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Use of living systems for clinical diagnostics by monitoring volatile chemicals

Autumn K. McLane-Svoboda [a,1], Simon W. Sanchez [a,1], Michael Parnas [a], Ehsanul Hoque Apu [b], Debajit Saha [a,c,*]

[a] Department of Biomedical Engineering and the Institute for Quantitative Health Science and Engineering, Michigan State University, East Lansing, MI, USA
[b] Department of Biomedical Sciences, College of Dental Medicine, Lincoln Memorial University, Knoxville, TN, 37923, USA
[c] Neuroscience Program, Michigan State University, East Lansing, MI, USA

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ABSTRACT

Volatile organic compounds (VOCs) emitted via exhaled breath and bodily fluids are indicative of a diseased state within the body and have the potential to be used as a diagnostic tool. The study of VOCs in disease detection is a concept that has seen excellent engineering progress recently, namely via developments of electronic noses that aim to replicate biological olfactory principles through cross-selective electrode arrays and sensors. However, there has yet to be an engineered device that can capture the full breadth, sensitivity, and versatility of biological olfaction. This review summarizes common principles in biological olfaction and provides an overview of recent techniques to incorporate living systems for VOC detection. We discuss the recent advances, strengths, and weaknesses of all three types of living systems-based disease detection approach, which include a) olfactory behavioral detection, b) bioelectronic noses and c) 'cyborg' biosensors for disease detection.

1. Introduction

Effective diagnostics are essential for the timely detection, prevention, and monitoring of health changes. For diseases, early diagnosis can make a tremendous difference in the mortality outcome of patients due to the swift administration of proper medical treatment. In addition, other areas of life, including financial and social burdens, are lessened when disease is caught and treated early. Volatolomics, the study of emitted volatile organic compounds (VOCs) from breath and other bodily secretions, provides a non-invasive tool for disease diagnostics. Blood contains VOCs produced through systemic metabolic processes which are then released from the body through the lungs, kidneys, and glands. Several diseases, including cancer, alter the concentrations of VOCs produced through metabolism [1–14]. As a result, multiple VOC concentrations in exhaled breath are changed in the parts-per-billion to parts-per-trillion range due to the presence of cancer or other diseases [15]. These altered VOC concentrations of disease states are reflected in samples easily collected from the body (i.e., breath, blood, urine, sweat); however, it is important to note that many studies have presented varying VOC profiles for diseases and yet there have been no singular compound marker agreed upon for a specific disease condition and prognosis. Nonetheless, the analysis of VOC profiles through bodily samples offers a reliable avenue for noninvasive and early disease detection.

Gas chromatography-mass spectrometry (GC-MS) has been extensively used for noninvasive disease detection [7,8,12,14]. However, in this component-wise detection approach, it is difficult to identify correct concentrations of several unknown VOCs at very low concentrations (in ppb to ppt range). Low mobility of the system, sample preprocessing time, and non-standardized analysis techniques also prove challenging for clinical applications of several GC-MS devices. Electronic noses (e-noses) attempted to replicate biological olfaction through sensors and pattern recognition algorithms, for example, Cyranose 320 (Sensigent) and Aeonose (The eNose Company) [16]. These relatively inexpensive and easy-to-use devices have been successful at diagnosing diseases [16], including infections [17–19] and cancers [20–25], but are frequently engineered for sensing a specific subset of VOCs, lacking the broad generalization of biological olfaction. Even with all the advances in chemical sensing devices, developing gas sensors that are sensitive to

* Corresponding author. Department of Biomedical Engineering and the Institute for Quantitative Health Science and Engineering, Michigan State University, East Lansing, MI, USA.
E-mail address: sahadeb3@msu.edu (D. Saha).
[1] These authors contributed equally to this work.

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Abbreviations

AL	antennal lobe
BSP	biosensor platform
C. elegans	*Caenorhabditis elegans*
CNT-FET	carbon nanotube field-effect transistor
COVID-19	Coronavirus disease
DDTS	detection dog training system
EIS	electrochemical impedance spectroscopy
e-noses	electronic noses
FRET	fluorescence resonance energy transfer
GC-MS	gas chromatography-mass spectrometry
GPCR	G-protein coupled receptor
hpDNA	hairpin DNA
hOPB	human odorant binding protein
KC	Kenyon cell
OB	olfactory bulb
OBP	odorant binding proteins
OR	odorant receptor
Orco	odorant receptor co-receptor
ORN	olfactory receptor neuron
pOBP	porcine odorant binding protein
PER	proboscis extension reflex
SPRi	surface plasmon resonance imaging
VOC	volatile organic compound

multiple chemicals at low concentrations, work well in natural environments, and perform reliably over time is still challenging.

Living systems have solved the problem of chemical sensing over millions of years of evolution and converged to a solution that is architecturally and functionally strikingly similar across different species. This indicates that there might be an optimal solution for gas sensing that is still elusive from an engineering standpoint. Living organisms (e.g., canines) have been proven to robustly detect multiple VOCs with high sensitivity and specificity. Therefore, it is important to learn how biology has solved the problem of chemical sensing which remains a longstanding challenge for engineered chemical sensors.

This review will first identify common principles that living systems employ for chemical sensing. We will review current approaches and results in which entire living systems have been employed for behavioral disease detection. Next, we will discuss how multiple state-of-the-art gas sensing systems have incorporated olfactory components from living systems. Finally, we will discuss a relatively new forward-engineered biosensing approach where biological olfactory brains are hijacked to perform chemical sensing tasks. These types of sensors are also called 'cyborg' sensors. Overall, this review highlights the power of incorporating biological components and biological neural computations in volatile chemical sensing for disease detection.

2. Common principles in biological olfaction

2.1. Olfactory sensory neuron – combinatorial code

At the periphery of the vertebrate olfactory sensory pathway, odor molecules first bind to specific G-protein coupled receptors (GPCRs) which transduce chemical signals into electrical impulses called action potentials or 'spikes.' It has been shown that a large family of GPCRs work as odorant receptors (ORs) [26] in vertebrates and are conserved across many species [27,28]. In insects, these ORs are shown to be ligand-gated ion channels, different from the vertebrate GPCRs [29]. These ORs form the main functional unit of the biological chemical detection array (e.g., dog's nasal epithelium, insect antenna). The neurons that contain these ORs are called olfactory receptor neurons (ORNs). Hundreds and thousands of ORNs are present in the vertebrate and invertebrate olfactory pathway. For example, in fruit fly antenna, about 60 ORs are expressed in 1200 ORNs, each containing only one type of OR and a universally expressed co-receptor [30,31]. In both vertebrates and invertebrates, a single ORN generally responds to many volatile chemicals in a cross-selective manner [32]. This is a common feature of biological sensory neurons, which employ combinations of sensory neuronal activity to encode odorants or mixtures. Biological olfaction prefers a group of sensors responding to one chemical over odor-specific single-sensor organization. These combinations of several cross-selective sensors help living organisms detect many chemicals using very few sensors (Fig. 1A). For example, using a combinatorial

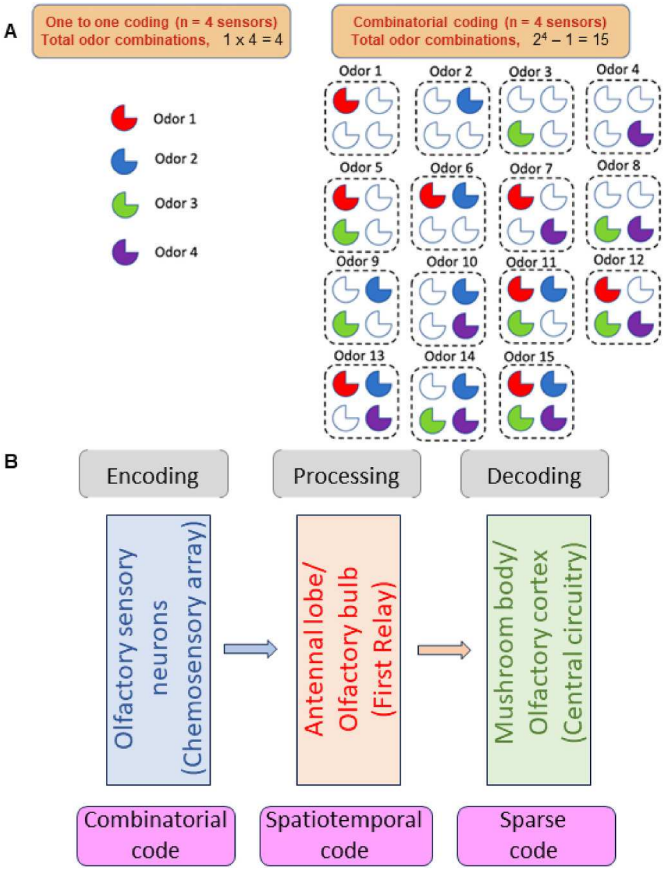

Fig. 1. General Principles in biological olfaction. A. Combinatorial coding scheme by cross-selective odor sensors [213]. **B.** Common principles in the biological olfactory pathway. Equivalent brain centers between vertebrates and invertebrates, their functional roles as well as general coding principles are shown.

coding scheme and only ON and OFF states of the sensor, 60 ORNs in the fruit fly antenna can detect $\sim 2^{60}$ chemicals which is more than one quadrillion odors or mixtures.

2.2. The first relay center – spatiotemporal odor code

The first relay of biological olfactory systems performs key roles for reliable detection of odorants in dynamic and natural environments [33]. In vertebrates and invertebrates, this odor-processing neural circuitry is known as the olfactory bulb (OB) and the antennal lobe (AL),

respectively (Fig. 1B). Intriguingly, like the sensory neurons, this neural center also shares many similarities across different species. More importantly, organizational and functional properties of the first relay have many similarities between vertebrates and invertebrates. Generally, the first relay (OB or AL) contains both excitatory principal neurons and inhibitory local neurons which form dense connections with the ORN axons in specialized neuropil areas called 'glomeruli' [34]. Within each glomerulus, usually, all the ORNs containing the same OR gene converge and make dense connections with one or several excitatory and inhibitory neurons [35]. In this neural circuitry, 'neural code' for each odorant is generated using complex spatiotemporal response profiles of the principal neurons that project their axons to the higher-order brain centers. The spatiotemporal coding scheme at this neural circuitry takes advantage of both the identity of the activated neurons and the temporal response dynamics of individual neurons [36]. Odor-induced evolution of principle neuron responses generate odor-specific manifolds in the neural response space which has been shown to be odor identity and intensity specific [37]. This spatiotemporal coding mechanism allows for background-invariant odor recognition, contrast enhancement, and novel odor detection [38–41]. The functional roles of the first relay are essential for odor detection in natural environments which is lacking in most of the current engineered gas sensors. Importantly, most engineered gas sensors account for only ON and/or OFF state of the sensor for odor detection and not the temporal response profiles of the sensor. However, biology tells us that the temporal response aspects are very important as odor stimuli are dynamic and both olfactory sensory neurons and central circuitry neurons track the temporal profile of a stimulus by eliciting complex patterns of spikes. Overall, looking at the first relay of biological olfaction, a general principle that emerges is that 'spatiotemporal' odor code is essential for odor stimuli and/or mixture detection in dynamic environments.

2.3. The central circuitry – signal sparsening and decoding

Spatiotemporal neural signals generated at the first relay (OB/AL) are transmitted to the central circuitry, which includes the olfactory cortex for vertebrates and mushroom body for invertebrates (Fig. 1B). In this central circuitry, several different functional roles are achieved that are essential for context-dependent stimulus decoding in natural settings including signal sparsening, gain control, learning, and memory [33,42]. Here, we will focus on signal sparsening as it is an important aspect of biological olfaction for reliable decoding of odor signals. In the insect mushroom body, a few hundred projection neurons from the AL converge to several thousands of the mushroom body neurons (Kenyon cells, KCs). This order of magnitude increase in the neuron numbers increases the coding space inside the mushroom body. Although the Kenyon cells (KCs), receive inputs from several presynaptic principal neurons from the AL [43], contrary to the high-firing and complex spatiotemporal response profiles of AL neurons, KCs remain mostly silent at resting potential (without any odor stimulus) and generate only a few spikes at the onset and offset of odor stimuli [44,45]. By reducing the odor stimulus-evoked spike numbers and by increasing odor specificity, KCs generate 'sparse' odor codes for respective stimuli in the brain. It is known that this sparsening of odor representation can achieve fast and reliable decoding of chemical stimuli in the presence of different background contexts. In several cases, KCs receive negative feedback from one or multiple inhibitory neurons which receive inputs from all KCs in the mushroom body. This gain control mechanism coupled with intrinsic excitatory properties of KCs and oscillatory synchronization helps limit KC firing rates to a low level and creates sparse odor representation [46]. Overall, biological organisms reformat odor-evoked neural responses in the central circuitry to generate sparse and odor context-specific representation that is used for behavioral outcomes.

3. Olfactory behavior-based disease detection

3.1. Potential of behavioral animal olfaction

Biological olfaction is powerful in its odor recognition capabilities, possessing a robust generalization for chemicals across varying concentrations and complex mixtures. When trained, animals can display a distinct olfactory behavior in response to a target stimulus throughout differing chemical backgrounds. Behavioral animal olfaction has been used in a variety of fields for chemical detection as they are capable of smelling odors at concentrations as low as parts per trillion [47–49]. Biological olfaction has been effective in many real-world applications, mentioned briefly, outside of disease detection. Canine sensing is at the forefront of applied animal olfaction with uses in explosives, narcotics, missing persons, and search and rescue detections [50–52]. Additionally, the use of other animal olfaction, such as rodents and insects, has gained popularity for real-world applications. Recently, honeybees have been successfully employed for passive explosive detection to locate residual landmines [53–55], while locusts and rodents have been used to actively detect explosive chemicals [56,57]. The potential of animal olfaction offers a non-invasive, accessible, and versatile method for detecting a variety of diseases. In this section, we will discuss the use of behavioral animal olfaction for clinical diagnostics including conditioning paradigms, animal models used, and recent results.

3.2. Behavioral conditioning paradigms and odor presentation methods

Though animals have an excellent sense of smell, they must first be trained for disease detection. Regardless of species, training was done through classical (Pavlovian) conditioning where a neutral stimulus was paired with a biologically significant one, shown in Fig. 2. Behavioral training usually comprised four main phases: habituation, association, indication, and discrimination. Habituation allowed the animals to familiarize themselves with the training environment and apparatuses used for odor delivery. Positive association consists of conditioning the animal to the target odor using reinforcement in the form of treats (dogs, mice, rats), sugar water (ants, honeybees, mice, rats), and/or clicker sounds. The next phase of training was indication, where a specific behavior was incorporated into identifying a positive sample. For example, dogs were trained to sit or stay-standing in front of a sample while honeybees produced a proboscis extension reflex (PER) when they identified a positive result [58]. Moreover, rats and mice were trained to go to a specific area; y-maze or ledges, corresponding to the target odor [59–61]. Finally, distracting odors, such as empty vials or samples from healthy patients, were incorporated into training for better discrimination between a positive and negative target. In some cases, a negative association was used for control/negative samples to further the discrimination between odors and could take the forms of puffs of water, unpleasant-tasting food, or loud noises [58,59,61]. Each animal model and study discussed in this section employed different training lengths varying from less than an hour to over a year with an exception for nematode studies that required no training at all. Once successfully trained, testing phases began where disease samples; positive, negative, and control, were presented to animals for diagnosis.

Samples used for training and testing were often collected directly from patients or created in the lab. The chemical makeup of these samples is an important indicator of health and disease progression. Urine, breath, and mouth/throat secretions were among the most common sample types used in behavioral studies. Other types included cultured cells, blood, sweat, smears, and cloth items that were worn. Samples were collected through third parties (hospitals, companies), frozen, shipped, and thawed/reheated during experimentation. For the studies mentioned in this section, each one had some type of apparatus that held either the animal or sample for detection, examples shown in Figs. 3 and 4. Invertebrates required simple and inexpensive apparatuses, such as Petri dishes or 3D-printed harnesses, to perform

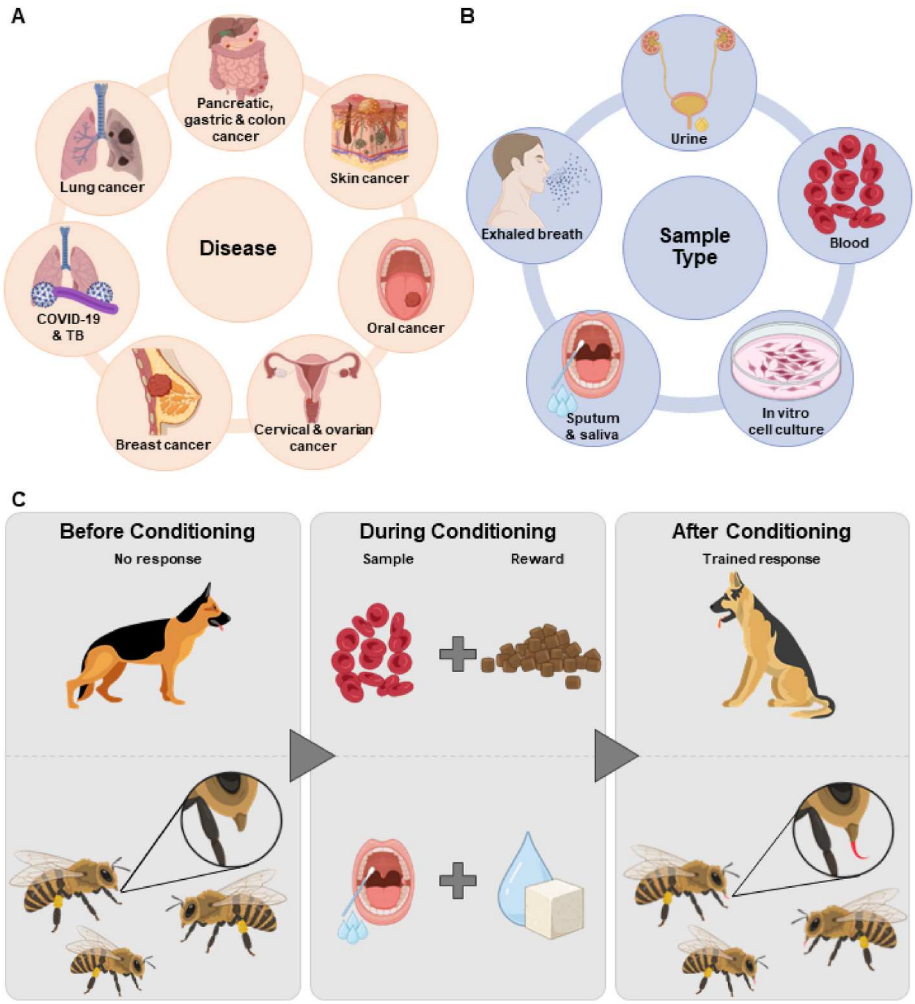

Fig. 2. Disease detection using behavioral olfaction. A. Different diseases detected by behavioral olfaction studies. TB is tuberculosis. **B.** Sample types collected from patients for disease detection. **C.** Overall process for conditioning target odors to a trained behavior. For example, before training, dogs and honeybees had no response to odor samples. They were then conditioned by pairing the odor sample with a reward (treats for canines and sugar water for insects). Once trained, animals displayed a specific response (sitting or proboscis extension reflex) when presented with the odor sample. (Images are created with Biorender.com and few images were collected from Freepik.com.).

experimentation. Samples were placed around the Petri dish using filter paper, small tubes, or solutions. However, the scale and complexity of odor delivery significantly varied for vertebrate studies. While some vertebrate studies used simplistic odor deliveries; y-mazes or lined canisters, others employed more technologically advanced means. For example, Wiesel et al. [62] utilized a novel biosensor platform (BSP) system created by EARLY Labs that allows for hands-off, automated sample odor presentation, monitoring, and data collection to multiple rats simultaneously. Likewise, Jendrny et al. [63], used another fully automated training and testing apparatus, the detection dog training system (DDTS) available through Kynoscience.

3.3. *Vertebrate models in behavioral diagnostics*

Canines are the most frequently used animal model for behavioral disease diagnostics due to their long history of olfaction-based detection in other areas (explosives, drugs, missing persons). Once trained, they are an effective diagnostic tool with high sensitivity and specificity, presented in Table 1. In the world of disease diagnostics, dogs are notably known for detecting several types and stages of cancer including lung, breast, and colorectal. In three separate studies [64–66], sniffer dogs were able to differentiate blood, breath, and urine samples from lung cancer patients with a sensitivity ranging from 65.5 to 96.7 %. The study by Feil et al. [66] found canine detection rate of lung cancer using breath and urine samples together (96.7 %) was comparable to commonly used diagnostic procedures such as CT (100 %), bronchoscopy (56.1 %), and tumor markers (65.4–93.1 %) (Fig. 3D). Another study, employing two dogs, showed very high sensitivities over in vitro cultures of breast (94.4 %) and colorectal (92.6 %) cancer cell lines [67]. In this study, both dogs were formally trained on one of the cancers; yet tested on both. Interestingly, the detection accuracy for the untrained cancer was comparable to the trained cancer (93 % vs 94 % average), giving insight into the relationship between breast and colorectal cancers while demonstrating that trained dogs can be used for multi-scent detection. While exciting, this could pose a challenge for diagnostics as dogs could be identifying a completely different disease that contains a similar VOC profile. This challenge was seen in a study by Guerrero-Flores et al. [68] where a dog trained for cervical cancer detection showed an interest in every sample containing endometrial cancer, endangering correct diagnosis. Recently, infectious disease detection using canines has increased with the rise of COVID-19. Multiple studies have shown that dogs are an effective diagnostic tool for COVID-19 with sensitivities ranging from 65 to 100 % [63,69–71], comparable to the commonly used rapid antigen test (~70 %) [72,73]. However, the study by Mutesa et al. [71] saw a decrease in detection sensitivity based on the COVID-19 variant used and speculated this was

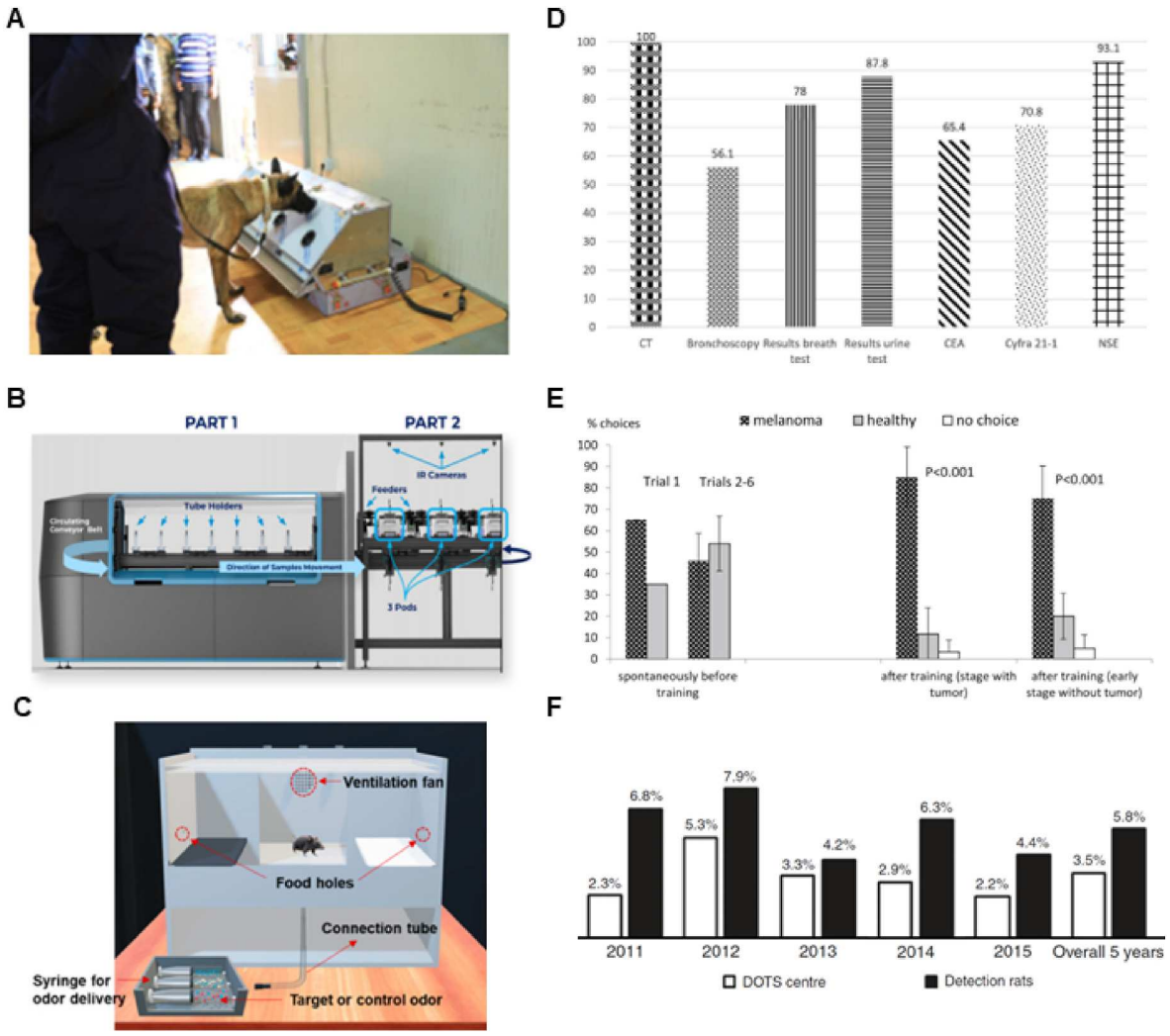

Fig. 3. Disease detection using vertebrate models. A. Detection dog training system (DDTS) built by Kynoscience and used for identification of COVID-19 samples by canine scent [63,71]. Position of positive sample automatically moves and rewards dog upon correct identification. Figure adapted with permission from Ref. [71]. **B.** Biosensor platform (BSP) system for training rats created by EARLY Labs and used by Wiesel et al. for detection of lung cancer by rat olfaction. The conveyor belt moves samples into place and automatically senses when a rat has correctly identified the sample. Figure reproduced with permission from Ref. [62]. **C.** Rat odor delivery system with two ledges for trained behavior on multi-discrimination of odors. Odor is delivered in one chamber and the rat must go to the correct corresponding ledge in a second chamber. Figure adapted with permission from Ref. [59]. **D.** Comparing the sensitivity of various diagnostic tests for lung cancer to that of canine detection. Canine detection using breath (78 %) and urine (87.8 %) was comparable to that of CT, bronchoscopy, and tumor marker diagnostics. Figure adapted with permission from Ref. [66]. **E.** Female mice detection of melanoma before and after training in the Y-maze. No significant spontaneous attraction towards healthy vs non-healthy urine samples. After training, mice were able to detect urine from cancer samples with and without a tumor. Figure adapted with permission from Ref. [61]. **F.** Rats were able to detect additional positive tuberculosis patients over 5 years compared to the directly observed treatments, short-course (DOTS) clinics. Figure adapted with permission from Ref. [77].

due to the difference in symptoms and viral load between the delta and omicron variants. Varied results seen between studies could be due to a variety of reasons including training lengths, sample sizes, and differences between the dog breeds used [74]. Additionally, handler dependency can become an issue as dogs pick up on the unintentional body cues from their handler; in some cases, this was overcome by performing double-blind testing where the handler did not know which samples were positive. Furthermore, a few studies used dogs that had been previously trained in areas such as explosives, hunting, and drug detection [63,68–70,75]. The difference in formal training experience could also contribute to diagnostic performance. Nonetheless, canines have exhibited a robust and versatile ability to detect multiple cancers and infectious diseases. With a dog's long lifespan, continuing disease detection can save a multitude of lives and better inform scientists about disease-altered VOCs.

Rats and mice are scavenger animals that rely on their sense of smell to detect food and prey from a distance. Their highly developed sense of smell makes rodents another avenue for disease detection, one that has been recently explored. Rodents have been used to detect cancers (lung, skin, bladder), bacteria, and tuberculosis with repeatable high sensitivities and specificities (Table 1). As a note, many of the studies mentioned here presented one odor at a time or one odor with one control, this directly contrasts with many canine studies which presented multiple odors simultaneously for testing. Three separate studies showed that rats were able to detect varying stages of lung cancer with sensitivity ranging from 83 to 93 % [59,60,62]. The two studies from Oh et al. displayed that rats could detect the odor in varying environmental conditions as well as up to 45 days after training [59,60]. Moreover, rats were able to discriminate target odors in the presence of distracting odors and at low concentrations [59,76], though there was a dependence of response time on odor concentration [59]. The ability to perform in different situations, generalize odors of varying

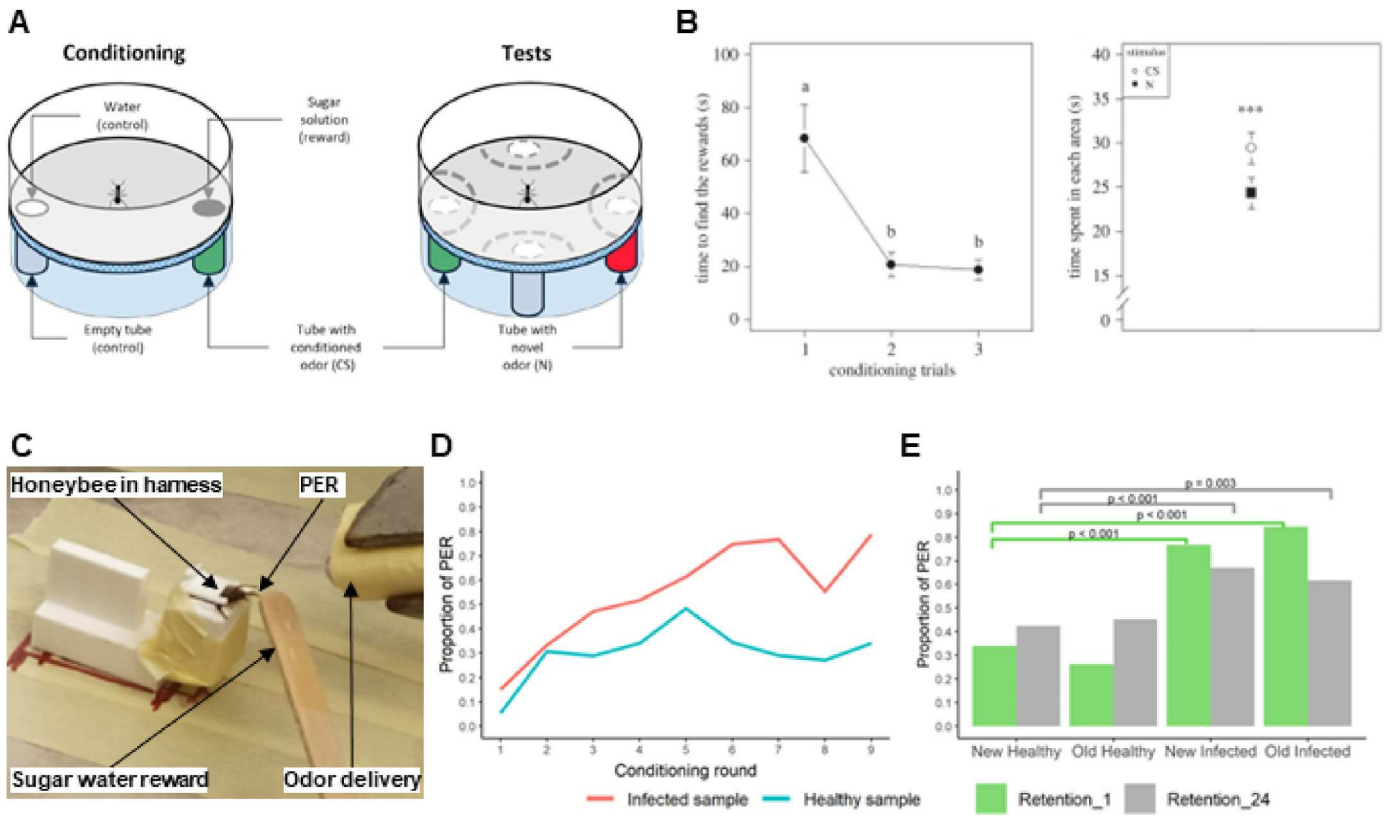

Fig. 4. Disease detection using invertebrate models. A. Ants placed in a dish with target odor and reward for conditioning and then transferred to a dish with conditioned odor and distracting odor for testing. Reproduced with permission from Ref. [95]. **B.** Ants conditioned to urine from patient-derived xenograft mice spent more time in the vicinity of the conditioned odor (CS) compared to the novel odor (N) after only three training trials. Reproduced with permission from Ref. [96]. **C.** Honeybees are contained in custom-made holders, exposed to a target odor, and given sugar water as a reward. **D.** Honeybees were conditioned to produce proboscis extension reflex (PER) towards positive COVID-19 samples (red line), only 9 conditioning rounds were needed for honeybees to learn the sample. **E.** Honeybees could discriminate between healthy and infected COVID-19 samples with an increased PER (green) and were able to retain training 24 h later (gray). D and E adapted with permission from Ref. [58].

concentrations, and retain long-term learning makes rats a reliable animal model for field work without the need for constant reinforcements. In some cases, relying solely on lab medical testing can lead to positive diagnoses being missed as seen in the study by Mgode et al. where trained rats increased tuberculosis detection by 67.6 %, finding 208 additional positive children those clinics had missed [77] (Fig. 3F). Though not as commonly used, mice are like rats in terms of sensitivity and disease detection. Mice were able to detect melanoma with a sensitivity of 82 % in its earliest stages before a tumor and any other clinical symptoms had become visible [61], (Fig. 3E). Another mouse behavioral detection study displayed a difference between the urine of bladder cancer patients pre- and post-surgery [78]. This remarkable ability can be used for early cancer detection and measuring treatment success. Due to the less expensive and shorter nature of raising and training rodents, studies were able to have a higher sample size for detection animals; however, their short lifespans are a limiting factor. Nevertheless, rodents have been used in a multitude of research areas leading to standardized approaches applicable to disease diagnostics and reproducible results.

3.4. *Invertebrate models in behavioral diagnostics*

Invertebrates are less conventional models used for disease detection research, yet recently these easily maintained models have caught the attention of scientists. One specific model is the nematode *Caenorhabditis elegans* (*C. elegans*) which has developed an innate attraction to cancer VOCs in urine and evasive behaviors toward healthy urine, using its highly developed chemosensory system [79]. *C. elegans* are easily maintained in lab settings and do not require any odor training, making them a useful model for rapid and accurate disease detection. It is important to note that all the nematode studies mentioned here, and displayed in Table 1, used VOC detection in a liquid state rather than in a gaseous state like other animals. For testing, chemotaxis assays were performed where nematodes were placed in the middle of a dish with one side containing a urine sample, the test would run for 30–60 min, and the chemotaxis index was then calculated using the number of nematodes on one side of the dish versus the other. As shown in multiple studies, *C. elegans* displayed an attraction to a variety of cancer types and stages with high sensitivity and specificity [80–85]. However, two cancers (Pancreatic and Lymphoma) tested in the study by Inaba et al. had sensitivities below 50 %, a direct contrast to previous results; this could be due to a low number of cancer samples (3 or fewer) tested for these types [85]. Types of cancers, namely pancreatic, can be extremely aggressive forms and early detection is key for many patients when it comes to survival. Nematodes were shown to accurately detect cancers from stages 0–4 with increased performance for earlier-stage samples even when a patient's tumor marker values were normal [81–83], proving extremely useful for early detection. Additionally, *C. elegans* were attracted to urine samples before surgery but not after surgery, displaying they can be employed for not only cancer detection but also treatment success [84]. Though *C. elegans* are a versatile cancer screening tool for urine, other sample types such as blood serum have not been successful; with no significant chemosensory response shown at any concentrations when employing blood serum [80]. Moreover, little research has been done on diseases outside of cancer using *C. elegans*. Even so, *C. elegans* show a promising approach for

Table 1
Studies utilizing behavioral olfaction for disease detection in the last few years.

Group	Animal (n)	Disease/Disorder	Sample Type	Sensitivity (%)	Specificity (%)	Reference
Vertebrate	Canine (1)	Cancer – Cervical	Bandages	96.36	99.55	[68]
			Cervical Smears	92.78	99.1	
	Canine (2)	Cancer – Breast	Cultured Cells	94.4	98.6	[67]
		Cancer – Colorectal	Cultured Cells	92.6	98.1	
	Canine (5)	Cancer – Ovarian	Blood Plasma	85	77	[98]
	Canine (3)	Cancer – Lung	Blood Serum	96.7	97.5	[64]
	Canine (2)	Cancer – Lung	Blood Serum	65.5	82	[65]
			Breath	68.5	80.5	
	Canine (1)	Cancer – Lung	Breath	78	90	[66]
			Urine	87.8	94.8	
	Canine (2)	Cancer – Prostate	Urine	71	70–76	[99]
	Canine (1)	Cancer – Bone	Cultured Cells	95.95	98.30	[75]
			Saliva	100	100	
	Canine (5)	Epilepsy	Breath/Sweat	86.8	98	[100]
	Canine (6)	COVID-19	Sweat	76–100	–	[69]
	Canine (6)	COVID-19	Throat Secretions	65	89	[70]
			Used Masks	86	92.9	
	Canine (8)	COVID-19	Tracheobronchial secretions/Saliva	82.83	96.35	[63]
	Canine (4)	COVID-19 – Delta Variant	Sweat	75–89.9	96.1–98.4	[71]
		COVID-19 – Omicron Variant	Sweat	36.6–41.5	95	
	Rat (3)	Cancer – Lung	Breath	83	81	[60]
	Rat (3)	Cancer – Lung and Diabetes	Breath	87	90	[59]
	Rat (18)	Cancer – Lung	Urine	93	91	[62]
	Rat (18)	Tuberculosis	Sputum	67.6 – (detection increase from clinical testing)		[77]
	Rat (8)	Tuberculosis	Sputum	–	–	[101]
	Rat (9)	Bacteria	Cultured Cells	93.56	97.65	[76]
	Mouse (40)	Cancer – Skin Visible tumor	Urine	90	N/A	[61]
		Cancer – Skin Nonvisible tumor	Urine	82	N/A	
	Mouse (23)	Cancer – Bladder	Urine	100	N/A	[78]
Invertebrate	Nematode (50–100)	Cancer – Pancreatic	Urine	71.4	83.3	[80]
	Nematode (100)	Cancer – Pancreatic	Urine	84.6	60	[81]
	Nematode (50–100)	Cancer – Pancreatic	Urine	–	–	[82]
	Nematode (50)	Cancer – Gastrointestinal	Urine	0.86 AUC	0.86 AUC	[83]
	Nematode (50–100)	Cancer – Colorectal	Urine	0.716 AUC	0.716 AUC	[84]
		Cancer – Gastric	Urine	0.765 AUC	0.765 AUC	
	Nematode (50–100)	All types (shown below)	Urine	87.5	90.2	[85]
		Cancer – Esophageal	Urine	100	–	
		Cancer – Gastric	Urine	100	–	
		Cancer – Colorectal	Urine	88.9	–	
		Cancer – Gallbladder	Urine	100	–	
		Cancer – Bile Duct	Urine	100	–	
		Cancer – Pancreatic	Urine	50	–	
		Cancer – Breast	Urine	100	–	
		Lymphoma	Urine	33.3	–	
		Leukemia	Urine	100	–	
	Honeybee (149)	COVID-19	Throat Swab	92	86	[58]
	Ant (70)	Cancer – Breast	Urine	–	–	[96]
	Ant (36)	Cancer – Breast	Cultured Cells	–	–	[95]
		Cancer – Ovarian	Cultured Cells	–	–	

Note. Sample size (n); area under the curve (AUC).

inexpensive easy-to-maintain cancer screenings.

Insects also rely heavily on olfaction for sensing food and predators from long distances as well as for communication. Honeybees and ants are incredibly social creatures that send information throughout colonies using chemicals and detect them using their antennae. Insects have been shown extensively to display specific behavioral responses towards a variety of odors including simple compounds, plant viruses, and floral odors [86–93]. Honeybees and ants can be trained within 30 min utilizing simple protocols. Interestingly, one trial for ants was enough to form long-term memory that can remain through multiple extinction trials [94]. Recently, ants have been shown to quickly identify breast and ovarian cancer using cultured cell lines [95] and urine from patient-derived xenograft mice [96], shown in Fig. 4B. In addition to ants, honeybees have been used recently for disease detection, specifically COVID-19 (Table 1). Over one hundred honeybees were quickly conditioned to exhibit PER to positive COVID-19 samples and once tested, could diagnose COVID-19 with 92 % sensitivity, although one day after training their ability to do so had decreased [58] (see Fig. 4D and **E**). Honeybees were able to detect COVID-19 with a higher sensitivity than many rapid antigen tests [72,73], displaying their potential for clinical diagnostics, especially in less developed regions where testing is not readily available. Though not as resistant to extinction as ants, the honeybee's ability to learn quickly allows for rapid reinforcement trials to retain training. Due to their social nature, one trained honeybee can reinforce a learned odor to an untrained honeybee through physical antennae communication, meaning that reinforcement to odors could be spread among a whole hive rapidly [97]. Insects are a promising avenue for disease detection because of their highly sophisticated olfaction, easy and inexpensive rearing, and the ability to be trained quickly.

4. Olfactory system component-based detection

4.1. Disease related volatile metabolites

Many volatiles are excreted (e.g. breath, urine) from the human body that contain insight into the disease states within. These volatiles are produced through metabolic processes in different regions of the body

where metabolites that enter the bloodstream, are carried to the lungs or kidneys, and transferred from blood to breath at the alveolar membrane or filtered through the glomerulus to end up in urine. The study of excreted volatiles concerning disease states of the human body is not a new research topic. In 1798 with his classic work "Cases of the Diabetes Mellitus" John Rollo, M.D., recorded the "odor of decaying apples" in the breath of patients with severe diabetes [102]. It wasn't until 1857 that this odor-producing substance was identified as acetone by Petters and in 1886 Dreschfeld stated that the odor of acetone in the breath was characteristic of diabetic coma [102]. Increases in the production of acetone indicate ketoacidosis, a dangerous metabolic state seen in type 1 diabetes where a shortage of insulin results in uncontrolled metabolism of fatty acids producing ketone bodies such as acetone [103,104]. Because there is no mechanism to convert acetone, it is either excreted through the urine or exhaled breath. Several VOCs have been recorded as accepted biomarkers of diseases due to their generation via metabolic pathways in the human body [104]. These volatiles have gone through extensive studies and regulation processes before being accepted as a biomarker; however, many other VOCs seen as putative biomarkers are currently being well established. For instance, aldehydes have been shown as a putative biomarker for cancer, Alzheimer's, and cardiovascular diseases [105–108] as it is an indicator of oxidative stress. Aldehydes are a secondary product of lipid peroxidation that occurs during oxidative stress where free radicals attack cell membrane lipids forming lipid peroxides and aldehydes. These then react with oxidizing agents resulting in oxidative stress and cell damage [106–108]. The incorporation of biomarkers and sensing technologies offers a powerful non-invasive diagnostic tool for monitoring many diseases.

Subtle changes in volatile metabolites have been shown to be indicative of certain types and subtypes of diseases [5,9,109–111]. Li et al. [105] found 4 aldehydes (hexanal, heptanal, octanal, and nonanal) in the exhaled breath of human patients with significant differences in concentrations between breast cancer and control groups. Not only can VOCs differ between breast cancer patients and healthy controls but also between genetically determined breast-cancer subtypes as shown in Barash et al. [112] where the authors found 23 VOCs that were significantly different including ethanol, benzene, heptane, and 1-hexanol. Buszewski et al. [113] found several VOCs concentrations that were significantly lower in the healthy group than in the lung cancer group including butanal, ethyl acetate, and ethyl benzene. With exhaled human breath containing over 3500 known VOCs, the complete list of disease related VOCs is beyond the scope of this review [114–116]. The VOCs mentioned demonstrate the extent and variety of VOC metabolites that have been found and proposed as potential biomarkers for disease detection. The incorporation of a biological components into sensors has been extensively studied due to the sensitivity and selectivity of these biological components for VOC detection. The ability for biological systems and components to probe and characterize chemical space has fueled many of these studies. Here, we discuss the incorporation of biological components into biosensors for disease and disease-related VOC detection.

4.2. Odorant binding protein

Several studies have employed odorant binding proteins (OBPs) as the biological component in bioelectronic sensors (Table 2). OBPs are involved in the initial step of odorant recognition and are small (~20 kDa) secreted globular proteins that act as transporters [117]. OBPs carry odorant molecules across aqueous nasal mucosa (vertebrates) or the sensilla lymph (invertebrates) to ORs [118]. OBPs form a complex with odorant molecules in the activation of ORs and additionally remove odorants from the nasal mucosa or sensillum lymph. OBPs from a variety of animals have been incorporated into bioelectronic sensors including pigs, honeybees, mosquitoes, rats, flies, moths, and human OBPs.

In the study by Capo et al. [119], the authors use porcine OBP (pOBP) for the detection of benzene using a competitive fluorescence resonance energy transfer (FRET) assay based on steady-state fluorescence spectroscopy. The developed assay displayed a high affinity for benzene detection with a limit of detection value of 0.05 μM (3.9μg/m3). In Calabrese et al. [120], the authors also use pOBP to detect three different volatile organic compounds (1-octen-3-ol, *trans*-2-hexen-1-ol, and hexanal) using an electrochemical biosensor via electrochemical impedance spectroscopy (EIS). Using EIS, the authors were able to detect each VOC sample at a minimum concentration of 0.1 μM. In another study utilizing a fluorescent assay, Dimitratos et al. [121] used the mosquito (*Anopheles gambiae*) AgamOBP1 for the detection of indole. The authors showed that indole could be detected at concentrations of 100 nM (~5 ppb). Interestingly, this sensor was also used to detect colony-forming units of *E. coli* and canine feces contamination in an aqueous solution. Soleja et al. [122] employed a FRET-based nanosensor for the detection of ethanol via a human OBP ($hOBP_{IIa}$). In this study, the authors used $hOBP_{IIa}$ sandwiched between two fluorophores (ECFP and Venus) that had an induced conformation change when bound to an odorant. The donor fluorophore transfers its excitation energy to a second acceptor fluorophore causing the second fluorophore to give off its characteristic fluorescence (Fig. 5A and **B**) [123].

An important advantage of using OBPs as the biosensing element in bioelectronic sensors is that the binding properties can be engineered through site-directed mutagenesis. In Hurot et al. [124], the authors used rat OBP3 to detect β-ionone, hexanoic acid, and hexanal.

Table 2
Odorant binding protein-based bioelectronic sensors.

Odorant Binding Protein	Derived from	Target Odorant	Detection range/limit	Detection with	Reference
$hOBP_{IIa}$	Human	Ethanol	500 nM−12 μM	FRET-based nanosensor	[122]
OBP2a	Human	Hexanal, heptanal, benzaldehyde, 2-octenal, decanal, β-cyclocitral, 2-isobutyl-3-methoxypyrazine, 2-Methylisoborneol	5–75 μmol/L	Fluorescence assay	[146]
pOBP	Porcine (*sus scrofa*)	Benzene	0.05 μM (3.9 μg/m^3)	Fluorescence assay	[119]
pOBP	Porcine (*sus scrofa*)	1-octen-3-ol, trans-2-hexen-1-ol, hexanal	0.1 μM	EIS	[120]
OBP3, OBP3-a, OBP3-c	Rat	β-ionone, hexanal, hexanoic acid	200 pM	SPRi	[124]
OBP1	Mosquito (*Anopheles gambiae*)	Indole	100 nM (~5 ppb)	Fluorescence assay	[121]
OBP5, OBP6, OBP7	Mosquito (*Anopheles gambiae*)	Hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, undecanoic acid, dodecanoic acid	2 ppb	SiNW array chip	[147]
OBP LUSH (OBP76a)	*Drosophila melanogaster*	Ethanol	10^{-6}% of EtOH	bio-FET	[148]
OBP3, I7 (OR)	Rat	Octanal, diacetyl, amyl butyrate	0.01 ppm	CNT-FETs	[139]

Note. Fluorescence resonance energy transfer (FRET); Electrochemical impedance spectroscopy (EIS); Surface plasmon resonance imaging (SPRi); Silicon nanowire (SiNW); Nanostructured bio-field-effect transistor (bio-FET); Carbon nanotube field effect transistors (CNT-FETs).

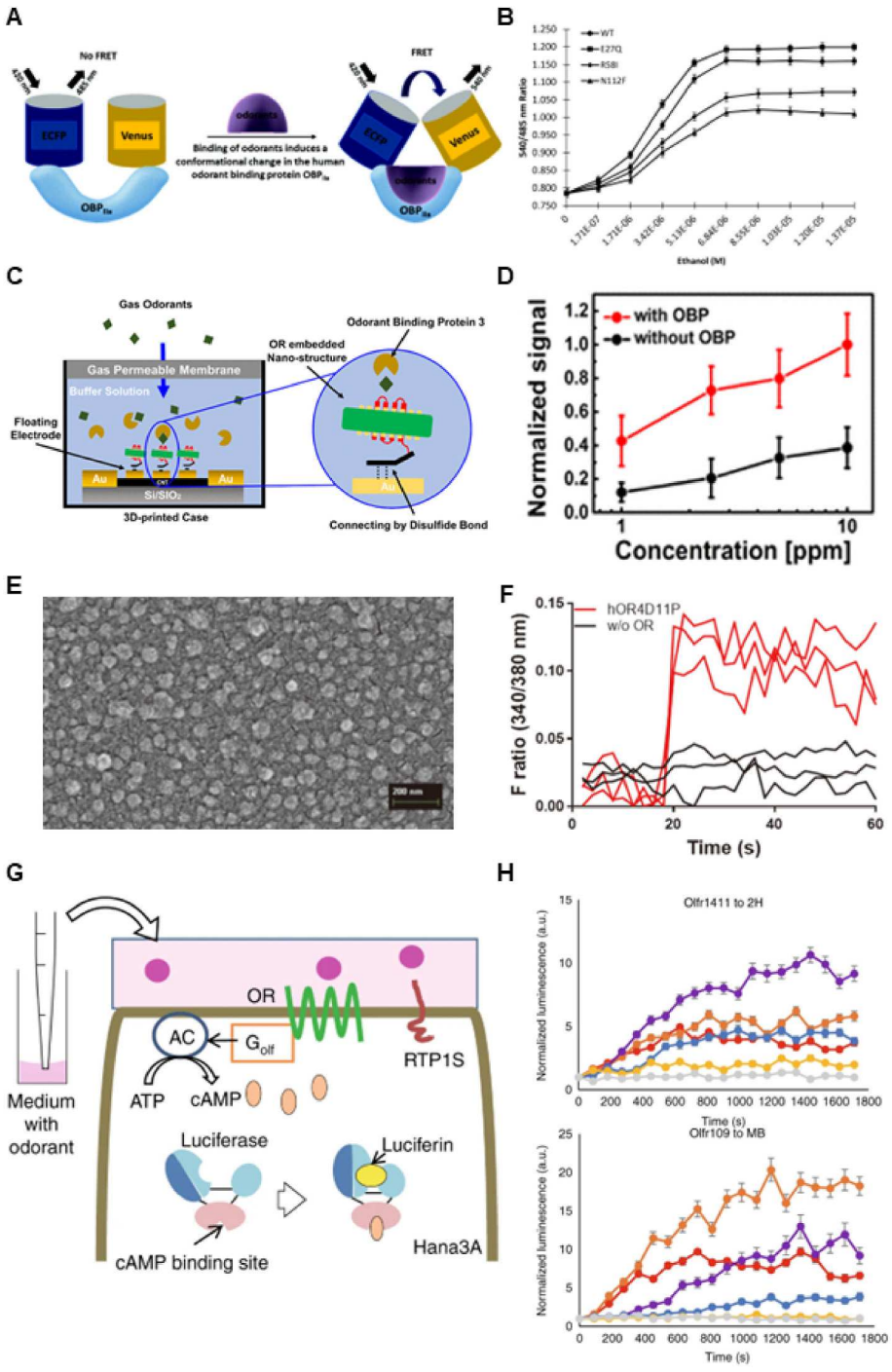

Fig. 5. Odorant binding protein and whole cell-based biosensors for the detection of volatile organic compounds. A. Schematic representation of OBP_{IIa} between two fluorophores ECFP and Venus in the presence of a ligand [122]. **B.** Comparison of wildtype (WT) and three engineered OBPs (E27Q, R58I, and N112F) and their concentration-dependent FRET ratio changes. Reproduced with permission from Ref. [122]. **C.** Bioelectronic sensor incorporating both OBPs and ORs. Schematic representation of a bioelectronic sensor platform [139]. **D.** Normalized signals of the bioelectronic sensor to different concentrations of diacetyl gas. Data points and error bars represent averages and standard deviations of the data measured from three devices, respectively. Reproduced with permission from Ref. [139]. **E.** OR nanovesicle-based sensor. SEM image of nanovesicles with OR, hORD11P, derived from HEK293 cells. (scale bar: 200 nm) [140]. **F.** Calcium signaling assay of hOR4D11P-expressing nanovesicles and empty-vector transfected nanovesicles exposed to 1 mM 2-ethyl-1-hexanol, a lung cancer biomarker. Reproduced with permission from Ref. [140]. **G.** Cell-expressing OR sensor schematic representation of GloSensor assay in Hana3A cells showing OR signal transduction pathway [153]. **H.** Real-time measurement of two ORs, Olfr109 and Olfr1411, to 2-heptanone(2H) and methyl benzoate (MB), respectively. Reproduced with permission from Ref. [153].

Additionally, two synthetic OBPs with various binding properties were created (OBP3-a and OBP3-c) by modifying the amino acid sequence of the OBP3 (wild type) binding pocket. This study showed for the first time the use of surface plasmon resonance imaging in combination with an OBP for odorant detection. Overall, the use of wildtype and engineered OBPs allowed the biosensors to detect odorants with detection limits in the picomolar range. The creation of engineered OBPs tuned to a desired odorant molecule represents an important advantage when

natural or wildtype proteins are either not known or have insufficient binding affinities for specific chemical detection [125–127]. There are several other advantages to using OBPs in bioelectronic sensors including their ability to be stable in high temperatures (around 70 °C) and resistant to changes in pH, solvents, and proteolytic digestion [128–131]. OBPs can be used in a wide range of environmental conditions. Additionally, OBPs can become denatured by chemicals and easily refold upon the removal of the denaturing chemicals to an active conformation [118,130–133]. OBPs can also be expressed and purified in high yields in eukaryotic and bacterial systems using established protocols [134,135].

OBPs are the natural transporters of VOCs in biological olfactory systems making them an ideal component to incorporate into bioelectric sensors for monitoring and detection applications in clinical diagnostics but also in other applications where chemical monitoring is needed. OBPs have been used for the detection of drugs (cannabinol, 3,4-methylenedioxy methamphetamine, cocaine hydrochloride) in a study by Cali et al. [136] where the authors used the mosquito (*Anopheles gambiae*) OBP1 and OBP47 immobilized on quartz crystal microbalance for detection. Additionally, Scorsone et al. [137] used an array of OBPs from the pig, mosquito (*Anopheles gambiae*), in combination with mouse major urinary proteins, another ligand binding protein, for drug and explosive detection. These studies show that OBPs can be employed in a wide variety of applications. While there are several advantages to employing OBPs in bioelectronic sensors there are some limitations. There are fewer OBPs in comparison to ORs. For example, there are around 1100 OR genes in mice, while there are only 5 known OBPs [138]. This limits the ability of OBPs to characterize the chemical space as extensively as ORs. The specificity of OBPs could be a limitation due to the broad detection range of chemical binding to OBPs.

An interesting study by Choi et al. [139] employed both OBPs and ORs in a single bioelectronic sensor that mimicked the biological olfaction in the nasal mucosa or the sensilla lymph. The authors used a carbon nanotube field-effect transistor (CNT-FET) that had been hybridized with the rat-I7 ORs. Furthermore, the CNT-FET with ORs was placed in a chamber covered by a gas-permeable membrane containing a solution with rat OBP3. This platform allowed odorant molecules to pass through the membrane, forming a complex with OBPs in the solution which are then transported to the I7 ORs for detection, just like biological olfaction (shown in Fig. 5C). The authors showed that the platform with OBPs had 10^4 times higher sensitivity and amplified signal intensity than the platform without the OBPs for the detection of octanal down to 0.01 ppm as shown in Fig. 5D. Overall, this study showed the importance of OBPs in the detection of odorant molecules that had applications in clinical diagnostics for VOC detection.

4.3. Odorant receptor

ORs are membrane-bound proteins found on the dendrites of olfactory neurons that bind to odorant molecules. Many of these bioelectronic sensors have incorporated ORs for odorant/chemical detection as presented in Table 3. Cho et al. [140] used the human OR 4D11P (hOR4D11P) and generated cell-derived nanovesicles to detect a lung cancer biomarker. The authors sampled headspace gas mixtures from lung cancer cell lines for the identification of a VOC biomarker, 2-ethyl-1-hexanol, that has been previously reported as a candidate biomarker for lung cancer [141]. A field emission scanning electron microscope image confirmed nanovesicle formation (Fig. 5E) and using a calcium signaling assay the nanovesicles showed significant response to 1 mM 2-ethyl-1-hexanol in comparison to nanovesicles without the

Table 3
Odorant receptor and whole cell-based bioelectronic sensors.

Odorant Receptor	Immobilized on/ Expressed in	Derived from	Target Odorant	Detection range/limit	Detection with	Reference
OR4D11p	Nanovesicles	Human	2-ethyl-1-hexanol	1 mM	Fluorescent imaging	[140]
OR2AG1	Graphene	Human	Amyl butyrate	500 fM	Resistance measurements	[142]
OR35a	Liposomes	*Drosophila melanogaster*	E2-hexenal	1 fM−1 μM	EIS	[145]
OR10a, OR22a, OR71a	Liposomes	*Drosophila melanogaster*	Methyl salicylate, methyl hexanoate, ethyl hexanoate, E2-hexenal, 4-ethylguaiacol	1 aM−1 μM	EIS	[149]
OR10a, OR22a, OR35a, OR71a	Lipid nanodiscs	*Drosophila melanogaster*	Methyl salicylate, methyl hexanoate, trans-2-hexen-1-al, 4-ethylguaiacol,	1 fM−10 pM	CNT-FETs	[150]
OR10a, OR22a, OR35a, OR71a, Orco	Liposomes	*Drosophila melanogaster*	Methyl salicylate, methyl hexanoate, 4-ethylguaiacol, E2-hexanal	1 aM−1 μM	EIS	[144]
OR10a, OR22a, Orco	Lipid nanodiscs	*Drosophila melanogaster*	Methyl salicylate, methyl hexanoate	1 fM	GFET	[151]
OR22a	Lipid nanodiscs	*Drosophila melanogaster*	Ethyl hexanoate	5.5 fM	EIS	[143]
OR8, Orco	Bilayer lipid membranes	Mosquito (*Aedes aegypti*)	1-octen-3-ol	0.01−0.2 ppm	Ion currents	[152]
Panel of 31 ORs	Hana3a cells	Mouse	Acetophenone, cyclohexanone, eugenol, heptanal, 2-heptanone, methyl benzoate, N-amyl acetate	10°–10^{-8} (vol/vol)	Luminescence	[153]
BmOR3	Sf21 cells	Silk moth (*Bombyx mori*)	Bombykal	1–10 μM	Fluorescent imaging	[154]
OR13a, OR56a	Sf21 cells	*Drosophila melanogaster*	Geosmin, 1-octen-3-ol	1 μM	Fluorescent imaging	[155]
OR13a	Sf21 cells	*Drosophila melanogaster*	1-octen-3-ol	10 μM	Fluorescent imaging	[156]
OR13a, OR56a, Orco	Sf21 cells	*Drosophila melanogaster*	Geosmin,1-octen-3-ol	0.1 % (vol/vol)	Fluorescent imaging	[157]
OR13a, Orco	Sf21 cells	*Drosophila melanogaster*	1-octen-3-ol, 2-heptanol, 1-hexanol, cis-3-hexen-1-ol, 1,8-cineole	0.1 % (vol/vol)	Fluorescent imaging	[158]
OR13a, OR56a, Orco	Sf21 cells	*Drosophila melanogaster*	Geosmin, 1-octen-3-ol	0.1 % (vol/vol)	Fluorescent imaging	[159]
OR13a, OR56a, OR49b, Orco	Sf21 cells	*Drosophila melanogaster*	Geosmin, 1-octen-3-ol, cis-3-hexen-1-ol, citral, decanal	0.1 % (vol/vol)	Fluorescent imaging	[160]

Note. Electrochemical impedance spectroscopy (EIS); Carbon nanotube field effect transistors (CNT-FETs); Graphene field effect transistor (GFET).

ORs (Fig. 5F). This study showed how the VOC profiles from lung cancer cell lines can have differences in specific volatiles that can be identified and displayed the ability of ORs for the detection of lung cancer-associated VOCs. However, this study only compares two lung cancer cell lines to a media control for the identification of the lung cancer VOC, and a more thorough analysis is needed for the comparison of a healthy cell line control. Goodwin et al. [142] also used a human OR, 2AG1, for the detection of amyl butyrate. Here the authors showed that a graphene-based sensor exhibits a linear response for amyl butyrate between 0.1 and 500 pM. Cheema et al. [143] used OR, Or22a, from the fruit fly, *Drosophila melanogaster*, for the detection of ethyl hexanoate with a limit of detection of 5.5 fM. The ORs were immobilized onto nanodiscs using phospholipids and membrane scaffold proteins that mimic ORs' native environment. The nanodiscs were then adhered onto a gold electrode and detection of ethyl hexanoate was measured via electrochemical impedance spectroscopy. Multiple ORs can be used in combination with the odorant receptor co-receptor (Orco), an ion channel-forming subunit. The OR functions to bind the odorant molecule which then causes the Orco ion channel to open allowing an influx of ions. Khadka et al. [144] used four ORs from the *Drosophila melanogaster*, OR10a, OR22a, OR35a, and OR71a, and Orco reconstituted into lipid bilayers of artificial liposomes to detect methyl salicylate, methyl hexanoate, and E2-hexanal. The biosensor could detect these target ligands down to sub-femtomolar concentrations which was an improvement from a previous study where the same ORs were used except without Orco subunit incorporated into the liposomes [145]. OR-based sensors have rapid response times and high sensitivity for target chemicals, however variability in the sensors' performance due to environmental conditions, such as temperature and humidity, is a challenge.

4.4. Whole cell

Whole cell-based systems provide another avenue for bioelectronic sensors in the detection of volatiles as presented in Table 3. These systems use cell cultures that either have olfactory components expressed in them or use genetically modified bacteria (without olfactory components) as the sensor. Regardless of the system, both approaches use the luminescence or fluorescence of the cell to monitor odorant detection. In Kida et al. [153], the authors use a panel of mouse ORs expressed in mammalian Hana3A cells where the luminescence activity of the cell depended on cAMP levels (see Fig. 5G). The authors conducted a large-scale screening of mouse ORs against a panel of seven odorants: acetophenone, cyclohexanone, eugenol, heptanal, 2-heptanone, methyl benzoate, and N-amyl acetate. The large-scale screen identified 29 ORs that responded to the panel of odorants. The results of two ORs, Olfr109 and Olfr1411, responding to methyl benzoate and 2-heptanone at several concentrations are shown in Fig. 5H, respectively. Also, the authors distinguished between structurally similar odorants using acetophenone and six of its analogs. This differential activation of structurally similar volatiles shows the robust nature of ORs in the detection of volatiles.

Several studies have used the insect Sf21 ovarian cells isolated from the Fall Armyworm (*Spodoptera frugiperda*) to express fruit fly, *Drosophila melanogaster*, ORs along with Orco. In these studies, the cells give off a fluorescence response via the calcium indicator fluorescent protein (GCaMP6). Briefly, when an odorant molecule binds to the OR, a cation channel consisting of the OR and Orco opens and allows for the influx of Ca^{2+}. The influx of Ca^{2+} binds to the GCaMP6 inside the cell causing a change in the fluorescence intensity. In the study by Sukekawa et al. [155], the authors used two ORs, Or13a and Or56a, and Orco from the *Drosophila melanogaster* expressed in Sf21 cells. The authors were able to use the specific pattern of multiple randomized cells for the identification of odorants, 1-octen-3-ol and geosmin. In the study by Deng et al. (2023) [160], the authors simultaneously expressed two ORs, Or56a and Or49b, in a single cell line. The usefulness of this system, however, is unclear as the partial quantification of a gas mixture (o-cresol and geosmin) was only able to quantify o-cresol.

Whole-cell biosensors can detect volatiles and analytes rapidly while also elucidating the response of these analytes on the cell's biological activity. Using genetically modified bioluminescence bacterial strains and analyzing the differences in the bacterial responses to general stresses (i.e., cytotoxicity, genotoxicity, oxidative stress, and quorum-sensing stress), these bacterial strains can be used to measure potentially hazardous substances [161]. Multiple studies have shown the application of whole-cell biosensors to test the toxic effects of volatile chemicals [162–167]. The wide range of volatiles detected in these studies include nonanal, 3-methyl-1-butanol, 1-octen-3-ol, 1-octanol, phenylethyl alcohol, 2-ethyl hexanol, ethyl propionate, 1-methyl-1 H-pyrrole, and 2,3 butanediol.

4.5. Peptide

Peptides offer another avenue for the creation of bioelectronic sensors for the detection of VOCs as presented in Table 4. Peptides and their affinity for VOCs can be tuned by engineering the peptide's amino acid sequence [168–170]. The main factor contributing to the bond is the chemical properties of the volatile compound [171]. There are several advantages for the use of peptides in biosensors including their high stability, simplicity of development from a combination of 20 amino acids, and ease of quality control [172,173].

Sim et al. [174] used peptides functionalized on CNT-FETs to discriminate four breath-related VOCs of isopropyl alcohol, acetone, isoprene, and toluene. The CNT-FETs functionalized with peptides were exposed to VOC concentrations of 10,000 ppm and the FET sensor showed distinct responses for each VOC. Gaggiotti et al. [175] used both peptides and hairpin DNA (hpDNA) in combination on the same biosensor for detection of six VOCs, 1-butanol, 1-pentanol, 1-hexanal, 1-nonanal, *trans*-2-nonenal, and 1-hexanoic acid, employing surface plasmon resonance imaging (SPRi) as the detection system. Combining the responses of peptides and hpDNA and by using hierarchical clustering analysis the biosensor showed perfect separation between the chemical classes and separation of VOCs within the same class with 1 carbon difference. Wasilewski et al. [176] designed a peptide sequence associated with an OBP, HarmOBP7 from the *Helicoverpa armigera* moth, that mimics its sites of molecular binding of ligands and immobilizes this peptide onto a piezoelectric transducer as shown in Fig. 6A. This sensor detected VOCs, octanal, decanal, undecanal, nonanal, and helional with the lowest limit of detection of 14 ppm for nonanal (Fig. 6B). In other applications, these studies have used OR-derived peptides for the detection of trimethylamine, a harmful gas, at concentrations as low as 0.01 parts per trillion and even parts per quadrillion levels [177,178]. While in other studies, pheromones β-ocimene and 4-vinylanisole were detected using *Hyphantria cunea* and the migratory locust (*Locust migratoria*) OR-derived peptides [179,180].

Overall, the possibility of synthesizing peptides in high yield assays, their stability as a biosensing element, and simple modification of amino acid sequences make peptides an attractive biological component in biosensors. However, the number of peptides enabling gas molecule analysis is narrow but can be overcome by understanding OBP binding sites for the creation of new peptide sequences that can successfully bind to VOCs and increase the efficiency of bioelectronic sensors.

4.6. Bacteriophage

Bacteriophage-based biosensors use filamentous phage, such as M13, that have been genetically modified to encode specific amino acid sequences (peptides) on the surface of the phage [181,182]. These peptides give the phage distinct surface chemistries that can bind and detect various targets. The outer coat of these phages is composed of thousands of copies of protein VIII for the formation of nanofibers with a high surface-to-volume ratio where the altered amino acid sequences or

Table 4
Peptide-based bioelectronic sensors.

Peptide Sequence	Identified from	Target Odorant	Detection range/limit	Detection with	Reference
IHRIC, LAWHC, TGKFC, WHVSC	–	1-butanol, 1-hexanol, 2-methyl-1-propanol, ethanol, hex-3-en-1-ol, ethyl acetate, ethyl-methyl-2-butyrate, isopentyl acetate	–	QCMs	[193]
NPAATMA, SIFPVSR, MPRLPPA	–	Benzaldehyde	–	Fluorescent intensity	[194]
KLLFDSLTDLKKKMSEC	HarmOBP7 (*Helicoverpa armigera*)	Octanal	37.5 ppm	QCMs	[195]
Panel of 4 peptides	–	Isopropyl alcohol, acetone, isoprene, and toluene	10,000 ppm	Field-effect transistors	[174]
TGKFC, KSDSC, IHRIC, WHVSC, LAWHC, LGFDC	–	1-butanol, 1-pentanol, 1-hexanal, 1-nonanal, trans-2-nonenal and 1-hexanoic acid	3.6−90 ppm	SPRi	[175]
KLLFDSLTDLKKKMSEC	HarmOBP7 (*Helicoverpa armigera*)	Octanal, decanal, undecanal, nonanal and helional	14 ppm	QCMs	[176]
Panel of 5 peptides	OR19a (*Drosophila melanogaster)*	Limonene	8 pM	Graphene field-effect transistor	[115]
GGGRGAGAGAR, FLLFGGGRGAGAGAR, RRWLLLW GGGRGAGAGAR	–	Limonene, methyl salicylate, menthol	10 pM−10 nM	Graphene field-effect transistor	[196]
20 peptides (bacteriophage)	Mammalian ORs	Breath from healthy and lung cancer patients	–	Colorimetric sensor	[186]
20 peptides (bacteriophage)	–	Emitted VOCs from diabetic cells, organoids, and mice	–	Colorimetric sensor	[188]

Note. Quartz crystal microbalances (QCMs); Surface plasmon resonance imaging (SPRi).

peptides exist [183]. These phages can be self-assembled onto colorimetric sensors composed of several bundles of these phages [184]. When white light is displayed onto the phage film, specific wavelengths of light are given off. When VOC molecules interact with the phages and if the interaction between the peptide and VOC is strong, the whole phage bundle swells and expands thus changing the wavelength or color of the bundle of phages [184]. Modifying the peptide sequence and thus altering the binding affinity to VOC molecules, will characterize the strength of the interaction between the peptide and VOC, which can be measured by the optical gap shift (color change) of the biosensor [185]. An array of these colorimetric sensors can be constructed with each sensor composed of a specific type of modified phage. The whole multi-array of colorimetric sensors can then be used as a sensor in combination with pattern recognition techniques to classify VOCs or VOC mixtures as presented in Table 4.

In Lee et al. [186], the authors use DNA sequences from mammalian ORs for the creation of 20 genetically modified phages expressing the reactivities of the ORs (Fig. 6C and **D)**. In this study, the authors used human breath (200 ml) from 31 healthy subjects and 31 lung cancer patients without pretreatment for classification. Using the phage-based biosensor the authors were able to achieve 87 % classification via hierarchical clustering analysis (Fig. 6E and **F**) [186,187]. In Jang et al. [188], the phage-based bioelectronic sensor also consisted of 20 genetically engineered M13 bacteriophages to detect diabetes via VOC gas mixtures emitted from cell culture, organoids, and mice. The bacteriophages contain peptides with properties of electrically charged side chains, polar uncharged side chains, hydrophobic side chains, and other special cases. Exhaled breath from four groups of mice (control, diabetes, cardiomyopathy, and diabetic cardiomyopathy) were collected for classification. Hierarchical cluster analysis with neural pattern separation achieved a classification success rate of 86.7 % indicating that this sensor was able to detect diabetes models and specific complications of diabetes (cardiomyopathy) via VOC gas mixtures. These studies display the ability to use bacteriophage biosensors for clinical diagnostics.

There are several advantages to using M13 bacteriophage-based biosensors including the ability to easily manipulate the sequence of amino acids via genetic engineering techniques and simple production of genetically engineering phage bundles through a spontaneous self-assembly process [183,184]. These sensors can respond within a few milliseconds to external stimuli and can detect concentrations as low as 1 ppb as shown in the study by Park et al. [189]. Beyond clinical diagnostics, these sensors can also be used for other chemical monitoring applications [190–192].

5. 'Cyborg'- 'part-brain-part-engineered' gas sensors for disease detection

5.1. The next generation of biosensors

Biosensors where the *whole* living olfactory brain is coupled with technology, such as electrophysiological or functional imaging setup, for the detection of volatile chemicals, are termed 'cyborg' sensors. Coupled with extensive biological detection and computational power, 'cyborg' sensors capture the complexities of odor-evoked neural activity dynamics and the ways in which the whole brain works to process information. There are current 'state-of-the-art' biohybrid disease detectors, where a part of the biological brain or a part of the biological olfactory pathway has been 'hijacked' by the researchers to perform the gas-based disease detection function. The advantages of these types of sensors are that they include biological chemical detection, chemical transduction, encoding, and decoding biological computations in one single part-brain-part-engineered device. However, this novel concept has been applied directly for disease detection only a handful of times, rather more work has been shown surrounding the detection of single compounds that make up the complex mixture of disease VOC profiles. Nonetheless, these sensors are also extremely sensitive for disease detection as they use the entire capability of a biological organism (e.g., insect brains) for volatile biomarker detection.

5.2. Cyborg disease sensors with electrical neuronal activity as readout

Several of these studies are conducted in insect brains. Insects have a powerful sense of smell and the neural coding of odorants in different neural circuits of the olfactory brain is well studied from a neuroscience perspective [37,39,42,56,197–202]. Insect brains are also accessible for physiological recordings from different parts of the olfactory sensory pathway. Farnum et al. [203] have created a novel method of combining cancer cell-evoked olfactory neural recordings from the locust (*Schistocerca americana*) antennal lobe with data acquisition and analytical techniques for the detection of human oral cancer using the 'smell' of cell cultures (Fig. 7). For these experiments, three different human oral

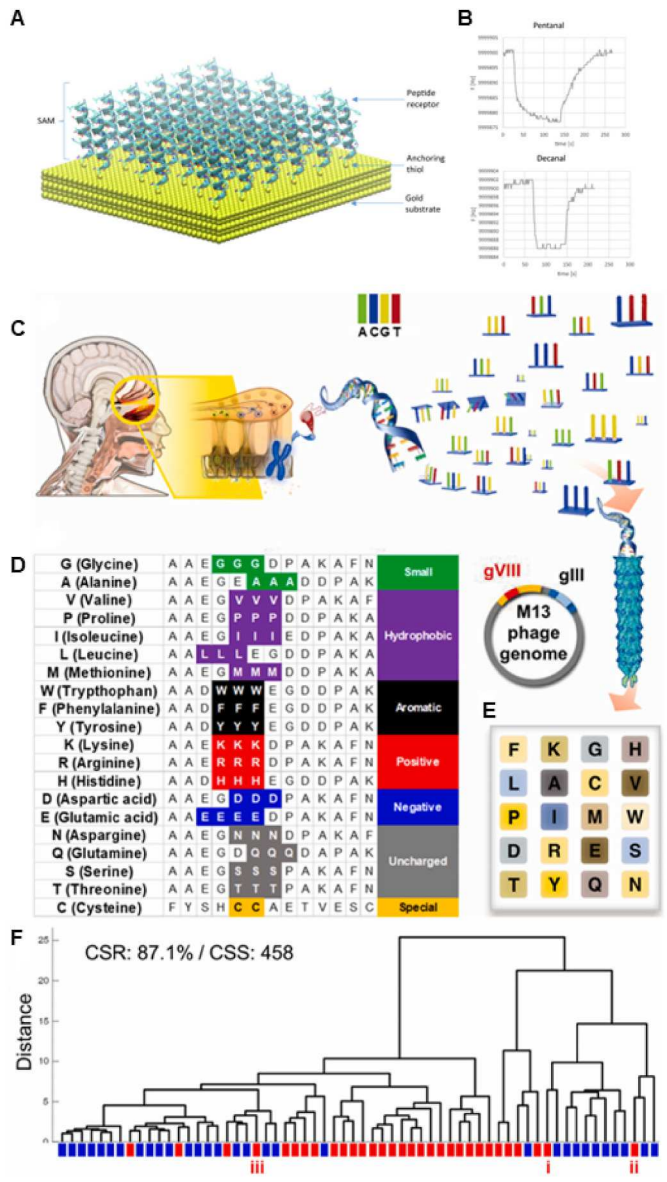

Fig. 6. Peptide- and bacteriophage-based biosensors for the detection of volatile organic compounds. A. Peptide molecules were anchored on the transducer using a bond with thiol group. The thiol group allows formation of self-assembled monolayers (SAM) on the gold surface [176]. **B.** Resonant frequency responses of the peptide-based biosensor to aldehydes for low concentration levels in the gas phase: pentanal – 105 ppm and decanal – 60 ppm. Reproduced with permission from Ref. [176]. **C.** Schematic representation showing the fundamental principle behind the construction of bacteriophage-based biosensors. Amino acid sequences related to DNA for mammalian odorant receptors were selected and implanted into phages via genetic engineering [186]. **D.** List of 20 peptide sequences expressed in the phages with associated properties [186]. **E.** Schematic representation of bacteriophage-based biosensor showing the array placement of each phage film [186]. **F.** Hierarchical clustering analysis (HCA) dendrogram with a classification success rate (CSR) of 87 % and classification success score (CSS) of 458. Blue indicates healthy patients and red indicates lung cancer patients. Reproduced with permission from Ref. [186].

cancer cell lines and a noncancer cell line were cultured using the same cell culture media. These cell cultures were kept in closed flasks and odor headspace above the cell cultures were delivered to a locust antenna using an olfactometer. Neural recordings were performed from the projection neurons in the antennal lobe. Using these odor-evoked population neural responses and biological neuronal computational scheme-based analyses, Farnum et al. demonstrated that all three human oral cancer cell lines can be distinguished from each other and the noncancer cell line with 100 % accuracy (using a leave-one-trial-out cross-validation method) [203]. This study also validated that the neural recordings from the locust olfactory brain were able to distinguish between human oral cancer cell lines over several days of culture. This detection technique was very fast as it was based on a biological neural computational approach, and the authors demonstrated that cancer cell classification can be achieved within 500 ms of odor exposure.

Furthermore, Parnas et al., demonstrated that human lung cancer biomarker-evoked neural recordings from the honeybee brain can be employed to differentiate between several different volatile cancer biomarkers [204]. Additionally, the authors showed that a 'synthetic lung cancer' breath mixture that mimicked the biological concentrations of lung cancer biomarkers found in patient's exhaled breath can be successfully distinguished from a synthetic healthy exhaled breath using neuronal recordings from the honeybee brain. To validate their technology, the authors applied their honeybee sensor to human cell lines by using the 'smell' of cell cultures, demonstrating that the honeybee brain could discriminate between healthy, small cell lung cancer, and non-small cell lung cancer cell lines. Employing this insect brain-based disease detection technology, cyborg sensors (locust or honeybee brain-based) have been used in other ongoing studies including early and noninvasive detection of endometriosis, differentiation between multiple types of cancers (e.g., lung cancer vs. breast cancer), and for gas-based detection of bacterial biofilm formation (unpublished data).

Another recent study, done by Neta et al., employed live *ex vivo* locust antennae coupled with electroantennogram recordings and machine learning-based data analysis for chemical detection [47]. The authors demonstrated that just the antennae of an insect can be used to detect several volatile chemicals and their mixtures at very low concentrations [47]. This innovative study establishes that only a part of the biological olfactory pathway (e.g., the biological chemosensory array or the antennae) can be successfully employed to differentiate between multiple volatile chemicals and their mixtures. Moreover, this study showed that the sensitivity of this antennae-based gas sensor is better than GC-MS-based detection thresholds as this device was able to detect the presence of 1 ng of volatile compounds. Due to the ability of this sensor to detect chemicals at incredibly low concentrations, this study opens the door for testing antennae-based cyborg devices for disease detection in the future.

5.3. *Cyborg disease sensors with functional imaging as readout*

Another methodology that can be applied for disease detection using olfactory neuronal excitability in the brain involves functional imaging. Using odor-evoked calcium imaging analysis, Strauch et al. [205] recorded from a large number of ORNs in the fruit fly antennae. As these sensory neurons are located close to the surface of the antennae, spatial imaging showed calcium activity in multiple olfactory neuron types. Cell culture VOCs corresponding to human breast cancer vs. healthy cell lines were used as the target stimuli. Next, the combinatorial patterns of activation of the neuronal population were analyzed, and the results showed distinction between human breast cancer vs. healthy cell lines. This early study demonstrated that human cancer can be detected by the combinatorial coding scheme of sensory neurons located in the fruit fly antennae. In a more recent study, Carcaud et al. [206] utilized a genetically encoded calcium sensor in honeybees to detect 16 different odorants. Neural responses were recorded from the AL, lateral horn, and mushroom body simultaneously with odor presentation and showed a biphasic response (increase after odor followed by undershoot). Odor fluorescent response patterns within the AL could be clustered based on functional group and carbon chain length, showing a clear neural coding and odorant type relationship. While not used for disease detection, this work shows promise for disease biomarker detection using cyborg gas sensors.

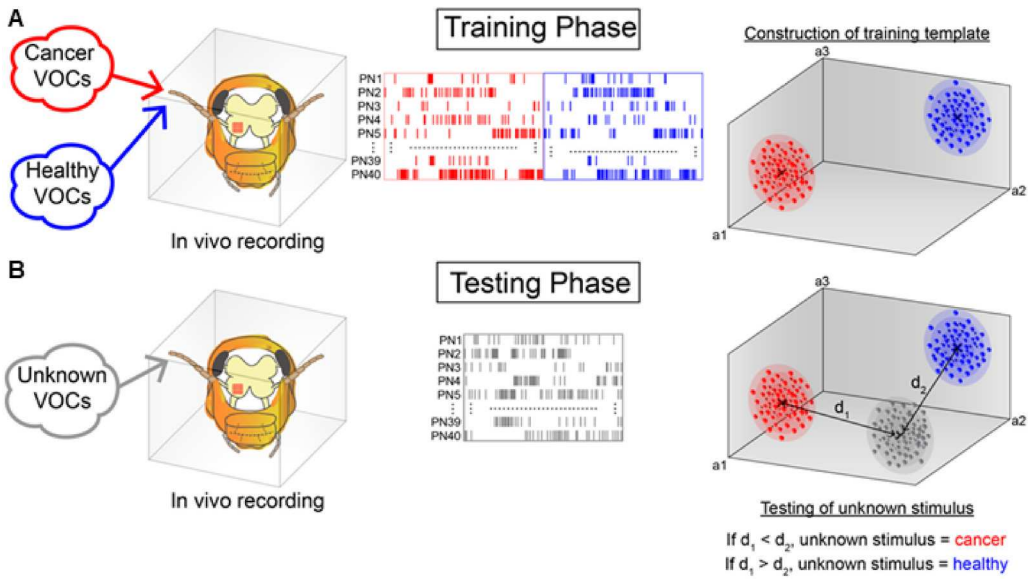

Fig. 7. Part-brain-part-engineered insect brain-based cyborg VOC sensor for disease detection. In this cyborg sensor, multi-channel electrophysiology recordings were combined with data acquisition and analysis platforms for human oral cancer detection. **(A)** training and **(B)** testing protocol of a cyborg sensor is shown here [203]. Briefly, during training, distinct population neuronal response fingerprints are generated corresponding to each target odor (e.g., cancer vs. non-cancer VOC mixtures). During testing phase, unknown gas mixtures were quantified based on the similarity between the unknown vs. training response fingerprints.

6. The future of VOC diagnostics

While VOC analysis has emerged as a promising new diagnostic tool, there are still many obstacles within the field. VOC analysis has the potential to be cheaper and faster than traditional diagnostic tools, while also being non-invasive. Because the VOC profile can carry information about the health of the entire body, these analyses can test for many different health concerns. However, before real devices can be implemented in medical settings, there are important challenges to overcome. Most biomarkers reported in the literature are still putative. This means that up- or down-regulation of the biomarker has been correlated with the presence of the disease, but a definitive link between the disease within the body and the biomarker has not been found. Also, unlike type-1 diabetes, which is associated with elevated acetone concentrations [207], most diseases have been correlated with complex changes to the breath VOC profile. Most notably, cancers are extremely heterogeneous with changes to hundreds of VOCs being observed that are not consistent between cancer types [208]. Therefore, a sensor that can only detect one or a few VOCs will not be relevant for diagnosing these more complex diseases. The two major types of engineered sensors have advantages and drawbacks: 1) GC-MS and other component-wise VOC detection instruments have the capability to sensitively detect an extremely broad spectrum of VOCs, however, they are expensive, large, and difficult to use. Unfortunately, the portability and inexpensiveness of VOC sensors is extremely important for early detection and long-term health monitoring. 2) E-noses are easy to use, portable, and relatively inexpensive, yet they have issues with long-term reliability, specificity, and generalizability across multiple diseases with different VOCs of interest [104]. Biological sensors, a third type of VOC detection platform and the newest of these three sensors are broadly sensitive, can rapidly detect many VOCs, and are small and portable. Their downsides being not enough longevity, difficulty calibrating, and lack of access outside of specialized laboratory settings. Each of these three VOC detection platforms can take advantage of the large amounts of health information carried by VOCs, yet each has their own weaknesses that need to be addressed prior to any clinically accepted device.

7. Concluding remarks

While senses other than smell, such as sight and hearing, have been reproduced using engineered systems, olfactory systems have not been well replicated so far [209]. These other senses can be fully replicated using just two dimensions, frequency and amplitude, while olfaction theoretically has an infinite number of dimensions as an odor can be the result of a single molecule, which could have widely varying chemical structures, or even a mixture of molecules at varying concentrations [209].

To overcome the natural difficulty of replicating olfaction, engineers have sought to employ biological rules of olfaction in e-noses. Cross-selectivity, when a single sensor can react to multiple stimuli, broadens the sensing capabilities using just a few sensing materials [210] and aids in combinatorial coding by having multiple sensors react to each molecule. In e-noses, combinatorial coding is achieved by using multiple different VOC reactive materials within the same device and analyzing the signals using machine learning algorithms such as dimensionality reduction and/or artificial neural networks to create a 'breath-print' for each odor [210]. Despite the many studies testing e-noses for medical purposes, there is extremely limited use of these instruments in clinical settings. E-noses have several limitations, including a trade-off between sensitivity to individual compounds and broad selectivity to many compounds, and difficulty dealing with humidity and natural conditions [211]. E-noses can be designed to be highly sensitive to a few compounds such as ammonia [212]; however, many diseases engender complex up- and/or down-regulation of multiple compounds. For these cases, e-noses have trouble maintaining the required sensitivity to all of the essential compounds [21]. To bypass this limitation, e-noses can be designed for a specific use [211], however then the e-nose is no longer a general sensor for many different diseases.

On the other hand, animal behavior and olfaction have recently been presented as a reproducible means for disease detection. Though medical testing has seen great advances with time, not all places have the means needed for medical equipment and expert personnel. Animals are an abundant resource located worldwide, offering many different models to be employed. Vertebrates: dogs, rats, and mice have been extensively shown to detect various diseases at high sensitivity and retention rates. These models may take longer to train behaviorally

compared to others but are effective in multi-odor presentations offering a long lifetime of real-world detection. Invertebrates: nematodes and insects are less conventional animal models but require less time and supplies to raise and train for scent detection with reliable sensitivities. Though they have been utilized less for in-field work compared to their vertebrate counterparts, large quantities of invertebrates can be employed for high throughput disease detection analysis. Even so, behavioral detection protocols between studies are not standardized, and confounding variables, such as similar VOC profiles, or comorbidities may skew data. Relying solely on behavior for biological olfaction produces binary results. It is worth diving deeper into biological olfaction complexities, studying internal processing and responses to specific diseases to gather a more complete picture of biosensing.

The implementation of biological components for constructing biosensors that mimic the olfactory system has been studied for many applications. The monitoring of VOCs via these biosensors provides an avenue for clinical diagnostics. Biosensors have the advantage of sensitivity and selectivity, owing to the specificity of biological materials. By employing olfactory elements, these biosensors make it possible to detect what the traditional electronic noses are not able to do. Bioelectronic sensors that have incorporated a biological recognition component into the system can respond in a concentration-dependent manner for VOC monitoring. These biological component-based bioelectronic sensors can provide numerous benefits including low cost, ease of operation, portability, and continuous monitoring of VOCs without the need for sample preparation. The integration of biological components with an analytical platform for signal measurement displays the robust nature of these components for use in clinical diagnostics and other chemical monitoring applications. While these biosensors have been extensively studied, clinical application of these biosensors is still lacking. The stability and compatibility of the biological components with fabricated transducers still pose limitations. In addition, the analysis of complex mixtures of VOCs does not compare to the robust nature of whole-olfactory systems. Only using components of biological olfaction limits the biosensor's ability to extensively characterize chemical space leading to suboptimal outcomes.

Cyborg sensors, which are part-brain-part-engineered biosensors, address VOC-based disease detection challenges by combining the entire olfactory sensory system of a biological organism with electrophysiology or other physiological recording and data analysis platforms. Cyborg sensors employ the entire biological chemosensory array (olfactory epithelium for vertebrates, antennae for insects), biological signal transduction, and biological neural computations all in one single device. All these advantages render cyborg sensors with extraordinary power of disease detection. Recent studies have demonstrated that insect brain-based cyborg sensors can not only detect human cancers but also differentiate between different cancer cell lines belonging to one type of cancer. Although this novel approach is promising for early and noninvasive disease and/or disease state detection, cyborg sensors also suffer from some limitations. Each cyborg sensor needs to be calibrated for the target stimuli of interest (e.g., healthy human exhaled breath vs. exhaled breath of cancer patients). However, with more research, this step can be accelerated, and we envision in the future, each sensor will require only a 15–30 min calibration. Performing surgery for this sensor development requires skilled personnel, which may not be easily available, and training. By employing precision robotic brain surgery this deficiency can be overcome. Currently, the insect brain-based cyborg sensor's lifetime is limited (1–2 days). Nonetheless, these brain-based devices are suitable for high-throughput testing with a test able to run every 60 s. Therefore, this type of sensor can be used to run hundreds of breath samples in a single day. Finally, like other biological sensors, cyborg sensors do not indicate which chemical compounds are different between two types of gas mixtures. Therefore, to identify disease-specific biomarkers, GC-MS-based component-wise analysis of gas mixtures is still necessary.

In summary, volatile chemical sensors that can be used for disease detection are rare to find to date. One reason for deficiencies in engineered VOC sensors is that most e-noses are not sensitive or specific to a broad range of volatiles and their concentrations. On the other hand, biological olfaction is both sensitive and specific, but we have not been able to back-engineer the components of olfactory sensory systems effectively. Therefore, the current approach is to incorporate biological components (primarily olfactory chemosensory array and its supporting elements) in engineered sensors to increase the efficacy of the sensors by the addition of cross-selectivity and combinatorial coding properties that biological sensors and components provide. Biological olfaction for disease detection has been used in mainly three different forms: whole animal (behavioral detection), components of biological olfactory system (e.g., few ORs combined with engineered sensors), and cyborg sensors, where the entire olfactory neural pathway has been hijacked for disease detection. All these living animal-based disease detection approaches are promising but also have individual advantages and disadvantages. More effort is needed in all forms of biological component-based disease detection (both new sensor development and clinical testing) which will tell us more about biological olfactory coding rules and help overcome current challenges faced by engineered chemical sensors.

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CRediT authorship contribution statement

Autumn K. McLane-Svoboda: Writing – original draft, Visualization, Conceptualization. **Simon W. Sanchez:** Writing – original draft, Visualization. **Michael Parnas:** Writing – review & editing, Writing – original draft. **Ehsanul Hoque Apu:** Writing – review & editing, Visualization. **Debajit Saha:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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