Biosensors and Bioelectronics 182 (2021) 113193

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: http://www.elsevier.com/locate/bios

Exhaled breath biomarker sensing

Alina Vasilescu [a,*], Borys Hrinczenko [b], Greg M. Swain [c,d], Serban F. Peteu [c,**]

[a] International Centre of Biodynamics, Bucharest, Romania
[b] Division of Hematology & Oncology, Breslin Cancer Center, Michigan State University, USA
[c] Department of Chemistry, Michigan State University, USA
[d] Neuroscience Program, Michigan State University, USA

ARTICLE INFO

Keywords:
Exhaled breath condensate
Biomarkers
Biosensors
Chemical sensors

ABSTRACT

This goal of this minireview is to introduce the reader to the area of research concerned with exhaled breath analysis for the purpose of detecting abnormal levels of physiologically-relevant chemical markers reflective of respiratory diseases. Two main two groups of sensing methods are reviewed: mass spectrometry and (bio)sensors. The discussion focuses on biosensor applications for EB and EBC analyses, which are presented in detail. The review finishes with conclusions and future perspectives, including recommendations for future near-term and long-term development of EBC biomarker sensing.

1. Introduction: What is exhaled breath (EB)?

The exhaled breath (EB) offers the most accessible biological samples for diagnostic analysis, since it is released spontaneously and in large quantities: approx. 10,000 L of air is inhaled and subsequently exhaled by an individual each day. The pathway of inhaled and exhaled molecules in the human body is illustrated in Fig. 1.

The oxygen/carbon dioxide respiratory cycle is the pulmonary gas exchange. Except for N_2 (78.04%), O_2 (16%), CO_2 (4–5%), and other inert gases, the exhaled breath (EB) contains water vapors and hundreds of different trace compounds of exogenous and endogenous origin in ppb to ppm levels (Das and Pal 2020).

An important distinction is noted between the parts of the exhaled breath deemed as “dead space air” (from mouth, trachea, bronchi) and alveolar air (from the lungs, reflecting the gas exchange with the blood) (Wallace and Pleil, 2018). The different composition according to the origin of VOCs sample in EB is relevant for specific diseases.

EB can be partly cooled and condensed into a liquid phase. This is referred to as “exhaled breath condensate” (EBC) which “simplifies the breath print”, since is constituted exclusively by water soluble volatiles and non-volatile compounds (Kuban and Foret, 2013). The analysis of EBC and VOCs from EB are thus complementary. Besides VOCs and EBC, exhaled breath aerosols (EBA) have been also used as a sampling media for breath analysis (Wallace and Pleil, 2018), particularly useful for larger analytes such as particles, viruses, bacteria, proteins or fatty acids. EBA constitutes a part of EBC, it does not require freezing therefore is simpler to analyze. The particles and aerosol-carried compounds are trapped on filters as a person is required to breath while wearing a mask and the filters are analysed afterwards. While intensively pursued, EBA analysis is not as advanced in terms of standardized sampling procedures and applications as EBC. In the following, the discussion will concentrate mainly on EB and EBC. More details on the different breath matrices and their analysis are found in comprehensive reviews such as the recent one by Wallace and Pleil (2018).

Compared to clinical blood and urine tests, EB monitoring offers several advantages, it is noninvasive, easily repeatable, has a very low infection risk, and is convenient for long-term clinical monitoring.

A brief historical perspective reveals that Linus Pauling is considered the father of modern breath analysis. In 1971, he analyzed frozen breath with gas chromatography and could differentiate more than 250 volatile features (Pauling, L., et al., 1971). Before him, one could mention Chinese medicine in antiquity whereby diseases were classified based on different odors (Maciocia, G., 2016). In addition, the Greek physician Hyppocrates cited characteristic odors in several types of medical imbalances/conditions: sweet (diabetes), fishy (liver problems), urine-like (kidney problems), or a putrid smell (lung abscess) (Neuburger, M., 1906). The frenchman Lavoisier is credited with one of the first laboratory approaches, when he described breath as a chemical reaction of respirable air (O_2) producing acid forming fixed air (CO_2) (Karamanou, and Androutsos, 2013).

* Corresponding author.
** Corresponding author.
E-mail addresses: avasilescu@biodyn.ro, amvasilescu@yahoo.com (A. Vasilescu), peteu@msu.edu, serbanfpeteu@gmail.com (S.F. Peteu).

https://doi.org/10.1016/j.bios.2021.113193
Received 1 June 2020; Received in revised form 3 March 2021; Accepted 19 March 2021
Available online 26 March 2021
0956-5663/

2. Biomarkers in EB and EBC

2.1. Biomarkers in EB

The exhaled breath contains a large number of volatile organic compounds (VOCs). One study identified 872 volatile organic compounds in the breath of healthy people (De Lacy Costello et al., 2014). These VOC's belonged to different classes of compounds including hydrocarbons, alcohols, aldehydes, ketones, esters, carboxylic acids, furans, ethers, chloro-biphenyls, sulfur-containing compounds, halogen-containing compounds, nitrogen-containing compounds etc. Another study reported a total of 3481 compounds detected in the breath upon screening 50 healthy people. Only a small number of volatiles (27) were found to be common to them all and therefore thought to reflect metabolic processes in the human body (Phillips et al., 1999).

Among VOCs considered as biomarker candidates for different medical conditions, the most important are ammonia, acetone, isoprene, nitric oxide, hydrogen sulfide, methane, ethane, pentane, and aldehydes, such as acetaldehyde, nonanal, and malondialdehyde. While there are no specific VOC biomarkers for a particular type of cancer, the levels of a series of volatiles in EB are significantly different in cancer patients compared to healthy people and can define disease-specific patterns or "breath print" (Altomare et al., 2020; Töreyin et al., 2020; Dent et al., 2013; Hakim et al., 2011).

Acetone concentrations in EB and served as an indicator of diabetes (Petters, 1857) and reflected the lipid metabolism. Methods for determining acetone in the breath of diabetic people were reported as early as 1898. In diabetic patients, insufficient amounts of insulin in the body trigger the production of energy from fat, instead of from glucose. In this process, acetone is produced from fatty acids by ketogenesis. Acetone concentrations in blood and in the breath are linearly correlated, with the levels in EB around 330 times lower than the concentrations in blood (Das and Pal, 2020). While for healthy people the level of acetone in breath is under 0.9 ppm, in diabetics the concentration can rise to tens of ppm (Das and Pal, 2020). Breath acetone concentration also increases with physical exercise, fasting and with a high fat/ketogenic diet.

Along with acetone, **isopropanol** levels increase in the EB of people suffering from ketoacidosis. In one recent study they found 85.44 ppb of IPA on average in a group of diabetic patients after a ketogenic diet, as compared to 17.99 ppb in the EB of healthy people (Li et al., 2017). IPA was thus proposed as a biomarker for the non-invasive diagnosis of diabetes via breathomics, even though the metabolic pathway for IPA formation is unclear. It has been suggested that IPA may form from acetone via the reverse reaction catalyzed by alcohol dehydrogenase, stimulated by elevated acetone levels produced by the beta-oxidation of fatty acids in the liver (Li et al., 2017). Besides diabetes, higher concentrations of IPA in breath were found in people suffering from liver diseases (Hanouneh et al., 2014) pulmonary arterial hypertension (Cikach Jr. et al., 2012) and lung cancer (Koureas et al., 2020).

Isoprene is formed during the production of cholesterol and is an indicator of lipid metabolism. Typical concentrations in the breath of healthy people are around 105 ppb (Das and Pal, 2020).

Hydrocarbons, such as methane, ethane and pentane, are biomarkers for asthma, breast cancer, liver diseases, intestine and colon related disease. Methane levels in breath can indicate carbohydrate malabsorption. Methane is produced by methanogenic bacteria in the gut of people suffering from inflammatory bowel disease, obesity or anorexia (Das and Pal, 2020).

Hydrogen together with methane, are indicators of carbohydrate maladsorption and small intestinal bacterial overgrowth (Rezaie et al., 2017).

Hydrogen sulfide levels in healthy people are in the range of 8–16 ppb and increase in the EB of people suffering from asthma, dental disease, oral malodor, and airway inflammation (Das et al., 2020).

Nitric oxide levels in the breath of healthy individuals are below 25 ppb, increased concentrations are found in the EB of individuals suffering from asthma, COPD, and other lung diseases (Das and Pal, 2020). Exhaled nitric oxide ("FeNO") is a model biomarker, whose determination in breath reached practical importance in clinical practice. Commercial devices measuring FeNO can also be applied to detect

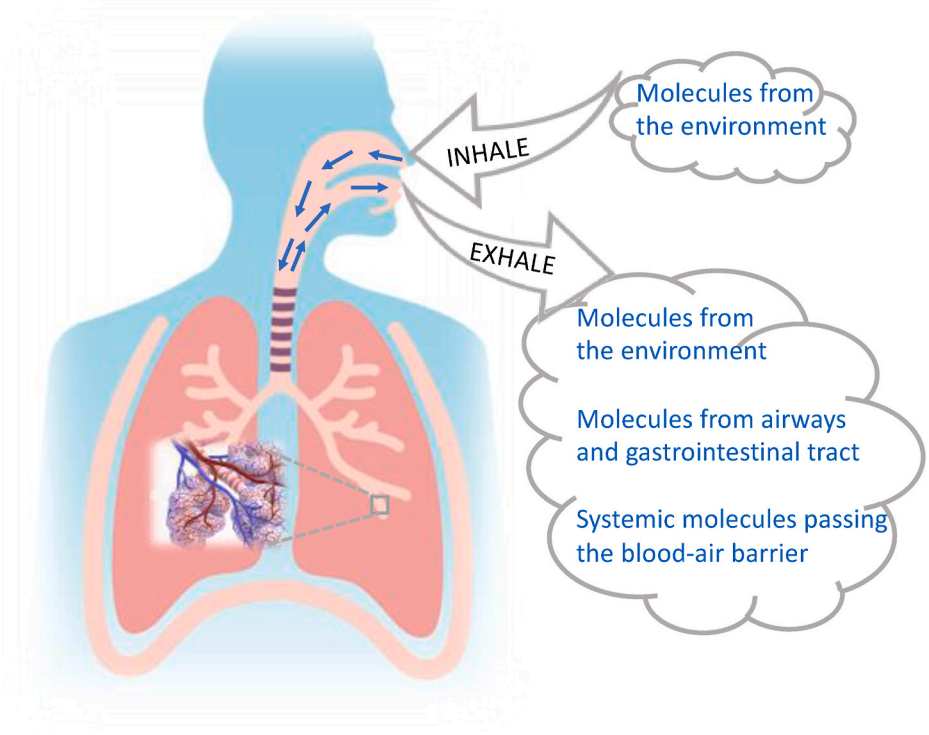

Fig. 1. The molecules from the environment are inhaled. The path of exhaled molecules in the body originates partly from the airways, gastrointestinal tract, or the whole body (i.e., systemic molecules passing the blood–air barrier in the lungs).

changes in the airway pH (Davis et al., 2013), which is relevant for medical conditions affecting the pulmonary tract.

Ammonia is a biomarker of renal failure, liver disease, peptic ulcers and halitosis. In the breath of healthy people, the concentration of ammonia is in the range of 425–1800 ppb (Das et al., 2020). Excess ammonia in the human body is eliminated through the urea cycle via the kidney and the liver. Consequently, higher levels of ammonia are indicative of dysfunction of either organ. Increased ammonia concentrations in breath were also detected in people suffering from pulmonary arterial hypertension (Cikach et al., 2012).

Hydrogen peroxide is known as a biomarker of oxidative stress. Breath concentrations of H_2O_2 are increased in patients with COPD (Das and Pal, 2020).

Carbon monoxide was measured in the EB as a marker of inflammation (Ryter et al., 2013) and as an indicator of hemolysis in sickle cell disease (Sylvester et al., 2005). Measurements of exhaled CO are successfully used for identifying smokers (Hung et al., 2006; Vasthare et al., 2018).

Exhaled **carbon dioxide** is useful to monitor ventilation, respiratory status and the efficacy of cardiac compressions in CPR (Siobal, 2016).

Ethanol concentrations in EB are proportional to its levels in the blood, and the use of breathalyzers for screening drivers has been well established for many years. Most of the ingested alcohol (95%) is metabolized in the liver. A portion of alcohol, carried by blood into the alveolar sacs in the lungs, is expelled in the exhaled breath. In addition, the analysis of breath ethanol is important for forensic purposes, in the diagnosis of metabolic diseases and in the monitoring of medical treatments.

Acetaldehyde is the first metabolite produced from ethanol during its metabolism in the liver, in a reaction catalyzed by alcohol dehydrogenase. It is also produced by microbes in the gastrointestinal tract and by oral microflora. Typical acetaldehyde concentrations in the breath of healthy people who have not consumed alcohol were 0.2–0.6 nmol L^{-1} (5–15 ppb) with higher levels found in smokers (Jones, 1995). The acetaldehyde concentrations increased after ingesting alcohol and peak concentrations of 200 and 500 nmolL^{-1} (5–12.6 ppm) were found. After drinking alcohol, the breath of people with ALDH2 polymorphism, or in individuals taking aldehyde dehydrogenase-inhibiting drugs showed acetaldehyde levels up to 1300 nmolL^{-1} (32.8 ppm, Jones, 1995).

Increased pentanal, hexanal, octanal and nonanal levels in breath were measured in lung cancer patients (Fuchs et al., 2010). More recently, a study focusing on the breath VOC profile of Covid patients reported higher acetaldehyde and octanal levels compared to healthy people, accompanied also by an increase of acetone and butanone and by a lowered concentration of methanol (Ruszkiewicz et al., 2020). Other studies reported methylpent-2-enal and nonanal (along with 2, 4-octadiene and 1-chloroheptane) among the most significant biomarkers differentiating Covid sufferers from other patients with acute respiratory diseases (Grassin-Delyle et al., 2021).

2.2. Biomarkers in EBC

EBC contains more than 99.9% water and water-soluble small non-volatile compounds, proteins, surfactants, macromolecules, bacteria, viruses and fungi (Horváth et al., 2005) (Kubáň and Foret 2013). As summarized by (Horváth et al., 2005; Kubáň and Foret 2013), the group of ions and small molecules in EBC include: Na^+, K^+, Cl^- ions, urea, together with prostanoids, leukotrienes, (involved in the inflammatory response), isoprostanes (8-iso-prostane in particular, marker of oxidative stress, relevant in lung diseases) glutathione (marker of oxidative stress), H_2O_2 (increased levels in inflammatory respiratory diseases (Teng et al., 2011)), reactive nitrogen species, such as nitrite, nitrate, 3-nitrotyrosine (which increase in asthma, COPD and cystic fibrosis), ammonia (smaller levels in asthma), adenosine (relevant in asthma and allergic rhinitis and malondialdehyde (formed by lipid peroxidation, a direct result of ROS in oxidative stress and potential biomarker in chronic obstructive pulmonary disease (COPD) (Corradi et al., 2003).

Proteins are present in trace quantities, totaling around 1μgmL^{-1}, thus, the determination of putative protein biomarkers in EBC poses some analytical challenges. The majority of proteins in EBC are cytokeratins. EBC proteomics is much less investigated compared to the analysis of small molecules, yet it is a very promising field of research for the discovery of novel disease biomarkers (Sun et al., 2019). Cytokins such as interleukins (IL), vascular endothelial growth factor (VEGF), and tumor necrosis factor α (TNF-α), representing non-specific markers of inflammation and are contained in EBC in the pgmL^{-1} range and were detected in higher concentrations in non-small cell lung cancer. The concentration of carcino-embryogenic antigen (CEA) in EBC was suggested as a potential biomarker for in the early diagnosis of non-small cell lung cancer (NSCLC) (Zou et al., 2013).

Presently, the detection of SARS-Cov 2 virus in the EBC is being intensively investigated due to the findings that EBC might provide more accurate detection, i.e. less false negatives, compared to nasal swabs (Ryan et al., 2021). Many teams are rushing to develop rapid cost effective tests and breath analyzers for the diagnosis of the Covid 19 infection Ryan et al. (2021); Shan et al., 2020 (The Sage Group 2021; DiagMetrics, 2021).

3. Medical conditions and diseases addressed by EB and EBC

Gastro-esophageal reflux. EBC has been studied in asthmatic patients with GERD (gastroesophageal reflux disease) in order to assess airway acidification. When gastric acid refluxes up toward the pharynx, acid enters the airways causing epithelial damage and therefore worsens asthma symptoms.

Cardiovascular. Bycova et al. (2019) studied several molecular compounds whose content varies in cardiovascular disease: pentane, isoprene, carbon monoxide, and trimethylamine.

Diabetes. Pamidipani (2010) investigated the direct measurement of glucose in EBC and noted the presence of a functional relationship between glucose in EBCs and also in blood. A very sensitive fluorometric assay was used to analyze and measure the low glucose concentrations in the EBC samples. This work describes a larger investigation into the feasibility of developing of a new, noninvasive glucose measuring device using EBC. A human subject study (Pamidipani, 2010) found that EBC glucose levels significant increased as blood glucose levels increased, and that also the level of blood glucose levels, the rate of breathing, as well as subject to subject variability, were significant factors in influencing EBC glucose levels. These results and others provide the motivation to continue investigations in the application of glucose EBC measurements in diabetes.

Respiratory Diseases (asthma, COPD, cystic fibrosis, pneumonia, sleep apnea, cancer). EBC is the natural matrix of the respiratory tract. Chronic respiratory diseases (CRDs) are complex multifactorial disorders involving the airways and other lung structures. The development of reliable biomarkers for early and accurate diagnosis, including disease phenotype, prediction of treatment response and adherence, are essential for the correct management of CRDs.

Concentrations of drugs in the lungs are difficult to measure, resulting in relatively unknown local pharmacokinetics. Kruizinga et al. (2020) studied the potential of EBC as a matrix for pharmacokinetic analysis of inhaled and also intravenous medications.

Drug pharmacokinetics. Although the airway surface is the anatomic target for therapy for many lung diseases however measuring drug concentrations and activities on these surfaces pose considerable challenges. Esther et al. (2014) reported that mass spectrometric analysis of the EBC could be utilized to non-invasively measure airway drug pharmacokinetics and to predict pharmacological activities.

4. Exhaled breath (EB) and exhaled breath condensate (EBC) collection methods

Exhaled Breath samples from humans or other mammals can be analyzed by two main methods– directly biosensing VOCs from EB, or instead cooling EB to obtain an EBC. A third, simple alternative is to collect EBA, by capturing aerosols on filters/surfaces at room temperature; however, the range of analytes in this case is mostly limited to particles and the largest compounds from EBC. EBC reflects a part of the EB, as it consists in 99.9% water and a matrix of water-soluble multiple biomarkers, similar to what might be found in blood, urine and the analysis of gases from EB.

The EB was either analyzed directly or more often was collected in various sampling bags or by using solid phase microextraction, sorbent traps etc (Das and Pal 2020; Wallace and Pleil, 2018). Storage time and other conditions (e.g., temperature) greatly influence the sample composition, thus sample degradation has to be prevented.

For EBA, the Gesunheit II device developed at Harvard University (USA), the SKC BioSampler (SKC Inc.,USA) and the ExaBreath sampler (SensAbues AB, Sweden) are examples of sampling devices that were used in studies analyzing the influenza virus and drugs, respectively from exhaled breath (Wallace and Pleil, 2018). These add to a series of breath aerosol collecting devices in development (Wallace and Pleil, 2018).

EBC is obtained as breath is exhaled from the lungs into a cooled collecting device, thereby condensing the vapor and also the aerosolized droplets emerging from the breath. The steps involved in using a device such as the R-tube are: 1-condense EB using the R-Tube; 2-store/transport the EBC; 3-use the Plunger to collect the EBC into a pool of liquid at the top of the R-tube; 4-analyze the EBC sample. The parts of the device are shown in Fig. 2.

There are currently several commercially available EBC collection devices with their advantages and drawbacks, see Table 1.

Additionally, various new technologies for EBC collection are developed as result of Covid 19 pandemic, targeting the efficient capture of viral particles from the exhaled breath. These include new technologies for the condensation of environmental water, e.g continuous dropwise condensation or the handheld devices allowing sampling and analysis "on the spot", such as Coronacheck® by Exhalation Technology (UK), built on the existing Inflammacheck® technology and seeking regulatory approval in EU and the UK.

Fig. 2. The parts of the R-tube (from Respiratory Research website, by permission).

5. EB and EBC sensing methods

Non-invasive EB and EBC analysis both have a huge diagnostic potential. From the discovery and validation of biomarkers to non-invasive monitoring of disease and also treatment progression, a whole realm of analytical possibilities exists. In targeted applications, dedicated sensors are used to monitor a specific compound and thus can be achieved with low-cost optical or chemical sensors. At the other end, in untargeted analysis, data is collected and analyzed to potentially discover new biomarkers or specific patterns indicative for certain health conditions. This corresponds to a complex, large pool of mass spectrometry data. The challenges of instrumentation, time, costs for data interpretation and validation, are different and are discussed below.

The analysis of gaseous biomarkers from EB poses specific experimental challenges and depending on the method, can be conducted at higher temperatures. EBC as a liquid matrix containing the water-soluble components from EB poses less difficulties, however its very variable dilution factor has to be considered in the measurements.

5.1. *Mass –spectrometry methods and sensors*

EB and EBC analysis is a rapidly developing field, fueled among other reasons by the current circumstances of the COVID-19 pandemic, which emphasized the global need for fast and noninvasive diagnostic methods. Various mass-spectrometry, sensors and biosensor-based methods have been described for EB and EBC analysis as summarized by recent reviews (Bruderer et al., 2019; Das and Pal 2020; Gaffney et al., 2020; Zhou et al., 2020).

Mass-spectrometry detectors enable unprecedented resolution in the analysis of complex samples, delivering a high power of identification and increased selectivity. This also leads to a much higher complexity of the data collection and interpretation and the necessity for validated libraries to enable accurate identification. Mass-spectrometry detectors have been interfaced with gas-chromatography (GC) for the off-line analysis of breath and with selected ion flow tube mass spectrometry (SIFT), proton transfer reaction mass spectrometry (PTR) and secondary electrospray ionization – (SESI) for on-line breath analysis (Bruderer et al., 2019). The limits of quantification for these methods are in the hundreds of pptv for SIFT-MS, even in the sub-ppqv range in the case of polar compounds analyzed by SESI-MS. Mass resolutions of $m/\Delta m = 10000$ achieved by PTR-TOF or even higher than 140000 reached with SESI-MS/MS enable unparalleled compound identification (Bruderer et al., 2019). Due to their resolution, accuracy, robustness and sensitivity, these mass-spectrometry methods have found many applications in EB analysis for studying the pathogenesis of different diseases, and also in screening patients and monitoring treatment efficacy. As details on these powerful analytical methods and their applications for EB and EBC analysis are beyond the scope of the current review, the readers are referred to recent reviews on this topic (Bruderer et al., 2019; Hayes et al., 2016; Wallace and Pleil, 2018). Of note are, however, the recent intensive efforts towards the application of GC with MS or ion mobility spectrometry detection, coupled with multivariate analysis for analysing the breathprints of Covid 19 patients for diagnosis, biomarker discovery and differentiation from other severe acute respiratory diseases (Grassin-Delyle et al., 2021; Ruszkiewicz et al., 2020).

As alternatives to the laboratory-based, complex and costly methods relying on mass spectrometry-relying procedures, various types of methods, more compatible with portable and miniaturized devices have

Table 1
EBC collection devices commercially available.

Device Brand	Maker	Advantages	Drawbacks
ALFA	Respiratory Research US	Collects EBC continuously throughout the course of ventilation. Gas standardizes and measures the EBC pH continuously. Compatible with most ventilators. Has both non-disposable and disposable parts.	Cited in fewer publications. Complex system, so requires a very skilled user. Only able to collect EBC at the exhaust port of the ventilator.
RTube		Overall, the preferred EBC collector. Multiple collections are possible concurrently. Disposable (no cleaning between patients). Useable with a standard freezer, enabling home use.	Maintaining a set condensing temperature requires an optional cooling unit. Otherwise, the condensation temperature is chosen by using a cooling sleeve prep temperature that increases during the collection.
RTube Vent		Can be utilized in-line with ventilator circuit/expiratory port.	
Eco-Screen	Carefusion Europe	Most frequently utilized EBC collection device in European centers. Optional package for determination of total exhaled volume. Has been used to collect EBC during mechanical ventilation.	Not readily portable. Cleaning between patients should be extensive to abide by standard respiratory care. Limited ability to control condensation temperature. No longer distributed in the US.

been developed for the analysis of EB and EBC.

However, these methods have lower sensitivities and variable selectivity (Bruderer et al., 2019; Das and Pal 2020; Zhou et al., 2020; Wallace and Pleil, 2018). The vast majority of these methods feature sensors based on semiconductor oxide, graphene and carbon nanotubes coupled with electrochemical, optical or acoustic analysis (Das and Pal, 2020). The main identified challenges are related to: the standardization of the sampling methods, solving interferences such as due to the moisture of EB (Das and Pal 2020) or from other volatiles in EB and EBC, and comparing the sensors with standard methods. These add to the issues of identifying and confirming specific compounds in EB and EBC as biomarkers for certain diseases, establishing disease-specific thresholds, and correlating breath levels with blood concentrations of biomarkers.

Metal oxide semiconductor (MOS) behaving as n-type or p-type conductors at high temperatures can sense traces of gaseous analytes by changes in their resistance. For example, ammonia was measured in the range from ppt to hundreds of ppm (Singh et al., 2017; Das and Pal, 2020) with MoO_3 and WO_3 based sensors, while WO_3 –based MOS detect NO at ppb level and CuO-based sensors determine trace H_2S at ppb levels. Acetone was most sensitively determined at tens of ppb levels with Pt-functionalized SnO_2, as summarized in (Das and Pal, 2020). The major parameters determining the sensitivity of the semiconductor metal oxide sensors are particle size, morphology, porosity, film thickness, deposition of noble metals on their surface and operating temperature. The selectivity is determined by the operating temperature but also by parameters such as doping, crystalline phase, composition, acid-base interactions, molecular filtration, or re-formation of the VOC within the sensing layer (Das and Pal 2020).

Carbon nanotubes and graphene act as p-type semiconductors and their high surface to volume ratio makes them attractive for the detection of VOCs. Compared to MOS, carbon nanomaterials/nanocomposites and semiconductor chalcogenides enable the detection of traces of VOC at lower temperature and are suitable for flexible sensors such as those needed in wearable devices (Das and Pal, 2020). Proof -of-concept chemoresistors incorporated in face masks for the detection of ethanol or formaldehyde has been described. They rely on multiwall carbon nanotubes (CNT)-polyaniline composites and on single wall CNT, respectively as sensing materials deposited on Nylon fibers (Bag and Lee 2021). However, they need further validation and addressing the effects of temperature and humidity.

Electrochemical sensors are frequently used to measure exhaled NO (Maniscalco et al., 2016; Obermeier et al., 2015), CO or low weight aldehydes (Obermeier et al., 2015), hydrogen peroxide (Maier et al., 2019), ethanol (Ozoemena et al., 2018) and volatile sulfur compounds (VSCs, hydrogen sulfide, methyl mercaptan and dimethyl sulfide (Kim et al., 2010) at ppb levels. To give one example, commercial voltammetric sensors (e.g., the Halimeter® Interscan Co, USA, introduced in 1997), can measure levels as low as 5 ppb of VSCs in 1 s and were used in clinical studies for the detection of halitosis (Kim et al., 2010). The analysis of nitrite in EBC was also reported with electrochemical sensors (Gholizadeh et al., 2017).

Besides electrochemical devices, many other types of chemical sensing modalities have been used for analyzing biomarkers in EB and EBC, including quartz crystal microbalance (QCM), surface acoustic wave (SAW) based sensors, field-effect transistors (FET), and various optical sensors, detailed among others by Das and Pal (2020), Zhou et al. (2020) and Bruderer et al. (2019).

Specifically for the analysis of EB, nanomaterials and their combinations provided multiple possibilities not only for enhancing the analytical signal but also for capturing more efficiently the VOCs more efficiently on the sensor surface (Qiao et al. 2018, 2019; Zhou et al., 2020). One illustrative example is a SERS sensor for the detection of aldehydes relevant to lung cancer, where Ag nanowires enhanced the intensity of the Raman signal while hollow Co-Ni layered double hydroxide nanocages assembled at the surface of the Ag wires contributed to capturing the volatile analytes (Qiao et al., 2019). The nanocages with a pore size of 15 nm greatly influenced the flow dynamics of the gaseous analytes, by enabling a more tortuous, longer trajectory towards the sensor surface thus providing longer interaction time with the SERS reporter p-aminothiophenol.

Selectivity was typically achieved by (i) modifying the surface with a chemical compound (ligand) binding preferentially the compound of interest and thus diminishing the interference from the other components of the EB, (ii) targeting an analyte present in excess compared to the potential interferents, (iii) applying multivariate analysis methods to isolate the specific contribution of the targeted compound or a combination of these approaches.

In addition to targeted analysis that relies on highly selective sensors for measuring specific biomarkers, electronic noses (e-noses) and arrays of QCM, SAW and colorimetric sensors have also been developed for capturing specific patterns indicative of respiratory disease, inflammatory diseases, different types of cancer, infectious diseases, cystic fibrosis (Farraia et al., 2019; Das and Pal 2020) among others. The VOC profile is compared to those pre-recorded for the people confirmed with the respective disease using various data interpretation algorithms to decide on the positive/negative diagnostic.

E-nose systems consisting in a set of low selectivity sensors coupled with a pattern recognition software, have demonstrated great potential as complementary diagnostic (Farraia et al., 2019) or pre-operative patient screening methods (Wintjens et al., 2020). They are cost-effective, simple to use, portable devices providing fast response (in under 2 min), the whole analysis process – including data interpretation – being performed in less than 15 min. These advantages are an incentive for their application for large scale traveller screening (e.g. the GeNose system for Covid 19 detection used in train stations in Jakarta, Indonesia at the end of 2020).

In one recent illustration of untargeted analysis, an array combining 8 chemiresistive sensors made from 3 to 4 nm Au nanoparticles linked to different organic ligands (Shan et al., 2020). captured the “breathprints” of 130 people, including healthy people, Covid patients and patients suffering from lung diseases other than COVID. By applying

discriminant analysis to the acquired breathprints, the authors noted a 76% accuracy of their method with regards to the evaluation of Covid patients/healthy people and a 95% accuracy in discriminating people with COVID-19 from those with other types of lung infections. These results emphasize the main challenges of the untargeted analysis systems for EB: standardized sample collection and analysis, increased accuracy and discriminating a specific disease from similar conditions (Farraia et al., 2019). To reach the sensitivity and specificity goals, calibration with a large representative set of samples tested in parallel by a standard reference method, larger clinical validation, selecting more variables/sensors and validated data interpretation algorithms are needed (Wintjens et al., 2020), as well as advances in sensor development.

Encouragingly, significant advances with respect to sample collection and data analysis were registered as result of the race to develop fast methods for the large-scale testing of infection with SARS-COV-2, e.g. aimed at applications such as traveller screening in airports or train stations. Many such untargeted detection methods are in development around the world, combining (1) devices for the fast breath sampling with (2) analysis by portable mass-spectrometers or e-nose systems and (3) various algorithms for recognizing the pattern of the VOCs of COVID infection in EB. In this context it is anticipated that solving breath sampling, standardization, calibration and stability issues will be fast tracked and adopted for the detection of other VOC biomarkers for wider diagnostics capabilities.

5.2. EB and EBC biosensing

Being based on combining a biorecognition event with a sensitive transducer, biosensors enable additional selectivity to the detection of various analytes compared to classic sensors and presume much lower costs and complexity compared to mass spectrometry methods.

These biosensors are based on enzymes or antibodies as biorecognition elements and use optical, electrochemical, piezoelectric or acoustic detection methods as summarized in Table 2.

Nevertheless, compared to other methods for analyzing biomarkers in EB and EBC, the number of biosensors developed so far remains very small, mainly due to the insufficient data on the stability of biological elements to warrant commercial applications and to the challenges of developing fast sampling devices that can be interfaced with biosensors. As the storage stability of biosensors was unfortunately rarely reported – mostly in older studies – it was not included in Table 2. Despite the unsolved stability issues, there are significant potential advantages in selectivity offered by enzymes, antibodies etc. compared to semiconductor oxide or carbon allotropes-based sensors. Enzymes from different sources have different substrate selectivity. The substrate preference can be altered by direct engineering. Enzymes from extremophilic microorganisms, thermophiles in particular have significantly better stability at ambient temperature compared to the mesophilic counterparts. Consequently, there is a potential for further improvement with regards to enzyme selectivity and stability. The biosensors for EB and EBC analysis are discussed in more detail in the following sections.

5.2.1. Analysis of EB

Compared to the analysis of liquids, the detection of gaseous analytes with biosensors poses significant additional challenges, mainly related to ensuring the appropriate hydration of the biorecognition element for sensitive and reproducible functioning, accounting for the biosensor's dependence on temperature, ensuring an appropriate stability. The experimental setup and the calibration using gaseous standards are also more complicated.

The development of biosensors for the detection of analytes in the gas phase stretches back to the 1980's when piezoelectric enzyme biosensors for formaldehyde (Guilbault, 1983) and immunosensors for parathion (Guilbault and Luong 1988) were first reported. The first amperometric enzyme-based biosensor for ethanol detection in the gas phase was reported in 1995 (Park et al., 1995) and targeted the detection of ethanol in breath. It used carbon screen printed electrodes modified with a printed coating layer containing alcohol dehydrogenase and the enzymatic cofactor NAD^+. Many of the biosensors for the analysis of target compounds in the gas phase were developed more than 20 years ago, nevertheless the research continued and gained attention in the past five years. The applications targeted with these biosensors are quite diverse, from analyzing air quality by determining toxic gases such as formaldehyde (Kudo et al., 2013; Achmann et al., 2008; Guilbault 1983), phenol (Kaisheva et al., 1997), acetone (Bohrn et al., 2013) etc, to detecting nerve gases for military applications (Arduini et al., 2007), volatile alcohols from fruit juice (Hämmerle et al., 2011), detecting the contamination of fruits and vegetables by fungi or bacteria (Schütz et al., 1999; Wang et al., 2019), measuring the oxygen level in packaged vegetables as an indicator of freshness (Gardiol et al., 1996), visualizing ethanol vapor emitted by the palm of the hand of people after drinking (Iitani et al., 2020) or visualizing the spatial distribution of ethanol vapors concentrations emitted by wine, in relation to the shape of the glass (Arakawa et al., 2015).

These biosensors rely on enzymes, intact insect antennae (Schütz et al., 1999) or odorant binding proteins (Zhao et al., 2012), DNA (Wang et al., 2019), whole cells (Naessens and Tran-Minh, 1998; Bohrn et al., 2013), genetically engineered bacteria (Gil et al., 2002) or artificial metalloenzymes (Vong et al., 2019) for specific recognition. The detection modes include electrochemical (amperometry by Achmann et al. (2008) or Hämmerle et al., (2011), impedance spectroscopy (Kaisheva et al., 1997), optical (colorimetric by Monkawa et al. (2015), fluorescence (Vong et al., 2019; Iitani et al., 2020), bio- and chemiluminescence (Gil et al., 2002; Arakawa et al., 2015) piezoelectric (Guilbault, 1983) and acoustic methods (Zhao et al., 2012).

The experimental setup for gas-phase analysis, appropriate calibration procedures to account for the effect of temperature on biosensor's performance and ensuring selectivity are ~~other~~ critical aspects to consider in the development of gas-phase biosensors. As the same challenges also apply to the analysis of EB, there are many lessons to be learned for improving EB biosensing from previous research on gas-phase biosensors. A significant contribution to this field of research in the past few years was done by Kobayashi's group, whose work was in part devoted to the development of fluorometric enzyme-based biosensors for the analysis of breath biomarkers such as isopropyl alcohol, acetone, acetaldehyde and ethanol (Chien et al., 2017a, 2017b; Ye et al., 2015; Iitani et al., 2018; Mitsubayashi et al., 2005).

Ethanol. The most extensive research efforts were dedicated to detecting ethanol in exhaled breath, aiming for the fast screening of drivers, to deter driving under the influence of alcohol or, more recently to study the pharmacokinetics of ethanol in relation to ALDH 2 polymorphism. Mono-enzymatic biosensors based on alcohol oxidase or alcohol dehydrogenase with a different design enabled the detection of ethanol down to 1 ppm and even to 0.1 ppm by amperometric (Mitsubayashi et al., 2005) and fluorometric detection (Iitani et al., 2019b) respectively. These devices were complemented by a several biosensors using alcohol oxidase and peroxidase, involving also the addition of an electrochemical mediator (e.g. ferrocyanide) or luminol to achieve amperometric and chemiluminometric detection, down to 20 ppm (Motooka and Uno 2018) and 10 ppm (Wang et al., 2011), respectively. Using more reagents did not improve the detection limit for ethanol vapor, nevertheless the studies with bienzymatic sensors brought out some advantages as discussed in the following.

Amperometric enzyme biosensors provide simple, fast, and cost-effective detection of breath alcohol with miniaturized portable equipment. For calibration, ethanol solutions of known concentrations were purged with N_2 to produce ethanol vapor and were then introduced in a flow-through measurement cell (Park et al., 1995) (Fig. 3 A). In other setups, either a gas.

generator was used to produce standard samples of ethanol vapor of

Table 2
Examples of biosensors for the detection of biomarkers in EB and EBC.

Analyte	Biorecognition element	Detection method	Dynamic/linear range (LR) and detection limit (DL)	Selectivity	Appli-cation	Refe-rence
Ethanol	Alcohol dehydro-genase	Electrochemical	LR up to 250 ppm (conti-nuous flow method); up to 800 ppm (syringe injection method).	Human breath samples from healthy volunteers are not interfering	EB	(Park et al., 1995)
Ethanol	Alcohol dehydrogenase	Electrochemical	20–800 ppm	No responses to ammonia, acetaldehyde, methyl mercaptan, acetone, carbon monoxide and tobacco smoking or the breath of healthy people that did not drink alcohol	Breath	(Park et al., 1999)
Ethanol	Alcohol oxidase	Electrochemical	LR: 15.7–1232 ppm DL: 1.57 ppm	n-pentane, benzene, methyl ethyl ketone, n-hexane, acetone not interfering	Ethanol vapor	Mitsu-bayashi et al. (1994)
Ethanol	Alcohol oxidase	Electrochemical	1–500 ppm	Reference to previous study (Mitsubayashi et al., 1994)	Measure-ment in breath after drinking	(Mitsu-bayashi et al., 2005)
Ethanol	Alcohol oxidase and peroxidase	Electrochemical	50–500 ppm	Not investigated	Ethanol vapor	Kawa-hara Shogo (2017)
Ethanol	Alcohol oxidase and peroxidase	Electrochemical	DL: 20 ppm LR: up to 150 ppm	Not investigated	Ethanol vapor	Moto-oka and Uno (2018)
Ethanol	Alcohol oxidase and peroxidase	Electrochemical	50–500 ppm	Not investigated	Ethanol vapor	Kure-take et al. (2017)
Ethanol	Alcohol dehydrogenase	Fluorescence	0.1–1000 ppm	Acetaldehyde, methanol, IPA, acetone, methylmer-captan do not interfere	EB of people with ALDH2 (+) and ALDH2 (-)	Iitani et al. (2019a)
Ethanol	Alcohol oxidase and peroxidase	Chemiluminescence	30–400 ppm DL: 10 ppm	Methanol interferes but typical levels in EB are low compared to ethanol; acetaldehyde, trimethylamine, acetone, methyl mercaptan, dimethyl sulfide don't interfere	EB of people with ALDH2 (+) and ALDH2 (-)	Wang et al. (2019)
Isopro-panol	secondary alcohol dehydro-genase	Fluorescence	1−9060 ppb	2-butanol interferes; methanol, ethanol, 1-propanol, 1-butanol, acetaldehyde, acetone and 2-butanone not interfering	Breath samples, healthy and diabetic people	Chien et al. (2017a)
Isopro-panol	secondary alcohol dehydro-genase	Fluorescence of NADH	1–9060 ppb	2-butanol interferes; 1-butanol, 1-propanol, ethanol, acetaldehyde, methanol, 2-buta-none and acetone not interfering	Breath samples in healthy subjects	Chien et al. (2017b)
Acetone	secondary alcohol dehydro-genase	Fluorescence	30−5468 ppb	2-butanone interferes; methanol, ethanol, 1-propanol, 1-butanol, 2-butanol, IPA and acetaldehyde not interfering	Breath samples, healthy and diabetic patients	Chien et al. (2017a)
Acetone	secondary alcohol dehydro-genase	Fluorescence	20–5300 ppb	2-butanone, 2-pentanone interfere; acetaldehyde, formaldehyde, methanol, methyl mercaptan, ethanol, 1-propanol, 2-propanol, 1-buthanol don't interefere;	Breath; Real time measurement during an exercise stress test on an ergometer;	(Ye et al., 2015)
Acetone	Secondary-alcohol dehydroge-nase, lactate dehydroge-nase, and pyruvate oxidase	Electrochemical	1.1–23.3 ppm in simulated breath	the enzyme sensitivi-ty ratio of secondary alcohols to primary alcohols was 50:1., ethanol did not inter-fere; 2 and 3-penta-none, 2-butanone and acetaldehyde interfe-re. But levels in healthy people that have not been drin-king were very low	Breath	Landini and Bravard (2009)
Acetal-dehyde	Aldehyde dehydrogenase	Electrochemical	0.11–10 ppm	methanol, ethanol, benzene not interfering	Measurement in breath after drinking	Mitsu-bayashi et al. (2005)
Acetal-dehyde	Alcohol dehydroge-nase	Fluorescence	0.2–10 ppm	Ethanol, methanol, IPA, acetone, methylmercaptan do not interfere	EB	Iitani et al. (2019a)
Acetal-dehyde	Alcohol dehydro-genase	Fluorescence	0.02–10 ppm	ethanol, methanol, IPA, acetone not interfering	EB	Iitani et al. (2018)
Carbon dioxide	Carbonic anhydrase	Electrochemical	160 ppm–2677 ppm (CO2 concentration dissolved in water)	O_2, CO, NO, NH_3 not interfering	Breath of healthy volunteer	Bagchi et al. (2017)
Lactic acid	Lactate oxidase	Electrochemical	150nM-1.1 mM	ascorbic acid, cholesterol, cysteine, glucose, glutamine acid, urea, and uric acid not interfering	Sprayed misty LA	Shimo-mura et al. (2012)
Glucose	Glucose oxidase		0.5 nM-125 μM	Not studied	Buffer	

(*continued on next page*)

Table 2 (*continued*)

Analyte	Biorecognition element	Detection method	Dynamic/linear range (LR) and detection limit (DL)	Selectivity	Appli-cation	Refe-rence
		High electron mobility transistor (HEMT)				Kang et al. (2007)
Glucose	Glucose oxidase	High electron mobility transistor (HEMT)	0.5 nM-125 μM	Not studied	Buffer	Chu et al. (2010)
Glucose	Glucose oxidase	Fluorescence	DL: 0.1 μM LR: 5 μM-1 mM	Not studied	Buffer	Cao et al. (2008)
Glucose	Glucose oxidase	Localised surface plasmon resonance	DL: 0.01 nM Dynamic range: 10^{-12}-10^{-3} M	Not studied	Buffer	Endo et al. (2008)
Glucose	Glucose binding protein	Fluorescence	DL: 0.05 μM	Not studied; the protein also binds galactose	Buffer	Salins et al. (2001)
Glucose	Concavalin A	Fluorescence	DL: 0.05 μM LR: 2–15 μM	Sucrose, D-fructose, lactose, maltose, mannitol, K^+, Na^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Al^{3+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, uric acid, bilirubin, glycine, arginine, l-phenylalanine, lysine, l-cysteine, tyrosine, ascorbic acid, cholesterol	Serum samples	Tang et al. (2008)
Lactate	Lactate oxidase	flow-injection (FIA) electrochemical system coupling with ion-exchange pre-concentration column	DL: 0.02 μM Dynamic range: 0.1–20 μM	H_2O_2 interference was eliminated by using the pre-concentration, anion exchange column	EBC (120 samples)	Karya-kina et al. (2016)
Carcinoembryo-nic antigen	Antibody	Love Wave mode surface acoustic wave	LR: 1–16 ng/mL DL: 1 ng/mL	Neuron specific enolase (NSE); squamous cell carcinoma (SCC) not interfering	EBC (28 samples)	Zhang et al. (2015)
Influen-za virus	Antibody	SiNW FET	DL: 29 viruses/μL	None for sample dilution factor higher than 100	EBC	Shen et al. (2012)

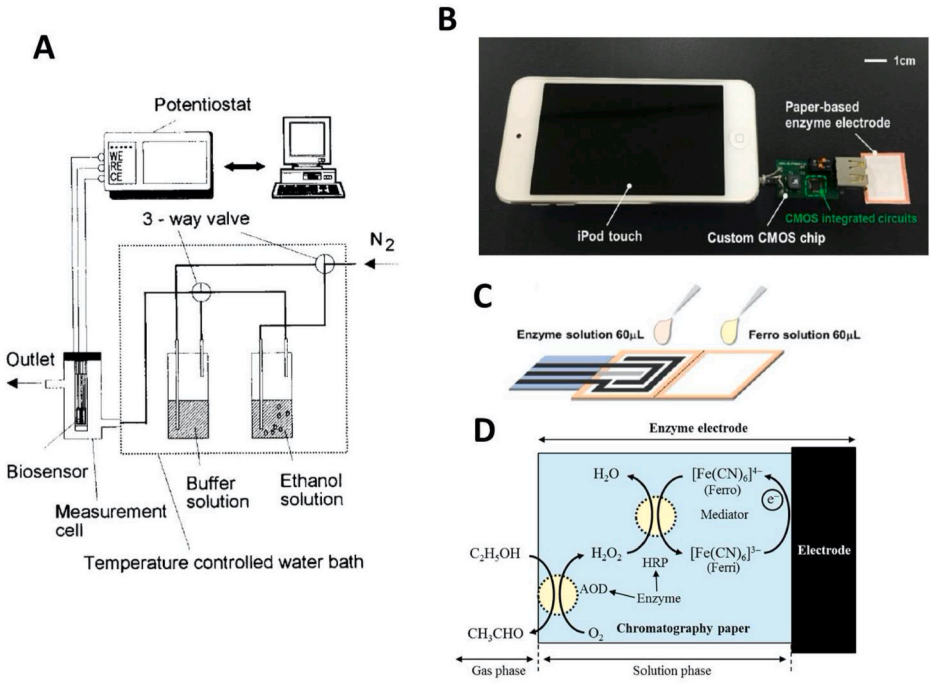

Fig. 3. A: Experimental setup of an electrochemical biosensor measurement system for ethanol including a thick film biosensor in a flow-through cell, the ethanol vaporization system, a potentiostat and a computer for data acquisition and interpretation respectively (Park et al., 1995). B: Experimental setup for ethanol gas analysis with enzyme-paper electrodes, including a CMOS chip and a smartphone for data acquisition and interpretation. (Kawahara et al., 2017). C: Illustration of paper enzyme electrodes drawn on chromatographic paper showing distinctive area pre-loaded with enzymes and the mediator (Kawahara et al., 2017). D: Functioning principle of a biosensor for the detection of ethanol vapors where the enzymes, mixed with the mediator were co-adsorbed on chromatographic paper, placed on top of carbon screen-printed electrodes. (Motooka and Uno, 2018). Reproduced from (Park et al., 1995; Kawahara et al., 2017) and (Motooka and Uno, 2018) with permission.

various concentrations (Mitsubayashi et al., 1994) or ethanol was vaporized in air-containing sampling bags (Kuretake et al., 2017). Vapor samples were blown through a syringe in the measurement cell (Park et al., 1999) or directly in the aqueous phase (Kawahara et al., 2017). The biosensors reported so far rely on screen-printed electrodes (Park et al. 1995, 1999; Kuretake et al., 2017; Motooka and Uno 2018) or electrodes drawn on chromatographic paper with a carbon pencil (Kawahara et al., 2017). Enzymes were mixed in screen-printing carbon ink (Park et al., 1995), immobilized in a polyacrylamide membrane placed on top of a Clark oxygen electrode (Mitsubayashi et al., 1994) or simply adsorbed on chromatographic paper (Motooka and Uno, 2018; Kuretake et al., 2017; Kawahara et al., 2017), placed in contact with the electrodes.

The studies reporting paper-based enzyme electrodes designed as single use devices reflect the emphasis in current research on simplicity and portability. As demonstrated by the authors (Kawahara et al., 2017),

the paper biosensors can be interfaced with a CMOS circuit chip and smartphone for data acquisition and interpretation (Fig. 3 B). Having the enzyme layer which contained alcohol oxidase and peroxidase in contact with the electrodes yet separated from the mediator layer including ferrocyanide (Fig. 3 C (Kawahara et al., 2017; Motooka and Uno 2018), leads to better detection limits compared to the alternative approach where the enzymes and the mediator were directly mixed in the same layer (Fig. 3 D). Yet, the detection limit of 20 ppm ethanol vapor is not very impressive compared to other biosensors, the characterization was limited to standard ethanol vapor samples and the paper biosensor had very limited stability, as the signal decreased drastically over 3 days of storage. Nonetheless, these proof–of-concept devices described in (Motooka and Uno 2018; Kuretake et al., 2017; Kawahara et al., 2017) illustrate the potential that can be achieved for the fast, simple, and cost-effective detection of volatile biomarkers, as they can be adapted for other analytes besides ethanol.

With regards to stability, alcohol dehydrogenase inclusion in the screen-printing carbon ink in an optimized enzyme to cofactor ratio led to biosensors that were stable for 35 days when stored at room temperature under nitrogen (Park et al., 1999). In one study, describing a biosensor where alcohol oxidase was immobilized in a polyamide membrane, the signal decreased to about 20% of the original value after 6 days of storage at below 10 °C (Mitsubayashi et al., 1994). While the storage stability depends on both the enzyme and the immobilization method, it must be noted that recent research in ethanol gas-phase biosensors did not place a particular focus on investigating this important aspect.

In comparison with electrochemical biosensors, optical methods used with ethanol biosensors not only provided sensitive detection down to 0.1 ppm but also facilitated the imaging of the distribution of volatile biomarkers in the gas phase. For example, “sniffer cameras” described by Kobayashi’s group enabled to visualize ethanol vapor distribution emitted by the palm skin (Iitani et al., 2020; Arakawa et al., 2019) or emitted by wine, poured in glasses with different shapes (Arakawa et al., 2015). A few studies have concentrated on monitoring the evolution of ethanol concentration in the breath of people after drinking (Iitani et al., 2019a; Wang et al., 2011). In the human body, aldehyde dehydrogenase 2 (ALDH2) is a major enzyme involved in ethanol metabolism, responsible for converting the toxic acetaldehyde, produced by ethanol oxidation, into acetic acid. Polymorphic variants of this enzyme have a huge (800 fold) difference in their catalytic power (Peng and Yin, 2009). In people with low activity of ALDH2, i.e., with ALDH2 (-) acetaldehyde accumulates in the body producing toxic, impairing effects. ALDH2 polymorphism is indicated as a genetic risk factor for alcoholism and was detected in a significant part (up to 35%) of some Asian populations (Peng and Yin, 2009).

The visualization of ethanol vapor distribution in the breath of ALDH2 (+) and ALDH2 (-) people was achieved with an imaging biosensor-based system, using chemiluminescence as detection method (Wang et al., 2011). Luminescence was produced via the well-known reaction between luminol and hydrogen peroxide, catalyzed by peroxidase. Hydrogen peroxide was co-produced in the alcohol oxidase catalyzed transformation of ethanol to acetaldehyde, thus ensuring the specific correlation between ethanol concentration in breath and the output luminescence signal. The linear range of the biosensor extended from 30 to 400 ppm ethanol, covering the medically relevant levels, from the threshold for drunk driving (78 ppm or 0.15 mg L^{-1}) to concentrations corresponding to intoxication. Analysis of human breath samples with this biosensor at different times after drinking indicated peak concentrations at around 30 min after drinking and slower detoxification over the next hours. Higher ethanol concentrations were measured in the breath of people with ALDH (-) compared to the ALDH (+) group. This agrees with expectations that a lower activity of ALDH2 and the accumulation of acetaldehyde in the body determine slower ethanol metabolism, non-metabolized ethanol being exhaled. Overall, as confirmed in the series of measurements with the biosensor the time required for ethanol to clear the body is longer in people with ALDH2 (-).

More recent research from the same group describes an ingenious fluorescence imaging system using a mono-enzymatic biosensor for the simultaneous monitoring of alcohol and acetaldehyde levels in breath (Iitani et al., 2019a). The device is based on alcohol dehydrogenase (ADH) and exploits both the direct catalysis of ethanol oxidation to acetaldehyde, at alkaline pH and the reverse reaction, i.e., transformation of acetaldehyde into ethanol, favored by a pH around 6.5. The experimental setup, illustrated in Fig. 4 A, involves two enzymatic meshes where ADH was immobilized in a matrix of bovine serum albumin by cross-linking with glutaraldehyde. The meshes were moistened with (1) NAD^+-containing buffer pH 9.5 (“EtOH mesh”) and with (2) NADH containing buffer pH 6.5 (“AcE mesh”), respectively. An UV-LED array used as light source and the camera for detection were coupled with two bandpass filters enabling selective excitation of NADH fluorescence at 340 nm and visualizing the emission at 490 nm. Breath samples from patients with low or high activity of ALDH2, who consumed alcohol, were collected in sample bags and analyzed with the imaging system. In the analysis of ethanol, as NADH is formed in the reaction, the fluorescence on the EtOH mesh increased while on the AcE mesh, the fluorescence decreased as NADH was consumed. The rate of change in fluorescence intensity was used to generate a differential image (Fig. 4 B).

Similar pharmacokinetic profiles were observed as in previous studies, i.e., in all patient breath samples, ethanol concentrations peaked at 30 min after drinking and diminished over the next couple of hours (Fig. 4 C). Breath ethanol and acetaldehyde concentrations of people with ALDH2 (-) were higher compared to those of people having ALDH 2 (+), i.e. [ethanol] = 163.3 ± 28.0 ppm, [acetaldehyde] = 8.4 ± 0.5 ppm compared to [ethanol] = 143.3 ± 13.5 ppm and [acetaldehyde] = 1.7 ± 0.2 ppm, respectively.

This study represents a model that can further drive the development of ethanol biosensors: it presents an application relevant for the medical field, where real patient samples were analyzed, and the results were confirmed by parallel measurements with commercial detection tubes. The authors noted that the sniffer camera, as it involves enzymatic reactions, has a much longer response time and lower stability compared to resistive gas sensors and fuel cells for ethanol. Nevertheless, it is advantageous in terms of selectivity and sensitivity. The authors suggested as a future improvement of the system a simpler sampling method, involving the patient breathing directly over the enzyme meshes, thus reducing the time for analysis and eliminating the need for a sample bag.

Selectivity of ethanol sensors. Electroactive components or co-existing enzyme substrates in breath may interfere with the response of enzyme biosensors for the detection of ethanol in EB. While EB contains more than 200 compounds, studies with alcohol dehydrogenase-based biosensors indicate that the devices were non–responsive to the breath samples from healthy people that have not ingested alcohol. To ensure accurate measurements, a differential system was suggested, implying a second sensor modified with bovine serum albumin instead of the enzyme (Park et al., 1999). The enzyme biosensors did not respond to carbon monoxide, methyl mercaptan, benzene, ammonia, acetaldehyde and several alkanes and ketones (Table 2). Notably, the commercial enzymes used in biosensors also responded to methanol and to other short chain primary alcohols. Specifically, the Michaelis-Menten constant (Km) of the yeast alcohol dehydrogenase (ADH) for ethanol is lower by a factor of 10 at least compared to that for methanol or for longer alcohols (Sigma-Aldrich, 2014). Alcohol oxidase from *Pichia pastoris* has a similar Km for methanol and ethanol in the mM range, the relative reaction rate with ethanol being 82% of the reaction rate with methanol (Sigma-Aldrich, 2018). Nonetheless, the concentrations of methanol and other potentially interfering alcohols are much lower compared to the concentrations of ethanol in the EB of people that ingested alcohol. Therefore these compounds are not affecting the accuracy of ethanol measurements with the biosensor, for this particular

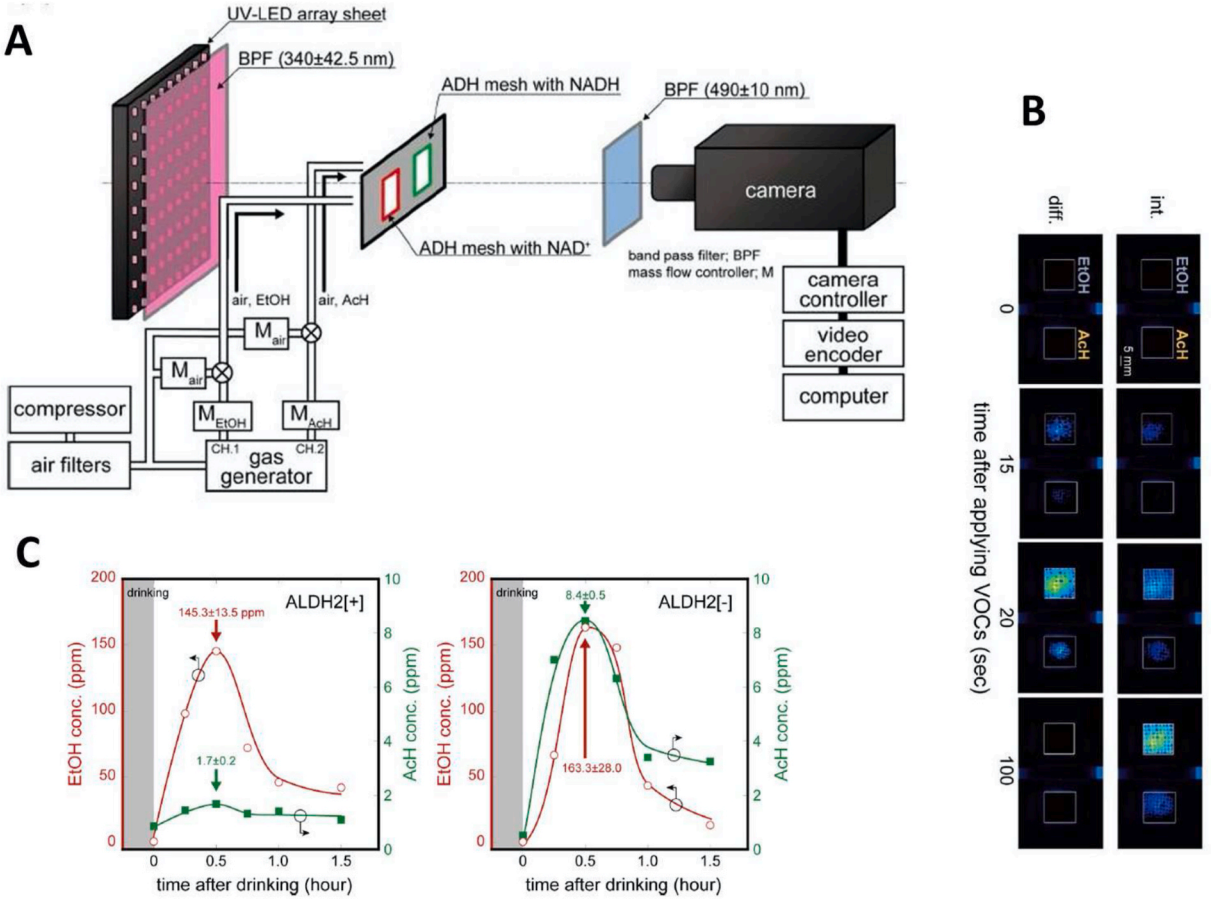

Fig. 4. A: Schematic representation of the experimental setup of the switchable sniff-cam for ethanol and acetaldehyde. B: Fluorescence ("int", right) and differentiation images ("diff", left) captured by the sniff cam at 0, 15, 20, and 100 s after application of EtOH and AcH. C: Variation of breath (○) EtOH and (■) AcH concentration in people with ALDH2 [+] and ALDH2 [-], respectively after consuming alcohol. Reproduced from (Iitani et al., 2019a) with permission from Elsevier.

application.

Reusability of ethanol gas biosensors. In general, for simplicity and to avoid problems related to fouling or degradation in performance due to enzyme activity, the biosensors were designed as single use devices. One study reported that the immobilization of alcohol dehydrogenase in PVA-SbQ at the surface of a carbon electrode enabled 20 repetitive assays (Mitsubayashi et al., 2005).

Using biorecognition elements in enzyme applications implies the existence of a hydration layer so that the enzymes can exert their function as a biocatalyst. To ensure optimum conditions for biosensing, designing an outer hydrophilic layer (e.g. a printed membrane of hydroxyethyl cellulose (Park et al., 1995) or chromatographic paper placed on the carbon electrodes (Motooka and Uno, 2018), dipping the sensor in buffer for less than 5 s (Park et al., 1999) or depositing a specified volume of buffer on the sensor before the analysis (Motooka and Uno, 2018) appears appropriate. Humidity and temperature significantly affect the results therefore proper controls and corrections must be envisioned. Breath samples must be maintained at 37 °C from collection until analysis since condensation at lower temperature will affect the result of the measurements. Park et al. proposed the preparation of calibration standards of ethanol vapor generated at 34 °C in a Breath Alcohol Simulator, followed by blowing a sample of EB through this simulator into a tube in the biosensor measurement chamber, kept at room temperature (Park et al., 1999). A different approach was used with patient samples analyzed with a "sniffer" camera, where the temperature of the breath samples collected from patients in sampling bags was maintained at 40 °C during the delivery through the gas flow lines into the fluorometric measurement cell (Iitani et al., 2019a).

Acetaldehyde in EB. Biosensors for the detection of acetaldehyde in breath via amperometric (Mitsubayashi et al., 2005) or fluorometric detection (Iitani et al. 2018, 2019a) were proposed as complementary tools to ethanol biosensors to investigate alcohol metabolism in relation to ALDH2 polymorphism. In the amperometric "sniffer stick" aldehyde dehydrogenase was immobilized by entrapment in polyvinyl alcohol bearing styryl pyridinium groups (PVA-SbQ) in a H-PTFE membrane at the surface of a sputtered Pt electrode. Similar sniffer sticks were developed in parallel for ethanol by immobilizing alcohol oxidase by entrapment in PVS-SbQ on a carbon electrode. A gas generator was used to produce calibration samples of different concentrations of acetaldehyde in sampling bags. Breath samples from volunteers were also collected in sampling bags. For analysis, the sniffer stick was quickly inserted into the mouth of the sampling bag. The biosensors were used for repetitive tests and were cleaned by rinsing with buffer between consecutive measurements. This approach enabled to observe the evolution of acetaldehyde levels in EB, by analyzing breath samples from healthy volunteers at different time intervals after the ingestion of alcohol. The pattern of acetaldehyde concentration changes was similar to that of exhaled ethanol emphasizing a peak concentration at 30 min (4 ppm acetaldehyde) and a decrease over the next hours (Mitsubayashi et al., 2005). Notably, the concentrations of exhaled acetaldehyde measured with the sniffer were confirmed by parallel measurements with commercial gas diffusion detection tubes. Two fluorescence-based enzyme biosensors for exhaled acetaldehyde were later described by the same research group, both using alcohol dehydrogenase as biocatalyst. One was designed as an imaging system for the simultaneous detection of ethanol and acetaldehyde (discussed above) and was characterized by a linear range between 0.2 and 10 ppm for acetaldehyde. Aldehyde dehydrogenase was immobilized on a cotton mesh by crosslinking with

glutaraldehyde in a matrix of bovine serum albumin. The cotton mesh was soaked in 0.1 M TRIS buffer pH 6.5 containing 0.5 mmol L^{-1} NADH and the decrease in the NADH fluorescence due to its conversion to NAD^+ in the enzymatic reaction provided the basis for the analytical signal. A differential image that considered the rate of change in fluorescence instead of the raw fluorescence was correlated with the concentrations of exhaled acetaldehyde. As the fluorescence signal did not revert to the baseline after the measurement, the cotton mesh had to be changed after each test.

The major advantage of this system is that it allows simultaneous monitoring of 2 biomarkers relevant for ethanol metabolism, i.e., ethanol and acetaldehyde and enables to visualize the spatial distribution of concentration levels. The second system featured a UV-LED as the light source, a photo-multiplier for the detection, and an optical fiber probe, fitted with a membrane with immobilized enzyme that was placed on top of a flow cell (Iitani et al., 2018). Immobilization of alcohol dehydrogenase in the H-PTFE membrane was achieved by physical entrapment in a polymer, of 2-metha-cryloyloxyethyl phosphorylcholine polymerized with 2-ethylhexyl methacrylate (PMEH). NADH-containing buffer was continuously supplied from a reservoir into the flow cell, at the same time as the reaction products NAD^+ and acetic acid were evacuated from the cell. This arrangement enabled to measure continuously gaseous acetaldehyde, in contrast to previous devices. The decrease in fluorescence intensity was correlated with the concentration of exhaled acetaldehyde. The sensitivity was better, the response time was around 2 min and the linear range was wider compared to the sniffer camera i.e., 0.02–10 ppm, covering both the concentration in the breath of people at rest and after drinking. (Iitani et al., 2018). A note should be made here on the systematic investigations of the research group, concluding that the reverse reaction of ADH leads to better sensitivity and shorter response time than the direct reaction catalyzed by ALDH (Iitani et al., 2017). Indeed, ALDH catalyzes the conversion of acetaldehyde to acetic acid with the simultaneous reduction of the enzymatic cofactor NAD^+ to NADH. The equilibrium of the enzymatic reaction is switched to the reactant side, therefore, to favor the conversion of acetaldehyde, high concentrations of NAD^+ and alkaline pH values are needed. In contrast, as discussed above, alcohol dehydrogenase catalyzes, at slightly acidic pH and in the presence of NADH, the transformation of acetaldehyde in ethanol, with simultaneous formation of NAD^+. Both the optical fiber biosensor and the sniffer camera were successfully used to monitor the changes in acetaldehyde levels in EB in people with different phenotypes of ALDH2, after the consumption of alcohol. The results confirmed the pharmacokinetic profiles observed with the amperometric biosensor for acetaldehyde and the similarity of pattern with exhaled ethanol. In people with ALDH (-) were observed higher levels of acetaldehyde compared to people with (ALDH2 (+) and the metabolism of ethanol was slower, considering the longer time required for breath acetaldehyde to decrease to insignificant levels (Iitani et al. 2018, 2019a).

Isopropanol and acetone. Isopropanol and acetone are considered as potential biomarkers for diabetes mellitus in exhaled breath, consequently the simultaneous detection of these compounds can be more relevant than each analysis alone. An ingenious biosensor for isopropanol and acetone combined the sensitivity of fluorescence detection with the specificity of secondary alcohol dehydrogenase, using pH as a switch mechanism between the forward and reverse reactions catalyzed by the enzyme (Chien et al., 2017). In acid media, the secondary alcohol dehydrogenase catalyzes the transformation of acetone in isopropanol with the concomitant oxidation of the presence of the reduced enzymatic cofactor NADH to NAD^+. The reaction can be driven in reverse by changing the pH to alkaline values as illustrated in Fig. 5.

The quantitative detection of acetone and isopropanol was achieved by measuring the fluorescence of the NADH consumed (for acetone) of formed (for isopropanol) in the respective reactions. The experimental setup included a UV-LED light source, a photomultiplier, the biosensor and a flow cell where the buffer containing enzymatic cofactor (NADH for acetone or NAD^+ for IPA detection) was circulated (Fig. 6A). The biosensor was built by securing a membrane with immobilized enzyme on the top of a flow cell containing an optical fiber. As acetone or isopropanol passed through the S-ALDH membrane into the flow cell, the enzymatic reaction proceeded and the fluorescence of NADH changed, promptly recorded via the optical fiber (Fig. 6A). The enzyme was immobilized in the H-PTFE membrane by entrapment in the PMEH polymer. Exhaled breath from patients was collected in sample bags that were transported to the lab, connected to an air pump and delivered to the biosniffer device. The biosensor reached a stable response at 40 s after introducing the sample. While this design presumes the continuous addition of cofactor in the buffer stream in the flow cell, the concentration of cofactor was very small, 50 μmol L^{-1}, resulting in low costs.

The dynamic range of the acetone biosniffer was 30–5468 ppb which covers the typical levels found in healthy and in diabetes-suffering patients. The study reports the analysis of acetone levels from healthy and diabetic people of different age and sex. Based on their screening the authors did not observe differences between men and women, but

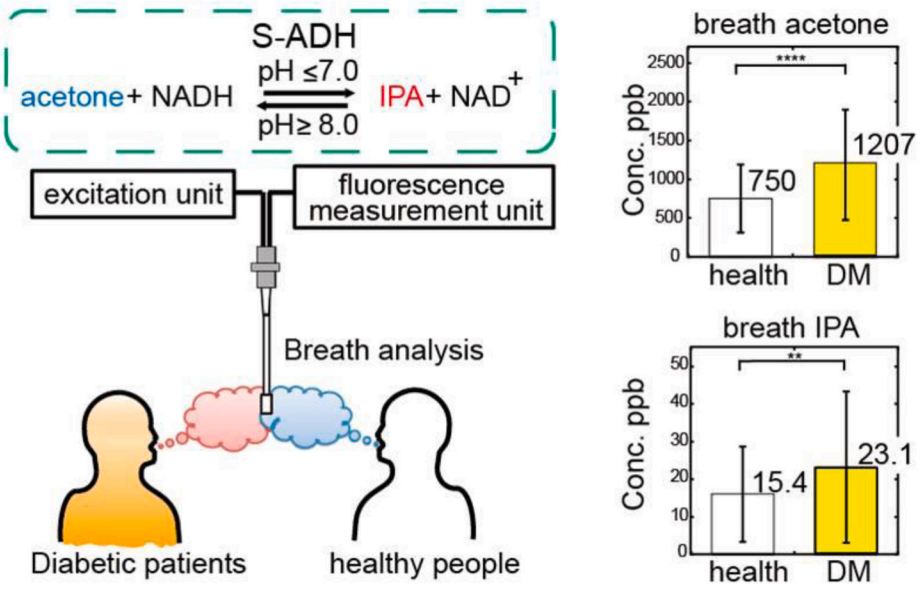

Fig. 5. A: Principle of the biosniffer for isopropanol and acetone based on secondary alcohol dehydrogenase. Reproduced from (Chien et al., 2017a) with permission.

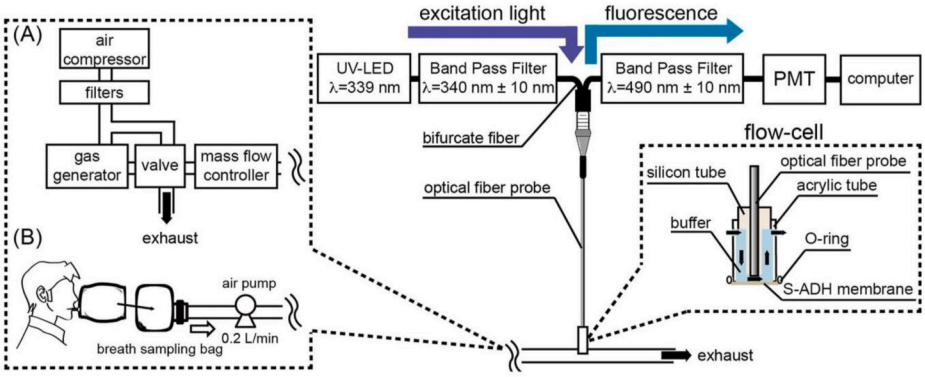

Fig. 6. A: Schematic representation of the breath measurement using biosniffers and the configuration of the flow cell. B: Schematic of the palm-size electrochemical breath acetone sensor, composed of an electronic handset and the electrode-containing mouthpiece and a schematic illustration of the electrochemical biosensor. Reproduced from (Chien et al., 2017a) with permission.

statistically significant differences were found between people with either type I (average: 1641 ppb) or Type 2 diabetes (average 1121 ppb) and healthy persons (average: 750 ppb).

With regards to IPA, the biosensor measuring range was 1–9060 ppb and the IPA levels in the patient groups screened using the biosniffer averaged 15.4 ppb and 23.1 ppb in healthy and diabetic people, respectively. Remarkably, while enzymes have group specificity and some compounds interfering with acetone or IPA determination with the biosniffer were found as well (2-butanone and 2-butanol, respectively), these are typically present at very low levels or not at all in exhaled breath. Consequently, the interferences were non-significant, supporting the feasibility of the proposed biosniffer approach. The accuracy of the biosniffer remains to be verified by comparison with a standard method and the operational and storage stability of the biosensor, given by the stability of the enzyme-immobilized membrane, are yet to be reported.

Prior to this study, the authors have optimized the detection of IPA using S-ALDH as biocatalyst in both liquid phase (Chien et al., 2016) and in air (Chien et al., 2017b). The same immobilization approach was followed in all studies: the enzyme was immobilized in PMEH polymer on a H-PTFE membrane.

The analysis of acetone was also optimized based on similar biosensor design and experimental setup using S-ADH in slightly acidic media: The biosensors were adapted to determine acetone in liquid phase (Ye et al., 2017) and in exhaled breath (Ye et al., 2015). The sensor for the determination of acetone in exhaled breath had a linear range of 20–5300 ppb that can be tuned according to the concentration of NADH (Ye et al., 2015). It can be adjusted to determine as low as 7 ppb (with 20 μM NADH) or up to 10000 ppb (with 100 μM NADH). The interference study focused on 10 other volatile compounds besides acetone, from which the biosensor detected also with high sensitivity the ketones 2-butanone and 2-pentanone. While the amounts of these compounds in exhaled breath of healthy people are low compared to acetone so they don't significantly impact the accuracy of its detection, the authors suggested that the preference for the two ketones can be exploited in the future in screening lung cancer patients, whose breath contains significant levels of these compounds. Real time monitoring of acetone in exhaled breath with the biosniffer was demonstrated in fasting patients, before and during exercise ("stress test") on an ergometer. Acetone concentration reached the maximum value at 15–30 min following the exercise, i.e., up to around 600–1200 ppb or up to 140% of the value before exercise.

Besides the mono-enzymatic sensors detailed above, a single use electrochemical assay for exhaled acetone was also reported, using S-ALDH, lactate dehydrogenase and pyruvate oxidase, deposited on a screen-printed electrode (Landini and Bravard, 2009). While more complicated (as it involves three enzymes) and showing inferior analytical performances compared to above discussed biosensors, this study presents a simple practical approach for analysis: the patient blows into a mouthpiece, that is coupled to the enzyme-modified screen-printed electrodes. Such approach can be investigated with other biosensors as well as it would simplify the analysis and reduce the time, compared to the alternative of collecting the samples in sampling bags and delivering later to the measurement system.

Electrochemical biosensors aimed at the analysis of the EB were also reported for **carbon dioxide** (Bagchi et al., 2017) and for **lactic acid** (Shimomura et al., 2012) based on carbonic anhydrase and lactate oxidase, respectively as biocatalysts. More details on electrochemical biosensors for breath analysis are given in (Gaffney et al., 2020).

5.2.2. *Analysis of EBC*

Glucose in EBC. The analysis of glucose in EBC was suggested as a non-invasive alternative to blood testing that can encourage the compliance of diabetic patients with regards to regular checks of their glycemia. Information on the levels of glucose in the EBC in healthy and diabetic patients is only starting to accumulate. While high glucose levels were found in hyperglycemic patients, the variability in the EBC collection methods and experimental approaches used in the different studies make it difficult to have a solid interpretation of currently available data (Tankasala and Linnes, 2019). Glucose diffuses from blood into the respiratory fluid, from there it passes in the EB and can be collected in the EBC. The dilution factor of the respiratory fluid with water vapor in the condensed breath varies in large limits, from 1000 to 50000, e.g., one study estimated a dilution of 1:20000 based on indicators such as total nonvolatile cations, urea and conductivity (Effros et al., 2003). As a consequence of the high and variable dilution, the reported glucose concentrations in EBC are in the nanomolar to low micromolar range (Tankasala and Linnes, 2019). Controlling the dilution factor but also the pH of EBC (strongly correlated with the concentration of CO_2) is critical for the accurate analysis of glucose and other biomarkers analysis in EBC.

Very few biosensors have been applied for EBC analysis, the main reason being their limited sensitivity. For glucose for example, as summarized by Tankasala and Linnes (2019), an adequate biosensor should have a detection limit in the nanomolar range, a fairly wide dynamic range and very good sensitivity to measure accurately both normal levels (0.2 μmol L^{-1}) and concentrations specific to hyperglycemic status (up to 2 mmol L^{-1}). A detailed review of the most sensitive biosensors for glucose with analytical performances that are adequate or close to matching the requirements for EBC analysis is presented in (Tankasala and Linnes 2019). It includes devices based on high electron mobility transistors (HEMT) (Chu et al., 2010; Kang et al., 2007), fluorescence (Tang et al., 2008; Cao et al., 2008) or localized surface plasmon resonance (Endo et al., 2008) and exploiting the specific recognition of glucose by the glucose oxidase (Kang et al., 2007; Endo et al., 2008; Cao

et al., 2008), glucose/galactose binding protein (Salins et al., 2001) or concanavalin A (Tang et al., 2008).

While none of the reviewed biosensors have been tested yet with samples of EBC, there are some specific advantages that are worth mentioning, besides their sensitivity. For example, one study described a miniaturized device with the potential to simplify EBC analysis. It is based on AlGaN/GaN HEMT and integrates a Peltier device, mounted underneath the biosensor for condensing the exhaled breath. The biosensor chip includes besides the glucose biosensor additional sensors for pH and for chloride ions that could serve to compensate via calibration the effects of pH variation and EBC dilution (Chu et al., 2010). To achieve sensitive detection of glucose, glucose oxidase was immobilized by electrostatic interactions with positively charged ZnO nanorods, grown on the gate of AlGaN/GaN HEMT. The nanorods enabled to both increase the active gate area and efficiently immobilize the enzyme. The charges generated in the conversion of glucose catalyzed by glucose oxidase were passed to the HEMT, causing changes in the drain current that were correlated with glucose concentration in the range 0.5 nmol L^{-1} to 125 μmol L^{-1}. In a different work, covalent binding of glucose oxidase to CdTe quantum dots (QDs) not only served to obtain a sensitive readout but also improved the Michaelis Menten (K_m) constant and the stability of the enzyme (Cao et al., 2008). With a view at practical applications, a good stability, i.e. 2 months at 4 °C was also observed for the glucose/galactose binding protein, conjugated with the fluorescent dye N-[2-(1-maleimidyl) ethyl]-7-(diethylamino) coumarin-3-carboxamide (Salins et al., 2001). It should be noted here that, although the challenges of glucose analysis in EBC are unique (variable dilution, pH, low concentrations) in comparison with other matrices suggested as less invasive alternatives to the glucose analysis in blood, many practical aspects related to the immobilization of biomolecules, signal amplification etc. can be inferred from point-of- care devices reported for glucose analysis of saliva, sweat and tears (Tu et al., 2020).

Selectivity of glucose biosensors. With regards to potential interferences with the response of glucose biosensors, one should note that despite the similar affinity of the glucose/galactose binding protein for two sugars, since galactose is not present in EBC, the accuracy of glucose determination will not be affected. Biosensors based on glucose oxidase need to consider the possible interferences due to the endogenous hydrogen peroxide in the EBC of healthy people and the significantly higher levels of hydrogen peroxide found in the EBC of children suffering from asthma or of lung cancer patients (Tankasala and Linnes 2019). A more detailed selectivity study was reported for a concanavalin A biosensor, however, the study was tailored to serum as the matrix of interest and not to EBC (Tang et al., 2008).

Lactate in EBC. Lactate concentrations in the EBC of healthy people and in people diagnosed with bronchial asthma, pneumonia and COPD were measured with an electrochemical biosensor based on lactate oxidase and Prussian Blue-modified, screen printed carbon electrodes, integrated in a flow injection analysis (FIA) setup and coupled with a pre-concentration cartridge (Karyakina et al., 2016). Although the differences in the lactate levels measured in healthy people versus people affected by various pulmonary conditions were not reported, concentrations measured with the biosensor were in the range 1.5-3x10^{-4} mol L^{-1}. A commercial condenser (EcoScreen, Erich Jaeger GmbH, Germany) was used for collecting the EBC and the samples were kept at -70 °C before the analysis. Remarkable in this study is not only the sensitivity of the biosensor, for which a detection limit of 0.8 μmol L^{-1} was reported, but the improvements in sensitivity and selectivity associated with the use of the pre-concentration cartridge. The cartridge consisting in an anion-exchange column enabled to eliminate the interferences due to endogenous H_2O_2 in the EBC and thus can be a solution for other oxidase-based biosensors. The detection limit was improved by a factor of 40, reaching 0.2 μmol L^{-1} and the sensitivity was 50 times higher (7.0 A cm^{-2} mol^{-1} L) compared to the system lacking the pre-concentration cartridge. Pre-concentration approaches, like the proposed cartridge, can ease the adaptation of existing biosensor systems optimized for other biological matrices to the lower concentration ranges specific to EBC, not only for lactate but also potentially for other biomarkers of interest.

Proteins and viruses in EBC. Carcino-embriogenic antigen (CEA), the influenza virus and more recently SARS-CoV-2 were determined in EBC with biosensors.

CEA is a glycoprotein involved in cell adhesion reported as a potential biomarker for lung cancer with a cutoff value in EBC of 1.85 ng mL^{-1} (Zou et al., 2013). To measure CEA concentrations in EBC, a specific capture antibody was immobilized on a Love Wave mode surface acoustic wave sensor (Zhang et al., 2015). The acoustic wave sensor with a synchronous frequency of 160 MHz, was obtained by depositing 20/200 nm Ti/Au interdigitated transducers on a ST-cut quartz substrate, followed by a 3 μm SiO2 guiding wave and a 200 nm Au layer (Fig. 7 A). Controlled immobilization of anti-CEA capture antibodies was achieved by functionalizing the biosensor with Recombinant Staphylococcal Protein A, which specifically binds to the Fc region of the antibodies. CEA in EBC was first bound to AuNPs functionalized with detection antibodies and then trapped at the antibody-functionalized biosensor surface. Signal amplification was achieved by injecting a gold staining solution, which increased the mass deposited on the AuNPs, bound to the CEA-antibody sandwich complexes causing a shift in the phase signal (Fig. 7 B). The biosensor was integrated in a flow cell and the decrease in phase was correlated with the amount of CEA in the sample. Selectivity for CEA was established by comparison with the much smaller shifts, recorded for the same concentration (10 ng. mL^{-1}) of other biomarkers in EBC, squamous cell carcinoma (SCC) and neuron-specific enolase (NSE), around 8% and 11%, respectively, of the signal for CEA.

A total of 28 EBC samples were collected via a commercial condenser (EcoScreen, Jaeger, Germany) and analyzed immediately. The results were confirmed by parallel analysis with a commercial chemiluminescence based instrument typically used in clinical labs, showing an excellent correlation and a slope very close to 1 (slope of 0.9974) between the CEA levels determined by the two methods. It was not mentioned whether the samples corresponded to healthy people or patients diagnosed with medical conditions. Moreover, the authors noted the need of larger studies uniformly covering the relevant clinical range of 1–10 ng mL^{-1} (Zhang et al., 2015). In a very recent study, a portable device based on an aptasensor for CEA integrated with a Love wave detector was demonstrated to enable distinguishing between healthy and lung cancer patients in clinical samples of EBC (Zou et al., 2020).

The influenza A virus was detected with a biosensor based on a silicon nanowire (SiNW) transducer functionalized with specific antibodies and coupled with microfluidics (Shen et al., 2012). Conductance changes were specifically correlated with the virus concentration in EBC down to 29 viruses/μL, only when the dilution of the EBC sample was 100 times or more. For samples with very low virus loads, pre-incubation with antibody-functionalized magnetic beads improved the selectivity and sensitivity. The authors demonstrated the feasibility of such strategy for detecting H3N2 and H1N1 viruses as well as the 8 iso PGF 2a biomarker using specific antibodies. The accuracy of viral infection detection was confirmed by parallel RT-qPCR analysis of samples from healthy people and patients with flu symptoms (Shen et al., 2012).

With regards to SARS CoV2, EBC was suggested to provide more accurate sampling than nasal swabs, strongly reducing the probability of false negative results (Ryan et al., 2021). The EBC of a Covid patient contains in excess of 20 copies of the virus/μL EBC. Among several tests in different development phases, the Coronacheck® developped by Exhalation Technologies (Cambridge, UK) is a POC device currently in clinical trials consisting in an electrochemical sensor modified with fragments of anti-SARS CoV2 antibody. It is advertised to provide results in less than 5 min with more than 90% specificity and detection limit of 1.5 copies of virus per μl EBC (The Sage Group, 2021). While the specific sensor design and more details are not available being IP protected, the manufacturers stated that CoronaCheck was challenged with

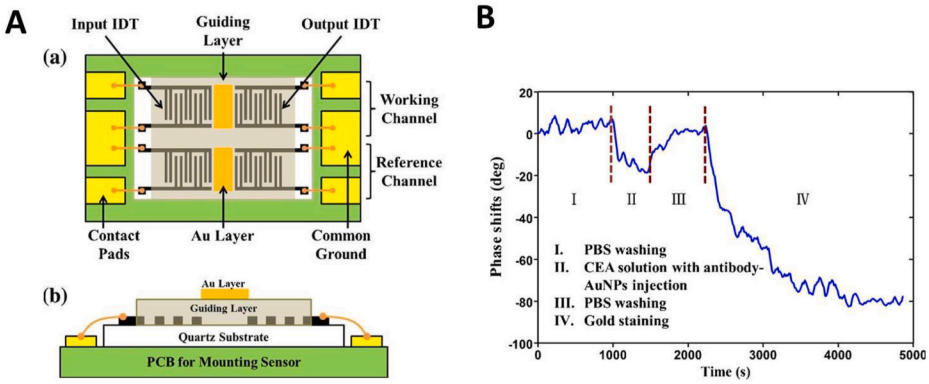

Fig. 7. A: (a) Top and (b) cross-sectional views of Love Wave sensor chip. B: Real-time response of the Love Wave immunosensor for the CEA detection. Reproduced from (Zhang et al., 2015) with permission.

live/inactivated virus, and SARS CoV2-spiked EBC, as well as with various respiratory viruses and bacteria. The device can discriminate between Covid 19 and common flu. Moreover, it is adapted from company's Inflammacheck® handheld system, a medical device approved for sales and use in the European Union and in the UK which enables to collect and to test EBC for specific biomarkers for inflammation relevant for COPD in under 5 min.

6. Conclusions and perspectives

EB and EBC collection and analysis have definite advantages in the clinical field, especially since noninvasive, uncomplicated, and painless collection of respiratory biological material is concerned. Also, EB/EBC are available in nearly unlimited quantities for analysis and allow for continuous monitoring in real-time. Specific VOC biomarkers and their patterns in the EB have been linked to a whole range of diseases. Also, with respect to respiratory diseases -such as Covid 19- which are spreading by expelled respiratory droplets with EB, EBC is a more representative sampling methods than nasal swabs or saliva, reducing the probability of false negative readings.

However, for EB and EBC analysis to be accepted by clinical practice, standardization is required, both in sampling and in interpretation. Reliable guidelines for EBC collection have been proposed (Horvath et al., 2005). The standardization of EBC-analysis necessitates a large cohort of samples to determine the normal-reference values of EBC-biomarkers (Koutsokera et al., 2008). Thus, a normal metabolic "fingerprint" of exhaled breath has not been adequately characterized yet. Furthermore, existing knowledge is mostly based on data provided by small scale studies, often demonstrating conflicting results, possibly attributable to the poor reproducibility of EBC biomarkers (Nowak et al., 1999). The complicated procedure of standardization implies the need for the development of suitable methods to process and store samples after collection so as to avoid decomposition and/or other changes in the samples.

Moreover, some newer – more sensitive and selective – analytical techniques are needed to detect the challenging low analyte content in EBC. The standardization of EBC methodology should lead to a database of normal physiological ranges of various EBC biomarkers as a baseline for clinical diagnostics. (Kubán and Foret, 2013).

Of particular promise are areas such as: the monitoring of disease and medications, disease phenotyping, diagnosis of inflammation and infections, novel applications, e.g., asthma diagnosis in young children, and chronobiology where continuous sampling is needed, e.g., sleep studies (Bruderer et al., 2019).

Technologies have improved tremendously in recent years. The most sensitive methods recently developed are based on mass spectrometry, while the simplest, lower performance ones are the use of chemical sensors or sensor arrays. In between in terms of cost and size are technologies such as laser spectroscopy. In this context, biosensors, delivering increased selectivity have significant advantages over other approaches, when it comes to the targeted analysis of specific biomarkers in EB and EBC.

While biosensors for EB and EBC analysis are very promising in terms of analytical performances, capabilities for real-time monitoring of metabolic processes and relevance for clinical applications, they face a number of challenges to overcome. The limitations in the expansion of biosensor development for EB/EBC are due to several issues, as follows:

(a) a lack of investigating the stability of the biosensor's response;
(b) difficulties in ensuring the accuracy of the measurements. The experimental setup and calibration are challenging, for EB in particular. Some of the solutions include for EB, maintaining the breath samples at 37 °C to avoid condensation which will affect the results, ensuring the appropriate hydration of the biosensing layer for optimum performance throughout the measurements and using differential sensors to account for any drift or unspecific response in EB analysis. Specifically, for EBC analysis, some reports mentioned including additional pH sensors (to enable corrections in the response of enzyme sensors due to pH changes), or chloride (for evaluating the dilution factor of EBC). All biosensor-based methods should include controls and corrections accounting for the effects of humidity and temperature;
(c) a need of confirmation by parallel measurements using already established analytical methods;
(d) eliminating the variability in EB/EBC collection to enable relevant comparison of the data reported by different groups. To the various sampling procedures used so far add many of the recently explored procedures investigated for SARS-CoV-2 detection which could be interfaced with biosensors. These processes need to avoid cross-contamination and contamination by environmental air. However, there is no unified validated procedure and no data on the variability of biosensor results wich is dependent on the sampling method.

While the technology of EB and EBC analyses-including biosensors-continuously improve, we are still years away from the mainstream application of diagnosing diseases. Most studies today are based on a proof-of-principle concept with a small patient sample size. (Bruderer et al., 2019). Nonetheless, renewed high promise and some general trends can be derived from the recent global efforts towards developing diagnostic tests for SARS-CoV-2.

Taking the example of diagnosing SARS-CoV2 infection, the untargeted analysis using the portable mass-spectrometry detectors, e-nose systems or sensor arrays appear to have the competitive edge over other approaches for large scale screening e.g., in airports, schools, malls, events etc. Due to the unproven stability, biosensors remained so far in

the shadow with respect to the EB analysis. Still as their potential for the selective targeted analysis of relevant biomarkers has been demonstrated in studies involving EB from patients, it is conceivable that the research will continue to advance in this direction as well. While little explored, the biosensors -enabled analysis of EBC appears as the best solution for the specific yet rapid and cost-effective detection of respiratory viruses. Various biosensors have been described for the detection of SARS CoV2 in serum and saliva and many more are in development, based on different recognition elements and detection methods. They could be easily adapted for EBC analysis, adding to the Coronacheck® biosensor developed by Exhalation Technology (UK), or the various Covid-19 testing system in development around the world. Besides viruses, the detection of H_2O_2 as inflammation biomarker relevant for respiratory diseases appears very promising in the near future as commercial devices are already on the market (e.g. Inflammacheck ®, by Exhalation Technologies, UK). Detection of other biomarkers in EBC is mostly limited by their needed confirmation as biomarkers, need to establish baseline levels and threshold relevant for various diseases, as well as their correlation with levels in blood or other biological matrices.

The published sampling procedures are diverse: from exhaling into sampling bags, to exhaling directly over the sensor surface, yet the need to include a "complete" sampling device with single use cartridges, saliva traps, HEPA filters and one-way valves to avoid contamination from environmental air and humidity. This shows a clear need for standardized, efficient, low cost, sampling, and avoiding cross-contamination systems to be interfaced with various detectors, sensor and biosensors platforms.

Controlling the temperature and humidity and addressing calibration are major hurdles for EB analysis while controlling pH, dilution, temperature and environmental humidity are some of practical challenges to overcome in EBC analysis, besides reproducibility of the sensors themselves to ensure accurate results.

Enhancing sensing capabilities is currently addressed on various levels, from (i) efficiently capturing the analytes on the sensor surface, to (ii) selective recognition by chemical or biological ligands or via multivariate analysis and to (iii) increasing the sensitivity of the detection method. Recent examples include: (1) hollow nanocages at the surface of a SERS sensor which was recently reported for the quantitative collection of aldehydes from EB, (2) studies on efficient collection of EBC e.g., designing a condenser included in a face masks, (3) engineering the collecting surface, (4) using a continuous dropwise condensation etc. In all of these, the surface functionalization, (nano) structuring and inclusion of nanomaterials brought fast advances (Zhou et al., 2020) and the trend should continue.

All things considered, in the short term, experts predict that the role of EBC analysis will become the method of choice for diagnosing a small number of niche applications, such as diagnosing asthma in very young children and infants, who cannot perform lung function tests easily. It is also conceivable that EB/EBC analysis will be utilized in the monitoring of disease progression, effectiveness of therapy, and adherence to treatment medications. In the long term, if all of the hurdles are met, EB/EBC diagnostics could replace or complement established tests relying on urine or blood samples, due to the ease and convenience of EBC sampling and real-time analysis.

As many scientists already pointed out, the best way for the field to succeed is to encourage close interdisciplinary collaborations between physicians, clinical researchers, analytical chemists, and engineers, this would lead to substantial progress in this exciting field.

Author's contribution

AV and SFP have designed the contents of the review.
BH has focused on the medical aspects of the review.
GS has focused on the sensor aspects of the review.
All authors contributed to the revised manuscript, both text and references.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge the financial support from their grants: UEFISCDI, PN-III-P2-2.1-PED-2019-2746, contract 266PED/August 03, 2020 (AV), the National Institutes of Health R01 DK094932 and the Innovation Discretionary Fund (DFI) Award through the MSU Office of the Senior Vice President for Research and Innovation (GMS and SFP) and the MSU Department of Medicine SEED Grant GS100060-C5BHS (BH).

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