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Electrochemical Quantification of H$_2$O$_2$ Released by Airway Cells Growing in Different Culture Media

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Abstract: Quantification of oxidative stress is a challenging task that can help in monitoring chronic inflammatory respiratory airway diseases. Different studies can be found in the literature regarding the development of electrochemical sensors for H$_2$O$_2$ in cell culture medium to quantify oxidative stress. However, there are very limited data regarding the impact of the cell culture medium on the electrochemical quantification of H$_2$O$_2$. In this work, we studied the effect of different media (RPMI, MEM, DMEM, Ham’s F12 and BEGM/DMEM) on the electrochemical quantification of H$_2$O$_2$. The used electrode is based on reduced graphene oxide (rGO) and gold nanoparticles (AuNPs) and was obtained by co-electrodeposition. To reduce the electrode fouling by the medium, the effect of dilution was investigated using diluted (50% v/v in PBS) and undiluted media. With the same aim, two electrochemical techniques were employed, chronamperometry (CH) and linear scan voltammetry (LSV). The influence of different interfering species and the effect of the operating temperature of 37 °C were also studied in order to simulate the operation of the sensor in the culture plate. The LSV technique made the sensor adaptable to undiluted media because the test time is short, compared with the CH technique, reducing the electrode fouling. The long-term stability of the sensors was also evaluated by testing different storage conditions. By storing the electrode at 4 °C, the sensor performance was not reduced for up to 21 days. The sensors were validated measuring H$_2$O$_2$ released by two different human bronchial epithelial cell lines (A549, 16HBE) and human primary bronchial epithelial cells (PBEC) grown in RPMI, MEM and BEGM/DMEM media. To confirm the results obtained with the sensor, the release of reactive oxygen species was also evaluated with a standard flow cytometry technique. The results obtained with the two techniques were very similar. Thus, the LSV technique permits using the proposed sensor for an effective oxidative stress quantification in different culture media and without dilution.

Keywords: H$_2$O$_2$; electrochemical sensor; cell culture media; graphene oxide; gold; bronchial epithelial cell; lung adenocarcinoma cell; oxidative stress; cigarette smoke extract; resveratrol

1. Introduction

The imbalance between the production of reactive oxygen species (ROS) from both endogenous and exogenous sources and antioxidant defense systems (including superoxide dismutase, catalase and glutathione peroxidase) leads to oxidative stress [1–4]. ROS production can be increased by various physiological or pathological conditions. If the antioxidant enzymes fail to rebalance this ROS production, an accumulation occurs, causing cell damage. This contributes to inflammation, aging, cancer and several chronic diseases, including chronic obstructive pulmonary disease (COPD) [5–7]. Cigarette smoke is a strong inducer of oxidative stress and represents the main risk factor for COPD. Accordingly,
therapies aimed at reducing oxidative burden or increasing antioxidant defences are useful in COPD management exacerbations and in preserving lung functions [8].

ROS include superoxide anion, hydroxyl radical and H$_2$O$_2$, among others [9–11]. Their half-life time is very short due to their high reactivity, and they are difficult to quantify [12,13]. Among ROS, H$_2$O$_2$ has the longest half-life time as well as the ability to cross biological membranes and induce damage in the extracellular space [14–16]. Thus, the quantification of H$_2$O$_2$ in cellular supernatants appears to be a convenient way to monitor the oxidative status of the cell [17,18].

Nowadays, H$_2$O$_2$ quantification is carried out by different laboratory-based techniques such as fluorometric and colorimetric assays, nuclear magnetic resonance spectroscopy and liquid chromatography [19–23]. Among the drawbacks of these techniques are their high cost, long analysis time, requirements of highly skilled personnel, and more importantly, their inability to facilitate in situ analysis to provide real-time results. Indeed, a sample must be collected from the cell culture to quantify H$_2$O$_2$ released by cells. This makes it very challenging to continuously monitor oxidative stress in real-time during cell growth [24]. Electrochemical sensors are perfect candidates to minimize all these drawbacks because they can be applied for in situ and real-time analysis, while offering good performances in terms of sensitivity, selectivity and limit of detection [25–35]. To improve the performance of an electrochemical cell, the use of nanostructured electrodes ensures a high active surface area and promotes a high current density [36–43].

In our previous work, we developed and studied the features of a nanostructured electrode made of reduced graphene oxide (rGO) and gold nanoparticles (Au-NPs) for the chronoamperometric detection of H$_2$O$_2$ released by the human macrophages cell line, THP1, grown in a Roswell Park Memorial Institute (RPMI) 1640 medium [44]. This sensor could be used to measure H$_2$O$_2$ release from other types of cell cultures as well. Different media can be used to grow different cells or the same cells, and the same culture medium can be used to grow different cells [45–47]. Usually, a medium contains a very wide range of chemicals, stabilizers and nutrients [48–51] and can differ from the other in nutrients and growth factors [48,52–54]. The most commonly used media include RPMI 1640 [55], Eagle Minimum Essential Medium (MEM) [49], Dulbecco’s Modified Eagle Medium (DMEM) [56], Ham’s F-10 & F12 [57], Medium 199 [58], and Iscove’s Modified Dulbecco’s Medium (IMDM) [59]. Considering their complex composition, an influence on the electrochemical detection of H$_2$O$_2$ by the different growth media can be expected. For this reason, we have carried out a systematic investigation of the effect of the cell culture media on the electrochemical response of the sensor for the detection of H$_2$O$_2$ released by epithelial cells from central and distal airways. In recent years, a number of different nanostructures have been studied for the electrochemical detection of hydrogen peroxide, such as Pt NPs [60], Cu$_6$(SC$_7$H$_4$NO)$_6$ nanoclusters [61], MnO$_2$ nanosheets [62] and Co$_3$O$_4$ nanowires [63]. Our sensor consists of an indium tin oxide/poly-ethylene terephthalate (ITO-PET) substrate modified by electrodeposited AuNPs and rGO. These active materials were selected because they were demonstrated to have improved detection sensitivity for H$_2$O$_2$ compared to sensors consisting of either AuNPs or rGO only [44,64–67].

To date, very few papers have reported on the influence of cell culture media on the performance of electrochemical sensors [68,69]. In this work, the effect of different culture media was studied in detail. Previously, AuNPs-rGO-based sensors were optimized and tested [44] in RPMI medium by chronoamperometry (CH). Using CH, it was necessary to dilute the sample with phosphate-buffered saline (PBS; pH 7.4) by 50% in volume to avoid fouling of the sensor surface, which could yield a low detection sensitivity. Starting from these results, the electrochemical sensor described in [44] was used here with the aim of performing a systematic investigation on the influence of the following parameters on the quantification of H$_2$O$_2$: - Cell culture medium (MEM, DMEM, RPMI, Ham’s F12 and bronchial epithelial cell growth media (BEGM)/DMEM); - Electrochemical quantification technique (CH and LSV);
- Medium dilution (undiluted vs. diluted in PBS (50% v/v));
- Operating temperature (25 °C and 37 °C);
- Interferents (uric acid, sodium chloride, lactic acid, glucose and HEPES);
- Storage condition (immersed in PBS or in deionized water and stored at 4 and 20 °C, sealed under vacuum and stored at 4 and 20 °C or stored at 4 and 20 °C in air).

Considering that the media have a very complex matrix, a fouling of the surface of the sensor is expected during its operation. To overcome this problem, a dilution of medium can be a solution. However, for a sensor that must operate in situ (in the culture plate during cell growth), a different approach is necessary, such as the use of a fast electrochemical technique that permits performing the analysis before the appearance of the fouling phenomena. For this reason, in this work, an LSV technique was also used, and the results were compared to those of conventional CH [70–73].

In addition, the sensor was validated quantifying the H$_2$O$_2$ released from different cell lines. In particular, human primary bronchial epithelial cells (PBEC), human bronchial epithelial cell line (16HBE) and adenocarcinoma alveolar basal epithelial cell line (A549) were cultured and stimulated with both pro-oxidant (cigarette smoke extract, (CSE) [74]) and antioxidant (resveratrol [75]) stimuli. The release of H$_2$O$_2$ release was also quantified with the electrochemical sensor, and results were compared with the data obtained by flow-cytometry using the same cells stained with Carboxy-H2DCFDA and MitoSOX Red probe, which detect intracellular ROS and mitochondrial superoxide, respectively. Both techniques revealed a significant increase in ROS in cells exposed to CSE. Resveratrol, an antioxidant molecule, reverted this effect. The AuNPs-rGO-based sensor offers high sensitivity and selectivity, a short response time (<60 s) and can be applied to real-time, in situ monitoring of H$_2$O$_2$ release.

2. Materials and Methods

2.1. Reagents

Flexible indium tin oxide/polyethylene terephthalate substrate (60 Ω cm$^{-2}$) and graphene oxide (4 mg mL$^{-1}$) were purchased from Sigma Aldrich (St. Louis, MO, USA) and Graphenea, respectively. The following reagents were purchased from AlphaAesar: KAuCl$_4$, 2-propanol, sodium acetate, glacial acetic acid, PBS tablet, 30% (v/v) H$_2$O$_2$, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), lactic acid, uric acid, sodium nitrate, sodium chloride, glucose.

MEM, RPMI-1640, DMEM, Fetal Bovine Serum (FBS), nonessential amino acids, L-glutamine, gentamicin, streptomycin and penicillin were obtained from Euroclone, while BEGM and Ham’s F12 Medium from Lonza Bioscience (see Supplementary Material for their composition). The probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, C-2938) and MitoSOX™ Red mitochondrial superoxide indicator were bought from Life Technologies.

2.2. Sensor Fabrication and Electrochemical Detection of H$_2$O$_2$

The working electrode, made of AuNPs and rGO, was synthesized as previously described [44]. Briefly, the ITO-PET substrate was ultrasonically cleaned with pure isopropanol and deionized water for 10 min. This electrode was then inserted into a homemade 3D printed cell (Figure S1), where the exposed active area of the electrode was 0.785 cm$^2$. In this cell, a Pt wire was used as counter electrode while a saturated calomel electrode (SCE) as reference.

A Princeton Applied Research potentiostat/galvanostat (PARSTAT, mod. 2273) was used for both fabrication and characterization. AuNPs and rGO were potentiostatically co-deposited on the working electrode at −800 mV vs. SCE for 200 s in a solution containing 0.5 mg mL$^{-1}$ GO and 0.5 mM KAuCl$_4$ in acetate buffer (pH 5.4).

The effect of 5 different culture media (MEM, DMEM, Ham’s F12, RPMI and BEGM/DMEM (B/D)) on H$_2$O$_2$ quantification was evaluated. The AuNPs-rGO-based sensor was calibrated using both CH (at a constant potential of −800 mV vs. SCE) and LSV (in the
potential range from +200 mV to −1200 mV, with a scan rate of 25 mV s⁻¹). For CH tests, the medium was diluted with 50% of PBS (pH 7.4), while for LSV measurement, the sensor was calibrated in both diluted and undiluted media. Each calibration was carried out at least 3 times with 3 different electrodes.

The effect of temperature on the sensor performance was also evaluated. Particularly, H₂O₂ was quantified at room temperature and 37 °C. This temperature was chosen because cells are conventionally cultured at 37 °C.

The sensor selectivity was studied using only LSV as an electrochemical technique because, in our previous work, it was already found that the electrode was selective also using CH. LSV was carried out in the presence of 0.5 mM of H₂O₂ and 5 mM of different interfering species (uric acid, sodium chloride, lactic acid, glucose and HEPES) that can be found in media.

The sensor stability was evaluated by measuring the output after 21 days of storing under different conditions (immersed in PBS or in deionized water and stored at 4 and 20 °C, sealed under vacuum and stored at 4 and 20 °C, stored at 4 and 20 °C in air).

2.3. Cell Culture Test

Cell line of immortalized human normal bronchial epithelial (16HBE) and cell line of lung adenocarcinoma (A549) (Interlab Cell Line Collection) were used.

16HBE were cultured in MEM with 10% fetal bovine serum (FBS) (heat-deactivated 56 °C, 30 min), 1% non-essential amino acids, 2 mM L-glutamine and 0.5% gentamicin [76]. A549 were cultured in DMEM with 10% FBS (heat deactivated at 56 °C, 30 min), streptomycin and penicillin, 1% nonessential amino acids and 2 mM L-glutamine. All cited components were obtained from Euroclone.

16HBE and A549 were seeded in 6-well plates (500,000 cells/well) and the adherent cell culture monolayers were maintained in a humidified ambient, at 37 °C and with 5% CO₂. Once reached the confluence (approximately 2 million cells/well), cells were stimulated with CSE at different concentrations (20% for 16HBE and 2.5% for A549) for 24 h. The 16HBE cells were also treated with resveratrol (40 µM), an antioxidant molecule, for 24 h in the presence or absence of CSE.

Human primary bronchial epithelial cells (PBECs) (American Type Culture Collection (ATCC), Manassas V.A.; PCS-300-010) were also used. PBECs were differentiated using the air–liquid interface (ALI) culture. Cells were seeded on 0.4 µm pore sized 12-well transwell plates (40,000 cells/well) (Corning Costar, Cambridge, MA, USA) coated with human fibronectin (Santa Cruz Biotecnology, Dallas, TX, USA), bovine albumin fraction V (Sigma Aldrich, MO, USA) and PureCol® (Advanced BioMatrix, Carlsbad, CA, USA) using B/D mixture (1:1, v/v BEGM/DMEM). The B/D medium contains 25 µM Hapes, bronchial epithelial cell growth supplement, 100 U/mL penicillin, 100 µg/mL streptomycin and 15 ng/mL retinoic acid receptor agonist EC23 (Tocris, Bristol, UK). Cells were cultured as submerged until confluence (approximately 500,000 cells/well), then the apical medium was removed and differentiated at ALI for 15 days. The differentiation of the cells into a more complex tissue containing different cell types is confirmed by the presence of cilia beating, by the mucus secretion and by the measurement of the trans-epithelial electrical resistance (TEER > 500 Ω cm²). After 15 days, PBECs were stimulated with CSE 20% for 24 h.

CSE was prepared by burning two cigarettes (3R4F-Kentucky—The Tobacco Research Institute, University of Kentucky) without any filter in 20 mL of PBS using a Watson–Marlow 323 E/D peristaltic pump (Rotterdam, The Netherlands). The solution was filter-sterilized (0.22 µm pore filter) and considered to be 100% of CSE. This was further diluted in the cell culture medium to reach the specific concentration for each experiment.

Flow cytometry was used to measure intracellular ROS and mitochondrial superoxide. After the stimulation, the cells were harvested and stained with 1 µM of 6-carboxy-2’, 7’-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA) probe to measure intracellular ROS (30 min, ambient temperature) and with 3 µM of MitoSOX™ Red probe, which
is specific for mitochondrial superoxide (15 min, at 37 °C) [77,78]. The flow cytometer 
CytoFLEX (Beckman Coulter, Brea, CA, USA) was used in these assays. The results were 
expressed as the mean of fluorescence intensity (MFI).

3. Results and Discussion

The sensor tested in this work consists of AuNPs and rGO that were co-electrodeposited 
on ITO/PET substrate. In this sensor, AuNPs and rGO act as active materials for the quantifi-
cation of H$_2$O$_2$, while ITO acts only as conducting material. Due to its poor electrocatalytic 
properties, ITO does not contribute to the sensitivity of the sensor but simply makes the 
PET substrate conductive, thus making possible the electrodeposition of Au and rGO and 
the subsequent electrochemical characterization tests. Thus, the sensing material consists 
of only Au-NPs (about 33 nm) and r-GO flakes (about 10 × 18 µm). According to the 
results obtained in [44], AuNPs-rGO-based sensors tested in RPMI as medium diluted 
with 50% of PBS displayed for the detection of H$_2$O$_2$ a limit of detection (LOD) of 6.55 µM 
and an average sensitivity of 0.064 µAµM$^{-1}$cm$^{-2}$. In the previous study [44], we also 
demonstrated that using CH, the dilution of medium (50%) is necessary to have good sen-
sitivity because, in medium alone, a decrease in sensitivity of about an 80% was observed 
due to fouling phenomena. In this work, the same experiments were carried out using 
other culture media. In particular, in each test, at the start of CH PBS alone was present in 
the cell. After the stabilization of the current, an equal volume of medium was added to 
have a dilution of 50%. The addition of the medium causes a spike in the current due to 
the instantaneous change in the solution composition at the electrode interface. After the 
transient, the current stabilizes, and the value is used as the blank current. In subsequent 
tests, the culture medium injected into the cell contains different amounts of H$_2$O$_2$. This 
causes a variation in the current, which depends on the concentration of H$_2$O$_2$.

Figure 1 (see also Figure S2) shows the effect of increasing H$_2$O$_2$ concentration on the 
CH experiments carried out using diluted MEM (1:1 v/v in PBS) as the solution. As 
expected, the higher the H$_2$O$_2$ concentration, the higher the output current density. A 
similar behavior was obtained in the other tested media. Considering that sensor calibration 
depends on the current value, careful and objective evaluations must be made. Thus, to 
build the calibration line, the output current density was selected based on the slope of 
the i-t curve. In particular, the current density corresponding to a slope equal to or lower 
than 50 nAs$^{-1}$ was selected. Operating in this way, the value of the current used for 
the calibration of the sensor is independent of the operator’s choice, and above all, it is 
independent of the precise time in which the measurement is made. With this procedure, all 
the i-t curves were processed, including that obtained for the blank. The current measured 
for the blank was subtracted from the values obtained by varying the concentration of H$_2$O$_2$, 
and the obtained data were used to construct the calibration lines, as shown in Figure 2.

![Figure 1](image_url) 
**Figure 1.** Effect of increasing H$_2$O$_2$ concentration on CH experiment carried out at −800 mV vs. SCE using diluted MEM medium.
Figure 1. Effect of increasing H₂O₂ concentration on CH experiment carried out at −800 mV vs. SCE using diluted MEM medium.

Figure 2 shows the calibration plots based on the CH signal obtained in different culture media at −800 mV vs. SCE. As can be observed, the electrode sensitivity (estimated by the slope of each linear calibration expression) was affected by the medium composition. Chronoamperometric detection of H₂O₂ maintains a similar sensitivity with MEM, DMEM and Ham’s F12, while the sensitivity dropped down to 0.0138 μAμM⁻¹cm⁻² using B/D medium. For each medium, the LOD was calculated by measuring the standard deviation of the blank using the following equation [79]:

\[
\text{LOD} = 3.3 \times \frac{\text{SD}}{S} \quad (1)
\]

where SD is the standard deviation of the blank, and S is the electrode sensitivity. The standard deviation of the blank using MEM, DMEM and RPMI was very similar (ranging from 0.128 to 0.157 μAcm⁻²), while it was much higher using Ham’s F12 and B/D (0.406 and 0.51 μAcm⁻²).

In Table 1, the analytical parameters estimated using CH carried out in diluted culture media are tabulated.

Table 1. Features of the AuNPs-rGO-based sensors using CH at −800 mV vs. SCE as the electrochemical technique in different culture media diluted with PBS.

| Medium            | Sensitivity μAμM⁻¹cm⁻² | Limit of Detection μM | Linear Range μM |
|-------------------|------------------------|-----------------------|-----------------|
| Diluted RPMI      | 0.064                  | 6.55                  | 25–5000         |
| Diluted MEM       | 0.0299                 | 13.05                 | 10–5000         |
| Diluted DMEM      | 0.033                  | 15.37                 | 10–5000         |
| Diluted Ham’s F12 | 0.0264                 | 51.61                 | 100–5000        |
| Diluted B/D       | 0.0138                 | 80.14                 | 250–5000        |
From the results reported in Table 1, it can be concluded that the best medium for chronoamperometry quantification of H$_2$O$_2$ is the RPMI. This result is attributable to the different media compositions.

The same experiments were carried out using LSV as the electrochemical technique. The potential was scanned from +200 to −1200 mV vs. SCE at a scan rate of 25 mV/s. The linear scan voltammograms obtained in a blank solution and in MEM de-aerated using a continuous N$_2$ flux are shown in Figure 3a,b, respectively. Figure 3b shows a featureless voltammogram, whereas Figure 3a depicts two peaks at −0.5 and −0.8 V, which are likely to be related to dissolved oxygen.

The first peak at −0.5 V is attributed to the reduction in dissolved oxygen to H$_2$O$_2$, following equation (2) [80,81]:

$$
O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^- \tag{2}
$$

The second peak at −0.8 V is related to the reduction in H$_2$O$_2$ to water, following Equation (3) [82,83]:

$$
H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \tag{3}
$$

Thus, even in the absence of H$_2$O$_2$, the presence of dissolved oxygen at about −400 mV generates a small amount of H$_2$O$_2$ that is then revealed at more cathodic potentials [84]. When different concentrations of H$_2$O$_2$ are spiked into the solution, the corresponding voltammograms are displayed in Figure 3c. In these voltammograms, the first peak at −0.4 V does not increase in intensity, while the peak at −0.8 V increases with H$_2$O$_2$ concentration [85]. To illustrate this point more clearly, only linear scan voltammograms for low H$_2$O$_2$ concentration are shown in Figure 3d. These results are a further confirmation that the peak at about −400 mV is attributable to the electro-generation of H$_2$O$_2$ from dissolved oxygen reduction. According to Nernst’s equation, at high H$_2$O$_2$ concentration,
the peak initially located at $-0.8$ V shifts to $-0.9$ V. A similar behavior was observed using both diluted and undiluted culture media. Figure 4 shows the obtained calibration lines using the media alone. The sensor performances are reported in Table 2. Interestingly, the sensitivity is much higher using LSV compared to CH (Table 1). For all studied media, the sensitivity increases by a factor of 3–4.

Using MEM, RPMI and DMEM, the effect of dilution is almost negligible (Table 2), while for Ham’s F12 and B/D, there is still an increase in sensitivity after dilution. Furthermore, in those media, better results were obtained with LSV compared to CH. This effect could be attributed to the fastness of the LSV compared to CH. In fact, the whole LSV experiments lasted less than 60 s, while CH experiments lasted for at least 600 s, due to the requirement for signal stabilization. During stabilization, H$_2$O$_2$ reacts at the electrode surface along with all the other chemical species present in the culture media. This may strongly affect the signal output due to a bio-fouling effect, especially with more complex mediums such as Ham’s F12 or B/D. Thus, the AuNPs-rGO-based sensor can work with every kind of medium with both LSV or CH measurements but, depending on the medium and the expected H$_2$O$_2$ concentration, it is necessary to use LSV or CH as the electrochemical technique to have a high sensitivity. In particular, the sensor can detect low concentrations of H$_2$O$_2$ in RPMI, MEM and DMEM medium with both LSV and CH, while for B/D, CH can be used when high concentrations of H$_2$O$_2$ are expected. LSV must be used to detect low concentrations of H$_2$O$_2$ (Tables 1 and 2). From these results, it can be concluded that the proposed sensor is adaptable for use in different media.

![Figure 4](image-url)

**Figure 4.** Calibration line obtained with LSV in: (a) RPMI alone, (b) MEM alone, (c) DMEM alone, (d) Ham’s F12 alone, (e) B/D alone ($n = 3$).
was carried out towards different chemicals that could be found in the different culture media or that could be generated by the different cell lines. Particularly, 5 mM of the interfering species (sodium chloride, sodium nitrate, glucose, lactic acid, HEPES and uric acid) were added to the solution containing 0.5 mM of H$_2$O$_2$. This is a conservative condition due to the high concentration of interfering species compared to the concentration of H$_2$O$_2$ ([H$_2$O$_2$]/[interfering] = 10) [89]. Figure 6 shows the results both as LSV curves (Figure 6a) and as a ratio between the peak current measured in the absence and in the presence of the interfering species (Figure 6b). In all conditions, the interference is negligible (lower than 5%) in terms of both current intensity and potential. This high selectivity of the sensor is expected because the potential at −0.8 V is way off the redox peaks of interferences. Only in the case of uric acid was a very small shift in potential value (−30 mV) observed.

Table 2. Performance of the AuNPs-rGO-based sensors using LSV as the electrochemical technique in different culture media diluted and not diluted with PBS.

| Medium            | Sensitivity μAμM$^{-1}$cm$^{-2}$ | Limit of Detection μM | Linear Range μM      |
|-------------------|----------------------------------|-----------------------|----------------------|
| Pure RPMI         | 0.134                            | 3.12                  | 20–6000              |
| Diluted RPMI      | 0.134                            | 3.12                  | 20–6000              |
| Pure MEM          | 0.125                            | 4.57                  | 20–6000              |
| Diluted MEM       | 0.125                            | 4.57                  | 20–6000              |
| Pure DMEM         | 0.108                            | 5.7                   | 20–6000              |
| Diluted DMEM      | 0.105                            | 5.8                   | 20–6000              |
| Pure Ham’s F12    | 0.11                             | 16.77                 | 50–6000              |
| Diluted Ham’s F12 | 0.08                             | 23.06                 | 50–6000              |
| Pure B/D          | 0.06                             | 26.71                 | 100–6000             |
| Diluted B/D       | 0.11                             | 15.2                  | 50–6000              |

Considering that the final goal of our research is to use the sensor directly on the plate during the culture of cells, the influence of operating temperature was also studied. Particularly, as the cell lines are cultured in incubators at a controlled temperature of 37 °C, the electrode was also tested at this temperature in order to simulate the operation in the plate [86]. Figure 5a,b shows the linear scan voltammograms obtained at 37 °C and the corresponding calibration line, respectively. The output current density increases with temperature, while electrode sensitivity is almost constant. In fact, a value of 0.127 μAμM$^{-1}$cm$^{-2}$ was obtained compared to 0.125 μAμM$^{-1}$cm$^{-2}$ obtained at room temperature (Table 2). The higher cathodic current density in Figure 5b, compared to that of Figure 4b, is expected at higher temperature due to the more favorable kinetics [87,88]. Thus, the sensor can also operate in the plate during the cell growth.

Figure 5. (a) LSV experiments at increasing H$_2$O$_2$ concentration and (b) corresponding calibration line. The tests were performed in pure MEM and at 37 °C (n = 3).

To fully characterize the electrode features for H$_2$O$_2$ quantification, a selectivity test was carried out towards different chemicals that could be found in the different culture media or that could be generated by the different cell lines. Particularly, 5 mM of the interfering species (sodium chloride, sodium nitrate, glucose, lactic acid, HEPES and uric acid) were added to the solution containing 0.5 mM of H$_2$O$_2$. This is a conservative condition due to the high concentration of interfering species compared to the concentration of H$_2$O$_2$ ([H$_2$O$_2$]/[interfering] = 10) [89]. Figure 6 shows the results both as LSV curves (Figure 6a) and as a ratio between the peak current measured in the absence and in the presence of the interfering species (Figure 6b). In all conditions, the interference is negligible (lower than 5%) in terms of both current intensity and potential. This high selectivity of the sensor is expected because the potential at −0.8 V is way off the redox peaks of interferences. Only in the case of uric acid was a very small shift in potential value (−30 mV) observed.
This interference was probably due to the different pH of the solution. In fact, uric acid must be solubilized in a 0.1 M KOH due to its low solubility, and thus, its injection in the electrochemical cell can modify the solution pH [90]. Similar behavior was found in the other media.

![Figure 6. (a) LSV experiments carried out in MEM alone in the presence of 0.5 mM H\textsubscript{2}O\textsubscript{2} and 5 mM of interfering species and (b) corresponding interference of each chemical on sensor output (n = 3).](image)

Finally, to study the stability of the AuNPs-rGO-based sensors over time, the effect of different storage methods was evaluated by measuring the current density for 5 mM H\textsubscript{2}O\textsubscript{2} before and after 21 days of storage. In particular, electrodes were stored in the following conditions:

- Immersed in PBS or in deionized water and stored at 4 and 20 °C;
- Sealed under vacuum and stored at 4 and 20 °C;
- Stored at 4 and 20 °C in air.

The results are summarized in Table 3. All the storage conditions with the electrode immersed in liquid solutions (PBS or deionized water) were found to be almost destructive for the electrode, with a signal reduction ≥20%. A similar result was found storing the electrode in air at room temperature, with a signal reduction of 36%.

| Sample                                | Current Density μA cm\textsuperscript{-2} | Difference % |
|----------------------------------------|-------------------------------------------|--------------|
| Fresh electrode                        | 240 ± 12                                  | 0%           |
| Immersed in deionized water at 4 °C    | 189 ± 8.5                                  | -21.2%       |
| Immersed in deionized water at 20 °C   | 212 ± 11.7                                 | -12%         |
| Immersed in PBS at 4 °C                | 157 ± 7.5                                  | -35%         |
| Immersed in PBS at 20 °C               | 171 ± 8.9                                  | -29%         |
| Stored in air at 4 °C                  | 245 ± 9.8                                  | +2%          |
| Stored in air at 20 °C                 | 153 ± 9.2                                  | -36%         |
| Vacuum at 4 °C                         | 259 ± 14.2                                 | +7.9%        |
| Vacuum at 20 °C                        | 222 ± 9.3                                  | -7.5%        |

The best storing conditions were found to be at 4 °C in air and vacuum at both 4 and 20 °C. In these conditions, the change in current density was lower than 10%. Considering that the sensor has a reproducibility of about 5% (see Figure 4), these storage methods are suitable for storing it without appreciable deterioration in performance. Thus, it can be concluded that the sensors stored in these conditions are stable for at least 21 days after their production. This result agrees with other studies [91,92], where the same storage method was used for similar sensors (based on AuNPs and rGO but on a different substrate).

The AuNPs-rGO-based sensor was used to quantify the H\textsubscript{2}O\textsubscript{2} released by A549, 16HBE and PBEC cells grown in RPMI, MEM and B/D, respectively. Considering the higher sensitivity obtained with LSV as the electrochemical technique, these samples were
analyzed using this technique. A549 and PBEC were tested in two different conditions: untreated cells (NT) and treated with CSE as a pro-oxidant stimulus. 16HBE cell line was tested after the treatment with CSE and with an antioxidant molecule resveratrol (RES) and a combination of pro- and antioxidant stimulus (RES + CSE). Figure 7a shows the linear scan voltammograms obtained in 16HBE cells. Particularly, Figure 7a shows the whole LSV curves obtained with 16HBE cell line, while background-subtracted voltammograms are shown in the inset. As expected, in all the studied cell lines, CSE treatment led to an increase in H$_2$O$_2$ production, while the treatment with RES, tested only in 16HBE cells, thanks to its antioxidant action, reverted the CSE-induced increase in H$_2$O$_2$ release. The results obtained in all experiments are summarized in Table 4.

![Figure 7](image.png)

**Figure 7.** (a) LSV experiments with 16HBE cell line and ROS detection by flow cytometry using Carboxy-H$_2$DCFDA probe in (b) 16HBE, (c) A549, and (d) PBEC and using Mitosox Red probe in (e) A549, and (f) PBEC. In the inset of (a) the LSV curves after baseline subtraction. *p value < 0.05 Unpaired t-test (n = 3).
Table 4. Real samples analysis tested using AuNPs-rGO-based sensors with LSV.

| Sample          | NT (µM)   | CSE (µM)   | RES (µM) | RES + CSE (µM) |
|-----------------|-----------|------------|----------|----------------|
| 16 HBE-MEM      | 10.63 ± 1.28 | 41.5 ± 11.8   | 9.7 ± 2.1   | 15.58 ± 3.58   |
| A549-DMEM       | 12.92 ± 1.04 | 15.8 ± 1.15   | -         | -              |
| PBEC-B/D        | 42.6 ± 7.1   | 129 ± 21.5    | -         | -              |

To confirm the results obtained with the sensor, we used the same cells in which we tested the release of H₂O₂ in the culture medium to evaluate cellular oxidative stress assessing the production of intracellular ROS and mitochondrial superoxide after stimulation with cigarette smoke (pro-oxidant) and RES (antioxidant), the latter only in 16HBE. For this purpose, we used Carboxy-H2DCFDA and Mitosox Red probe, commonly used in research laboratories, which bound the intracellular ROS and mitochondrial superoxide, respectively, and evaluated their expression by flow cytometry. As Figure 7 shows, treating 16HBE (b), A549 (c, e) and PBEC (d, f) with CSE increases the production of intracellular ROS and mitochondrial superoxide, while RES reduces cigarette smoke-induced oxidative stress in 16HBE (b). These results are in line with those obtained with AuNPs-rGO-based sensors, confirming the possibility of using the latter as a replacement for more expensive and time-consuming laboratory techniques for monitoring cellular oxidative stress.

4. Conclusions

In this work, we have shown the effect of different parameters on the quantification of H₂O₂ using a AuNPs-rGO-based sensor. In particular, the detection was performed using different cell culture media (diluted and undiluted), different electrochemical techniques (CH and LSV) and temperature operations (25 and 37 °C). Furthermore, the effect of different interferents and storage conditions was studied. Using CH, the AuNPs-rGO-based sensor showed a sensitivity ranging from 0.033 to 0.064 µA/µM cm⁻² using MEM, DMEM or RPMI as medium, while the sensitivity decreased using Ham’s F12 or B/D. The LOD was about 10 µM with RPMI, MEM and DMEM, while it increased to up to 50–80 µM in Ham’s F12 and B/D. LSV was also used to quantify H₂O₂, and results showed a higher sensitivity (about four times) in all the studied medium, with a consistent decrease in LOD. The selectivity test showed an excellent anti-interference property of the AuNPs-rGO-based sensor. The effect of electrode storage for 21 days was also studied in different conditions. The results showed that the best storage method consists of in storage at 4 °C in air and in the dark. In these conditions, the current density changed by only 2% after 21 days, confirming the long-term stability of AuNPs-rGO-based sensor. To use the electrode directly in the cell culture plate, the effect of operating temperature was studied, and a negligible effect was found, showing an increase in current density with a constant sensitivity.

The sensor was used to quantify H₂O₂ in different cell cultures. In particular, the H₂O₂ released from A549, 16HBE and PBEC cells exposed to pro-oxidant and antioxidant treatments was measured. The sensor was able to quantify the variation of H₂O₂ released from different cell types after different treatments. These results were validated by flow cytometry, a technique providing a quantitative measure of intracellular oxidative stress. Thus, the AuNPs-rGO-based sensor can be effectively used to quantify oxidative stress in cell culture medium in a fast, easy, cheap, reproducible, and sensitive way.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/mi13101762/s1, Tables with media composition. Figure S1: Photo of the 3D printed cell. Figure S2: Effect of increasing H₂O₂ concentration on CH experiment carried out at −800 mV vs. SCE using diluted MEM medium.

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**Abbreviations**

- AuNPs-rGO sensor
- 16HBE human normal bronchial epithelial cell line
- A549 lung adenocarcinoma cell line
- ALI air-liquid interface culture
- AuNPs gold nanoparticles
- BEGM bronchial epithelial cell growth media (BEGM)
- B/D BEGM/DMEM (1:1) medium
- Carboxy-H2DCFDA 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate
- CH chronoamperometry
- COPD chronic obstructive pulmonary disease
- CSE cigarette smoke extract
- DMEM Dulbecco’s modified eagle medium (DMEM)
- FBS fetal bovine serum (FBS)
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- ITO-PET indium tin oxide/polyethylene terephthalate substrate
- LOD limit of detection
- LSV linear scan voltammetry
- MEM Eagle’s minimum essential medium (MEM),
- MFI mean of fluorescence intensity
- PBECs human primary bronchial epithelial cells
- PBS Phosphate-buffered saline
- RES resveratrol
- rGO reduces graphene oxide
- ROS reactive oxygen species
- SCE saturated calomel electrode
- RPMI-1640 Roswell Park Memorial Institute-1640 medium,
- TEER trans-epithelial electrical resistance

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