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Non-Invasive Glucose Detection in Exhaled Breath Condensate

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Abstract

Two thirds of patients with diabetes avoid regularly monitoring their blood glucose levels because of the painful and invasive nature of current blood glucose detection. As an alternative to blood sample collection, exhaled breath condensate (EBC) has emerged as a promising non-invasive sample from which to monitor glucose levels. However, this dilute sample matrix requires sensors capable of detecting glucose with high resolution at nanomolar and micromolar concentrations. Recent developments in EBC collection methods and highly sensitive glucose biosensors provide a path towards enabling robust and sensitive glucose detection in EBC. This review addresses current and emerging EBC collection and glucose sensing modalities capable of quantifying glucose in EBC samples. We highlight the opportunities and challenges for development and integration of EBC glucose detection systems that will enable clinically robust and accurate EBC glucose measurements for improved glycemic control.

Introduction

Diabetes mellitus is a serious metabolic condition caused by hyperglycemia, which results from either insufficient insulin secretion or defects in insulin action.[1] According to the 2017 National Diabetes Statistics Report from the CDC, approximately 30.3 million people (over 9.4% of the U.S. population) are afflicted with this disease. Diabetes is classified into two primary types: type 1 – insulin-dependent/juvenile-onset diabetes, and type 2 – non-insulin dependent/adult-onset diabetes. Type 1 diabetes affects 5–10% of the individuals diagnosed with diabetes and those individuals are usually under 20 years old. Type 2 diabetes, on the other hand, accounts for 90–95% of the diabetic population and is commonly diagnosed in adults over the age of 20. Furthermore, around 84.1 million individuals have pre-diabetes, which if untreated, can lead to type 2 diabetes within five years[2].

Methods exist to manage diabetes and lower blood glucose levels but there is currently no definitive cure for either type. Glycemic control options can reduce the risk of developing severe complications associated with hyperglycemia, such as retinopathy (loss of vision), peripheral neuropathy, and cardiovascular symptoms.[1] However, if medications or insulin injections are not administered properly, patients can develop hypoglycemia. Despite the

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recent advances in the self-monitoring of blood glucose (SMBG) at the point of care, glycemic control is unfortunately still a challenge for many diabetic patients[3,4].

As many as 67% of diabetic patients fail to monitor their blood glucose; citing finger soreness, pain, and inconvenience as the most common reasons for noncompliance[5]. To mitigate this issue, many researchers are working to develop non-invasive glucose sensors that possess the same level of accuracy as traditional blood-dependent measurement systems. The non-invasive measurement of glucose may enable patients to conveniently check their glucose levels without the painful side effects of finger prick measurements.

It is important to note that Type 1 and Type 2 diabetic patients have different needs when it comes to how they choose to maintain glycemic control. Type 1 diabetes is caused by an absolute deficiency of insulin secretion whereas type 2 diabetes, which is caused by a combination of insulin resistance and inadequate insulin secretory response[6]. Therefore, most patients with Type 1 diabetes rely on insulin injections in addition to monitoring their blood glucose levels. To ensure that glucose levels are within the normoglycemic range at all types, especially during insulin injections, continuous glucose monitoring systems (CGMs) are beneficial[7]. On the other hand, most Type 2 and insulin-free diabetic patients do not require continuous monitoring of glucose levels. After performing informal interviews with 100 stakeholders in the glucose biosensors market, including diabetic patients (Type 1 and 2), ICU/NICU nurses, nursing home caretakers, and medical device companies, we found that most Type 2 patients prefer to only check their blood glucose levels when they deviate from their diet or have not eaten regularly. According to the American Diabetes Association, at least three finger prick blood glucose measurement should be taken daily to maintain glycemic control[8]. From our findings, we noticed that this number varies significantly across Type 2 patients; from two times a day to once in two months, depending on how well-controlled they feel their blood glucose levels are. Many patients mentioned that the inconvenience of finger-prick whole-blood sample collection prevents them from wanting to check more frequently, which is consistent with literature findings.

Therefore, while CGM devices are extremely beneficial for the percentage of the diabetic patient population that requires readouts consistently to balance glucose and insulin concentrations, the larger percentage of the population (Type 2 and insulin-free patients, pre-diabetic patients) can benefit from a non-invasive, intermittent method of glucose detection. This will potentially encourage them to check glucose levels more regularly and improve glycemic control.

Glucose passively diffuses form the blood into other less-complex physiological fluids, including: interstitial fluid (ISF), sweat, tears, aqueous humor, saliva, and respiratory fluid[9–14]. Each of these fluids are associated with a specific glucose concentration range but not all are well correlated with blood glucose due to differences in both the ratio and time-lag between changes in blood glucose and these other fluids[15,16]. Respiratory fluid is believed to have a rapid and stable glucose exchange with plasma, making it a promising candidate for non-invasive glucose detection.

In the lungs, sodium-glucose transporter pumps actively remove glucose from the respiratory fluid lining of the airways in order to prevent pulmonary edema and infection[17–19]. The average glucose concentration in respiratory fluid for a healthy human subject is reported to be 0.4 mM, approximately 12-fold lower than that of blood glucose (~4.8 mM)[18,20]. Because of the high concentration gradient between capillaries and respiratory fluid, glucose transport and equilibrium between the blood plasma and lungs is hypothesized to occur more rapidly than in other compartments.

The rapid glucose exchange that occurs between the respiratory fluid and plasma makes it a promising indicator of blood glucose levels[21,22]. However, collection of respiratory fluid from the alveoli is an invasive process and instead, analytes are collected as either exhaled gas for volatile organic compounds (VOCs) or as exhaled breath condensate (EBC) [14]. EBC, in particular, has been known to possess various biomarkers indicative of a host of diseases, including: lung cancer, chronic obstructive pulmonary disorder (COPD), and cystic fibrosis[14,23]. In addition, it has sparked recent interest as a potential non-invasive sample for glucose detection and diabetes monitoring[17]. While results have shown elevated respiratory glucose concentrations in hyperglycemic subjects, very few studies have characterized glucose concentration ranges across normal and diabetic subjects – rendering reported values as highly variable. This is largely due to the inconsistency in the method of collection and the detection methods used to quantify glucose concentrations[17,19].

The purpose of this review is to examine EBC as a potential non-invasive sample for glucose detection. The scope will review current methods of collection and the related drawbacks as well as the various detection methods used to quantify glucose within the expected range of EBC glucose concentrations, including sensing modalities and corresponding recognition elements. In doing so, we present a holistic overview of current state-of-art technologies, the challenges, and future directions for developing robust and accurate non-invasive glucose detection systems for EBC.

Respiratory Fluid Dilution

The thin layer of fluid covering the mucosa of the alveoli as well as both the small and large airways is interchangeably referred to as the airway surface liquid (ASL), alveolar lining fluid (ALF), epithelial lining fluid (ELF), and respiratory fluid (RF) in the litterature[24–26]. For simplicity, we will refer to it as respiratory fluid. This fluid serves numerous purposes and is the first layer of defense between the lungs and the outside world.

Respiratory fluid in the conducting zone of the airway (from nose to proximal bronchioles) exists as a sol-gel compartment whereas fluid in the respiratory zone of the airway (from distal bronchioles to alveoli) exists as a soluble layer to prevent collapse of alveoli[27]. Nasal secretions of respiratory fluid do not normally contain glucose, but a study by Phillips *et al.* [28] found that glucose concentrations at 2–7 mM are present in nasal secretions during episodes of hyperglycemia and epithelial inflammation. However, nasal secretions are not necessarily representative of blood glucose concentrations due to the disturbance of normal glucose absorption across the epithelium during these episodes. As such, the composition of the respiratory fluid is highly varied across the respiratory tract. Respiratory fluid in alveoli

contains glucose concentrations that are ~12-fold lower than that of plasma glucose whereas concentrations in the conducting zone are negligible since the air in that region (referred to as anatomical dead space) does not participate in gas exchange of any sort[18,29]. Droplets of respiratory fluid are released from the epithelial cell surfaces throughout the airway; the rate of droplet formation is dependent on the epithelial cell permeability in the various regions of the tract and the glucose transport from the alveoli. During turbulence in the airways caused by the reopening of bronchioles and alveoli, non-volatile compounds in the respiratory fluid, such as glucose, undergo aerosolization [23,30] (Figure 1). Furthermore, fluid in the respiratory tract is diluted by water vapor as it travels from the respiratory zone to the conducting zone.

Thus, when respiratory fluid is ultimately collected as exhaled breath condensate (via cooling of the air exhaled by a subject), it is greatly diluted by water vapor. While EBC glucose is assumed to have a theoretical dilution factor from plasma glucose of 1:10000[14,22], the variable nature of water vapor dilution and droplet aerosolization has resulted in reported EBC glucose concentrations varying significantly from 0.24–5.5 μM[21,22,31–33]. There is a need for studies that combine a standardized collection method accounting for variable dilution factors with a robust detection system to measure glucose at low concentrations in order to demonstrate reliable and reproducible EBC glucose measurements.

Sample Collection Methods, Issues, and Controls

The appeal of using EBC as a sample for biomarker detection and biomolecule analysis is its non-invasive collection method. Compared to bronchoalveolar and nasal lavage, EBC collection samples a much larger area of the lung respiratory fluid and does not alter the physiology of the respiratory tract lining[34]. Despite this ease of accessibility, there are a number of physiologic and environmental factors affecting EBC glucose concentrations which must be considered and controlled for during collection.

Most commercially-available devices for EBC collection, such as RTube and EcoScreen, rapidly cool the exhalate so that aerosol particles can adhere to cooled surfaces such as silicone or Teflon[23,35]. In a typical procedure, a subject is asked to exhale into a collection tube that is maintained at a much lower temperature than the ambient air (between 5–10°C) (Figure 2). Cooling is normally achieved via an ice bath or dry ice immersion of the collection surface prior to collection. The surface contacting the droplets does not directly interface with the ice to avoid contamination and additional dilution. This allows the aerosolized droplets of exhaled air to condense into a liquid that can be collected and analyzed. A drawback to current collection methods is the time required for a significant sample volume to be collected. For many standard detection systems for small molecules (described below), at least 1 mL of sample is required for thorough and accurate analysis. To condense 1 mL of EBC, the subject needs to breathe for at least 10 minutes[14,21]. This is not only inconvenient for rapid glucose detection applications but would also average glucose concentrations that vary widely within this short window – for example, directly after a meal or during a diagnostic glucose tolerance test. Thus, studies correlating blood glucose to EBC glucose should first perform measurements at baseline sugar levels and then measure blood glucose and collect EBC simultaneously at short time increments (e.g. five minutes) to estimate any physiological time lag due to the glucose transport through respiratory

epithelia. Five minutes is recommended because the average time lag from glucose in blood to interstitial fluid is around 15 minutes[36]; therefore, shorter increments would enable estimates of whether the EBC time lag is greater or less than this amount.

A major issue affecting the reliability of EBC glucose measurements is the variable dilution factors due to water vapor when respiratory fluid is collected as EBC. While respiratory fluid is secreted throughout the epithelial cell lining of the respiratory tract, it is aerosolized and diluted by droplets of water vapor as it travels up from the lower airways to the mouth. Thus, all non-volatile solutes, such as glucose, are diluted to a certain degree by water vapor when they are collected as EBC. This makes it difficult to determine whether changes in reported EBC glucose concentrations reflect fluctuations in water vapor droplet formation or the concentration changes of glucose in the fluid lining[22]. However, estimating the dilution factor of several reference non-volatile solute concentrations from blood to EBC might provide a more accurate method of glucose quantification. According to a study done by Effros *et al.*[22], the dilution of respiratory droplets by water in EBC have equivalent dilutions of Na^+, K^+, and Cl^- ions and thus, the sum of Na^+ and K^+ concentrations can be used as dilution markers. This is based on the assumption that respiratory fluid has the same osmolality as plasma; meaning that the sum of the cation concentrations in respiratory fluid is the same as that in plasma. The sodium and potassium cations were chosen particularly because along with their respective anions, they are the principal solutes in plasma, respiratory fluid, and EBC. In their study, they concluded that the dilution of respiratory droplets by water vapor in condensate can be calculated by Equation 1:

$$D = \frac{[Na^+]_{plasma} + [K^+]_{plasma}}{[Na^+]_{EBC} + [K^+]_{EBC}} \quad (1)$$

In Effros's study, approximately 20 subjects were enrolled (male and female, age range: 22–53); none had histories of pulmonary disease. However, a larger sample size is needed to demonstrate the reproducibility and reliability of using these cations as dilution markers for EBC glucose quantification in both in normoglycemic populations and patients with diabetes, as using this approach requires sodium and potassium electrodes that need to measure blood and EBC cation concentrations. Other studies have also used conductivity to measure total nonvolatile cation concentrations but this requires the lyophilization of each sample to remove ammonia, the primary contributor to the overall conductivity of the sample – rendering this method impractical for rapid point-of-care detection[14]. Urea may also be useful as a dilution marker, however high concentrations of ammonia (the product of many enzymatic detection assays for urea) in EBC samples make it difficult to measure accurate concentrations[22]. If a larger set of studies can demonstrate the benefit of using cationic dilution markers for accurate glucose detection, one can establish the frequency at which cation measurements and subsequent dilution factor calibrations should be performed on an exhaled breath condensing device.

Another possible explanation for the large variability in dilution factors of EBC is the inclusion of anatomical dead space air in the condensed exhaled air sample. Anatomical

dead space is the portion of air from the upper respiratory tract (mouth, nose, trachea), that does not participate in gas exchange and thus does not contain solutes of clinical relevance from the alveolar epithelial lining[37] (Figure 3). Therefore, the inclusion of respiratory fluid from the dead space air in EBC can dilute solute concentrations to levels that are below the limit of detection for many assays. The EcoScreen 2 device has come close to addressing this issue by allowing fractionated collection of EBC from different areas of the bronchial tree so that dead space condensate can be collected separately and discarded. While of use for clinical research studies, this device weighs over 20 kg and is not portable[38].

To test the effects of dead space air on total EBC glucose concentrations, Tankasala *et al.* developed a temperature-based selective condenser that collects and condenses exhaled breath from only the deep lung circuit[39]. This was based on the theory that air exchanged with the lungs is characterized by a rise and plateau in CO_2 levels compared to dead space air which does not contain CO_2[40]. Figure 3 illustrates the portion of exhaled air that is relevant for biomarker and non-volatile solute analysis. Previously, Schubert *et al.*[41] had demonstrated that it was possible to sample alveolar gas in mechanically ventilated patients using a CO_2-controlled sampling method to separate dead space gas from alveolar gas. Instead of using a CO_2 sensor, a temperature sensor was used to distinguish between dead space and deep lung air after confirming that the temperature and CO_2 profiles of human exhaled breath are well correlated (Figure 4a).

By setting a threshold temperature (Figure 4b) at which exhaled breath would be collected and condensed, the study aimed to collect EBC that possesses higher concentrations of the solute of interest – which in this case, was glucose. The EBC collected using a selective threshold was analyzed for glucose content using a commercially available glucose oxidase-based assay and compared against EBC collected without a threshold; it was found that the selectively collected EBC contained significantly higher glucose concentrations (Figure 4c–d). It is important to note that the study's sample size was small and there were no additional controls such as cation dilution comparison to correct for variable dilution factors or residual glucose in the device.

To address the issue of variable dilution factors due to water vapor and dead space air inclusion, a combination of the approaches used by the previously described studies may be beneficial. By using reference non-volatile cations or any solute that has a known and stable plasma concentration and high diffusion rate through the epithelial membrane, the water vapor dilution factor can be correct for glucose concentrations in each collected sample[38]. Furthermore, by eliminating dead-space air from EBC collection, the samples will be less dilute. There is no diffusion occurring between the epithelial cells and plasma in the dead space region; which would mean that there is no dependence on an ionic gradient to propel the gas exchange between oxygen and carbon dioxide[29]. As a result, the calculated dilution ratio (D) of non-volatile cations would decrease upon selective collection – which would support the claim that the elimination of dead space air is necessary in increasing EBC solute concentrations. Other parameters to control include the subject's rate of breathing and the total volume air exhaled and collected into the device. Deeper breaths may produce larger condensate volumes than shallow breaths, which may affect glucose dilution factors.

Next, the material chosen for droplet condensation and collection should be sufficiently hydrophobic to allow for ease of droplet flow while also not trapping small molecules or proteins on its surface. Fouling of the collection surface with biomolecules can affect the volume of EBC sample collected and potentially, the concentration of glucose in the sample if residual glucose remains on the collection surface. Any material used in collection surfaces should also be tested to ensure it does not leach or contaminate the EBC sample as certain surfactant adhesive properties may affect the concentration of biomarkers and small molecules collected. Copper, for example, has been shown to leach into EBC samples; since it is also a highly reactive material, it can interfere with glucose concentrations[42].

The current devices on the market for EBC collection, EcoScreen and RTube, use different materials for trapping and cooling exhaled breath condensate. The RTube consists of a polypropylene tubing with an outer aluminum cooling sleeve to collect EBC whereas the coating material for EcoScreen is very similar to Teflon. While no studies have compared the effect of coating materials for EcoScreen and RTube on glucose concentration in collected EBC, Rosias *et al.* found that variation of the coating material of these devices did have an impact on amounts of protein and biomarkers[43]. This study found that a silicone or glass coating is more efficient for measurement of 8-isoprostane or albumin in exhaled breath rather than aluminum, polypropylene, or Teflon. However, protein recovery may have different material requirements than the recovery of small molecules such as glucose.

Although not tested on RTube and EcoScreen specifically, one study analyzed the interaction effects of four different collection materials on glucose measurements[44]. Teflon appeared to have the most consistent and well-correlated glucose measurements compared to the original glucose solutions; making it the most appropriate material for glucose collection. While stainless steel and polyethylene did not significantly alter glucose concentrations, glass introduced high variability in the glucose measurements. It is important to note that the sample size was small (n=3) and only four materials were tested for glucose variability in stock solutions rather than EBC. Therefore, more studies are needed to assess the effect of materials such as aluminum, silicone, and brass on glucose recovery in collected EBC.

A larger surface area may allow for increased interaction and cooling to enable droplet formation and condensation. However, this larger area can also hinder collection of the condensate if the droplets are too small to move or coalesce. Therefore, an optimal design for condensation and collection should allow enough surface area for droplet formation but in a smaller geometry that can allow for droplet coagulation and collection.

Lastly, salivary contamination is an issue when collecting EBC orally. Several studies have shown that salivary glucose does not accurately represent blood glucose[13,14,45–47]. To combat salivary contamination, subjects should rinse their mouth briefly prior to exhalation into the collection device as certain food or drinks present in saliva may affect the concentration of glucose as well as other biomarkers. However, during the collection, the device should also be designed with a saliva trap and the subject should swallow periodically to avoid excess saliva from escaping the saliva trap. Because amylase is found only in the saliva and not the rest of the respiratory circuit, some studies have assayed the concentration of amylase in the saliva as well as EBC collected to determine the extent of salivary

contamination in EBC. If the amylase concentration in EBC is at least 10-fold lower than that of the saliva, then salivary contamination is considered minimal[38,48].

Effects of Co-Morbidities and Interferents on EBC Glucose Measurements

For glucose detection, the EBC sample must be sufficiently stable and free of interferents that may affect the accuracy of any assays used. However, it is not feasible to do an extensive sample preparation step prior to detection for every measurement. Therefore, it is important to note that many respiratory solute concentrations may change according to patient disease states and any comorbidities. Furthermore, the assays used to detect glucose should not be affected by other non-volatile solutes existing in the sample.

Previous studies have shown that patients with lung-related diseases such as chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) have altered glucose concentrations in their exhaled breath condensate[14,17,23,38]. In a study done by Baker *et al.*, it was found that CF patients have elevated EBC glucose levels compared to diabetic patients without any lung-related ailments. Compared to both CF and diabetic patient groups, patients with CF-related diabetes had significantly higher concentrations of glucose in exhaled breath. In other studies, while EBC itself wasn't sampled, it was found that in other airway samples (nasal lavage fluid, sputum, bronchoalveolar lavage fluid) from patients with COPD, airway glucose concentrations were increased compared to normal subjects[49,50]. This means that lung-related comorbidities may influence the reliability of using EBC glucose measurements as an alternative to blood glucose measurements.

As previously mentioned, ammonia is present in high concentrations in EBC samples – thus, using conductivity or urea as dilution markers are not feasible without of the sample lyophilization to remove volatile organics such as ammonia for each measurement. In addition, the partial pressure of CO_2 passing through the condensing device surface can increase the amount of ammonia in the collected condensate. This is because high pCO_2 results in a relatively acidic environment and if the aqueous solution falls below a pH of 6, efficient trapping of ammonia occurs in the condensate[22]. In alveolar surface linings, the pCO_2 is estimated to be around 5.33 kPa. Kullman *et al.* demonstrated that when EBC is collected at this partial pressure, the pH is around 6.54 and that there is a strong negative correlation between CO_2 levels and EBC pH[51]. Therefore, by eliminating dead space air, it is expected that the total pCO_2 of expired air passing through the collection port of the device will increase, and as a result, the EBC pH will drop. When using biologic or enzymatic assays for EBC glucose analysis, it is important to ensure the pH of the EBC doesn't drop below 6 as it can affect enzymatic activity and/or protein stability.

Another potential interferent in EBC is hydrogen peroxide. Hydrogen peroxide is present in high concentrations in asthmatic children and lung cancer patients and is a detectable in EBC samples of healthy subjects. Many enzymatic assays for glucose rely on glucose oxidase (GOx), an enzyme that has been studied for the last fifty years and is known for its high glucose selectivity and robustness. GOx catalyzes the oxidation of glucose by molecular oxygen and produces D-glucono-delta-lactone and hydrogen peroxide. The characteristics and performance criteria of GOx will be discussed later in this review.

However, hydrogen peroxide is often the oxidation product that is quantified via colorimetric or fluorometric assays to determine the concentration of glucose[52]. Further studies are needed to understand whether native hydrogen peroxide concentrations affect the accuracy of GOx assays for EBC glucose.

Table 1 summarizes the current issues with EBC collection for accurate glucose quantification and the proposed controls to resolve or ameliorate them. If these controls are addressed, a device for reliable collection of EBC can be used for not only glucose detection, but also used as a platform for the detection of many other analytes at the point-of-care. Point-of-care devices that can be used by patients within their home are the most appropriate for commercialization since there is a significantly larger diabetic patient population outside clinical settings. Furthermore, the influence of co-morbidities on EBC glucose content limits utilization in a hospital environment. Both ICU and NICU hospital staff use established protocols and medical grade equipment to monitor glucose levels; introduction of a new detection method into hospitals is difficult due to issues related to workflow integration, personnel training, and meeting the accuracy of existing hospital grade equipment.

There are still many hurdles to cross prior to the commercialization of an EBC glucose detection system. Specifically, a biosensor with high sensitivity and specificity for glucose at the minute concentrations present in EBC is required. Without an appropriate detection system, even the most robust and efficient collection device is impractical. This is because existing commercially available glucose sensors do not possess the sensitivity and resolution required to distinguish between sub-micromolar changes in glucose concentration at EBC. In addition, with the introduction of any new detection system, sufficient calibration against blood glucose measurements is needed to ensure accuracy.

Detection Methods

Biosensor Specifications

In addition to proper controls and standardized methods for collecting EBC, a number of key design and performance criteria are required for accurate and rapid sensing of glucose at these low concentrations. Most of the previously published studies on EBC glucose detection use small molecule detection instruments to quantify glucose concentration. Common laboratory-grade systems used for detection of small molecule carbohydrates include but are not limited to: high-performance liquid chromatography and ion-exchange chromatography, pulsed amperometric detection, time-of-flight mass spectrometry, and [14,21,33,53]. These systems are highly accurate and are often used as the gold standard for small molecule detection.

In contrast to chromatography-based laboratory instruments, biosensors which do not require laboratory equipment are commonly used for rapid, point-of-care glucose detection in blood and interstitial fluid. Glucose biosensors currently make up 85% of the global biosensor market, which is worth approximately $11.5 billion USD[54]. A biosensor is an analytical device that incorporates biologically-derived recognition elements to detect analytes through a physiochemical transducer[55]. The biological or chemical reactions are

quantified through the signals generated in proportion to the concentration of the analyte of interest. The main components of a biosensor include: the analyte (glucose), a bioreceptor or recognition element, and a transducer, which converts the biorecognition element into a measurable signal. There are many different molecular recognition elements but commonly used glucose biosensors include proteins (enzymatic and non-enzymatic), lectins, and molecular imprints[56]. The type of recognition element is determined by the type of physiological sample and signal of interest.

Few studies have demonstrated successful glucose biosensing in EBC samples – this is largely due to the lack of sensitivity and appropriate resolution required to detect and distinguish between normo-, hyper-, and hyperglycemic glucose ranges in EBC. The limitations addressed in the previous section regarding accurate characterization of EBC and blood glucose dilution factors also contribute to this shortcoming. However, some recognition elements and sensing modalities are promising for low-concentration glucose detection. For successful application in exhaled breath condensate, the limit of detection of a glucose biosensor should be at the submicromolar level because of the high dilution factors reported in literature. The dynamic range should cover normal and hyperglycemic ranges for all literature values: ideally between 0.2 μM and 2 mM. Because the differentiation between normal and hyperglycemic ranges are reported to be at the sub-micromolar range for some studies, the resolution should be 0.1 μM or smaller[33].

Recognition Elements and Appropriate Sensing Modalities

The following sections will discuss specific recognition elements used for glucose detection, appropriate sensing modalities for each, and necessary controls and design for EBC-specific glucose sensing. While previous reviews have extensively covered many recognition elements and sensing modalities for glucose detection, this review will only discuss biosensors that have exhibited limits of detection and linear detection ranges that are suitable for EBC. The biosensor characteristics will be compared to those of small molecule detection systems mentioned previously.

Protein-Based Enzymatic Elements

The most widely-used method for glucose detection is based on the interaction of the analyte with glucose-specific enzymes. Glucose oxidase (GOx) and glucose dehydrogenase (GDH) are the two enzymes used in monitoring blood glucose. GOx has been shown to have high selectivity for glucose in blood and other fluids such as interstitial fluid, tears, sweat, and saliva[57–60]. GOx catalyzes the oxidation of β-D-glucose into D-glucono-1,5-lactone using molecular oxygen as an electron acceptor[61]. As previously, mentioned, hydrogen peroxide is a by-product of this enzymatic reaction (Eq. 2).

$$glucose + O_2 \xrightarrow{glucoseoxidase} D\text{-glucono-}1,5\text{-lactone} + H_2O_2 \quad (2)$$

While GOx is the gold standard of glucose sensing due to its high stability, it uses molecular oxygen as the primary electron acceptor and is unable to transfer electrons efficiently to electrode surfaces[62,63]. GDH combined with redox cofactors to transfer electrons to

electrode surfaces are an alternative to GOx based biosensors. GDHs are further categorized according to redox cofactors used which act as primary electron acceptors. Cofactors used with GDH include nicotine adenine dinucleotide (NAD), nicotine adenine dinucleotide phosphate (NADP), and pyrroloquinoline quinone (PQQ)[64] (Eq. 3).

$$glucose + PQQ(ox) \xrightarrow{glucose\ dehydrogenase} gluconolactone + PQQ(red) \quad (3)$$

Traditionally, the enzyme of choice is immobilized on a surface which interacts with the biological sample of interest[52,54,65]. Enzyme-based glucose biosensors are quantified electrochemically (amperometrically), colorometrically, or, more recently, fluorometrically[59,66–68]. The presence of a redox center in the enzyme and the mechanism of electron transfer make electrochemical or amperometric detection the most popular application of GOx and GDH enzymes[52]. Originally introduced by Clark and Lyons[69] for use in glucose monitoring, first generation glucose biosensors functioned by using natural oxygen as the primary electron acceptor in an enzyme-catalyzed (GOx) glucose reaction and glucose concentration was amperometrically determined by monitoring either the decrease in oxygen or the production of hydrogen peroxide[70]. GOx and GDH have similar affinities for glucose (3.3 mM and 10 mM, respectively) and are quite stable in a wide range of concentrations[64,71].

Second generation glucose biosensors used GDH as the sensing enzyme because instead of oxygen, it used artificial electron acceptors in the form of mediators or redox dyes[64]. These mediators transport electrons from the redox center of the enzyme to the center of the electrode. Third generation glucose sensors were developed to achieve mediator-less, reagent-less biosensors that required low operating potentials closer to the redox potential of the actual enzyme. Sensor development focused on modifying the electrochemistry of the actual GOx enzyme itself[72–74]. Figure 5 illustrates the evolution of the three generations of electrochemical glucose biosensors. Although these sensors claimed to avoid errors due to oxygen fluctuations while maintaining high selectivity, these reports should be taken with caution[54]. Chen *et al.*[75] reviewed third generation amperometric glucose biosensors and concluded that most of the reports on the modified enzyme do not necessarily demonstrate that the enzyme was fully functioning. The electrochemical modifications can affect the enzyme conformation and potentially deactivate it or significantly reduce its activity[76].

Modification to GOx and GDH-based electrochemical sensors have been made in order to achieve the low limit of detection and reasonable resolution required for non-invasive fluids with sub-micromolar glucose concentrations. Two studies from the Ren group have demonstrated GOx-based electrochemical glucose sensing for application in EBC[77,78]. These studies used zinc oxide (ZnO) nanorod arrays selectively grown on the gate of aluminum gallium nitride/gallium nitride (AlGaN/GaN) high electron mobility transistors (HEMTs) to immobilize GOx for electrochemical glucose detection. In the first study by Kang *et al.*, the ZnO nanorod array was deposited on the gate area of the HEMT to increase the total sensing area for high detection sensitivity (Figure 6a). The array provided a positively charged microenvironment to not only immobilize negatively charged GOx, but to also pass the charges produced during the oxidative reaction with glucose to the AlGaN/GaN

HEMT (Figure 5). Thus, glucose could be detected by GOx via the changes in electrostatic interactions between GOx and the nanorods; those signals were measured through the drain current of the HEMT and subsequently amplified[77]. Using the changes of drain current as a function of glucose concentration, a linear range of 0.5 nM to 14.5 μM with a limit of detection of 0.5 nM[77] (Figure 6b).

Neither study tested the sensor on collected EBC samples from human subjects but the second study by Chu *et al.* integrated the sensor into a condensing unit comprising of a Peltier (thermoelectric) cooler to demonstrate condensation of an aerosolized solution and direct detection of glucose from the condensed sample. They found that only 3 μL of condensed sample was required to cover the sensing area, which took less than 2 seconds to form when the temperature of the cooling surface was maintained at 7°C[78]. This is a significant improvement from traditional EBC collection methods and shows promise that shorter collection times and smaller volumes can be used for glucose quantification in EBC samples. After integrating their previously developed ZnO/HEMT GOx sensor with the collection device, they found that real-time first-order glucose detection was possible with the same linear range as the previous study. The authors did not test on physiological EBC samples since the immobilized GOx enzyme is very dependent on pH and its activity can be reduced by 80% if the pH falls outside the range of 6–8. This affected the drain current as it gradually decreased over time, causing a drift in the sample measurements. While this device shows promise with low detection limits and a physiologically relevant linear range, unless it uses a pH-controlled environment for glucose detection in EBC, it is very difficult to produce robust, rapid, and accurate measurements[78].

In an effort to overcome some of the drawbacks associated with non-invasive electrochemical glucose detection, researchers have used enzyme-based elements with various sensing modalities such as fluorescence, surface plasmon resonance, and colorimetry[66–68,79,80]. Due to the higher glucose selectivity and stability of GOx over GDH, most studies for non-invasive glucose detection at low concentrations use GOx as the recognition element. Steiner *et al.*[81] have extensively reviewed various fluorescence-based detection schemes using GOx; most of those reviewed do not achieve submicromolar limits of detection and thus will not be discussed in this review.

Apart from traditional organic fluorophores and luminescent probes such as ruthenium and platinum, some studies have used quantum dots to achieve higher sensitivity with GOx-based glucose detection. Quantum dots are inorganic, semiconducting nanocrystals which have well-defined energy levels and are used in a wide range of applications. Unlike organic dyes, which operate over a limited range of colors and are susceptible to photobleaching, quantum dots are significantly brighter, more photostable, and can be tunable to produce any color of visible light. They have been employed in enzyme-based glucose biosensors which have achieved micromolar and submicromolar detection limits *in vitro*[80,82–84].

The most successful study was done by Cao *et al.*[85] in which a detection limit of 0.10 μM was achieved. They developed a complex consisting of CdTe QDs bound by four GOx structures (Figure 7). When glucose was introduced to the complex, the GOx would produce hydrogen peroxide that quenched the QDs in a proportional manner to the amount of

glucose detected. The hydrogen peroxide was immediately reduced to oxygen when the electron transfer occurred on the surface of the QDs. The oxygen is then stored in electron hole traps on the QDs and can be used as an acceptor for future GOx reactions; this allows for greater temporal resolution. Their complex exhibited better thermal stability compared to native GOx as it was stable between 20–80 °C and had maximum activity in the 40–50 °C range. When tested for glucose detection, a linear range of 5 μM to 1 mM was demonstrated along with an experimental detection limit of 0.1 μM[80]. It should be noted that only under optimized pH conditions was the sensor able to achieve the desired detection limit and linear detection range.

GOx has also been conjugated to gold or silver nanoparticles (NPs) for glucose detection via surface plasmon resonance (SPR). Endo *et al.*[79] developed an optical GOx biosensor with a stimuli-responsive hydrogel with silver nanoparticles (AgNPs) that could quantify glucose via SPR. The GOx was immobilized into this hydrogel and when glucose was added to the surface, the interparticle distances of the AgNPs increased, which subsequently decreased the localized SPR absorbance strength. The hydrogen peroxide produced by the GOx reaction played a role in signal amplification by inducing the degradation of highly clustered AgNPs, which resulted in drastic SPR absorbance changes proportional to glucose concentrations. Thus, a detection limit of 10 pM was achieved – far lower than any study until date[79,81]. This is a promising application of GOx-based detection for EBC. However, more work should be done on testing this sensor in physiological conditions to determine its stability.

It is evident that significant work has been done in developing enzyme-based sensors for non-invasive glucose detection. However, few studies have demonstrated submicromolar sensitivity and detection ranges suitable for EBC[67,68,86]. The high dissociation constant (3.3 mM) of GOx prevents it from being suitable for EBC detection unless expensive modifications are made for signal amplification. Furthermore, the enzymatic activity of GOx is greatly dependent on pH and temperature – both of which can fluctuate greatly in physiological conditions. In addition, byproducts such as hydrogen peroxide and gluconic acid (hydrolyzed form of D-glucono-1,5-lactone) can deactivate the enzyme, affect the stability of electrodes when used in electrochemical detection, and ultimately reduce the shelf-life of the device[78,87,88]. The presence of baseline hydrogen peroxide in normal EBC samples also questions the accuracy of enzymatic sensors that rely on glucose quantification through the production of hydrogen peroxide. Lastly, not only do enzymes run the risk of being easily “poisoned”, they are also expensive to obtain since they have to be carefully extracted from natural sources[88]. Thus, for an accurate and sustainable EBC-specific glucose sensor, researchers should 1) avoid electrochemical sensing modalities and 2) consider using alternative recognition elements that are inexpensive, highly stable, reagent-less, and possess higher affinity for glucose.

Protein-Based Non-Enzymatic Elements

The glucose binding protein (GBP) is a periplasmic binding protein found in Gram negative bacteria. In particular, it is natively expressed in *E. coli,* which makes it an easily acquirable recognition element. This protein undergoes a conformational change from “open” to

"closed" upon binding with glucose (Figure 8). Due to its high affinity for glucose (K_d = 0.14 μM), it has been integrated into glucose sensors to detect concentrations at the μM range without much modification or signal amplification, which is a significant advantage over other recognition elements. Unlike enzymatic sensors which have byproducts that can potentially limit their activity, GBP does not alter the chemistry of glucose with its conformational changes. Because it does not possess electron transfer capabilities, fluorescent detection is the sensing modality used for this protein. The Daunert group engineered the GBP with fluorescently labeled amino acid residues near the binding site. The fluorescent quenching was demonstrated to be proportional to the concentration of glucose and the limit of detection for one mutant GBP was reported to be 0.05 μM, which is very well-suited for detection at EBC concentrations[89]. While their sensor was not tested *in vivo,* it was found that the GBP mutant was stable at 37 °C for over 3 months, meaning that this protein has a higher stability than traditional GOx and GDH enzymes[90]. Other groups have used similar methods of engineering the GBP binding site but have focused on altering the range of detection to be suitable for glucose concentrations in interstitial fluid, which is 1.7–33 mM[91,92].

Fluorescent intensity-based measurements based on single fluorophores significantly fluctuate due to leaching or photobleaching of the fluorophore and the positioning of the sample. To address this, Ge *et al.*[93] labeled GBP with two fluorophores; an environmentally sensitive acrylodan fluorophore at a cysteine mutation near the binding site and a long-lifetime ruthenium complex at the N-terminus. In the presence of glucose, the fluorescent emission of acrylodan is quenched since it is near the binding site. On the contrary, ruthenium emission remained constant; making it a stable reference fluorophore. Glucose detection at the micromolar range was quantified by the fluorescence intensity ratios of the two fluorophores.

This sensor was also integrated into a custom-made miniature fluorometer for point-of-care measurement of glucose concentrations sampled from the skin surface. The microfluorometer system was able to achieve a limit of detection of 0.08 μM and was also tested on three human subjects with a resolution of 0.3 μM distinguishing baseline glucose levels vs. glucose levels after a meal[94]. While it was unclear whether the glucose measurements were correlated with blood glucose, it demonstrated a promising application of GBP as a point-of-care biosensor for samples with lower glucose concentrations such as EBC.

Recently, the Tolosa and Rao groups implemented fluorescently labeled GBP into a fiber optic biosensor for transdermal glucose detection and was able to detect concentrations in the micromolar range after demonstrating its application using a pig skin model[95]. In this study, the GBP was labeled with a polarity-sensitive probe (BADAN) to a single cysteine mutation near the binding pocket to allow for fluorescent detection of open and closed conformations. The BADAN-GBP was then conjugated to Ni-NTA beads, which were attached to the tip of optical fibers via nylon mesh and tubing (Figure 9a). In-house optics and electronics were used to develop a mini-fluorometer that can quantify fluorescent intensity upon glucose binding in mV (Figure 9b). While the study was able to acquire a linear detection range of 2–10 μM, it was not able to achieve the sub-micromolar detection

limit as demonstrated in their previous study with acrylodan-labeled GBP. However, this was a result of compensating for the loss of GBP activity and the photobleaching effects caused by the reference dye used in the previous study. In using Ni-NTA immobilized GBP labeled with only one probe, they were able to achieve better photostability and activity after continuous hours of usage. In the future, this sensor can be modified to achieve even lower detection limits while maintaining stability.

Another common method used for fluorescent glucose detection with GBP is Förster (fluorescence) resonance energy transfer (FRET), in which the distance between the donor and acceptor molecules determines the rate of energy transfer. Many of the studies published on FRET-based glucose detection with GBP have wide-ranging reported sensitivity values. This is due to the wide variety in the positioning of fluorescent labels and the types of fluorescent dyes used. Khan *et al.*[96] developed a GBP-FRET system using an Alexa fluorophore conjugated to the N-terminus of the protein and a non-fluorescent acceptor, QSY7, attached via a thiol group to a cysteine residue at one of two different locations near the binding site. This sensor was able to detect glucose at micromolar concentrations with K_d = 0.1 μM but with sub-optimal resolution due to small fluorescence intensity changes. When GBP was labeled with Alexa 555 at the N-terminus and QSY7 at the Cys 182 residue, only a 7% maximum fluorescence increase was observed in the presence of glucose. To improve the signal change, GBP was labeled with Alexa 488 at N-terminus and QSY7 at Cys 152 via a His mutation. However, this only improved the fluorescent signal change by 16%. As a comparison, an environmentally sensitive dye, badan, was linked to one of the two cysteine residues to analyze glucose sensing by GBP. While badan at Cys 182 had negative fluorescence change in the presence of glucose, when attached to Cys 152, it had a fourfold change in fluorescence. This points to the fact that an environmentally sensitive dye can provide higher resolution glucose measurements.

FRET based on the conformational change of GBP results in insufficient fluorescence intensity changes due to the limited range of motion of the N-terminus with respect to the protein domains upon glucose binding[96]. To address this, Hsieh *et al.*[97] developed a dual-labeled GBP sensor with an environment-sensitive fluorophore, nitrobenzoxadiazole (NBD) on the outer surface of the binding pocket and a Texas Red (TR) fluorophore on the inner surface of the binding site. They employed a ligand-protection strategy to sequentially label the protein via two cysteine mutations. When GBP was bound to glucose, the inner surface of the binding site was inaccessible; making it possible for the outer cysteine residue to be labeled with NBD. Subsequently, when bound glucose was removed, the inner cysteine residue was labeled with TR. In the absence of glucose, the NBD-TR constructs showed reasonable FRET efficiency as the donor molecule (NBD) had low fluorescence and the acceptor molecule (TR) had high fluorescence. However, rather than any quenching occurring, both molecules had increased fluorescence upon glucose binding, with the TR having a slightly larger increase than NBD. As a result, a ratiometric measurement of the two molecules was used to determine fluorescence response curves; glucose was detectable in the millmolar range (1–30 mM) with a $K_{apparent}$ of 1.7 mM. While this method improved the fluorescent intensity changes in FRET by avoiding the dual-terminal labeling of the protein, the sensitivity was significantly compromised, as it would not be suitable for micromolar glucose detection[97].

Moving forward, FRET detection based on a single fluorophore attached to the protein and a competing ligand with an acceptor fluorophore may produce an even larger intensity change upon competitive displacement by glucose. This will potentially enable reliable detection sensitivity and resolution at the submicromolar level.

Numerous studies have used fluorescence modalities for GBP-based glucose sensing and have achieved submicromolar limits of detection[81]. However, a drawback to the currently reported fluorescence-based GBP sensors is the use of organic fluorophores which are susceptible to photobleaching, pH dependence, and narrow excitation with broad emission spectra.[9,98] Thus, photostable nanomaterials such as quantum dots may provide a more robust option for non-invasive GBP-based biosensors.[98] In terms of FRET, the quantum dots also make for excellent fluorescent donor molecules since they have broad absorption and narrow emission spectra.

While GBP is a promising candidate for a highly stable, reagent-less, intramolecular fluorescence sensor, it also binds to galactose, albeit at a slightly lower affinity ($K_{d,galactose}$ = 0.25 μM vs. $K_{d,glucose}$ = 0.14 μM)[99]. Thus, sensor design should be designed to maintain high selectivity towards glucose, especially in samples with already low glucose concentrations, such as exhaled breath condensate. Fortunately, high galactose levels have not been indicated in studies examining EBC composition[21,22,38]. As an alternative to FRET using dual-labeled GBP termini, galactose can potentially be used as a competitively binding quencher molecule in GBP-FRET using stable GBP-bound nanomaterials, such as QDs (Figure 10). When samples containing glucose are introduced, the galactose-quencher would be displaced by the glucose and the fluorescence of GBP-QD would increase proportionally. This method would also allow for adjustable linear detection ranges based on the molar ratio of the galactose-quencher to the GBP-QD. While this has yet to be demonstrated in GBP, similar mechanisms have been shown in maltose binding protein and sensors for 2,4,6-trinitrotoluene (TNT)[100].

Lectins

Concanavalin A (ConA) is another popular molecular receptor for fluorescence-based glucose detection. It is a plant-derived lectin, which is a family of proteins with strong binding affinities for glycans due to their multivalent interactions.[56] In particular, it contains four binding sites for glucose and competitively binds to glucose in biosensor schemes. Usually, ConA is bound to an existing labeled carbohydrate derivative such as dextran but is displaced from the molecule when glucose preferentially binds to it. The earliest example of this affinity sensor was developed in 1984 by Mansouri and Shultz in which fluoresceine isothiocyanate (FITC)-labeled dextran was bound to ConA, allowing a charge transfer to occur which quenched the fluorescence intensity of the FITC.[101] When glucose was introduced to the sensor assembly, it preferentially bound to ConA, displacing the FITC-dextran molecule. This increased the fluorescence intensity in proportion to the concentration of glucose.

ConA has been extensively studied in various sensing schemes involving different combinations of fluorophores and fluorescent particles[102–106]. Most of these systems showed a linear detection range of sub-micromolar to 25 mM of glucose, but with varied

response times depending on the type of sensor. Tang *et al.* demonstrated a promising non-invasive application of ConA when it was integrated with quantum-dot FRET (Figure 11). In their study, they were able to successfully detect glucose concentrations with high resolution within 0.10–50 μM and a lower detection limit of 50 nm under optimized experimental conditions[80]. However, ConA has exhibited problems with aggregation and leakage, limiting its performance in physiological conditions. This can be especially problematic in low-concentration glucose samples such as EBC. Furthermore, none of these systems have been successfully integrated into *in vivo* monitoring devices due to the complexity of physiologically relevant matrices such as blood, serum, or plasma[91]. More work should be done to control for aggregation and leakage issues in physiological samples before using ConA as a recognition element for glucose detection in EBC.

Table 2 summarizes the current biosensor systems that can potentially be used for EBC detection along with the specific controls required for feasibility.

Liquid chromatography-based separation and detection systems

Liquid Chromatography (LC) instruments have been considered the gold standard for separation of low concentration carbohydrate solutions, including exhaled breath glucose. These instruments perform charge or mass-based separations of analytes of interest. When combined with appropriate detectors, controls and standards, LC-coupled instruments can not only detect exquisitely small concentrations of glucose but differentiate glucose from other similar carbohydrates separated via LC. These methods are highly sensitive but can require hours for the separation and analysis of each sample. With large numbers of samples, the analysis can be automated, mitigating for high throughput. Sample preparation techniques as well as column flow rates, temperatures, and elution profiles of known standards are critical for proper analyses.

HPAEC-PAD

Electrodes made of gold, silver, or carbon can be used for enzyme-free detection of carbohydrates due to electrocatalytic oxidation of carbohydrates at relatively high pH onto the surfaces of these materials. Nanostructured electrocatalytic biosensors are of growing interest for non-enzymatic glucose detection at the mM levels found in blood and in some cases μM concentrations. However, recent reviews caution the lack of studies on selectivity for glucose[107]. Because carbohydrates, such as glucose, are weak acids and can be separated at high pH using by high performance anion exchange chromatography (HPAEC), HPAEC can be coupled with pulsed electrochemical detection (PED), to provide the required specificity for individual carbohydrates. When the retention time of each carbohydrate of interest is determined *a priori,* this technique provides high resolution separation and differentiation of even similar monosaccharides such as glucose, galactose, and fructose. For this reason, HPAEC-PED and has been used in carbohydrate monitoring of many foods. Because EBC provides a relatively simple aqueous matrix, EBC itself can be used as the mobile phase during separations and requires little pre-processing compared to food-based HPAEC-PED samples.

Pulsed amperometric detection (PAD), is a subset of PED which induces an electrical pulse at not only the detection potential, includes additional pulses of voltage potentials to induce oxidation and then reduction to mitigate fouling of the detection electrodes. Baker and colleagues[14] and used HPEAC-PAD to evaluate exhaled breath glucose concentrations from patients with cystic fibrosis to evaluate glucose as a biomarker of pulmonary inflammation in CF patients. Early studies of glucose appeared to indicate breath glucose concentrations in the mM range. However, by first lyophilizing the sample to remove volatile NH_4 and accounting for dilution during exhalation and reconstitution via conductivity measurements, Srivastava *et al.*[108] later determined that glucose concentrations in the breath were nearer to 0.72 μM. Indeed, our own preliminary measurements have indicated breath glucose concentrations at 3.6 ± 0.2 μM[39]. Of note, EBC sample volumes for HPAEC-PAD should be a minimum of 50 μL to avoid inducing air bubbles into the system and skewing detection measurements.[109]

Mass Spectrometery (MS)

Mass spectrometry enables detection of separated small molecules based on mass of the molecules. Eugenia Monge *et al.*[33] performed investigations of three different electrospray ionization (ESI)-MS techniques to evaluate their appropriateness for detection of endogenous EBC glucose when known concentrations of glucose were spiked into the samples prior to lyophilization and reconstitution with acetonitrile and water (80:20 v/v)[110]. These studies indicate that all three methods, ultra-high-performance liquid chromatography (UHPLC) coupled to time-of-flight (TOF) MS or to triple quadrupole (QqQ) tandem MS (MS/MS) and direct-infusion traveling wave ion mobility spectrometry (TWIMS) with TOF-MS have the required limits of detection (0.07 μM, 0.03 μM, and 0.1 μM respectively) and linearity (0.09–50 μM, 0.2 to 50 μM, and 0.2 to 80 μM, respectively) required for detection of EBC samples. The linearity over these three orders of magnitude span most of the expected ranges of EBC glucose. Of note, while the spiked glucose concentrations were fairly similar between the methods, the endogenous EBC glucose concentrations ranged between 0.24 μM to 5.4μM. Further, there was significant drift over time in the TWIMS-TOF-MS, which must be accounted for during experiments.

Future Directions, Challenges, and Opportunities

Advances in EBC collection and glucose sensing could enable convenient, non-invasive methods for patients to detect and monitor their glucose levels. Many of the technologies and methods reviewed above provide important proof-of-concepts for EBC glucose detection technologies. These promising studies provide opportunities to further understand EBC glucose concentrations and ultimately develop important non-invasive tools for glycemic control.

For accurate and reliable platforms for EBC glucose detection, collection methods must be standardized and account for the highly variable nature of glucose dilution from the respiratory fluid to the condensing device. Fundamental research into the variability in glucose dilution from the respiratory fluid due to individual and environmental factors is needed to understand and develop reproducible EBC collection across subjects. Key controls

such as incorporating reference dilution markers and monitoring relative humidity and temperature, as well as evaluations of EBC sample stability are critical to advancing EBC glucose detection research. These will enable critical studies to understand the time-lag between blood and EBC glucose and establish standardized models of the blood-to-EBC glucose ratio.

Numerous glucose sensing proteins including GOx, ConA, and GBP combined with sophisticated electrochemical and fluorescent transducers have shown promise for detecting the required micromolar concentrations of glucose from EBC. However, these sensors have not yet been tested in EBC samples and further development is required to increase sensor reliability and robustness. GOx remains the most studied protein for glucose detection and provides flexible detection modalities ranging from electrochemical, to fluorescence, and plasmonic resonance. In particular, novel electrochemical transduction methods have pushed the detection limits of GOx sensors into relevant ranges for EBC glucose. These enzymes present the advantage of being well studied and robust, but dependence on specific pH and temperature ranges may limit their translation to physiologic EBC glucose detection. Non-enzymatic elements, such as GBP and ConA, are promising for highly sensitive glucose detection because of their greater affinity for glucose over GOx. They are limited to optical detection mechanisms which possess higher detection limits than electrochemical and SPR GOx sensors. However, the detection limits for GBP and ConA are well within the range of EBC glucose concentrations. While liquid chromatography-based instruments using PAD and MS have been demonstrated for EBC glucose detection, their complex instrumentation and intensive sample preparation requirements limit their use to laboratory research. Nevertheless, these systems provide an important benchmark for EBC glucose detection in terms of limit of detection, detection resolution, and linear dynamic range. Moving forward, new biosensors should be compared to these more studied methods when determining biosensor performance.

To move EBC glucose detection beyond a research tool and into patient-centered point-of-care applications, biosensor integration into an appropriate condensing device will be critical. The device will need to separate alveolar air from dead space air and include efficient cooling to enable rapid and consistent condensation surface temperature for droplet formation. Further, the collection surface of the device should seamlessly direct the collected condensate to the biosensor, either through automated fluidic controls or condensation on the biosensor itself. Because reaction kinetics are temperature and pH dependent, the environment of the sensor must be controlled for temperature, pH, and other reaction interferants and binding kinetics should be carefully characterized. Finally, the signal from the biosensor will need to be transduced through a low-cost, but quantitative device, such as a potentiostat or fluorometer that enables signal-to-noise ratios capable of detecting sub-micromolar resolution in glucose binding events. Long-term stability, reliability, and robustness of these integrated sensors will be required and systems will need to be compared to gold-standard blood glucose detection to ensure clinical sensitivity and specificity. In addition to technical improvements and clinical evaluation, the economics, manufacturability, and usability of these systems will all be key considerations in developing scalable and impactful EBC glucose detection.

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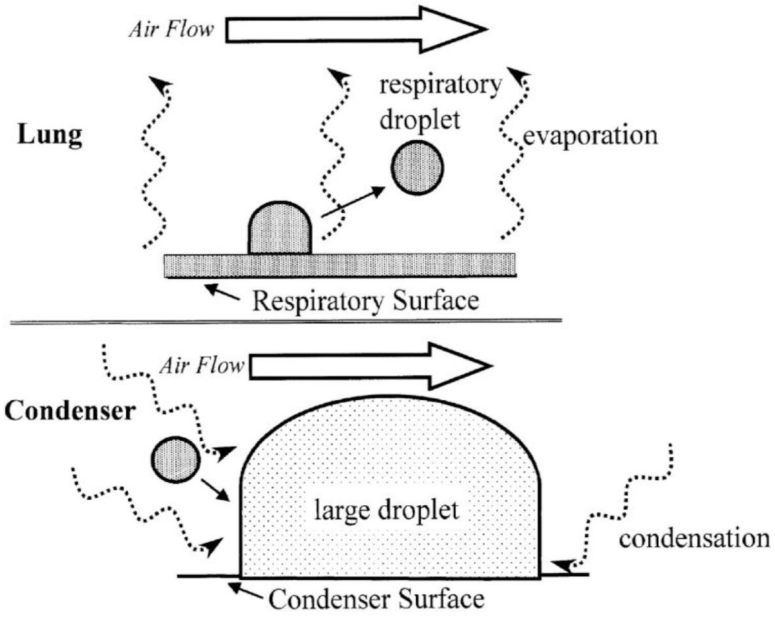

Figure 1. Respiratory droplet aerosolization and dilution.

When droplets are released from the surface of the airway lining, they undergo evaporation. However, as they travel up the respiratory tract, they are also diluted by water vapor. Hence, the droplets are much larger when they are collected as exhaled breath condensate. Reprinted from Effros *et al.*[21] with permission from [Permissions pending from American Journal of Respiratory and Critical Care Medicine (permissions@thoracic.org)].

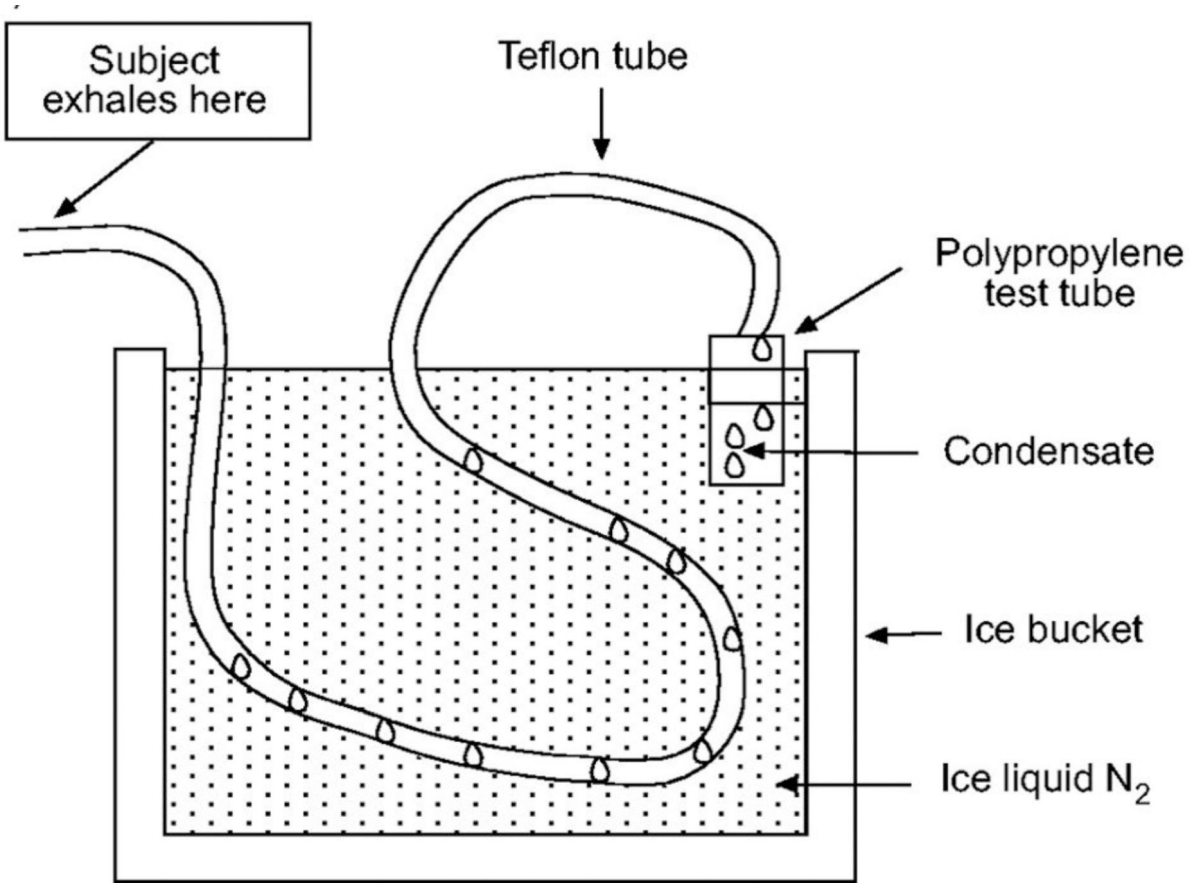

Figure 2. Typical collection method and relevant components for condensation of exhaled breath.
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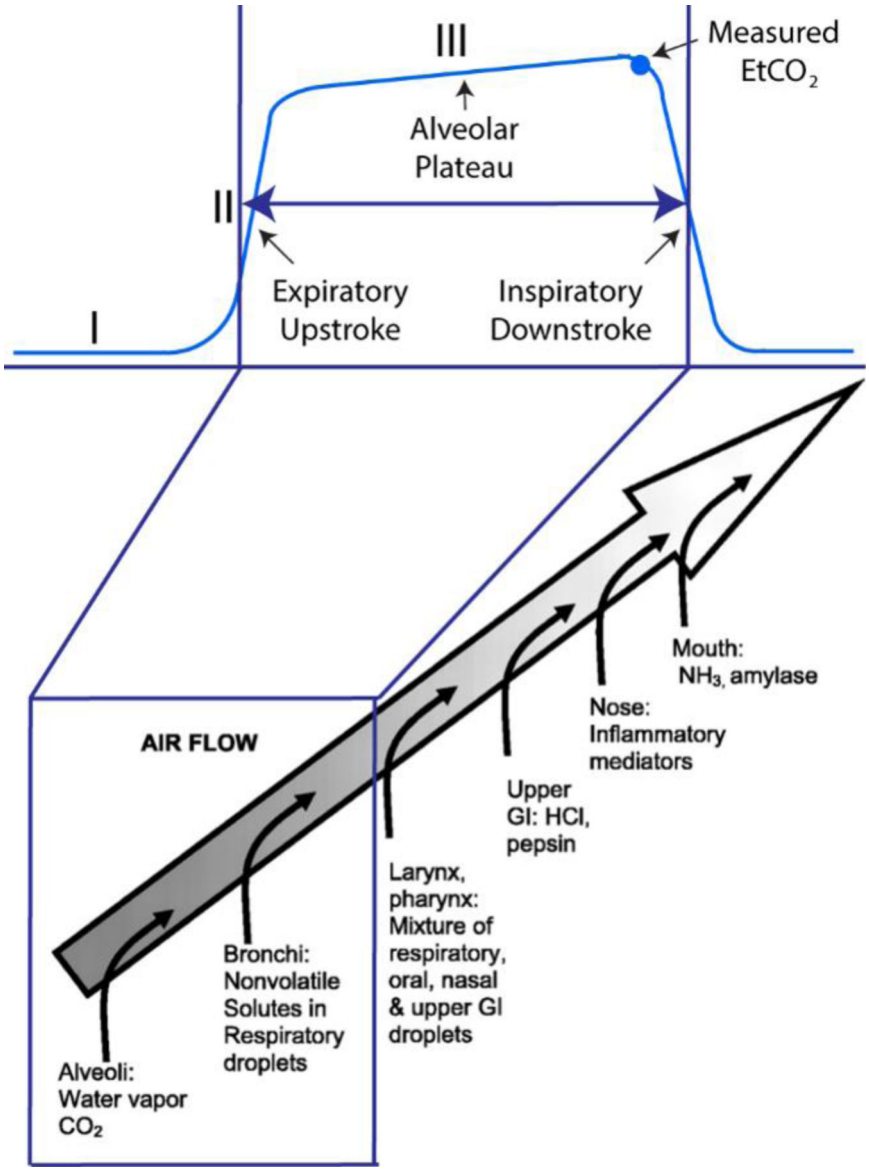

Figure 3. Components of exhaled air from various regions of respiratory tract.

Adapted from Effros *et al.*[111] The box indicates the respiratory zone of the airway tract in which solutes of interest are present in the alveolar fluid lining and respiratory droplets in the bronchi. This region is represented by phase III of the capnography plot above, which is characterized by the CO_2 plateau. Anatomical dead space air is present in the upper region of the respiratory tract (outside of the box). It does not participate in gas exchange and is represented by phase I and II of the capnography plot. Airway diagram reprinted with permission from [Permissions pending from American Journal of Physiology – Lung Cellular and Molecular Physiology https://www.physiology.org/author-info.permissions].

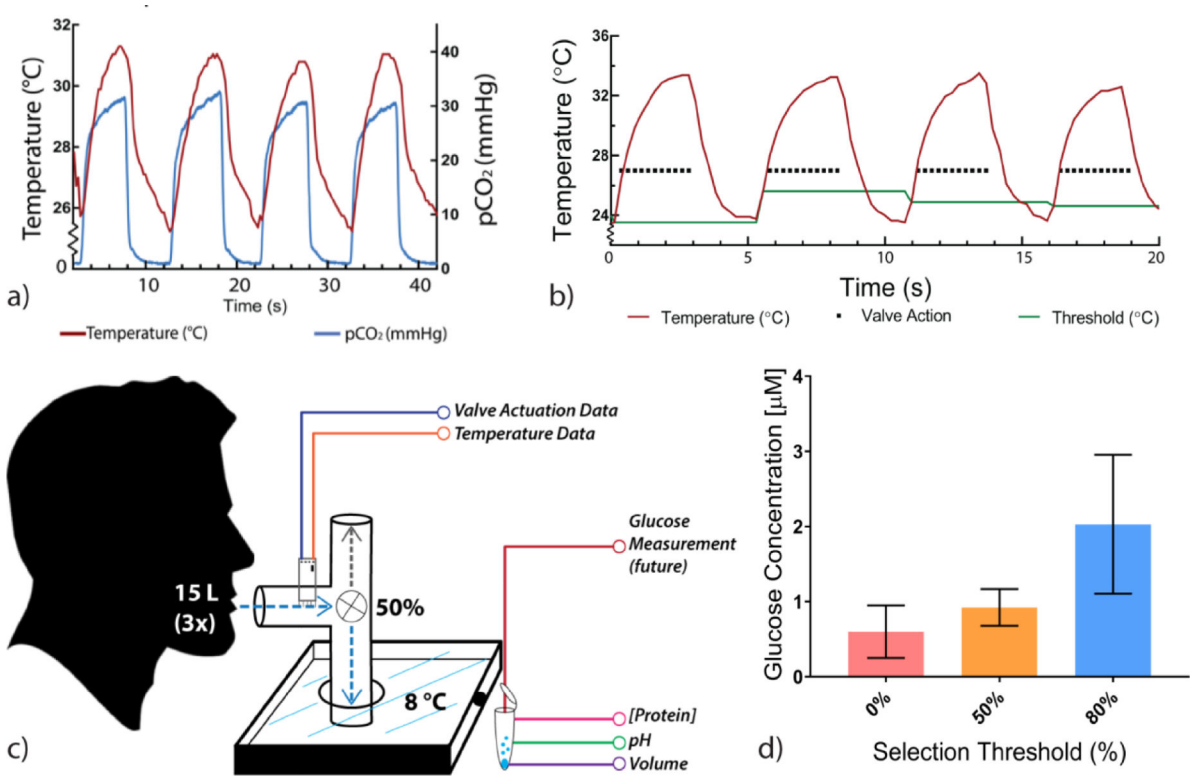

Figure 4. Temperature-based selective condensing mechanism for EBC developed by Tankasala *et al.*[39].

a) Temperature and CO_2 profile comparison from breathing profile of a human subject; b) Temperature-based valve actuation. The valve action (black) is displayed for periods where the valve is open. The threshold (green) is continuously updated based on the average temperature range of the last three breaths; c) Device set-up for exhaled breath condensate collection and analysis. Temperature, valve actuation, and time for collection are recorded. The sample is then analyzed for glucose content. Sample can also be analyzed for total protein concentration and pH; d) Glucose concentrations from EBC samples collected with different temperature selection threshold. Reprinted with permission from Tankasala *et al.*[39] [Permissions pending from 2018 40th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC) https://ieeexplore.ieee.org/document/8513393].

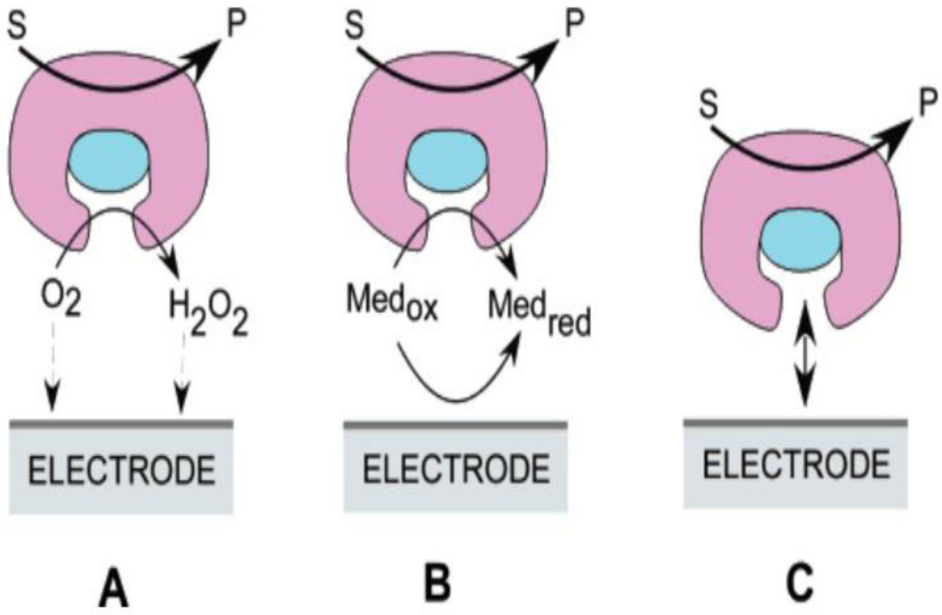

Figure 5. Schematic representation of the progression of electrochemical glucose biosensors.
a) First generation sensor that uses molecular oxygen as a cofactor; b) second generation sensor that uses artificial redox mediators; c) third generation sensor that relies on direct electron transfer between the enzyme and electrode. Reprinted from Wang[52] with permission from [Permissions pending from Chemical Reviews https://pubs.acs.org/doi/10.1021/cr068123a].

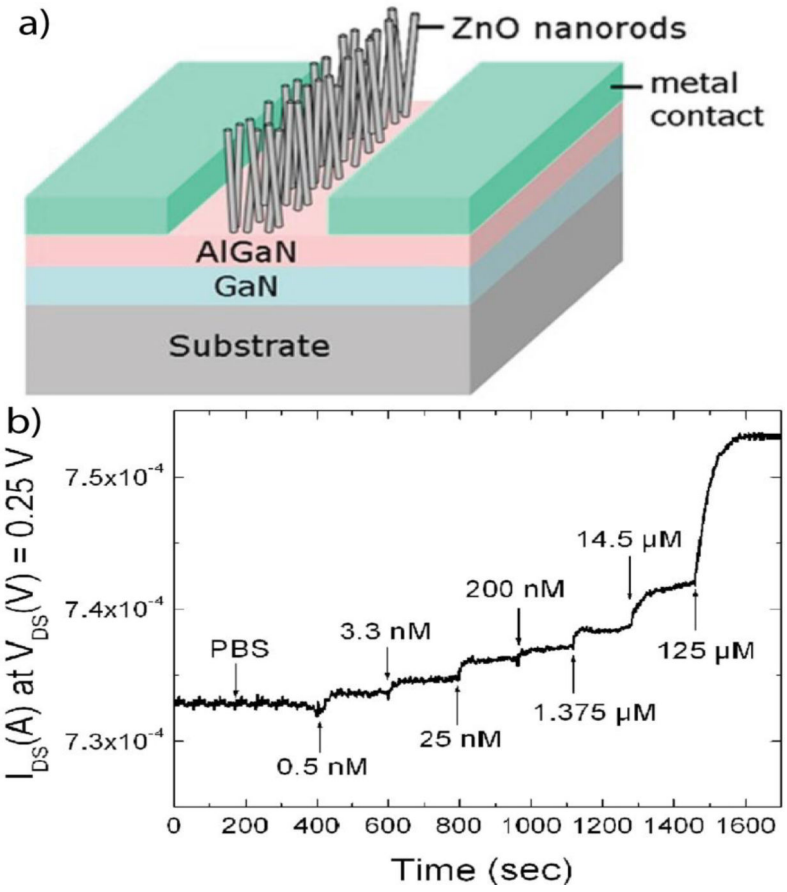

Figure 6. Schematic of the ZnO nanorod array deposited on the gate area of the AlGaN/GaN HEMT sensor.

a) The HEMTs were fabricated through several steps of molecule beam epitaxy, chemical vapor deposition, inductively coupled plasma etching, and *e*-beam deposition GOx is immobilized on the nanorods. b) Glucose was detected through the changes in the electrostatic interactions between GOx and the nanorods, which were measured by the drain current of the HEMT. Reproduced from Kang *et al.*[77] with permission from [Permissions pending from Journal of Diabetes Science & Technology]

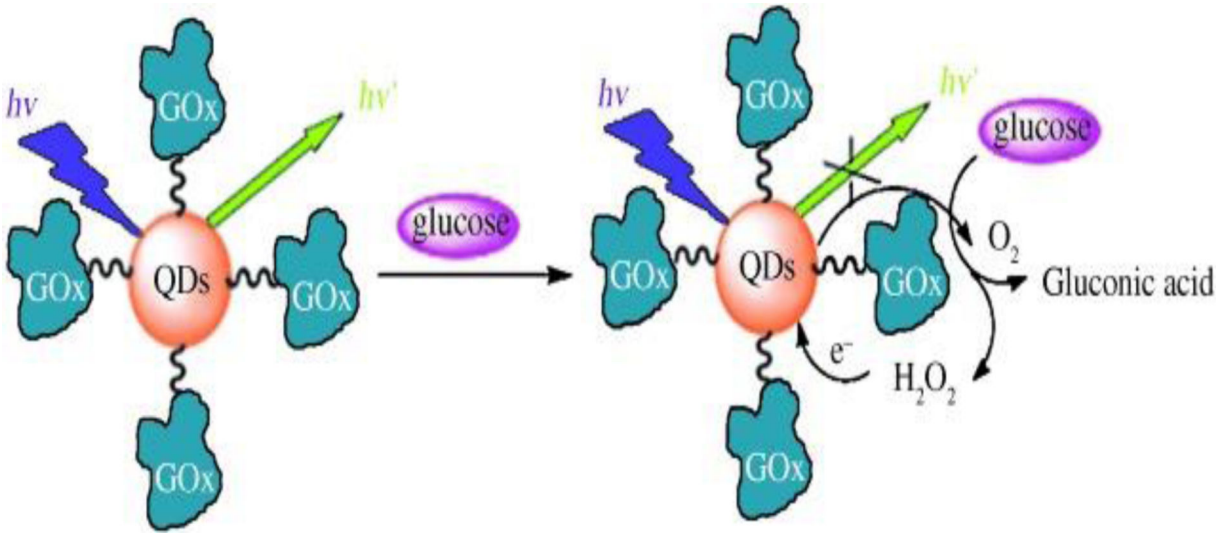

Figure 7. Assembly of CDTe Quantum Dots complexed with glucose oxidase and schematic of glucose sensing.

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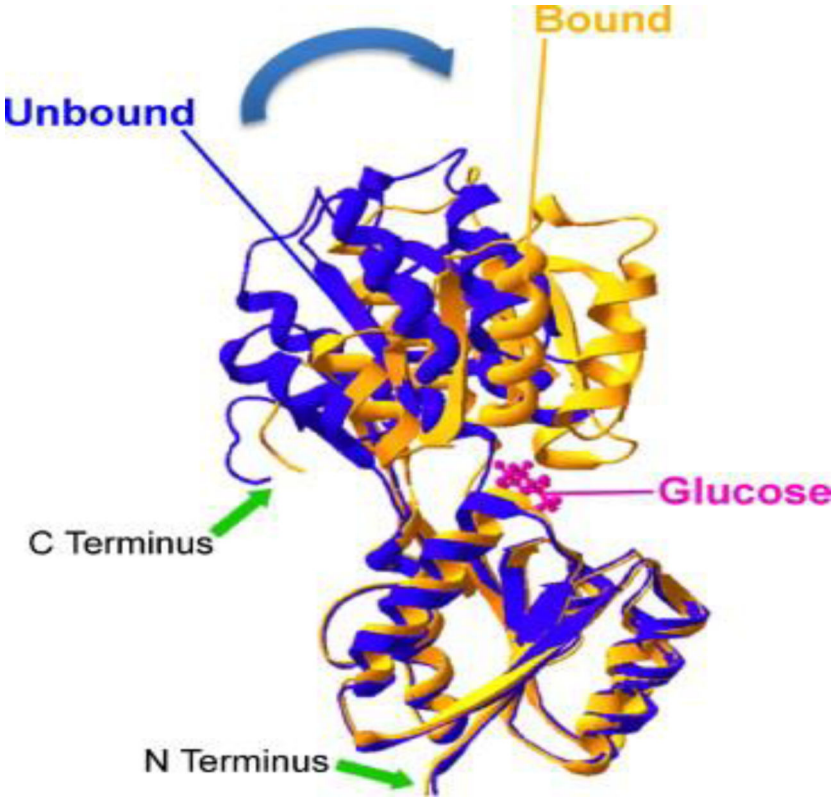

Figure 8. Representative image of the glucose binding protein and its conformational change upon binding to glucose.

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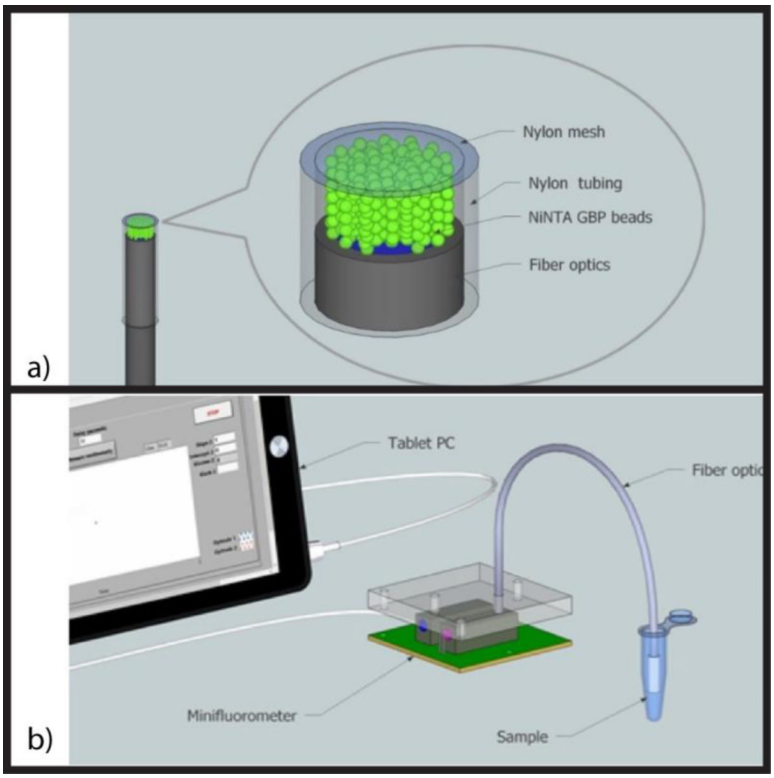

Figure 9. Schematic of Ni-NTA immobilized GBP for fiber optic glucose detection.

a) Incorporation of immobilized GBP on optical fiber. The beads are entrapped within a nylon tube on the tip of an optical fiber and secured in place with nylon mesh; b) Set-up of fiber optic GBP sensor with min-fluorometer for signal acquisition and image processing. Image reproduced from Tiangco *et al.*[95] with permission from [Permissions pending from Sensors and Actuators B: Chemical].

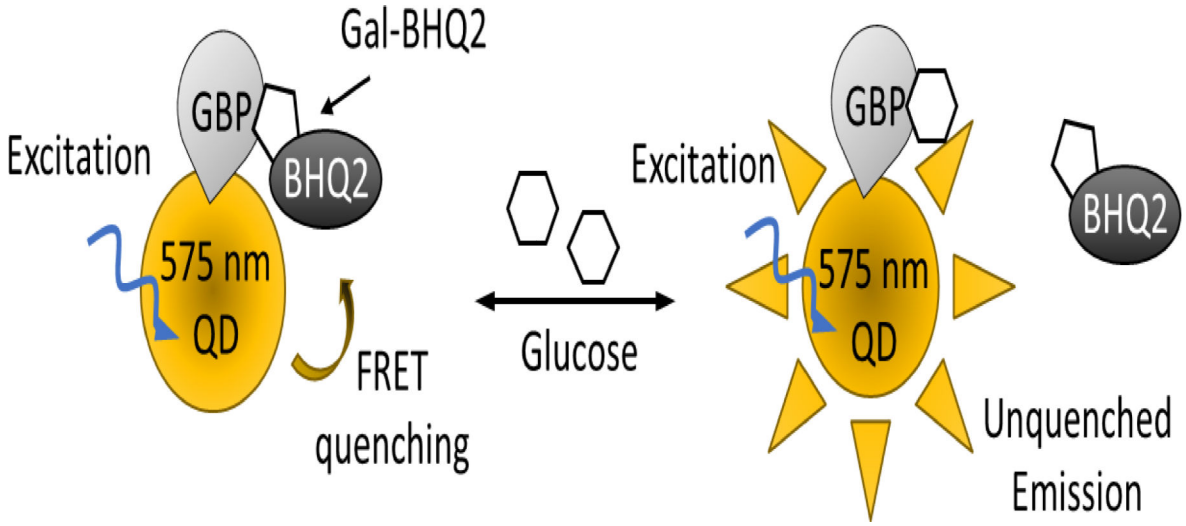

Figure 10. Application for QD-FRET in GBP glucose biosensor.

GBP can be covalently conjugated to a QD. Initially, a galactosamine quencher (gal-BHQ2) will be bound at the binding pocket of GBP and result in FRET quenching of the QD luminescence. When samples containing glucose are introduced, the gal-BHQ2 is displaced by glucose and photoluminescence of the QD will increase.

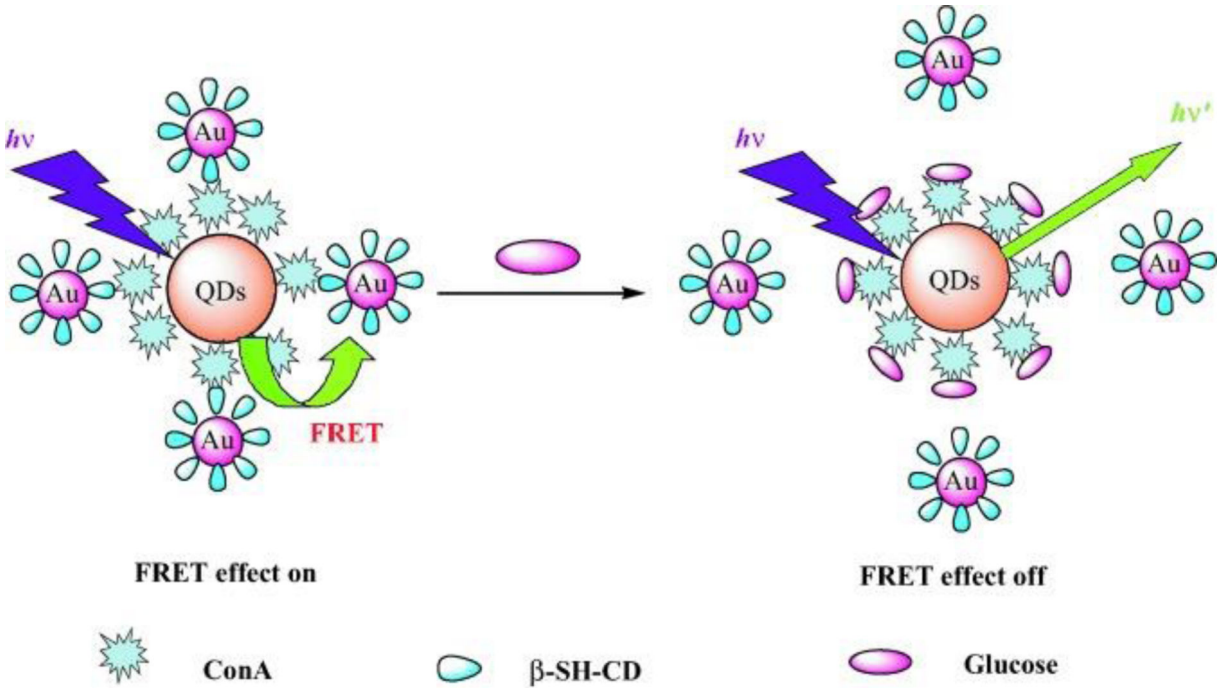

Figure 11. Chemical structure of the CdTe quantum dots complexed with Concanavalin Abound gold nanoparticles for FRET-based detection of glucose.

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Table 1

Necessary controls for standardized collection and quantification of exhaled breath condensate glucose

Category	Current Issue	Controls	Reasoning
Glucose Dilution	Variable dilution factor from water vapor	Using reliable reference dilution markers that have similar concentrations in plasma and respiratory fluid[22]	Can determine whether variable dilution rate is caused changes in respiratory fluid solute concentration vs. water vapor dilution
	Non-homogeneous solute concentrations throughout respiratory tract	Elimination of dead space air via a temperature-based or CO2-based threshold that is continuously calibrated for most recent exhalations;	Elimination of dead space and inclusion of only deep-lung air may increase total concentration of solutes in collected EBC[37,44]
	Variable droplet volume and formation rate	Controlling for relative humidity[23,38]	Relative humidity may affect droplet size distribution. Lower relative humidity may increase droplet evaporation during collection[113].
Sample Stability	Hydrogen Peroxide in EBC	Calibration of hydrogen peroxide levels prior to assaying via glucose oxidase[19,38]	Asthma and lung-cancer patients have elevated levels of hydrogen peroxide that may affect accuracy of glucose oxidase assays[114]
	Low pH of collected EBC	Measuring pH before protein or enzyme-based assays[19,21]; control total CO_2 passing through collection device	Increased total CO_2 passing through device surface lowers pH of collected EBC[51]
	Salivary glucose contamination	Use saliva trap on collection device and perform amylase assay on EBC samples to test effectiveness of saliva trap	Saliva contains amylase concentrations but EBC does not[38]
	Residual glucose on collection device	Material that does not bind to solutes; effective cleaning process before and after each collection	Material should not leach into sample; may need hydrophilic/hydrophobic hybrid material to allow droplet formation and flow to collection site
Subject Variability	Variable collected EBC glucose concentration	Subjects should exhale the same total volume of collected air[39]	Variability in total volume exhaled may affect measured glucose concentrations
		Subjects should breath at similar breathing rates and avoid deep or shallow breaths	Shallow breathing may result in higher CO_2 levels in EBC[115]; deep breathing can increase dilution factor of glucose

Table 2.

Types of glucose biosensors with detection limits suitable for EBC glucose.

Recognition Element	Detection Category	Sensing Modality	Technique	Detection Limit	Controls for EBC
GOx	Electrochemical	ZnO Nanorods + HEMTs	Changes in electrostatic interactions between GOx & ZnO nanorods upon GOx-mediated glucose reaction	0.5 nM[77,78]	Controlled pH environment between 6–8 and controlled temperatures
	Fluorescence	CdTe QDs conjugated to GOx	QD quenching via H_2O_2 production through GOx-mediated glucose reaction	0.1 μM[85]	Optimized pH conditions
	SPR	AgNPs in stimuliresponsive hydrogel	Interparticle distance changing SPR strength; amplified by H_2O_2 byproduct from GOx-mediated glucose reaction	0.01 nM[79]	Test hydrogel stability in physiological pH & temperatures; EBC glucose should penetrate through hydrogel
GBP	Fluorescence	Fluorescently-labeled amino acid residues on binding site	Change in fluorescent intensity upon glucose binding	0.05 μM[89]	Use stable fluorescent particles; determine if resolution can distinguish between fasting and normal EBC glucose levels
		Dual-labeled fluorescent tags on GBP	FRET between fluorophores	0.08–0.5 μM[94]	Use stable fluorophores; determine correlation between blood and EBC glucose levels
		Single polaritysensitive fluorescent probe on binding site; attached to optical fiber	Change in fluorescent intensity upon glucose binding	2–10 μM[95]	Use dual-labeled GBP or a FRET quencher to achieve higher signal intensity and detection sensitivity
ConA	Fluorescence	ConA-conjugated CdTe QDs with AuNPs	FRET between QDs and AuNPs; fluorescence emission upon glucose displacement of AuNPs	0.05 μM [80]	Control for aggregation issues in physiological fluids at varying pH and temperature ranges

GOx: glucose oxidase; **ZnO:** zinc oxide; **QD:** quantum dots; **SPR**: surface plasmon resonance; **AgNPs:** silver nanoparticle; **GBP:** glucose binding protein; **FRET:** förster-resonance energy transfer; **ConA:** concanavalin A; **AuNP:** gold nanoparticle