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What will be the Future Development of Electrochemical Biosensors for the Detection and Quantification of Biomarkers?

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The presence of a tumour often leads to the alteration in levels of specific proteins in the blood, known as biomarkers [1,2]. In some instances it has also been shown that specific tumours can be associated with specific biomarkers and therefore if detected and measured would able to confirm the presence of tumours. Examples of such biomarkers are the Prostate Specific Antigen (PSA) in the case of prostate cancer [2], Human Chorionic Gonadotropin (hCG) in breast cancer [1], cervical cancer, gastric cancer [3], and colorectal cancer Carcinoembryonic Antigen (CEA) in colorectal cancer [4], gastric cancer [5], lung, and breast cancers [6]. The production of substances as a response to a change in the human body is not limited to cancer and various markers have been identified for other disease states; this is the basis of clinical laboratory medicine. For example, troponin (a family of three types of protein, troponin C, T, and I) is a protein which is important in muscle contraction in skeletal and cardiac muscles, and troponin I and T are specific to heart muscles. Normally they are of trace amounts in the blood, but in case of damage to the heart muscle cells they are released into the circulation and can be used as an indicator of heart attack, or injury to the heart muscle that might result in a heart attack [7].

Since different diseases are known to produce or alter the levels of different biomarkers, it is very important that appropriate and rapid diagnostic methods are used for their detection, as this lead to appropriate and timely, often live saving, therapeutic intervention. Whilst much research is being funded to identify more disease related biomarkers, we often forget the important applied research necessary to devise the best ways of detecting such markers. A biomarker test, to be of any clinical value, needs to be quick, simple to perform, inexpensive reliable and robust. Specificity for any given biomarker is often achieved by the use of antibodies and for decades now they have been the biological chemistry at the centre of instrument systems developed for detecting antigens/biomarkers.

The invention of immunoassays is where a specific antigen can be recognised amongst abundant and complex mixtures of other molecules based on the interaction between a specific binder molecule and the antigen. Such is the specific binding of an antibody to its target antigen that in a complex mixture such as serum and plasma the detection and quantification of molecules like hormones at levels as low as Picograms (pg) is routinely achieved. Immunoassays are widely used in medical and research laboratories, and in the past they were manually performed by skilled technicians, and were very time consuming. Examples of two common types of immunoassays are the Radioimmunoassay (RIA) and the sensitive optics based Enzyme-Linked Immunosorbent Assay (ELISA). Technological development in techniques using antibodies has lead to the invention of highly automated systems such as the Roche Cardiac 200 lateral flow immunoassay testing system, which provides quantitative cardiac marker test results on whole blood samples with a turnaround time of 10-19 min. This helps to meet the best practice guidelines for emergency department settings, by processing up to 36 tests per hour, and offers a full cardiac marker menu that includes N-terminal prohormone brain natriuretic peptide (NT-proBNP), Troponin I (TnI), creatine Kinase (CK-MB), and Myoglobin. Such systems use advanced immunochemistry and are well suited instruments for both research and diagnostic use due to their ability to process large numbers of samples.

Unfortunately, despite their huge success, there are drawbacks, for example, in diagnostic laboratories most tests are rapidly done, but since the analyzers need to process a large number of samples and are under very strict quality control procedures, it is a matter of hours before the test result leaves the lab to reach the clinician, and probably more hours or even days for the patient to know the outcome. One such example is the Prostate Specific Antigen test, which in the UK NHS, has a turnaround time (from receipt of sample to availability of results to clinician) of about one and half days. Thus, despite the fact that an automated analyser has the capability of processing samples in a short time, the time taken from when the patient gives a sample to bedside result is very difficult to lower due to remote processing logistic and the volume required for justify large expensive automated equipment. Simpler ward based analyzer are now common for blood gases, full blood count and blood ketones. However, even these instruments do not allow for real time measurements to be taken, and therefore cannot be used as a continual measurement system.

The past few decades have seen great advances in the development of biosensors and biochips capable of characterizing and quantifying biomolecules [8]. One of the main advantages of developing biosensors and biochips is that they would be suitable for use as Point of Care (POC) instruments by the bedside and give immediate results. This new development forms part of the novel Lab-on-a-chip (LOC) technology, which aims to integrate one or more laboratory functions on a single chip of up to few square centimetres in size. The major obstacle in producing miniature and truly nano detection and quantification devices, which could even be implanted, is the current reliance on optics. This is a major component of most biomarker measurement systems and converts biochemical reactions (resulting in absorbance, luminescence, fluorescence, diffraction) into a quantifiable electrical signal i.e, is a transducer. Reaction resulting in a directly measurable and concentration dependant electrical signal eliminates the need for such a major and bulky transducer [9-11].

The biological component of a biosensor can be classified into two main groups, which are catalytic and non-catalytic [12]. The catalytic group includes enzymes, microorganisms, and tissues, while the non-

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catalytic group consists of antibodies, receptors, lectins, and nucleic acids [10,12]. Catalytic sensors detect the change in the concentration of a component resulting from the catalysed reaction, to produce a signal. In non-catalytic sensors the binding event that occurs between the receptor and target analyte produces the signal [13].

Piezoelectricity was first investigated in 1880 by Jaques and Pierre Curie, and they observed that when a mechanical stress was applied to the surfaces of various crystals, it resulted in an electrical potential across the crystal, in which the magnitude was proportional to the applied stress. The application of an alternating electric field across the crystal substrate results in an alternating strain field. This causes a vibration or oscillatory motion in the crystal, resulting in the generation of acoustic standing waves [14]. Piezoelectric effects can be exploited by coating a piezoelectric crystal with a biological material such as antibodies that would interact with the target molecule. Once the crystal is exposed to the substance of interest, adsorption will occur; causing a frequency change that could then be used to determine the amount of materials adsorbed [15]. The use of piezoelectric crystals for studying immunological reactions was demonstrated by Shons et al. [16]. The study showed that immobilisation of the antigen to the crystal lead to successful determination of antibody activity in solution, as only specific antibodies for the antigen formed an additional protein layer on the crystal. The thickness of that layer was then determined by measuring the frequency shift of the crystal, which was proportional to the concentration of specific antibody in solution. This technique provided both qualitative and quantitative assays of antibody activity [16]. Since then, the use of antibodies, antigens, and enzymes as specific coatings in piezoelectric biosensors is well known, and are thought to be among the most sensitive analytical instruments that have been developed with the capability of detecting antigens in the picogram range both in gas and liquid phase [15,17].

Electrochemical biosensors are so called because they are molecular sensing devices which intimately couple a biological recognition element such as antibodies, to an electrode transducer. These types of biosensors rely exclusively on the measurements of currents and/or voltage to detect binding between the recognition element and analyte of interest. Electrochemical biosensors can be subdivided into categories depending on the transducer and measurement implored. Amperometric and potentiometric transducers are the most common types in electrochemical biosensors.

In potentiometric measurements, the potential difference between either an indicator and a reference electrode, or two reference electrodes is determined by a voltmeter when there is no significant current flowing through them [18]. The potential difference measured is due to the oxidation and reduction of the species in the sample solution rather than their concentration [19]. The transducer may be an Ion-selective Electrode (ISE) based on thin films or selective membranes as recognition elements [18], and the analytical information is obtained when the ISE converts the biorecognition process into a potential signal [20].

The most commonly used potentiometric ISE based biosensors are the pH electrodes, and one example of its use is in glucose biosensors, where glucose oxidase is immobilised on the surface of the pH electrode. Although glucose has not got much influence on pH in the working medium, the enzymatic reaction that results in the formation of gluconate causes acidification leading to a change in pH [21].

Amperometric biosensors are based on the measurement of current that results from the electrochemical oxidation or reduction of an electroactive species. This type of measurement is taken by maintaining a constant amplitude voltage at platinum, gold or carbon based working electrode, or an array of electrodes with respect to a reference electrode [18,22]. The application of a fixed potential causes the analyte in the sample to react and a current to pass through the sample. An example of an amperometric biosensor is that for tumour markers first developed in 1979 for the detection of the hormone hCG based on a competitive immunoassay. Developing such a biosensor involved the immobilisation of monoclonal anti-hCG on an amperometric oxygen electrode. Catalase labelled hCG as well as pure hCG was made to compete with each other for the binding sites of the antibodies bound on the electrode surface. Washing the sensor removed all unbound hCG, and the addition of a substrate lead to the catalase labelled hCG reacting with the substrate, releasing oxygen. The oxygen generated was then sensed by the oxygen electrode [23,24].

Voltammetry involves the application of a time dependent potential to an electrochemical cell, and the resulting current is measured as a function of that potential [25]. In voltammetry, different potentials are applied by sweeping through a range of voltages, and as different species react at different potentials, the concentration of different species would be determined in the same experiment, therefore making it unnecessary to separate the different species and then conducting several experiments [19]. Cyclic voltammetry is a common version of voltammetry, and in this method, the voltage is swept, but at a predetermined voltage level this sweep is reversed ending the cycle at the starting voltage [22].

Recently electrochemical affinity biosensors, namely DNA or immunosensors have shown the potential for monitoring cancer related protein markers, and DNA mutations [20]. Limbut et al. [26] have developed a reusable capacitive immunosensor based on a self-assembled monolayer (SAM) of thiourea on a gold electrode for the detection of Carcinoembryonic Antigen (CEA) detection. CEA is a highly glycosylated cell surface glycoprotein, a subset of the tumour-associated antigens, and its measurement has been very successful in the diagnosis of colorectal and breast tumours. The normal blood serum concentration of CEA in healthy individuals is <2.5 ng/ml, with increased levels suggesting the presence of a tumour. Detection of CEA using this biosensor was shown to strongly correlate with serum levels measured by a convention CEA Enzyme Linked Fluorescent Assay (ELFA).

Kerman et al. [27] reported the development of an electrochemical biosensor for the detection of Human Chorionic Gonadotropin (hCG) in urine based on voltammetry. Briefly; a carbon electrode was dipped in phosphate buffer containing 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-Hydroxysuccinimide (NHs) followed by incubation of the modified electrode with Protein A which has high binding affinity for the Fc region of Antibodies. Monoclonal antibody for hCG was then immobilised on the Protein A-linked modified carbon electrode. After incubation and appropriate washing, the sensor was ready to be tested with synthetic hCG, and later urine samples. Measurements were taken using Square Wave Voltammetry (SWV), and the limit of detection of this method was calculated to be 15 pm (15 mIU/mL) in synthetic hCG samples and 20 pm (20 mIU/mL) in human urine. The electrochemical results obtained were in agreement with the results obtained using ELISA.

A new approach is the use of Electrical Impedance Spectroscopy (EIS), to measure a change in impedance when the analyte forms a complex with the biological recognition molecule upon small surface electrode. EIS is a technique based on the measure of the degree to which an electric circuit resists the flow of an alternating current when a voltage is impressed across its terminal. In EIS a current is applied to the counter electrode and the impedance change is measured at the working electrode. This technology delivers faster results than conventional testing equipments while using a fraction of the material saving both time and money. This innovation holds great promise in the medical and environmental monitoring [28].

In this type of biosensor the biological signal is transduced to an electrochemical signal as a measure of impedance. The numerous impedance measurements, or as it is also known Electrochemical Impedance Spectroscopy (EIS), involves the analysis of both the resistive and capacitive properties of materials when there is a minor change in the physical properties of a system at equilibrium due to a small amplitude sinusoidal excitation signal [29].

In contrast to voltammetry and amperometry which measures current at an electrode as a function of applied electrode-solution voltage and intentionally change the electrode conditions due to their DC approach, in impedance biosensors, the electrical impedance of an interface is measured in a steady AC steady state with constant DC bias conditions. This is achieved by applying a small sinusoidal voltage at a particular frequency and measuring the resulting current, and this current-voltage ratio gives the impedance. A change in impedance of the electrode-solution interface after the binding of the target analyte to the recognition element indicates the efficiency of EIS as an effective way of detecting impedance change in such instances [30]. Literature has also reported that potentials lower than 0.5 V and current lower than 100 μAcm-2 are very likely to cause alterations in protein binding, which suggests that a very small potential should be used in impedance biosensors [31].

This method allows the impedance of a system to be measured over a wide range of Alternating Current (AC) frequencies (spectroscopy) [29]. Taking impedance measurements at different frequencies gives an impedance spectrum that then allows the characterisation of surfaces, layers or membranes as well as exchange and diffusion processes [32]. Usually the impedance spectrum is analysed using an equivalent circuit, which consists of resistances and capacitances. These resistances and capacitances represent the different physicochemical properties of the system under investigation. In this regard a wide frequency range to take impedance measurements is scanned to identify the particular range that gives the best impedance signal on building up different layers of the sensor and which frequency range is best to use for detecting the substance of interest.

Unlike the techniques described above, the construction of enzyme based impedimetric biosensors is slightly different, as in this case instead of the interaction between the enzyme and the substrate, the effect of that interaction on the substrate is measured, that is, the degradation of the substrate when the enzyme reacts with it. However, different ways of measuring that degradation process of the substrate is employed in different studies. One such example of the biological interface of an enzyme based was developed by Cortina et al. [33]. Their sensor was constructed for potential urea analysis in urine. The working electrode was modified by coating it with a polymer layer (Eutragit s-100), followed by the loading of a cellulose membrane on its surface, whereby this membrane was immobilised with Urease. The Urease reacts with the polymer substrate, which then results in an increase in pH which subsequently causes its degradation. The degradation is then monitored by EIS through its geometric capacitance (Cg) changes, such that a larger Cg value indicates faster polymer degradation [33].

In 1998, Saum et al. [34] developed a biosensor for the detection of collagenase. This biosensor worked by detecting the change in impedance caused by the proteolytic digestion of gelatine coated interdigitated gold electrodes. It was shown that as enzyme degradation of the layer occurred; there was a sudden increase in the impedance on reaching a critical thickness of the gelatine layer. The change in impedance due to the protease digestion was correlated with the degradation of the gelatine layer from the surface of the sensor by measuring weight loss of the layer. However, it was noticed that the ability to detect the gelatine coating on the sensor was severely impaired by the presence of electrolytes [34]. Following this work the group moved on to detecting protease activity in the wetted surface of gelatine coated electrodes in humid air by AC impedance spectroscopy, and they showed that the addition of glycerol to the gelatin caused a lower impedance response and smaller loss in weight. Also with glycerol-enhanced gelatin, there was a clear distinction between the extent of impedance change with different collagenase concentrations. These studies lead to the possibility of using this method in the field of bioaerosol sampling [35].

Electrical impedance is a common and efficient application in the microbiology field as a means of detecting, quantifying and even identifying bacteria in microorganism based biosensors; it involves immobilising specific antibodies to bacteria on the surface of an electrode. On testing the sensor with diluted bacterial samples, the binding of the bacterial cells with the antibodies causes a change in impedance which is recorded. Microorganisms based impedimetric biosensors have been used mainly for the detection and quantification of microorganisms present in milk and dairy products. This idea was first proposed in 1978 by Cady [36], following which, several systems based on this method were developed by a number of companies for use on dairy products. The use of such biosensors moved on to other applications such as monitoring the total microbial count in fish, meat, wine, fruit juices, and potable water [29]. An example of the development of such a biosensor is for the detection of salmonella enteritidis in food samples. This was achieved by immobilising anti-salmonella antibodies on the surface of an interdigitated microelectrode via an avidin-biotin layer. The bacterial cells to be tested were diluted to different concentration, and on testing the sensor, it was noticed that the higher concentration, that is, more bacterial cells, the bigger was the impedance signal. This impedimetric biosensor could detect 103 cfu/mL of Salmonella in pork meat extract with an incubation time of 5 minutes, suggesting that this method may provide a simple, rapid and sensitive method to detect food borne pathogens [37].

This method was also developed for use in biomedicine and given the new name Electric Cell-substrate Impedance Sensing (ECIS), based on measuring changes in AC impedance of small gold electrodes which are deposited on a culture dish and used as a growth substrate. The use of this method extends to the monitoring of attachment and spreading of mammalian cells quantitatively and in real time [38]. The use of different culture media was tested and it was found that a low conductive medium was optimal for the monitoring of bacterial growth. From this study it was concluded that the solid medium biosensor is useful for the detection of pathogenic microorganisms, and offers the possibility of a new analytical tool in microbiology [39].

Immuno-binding based impedimetric biosensors are attracting great interest as they are direct and do not require labels, thus having the advantages of being quick to perform and take measurements, easy to use and do not necessarily require skilled technicians for operation.

This method involves immobilising specific antibodies to the test analyte, on an electrode via suitable biochemistry techniques so that they interact with the target analyte on testing, resulting in a change in impedance. This method however, although seems fairly simple as it involves an antibody/antigen interaction (immunoassays) and is extensively used routinely in biochemistry and immunology laboratories, is not as straightforward when combined with electronics. This is because it involves interaction of biological layers on solid surfaces rather than in solution as in immunoassays. Also, the detection techniques are completely different to the simple colour reaction as in ELISA, which can just be read by a simple instrument to give the concentration of the antigen of interest in a sample. In impedimetric based biosensors, the interaction between the antibody and antigen needs to be converted to an electrical signal in order to be detected, a method which is proving to be quite challenging due to the complexity of the antibody immobilisation process.

In 1999, Jie et al. [40] reported an impedance immunosensor for the specific detection of the interaction between human mammary associated tumor glycoprotein and its monoclonal antibody (GP1D8). This sensor was developed by immobilising the antibodies onto a gold electrode by spontaneous adsorption, and the binding of the specific antigen to the antibodies lead to a change in electrochemical properties, which caused an increase in the charge transfer resistance (Rct). The specificity of this sensor was shown by incubating the monoclonal antibody coated electrodes with a non specific antigen and taking impedance measurement. The resulting Nyquist plot showed that no apparent change occurred compared to the instant where the specific antigen was added, and a change in impedance was noticed [40]. Kassanos et al. [19] reported findings regarding the first two stages in the development of an electrochemical biosensor based on impedance spectroscopy for the detection of the hormone hCG-β using commercially available gold microelectrodes. This paper describes the application of a Self Assembled Monolayer (SAM) formed by the dissociative chemisorptions of the dialkyl disulphide, dithiobis (succinimidyl propionate) on the gold surface, the immobilisation of anti-hCG-β and ultimately hCG-β. Impedance measurements were taken at each step in the construction of the sensor and the paper reports an increase in impedance on the addition of each layer in the sensors, demonstrating successful biosensing of hCG-β with this method.

Multiple approaches are currently being developed to replace optics as the transducers in immunoassays and other biosensors. No one approach is likely to be as widely adopted as optics has in laboratory based biomarker detection and measurement instruments. However, some of the approaches such as electrical impedance may truly lead to nano-scale, point of care, implantable biosensors.

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