Review

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Towards Point-of-Care Insulin Detection

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Abstract

Good glucose management through an insulin dose regime based on the metabolism of glucose, helps millions of people worldwide manage their diabetes. Since Banting and Best extracted insulin, glucose management has improved due to: the introduction of insulin analogues that act from 30 minutes to 28 days, improved insulin dose regimes, and portable glucose meters, with a current focus on alternative sampling sites that are less invasive. However, a piece of the puzzle is still missing - the ability to measure insulin directly in a Point-of-Care device. The ability to measure both glucose and insulin concurrently will enable better glucose control by: providing an improved estimate for insulin sensitivity, minimising variability in control, and maximising safety from hypoglycaemia. However, direct detection of free insulin has provided a challenge due to the: size of the molecule, the low concentration of insulin in blood, and selectivity against interferants in blood. This review summarises current insulin detection methods from immunoassays to analytical chemistry, and sensors. We also discuss the challenges and potential of each of the methods towards Point-of-Care insulin detection.

Since, Fredrick Banting and Charles Best successfully extracted insulin from the pancreas islets of dogs in the 1920s, millions of individuals with diabetes have benefited.[1-4] The World Health Organisation (WHO) statistics from 2014 indicate that approximately 422 million people worldwide have diabetes - with several million people dying annually as a consequence.[5] Wild *et al.* predicted that the prevalence of diabetes will be 4.4% worldwide in 2030 (over all age groups) – increasing from 2.8% in 2000.[6]

How is insulin produced? Insulin is first synthesized as preproinsulin **(Figure 1a)** in the beta cells of the pancreas islets. Preproinsulin contains a signal peptide and three peptide chains. After a series of conversions, C-peptide **(Figure 1b),** and human insulin **(Figure 1c)** are secreted by the beta cells. Insulin is a hormone, comprising of a sequence of 51 amino acids in the form of two peptide chains, which are connected by two disulfide bonds.

What is the role of Insulin? Insulin regulates the uptake and metabolism of glucose, commonly referred to as sugar, which is required for muscle energy and is essential for brain function.[7] This process is influenced by many factors, for instance, exercise, sleep, stress, and even the type of food consumed. Which all act in conjunction with a person's phenotype, such as gender, ethnicity, and age.

Why is insulin so important for an individual with diabetes? Diabetes is a metabolic disorder, which presents in several different forms: (1) Type 1 Diabetes Mellitus (DM1), previously known as insulin-dependent diabetes (IDDM), is an autoimmune disorder in which the pancreas is unable to produce insulin. (2) Type 2 Diabetes Mellitus (DM2), previously known as non-insulin dependent diabetes (NIDDM), is when the body becomes resistant to insulin. (3) Gestational Diabetes, is a condition in which a woman presents with heightened blood glucose levels during pregnancy. The occurrence of gestational diabetes also increases the chance of both mother and child developing DM2.[7-8]

How is diabetes managed? Diabetes is managed predominantly via the administration of insulin. In 1922, the first diabetic patients were given insulin from foetal calves **(Figure 1d)**. This reduced their symptoms of diabetes and forestalled what was then a fatal disease. One of these patients, Elizabeth Hughes, saw her life expectancy increase from several years to several decades due to the administration of regular insulin doses.[1-4] Since 1922, insulin purity, demand, and production have increased significantly. Which was made possible by the introduction of biosynthetic 'human' insulin by *Eli Lilly and Company* in the 1980s. In the following decade insulin analogues began appearing

on the market – with several analogue options now available. Compared to human insulin these analogues differ slightly in the amino acid sequence **(Figure 1e,f)**. These differences enable insulin to act anywhere from 30 minutes (very rapid-acting) to several hours (long-acting) after injection; thus, enabling better mimicking of the physiological insulin response to various daily activities. Recently, ultra long-acting analogues lasting up to 28 days have started to enter the consumer market.[9-12]

The second major improvement in diabetes management resulted from the development of the blood glucose meter, which offered improved control over an insulin dose based on more exact knowledge. Similar to the evolution of insulin, the glucose meter underwent considerable development. From the first test strip developed by *Ames Company* in the 1960s, glucose meters have developed to portable self-monitoring devices with improved accuracy, affordability, and portability.[12-14] Continuing technological advances have enabled monitoring devices to become more compact, offer smaller blood sample volumes, and quicker response times.

More recently, a need has been expressed for devices that are less invasive.[11-13] Innovative approaches with alternative glucose sampling and insulin delivery sites are emerging to meet this need. For instance, Ghu's research group have proposed a 'smart insulin patch', which in essence will detect an increase in glucose levels and release insulin accordingly.[15-16] In addition, for some individuals with diabetes the stress of diabetes management has been reduced, with the use of continuous glucose monitoring devices. Furthermore, some glucose monitoring devices are able to communicate with a continuous insulin pump. Thus, resulting in automated diabetes management.[13-14] Beyond technological advances, improvements are being made in insulin therapy regimes to control blood glucose levels.[17-19] Insights are being gained by examining the influence of different factors in glucose management, such as exercise and diet. In addition, the presence of critical illness or disease (such as cardiac disease), and the impact this has on glucose management is being investigated.[20-26]

However, despite these advances one significant piece of the puzzle is still missing – *the ability to directly measure free insulin using a commercial Point-of-Care device*. This missing piece is of significance, as modern models use an insulin dose to control blood glucose levels - based on estimates of glucose uptake in relation to insulin.[24, 27] The ability to measure both glucose and insulin concurrently will enable better glucose control, by enabling: better estimates of insulin sensitivity, minimising variability in control, and maximising safety from hypoglycaemia and hyperglycaemia. Hypoglycaemia and hyperglycaemia occur when blood glucose levels are either dangerously low or high, respectively. Hypoglycaemia is of a greater concern, as it is the main cause of the occurrence of disease (i.e. cardiac diseases) for individuals with diabetes, and in extreme cases can cause the

sudden onset of diabetic coma or fatality.[28] Insulin detection would help limit the insulin dose, to ensure that an individual with diabetes would not enter dangerously low blood glucose levels. Thus, increasing the ability of personalised healthcare for individuals with diabetes. As the allowable insulin dose range/level would be specific for each individual with diabetes, whilst accommodating everyday activities.

How readily can free insulin be detected? Not as easily as glucose. The ability to measure free insulin directly is not an easy feat due to: (1) the size of the insulin molecule; (2) the picomolar concentrations of free insulin in blood, blood plasma or interstitial fluid (normal insulin serum level under fasting is 50 pM); and (3) the required selectivity against interferants in the blood, such as: glucose, ascorbic, uric acid, and C-peptide depending on the detection method used.[29-31] As with any other device, a Point-of-Care insulin detection device would be required to undergo rigorous clinical testing, and meet local regulations. However, as models are not entirely reliant on insulin measurements but glucose measurement as well, the requirements of the sensitivity towards insulin will not be critical down to the fraction of a picomolar.

What methods exist that can detect free insulin? Immunoassays, conventional analytical chemistry method, and biosensors have been used to detect insulin.[32-44] Progress in direct insulin detection was first reported by R. S. Yalow and S. A. Berson, by a radioimmunoassay method - *Immunoassay of Endogenous Plasma Insulin in Man.*[45] Over half a century has passed since Yalow and Berson directly measured insulin in plasma. In this time, many advancements have been made in device technology and insulin detection methods, indicating the potential to develop a Point-of-Care device capable of measuring free insulin for improved blood glucose management. Several reviews have been published by various groups over the years, with each existing review focused on a particular detection method. The review by P. M. Clark in 1999 for example focused on immunoassays for insulin, proinsulin(s), and C-peptide.[36] In the same year a review by Chevenne *et al.* also focused on insulin immunoassays.[46] A more recent review published in 2013, discussed the advances and challenges associated with the quantification of insulin analogues, using liquid chromatography separation with tandem mass spectrometric detection.[47] The most recent review published in 2018 discusses the use of surface plasmon resonance and electrochemical methods for the detection of insulin.[48]

In contrast, in this review we provide a comprehensive overview of the development of free insulin detection research from conventional methods to biosensors, since Yalow and Berson detected insulin in 1960. We believe this is critical for moving the detection of insulin towards Point-of-Care, as we observed limited citation occurring between the different detection methods, and the development of insulin biosensors can benefit from the knowledge transfer between the different methods. For

instance, the use of a particular antibody in an immunoassay or an aptamer in a conventional analytical chemistry approach may assist in the development of an insulin biosensor. In addition, we discuss the challenges and potential of each of the methods towards Point-of-Care insulin detection. In this context, a Point-of-Care device is a device which enables detection to be completed with instantaneous results displayed for the user. These devices are routinely used in clinics, hospitals, and at home. Point-of-Care devices offer portability, quick detection times, small sample volumes, and are inexpensive. Whilst not compromising accuracy, to produce a device which is convenient and reliable for the user.[49-50] Point-of-Care devices are well established in the field of diagnostics, and are routinely utilised for: (1) nucleic acid testing for infectious diseases, such as human immunodeficiency virus (HIV); (2) monitoring of blood coagulation, primarily for those that take blood thinning drugs; (3) measuring glucose levels for diabetes management; and (4) the most well-known in home test, the pregnancy test, which measures the pregnancy hormone – human chorionic gonadotropin (hCG).[14, 49-53] In addition, Point-of-Care testing will continue to play a substantial role in diagnostics in developing countries, where clinics are not as well equipped as developed countries and access to laboratories is few and far between.[54]

In this review, we discuss the potential of each detection method for Point-of-Care insulin detection in terms of portability, detection time, expense, and performance. In terms of performance, interferants are acknowledged for a method, and the linear range and sample media is indicated for different approaches using each method. A linear range is used for comparing the performance, as this parameter is consistently used by researchers reporting their respective insulin detection methods. It is worth noting that the majority of approaches discussed in the review use human insulin (**Figure 1c**) to test their approach; however, bovine insulin (**Figure 1d**) which differs only slightly in its amino acid sequence is routinely used, and porcine insulin to a lesser extent.

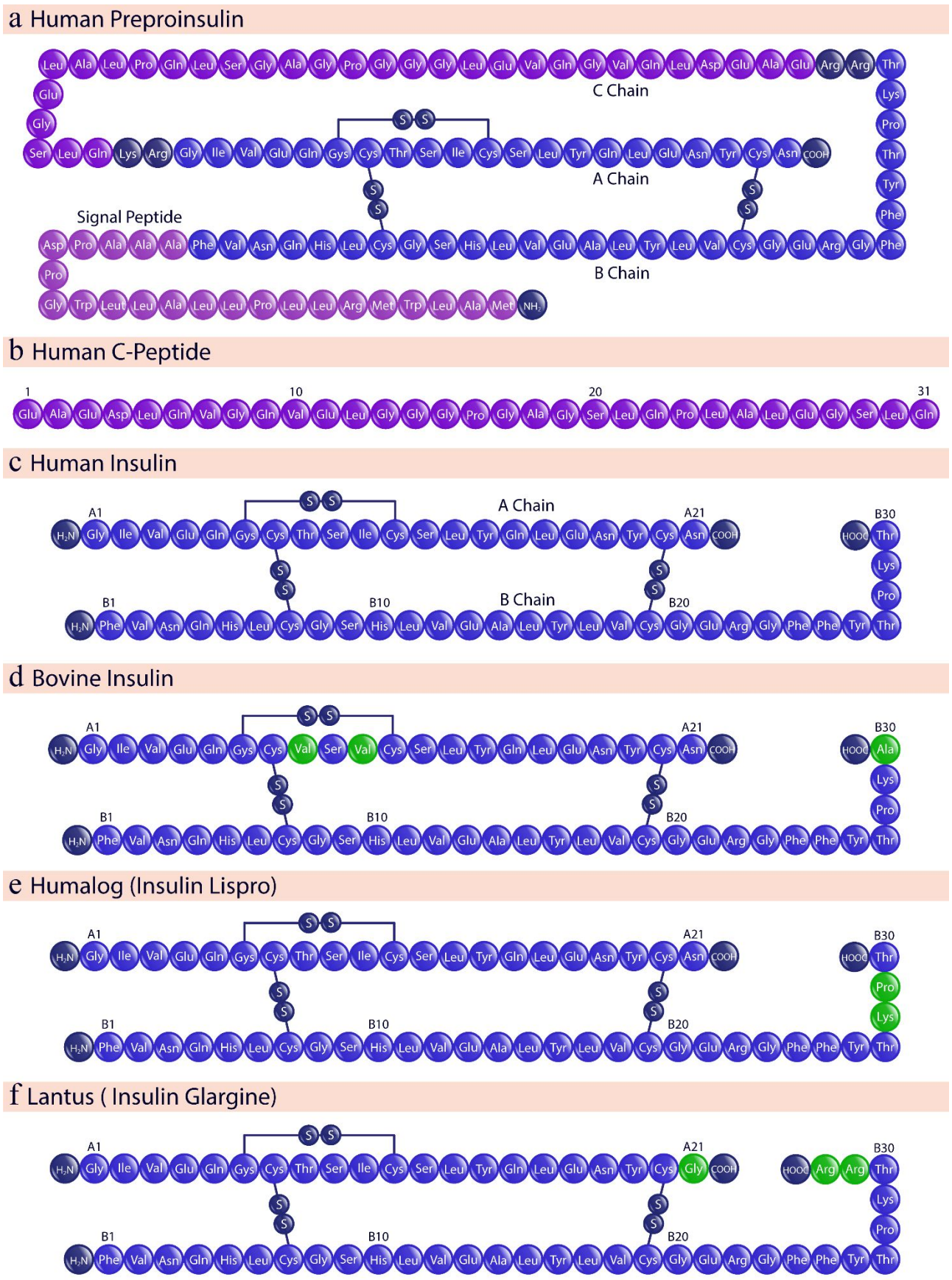

Figure 1: Amino Acid Sequences. For (a) human preproinsulin, (b) C-peptide, (c) human insulin, (d) bovine insulin, (e) insulin lispro (Humalog) a rapid-acting insulin analogue, and (f) insulin glargine (Lantus) a long-acting insulin analogue.

Conventional Methods

We begin our discussion on insulin detection methods with conventional biological and chemical laboratory methods, as these were the first methods used to detect free insulin. Initially, we discuss immunoassays, a conventional biological method that is used extensively for insulin detection. Since Radioimmunoassays (RIA) were developed by Yalow and Berson, other immunoassays, such as Enzyme-Linked Immunosorbent Assays (ELISA) and Chemiluminescence Immunoassays (CLIA) have been developed and subsequently used for insulin detection. [32-35] In addition to immunoassays, analytical chemistry methods are discussed, with a primary focus on methods involving liquid chromatography with either ultra violet/visible light detection or mass spectrometric detection.[37-41, 55-57]

Immunoassay Methods

Immunoassays are a set of methods that detect or measure a specific biomolecule, an analyte, in body fluids such as blood or urine. Immunoassays are used extensively in pharmaceutical analysis and medical diagnosis for the diagnosis of diseases, therapeutic drug monitoring, and drug discovery.[58-63] Immunoassays take advantage of the immunometric reaction between a labelled antibody and an antigen (the analyte). In some rare cases, an antibody can be the desired analyte.[63-64] The first immunoassay, a RIA developed by Yalow and Berson, utilised a radioactive isotope label - Iodine-131 bound to bovine insulin. Human plasma insulin would then compete with the bovine insulin to bind to insulin antibodies, in sera from guinea pigs immunised with bovine insulin. This approach enabled a radioactive change to be measured, which is proportional to the concentration of human insulin.[45]

However, as time progressed, safer alternatives to radioactive isotope labels were sought, whilst maintaining sensitivity and selectivity comparable to RIA. This led to the discovery of ELISA in the 1970s, in which an enzyme is utilised as a label. Commonly used labels include horseradish peroxidase (HRP), alkaline phosphatase, and glucose oxidase.[65] Various approaches are used for the detection of insulin using ELISA such as, enrichment processes prior to detection, and the use of aptamers or polyclonal and monoclonal antibodies (**Table 1**).[33, 66-68] For instance, Lei *et al.* modified mesoporous silica with an insulin-binding aptamer, a single stranded oligonucleotide from either RNA or DNA, and introduced hydrophobic methyl groups using aminopropyl(diethoxy)methylsilane. Aptamers offer high affinity towards a specific analyte, good stability, and can be readily chemically modified.[69-71] This approach enabled the enrichment of insulin from serum prior to an ELISA utilising horseradish peroxidase.[68] A linear range of 0.04 to 0.09 pM was achieved using this approach, which is well below the detectable levels of insulin in blood - around 50 pM under normal fasting conditions **(Table 1)**.

The development of monoclonal antibodies in which all antibodies derive from a single parent cell, has improved the development of immunoassays. Monoclonal antibodies offer improved selectivity towards an analyte. Consequently, monoclonal antibodies have been utilised for insulin detection since the 1980s.[33, 67, 72] Monoclonal insulin antibodies are produced in murine through the immunisation of guinea pigs, porcine, or bovine with insulin, as well as the fusion of lymphocytes from lymph nodes with murine myeloma lines.[72] Comitti *et al.* utilised either bovine or porcine insulin with lymph nodes from the P3x63 murine myeloma line to produce monoclonal antibodies. In this work, one monoclonal antibody was bound to the wells of the microtiter plate, with the second monoclonal antibody conjugated to alkaline phosphate, forming a two-site immunoassay. A linear

range of 14 pM to 1 nM was reported, which includes detectable levels of insulin in blood. However, experiments were carried out in a sodium carbonate buffer, and took three to four hours to complete **(Table 1)**.[72]

Similar to ELISA, CLIA assays were developed in which chemiluminescence labels are used in replace of radioactive labels.[73] Chemiluminescence labels originate from A. K. Campbell's work on bioluminescent jellyfish *Obelia,* which aimed to replace the radioactivity in immunoassays and DNA technology.[73-74] Much like ELISA, various approaches are used in insulin detection using CLIA.[34, 75] For example, Tanaka and Matsunaga utilised bacterial magnetic particles coated with lipids for improved dispersion in solution compared to artificial magnetic particles. They used bacterial magnetic particles attached to antibody-protein A in a sandwich immunoassay configuration, with an alkaline phosphate conjugated secondary antibody **(Table 1).**[34] In another example, Poulsen and Jensen, used a luminescent oxygen channelling immunoassay to detect insulin in a sandwich immunoassay configuration. Poulsen and Jensen reported a linear range of 1 to 10 pM, which is just below the detectable levels of insulin found in blood **(Table 1)**.[76]

However, immunoassays possess a few shortcomings, such as: a lack of standardization, cross-reactivity in particular with proinsulin (preproinsulin without the signalling peptide) and C-peptide (**Figure 1**), a limited linear range, and considerable sample preparation steps. Consequently, due to issues plaguing insulin immunoassays, in particular regarding standardization, workgroups were assembled by the American Diabetes Association.[36, 77] The idea behind these workgroups was to provide recommendations to improve the performance of immunoassays.[77-79] The initial task force report from 1996 found that when different laboratories were given identical samples, results would include a variance. However, the group was unable to identify a specific assay characteristic as the cause of this variance. The task force report concluded with an assessment and certification process for the performance of an insulin assay, and guidelines for the validation of insulin immunoassays.[77] Two other reports have been assembled for the Insulin Standardization Workgroup since 1996. These reports focused on testing commercially available kits. The results from these reports also indicated a variance, and the outcomes of these reports were in agreeance with the earlier report.[78-80]

These shortcomings identified by the working group reports, together with the inherent complexity of the preparation steps in conjunction with the preparation/detection duration required, make immunoassays unsuitable for point-of-care free insulin detection. For instance, the use of a second antibody required to produce sandwich assays for improved detection adds an extra degree of complexity **(Table 1)**.[34, 67, 72, 76] Furthermore, the majority of people may not have the expertise required to carry out an immunoassay, and the reported assay times for the detection of insulin take

anywhere from one hour to four hours.[33, 67, 72, 76, 81] When immediate action is required, waiting several hours for an insulin level could result in the patient's life being put at risk. These aspects would restrict the impact in Point-of-Care applications, where a user requires instantaneous feedback, ideally with no additional sample preparation.

Table 1: Examples of Immunoassay Methods for Insulin Detection. RIA – Radioimmunoassay, ELISA – Enzyme-Linked Immunosorbent Assay, and CLIA Chemiluminescence Immunoassay.

Immunoassay	Detection Approach	Linear Range	Ref.
RIA	**Equilibrium RIA.** This approach employs three monoclonal antibodies. Two antibodies are utilised in the preincubation step, for the separation of insulin precursors (proinsulin and C-peptide). This enabled the third antibody to react with insulin without interference from the insulin precursors.	10 – 1240 pM (plasma)	33
ELISA	**Flow-injected Thermometric ELISA.** This approach employs a lactate dehydrenanse/lactate oxidate substrate recycling system for signal amplification.	8.7 pM – 348 nM (phosphate buffer)	66
	Sandwich ELISA. In this approach two murine monoclonal antibodies are employed, which bind to two different epitopes on insulin.	5 – 600 pM (serum)	67
	ELISA with an Enrichment Process. Mesoporous silica was modified with both an insulin-binding aptamer, and hydrophobic methyl groups. The modified mesoporous silica enables improved enrichment of insulin prior to an ELISA.	0.04 – 0.09 pM (serum)	68
	Two-site Immunoassay. In this approach one monoclonal antibody was bound to the wells of the microtiter plate, and the second monoclonal antibody conjugated to alkaline phosphate. The monoclonal antibodies were produced in murine.	14 pM – 1 nM (sodium carbonate buffer)	72
CLIA	**Chemiluminescence Enzyme Immunoassay.** Two conjugated antibodies were employed in this approach, (1) antibody-protein A - bacterial magnetic particle complex, and an (2) alkaline phosphatase conjugate secondary antibody.	156 – 2082 pM (serum)	34
	Homogenous Liposome Immunoassay. In this procedure phospholipase C was conjugated to insulin. The concentration of phospholipase C was measured through the release of calcein (a fluorescent complex), as a result of the lysis of the liposomes.	33 – 1070 pM (phosphate buffer)	35

	Luminescent Oxygen Channelling Immunoassay. A two-step sandwich process was used: (1) HUI018 (monoclonal antihuman insulin antibodies) coupled to unconjugated Eu-acceptor beads were incubated with insulin and biotin OCXI005 (antibody). (2) Streptavidin-coated donor beads were then added, then the assay buffer was incubated further.	1 pM – 10 pM (plasma)	76

Analytical Chemistry Methods

Commonly used conventional analytical methods for insulin detection combine separation carried out by liquid chromatography, with either UV (ultra violet)/visible light detection or mass spectrometry (**Table 2**).

When botanist Mikhail Tswett, reported the separation of different plant pigments in liquid into a series of coloured bands inside a column in 1906, he laid the foundation for *chromatography*.[82] Over the course of a century, different chromatography methods have flourished, including gas chromatography, in which the mobile phase carrying the analyte sample is gaseous. However, this technique was not suitable for all biomolecules and high pressure liquid chromatography, also known as high performance liquid chromatography (HPLC) was developed as a result.[83] In general, a good chromatographic separation is dependent on the difference in strength between the physical interactions of the analyte in the two phases, including the transport properties. In HPLC, a solution containing the analyte will be combined with the *mobile phase* (a solvent). The mobile phase under high pressure will be flowed into a HPLC column. The column contains the *stationary phase* (porous material), which influences the separation of the mobile phase.[83] Once separation has occurred inside the column, the sample undergoes detection. In the case of insulin detection, either UV/visible light or mass spectrometric detection is generally used (**Table 2**).[37-41]

HPLC with a UV/visible light detector is a non-destructive detection method. Three different detectors are commonly used: fixed wavelength, variable wavelength, and photodiode array (PDA) detection. PDA detectors are becoming the preferred detector, as they are comparably inexpensive and enable a variable wavelength. A variable wavelength enables a detector to be tuned to operate at the absorbance maximum of the desired analyte.[83-84] For instance, for insulin detection, Moslemi *et al.* utilised a C18 column for HPLC separation with UV detection. Octadecylsilica and tetramethylammonium hydroxide in water and acetonitrile were used as the mobile and stationary phases, respectively. With this approach Moslemi *et al.* were able to achieve a linear range of 0.46 to 16.1 μM (**Table 2**), which is above the levels of insulin found in blood.[57] A linear range comparable to the level of insulin found in blood, might be possible with HPLC with PDA detection; however, the capacity of HPLC separation with PDA detection is still relatively unknown for insulin sensing.

In addition to PDA detection, mass spectrometric detection is commonly used for insulin detection. Although mass spectrometry is a destructive method, the high sensitivity of mass spectrometry makes it useful for measuring insulin, especially considering free insulin appears in very low concentrations (around 50 pM under fasting conditions).[37-39] A mass spectrometer comprises of an ion source, a mass analyser, and a detector. Electrospray ionization (ESI) and Matrix-assisted laser desorption/ionization

(MALDI) are ionization processes commonly utilised for the detection of proteins and peptides, including insulin. The aforementioned ionisation processes require different sample preparation procedures and delivery conditions. For instance, ESI requires a solvated sample, which is infused into the mass spectrometer; whereas, for MALDI the sample/analyte is immobilised onto the target plate. [39, 41, 85-91]

For example, Zhang *et al.* modified the MALDI target plate with coagulated gold nanoparticles and an insulin binding aptamer – In1: 5'-SH-$(CH_2)_6$-ACAGGGGTGTGGGGACAGGGGTGTGGGG-3'. Insulin binding aptamers are determined through the aptamer selection process called SELEX (systematic evolution of ligands by exponential enrichment). Several aptamers have been determined through this process, with aptamers IGA3 (5'-SH-$(CH_2)_6$-GGTGGTGGGGGGGGTTGGTAGGGTGTCTTC-3') and In1 commonly being used for insulin detection.[92-93] Zhang *et al.* incubated the insulin solution on the gold aptamer modified target plate for an hour shortly before undertaking mass spectrometric analysis. They achieved a linear range of 872 to 174 pM (**Table 2**).[41] Similarly, Liang *et al.* modified the MALDI target plate, although, in this case the plate was modified with gold nanoparticles and insulin antibodies. This approach took around an hour to determine the level of insulin in the range of 0.9 to 48 nM (**Table 2**).[86] One hindrance of mass spectrometric detection is the serial process of detection, which limits high-throughput analysis. To overcome this, different strategies are being employed. One strategy is to interface the output liquid chromatography column directly to the ESI inlet of the mass spectrometer, thus, offering potential for the automation of sample separation and detection. Darby *et al.* employed this strategy for insulin detection. In addition, the insulin spiked blood samples underwent solid phase extraction for sample purification, before liquid chromatographic separation was carried out. In an unspecified duration a linear range of 70 pM to 14 nM was achieved, which includes the top range of insulin concentration found in blood (**Table 2**).[39] In addition, approaches using liquid chromatography in conjunction with mass spectrometry, have reported the capability of being able to distinguish between human insulin and different insulin analogues with high success.[40, 94-96] For example, Thomas *et al*. reported a method with liquid chromatography coupled to ion mobility mass spectrometry, which was able to distinguish between human insulin, bovine insulin, and different insulin analogues in less than eight minutes.[40] The short detection duration and the ability to distinguish between different insulin analogues would be beneficial in Point-of-Care applications, especially with individuals that use both fast-acting and long-acting insulin analogues.

Recently, using a different analytical chemistry method, surface-enhanced Raman spectroscopy (SERS), Cho *et al.* measured insulin in phosphate buffer saline, and pancreas islet secreted insulin suspended in Krebs-Ringer bicarbonate buffer with 1 % human serum albumin. For their SERS

substrate they used silicon functionalised with dense clusters of pillars comprising of gold nanoparticles. A linear range of 100 pM to 50 nM was obtained. Although label-free, detection was achieved after the SERS substrate was incubated for 12 h with the desired insulin sample.[97] This long incubation time is not suitable for Point-of-Care insulin detection, where instantaneous results are required. However, the potential of SERS for insulin detection is relatively unknown, as only a couple of approaches have been published.[97-98] This is attributed to the limited success of SERS for the detection of hormones in general.[97-100]

Detection generally takes around an hour for analytical chemistry methods, which is a considerable improvement towards Point-of-Care insulin detection compared with immunoassays that take several hours. However, the extent of time required for sample preparation in some approaches is not always identified. Analytical chemistry **(Table 2)** and immunoassay **(Table 1)** methods have comparable linear ranges. In addition, both methods require specialised expertise to undertake the sample preparation steps and interpret the results. Conversely, improved sensitivity and selectivity is capable with analytical methods, especially those that use liquid chromatography separation in conjunction with mass spectrometric detection. Although, most people would probably think a portable mass spectrometer would be nice to have in a clinic, or even at home, currently this level of portability is not possible. In addition, a portable mass spectrometer would more than likely not be inexpensive, which is another requirement for regular Point-of-Care measurements. However, progress is mainly hindered by the need to maintain performance comparable to a laboratory mass spectrometer, in terms of sensitivity and selectivity while improving portability and cost.[101] On the other hand, PDA detectors might be more suitable for Point-of-Care. However, they are potentially limited in their ability to discriminate the analyte against other similar interferants in a sample. In summary, the knowledge obtained with these methods can be transferred into more compact technologies (such as biosensors), which may provide a pathway to Point-of-Care insulin sensing.

Furthermore, HPLC coupled with mass spectrometric approaches are being actively utilised for insulin detection in drug doping.[37, 40, 85, 96] Although the performance-enhancing properties of insulin are still up for discussion, athletes looking for something difficult to detect may turn to insulin to improve their performance. Since 1999 the World Anti-Doping Agency has included insulin in the prohibited list, citing its anti-catabolic and chalonic properties.[102] Insulin doping is not limited to humans, as there have been reports of horses being doped to improve their performance.[103] A research group at the Centre for Preventive Doping Research and Institute of Biochemistry, Germany, investigates methods of insulin detection for drug testing of athletes. In a research article published in 2007 they used mass spectrometric detection to identify the degradation products of insulin, and insulin analogues in human urine samples. Drug doping faces different challenges compared to Point-

of-Care devices, such as: (1) urine samples are commonly taken from athletes instead of blood; and (2) insulin metabolism and systematic removal of insulin by the kidneys and liver needs to be detected (in conjunction with free insulin) to determine if insulin abuse has occurred.[40, 96, 104]

Analytical methods in particular are being used to detect insulin not only for therapeutic uses, but also for the detection of *biosimilars* and for designing innovative insulin formulations. Biosimilars are a biological medical product produced by a different manufacturer that closely resembles the original product. The biosimilar market is expected to grow and has the potential to affect the insulin analogue market with patents expiring in the near future.[95, 105-107] In the case of insulin formulations, Mercolini *et al.* utilised reverse phase HPLC with fluorescent detection to evaluate the best formulation of insulin for oral and nasal delivery.[108] Similarly, Sarmento *et al.* utilised HPLC with UV detection for the determination of insulin in nanoparticulate dosage forms.[55]

Table 2: Examples of Analytical Chemistry Methods for Insulin Detection. LC – Liquid Chromatography, HPLC – High Performance Liquid Chromatography, RP-HPLC Reverse-Phase HPLC, MISPE – Molecularly Imprinted Solid-Phase Extraction, PDA – Photodiode Array MS – Mass Spectrometry, MS/MS – Tandem Mass Spectrometry, SPE – Solid Phase Extraction, ESI – Electrospray Ionization, MALDI – Matrix-Assisted Laser Desorption/Ionization, TOF – Time of Flight, and SERS – surface-enhanced Raman spectroscopy.

Detection Method	Detection Approach	Linear Range	Ref.
Fluorescent Detection	**RP-HPLC.** Insulin was reacted with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole. Insulin separation was then carried out in a C18 column, with a mobile phase of acetonitrile in water containing 0.1% trifluoroacetic acid.	0.46 – 16.1 μM (serum)	109
Ultraviolet/Visible Light Detection	**Ion-pair RP-HPLC.** Insulin separation was carried out in a C18 column, where Octadecylsilica was used as the stationary phase. For the mobile phase a solution of 3.0% tetrametylammonium hydroxide in water and acetonitrile was used. Insulin was then detected with UV detection.	1.8 – 17 nM (0.1 M HCl)	57
	MISPE-HPLC-PDA. Water compatible molecularly imprinted polymer cartridges were prepared using methylacrylic - with insulin as the template. The cartridges were used to improve sample extraction prior to HPLC.	12 pM – 44 nM (plasma) 17 pM – 48 nM (urine)	110
	HPLC-PDA. Separation of insulin in human plasma was carried out in a C18 column. With a mobile phase consisting of acetonitrile and 0.2 M sodium sulfate.	22 – 26 nM (plasma)	111
Mass Spectrometric Detection	**LC-MS.** Immunoaffinity extraction was applied prior to LC-MS. Secondary antibody coated magnetic nanoparticles were utilised in the immunoaffinity extraction.	0 – 1.75 nM (blood)	37
	LC-MS/MS. Monoclonal antibodies immobilized on magnetic beads were used to enrich insulin, which was processed on a robotic liquid handler. The eluted peptides were then analysed by LC-MS/MS.	18 - 1920 pM (serum)	38
	SPE-LC-ESI MS. SPE was utilised to purify and concentrate insulin prior to LC and MS. For MS a trifluoroacetic acid mobile phase was utilised.	70 pM – 14 nM (plasma)	39
	MALDI-TOF-MS. In this approach the MALDI target plate was modified with coagulated gold nanoparticles and an insulin binding aptamer.	872 pM – 174 nM (serum)	41

	MALDI-MS. In this approach the target plate was modified with gold nanoparticles and functionalized with human insulin antibodies.	0.8 – 48 nM (serum)	86
	MALDI-TOF-MS. Insulin was captured by aptamer conjugated magnetic mesoporous silica – gold nanocomposites. The insulin was then released prior to mass spectrometry.	3.5 nM – 87 nM (serum)	87
Raman Spectroscopy	**SERS.** In this approach the SERS substrate was functionalised with dense clusters of pillars comprising of gold nanoparticles. The substrate was incubated for 12 h prior to SERS detection.	100 pM – 50 nM (buffer)	[97]

Biosensors

Since the conception of biosensors in 1956 by the "Father of Biosensors" Leland C. Clark Jr., who developed the first biosensor which rapidly measured glucose, the field of biosensors has seen extensive growth.[112-113] Biosensors typically provide a favourable alternative to conventional laboratory analytical techniques when the goal is to produce quick, stable, sensitive, cheap, and portable Point-of-Care devices. While glucose monitors and pregnancy tests are probably the most well-known commercial examples, biosensors are utilised in many other applications, with their use extending to environmental and food quality testing.[14, 114]

In essence, a biosensor **(Figure 2)** detects the presence or concentration of a biological analyte, such as insulin, through a bioreceptor connected to or integrated with a transducer.[115-117] Thus, producing an *active surface* to enable the detection of an analyte being bound to the bioreceptor. Once an analyte is bound, a biological response will occur. For instance, this response can take the form of a change in mass, an emittance of light, or a change in electroactive behaviour, which is subsequently detected by an appropriate transducer. Consequently, an electronic response is produced which undergoes signal processing to produce a result that is proportional to the concentration of analyte. Alternatively, a result can indicate the presence or absence of the analyte. The measurement is then displayed to the user in some manner, for example, a number is displayed when using a glucose meter or a series of coloured strips as in the case of pregnancy tests.[117-122]

Modern day transducers have increased the diversity and development of biosensors, a development largely facilitated by nano- and micro- technological advancements and novel bioreceptors.[14, 114, 116, 123-127] Similarly, advancements in Lab-on-a-Chip and microelectromechanical systems (MEMS) fabrication have enabled complex processes requiring large pieces of equipment, to be translated into portable devices.[49-50, 125, 128-131] These technologies have also been used to investigate the physiology of cells, diagnose diseases, and to create organs-on-a-chip, for instance.[132-137] As with any technology, many hurdles must be overcome to translate a biosensor from a laboratory environment to a commercial product. For instance: (1) the lifetime performance after extended storage (several months); (2) ethics surrounding the use of the biosensor; and (3) the cost and ease of fabrication of the biosensor. As time progresses, these hurdles are becoming smaller and insulin detection should be able to capitalise on these improvements, to expedite the realisation of a Point-of-Care device.[14, 114] In the following sections, we discuss biosensor detection methods being investigated for insulin detection. To date optical, micromechanical, and electrochemical methods have been reported for insulin detection **(Table 3)**.

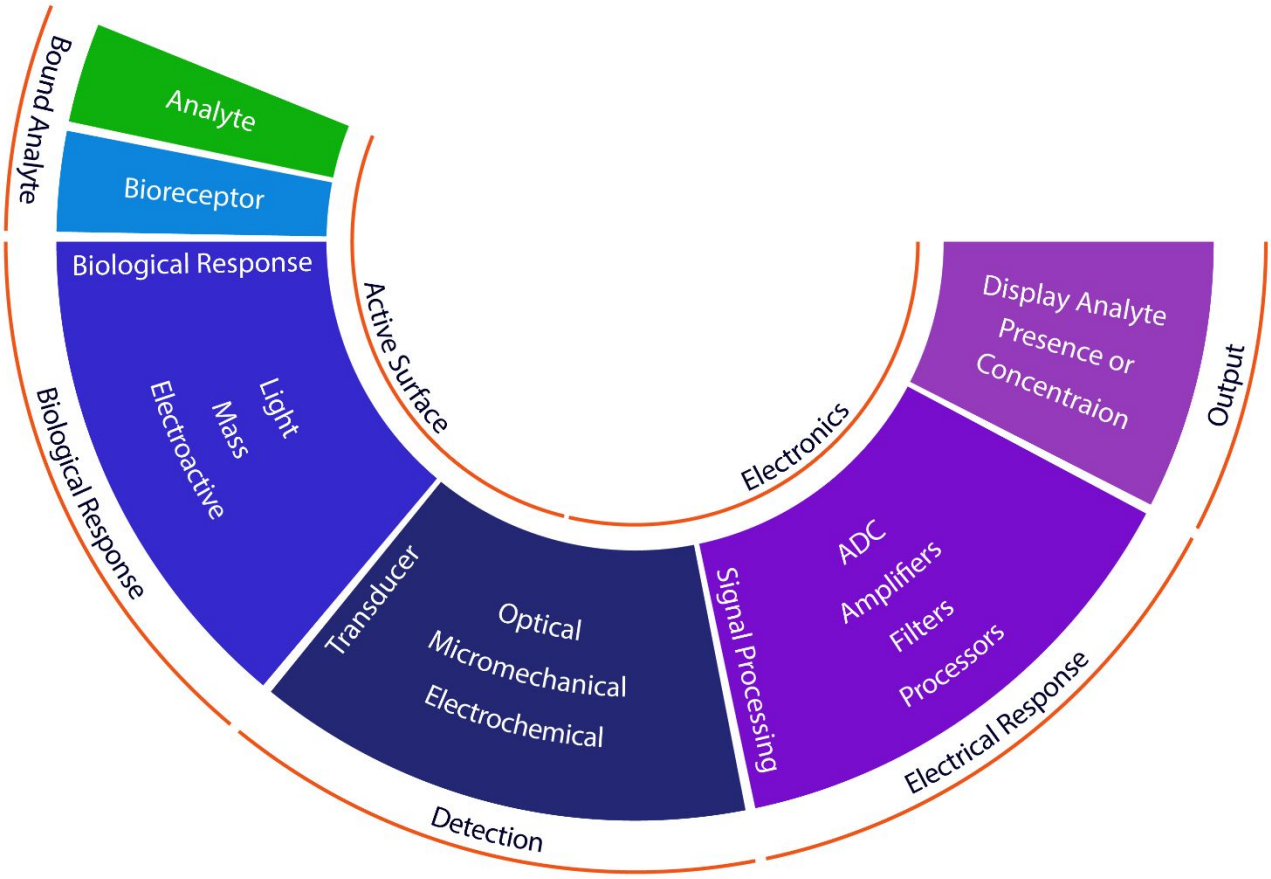

Figure 2: Principle of a Biosensor. Three biosensor technologies have been utilised so far for insulin detection – optical, micromechanical, and electrochemical. These three technologies detect a change in light, mass, or an electroactive response, respectively. Once detection has occurred, signal processing is undertaken. Electronic components are utilised in signal processing, for instance: analog-to-digital converters (ADC), amplifiers, filters, and processors. Once the presence or concentration of the analyte is determined, the results are displayed accordingly.

Table 3: Examples of Biosensing Methods for Insulin Detection. SPR – Surface Plasmon Resonance, FRET – Förster/Fluorescence Energy Transfer, CLIA – Chemiluminescence Immunoassay, QCM – Quartz Crystal Microbalance, and eQCM – Electrochemical QCM.

Detection Method	Detection Approach	Linear Range	Ref.
Surface Plasmon Resonance	**Microfluidic SPR.** A microfluidic platform included a reservoir to allow for SPR detection. The SPR substrate was modified with silica nanoparticles modified with amino groups and then coated with a gold layer to cap the silica nanoparticles.	17.5 pM – 1.75 nM (phosphate buffer)	42
	Dendrimer-based SPR. Polyamidoamine dendrimer encapsulated gold nanoparticles were synthesized and immobilised onto the gold surface. Insulin was then bound to the surface. A competitive immunoassay approach was utilised with SPR detection.	0 – 86 pM (phosphate buffer)	138
	SPR Immunosensor. Insulin was bound to a polyethyleneglycol monolayer on gold sensor surface. Antibody binding to insulin was detected with SPR.	175 pM – 52 nM (phosphate buffer)	139
Förster/Fluorescence Energy Transfer	**Aptamer.** A triple-helix molecular switch comprising of a label-free insulin specific aptamer and a dual-labelled oligonucleotide (signal transduction probe) was utilised to detect insulin. Upon insulin binding to the aptamer the molecular switch dissembled and the signal probe was released.	0 – 100 nM (phosphate buffer)	140
	Molecular Pincer. Two antibodies were modified with fluorochrome-labelled oligonucleotides. In the presence of insulin, the antibodies bound to two different epitopes, which resulted in an increase in the concentration of signalling oligonucleotides.	0 – 50 nM (tris assay buffer)	141
Fluorescence	**Microfluidic CLIA.** The continuous flow microfluidic platform enabled analyte capture through antibody conjugated microspheres. Detection was achieved through fluorescent conjugated secondary antibodies, in a sandwich immunoassay approach.	50 – 180 pM (serum)	142
	Liposomal Immunosensor. This approach was based on sandwich immunoassay, with liposomal sulforhodamine B utilised as a fluorescent label. In addition, patterning of anti-insulin antibodies in a microcapillary via protein A was employed for improved separation.	10 pM – 10 nM (tris-saline buffer)	143

QCM	**eQCM Immunosensor.** Insulin antibodies were covalently attached to 3-mercaptopropionic acid, which formed a self-assembled monolayer on the gold quartz crystal resonator. Insulin was conjugated to magnetic nanoparticles, which would in turn bind the monoclonal insulin antibodies – resulting in a change in mass and in turn frequency.	1 – 100 pM (phosphate buffered saline)	43
	Sandwich Microgravimetric Immunoassay. Six interdigitated piezoelectric quartz crystals were modified with insulin antibodies. Insulin would bind to the antibodies and a second antibody was introduced to form a sandwich immunoassay. The shift of the resonant frequency of QCM was measured before and after the second antibody bound to insulin.	1.75 nM – 1.75 μM (phosphate buffer)	144
	Molecularly Imprinted Polymer. An imprint of insulin crystals in polyurethane acted as a receptor for insulin on the surface of the QCM gold crystal surface.	176 nM – 17.6 μM (phosphate buffer)	145
Microcantilever	**Microcantilever with Antibodies.** A microcantilever was cut out of the bottom of the microchannel with a meniscus forming around the microcantilever due to surface tension. One side of the cantilever was functionalised with insulin antibodies. When insulin bound to the antibodies a mass change occurred on the cantilever, which resulted in a detectable shift in the resonance frequency.	70, 350, 1100 pM (phosphate buffered saline)	146
Cyclic Voltammetry	**Interface Between Two Immiscible Electrolyte Solutions.** Lithium chloride was utilised as the aqueous phase and bis(triphenylphosphoranylidene) ammonium tetrakis(4-chlorophenyl)borate as the organic phase.	10 – 50 μM (lithium chloride)	44
	Glassy Carbon Electrode surface modified with an insulin aptamer (insulin-linked polymorphic region).	10 – 200 nM	93
Amperometry	**Carbon Electrode** surface modified with carbon nanotubes, nickel-cobalt oxide and a nafion film.	0.1 – 31.5 nM (phosphate buffer)	30
	Glassy Carbon Electrode surface modified with nickel-oxide nanoparticles and guanine.	0.1 – 31.5 nM (phosphate buffer)	147
	Screen Printed Electrode surface modified with multi-walled carbon nanotubes and nickel oxide nanoparticles.	20 nM – 0.26 μM (sodium hydroxide)	148

Differential Pulsed Voltammetry	**Magnetic Glassy Carbon Electrode** surface modified with self-assembled electromagnetic imprinted polymers. Insulin was utilised as the template to produce the imprinted polymers.	0.1 – 10 nM (phosphate buffer)	149
	Carbon Paste Electrode surface modified with acetylene black nanocarbon particles.	20 – 1000 nM (sodium carbonate)	150
Electrochemical Impedance Spectrometry	**Interdigital Indium Tin Oxide Film Electrode** surface modified with reduced graphene oxide, bovine serum albumin, and insulin antibodies.	0.17 – 172.1 nM (phosphate buffer)	151
	Gold Electrode surface modified with polyethylene glycol and anti-insulin monoclonal antibodies.	5 – 10 pM (phosphate buffer)	152
	Gold Electrode surface modified with zwitterionic polymer, carboxybetaine methacrylate, and antibodies.	0.1 – 200 pM (phosphate buffer)	153

Optical Methods

In the case of optical biosensing, three methods have been used for insulin detection – surface plasmon resonance (SPR), Förster/fluorescence energy transfer (FRET), and fluorescence **(Table 3)**. Optical methods commonly utilise an immunoassay approach.[42, 139, 142-143, 154] In an optical biosensor, a change in the behaviour of a light wave or a chemiluminescent reaction occurs, which can then be detected by an appropriate transducer **(Figure 2)**. Details of each of the three methods are given below, in conjunction with an example of the method being utilised for insulin detection.

SPR is a quantum optical phenomenon which can occur at the interface between two dielectrically opposing materials. Generally, this will occur at the interface of a thin film (~ 50 nm) of a noble metal (typically gold or silver) with a dielectric material (typically quartz or glass). A surface plasmon is excited when the oscillations of the electrical component of the optical wave, and the resonant oscillations of the free electrons at the metal interface match. A common method of excitation utilised in biosensors is the method of attenuated total reflection, which was initially demonstrated by both Kretschmann and Otto.[155-156] This method of excitation is undertaken using a prism-coupler to enhance the momentum of the incident optical wave, to ensure that the two resonant oscillation frequencies match. The excitation of the plasmon results in a reduction of optical power of the optical wave, which can then be measured by an appropriate detector. The resonant oscillations of the free electrons at the metal interface depends strongly on the refractive index of the surrounding medium. In SPR biosensors the change in the refractive index of the surrounding medium is achieved, when an analyte binds to a bioreceptor immobilised on the metal surface. Consequently, this makes SPR biosensors extremely sensitive and offers label-free detection in real time.[157]

For example, Gobi *et al.* used SPR with an immunoassay approach to detect insulin. They functionalised the gold thin films with heterobifunctional oligo(ethyleneglycol)-dithiocarboxylic acid derivative - containing dithiol and carboxyl end groups. Insulin was then bound to the surface. To form an immunoassay, an insulin antibody was introduced into the system. A measurable change in the surface plasmon wave occurred when the antibodies bound to the insulin. Detection took five minutes and was carried out in a phosphate buffer **(Table 3)**.[139] In addition, SPR has also been utilised for epitope mapping and binding investigations of insulin specific monoclonal antibodies.[158]

FRET is a physical phenomenon first reported by Theodor Förster in 1948 in a paper appropriately entitled *Zwischenmolekulare Energiewanderung und Fluoreszenz* – intermolecular energy transfer and fluorescence.[159] In essence, the energy transfers from a donor molecule (a fluorophore) in an excited state to the acceptor fluorophore - through dipole-dipole coupling. For FRET sensors, analyte binding to a bioreceptor results in a change in the acceptor emission, which in turn is quantified and

related to the concentration of the analyte.[160] For example, Heyduk *et al.* designed a FRET sensor to detect either free insulin or C-peptide **(Figure 1)** in around 20 minutes. They designed the sensor with the intention to instantaneously measure the secretion of either insulin or C-peptide from beta cells. For this, a molecular pincer design approach was utilised, in which a pair of antibodies are modified with short fluorochrome-labelled oligonucleotides. In the presence of insulin, the antibodies bound to two different epitopes. In consequence, an increase in the concentration of signalling oligonucleotides occurred, which was measured with fluorescent spectroscopy **(Table 3)**.[141]

Fluorescent sensors used in insulin detection often employ an immunoassay approach with a chemiluminescent label. The principals discussed in the immunoassay section are essentially downscaled to portable solutions, such as microfluidic platforms.[161] For instance, Cohen *et al.* use a continuous-flow microfluidic platform, where insulin is captured with antibody conjugated microspheres. Cohen *et al.* achieved a linear range of 50 pM to 180 pM, which includes the upper range of insulin found in blood **(Table 3)**.[142] In another example, Ho *et al.* based their approach on a sandwich immunoassay, which used a microcapillary system for improved separation. Their approach took around 30 minutes and they achieved a linear range of 10 pM to 10 nM - which includes the levels of insulin found in blood. However, their experiments were conducted in a tris-saline buffer. Ho *et al.* identified that translating their immunoassay approach into a microfluidic platform, would be the next step in developing their sensor **(Table 3)**.[143]

Recently, alternative approaches have been reported using optical methods for the detection of insulin.[162-163] Wang *et al.* report a ratiometric fluorescent sensor using metal-organic frameworks for detecting insulin between 0 and 1.8 μM.[162] Whereas, Strano and co-workers use corona-phase molecular recognition to detect insulin in phosphate buffer saline or serum. In which the corona phase of a fluorescent nanoparticle is used for the detection and quantification of an analyte. Strano *et al.* have previously displayed the capacity of corona-phase molecular recognition for detecting fibrinogen concentrations in human blood. In the case of insulin, they developed a complex of fluorescent single-walled carbon nanotubes suspended in a PEGylated lipid amphiphilic heteropolymer, which specifically binds to insulin and causes a decrease in fluorescent intensity with increasing insulin concentration. Strano and co-workers identified that their sensor is limited by the detection range being higher than insulin levels present in blood. Furthermore, they highlighted the importance of considering the three-dimensional conformation of insulin.[163-164]

Currently, the main limiting factor of optical methods towards Point-of-Care is the expense of the optical components. In the case of SPR, the bench-top equipment is considerably more effective than the portable counterparts. In addition, the functionalisation of the surfaces used in SPR for insulin

detection still has room for improvement. On the other hand, FRET sensors remain limited in fluorescence resolution and selectivity towards the analyte.[160] Furthermore, the linear detection range achieved with optical methods **(Table 3)** is comparable to both immunoassays **(Table 1)** and analytical methods **(Table 2)**. In the case of insulin detection, most optical biosensors are based on immunoassays. Hence, presenting similar limitations regarding cross-reactivity with insulin precursors. However, immunoassays are simplified when using an optical sensor approach. This means the detection time can be reduced, with detection durations of less than an hour being reported, in comparison to several hours required for conventional immunoassay methods.[33, 67, 72, 76, 81, 139, 141-143] Moreover, in the reported optical sensor approaches the sensors are commonly tested using insulin spiked phosphate buffers, rather than serum or patient samples.

Micromechanical Methods

Although not employed to the same extent as optical methods **(Table 3)**, micromechanical approaches utilising quartz crystal microbalances (QCM) and microcantilevers have also been used to detect insulin **(Figure 2)**.

The piezoelectric effect in crystals (predominantly quartz) is the foundation of the transducer in a QCM. In 1880 the piezoelectric affect was discovered by brothers Jacques and Pierre Curie, when they realised that putting pressure on crystals produced electricity.[165] QCM sensors utilise the effect Lord Rayleigh reported in 1888, that a change in inertia of a vibrating crystal would modify the resonant frequency of the crystal.[166] Exploiting this phenomenon for insulin sensing, Saha *et al.* modified six interdigital piezoelectric quartz crystals with insulin antibodies. Insulin would bind to an antibody and a second antibody was introduced to form a sandwich immunoassay. The shift of the resonant frequency of QCM was measured before and after the second antibody bound to insulin. The concentration of the insulin was subsequently determined from the frequency shift **(Table 3)**.[144] Although QCM biosensors in general are limited to low throughput analysis, the main disadvantages for Point-of-Care insulin detection are due to the crystal being sensitive to heat, and the presence of liquid samples, such as blood, which have a dampening effect on the crystal.[167-168]

Another micromechanical method employed for insulin detection uses microcantilevers, which are sensitive to molecular adsorption.[169] Upon adsorption, the resonant frequency of the microcantilever shifts and the cantilever deflects. In addition, when the two surfaces of a cantilever are chemically different, shear stress will be produced when molecules are adsorbed, which again can be observed via the deflection of the cantilever. This deflection itself is typically measured using optical deflection, in which an optical beam is focused at the tip of the cantilever. The reflected light from the tip will be detected by a position sensitive detector, and the resulting cantilever movement can be related back to the molecular absorption. In essence, for a biosensor the analyte will bind to a bioreceptor attached to the surface of the cantilever, which will result in a resonant frequency shift and cantilever deflection. The amount of this shift or deflection will enable the analyte concentration to be determined.[170-171]

Using this principle, Park *et al.* measured insulin concentration by measuring a shift in the resonant frequency. They designed a microcantilever that was cut out of the bottom of a microchannel, with a meniscus forming around the microcantilever due to surface tension. One side of the cantilever was functionalised with insulin antibodies. Upon insulin binding, a mass change occurred on the cantilever, which resulted in a shift in the resonant frequency **(Table 3)**.[146] As with micromechanical technologies, microcantilevers can also be hindered by the chemical and physical properties of the

cantilever, such as thermal stress.[172] Currently, microcantilevers are mainly hindered by their lack of selectivity towards an analyte. Even when the surface of the cantilever is modified. This issue is not limited to insulin, but to many other analytes.[170-171] Furthermore, expensive laboratory equipment, such as atomic force microscopy (AFM) or optical equipment are required to assist in device calibration and/or analyte detection.

As with optical methods, micromechanical methods are being tested with spiked phosphate buffer solutions and utilise immunoassay approaches. Micromechanical methods in general have been unable to achieve a linear range comparable to insulin levels found in blood – with ranges reported in the nanomolar to micromolar range **(Table 3)**.[144-145] Which is noticeably higher than what has been achieved with optical methods also using an immunoassay approach **(Table 3)**.

Electrochemical Methods

Electrochemical biosensors have been used more extensively than either optical or micromechanical biosensors for the insulin detection. However, most electrochemical detection approaches for insulin are currently still on a larger scale, with benchtop equipment such as a potentiostat being utilised. Aside from the current status of insulin detection using electrochemical methods, a considerable amount of knowledge in regard to transposing electrochemical setups towards portable devices exist in the commercial sphere, in part due to the development of glucose meters.[14, 122] In general, Point-of-Care electrochemical biosensors can be manufactured at a significantly less expense, compared to optical and micromechanical sensors.

Conversely, electrochemical sensors tend to be more prone to fouling due to the adsorption of oxidation products. As a consequence the stability and sensitivity of the sensor is affected.[173-174] However, this is more of a problem for continuous measurement devices, rather than single use devices. For Point-of-Care applications, the use of single use electrodes, which can be readily disposed after use is preferred - as this minimises concerns relating to blood contamination. Through modifying the working electrode surface the effects of fouling can be minimised. [173, 175] In addition to fouling, electrochemical sensors for insulin detection are also hindered by the slow oxidation kinetics of insulin.[176] Various bioreceptors have been utilised for the insulin detection, such as nanoparticles (for example silica, nickel, and cobalt hydroxide), nanosheets (reduced graphene oxide), carbon nanotubes, aptamers, and molecularly imprinted polymers. These bioreceptors are typically applied in conjunction with different electrodes, such as gold, indium tin oxide (ITO), and carbon based electrodes.[30-31, 44, 93, 147-153, 177-179] More importantly, bioreceptors need to be able to discriminate insulin from other molecules that appear in body fluid, such as glucose, uric acid, and ascorbic acid, all of which oxidise more readily than insulin. In conjunction with poor electrode stability, these limitations are a major hindrance towards the development of electrochemical sensors towards Point-of-Care insulin detection.[30, 180]

In an electrochemical setup, three electrodes are generally utilised: (1) a working electrode, which is modified with bioreceptors that interact with the analyte; (2) a reference electrode, commonly either silver/silver chloride electrode or saturated calomel electrode; and (3) a counter electrode, made from an inert material such as gold, silver, or platinum. Furthermore, one must take care to ensure no current flows through the reference electrode, this is done to ensure that the: (1) reference electrode does not hinder current flow between the working and counter electrode; and (2) the reference electrode remains stable. These can be done by ensuring that the surface area of the counter electrode is larger than the working and reference electrodes.

Out of all the electrochemical methods cyclic voltammetry is the most well-known method. In cyclic voltammetry, a changing potential is applied to the working electrode and a subsequent measurement of current is made. The current is a result of the reaction of the analysed species at the working electrode.[181-182] For example, Gerasimov *et al.* modified a glassy carbon electrode with an insulin binding aptamer: insulin-linked polymorphic region sequence. In the presence of insulin, the G-rich sequence underwent ligand-induced folding to form a G-quadruplex. Alternating current (AC) cyclic voltammetry was used to quantify insulin concentrations in the range of 10 to 200 nM **(Table 3)**.[93]

In the case of sensors, amperometric detection is the preferred electrochemical technique. In fact amperometric detection is commonly used in glucose meters, where glucose oxidase, hexokinase or glucose-1-dehydrogenase are utilized as the oxidative species.[14] In amperometric detection, the applied potential is kept constant between the working and reference electrode, to enable the cell current to be measured. The current arises due to the oxidation or reduction of an electroactive species. The measured current is representative of the bulk concentration of the electroactive species, the analyte.[183] Amperometry was used by Salimi *et al.* for insulin detection at an applied potential of 0.8 V. Using a rotating electrode configuration, with the working electrode spinning at 1000 rpm. The glassy carbon electrode surface was modified with nickel-oxide nanoparticles and guanine. A linear range of 0.1 to 31.5 nM in phosphate buffer was achieved **(Table 3)**.[147]

Another electrochemistry technique used for biosensors is electrochemical impedance spectrometry (EIS), which forms the foundation of impedimetric sensors. In EIS, both the resistive and capacitive properties of the materials are analysed over a wide frequency range. This analysis is undertaken while the system is in equilibrium, by applying a perturbation in the form of a small amplitude sinusoidal signal.[184] Xu *et al.* used polyethylene glycol and monoclonal anti-insulin antibodies (mouse IgG1 isotope) to modify the surface of a gold electrode. A frequency range of 0.01 to 10 kHz was used to determine insulin concentrations in the range of 5 to 10 pM – well below the level of insulin present in blood **(Table 3)**.[152] Advancements in nanofabrication and microfabrication have attributed to improvements in impedimetric sensor performance. Consequently, the use of microelectrodes in impedimetric sensors has enabled improved sensitivity in comparison to conventional electrochemical electrodes.[184] In particular, the use of interdigital electrodes for an impedimetric sensor was highlighted by Yagati *et al.* for insulin detection. Thin film ITO microelectrodes were fabricated onto a glass microscope slide, and the surface was modified with reduced graphene oxide and monoclonal anti-insulin antibodies (goat anti-rabbit IgG-TR). Insulin in a phosphate buffer was able to be detected between 0.17 and 172.1 nM. However, insulin was introduced to the sensor 60 minutes prior to detection **(Table 3)**.[151]

In summary, the electrochemical methods discussed here utilise different surface modification approaches, using various chemicals and/or biologicals. Electrochemical approaches have been reported to achieve detection consistently in the picomolar and nanomolar ranges **(Table 3)**. Which is comparable to the linear ranges achieved with immunoassays **(Table 1)**, analytical chemistry methods **(Table 2)**, and optical biosensors **(Table 3)**. However, experiments conducted to characterise the electrodes were generally preformed using an insulin spiked phosphate buffer. In addition, not all papers investigated the influence of the presence of interferants, such as glucose and uric acid,and the life-time stability of the modified electrode. Unlike other sensors, considerable commercial development knowledge exists regarding the production of electrochemical biosensors, due to the extensive development of glucose meters.

Outlook and Perspective

Insulin detection using a Point-of-Care device offers significant potential to minimise the impact of the impending diabetes epidemic. On a personal level this will improve the quality of life for individuals with diabetes, in combination with emerging personalised glucose metabolism models for improved glucose management. In this review, we have summarised the current status of insulin detection using immunoassays **(Table 1)**, conventional analytical chemistry methods **(Table 2),** and biosensors **(Table 3)**. In addition, we discuss the challenges and limitations towards Point-of-Care insulin detection. Furthermore, we summarise the potential of the discussed methods towards Point-of-Care insulin detection in **Table 4**. In summary, immunoassays and conventional analytical chemistry methods offer suitable linear ranges; although, they are hindered by cross-reactivity and expensive large specialist equipment, respectively. Biosensors offer a reduced detection duration, generally less than an hour. Whilst, immunoassays and analytical methods can take several hours. Furthermore, electrochemical biosensors in particular offer inexpensive portable solutions for insulin detection **(Table 4)**.

Going forward, the recommendations made by the American Diabetes Association task force need to be translated to other detection methods. In particular, this applies to how results are presented and validated, and how performance requirements are assessed. Based on our review we recommend that, at a minimum, the linear range, sensitivity, and limit of quantification in molar concentration should be reported. We suggest molar concentration, as this measure can be universally reported for the different detection methods discussed in this review; thus, enabling for more informed comparing and contrasting of approaches used for insulin detection. Furthermore, while assembling this review we noticed key performance terms were used interchangeably, although they have statistically different definitions. For examples: (1) *limit of detection* and *limit of quantification*, and (2) *specificity* and *selectivity*. To that extent we have taken the time to assemble a list of relevant definitions for measuring analytes - at the end of this review. These definitions are accompanied with relevant references and are taken from definitions given by the International Union of Pure and Applied Chemistry (IUPAC), and the American Chemical Society (ACS). In addition, we recommend that the type of insulin (for example human, bovine, or porcine) used in experiments is indicated, along with the specific supplier information. This is due to the properties of insulin not being specified in some research articles.

Based on existing literature we conjecture that electrochemical detection holds the most promise towards a Point-of-Care device. This is due to the amount of existing commercial knowledge in electrochemical sensors and continuing improvements made to selectivity and sensitivity for insulin

detection. However, we suggest going forward that the performance of an electrochemical approach needs to include characterisation pertaining to: the lifetime stability of the modified electrode, including extended periods of time, such as a few months; selectivity against interferants, such as C-peptide, glucose, and uric acid; and the examination of the electrode performance in solutions other than phosphate buffer.

Furthermore, we recommend some potential improvements for these aforementioned performance parameters, not only for electrochemical-based sensors, but for any sensor designed to detect insulin. For instance, incorporating a sensor into a microfluidic platform with hydrodynamic separation could facilitate the removal of larger interferants in a sample. In addition, aptamers could be used instead of antibodies or other materials as a bioreceptor, as aptamers offer high affinity towards a specific analyte. Aptamers also offer good stability and can be readily chemically modified, which has the potential to extend lifetime stability of the active surface of a biosensor. However, the use of aptamers for insulin detection remains relatively unexplored. For example, the selectivity of insulin aptamers against common interferents in blood requires further studies.

Only by engaging in constructive interdisciplinary dialogue between scientists, engineers, and clinicians the current limitations towards Point-of-Care insulin detection will be overcome.

Table 4: Summary of the advantages and limitations of the detection methods presented in the review towards Point-of-Care insulin detection.

Detection Method	Advantages	Limitations
Immunoassays	▪ Existing commercial laboratory kits ▪ Assays exist for insulin and C-peptide	▪ Cross reactivity with insulin precursors ▪ Considerable sample preparation steps ▪ Analysis can take several hours
Analytical Chemistry	▪ Excellent sensitivity ▪ Ability to distinguish between insulin analogues ▪ Potential for devices to be portable in a clinical setting	▪ Expensive specialist equipment ▪ Large equipment ▪ Considerable sample preparation steps ▪ Analysis can take several hours
Biosensor – Optical	▪ Quick detection ▪ Potential for devices to become portable ▪ Potential for good sensitivity	▪ Expensive peripheral optical equipment ▪ Generally based on immunoassays ▪ Selectivity
Biosensor – Micromechanical	▪ Quick detection ▪ Potential for devices to become portable ▪ Potential for good sensitivity	▪ Selectivity ▪ Thermal sensitivity ▪ Liquid samples causing dampening affects
Biosensor – Electrochemical	▪ Oxidation can be overcome with surface modification ▪ Commercial microelectrode fabrication exists ▪ Quick detection and inexpensive ▪ Portability for Point-of-Care possible	▪ Slow oxidation of insulin ▪ Selectivity ▪ Surface fouling of electrodes ▪ Interference species (i.e. glucose, uric acid)

Definitions of Performance Parameters

Selectivity. The ability to detect an analyte (or a group of analytes) without interference/cross-reactivity.[185-186]

Specificity. The ability to detect an analyte (or a group of analytes) without interference/cross reactivity with 100% certainty. On some occasions the specificity is defined to lesser certainty (for example 80%), with the specific interferants indicated. For example, with an immunoassay approach, one may state that the assay has a specificity of 85% due to a cross reactivity with proinsulin(s).[185]

Calibration Curve. Where the measured signal (y-axis) is related to the concentration of the analyte (x-axis), and can be described by a linear relationship.[187]

Sensitivity. The gradient of the calibration curve gives the sensitivity of the approach towards the desired analyte.[186-187] Note that sensitivity can also be specified to a particular part of a piece of equipment, and this should be made explicit.

Dynamic/Linear Range. Depending on the detection method and author preference, this range can be referred to either the dynamic or linear range, and to a lesser extent the detection range. The linear range is defined as the range in which the measured signal intensity (applicable to the detection method) is proportional to the concentration of the analyte.[187-188]

Limit of Detection (LOD). The limit of detection is the lowest concentration of an analyte that can be significantly different from an averaged analytical blank. Generally, a signal-to-noise ratio or standard deviation of three is used.[186-189] In essence, this is the lowest concentration that can be determined with some reliability from background noise.

Limit of Quantification (LOQ). IUPAC provides no definition for LOQ. However the ACS defines LOQ as the smallest concentration of an analyte, which can be quantitatively analysed with reasonable reliability.[188-189] Note that the LOQ should be higher than the LOD for a given experiment. For statistical definitions, consult the following publications:

(1) *Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry*.[189]

(2) *A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: application to voltammetric and stripping techniques (technical report)*.[188]

Accuracy. Accuracy indicates the agreement of the measured concentration to the true concentration of the analyte.[186]

Precision. Precision indicates the agreement of independent experiments or measurements undertaken under the same conditions. Essentially this describes how many sensors would adequately detect the desired analyte to a desired level.[186]

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Vocabulary

Analyte, a desirable biological or chemical species that is being detected or analysed, Antibody, also known as an immunoglobulin, is a protein produced by the immune system due to the presence of an antigen; Antigen, a substance such as bacteria, which induces the production of antibodies; Aptamer, a single stranded oligonucleotide from either RNA or DNA; bioreceptor, a compound, such as an antibody or aptamer, that is part of the active surface of a biosensor, and recognises the desired analyte.

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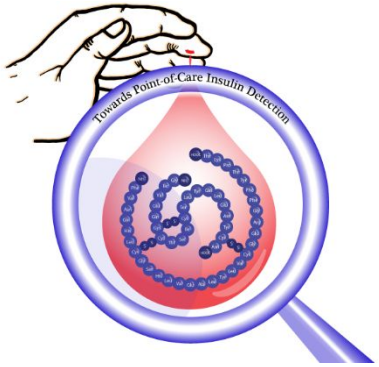

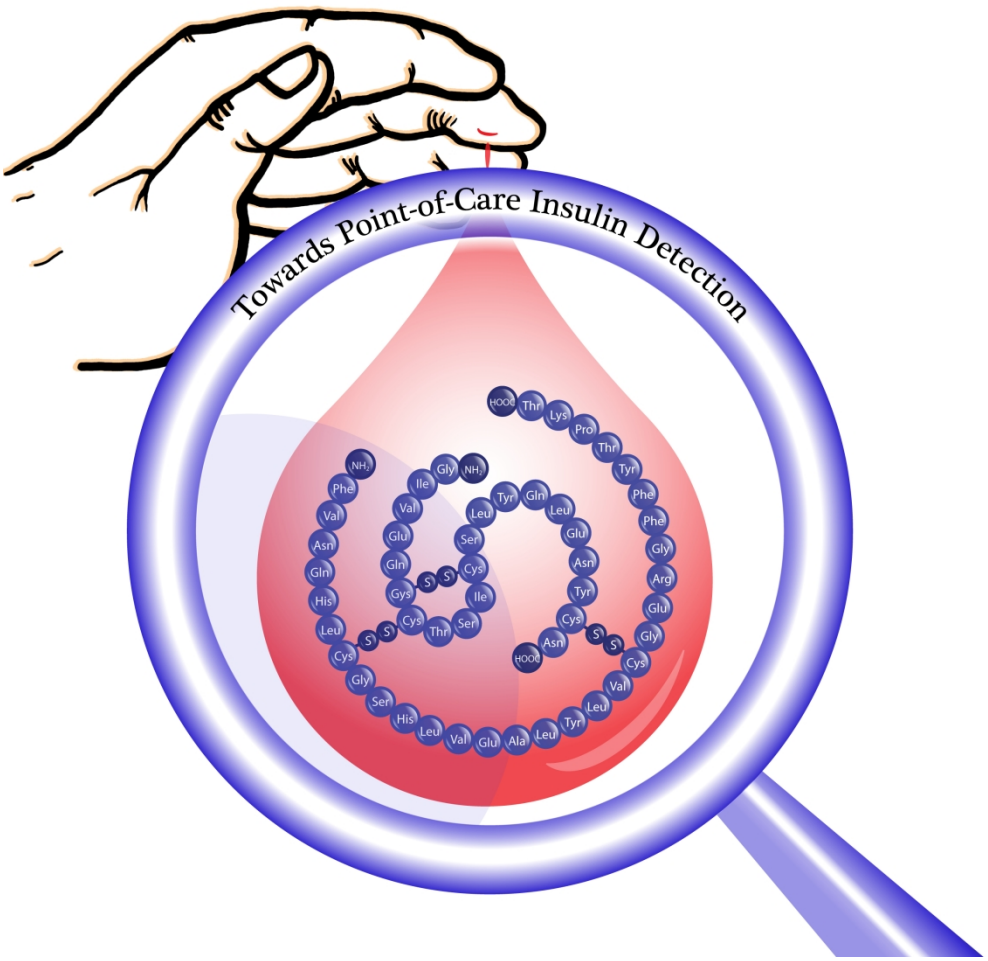

Towards Point-of-Care Insulin Detection

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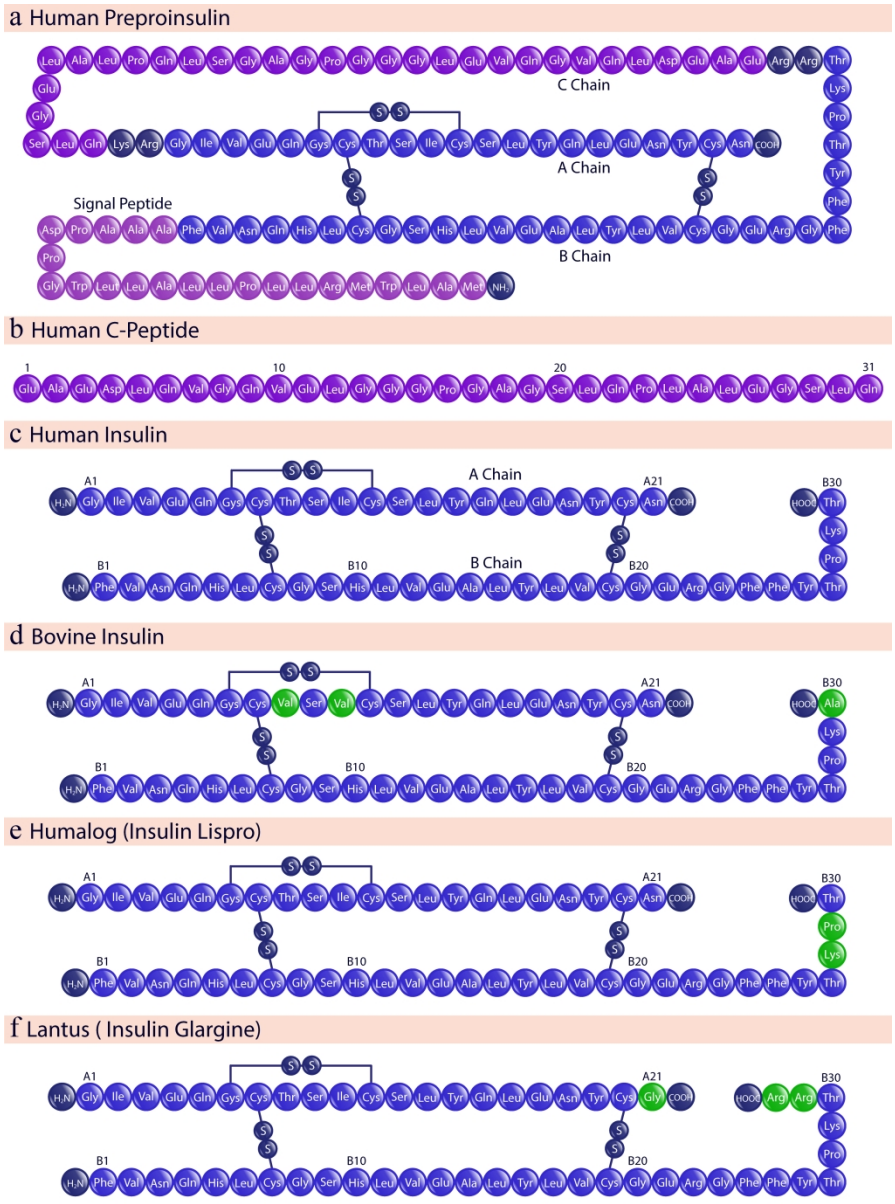

Figure 1

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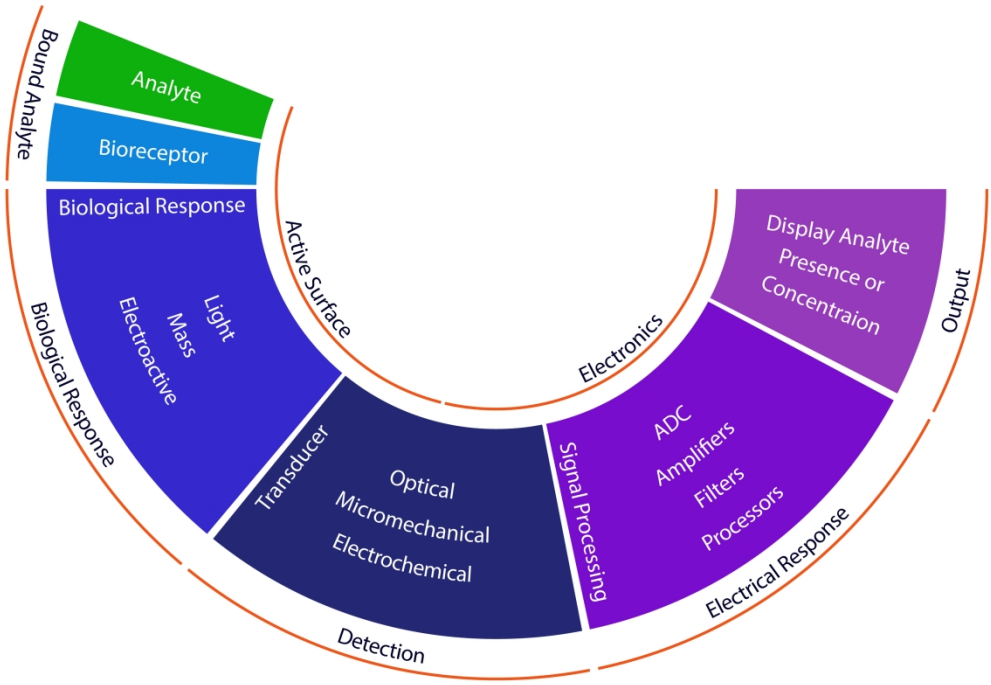

Figure 2

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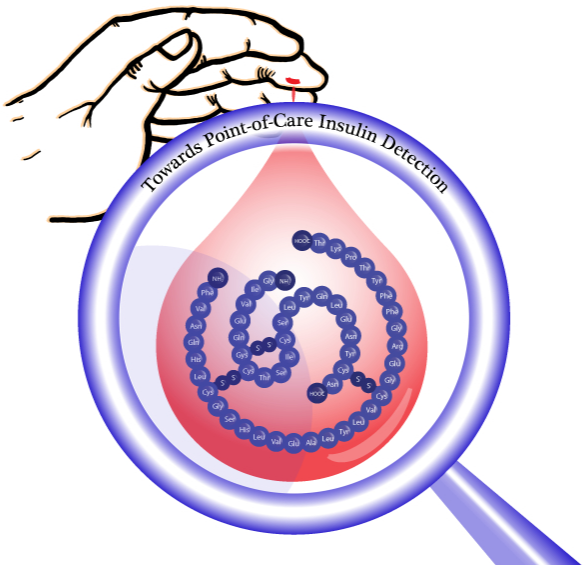

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84x47mm (300 x 300 DPI)