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Review

# Hydrogen Peroxide Sensors for Biomedical Applications

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**Abstract:** Hydrogen peroxide ( $H_2O_2$ ) is an important molecule within the human body, but many of its roles in physiology and pathophysiology are not well understood. To better understand the importance of  $H_2O_2$  in biological systems, it is essential that researchers are able to quantify this reactive species in various settings, including in vitro, ex vivo and in vivo systems. This review covers a broad range of  $H_2O_2$  sensors that have been used in biological systems, highlighting advancements that have taken place since 2015.

**Keywords:** hydrogen peroxide; sensors; biomedical engineering; biological applications

## 1. Introduction

Hydrogen peroxide ( $H_2O_2$ ) is a reactive oxygen species (ROS) that is present throughout the body, playing various roles in physiological processes, including cellular signaling, where it regulates cell growth, immune activation, and apoptosis [1–4]. However, at high levels,  $H_2O_2$  can be detrimental to the body, causing cell damage [5], inflammatory disease [6], and cancer [7].  $H_2O_2$ 's reactivity and low physiological concentration makes accurate detection difficult, leading to confusion over the roles of  $H_2O_2$  within the body. To improve our understanding of  $H_2O_2$ 's role in biological systems, researchers are developing sensors to detect and quantify  $H_2O_2$  under various conditions. This paper reviews some of the  $H_2O_2$  sensors that have advanced the field, with a focus on advancements made since 2015.

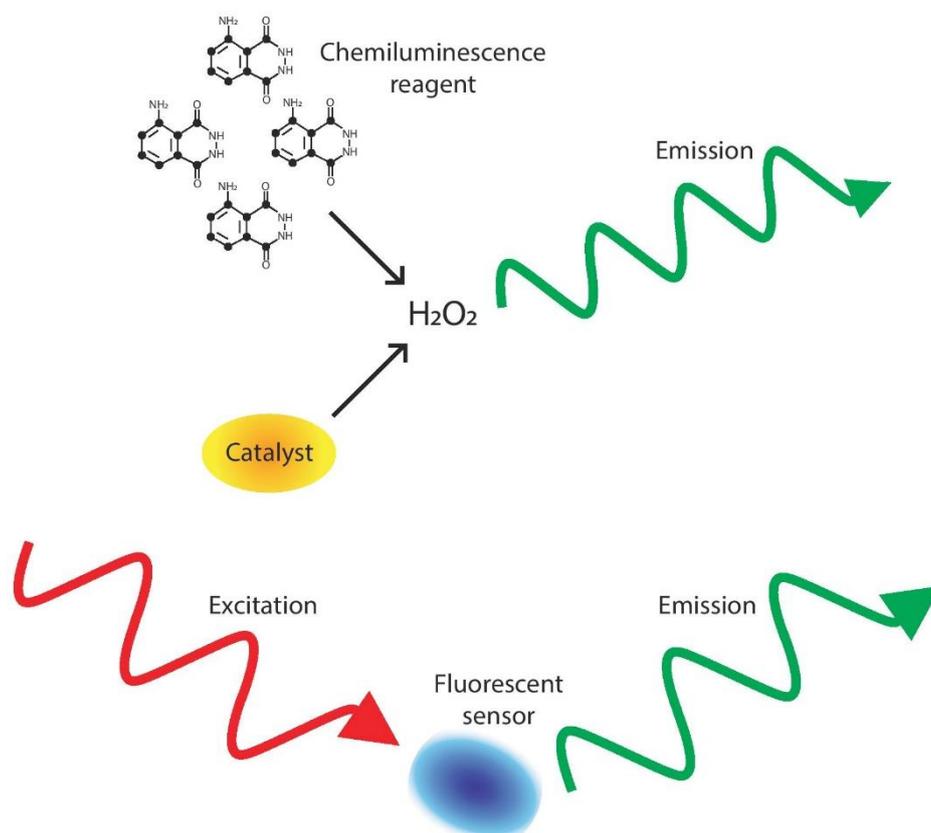
There are a variety of methods to detect and quantify  $H_2O_2$  that have been in use for many years, including titration, chromatography, light detection, and electrochemical sensors [8–13]. While titration and chromatography can be used to quantify  $H_2O_2$  levels, they are not ideal for in vitro and in vivo testing [14,15]. The two classes of sensors that are typically used for  $H_2O_2$  detection in biological systems are light detecting sensors and electrochemical sensors [16–24]. Light detection sensors function through the detection and analysis of light emitted from a sample; besides that, they can greatly vary in their method of excitation, method in which light is changed, and emission wavelengths [16,17]. Chemiluminescence sensors use a chemical compound to excite or alter the  $H_2O_2$ , while fluorescence sensors use an external energy source, typically light [16,17]. Sensors frequently exhibit changes in emission intensity, but they can also function through shifts in their emission profiles, such as a red or blue shift of peaks [10,25–27].

Electrochemical sensors function through the quantification of changes in chemical energy through an electrical transducer [18,20,23]. Potentiometric sensors measure the potential (voltage) between probes for which there is no current [18,20,23], and amperometric sensors measure the current while the potential (voltage) is maintained [18,20,24]. Both potentiometric and amperometric sensors have been used to quantify  $H_2O_2$  levels and are discussed in greater detail in this paper [12,13].

## 2. Light Detecting Sensors

### 2.1. Chemiluminescence

Chemiluminescence (Figure 1) is a versatile detection method in which an  $\text{H}_2\text{O}_2$  sensitive reagent promotes a chemical reaction for which the emission's signal intensity or wavelength change can be measured to determine the concentration of  $\text{H}_2\text{O}_2$  [16]. There are many different chemiluminescence  $\text{H}_2\text{O}_2$  sensors that could be used by researchers, including those outlined below [10,25,28–38].



**Figure 1.** Process of light-based detection. In chemiluminescence detection, a substance (such as luminol) and catalyst (except in the enzyme free reactions) react with  $\text{H}_2\text{O}_2$ , causing an emission of light that can then be read to determine  $\text{H}_2\text{O}_2$  concentration [16]. In fluorescent detection, an outside photon source excites a sample, causing an emission that corresponds to  $\text{H}_2\text{O}_2$  concentration [17].

#### 2.1.1. Developments Prior to 2015

Chemiluminescence methods include using luminol [10], eosin [28,29], peroxalate nanoparticles [25], and D-aminoluciferin [30] for  $\text{H}_2\text{O}_2$  detection and have been used to detect  $\text{H}_2\text{O}_2$  in the peritoneal cavity of a mouse [25], in mouse tumor xenografts [30], as an intermediate step to determine glucose levels in human serum [10], and to determine the peroxidase activity of human red blood cell membranes [29].

Of note is Lee et al.'s *in vivo* chemiluminescent  $\text{H}_2\text{O}_2$  sensor created with peroxalate esters and fluorescent dyes [25]. Lee et al.'s  $\text{H}_2\text{O}_2$  sensor can detect intramuscular exogenous  $\text{H}_2\text{O}_2$  at a depth of 3 mm, as well as  $\text{H}_2\text{O}_2$  production in the peritoneal cavity during lipopolysaccharide-induced stress [25].

Reverse micelles were used to develop a luminol-based  $\text{H}_2\text{O}_2$  sensor that allows reactions to be conducted in a low pH setting [10]. Igarashi et al. used their hexadecyltrimethylammonium bromide reverse micellar system to determine the substrate concentrations of L-phenylalanine and glucose, as well as to find the concentration of glucose in human serum [10].

### 2.1.2. Developments from 2015 to 2019

In 2015, Yu et al. discovered that the luminol reaction could be catalyzed by iodophenol blue, providing a less expensive method for  $\text{H}_2\text{O}_2$  detection than the commonly used horseradish peroxidase (HRP) assays; the method also increased reproducibility compared to peroxidase-mimicking nanoparticle assays, whose detection properties depend on particle size [31].

He et al. used the luminol– $\text{H}_2\text{O}_2$  reaction to spatially position an electrochemical probe in order to record intracellular  $\text{H}_2\text{O}_2$  levels [32]. Previously, a nanometer-sized electrode had been inserted into a cell for the electrochemical measurement of  $\text{H}_2\text{O}_2$ , but this method suffered from a lack of awareness of where the probe was located within the cell [39,40]. He et al. designed a chitosan-luminol probe attached to the electrochemical probe in order to provide spatial information about the probe's location within the cell, allowing for more precise data collection [32].

In 2016, Koren et al. used Prussian white's ability to convert to Prussian blue when oxidized to form a rechargeable optical sensor for  $\text{H}_2\text{O}_2$  [33]. Prussian blue's ability to be recharged in a 0.05 M ascorbic acid doped agarose gel allows the components to be reused multiple times [33]. Koren et al. demonstrated the ability of the Prussian white/Prussian blue assay to detect biologically-relevant samples by quantifying  $\text{H}_2\text{O}_2$  levels in activated neutrophils [33].

In the following year, Sheng et al. used silver nanocluster-capped bovine serum albumin (BSA) as a catalyst for the luminol– $\text{H}_2\text{O}_2$  reaction [34]. The silver nanocluster capped BSA is a small alteration that allows for variable emission wavelengths, creating a tunable sensor [34].

Moßhammer et al. combined a previous luminescence flow injection assay technique by King et al. [38] with microdialysis probes to decrease the impact of pH on readings as well as to allow for the continuous monitoring of a system [35]. Moßhammer et al. demonstrated the probe's ability to detect  $\text{H}_2\text{O}_2$  concentration in biological solutions by detecting the  $\text{H}_2\text{O}_2$  created due to the reaction of glucose oxidase and a catalyst in a phosphate buffered saline (PBS) glucose solution. This method was used to determine the change in  $\text{H}_2\text{O}_2$  levels in a *Pseudomonas aeruginosa* solution, which is known to cause acute and chronic infections in many systems throughout the body, including the urinary tract, the dermal system, and the respiratory system [35,41,42].

In 2019, Wang et al. decreased the  $\text{H}_2\text{O}_2$  detection limit of the luminol reaction via the synthesis and use of a hemin and poly(ethylene glycol) methyl ether catalyst [36]. The new catalyst resulted in a detection limit of 1.8 nM [36], lower than other luminol– $\text{H}_2\text{O}_2$  detection levels [31,36,43–45].

Additionally in 2019, Jiao et al. detected intracellular  $\text{H}_2\text{O}_2$  in cervical cancer cells (HeLa) by using iron–nitrogen–carbon single-atom nanozymes, which exhibit peroxidase-like activity, allowing for the catalyzation of  $\text{H}_2\text{O}_2$  [37].

## 2.2. Fluorescence

Fluorescent signal detection (Figure 1), another widely used method for  $\text{H}_2\text{O}_2$  detection, involves the quantification of a signal that is emitted from the excitation of electrons by light [17]. For fluorescence sensors, the excitation is caused by an external photon source, rather than a chemical reaction, as is the case in chemiluminescence [17].

### 2.2.1. Developments Prior to 2015

There have been several fluorescent probes using different materials made over the years, including naphthofluorescein disulfonate [46], homovanillic acid [47,48], peroxyfluor-1 [49,50], *Escherichia coli* OxyR [51], peroxyresorufin-1 [49], single-walled carbon nanotubes [11,52], peroxyxanthone-1 [49], and phosphine-based fluorescent reagents [53]. Fluorescent sensors have been used to detect intracellular  $\text{H}_2\text{O}_2$  in mice peritoneal macrophages [46], to detect intracellular  $\text{H}_2\text{O}_2$  levels when human embryonic kidney cells are bathed in  $\text{H}_2\text{O}_2$  [49], and to measure single molecule efflux from human umbilical vein endothelial cells [11].

An important advancement in  $H_2O_2$  detection occurred in 2005 with the development of three fluorescent probes from the peroxysensor family [49]. The probes are detectable via confocal and two-photon spectroscopy, and each emits at a different wavelength, allowing for different uses depending on the desired emission wavelength [49]. Miller et al. demonstrated that the probes were taken up by live human embryonic kidney (HEK) cells, where they responded to the introduction of extracellular  $H_2O_2$ ; they also demonstrated that the probes can detect simulated conditions of oxidative stress in embryonic rat hippocampal neurons [49].

In 2006, Belousov et al. developed an  $H_2O_2$  sensor named HyPer that can detect intracellular  $H_2O_2$  levels [51]. HyPer was created from the insertion of a yellow fluorescent protein into *Escherichia coli* [51]. HyPer was able to detect an increase in  $H_2O_2$  levels in HeLa cells during Apo2L/TRAIL protein-induced apoptosis and in rat adrenal medulla (PC-12) cells exposed to nerve growth factor [51].

### 2.2.2. Developments from 2015 to 2019

In 2015, Xu et al. developed Mito- $H_2O_2$ , a probe specifically designed for the detection of mitochondrial-associated hydrogen peroxide [27]. After confirming the location of Mito- $H_2O_2$  within the mitochondria with MitoTracker Deep Red and the selectivity of the probe against several different reactive oxygen and nitrogen species, Xu et al. delivered Mito- $H_2O_2$  to HeLa cells and recorded its response to the addition of  $H_2O_2$ , confirming the creation of a mitochondrial-targeted sensor with a high selectivity and rapid response time [27].

In the following year, Xiao et al. developed two fluorescent probes (MI- $H_2O_2$  and ER- $H_2O_2$ ) that were capable of targeting the mitochondria and the endoplasmic reticulum, respectively, have high specificity for  $H_2O_2$ , and have a fast response time [26]. MI- $H_2O_2$  and ER- $H_2O_2$  have different emission wavelengths and can therefore be used together to simultaneously measure  $H_2O_2$  associated with the two organelles [26]. Xiao et al. was able to detect  $H_2O_2$  associated with each organelle during L-buthionine sulfoximine-induced apoptosis [26].

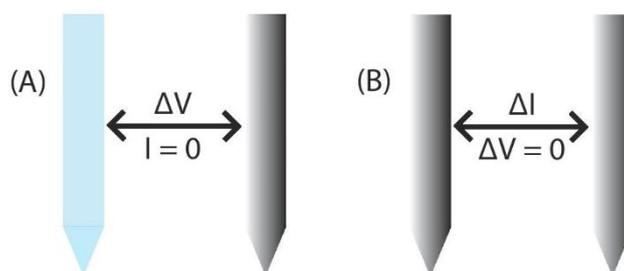
Qian et al. developed a ratiometric  $H_2O_2$  sensor in 2019 in an attempt to decrease the false positive and false negative readings that frequently occur with non-ratio sensors [52,54]. Using a cobalt/carbon nanotube hybrid nanocomplex as a catalyst for Amplex Red and fluorescent scopoletin, Qian et al. created a cost-effective and sensitive ratiometric  $H_2O_2$  sensor capable of detecting hydrogen peroxide concentrations as low as 100 nM [52].

## 3. Electrochemical Probes

Two types of electrochemical probes are frequently used for  $H_2O_2$  detection, specifically potentiometric and amperometric [12,55]. The way in which the two classes of probes function is different. Potentiometric probes measure the potential (voltage) between a working and reference electrode in a system that has no significant current flow [18–23]. The working electrode needs to be modified so that changes in potential correlate to changes in  $H_2O_2$  concentration, and the reference electrode must remain constant so that it can serve as a reference, or comparison, to the working electrode [18–23]. Amperometric sensors rely on the principle that changes in current are correlated to change in concentration [18–20,22,24]. Therefore, amperometric sensors use two or three electrodes to measure the change in the current of a sample while the potential (voltage) is held constant [18–20,22,24].

### 3.1. Potentiometric

Potentiometric sensors (Figure 2) measure the electrical potential of an electrode when there is no significant current in the system by using a reference electrode and a functional electrode [18–23]. Electrodes are tuned to detect specific analytes with membranes that surround their surface [20,23]. When the target analyte reacts with the membrane, the corresponding change in electrical potential can be read by the electrode [20,23].



**Figure 2.** Two different methods of electrochemical detection. (A) Potentiometric probes measure the potential between a working and reference electrode in a system with no significant current flow [18–23]. (B) Amperometric sensors use two or three electrodes and measure the change in current while the voltage is held constant [18–20,22,24].

### 3.1.1. Developments Prior to 2015

There have been several potentiometric sensors for  $H_2O_2$  developed over the years; unfortunately, very few have been used in biomedical systems [56]. One of the potentiometric  $H_2O_2$  sensors to be used in biomedical research is the  $N,N',N,N'$ -Tetramethylbenzidine with horseradish peroxidase system that has been used to detect  $H_2O_2$  as an indicator of glucose levels in human blood samples [12].

### 3.1.2. Developments from 2015 to 2019

Parrilla et al. created a potentiometric  $H_2O_2$  sensor in 2017 by coating platinum electrodes with Nafion, a sulfonated tetrafluoroethylene-based fluoropolymer-copolymer [57]. Parrilla et al. showed that the Nafion coating decreased signal interference from ascorbate, which commonly interferes with signal detection in biological systems, and acted as a permselective barrier, increasing the sensor's sensitivity in comparison to a bare electrode [57].

Cánovas et al. created a different Nafion-based  $H_2O_2$  sensor in which paper coated with a Nafion membrane containing glucose oxidase was used to detect blood glucose levels [58]. The sensor is a low-cost alternative that can detect glucose levels in both human serum and whole blood samples [58].

In 2018, Iwata et al. used a previously-reported glutamate sensor, which was based on the redox reaction of a gold electrode [59], as an  $H_2O_2$  sensor that functioned independently of the pH of the solution [60]. Iwata et al. showed that both a ferrocenyl methanol solution and a 11-ferrocenyl-1-undecanethiol-coated electrode were capable of  $H_2O_2$  detection but that the ferrocenyl methanol solution had a lower detection limit [60].

## 3.2. Amperometric

Amperometric sensors (Figure 2) are similar to potentiometric sensors in that they use electrodes, but amperometric sensors use two or three [24] electrodes to measure the current at a fixed potential and therefore rely on analyte diffusion to perform their measurements [18–20,22,24].

### 3.2.1. Developments Prior to 2015

Amperometric sensors are common for  $H_2O_2$  detection systems, and many different methods, including hemoglobin adhered to gold nanoparticle hybrid microspheres [55], graphene/platinum nanoparticles on glassy carbon electrodes [61], nanoceria capped with hexamethylene-tetra-amine or fructose [13], and (3-aminopropyl) triethoxysilane functionalized reduced graphene oxide [62] have been used in biological systems. Some of the biomedical applications for the use of amperometric sensors include quantifying  $H_2O_2$  in disinfectants [63], determining the antioxidant activity of cerium oxide nanoparticles with rat cardiomyoblast cells (H9c2) [13], and the release of  $H_2O_2$  from rat adrenal medulla pheochromocytoma cells (PC12) [61].

### 3.2.2. Developments from 2015 to 2019

Amperometric sensors have experienced a lot of improvement over the past five years, including improvements to stability [64,65], detection limit [65], and cost [66]. Since  $H_2O_2$  can decay in rapid and unpredictable ways, Draminska et al. developed a bienzymatic system to detect the catalase reaction as well as the decay of the  $H_2O_2$  [67]; their bienzymatic sensor is created by coating a glassy carbon electrode in multi-walled carbon nanotubes with absorbed catalase and either laccase or bilirubin oxidase [67]. Draminska et al. demonstrated the use of their sensor by measuring  $H_2O_2$  concentration in pharmaceutical formulations [67].

Since amperometric sensors frequently rely on enzymes and mediators to facilitate electron transfer, they often suffer from instability, which can lead to inconsistent results [64,68]. A common cause of instability is enzyme dependence on pH [64]. Thenmozhi et al. created a more stable  $H_2O_2$  sensor by covalently linking enzymes and mediators, specifically 3-aminopropyl trimethoxy silane with HRP and toluidine blue, which was deposited onto a graphite powder electrode [65]. Thenmozhi et al. determined that the sensor retained 86.7% of its initial response after being stored at 4 °C for three months, and they were able to use their stable sensor to quantify  $H_2O_2$  concentrations in pharmaceutical preparations [65].

#### Enzyme-Free Sensors

Many amperometric sensors use enzymes, but this unfortunately leads to increased costs due to the extraction and purification techniques that are necessary to acquire the enzymes [69,70]. In 2015, Sekar et al. circumvented this extraction and purification issue by immobilizing raw turnip peroxidase and potassium hexacyanoferrate into a cellulose paper that is both disposable and biodegradable [66]. Sekar et al.'s sensor, which can be used to detect  $H_2O_2$  in a commercial wound disinfectant, does use an enzyme, but is able to do so in a more cost-effective manner [66].

In 2016, Bai et al. created an enzyme-free  $H_2O_2$  sensor with platinum nanoparticles and reduced graphene oxide–chitosan–ferrocene carboxylic acid nanohybrids [71]. Bai et al.'s sensors showed a negligible response to ascorbic and uric acid (two electroactive species that can interfere with  $H_2O_2$  sensing), retained 80% of their initial value after 22 days, and successfully detected  $H_2O_2$  released from adenocarcinomic human epithelial (A549) and stimulated human liver cancer cells (HepG2 and LO2) [71].

In 2017, Liu et al. used a porphyrinic iron metal–organic framework decorated with ordered mesoporous carbon to create an enzyme-free  $H_2O_2$  sensor [72]. Liu et al.'s sensor was successfully used to observe the  $H_2O_2$  levels in HeLa cells after exposure to CdTe quantum dots, which cause cells to produce increased levels of ROS [72–75].

Recently, in 2019, Liu et al. developed an immobilization-free  $H_2O_2$  sensor, which can be created much faster than an immobilized sensor, by using the difference in diffusivity between single-stranded DNA and  $CeO_2$  nanoparticles [76]. Liu et al.'s sensor was used to detect both intercellular and extracellular  $H_2O_2$  levels in stimulated human breast cancer cells (MCF-7) [76].

## 4. Sensor Specifics

Sensors and Biosensors Discussed in this Review as follows (Table 1):

Table 1. Sensors and Biosensors Discussed in this Review.

Probe	Detection Limit	Linear Detection Range ( $\mu\text{M}$ )
<b>Chemiluminescence</b>		
Luminol– $\text{H}_2\text{O}_2$ catalyzed by iodophenol blue [31]	14 nM	0.025–10
Chitosan and luminol coated in polyvinyl chloride/nitrophenyl octyl ether [32]	1 mM	Not tested
Prussian blue/white rechargeable optical sensor [33]	0.4 $\mu\text{M}$	1–100
Luminol– $\text{H}_2\text{O}_2$ catalyzed by bovine serum albumin capped silver nanoclusters [34]	0.016 $\mu\text{M}$	0.14–100
Flow injection analysis with microdialysis probes [35]	Varies from 0.01 to 1.5 $\mu\text{M}$ depending on the medium, injection mode, and quantity of reagent	Varies from 1 to 100 depending on the medium, injection mode, and quantity of reagent
Luminol– $\text{H}_2\text{O}_2$ catalyzed by hemin and poly(ethylene glycol) methyl ether [36]	1.8 nM	0.002–3
Iron–nitrogen–carbon single-atom nanozymes [37]	0.5 $\mu\text{M}$	500–100000
<b>Fluorescence</b>		
Mitochondria-targeted cationic probe [27]	0.04 $\mu\text{M}$	0.2–10
Mitochondria-targeting probe [26]	80 nM	0.5–15, 15–40
Endoplasmic reticulum-targeting probe [26]	120 nM	0–40
Cobalt/carbon nanotube hybrid nanocomplex [52]	100 nM	0.2–20
<b>Potentiometric</b>		
Nafion-coated platinum electrode [57]	3.981 $\mu\text{M}$	10–1000
Redox and enzymatic reactions with a gold electrode (ferrocenyl methanol) [60]	10 $\mu\text{M}$	10–1000
Redox and enzymatic reactions with a gold electrode (11-ferrocenyl-1-undecanethiol) [60]	100 $\mu\text{M}$	100–10000
<b>Amperometric</b>		
Turnip tissue, paper-based sensor [66]	4.1 $\mu\text{M}$	20–500
Multi-walled carbon nanotube and absorbed enzyme-modified electrode [67]	54.4 $\mu\text{M}$ (bilirubin oxidase) 33.1 $\mu\text{M}$ (laccase)	0.03–0.62 mM (laccase) 0.05–0.99 mM (bilirubin oxidase)
Modified silane and graphite powder electrode [65]	0.171 $\mu\text{M}$	0.429–455
Platinum nanoparticles/reduced graphene oxide–chitosan–ferrocene carboxylic acid nano-hybrids [71]	20 nM	0.02–3
Porphyritic iron metal–organic framework-decorated with ordered mesoporous carbon [72]	0.45 $\mu\text{M}$	0.5–1830.5
Single-stranded DNA and $\text{CeO}_2$ nanoparticles [76]	35 nM	0.1–1

## 5. Conclusions

Hydrogen peroxide is an important molecule within the human body, but its roles and interactions are not well understood. Before we are able to understand H<sub>2</sub>O<sub>2</sub>'s role in the body, there must be reliable, fast, and versatile methods of detection with appropriate range and detection limits to function within biological systems and to detect biologically-relevant concentrations. In the past five years, researchers have continued to improve existing H<sub>2</sub>O<sub>2</sub> sensors [26,27,33,34,36,52,57,65,66,71,76] and developed novel methods of H<sub>2</sub>O<sub>2</sub> detection [26,27,32,66,67]. Sensor developments have occurred in the improvement of H<sub>2</sub>O<sub>2</sub> detection limits, with researchers able to detect much smaller concentrations than in the past; longevity, with researchers developing sensors that function over multiple weeks/months; and cost, with prices dropping in sensor development, this making sensors easier to manufacture at desired price points [65,66,71]. These improvements, and many more, have been accomplished by researchers who have expanded their materials and techniques in sensor design and manufacturing. Despite the advances that have been made in H<sub>2</sub>O<sub>2</sub> detection over the past five years, there is still room for growth in the field. Between fluorescent, chemiluminescent, amperometric, and potentiometric systems, researchers have been able to detect H<sub>2</sub>O<sub>2</sub> in within single cells (in vitro), solutions (ex vivo), and animal models (in vivo) [26,27,32,46,51,58,61,71]. With the continued development and refinement of H<sub>2</sub>O<sub>2</sub> sensors, we predict that even more knowledge about H<sub>2</sub>O<sub>2</sub>'s impact on cellular signaling and biological processes will soon be discovered.

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