS was performed by mixing 1 mL of GOx-ADH with 500 μ L of activated cortisol. Mixture was incubated at 4 °C overnight and filtered through Sephadex G-25. HiTrap Desalting columns with Sephadex G-25 resin were purchased from GE Healthcare. Desalting was done manually with syringe. The scheme of conjugation is shown in Fig. 3.3.5.1.

Figure 3.3.5.1 Conjugation scheme between GOx and cortisol-21-HS

3.3.6 Test of conjugation

SDS-PAGE which serves as an analytical tool for the separation of proteins by mass in an electric field was executed with the aim to see if any changes happened to native GOx protein. It uses sodium dodecyl sulfate (SDS) to help identify and isolate protein molecules. SDS acts as a surfactant, covering the proteins' intrinsic charge and conferring them very similar charge-to-mass ratios [17]. Upon application of a constant electric field, the protein migrates towards the anode, each with a different speed, depending on its mass. The SDS-PAGE method is composed of gel preparation, sample preparation, electrophoresis, protein staining or western blotting and analysis of the generated banding pattern. The gel was produced by radical polymerization in a mold consisting of two sealed glass plates with spacers between the glass plates. 30% AA was mixed with methylenebisacrylamide as a cross-linker, 1 M Tris buffer (pH 6.8), water, 10% SDS, TEMED and 10% ammonium

persulfate as the radical initiator. The solution was poured between the glass plates without creating bubbles. Polymerization lasted for 1 h. Separating gel was poured first and covered with a few drops of isopropanol. After the polymerization of the separating gel, the alcohol was discarded and the residual alcohol was removed with filter paper. Stacking gel solution was poured on top of the solid separation gel. Afterwards, a sample comb was inserted between the glass plates without creating bubbles and carefully pulled out after polymerization. For sample preparation, excess of SDS and β -mercaptoethanol as a disruptor of disulfide bonds were added to Tris buffer and heated for 5 min at 70 °C. After cooling to room temperature, GOx and GOx-cortisol-21-HS were pipetted into their own wells in the gel. In addition to the samples, a molecular-weight size marker is usually loaded onto the gel. This consists of proteins of known sizes and thereby allows the estimation (with an error of $\pm 10\%$) of the sizes of the proteins in the actual samples, which migrate in parallel in different tracks of the gel [18]. Bromophenol blue was added to sample buffer. After all proteins were sorted by size, they were stained by Coomassie [19, 20].

3.3.7 Studies on activity of GOx-cortisol-21-HS

The activity of GOx-cortisol-21-HS in buffers was tested in the following way: 80 μ L of GOx-cortisol-21-HS in 50 or 100 mM acetate, phosphate or MES buffer with pH 5.4, 6.1, 7.0 and 9.2 or in water was added to 10 μ L of 0.1 g/L peroxidase (POx) and 1 g/L o-dianisidine and 10 μ L of 0.1 M β -glucose. Optimization of the detection assay was performed by variation concentrations of active components: β -glucose (0.05, 0.1, 0.5 M), POx (0.01, 0.05, 0.1, 0.5 g/L) and o-dianisidine (0.1, 1, 10 g/L). The absorbance spectra of enzymatic reaction was recorded using plater reader Infinite M200 Pro (Tecan, Switzerland).

3.3.8 Control of GOx-cortisol-21-HS binding to the cavities in MIP layer

MIP@paper and bare paper membrane (all 10 x 10 mm) were mixed with 50 μ L of 0.1 g/L GOx-cortisol-21-HS or GOx, incubated for 5 min and left to dry in air. The membranes were washed in 80 μ L of 10 mM MES buffer (pH 6.1). 5 μ L of 0.1 g/L POx, 5 μ L of 1 g/L

o-dianisidine and 10 μL of 0.1 M β -glucose were added to the resulting solution. The kinetics of the enzymatic reaction was recorded using plate reader Infinite M200 Pro (Tecan, Switzerland).

Quartz crystal microbalance with dissipation (QCM-D) monitoring was chosen as another confirmation method of formation of cavities complementary to cortisol in MIP layer and as a proof of concept that GOx was conjugated with cortisol molecules. SiO_2 crystal has been chosen as the one that mimics chemistry of paper with hydroxyl groups introduced to the surface in a simple way. Cleaning of the crystal involved ozoning for 10 min, immersing crystals in the solution of 2% SDS for 30 min, rinsing with water, drying in N_2 stream and final ozoning for 10 min. With the aim to introduce OH groups to the surface of crystal, its treatment with 30% H_2O_2 and H_2SO_4 (v/v 1:3) was conducted for 30 min. Afterwards, crystals were washed with water. With the aim to introduce C=C bonds on the surface of crystals, they were immersed for 1 h in 95 μL of 3-(methacryloxypropyl)-trimethoxysilane in ethanol and water (4:1). Crystals were rinsed with ethanol 3 times and immersed in polymerization mixture that consisted of 20 mM EGDMA, 20 mM AA, 50 mM cortisol and 0.8 mM AIBN in 40 mL of ethanol and water (4:1), that was beforehand sonicated 5 min placed on ice and bubbled with N_2 for 3 min. Polymerization mixture was kept at water bath at 60 $^\circ\text{C}$ for 2 h. Crystals were washed multiple times by ethanol. All QCM measurements were conducted at Q-Sense set up (Biolin Scientific) connected to a pump (Ismatec).

The measurement started with injecting 50 mM acetate buffer to establish flat baseline. After that, cortisol was removed from the cavities by washing the crystals with methanol and acetic acid (90% methanol, v/v). Washing by buffer followed cortisol removal. With the view to test the capacity of formed cavities in MIP layer, 250 μL of 0.5 g/L of cortisol in 50 mM acetate buffer were introduced to the surface of chips. Bound cortisol was removed by washing with methanol with acetic acid (90% methanol, v/v). Acetate buffer was added to the chips to establish a flat line. In order to test binding ability of GOx and GOx-cortisol-21-HS, 0.05 g/L solutions of GOx and GOx-cortisol-21-HS in 50 mM acetate buffer were flushed through 2 chips. The frequency shift was recorded. The same steps

were repeated with GOx in different chip. Displacement of GOx-cortisol-21-HS by cortisol was also tested. After washing chip with acetate buffer, 0.5 g/L cortisol was introduced. Concentration of cortisol was chosen to be high with the view to evaluate if cortisol molecules tend to bind to cavities unoccupied by conjugate firsthand and then to replace a conjugate. The frequency shift observed in this case would potentially indicate about replacement and removal of heavy weight conjugate from the cavity. The scheme of modification of QCM chip is shown in Fig. 3.3.8.1.

Figure 3.3.8.1 Schematic representation of a displacement mechanism of GOx-cortisol-21-HS by cortisol

3.3.9 Binding of GOx-cortisol-21-HS to MIP@paper

MIP@paper membranes were incubated in 0.1 g/L solution of GOx-cortisol-21-HS for 5 min to occupy free cavities of MIP layer. Paper membranes were rinsed with 10 mM MES buffer (pH 6.1) to remove loosely bound GOx-cortisol-21-HS.

3.3.10 Synthesis of cortisocosterone (CS-HS) and testosterone hemisuccinates (T-HS)

25 mg corticosterone (CS) or testosterone (T) and 50 mg succinic anhydride were dissolved in diethyl ether and cooled in the ice bath. 55 μ L pyridine was added dropwise while stirring. The mixtures were then warmed to room temperature and acidified by 0.1 M HCl. The precipitates were collected by filtration and washed by cold ethanol. The confirmation of obtained structures was done by MALDI/ToF (MALDI Shimadzu Performance)

(Fig.3.3.10.1). Subsequent purification of CS-HS and T-HS was done by HPLC/UV (1260 Infinity II LC, Agilent).

Figure 3.3.10.1 Mass spectrum of synthesized (a) CS-HS, m/z (CS) = 346, m/z (CH-HS) = 446; and (b) T-HS, m/z (T) = 288, m/z (T-HS) = 388

3.3.11 Synthesis of GOx conjugates with cortisol homologues

5 mg of CS-HS, T-HS or testosterone 3-(O-(carboxymethyl)oxime) (TO) were mixed with 10 mg of NHS and 20 mg of EDC in 200 μ L DMF, 200 μ L of 1,4-dioxane and 100 μ L water. The mixture was incubated at 4 $^{\circ}$ C overnight. Modification of mannose on the surface of GOx occurred upon mixing 10 mg of GOx with 10 μ L NaIO₄ in 1 mL of water. The solution was incubated for 30 min at room temperature. Sephadex G-25 column was equilibrated with 10 mM ammonia carbonate (pH 9.3), and the solution was filtered through it to remove low weight compounds. Resulting yellow fraction was collected into a vial. Next, 1 mL of yellow fraction was mixed with 100 mg ADH and incubated at 4 $^{\circ}$ C overnight. Then, 10 μ L of 5 M NaBH₃CN in 1 M NaOH were added to a solution, incubated for 3 h at 4 $^{\circ}$ C and filtered through Sephadex G-25, equilibrated with 10 mM phosphate

buffer. Conjugation between GOx-ADH and activated CS-HS, T-HS or TO was performed by mixing 1 mL of GOD-ADH with 500 μ L of activated CS-HS, T-HS or TO. Mixture was incubated at 4 °C overnight and filtered through Sephadex G-25.

3.3.12 Control of GOx conjugates with cortisol homologues binding to the cavities in MIP layer

MIP@paper (10 x 10 mm) was mixed with 50 μ L of 0.1 g/L GOx-conjugate with cortisol homologues, incubated for 5 min and left to dry. Each membrane was washed in 80 μ L of 10 mM MES buffer (pH 6.1). 5 μ L of 0.1 g/L POx, 5 μ L of 1 g/L o-dianisidine and 10 μ L of 0.1 M β -glucose were added to the resulting solution. The kinetics of the enzymatic reaction was recorded using plate reader Infinite M200 Pro (Tecan, Switzerland).

3.3.13 Optimization of a design of a lateral flow displacement assay

MIP layer was synthesized on qualitative filter paper (Toyo Roshi Kaisha, Japan). Qualitative filter paper was also tested as a loading membrane and detection pad. Fusion 5 (GE Healthcare, UK) was tested as adsorption pad and detection zone. Cellulose fiber pad (EMD Millipore, USA) was tested as a loading and a detection pad. Hi-Flow Plus 75 membrane cards (EMD Millipore, USA) were used as backing strip to test applicability of nitrocellulose as flow equalizer pad. Glass fiber (EMD Millipore, USA) was tested as flow equalizer, reaction zones impregnated with reagents, detection and sample pad. Laminated cards (EMD Millipore, USA) were used as backing strip.

The width of lateral flow displacement strip is 30 mm. Lengths of membranes on the laminated backing strip varied. All membranes were treated separately and air-dried before placing on the backing strip. Cellulose membrane (40 mm) was followed by bare glass fiber (30 mm) that aimed to equalize the flow and MIP@paper (30 mm) saturated with 0.1 g/L GOx-cortisol-21-HS and used as a recognition site and displacement zone. Glass fiber (150 mm) with 0.1 M β -glucose aimed to serve as a flow equalizer for removal of displaced conjugate and subsequent oxidation of impregnated β -glucose. Flow equalizer zone was

followed by glass fiber in reaction zone (30 mm) with 0.1 g/L POx that catalyzed reaction between H_2O_2 released as a by-product of enzymatic reaction between GOx-cortisol-21-HS and β -glucose and 1 g/L o-dianisidine impregnated on the surface of cellulose fibers pad (120 mm) utilized as detection pad. To test the ability of pre-deposited H_2O_2 on the detection pad to amplify produced signal and to trigger o-dianisidine oxidation, o-dianisidine was mixed with 0.03, 0.15, 0.3 and 3 mM H_2O_2 in the ratio 10:1 (v/v), and after that cellulose fiber pad was vertically immersed into the resulting solutions.

3.3.14 Preparation of test lateral flow displacement strip

Test lateral flow displacement membranes were prepared in the following way. MIP@paper (30 mm) was immersed in 100 μL 0.1 g/L GOx-cortisol-21-HS to achieve high saturation of MIP cavities by conjugate, incubated for 5 min, washed by 10 mM MES buffer (pH 6.1) and air dried. Glass fiber (150 mm) was immersed into 20 μL of 0.1 M β -glucose and air dried. 15 μL of 0.1 g/L POx was added to another glass fiber (30 mm) and air dried. Cellulose fiber pad (120 mm) was vertically immersed into 30 μL of solution of 1 g/L o-dianisidine and 0.3 mM H_2O_2 (10:1, v/v) and left to air dry. After assembling a lateral flow displacement strip with constituent parts, it could be utilized for analysis of cortisol concentration.

3.3.15 Preparation of standard lateral flow displacement strip

Standard lateral flow displacement membranes were prepared in the following way. MIP@paper (30 mm) was immersed in 100 μL 0.1 g/L GOx-cortisol-21-HS to achieve high saturation of MIP cavities by conjugate, incubated for 5 min, washed by 10 mM MES buffer (pH 6.1) and air dried. Glass fiber (150 mm) was immersed into 20 μL of 0.1 M β -glucose and air dried. 10 μL of 0.1 g/L POx was added to another glass fiber (30 mm) and air dried. Cellulose fiber pad (120 mm) was vertically immersed into 30 μL of solution of 1 g/L o-dianisidine and 0.3 mM H_2O_2 (10:1, v/v) and left to air dry. After assembling a lateral flow displacement strip with constituent parts, 40 μL of standard solution of cortisol (0.1, 0.5, 1.0, 5.0, 10.0, 20.0 ng/mL) was introduced to the cellulose loading pad. 20 μL of

water was subsequently added to facilitate removal of displaced conjugate molecules. The image was taken after 2 min of reaction. Obtained color intensities of the detection membranes were used as standard membranes to obtain semi-quantitative estimation of cortisol concentration in saliva samples.

3.3.16 RGB analysis of detection pad in lateral flow displacement strip

For digital image analysis, tested lateral flow displacement strips were placed on a white background and the images of lateral flow strips were taken with a digital camera ILCE 7R (Sony) fixed at a constant distance and angle from the samples. Subsequently, the images were transferred to a computer for further analysis. Full area of detection pad was cut for RGB analysis. The image of detection pad was afterwards reduced to 75x150 pixels and analyzed by ImageJ software for quantification of obtained colorimetric responses.

3.3.17 Interferences from cortisol homologues present in saliva

0.5 and 1 ng/mL of T, CS and TO were prepared to be tested for their affinity towards designed cavities. 40 μ L of each solution were introduced to the prepared test lateral flow displacement strips with subsequent washing by 20 μ L of water. The images of detection pads were taken after 2 and 5 min. The same steps were repeated with cortisol solution.

3.3.18 Optimization of saliva pre-treatment

Collected saliva was separated into two parts and transferred to 1.5 ml tubes. One part was diluted by PBS 2 times. The amount of cortisol in whole and diluted saliva was immediately determined by HPLC/MS for proper calculation of concentration of spiked cortisol. HPLC/MS detection of cortisol in saliva included prior solid phase extraction of cortisol from saliva based on modified protocol suggested by Tsyrulneva et al [21]. MIP particles (100 mg), collected in 1 h after removal of MIP@paper from polymerization mixture and treated with mixture of methanol and AcA (90% methanol, v/v) to remove bound cortisol, were placed in 1 mL syringe with glass fiber on the bottom and conditioned

with water. Whole or diluted saliva was run through the syringe followed by subsequent washing with ACN, water, methanol, water and AcA in order to remove numerous salivary compounds that could potentially complicate HPLC/MS detection of cortisol. After most of interferences were eluted in this way, the syringe was washed with a mixture of methanol and AcA (90% methanol, v/v). The final extract was used for HPLC/MS analysis of cortisol after evaporation in N₂ stream and reconstitution in 0.5 mL water. Three syringes were used simultaneously and were reused several times without noticeable loss of recognition properties. HPLC/MS detection method was the same as used for control of equilibrium cortisol.

10 µL of 130 ng/mL, 900 ng/mL and 1.8 µg/mL of cortisol were added to whole and diluted saliva to obtain final concentration of 5, 10 and 20 ng/mL, respectively. Images of resulting saliva samples were used for visual and semi-quantitative detection. Cortisol concentration in spiked whole and diluted saliva was also confirmed by HPLC/MS detection. 40 µL of pre-treated saliva was added to the loading pad of developed lateral flow displacement test strip. Subsequently, 20 µL of water were introduced to assist in removal of displaced conjugate. The concentration of salivary cortisol was then estimated based on the obtained color intensities of the test membranes with reference to color intensities of standard membranes.

3.3.19 Validation of developed assay

Saliva samples were collected from 4 volunteers in different physiological conditions in order to cover a wide range of salivary cortisol concentration levels. The samples were collected upon their verbal consent. Volunteers were asked to avoid consumption of food at least for 1 h (unless stated different) and to rinse their mouths with water prior to saliva collection. Collected saliva samples were either analyzed within 30 min or, in case of saliva that was collected before sleep, were kept at -4 °C fridge overnight and were used for analysis next morning after defrosting. Saliva samples were diluted 2 times by PBS and 40 µL of resulting solution were added to the loading pad of developed lateral flow displacement test strip. Subsequently, 20 µL of water were introduced to assist in removal

of displaced conjugate. In order to estimate cortisol level semi-quantitatively, after 2 min of reaction, the resulting color was compared to the standard lateral flow displacement strip. The image of detection pad was also taken for subsequent quantitative analysis. Diluted saliva after SPE was used for detection of cortisol by HPLC/MS that is considered a standard in detection of small molecules in complex matrices.

3.4 Conclusions

Protocols that were used to investigate the degree of heptapeptides extraction from saliva and to develop an optimized QCM platform for their detection were described here. Step-by-step elaboration of colorimetric paper-based test for detection of α -amylase in saliva, including description of involved instrumental and visual methods to confirm its concentration is provided. Description of procedures for synthesis of MIP@paper with specificity towards cortisol and conjugation between GOx and cortisol-21-HS is postulated here. Methods used to confirm MIP synthesis at paper, to control specificity of the cavities and conjugation process are described here. Detailed description of stages for optimization of lateral flow displacement strip is provided. Detection of α -amylase and cortisol in saliva was validated with the help of standard detection methods that are currently employed. Study of salivary interferences is done for three developed systems.

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Chapter 4

Exploring extraction protocols for heptapeptides and elaboration of a QCM platform for their detection in saliva

Recently, the ratio of two heptapeptides was proposed as an objective biomarker responsible for overtraining and fatigue in sportsmen. Thus, this chapter focuses on the development of efficient extraction protocols of target peptides from saliva, exploring methods and techniques for confirmation of their presence, followed by the elaboration of a platform capable of recognizing and quantifying the peptide biomarkers with potential to be implemented for overtraining detection.

To achieve these goals, conjugation and surface chemistry for stable and efficient immobilization of target analytes required optimization. The QCM platform was tested in real saliva samples. Although the antibodies raised against target peptides are characterized by sufficient selectivity in buffer solutions, they demonstrate poor recognition towards peptides in salivary extract.

4.1 Heptapeptides as biomarkers of overtraining in saliva

Recently, the ratio of two heptapeptides was proposed as an objective biomarker responsible for overtraining and fatigue in sportsmen. Michael D. et al [1] collected saliva samples of 9 sportsmen-cyclists before, during and after their training program, analyzed them by HPLC/MS and found that only 2 peptides (GGHPPPP and ESPSLIA) exhibit specific pattern in the saliva samples of overtrained sportsmen. The ratio of these heptapeptides, derived from acidic and basic proline-rich proteins (aPRP and bRP), was identified as an objective biomarker for the evaluation of fatigue in sportsmen. These peptides are suggested to be good biomarkers of overtraining. Fingerprint of two salivary heptapeptides has the perspective to be used for confirmation of fatigue and overtraining in sportsmen. Constant monitoring of these biomarkers can serve as a tool for investigating the mechanisms of overtraining development. Therefore, the main focus of this thesis chapter is on extraction and detection of these peptide sequences using specific recognition molecules and a piezoelectric detection platform.

4.2 Extraction of heptapeptides from saliva

The GGHPPPP and ESPSLIA peptides that recently were found to be relevant biomarkers for overtraining and fatigue of sportsmen belong to the family of basic salivary proline-rich protein 1 and 2 (P04280 and P02812, respectively, UniProt) and acidic proline-rich phosphoprotein 1/2 (P02810, UniProt). The mechanism of their appearance in saliva is to the best of our knowledge still uninvestigated. However, peptides with related structures were identified in whole and parotid saliva. The researchers lean toward the idea that the increase of the concentration of catecholamines produced when salivary glands are innervated by neurons from SNS [2], along with the raise of endurance, leads to the synthesis of specific salivary peptides [3]. The two peptides have different physical and chemical properties. A brief description of each peptide is shown in Table 4.2.1.

Table 4.2.1 Physical and chemical properties of peptides

Property	GGHPPPP	ESPSLIA
Protein family	acidic proline-rich phosphoprotein	basic salivary proline-rich protein 1 and 2
Molecular weight, Da	657	717
pI	6.74	4
Hydropathy index	-1.5	0.5

Since the peptides possess different characteristics, their detection and extraction is always a compromise between various techniques, which implies that high recoveries for one peptide can be achieved, while recovery of another one could be lost. Besides, the biological liquid – saliva – comprises of large amount of other peptides that possess similar sequences as peptides of interest that complicates the elaboration of an extraction procedure for peptides. As it was established by Vitorino et al. [4], most of salivary peptides are in the range of 1000-3000 Da.

The ultrafiltration by using membranes with 3 kDa cut-off made of regenerated cellulose was chosen. The extraction procedure combined several steps: spiking non-diluted saliva with different concentrations of standard solutions of GGHPPPP and ESPSLIA, shaking, centrifugation for 20 min at 14000x g and collecting supernatant for subsequent HPLC analysis. The influence of ultrafiltration time on the recovery of peptides was tested (Fig.4.2.1).

Figure 4.2.1 Recovery of the target peptides as a function of ultrafiltration time

As can be seen in Fig. 4.2.1, the highest recovery (approx. 100%) is achieved after 20 min of ultrafiltration at centrifugation speed of 14000x g. Besides, there is no need to choose shorter ultrafiltration time because under such conditions the volume of filtrated solution decreases significantly which further complicates sample manipulations.

In order to identify the peptides in saliva extract, the supernatant was collected for HPLC/UV analysis using a C18 column. The fractions in the range of time retentions of both peptides were collected for detection of the target peptides using MALDI/ToF. A representative chromatogram of spiked saliva is shown in Fig. 4.2.2.

Figure 4.2.2 Chromatogram of saliva sample (black curve) and of saliva spiked with 0.2 mg/mL of peptides (red curve)

The calibration curves are linear in the range of 0.02-0.5 mg/mL for both heptapeptides, with linear fit parameters $Y = 19 \pm 16 + (9890 \pm 72) \cdot x$ (mg/mL) and $Y = 18 \pm 12 + (1592 \pm 56) \cdot x$ (mg/mL), respectively. The estimated limits of detection, calculated by 3σ -criteria, equal 0.005 and 0.02 mg/mL for GGHPPPP and ESPSLIA, respectively.

Saliva is considered a complex biological fluid and matrix effects usually interfere with the detection of a target compound by enhancing or reducing analytical signal. The components may interfere with the detection by introducing distortions or contributing to changes to signal read out. One of the simplest methods widely applied in analytical

chemistry is the method of standard additions that provides a way to relate the analytical signals from both unknown sample and standard solutions with the help of calibration curve [5]. This method found its wide application in atomic absorbance spectroscopy and gas and liquid chromatography. Standard addition method stands for spiking the same volume of an unknown sample by known amounts of analyte standard solution, diluting the sample with solvent to the same volume and measuring detector signals as a response to every added amount. Given the linear change of analytical signal towards change of spiked concentrations, the concentration of a target compound can be identified from the linear regression by extrapolating it to x-axis where $y=0$. The intercept obtained with the negative sign indicates the amount of target compound in the sample. The concentration of an analyte is calculated according to equation (1), where C_x is actual concentration of the analyte in the sample, C_0 is the concentration of a standard, V_x is the volume of a sample, C_{std} is the concentration of standard solution, V_{std} is the volume of standard solution added to each flask, V_{tot} is the total volume of solution:

$$C_x = \frac{C_0 V_x + C_{std} V_{std}}{V_{tot}} = \frac{C_0 V_x}{V_{tot}} + \frac{C_{std} V_{std}}{V_{tot}}. \quad (1)$$

C_{SA} is the concentration of standard aliquot and is determined by C_{std} , V_{std} and V_{tot} (2):

$$C_{SA} = \frac{C_{std} V_{std}}{V_{tot}}. \quad (2)$$

R is the detected signal dependent on instrumental sensitivity (K) and concentration that gives the following equation (3):

$$R = K \frac{C_0 V_x}{V_{tot}} + K C_{SA}. \quad (3)$$

This equation resembles linear fit equation (4) where a is the intercept and b is the slope.

$$y = a + bx. \quad (4)$$

Given that $y = 0$, equation looks as follows:

$$C_0 = -C_{SA} \frac{V_{tot}}{V_x}, \quad (5)$$

that allows to calculate V_x .

Preliminary analysis of saliva samples with the aim to identify target peptides was conducted using saliva collected from 4 healthy humans (3 males, 1 female) before and after training of different types (2 – endurance (jogging at 9 and 12 km/h, distance 10 km) and 2 – strength (weightlifting, exercise time 1 h) trainings). For this, different aliquots of separate peptide standards were added to saliva and full extraction protocol was performed. The results can be found in Table 4.2.2. Here, the examples of calculating initial concentrations of both peptides are shown for four sportsmen. The value of a refers to the interception, according to equation (3), which in turn is dependent on the concentration of target peptides in the unknown sample. The value of b represents the sensitivity of the method. Approach of standard addition method applied to saliva samples demonstrates positive values of a almost in all cases which connotes the evidence of peptides presence in saliva, as it can be seen from the Table 4.2.2. However, standard error for a (Δa) that in most cases is commensurable or excessive as compared to a negates the reliability of coefficient a and cannot be recommended for confirmation of peptides presence in saliva. Calibration curves of standard additions of peptides for all sportsmen can be obtained from Fig. 4.2.3.

Figure 4.2.3. Calibration curves obtained by method of standard addition of GHPPPP (1) and ESPSLIA (2) solutions to saliva samples before (a) and after training (b) for 4 sportsmen

Table 4.2.2. Application of standard addition method to saliva samples with the aim to identify the peptides of interest. $V=0.5$ mL, $C(\text{in}) = 1$ mg/mL. BT – before training, AT – after training.

N	V_{st} μL	C_{st} mg/mL	Sportsman 1			Sportsman 2			Sportsman 3			Sportsman 4		
			S_{peak} ESPSLIA	S_{peak} GGHPPPP	S_{peak} ESPSLIA	S_{peak} GGHPPPP	S_{peak} ESPSLIA	S_{peak} GGHPPPP	S_{peak} ESPSLIA	S_{peak} GGHPPPP	S_{peak} ESPSLIA	S_{peak} GGHPPPP	S_{peak} ESPSLIA	S_{peak} GGHPPPP
BT	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BT1	10	0.02	21	20	10	16	14	16	14	16	18	18	18	18
BT2	25	0.05	59	51	48	39	56	47	50	48	48	48	48	48
BT3	50	0.1	126	102	112	88	120	87	105	99	99	99	99	99
BT4	100	0.2	256	206	250	188	252	189	241	1989	1989	1989	1989	1989
BT5	250	0.5	631	498	580	4018	600	433	578	4469	4469	4469	4469	4469
	$a \pm \Delta a$		-2 $\pm$ 2	2 $\pm$ 2	-8 $\pm$ 8	6 $\pm$ 9	-3 $\pm$ 5	3 $\pm$ 5	-5 $\pm$ 5	79 $\pm$ 69	79 $\pm$ 69	79 $\pm$ 69	79 $\pm$ 69	79 $\pm$ 69
	$b \pm \Delta b$		1270 $\pm$ 8	996 $\pm$ 8	1220 $\pm$ 30	804 $\pm$ 36	1210 $\pm$ 20	866 $\pm$ 20	1170 $\pm$ 20	889 $\pm$ 24	889 $\pm$ 24	889 $\pm$ 24	889 $\pm$ 24	889 $\pm$ 24
AT	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
AT1	10	0.02	19	20	12	15	20	16	18	18	18	18	18	18
AT2	25	0.05	50	52	40	38	63	49	61	49	49	49	49	49
AT3	50	0.1	103	104	118	77	131	87	124	98	98	98	98	98
AT4	100	0.2	210	208	253	170	253	183	249	190	190	190	190	190
AT5	250	0.5	518	500	594	399	621	445	600	460	460	460	460	460
	$a \pm \Delta a$		-1 $\pm$ 1	3 $\pm$ 2	-6 $\pm$ 8	0 $\pm$ 3	1 $\pm$ 3	2 $\pm$ 2	1 $\pm$ 4	4 $\pm$ 2	4 $\pm$ 2	4 $\pm$ 2	4 $\pm$ 2	4 $\pm$ 2
	$b \pm \Delta b$		1039 $\pm$ 5	998 $\pm$ 10	1180 $\pm$ 30	803 $\pm$ 16	1240 $\pm$ 10	889 $\pm$ 9	1200 $\pm$ 20	915 $\pm$ 9	915 $\pm$ 9	915 $\pm$ 9	915 $\pm$ 9	915 $\pm$ 9

It is widely recognized that UV detection is not very sensitive, especially towards salivary matrices where interference can be caused by significant amounts of other peptides with similar structures that also are extracted after ultrafiltration. Presence of target peptides in the collected fractions was double confirmed by MALDI/ToF, which is recognized as one of the most sensitive and accurate methods for analysis of biological objects. Extensive application of MALDI/ToF for detection of peptides, proteins and oligonucleotides makes its application for analysis highly desirable.

However, saliva is a complicated object for MALDI analysis because the rate of ionization induced by matrix differs for the various peptides. It affects the signal intensity causing discrepancies between the amount of compound and intensity of a signal. Moreover, high viscosity of saliva and its multicomponent system intricate direct analysis of peptides. Several extraction protocols of peptides from saliva were examined with the aim to confirm if peptides of interest are extracted from spiked and from non-treated saliva. Extraction by ACN and ethanol demonstrated that peptides were recovered from spiked saliva. However, the same extraction protocol applied to whole non-treated saliva didn't reveal any trace of peptides after analysis by MALDI/ToF. As there was no particular difference between extraction by ACN or ethanol, all future experiments that involved extraction of peptides from saliva for MALDI/ToF analysis were performed with ethanol.

In order to obtain full picture of saliva recording mass-spectra in both positive and negative modes and study on matrix type impact is admitted as a best technique. It was established that among all available matrices, α -cyano-4-hydroxycinnamic acid significantly influenced the ionization of the components of saliva samples, producing highly ionized species. Linear and reflectron positive mode as compared to negative mode demonstrated highest sensitivity towards target analytes. Mass-spectra of standard solution of peptides are shown in Figure 4.2.4. As it can be seen, GGHPPPP produces molecular ion with m/z 656, while ESPSLIA – 718.

Figure 4.2.4. Mass-spectra of standard solutions of GGHPPPP and ESPSLIA

Mass spectra of non-spiked and spiked pretreated saliva of one volunteer are shown in Fig. 4.2.5 (a) and (b), respectively. The results obtained for other volunteers are similar and are not highlighted here.

It can be seen that no peptides of interest could be found in saliva of volunteers that could be taken as evidence of low amount of target analytes in saliva which cannot be detected by MALDI/ToF, or as an assumption that peptides are not present in saliva. With the view to test first hypothesis, the lowest concentration of peptides in spiked saliva that can be detected by MALDI/ToF was determined. A range of concentrations varied from 0.01 ng/mL to 1 µg/mL in saliva diluted twice by ethanol and in water. While aqueous solutions enable detection of the concentrations equal to 0.05 ng/mL of both peptides, saliva samples (diluted twice by ethanol) demonstrated the reliable results for confirmation of ESPSLIA presence at the level of 1 ng/mL and of GGHPPPP – at the level of 0.5 ng/mL. Big difference in levels of detection in aqueous solutions and in pre-treated saliva can be explained by high variety of compounds extracted from complex salivary matrix and by low ionization of target peptides due to strong interference from other constituents. Although being a highly sensitive technique, MALDI/ToF is not the best choice for analysis of biological liquids for trace amounts of peptides.

a)

b)

Figure 4.2.5 Mass-spectra of pre-treated non-spiked (a) and spiked with peptides (b) saliva

Therefore, there is a need to develop another approach to detect peptides with high sensitivity and selectivity. Microgravimetric platform with antibodies as recognition molecules is supposed to fulfil these requirements.

4.3 Development of QCM-D method for detection of heptapeptides

As it was demonstrated previously, it was not possible to detect target peptides in saliva by employed methods. The reason for this lies in low sensitivity of HPLC/UV and difficulties in ionization of peptides by MALDI/ToF. Development of a platform that utilizes label-free antibodies raised against target peptides could possibly ensure specific recognition and be a perspective tool for selective detection of peptides in saliva.

In order to obtain a selective platform for detection of a peptide, a surface of gold chip needs to be modified. As far as QCM method is not applicable to the direct detection of short peptides due to low sensitivity to small changes in mass, a competitive immunoassay which is based on reaction of peptides with excess of antibodies in the sample, followed by reaction of unreacted antibodies with immobilized on gold chip peptide, was proposed as an approach to tackle this shortcoming.

The effect of hydrophobic linker (mercaptohexadecanoic acid, $\text{SH-C}_{15}\text{-COOH}$), chemically activated by EDC with subsequent coupling of target peptide, was checked. Modification of surface was performed in the presence of different blockers. Step of antibody binding to linker, blocker and their mixture is demonstrated in Fig. 4.3.1.

Figure 4.3.1 Antibody binding to an immobilized peptide as a function of linker (16-mercaptohexadecanoic acid – black curve), blocker (1-octadecanethiol – blue curve) nature and their mixture in ratio 1:1 (red curve)

Figure 4.3.1 represents different behavior of antibody dependent on type of linker and blocker. 16-Mercaptohexadecanoic acid ($\text{SH-C}_{15}\text{-COOH}$) (black curve) upon activation by EDC is expected to exhibit high values of binding. The experiment with hydrophobic 1-octadecanethiol (SH-C_{18}) (blue curve) aimed at blocking active surface sites and at elimination of any non-specific interactions. A frequency drop caused by antibody binding proved that binding to the peptide is caused by non-specific forces. This unwanted result encouraged to test a different system. Thus, an alternative system with a hydrophilic PEG linker was also examined. Hydrophilic linker aims to provide only specific binding,

therefore, the reduction of binding of antibody to a chain of a linker is expected. A monolayer of SH-PEG-COOH was formed on a surface of gold chip and peptide was covalently bound to a chemically activated carboxylic group. In order to assure specific binding, another gold chip was covered by a monolayer of SH-PEG that does not exhibit any reactivity towards peptide. All steps of peptide immobilization were also conducted with the aim to eliminate any non-specific binding of a peptide to either blocker or chip surface. The frequency shifts observed upon antibody binding to an immobilized peptide are demonstrated in Fig. 4.3.2 (a).

Figure 4.3.2 Frequency shift upon antibody binding as a function of hydrophilic linker (SH-PEG-COOH – red curve) and blocker (SH-PEG – black curve) used (a); The effect of blocking agents on frequency drop upon antibody binding (b)

As it can be seen from Fig. 4.3.2 (a), no frequency shift upon injection of antibody to a gold chip modified by a hydrophilic blocker is observed. It can be assumed that modification of surface with PEGs that work as blockers is highly effective, since no non-specific interaction between an antibody and the blocker is observed. With a view to develop optimal surface chemistry, different ratios of linker to blocker were tested. Blocking of free unoccupied sites on gold surface aimed to reduce non-specific binding. The linker diluted by blocker in proportion 4 to 1 caused the frequency drop upon binding of antibody to activated carboxylic group, whereas introduction of more blocker to the system didn't affect the binding (Figure 4.3.2 (b)). An increase in dissipation energy ($0.6 \cdot 10^{-6}$) upon interaction with antibody was observed attesting viscoelastic properties of the monolayer. Else, two different modes of surface blocking were compared: diluting of

the linker by a blocker and blocking surface after immobilization of a peptide. No significant difference in frequency shift is observed but the reproducibility of blocking free sites is higher when conducted after immobilization of peptide. That's why this way of blocking was chosen for future investigation.

After having optimized immobilization protocol, a series of antibodies raised against every peptide was flown through system in order to establish the antibody with the highest specificity towards a given peptide. Antibody that has higher affinity towards peptide would demonstrate a higher frequency shift. The list of all antibodies with the tested for their specificity against target peptides is shown in Fig. 4.3.3.

Figure 4.3.3 Specificity of antibodies raised against GGHPPPP (Ab6-Ab10) and ESPSLIA (Ab1-Ab5) towards GGHPPPP (a) and ESPSLIA (b) immobilized on the gold surface

As it can be seen from Fig. 4.3.3, Ab 5 and 9 cause the biggest frequency drop that indicates about their higher specificity towards ESPSLIA and GGHPPPP, respectively. At the same time, certain cross-reactivity towards other peptide is also observed that can be foreseen as a complication for future analysis.

4.4 Validation of developed QCM platform

In order to verify the applicability of developed assay for detection of peptides, the competitive approach was tested. Each peptide with concentration 0.1 μM was mixed with 0.6 μM antibody (Ab 5 or Ab 9). Antibody was added in excess in order to bind all peptide

molecules, while unreacted antibodies are expected to bind to immobilized on gold surface peptides that would cause a frequency shift. The same experiment, but with higher concentration of peptide ($10\ \mu\text{M}$), aimed to test if antibodies are characterized by high specificity to bind target analytes. The binding of unreacted antibodies to immobilized on gold surface peptides is shown in Fig. 4.4.1.

Figure 4.4.1 Binding of excess of antibody to the surface with immobilized ESPSLIA (a) and GGHPPPP (b) after its mixing with ESPSLIA and GGHPPPP, respectively. $C(\text{peptide}) = 0.1\ \mu\text{M}$ (black curve), $10\ \mu\text{M}$ (red curve), $C(\text{Ab}) = 0.6\ \mu\text{M}$

As it can be seen from Figure 4.4.1 (black curve), low amount of peptide binds readily to antibody causing the reaction of left amount of antibody with immobilized on surface peptide. Contrarily, high amount of peptide binds to all antibodies present in solution and disables them from recognizing immobilized peptide as a target (red curve).

When same antibodies were added to pre-treated, spiked with peptides saliva, and the resultant solution was run through the modified chip, no frequency shift was observed. This phenomenon can be explained by low specificity of antibodies that bind all peptides with similar structures that are present in saliva instead of peptides of interest.

Thus, recognition molecule that has higher affinity towards target peptides should be found to enable analysis of salivary samples. Moreover, extraction protocol that would enable recovery of peptides of interest and minimize the interfering effect of other peptides should be elaborated.

4.5 Conclusions

Although the mechanism of appearance of heptapeptides is not established yet, their ratio is believed to be a biomarker of overtraining and thus, they represent a good object for elaboration of lab-on-a-chip devices. Piezoelectric platform based on immunoassay for quantitative detection of heptapeptides was elaborated. The surface chemistry was optimized with the aim to ensure only specific binding of antibodies to immobilized on the surface peptides. Developed platform can potentially serve as a reference method in future experiments devoted to production of onsite testing paper-based immunosensor for detection of peptides.

The application of the assay for analysis of peptides in water demonstrated good results, whereas validation of immunoassay in saliva revealed ambiguous findings. Binding of antibodies raised against GGHPPPP and ESPSLIA with other peptides present in salivary extract indicated high cross-reactivity that significantly complicates the analysis. Development of a more precise extraction procedure that would target recovery only of a small amount of salivary peptides and search for recognition molecules that can provide more selective binding of heptapeptides is a part of future experiments.

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Chapter 5*

Visual Detection of Salivary α -Amylase using Maltose as Inhibitor for Polysaccharide Cleavage

This chapter reports about development of an assay for naked eye detection of salivary α -amylase, one of the biomarkers of overtraining. The ability of α -amylase to cleave α -bonds of polysaccharides is utilized for developing a colorimetric assay. In the proposed approach, 2-chloro-4-nitrophenyl- α -D-maltotrioside (G3-CNP) as substrate releases a colored by-product upon cleavage by salivary α -amylase. Introduction of maltose as product inhibitor yielded desirable linear responses in the clinically relevant range 20-500 $\mu\text{g/mL}$, and a limit of detection of 8 $\mu\text{g/mL}$ (in aqueous solution). Subsequently, a facile paper-based assay is proposed for analysis of human saliva samples with minimum interference from saliva components. The proposed paper-based assay is rapid, specific and easy-to-implement for onsite testing of saliva samples within 20-500 $\mu\text{g/mL}$ at a LOD of 11 $\mu\text{g/mL}$ (upon visual detection). The proposed assay is benchmarked against Phadebas test kit that is the gold standard for spectrophotometric detection of α -amylase. Stability of developed assay, its precision and accuracy were thoroughly tested and reveal high potential of the paper-based assay for onsite testing.

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5.1 Background information about α -amylase

Unlike other biomarkers associated with fatigue, fluctuations in salivary levels are directly caused by ANS response to parasympathetic and sympathetic stimulation. α -Amylase steadily diminishes after start of the training and, upon reaching a certain point when organism switches to anaerobic training via activation of the sympathetic adrenal system, its level grows [1]. The elevated amount of α -amylase in saliva after physical exercises is linked to the growth of plasma catecholamines, proving adrenergic nature of training [2]. With the adaptation of organism to the increased stress after training, the level of α -amylase returns to a baseline. However, if it remains at the same level for prolonged time, it can be an indicator of overtraining development and a closer attention to the health condition of an athlete is required. Thus, in order to carefully control the training conditions of a sportsman and to avoid overtraining it is important to evaluate the concentration of salivary α -amylase at every time point of training as well as during periods of recovery.

The physiological range of α -amylase in saliva typically varies from 50 to 500 $\mu\text{g/mL}$, presented usually in units/mL [3]. However, expression of enzymatic units depends on type of substrate that is used for detection under certain temperature, thus, the numbers may vary significantly. Given, that conditions for maintaining constant activity of the enzyme are abided all the time, utilization of mass units is more applicable in this study. It has been reported that the evaluation of salivary amylase concentration is influenced by the adopted sample collection and analytical methodologies except for enzymatic assays that provide reliable quantification of amylase [3].

The main function of amylase is to cleave α -bonds of polysaccharides, yielding short chain by-products such as maltose and maltotriose [4]. G3-CNP is utilized as substrate in this study as it possesses good cleavage efficiency of the dye-glycoside bond, concurring with previous reports [5]. The schematic mechanism of the cleavage reaction is illustrated in Fig. 5.1.1.

Figure 5.1.1 Schematic mechanism of cleavage of G3-CNP by α -amylase

5.2 Study of kinetics of α -amylase

In order to understand the substrate cleavage by α -amylase, the kinetics of enzymatic reaction is examined. The Michaelis-Menten kinetics provides the description of the velocity of reaction between enzyme and substrate. Given that the amount of enzyme is fixed, the increase of substrate concentration yields linear augmentation of the reaction rate until saturation of enzyme with substrate molecules, resulting in a plateau after which no further change in velocity is observed. Thus, when the substrate is in excess to the enzyme, all active sites are occupied by substrate molecules and the enzyme works at a maximum possible rate. The reaction velocity in this case is independent of substrate concentration. On the other hand, for experiments that aim to study the reaction rate as a function of increasing enzyme concentration, the substrate concentration should be significantly higher than the enzyme concentration so that the reaction is independent of substrate concentration. This will result in release of products in accordance with the enzyme concentration.

With the view of investigating the reaction velocity with increasing substrate concentration, the absorbance values of the released product (CNP) were monitored at 405 nm for a fixed concentration of α -amylase (200 $\mu\text{g/mL}$) cleaving G3-CNP in the concentration range of 0.01 – 5 mg/mL (Fig. 5.2.1). The absorbance values display a complex (non-linear) behavior with increasing substrate concentration, and they reach a plateau at around 4 mg/mL. The absorbance curve does not resemble a classic hyperbolic that is inherent for enzymes that obey Michaelis-Menten kinetics. On the contrary, the absorbance curve displays intermediate velocity profiles, which could be attributed to cooperative binding of by-products (oligosaccharides) formed during enzymatic reaction. These by-products are prone to compete with the substrate molecules during binding to the enzyme.

Figure 5.2.1 Absorbance curve of released dye (CNP) illustrating the enzymatic digestion of increasing concentration of substrate (G3-CNP) by α -amylase (200 $\mu\text{g/mL}$). The curve is recorded after a reaction time of 3 min. The samples for were prepared for $n=3$

Although oligosaccharides with less than three residues are not recognized as a substrate [6], they could sterically block the active site of α -amylase, thus inhibiting the access for substrate molecules. When products bind to the active site and thereby inhibit the binding of a substrate molecule, the interaction becomes negatively cooperative. The shape of $V/[S]$ vs V (modified Scatchard plot) was examined in order to evaluate cooperativity. In the presence of cooperativity, the curve is no longer linear; instead, it is concaved downwards or upwards, in the case of positive or negative cooperativity, respectively [7]. The results of calculation are shown in Fig. 5.2.2.

Figure 5.2.2 Modified Scatchard plot to address the nature of the cooperative binding between α -amylase (200 $\mu\text{g/mL}$) and G3-CNP

The shape of the curve shown in Figure 5.2.2 undoubtedly points toward negative cooperative binding caused by an excess of by-products in the solution. Typically, a longer reaction time could overcome inhibition, as the substrate molecules would have sufficient time to reach and bind to the active sites of the enzyme. This assumption is confirmed by the kinetic curve recorded after 20 min of reaction (Fig. 5.2.3).

Figure 5.2.3 Absorbance curve of enzymatic reaction between α -amylase (200 μ g/mL) and increasing concentration of substrate. The curve is recorded after a reaction time of 20 min. The samples for were prepared for $n=3$

Absorbance curve of enzymatic reaction between α -amylase and increasing concentration of substrate, recorded after a reaction time of 20 min, indicates about negative cooperative binding between α -amylase and G3-CNP molecule. Increasing reaction time provides more chances for substrate molecules to reach the active site of enzyme, resulting in a less pronounced step in the intermediate area between 1 and 4 mg/mL of substrate.

5.3 Effect of product inhibitor

For accurate determination of α -amylase amount in the sample, the dynamic range of developed system should be established. For this purpose, the concentration of α -amylase was varied between 10 and 500 μ g/mL with the intent to evaluate response for physiological concentration ranges of α -amylase in saliva. The results are shown in Fig. 5.3.1.

As observed from Figure 5.3.1, the curve is still non-linear, preventing accurate determination of α -amylase over the entire physiological range. Therefore, in order to reduce enzymatic reaction rate and thereby to broaden the dynamic range, maltose is evaluated as a non-competitive inhibitor [8]. Maltose binds nonspecifically to the enzyme (not to the active site), thereby changing its morphology that indirectly influences the binding and cleavage rate of the substrate.

Figure 5.3.1 Absorbance curve of released dye as a function of amount of α -amylase ($C(\alpha\text{-amylase}) = 5\text{-}500 \mu\text{g/mL}$), concentration of substrate $C(\text{G3-CNP}) = 5 \text{ mg/mL}$. The curve is recorded after a reaction time of 3 min. The samples for were prepared for $n=3$

When 1, 2 or 5 mM solutions of maltose are added to the reaction mixture, a decrease in absorbance at low α -amylase concentrations is observed, which implies that less products are released from substrate molecules. The results of the experiment can be seen in Fig. 5.3.2.

Figure 5.3.2 Influence of maltose concentration (1, 2 or 5 mM) on the velocity of the enzymatic reaction for $C(\alpha\text{-amylase}) = 200 \mu\text{g/mL}$ and $C(\text{G3-CNP}) = 5 \text{ mg/mL}$

While 1 and 2 mM maltose appears to have a marginal effect on reaction velocity, addition of 5 mM maltose drastically reduces the product formation (Figure 5.3.2). Although the kinetic curves for reaction between α -amylase and substrate with 1 mM or 2 mM maltose almost coincide, better visual perception of colorimetric responses and higher linearity within the physiological range was observed with the inhibitor concentration of 2 mM (Fig. 5.3.3). This observation is in agreement with the colorimetric response and RGB analysis (Appendix A).

Figure 5.3.3 Absorbance curve of released dye as a function of amount of α -amylase ($C(\alpha\text{-amylase})=5\text{-}500\text{ }\mu\text{g/mL}$) with 2 mM maltose as non-competitive inhibitor, concentration of substrate $C(\text{G3-CNP})=5\text{ mg/mL}$. The curve is recorded after a reaction time of 3 min. The samples for were prepared for $n=3$

The best linearity for the system is observed for 2 mM maltose after a reaction time of 3 min, with the linear fit equation: $A = 0.03 \pm 0.02 + (7.43 \pm 0.08) \cdot 10^{-3} \cdot C(\alpha\text{-amylase})$ (Figure 5.3.3). The LOD is evaluated as $8\text{ }\mu\text{g/mL}$ based on 3-fold standard deviation ($n=3$) of the mean value divided by a slope in the linear fit equation and is lower than natural variation of α -amylase levels in saliva. It is important to stress that the adopted approach enables to expand the dynamic range of α -amylase assay and to obtain a linear calibration curve (Figure 5.3.3), which was not feasible in the absence of a non-competitive inhibitor (Figure 5.3.1). Moreover, visual inspection of the corresponding solutions confirms the importance of using the maltose inhibitor to maintain linearity over a broader dynamic range (Figure 5.3.4).

Figure 5.3.4 Colorimetric response of enzymatic reaction between 5 mg/ml G3-CNP and α -amylase (20, 50, 75, 100, 200, 300, 400 and 500 $\mu\text{g/mL}$, from left to right) without maltose (a) and with 2 mM maltose inhibitor (b). The images are recorded after a reaction time of 3 min

Figure 5.3.4 (a) demonstrates that in the absence of maltose it is difficult to differentiate the color intensities at low concentrations and they are almost indistinguishable at higher concentrations of α -amylase ($>100 \mu\text{g/mL}$). On the contrary, color changes upon addition of different concentrations of α -amylase within the physiological range could be readily distinguished by naked eye using 2 mM maltose as inhibitor, Figure 5.3.4 (b). Thus, the use of maltose as non-competitive inhibitor undoubtedly provides an avenue to improve the linearity over broad dynamic range and to obtain a competitive LOD of 8 $\mu\text{g/mL}$ for α -amylase.

With the view to enable utilization of developed test onsite, the assay requires to be transferred to a platform, and paper was taken as an initial choice due to its accessibility, ease of use and modification and low cost.

5.4 Development of colorimetric paper-based test

Based on the promising spectrophotometric and colorimetric responses obtained in homogeneous solutions, the assay was then transferred to a paper membrane with an aim to establish a platform for onsite analysis of saliva samples. Again, a series of experiments to establish the most favorable assay conditions for naked eye estimation of α -amylase concentrations was carried out. This optimization was necessary as the exact concentration of inhibitor might vary when applied to paper membranes because of unknown sorption phenomenon, if any. The concentrations of the G3-CNP and maltose were 5 and 20 mg/mL,

and, 0, 2 and 5 mM, respectively. Images were captured after 3 min and 15 min of reaction time for further analysis. The results are shown in Figure 5.4.1.

As observed from Figure 5.4.1, the difference in colorimetric responses recorded after 3 min of reaction time could not be clearly distinguished by naked eye although most linear responses are recorded in aqueous solutions under similar test conditions. This is attributed to the difference in velocities of enzymatic reactions in homogeneous solutions and on paper membranes. Paper membranes may restrict the reaction rate by lowering degrees of freedom and/or by steric hindrance caused by the three dimensional morphology of the paper.

Figure 5.4.1 Colorimetric response of paper membranes with concentration of G3-CNP a) 5 mg/mL; or b) 20 mg/mL, maltose 0, 2 or 5 mM and with reaction time of 3 or 15 min to variation of α -amylase concentrations (20, 50, 75, 100, 200, 300, 400 and 500 $\mu\text{g/mL}$, from left to right)

However, colorimetric responses recorded after 15 min of reaction time appear to be more applicable for the assay as significant differences in color intensities are observed. Figure 5.4.1 illustrates that the systems comprising of 5 mg/mL G3-CNP and 5 mM maltose, as well as 20 mg/mL G3-CNP and 2 mM maltose possess distinct and gradual changes in colors, enabling semi-quantitative detection of α -amylase at physiological ranges. It is also evident from Figure 5.4.1 that the use of maltose inhibitor is crucial for obtaining a gradual color change over the entire dynamic range.

It is important to note that the visual estimation of color is highly susceptible to light intensity. Illumination plays an important role in accuracy of visual estimation of paper membrane color. In order to investigate optimal lighting conditions for assay, a series of test membranes with deposited 20 mg/mL of G3-CNP and 2 mM maltose and increasing concentration of α -amylase was prepared. The images were taken at three different illuminations (540, 110 and 42 lum) after 15 min of reaction (Table 5.4.1).

Table 5.4.1 Colorimetric response of test membranes with gradually increasing α -amylase concentration from left to right from 20, 50, 75, 100, 200, 300, 400 to 500 μ g/mL, respectively, for different illumination

	C (α -amylase), μ g/mL							
	20	50	75	100	200	300	400	500
Light intensity, lum								
540
110
42

As observed from Table 5.4.1, the visual perception of difference in obtained color intensities with respect to varying α -amylase concentration drops with the decrease of light intensity. Therefore, utilization of test paper membranes in normal lighting conditions (in the range of 500 lum) is recommended. Experiments to amend the calibration curves with respect to the ambient light is a part of future work.

5.5 RGB analysis of colorimetric response

In order to objectively select the most appropriate test strip for calibration purposes a detailed RGB and Delta-E analysis was performed. The Delta-E (ΔE) parameter (calculated according to CIE76 algorithm [9]) for the two assay conditions was calculated (Fig.5.5.1). A ΔE value of 2 is normally regarded as a cut-off indicator above which the human vision

system is capable of differentiating two colors [10]. Therefore, larger differences in ΔE values enable more accurate estimation of α -amylase concentrations.

Figure 5.5.1 demonstrates a larger difference in ΔE values for the sample containing 20 mg/mL G3-CNP with 2 mM maltose, as compared to the sample with 5 mg/mL G3-CNP and 5 mM maltose. Specifically, the ΔE values for the assay with 5 mg/mL G3-CNP and 5 mM maltose varies between 5.4–6.9 in the concentration range from 20 to 100 $\mu\text{g/mL}$.

Figure 5.5.1 Estimation of expected color difference of paper membranes for visual α -amylase detection assays that include 5 mg/mL G3-CNP with 5 mM maltose (white box) or 20 mg/mL G3-CNP with 2 mM maltose (black box) with reaction time 15 min

Although being sufficient for the human eye to distinguish the difference between colors, the assay with 20 mg/mL G3-CNP and 2 mM maltose provides more distinctive changes in ΔE values (4.6–14.3) within the same concentration range. The difference in the ΔE -values between the two membrane preparations remain the same for concentrations above 200 $\mu\text{g/mL}$.

RGB analysis also was utilized for evaluation of the system with best linearity, which could be used for quantitative estimation of α -amylase in saliva samples. In order to identify color components responsive towards change of α -amylase concentration, the histogram of distribution of values of color components for each membrane was calculated with the results shown in Figure 5.5.2.

Figure 5.5.2 Distribution of RGB values of standard membranes within the dynamic range of α -amylase (20-500 $\mu\text{g/mL}$). Red curves correspond to the value of red component, green curves – green component, blue curves – blue component. Each concentration point refers to 3 curves – red, green and blue

The histogram in Fig. 5.5.2 is characterized by uneven distribution of colors that can be explained by heterogeneity of color development on a membrane. It can be clearly seen that red and green components demonstrate marginal variation in response to concentration change, whereas blue component deviates significantly. In order to account for the simultaneous shift in color components' value, the ratios of colors were tested to fulfil this requirement. The R/G ratio demonstrates insignificant change with increasing α -amylase concentration in contrast to the R/B and B/G ratios. Ratio B/G is chosen for analysis due to its distinctive dependence on concentration of α -amylase. The ratio B/G is then calculated for images of membranes with G3-CNP concentrations of 5 and 20 mg/mL , and with 0, 2 and 5 mM maltose, after 3 and 15 min of reaction time. The results are shown in Figure 5.5.3.

Figure 5.5.3 Analysis of B/G ratio as a variable of colorimetric response of evaluated assays that consist of (a) 5 mg/mL and (b) 20 mg/mL substrate and inhibitor to different concentrations of α -amylase

Although the dynamic range of every studied system lies within natural distribution of salivary α -amylase, the optimized assay system should possess a viable combination of three parameters (besides linearity): low standard deviation, low LOD and distinct differences in B/G values obtained from RGB analysis.

Figure 5.5.3 illustrates that the most linear responses are obtained for the strips prepared using 5 mg/mL of G3-CNP with 5 mM maltose, with reaction time 15 min (Figure 5.5.3 (a)) and for 20 mg/mL of G3-CNP with 2 mM maltose after 15 min (Figure 5.5.3 (b)) within dynamic range 20 – 500 $\mu\text{g/mL}$ of α -amylase.

Moreover, the assays based on the above experimental conditions provide the lowest standard deviation for $n=3$ and yield the lowest LOD, calculated based on 3-fold standard deviation ($n=3$) of the mean value divided by a slope in the linear fit equation, as 10 and 11 $\mu\text{g/mL}$, respectively. These LODs are substantially lower as compared to those obtained for experiments with the same amount of substrate but without maltose (60 and 35 $\mu\text{g/mL}$, respectively, calculated from the black curves in Fig. 5.5.3 (a and b)). Based on the analysis of B/G ratio, these two systems appear to satisfy all three parameters and therefore suitable for semi-quantitative estimation of α -amylase in buffer.

Having investigated all assays that consisted of different amount of G3-CNP, maltose and with variable reaction time, it was established that 20 mg/mL G3-CNP and 2 mM maltose as product inhibitor fulfills requirements applied to the optimization of an assay: low standard deviation, low LOD and distinct differences in certain colors obtained from RGB analysis, as compared to other systems. RGB analysis of all systems confirms visual observation that enables utilization of developed assay for visual estimation of α -amylase in a saliva sample (Fig. 5.4.1 (b), 5.5.1 and 5.5.3 (b)).

Optimized assay enables visual semi-quantitative estimation of α -amylase amount in saliva that holds the promise to be successfully implemented as a test strip for onsite testing due to its fast response and absence of sophisticated reagents or equipment.

5.6 Semi-quantitative detection of α -amylase in saliva samples

Saliva samples are known for their matrix complexity, where different constituents could interfere with the detection of target analyte. However, enzymatic reactions are characterized by high specificity of substrate recognition that enables assaying without extensive sample cleanup and pretreatment. Phadebas test was utilized for benchmarking of the results obtained by semi-quantitative estimation of α -amylase concentration in saliva. For validation of the proposed methodology saliva samples that span the entire physiological range were selected from a study involving a group of volunteers. More information on volunteers is provided in Chapter 3, section 3.2.5.

Colorimetric responses obtained for saliva samples from the volunteers are then assessed using the optimized assay condition with 20 mg/mL G3-CNP and 2 mM maltose with a reaction time of 15 min (Fig. 5.4.1 (b)). The Phadebas test was carried out concurrently for validation of obtained colorimetric responses. The results are shown in Table 5.6.1.

As illustrated in Table 5.6.1 (column 4), the concentration of α -amylase estimated by the paper based test correlates well with responses obtained using the Phadebas test (employed in homogeneous solution), ascertaining that visual estimation of α -amylase concentration

is feasible for rapid screening. Moreover, it is evident from the Table 5.6.1, column 3, that variations in visual responses among 3 membranes are not significant. It is to be noted, however, that semi-quantitative estimation implies lower resolution of the assay.

Table 5.6.1 α -Amylase concentration in human saliva collected from volunteers, n=3 (different membranes), arranged in increasing concentration as determined using the Phabebas test, visual read out and B/G ratio analysis of test strips

#	Concentration of α -amylase according to Phadebas test, $\mu\text{g/mL}^*$	Image of a colorimetric response from test strip	Concentration of α -amylase obtained by visual read out of the developed strip test, $\mu\text{g/mL}^{**}$	Concentration of α -amylase obtained by RGB analysis of the strip test, $\mu\text{g/mL}$
1	70 $\pm$ 1		75	73 $\pm$ 7
2	157 $\pm$ 1		150 ¹	166 $\pm$ 5
3	202 $\pm$ 1		200	192 $\pm$ 20
4	306 $\pm$ 1		300	297 $\pm$ 27
5	345 $\pm$ 1		350 ²	348 $\pm$ 17
6	407 $\pm$ 1		400	401 $\pm$ 1
7	429 $\pm$ 1		450 ³	443 $\pm$ 1

* Obtained by Phadebas test kit using benchtop UV-spectrometer

** Obtained visually using the calibration strip test in Figure 5.4.1 for 20 mg/ml G3-CNP and 2 mM maltose (15 min).

¹ Obtained colorimetric response was estimated to fall in between 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$

² Obtained colorimetric response was estimated to fall in between 300 $\mu\text{g/mL}$ and 400 $\mu\text{g/mL}$

³ Obtained colorimetric response was estimated to fall in between 400 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$

Concentrations of α -amylase can be evaluated only approximately because of variations in subjective judgment of coloration between users, differences in the illumination, etc. The variation in subjective judgement is also a limiting factor for inclusion of intermediate concentrations in the calibration strip (standard paper membranes) to improve the resolution of the assay. Nevertheless, the developed paper-based colorimetric assay provides a rapid, specific and reagents-free assessment of α -amylase concentration in saliva, which facilitates applications in field, without requiring trained personnel and sophisticated instrumentation.

5.7 Quantitative detection of α -amylase in saliva samples

In order to quantitatively evaluate the obtained colorimetric responses, RGB analysis of paper membranes with saliva samples was carried out. Ratio B/G was calculated for all the membranes with saliva samples from volunteers and the concentrations of α -amylase were then estimated according to the linear equation ($B/G = 0.962 \pm 0.004 - (109 \pm 2) \cdot 10^{-5} \cdot C$ (α -amylase)) obtained for assay with 20 mg/mL and 2 mM maltose (Figure 5.5.3 (b), purple curve). Table 5.6.1 (column 5) reveals good correlation between the concentrations of α -amylase obtained by the RGB analysis and by Phadebas test with an accuracy of >95% and an average precision of 5.4%. Within the assay's linear range (20-500 μ g/mL), there was close agreement between semi-quantitative estimation of α -amylase levels in saliva based on visual estimation of color intensities of paper membranes, quantitative estimation of α -amylase based on RGB analysis of colored membranes and instrumental analysis performed with the help of Phadebas kit taken as a reference. The correlation among three approaches to estimate α -amylase concentration is demonstrated in Figure 5.7.1.

As it can be seen from Fig. 5.7.1, results obtained by visual estimation and by RGB analysis demonstrate excellent correlation ($R^2 = 0.98-0.99$) with the measurements obtained from the laboratory-based Phadebas kit. The slope of the fitted linear regression model is very close to best fit line for the measured standards.

Figure 5.7.1 Correlation among the results obtained from quantitative estimation of α -amylase level (by RGB analysis) and from semi-quantitative visual estimation versus Phadebas as the gold standard for detection of α -amylase

The standard deviation of α -amylase amount estimated by Phadebas test (used as gold standard) for three measurements of each concentration varies from 0.2 to 1.4% (with average 0.5%). The standard deviation for three measurements at each concentration in the dynamic range for developed assay varies from 0.2% to 10.4% (with average 5.4%). Thus, our method displays larger variation of α -amylase concentration but it still provides sufficient precision. Accuracy of the developed assay is higher than 95% across the concentration range. The results are shown in Table 5.6.1.

The LOD of Phadebas test (2.5 $\mu\text{g/L}$, as calculated according to the specifications provided in the test kit) is much lower than that of a developed assay (11 $\mu\text{g/mL}$). However, it is necessary to emphasize that detection of high concentrations of α -amylase within physiological range of saliva required an additional dilution step.

5.8 Interference of saliva components

Enzymatic reactions are highly specific in substrate recognition, thus, they are preferable for detection of analytes in complex matrices, if possible. α -Amylase is specific towards polysaccharides that contain α -bonds. α -Amylase is the only enzyme produced by salivary glands that can recognize polysaccharides with α -bond as a substrate. Human saliva is composed mainly of water (94–99%), however an important fraction of saliva is made up

of proteins (especially glycoproteins) and lipids. Saliva is also rich in carbohydrates, salts and contains non-protein nitrogen (urea, uric acid, amino acids and creatinine). Besides the excretions of salivary glands, saliva includes gingival fluid, serum components, bacteria and bacterial metabolites, exfoliated epithelial cells and leukocytes. Saliva derived from parotid glands (26% of total salivary volume) contains α -amylase and other digestive enzymes. Saliva derived from submandibular glands (69% of total salivary volume) contains glycoproteins. Macromolecular glycoproteins (mucins) make up about 26% of salivary proteins. Salivary mucins play a significant role in the homeostasis of the oral cavity by creating an acquired membrane, covering the soft and hard tissues lining the oral cavity. Oligosaccharide chains of salivary mucins are built by individual sugar molecules linked to each other O-glycosidically. The majority of oligosaccharide chains of human salivary mucins are O-glycosidically attached to polypeptide core. O-glycosidic linkages of glycoconjugates (glycoproteins, proteoglycan and glycolipids) are hydrolyzed by lysosomal exoglycosidases [11-16].

Interference also might come from the traces of food high in polysaccharides left in the mouth. It is to be noted that, in a typical assay setting, saliva samples are collected after rinsing the mouth to remove any food traces. It is to be noted that cross-reactivity from other salivary enzymes have not been observed and reported (refer to the list of salivary enzymes in Table 5.8.1).

Table 5.8.1 Salivary enzymes, their role and substrates

Enzyme	Role	Substrate
α-Amylase	α -Amylase initiates the digestion of long chains of sugars found in bread and crackers known as starches into smaller maltose sugar molecules	Polysaccharides with α -bonds.
Lysozyme	Lysozyme breaks down the polysaccharides in the cell walls of many bacteria.	1,4- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell wall.

Lingual lipase	Breaks down fats and is specifically associated with triacylglycerols such as the ones found in milk.	Fatty acids
Salivary kallikrein	Protease, breaks down some proteins into bradykinin	High molecular weight proteins
Lactoferrin	Each lactoferrin molecule can reversibly bind two ions of iron, zinc, copper or other metals. Lactoferrin's primary role is to sequester free iron, and in doing so remove essential substrate required for bacterial growth	Lactoferrin binds to lipopolysaccharide of bacterial walls, and the oxidized iron part of the lactoferrin oxidizes bacteria via formation of peroxides.
Lysosomal exoglycosidases	Hydrolyze mucins - macromolecular glycoproteins make up about 26% of salivary proteins. Oligosaccharide chains of salivary mucins are built by individual sugar molecules linked to each other O-glycosidically. The majority of oligosaccharide chains of human salivary mucins are O-glycosidically attached to polypeptide core.	β -anomerically linked N-acetyl-D-hexosamines, β -anomerically linked galactose, α - or β -anomerically linked mannoses, α -anomerically linked fucose and β -anomerically linked glucuronic acid in glycosaminoglycans

Developed assay was additionally tested for possible interferences from salivary components by performing a series of experiments. The experiments were designed to study the effect of varying salivary components that differ between volunteers and the interferences from salivary components that could potentially hydrolyze the α -bonds of the polysaccharide substrate.

It is to be emphasized that the amount of salivary components varies from volunteer to volunteer. If α -amylase experienced any kind of interference (sterical blocking of active sites, inhibition/activation) by other salivary components, estimation of its amount in buffer would differ significantly compared to the assay in saliva samples. To address this point, buffer solutions with varying concentrations of α -amylase that match the levels obtained in the saliva samples from 7 volunteers as estimated using Phadebas test (Table 5.6.1) were prepared. The colorimetric response was assessed visually (semi-quantitatively) and by RGB analysis. The results of the experiment are shown in Table 5.8.2.

Table 5.8.2 α -amylase concentration in MES buffer versus concentration in saliva samples from 7 volunteers, n=3 (different membranes), as determined using visual read out and B/G ratio analysis of test strip

#	Concentration of α -amylase in buffer, $\mu\text{g/mL}$	Image of a colorimetric response from strip test, in buffer	Image of a colorimetric response from strip test, in saliva	Concentration of α -amylase obtained by visual read out of the strip test in buffer (results in saliva), $\mu\text{g/mL}$	Concentration of α -amylase obtained by RGB analysis of the strip test in buffer (results in saliva), $\mu\text{g/mL}$
1	70			75 (75)	80±7 (73±7)
2	157			150 ¹ (150 ¹)	164±6 (166±5)
3	202			200 (200)	216±5 (192±20)
4	306			300 (300)	308±6 (297±27)
5	345			350 ² (350 ²)	340±4 (348±17)
6	407			400 (400)	404±11 (401±1)
7	429			450 ³ (450 ³)	437±15 (443±1)

¹ Obtained colorimetric response was estimated to fall in between 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$

² Obtained colorimetric response was estimated to fall in between 300 $\mu\text{g/mL}$ and 400 $\mu\text{g/mL}$

³ Obtained colorimetric response was estimated to fall in between 400 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$

There is a slight difference observed in coloration of membranes especially exposed to higher concentrations of α -amylase that can be associated with a marginal effect of salivary components that vary within each volunteer. Nevertheless, RGB analysis of the membranes provides sufficient correlation of α -amylase concentrations obtained in saliva and buffer.

Potential interference from salivary components can also be studied by maintaining the concentration of salivary components (in order to preserve the interferences, if any) and by gradually increasing the concentration of α -amylase by spiking saliva sample with standard

α -amylase solution. In case of positive interference from salivary components on α -amylase activity, the assay would yield erroneous estimation of α -amylase levels. Saliva collected in the morning from one volunteer had 81 $\mu\text{g/mL}$ as determined by Phadebas. It was spiked with 5 μL of increasing concentration of α -amylase in order to obtain its final concentration of 100, 200, 300 and 400 $\mu\text{g/mL}$. The final concentrations were chosen as mentioned in accordance to the concentration steps prepared for standard membrane. The results of visual and RGB analysis as well as of Phadebas test are given in Table 5.8.3.

Table 5.8.3 α -Amylase concentration in whole saliva (1) and saliva with 5 μL aliquot of α -amylase (2-5), $n=3$ (different membranes), as determined using Phadebas test kit, visual read out and B/G ratio analysis of test strips

#	Final concentration of α -amylase in sample, $\mu\text{g/mL}$	Image of a colorimetric response from strip test			Concentration of α -amylase according to Phadebas test, $\mu\text{g/mL}$	Concentration of α -amylase obtained by RGB analysis of the strip test, $\mu\text{g/mL}$	Concentration of α -amylase obtained by visual read out of the strip test, $\mu\text{g/mL}$
1	81				81 $\pm$ 1	84 $\pm$ 1	75
2	100				102 $\pm$ 1	97 $\pm$ 2	100
3	200				199 $\pm$ 2	202 $\pm$ 8	200
4	300				302 $\pm$ 2	305 $\pm$ 6	300
5	400				406 $\pm$ 1	406 $\pm$ 9	400

As shown in Table 5.8.3, the concentrations of α -amylase estimated by the proposed assay are in agreement with the α -amylase concentration of spiked saliva samples, further indicating marginal interferences from salivary components on α -amylase activity.

Yet another experiment was performed to address the effect of salivary enzymes on the substrate used in the developed assay. Saliva collected from one volunteer in the morning contained 103 $\mu\text{g/mL}$ of α -amylase (as determined by Phadebas test). Saliva was then divided into 3 parts (whole saliva, saliva diluted by MES buffer 2x times and 5x times). Dilution of saliva aimed to decrease the effect of interfering substances. 5 μL of different

concentrations of α -amylase were added to whole and diluted saliva to achieve concentrations of α -amylase as 103 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$. Simultaneous increase of α -amylase concentration in the sample in the presence of reduced amount of interfering components could lead to different coloration of membrane as compared to standard membrane test. The results of α -amylase estimation obtained by Phadebas test, visual estimation and RGB analysis are demonstrated in Table 5.8.4.

Table 5.8.4 α -Amylase concentration in whole saliva (1), saliva diluted 2 times (2) and 5 times (3), with addition of aliquots of saliva, $n=3$ (different membranes), as determined using Phadebas test kit, visual read out and B/G ratio analysis of test strips

#	Image of a colorimetric response from strip test			Concentration of α -amylase according to Phadebas test, $\mu\text{g/mL}$	Concentration of α -amylase obtained by RGB analysis of the strip test, $\mu\text{g/mL}$	Concentration of α -amylase obtained by visual read out of the developed strip test, $\mu\text{g/mL}$
1-1				103 $\pm$ 1	105 $\pm$ 15	100
2-1				55 $\pm$ 1	62 $\pm$ 10	50
3-1				21 $\pm$ 1	27 $\pm$ 5	20
2-2				102 $\pm$ 1	93 $\pm$ 11	100
3-2				99 $\pm$ 2	100 $\pm$ 3	100
1-2				204 $\pm$ 2	211 $\pm$ 14	200
2-3				204 $\pm$ 2	208 $\pm$ 16	200
3-3				208 $\pm$ 3	217 $\pm$ 15	200

By comparing results of standard addition method to achieve concentration 200 $\mu\text{g/mL}$ obtained for whole saliva (1-2) and the most diluted saliva (3-3) by Phadebas test, RGB

analysis and visual estimation, it can be concluded that no interference is observed from salivary enzymes that could potentially utilize substrate used in the developed assay or inhibit α -amylase activity. Results in Table 5.8.4 indicate very marginal or no interference from salivary components on α -amylase activity as there is a good correlation between concentrations estimated by the proposed assay and the α -amylase concentration of spiked saliva samples.

Moreover, activity of α -amylase strongly depends on the presence of Ca^{2+} and Cl^- ions that maintain enzyme's structure to facilitate glycolysis of α -bonds. Decrease in α -amylase activity might affect proper representation of concentration. Any Ca^{2+} binding constituents or Cl^- consuming reactions might affect the estimation of α -amylase representation in saliva. For example, it is known that Ca^{2+} binds to Protein A in amount 664 nmol of Ca^{2+} /mg of protein [17]. Given very low concentration of phosphoprotein and high amount of Ca^{2+} in saliva 1.3-1.7 mM/L [18], it is unlikely to expect loss of α -amylase activity due to Ca^{2+} removal. Parathyroid hormone is also known to regulate Ca^{2+} . So any chronic health condition that affects formation of parathyroid hormone can potentially alter Ca^{2+} regulation. Altered activity of proline-rich proteins and statherin is known to increase precipitation of Ca^{2+} on teeth which in turn can affect α -amylase activity by creating lack of Ca^{2+} ions in the environment. However, it is challenging to study the effect of complex nature of Ca^{2+} and Cl^- regulation due to many physiological processes involved. Nevertheless, the results provided in Tables 5.8.2-5.8.4 demonstrate that salivary components do not have a pronounced effect on the α -amylase activity.

5.9 Storage stability of membranes and saliva

Different concentrations of α -amylase (20, 100, 400 $\mu\text{g/mL}$) were added to membranes and stored in dark for 1 (freshly prepared), 42 and 70 days at room temperature. Storage stability of prepared assay membranes was verified by comparing the colorimetric responses of membranes stored for 42 and 70 days with that of responses obtained using freshly prepared membranes. The results are demonstrated in Fig. 5.9.1.

Figure 5.9.1 RGB analysis of developed assay membranes stored for 1, 42 and 70 days to different concentrations of α -amylase showing stable B/G ratio, indicating good storage stability for up to 70 days (a). Images of membranes showing minimum variations between colorimetric responses of freshly prepared membranes and membranes stored for 42/70 days upon exposure to various concentrations of α -amylase (20, 100 and 400 $\mu\text{g/mL}$), further confirming the storage stability (b)

The average percentage drift, quantified by difference between B/G ratio obtained for 42/70 days and for 1st day, divided by B/G ratio obtained for 1st day, was 0 and 0.4%, 0.1 and 1.6%, 3.4 and 3.6% for concentrations of α -amylase equal to 20, 100 and 400 $\mu\text{g/mL}$, respectively (Figure 5.9.1 (a)). No significant variation in coloration of the membranes stored up to 70 days upon exposure to different concentrations of α -amylase (Figure 5.9.1 (b)) illustrates that the membranes are stable to perform visual and RGB analysis up to 70 days. Storage tests beyond 70 days is a subject for further studies.

Storing saliva in room temperature for a prolonged time can affect α -amylase activity. Whole saliva contains bacteria, debris and various substances that can influence α -amylase activity [19]. However, no particular increase/decrease in activity has been reported in the literature upon storage of saliva at various temperatures. We have attempted to test the activity of α -amylase in a saliva sample that was stored for different periods of time at room temperature. Saliva sample from a volunteer was analyzed immediately, after 30 min, 1 h, 5 h and 12 h after collection with no prior pre-treatment. The results are shown in Table 5.9.1 and Figure 5.9.2.

Table 5.9.1 α -Amylase concentration in human saliva collected from a volunteer, n=3 (different membranes), as determined using the Phabebas test, visual and B/G ratio analysis of test strips after storing at room temperature

Sample	Time of storage, h	Image of a colorimetric response from strip test	Concentration of α -amylase according to Phadebas test, $\mu\text{g/mL}$	Concentration of α -amylase obtained by RGB analysis of the strip test, $\mu\text{g/mL}$	Concentration of α -amylase obtained by visual read out of the developed strip test, $\mu\text{g/mL}$
1	0		186 ± 1	172 ± 1	200
2	0.5		187 ± 1	179 ± 2	200
3	1		212 ± 2	182 ± 1	200
4	5		168 ± 1	156 ± 27	150
5	12		112 ± 3	96 ± 12	100

As it can be seen, saliva is exposed to various unknown factors that affect its activity during prolonged periods of time. Activity of α -amylase does not vary significantly if the analysis is performed within 1 h (Figure 5.9.2).

Figure 5.9.2 Variation of α -amylase amount as a function of storage time of whole saliva at room temperature

5.10 Effect of temperature on α -amylase detection in saliva sample

It has been reported that the activity of human salivary α -amylase is not greatly affected by changes in temperature within 20-37 °C, but that the activity drops significantly upon incubation for prolonged time at temperatures higher than 40 °C [20, 21]. In order to verify this hypothesis, an experiment for the detection of α -amylase at room temperature 24 °C and at 31 °C was performed. The membranes were kept at mentioned temperatures 15 min before the start of experiment and during the assaying (15 min). The results are shown in Table 5.10.1.

Table 5.10.1 The effect of temperature on the measurement of α -amylase in saliva sample

Temperature, °C	Image of a colorimetric response from strip test			Concentration of α -amylase according to Phadebas test, µg/mL	Concentration of α -amylase obtained by RGB analysis of the strip test, µg/mL	Concentration of α -amylase obtained by visual read out of the strip test, µg/mL
24				248±1	239±9	250
31				254±2	231±9	250

No significant variation in visual and RGB responses is observed, indicating that the developed assay could be utilized for measuring α -amylase levels at temperature range 24-31°C. Therefore, the developed assay could be utilized for onsite estimation of α -amylase levels, for instance at users' homes or training sites, at typical room temperature conditions (around 24 °C). However, if the developed assay is utilized in adverse weather conditions, then a calibration may be required, which will be investigated in our future studies.

5.11 Conclusions

Having investigated the catalytic behavior of α -amylase, the effect of a non-competitive inhibitor that was introduced into the proposed assay in order to extend the linearity for assay in physiological range was evaluated. The analysis of colorimetric response to

variation in concentration of substrate and inhibitor as well as with time was conducted, which enabled optimization of α -amylase assay using membranes. Benchmarking was performed with state of art Phadebas test that requires benchtop spectrophotometer to determine actual amount of α -amylase in saliva. The results of visual (semi-quantitative) and RGB-based (quantitative) estimation of level in saliva samples demonstrated good correlation and repeatability with respect to Phadebas test. Developed assay demonstrated LODs and dynamic range that is sufficient for routine analysis of α -amylase in saliva. Thus, the developed paper membrane can be used onsite in field settings or at home without requiring sophisticated instrumentation and trained personnel for analysis. Moreover, the designed enzymatic assay is characterized by negligible interference from salivary components. It was demonstrated that salivary components do not inhibit the activity of α -amylase and do not consume the substrate which furnishes the strip test by high specificity of analysis. It was also affirmed that elaborated strip tests remain stable for more than 2 months and α -amylase in saliva preserves its activity at room temperature for 1 h.

It is at this stage important to emphasize that conclusions on the effect of cognitive or physical activities on salivary α -amylase levels are not being done intentionally. However, the observations herein provide an opportunity to assess the response of α -amylase level to different stressors and potentially estimate and predict onset of fatigue using a simple and fieldable methodology. Future work will emphasize on evaluation of α -amylase response to ANS activity caused by external factors for assessment of fatigue.

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Chapter 6

Lateral Flow Displacement Assay for Detection of Cortisol in Saliva

This chapter describes the development of a MIP@paper based lateral flow displacement assay for detection of cortisol, one of the biomarkers of fatigue, overtraining and a stress indicator. In the suggested displacement approach, MIP layer with cavities complementary in shape and chemical properties to cortisol molecule is synthesized on cellulose paper. The cavities are then saturated with cortisol-21-hemisuccinate (cortisol-21-HS) conjugated to glucose oxidase (GOx). Displacement is based on a higher specificity of the cavities towards cortisol than cortisol-21-HS. Conjugate, when displaced, catalyzes oxidation of β -glucose impregnated on glass fiber with the release of H_2O_2 . Upon the capillary flow, peroxidase (POx) is released, which catalyzes the oxidation of impregnated o-dianisidine dye by H_2O_2 . The amount of oxidized dye is proportional to the concentration of cortisol. Various materials for membranes were tested in order to ensure stable capillary flow along the test strip, release of impregnated reagents and reproducible development of a color in the detection pad. Introduction of 0.3 mM H_2O_2 to the detection pad as a signal amplifier enabled expansion of dynamic range of cortisol detection (within 0.1-20 ng/mL) with a limit of detection 0.3 ng/mL. Subsequently, paper-based assay was used for test of human saliva samples. The proposed assay was validated by HPLC/MS. The precision and accuracy of the assay were then tested, which indicated the potential of the proposed approach for onsite applications.

6.1 Background information about cortisol

One of the main mediators to stress development is hypothalamic–pituitary–adrenal (HPA) axis that maintains adaptive to external factors and regulates release of cortisol – a steroid hormone that plays an important role in stimulating immune system to prevent inflammation. Cortisol secretion also increases markedly in response to stress, whether physical (illness, trauma, surgery, fever, physical exercise and extreme temperatures) [1] or psychological [2-5]. Alterations in HPA axis regulation in comparison to healthy populations have been documented for various conditions like panic disorder [2], depression [3] and posttraumatic stress disorder [4].

Moreover, as it was mentioned previously, cortisol demonstrates particular behavior during training that provides an avenue for investigation of overtraining syndrome development [6]. Screening of cortisol amount during training season is widely utilized by sports doctors to predict the stress level in sportsmen [7, 8]. It is recognized that when sportsmen follow a well-balanced exercise program, cortisol produced after each workout is removed from the body within 24 h. Failure to return to initial baseline can be the evidence of stress accumulated during the season [9]. Moreover, prolonged elevation of cortisol levels can cause adverse negative effects such as hippocampal atrophy and cognitive impairment [10] followed by brain aging [11], osteoporosis [12] or immune functional disease [13].

Thus, constant observation of cortisol response to internal and external factors can provide a picture of physiological conditions. It also can be used as a tool to predict development of stress and devise actions to prevent it.

Changes in the level of cortisol as a response to physical exercises were proved to be more pronounced in saliva than in blood [14, 15]. Foremost, its amount correlates well with blood cortisol and varies from 0.5 to 20 ng/mL [16, 17]. Cortisol in saliva consists only of unbound, biologically active cortisol, which is not affected by salivary flow. Cortisol bound to corticosteroid-binding globulin and albumin is not released to the saliva [18]. Moreover, assessing of cortisol in saliva is characterized by non-invasiveness of analysis that aids to

avoid development of additional stress and increase in cortisol concentration representation. Cortisol molecule is relatively small, with molecular weight 363 Da. The structure of cortisol is shown in Fig. 6.1.1. Presence of cortisol homologues in saliva (testosterone, cortisone, dehydroepiandrosterone etc) complicates the specificity of assays significantly.

Figure 6.1.1 Molecular structure of cortisol

Recognition molecules that can be potentially used for visual detection of cortisol include aptamers, antibodies or MIPs. Current methods that are being used are mostly chromatography [19-22] and immunoassays based on surface plasmon resonance [23-32] that are time and labor consuming, costly and not ideal for field applications. However, high sensitivity and specificity of these methods provide accurate and robust detection of cortisol and the outcome from these investigations will be used for benchmarking purposes.

Available in market tests for onsite electrochemical detection of cortisol widely utilize antibodies as recognition entities and involve electrochemical molecular detection with gold nanowires coupled with cortisol Abs [33], GOx conjugated with cortisol molecule and attached to self-assembling monolayers [34], and modified interdigitated μ -electrodes [35]. Current assays for the visual detection of cortisol utilize anti-cortisol antibodies and labeled cortisol-glucose oxidase conjugates. For example, Yamaguchi [34] proposed a lateral flow device with immobilized anti-cortisol antibodies that is based on competitive replacement of cortisol conjugated with glucose oxidase and by the free cortisol molecule. Visual evaluation of color intensity was performed with bench top reflectance reader with a linear detection range of 1-10 ng/mL. However, no information about time of analysis, interference and application of the approach for assaying real samples was provided. Visual methods are not widespread for the detection of low amounts of cortisol given the slow and uneven development of color, low sensitivity and lack of specificity for analysis of

complex matrices. Moreover, visual assessment of cortisol amount usually requires utilization of special equipment for precise read out. Among all available recognition strategies, MIPs were chosen because of their high tunable affinity, robustness and low cost of manufacturing.

In this study, it is hypothesized that displacement of conjugate between GOx and cortisol-21-HS by cortisol from MIP cavities, complimentary in shape and in chemical properties to cortisol, occurs with sufficient reproducibility and specificity. Subsequent reaction of displaced conjugate with its substrate, β -glucose, results in production of H_2O_2 that is utilized by POx as a substrate to catalyze oxidation of o-dianisidine (Fig.6.1.2). In turn, the exceptional properties of o-dianisidine as a bifunctional dye [36] involve gradual change in color (from colorless through blue to red) based on its sensitivity to the amount of H_2O_2 . This color response can be utilized for detection of low amounts of displaced conjugate that commensurate with the amount of cortisol.

Figure 6.1.2 Structure of reduced and oxidized o-dianisidine

The approach to analyze the color pattern of the detection pad in the final lateral flow device is based on the estimation of ratio of prevailing color components rather than on the color intensity of produced dye. This methodology improves the robustness of the assay and enables detection of very low amounts of cortisol. Study on the functions and the effect of various membranes as separate parts of the assay results in optimized separation of components (interferents), reduced time of analysis and clear distribution of color in detection zone. A simplified depiction of the displacement assay is shown in Fig. 6.1.3.

Figure 6.1.3 Scheme of displacement assay for visual detection of cortisol. (a) Synthesis of MIP@paper followed by removal of cortisol and saturating of cavities by GOx-cortisol-21-HS; (b) Displacement of GOx-cortisol-21-HS by low amount of cortisol; (c) Displacement of GOx-cortisol-21-HS by high amount of cortisol

6.2 Characterization of MIP@paper

In order to achieve covalent binding of MIP to the cellulose fibers, they were initially activated by silanization using 3-(methacryloxypropyl)-trimethoxysilane. Introduction of double bonds on the surface of fibers aimed to create centers of polymerization. Figure 6.2.1 (a) demonstrates that fibrous structure of paper was not disrupted during silanization. Activated paper was subsequently immersed into polymerization mixture, during which the network of interconnected MIP particles (~100-400 nm) formed on the surface of fibers (Fig. 6.2.1 (b-c)), which was not removed from the surface after washing papers by methanol and acetic acid (AcA), indicating strong covalent binding of MIP particles to paper.

Figure 6.2.1 SEM images of paper activated by 3-(methacryloxypropyl)-trimethoxysilane (a) and of MIP@paper after removal of cortisol from MIP cavities (b and c), note different scale bars.

6.3 Selection of a solvent for MIP polymerization

Solvent plays a vital role in the MIP polymerization process. It controls the ability to properly dissolve constituents of polymerization mixture and should be chosen to ensure low interaction with the constituents. This favors formation of highly specific recognition cavities and reduces the risk non-specific interactions to the polymer itself. Based on the solubility of cortisol in various solvents, an ethanol/water mixture and DMF were chosen as polymerization solvents.

To test the ability of MIP cavities formed in different solvents to bind cortisol, MIP@paper (10 mm x 10 mm) was immersed into 0.5 mL of 0.5 g/L of cortisol and incubated for 5

min. MIP@paper was removed from the solution and leftover concentration of cortisol was determined by HPLC/MS. Subsequently, MIP@paper was immersed into the solution of 0.5 mL of water with the intention to remove non-specifically bound cortisol. The leftover concentration of cortisol removed by water was also determined by HPLC/MS. It enabled estimation of the amount of cortisol bound to MIP@paper through specific interactions that were not perturbed upon washing with water (Figure 6.3.1).

Figure 6.3.1 Degree of cortisol bound to MIP@paper that was synthesized in different solvents before (white box) and after washing by water (grey box). C (cort) = 0.5 g/L. Sample size = 10 mm x 10 mm

Rebinding of cortisol demonstrated that high amount of cortisol (80 and 90%) binds to the MIP synthesized in ethanol/water mixture and DMF, respectively (Fig. 6.3.1). However, washing by water removed around 10% and 80% of bound cortisol from MIP synthesized in ethanol/water mixture and DMF, respectively. High amount of removed cortisol in case of a MIP synthesized in DMF indicates poor recognition properties of the synthesized cavities or loose polymer network. At the same time, washing by water MIP@paper synthesized in ethanol/water mixture removes low amount of loosely bound cortisol (<10%), indicating that the rest of cortisol is retained in the MIP cavities via specific forces. In conclusion, MIP@paper synthesized in mixture of ethanol/water was used for subsequent experiments as it resulted in polymer characterized by low non-specific binding of cortisol.

6.4 Specificity of synthesized MIP@paper

Binding of cortisol to MIP@ paper occurs with involvement of various forces due to MIP structure and presence of charged/polar/apolar entities in the cavities and on the non-imprinted parts of the polymer surface. Thus, binding does not necessarily occurs to specifically designed cavities. The amount of cortisol that binds directly to the cavities of MIP@paper can be explored by washing with methanol and AcA and compared with an identical set of experiments undertaken on non-imprinted polymer NIP@paper and non-modified paper, respectively. In order to estimate the type of binding involved in retention of cortisol on the polymer surface, washing with methanol was chosen as it has lower dielectric constant than water (33 vs 80) and thus, facilitates disruption of van der Waals forces [37], and with acetic acid that breaks existing hydrogen bonds by changing the protonation state of polymer [38].

Again, MIP@paper was immersed into 0.5 mL of 0.5 g/L solution of cortisol, incubated for 5 min and subsequently exposed to the various rinsing solvents. As before, water was used to remove loosely bound cortisol. Washing with methanol attempted to decrease effectively the strength of van der Waals forces between the polymer and cortisol as reducing dielectric constant of the solvent results in partial reduction of the electric field of a charged particle. Addition of AcA aimed to disrupt existing hydrogen bonds between cortisol and polymer backbone and potentially to create new hydrogen bonds. A mixture of methanol and AcA (9:1) was utilized to weaken a broader spectrum of bonds including electrostatic and hydrogen bonds as well as van der Waals bonds inside MIP cavities. A combination of these bonds is presumably responsible for the affinity between cortisol and the cavity. The results from the rinsing experiments are shown in Figure 6.4.1.

Washing MIP@paper in 0.5 mL of water removes up to 10% of bound cortisol, illustrating low non-specific binding of cortisol to the MIP. This is in good correlation with the results obtained from Figure 6.3.1. Washing with methanol demonstrated negligible amount of removed cortisol that points towards low amount of cortisol retained only due to van der Waals forces. Immersing MIP@paper in 0.5 mL of AcA removed up to 10% of bound

cortisol indicating that binding is partly due to the involvement of hydrogen bonds. Washing MIP@paper with the mixture of methanol and AcA removed the rest of bound cortisol (>80%), which provides evidence for strong electrostatic binding of cortisol to the MIP@paper with the involvement of van der Waals interactions.

Figure 6.4.1 Degree of cortisol removed from MIP@paper after its rebinding by alternation of washing steps as determined by HPLC/MS. Each washing step was followed by water rinsing. C (cort) = 0.5 g/L. Sample size = 10 mm x 10 mm

These findings provide evidence for strong binding and specific recognition between the cavity and cortisol. Moreover, the results obtained enable calculation of overall loading capacity of MIP cavities per cm^2 , which can be considered as the amount of cortisol that was removed by mixture of methanol and AcA and equals to ~80% from loaded amount (250 μg) or 200 μg per cm^2 .

However, the question arises here if binding of cortisol exclusively occurs to the cavities or elsewhere to the polymer surface, or even to the supporting paper. To address this issue, the same series of experiments was performed using NIP@paper and non-modified paper. It is expected that cortisol is retained on these surfaces due to non-specific forces and it will be removed by washing with water. The results are shown in Figure 6.4.2.

Figure 6.4.2 Degree of cortisol removed from NIP@paper (a) and non-modified paper (b) by different washing steps as determined by HPLC/MS. Each washing step was followed by rinsing by water. C (cort) = 0.5 g/L, sample size = 10 mm x 10 mm

As it can be seen from Fig. 6.4.2, washing by water removes more than 60 and 80% of bound cortisol from NIP@paper and non-modified paper, respectively, which indicates that a large amount of cortisol is loosely bound to the surface of the polymer. Washing by methanol, followed by AcA reveals that binding of cortisol to NIP involves approximately equal and small amounts of van der Waals and hydrogen bonds (Fig. 6.4.2 (a)), whereas binding of cortisol to non-modified paper unlikely occurs due to involvement of these forces (Fig. 6.4.2 (b)). Notably, around 20% of cortisol appears to bind to NIP@paper via electrostatic/van der Waals forces (as is demonstrated after washing NIP@paper with a mixture of methanol and AcA) even though it does not have specific cavities. Finally, non-modified paper demonstrates negligible binding of cortisol as confirmed by washing it with methanol, AcA and their mixture.

6.5 Conjugation between GOx and cortisol-21-HS

Glucose oxidase (GOx) was chosen as an enzyme for conjugation due to its ability to catalyze transformation of β -glucose to D-glucono-1,5-lactone with H_2O_2 as a by-product which in turn, being catalyzed by POx, oxidizes the o-dianisidine dye to develop bluish/reddish colors. GOx, a dimeric protein with molecular weight 150 kDa, is an enzyme

that works outside cells. It contains two polypeptide chains (75 kDa each) held together via disulfide bonds (Fig. 6.5.1). Its essential component is a flavin adenine dinucleotide (FAD) that acts as electron acceptor to be reduced to FADH₂. In the presence of stronger acceptor, as molecular oxygen, FADH₂ is oxidized to FAD whereas oxygen is reduced to H₂O₂. As an extracellular enzyme, GOx is covered with carbohydrate chains. According to Swoboda et al. [39], carbohydrate chains contribute to approximately 16% of total GOx molecular weight and can serve as targets for chemical modification as it provides easily accessible spot for conjugation with small molecules.

Figure 6.5.1 Structure of dimeric GOx with carbohydrates on the surface [40]. The active site where glucose binds is in a deep pocket

As most of carbohydrates on the surface of GOx contain mannose, glucose and hexosamine [41], mannose was chosen as the target for conjugation. Oxidation of diols from mannose sugars was performed via Malaprade reaction [42, 43] that resulted in glycol cleavage in the mannose molecule and formation of an aldehyde, ensuring the possibility of reaction with amines to form Schiff's base that can react with activated carboxyl groups of cortisol homologues. Given the approach used for conjugation that aimed at full oxidation of diols to aldehydes and guaranteed excess of cortisol or its homologues in the reaction solution, it can be assumed that all mannose molecules formed a bond with cortisol or its homologues. The activity of the conjugate was checked and no alterations as compared to the activity of GOx was observed.

To the best of my understanding, it is virtually impossible to establish the exact stoichiometry of high weight molecule with small molecules attached as a resulting conjugate. MALDI/ToF of diluted conjugate solution demonstrated high signal-to-noise ratio that disabled accurate estimation of conjugate structure/composition. SDS-PAGE is not capable of capturing small differences in weight, however, it provided us with an interesting observation: a band of 150 kDa appears in GOx-cortisol-21-HS while it is absent in GOx. This points towards dimerization, most likely triggered by the exposure to oxidative agents during the harsh chemical conjugation of cortisol-21-HS to GOx (Fig. 6.5.2). The broad and blurred bands observed at 75 kDa (Fig. 6.5.2) can be associated with uneven distribution of SDS molecules at GOx due to the differential levels of its glycosylation that affected overall charge-to-mass ratios, as reported before by Jensen et al. [44].

Figure 6.5.2 SDS-PAGE of 20 $\mu\text{g/mL}$ GOx (column 1) and GOx-cortisol-21-HS conjugate (column 2) versus molecular-weight size marker (column 3)

The degree of conjugation of cortisol-21-HS to GOx can be addressed indirectly by exploring the specific interaction between cortisol and MIP cavities. If cortisol-21-HS has been successfully linked to GOx, MIP cavities are expected to fully bind with the conjugate. On the contrary, insufficient conjugation to GOx results in reduced binding to the surface. To test this assumption, QCM-D method that involved synthesis of MIP on the surface of crystal was elaborated [45-47].

SiO₂ has been chosen as the chip because of its resemblance to the chemical properties of cellulose (hydrophilic and exposure of silanol groups). The same polymerization process of MIP layer on the surface of SiO₂ chip was employed as for the synthesis of MIP@paper. After polymerization, the chip was washed 3 times by ethanol to remove any loose polymer. QCM measurement was started by establishing a baseline in acetate buffer (Figure 6.5.3 (I)). The mixture of methanol and AcA (9:1) was added in order to remove cortisol (II). The chip was subsequently washed with buffer (III) to remove solvent. A positive frequency shift observed after 1050 s (~35 Hz) implies that substantial amount of cortisol, including any loosely bound MIP particles, was removed at this stage. In order to test the ability of empty MIP cavities to bind cortisol, solution of 0.5 g/L cortisol was introduced to the chip (IV). However, no noticeable frequency shift was observed that points toward low amount of cortisol capable to occupy the cavities. Bound cortisol was removed from MIP@SiO₂ by washing it with mixture of methanol and AcA (9:1) (V), followed by buffer (VI). Noticeably, marginal frequency shift was observed at this stage indicating about low concentration of cortisol that was removed by this solvent and by MIP stability. In order to test the specificity of synthesized cavities towards conjugate, 0.1 g/L of GOx-cortisol-21-HS was added to the chip with MIP layer and the frequency change was recorded (VII). If GOx is not conjugated to cortisol-21-HS, then no specific binding to the cavities is expected. On the contrary, GOx-cortisol-21-HS would enhance specific binding of cortisol to the MIP cavities resulting in a significant frequency drop. As it can be seen From Fig. 6.5.3 (VII), a distinct frequency drop (~40 Hz) was observed upon addition of GOx-cortisol-21-HS to the MIP layer pointing towards high affinity of conjugate to cavities in MIP layer that confirms our prior observations. Increase in dissipation (up to $1 \cdot 10^{-6}$) is observed upon addition of GOx-cortisol-21-HS to the MIP layer indicating about flexibility of the polymer layer on the surface of SiO₂ chip. This phenomenon indicates about loose polymer network created during synthesis. Washing chip with buffer (VIII) did not result in removal of conjugate that indicates strong binding to MIP. These experiments reveal that GOx-cortisol-21-HS specifically binds to designed cavities in MIP@SiO₂. Furthermore, QCM is also a suitable tool to monitor displacement of GOx-cortisol-21-HS from cavities by cortisol. High amount of cortisol (0.5 g/L) was then added to the MIP@SiO₂ to facilitate complete removal of conjugate. Interestingly, upon addition of

cortisol a short lag is observed between 2400 and 2450 sec (IX). This phenomenon can be explained by the fact that cortisol molecules do not immediately replace conjugate but, instead, occupy cavities free from conjugate. Having saturated free cavities, cortisol molecules trigger the displacement of conjugates that causes a rapid (~ 40 Hz) increase in frequency. Meanwhile, displacement of conjugate by cortisol is characterized by drop in dissipation energy that additionally indicates about removal of bulky conjugates from loose MIP network and returning to initial rigid state.

Figure 6.5.3 Frequency shift and dissipation upon removal of cortisol from synthesized MIP@SiO₂, addition of GOx-cortisol-21-HS conjugate to MIP synthesized on surface of SiO₂ chip, and its replacement by cortisol molecules. I – washing by buffer; II – washing by mixture of methanol and AcA (9:1); III – washing by buffer; IV – addition of 0.5 g/L cortisol; V – washing by mixture of methanol and AcA (9:1); VI – washing by buffer; VII – addition of 0.1 g/L GOx-cortisol-21-HS; VIII – washing by buffer; IX – addition of 0.5 g/L solution of cortisol; X – washing by buffer

However, the frequency and dissipation shifts observed upon addition of conjugate to MIP@SiO₂ can be caused by binding of GOx to the MIP due to non-specific interaction. In order to address this issues, the same experiment was repeated with GOx (Figure 6.5.4).

Figure 6.5.4 Frequency shift and dissipation upon removal of cortisol from synthesized MIP@SiO₂ and addition of GOx to MIP synthesized on surface of SiO₂ chip. I – washing by buffer; II – washing by mixture of methanol and AcA (9:1); III – washing by buffer; IV – addition of 0.5 g/L cortisol; V – washing by mixture of methanol and AcA (9:1); VI - washing by buffer; VII - addition of 0.1 g/L GOx; VIII - washing by buffer

As it can be observed from Figure 6.5.4, the same frequency shifts occur for this chip. Addition of 0.1 g/L GOx (VII) resulted in marginal frequency drop (~ 5 Hz) that indicates low binding of GOx to the MIP@SiO₂. However, prolonged washing with buffer removes adsorbed GOx that can be the evidence of its non-specific binding.

These observations validate high affinity of MIP cavities towards cortisol and at the same time confirm the completeness of conjugation between GOx and cortisol-21-HS.

6.6 Control of specificity of GOx-cortisol-21-HS binding to MIP@paper

The mechanism of specific binding of conjugate to MIP cavities and its displacement by cortisol already has been confirmed by QCM experiments. However, binding of conjugate to MIP@paper involves a plethora of non-specific interactions that are not straightforwardly explored with the help of QCM. Besides specific interaction between cortisol in the conjugate and MIP cavities, binding of conjugate can occur via interaction

between the cortisol or GOx portion of conjugate and the polymer surface and the cellulose fibers. To estimate possible interactions, 50 μL of 0.1 g/L GOx-cortisol-21-HS solution was added to 10 mm x 10 mm piece of MIP@paper and non-modified paper, respectively, incubated for 5 min and left to dry. After drying, membranes were immersed into 80 μL of 10 mM MES buffer (pH 6.1) that aimed to completely remove non-specifically bound conjugate molecules. MES buffer was chosen (instead of water) to ensure minimal salt effect on the activity of GOx that is highly susceptible to the amount of salts. Moreover, only MES buffer demonstrated complete removal of non-specifically bound conjugate molecules. After incubation, papers were removed from resulting solution, 10 μL of 0.1 M β -glucose, 5 μL of 0.1 g/L POx and 5 μL of 1 g/L o-dianisidine were added to the solution and the kinetic curve of the enzymatic reaction was recorded. High amount of conjugate removed from the surface of paper by MES buffer would indicate high levels of non-specific binding of conjugate to the surface and therefore, marginal binding to the MIP cavities. Whereas low amount would confirm more specific binding of the conjugate to the surface (including the cavities) that could not be overcome by washing with MES buffer. The absorbance curve of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS and 0.1 M β -glucose, 5 μL of 0.1 g/L POx and 5 μL of 1 g/L o-dianisidine was recorded for reference. The absorbance curves of enzymatic reactions are shown in Figure 6.6.1.

Figure 6.6.1 Kinetic curve of enzymatic reaction of GOx-cortisol-21-HS that was removed from non-modified paper (red curve) or from MIP@paper (blue curve) compared to enzymatic reaction of GOx-cortisol-21-HS in solution (without being in contact with paper or MIP@paper) (black curve). C (GOx-cortisol-21-HS) = 0.1 g/L. Sample size for MIP@paper and non-modified paper = 10 mm x 10 mm and $\lambda = 405$ nm

As it can be observed from Figure 6.6.1, the velocity of the reaction remains essentially unchanged after 100 s. To ensure reading the result when the enzyme has consumed most of the substrate, that is when the absorbance values of colored product do not change with time, the comparison of the responses is done at 180 sec.

It can be clearly observed from Figure 6.6.1 that the absorbance values of GOx-cortisol-21-HS on non-modified paper (red curve) at 180 s is the same as for standard solution of GOx-cortisol-21-HS (black curve). It is evident that the binding between conjugate and non-modified paper is due to weak non-specific forces as confirmed by total removal of conjugate by MES buffer. On the contrary, the amount of conjugate removed by washing with MES buffer from MIP@paper is much lower (blue curve) indicating that a substantial fraction of the conjugate is bound specifically to the MIP@paper.

However, binding of conjugate to MIP@paper can be caused by another reason, for instance, via GOx attachment to paper or polymer. In order to exclude this possibility, similar experiment was repeated with non-modified GOx to estimate if it binds to MIP@paper due to non-specific forces that also contribute to the binding of GOx-cortisol-21-HS. The results are given in Fig. 6.6.2.

Figure 6.6.2 Kinetic curve of enzymatic reaction of GOx that was removed from non-modified paper (red curve) or from MIP@paper (blue curve) compared to enzymatic reaction of GOx in solution (black curve). $C(\text{GOx}) = 0.1 \text{ g/L}$. Sample size MIP@paper and non-modified paper = 10 mm x 10 mm and $\lambda = 405 \text{ nm}$

It can be observed from Figure 6.6.2 that the amount of GOx that did not react with MIP@paper (blue curve) coincides with the standard solution of GOx (black curve) after 180 s of reaction, suggesting marginal adsorption of GOx to MIP@paper. At the same time, red curve in Figure 6.6.2 points toward partial specific binding of GOx to non-modified paper. Higher affinity of GOx towards cellulose rather than MIP backbone can be explained by the contribution of GOx carbohydrate chains that have tendency to bind to paper.

In summary, observations from Figures 6.6.1-6.6.2 confirm the assumption that GOx was successfully conjugated with cortisol-21-HS and validate sufficient specificity of the cavities in MIP@paper towards cortisol.

6.7 Optimization of developed assay in homogenous solution

GOx was reported to be sensitive to the variability in its activity as the response to salt content and pH [48]. In order to estimate the effect of buffers on the activity of conjugate, series of buffers of various nature and pH was tested. GOx-cortisol-21-HS was prepared in various buffers: 50 or 100 mM carbonate, phosphate or MES buffer with pH 5.4, 6.1, 7.0 or 9.2. It was noticed that buffers with high amount of salts (100 mM) and with pH higher than 8.0 reduced the activity, thus, their utilization should be avoided. Conjugate demonstrated uniform activity in the range of pH from 5.4 to 7.0 with the highest activity observed for water. However, as demonstrated in section 6.6, MES buffer was used for removal of GOx-cortisol-21-HS that are non-specifically bound to the MIP@paper as other solvents did not demonstrate the ability to completely remove excess conjugate. The results are shown in Appendix B.

With the aim to optimize reaction time and to achieve the highest activity of conjugate, the effect of the amount of β -glucose (as substrate for GOx), POx (as a catalyst of o-dianisidine oxidation) and o-dianisidine (as a reporting molecule) was evaluated. GOx-cortisol-21-HS was mixed with varying concentrations of the reagents and the absorbance curves of kinetic reactions were recorded. The results are shown in Figs. 6.7.1-6.7.3.

Figure 6.7.1 Kinetic curves of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS, 0.1 M β -glucose and 0.1 g/L POx for varying concentrations of o-dianisidine (0.1, 1 and 10 g/L)

Figure 6.7.2 Kinetic curves of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS, 1 g/L o-dianisidine and 0.1 g/L POx for varying concentrations of β -glucose (0.01, 0.05, 0.1 and 0.5 M) as a substrate for GOx

Figure 6.7.3 Kinetic curves of enzymatic reaction between 0.1 g/L GOx-cortisol-21-HS, 1 g/L o-dianisidine, 0.1 M β -glucose and varying concentrations of POx (0.01, 0.05, 0.1 and 0.5 g/L) as a catalyst for the reaction of oxidation of o-dianisidine by H_2O_2

As it can be seen from Figures 6.7.1-6.7.3, the highest activity achieved within 180 s is observed for systems comprising 1 g/L of o-dianisidine (Fig. 6.7.1, red curve), 0.05-0.1 M of β -glucose (Fig. 6.7.2, red and blue curves) and 0.05-0.5 g/L of POx (Fig. 6.7.3, red, blue, magenta curves). For the future experiments 1 g/L of o-dianisidine, 0.1 M of β -glucose and 0.1 g/L of POx were utilized, unless differently stated.

6.8 Affinity of GOx conjugate to cortisol homologues towards MIP@paper

Although developed assay is characterized by good recognition properties, the affinity of conjugate between GOx and cortisol homologues towards MIP cavities was tested with the aim to verify if recognition properties of MIP@paper can be further optimized. Potentially, if a MIP cavity is saturated by a conjugate between GOx and cortisol homologue, free cortisol in solution would displace it faster due to its higher affinity. In order to achieve this, synthesis of GOx with cortisol homologues was performed. The homologues were chosen based on their structural similarity, availability or easiness of synthesis and cost affordability (Fig. 6.8.1).

Figure 6.8.1 Cortisol homologues that were chosen to be conjugated with GOx

In order to activate corticosterone (CS) and testosterone (T) and to introduce COOH groups that potentially would react with Schiff's base formed during chemical modification of GOx, they were transformed to hemisuccinates. Testosterone 3-(O-carboxymethyl)oxime possesses a reactive group that provides us with an opportunity to establish the mechanism of recognition that occurs in cavity. Yet again, the binding process of resultant conjugate to MIP@paper required investigation. If GOx was not modified through conjugation process, no specific binding could be observed to MIP@paper. To perform the experiment, 50 μL of 0.1 g/L conjugate was added to 10 mm x 10 mm piece of MIP@paper, incubated for 5 min and left to dry. It is assumed that adsorption of conjugate to MIP@paper will include binding to the specific MIP cavities, to the polymer backbone and to the cellulose fibers. After drying, membranes were immersed into 80 μL of 10 mM MES buffer (pH 6.1) that aimed to completely remove non-specifically bound conjugate molecules. After incubation, papers were removed from resulting solution, 10 μL of 0.1 M β -glucose, 5 μL of 0.1 g/L POx and 5 μL of 1 g/L o-dianisidine were added to the solution and the kinetic curve of the enzymatic reaction was recorded. High amount of conjugate removed from the surface of paper by MES buffer indicate the prevalence of non-specific binding of conjugate to the surface. Whereas low amount would confirm more specific binding of the conjugate to the surface that could not be overcome by washing with MES buffer. The absorbance curves of enzymatic reactions are shown in Figure 6.8.2.

Conjugates exhibit different behavior towards adsorption to MIP@paper as it can be noticed from Figure 6.8.2. The highest binding to MIP@paper is demonstrated by GOx-TO (red curve). This can be the evidence of better recognition properties of a cavity towards

TO rather than to cortisol-21-HS that is used for conjugation with GOx. It is interesting to note, that TO possesses carboxymethyloxime group instead of C=O that enabled its conjugation with GOx. Easier binding to the cavity can be explained by involvement of non-specific bonds, along with formation of hydrogen bonds between polymer in a cavity and a TO. A significantly lower degree of binding is observed for GOx-CS-HS indicating lower specific binding of conjugate to the MIP@paper cavities. At the same time, GOx-T-HS demonstrated very marginal binding to cavities (green curve) that is an evidence of poor recognition of T backbone by cavity. Furthermore, activity of GOx-T-HS conjugate remains low during reaction time, which can be the result of harsh conjugation procedure that could possibly affect the activity of enzyme.

Figure 6.8.2 Kinetic curve of enzymatic reaction of GOx-CS-HS, GOx-T-HS or GOx-TO that was removed from MIP@paper (navy, green and red curves, respectively) compared to enzymatic reaction of GOx-CS-HS, GOx-T-HS or GOx-TO in solution (pinkish, black and blue curves, respectively). C (conjugate) = 0.1 g/L. Sample size MIP@paper and non-modified paper = 10 mm x 10 mm and $\lambda = 405$ nm

Based on the obtained results, GOx-TO along with GOx-cortisol-21-HS, have been chosen to test their affinity towards cortisol-shaped cavities in MIP@paper.

6.9 Design of a Lateral Flow Displacement Assay

Based on the promising spectrophotometric and colorimetric responses obtained in homogeneous solutions, the assay had to be transferred to a membrane based strip with the aim to establish a platform for onsite analysis of saliva samples for cortisol quantification.

Various designs of paper-based assay were examined, with alternating types of membranes for each design of displacement assay. One of the most crucial tasks was to choose membranes with properties that correlate well with the requirements of the lateral flow displacement assay. Being placed on one platform, they should complement each other's properties, enhance robustness and reproducibility and demonstrate homogenous color of detection pad for visual read out.

To account for the applicability of developed assay for saliva analysis in field conditions, membrane strip had to fulfill several requirements: quick clean-up of saliva sample, smooth flow of displaced conjugate along the strip, solubilization of impregnated reagents and channeling them to the detection pad. Moreover, it is important to mention that homogenous development of a color is one of the most essential requirements as the color pattern is representative of cortisol concentration and is aimed to be read by non-trained individuals. A strip that could potentially demonstrate sufficient flow and separation of assay components is shown in Fig. 6.9.1.

Figure 6.9.1 Scheme of a lateral flow displacement assay for detection of cortisol that comprises of following zones (from left to right): 1) loading zone, 2) flow equalizer, 3) displacement zone (MIP@paper), 4) flow equalizer, 5) reaction zone, and 6) detection zone. Overall length of a strip is 4 cm

Three components of enzymatic reaction must be present along the strip: β -glucose that acts as a substrate for GOx-cortisol-21-HS and releases H_2O_2 ; POx that catalyzes oxidation of o-dianisidine by H_2O_2 ; and o-dianisidine itself that changes its color from colorless via

blue to red transition depending on the concentration of H_2O_2 . The sequence of reagents disposition at the strip is critical for maintaining sufficient separation of displaced GOx-cortisol-21-HS from bound to the MIP@paper and for ensuring homogenous development of color in detection pad.

While electing proper position for each component of the assay, following criteria were adhered to:

1. The main requirement for disposition of β -glucose as a substrate for GOx-cortisol-21-HS was to be along the flow direction of the displaced conjugate. Thus, the reaction with adsorbed conjugate should be avoided. Disposition of β -glucose was varied: it was placed along flow equalizer, reaction zone and detection zone.
2. POx, given its high weight and size, is characterized by low mobility. Moreover, it can cause distortion of image due to steric hindrance that it might create for other reaction constituents. Therefore, its optimal position would be after membrane with β -glucose but before membrane with o-dianisidine. It is expected that POx will be removed by flow, catalyze oxidation of o-dianisidine by H_2O_2 , but due to the size of the molecule it would be captured by 3D fibrous structure of membrane with impregnated dye.
3. Detection zone requires homogeneity of o-dianisidine distribution on it to enhance reproducibility of analysis, so avoiding any other reagents and reactions rather than o-dianisidine and H_2O_2 in detection pad should be advantageous.

Furthermore, regular lateral flow strip implies utilization of adsorption pad that aims to adsorb all small components of the assay. However, it is not straightforwardly applicable in this particular case as it might also adsorb impregnated o-dianisidine placed in detection zone, which eventually leads to altered readout results. The list of membranes that were tested for working zones is provided in Table 6.9.1.

Two separate membranes: for POx and glucose		Displaced conjugate does not have sufficient time to digest β -glucose in small pad that leads to uneven development of color in detection pad.		Given the function of reaction pad is to ensure solubilization of impregnated components, no other membranes were evaluated, as glass fiber ensures low sticking of reagents to its fibres.	
Reaction pad	Glass fiber	One membrane with POx and β -glucose	Same		
		POx	Fast removal of POx by flow, reaction time is within 3 min, does not block detection pad		
		Immersing	Immersing		
Detection pad	Cellulose	o-Dianisidine*	Uneven development of color, time-dependent, final color is developed within 30 min.	o-Dianisidine is quickly removed from cellulose	
		o-Dianisidine*	Uneven development of color due to bulky POx molecule, final color is developed within 30 min	detection pad due to capillary flow and distorts color	
		o-Dianisidine, POx, glucose	Immersing in mixture of reagents	POx shouldn't be impregnated within detection pad as it leads to heterogeneous color development	
		o-Dianisidine and POx	Immersing in mixture of reagents	detection pad as it leads to heterogeneous color development	
		o-Dianisidine	Patterning	Use of hydrophilic dye is not possible for patterning	
				line	

Hydrophilic PVDF	Mixture of 3 reagents	Centrifugation, three times	POx was centrifuged with membrane first to utilize its size to cover the surface. Centrifugation of glucose and o-dianisidine on top of adsorbed POx was alternated. Membranes acquire yellowish color upon addition of reagents. Color develops heterogeneously only for high amounts of replaced GOx-cortisol-21-HS.	The mechanism of reagents retention is not clear and not reproducible
	POx, glucose, o-dianisidine,	Centrifugation in different order		
		Immersing in the mixture of reagents		
Nitrocellulose	o-Dianisidine	Patterning	Hydrophilic dye is distributed unevenly within patterned line. Heterogeneous development of color	Use of hydrophilic dye is not possible for patterning
		Patterning and blocking	Capillary forces are low, flow is slow	
Adsorption pad	o-Dianisidine	Immersing in mixture	Water is quickly adsorbed that leads to uneven development of color.	Heterogeneous development of a color
		Vertical flow	Even development of color with clear border between tow colors, blue and red.	
		Immersing	Slightly uneven development of color. Lower reproducibility of results	
			Uneven development of color, quick removal of a dye from a patterned dye due to its low adsorption properties. Patterned o-dianisidine occupies wide line due to its low viscosity and high hydrophilic properties.	
Cellulose fibers	o-Dianisidine			Cellulose fibers pad was adopted as a detection pad due to its high thickness and potential possibility to capture dye
		Patterning		

*Detection pads that had only o-dianisidine utilized glass fiber impregnated with β -glucose as flow equalizer and a glass fiber with POx as a reaction pad.

After evaluating possible combinations of membranes, accounting for the enlisted earlier requirements to the lateral flow displacement strip, the following parameters for the assay were adopted. The best membranes for each zone are the following: loading zone – cellulose, flow equalizer and reagent pad – glass fiber, and cellulose fiber for detection pad, as it can be seen from Table 6.9.1.

Glass fiber used as flow equalizer and placed after MIP@paper was impregnated by 0.1 M β -glucose in order to provide area for displaced GOx-cortisol-21-HS to react with its substrate. Reaction pad (another piece of glass fiber) was impregnated by 0.1 g/L POx to enable its removal close to detection pad in order to avoid any loss during the capillary flow due to the high weight of molecule. Cellulose fiber pad demonstrated the best properties in even development of color that could be associated with its higher thickness, better retention properties and proper fixation of a dye that prevents its leakage and heterogeneous development of color. Final design of a strip with reagents is shown in Fig. 6.9.2.

Figure 6.9.2 Optimized lateral flow displacement strip for detection of cortisol. Membrane components are as following: 1 – cellulose, 2 – glass fiber, 3 – MIP@paper saturated with GOx-cortisol-21-HS, 4 – glass fiber impregnated with 0.1 M β -glucose, 5 – glass fiber impregnated with 0.1 g/L POx, 6 – cellulose fiber with 1 g/L o-dianisidine deposited via vertical flow

6.10 Employing lateral flow displacement strip assay for detection of cortisol

Promising spectrophotometric and QCM results that demonstrated specificity of synthesized MIP cavities towards cortisol and GOx-cortisol-21-HS, and optimized design of a lateral flow displacement strip permitted investigation of a designed membrane based platform towards its ability to detect cortisol in solutions.

A series of experiments to establish the most favorable assay conditions for naked eye estimation of cortisol concentrations was carried out. This optimization was necessary as

the amount of GOx-cortisol-21-HS displaced by low amounts of cortisol could be too negligible to produce sufficient amount of H_2O_2 to enable oxidation of o-dianisidine to obtain naked eye read out. To perform this test, 40 μ L of cortisol solution (0.1, 0.5, 1.0, 5.0, 10.0, 20.0 ng/mL) was introduced to the loading pad, washed by 20 μ L of water and the image was captured after 2 min of reaction. The image of membranes' response to the addition of increasing amount of cortisol is shown in Figure 6.10.1.

Figure 6.10.1 Colorimetric response of lateral flow displacement strip after addition of 0.1, 0.5, 1.0, 5.0, 10.0 and 20 ng/mL of cortisol, from left to right. Images are taken after 2 min of reaction. For RGB analysis detection pad marked by red box was resized to 75x150 pixels

Visual recognition of differences in membranes' coloration is based on the increase of red component and decrease in blue upon addition of cortisol solutions with various concentrations. As it can be seen from Fig. 6.10.1, visual detection of cortisol is possible only at concentration >1 ng/mL, which is higher than the lowest possible amount of cortisol present in saliva. The reason for low colorimetric response lies in the low amount of GOx-cortisol-21-HS replaced by cortisol and, evidently, in low amount of produced H_2O_2 as a result of enzymatic reaction between conjugate and 0.1 M β -glucose. One of the approaches to induce production of H_2O_2 is to increase the amount of impregnated β -glucose along the flow equalizer strip. The results obtained are shown in Fig. 6.10.2.

Figure 6.10.2 Colorimetric response of lateral flow displacement strip with 0.5 M β -glucose impregnated in glass fiber instead of 0.1 M, after addition of 0.1, 0.5, 1.0, 5.0, 10.0 and 20 ng/mL of cortisol, from left to right. Image is taken after 5 min of reaction. For RGB analysis detection pad marked by red box was resized to 75x150 pixels

As it can be seen from Figure 6.10.2, low amounts of cortisol are still undetectable, whereas coloration of pads obtained at high amounts of cortisol is uneven and complicates visual detection. This effect can be caused by increased concentration of β -glucose that in turn has the ability to block active sites of enzyme, and thus inhibit the enzymatic reaction. It could also affect time of assaying, as color developed much slower and did not change after 5 min.

Another approach to address low sensitivity of o-dianisidine to released H_2O_2 after reaction of low amounts of displaced GOx-cortisol-21-HS with β -glucose was to introduce small quantity of H_2O_2 to the detection pad along with o-dianisidine. It is important to note that H_2O_2 does not react with o-dianisidine in the absence of POx. This procedure could potentially amplify low signal obtained from low concentration of cortisol that could enhance visual response. The results of introduction of increasing amounts of H_2O_2 to the detection pad are shown in Table 6.10.1.

Table 6.10.1 Colorimetric response of detection paper membranes with concentration of o-dianisidine 1 g/L and varying concentration of H_2O_2 . The reaction time was 2 min and the cortisol concentration increases from left to right from 0.1 to 20 ng/mL

C (H_2O_2), mM	C (cort), ng/mL					
	0.1	0.5	1	5	10	20
0.03
0.15
0.3
3

As it can be seen from Table 6.10.1, the difference in colorimetric responses of detection pads deposited with 0.03 and 3 mM H_2O_2 could not be clearly distinguished by naked eye. Visual recognition in these cases is suitable for concentrations of cortisol higher than 1 ng/mL or lower than 0.5 ng/mL for the 0.03 and 3 mM H_2O_2 , respectively. This can be also associated with critical amount of H_2O_2 that triggers disproportionate oxidation of o-dianisidine. Table 6.10.1 also demonstrates that detection pads with pre-deposited 0.15 and 0.3 mM H_2O_2 possess well-defined and gradual changes in colors, enabling semi-quantitative detection of cortisol at physiological ranges.

6.11 RGB analysis of colorimetric response

In order to objectively select the most appropriate test strip for calibration purposes, a detailed RGB and Delta-E analysis were performed. The entire area of detection pad (75x150 pixels) was taken for RGB analysis. The Delta-E (ΔE) parameter (calculated according to CIE76 algorithm [49]) for the two assay conditions (1 g/L o-dianisidine and 0.15 or 0.3 mM H_2O_2 , Table 6.10.1) was calculated. A ΔE value of 2 is normally regarded as a cut-off indicator above which the human vision system is capable of differentiating two colors [50]. Therefore, larger differences in ΔE values enable more accurate estimation of cortisol concentrations. The results are shown in Fig. 6.11.1.

Figure 6.11.1 Estimation of expected color difference of two membranes for visual cortisol detection assays that include 0.15 mM (black box) or 0.3 mM (white box) H_2O_2 , pre-deposited on detection pad, with reaction time 2 min

Figure 6.11.1 demonstrates a larger difference in ΔE values for samples containing 0.15 mM of pre-deposited H_2O_2 for most of concentrations as compared to the samples with 0.3 mM H_2O_2 . However, the color differences that are caused by higher concentrations of cortisol (5-20 ng/mL) are almost indistinguishable, which correlates well with the observations from Table 6.10.1. Thus, better visual recognition of color differences for higher concentrations of cortisol is inherent for membranes pre-deposited with 0.3 mM H_2O_2 (Delta-E varies in between 53 to 69 for 0.3 mM H_2O_2 versus 61-63 for 0.15 mM H_2O_2).

Although the dynamic range of every studied system lies within natural distribution of salivary cortisol, the optimized assay system should possess a practical combination of three parameters (besides linearity): low standard deviation, low LOD and distinct differences in RGB values obtained from RGB analysis. The calculation of R, G and B values for the images of two systems with 0.15 mM and 0.3 mM of pre-deposited H_2O_2 is provided in Fig. 6.11.2.

Figure 6.11.2 RGB analysis of detection pad with pre-deposited 0.15 mM (a) and 0.3 mM (b) H_2O_2 in response to cortisol with increasing concentration

Figure 6.11.2 illustrates that blue and green components are more sensitive than red to the change in concentrations. However, detection pad that contains 0.15 mM H_2O_2 hardly demonstrate changes in RGB values at high concentrations of cortisol that disables its detection. This observation is in correlation with results obtained in Fig. 6.11.1.

The ratios of obtained RGB values were calculated in order to estimate linearity of developed system and to understand its sensitivity limitations (Fig. 6.11.3).

Figure 6.11.3 (b) illustrates that ratio R/G demonstrates the steepest slope for both systems, however, its linearity is low. The linear fit equations are as following: $R/G = 1.2 \pm 0.1 - (0.10 \pm 0.02) \cdot C(\text{cortisol})$, and $R/G = 1.20 \pm 0.09 - (0.09 \pm 0.02) \cdot C(\text{cortisol})$, for 0.15 mM and 0.3 mM H_2O_2 , respectively. LOD calculated based on 3-fold standard deviation ($n=3$) of the mean value divided by a slope in the linear fit equation equals 3 ng/mL for both

cases, which is far below expected sensitivity of the assay. Nevertheless, ratio R/B seems promising in case of system that contains pre-deposited 0.3 mM H_2O_2 . The linear curve for this ratio is characterized by following equations: $R/B = 1.04 \pm 0.01 + (0.088 \pm 0.003) \cdot C(\text{cortisol})$. This system yields the lowest LOD of 0.3 ng/mL and is almost a factor of 2 lower than minimum amount of cortisol found in saliva.

Figure 6.11.3 Analysis of RGB ratios as a variable of colorimetric response of evaluated assays that consist of 0.15 mM (a) and 0.3 mM (b) H_2O_2 pre-deposited in detection pad

Having investigated assays that had different amount of H_2O_2 pre-deposited in detection pad, it was established that 0.3 mM H_2O_2 fulfills requirements applied to the optimization of an assay: low standard deviation, low LOD and distinct differences in certain colors obtained from RGB analysis, as compared to other systems. Based on visual estimation, studies of Delta-E and RGB analysis of deployed approaches (Fig. 6.11.1, 6.11.2 (b) and 6.11.3 (b)), assay that comprises 0.3 mM H_2O_2 pre-deposited on detection pad will be utilized for future studies. It is important to mention that similar set of experiments was conducted with GOx-TO as well. However, obtained results were characterized by low reproducibility that could be explained by different retention mechanism of TO inside the cavity due to its structure. Results are not shown here.

Optimized assay potentially enables visual semi-quantitative estimation of cortisol amount in saliva that holds the promise to be successfully implemented as a point-of-care device due to its fast response and absence of sophisticated reagents or equipment.

6.12 The interference from other hormone-like substances

Saliva comprises a lot of substances alike cortisol that potentially can interfere with its detection. MIP@paper cavities due to their specific shape can potentially bind hormone-like substances that possess similar aromatic backbone, leading to complication of analysis. However, other salivary hormones are excreted in much lower amounts compared to cortisol. For example, DHEA and T, the most abundant hormones in saliva after cortisol appear at the maximum level of 0.2 ng/mL that is half of the minimum possible amount of cortisol found in saliva [16, 51]. This is a good evidence that hormones in saliva may not interfere with the detection of cortisol. However, in order to test the specificity of designed cavities in MIP towards hormones, T, TO and CS (0.5 and 1 ng/mL) have been chosen as the compounds that are characterized by the highest structural similarity to cortisol. The concentrations were taken higher than usually present in natural range in order to make it comparable to the amount of salivary cortisol. A solution (40 μ L) of T, TO or CS was introduced to the loading pad, washed by 20 μ L of water and the image was captured after 2 min of reaction. The results are shown in Table 6.12.1.

Table 6.12.1 Colorimetric response of detection paper membranes with concentration of o-dianisidine 1 g/L and 0.3 mM H₂O₂. Amount of interfering substances varies from 0.5 to 1 ng/mL. The reaction time was 2 min

C, ng/mL	CS	TO	T	C
0.5
1

As it can be seen from Table 6.12.1, no color response is observed for membranes where 0.5 ng/mL of T, TO or CS were introduced. A slight development of red color can be noticed after MIP membrane reacted with 1 ng/mL solution of interfering compounds. However, it is very unlikely that these hormones will be present in saliva at this level and

moreover, the total amount of all salivary hormones (except cortisol) will be substantially lower than 1 ng/mL. This observation enables dismissing interference from cortisol structural homologues. It is important to mention that one of pre-cursors for synthesis of cortisol (cortisone) is also released to saliva in amount commensurate to cortisol and is considered to be its active metabolite [16]. However, cortisone molecule possesses one distinct difference with cortisol molecule, which is dehydrogenized OH group in position 11 that presumably is used as one of recognition sites in cavities. Variations in group structure reduce recognition properties that is beneficial for excluding interference from other substances.

It is foreseen that complex constitution of saliva potentially can trigger unaccounted effects of other constituents that can affect enzymatic ability of developed assay, sterically block the cavities or oxidize o-dianisidine. Application of developed lateral flow displacement assay in saliva was required in order to estimate any potential interfering factors.

6.13 Semi-quantitative detection of cortisol in saliva samples

Saliva samples are known for their matrix complexity, where different constituents could interfere with the detection of target analyte. For validation of the proposed methodology, saliva with tentatively the lowest amount of cortisol was collected in the evening from one volunteer. The amount of cortisol was determined by HPLC/MS and was 2.4 ng/mL. Whole and diluted saliva (in PBS) were spiked with increasing concentrations of cortisol to achieve total concentration of cortisol 5, 10 and 20 ng/mL in order to estimate the response to cortisol addition and to relate it visually and quantitatively to the coloration of standard lateral flow displacement strip. It is assumed that if the intensity of detection pad in test membrane coincides with standard membrane, then saliva has marginal effect on detection of cortisol. On the contrary, any deviations in coloration of test membrane as compared to the standard membrane are considered to be a demonstration of impeding salivary matrix. Developed method for HPLC/MS detection of cortisol in saliva was employed for benchmarking of the results obtained by optimized assay condition with pre-deposited 0.3 mM H₂O₂ and with reaction time of 2 min. Visual estimation of colorimetric response was

done by comparing detection pads of test lateral flow displacement strips with standard strips that were prepared freshly before the experiment by adding 0.5, 1, 5, 10 and 20 ng/mL of cortisol to strips that already contained MIP@paper with adsorbed GOx-cortisol-21-HS, glass fibers impregnated with 0.1 M β -glucose and 0.1 g/L POx and detection pad with pre-deposited 0.3 mM H_2O_2 . Images of detection pads of standard lateral flow displacement strips are shown in Table 6.13.1.

Table 6.13.1 Images of detection pads of standard lateral flow displacement strips taken after 2 min of reaction

C (cortisol), ng/mL	0.5	1	5	10	20

The results of saliva analysis are shown in Table 6.13.2.

Table 6.13.2 Concentration of cortisol in whole (1), diluted twice by PBS (2) and spiked whole and diluted (3-8) saliva, n=3 (different membranes), as determined using HPLC/MS confirmation, visual read out and R/B ratio analysis of test strips

#	Samples	Concentration of cortisol according to HPLC/MS, ng/mL	Image of a colorimetric response from test strip	Concentration of cortisol obtained by visual read out of the developed assay, ng/mL	Concentration of cortisol obtained by RGB analysis of the developed assay, ng/mL
1	Whole saliva	2.4±0.4		5	10±1
2	Saliva diluted 2x by PBS	0.8±0.2		1	1.1±0.2

3	Saliva spiked to final concentration 5 ng/ml	5.4±0.4		10	10±2
4	Saliva spiked to final concentration 10 ng/mL	11±1		Between 10 and 20	16.0±0.8
5	Saliva spiked to final concentration 20 ng/mL	19.8±2		20	26±3
6	Saliva diluted 2x by PBS and spiked to final concentration 5 ng/ml	5.1±0.4		5	6±1
7	Saliva diluted 2x by PBS and spiked to final concentration 10 ng/ml	11±2		10	13±3
8	Saliva diluted 2x by PBS and spiked to final concentration 20 ng/ml	21±3		20	19±3

As illustrated in Table 6.13.2 (#1, 3, 4, 5), spiking cortisol to whole saliva results in excessive overestimation of its amount by visual and RGB analysis as compared to HPLC/MS. Saliva, comprising a lot of interfering substances, can potentially affect representation of cortisol concentration by developing excessive coloration in detection pad. One of possible interferences might come from α -glucose that could be present in saliva. Although GOx is selective towards β -glucose, it can catalyze oxidation of α -anomer in the absence β -glucose at much slower rate. According to Raba et al. [52], β -anomer of glucose reacts about 150 times faster than α -anomer. Additionally, the amount of β -glucose in the developed system is much higher than any possible concentration of α -glucose in saliva that indicates minor interference from α -glucose. Another complication from utilizing non-treated saliva might come from salivary peroxidases that catalyze oxidative processes by H_2O_2 . Peroxidases play active role in mucosal antibacterial protection [53,

54]. It is reported that two major peroxidases produced by glands are myeloperoxidase and lactoperoxidase with total amount that varies within 2-13 $\mu\text{g/mL}$ [55], which is much lower than peroxidase impregnated on glass fiber (0.1 g/L). Moreover, human salivary peroxidases tend to have higher affinity towards oxidation of halides [56, 57] implying lower catalytic affinity towards o-dianisidine. H_2O_2 is also present in human saliva, where it is produced and released by oral microorganisms [58]. The level of released H_2O_2 slightly exceeds 10 μM , which is significantly lower than the amounts of H_2O_2 produced and pre-deposited in membrane paper-test. Notably, H_2O_2 is not produced by salivary glands, which infers that rinsing mouth with water would temporarily remove produced amount of H_2O_2 that was not consumed by salivary peroxidases.

Dilution of saliva by PBS 2-fold demonstrated good results in relating colored membrane to final concentration of cortisol, as it can be seen from rows 2, 6, 7 and 8, Table 6.13.2. The reason for this can lie in effective decrease of saliva viscosity that facilitated flow of cortisol, dilution of proteins that could cause cortisol sticking and in removal of excessive amount of salivary components that could potentially interfere with cortisol. As described earlier, one of saliva compounds that could potentially interfere is cortisone that is present in commensurate amount in saliva as cortisol. Dilution of interfering substances twice demonstrated positive effect and assisted in diminishing interfering effect of cortisone.

As a result, the concentration of cortisol in saliva diluted two times by PBS estimated visually by the membrane based test correlates well with responses obtained using HPLC/MS asserting that visual estimation of cortisol concentration is feasible for rapid screening.

It is to be noted, however, that semi-quantitative estimation implies lower resolution of the assay. Concentrations of cortisol can be evaluated only approximately because of variations in subjective judgment of coloration between users, differences in the illumination, etc. The variation in subjective judgement is also a limiting factor for inclusion of intermediate concentrations in the calibration strip (standard membranes) to improve the resolution of the assay. Nevertheless, the developed lateral flow displacement colorimetric assay

provides a rapid and specific assessment of cortisol concentration in saliva with its minimal prior preparation, which facilitates applications in field, without requiring trained personnel and sophisticated instrumentation.

6.14 Quantitative detection of cortisol in saliva based on RGB analysis.

In order to quantitatively evaluate the obtained colorimetric responses, RGB analysis of membrane based test with saliva samples was carried out. Ratio R/B was calculated for all the membranes with saliva samples and the concentration of cortisol was then estimated according to the linear equation: $R/B = 1.04 \pm 0.01 + (0.088 \pm 0.003) \cdot C(\text{cortisol})$, obtained for assay with pre-deposited 0.3 mM H₂O₂ (Fig. 6.11.3 (b), black curve).

Table 6.13.2 (column 6) reveals good correlation between the concentrations of spiked cortisol in saliva diluted 2 times by PBS obtained by the RGB analysis and by HPLC/MS with an accuracy of >85%. Within the assay's linear range (0.5-20 ng/mL), there was close agreement between semi-quantitative estimation of cortisol levels in saliva based on visual estimation of color intensities of detection paper membranes, quantitative estimation of cortisol based on RGB analysis of colored membranes and HPLC/MS analysis taken as a reference.

6.15 Effect of different saliva samples on the method reproducibility.

Having elaborated sample preparation, sufficient for detection of spiked cortisol in its natural range, it was necessary to test applicability of lateral flow displacement strip using real saliva samples that span within natural range of salivary cortisol. It is recognized that interferences in saliva can vary interpersonally and can potentially cause biased result. Saliva was collected from volunteers upon awakening, after resistance training, after lunch and before going to sleep. These physiological conditions are recognized to have certain impact on cortisol concentration. Saliva was diluted twice by PBS, added to the developed membrane based test and the coloration of detection membrane was assessed quantitatively

and semi-quantitatively. The amount of cortisol was confirmed by HPLC/MS. The results of analysis of saliva samples are shown in Table 6.15.1.

Table 6.15.1 Concentration of cortisol in saliva, diluted by PBS (1:1), n=3 (different membranes), as determined using the HPLC/MS confirmation, visual read out (refer to Table 6.13.1) and R/B ratio analysis of test strips

#	Samples	Concentration of cortisol according to HPLC/MS, ng/mL	Image of a colorimetric response from the developed test	Concentration of cortisol obtained by visual read out of the developed test, ng/mL	Concentration of cortisol obtained by RGB analysis of the developed test, ng/mL
1	Before sleep	1.2±0.2		1	1.5±0.2
2	After lunch	3.7±0.4		5	4.6±0.5
3	After training	6.2±0.8		Between 5 and 10	8.0±0.7
4	In the morning	8.9±0.9		10	11±1

It can be clearly observed from Table 6.15.1, that both visual estimation and RGB analysis of detection pad in membrane based test slightly overestimate values of cortisol level in saliva that can be the evidence of interfering effect of salivary components preserved even upon dilution. However, the amount of cortisol obtained by HPLC/MS is in good correlation with results obtained by visual readout of detection pad (Fig. 6.15.1).

Figure 6.15.1 Correlation among the results obtained from quantitative estimation of cortisol level (by RGB analysis) and from semi-quantitative visual estimation versus results obtained by HPLC/MS taken as a standard for detection of cortisol

As it can be seen from Fig. 6.15.1, results obtained by visual estimation and by RGB analysis demonstrate sufficient correlation ($R^2 = 0.96$) with the measurements obtained by HPLC/MS. The slope of the fitted linear regression model is close to best fit line for the measured standards. The standard deviation of cortisol amount estimated by HPLC/MS, taken as a standard for detection of cortisol, for three measurements of each concentration varies from 10 to 16% (with average 12%). The standard deviation for three measurements at each concentration in the dynamic range for developed assay varies from 8% to 13% (with average 10%). Both methods display similar variation of cortisol concentration. Accuracy of the developed assay, calculated as the difference between average values obtained by RGB analysis and HPLC/MS divided by average value obtained by HPLC/MS is higher than 75% across the tested concentration range.

It is at this stage important to emphasize that no conclusions on the effect of cognitive or physical activities on cortisol levels in saliva are done. However, the observations herein provide an opportunity to assess the response of cortisol concentration fluctuations to different stressors and potentially to estimate and predict onset of fatigue using a simple and fieldable methodology.

6.16 Conclusions

Having optimized synthesis of MIP nanoparticles on cellulose fibers, the recognition properties of designed cavities were investigated towards their specificity to cortisol and GOx-cortisol-21-HS. While the molar ratio between GOx and cortisol-21-HS could not be confirmed, conjugate demonstrated sufficient binding to the cavities. The mechanism of conjugate displacement from cavities by cortisol molecules was studied by QCM. Different membranes and their combinations were tested in order to ensure proper removal of displaced conjugate, oxidation of β -glucose with release of H_2O_2 , and its utilization by POx to oxidize o-dianisidine that develops color according to the amount of added cortisol. The analysis of colorimetric response to variation in concentration of pre-deposited H_2O_2 used for signal amplification enabled optimization of lateral flow membrane based test strip for detection of cortisol. Validation of developed test was performed with HPLC/MS. Whole saliva demonstrated overestimated cortisol amount. However, diluting saliva by PBS facilitated detection of cortisol and diminished the effect of interfering substances. The results of visual (semi-quantitative) and RGB-based (quantitative) estimation of cortisol level in saliva samples demonstrated good correlation and repeatability with respect to HPLC/MS. Developed assay demonstrated LODs and dynamic range that is sufficient for routine analysis of cortisol in saliva. Thus, the developed lateral flow displacement test can be used onsite in field settings or at home without requiring sophisticated instrumentation and trained personnel for analysis.

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Chapter 7

Conclusions and Future Recommendations

Chapter 7 concludes the results obtained in this thesis work and reports on several reconnaissance studies on identification of salivary peptides. It describes the milestones for future optimization of lateral flow displacement strip for detection of cortisol and articulates the possibility of development test strips for detection of other small molecules. It also suggests the design of onsite testing device for simultaneous detection of α -amylase and cortisol in saliva and highlights the necessity of its approbation for saliva samples collected from sportsmen or volunteers. The aim is to establish their individual response to various internal and external factors via simultaneous control of ANS and HPA activity and to use this baseline to identify the unsynchronized responses that testify the alterations in hormonal adaptation. Moreover, screening of saliva samples in the search of other potential biomarkers that are present across all sportsmen and at the same time demonstrate fluctuations in concentrations as a response to physical exercises would provide a good foundation for deeper understanding of the onset and development of overtraining.

7.1 Summary

The main issue in diagnosing overtraining lies in its confirmation after actual development which implies that an athlete continued training in the lack of physiological adaptation to the loads that led to the incessant accumulation of stress. After being diagnosed with overtraining, a sportsman is recommended to take a rest for a prolonged period of time aiming to resume proper mechanism of hormonal adaptation that significantly reflects on his training schedule. Obviously, late confirmation of overtraining is as bad as no confirmation at all. Thus, the efforts must be put in preventing overtraining.

One of the approaches is to control physiological changes of an athlete during both off-season and competition season. While no unique biomarker exists to indicate about overtraining development, a combination of several markers can provide a full picture of organism functioning during training and grant an opportunity to study the factors that influence overtraining development. Cortisol, controlled by HPA axis, and α -amylase, a marker of ANS activity, are unique biomarkers since many processes that happen with athlete's body during exercises get reflection in concentration fluctuations of these substances.

Continuous monitoring of changes in the metabolic and hormonal response of cortisol and salivary α -amylase - two major mediators in stress and fatigue development - to the type of exercise enables early detection of overtraining, provides knowledge about their specific behavior for its prediction and thus, assists an athlete in customization of his training in order to ensure high performance. Salivary heptapeptides GGHPPPP and ESPSLIA, accompanying cortisol and α -amylase, were hypothesized to provide a specific tool in detecting overtraining and fatigue in addition to monitoring ANS and HPA responses to the internal and external factors. The detection method should be able to rapidly record the responses, be cost efficient and easy-to-use. Visual methods of detection were chosen due to fast readout, simplicity, no need in special instrumental support or training and being intuitively clear. Paper strips were selected as the base for visual methods due to their

disposability, low cost and long shelf life (critically dependent on the nature and robustness of the surface chemistry and immobilized target molecules).

It was hypothesized that paper-based assay for visual detection of α -amylase based on its specific property to cleave α -bonds of polysaccharides coupled to a dye would be characterized by high specificity and sensitivity. It was also proposed that an assay comprised of a glucose oxidase – cortisol conjugate and molecularly imprinted polymers as recognition entities would be ideal for detection of cortisol. Visual detection was chosen as interrogation principle for both assays.

The developed assay for visual detection of α -amylase demonstrated a LOD and dynamic range that are sufficient for routine analysis of α -amylase in saliva. The developed paper membrane can be used onsite in field settings or at home without requiring sophisticated instrumentation and trained personnel for analysis. It is suitable for both semi-quantitative detection of α -amylase as well as for quantitative analysis using RGB analysis as it demonstrated excellent correlation within Phadebas tests. The stability of paper membranes and reproducibility of the colorimetric responses offered great potential to be used onsite in stated conditions by a non-trained patient.

The results of visual (semi-quantitative) and RGB-based (quantitative) estimation of cortisol level in saliva samples with the help of lateral flow displacement strip demonstrated good correlation and repeatability with respect to HPLC/MS. Developed assay demonstrated low LOD (0.3 ng/mL) and dynamic range (0.5-20 ng/mL) that is sufficient for routine analysis of cortisol in saliva, including 2-fold dilution. The small size of cortisol molecule and complexity of salivary matrix imply high interferences and competition for the specific cavities in MIP@paper. While 2-fold dilution by PBS improved the response in spiked saliva, quantitative estimation of cortisol in saliva by RGB analysis demonstrates slightly overstated results that is the evidence of highly interfering salivary matrix. Nevertheless, visual estimation of colorimetric response provides good correlation with the results obtained via HPLC/MS, which confirms validity of the elaborated test. Thus, the developed lateral flow displacement test can be used onsite in

field settings or at home without requiring sophisticated instrumentation and trained personnel for analysis. Moreover, the visual response can be obtained within 2 min.

A piezoelectric platform with optimized surface chemistry for quantitative detection of heptapeptides based on their interaction with antibodies as recognition molecules was employed and it demonstrated sufficient specificity in buffer solution. However, it was found that complex salivary matrix greatly interfered with their detection even after extraction performed at 3 kDa membranes. The ample amount of peptides in salivary extract triggers cross-reactivity of the antibodies. Nevertheless, the approach in optimization of surface chemistry established for developed QCM platform can potentially serve as a reference method in future experiments devoted to production of a paper-based immunosensor for onsite detection of diverse peptides.

Two membrane based tests for detection of salivary α -amylase and cortisol demonstrated sufficient correlation with reference methods which can be taken as an evidence of their applicability in field settings. Moreover, the level of α -amylase and cortisol tested after certain physiological condition is in good correlation with reported earlier pattern of the biomarkers' fluctuation.

7.2 Reconnaissance studies and future work

Although the mechanism of appearance of heptapeptides is not established yet, their ratio is believed to be a biomarker of overtraining and thus, they represent a good object for elaboration of lab-on-a-chip devices. The investigation of more specific antibodies or examination of better extraction protocols that would enable specific recovery of target heptapeptides would benefit the detection system. Future work will focus on experiments with the best recognition molecule to identify heptapeptides and development of a lateral flow immunosensor.

However, failure to identify heptapeptides in saliva using multiple detection methods and absence of subsequent works on the ratio of heptapeptides as a fatigue biomarker index aid

the fact that these heptapeptides are not common for all sportsmen and might be produced by specific unaccounted factors that volunteers in the research [1] were exposed to. Several reconnaissance studies were performed with saliva of sportsmen collected before, during and after training with the aim to identify peptides of interest. The extraction protocol developed in this thesis was applied on saliva samples but no peptides were identified by MALDI/ToF and HPLC/MS. There is a possibility that natural peptides in saliva could be retained at the ultrafiltration membrane being bound to a protein and thus, they will be undetectable in the supernatant. To address this issue, addition of 3% SDS to saliva was performed before MALDI/ToF analysis. The usage of SDS aimed to denature proteins and to prevent from forming hydrogen bonds between peptide and protein. Ultrafiltration of saliva with SDS did not reveal presence of heptapeptides that can be an evidence of their absence in saliva of selected sportsmen.

Nevertheless, the developed approach to the immobilization of a peptide on a surface of a gold chip can be utilized for immobilization of other peptides and the QCM platform can serve as a confirmation method for visual membrane based tests. Development of a more precise extraction procedure that would target recovery only of a small amount of salivary peptides and search for recognition molecules that can provide more selective binding of heptapeptides is a part of future experiments.

Satisfying results of cortisol detection in saliva open an avenue for development of similar lateral flow displacement platforms for detection of small molecules in biological liquids. One of the next steps would be to elaborate similar platform for detection of testosterone in saliva. This platform could potentially comprise of MIP@paper as recognition entities that have already proved their efficiency towards recognition of small molecules. One of the main pitfalls in this case will be low concentration of testosterone in saliva which is 10 times lower than of cortisol. Preliminary pre-concentration of testosterone from saliva or elaboration of more sensitive system might be required to omit this pitfall.

There are several opportunities to prevent non-accounted binding of small molecules and to increase the sensitivity of the assay that can serve as milestones for both optimization of

developed lateral flow displacement strip for cortisol detection and development of a strip for testosterone detection that utilizes similar approach:

1. Selection of a light-weight enzyme for conjugation with a single point for conjugation. Less bulky molecule would occupy less space and will prevent steric blocking of free sites. Smaller molecule will also decrease the possibility of being lost after displacement and along the removal way.
2. Covalent binding of only one molecule to the enzyme will ensure stoichiometric conjugation and will secure the fact that one molecule of conjugate occupies one cavity that will result in highly reproducible result. GOx due to its specific structure is covered by chains of carbohydrates that appeared to be an attractive point for conjugation. While elaborated conjugation protocol targeted mannose, it was impossible to find information about the exact amount of mannose molecules on the surface of GOx that complicated estimation of final stoichiometry.
3. Alternation of loading membranes and experimenting with various impregnated substances in loading pad is expected to assist in eliminating step of saliva dilution and would be ideal for saliva clean-up or for preconcentration of small molecules before reaching membrane with MIP@paper.
4. The design of the strip enables development of florescent assay with labeled components to simplify the complexity of the system and to increase the sensitivity. However, implementation of this strip would require a device for fluorescence readout.
5. MIP@paper after saturation with conjugates can be blocked by BSA to create steric hindrance for small molecules, or by homologue molecules, that, as it was demonstrated in section 6.12, do not replace conjugate but instead, potentially occupy free cavities. There is a possibility that they can be displaced by cortisol as well as the enzyme-conjugate which implies the necessity of QCM studies of surface behavior.

6. o-Dianisidine that is utilized as a dye in the developed system due to its specific property to develop color gradually, from colorless via blue to red, might have toxic effects on human health. Other dyes can be tested to replace o-dianisidine, such as ABTS, that is practically insensitive towards light and does not undergo autooxidation [2]. It is also reported to be more sensitive to the amount of release H_2O_2 than o-dianisidine.

Developed tests open the avenue for investigation of stability and storage conditions to specify the usage possibilities of the developed paper-based tests. While storage tests of the paper based assay for detection of α -amylase demonstrated its stability for more than 2 months in simple conditions, involvement of enzymes to the preparation of membrane based assay for detection of cortisol could possibly decrease the overall stability and reproducibility of the assay with time as they might lose activity upon prolonged storage. Moreover, o-dianisidine used as a dye has a tendency to being oxidized by air with time and thus, changing its color which might affect the results of visual readout. One of possible solutions could be storing the test strip in neutral non-reactive environment in the lack of oxygen access that could help prevent the dye from oxidation. Humidity should be also kept under control, as wetting membrane with impregnated POx could lead to its flow towards detection pad and to the catalyzed reaction between o-dianisidine and pre-deposited H_2O_2 .

Some preliminary tests on the storage of lateral flow displacement test for cortisol detection were done. Assembled strips with all reagents deposited were kept in dark for 5 days at room temperature and no alterations in subsequent analysis of cortisol were observed. Nevertheless, to ensure prolonged storage the test strips should be kept sealed and covered by inert material to protect oxidation of enzymes and o-dianisidine. It is to be noted that the optimum temperature for GOx storage without significant loss of its activity is within 25-30 °C [3]. The study on storage conditions of lateral flow displacement assay is a subject for further investigation.

7.3 Required tests for suitability of developed assays onsite

It is known that both HPA axis and ANS are controlled by hypothalamus – organ that links nervous and endocrine systems and is responsible for the circadian rhythms, development of fatigue, hunger etc. Thus, simultaneous evaluation of biomarkers of ANS and HPA axis could provide information about systems' reaction to various stressors [4]. Nervous and endocrine systems are thought to react synchronously to ensure appropriate reaction to internal and external stimuli. However, discrepancy in their responses can lead to an asynchronous release of biomarkers pointing towards stress development [5].

Therefore, it is recommended to constantly monitor biomarkers of both systems with the aim to investigate their interaction and to analyze the level of their contribution to the development of fatigue and overtraining. Cortisol and α -amylase as biomarkers of HPA axis and ANS, respectively, can be assessed foremost by analyzing variations in their levels as a response to circadian rhythm, hunger or satiety, environmental factors, physical exercises, cognitive tasks, etc. Once the baseline for each sportsman is established, the decision about response deviation can be made after unexpected reaction of the biomarkers' level to a stressor.

As a part of future work, clinical saliva samples that were collected from volunteers and elite sportsmen after their exposure to certain controlled factors should be analyzed. Their individual response of HPA axis and ANS to the factors as a fluctuation in cortisol and α -amylase level, respectively, will be analyzed and compared to each other with the aim to find patterns and relations. Besides, changes in relative concentrations of hormones and α -amylase can indicate health disorders that should not be confused with overtraining. Therefore, this study also should be based on knowledge of chronic diseases and their effects on fluctuations of biomarkers. It is necessary to emphasize that one of the main problems in diagnosing overtraining is late confirmation of overtraining which is as bad as no confirmation at all. Thus, the efforts must be put in preventing overtraining.

As both developed systems have demonstrated good reliable results in detection of α -amylase and cortisol, they should be tested for the ability to monitor saliva from one volunteer, collected during the day, as a response to various external and internal factors with the aim to establish individual baseline. The main goal of constant monitoring of essential biomarkers with recording a response to various external and internal factors is to provide a doctor with a baseline of a healthy person's individual reactions to stimuli.

The elaborated colorimetric membrane-based assays for detection of α -amylase and cortisol have proved their effectiveness in detection of these biomarkers in saliva. They can be potentially transferred to one multichannel platform and serve as a tool for simultaneous assessment of α -amylase and cortisol amounts in saliva. Utilization of similar paper-based membranes and visual read out as method of concentration estimation greatly facilitates its assembly. The balancing of saliva preparation might be also required as the developed tests utilize different protocols. However, further improvement of membrane-based strip for detection of cortisol according to the steps described previously might provide this opportunity. Otherwise, dilution of saliva in the same manner with subsequent detection of α -amylase using developed test should be checked. Prior results demonstrated that dilution by MES buffer does not affect representation of α -amylase in saliva. After recalibration of assays, the following design of onsite testing device that would enable simultaneous detection of α -amylase and cortisol upon addition of saliva is expected to be working (Fig. 7.3.1).

Figure 7.3.1 Schematic representation of onsite testing device for simultaneous colorimetric detection of α -amylase and cortisol in saliva

The conclusion on the biomarker concentration is supposed to be done by comparison of the obtained color of test membrane to standard membrane. The gamut of standard membranes was created from averaged RGB values obtained for each standard membrane with the aim to account for heterogeneous distribution of colors. The generated gamut, obtained from the membranes in Fig. 5.4.2 (b), is plotted versus real images of standard membranes (Fig. 7.3.2). As can be clearly seen from Fig. 7.3.2, marginal difference in color gradient is observed for two gamuts, pointing towards low heterogeneity of color distribution within one membrane. The findings attest the robustness of developed assay.

Figure 7.3.2 An example of gamut of standard membranes (1) and real images of standard membranes (2) as a response to increasing concentration of α -amylase (from 20 to 500 $\mu\text{g/mL}$)

Calculation of a gamut based on the principle of averaging each color component for each membrane used for detection of cortisol is not suitable as a different principle of visual estimation of color distribution is utilized. Here, region of interest is area 6 (Fig. 6.9.2) where the color developed in response to concentration of cortisol is heterogeneous. However, the heterogeneity is caused by a specific property of o-dianisidine to develop red color gradually from colorless via blue that enables detection of low concentrations of cortisol. O-dianisidine turns blue due to impartial oxidation by low amounts of H_2O_2 and gradually turns red following full oxidation by high amounts of H_2O_2 . The semi-quantitative estimation of cortisol is possible by the evaluation of the amount of red color

developed within full region of interest. It is expected that the conclusion on the biomarker concentration to be done by comparison of the amount of red color in test membrane to the same in standard membrane. However, calculation of averaged RGB values from each membrane provides a gradient colors shift, indicative of a color change sensitive to the amount of cortisol. The gamut is shown in Fig. 7.3.3.

Figure 7.3.3 Color gamut for lateral flow displacement assay for detection of cortisol (0.1-20 ng/mL)

A subsequent study that involves big numbers of volunteers, including sportsmen and those diagnosed with fatigue will be required with the aim to collect dataset and to establish baseline of the investigated biomarkers. This data will enable assessment of factors that inevitably change the baseline, have the most impact on fatigue development with variation from person to person and would serve as a manual for practitioners to predict fatigue or overtraining development. When abnormality in reaction to same factors is observed, decision about disrupted signal exchange between body systems can be taken and a sportsman can be asked to refrain from further exercises until results are confirmed. Fast reaction can help a sportsman prevent overtraining development.

The access to sportsmen saliva will assist in performing screening of saliva for detection of potential biomarkers which release is dependent on physical exercises. Establishing the baseline and control of fluctuations will provide a picture of body's response to the stressors. Some preliminary MALDI/ToF studies with sportsmen saliva have been done

and it was noticed, that certain substances are present across all sportsmen, while some of them are specific only for several athletes (Figure 7.3.4).

Figure 7.3.4 Mass-spectra of saliva collected from 6 sportsmen (a) before and (b) after training in a range of m/z 600-800

As it can be seen, ions with m/z 688, 727 and 771 are present in almost all samples collected before training, while the picture changes for saliva collected after training. In this case, the prevalence of ion with m/z 744 and 769 is peculiar for all samples. The establishment of structures of these compounds can aid in identifying specific biomarkers. Control of fluctuations in their concentration levels during the training period with LC/MS assistance will be the aim of my future studies.

Moreover, screening of saliva samples and looking for new potential biomarkers that are present across all sportsmen, demonstrate fluctuation in concentrations as the result of physical exercises and are not dependent on other external factors except physical load will provide us with a fundamental avenue for better understanding of the molecular processes involved in overtraining development. Future work in this direction will aim at exploring processes related to the onset of overtraining of sportsmen and types of physiological markers that can be relevant for overtraining diagnosing.

7.4 Conclusions

The reflections on applicability of cortisol, mediated by HPA axis, and α -amylase, a marker of ANS activity, for assessing adaptability of the organism to external factors are described. It is assumed that continuous monitoring of changes in the metabolic and hormonal response of cortisol and salivary α -amylase to the type and intensity of training load enables early detection of overtraining. It also provides a facile tool to screen the synchronicity of responses of both systems to diverse factors that can be also utilized for prediction of mental issues or chronic fatigue. Colorimetric membrane based tests for detection of α -amylase and cortisol that were developed in this thesis have the potential to be applied for rapid analysis of α -amylase and cortisol in saliva. They demonstrated reproducible results in assessing of the biomarkers concentration and were validated by laboratory based methods. Their reliability enables their utilization onsite for rapid and reagents free determination of saliva.

The directions of future work that include analysis of saliva samples collected from sportsmen and volunteers for the presence of α -amylase and cortisol as well as general screening and search for new potential biomarkers of overtraining and fatigue are also outlined.

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APPENDIX A

Although the kinetic curves for reaction between α -amylase and substrate with 1 mM or 2 mM maltose almost coincide (Fig. 5.3.2), better visual perception of colorimetric responses and higher linearity within all physiological range is observed with the inhibitor concentration of 2 mM that is supported by colorimetric response (Figure A1a). The ratio B/G is calculated for each colorimetric response obtained after 3 min of reaction time between various concentrations of α -amylase and fixed concentration of G3-CNP equal to 5 mg/mL with 0, 1, 2 and 5 mM maltose in solution (Figure A1b).

Figure A1 Colorimetric response of enzymatic reaction between 5 mg/ml G3-CNP and α -amylase (20, 50, 75, 100, 200, 300, 400 and 500 μ g/mL, from left to right) with 1, 2 and 5 mM maltose as inhibitor, the images are recorded after a reaction time of 3 min (a); RGB analysis of colorimetric response in a) (ratio of the B and G channels) vs C(α -amylase) for different concentrations of maltose.

Appendix B

The effect of buffers, ion strength and pH on the activity of GOx-cortisol-21-HS

In order to estimate the effect of buffers on the activity of conjugate, series of buffers of various nature and pH was tested. GOx-cortisol-21-HS was prepared in various buffers: 50 or 100 mM acetate, phosphate or MES buffer with pH 5.4, 6.1, 7.0 or 9.2. The results are shown in Figures B1-B4.

Figure B1 Absorbance recorded after 3 min of enzymatic reaction between of 0.1 g/L GOx-cortisol-21-HS prepared in different buffers (pH 5.1), 1 g/L o-dianisidine, 0.1 g/L POx and 0.1M β -glucose

Figure B2 Absorbance recorded after 3 min of enzymatic reaction between of 0.1 g/L GOx-cortisol-21-HS prepared in different buffers (pH 6.1), 1 g/L o-dianisidine, 0.1 g/L POx and 0.1M β -glucose

Figure B3 Absorbance recorded after 3 min of enzymatic reaction between of 0.1 g/L GOx-cortisol-21-HS prepared in different buffers (pH 7.0) or water, 1 g/L o-dianisidine, 0.1 g/L POx and 0.1M β -glucose

Figure B4 Absorbance recorded after 3 min of enzymatic reaction between of 0.1 g/L GOx-cortisol-21-HS prepared in different buffers (pH 9.2), 1 g/L o-dianisidine, 0.1 g/L POx and 0.1M β -glucose

As it can be seen from the Figures B1-B4, buffers with high amount of salts (100 mM) and with pH higher than 8.0 reduce GOx-cortisol-21-HS activity, thus, their utilization should be avoided. Conjugate demonstrated uniform activity in the range of pH from 5.4 to 7.0 with the highest activity observed for water.