Biosensor Development for Real-time Measurement of Neurotransmitters Related to Pain in Biofluids

Vitella Fomenko

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I, Vitella Fomenko, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the work.

Abstract

Saliva is considered a noninvasive diagnostic biofluid and its use within the medical field is increasing. As a biofluid, it contains a large variety of biomarkers, which can be used for the detection of disease. For example, glutamate, an important neurotransmitter, has been linked to various diseases, as well as pain. Salivary glutamate concentration has been shown to vary during pain induced states. However, technology for detecting this change has yet to be developed. Therefore, the main aim of this study was to develop the design of a wireless wearable intraoral glutamate sensor that will measure glutamate continuously in real-time whilst meeting the derived user requirements. This was conducted by reviewing previously published recipes for the glutamate biosensor and methods for wireless telemetry, instrumentation electronics and biocompatible packaging. Hence, the optimal glutamate biosensor recipe was determined based on high sensitivity (279.4 ± 2 μA mM^{-1} cm^{-2}), low limit of detection (0.005 μM), wide linear detection range (0.5-100 μM) and fast response time (10s). Biocompatibility and size (0.5 mm length) were equally important. For wireless communication and real-time continuous measurements, an amperometric printed circuit board should be fabricated. This should consist of an on-board potentiostat, Analogue Front End, driven by a Bluetooth low-energy system-on-chip. To enable wireless transmission, a chip antenna and an impedance-matched balun should be incorporated and a lithium battery used as a power source. Polyethylene terephthalate was established as the optimum biocompatible material. Consequently, the mouthguard platform and packaging of the device should use this material to meet the user requirements. Together, these results allowed for the development of the design for the proposed device. Further investigation into designing a product development protocol and consequently the fabrication of this device would be necessary to evaluate functionality.

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Contents

1. Introduction

The importance in early detection and diagnosis of diseases has always been of great interest to the scientific community. Thus, various biofluids, such as blood, saliva and urine have been employed to aid in diagnosing and detecting various disorders. This is due to the presence of disease signaling molecules within these diagnostic mediums. The detection of these, also knowns as biomarkers, and the consequent changes in their concentration can help specify the state of a patient (Gug et al., 2019). The World Health Organization (WHO) has defined biomarker as any substance that is measurable and can therefore detect or predict the prevalence of a disease (Gug et al., 2019).

Currently, invasive methods of biomarker detection are employed, such as blood collection. However, noninvasive methods, for example collection of saliva, are currently being more widely explored. Human saliva is a unique fluid and it highly important as a diagnostic medium. The composition of various biomarkers, such as proteins, hormones, small biomolecules, are all present within this medium (Pfaffe et al., 2011). Small biomolecules, such as glutamate have also been detected within saliva. Glutamate is an important neurotransmitter, which has been described to be responsible for the functionality of cognition. The mechanism of how glutamate works mainly occurs within the central nervous system (Schultz et al., 2020). However, its presence has been detected throughout the whole human body. Glutamate and its overproduction, known as excitotoxicity, has been linked to various neurodegenerative disorders, including Alzheimer's disease (Schultz et al., 2020). In addition, elevated glutamate levels have been detected during pain states.

Currently, the quantification of pain and its detection is only through subjective methods. Thus, using saliva as a diagnostic medium, the detection of glutamate could be done noninvasively. By applying an electrochemical technique could measure the change in glutamate concentration in real-time. Therefore, the main of this study is to develop a design of a wireless wearable intraoral glutamate biosensor that will be able to measure continuously in real-time, whilst meeting the derived user requirements. Thus, the research question is if this would be feasible based on already published recipes for glutamate biosensors and methods for instrumentation electronics, wireless communication and biocompatible packaging.

This study will conduct a literature review emphasizing on the existing salivary biomarkers and technologies used for their detection. Furthermore, potential salivary biomarkers which have not been used in clinical settings are also identified. The focus will be on potential biomarkers of pain, such as glutamate and their detection.

Moreover, to answer the research question, user requirements and device specifications are derived to allow for the development of the proposed device.

2. Literature Review

2.1 Salivary Composition, Secretions and Roles

The human body creates a variety of fluids and more attention has currently been drawn to saliva. Normally, a healthy adult can produce up to 1500 ml of saliva per day, although this may vary due to a wide spectrum of conditions (Chiappin et al., 2007). Saliva is a slightly acidic (pH 6-7) clear biofluid consisting of about 99% of water. The remaining percent comprises of inorganic molecules, such as various ions, and organic molecules, for example hormones, proteins and lipid molecules (Humphrey and Williamson, 2001; Chiappin et al., 2007). These biological variables, known as biomarkers, can be measured and quantified to assist in the identification of local and systemic disorders, thus yielding saliva as a highly attractive diagnostic medium (Yoshizawa et al., 2013).

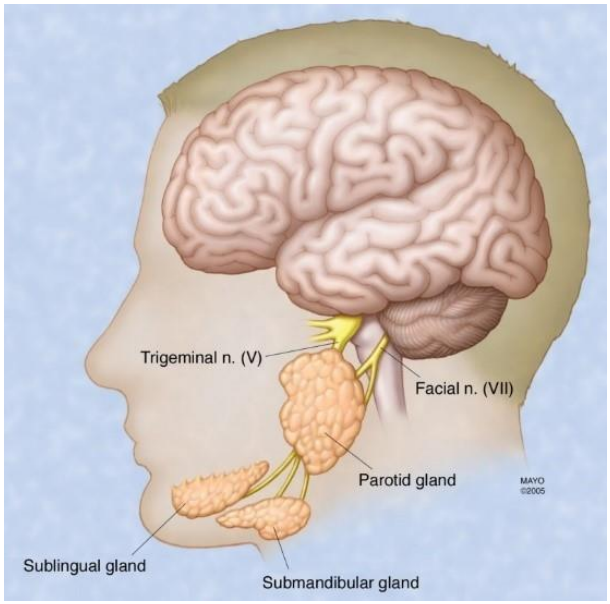

Figure 1. Location of major salivary glands (Figure taken from Yoshizawa et al., 2013.)
The major glands are all paired. The largest glands are the parotid glands, which are situated in front of each ear. The sublingual glands are located underneath the tongue and the submandibular glands are located more posteriorly, below the mandible (lower jaw). The nerves innervating the glands are the trigeminal and facial nerves (Yoshizawa et al., 2013).

Generally, oral fluid is generated from the paired major salivary glands, the parotid, sublingual and submandibular glands, alongside numerous minor salivary glands (Figure 1.). The minor glands are anatomically located around the lower lip, tongue, cheeks, palate and pharynx. Due to the anatomic size of the major glands, the amount of saliva secreted is greater than the minor glands. However, the types of biomolecules released, i.e. blood-derived molecules, and the type of function they provide, varies (Yoshizawa et al., 2013; Humphrey and Williamson, 2001). These glands consist of specialized epithelial cells called acinar cells, myoepithelial cells and duct system cells. Acinar cells uphold the key function of saliva secretion. The type of secretion (mucous, serous or mixed) depends with each salivary gland. For example, most serous secretions are produced from the parotid glands, whereas mucous

secretions are mainly produced from the minor glands. Thus, the mixed is secreted from the sublingual and submandibular glands (Humphrey and Williamson, 2001; Pfaffe et al., 2011). The proteomic expression varies between these glands, for example the protein cystatin C (biomarker of) is expressed in the secretions from the submandibular gland, whereas MUC5B mucin is secreted from the sublingual gland (Chiappin et al., 2007). The composition of saliva, such as the amount of proteins or concentration of ions, differs depending on the type of secretion released. Due to this constant variation in composition, saliva is considered multifunctional; however, the main role is to maintain suitable oral hygiene. Thus, functions like lubrication, protection, antibacterial activity all assist with the main role. Other functions including digestion and taste, maintaining tooth integrity, clearance and buffering action are also essential for maintaining homeostasis and oral health (Humphrey and Williamson, 2001). The performance of some of these functions highly rely on the unique variety of proteins in saliva. Thus, any alterations in the proteomic concentration can cause a wide range of diseases.

Furthermore, salivary secretions are controlled by the autonomic nervous system, more specifically the salivary center located in the medulla. Therefore, the salivary glands are controlled both by the parasympathetic system and sympathetic system. Consequently, various hormones and neurotransmitters stimulate the different receptors and salivary glands, therefore obtaining diverse responses. If the sympathetic system is primarily innervated, then the acinar cells will produce secretions with a higher protein concentration, whereas if mainly parasympathetic is innervated, then secretions have a higher concentration of water (Humphrey and Williamson, 2001). Different triggers including both neural and/or pharmacological, can stimulate the production of saliva, hence altering its viscosity, volume and content of biomarkers (Chiappin et al., 2007). Such variations can also be affected by the circadian rhythm, as the flow of saliva decreases during sleep and increases during high stimulation periods (Humphrey and Williamson, 2001).

Nonetheless, oral fluid (whole saliva) is not only the secretions produced by the salivary glands, but also contains fluids from oropharyngeal mucosae, gingival crevicular fluid, food debris and blood-derived compounds (Figure 2.; Chiappin et al., 2007).

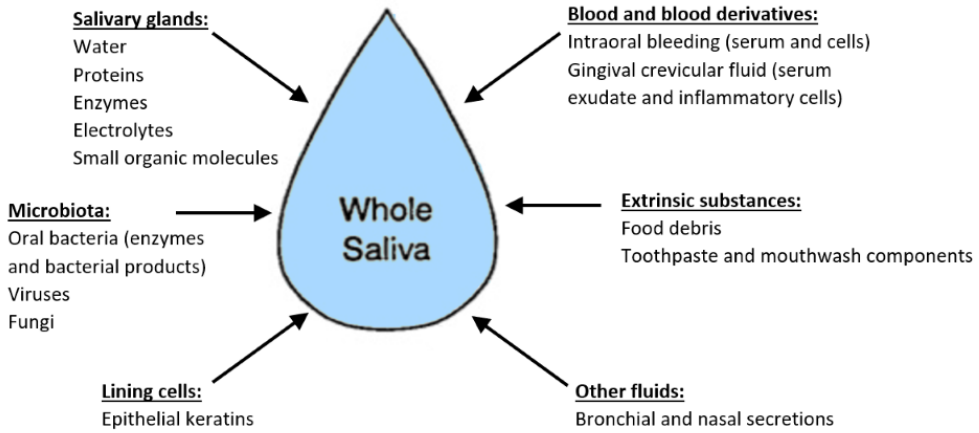

Figure 2. Oral fluid composition (Figure modified from Thavarajah, Ranganathan and Nair, 2012) Whole saliva contains a wide variety of endogenous and exogenous components. In addition to salivary gland secretions, oral fluid consists of secretions from various non-glandular origins. For example,

secretions from oropharyngeal mucosae, which consists of oral bacteria, viruses, fungi, upper airway secretions, oral mucosal transudate cells and gastrointestinal flux). In addition, an extracellular fluid which is derived from gingival crevice epithelia, gingival crevicular fluid, is also present. Various other extrinsic substances, blood-derived compounds and other fluids are also present in whole saliva (Mamta et al., 2013).

Subsequently, the attaining of such blood-derived compounds occurs due to each salivary gland being highly permeable to the surrounding capillaries. These molecules enter the acinar cells through three different routes known as active transport through protein channels and passive transport, more specifically the diffusion of lipophilic molecules. Additionally, another route by which these compounds enter saliva from plasma, is extracellular ultrafiltration, however for molecules to be able to pass through the gap junction, their molecular weight must be below 1900 Da (Figure 3; Pfaffe et al., 2011).

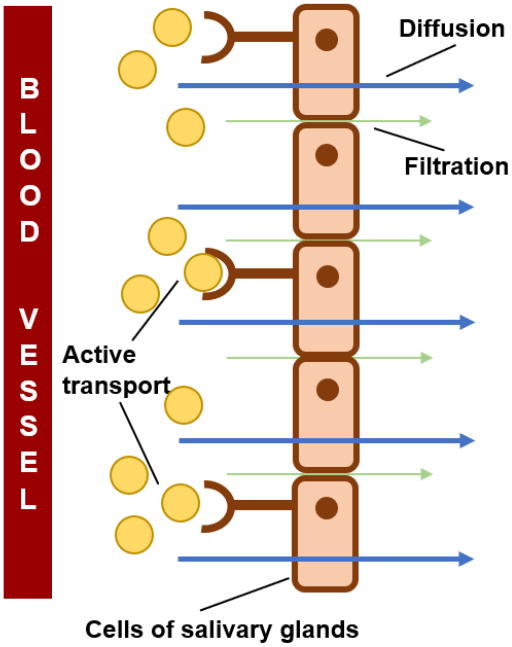

Figure 3. Transport of molecules from blood to saliva (Figure adapted from Pfaffe et al., 2011)

Most small molecules pass into saliva from blood through passive diffusion, as the salivary glands are surround by porous capillaries. The size and electrical charge affect the ability of the small molecule to passively diffuse through cell membranes, thus molecules small in size and nonpolar are able to pass relatively easily. Molecules can also enter the saliva through the secretory cells of salivary glands by active transport. Molecules bind to receptors present on the secretory cells and are then released into saliva. The last route is extracellular ultrafiltration through the spaces between the secretory cells (acinus and ductal cells), as well as through the gap junctions between cells of secretory units. Molecules must be very small to follow this route (<1900 Da), for example water, ions and specific hormones (steroids and catecholamines) (Pfaffe et al., 2011).

2.2 Saliva versus Blood

As previously mentioned, salivary composition consists of varying molecules with some of them being blood-derived molecules. Thus, demonstrating both human serum and saliva containing similar biomarkers. For example, around 27% of protein biomarkers in whole saliva are also located in plasma (Yan et al., 2009; Loo et al., 2010). In addition, 40% of protein biomarkers detected for diseases such as stroke and cardiovascular disease have been identified in whole saliva (Loo et al., 2010). Blood serum has been used as a diagnostic tool for many years, however recently saliva has been described as a more useful diagnostic medium due to its advantages. One of these includes an inexpensive and painless procedure. Another advantageous point is the simple noninvasive collection, which can be performed individually (by oneself) with a decreased risk of obtaining an infection or spread of viruses. In addition, the storage and manipulation of saliva is also easier (Yoshizawa et al., 2013). Although saliva provides many advantages and there is a good relationship of biomarkers between saliva and serum, serum is still the preferred diagnostic fluid. Compared to serum, saliva has reduced levels of biomarkers, thus making their detection and interpretation for pathological diseases more difficult. Therefore, the production of highly sensitive technology is necessary (Pfaffe et al., 2011). Alongside, saliva is more vulnerable to biochemical changes due to the circadian rhythm, various oral stimuli and method of collection (Pfaffe et al., 2011). Nevertheless, saliva provides a real-time diagnostic approach, which is important for monitoring the health of patients. Thus, saliva is a valuable diagnostic fluid providing multiple opportunities for the assessment of diseases and clinical applications.

2.3 Salivary biomarkers and their use in disease diagnosis

2.3.1 Salivary proteins

Cardiovascular diseases (CVDs) are the most prevalent causes of death worldwide and thus numerous studies have investigated the correlation between salivary proteins and CVDs. CVDs incorporate multiple diseases affecting the heart and blood vessels and is usually associated with atherosclerosis. This occurs when there is an increase in lipid deposits within the arteries and is mainly triggered due to inflammation. Consequently, this increases the risk of blood clot formation and thus, the development of acute myocardial infarction (AMI), also known as heart attack (Libby, Ridker and Maseri, 2002; Pfaffe et al., 2011). Studies have shown a connection between AMI and the detection of C-reactive protein (CRP) in saliva, which is a protein produced in the liver. CRP levels in plasma increase during inflammation and this has also been illustrated in saliva. Additionally, CRP was demonstrated to be the best biomarker for the prediction of AMI with 80% sensitivity and 100% specificity by correlating an electrocardiogram with the protein levels (Miller et al., 2014). Moreover, correlation between plasma and salivary CRP levels in ischemic heart disease patients was proven to be strong, positive and significant, further validating CRP as an important biomarker for detecting CVDs (Labat et al., 2013). Multiple other proteins have also been identified for the detection of CVDs, such as higher levels of myoglobin in saliva for patients with AMI (Miller et al., 2014).

Sjögren's syndrome (SS) is an autoimmune disease that affects the salivary and lachrymal glands by lymphoplasmacytic infiltration, therefore causing dry mouth and eyes. Due to this, the salivary flow rate decreases and the composition alters (Ryu et al., 2006). Possible biomarkers within both the transcriptome and proteome of patients with SS were investigated. Studies using proteomic methods obtained significant increases in various proteins, however β-2-microglobulin was most commonly detected and therefore can potentially act as one of the main protein biomarkers for the diagnosis of SS (Ryu et al., 2006; Hu et al., 2010). Additionally, SS has presented with the most consistent biomarker findings, hypothesizing that this is due to the syndrome being directly linked to the salivary glands (Al-Tarawneh et al., 2011).

2.3.2 Salivary RNA and DNA molecules

Salivary proteins are not the only potential biomarkers researched, but also RNA molecules and specific saliva-based DNA. Oral cancer refers to malignancies affecting all intraoral regions with most being in the oral cavity. Around 90% of those are represented by oral squamous cell carcinoma (OSCC) and is considered to be the eight most common cancer globally (Gug, Tertis, Hosu and Cristea, 2019). Obtaining an early diagnosis for OSCC could improve the high mortality rate. Hence, several biomarkers have been identified in correlation with OSCC such as p53 protein or salivary RNAs like interleukin-8, which presented with 81% predicition accuract for OSCC (Li, 2004). Moreover, the existence of tumor-specific DNA was observed in 100% of the oral cancer patients. Additionally, around 47-70% of the patients presented salivary tumor-specific DNA markers related to tumors in other locations (Wang et al., 2015). Therefore, even though there are no currently clinically validated salivary biomarkers for oral cancer, this highlights the high potential of using tumor-specific DNA for diagnosis.

2.3.3 Salivary small biomolecules

Moreover, saliva is also rich in small biomolecules, known as metabolites, which can either be endogenous or exogenous. The measurement of endogenous metabolites, such as amino acids, organic acids, vitamins and more, could provide for a better understanding of the different diseases and even possibly for their early detection (Zhang, Sun and Wang, 2012).
This includes periodontal diseases (PD), which occurs due to bacterial infection, thus triggering inflammation of the periodontium and eventually resulting in tooth loss. Periodontitis can either be classified as aggressive, directly affecting the oral cavity functions or chronic, which has been linked to various other systemic diseases, specifically CVD and type 2 diabetes mellitus (Barnes et al., 2014). Increased levels of nitric oxide, an important radical for maintaining neutrophil and macrophage functions, has been detected in both chronic and aggressive periodontitis (Hussain, McKay, Gonzales-Marin and Allaker, 2015). Additionally, the end products of nitric oxide metabolism in saliva have shown to be important for diagnosing PD (Bejeh-Mir et al., 2014). Even though various studies have produced inconsistent biomarkers for PD, in particular, the upregulation and downregulation of lactate, there are similarly observed upregulations of valine (Gardner, Carpenter and So, 2020).

2.3.4 Hormones and enzymes

Hormones and enzymes are also crucial salivary biomarkers for detecting chronic diseases such as Cushing's syndrome and Addison's disease. These diseases are linked to physiological stress and the steroidal stress hormone, cortisol; a diagnostic marker for both diseases. Decreased salivary cortisol levels have been observed in Addison's disease patients and increased in Cushing's syndrome patients. Numerous studies using late-night salivary cortisol to diagnose Cushing's have presented with very high sensitivity and specificity, thus solidifying cortisol as a key biomarker (Ceccato et al., 2013; Raff, 2009).
Additionally, gastroesophageal reflux disease (GERD) is a common digestive disease affecting the movement of gastric contents. A crucial effect of GERD on oral health is the alteration of the oral mucosal pH as the acidity increases, thus damaging the oral cavity (Sujatha et al., 2016). The enzyme pepsin is solely excreted from gastric chief cells, therefore, its detection within the esophagus or airways indicates gastro-oesophageal reflux. Salivary pepsin has been investigated as a diagnostic marker for GERD, although studies show that it provides moderate diagnostic value (Du et al., 2017; Guo et al., 2018).

2.4 Potential biomarkers of Pain in saliva

Pain and its quantification have recently gained traction within the scientific field. Specifically, because there are currently no objective methods for the detection of pain and exiting methods are subjective and based on self-reporting and observing behavior (Cantón-Habas et al., 2019). Thus, research into identifying and measuring salivary biomarkers could aid in the quantification of pain and thus, disease diagnosis. As well as monitoring health conditions and post operational progress, such as operations for musculoskeletal disorders or amputations. Several pain biomarkers have already been measured, for example secretory IgA, tumor necrosis factor receptor type II (sTNF-RII) and salivary a-amylase are most commonly detected (Ferrara et al., 2013; Sobas et al., 2016; Lopez-Jornet et al., 2020). Nonetheless, sIgA and sTNF-RII displayed the highest intra-individual reproducibility, which is vital if measurements are required daily (Sobas et al., 2020).
Additionally, various neuropeptides and neurotransmitters have also been detected whilst measuring pain biomarkers, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glutamate (Jasim et al., 2018; Jasim et al., 2020). Salivary glutamate concentration showed the highest correlation with the glutamate levels in plasma (Jasim et al., 2018). Thus, glutamate could potentially act as a salivary biomarker of pain.

2.5 Technology used for biomarker quantification

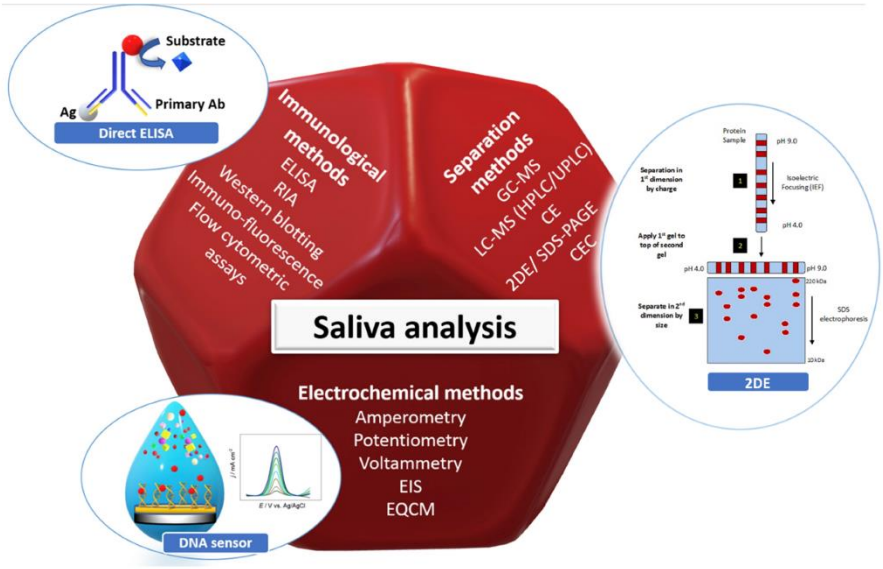

Figure 4. Saliva analysis techniques (Figure adapted from Gug et al., 2019; Overview of SDS Compatible 2D Electrophoresis Method, 2020).
Immunological, electrochemical and separation methods are commonly used for the detection of salivary biomarkers.

Due to the number of biomarkers, different types of techniques are utilized for their detection using either immunological, separation, or electrochemical methods (Figure 4.; Gug et al., 2019). Described briefly below are the various methods and the most suitable when real-time measurement of the biomarker is needed.

2.5.1 Separation techniques

Separation techniques such as liquid chromatography or gel electrophoresis have been successfully applied to study the proteome expression in saliva. Most studies use two-dimensional gel electrophoresis (2DE), which is a protein separation method that applies IEF (isoelectric focusing) and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) separating proteins based on their isoelectric point and mass accordingly (Figure 4.). This is then followed by enzymatic digestion of these proteins with trypsin resulting in peptide fragments, which are further analyzed by mass spectrometry to identify the proteins (Hu et al., 2005; Al-Tarawneh et al., 2011).

2.5.2 Immunological techniques

Immunological techniques are most commonly applied for the detection of biomarkers, specifically regarding tumoral and cellular immune responses. The most widespread techniques using immunological analysis, is enzyme-linked immunosorbent assay (ELISA) and immunoblotting, more specifically Western blotting (Gug et al., 2019). Whilst

studying the salivary composition, it is necessary for the technology to be highly sensitive and specific, thus ELISA is most frequently applied when quantifying specific antibodies, cytokines, proteins and hormones. This occurs by immobilizing an antigen of interest through a previously attached capture antibody and further detecting it either directly by applying a labelled primary antibody or indirectly using a labelled secondary antibody (Gug et al., 2019). The proteomic expression in saliva can also be investigated using western blotting, which also separates proteins by electrophoresis, but then uses an antibody to probe the proteins and applies similar detection techniques as ELISA to detect the antigen protein (Gug et al., 2019).

2.5.3 Electrochemical techniques

Electrochemical techniques provide copious amount of advantages within the medical field and can further the development of point-of-care devices (POC). Sensing techniques such as amperometry, potentiometry and voltammetry can be highly sensitive, simple, low cost, portable and, most importantly, allow for real-time detection of biomarkers, unlike the previously mentioned assays. However, these techniques are less frequently used (Gug et al., 2019). These electrochemical assays can be applied through biosensors for the detection of the salivary biomarkers. The sensor often requires voltage actuation and current readout. Numerous techniques exist, for example cyclic voltammetry, has been applied to a glucose biosensor (Rassas et al., 2019). Cyclic voltammetry varies the voltage applied by sweeping it cyclically from a minimum to a maximum value between electrodes. A current is then measured between two electrodes and current vs voltage is plotted (voltammogram). Identification and concentration of the analyte from saliva can be gathered from the voltammogram (Mirceski, Skrzypek and Stojanov, 2018).
Electrochemical biosensors are a promising tool for the detection of salivary biomarkers, especially when comparing to the traditional use of ELISA. Although, there are still many drawbacks including interferences affecting the electrochemical assays and the difficult circuitry used when miniaturizing the system. Nonetheless, the advantages previously mentioned, particularly the real-time detection alongside decreased limits of detection and higher selectivity for analytes outweigh the advantages presented by ELISA (Gug et al., 2019). Thus, the importance of developing electrochemical sensors is rising as it could provide faster, more reliable disease diagnosis and monitoring.

2.6 Summary

In this chapter, I briefly review the potential of salivary biomarkers for the detection of disease and health conditions. I also discuss the potential of salivary glutamate as a biomarker for pain and TBI. There are currently only subjective methods of identifying pain, thus measuring biomarkers, such as glutamate, could allow for better pain detection (Cantón-Habas et al., 2019). Studies have shown that the levels of glutamate concentration within saliva change with pain (Amrapala, 2019), however, there is currently no technology that can measure this change in real-time. Therefore, to develop a non-invasive point-of-care biomolecule measurement device, the use of saliva as a diagnostic medium is ideal. Here the best measurement method, electrochemical technique, should be selected to allow fast, real-

time measurement. This project will focus on designing a wireless device for electrochemical measurement of glutamate in saliva.

2.7 Aim, Research Question and Hypothesis

To date, there is no study specifically focusing on the development of an intraoral glutamate biosensor. Thus, the main aim of this study is to develop the design of a wireless wearable intraoral glutamate sensor to measure glutamate continuously and real-time, whilst meeting the derived user requirements.

Hence, the research question is: based on already published glutamate sensor recipes and methods to develop the instrumentation electronics, wireless telemetry and biocompatible packaging, would it be possible to develop a wireless wearable intraoral glutamate sensor that satisfies the user requirements?

Our hypothesis is that: it is feasible to design a wireless intraoral glutamate biosensor device for continuous real-time measurements based on already published recipes and methods for the individual building blocks.

3. Methods

In this chapter, I explain how I have conducted this project, including how I found and designed the different building blocks of the intraoral wearable device.

Firstly, I derived the user requirements for a device that is capable of real-time and continuous measurements of glutamate. These were drawn mainly from literature and from previously submitted coursework. Consequently, based on the user requirements, the device specifications were developed. Subsequently, I considered the building blocks of the device; the biosensor itself, the electronics, and packaging, each being designed individually.

For the biosensor design, I conducted a literature review on previously published recipes for glutamate sensor fabrication. Based on the user requirements, I selected the most suitable recipe.

Furthermore, for the instrumentation, data communication, and power source, I conducted a separate literature review and summarized the outcomes. This comprised of already published designs for intraoral devices, either with or without embedded electronics. Subsequently, I selected the most suitable design to start with. Furthermore, improvements to the power supply were made, based on other previously designed wearable wireless devices.

The combination of these components, alongside the mouthguard platform were designed based on the previously published intraoral devices. The design for the packaging of the device was also investigated by conducting another literature review. This focused on biocompatible materials, which can be used for packaging medical devices, specifically the instrumentation, and withstand the harsh environment of the mouth.

The image of the proposed device was designed by combining components from the literature. The mouthguard platform and instrumentation were sourced from previously designed intraoral devices. The biosensor design was also established from previously published literature on glutamate sensors. Lastly, I combined the components and designed the device using tools within Microsoft Word.

4. User Requirements and Device Specifications

The information described below has been in part previously submitted as coursework (SURG0091), describing the user requirements and device specifications. Thus, the same justifications and parameters are presented below (Fomenko, 2019).

4.1 User requirements

Similar to the previously designed intraoral devices, the biosensor along with the electronics and wireless transmission, will be packaged together. To provide comfort for the user, the device should be attached on to the back of a flexible mouthguard for the lower jaw. This would also allow for easier access to the salivary glands, thus sufficient saliva would contact the sensor (Arakawa et al., 2016). However, the glutamate sensor must also be highly flexible so when it is attached, it can follow the natural oral cavity and the mouthguard's curvature. For it to remain non-invasive, the sensor and device itself must be small, thus large volumes of saliva would not be required by the user. Additionally, the device should be cheap.

Furthermore, rapid measurement and detection can be achieved by rapid response time, as well as high selectivity and sensitivity to the low salivary glutamate levels. It is essential for the mouthguard, as well as the device to be biocompatible to prevent excretion of toxic materials and the consequential inflammatory response (Gray et al., 2018). To reduce this risk overall, the sensor should be disposable, more precisely it can be taken out and replaced. This would ease the use, increase safety of the device, and improve hygiene, as the mouthguard can be washed. Although, the device should still have a lifetime long enough to provide accurate measurements and withstand the experiment (>2 hours). Another crucial requirement is the biostability of the device. The physiological pH range of saliva can drop either below 5.5 or above 8 (Kubala et al., 2018). Consequently, increasing the risk of damage to the sensor and inaccurate results. Equally important is high specificity as secreted proteins, alongside active chemicals produced from food residues, can interfere with glutamate detection, thus providing incorrect measurements (Yang and Gao, 2019).

The device specifications are based on the user requirements and can act as targets throughout the device development. Most of the specifications presented are quantitative, however, some qualitative information has been described as a result of limited information available.

4.2 Device specifications

The device should consist of a custom-fitted mouthguard platform in respect to the normal human mandible size with females ranging from 16.44-18.67 cm and males from 17.22-19.33 cm (Olayemi, 2011). The mouthguard support material should comprise of a 2mm thick transparent

polyethylene terephthalate (PET) substrate from Sigma-Aldrich, as this provides flexibility, biocompatibility and low cost (158 GBP), meeting the user requirements (Jeerapan and Poorahong, 2020; Wang, Hou and Wang, 2020).

To achieve high sensitivity, the device must detect the low levels of salivary glutamate, during normal, 7.51 ng/μL (+/- 2.5), and pain induced, 7.43 ng/μL (+/- 4.2), states (Amrapala, 2019). Conversely, in μmol/L 51 (+/- 17) μM and 50 (+/- 29) μM. In contrast, other studies have presented lower normal baseline glutamate concentrations in saliva, varying from 10-33 μM (Shimada et al., 2016; Jasim et al., 2014; Jasim et al., 2018). The lowest detected concentration of normal salivary glutamate was 0.232 (+/- 0.177) μM and the highest concentration of salivary glutamate during pain states was 70.2 μM (Jasim et al., 2018; Jasim et al., 2014). Thus, the sensors limit of detection, which is the minimum accurately measured glutamate concentration, should be lower than 0.232 (+/- 0.177) μM. A wide linear detection range is also crucial to incorporate both normal and pain states, thus if the concentration of glutamate is varied from 0-200 μM, the desirable linear detection range would be from 0.5-150 μM.

The surface area of the working electrode (WE) should be large enough (~20mm^2) so the noise produced is reduced and sensitivity is increased (Schultz et al., 2020; Ciui et al., 2019; Arakawa et al., 2016). As the sensor should be small to satisfy the user requirements, the dimensions should follow the length and width of the second mandibular molar. Thus, the maximum dimensions of the sensor would comprise of 1.5x1cm (length x width) (Singh and Goyal, 2006; Barbería et al., 2009). The device should also include a fabricated amperometric printed circuit board with dimensions 1.8x1.9cm (length x width), thus when combined with the sensor, the device can be considered small and non-intrusive (Kim et al., 2014). In addition, the required response time should be <10 seconds when the concentration of glutamate is ~ 20 μM (Schultz et al., 2020).

5. Literature on existing devices

In this chapter, I briefly overview the current existing intraoral wearable devices and their building blocks. The gaps in the literature are identified and discussed. Consequently, referring to the existing literature, the projects' aim and hypothesis for the development of a wireless device for glutamate measurement are stated.

5.1 Existing intraoral wearable devices

A review of the literature shows an exceptionally low amount of previously published work on intraoral wearable devices. The table below lists and describes all of the successful devices and their features, including the various biomarkers detected, ranging from metabolites to electrolytes (Table 1.; Figure 5). As intraoral wearable devices are an emerging technology, there are many diseases and their biomarkers are still to be explored, such as glutamate and its detection as a result of pain. However, there are many gaps in the literature concerning e.g. how to further miniaturize the circuit, wireless transceiver, and battery for acquiring and processing

data during in-mouth monitoring. Also, the application of anti-fouling materials to provide protection, increasing biocompatibility and reducing toxicity.

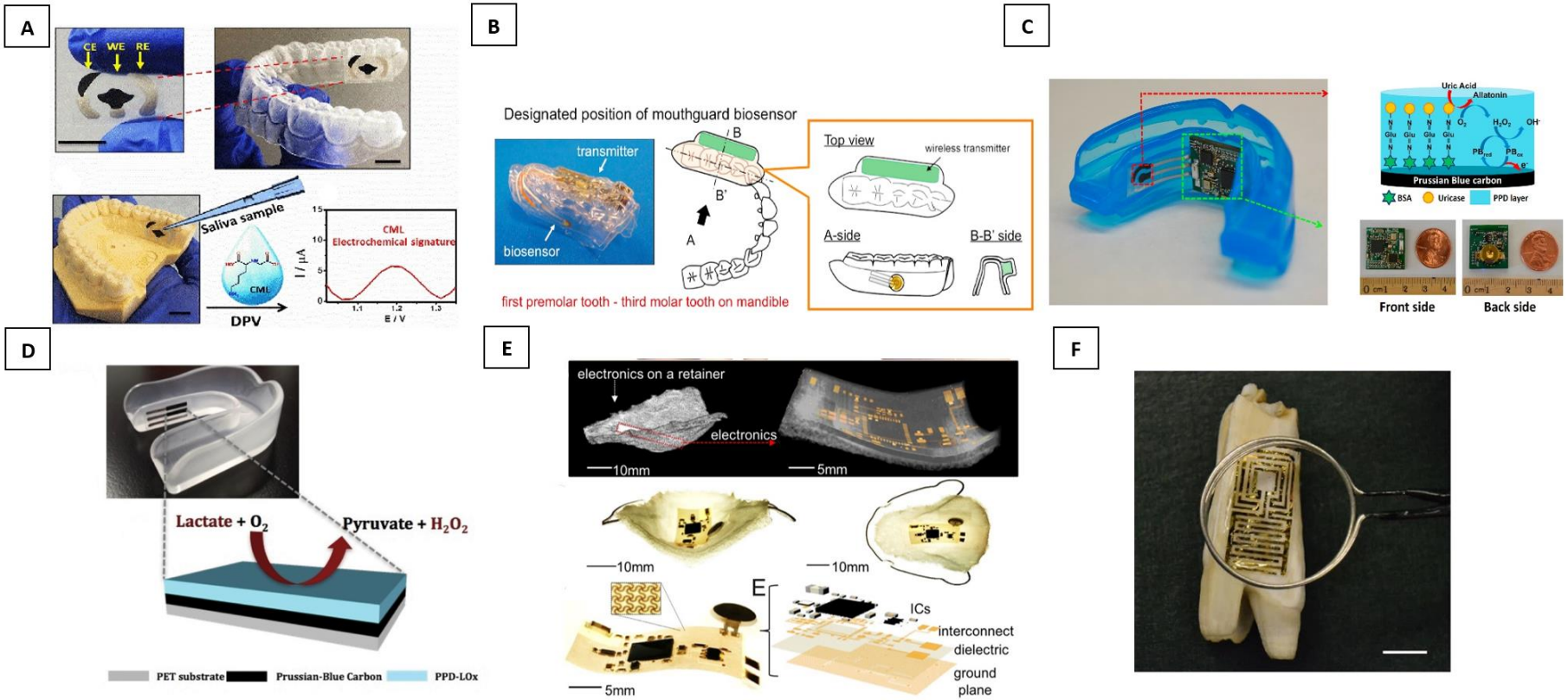

Figure 5. Existing intraoral devices (Figure adapted from

A) CML biosensor consisting of the screen-printed three-electrode system and further illustrated to be placed on the lower jaw of the mouthguard. The mouthguard biosensor was placed onto a phantom of a human mandible. To detect CML, the DPV technique was applied and an electrochemical signature was recorded.
B) Mouthguard biosensor device, consisting of the glucose biosensor and a polyethylene terephthalate glycol encased wireless transmitter incorporating a potentiostat. The mouthguard biosensor is designed to be placed on the lower jaw dentition, starting from the first premolar and ending at the third molar.
C) Mouthguard salivary uric acid biosensor, consisting of a Prussian-Blue carbon working electrode encompassing uricase. Alongside, an integrated wireless amperometric circuit board.
D) Mouthguard biosensor with the combined screen-printed three-electrode system and a schematic illustrating the Poly-orthophenylene diamine (PPD) – Lactate oxidase (Lox) layer coated onto the Prussian-Blue working electrode.
E) An X-ray of the oral retainer encompassing the intraoral electronics and the porous membrane with the colorized circuit interconnects. The oral retainer with the electronics shown from top and back, alongside zoomed-in photographs of the wireless electronics, consisting of ICs, mesh interconnects, a dielectric layer, and ground plane.
F) Graphene-based wireless nanosensor on the surface (enamel) of a human tooth.

Table 1. Summary of previously published intraoral devices with and without embedded electronics.
WE – working electrode, RE – reference electrode, CE – counter electrode, ISEs – ion selective electrodes, ADC – analogue to digital converter Pt – platinum, PMEH - poly(MPC-co-EHMA), PDMS- Polydimethylsiloxane, PPD - poly-orthophenylenediamine , Pd - palladium

Reference	Measured analyte	Electrochemical method	Sensor material	Instrumentation	Data communication	Packaging	Power	Wearable	Design
(Ciui et al., 2019)	N-epsilon (Carboxymethyl)lysine (CML)	Differential pulse voltammetry (DPV)	Screen-printed on a flexible foil: RE and contacts for interfacing the electrodes to the electrochemical analyzer – Ag/AgCl conductive ink WE and CE – carbon ink	No electronics embedded	No	No	No	Mouthguard	Figure 5A
(Arakawa et al., 2016; Mitsubayashi and Arakawa, 2016)	Glucose	Amperometry	Sensor electrodes: Pt WE – glucose oxidase and PMEH, insulated with PDMS Ag/AgCl RE/CE – Insulated with PDMS	No electronics embedded	Wireless transmitter (Blueetooth 4.0 low-energy) with an ADC	Transmitter – encased in PETG and sealed using a dental composite resin	LR41 1.5 V battery	Monolithic mouthguard	Figure 5B
(Kim et al., 2015)	Uric acid	Chronoamperometry	Screen-printed on a flexible PET substrate: RE and current collector – Ag/AgCl conductive ink WE and CE – Prussian-blue-graphite ink WE - uricase enzyme,	Embedded electronics: LMP910000 AFE, program through the I^2C interface	CC2451 BLE system-on-chip 2.45GHz chip antenna (2450AT42A100) and impedance matched balun (2450BM15A0002)	Medical adhesive	Two 396/397 watch batteries (2 x 1.55 V, 33 mAh each) in series TPS61220 boost converter and LM4120 low-dropout voltage regulator	Mouthguard	Figure 5C

			antibiofouling membranes, PPD Insulator layer – dielectric paste						
(Kim et al., 2014)	Lactate	Chronoamperometry	Screen-printed on a flexible PET substrate: RE and contacts for interfacing the electrodes to the electrochemical analyzer – Ag/AgCl conductive ink WE and CE – Prussian-Blue-graphite ink WE - lactate oxidase and PPD film Insulator layer – dielectric paste	No electronics embedded	No	No	No	Mouthguard	Figure 5D
(Lee et al., 2018)	Sodium	No	ISEs were made by electroplating Pd (WE) and Ag/AgCl (RE) on a copper membrane	Electronics embedded: Single channel Op-Amp IC (AD8603AUJZ-REEL7) with low pass filter	2.4 GHz wireless System on chip for Bluetooth (NRF51422-QFAC-R7) Antenna chip (2450AT18A100E) with impedance matching network, Balun filter (2450BM14E0003T)	Breathable elastomeric membranes (silicone elastomer)	Micro-lithium rechargeable battery (3.3 V with 5.5 mAh) (MS621FE-FL11E)	Dental retainer	Figure 5E

					ADC and Microcontroller				
(Manoor et al., 2012)	Bacteria	No	Graphene nanosensor – printed onto water-soluble silk thin-film substrates and contacted by interdigitated electrodes	No	LRC resonant circuit with a gold two-turn inductive coil	No	Inductive two-turn coil antenna and a frequency response analyzer (HP 4191A RF impedance analyzer).	Silk dental tattoo platform on tooth enamel	Figure 5F

6. Results

6.1 Glutamate sensor

Biosensors can either be fabricated on a traditional rigid electrode or on a flexible screen-printed electrode (SPE). If using the rigid electrodes, movement and integration with the mouthguard will be restricted. In contrast, screen printing onto a flexible substrate, e.g. PET, would satisfy the user requirements and increase sensor flexibility within the mouthguard. Screen-printing techniques can rapidly produce (~30 min) highly uniform, reliable and reproducible sensors (Couto, Lima and Quinaz, 2016; Ciui et al., 2019). As the sensor should be disposable, sensor reproducibility is imperative. Moreover, SPE-based sensors are inexpensive, have low detection limits and can be potentially miniaturized (Couto, Lima and Quinaz, 2016). Consequently, this potential and the ability to control the dimensions of the SPEs could produce a small sensor with the desirable dimensions (Ciui et al., 2019). Hence, SPEs-based sensors meet the user requirements proficiently. SPEs should be printed using Ag/AgCl ink for the RE, as it is easy to print (Jeerapan and Poorahong, 2020). Carbon or graphite ink should be employed for the WE and CE, as carbon materials are multipurpose, have a lower background current and are chemically inactive (Couto, Lima and Quinaz, 2016). The SPEs should be printed directly onto the flexible PET substrate, aligned against the curvature of the mouthguard support and improving detection of biomarkers (Jeerapan and Poorahong, 2020; Wang, Hou and Wang, 2020).

High selectivity and sensitivity were attained by integrating Prussian-blue (PB) into the printable ink for the WE. This is due to PB acting as a ‘artificial peroxidase’ increasing selectivity in hydrogen peroxide reduction and lowering working potential (Karyakin, Karyakina and Gorton, 2000; Kim et al., 2014). Biofouling around the sensor commonly occurs and could potentially also trigger inflammation. Subsequently, to prevent this, anti-biofouling membranes have been applied (Rocchitta et al., 2016). Highly biocompatible materials and thin membranes (~0.1 μm), such as Nafion (perfluorinated sulfonated membrane) or poly-orthophenylenediamine (PPD) have shown to decrease electroactive interferences (e.g. ascorbic acid), protect the biosensor surface, and restrict biofouling through immobilization of enzymes, such as glucose oxidase or glutamate oxidase (Rocchitta et al., 2016, Hamdan and Zain, 2014; Clay and Monbouquette, 2017). Subsequently, the lifetime, biostability, specificity, and selectivity of the sensor increases. The use of sol-gels has also shown to enhance the lifetime and sensitivity of the biosensor (Rocchitta et al., 2016).

Analysis of the literature presented multiple studies incorporating glutamate sensors in various biofluids, however only a limited number have been tested in human biofluids. As there are many gaps concerning the biocompatibility of the sensor and device itself, it would be necessary to choose a recipe to meet the user requirements and bridge this gap. Therefore, a summary of previously published electrochemical enzymatic glutamate sensors was created to find the most

suitable glutamate biosensor recipe (Appendix A). Following the recipe provided by Tian et al. (2009), the optimum desired glutamate biosensor for real-time monitoring can be designed (Figure 6.). Tian et al. (2009) presented a glutamate microbiosensor with a low LOD (0.005 μM) and a wide LDR (0.5-100 μM), whilst varying the concentration from 0-140 μM. In addition, the sensor presented high sensitivity (279.4 ± 2 μA mM^{-1} cm^{-2}), high selectivity and a fast response time of 10 seconds, meeting the user requirements. Biocompatibility and long-term stability were also ensured by entrapping the L-glutamate oxidase enzyme in a silica gel layer using a sol-gel electrodeposition method.

Hence, following the recipe provided by Tian et al. (2009), the glutamate biosensor should be fabricated as followed. A three-electrode cell encompassing a platinum foil CE and an Ag/AgCl RE should be used. The working electrode should consist of a platinum microelectrode with a length of 0.5mm, 50 μm diameter and 7.85 x 10^{-4} cm^2 surface area. It should then be coated with poly(phenylenediamine) through the use of cyclic voltammetry, scanning from 0.2-0.8 V for 6 cycles at a scan rate of 10 mV s^{-1} . This should be done in a 10 mM phenylene diamine solution in 100 mM phosphate buffer pH 7.4. Afterwards, under a mild chemical environment, entrapped L-glutamate oxidase in a silica gel layer should be electrodeposited onto the WE using a fast sol-gel electrodeposition method. This method is described by Vasylieva and Marinesco (2013). Consequently, all three electrodes should be inserted into a glass capillary consisting of the defined solution by Vasylieva and Marinesco (2013) for the robust gel layer. Subsequently, under potentiostatic conditions, a reduction potential should be applied between -0.9 to – 1.2 V for the time range of 10-40 s to complete the electrodeposition. The biosensors should be stored in phosphate buffer solution, pH 7.4.

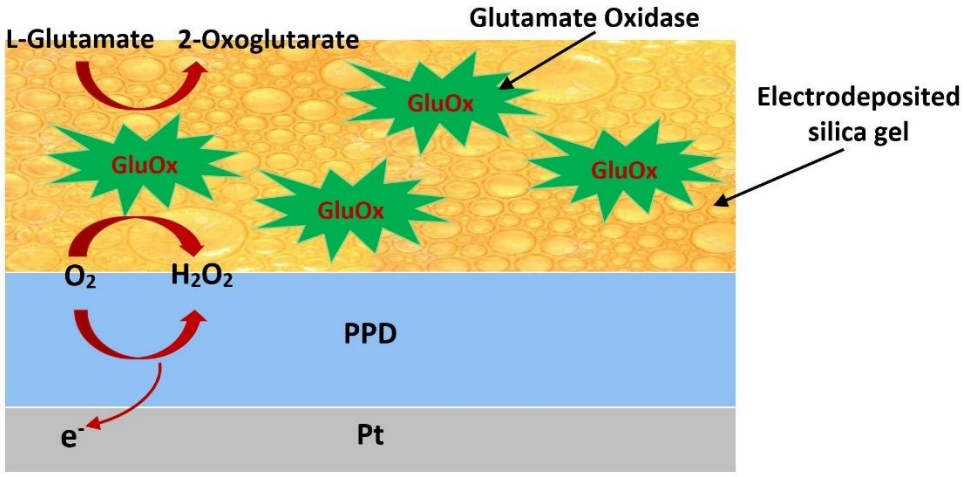

Figure 6. Glutamate biosensor consisting of a platinum microelectrode, PPD layer and silica gel layer with entrapped glutamate oxidase (Adapted from Vasylieva and Marinesco, 2013).

6.2 Instrumentation, data communication and power

For wireless real-time continuous measurement of salivary glutamate, a wireless ameprometric printed circuit board (APCB) should be fabricated (Kim et al., 2015). To enable wireless communication to a laptop, smartwatch, or smartphone, a Texas Instrument (TI) CC2541 Bluetooth low-energy (BLE) system-on-chip combined with a microcontroller should be employed (Søpstad, 2019; Imani et al., 2016; Kim et al., 2015). The CC2541 employs a built-in 12-bit analog-to-digital converter. To determine glutamate concentration, an on-board potentiostat must be implemented. Thus, an amperometric and voltammetric LMP91000 Analog Front End (AFE), which is programmable through an I^2C interface driven by the CC2541, should be used (Figure 7; Søpstad, 2019; Imani et al., 2016; Kim et al., 2015). The AFE is based upon a control amplifier, controlling the cell potential, and a transimpedance amplifier, which reads out the current as a voltage (Søpstad, 2019). It interfaces with both the electrochemical sensor and CC2541 (Figure 7). The CC2541 firmware can be calibrated and configured for the specific scenario through the I^2C interface. The board should be programmed to take measurements every 2 minutes (Kim et al., 2015). Furthermore, to achieve wireless transmission A Johanson Technology 2.45 GHz chip antenna (2450AT42A100) and impedance-matched balun (2450BM15A0002) should be used (Imani et al., 2016; Kim et al., 2015). Acting as a power source, a CR2032 button cell lithium battery (3 V, 220 mAh) should be incorporated. The battery voltage can be regulated via a TPS61220 boost converter (Imani et al., 2016).

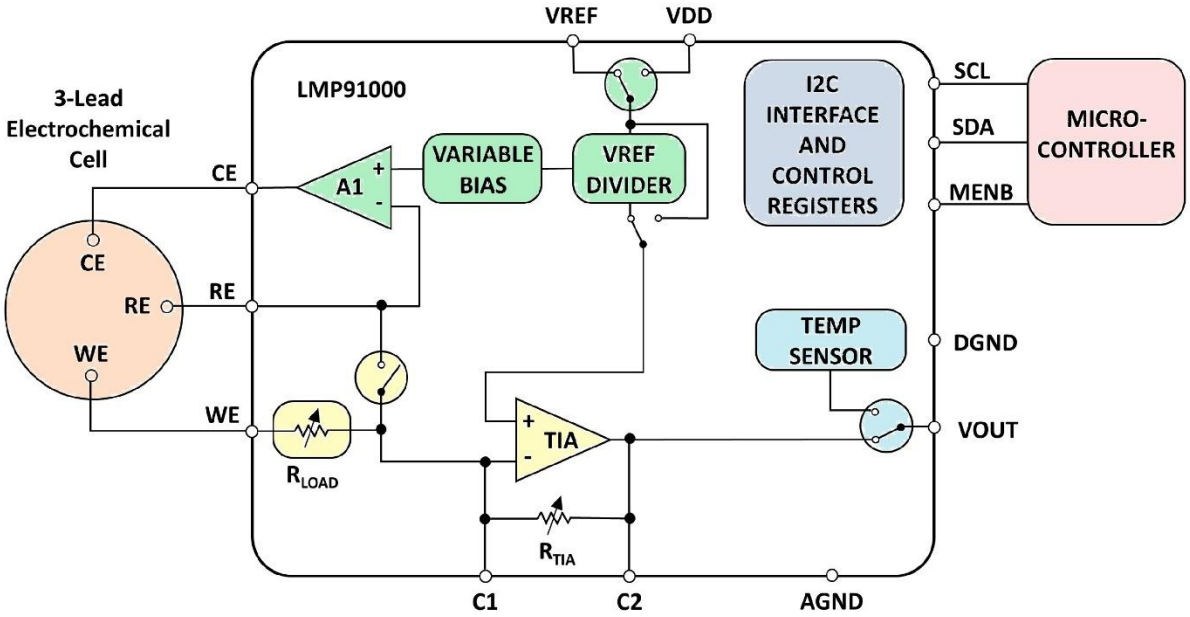

Figure 7. System block diagram of AFE

6.3 Packaging

The packaging film enclosing the complete device must satisfy the user requirements, including the lifetime and survival of the device. It is crucial to have an insulated, sealed biocompatible packaged device so no harm is caused to the user by electrical leaks. In addition, to prevent saliva from entering and damaging the device itself, more specifically the instrumentation (Teo et al., 2016).

Consequently, to securely immobilize and attach the fabricated biosensor and APCB on the interior site of the PET mouthguard platform, a medical adhesive (Loctite) should be employed (Kim et al., 2015). Each electrode should be attached to a stainless-steel conductive wire and soldered to connect to the APCB (Kim et al., 2015). Insulator tape should be employed to cover interconnects, thus decreasing the risk of any short-circuits (Ciui et al., 2019). An example of how the envisioned device would look before application of the packaging is demonstrated in Figure 8. The APCB should be encapsulated in the same material as the mouthguard platform (PET). Similarly, the biosensor should also be encased in PET, as it is incorporated within a small glass capillary, which could potentially harm the user. However, the biosensor needs to be in contact with saliva for glutamate detection, thus, a PET mesh covering the biosensor, with pores small enough for glass to not leak out and big enough for glutamate molecules to pass through, should be employed. To seal the edges of the packaging, a biocompatible dental flowable composite resin should be used and then dried under Ultraviolet light for 20 seconds (Ciui et al., 2019). This would prevent the glass and components from the biosensor to leak out into the body.

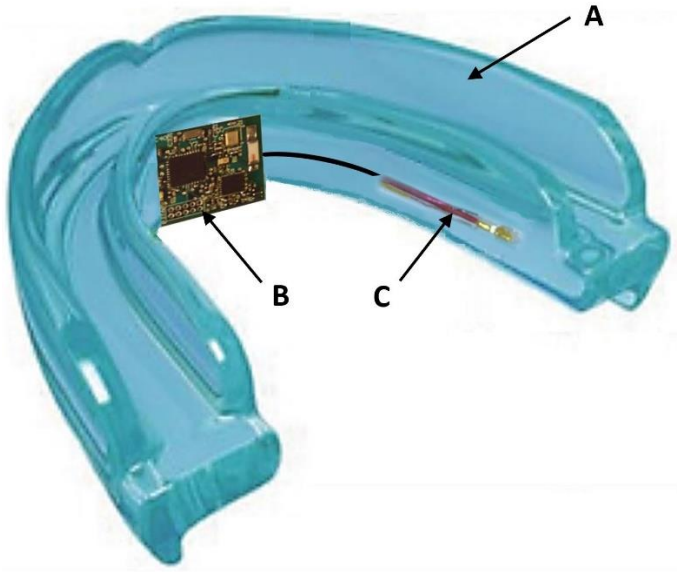

Figure 8. Design of the proposed device before packaging (Adapted from Kim et al., 2015)
A. PET mouthguard platform
B. Fabricated amperometric printed circuit board
C. Glutamate biosensor (in a glass capillary)

7. Discussion

The main aim of this study was to develop a design of a wireless, wearable, intraoral glutamate biosensor that can measure glutamate continuously in real-time, whilst meeting the derived user requirements. Hence, the research question was if this is possible based on already published glutamate sensor recipes and methods to develop instrumentation, wireless telemetry and biocompatible packaging. Consequently, the analysis of the literature allowed for the development of the proposed device that satisfies the user requirements. Additionally, the study demonstrates the design of the device following previously published methods and recipes for the individual building blocks (Figure 8).

It was hypothesized that it would be feasible to develop a wireless wearable intraoral glutamate biosensor device for continuous, real-time measurements based on already published recipes and methods for the individual blocks.

7.1 Glutamate sensor

The academic field has extensively explored the development of glutamate sensors, as glutamate is an important neurotransmitter that has been linked to pain and various diseases. As result of this, various differing recipes have been used to fabricate glutamate sensors. Thus, a range of these have been summarized based on a few of the derived user requirements (Table). Analyzing the literature and table, the highest sensitivities, lowest LOD, widest LDRs and fastest response times were demonstrated by Batra, Kumari and Pundir (2014), Tian et al. (2009) and Liu et al. (2014).

Batra, Kumari and Pundir (2014) constructed their glutamate sensor by immobilizing glutamate oxidase (GluOx) on a gold (Au) electrode modified with polypyrrole nanoparticles (PPyNPs) on a polyaniline composite film. The sensor presented with a very high sensitivity of 533 nA μM^{-1} cm^{-2}, a very low LOD (0.0001 μM), a fast response time (3s) and a wide LDR (0.02-400 μM), whilst varying the concentration from 0.02-1000 μM. Liu et al. (2014) fabricated the biosensor using a graphite electrode modified with Prussian blue (PB) film and immobilized GluOx. The sensitivity obtained was 238 nA μM^{-1} cm^{-2}, the LOD was 0.01 μM, the response time was 3 seconds and the LDR was 10-100 μM, whilst varying the concentration from 0-500 μM. In contrast, Tian et al. (2009) fabricated a glutamate biosensor using a platinum (Pt) microelectrode coated with poly(phenylene diamine) and electrodeposited silica gel layer with entrapped L-GluOx. Thus, the sensitivity of the sensor was 279.4 ± 2.0 μA $(mmol\ L^{-1})^{-1}$ cm^{-2}, the LOD was 0.005 μM, the response time was 10 seconds and the LDR was 0.5-100 μM, while the concentration was varied from 0-140 μM.

As previously stated, the lowest detected salivary glutamate concentration was 0.232 (+/- 0.177) μM. Therefore, if the error (+/-0.177) is considered, the LOD should be lower than 0.055 μM.

All three recipes meet this requirement, with the lowest being presented by Batra, Kumari and Pundir (2014). Thus, to evaluate the optimal glutamate sensor, the LDR, sensitivity and response time are compared. Liu et al. (2014) in comparison to the other two recipes, exhibited the lowest sensitivity and the highest LOD, thus was excluded. In addition, biocompatibility and prevention of biofouling are also crucial requirements. Both recipes ensure this by applying polymeric films, blocking interferents and restricting biofouling via immobilization of GluOx (Rocchitta et al., 2016, Hamdan and Zain, 2014; Clay and Monbouquette, 2017). Based on these requirements, Batra, Kumari and Pundir (2014) present a glutamate sensor with the most desirable properties. However, it was stated that the fabricated sensor experienced challenges when in the detection of glutamate in saliva (Schultz et al., 2020).

Additionally, the flexibility and size of the sensor are also critical requirements. However, neither recipes fabricated the sensor on a flexible substrate. An alternative to a flexible sensor, could be a sensor small enough so there is no need to follow the curvature of the mouthguard as well as to maintain non-invasiveness. Therefore, the optimum glutamate sensor recipe chosen was by Tian et al. (2009). This is due to the size of the Au electrode being 2cm x 1mm, whereas the Pt microelectrode had a length of 0.5mm and a diameter of 50 μm (Batra, Kumari and Pundir, 2014; Tian et al. 2009). Thus, the size of the Pt working electrode was considerably smaller. The smaller sensor size allows more volume of saliva to come into contact with it, thus increasing the possibility of better detection of salivary glutamate.

In summary, the optimal recipe for developing an intraoral glutamate biosensor whilst meeting the user requirements, was provided by Tian et al. (2019). The sensor provides high sensitivity, low LOD, wide LDR and a fast response time. It is also biocompatible and prevents biofouling due to the polymeric membrane and silica-gel layer. Lastly, the size of the sensor is also imperative, satisfying the requirements. Thus, using already published glutamate sensor recipes, it is possible to fabricate an intraoral glutamate biosensor, whilst meeting the user requirements. This answers the research question and confirms the hypothesis in part.

7.2 Instrumentation, data communication and power

Reviewing the literature on already published intraoral devices, allowed for identification of an amperometric printed circuit board (APCB) (Kim et al., 2015). This consisted of a BLE system-on-chip combined with a microcontroller, thus removing the requirement for an additional integrated circuit. The built-in 12-bit ADC allows signals from the sensor to be relayed to a Bluetooth transceiver. BLE is most commonly employed in wireless biosensors for continuous real-time measurements due to the decreased power consumption. As well as, the ability to send information wirelessly to a distance of about 100 meters. In contrast, near field communication has also been employed in wireless sensors, however communication requires close proximity (Kim et al., 2019). Comparing the two, provided identification that Bluetooth is the best option to allow real-time continuous measurements with good spatial range and low power consumption (Taffoni et al., 2018). The Texas Instrument BLE is also small in size with dimensions being 6x6mm, meeting the user requirements. The amperometric AFE driven by the BLE is employed, as it is one of the only commercially available on-board potentiostats. The AFE has a low power consumption, ensuring long battery life, low cost and small in size (4x4mm) (Jasinski, Strzelczyk

and Koscinski, 2016;). The transimpedance amplifier gain and cell bias can be adjusted and programmed through the I^2C interface. For wireless transmission (BLE transmission line), a chip antenna and impedance matching balun are incorporated. Thus, the method for fabricating an APCB provided by Kim et al. (2015) satisfies the user requirements by providing real-time continuous measurements. However, an improvement to the power source could be by incorporating one lithium cell-battery, instead of two 396/397 watch batteries (Imani et al., 2016). This is due to the lithium battery having a higher energy density and lower cost (Lisbona and Snee, 2011). A boost converter is employed to step up the voltage from the input to the output.

To sum up, an APCB can be fabricated from existing wireless devices and previously published designs of intraoral devices. Thus, providing wireless real-time continuous measurements. In addition, due to the BLE, AFE and battery (20x3.2 mm) all having small dimensions, the user requirements are satisfied. Therefore, the hypothesis is confirmed in part.

7.3 Packaging

Whilst reviewing the literature, limited information could be found regarding the packaging of intraoral biosensors. However, polymeric biomaterials have been studied for packaging medical implants and devices. Hence, by encasing the device with biocompatible and biostable polymers, salivary components and moisture would be prevented from interfering with the devices' electronic circuit (Teo et al., 2016; Modjarrad and Ebnesajjad, 2013). Polyethylene or a polymer blend, such as PET, are commonly employed as flexible substrates and packaging for wearable sensors (Teo et al., 2016; Modjarrad and Ebnesajjad, 2013). Thus, PET, which provides good chemical resistance and moderate moisture absorption, can be employed not only as the mouthguard platform, but also as the packaging of the device (Wang, Hou and Wang, 2020). Subsequently, toxic materials would not leak out and biocompatibility and biostability of the device would be ensured. In addition, as the mouthguard platform and the packaging would both consist of the PET substrate, the packaging will adhere to the platform more successfully.

Furthermore, from previously published intraoral designs, components to enable attachment and sealing, were chosen based on biocompatibility. Thus, a medical adhesive would provide attachment of the sensor and device to the mouthguard platform (Kim et al., 2015). Whereas, a biocompatible dental resin would tightly seal the edges of the packaging (Ciui et al., 2019).

Moreover, the biosensor fabricated using the recipe provided by Tian et al. (2009) is incorporated within a small glass capillary. Thus, to ensure user safety and meet the user requirements, the intraoral glass capillary sensor should also be packaged. This packaging for the sensor should prevent damage to the user, whilst keeping sufficient contact between the biosensor and saliva. Since this has not been previously investigated, I propose the use of PET mesh. Similarly, by applying the same biocompatible polymer material, the adhesion to the mouthguard will be simpler. The mesh should have pores small enough to prevent the glass from leaking out, in case of damage, but big enough to allow salivary glutamate molecules to easily pass through. An example of PET mesh application has been demonstrated in for patients with acute myocardial infarction, where a PET micronet mesh was used to cover metal stents (Stone et al., 2012). Consequently, to ensure safety due to the glass element, the application of PET

mesh could successfully meet the user requirements whilst protecting the user. Given these points, a biocompatible packaging can be developed based on the literature and previously published designs. Thus, partially confirming the hypothesis.

Overall, the results have indicated that it would be feasible to develop a wireless wearable intraoral glutamate biosensor device for continues real-time measurements based on already published recipes and methods for the individual blocks, confirming the hypothesis.

7.4 Limitations and Future Work

Although the hypothesis was confirmed and the main aim of the project was met, there are many limitations to be addressed before fabricating this device.

Firstly, following the chosen optimal recipe, the glutamate biosensor would be fabricated within a small glass capillary. For in-vitro monitoring or monitoring in rat ECF, the use of a glass capillary microelectrode would be satisfactory. However, as the hypothesized device is for intraoral use, a glass capillary would not be satisfactory. Not only would this decrease user safety, but also decrease flexibility of the whole device, and subsequently diminishing user comfort. Although, the proposed PET mesh package could potentially reduce the risk of harm, it could also present with further consequences. For example, the application of the PET mesh would reduce the amount of saliva the biosensor comes into contact with. Thus, potentially hindering the sensitivity, LOD, LDR and response time of the glutamate sensor. Therefore, I would propose to use SPEs as an alternative method to the Pt microelectrode but using the same recipe. Initially, platinum inks for both the WE and CE and Ag/AgCl ink for the RE would be used to print the electrode. As previously mentioned, SPEs have many advantages. Thus, for better flexibility, the SPE can be printed directly onto a PET substrate. Consequently, integration of sensor onto mouthguard platform would improve by following the natural oral cavity curve (Jeerapan and Poorahong, 2020; Wang, Hou and Wang, 2020). To maintain consistency, the SCE can match the dimensions of the Pt microelectrode (0.5mm length, 50 μm diameter). After screen-printing, the WE can be modified following the same recipe, forming the PPD and silica gel layer with entrapped GluOx.

Secondly, the use of a lithium-based battery reduces mechanical flexibility of the APCB and potentially provide toxicity issues (Jeerapan and Poorahong, 2020; Kim et al., 2019). An alternative to this could be applying batteries, which have been fabricated on flexible and stretchable materials. However, these do not exhibit suitable energy densities over a long time period (Kim et al., 2019). Thus, further research should be conducted into finding a flexible energy source, which matches the energy density of the proposed lithium battery.

Lastly, the glutamate sensor has only been tested in Rat ECF, therefore there may be a possibility that the sensor will have issues with the detection of salivary glutamate. If so, this can be improved by rendering the recipe using other electrode materials, such as screen-printed carbon electrodes. It is also important to consider the interface between the sensing material and substrate. A mismatch in surface-energy between these could decrease the stability of the biosensor (Jeerapan and Poorahong, 2020).

Future work into developing a product development protocol would be necessary to fabricate the envisioned device. Specifically, describing step by step how to construct the device, starting from the sensor and ending with the biocompatible packaging. Afterwards, the testing of the device should be conducted. This would include testing the sensitivity, specificity, response time, LOD and sensor to sensor reproducibility. The device should then be validated by comparing the biosensor outputs to the outputs generated by the traditional ELISA method. The following data analysis should be performed. Afterwards, even though all the user requirements have been addressed and met, an assessment on safety should be conducted, focusing on the biocompatibility, biostability, sterilization and risk of toxicity of the device.

8. Conclusion

This study hypothesized if it would be feasible to design a wireless intraoral glutamate biosensor device for continuous real-time measurements based on already published recipes and methods for the individual blocks. Analysis of the literature allowed for the detection of the best glutamate biosensor recipe, which satisfies the user requirements. This biosensor consisting of a platinum microelectrode presented high sensitivity, a low limit of detection, a wide linear detection range and a fast response time. It also prevents biofouling and ensures biocompatibility through the use of polymeric membranes, such as PPD. This was chosen as the best glutamate biosensor recipe due to the small dimensions of the electrode, thus providing better integration within mouthguard and remains noninvasive. The best method, with an improvement to the power source, to enable wireless, real-time continuous measurements was also described. This was employed by an amperometric analogue front end driven by a Bluetooth low energy (BLE) system-on-chip on a printed circuit board, powered by a lithium battery. The BLE transmission line consisted of an impedance-matched balun and a chip antenna. These findings were based on previously published wireless intraoral devices. The biocompatible packaging for the device ensures saliva from entering and damaging the electronics, as well as preventing toxic materials from leaking out. Thus, PET was selected to encapsulate the device, matching the mouthguard platform. However, due to the glass element within the glutamate biosensor, a PET mesh should be applied as a cover to provide user safety. Overall, these results have confirmed the hypothesis, however crucial limitations need to be addressed. The glass element within the intraoral device presents high risk to user safety and thus the biosensor should be altered to have a SPE. Although the design was developed, extensive future work into the device fabrication must be conducted to depict functionality.

In conclusion, this study developed the design of the first wireless wearable intraoral glutamate biosensor for continuous real-time measurements. The development of this design for the proposed device could expand the diagnostic field by introducing a noninvasive method for monitoring biomarkers of pain.

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Appendix A

Table 2.

Working electrode	**Enzyme**	**Application (biofluid)**	**Limit of detection (µM)**	**Linear detection range (µM)**	**Response time (s)**	**Sensitivity (nA μM^{-1})**	**Applied potential (V)**	**Reference**
Pt-MEA/BSA/GluALD/ *m*PD	GluOx	Rat ECF	0.16 ± 0.02	10-570		270 ± 8cm^{-2}	0.7	(Scoggin et al., 2019)
Pt/PoPD/AscOx/BSA	GluOx	Rat ECF	0.044	5-150	2	0.097 ± 0.001	0.6	(Ganesana et al., 2019)
Pt-CNF	GluOx	Rat ECF		1-100		266cm^{-2}	-0.15	(Isoaho et al., 2018)
Pt/SiO_2/BSA/GluALD	GluOx	HEPES buffer	1	2.5-450	20-30	0.5	0.6	(Soldatkina et al., 2017)
Pt/PI	GluOx	Processed foods	0.150			1	0.45	(Weltin, Kieninger and Urban, 2017)
CFE/PB/PoPD/PEI/GluALD	GluOx	Electrolyte solution	1.5	0-150		135 ± 2cm^{-2}	0.05	(Salazar, Martín, O'Neill and González-Mora, 2016)
PG/ZnONR/PPy	GluOx	Food stuffs	0.00018	0.02-500	<5		0.065	(Batra, Yadav and Pundir, 2016)
GCE/Naf/Gldh-bacteria/PEI-MWNT	GLDH	PBS	2	10-1000 & 2000 – 10000			0.52	(Song et al., 2015)
MEA/PtNP/ *m*PD/GluALD/BSA	GluOx	Rat ECF	0.5	5-30	<8	0.056	0.7	(WEI et al., 2015)
SPCE/MB/chit/MWCNT	GLDH	In vitro proof of concept	3	7.5-105	20-30	0.39	0.1	(Hughes, Pemberton, Fielden and Hart, 2015)
BDD/PtNP/PPD	GluOx	PBS	0.35	0.5-50	4	24 cm^{-2}	0.5	(Hu, Wisetsuwannaphum and Foord, 2014)

GCE/Pt-GNPs/PPD	GluOx	PBS	0.75	0.2-100	4	174 cm^{-2}	0.5	(Hu, Wisetsuwannaphum and Foord, 2014)
Pt/CeO_2NP/TiO_2NP/chit/oPD/BSA (oxygenated conditions)	GluOx	Rat CSF	0.594	5-90	2	0.793	0.6	(Özel et al., 2014)
Pt/CeO_2NP/TiO_2NP/chit/oPD/BSA (deoxygenated conditions)	GluOx	Rat CSF	0.493	5-50	5	0.395	0.6	(Özel et al., 2014)
Au/PPyNPs/PANI/GluALD	GluOx	Food stuffs	0.0001	0.02-400	3	533 cm^{-2}	-0.13	(Batra, Kumari and Pundir, 2014)
Pt/PPy/Naf/chit	GluOx	Rat ECF	2.5 ± 1.1	20-217	<2	34.9 ± 4.8 cm^{-2}	0.7	(Tseng, Chang and Chan, 2014)
Pt/PPy/Naf/BSA/GluALD	GluOx	Rat ECF	6.5 ± 1.7	20-352	<5	86.8 ± 8.8 cm^{-2}	0.7	(Tseng, Chang and Chan, 2014)
Pt/PI/ *m*PD/BSA/GluALD	GluOx	Rat ECF	0.22	<150	4.9 ± 1.9	2.16 ± 0.08 mm^{-2}	0.45	(Weltin et al., 2014)
Nano-PPCPE/Cat/BSA	GluOx	PB	0.25	0.5-10				(Deng et al., 2013)
MEA/Pt/PI/ *m*PD/BSA/GluALD	GluOx		0.6				0.7	(Burmeister et al., 2013)
Pt/SiO_2/Ti	GluOx		50	50-10000		3.68	0.6	(Bäcker et al., 2013)
Pt/PPD	GluOx	Human serum	21		15	8.077	0.4	(Windmiller et al., 2011)
Pt/PAA/SWCNT	GluOx	PBS	0.0046	0.05-1600	5	72.4 cm^{-2}	0.35	(Claussen et al., 2011)
Au/EDC/TGA/SAM	GluOx	PBS	0.072	0.1-10000	10	17.89cm^{-2}		(Rahman et al., 2009)
Pt/PPy/MWCNT/PU	GluOx		0.3	<140	7	3.84mm^{-2}		(Ammam and Fransaer, 2010)
Pt-PPD/Silica gel	GluOx	Rat ECF	0.005	0.5-100	10	279.4 ± 2cm^{-2}	0.5	(Tian et al. 2009)
SPCE/MB/ CHIT	GLDH	FBS	1.5	12.5-150	2	0.44	0.1	
Pt-MEA/PPy/Naf	GluOx	Rat dorsal striatum	<1	10-100	<1	2.46 ± 0.48 pA µM-1	0.7	(Wassum et al., 2008)
O_2/GluALD/BSA	GluOx and GLDH	In vitro (food)	0.02 mg/L	0.02-1.2 mg/L	120			(Basu, Chattopadhyay,

								Roychudhuri and Chakraborty, 2006)
GCE/SWCNT/thionine	GLDH	NADH	0.1	0.5-400	5	137.3 ± 15.7 µA mM^{-1} cm^{-2}	0.19	(Meng et al., 2009)
AuPNC/CHIT/MWCNT/ferricyanide	GLDH and diaphorase	In vitro (food)	5.4	10-3495	<60	28 cm^{-2}		(Monošík, Streďanský and Šturdík, 2012)
PB/graphite	GluOx	PBS	0.01	10-100	3	238	-0.05	(Liu et al., 2014)
cMWCNT/AuNP/chit	GluOx	In vitro (sera)	1.6	5-500	2	155 cm^{-2}	0.135	(Batra and Pundir, 2013)
VACNT-NEA	GLDH	Proof of concept	0.01	0.01-20 & 20-300		2.2 Am M^{-1} cm^{-2} & 0.1 Am mM^{-1} cm^{-}	0	(Gholizadeh et al., 2012)
Pt/CHIT/GDI gels	GluOx	Proof of concept	0.10	0.10-500	2	100 mA M^{-1} cm^{-2}	0.6	(Zhang, Mullens and Gorski, 2005)
Pt/CHIT	GluOx	In vitro (food)	0.10	1-10	2	85 mA M^{-1} cm^{-2}	0.4	(Zhang, Mullens and Gorski, 2006)
Pt/GluALD/SiO_2/PCB	GluOx	Cell culture fermentation	0.0002	0.00022500		96 nA mM^{-1}	0.6	(Bäcker et al., 2011)
SAM/ECD/TGA	GluOx	Proof of concept	0.089	0.1-10000		20.75 ± 1.0		(Rahman, 2011)
Pt/GluALD	GLuOx	In vitro (brain tissue) uptake	0.5	2-800	15-20	250-300 nA mM^{-1}	0.6	(Soldatkin et al., 2015)
PtD/PPD-BSA/PEA/PEI/PPD-BSA	GluOx	In vitro	2.5	0-50	<5	71 ± 1 cm^{-2}	0.5	(Govindarajan and McNeil, 2009)
Pt/PEI/PPD	GluOx					100 ± 13 cm^{-2}		(McMahon et al., 2006)
Pt electrode	SHL/GLDH	Tris-HCL buffer	3	10-1500	1	86.6 nA mM^{-1}		(Cui, Barford and Renneberg, 2007)
MWCNT/CHIT/MDB	GLDH		2		4	0.71 ± 0.08	-0.1	(Chakraborty and Retna Raj, 2007)

MWCNTs/PAMAM/Pt/Nafion	GluOx	Striatum - rats	0.5	1-50		1.74 ± 0.02	-0.2	(Yu et al., 2011)
[C3(OH)2mim][BF4]-Au/Pt-Nafion	GluOx	Subthalamic nucleus area (STN) - rats	0.17	0.5-20		1.60 ± 0.56	-0.2	(Yu et al., 2011)
Pt Blk/OPPy/ GA	GluOx	Striatum	2	0-250	1-2	80 ± 10 cm^{-2}	0.45	(Hamdi, Wang and Monbouquette, 2005)