ISTANBUL BILGI UNIVERSITY
INSTITUTE OF GRADUATE SCIENCES
ELECTRICAL AND ELECTRONICS ENGINEERING
MASTER'S DEGREE PROGRAM

APTAMER BASED BIOSENSOR DESIGN FOR GLUTEN DETECTION

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ISTANBUL
2019

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Glüten Tespiti için Aptamer Tabanlı Biyosensör Tasarımı

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Tezin Onaylandığı Tarih : .....17/09/2019.....

Toplam Sayfa Sayısı: .....88.....

Anahtar Kelimeler (Türkçe)

1) Biyosensör
2) Glüten
3) Aptamer
4) Çölyak Hastalığı
5) Gıda İntoleransı

Anahtar Kelimeler (İngilizce)

1) Biosensor
2) Gluten
3) Aptamer
4) Celiac Disease
5) Food Intolerance

ACKNOWLEDGEMENT

I would like to mention about my gratitude to whom make this thesis possible to complete.

Firstly, I would like to give my sincere gratitude to my advisor Asst. Prof. Dr. Özgür Gül and my co-advisor Asst. Prof. Dr. Y. Dağhan Gökdel. Throughout of my thesis, I am motivated and encouraged with their patience and guiding. Their guiding is not only aspect of project, they also lead me to my life plans and encourage me to relief my best. I would also thank again to Asst. Prof. Dr. Özgür Gül to encourage me to study at master degree without his support, it would not be possible to complete this project.

Being a part of Protein Engineering Lab make me feel lucky. I would like to thank all the members in Protein Engineering Lab. I am gratitude to have such laboratory team mates Ali Sinan Kara, Lara Naseri and İlayda Amanoğlu. I am thankful for their help and motivation.

Finally, I would like to express my appreciation to my family. I owe my parents Hilal Yeşilyurt and Gökhan Yeşilyurt so much for their tolerance, understandings and supports. I am grateful Nezih Sipahioglu for his continuous pertinacity to motivate and support me.

ABSTRACT

A lot of people are suffering because of gluten intolerance or celiac disease which is caused by gluten. Additionally, there is a growing demand for the gluten-free diet as a part of healthy lifestyle. The only treatment for the celiac disease or gluten intolerance is to follow a strict gluten-free diet. As a result, there is an increasing need for the gluten detection for not only raw food but also for the processed food.

The goal of our study was to design an aptamer-based biosensor for the gluten detection. To achieve this goal, we have optimized sample preprocessing and compared the results with industry standard gluten from Prolamine Working Group (PWG). We have also, modified some aptamers that are already used in some scientific reports with biotin and tested their ability to recognize gluten using ELISA.

Finally, we have used lateral flow assay format to detect gluten using biotinylated aptamers on our homemade strips.

Therefore; we designed specific biosensor that has the potential to detect and quantify different sources of gluten. We choose aptamer as the biological recognition element because it is easy to produce, it is stable under different conditions and it is fast for the detection of proteins. Since aptamers are new generation of biosensor, which is called aptasensors, they will be well adapted to our conditions.

ÖZET

Birçok insan glüten kaynaklı olarak intolerans veya çölyak hastalığı çekmektedir. Bununla beraber, sağlıklı yaşam için de glüten içermeyen beslenme şekline giderek artan bir talep oluşmuştur. Çölyak hastalığının veya glüten intoleransının tek tedavisi, glüten içermeyen beslenme şekli olmaktadır. Bunların sonucunda, çiğ ve işlenmiş yiyeceklerde glüten tespiti ihtiyacı artmaktadır.

Bu çalışmamızın amacı, glüten tespiti için aptamer tabanlı bir biyosensör tasarımı yapmaktır. Bu amaca ulaşabilmek için, örneklerin ön işlemesini gerçekleştirdik ve bunu Prolamine Working Group (PWG)'tan elde ettiğimiz standart ile karşılaştırdık. Buna ek olarak bilimsel literatürde daha önce kullanılmış olan aptamerlere biotin ekleyerek bunların glüteni tespit etme özelliklerini ELİZA yöntemiyle test ettik.

Son olarak, yanal akımlı test (Lateral Flow Test) formatında kendi ürettiğimiz stripler üzerinde biotin işaretlenmiş aptamerleri test ettik.

Sonuç itibariyle, potansiyel olarak farklı kaynaklardan glüten varlığını tespit edebilecek ve miktarını ölçebilecek bir biyosensör tasarlamış olduk. Biyolojik tanıma elementi olarak, kolay üretilebilmesi, farklı koşullarda stabil olması ve hızlı sonuç vermesi sebebiyle aptamerleri tercih ettik. Aptamerler yeni jenerasyon biosensörler olduğu için, aptasensör olarak adlandırılmakta ve bizim koşullarımıza iyi adapte olmuştur.

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LIST OF SYMBOLS/ABBREVIATION

Tris-HCl	Tris(hydroxymethyl) aminomethane hydrochloride
DTT	Dithiothreitol
SDS	Sodium Dodecyl Sulfate
PWG	Prolamin Working Group
ELISA	Enzyme-linked immunoabsorbent assay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
HRP	Horse-Radish Peroxidase
PVC	Poly Vinyl Chloride
TMB	3,3',5,5'-Tetramethylbenzidine
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
DIY	Do it yourself

INTRODUCTION

1.1. BIOSENSORS

The biosensor is an analytical device that transforms biological response into an interpretable signal. With the production of an interpretable signal, biosensors measure the concentration of an analyte in the reaction. Biosensors are used for biological applications. Those applications can be disease monitoring, drug discovery, and detection of pollutants etc. Figure 1.1; describes the typical form of biosensors and its components.

• **Analyte**: A substance of interest that needs detection. For instance, glucose is an 'analyte' in a biosensor designed to detect glucose.

• **Bioreceptor**: A molecule that specifically recognizes the analyte and comes from a biological source is known as a bioreceptor. Enzymes, cells, aptamers, deoxyribonucleic acid (DNA) and antibodies are some examples of bioreceptors. The process of signal generation (in the form of light, heat, pH, charge or mass change, etc.) upon interaction of the bioreceptor with the analyte is termed bio-recognition.

• **Transducer**: The transducer in biosensors is an element that interprets the signal generated from analyte-bioreceptor interaction and produces a measurable signal. Most transducers produce either optical or electrical signals that are usually proportional to the amount of analyte–bioreceptor interactions.

• **Signal-processing**: This is the part of a biosensor that processes the transduced signal and prepares it for display. It consists of complex electronic circuitry that performs signal conditioning such as amplification and conversion of signals from analogue into the digital form. The processed signals are then quantified by the display unit of the biosensor.

• **Display**: The display consists of a user interpretation system such as the liquid crystal display of a computer or a direct printer that generates numbers or curves understandable by the user. This part often consists of a combination of hardware and software that generates results of the biosensor in a user-friendly manner. The output signal on the display can be numeric, graphic, tabular or an image, depending on the requirements of the end user [1].

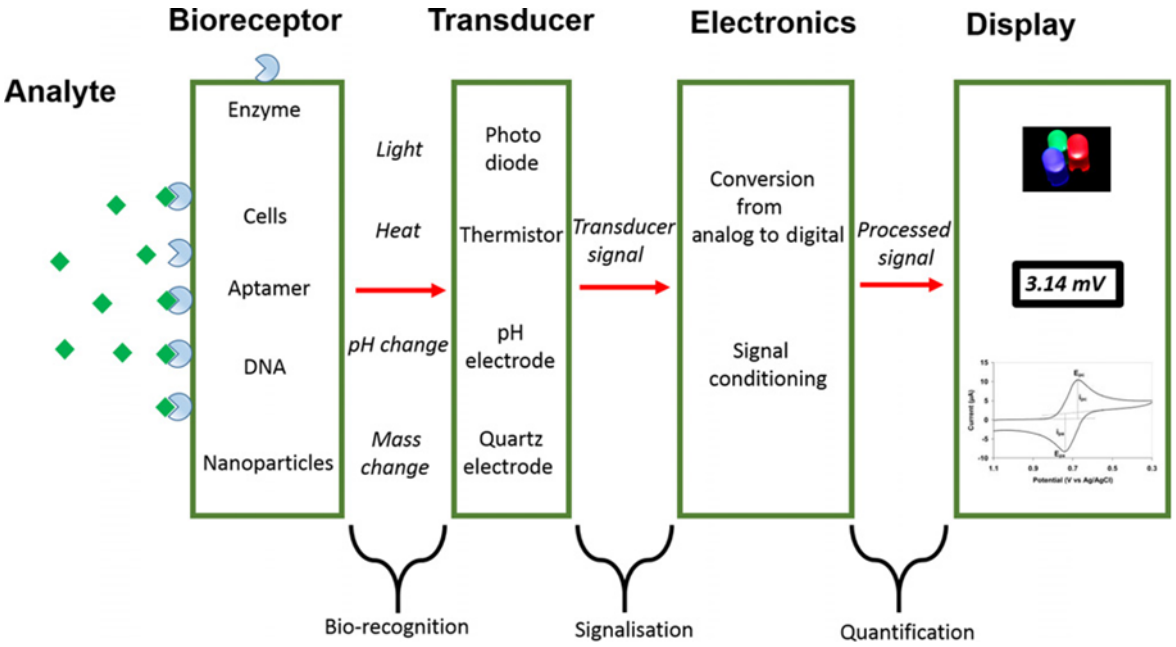

Figure 0.1 Schematic show of a biosensor [1].

1.1.1. Historical background

The history of biosensors starts in 1906, when M. Cremer[2,3] demonstrated that the concentration of an acid in a liquid is proportional to the electric potential that arises between parts of the fluid located on opposite sides of a glass membrane[3,4] The first "functional" biosensor was invented for oxygen detection by Leland C. Clark, in 1956. Scientists know Clark as the 'father of biosensors' and to this day his invention is known as the: "Clark electrode".[5,6] He is also the inventor of the first glucose biosensor. Following Leland C. Clark the first potentiometric biosensor for detecting urea was discovered in 1969 by Guilbault and Montalvo, Jr[7]. In 1975 the first commercial biosensor was produced by Yellow Spring Instruments (YSI). Table 1.1 shows the history of biosensors. Ever since the development of the i-STAT sensor, remarkable progress has been achieved in the field of biosensors. The field is now a multidisciplinary area of research that bridges the principles of basic sciences (physics, chemistry and biology) with fundamentals of micro/Nano-technology, electronics and applicatory medicine. The database 'Web of Science' has indexed over 84000 reports on the topic of 'biosensors' from 2005 to 2015[8-15].

Table 0.1 The development of biosensors during the period 1970–1992 [15].

1970	Discovery of ion-sensitive field-effect transistor (ISFET) by Bergveld
1975	Fibre-optic biosensor for carbon dioxide and oxygen detection by Lubbers and Opitz
1975	First commercial biosensor for glucose detection by YSI
1975	First microbe-based immunosensor by Suzuki et al.
1982	Fibre-optic biosensor for glucose detection by Schultz
1983	Surface plasmon resonance (SPR) immunosensor by Liedberg et al.
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for glucose detection
1990	SPR-based biosensor by Pharmacia Biacore
1992	Handheld blood biosensor by i-STAT

Figure 1.2 shows the trends in the annual numbers of scientific articles covered by Scopus from 2010 to 2018 in the field of bioreceptor-based biosensors [15].

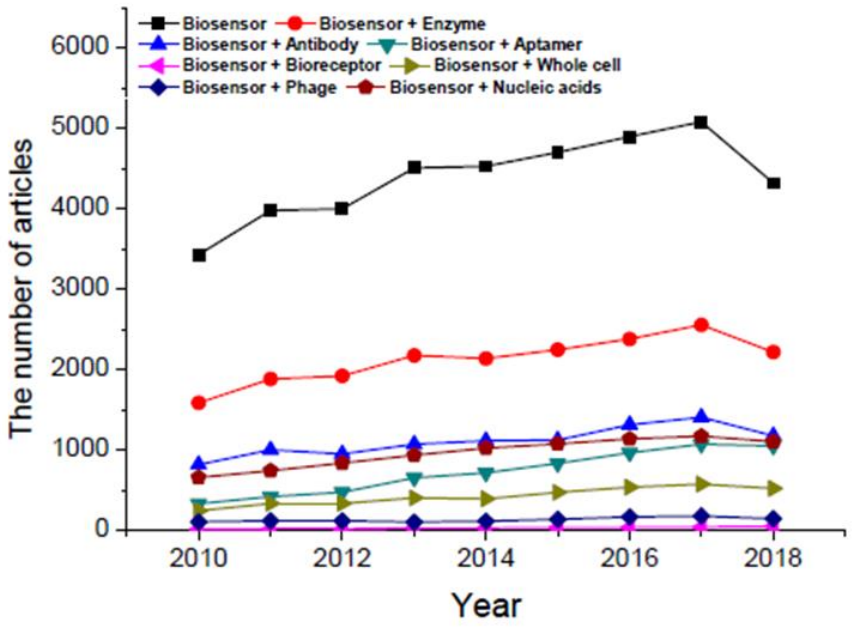

Figure 0.2 Graphical representation of numbers between 2010 and 2018 of biosensors related studies [15].

1.2. TYPES OF BIOSENSORS

Biosensors are generally based on components. They can used enzymes, antibodies, cells, DNA or RNA, and classified based on the components or they can classified by type of the transducers like electrochemical, mass based etc. also they can classified by the interactions between analytes and biological materials that used[15].

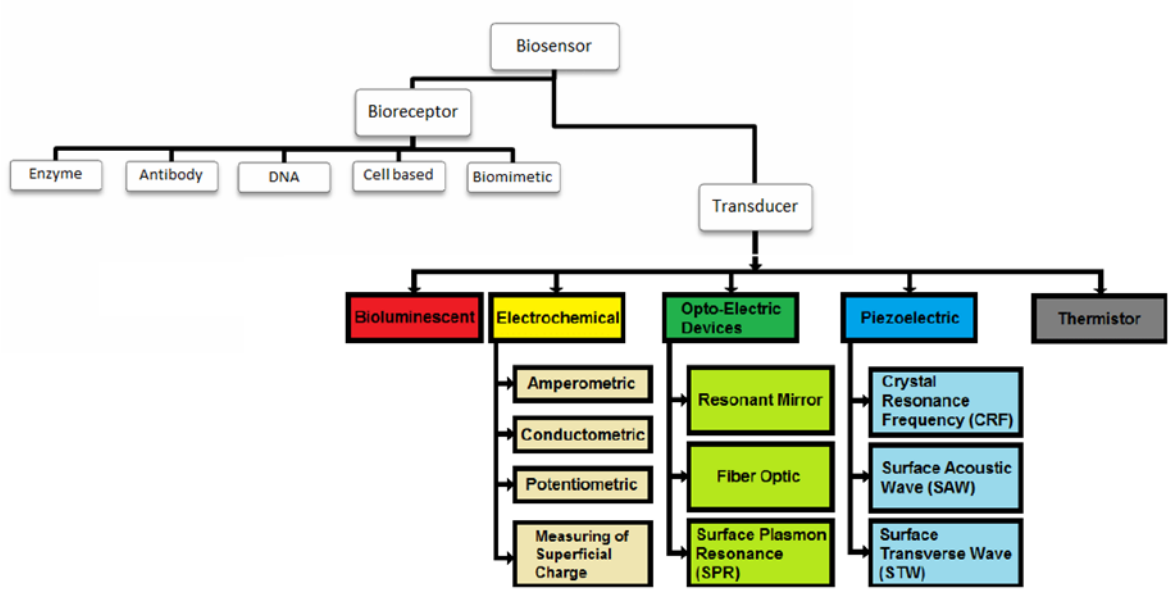

Figure 0.3 Categorization of biosensor[15].

Biosensors can be examined under five groups based on bioactive layer-transmission and detection methods. As it is seen in Figure 1.3, Bioreceptor groups are;

(a) Enzyme

(b) Antibody

(c) DNA

(d) Cell based

(e) Biomimetic

Transducer groups are as follows;

(a) Bioluminescence biosensors

(b) Electrochemical biosensors including; amperometric, potentiometric, voltametric, voltamperometric and conductometric biosensors

(c) Optic based namely photometric, fluorometric

(d) Piezoelectric based biosensors

(e) Calorimetric in other words thermistors [16, 17].

1.2.1. Analyte based classification

1.2.1.1.Enzymes

Enzymes are frequently using for the development of biosensors. Enzymes are known as catalyzers and when they used in biosensors they catalyze the production which is specific for the desired molecules. 90 per cent of the biosensor market is composed of glucose biosensors, which are used for detecting glucose in blood samples [18].

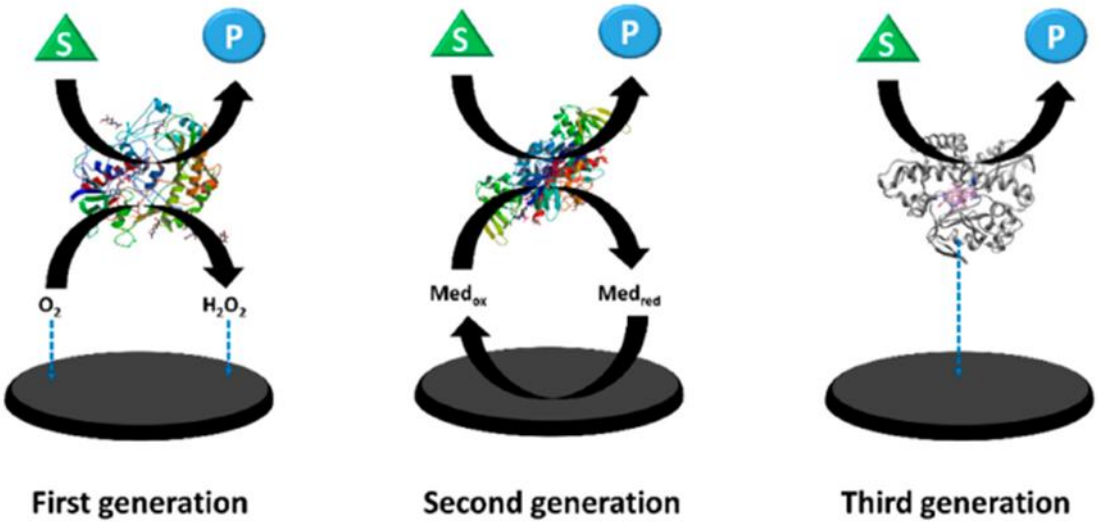

Figure 0.4 Schematic illustration of first-, second-, and third-generation amperometric enzymatic biosensors. First generation is based on the electroactivity of the receptor substrate or the product. Second generation is based on the use of artificial redox mediators. Third generation is based on the direct electron transfer between the redox-active biomolecule and the electrode [19].

Variety of enzymes were used for biosensor construction, for example oxidoreductase enzymes were used for lactate, malate, ascorbate, amino acids, alcohol, cholesterol, glycerol, fructose and transferase can be utilized in biosensor assisted analysis of acetic acid, determination of xenobiotics such as captan or atrazine, hydrolase in sucrose, lyase in citric acid analysis, ligase in DNA point mutation detection, isomerase for 19-norandrostenedione, etc. There are many different factors that can affect the performance of the biosensor those

can be pH, temperature, density, etc. Also thickness of the enzyme layer can be a parameter [18].

1.2.1.2.Antibodies

Hundreds of amino acid sequences bind to each other and these different combinations produces different complex biomolecules known as antibodies. An antigen-specific antibody fits its unique antigen in a highly specific way. High antigen specificity makes antibodies a great bioreceptor molecule for biosensor development.

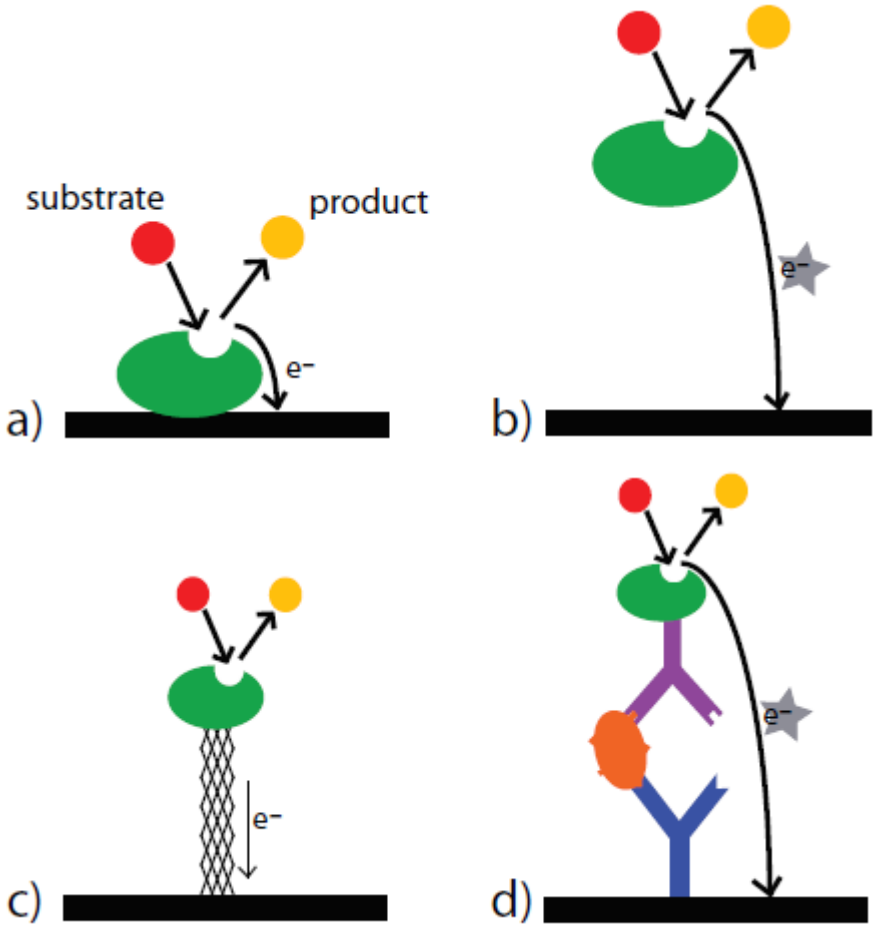

Figure 0.5 Scheme of direct and indirect electron transduction. a) Direct transduction: the electrons only generate a measurable current if the reaction takes place close to the surface. b) Indirect transduction: with the help of a mediator, that shuttles the electrons between the reaction site and the surface, much larger distances can be overcome. c) Enzyme bound via

conducting CNT. d) ELISA-like sandwich setup, where an enzyme labelled antibody binds to the detected antigen [20].

These unique properties of antibodies are crucial to their usefulness in immunosensors, where only the Specific analyte of interest, the antigen, fits into the antibody-binding site. There are two categorize for biomolecular interactions based on test format performed. The firs one is direct and the second one is indirect. Direct format is based on interaction between the immobilized target molecule and a ligand molecule or the immobilized ligand interacts with a target molecule directly. In indirect format, a mediator is used to shuttle the electrons between the reaction site and the surface [19, 20].

1.2.1.3.Nucleic acids

When DNA, RNA and peptide nucleic acid used in biosensors they have a strong base pair affinity and that is the reason of their high sensitivity and selectivity. Nucleic acids (NA) are used for biological recognition element in NA-based biosensors. DNA hybridization sensors are using synthetic oligodeoxyribonucleotides (ODNs) as probes. For immobilizing of ODNs to transducer surface, end-labels such as thiols, disulfides, amines, or biotin, are incorporated. A long flexible spacer is usually added by means of hydrocarbon linkers to provide sufficient accessibility for surface attachment. The electrochemical DNA biosensors, which rely on the conversion of the base-pair recognition event into a measurable electrical signal, are regarded to be suitable candidates for the rapid and inexpensive diagnosis of genetic diseases, the detection of pathogenic biological species of clinical interest, and for the compatibility with microfabrication technology. The complementarity of adeninethymine and cytosine-guanosine pairing in DNA forms the basis for the specificity of biorecognition in DNA biosensors. For the known sequence of bases in DNA molecule the complementary sequence, called a probe, can be synthesized and subsequently labelled with an optically detectable compound (e.g., a fluorescent label). The labelled probe will hybridize to its complementary sequence on the target molecule once the double-stranded DNA is unwound into single strands, then the probe is added, and finally the strands annealed. The formation of the duplex may be considered as evidence that the target has the expected nucleotide sequence. Electrochemical (EC) detection of the formation of a DNA

duplex, called hybridization event, is based on the EC signals due to NA electro activity, labelling of the target or the probe with covalently bound electroactive species (e.g., nanoparticles), or changes in various electrochemically detectable DNA properties related to changes in the DNA structure resulting from the hybridization step[21,22]. Aptamers, artificial single-stranded DNA or RNA oligonucleotides (typically <100mer) which are selected from randomized oligonucleotide libraries by SELEX (systematic evolution of ligands by exponential enrichment) are also used to specifically bind with various targets such as proteins, cells, viruses, bacteria, as well as small molecules such as organic dyes, metal ions, amino acids[23].

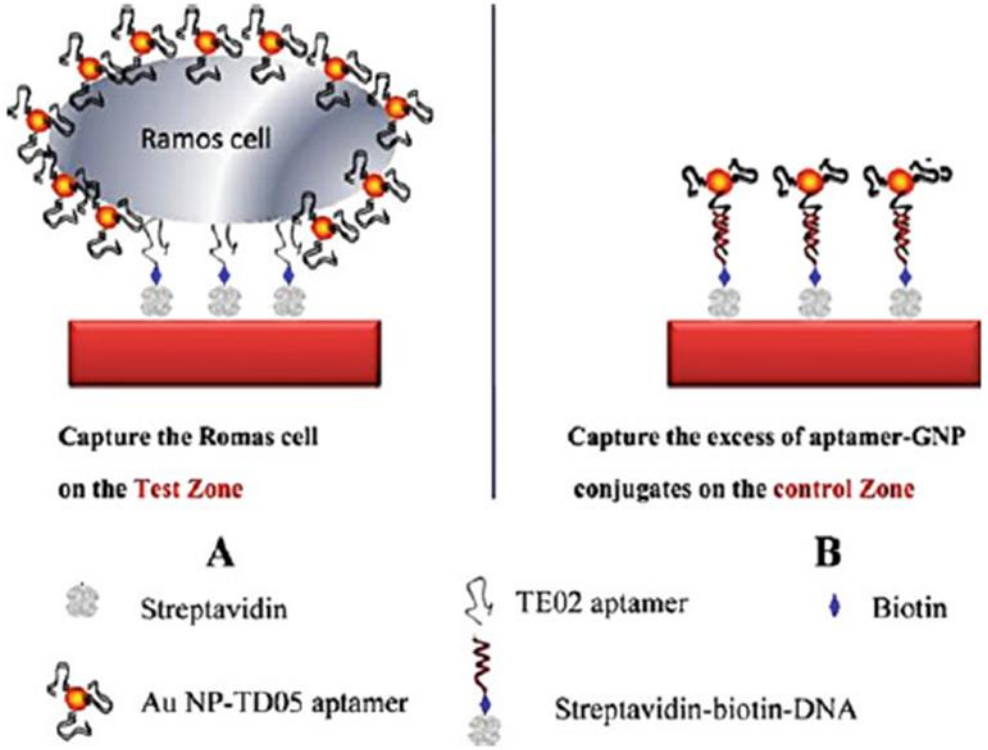

Figure 0.6 Scheme of the detection of Ramos cells on aptamer-nanoparticle strip biosensor (a) Capturing Au-NP-aptamer-Ramos cells on the test zone through specific aptamer-cell interactions (b) Capturing the excess of Au-NP aptamer on the control zone through aptamer-DNA hybridization reaction.[24].

Basically, their notable and modifiable stability functions are led up to the development of a new biosensor generation. Aptamers are more resistant to degradation and denaturation unlikely monoclonal antibodies; moreover aptamers and monoclonal antibodies binding

affinities are identical. Furthermore, in case of rational design or molecular evolution techniques, binding affinity and properties can also be modified. For this purpose, many functional groups or tags that allow covalent, directed immobilization on biochips, resulting in highly ordered receptor layers are used. Aptamers can act as a chiral selector and therefore can distinguish chiral molecules and recognize a different epitope of a target molecule [25].

1.2.1.4.Whole Cells

These bioreactors are based on biological recognition by either a whole cell/microorganism or a particular cellular component that can specifically bind to a particular species. One of the great advantages resulting from use of whole cells as bioreceptors is low detection limit due to the limitation signal. A lot of biosensors developed with this kind of bioreceptor are based on their catalytic or pseudo catalytic properties. For instance, viable or non-viable microbial cells are using for microbial biosensors. Non-viable cells obtained after permeabilisation or whole cells containing periplasmic enzymes have been used as a cheaper alternative for enzymes. Viable cells utilize the respiratory and metabolic functions of the cell, thus the analyte may be monitored being either a substrate or an inhibitor of these processes [26]. The sensitivity of the cell-based biosensors (CBBs) for certain agonist can be deduced by the receptor-ligand combination constant. CBBs may be applied to analyze the effect of pharmaceutical compound on a given physiological system [27]. There are many complex obstacles when living cells were treated as the primary biosensor, including the selection, the culture and the maintenance of living cells. The coupling of living cells and the secondary sensor represents one of challenges [28]. On the other hand, cell-based biosensors can perform real-time bioassays dynamically and rapidly, and have numerous applications ranging from biomedicine to the environment, for example detection of pathogens and toxins.[29].

1.2.1.5.Biomimetic

The synthetic or artificial bioreceptors that mimic the function of a natural bioreceptors are called biomimetic biosensors. Biomimetic biosensors include aptamers in which they are used as bioreceptors.[30]. Firstly, aptamers were reported as artificial nucleic acid ligands in the early 1990s. Aptamers are chemically associated with nucleic acid probes, but they act

like antibodies and show amazing versatility according to other bio-recognition components. Aptamers can be contemplated to recognize amino acids, peptides, proteins and oligosaccharides, namely aptamers are synthetic strands of nucleic acids. An aptamer has very little advantages over antibody-based biosensor such as high binding efficiency, avoiding the use of animal, smaller and less complex, and etc. accordingly, the properties of nucleic acids such as structural pleomorphic and chemical simplicity which reduce assay efficiency and also increase the cost of production, are the common challenges facing the aptasensors. After that, some efforts were made to characterize and optimize the aptamer to overcome this limitation. Aptamer properties used successfully to optimize various bio-sensing formats include; small size, modification and immobilization versatility, high specificity, regeneration, or conformational change induced by target binding [31]. Aptamer based biosensor has been widely used in various application. Biomimetic sensor and aptasensor have recently made adequate progress for clinical practice [32].

1.2.2. Biosensors – Detection Methods Detailed

1.2.2.1.Electrochemical Biosensors

The basic principle of electrochemical biosensor class is that the chemical reactions between inactive biomolecule and target analyte produce or consume ions or electrons that affect the measurable electrical properties of the solution, such an electric current or potential [34].

A)Amperometric Biosensors

Amperometric biosensors are the most common biosensor class. In an enzyme-based amperometric biosensor, the measured signal is the current generated by the oxidation or reduction of the electroactive species at the working electrode (i.e., gold, carbon, platinum, etc.). When a constant potential is applied between the two electrodes, it is observed that the magnitude of the current produced on the surface of the working electrode is proportional to the concentration of analyte in the test solution after the substrate has been added. Enzyme-based amperometric biosensors have been extensively studied because of their advantages such as ease in robustness, miniaturization, and small sample volumes to work with highly complex matrices [35, 36].

B)Potentiometric Biosensors

In many enzyme reactions, the ion-selective electrode can be used to monitor these processes as it involves the release or absorption of hydrogen ions that cause the ionic concentration to change. In a potentiometric biosensor, the difference in potential (voltage) between the working electrode and the reference electrode measured under equilibrium conditions is considered signal to avoid interference with the reaction. The measured signals form a function of target analyte concentrations in a logarithmic manner, and are used for quantification. Potentiometric biosensor divided to tree categorizes which is Ion-selective electrode (ISE), enzyme field-effect transistor (EnFET) and light-addressable potentiometric sensor (LAPs) [30].

Electrode Types Used in Electrochemical Biosensors

A. Ion Selective Electrodes

An ion-selective electrode converts the activity of a specific ion in a test solution into a voltage and generated voltage can be measured with pH / mV meter. The electrode is generally based on two components: (1) An ion specific membrane for specific ions in the analyte solution, and (2) reference electrode. After the ion transition, electrochemical balance is formed, and a potential difference occurs between the two phases (the reaction solution and the inner/measuring solution). Because of the membrane specificity, this potential difference is controlled only by the activities of a specific ion in these phases. Ion-selective electrodes divided to five main types, which are classify by the nature of the material used to build the electrode; these are: glass membrane electrode, solid-state membrane electrode, polymer membrane electrode, gas-permeable membrane electrode, enzyme electrode. Differences in membrane structure are properties that make an electrode selective for a particular ion. Gas-sensing electrodes and enzyme electrodes are the most commonly used electrodes in electrode biosensors between electrode types. Gas-sensing electrodes have gas-permeable membranes separating an enzyme reaction solution from an internal solution. When gas molecules diffuse across the gas-permeable membrane, they hydrolyze in the thin film of internal solution, leading to variations of some ion concentrations (generally H+), which results in a pH change that could be detected by a pH electrode. Therefore, the potential changes are directly related to the concentration of gas existing [37, 38].

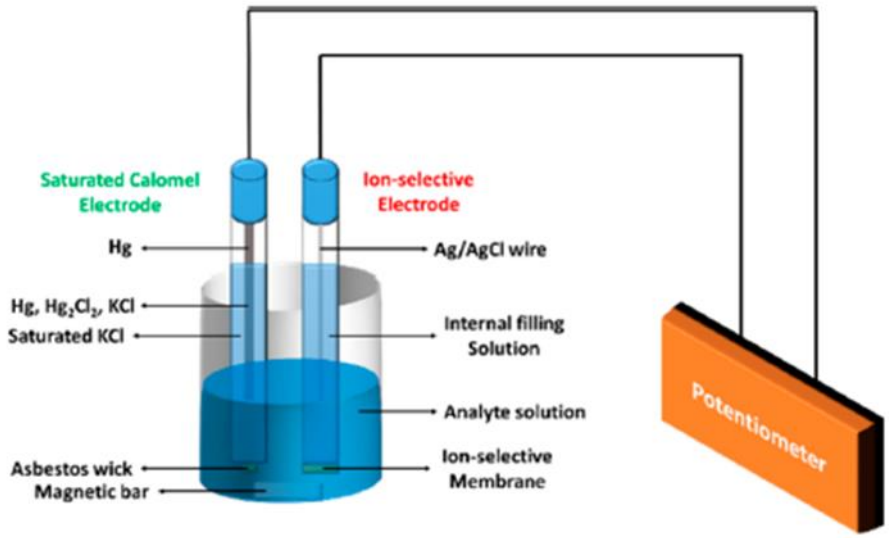

Figure 0.7 Schematic diagram of a conventional ion selective electrode [38].

B. Enzyme Field-Effect Transistors

An EnFET sensor is constructed based on an ion-sensitive field-effect transistor (ISFET), which is built by separating the metal gate of a classical MOSFET (metal oxide semiconductor field-effect transistor) from the device, and reconstructing the gate in the form of a reference electrode inserted in an aqueous solution, which is separated with the gate oxide by an enzymatic membrane[38]. When such ISFETs are coupled with the immobilization of a thin enzyme membrane at the gate surface, they become EnFETs. The types of membranes were relatively similar to ion-selective electrode classification, stated in the previous section. The EnFET sensor originates from a pH-sensitive detector, in which the enzymatic catalytic activity is sensitive to the pH level [38, 39].

C. Light-Addressable Potentiometric Sensors (LAPS)

In LAPS, a modulated light from a light-emitting diode (LED) is used for semiconductor activation instead of applying an alternating current (AC) voltage. Under illumination, electron-hole pairs are generated on parts of the semiconductor surface. As a result, a photocurrent is produced and measured under a fixed bias voltage. The LAPS is a semiconductor-based chemical sensor which is built following the electrolyte–insulator

semiconductor (EISC) structure. PH-sensitive LAPS that uses LEDs in combination with silicon as semiconductor and SiO2/Al2O3 as pH sensitive insulator was developed to build an EISC-based sensor for the detection of urea, penicillin and glucose [39].

1.2.2.2.Conductometry

Enzymatic reactions often change the electrical conductivity of the electrolyte solution it includes changes in ionic concentration. The conductivity of this solution can be measured by means of a conductive biosensor by applying a potential difference between two parallel electrodes. As a result, ion mobility is increased due to the correct movement of negatively charged ions to the anode and to the cathode towards the positively charged ions. The conductivity of the electrolyte solution only depends on the ion concentration and mobility, so that measurement can be useful when there is no incoming or insignificant electrochemical reactions occur at the electrodes. In a similar manner to other electrochemical based biosensors, the principles and methods of enzyme immobilization on electrodes for an amperometric biosensor are suitable for conductometric transducers. For example, the electrode surface was immobilized, with the enzyme included inside an albumin gel film by the means of covalent attachment using glutaraldehyde [40]. Also, the enzyme was immobilized on an electrode surface by using the sol-gel entrapment method [41], covalent binding with a collagen membrane [42], electrochemical polymerization [43], or cross-linking with bovine serum albumin using glutaraldehyde [44].

1.2.2.3.***Impedimetric Enzyme Based Biosensors***

The impedance of the electrode in an electrochemical impedimetric biosensor is defined as and measurable change in the impedance value of the circuit.. Electrochemical impedance spectroscopy (EIS) is used to investigate changes in interface properties due to biodiversity events occurring on modified surfaces. The resulting impedance spectrum can then be used to determine the quantitative parameters of electrochemical processes. In enzyme-based biosensors, this impedance measurement technique is used less when in comparison to potentiometric and amperometric techniques, due to the time consumption of a full impedance spectrum record within a wide frequency range. In addition, in order to obtain a valid impedance spectrum in the EIS technique, various requirements such as linearity, stability and causality are met. Therefore, EIS techniques are commonly used as methods

that can be characterized for most of the enzyme-based impedimetric biosensors. Shervedani et al. developed an impedimetric biosensor for the determination of glucose based on EIS measurements [45].

1.2.2.4.Optical Transducer Based Biosensor

In recent years, the optical biosensors have undergone rapid advances and have been implemented in a number of key areas such as food safety, life science, environmental monitoring and medical diagnosis [46]. Medically, the optical transducer was established for both routine medical diagnostics and medical research applications [47]. The word "optrode" is a combination of the words "optical" and "electrode" is sometimes used to define optic based device [48]. This method of transduction has employed in many class of biosensor due to the many different types of spectroscopy such as absorption, fluorescence, phosphorescence, Raman, SERS, refraction and dispersion spectroscopy. These transduction methods are capable of measure different properties of analyte/target. Optical-based biosensor with no labeling, real-time and parallel detection [49]. Surface plasmon resonance or fluorescence integrated with optical fiber is the most popular method for biosensors that utilize optical transducers.. It is understood that the optical fiber based sensor is interested in research and used in biosensor studies [50]. This type of biosensor may be based on optical diffraction or electro chemiluminescence principle that a silicon wafer coated with a protein. Covalent then submitted to UV light through a photo veils bond, and antibodies remain idle in uncovered areas. At the point where the chopped wafer chips are incubated in an analyte, the antigen-counteracting agent bonds are formed in dynamic loci and a diffraction milling is performed. This grinding creates a break flag when illuminated by a light source. Optical biosensors consist of various optical sections and also from a light source to produce a light bar with shortcuts to specific features and make a determination that a balancing operator head this light is set next to a photo detector [51]. These biosensors measure both reactant What's more regular slant reactions. They measure an advance for fluorescence on the other hand on absorbance expedited toward the outcomes created toward reactant reactions. On the other hand, they measure those movements provoked in the inborn optical properties of the biosensor surface in view of stacking on it for dielectric particles[51].

1.2.2.5.Piezoelectric Transducer Based

Piezoelectricity is discovered in 1880 by the Curie brothers [52]. Piezoelectric structures create a gem of a combustion characteristic recurrence which can be used to generate a signal [53].Consequently, in piezoelectric biosensor the transducer is made of piezoelectric material (e.g., quartz) and the biosensing material that secured on the piezoelectric material which vibrate at the basic repeat. The recurrence is controlled by the outer electrical flag which delivers a specific estimation of current, when the objective analyte is presented to the detecting material the connection/response will cause the recurrence move which will create changes in current perusing that can be examined to the mass of the analyte of intrigue. There are two essential sorts of piezoelectric sensors: mass wave (BW) and surface acoustic wave (SAW). In any case, writing indicates piezoelectric sensors are not getting much consideration and second rate contrasted with electrochemical and optical based biosensing. Bulk waves gemstone quartz microbalance and surface acoustic wave transducer, a very basic level for the piezoelectric effect. The unique properties of piezoelectric material are used in this sort of detecting. Quartz is the most customarily used piezoelectric since it is unassuming, can be taken care of to yield single valuable stone and can withstand creation, warm and mechanical weight; regardless, there is report that lithium niobate and lithium tantalate can also be used[54-56]. An existing questionnaire has shown that this strategy is exceptionally interesting when coordinated with Microelectromechanical frameworks (MEMS) for the application of biosensing. In addition, the inspection indicates that such a transmission is suitable for touching, compact and continuous biosensitivity [57]. The most common type of piezoelectric biosensor is quartz crystal microbalance (QCM), which is able to determine nanograms of material. The sensor consists of a thin wafer of quartz-sensing crystal plated with metallic electrodes on either sides of the crystal by means of vapor deposition. When an AC voltage is applied across the crystal-induced piezoelectric effect causes the release of the resonance frequency. Any adsorption of molecules to the surface of the oscillating crystal will cause its frequency to decrease. By measuring this frequency change, the amount of mass per unit area deposited on the surface can be determined with great precision (down to a few billionths of a gram). In an enzyme-based QCM biosensor, the resonance frequency decreases upon the adsorption of the enzymatic product onto the sensor surface. The frequency change (ΔF) is proportional to the mass (Δm) of the adsorbed molecules per unit area. A QCM-based piezoelectric biosensor was developed for urea detection by immobilizing urease onto Nano porous alumina membranes by the means of physical adsorption and cross-linking. Piezoelectric sensors have been attractive, due to their

simplicity, real-time measurement, high sensitivity, and cost-effectiveness. However, the major drawbacks of these devices are the interference from atmospheric humidity, and the difficulty in applying for the determination of the material in solution [58].

1.2.2.6.Calorimetric (thermometric)

Calorimetric biosensors are created by immobilization of biomolecules on temperature sensors. When the analyte is in contact with the biological component, the reaction temperature becomes proportional to the analyte concentration which in turn is measured. The total heat produced or absorbed is proportional to the total number of molecules in the molar enthalpy of the reaction. Temperature measurement is made by means of a thermistor, and this enzyme is called to devices such as thermistors. Thermal biosensors do not require frequent recalibration and are insensitive to the optical and electrochemical properties of the sample [59]. Calorimetric biosensors are used in analysis of food, cosmetics, pharmaceuticals [60, 61].

1.3. BIOSENSORS – FORMAT

1.3.1. Microfluidics Format

Many microfluidic devices have been advanced for different chemical and biological applications. Conventional microfluidic systems are based on the continuous flow regimes in micron – sized channels. These microchannels are produced via using soft – lithography methods [62].

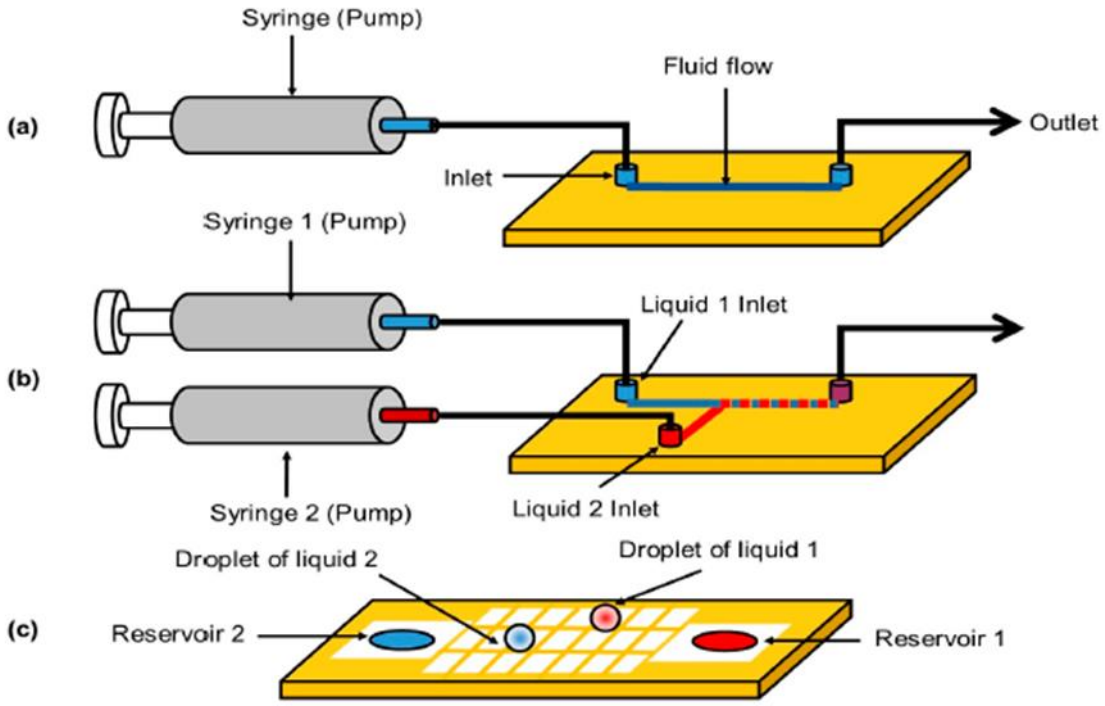

Figure 0.8 The scheme of three microfluidic systems. (**a**) Continuous; (**b**) Drop – based; and (**c**) Digital [62].

To reduce the sample consumption, and also create isolated reaction sites, droplet-based microfluidic systems have been developed. The first generation of the droplet-based microfluidic systems utilized the continuous stream of two or more fluid mainly intersected at a T – junction to create discrete droplets which are isolated from each other using an immiscible fluid. To further reduce the volume of consumption in the early 2000s, Digital microfluidic (DMF) called droplet based microfluidic system was introduced. DMF systems form droplets on an electrostatic – actuated electrode array rather than having continuous droplets flow through the micro channels. The most widely used operating mechanism array of droplets using an electric field based on modifying the interfacial properties of the dielectric fluid (EWOD) makes electrolysis technique. EWOD technique provides higher localization compared to the other technique. In addition, low power consumption and scalability. This unique feature of these systems makes them a very viable option to apply additional sensing modules. In particular, in recent years, these systems of multiple samples on the same chip are used for numerous applications bio sensing for allowing the parallel

processing with high efficiency. Table 1.2 summarizes the operating and actuation methods, advantages and disadvantages of the three different types of microfluidics [62].

Table 0.2 The comparison between the three types of microfluidics [62].

	Continuous-Flow Microfluidics	Droplet-Based Microfluidics	Digital Microfluidics
Operating Method	Motion of continuous fluid in micro-channels	Motion of droplets in micro-channels using streams of immiscible fluids	Motion of discrete droplets on an array of planar electrodes
Flow Actuation	Mechanical (syringe) pumps, Pneumatic pressure, Electrokinetic	Mechanical (syringe) pumps, Pneumatic pressure	Electrowetting On Dielectric, Dielectrophoresis
Advantages	Ease of fabrication and operation, suitable for applications that require a continuous flow with relatively high sampling volume, and being compatible with most of current screening and sensing mechanisms	Ease of fabrication and operation, suitable for a applications that require isolated reaction sites to avoid cross contamination	Lower sample consumption, scalability, better localization, reconfigurability, and portability
Disadvantages	High sample volume consumption compared to other microfluidic systems, possible contamination, and not being scalable due to fabrication and physical limitations	No control over individual droplets, challenging to create droplets of different sizes using the same setup, and challenging to implement stable gas-liquid systems	Complicated fabrication procedure, and bio-adsorption and evaporation

1.3.2. Lateral Flow Assays

The lateral flow assay (LFA) is a paper-based platform for the detection and quantification of analytes in complex mixtures, where the sample is placed on a test device and the results are displayed within 5-30 minutes. Low development costs and ease of production of LFAs have resulted in the expansion of its applications to multiple fields in which rapid tests are required. LFA – based tests are widely used in hospitals, physician's offices and clinical laboratories for the qualitative and quantitative detection of specific antigens and antibodies, as well as products of gene amplification. A variety of biological samples can be tested using LFAs, including urine, saliva, sweat, serum, plasma, whole blood and other fluids. Further industries in which LFA-based tests are employed include veterinary medicine, quality control, product safety in food production, and environmental health and safety. In these areas of utilization, rapid tests are used to screen for animal diseases, pathogens, chemicals, toxins and water pollutants, among others [63].

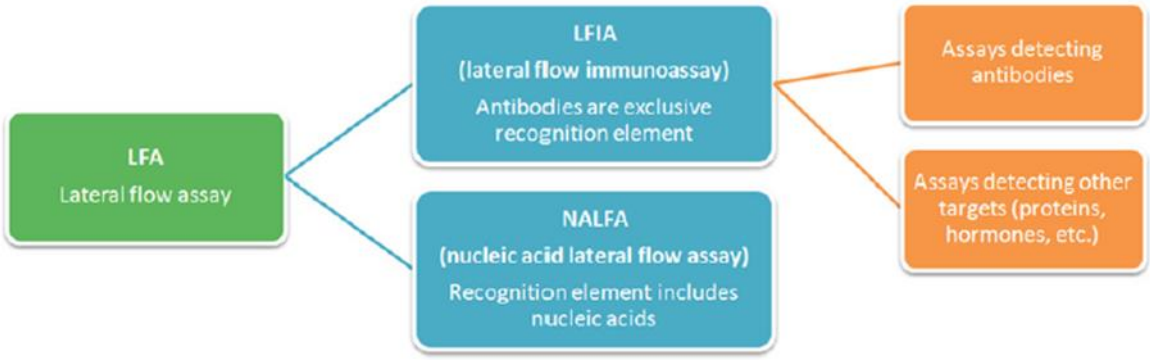

Figure 0.9 Classification of lateral flow assay[63].

In recent years, with multiple test lines to be perceived as allowing fast and simultaneous multi – analyte present in the sample has been an increasing demand for multiple diagnostic tests for point of care. This type of analysis (potential as a single LFA), laboratory investigations or persons trained in chemical analysis performed should be easy to use. LFAs are very good candidates as they are cheap to produce, easy to use and, importantly, widely accepted by users and regulatory authorities. As the pathway for the development and introduction of novel technologies to the clinical diagnostics market requires hundreds of millions of dollars and decades of work, the improvement and further development of already established LFA technologies is a favorable alternative. This process has the potential to produce devices that may become powerful tools for new challenging applications such as early cancer detection. Moreover, because of the long shelf life and the fact that refrigeration is not required for their storage, LFA are very well adapted for use in developing countries, small ambulatory care settings, remote regions and battlefields. Depending on the elements of recognition used, LFAs can be categorized into different types (Figure 1.9) [63].

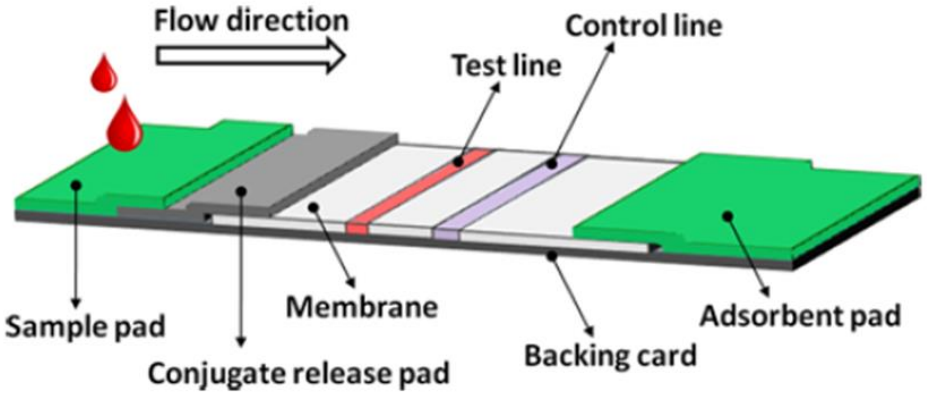

Figure 0.10 Typical configuration of a lateral flow immunoassay test strip[63].

The principle behind the LFA is simple: a liquid sample (or its extract) containing the analyte of interest moves without the assistance of external forces (capillary action) through various zones of polymeric strips, on which molecules that can interact with the analyte are attached. A typical lateral flow test strip (presented in Figure 1.10) consists of overlapping membranes that are mounted on a backing card for better stability and handling [63].

1.3.3. Paper – Based Biosensor

Biomarkers such as reagents and environmental analytes provide fast, simple, accurate and cost-effective detection which is an important need in biochemical research. For example, methods currently utilized in immunological assays (e.g., enzyme-linked immunosorbent assay {ELISA}) for the detection of biomarkers in bodily fluids (e.g., blood, urine, serum) provide sensitive and reliable results. Likewise, Spectrophotometric methods and environmental reagents (e.g., heavy metal ions in industrial waste water) is used for quantitatively analyzing traditional ELISA, also provides consistent and accurate results. However, sophisticated and lengthy procedures for conducting experiments and evaluate the results highly trained staff requirements and the need for large amounts of reagent and sample, these analytical tools to prevent its use in resource poor environments [64]. Therefore, unskilled users to easily identify analytes and unmet resources for the development of simple and low-cost detection system to provide the ability to assess the results in restricted environment there is a need. Paper-based diagnosis of diseases in

applications of biosensors, monitoring of health conditions, environmental agents or exposure to sunlight, detection of pathogens (e.g., bacteria, fungi), and controlling food safety and quality water [65]. Low cost paper-based microfluidic approaches enables the production of flexible and portable diagnostic platform [66]. This simple technology, microfluidic channels to form hydrophobic materials in hydrophilic by modeling paper is used as paper substrates. To test a biological substance (e.g., blood, urine, saliva, sweat, tear) or an environmental reagent (e.g., heavy metal ion, hydrogen sulfide gas) that contains an analyte of interest, the sample is applied to the device and wicked to a detection zone by capillary action without the need to use an external pump[65,67]. Analyte detection from the sample is facilitated by a chemical reaction which induces a change in color, electrochemical properties and light absorption or emission. Among these methods, the most frequently used detection approach is based on colorimetric change. In this method, results can be simply evaluated by formation of a color product generated by ligand-analyte binding (e.g., antibody-antigen) which can be quantified using low-cost benchtop scanners, single-lens reflex cameras, or cellphones [68]. The main advantages of paper include; (i) high surface to volume ratio, (ii) adsorption properties, (iii) capillary action, (iv) compatibility with biological samples, (v) chemical functional groups for immobilization of proteins and antibodies and (vi) straightforward sterilization[65]. Paper also allows for easy disposal via incineration. Furthermore, the ability to store and transport reagents within the paper matrix eliminates the need for users to handle chemical solutions. In addition, paper is lightweight and accessible globally [69]. Finally, paper-based microfluidic devices can be easily fabricated at low-cost using practical fabrication techniques such as wax printing [70]. Paper-based dipstick assays, lateral flow and vertical flow immunoassay test is conducted as a routine for the rapid detection of target analytes. Simple dipstick techniques were initially utilized to quantify the glucose in urine in 1956[71, 72]. Dipstick technique, although convenient and easy to interpret, the main disadvantage of a long analysis times and contained mistakes. Therefore, a variety of lateral flow devices to achieve better performance parameters have been developed in the 1980s. These devices are mostly used for pregnancy tests [73]. Over time, lateral flow assays, screening of blood clots, the detection of samples in pesticides and food and drink has become standard platforms for other applications such as the detection of pathogens (such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*)[65].

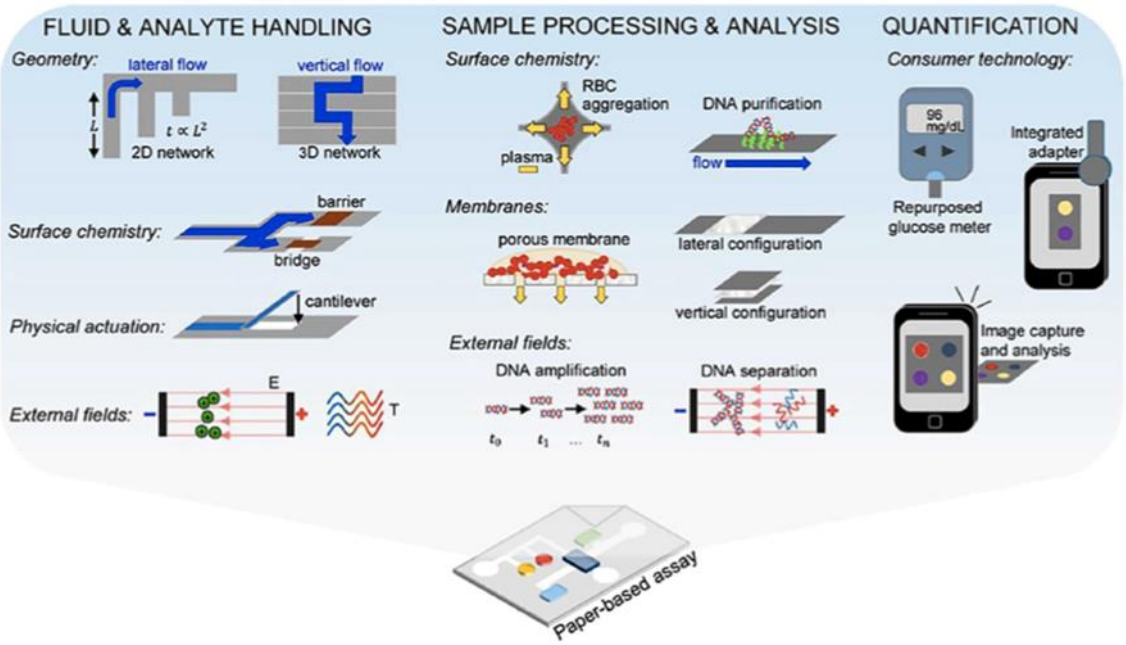

Figure 0.11 Paper-based sensors used in analytical capabilities overview. Processing strategies and the analyte fluid sample processing and analysis and experiments with paper-based processing strategy and the analyte fluid, sample processing and analysis, quantification [65].

The lateral flow configuration, fluid is pulled horizontally towards a sample detection zone; the fluid flow is directed vertically through the vertical flow immunoassay. Although the direction of fluid flow is the most apparent difference between these two assay formats, vertical flow provides detection more rapidly (as short as 5 min) with a higher detection sensitivity in nanograms per mL. Moreover, Hook's effect (a phenomenon that is caused by the presence of excess amount of antibodies preventing agglutination), which can lead to inaccurate results in lateral flow assays, is also eliminated in the vertical flow configuration [74]. Thus, more recent studies use vertical flow assays for detection of analytes. For example, Rivas et al. fabricated a paper-based vertical-flow device to analyze microarrays of DNA for detection of N. meningitides [75]. Despite promising Paper-based devices and applications of the sensors, the sensitivity of multiple analytes at the same time, there are some limitations on the accuracy and detection. Recently, paper-based sensors are manufactured by molding to eliminate these drawbacks. In addition to colorimetric technique, chemiluminescence, electroluminescence, other approaches such as fluorescence and electrochemical detection is used to monitor analyte. The analytical approach was

applied to the paper-based microfluidic platform; however, the cost of these techniques can also exhibit drawbacks associated with the simplicity and precision [76].

1.3.3.1.Paper Types

Depending on the fabrication method and sensor applications have different types of paper used in paper-based sensor. The most commonly used due to its superior wicking ability of material and paper of Whatman brand chromatography [65]. This particular type of paper has medium retention and flow rate owing to its thickness (180 μm) and pore size (11 μm). Other types of paper such as the Whatman filter paper No. 4, was used due to its larger pore size of 20–25 μm and higher retention rate [77]. More recently, filter paper has been used in paper-based sensors, which is also manufactured by Whatman (Maidstone, United Kingdom). This type of paper has been used for its relatively uniform thickness and wicking properties as well as superior adsorption and retention of reagents compared to the similar types of paper[78]. Biosensors for different physical and / or chemical properties may be subject to other paper categories were examined depending on the target application. Nitrocellulose membranes have been used due to their chemical functional groups that enable covalent immobilization of biomolecules. Nitrocellulose allows for charge-charge interactions, weak hydrogen bonds, and van der Waals interactions with protein-based substrates [65]. As a result of their high protein-binding abilities, these membranes are commonly used in ELISA and gold nanoparticle-based assays [79]. These membranes have also been used, since it prevents the diffusion and infiltration of the samples through the membrane, which leads to a higher degree, and then holding the reaction for a longer period of time allows the sensor. The nitrocellulose membranes are smooth and have a uniform pore size of 0.45 pm. These membranes can be modified by heating the wax after printing, but the nitrocellulose membrane is slower compared to the filter paper permeated wax [77]. In addition to this type of paper it is used in paper of bioactive biosensor. Bioactive paper is replaced by some paper matrix biomolecules. Without modification, only the cationic molecules absorb the wet cellulose fibers forming the paper. In addition, when proteins adsorbed on cellulose, the rate and extent of this process was not effective in comparison to other hydrophilic surfaces [80]; therefore, the cellulose fibers must be modified to absorb biomolecules. This is done by activating the surface of the paper (for example, aldehydes, amides) and then can be covalently conjugated with biomolecules. Furthermore, bioactive paper-based device, supported by paper, the piezoelectric of the enzymes within the

biocompatible sol-gel silica layers were produced by inkjet printing [81]. The vaccine also copolymerization of cellulose filter paper glycityls methacrylate, point and gold nanoparticles have been shown to allow ELISA-based test for the immobilization of biomolecules permitted [82]. Glossy paper, were examined at the same time as a viable option for paper-based sensors. Bright paper is composed of inorganic fillers blended from cellulose fibers and paper matrix. Arena et al. used glossy paper in order to develop a flexible paper-based sensing device for detection of ethanol [83]. This special type of paper is used instead of filter paper, because it is easier to change the surface properties of the glossy paper. Common paper of different types in the production of paper-based sensors has been used successfully. A wearable device, folding and was selected to be flexible conventional printing paper intended [84]. The paper was subjected to printing wax and carbon black ink was then spread across the surface of the paper. Additionally, the paper towel was used as a surface for the printing of carbon black modified electrode [85]. Paper towel has also been used as wicking layer for a biosensor produced from filter paper. Paper towel is cheaper than filter paper and possesses a high porosity, which makes it a viable material for analysis of a wide range of analytes [65].

1.3.3.2.Printing and Fabrication Methods

Production method used to produce the biosensor can affect and simplicity of application. There are numerous approaches available that involve chemical modification or physical deposition onto the paper, both of which alter the material characteristics of the cellulose matrix. The approaches that will be covered in this review are wax printing, photolithography, inkjet printing, laser cutting, polydimethyl–siloxane (PDMS), hot embossing, hydrophobic salinization, and the use of origami and kirigami–based approaches. Main fabrication techniques, several advantages and disadvantages of each procedure are listed in Table 1.3 and Table 1.4[65].

Table 0.3 Advantages and disadvantages of the main fabrication techniques for paper-based sensors [65].

Procedure	Advantages	Disadvantages
Wax Printing	Low-cost, easy fabrication, short fabrication time	Low resolution, unstable upon heating
Photolithography	High resolution, suitable for large-scale production	Expensive and sophisticated equipment, unstability against bending or folding
Inkjet Printing	Efficient, reduced cross-contamination, rapid fabrication, high resolution	Expensive bio-ink printer
Laser Cutting	Simple, inexpensive	Specialized equipment
PDMS	Low-cost, flexible	Low resolution, sophisticated equipment for fabrication of molds
Hot Embossing	Short fabrication time, efficient	Specialized equipment
Hydrophobic Silanization	Low-cost, rapid fabrication	Limitation with simple designs
Origami and Kirigami	Intricate and innovative designs, simple fabrication	

Table 0.4 Main fabrication techniques for paper-based sensors [65].

Fabrication Technique	Procedure	Detection Method	Recognition Element	Analyte Detected
Wax printing	Melted wax	Electrochemical	Glucose oxidase	Glucose
	Melted wax	Colorimetric	Ascorbic acid and 1,10-phenanthroline	Iron
	Melted wax	Colorimetric	Phenolphthalein	Limiting reagent of acid-base reaction
	Melted wax	Colorimetric	Anti-Human IgG	Human IgG
Photolithography	Hydrophobic photoresist	Colorimetric	Oxidase enzymes that produce H_2O_2	Glucose, lactate, uric acid
	Hydrophobic photoresist	Colorimetric	Glucose oxidase, potassium iodide, trehalose, horseradish peroxide	Glucose
	Hydrophobic photoresist	Colorimetric	Glycine, sodium nitroprusside	Acetoacetate
	Hydrophobic photoresist	Colorimetric	Sulfanilamide, citric acid, and n-(1-napthyl)ethylenedi-amine	Nitrite
PDMS	Hydrophobic PDMS	Colorimetric	Glucose oxidase, potassium iodide, horseradish peroxide	Glucose
	Hydrophobic PDMS	Colorimetric	Bromothymol Blue	pH
	Hydrophobic PDMS	Colorimetric	Tetrabromophenol blue	Proteins
	Hydrophobic PDMS	Fluorescence	Tetrabromophenol blue and NBT alkaline Phosphatase Color Development Kit	Total serum protein, alkaline phosphatase, aspartate aminotransferase
	Hydrophobic PDMS	Colorimetric	Detection antibodies and capture antibodies	C-reactive protein (from human serum)
Inkjet printing	Printed biomolecules	Colorimetric	3,3′-diaminobenzidine	Horseradish peroxidase
	Printed conductive electrodes	Electrochemical	Change in capacitance	Humidity
Laser cutting	Hydrophilic patterns printed using CO_2 laser cutter	Chemiluminescence	Catalytic action of iron in hemoglobin	Luminol-based hemoglobin
Hot embossing	Embossed paper with layer of rubber and poly(vinyl)alcohol	Colorimetric	Glucose oxidase, horseradish peroxidase, and o-Dianisidine	Glucose
	Parafilm and paper heated in an oven	Colorimetric	Cholinesterase and dithiodipropionic nitrobenzene acid	Methomyl
	Parafilm and paper heated in an oven	Colorimetric	Uricase, horseradish peroxidase, tetra-methyl benzidine	Uric acid
Hydrophobic silanization	Selective wet etching of hydrophobic filter paper	Colorimetric	Glucose oxidase, potassium iodide, horseradish peroxide	Glucose
Origami	Printed electrodes followed by baking	Electrochemical	Glucose oxidase	Glucose

1.4. GLUTEN

Wheat flour is unique among edible grains, because wheat flour, which can be made into a paste with rheological properties, has a protein complex called "gluten"[86]. Gluten on the rheological properties not only for the production of bread, but also just wheat, vid., Noodles, pasta, mobile breads, pastries, cookies and needs within the broader food coverage that can be made from other products is heard[87]. In practice, the "gluten" as used herein, refers to proteins. Gluten determines the water absorption capacity of dough stickiness, viscosity and elasticity of wheat and plays a key role in determining the unique cooking quality [88]. Gluten, oligo- or monomer, with inter-chain disulfide bonds and contains hundreds protein component linked polymer [89].

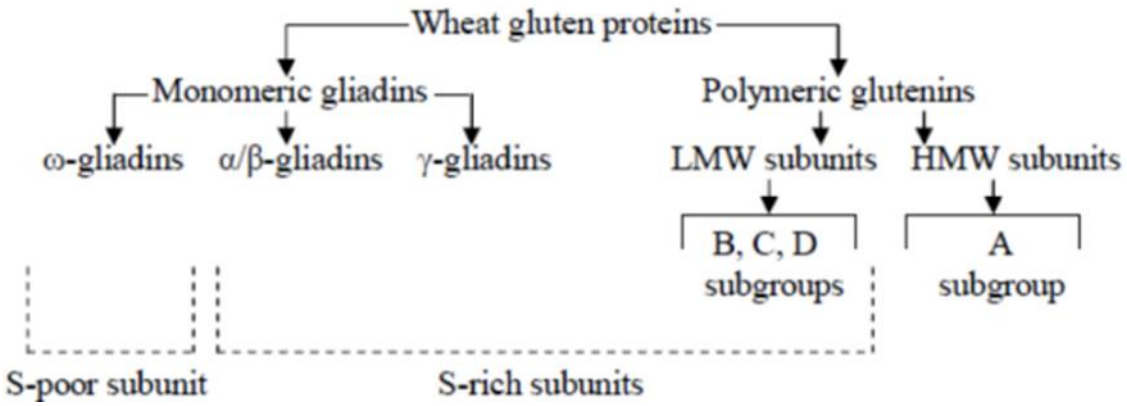

Figure 0.12 Classification of gluten proteins. Adapted from [94].

One approach to apply effectively the wheat gluten in the food industry, functional properties (such as solubility, emulsification and film forming properties) improve or hypoallergenic infant diet and nutrition therapy intensively for preparing hydrolyzed proteins are widely used in the enzymatic hydrolysis used[91]. However, it has been reported that there were few researchers in antioxidant peptide wheat gluten hydrolysate [91, 92]. The native gluten protein had a high antioxidant capacity of 74.39 mmol Trolox/kg [93]. Gluten, wheat dough when the starch granules and washed to remove water-soluble components can be defined as the rubber mass. Durum average, contained much higher amounts than in the wet milling wheat gluten. Wet gluten ranged from 17.35 to 29.65 per cent and 20.00 to 32.20 per cent in bread and durum genotypes, respectively [94].

Depending on the thoroughness of washing, the dry solid contain 75–85 per cent protein and 5–10 per cent lipids; most of the remainder is starch and nonstarch carbohydrates [88, 91]. Gluten, which is present as monomers or inter-chain disulfide linkages and hundreds of oligo protein component linked polymer. Traditionally, gluten protein can be roughly divided into equal fractions: monomeric gliadins and the polymeric glutamines [94]. According to the alternative classification, wheat gluten can be separated into three large groups: Sulphur-rich (Mw of ~50 kDa; α-, β-, γ-gliadins and B- and C-LMW glutenins), Sulphur-poor (Mw ~50 kDa; ω-gliadins and D-LMW glutenins) and high molecular weight (Mw ~100kD; HMW glutenins) proteins. Glutenins and gliadins are recognized as the major wheat storage proteins, constituting about 60-90 per cent of the total grain proteins [94, 95] and they tend to be rich in asparagine, glutamine, arginine or proline but very low in nutritionally important amino acids lysine, tryptophan and methionine [96]. Cysteine, although belonging to minor amino acid of the protein gluten (≈ 2 per cent), is extremely important for the gluten structure and functionality [97].

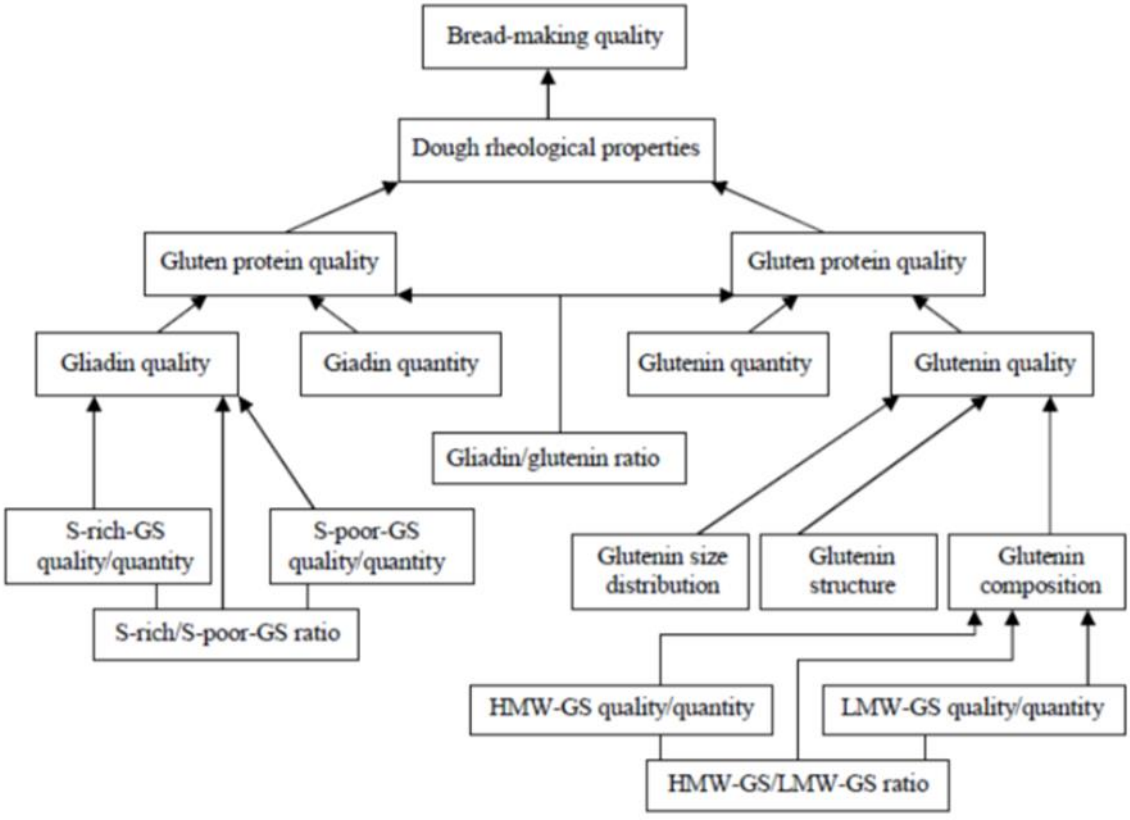

Figure 0.13 Factors affecting wheat dough rheological properties [94].

Most of the system generates an intra-chain disulfide bonds between the protein and proteins in the intra-chain disulphide bonds. Dough during development, it can be activated by disulfide bonds, disulfide exchange reactions [98]. Wheat gluten, as the most complex protein, had a high concentration of disulfide bonds (45.37 nmol/mg) [99]. Additional covalent bonds formed during break making are tyrosine–tyrosine crosslinks between gluten proteins and tyrosine–dehydroferulic acid crosslinks between gluten proteins and arabinoxylans. The covalent structure of the gluten network is superimposed by non-covalent bonds (hydrogen bonds, ionic bonds, hydrophobic bonds). Although hydrogen bonds are individually weak, they create stability to the dough when large numbers of bonds are established during the dough development [100]. Hydrophobic and ionic bonds, although present in very small quantities, the interactions between the biopolymers resulting in bread dough to increase dough stability play an important role [101]. The glutenin to gliadin ratio clearly affects the mechanical properties of gluten dough. Expanding gliadin content, is lower than the elongation resistance, extensibility has to be higher. Based on measurements on glutens reconstituted at various glutenin/gliadin ratios, they found that at the constant protein content the main factor determining the rheological behavior of hydrated gluten is the glutenin to gliadin ratio. The effect of gluten proteins on wheat dough rheological properties and bread-making quality is shown in Figure 1.13 [102].

Celiac disease (CD) is a genetic, autoimmune disorder that occurs in reaction to the ingestion of gluten. To develop celiac disease a person must inherit the genetic predisposition, be consuming gluten, and have the disease activated. Activation triggers include stress, trauma (surgeries, etc.) and possibly viral infections and other environmental factors. While approximately 33% of the general population has the genetic predisposition for CD, only about 1% will develop the condition. The reaction to gluten causes inflammation and villous atrophy or flattening of the cells lining the small intestine, which can lead to malabsorption of nutrients and related health issues. There are over 200 identified symptoms of CD, which include those listed above as well as anemia, behavioral changes, stunted growth and infertility. Dermatitis herpetiformis is celiac disease that manifests as a skin rash, and affects approximately 10% of individuals with CD. The rate of CD is higher among relatives of those who are diagnosed, but anyone with the genetic predisposition can develop celiac disease at any age. The majority of individuals with celiac disease are still

undiagnosed. At this time the only treatment for celiac disease is to maintain a gluten-free diet for life.

Non-Celiac Gluten Sensitivity (NCGS), also referred to as gluten sensitivity (GS) or non-celiac wheat sensitivity (NCWS), is not well defined. It is not an immunoglobulin E (IgE) (as with wheat allergy, see below) nor autoimmune reaction (as with CD, see above). NCGS may have an innate immune component, but this has not been firmly established. There are no tests or biomarkers to identify GS. Since GS is not well understood it is still not clear whether other components of gluten-containing grains may be involved in causing symptoms, at least in some cases. In order for gluten sensitivity to be diagnosed, it is first necessary to rule out CD, wheat allergy, and other possible causes of symptoms. Then, if improvement is seen when following a gluten-free diet, gluten sensitivity may be diagnosed.

Wheat allergy is an immune reaction to any of the hundreds of proteins in wheat. When a person has a wheat allergy, one type of white blood cells, called B-cells, send out immunoglobulin E (IgE) antibodies to "attack" the wheat. At the same time, local tissues in the body send out natural chemical messengers to alert the rest of the body that there is a problem. This reaction happens very fast (within minutes to a few hours) and can involve a range of symptoms from nausea, abdominal pain, itching, swelling of the lips and tongue, to trouble breathing, or anaphylaxis (a life-threatening reaction). A person with a wheat allergy must avoid eating any form of wheat, but does not have trouble tolerating gluten from non-wheat sources. (It is possible for a person to be both allergic to wheat and have CD or NCGS.) In the United States, wheat is one of the eight most common foods to which people are allergic. Children who are allergic to wheat may outgrow the allergy, but adults with an allergy to wheat usually have it for life. The only treatment is a wheat-free diet.

1.4.1. Importance of Gluten Detection

1.4.1.1.International Legislation - Codex Alimentarius

Grains that contain gluten (ie, wheat, rye, barley, oats, sourced or hybridized strains and their products) as one of the ingredients and components known to cause hypersensitivity should always be indicated on the label of pre-packaged foods [103]. Cereal, as well as contain gluten, the standard 10 mg / kg or greater than concentration of the main food allergens shellfish, eggs, fish, peanuts, soybeans, milk, includes tree nuts and sulfites. More specific

legislation regarding gluten-free products, in 2008, the latest revised gluten is defined in the Codex Standard for Foods for Special Diet Used in retaining the People. [104]. This standard defines that "gluten-free foods are dietary foods consisting of or made from one or more ingredients which do not contain wheat (i.e., all Triticum species, such as durum wheat, spelt, and kamut), rye, barley, oats or their crossbred varieties, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer." Alternatively, consisting of cereal foods containing gluten, specially processed to remove gluten, so that the level 20 mg / kg does not pass. Regarding Oats, national arrangements can be made, because the majority of uninfected people who are not gluten intolerance can tolerate oats are accepted. In subsidiary definitions "gluten is defined as a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl". "Prolamins are defined as the fraction from gluten that can be extracted by 40e70 per cent of ethanol" and the "prolamin content of gluten is generally taken as 50 per cent". The decision on whether or not to use the term "gluten-free" on the label lies with each manufacturer and the product is only subject to the respective regulatory framework if a voluntary gluten-free claim is made [105].

1.4.1.2.European Union Regulations

Definitions, thresholds, and labelling currently specified in the European Commission Regulation (EC) No 41/2009 of 21 January 2009 are equivalent to those in Codex Standard 118-1979 (2008) [104,106]. The new Regulation of the European Parliament and of the Council (EU) (2013), No 609/2013 'on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control' to be implemented from 20 July 2016 will repeal EC No 41/2009 [107]. As stated in article 41 of No 609/ 2013, the rules on the use of the statement "gluten-free" are to be regulated under Regulation of the European Parliament and of the Council (EU) (2011), No 1169/2011 'on the provision of food information to consumers' [108]. The EU 1169/2011, which entered into force on 13 December 2014, aims to provide a high level of consumer protection by setting requirements for food information and labeling of prepackaged and unpackaged foods. In accordance with this regulation, it will be necessary to indicate that any component or processing aid listed in Annex II will cause allergies or intolerances to be used in food

preparation and still in altered form in the finished product. The substances listed in Annex II include gluten, that is, wheat, rye, barley, oats, sliced, kamut or their hybridized strains and products derived there from. The rules on the use of /2011 gluten-free lu in 41/2009 / EC will be transferred to EU No 1169/2011 to provide at least the same level of protection for persons with gluten intolerance currently provided. It should also be noted that CD patients are sufficiently aware of the difference between a specially processed food to reduce gluten content and other foods derived from naturally only gluten-free content [105].

1.4.2. How It Is Measured?

1.4.2.1.Legislation Regarding Gluten Measuring

Codex Standard 118-1979 (2008) specifies the general requirements for gluten analysis and sampling methods. The quantitative determination of gluten in foods and components in an immunological method or providing at least the same sensitivity and specificity should be based on another method. The antibody used by cereal protein fractions that are toxic to people are intolerant to gluten must react and should not cross-react with other components. The methods should be approved and calibrated according to an approved reference material (RM), if any. The limit of detection according to the technology must be suitable and 10 mg gluten / kg or less should be. The qualitative analysis that indicates the presence of gluten shall be based on relevant methods (e.g., ELISA-based methods, DNA methods). More specifically, Enzyme Linked Immunoassay R5 Mendez Method is expressed in a type I method for determining gluten. A type I method, also called defining method, is defined as "a method which determines a value that can only be arrived at in terms of the method per se and serves by definition as the only method for establishing the accepted value of the item measured". However, in February 2015, the American Association of Cereal Chemists International (AACCI) successfully proposed changes to Codex Standard 234-1999 (2014) on 'Recommended Methods on Analysis and Sampling'. Because of the recent developments in method development and validation, two immunological tests will be determined as type I methods. The first is based on the R5 monoclonal antibody (mAb) and recommended for gluten analysis in maize matrices and the second is based on the G12 mAb and recommended for rice matrices. Both methods fulfil the requirements for gluten analysis, but effects of matrices on recovery should be taken into account when using these methods

for other than maize- or rice-based matrices, respectively. In particular, a major concern expressed by celiac communities, is allowed to have more than one registered suitability of the ELISA method for the measurement of gluten. At present, it is not clear how to proceed in case of conflicting results. Furthermore, the possibility of having two types of methods has to be questioned, because its definition as “the only method” should preclude the establishmENT of a second type I method [105].

1.4.2.2.Gluten Measurement

The requirements of reliable analytical method to quantify the amount of gluten in food are specificity, reproducibility, and reliability verification [105]. Gluten detection is needed in heated or drawn products such as bread and pasta [109], and in products containing partially hydrolyzed gluten.[110]. as the first step, the extraction of gluten proteins and/ or peptides from the food matrix should be as complete as possible. The assay itself must be correctly and appropriately calibrated against a suitable and representative reference protein. Most methods rely on the measurement of the amount of alcohol soluble gluten prolamin fraction. The alcohol-insoluble glutelin fraction is often not targeted, although both prolamins and glutelins contain immunogenic peptides [111]. Because the prolamin content of gluten is taken as 50 per cent [104], the determined prolamin content is usually multiplied by a factor of 2 to obtain the gluten content. This prolamine / glutelin ratio is assumed to be one. However, a comprehensive analysis of wheat, spelt, emmer, einkorn, rye, and barley flours as well as wheat starches showed that the true prolamin/glutelin ratios were highly variable ranging from 0.2 in wheat starch to 13.9 in einkorn. Therefore, the gluten content is either overestimated, or, more seriously for CD patients, underestimated by duplication of the prolamin content [105].

1.4.2.3.Extraction of Gluten from the Food Matrix

The complexity of the food matrix containing gluten and gluten analytical creates significant difficulties. Natural gluten proteins are characterized via their insolubility in water or saline solution, their high molecular weights, their heterogeneous surface properties, intermolecular and molecular disulfide bonds, and their limited stability when dissolved. In contrast, partially hydrolyzed gluten and salt is soluble in water or aqueous alcohol solution.

The most commonly used solvent in gluten detection methods is aqueous alcohol (60 per cent ethanol or 50 per cent propanol), which extracts mainly the prolamin fraction from nonprocessed materials such as flours. However, aqueous alcohol is insufficient to solubilize prolamins from processed materials, because prolamins and glutelins aggregate through heat-induced formation of interchain disulphide bond. For this reason, reducing agents such as 2-mercaptoethanol or tris (2-carboxyethyl)-phosphine (TCEP) (for disruption of disulphide bonds) and disaggregating agents such as guanidine or sodium dodecyl sulphate (SDS) (for enhanced solubility) are used in combination with aqueous alcohols to extract prolamins together with glutelins from raw and processed materials. The extraction at 50°C with the so-called cocktail, which contains 2-mercaptoethanol and guanidine in a phosphate buffer, is part of the sandwich R5 ELISA method. Combinations of 2-mercaptoethanol and SDS, TCEP and guanidine, or TCEP and the anionic surfactant N-lauroyl-sarcosine in a phosphate buffer, the so-called universal prolamin and glutelin extractant solution (UPEX) were shown to be suitable for the extraction of gluten proteins and peptides from various food samples. Depending on the food matrices, additional steps may be necessary. Defatting with n-hexane is recommended for products with more than 10 per cent fat. For products containing high amounts of polyphenols, it may be necessary to add fish gelatin and/or polyvinylpyrrolidone, or skim milk powder to the extraction solution to disrupt gluten protein-polyphenol interactions. The compatibility of the extraction solvent with the subsequent analytical procedure obviously needs to be verified for each procedure [105].

1.4.2.4.Reference Materials

Assays compare a generally obtained analytical signal with a standard curve produced by a set of calibrators at different concentrations of the target molecule. These calibrators are associated with an RM to determine the actual amount of gluten in the sample. Although the calibrators provide an assay (eg, ELISA test kits commercially available for the determination of gluten or gliadin), the generally accepted RM is minimal and is not always commercially available. According to the definition of ISO (Guideline 30, 2015), an RM should be sufficiently homogeneous and stable to one or more specific features specified in a measurement to suit the intended use. RM, to determine the accuracy of analytical results (validation studies, proficiency testing, method validation) and also different methods of quality assurance and / or is important for comparability of analytical results between

laboratories. Because of food processing and food matrix to affect the analytical results and to provide a better use of portability to real food samples prepared material is recommended. Incurred materials, in which a known amount of gluten/gliadin or the respective prolamin-containing flour has been incorporated during processing, mimicking as closely as possible the actual conditions under which the sample matrix would normally be manufactured, are difficult and costly to obtain. Therefore, the addition of extracts or solutions of the analyte is still considered an acceptable way to test the performance of a method that includes information on matrix effects. The best-defined RM for gluten analysis is the Prolamine Working Group (PWG) gliadin standard to date. PWG gliadin, 28 derived from a highly pure mixture of European wheat gliadin. However, PWG gliadin represents only the total gluten and alcohol-soluble part of cereal proteins, thus limiting its use to methods that target the prolamine portion of the cereals involved. In an effort to develop an improved RM containing all gluten protein fractions based on the Codex definition of gluten, a new task force on RM for food allergen and gluten-free analysis coordinated by the MoniQA Association is currently characterizing minimally processed materials (flours from wheat, rye, and barley). These materials and materials containing various concentrations of wheat, rye and barley flour will be available in late 2015 or early 2016. RM, which is generally accepted for gluten analysis in food products, will enable adaptation, standardization and calibrating the corresponding methods, and international traceability, i.e. the ability to achieve comparability of such measurement results worldwide. In addition, this RM may be the basis for further development or the production of innovative analytical tools [105].

1.4.2.5.Immunological Methods

Immunoassays are, antibody specific methods for the detection of the corresponding antigen (immunoglobulin). Antibodies are produced by immunization of mammals such as mice, rabbits or goats after injection of the antigen. Polyclonal antibodies (pAb) are secreted by different B cell lineages within the mammal and are a mixture of immunoglobulins that react against a specific antigen, each binding to a different epitope. In case gluten analysis made with PAP, the cereal species during processing less affected by a change of varieties and modifications, but limited supplies of the same pAb and less extensive characterization are great disadvantages. In contrast, mAbs, a myeloma cell and an antibody-producing B cells is carried out by assembling the hybrid cells to obtain so-called hybridomas. All of these

mAbs define the same epitope and can be produced in an almost unlimited amount with repeatability. ELISAs are recommended by the Codex and most frequently used in gluten analysis, but new techniques like immunosensors or immunomagnetic beads for multiplex analyses are reported to being developed [105,112].

1.4.2.6.Principles of ELISA

ELISAs are based on coordinating antibodies that covalently bind to an enzyme such as horseradish peroxidase or alkaline phosphatase and produce a colored, chemiluminescent or fluorescent product for measurement. Two principles of sandwich and competitive ELISA can be applied for gluten analysis [113]. In sandwich ELISA (Figure 1.14A), the plate is coated with a known quantity of the capture antibody and nonspecific binding sites on the surface are blocked. Then, the antigen-containing sample is applied and the antigen/antibody complex is formed (step 1). After removal of the excess by washing the antigen is then added to the antibody detection reagent labeled with an enzyme binds to a second antigen binding. Therefore, the antigen is "compressed" between the capture antibody and the detection antibody (step 2). Non-binding detection antibodies are then washed by addition of the enzymatic substrate which is converted to a colored product whose absorbance can be measured in a plate reader (step 3). The measured absorbance is directly proportional to the concentration of antigen in the sample extract, which can be calculated from the calibration curve using a gluten reference protein. Since the antigen must have two spatially separated binding sites for the capture and detection antibody, the sandwich ELISA is only suitable for larger antigens, such as intact gluten proteins. This requirement is not suitable for gluten analysis in products containing partially hydrolyzed gluten, such as beer, sourdough products or malt extracts, since these gluten peptides may have only one binding site [105].

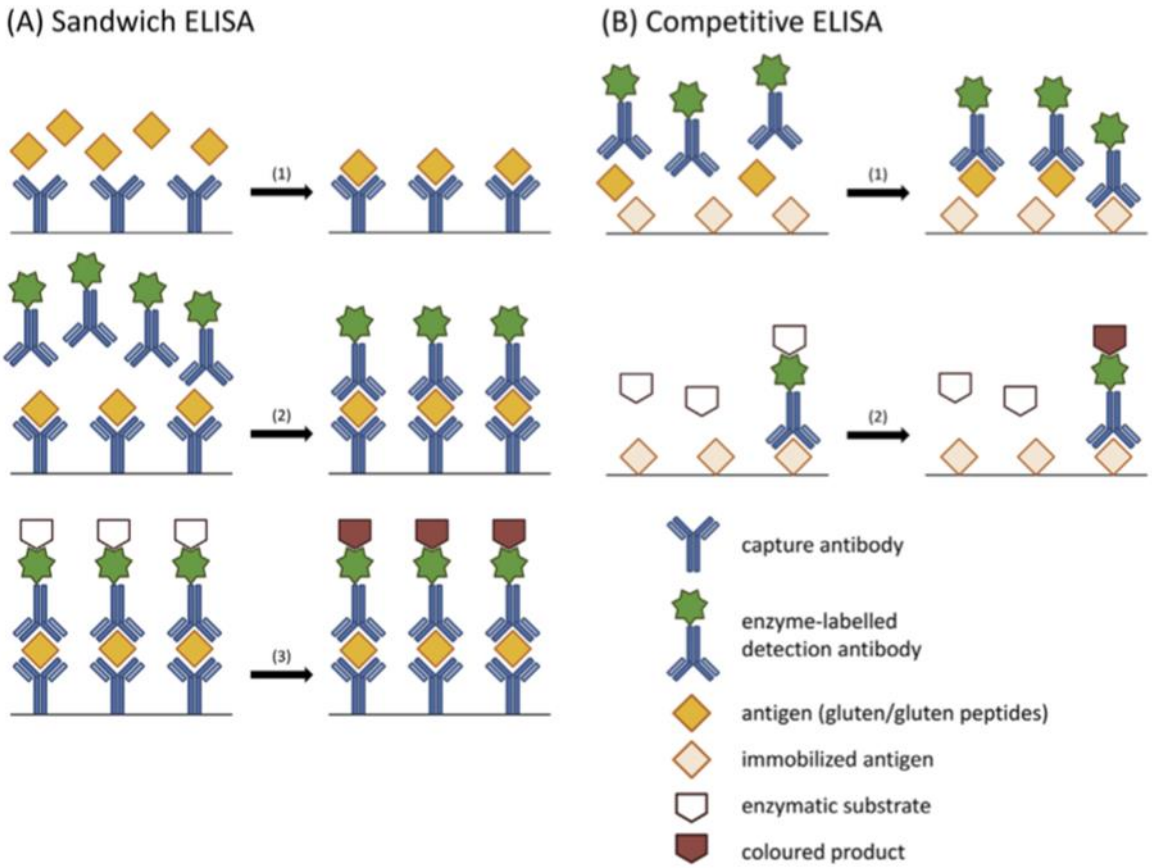

Figure 0.14 Principles of ELISA. A: sandwich ELISA, B: competitive ELISA. For explanation of the different steps [105].

The competitive ELISA (Figure 1.14B) may be used for both intact proteins as well as smaller antigens (gluten peptides), because it requires only one binding site. A known quantity of antigen is immobilized on the surface of the microtiter plate. The antigen-containing sample and a limited, constant amount of enzyme-labeled antibody are added to the well at the same time. During the incubation, the immobilized and free antigens compete for antibody binding sites (step 1). If more antigens are present in the sample, fewer antibodies will bind to the immobilized antigens. Unbound antibodies, antigens and antigen / antibody complexes are removed by washing. A colored product is formed after addition of the enzymatic substrate (step 2). In this case, the measured absorbance is inversely proportional to the antigen concentration in the sample extract. Again, samples with a gluten peptide or peptide mixture, a calibration curve are used to calculate the antigen concentration in the extracts [105].

1.4.2.7.Antibodies Used in ELISA Test Kits for Gluten Detection

Several gluten or gliadin-specific antibodies have been improved [114], but the majority of commercially available ELISA test kits for gluten detection is based on the Skerritt (401.21) [115], R5 [116], G12 [117], and a20 [118], mAbs, or various pAbs (Table 1.5).

Table 0.5 List of commercially available ELISA kits for gluten detection [105].

Manufacturer	ELISA kit	Principle	Antibody
Abnova	Gluten/Gliadin ELISA Kit	Sandwich	pAb
Astori Tecnica	Gluten ELISA Kit	Competitive	pAb
Biomedal Diagnostics	GlutenTox ELISA Sandwich	Sandwich	A1/G12 mAb
	GlutenTox ELISA competitive	Competitive	G12 mAb
	GlutenTox Sticks	Dipstick	G12 mAb
Biocontrol	Transia Plate Prolamins	Sandwich	R5 mAb
BioCheck (UK)	Gluten-Check ELISA kit	Sandwich	401.21 mAb
Diagnostic Automation	AccuDiag™ Gliadin/Gluten ELISA	Sandwich	pAb
ELISA Systems	ELISA Systems Gliadin assay	Sandwich	401.21 mAb
ELISA Technologies	Gluten Aller-Tek	Sandwich	401.21 mAb
	EZ gluten®	LFD	401.21 mAb
Elution Technologies	Gluten Rapid Kit	LFD	pAb
EuroProxima	Gluten-Tec® ELISA	Competitive	α20 mAb
Immunolab	Gliadin/Gluten	Sandwich	pAb
Imutest	Gluten-Check ELISA Kit	Sandwich	401.21 mAb
	Gluten-in-Food Test	Screening test	401.21 mAb
InCura	GlutenAlert ELISA	Competitive	pAb
Ingenasa	Ingezim Gluten®	Sandwich	R5 mAb
	Ingezim Gluten® SemiQ	Sandwich	R5 mAb
	Ingezim Gluten® Hidrolizado	Direct	R5 mAb
Morinaga Institute	Wheat Protein ELISA Kit	Sandwich	pAb
Neogen	Alert for Gliadin	Screening test	401.21 mAb
	Alert for Gliadin R5	Screening test	R5 mAb
	BioKits Gluten Assay Kit	Sandwich	401.21 mAb
	Veratox® for Gliadin	Sandwich	401.21 mAb
	Veratox® for Gliadin R5	Sandwich	R5 mAb
	Reveal 3-D for Gluten	LFD	401.21 mAb
R-Biopharm	Ridascreen® Gliadin	Sandwich	R5 mAb
	Ridascreen® Fast Gliadin	Sandwich	R5 mAb
	Ridascreen® Gliadin competitive	Competitive	R5 mAb
	Rida®Quick Gliadin	Dipstick	R5 mAb
Romer Labs	AgraQuant® ELISA Gluten G12	Sandwich	G12 mAb
	AgraQuant® ELISA Gluten	Sandwich	pAb
	AgraStrip® LFD Gluten G12	LFD	G12 mAb
	AgraStrip® LFD Gluten	LFD	pAb
Zeulab	Proteon Gluten Express	Dipstick	G12 mAb

LFD: lateral flow device; mAb: monoclonal antibody; pAb: polyclonal antibody; 401.21 mAb is also known as Skerritt mAb

1.4.2.8.Measured with 33-mer Peptide

Major immunogenic component of wheat gluten, high proline and glutamine residues, characterized in that a family of proteins, the gliadins content, 15 per cent and 35 per cent, respectively. Two monoclonal antibodies (moAbs), G12 and A1, were developed against 33-mer, a major immunotoxin peptide from α-2 gliadin. These antibodies also wheat, barley and rye allow high precision from other immunotoxin peptides. Analysis of T-cell reactivity and detoxification proteins, potentially toxic for celiac sufferers of samples of these antibodies revealed that the signal is correlated with. In those studies, the G12 antibody showed cross-

reactivity that was used to detect avenin in oats, although with lower sensitivity than for the prolamins of wheat, barley, or rye. The monoclonal antibody G12 has three recognition epitopes along the sequence of the 33-mer. Therefore, the low sensitivity against oat-free prolamines by the G12 antibody may be due to low affinity for epitopes present in avenins.

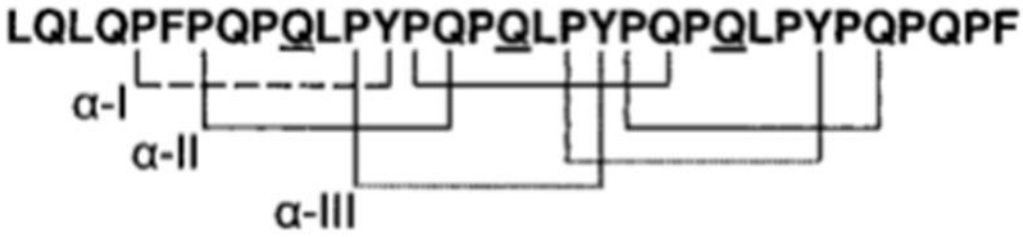

Figure 0.15 The 33-mer and its epitopes [120].

Cultivated oats are hexaploid cereals belonging to the genus Avena L., which is found worldwide in almost all agricultural environments. Recently, oats have increased interest in human feed, mainly because cereal may be suitable for consumption of celiac patients. Oats have other nutritional attributes such as those derived from β-glucan content, or the protein amino acid composition. Oat "gluten-free" is controversial inclusion in food, because it showed conflicting results in previous toxicity studies. Some researcher's intestinal inflammation without symptoms of celiac patients claims that oats can be tolerated. However, other studies have confirmed the toxicity of oats in some celiac patients. More recently it has been reported to be used to identify potentially toxic oats in celiac G12 antibody. This finding allowed classification of oat varieties into three groups based in their degree of affinity for the G12 antibody: a highly recognized group, one of moderate recognition, and one with no reactivity. The reactivity that T-cells isolated from celiac patients exhibited with three oat varieties (one from each of the classified groups) correlated directly with the Moab G12 reactivity. The diversity observed in the reactivity to the different oat cultivars suggests variations in the avenin composition, and therefore in the amount of immunotoxin epitopes similar to the 33-mer present in these varieties. Compared to wheat gliadins, avenins have been studied little and the number of genes that are currently available in the databases is limited, and the variability of the genes of avenin in oats has not been well demonstrated from a number of genotypes. With this background, the aim of the present work was to obtain further gene sequences from different toxic and non-toxic

varieties of oats in order to provide more information on the structure of avenin genes and on the evolutionary relationships with the prolamins and glutenins of wheat and other cereals. It will also facilitate identification of toxic epitopes described in other cereals that may be present in oats. In addition, these sequences may lead to the discovery of newly unspecified toxic epitopes in cereals, and may explain why some oat varieties are toxic for celiac patients and others are not [119].

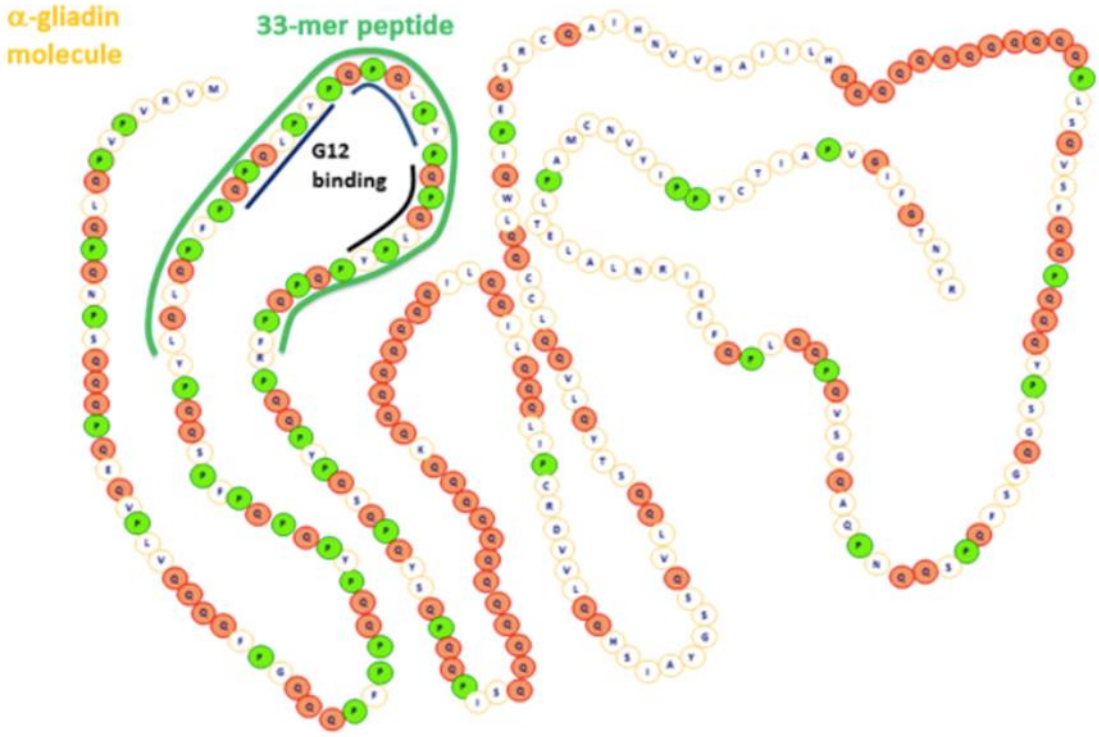

Figure 0.16 An illustration of 33-mer peptide on a-gliadin molecule [121].

1.4.2.9.Detection of Gliadin Immunogenic Peptide by Anti-33-mer MoAbs

The 33-mer peptide from a-2 gliadin is a principal contributor to gluten immunotoxicity. Thus, production of moAb against this toxic gluten peptide can be of great importance for both research and diagnosis. To test the relative sensitivity of each moAb for the 33-mer peptide, they immobilized different concentrations of the C-LYTAG-33-mer polypeptide,

and detected with A1 and G12 moAb in an indirect ELISA. The affinity of each moAb for the antigen was quantified by calculation of the concentration of the antigen giving a 50 per cent reduction of the peak signal in the ELISA (IC50). The sensitivity of the G12 moAb for the toxic 33-mer peptide was about eight times higher than that of A1. To test for moAb specificity, they studied the cross-reactivity values (CR) of these moAb against commercial gliadin, also by indirect ELISA. The G12 moAb presented an IC50 of almost double that obtained with the A1 moAb, suggesting that A1 had broader reactivity with gliadin epitopes than G12, which is more specific for the 33-mer [117].

1.4.3. Celiac (coeliac) Disease

Celiac disease is a genetic, autoimmune disorder that occurs in reaction to the ingestion of gluten. To develop celiac disease a person must inherit the genetic predisposition, be consuming gluten, and have the disease activated. Activation triggers include stress, trauma (surgeries, etc.) and possibly viral infections and other environmental factors. While approximately 33% of the general population has the genetic predisposition for Celiac disease, only about 1% will develop the condition. The reaction to gluten causes inflammation and villous atrophy or flattening of the cells lining the small intestine, which can lead to malabsorption of nutrients and related health issues. There are over 200 identified symptoms of Celiac disease, which include those listed above as well as anemia, behavioral changes, stunted growth and infertility. The majority of individuals with celiac disease are still undiagnosed. At this time the only treatment for celiac disease is to maintain a gluten-free diet for life.

Celiac disease is genetically determined and is more common in women than men. Disease is caused by gluten in insoluble wheat, barley or rye triggered by receipt of the protein fractions is an autoimmune condition. Clinical data show that the majority of celiac patients tolerate oats, but there are some concerns about the safety of some cereals for celiac disease [122], and oats remain currently on the Codex list of gluten containing cereals. Abnormal immunological response, which leads to flattening of the mucosa in the small intestine is characterized by an inflammatory reaction. The accepted diagnostic criteria for celiac disease, as defined by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), are based on detection of flat mucosa on small bowel biopsy and

disappearance of symptoms following a gluten-free diet [123]. For many years, celiac disease was considered a disease of very young infants. Malabsorption symptoms and signs occur months after starting a diet containing gluten. Chronic diarrhea or loose stools, vomiting, swollen abdomen and inability to develop were frequent presentations. Likewise, diarrhea, weight loss and general fatigue were the most common symptoms in adults [124]. Celiac disease presenting with malabsorption syndrome is not a general rule [122]. Indeed, when the serological tests based on the detection of autoantibodies bound to celiac disease developed as a safe browsing tool, which is not limited to the gastrointestinal tract manifestations of celiac disease was understood to be a complex disease [122-115]. Isolated iron deficiency anemia is the most common example. Other manifestations include permanent tooth enamel defects, epilepsy and cerebral calcification, isolated liver diseases osteopenia, idiopathic ataxia, infertility, non-Hodgkin's lymphoma and myocardiopathy [122-126]. It also relates to specific conditions such as celiac disease, selective IgA deficiency [127] and Down's syndrome [128]. At the same time, it is also often associated with other autoimmune diseases [129] of which type I diabetes is the most frequently studied [130]. In most cases, the mechanisms underlying this relationship have not been established. Screening programs in populations show insufficient disease. Serological tests detect the undiagnosed disease in one out of 100 people. Biopsy revealed a flattened mucosa in the majority of patients that tested positive. A life-long gluten-free diet is in fact the only effective treatment for celiac disease. Wheat, rye and barley-based products should be avoided. Oat-based products, is tolerated by most patients unless they are contaminated with other gluten grains. In a gluten-free diet, the small bowel mucosal lesions of celiac patients heal and symptoms disappear [122].

Table 0.6 Immonologic difference between allerfy, celiac disease and non-celiac gluten sensitivity.

	IgE Allergy	Celiac Disease	Non-Celiac Gluten Sensitivity
Reaction to:	Proteins (IMMUNE)	Proteins - gliadin and glutenins (AUTOIMMUNE)	Gluten Possibly other components of gluten-containing foods (Possibly INNATE IMMUNE)
Reaction time:	FAST Immediate: Minutes to hours Exposure – reaction may vary in severity, time of onset, and may be affected by when the food was eaten Can be exercise-induced in some	OFTEN SLOWER Can be fairly immediate, or up to 24 hours; can last for days. In some cases no obvious reaction occurs, but intestinal damage still occurs	OFTEN SLOWER Can be fairly immediate or up to 24 hours; can last for days.
Reactions: (reactions vary; could include those listed)	May affect different areas of the body with different exposures. Potentially deadly. **GI** - IBS, indigestion, abdominal pain, bloating, nausea, vomiting, and diarrhea **Systemic** - fever, fatigue, sweating, and chills **Lungs** - food-induced bronchitis and asthma, sneezing, runny nose, and shortness of breath **Joints** - pain/achiness **Muscles and connective tissue** - pain, stiffness, and swelling **Skin** - itching, rashes, hives, redness, swelling, and scaling as in eczema and psoriasis **Brain** - disorganized, disturbed or foggy thinking, headaches, migraines. **Anaphylaxis** - affects several areas of the body at the same time. These might include the skin: flushing, itching, or hives; the airway: swelling of the throat, difficulty talking or breathing; the intestines: nausea, vomiting, or diarrhea; and the heart – low blood pressure or unconsciousness and possibly death.	May affect different areas of the body. Damages the intestine. Does not cause death **GI** – IBS-like symptoms may include indigestion, abdominal pain, bloating, nausea, vomiting, and diarrhea **Systemic** - fatigue, achiness, sweating, and chills **Lungs** - food-induced bronchitis and asthma, sneezing, runny nose, and shortness of breath **Joints** – pain/achiness **Muscles and connective tissue** - pain, stiffness, and swelling **Skin** – blistering in cases of dermatitis herpetiformis, the skin manifestation of CD **Brain** - disorganized, disturbed or foggy thinking, headaches, migraines.	May affect different areas of the body. An irritant. Likely does not cause damage to the intestine. Does not cause death. **GI** – IBS-like symptoms may include, indigestion, abdominal pain, bloating, nausea, vomiting, and diarrhea **Systemic** - fatigue, achiness, sweating, and chills **Lungs** - food-induced bronchitis and asthma, sneezing, runny nose, and shortness of breath **Joints** - pain/achiness **Muscles and connective tissue** - pain, stiffness, and swelling **Brain** - disorganized, disturbed or foggy thinking, headaches, migraines.

1.4.3.1.Eliciting Dose

Life-long gluten-free diet is the only known medical treatment of celiac disease. However, gluten sensitivity of celiac disease varies individually. This makes it difficult to determine the acceptable limit for the gluten in gluten-free food in trace amounts. In general, the in vivo gluten challenge is considered to be the gold standard for the assessment of the gluten

tolerance level. Both acute and long-term in vivo tests were used to investigate the effects of small amounts of gluten in the diet [122].

1.4.4 Genetics of Celiac Disease

The gene test for celiac disease does not diagnose celiac disease. Rather, if the result is positive, it places individuals into an at-risk group for celiac disease. People who carry a gene for celiac disease who also have a first degree relative with it should be tested every three years, or sooner if symptoms develop. Those without a diagnosed close relative can be checked if and when symptoms develop. The HLA gene test for celiac disease can be performed at any time after birth (and even on cord blood at birth). People are born either with or without these genes, and that does not change over time. Every case of celiac disease studied has been found to show these genes; therefore, someone with a negative gene test will not ever develop celiac disease. The HLA test is not affected by diet.Thus, for a person who is already on a gluten-free diet and who is reluctant to go through a gluten challenge, having an HLA gene test can help rule out celiac disease. It is impossible for someone with a negative gene test to develop celiac disease. [154]

1.5. QUARTZ CRYSTAL MICROBALANCE

There is a growing demand of highly selective and precise analytical techniques that allow direct monitoring in analytical and physical chemistry, medical diagnostics and biotechnology, real-time in real-time with easy-to-use, reliable and miniaturized devices. Antigen-antibody, pathogen detection, cell adhesion, oligonucleotides adsorption and DNA and are typical applications in biomolecular interactions such as the retention of the complementary strand RNA interactions of adsorbed protein characterization, and other bacteria and viruses as well as the art [131]. The quartz crystal microbalance (QCM) technique is currently experiencing rapid expansion in application and understanding-oriented work. After the pioneering work of Sauerbrey, which demonstrated proportionality between a mass change on a quartz oscillator’s electrode(s) and the associated frequency shift, the main use of the QCM has been as a film thickness monitor in evaporation and sputtering systems. Other minor application areas in vacuum environments included monitoring metal oxidation, gas adsorption, dry etching, and catalytic reactions and, more

recently, friction studies at the atomic level. Eventually, the QCM was used also in gaseous environments, e.g. as gas and humidity sensors and for the detection of aerosols [132]. An important new step was the demonstration that the QCM can be reliably operated in the liquid phase, preferably in an arrangement where one face of the sensor is exposed to the liquid and the other to the gas phase. This led to the electrochemical quartz crystal microbalance (EQCM)[133] which has been used for studies of electrochemical processes such as electropolishing, electrodeposition and corrosion. A parallel and rapidly developing application is the use of the QCM in the liquid phase as a biosensor for the study of processes such as protein adsorption, antibody-antigen binding, and even the adhesion of much larger biological entities such as cells [186]. A QCM sensor makes use of the piezoelectric effect of quartz crystal materials. Piezoelectricity literally means “pressure electricity” (“piezo” is Greek for pressure)[134]. Gliadin analysis systems are time intensive, inconvenient, and expensive and require that their operators have been extensively trained. Therefore, a rapid, sensitive, user-friendly, and environmentally friendly analytical system must be developed for detecting gliadin in foods. Some label-free methods, including surface Plasmon resonance (SPR) and quartz crystal microbalance (QCM), have been developed. In 1959, Sauerbrey established the relationship between the change in resonant frequency of quartz and the change in mass of attached molecules on the surface of a gold electrode in a QCM, leading to the development of QCM as a commonly used biosensor that depends on the increase in mass that is caused by the absorption of target molecules. As a directly responsive microsensor, QCM is extensively applied in the liquid phase because it supports rapid analysis and free labeling, is relatively easy to use, and exhibits both high selectivity and high sensitivity. Therefore, QCM has been used as an immunosensor in clinical diagnosis, food toxin analysis, microorganism pollution detection, environmental protection, and agricultural monitoring. Recently, fundamental studies and technological applications have exploited biosensing assays that are based on gold nanoparticles (AuNPs) because they are low-cost, provide a high surface area, and are easy to process. Some novel analytical methods that use AuNPs have been found to have a better detection limit. For example, the QCM DNA sensing system uses AuNPs as carriers and is adopted in the investigation of DNA hybridization, and layer-by-layer AuNP hybridization has been used to detect the dengue virus. In another study they developed a novel, rapid, and sensitive method for detecting gliadin in gliadin-free food using a QCM. They basically increase the number of anti-gliadin antibody binding sites and the subsequent target mass on a QCM chip by

modifying the surface of the gold electrode in the QCM with AuNPs. The changes in frequency (ΔF) and assay sensitivity of this developed QCM were compared with those of the traditional QCM with an unmodified gold electrode [135].

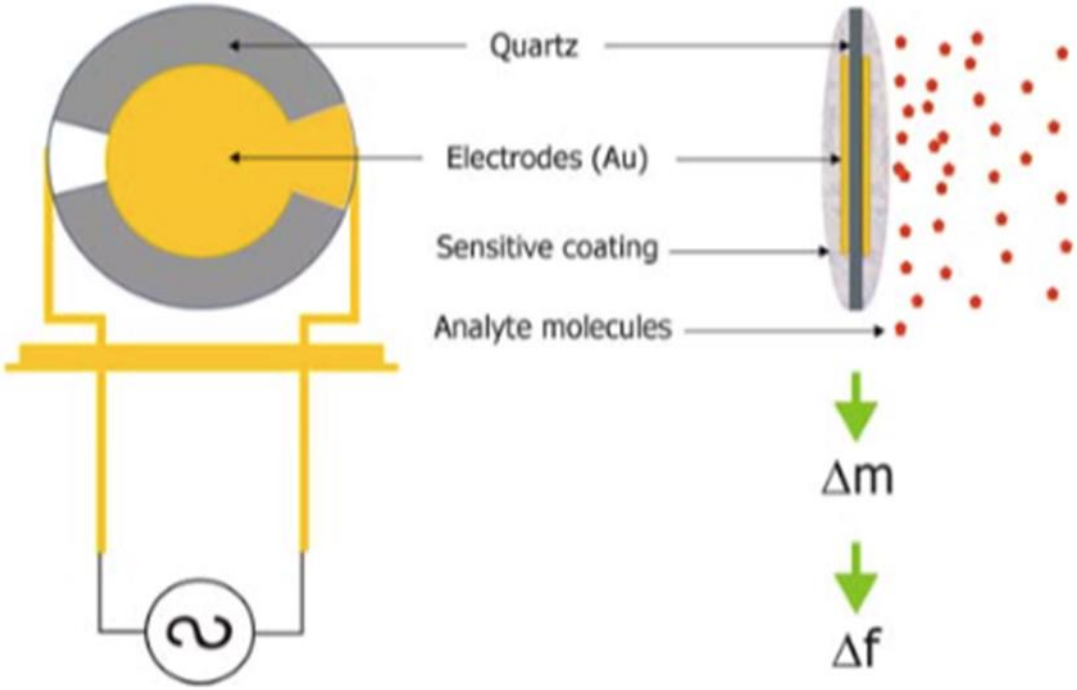

Figure 0.17 Basic working principles of a quartz crystal microbalance (QCM) sensor [134].

Using an equation derived by Sauerbrey, a mass change on a QCM sensor surface due to adsorption of any analyte by sensitive coating material can be expressed in a frequency change quantity as follows[134]:

$$\Delta f = -2.3\ x\ 10^6 F^2(\Delta m/A)$$

Where;

Δf = the frequency change [Hz]

F = the oscillating frequency of the quartz crystal [MHz] (for a typical AT-Quartz, F = 10 MHz)

Δm = the mass change of the adsorbed analyte, i.e. odor substance [g]

A = the area coated by the film [cm2]

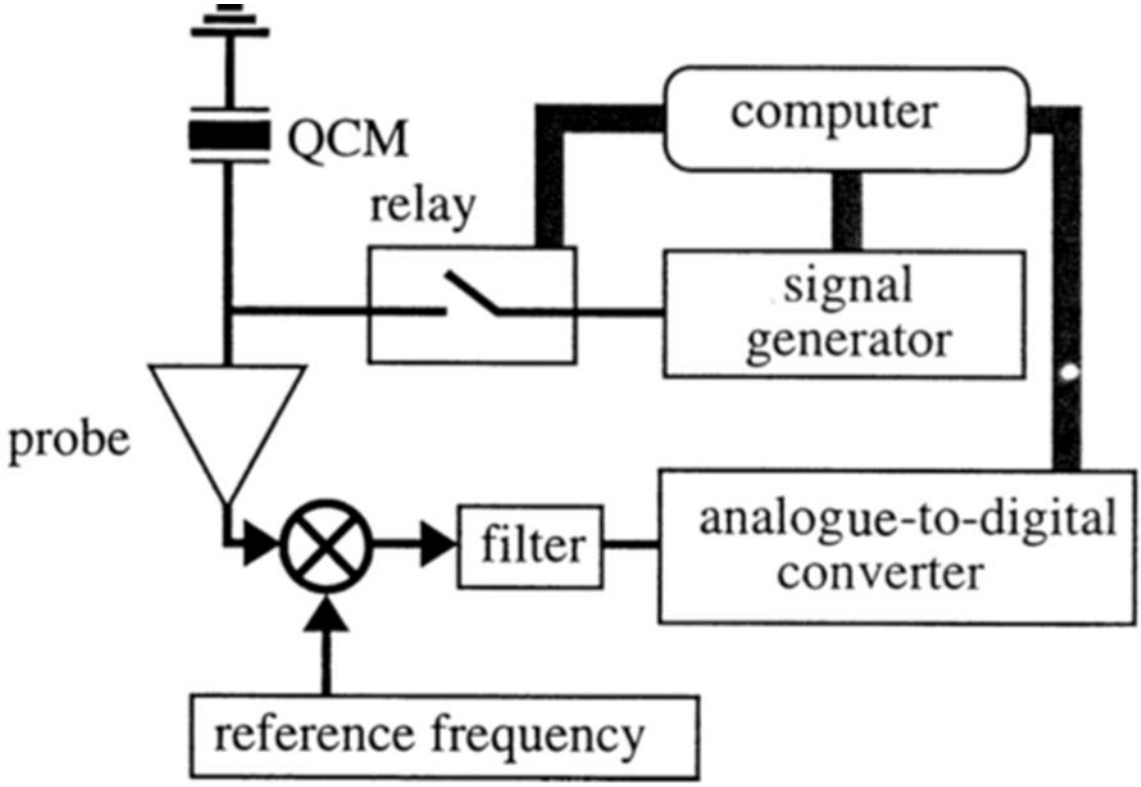

Figure 0.18 Schematic illustration of the QCM measurements [132].

The advantages of the use of sensors in an array form are technical conditioning, i.e. control of temperature stability, sample mass flow rate, etc. are simpler, a more compact measuring chamber, i.e. a single measuring chamber is used by all sensors, and better description of the measured, i.e. the measured can be described in a better way by a series of sensors (in form of a pattern) than if it were described by a single sensor. The quartz crystal microbalance sensor has been used in a numerous fields of application including gas mixture analysis, detection of solvent vapors, detection of organic vapors, detection of carbon dioxide (CO2), discrimination of aromatic optical isomers, discrimination of odorants, detection of mutagenic polycyclic compounds, detection of organic pollutants in water, detection of L-glutamic acid, and discrimination of aromas from various Japanese sake [134].

1.6. APTAMER

Natural selection is one of the fundamental evolutionary mechanisms discovered by Darwin 150 years ago. However, artificially, the importance of in vitro selection was accepted later [135]. Aptamers are an excellent example of the functional molecule selected in vitro. In 1990, two groups independently developed in-vitro selection and amplification for the

isolation of RNA sequences that could specifically bind to target molecules. These functional RNA oligonucleotides were then termed aptamers, derived from the Latin aptus, meaning ‘‘to fit’’. After that DNA-based aptamers were found. Since its discovery, the aptamer technology has attracted great attention in scientific and industrial communities. After nearly 20 years’ endeavor, DNA and RNA aptamers have been identified as binding tightly to a broad range of targets (e.g., proteins, peptides, amino acids, drugs, metal ions and even whole cells), especially with the development of rapid, automated, selection technologies. Aptamers generally have high affinity for the target are derived from folding upon binding ability with the target molecule (i.e., they may include small molecules to nucleic acid constructs or macromolecules (such as proteins) can be integrated into the structure. Aptamers, molecular tools for diagnosis and has become increasingly more important for therapeutics. In particular, the aptamer-based biosensors, such as antibodies and enzymes have previously unprecedented advantages compared to biosensors using natural receptors [136]:

- First, aptamers with high specificity and affinity to any given target in principle, the large proteins from small molecules and even cells as selected in vitro for any target variable, thereby enabling large an aptamer-based biosensor development.
- Secondly, aptamers, once selected it can be synthesized with high reproducibility and purity from commercial sources. Furthermore, unlike protein-based antibody or enzyme, DNA aptamers are often very chemically stable.
- Third, aptamers will usually important conformational changes upon binding target. This offers great flexibility in the design of new biosensors with high detection sensitivity and selectivity.

In recent years, deep understanding of nucleic acid aptamers in terms of their conformational and ligand binding properties has aroused interest and led to a series of bioassay methods based on aptamer receptors [136].

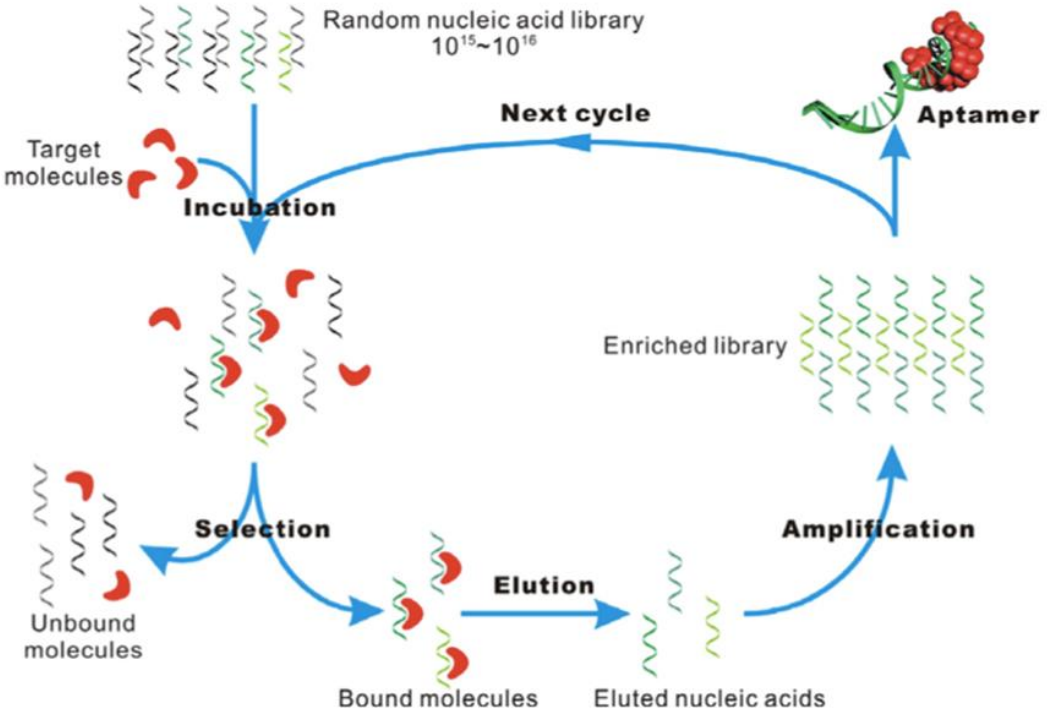

Figure 0.19 Scheme for the Systematic Evolution of Ligands by exponential (SELEX) enrichment process. A random nucleic acid library is incubated with a target molecule, and unbound molecules are separated from bound molecules. Bound nucleic acids are eluted, amplified by PCR (polymerase chain reaction) and serve as an enriched library for the next cycle. For every target, 6–12 consecutive cycles are performed and the final enriched library is cloned and sequenced [136].

Aptamers are also termed ‘‘chemical antibodies’’ because of their artificial process in vitro based on Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Figure 1-19). In contrast to the preparation of antibodies based on stimulation of an animal immune system, the SELEX process allows the manufacture of aptamers for non-immunogenic and toxic targets, which is impossible to obtain by the immune system [138]. Until now, aptamers have been selected toward a broad range of targets, including metal ions (e.g., K+, Hg2+ and Pb2+), small organic molecules (e.g., amino acids, ATP, antibiotics, vitamins and cocaine) organic dyes, peptides and proteins (e.g., thrombin, growth factors and HIV-associated peptides) and even whole cells or microorganisms (e.g., bacteria). Importantly, the presence of such a large pool of aptamers makes it possible to develop new bioassay tools that include areas of diagnosis, anti-bio-terrorism, and environmental and food analysis [139]. Aptamers often possess high selectivity and affinity toward their targets. Analogous to that of

antigens/antibodies, interactions between aptamers and their molecular targets are usually so specific that even small variations in the target molecule may disrupt aptamer binding (e.g., the aptamers for theophylline and L-arginine can discriminate closely related chemical structures by factors as high as 4 orders of magnitude[136,140]. In addition to this high selectivity, aptamers bind to their targets with high affinity, particularly with macromolecules (e.g., proteins), which often possess remarkable dissociation constants (Kd) ranging from picomolar to nanomolar [140].

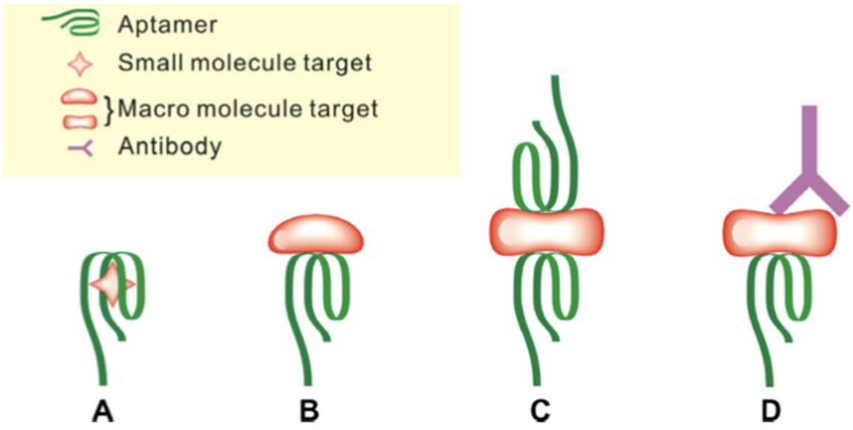

Figure 0.20 Aptamer-based assay formats. (A) Small-molecule target buried within the binding pockets of aptamer structures; (B) single-site format; (C) dual-site (sandwich) binding format with two aptamers; and, (D) ‘‘sandwich’’ binding format with an aptamer and an antibody [140].

Similar to immuno-assay based on antigen-antibody interaction, aptamer-based bioassays can take different assay configurations to transmit bio-recognition events. A variety of assay configurations have been designed and reported, since aptamers are chosen to bind a wide range of targets from small molecules to macromolecules, such as proteins. However, many of these designs fall into two configuration categories (Figure 1.20). the design for the sensors is largely based on naturally different recognition modes of each aptamer – target pair. For small molecular targets, nuclear magnetic resonance (NMR) studies have indicated that they are often buried within the binding pockets of aptamer structures (Figure 1.20A), leaving little room for the interaction with a second molecule. Because of this limitation, small molecule targets are usually tested using a single-region binding configuration. By contrast, the protein targets are structurally complicated, allowing the interplay of the various discriminatory contacts (e.g., stacking, shape complementarity, electrostatic interactions,

and hydrogen bonding). As a result, protein targets can be assayed via both single-site binding (Figure 1.20B) and dual-site binding (Figure 1.20C). as a note, double-zone binding is also based on the presence of a pair of aptamers that bind to different regions of the protein. The double-binding binding assay, also known as "sandwich", is one of the most commonly used assay formats. In this approach, the analyte is sandwiched by a pair of aptamers (Figure 1.20C), one capture probe and the other reporter probe. Capture probes are often immobilized on the surface of solid supports (e.g., electrodes, glass chips, nanoparticles or micro-particles), while reporter probes are often conjugated with signaling moieties (e.g., fluorophores, enzymes or nanoparticles (NPs)). Generally, capture and reporter probes have different nucleic acid sequences; however, in limited cases, some proteins (e.g., dimeric) contain two identical binding sites, thus allowing the use of a single aptamer for the sandwich assay. Also, in cases when there are no two aptamers sharing identical or overlapping binding sites on the target of interest, it is possible to use an antibody as the second ‘‘aptamer’’ (Figure 1.20D). Apparently, it also emphasizes the importance of a single molecular target identification for more than one aptamer[140].

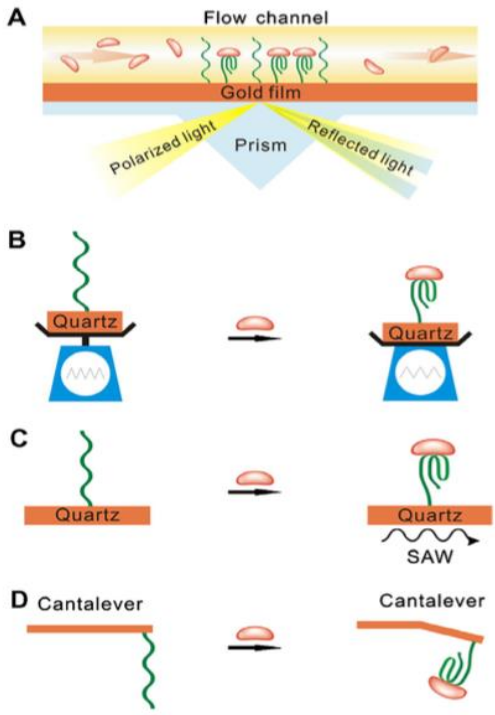

Figure 0.21 -sensitive aptasensors. (A) SPR-based aptasensor; (B) QCM-based aptasensor; (C) SAW-based aptasensor; and, (D) micromechanical cantilever-based aptasensor [140].

1.7. WHY WE USED APTAMERS

Aptamers have been developed for different applications. Their use as new biological recognition elements in biosensors promises progress for fast and easy detection of proteins. This new generation of biosensor (aptasensors) will be more stable and well adapted to the conditions of real samples because of the specific properties of aptamers.[142]

Until now, proteins are detected mostly by antibodies in analytical formats like ELISA, immunobead assay, western blotting, microarrays and also biosensors. Aptamers are equal to monoclonal antibodies concerning their binding affinities, but furthermore, they provide decisive advantages. They are more resistant to denaturation and degradation, their binding affinities and specificities can easily be manipulated and improved by rational design or by techniques of molecular evolution, and they can be modified with functional groups or tags that allow covalent, directed immobilization on biochips, resulting in highly ordered receptor layers [141, 143].

1.8. THE ASSURED CRITERIA

Laboratory testing has been undergoing a major paradigm shift in recent years to adapt to the new requirements that are driven mainly by ever-changing health care scenarios, technology, regulations, and various market forces. According to a report published by the Milken Institute, data from US Centers for Disease Control and Prevention (CDC) indicate that chronic illnesses affect one of every two adults in the United States, and are responsible for per cent 75 of health care costs. [142] In 2008, health care costs in the United States were per cent 16.8 of GDP, and by 2022 they are projected to be approximately per cent 20.1 Even though the urbanization trend around the globe has been increasing, per cent 55 of inhabitants in the developing world still live in rural areas.. According to the World Health Organization (WHO), after cardiovascular disease, infectious diseases are the second leading cause of mortality around the world [143,144]. This problem is greater in resource-limited settings in developing countries with poor hygiene. The burden of disease is typically measured in terms of disability adjusted life years (DALYs), a unit that accounts for the years of life lost due to both mortality and disability caused by the disease. More than per cent 95 of deaths due to major infectious diseases that include acute respiratory infections

(ARIs), malaria, HIV, and tuberculosis (TB) occur in developing countries, with by far the largest burden on Africa [145]. In developed countries, in spite of many advances in health care, there are many challenges related to pathogen outbreaks, sexually transmitted diseases, and food safety that need to be addressed. In the past decade, the demand for rapid and accurate on site detection of plant disease diagnosis has increased due to emerging pathogens with resistance to pesticides, increased human mobility, and regulations limiting the application of toxic chemicals to prevent spread of diseases. Many portable devices have been developed for plant pathogen detection, but current Technologies are limited to detecting known pathogens with limited detection accuracy [146]. Diagnosis is the first step to treat a condition or take preventive steps. However, one of the major hurdles in monitoring and controlling diseases and contaminations is the availability of easy-to use, low-cost, and robust diagnostic tests. Diagnostics for limited-resource settings does not get the required attention from large companies due to its low profit margins, whereas the limited research contribution from smaller companies and academia is not sufficient to make a major impact in developing diagnostic products. In many low-resource settings without diagnostic tests, disease is often treated based on clinical symptoms and local prevalence of disease. Such syndromic disease management can be cost-effective and has been recommended by the WHO for certain diseases, such as malaria and sexually transmitted infections. However, this approach sometimes unnecessarily treats patients who do not require treatment, and thus may accelerate drug resistance. The WHO Sexually Transmitted Diseases Diagnostics Initiative has developed a set of generic guidelines for the development of diagnostic tests that can be summarized under the acronym ASSURED: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and delivered to those who need it. Apart from the design focus toward low-cost platforms, there is also a demand for higher sensitivity and specificity from rapid diagnostic assays [147,148].

1.8.1. Ideal point-of-care tests

The ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users) criteria, outlined by the World Health Organization (WHO), provide a good framework for evaluating POC devices for resource-limited environments. Tools that satisfy the ASSURED criteria primarily aim to provide same-day diagnosis and facilitate immediate decision-making [150]. Quick receipt of results increases

the number of people who know their HIV status, assists in delivering timely ART (especially to women in labor) and minimizes the number of patients lost to long-term follow-up [151]. In addition, devices whose use requires minimal training and that have a high throughput can widen the pool of end users and alleviate congestion at HIV testing centers [152]. Finally, such a device should be operable in resource-limited environs, which include those with unreliable electricity, non-sterile conditions and a lack of trained personnel to perform the duties typically reserved for nurses and health workers. Further examples of target specifications, listed in Table 1, will vary on the basis of the needs and setting in which the device will be used.

Table 0.7 ASSURED characteristics (WHO) and examples of target specifications for the evaluation of point-of-care devices [152].

Characteristic	Target specification
Affordable	Less than US$ 500 per machine, less than US$ 10 per test
Sensitivity, specificity	Lower limit of detection: 500 HIV RNA copies per mL, 350 CD4+ T-cells per µL
User-friendly	1–2 days of training, easy to use
Rapid and robust	< 30 minutes="" for="" diagnosis,=""><< 1.5 hours for="" hiv="" load="" monitoring,="" minimal="" consumables="" (i.e.="" pipettes),="" shelf="" life="">> 1 year at room temperature, high throughput
Equipment-free	Compact, battery powered, on-site data analysis, easy disposal, easy sample handling, no cold chain
Deliverable	Portable, hand-held

For example, a device that detects a lower viral load may be less useful than a device that provides semi quantitative but clinically useful viral load ranges (e.g. levels denoting a low, medium and high risk of virology failure). Hence, consultation with clinicians, health workers and other end users can result in well-defined performance specifications that complement the ASSURED criteria and suit the specific needs of health professionals and patients [153].

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

- Pure Isopropanol from Thermofisher
- Sulfuric Acid from Sigma
- Pure Ethanol from Thermofisher

2.1.2 Aptamers

Table 0.1 Aptamers and their sequences[155][156].

Oligo Name	Oligo Seq	Mer(s)	Amount	Purification	Modification
Gli4-5btn	CCA GTC TCC CGT TTA CCG CGC CTA CAC ATG TCT GAA TGC C	40	0.2	MOPC	5`Biotin
Gli1-5btn	CTA GGC GAA ATA TAG CTA CAA CTG TCT GAA GGC ACC CAA T	40	0.2	MOPC	5`Biotin
g33-5btn	AAA CTA CTA ACT AGG TAA GAT CAC GCA GCA CTA AAC GAC GTA GTT GCC A	49	0.2	MOPC	5`Biotin

2.1.3 Enzymes and Substrates

- TMB from Thermofisher
- BCIP/NBT from Thermofisher
- HRP-Streptavidine from Rockland INC.
- PWG Gliadin from PWG
- Oat, wheat, siyez, rye, bread and whole-wheat bread sourced from local supermarkets

2.1.4 Lateral Flow Assay Components

- Whatman HP170 nitrocellulose membrane
- Whatman CF3 paper
- Whatman Cf4 paper

- Whatman Fusion 5 paper
- 3M 468MP double sided adhesive
- PVC substrate 100x200cm

2.1.5 Buffers and Solutions

- Coating Buffer (for 10 ml 10x)
    - Na2CO3 0.15g
    - NaHCO3 0.3g
    - pH adjusted to 9
- 1x Blocking Buffer
    - BSA 1g
    - 10% tween 20 250µl
    - 10x TBS 5ml
    - DDW Up to 50ml
- 1x Wash Buffer
    - 10x TBS 20ml
    - 10% tween 20 100µl
    - DDW 180ml
- 10xSDS Running Buffer
    - 30.28g Tris
    - 144.14g Glycine
    - 10g SDS
    - Up to 1L DDW
- 3x Sample Buffer
    - 5ml Beta-mercaptoethanol
    - 10mg Bromophenol blue
    - 3g SDS
    - 10ml Glycerol
    - 6.25ml Upper Tris
    - Up to 100ml DDW
- Lower SDS-Page Gel
    - 1.5M Tris pH=8.8 + SDS

    - 90.75g Tris
    - Up to 500ml DDW
- Upper SDS-Page Gel
    - 1M Tris pH=6.8 + SDS
    - 60.5g Tris
    - 3.7g SDS
    - Up to 500ml DDW
- Staining
    - 2g Coomasie BB R-250
    - 500ml Methanol
    - 100ml Acetic Acid
    - 400ml DDW
- Destaining
    - 140ml Acetic Acid
    - 400ml Methanol
    - 1460ml DDW

2.1.6 Equipment

- Varioskan Flash Spectrofotometer from Thermofisher
- Electrophoresis tank from BioRad
- Powersupply
- Micro-centrifuge from Thermofisher
- STS5 Fusion Gel Imaging System
- Ideal Guiotine cutter
- Pipette Set from Axigen
- ELISA Plate from Nunc

2.1.7 Software

- Microsoft Office Word
- Microsoft Ofiice Powerpoint
- Microsoft Office Excel
- Inkscape

- Variokan Flash SkanIt Software
- Google Drive

2.2 Methods

2.2.1 Gluten Extraction Methods

2.2.1.1 Gluten Extraction Procedure (A):

First sample extracted from 200 mg grinded wheat (with Lavion multipurpose disintegrator) by continuous shaking for 30 minutes at 1000 rpm with addition of 1 mL buffer 1 (%50 isopropanol). Then, the centrifugation is repeated at 2500 rpm for 15 minutes and the supernatant is taken to a 1.5 eppendorf tube (A1). These steps are repeated with the remaining residue for A2 and A3. Followed by addition of 1 mL buffer 2 [50 per cent isopropanol, 50 mM Tris-HCl (pH 7.5) with 1 per cent DTT] to the residue and sample is incubated at 60 °C for 30 minutes with mixing every 5-10 minutes. Then the sample is centrifuged at 10.000 rpm for 10 minutes and the supernatant is obtained (A4).

2.2.1.2 Gluten Extraction Procedure (B):

50 mg of sample is extracted with the addition of 0.5 mL of buffer 2[50 per cent isopropanol, 50 mM Tris-HCl (pH 7.5) with 1 per cent DTT] and the sample is incubated at 60 °C for 30 minutes with mixing every 5-10 minutes. Then the sample is centrifuged at 10.000 rpm for 10 minutes and the supernatant is obtained (B1).

2.2.1.3 Gluten Extraction Procedure (C):

50 mg of sample is extracted with addition of 1.5 mL buffer 3 [60 per cent ethanol] and mixed continuously at 1000 rpm for 1 hour followed by centrifugation at 10000 rpm for 10 minutes. Supernatant referred as C1. 1.5 mL of buffer 3 added to the residue and this procedure is repeated for C2.

2.2.1.4 Gluten Extraction Procedure (D):

100 mg of sample is extracted with addition of 1 mL buffer 1(50 per cent isopropanol) and they are mixed continuously at 1000 rpm for 30 minutes. Then the sample is centrifuged at 10000 rpm for 10 minutes and the supernatant is obtained (D1).

Residue is sonicated for 10 minutes with the addition of 1 mL buffer 2. Then, the sample is incubated at 60 °C for 30 minutes followed by centrifugation at 10000 rpm for 10 minutes and the supernatant is obtained (D2). These steps are repeated with the remaining residue for D3. 2 mL buffer 4 [95 per cent 25mM Tris-HCl (pH 8.0) with 5 per cent SDS] is added to the residue and incubated at 60 °C for 30 minutes followed by centrifugation at 13300 rpm for 10 minutes and the supernatant is obtained (D4). Followed by addition of 2 mL of buffer 5 [94 per cent 25mM Tris-HCl (pH 8.0) with 5 per cent SDS and 1 per cent DDT] to the residue and sample is incubated at 60 °C for 30 minutes. Then the sample is centrifuged at 13300 rpm for 10 minutes and the supernatant is obtained (D5).

Table 0.2 Buffers and their contents.

BUFFER	1	2	3	4	5
CONTENT	50 per cent aqueous iso-propanol	50 per cent aqueous iso-propanol + 50 mM Tris-HCl(pH 7.5), 1 per cent DTT	60 per cent ethanol	25 mM Tris-HCl (pH 8.0), 2 per cent SDS	25 mM Tris-HCl (pH 8.0), 2 per cent SDS, 1 per cent DTT

Table 0.3 Samples and their contents.

SAMPLE	A1	A2	A3	A4	B1	C1	C2	D1	D2	D3	D4	D5
CONTENT	1st gliadin extract	2nd gliadin extract	3rd gliadin extract	Glutenin extract	DTT extract	60 per cent ethanol extract	2nd 60 per cent ethanol extract	1st gluten extract	2nd gluten extarct	3rd gluten extract	1st residual extract	2nd residual extract

2.2.2 ELISA Method

2.2.2.1 Calculations

Calculation for gliadin concentration:

PWG gliadin: 2630 µg . x = 10 ml . 1 µg/ml = x= 1000µl / 263 = 3.8µl

Calculation for aptamer concentration:

100.000nM . X = 440µl . 800nM X = 3.52µl

For the first tube (for 440µl) = 3.52µl aptamer + 436.48µl Binding buffer

2.2.2.2 Dilution steps for gli1 and gli4

800nM >400nM>200nM>50nM>25nM>12.5nM>0nM

2.2.2.3 Procedure

1) Put 100µl coating buffer to the each well for overnight at 4 C°.

2) Removed coating buffer, put 100 µl Blocking Buffer to each well for overnight at 4 C°.

3) Removed blocking buffer, wash 3 times full wells with Wash Buffer.

4) Put diluted gli1 and gli4 to wells for 1 hour at room temperature.

5) Washed 3 times full wells with Wash Buffer.

6) Added 100 ul TMB color reagent to each well and observe the color until it remains constant.

7) Added 50 ul 1M H2SO4 to end reaction.

8) Measured directly at 450 nm.

2.2.3 Lateral Flow Assay Strip Production

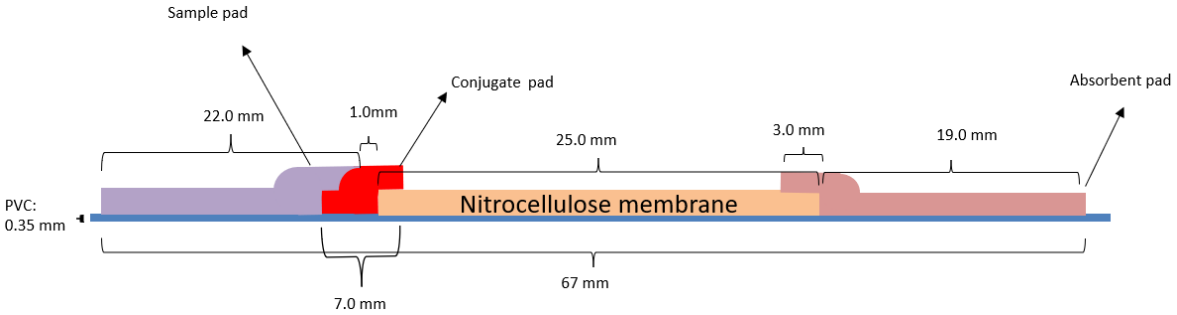

Figure 0.1 Dimensions of the lateral flow strips.

Dimensions of our strips are given in figure 2.1. Production technique of our strip is explained in figure 2.2. Production starts with a PVC substrate which a 2 sided adhesive is applied upon. Then some cut sites are applied for easy removal of adhesive cover. After removal of the adhesive cover, strips are transferred to a DIY assembly tool. Here first nitrocellulose padding is applied to the middle. Conjugate pad it added with keeping contact with nitrocellulose padding. Then a sample pad is applied to the left side of the strips while keeping contact with conjugate pad. Last part to be added is the absorbent pad which applied with similar fashion while being in contact with nitrocellulose membrane.

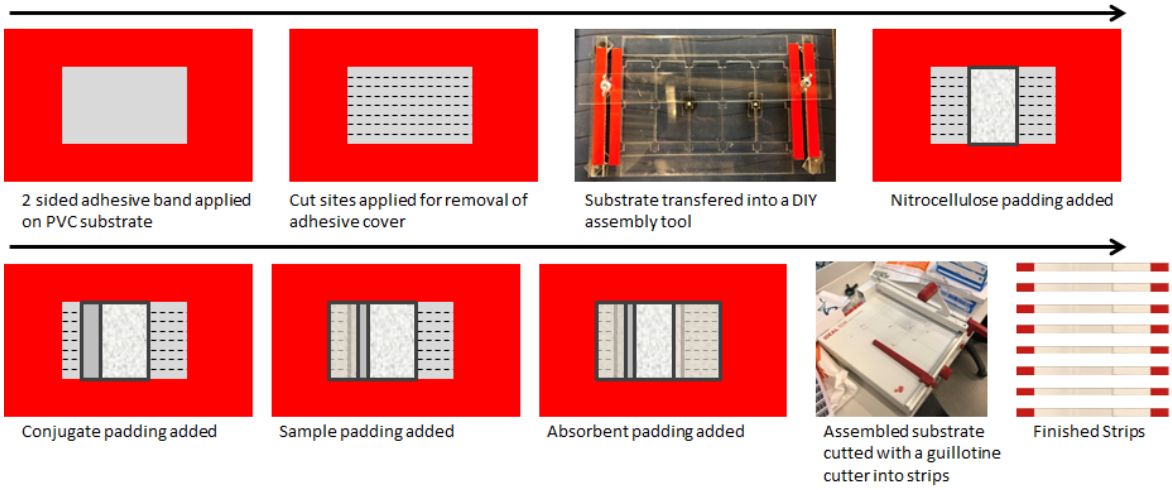

Figure 0.2 Lateral flow strip construction process.

2.2.4 Lateral Flow Test Procedure

We used a simple design to test our ability to detect gluten in food samples. We prepared 0ng Gluten solution in an Eppendorf tube and 100ng Gluten solution in another one. Gluten is extracted from oat, siyez and wheat. Then we prepared HRP our strips with dripping 1µl of HRP-streptavidin on the nitrocellulose membrane. We mixed our 0ng and 100ng samples with biotin tagged aptamers. Then we loaded 50µl on to the sample pad and waited fluid to reach to the absorbent pad which took 5 minutes. Lastly we add 50ul of substrate to each strip with increasing concentration of BCIP/NBT starting from 0. For 8 strips concentrations were; 0ul >1ul >2ul>3ul>4ul >5 ul >6ul >7ul. We expected increasing color change on each strip. Whole process is explained in figures 2.3 and 2.4

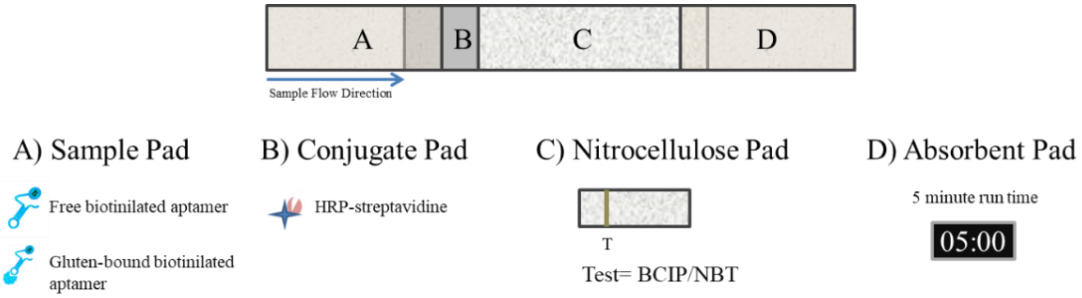

Figure 0.3 Gluten test design on lateral flow strip. A) Sample pad which sample/aptamer solution is applied. B) Conjugate pad which HRP-streptavidin is placed. C) Nitrocellulose

pad which BCIP/NBT is applied. D) Absorbent pad which determines the run time and observed to be 5 minutes.

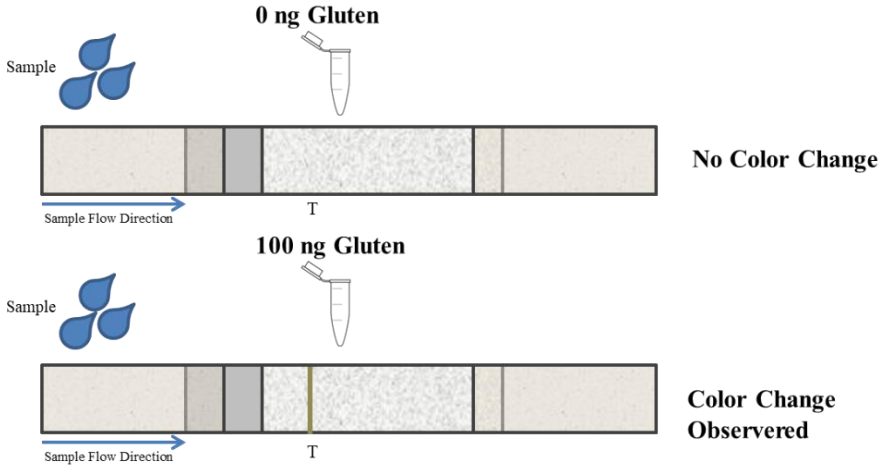

Figure 0.4 Expected result of gluten testing in lateral flow strips.

With 0ng gluten samples we expected to see no color change on nitrocellulose membrane. On the contrary, with 100ng of gluten we expected a color change on the nitrocellulose membrane when substrate BCIP/NBT applied.

RESULTS

3.1. OPTIMIZATION OF GLUTEN EXTRACTION RESULTS

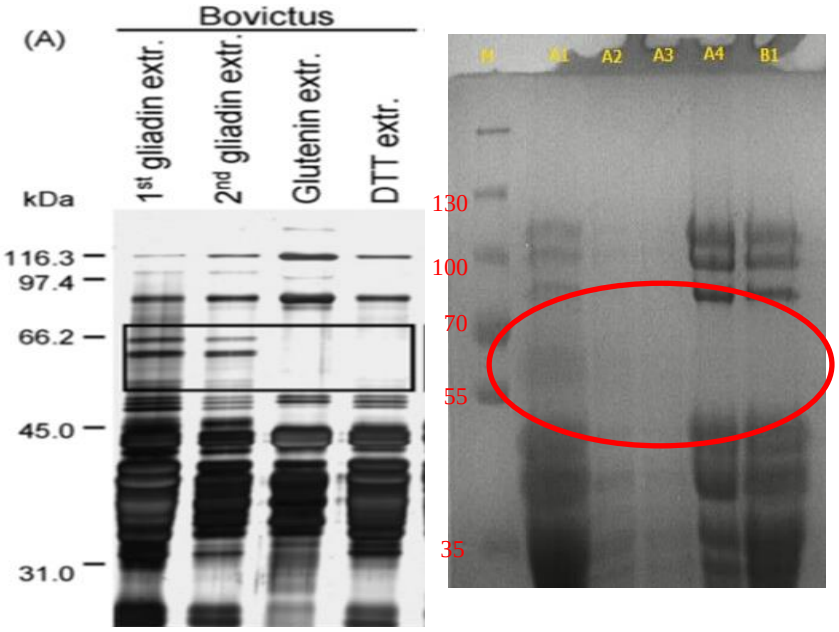

Figure 0.1 SDS-PAGE analysis of Gluten extraction from oat with A and B procedures (A1, A2, A3, A4, B1). Omega gliadins are marked with red circle.

As seen in the Figure 3.1, when we performed SDS-PAGE analysis to the extracts of oat using extraction methods A and B, they failed to extract significant amount of omega gliadins from oat sample. We decided that A and B is not suitable extraction methods to extract omega gliadins from oat samples.

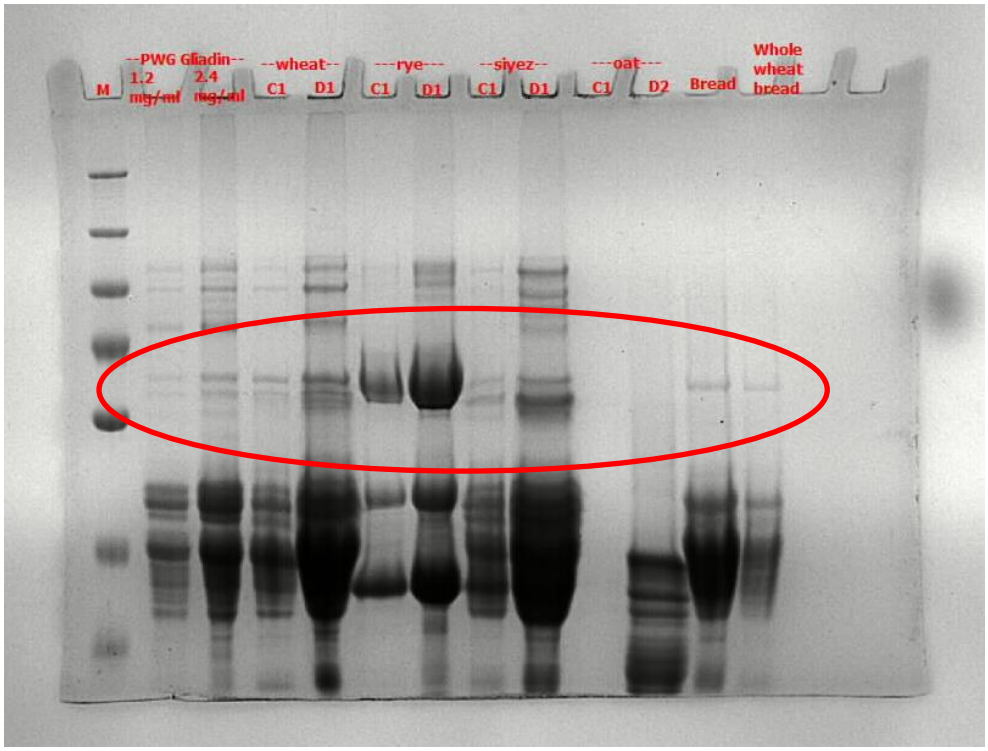

Figure 0.2 SDS-PAGE analysis of gluten extracts from PWG gliadin, wheat, rye, siyez, oat, bread and whole-wheat bread using extraction methods C and D. Omega gliadins are marked with red circle.

As seen in the Figure 3.2; when we performed SDS-PAGE analysis on the products of C and D extraction methods on wheat, rye, siyez and oat, we managed to extract significant amounts of omega gliadins from wheat, rye, siyez and oat samples. We failed at extracting significant amounts of omega gliadins from bread and whole-wheat bread samples with extraction methods C and D.

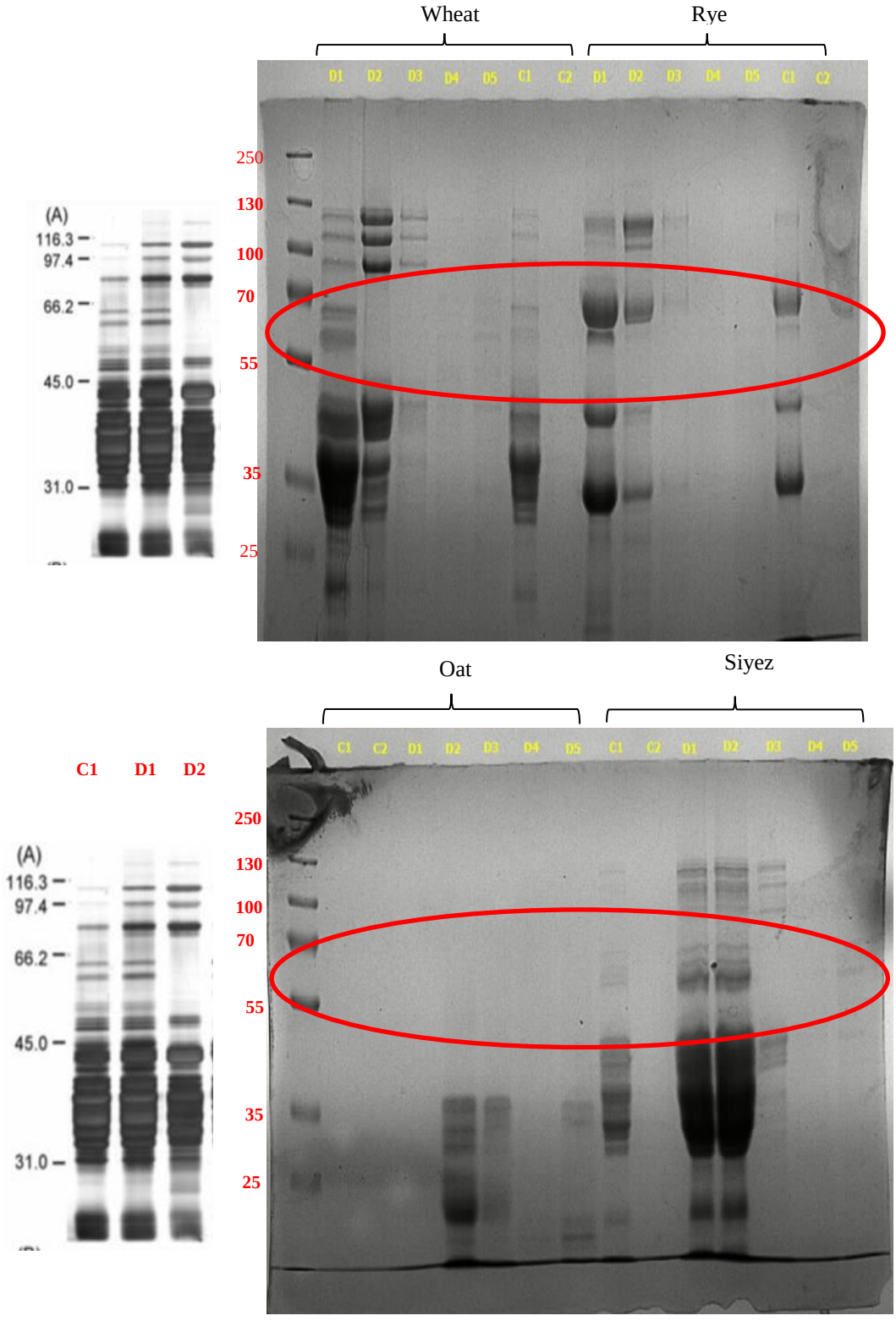

Figure 0.3 SDS-PAGE analysis of gluten extraction from wheat, rye, oat and siyez using extraction methods C and D. Omega gliadins are marked with red circle.

As seen in the Figure 3.3, between the extraction methods C and D, method D showed best result for extracting omega gliadins from wheat, rye, oat and siyez samples. According to the SDS-PAGE analysis we performed, with method D both siyez and oat omega gliadins can be extracted. Method C managed to extract omega gliadins from siyez sample 1, but the amount of protein extracted was low.

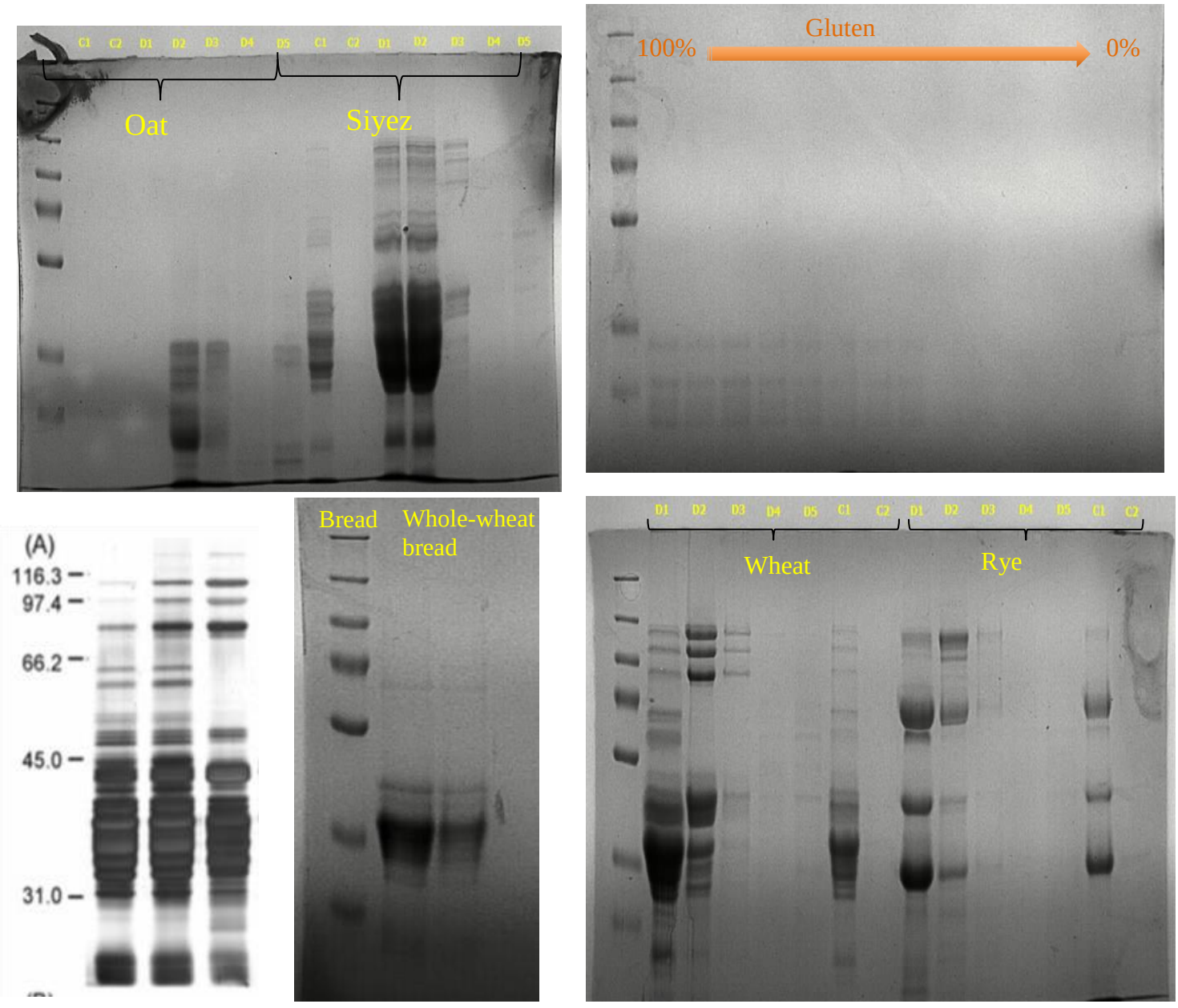

Figure 0.4 SDS-PAGE Gluten extraction from oat, siyez, bread, whole-wheat bread, wheat and rye using extraction methods C and D. Top right shows gluten dilution assay.

As seen in the Figure 3.4, extraction method D was able to extract large amount of omega gliadins from siyez, wheat and rye when compared to method B on SDS-PAGE analysis of

extracts. When extraction methods C and D applied to oat, bread and whole-wheat bread, they failed to extract significant amounts of omega gliadins from samples.

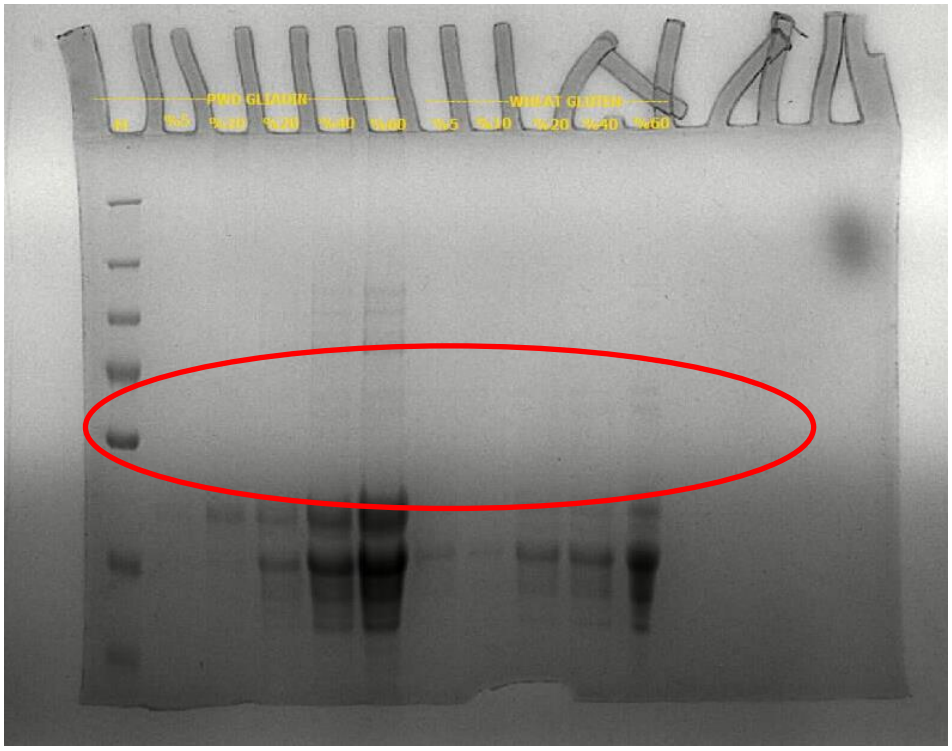

Figure 0.5 SDS-PAGE analysis of PWD gliadins and wheat gliadins
Omega gliadins are marked with red circle.

As seen in the Figure 3.5, reference glutenin acquired from PWG shows similar glutenin composition with glutenins extracted from wheat when compared in SDS-PAGE analysis. This experiment is performed to show our extraction methods ability to extract different subunits of gluten.

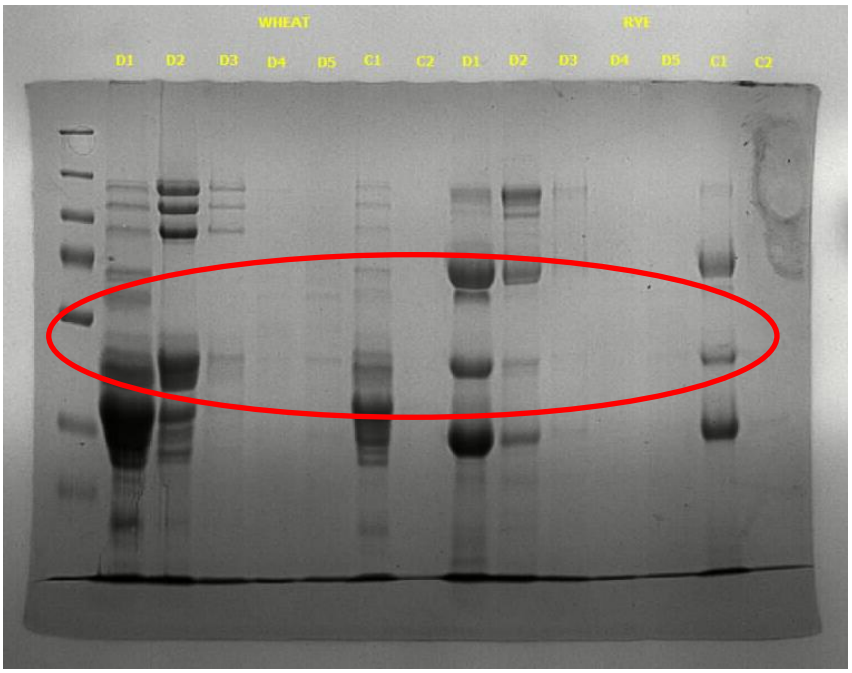

Figure 0.6 Gluten Extraction for wheat and rye in C and D procedures. Omega gliadins are marked with red circle.

As seen in the Figure 3.6, SDS-PAGE analysis of extracts of extraction method D showed better extraction ability then extraction method C when applied to wheat and siyez. D becomes apparent choice for extracting omega glutenins from food samples according to the SDS-PAGE analysis of wheat and siyez extracts.

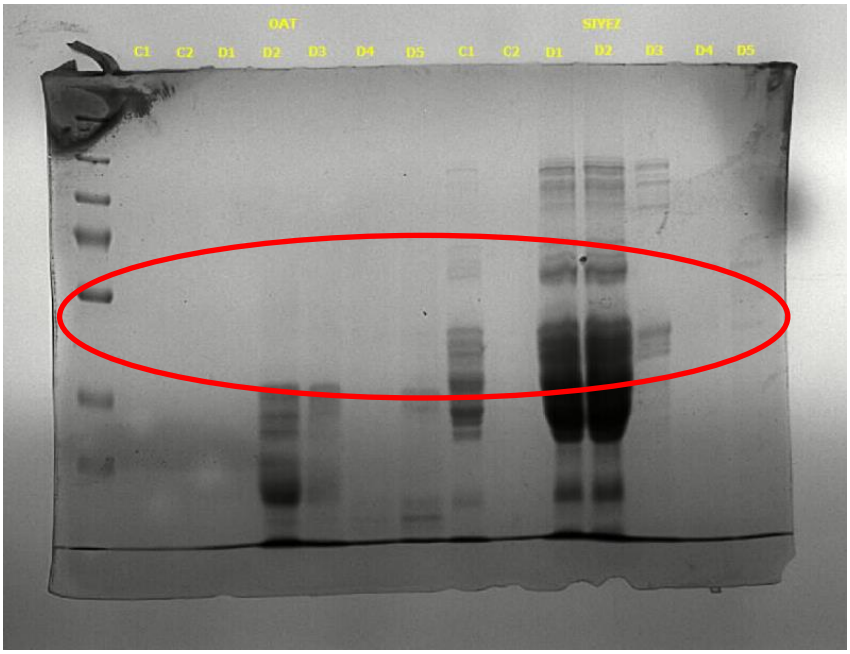

Figure 0.7 Gluten extraction using C and D methods on oat and siyez. Omega gliadins are marked with red circle.

As seen in the Figure 3.7, extraction method D managed to extract large amounts of omega gliadins from siyez samples, but failed to extract large amounts with oat extracts when we performed SDS-PAGE analysis on the extracts of methods D and C. We decided to use method D for all future extracts.

3.2 ELISA RESULTS

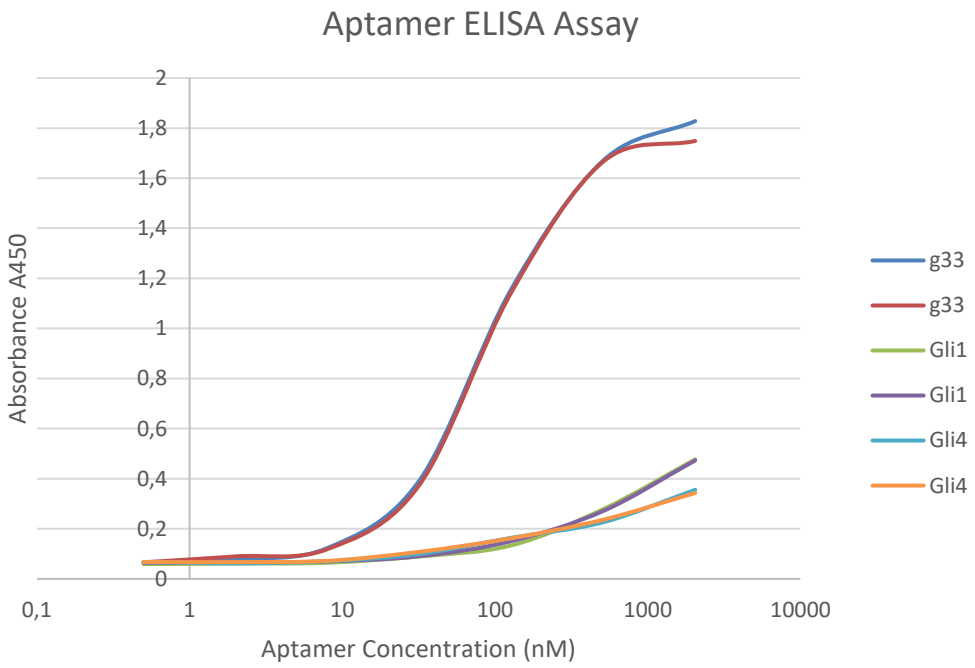

Figure 0.8 Aptamer ELISA results.

Binding abilities of different 5'biotinylated aptamers were measured using ELISA method. After incubating 1 hour at room temperature, wells were filled with streptavidin-HRP solution and results were visualized using TMB as a substrate. In Figure 3.8, we have obtained best results from g33 aptamer which is biotinylated at 5' end.

We did not perform any Km calculations for the ELISA results, because our aim here is to find out which aptamer is the best for our conditions.

So, g33 aptamer was chosen for further studies because of its high binding constant.

3.3 STRIP RESULTS

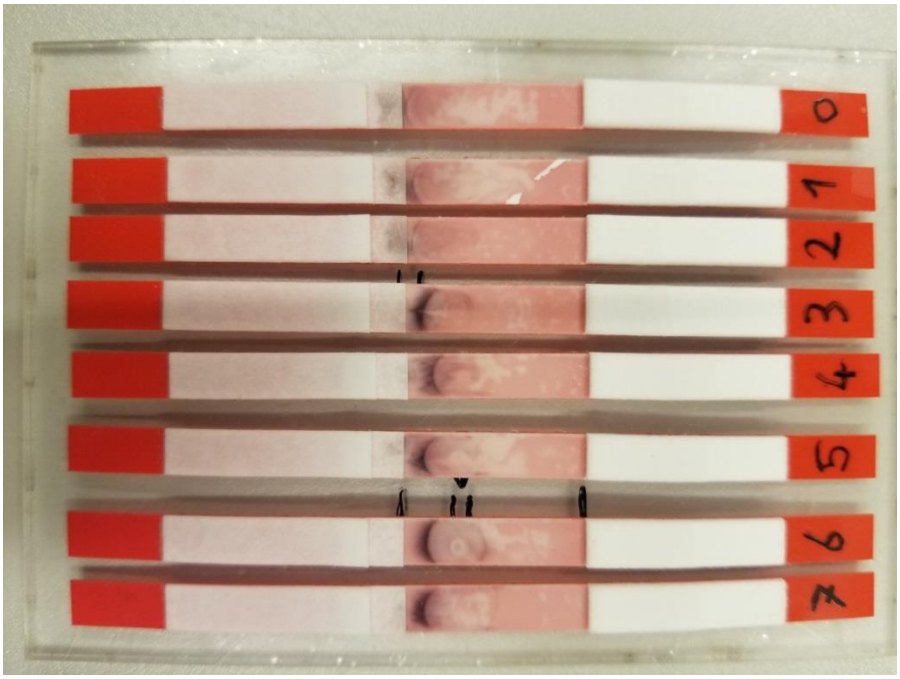

Figure 3.8: Lateral flow assay with gluten sample. Sample 0 shows unspecific binding on the conjugate pad. Samples 1-7 shows color change at the nitrocellulose membrane caused by the detection of gluten in the sample

We have applied increasing amount of PWG gluten on a nitrocellulose surface to test the ability of g33 aptamer to bind gluten on the surface using lateral flow test system. As seen in Figure 3.8, we have obtained brown precipitate after reacting streptavidin-HRP with BCIP/NBT substrate. Gluten samples were applied using micropipette so that we have circular pattern other than classical linear lines in our setup.

DISCUSSION AND CONCLUSION

Towards the detection of gluten from raw and processed food, we have optimized the preprocessing methods to extract gluten from different samples. Obtained samples are compared with the industry standard gluten from Prolamine Working Group and results were analyzed using SDS-PAGE. We have obtained similar band patterns and this finding encouraged us to use aptamers.

Detection of gluten is carried out by antibodies, traditionally. Recently, there are some reports showing good aptamers are obtained using gluten and selection via SELEX methodology. We have ordered 3 different aptamers with 5'biotin label and we have tested their ability to recognize PWG gluten in ELISA format. The aptamer, named g33, was the one with highest recognition ability and we choose g33-5'biotin aptamer for the lateral flow test studies.

As a result, we have optimized the preprocessing method and aptamer solution that can be used to generate biosensor for the gluten detection in raw and processed food samples.

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