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# Effects of Sterilization on Cellobiose Dehydrogenase and Glucose Oxidase Based Glucose Biosensors

Richard Bennett, Arvind Rathore, Sébastien Gounel, Anna Lielpetere, Thomas M.B. Reichhart, Kavita Jayakumar, Roland Ludwig, Alfons K.G. Felice, Dónal Leech, Wolfgang Schuhmann, Andrew Mount, Nicolas Mano, and Claudine Boiziau\*

Research on implantable glucose biosensors is driven by the need for innovative medical devices for continuous glucose monitoring in patients with diabetes mellitus. However, biosensor sterilization is a step that is widely omitted during the process of innovation. To compare the effects of gamma irradiation and chemical treatment with ethylene oxide (carbon microarray electrodes are fabricated, functionalized with glucose oxidizing enzymes (cellobiose dehydrogenase CDH or glucose oxidase GOx), and coated with a specifically designed zwitterionic polymer prior to the sterilization step. Cyclic voltammetry in the presence of 100 mM glucose of the biosensors before and after sterilization shows that gamma irradiation with a low radiation rate (25 kGy, 260 Gy h<sup>-1</sup>) does not induce a sensor performance loss, unlike the EtO treatment. In addition, no cytotoxic by-products are released after gamma sterilization. Based on these results obtained with both glucose oxidizing enzymes (CDH and GOx), gamma irradiation of the glucose biosensors with a low dose rate is preferable to exposure to EtO for biosensor terminal sterilization.

## 1. Introduction

The increase in the number of patients with diabetes is driving research to improve diabetes management. In this context, accurate glucose monitoring is important to improve the patient's health and quality of life. The most common method to measure glycemia is based on a blood drop analysis after a finger prick, termed self-monitoring blood glucose (SMBG). However, this method gives access to only a few measurements per day, does not provide precise information on the glycemia evolution tendency, and is also painful. Compared with a finger prick, continuous glucose monitoring (CGM) in interstitial fluids has a demonstrated positive effect on the overall health status.<sup>[1,2]</sup> The domain of CGM

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is growing in interest (with an estimated market of \$ 31 billion by 2031).<sup>[3]</sup>

Electrochemical glucose sensing devices are generally classified according to four categories, depending on the mode of glucose detection.<sup>[4,5]</sup> Whereas the fourth generation is metal electrode-based glucose sensing, the first three generations are based on glucose oxidizing enzymes,<sup>[6]</sup> either glucose oxidase (GOx) produced by the fungus *Aspergillus niger* or glucose dehydrogenase from a range of bacterial origins. In the second generation (the approach used in this article), a redox mediator is used as an electron acceptor to allow for an efficient electron transfer from the enzyme-active center to the electrode.<sup>[7]</sup> Third-generation biosensors operate without artificial electron mediators by taking advantage of oxidoreductases capable of direct-electron transfer between the enzyme and the electrode, such as cellobiose dehydrogenase (CDH). As such, CDH is an oxidoreductase composed of a sugar-oxidizing dehydrogenase domain and an electron-transferring cytochrome domain. Both domains are capable of electron transfer, making CDH a promising bioelectrocatalyst for both second- and third-generation glucose biosensors.<sup>[8]</sup>

However, new challenges have arisen with SMBG. Indeed, implanted medical devices (IMDs) face biological barriers, produced by the inflammatory response, and fibrosis designed to isolate the foreign body. Therefore, many studies have been conducted to improve the properties of these devices: device design to minimize biosensor size and limit the damage due to subcutaneous implantation, and tuning the stability<sup>[9]</sup> and specificity<sup>[10]</sup> of the glucose oxidizing enzyme, the nature of the redox mediator,<sup>[11,12]</sup> and of the coating to minimize the foreign body reaction (FBR).<sup>[13,14]</sup> One bioinspired coating strategy utilizes the zwitterionic phosphatidylcholine headgroups from the phospholipids of cell membranes, whose anionic and cationic groups provide antifouling properties,<sup>[15]</sup> which has strongly stimulated research in this field.<sup>[16]</sup> We have developed such molecules and have demonstrated that poly(2-methacryloyloxyethyl phosphorylcholine-co-glycidyl methacrylate) (MPC polymer) efficiently limits fibrinogen adsorption and fibroblast adhesion in vitro,<sup>[17]</sup> which has prompted us to pursue the development of this polymer in association with electrodes functionalized with glucose oxidizing enzymes.

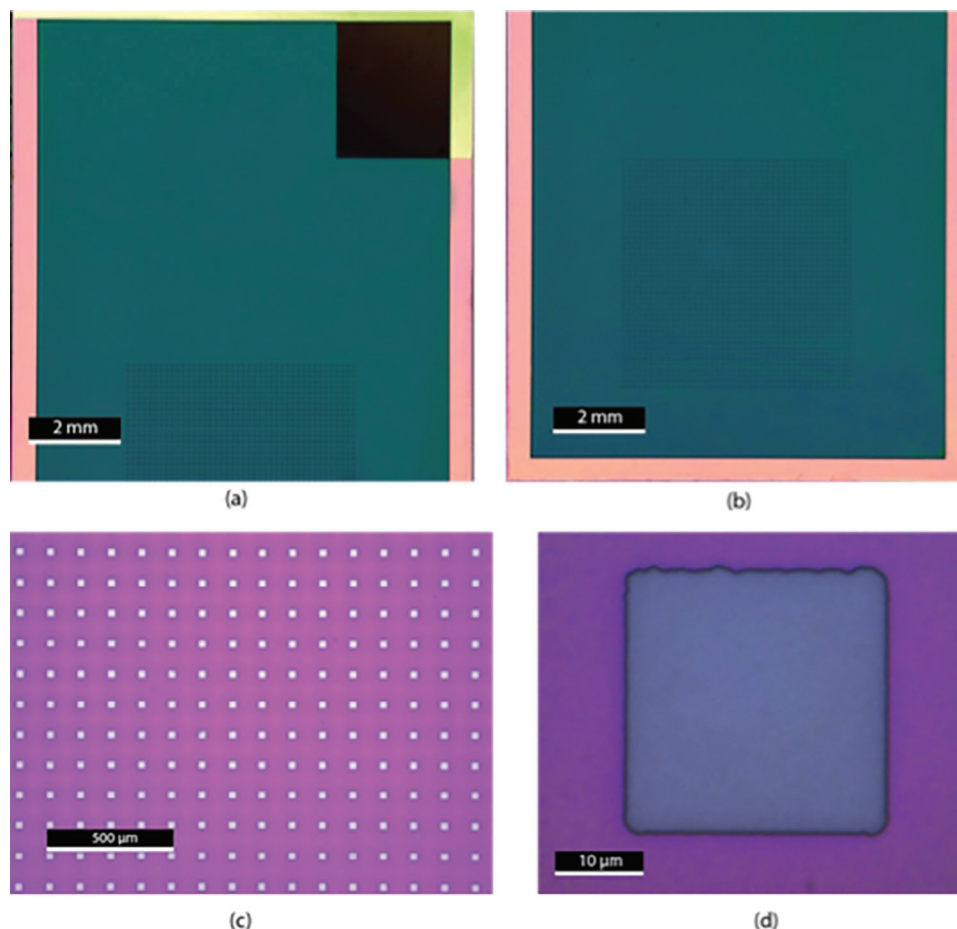
One endpoint that is widely omitted at the development stage is sensor sterilization. Sterility, considered as the absence of viable microorganisms, is determined by probability: the sterility assurance level for an IMD in contact with blood is  $10^{-6}$ , i.e., less than one microorganism in a million should survive the sterilization procedure.<sup>[18]</sup> Unlike aseptic processing (sterilization of all the IMD components and their assembly under aseptic conditions) terminal sterilization is strongly recommended since it is less expensive, and more effective in limiting the risk of accidentally introducing infectious contaminants onto implantable medical devices during their last preparation steps.<sup>[19]</sup> However, depending on the IMD, this last step can induce some undesirable property changes, as described by Galante et al. when sterilizing hydrogels designed for various medical applications.<sup>[20]</sup>

Terminal sterilization of IMDs is commonly based on either a physical or a chemical method. Among the physical processes, heat (either dry heat or steam heat) cannot be used with enzymatic glucose sensors since it leads to protein denatura-

tion. Thus, low-temperature sterilization methods are preferred. Gamma irradiation has long been used since such ionizing radiation is efficient in inactivating microorganisms by promoting DNA fragmentation; the standard exposition commonly requires an absorbed radiation dose of 25 kGy;<sup>[19]</sup> Its efficiency is due to good penetration into the IMD structure, and it also has the advantage of leaving no chemical residues after sterilization. Chemical methods are good alternatives when neither heat nor radiation can be used. Among the different options, ethylene oxide (EtO) is mostly used. As a liquefied gas, EtO is volatile, small enough to have excellent diffusion into the materials, and readily inactivates microorganisms by hydroxyethylation of amino acids, RNA, and DNA bases.<sup>[21]</sup> However, one of the limits of such chemical sterilization processes is the risk of toxicity due to the presence of remaining chemical residues in the IMD.<sup>[19]</sup> Together, gamma irradiation and ethylene oxide are used to sterilize  $\approx 90\%$  of all manufactured medical devices.<sup>[22]</sup> Other methods are receiving increasing attention for the sterilization of implantable medical devices,<sup>[23–25]</sup> such as vaporized hydrogen peroxide (VH<sub>2</sub>O<sub>2</sub>) or UV-C light (200–280 nm): VH<sub>2</sub>O<sub>2</sub> is a strong oxidizing agent, based on hydrogen peroxide whose biocidal properties are long recognized.<sup>[26]</sup> UV irradiation in the wavelength range of 260 nm (absorbed by microorganism nucleic acids and inducing thymine dimers), is approved by the FDA in the field of food industry and might offer an alternative to gamma irradiation for IMD surface sterilization.<sup>[27,28]</sup> The use of UV-C light has recently shown promising results in the context of NO-releasing implantable glucose sensors.<sup>[29]</sup> Its use to sterilize surfaces in healthcare facilities, and its short sterilization time (less than one hour) might also be time-saving and cost-effective, as claimed by the Lifecare ASA organization.<sup>[30]</sup>

The question of sterilization of glucose sensors has been investigated for over 30 years,<sup>[27]</sup> ever since the standard procedure of heat sterilization was found to be detrimental to enzyme activity. Different procedures have been developed, using gamma irradiation and various chemical methods;<sup>[27,31–35]</sup> however authors do not agree on the procedure that will induce the smallest performance loss: whereas some claims evidence a loss of GOx activity after a 25 kGy irradiation of the sensor,<sup>[27,31]</sup> ethylene oxide is recommended by others as a procedure as they claim it induces less changes to sensor properties.<sup>[32,35]</sup>

In this article, we address this issue and attempt to identify the main reason(s) for activity loss after sterilization with the two dominant methods, a 25 kGy gamma irradiation and chemical exposure to EtO. Given the common electrode substrate of choice is conducting carbon, we have employed our original in-house fabricated carbon micro square array electrodes of controlled and defined size and placement (30  $\mu\text{m}$   $\times$  30  $\mu\text{m}$  square electrodes, arranged in a square array, with row, column interelectrode spacing of 90  $\mu\text{m}$ ) formed by controlled polymer deposition and pyrolysis as the basis of the enhanced response small footprint benchmark sensor system to be tested for glucose sensing. Their fabrication and characterization are reported elsewhere (Bennett et al., personal communication). These were then modified with osmium-complex-based redox polymers<sup>[36]</sup> containing an engineered cellobiose dehydrogenase (CDH),<sup>[9]</sup> and then further coated with a specifically designed polymer<sup>[37]</sup> to ensure biocompatibility. Various effects of sterilization on the combined system were monitored in combination. Current responses of the CDH



**Figure 1.** Typical images of the carbon microarray electrode after the microfabrication process. The overall electrode view of microarrays, contact pad, and surroundings are shown in (a and b), a magnified image of the carbon microarray is shown in (c) and a single micro square electrode is shown in (d). Scale bars: 2 mm a,b), 500  $\mu\text{m}$  c), 10  $\mu\text{m}$  d).

functionalized electrodes were then assessed by cyclic voltammetry, in parallel with monitoring the release of cytotoxic by-products. In addition, changes in enzyme conformation induced by sterilization of solubilized CDH were evaluated by circular dichroism. In parallel, comparison analyses were done using the widely used glucose oxidizing enzyme GOx.

## 2. Result

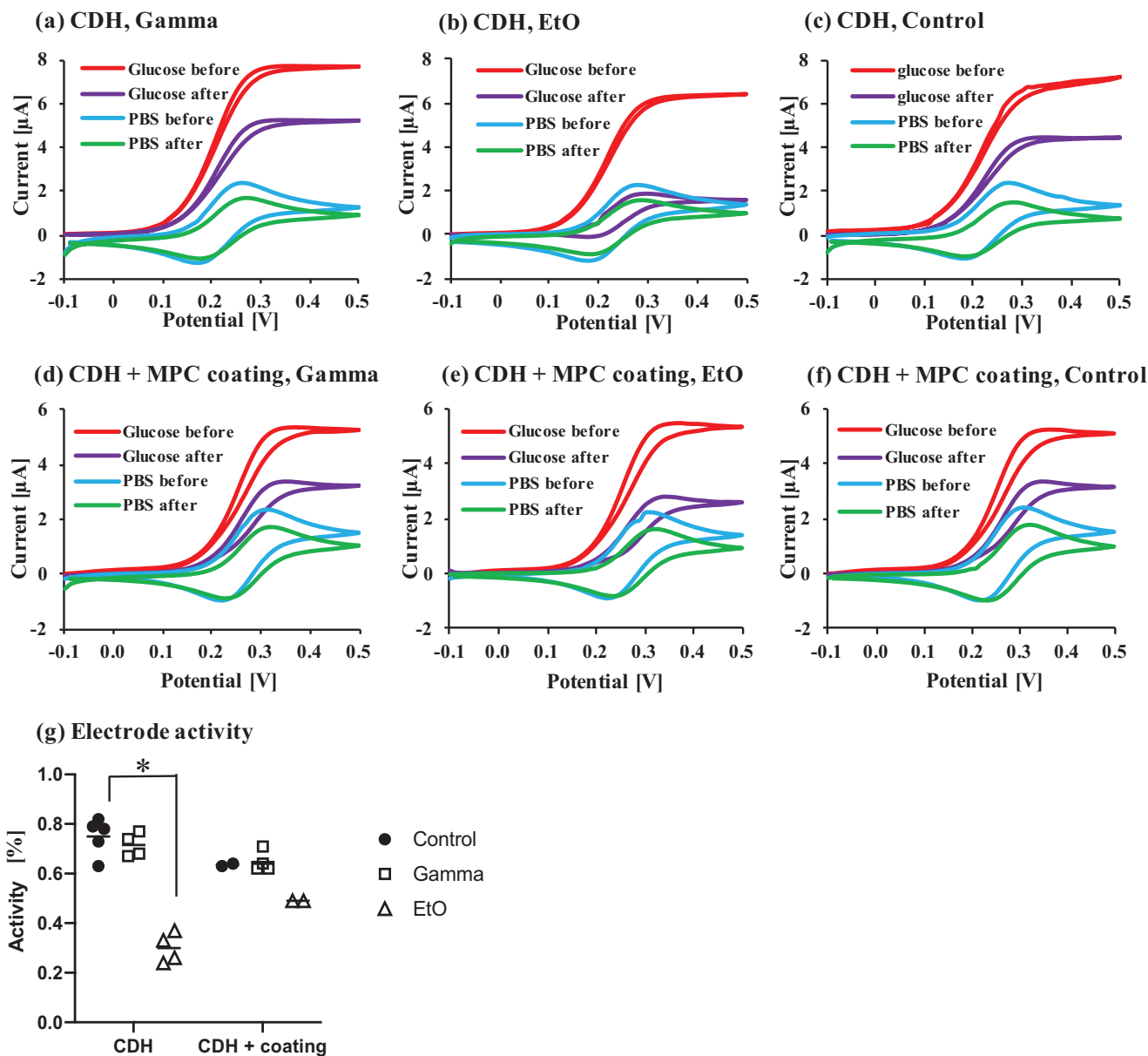
### 2.1. Choice of a Sterilization Procedure of the Electrodes

**Figure 1** shows our designed and microfabricated bespoke high-fidelity carbon microsquares array electrodes, as confirmed by the expected resulting square and array placement and dimensions (Figure 1c,d). The biosensors have been developed to work as second-generation sensors, requiring deposition of enzyme and mediator on each carbon surface; thus, the glucose oxidizing enzymes were mixed with an osmium-complex modified redox polymer crosslinked with PEGDGE (poly(ethylene glycol) diglycidyl ether), as previously suggested.<sup>[17]</sup> Two glucose oxidizing enzymes were compared: cellobiose dehydrogenase (CDH)<sup>[8]</sup> and

the commercial glucose oxidase produced in *Aspergillus Niger* (GOx) that we purified to increase its specific activity.<sup>[38]</sup> Consistent with our combined analysis approach, a coating layer of zwitterionic molecules (“MPC polymer”) was also added on some electrodes, whose presence was expected to limit the biofouling in the perspective of implantation.<sup>[17]</sup>

Gamma irradiation was performed using a Cesium source (<sup>137</sup>Cs), at a low radiation flow rate (25 kGy applied at 260 Gy h<sup>-1</sup> for 96 h). In parallel, other electrodes were submitted to EtO sterilization for 12 h, and then degassed for 3.5 days before testing; the set of control electrodes was left at room temperature for the same time period (96 h). Cyclic voltammetry was performed and cyclic voltammogram (CV) responses were compared before and after sterilization to assess the activity loss.

First, the currents produced by electrodes functionalized with CDH, without or with MPC coating, were quantified, with representative CVs shown in **Figure 2**. The CVs in the absence of substrate typically gave redox peaks centered  $\approx +0.25$  V versus Ag/AgCl/3 M KCl characteristic of the polymer-bound osmium complex. The CVs observed in PBS before (blue curves) and after (green curves) treatment were typically almost identical

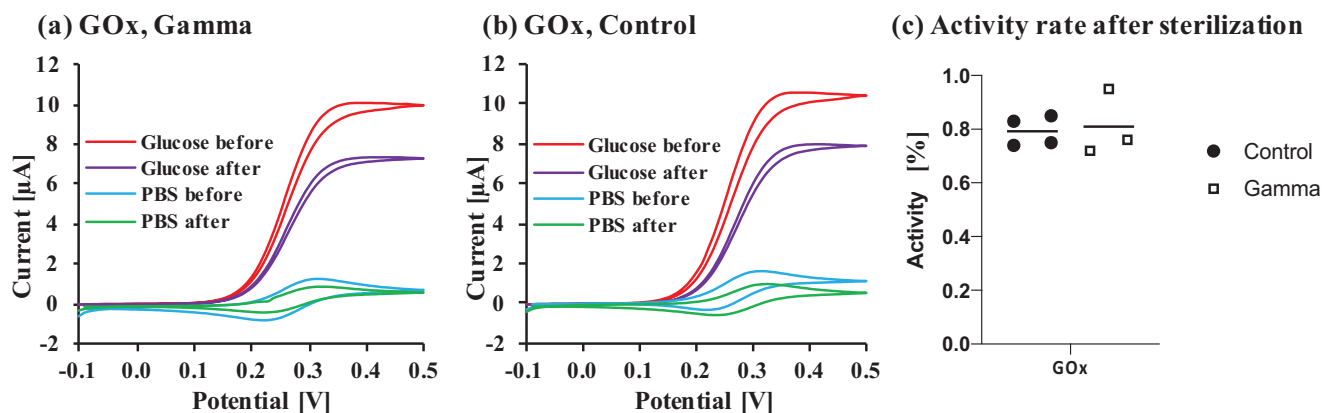


**Figure 2.** Effect of gamma irradiation and EtO on electrode activity functionalized with CDH. Cyclic voltammograms ( $5 \text{ mV s}^{-1}$ ) were recorded in PBS (green and blue curves) and in 100 mM glucose (red and purple curves), before (blue, red) or after (green, purple) the sterilization procedures: electrodes functionalized with CDH, without a–c) or with the addition of a zwitterionic coating (MPC polymer) d–f) were submitted to gamma irradiation a,d), EtO sterilization b,e), or left at  $18^\circ\text{C}$  during the same 4-day period c,f). g) The currents obtained at 0.4 V with the electrodes of each group after sterilization (or the equivalent period of 96 h) are reported as a percentage of the current before sterilization (or the 96-h period). The total number of electrodes was 20 (a:  $n = 4$ ; b:  $n = 4$ ; c:  $n = 5$ ; d:  $n = 3$ ; e:  $n = 2$ ; f:  $n = 2$ ). Statistics: non parametric statistical analysis was performed (Kruskal-Wallis and Mann-Whitney tests) to assess the effect of sterilization (Gamma/EtO) versus Control ( $*p = 0.016$ , 2-tailed).

indicating that the leaching of redox species was not the main reason for any decays in current observed. Upon addition of 100 mM glucose and before sterilization (red curves), the modified electrodes with CDH (Figure 2a–c) gave peak currents between 6.5 and 8.0  $\mu\text{A}$  which indicates the scale of signal variation across experiments. After sterilization, these currents declined by  $29\% \pm 5\%$  (gamma irradiation) and by  $70\% \pm 6\%$  (EtO), while a decline of  $25\% \pm 7\%$  was observed for the non-sterilized controls (Figure 2a–c, purple curves). This indicates a

significant loss of activity after EtO sterilization compared with the controls ( $p = 0.016$ ), but no significant effect from gamma irradiation over and above that of the control, indicating this to be an effective sterilization method for the electrodes functionalized with CDH (Figure 2g). Similar trends were also observed for CDH electrodes coated with MPC, although with a smaller decrease in signal upon exposure to EtO (Figure 2d–g). Electrodes modified with GOx were also fully resistant to gamma irradiation (Figure 3).





**Figure 3.** Effect of gamma irradiation on electrode activity functionalized with GOx. Slow scan cyclic voltammograms ( $5 \text{ mV s}^{-1}$ ) were recorded in PBS (green and blue curves) and 100 mM glucose (red and purple curves), before (blue, red) or after (purple, green) the sterilization procedure: electrodes functionalized with GOx were submitted to gamma irradiation (a,  $n = 4$ ), or let at  $18^\circ \text{C}$  during the same 4-day period (b,  $n = 3$ ). The currents obtained with the electrodes of each group at 0.4 V are reported in graph c). Statistics: no statistical difference was observed (Mann–Whitney test).

## 2.2. Effect of the Sterilization Procedures on Enzyme Conformation

To explain the observed activity difference after gamma irradiation and EtO treatments, we hypothesized that the latter could be inducing protein conformation changes caused by chemical reaction/functionalization. Therefore, we combined activity tests with circular dichroism (CD) to characterize the activity and enzyme conformation after sterilization. Both enzymes were sterilized in an aqueous solution following the same procedures (or left at room temperature for the same time as controls) and their conformation was assessed (Figure 4).

The circular dichroism of CDH indicated that upon sterilization with gamma irradiation (green curve), a change in the secondary structure was induced (Figure 4a) leading to a loss of heme ( $-25\%$ ) and thus a loss of 70% in activity (Figure 4b), which was not observed after EtO sterilization: the secondary structure remained the same (Figure 4a) but a 40% loss of activity was observed without any loss of the cofactor (Figure 4b). In contrast with CDH, as evidenced by the CDs, the secondary structure of GOx remained the same whatever the method of sterilization used (Figure 4c). However, a small loss of FAD cofactor was observed after gamma irradiation ( $-25\%$ ) associated with a 40% loss of activity (Figure 4d). UV visible was performed to identify the reason for such decay in activity (Figure 4e). As evidenced by a shoulder appearing in the UV–vis spectrum at 320 nm, the decrease in activity may be attributed to the formation of micro-aggregates without precipitations, shown to promote light scattering above 320 nm<sup>[39]</sup> (Figure 4e).

## 2.3. The Sterilization Procedures do not Promote the Release of Cytotoxic by-Products

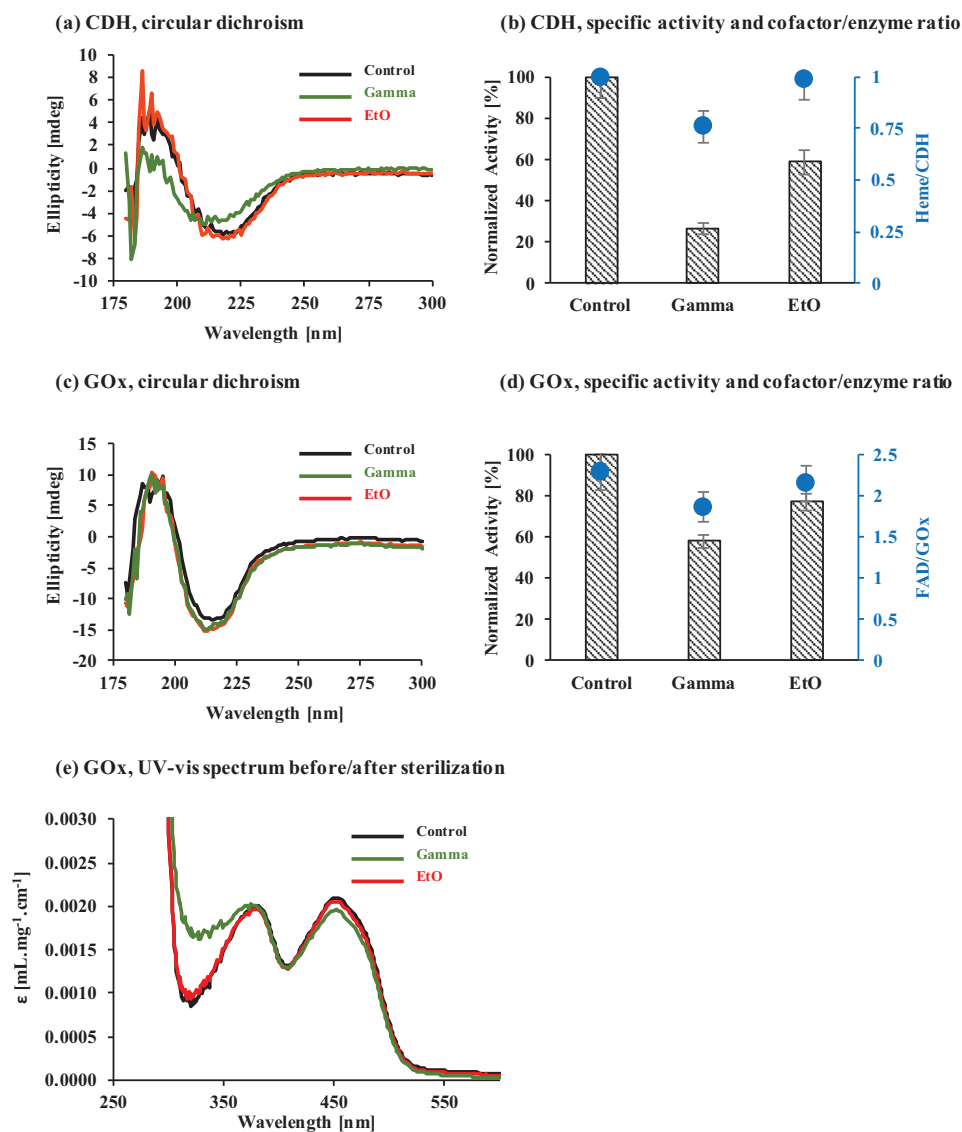
Since terminal sterilization is recommended, i.e., sterilization of the assembled device containing all its components (electrode, enzyme, redox polymer, zwitterionic coating), we checked to confirm that the sterilization procedures did not induce the generation of cytotoxic products in these functionalized electrodes.

Therefore, to ensure that the functional electrode (CDH/Os polymer/MPC coating) was not releasing toxic products, extracts were prepared during three successive days (days 1–3) and added to a cell culture, following European standard 10 993–05. As shown in Figure 5, neither cell survival nor cell metabolism were significantly affected by leachables. Comparable results were obtained in the absence of MPC coating, except for low toxicity of the day 1 extract of electrodes sterilized with EtO (40%, and 58% of the control survival and metabolism, resp., not shown), which may be due to the presence of some chemical residues remaining in the device after the 4-day aeration and released in the first extract. In addition, no toxicity was observed with GOx functionalization (not shown).

## 3. Discussion

In previous work, we engineered a modified CDH for in vivo applications to be more stable and with a limited oxygen reactivity,<sup>[9]</sup> which we use to produce glucose sensors by deposition on originally designed carbon electrodes. Sterilization of implantable glucose sensors is a central issue, mainly addressed by using the typical enzyme GOx in the literature, but for which the best procedure that limits enzyme inactivation is not clearly established. As described by Chen et al in a patent,<sup>[40]</sup> a high irradiation rate ( $20 \text{ kGy h}^{-1}$ ) inactivated GOx by 80%, whereas a lower rate ( $6 \text{ kGy h}^{-1}$ ) was less deleterious (50% loss). Based on this result, we chose a lower radiation rate ( $0.26 \text{ kGy h}^{-1}$  for a 25 kGy total irradiation); this showed no electrode activity loss due to gamma irradiation (i.e., a comparable loss with and without irradiation), both with CDH and GOx. This result is in accordance with the observations made by von Woedke et al and Ahmed et al,<sup>[27,31]</sup> but not with Sharma et al<sup>[34]</sup> who described a 40–50% activity loss after gamma irradiation. These discrepancies could be due to the different irradiation rates, which are often not reported in such publications.

In contrast with these publications, EtO was recommended by Tipnis et al<sup>[35]</sup> who showed that, despite an activity loss of  $\approx 50\%$  (to be compared with the 70% loss we observed for the electrode functionalized with CDH, Figure 2g), EtO sterilization

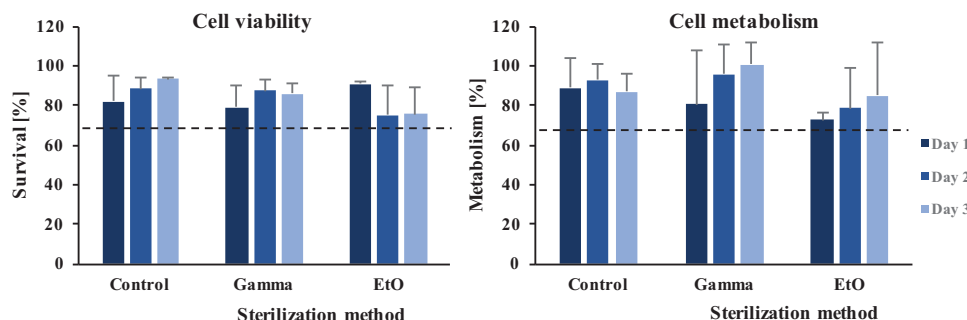


**Figure 4.** Assessment of enzyme conformation after sterilization. Circular dichroism (CD) and CDH activity: Circular Dichroism for CDH a) and GOx c) after 96 h at room temperature (black curves) and after gamma (green curves) or EtO sterilization (red curves). Acquisition time: 0.5 s, protein concentration: 0.9 mg mL $^{-1}$  (triplicates). b) Bar and dot chart showing the normalized enzyme activity (left y-axis) and ratio cofactor/enzyme (right y-axis) for CDH b) and GOx d) (mean  $\pm$  s.d). e) Zoom in the 250–600 nm range of a solution of GOx before (black curve), and after gamma (green curve) or EtO sterilization (red curve). Three series of ten scans were made and averaged for each spectrum.

maintained the linearity of glucose sensor response; it should be noted that this is a parameter that was not explored in this present study.

Our first goal was to explain the loss of CDH activity after EtO sterilization (Figure 2g). We first hypothesized that a conformational change might occur as a result of chemical modifications. Since a very low amount of protein would recover from the surface of the electrodes (with potential contamination of the CD signal by other molecules), the enzyme conformation was assessed by circular dichroism after EtO treatment of a pure solution containing CDH. The CD curve was not modified and the heme/CDH ratio also remained the same (Figure 4a,b). In parallel, experiments were carried out with the second sterilization method. In contrast to EtO treatment, gamma irradiation

did not promote loss of activity of CDH immobilized on the electrode surface, whereas conformational change, decrease of heme/enzyme ratio, and activity loss of the pure enzyme were observed (Figure 4c,d). These apparently contradictory results could be explained by the difference in experimental conditions. Gamma-ray photons are absorbed readily by H $_2$ O molecules, inducing water radiolysis and the production of oxygen radicals<sup>[41–43]</sup> which may lead to further chemical reactions, functional changes, and hence conformational change of the enzymes when in solution. In contrast, when CDH was immobilized on the electrode surface in a semi-dry environment, the effect of gamma irradiation was minimized due to the lower amount of water and hence oxygen radical generation. The opposite effect was obtained with EtO treatment: both the diffusion



**Figure 5.** Assessment of cytotoxicity induced by the sterilization procedures. Electrodes were functionalized with CDH and left at 18 °C (“Control”), or submitted to gamma irradiation (“Gamma”) or EtO sterilization (“EtO”). Four electrodes of each group were used to prepare extracts, during three successive periods of 24 h (Day 1, 2, 3). The extracts containing the leachables were added to L929 cells (5–7 replicates for each extract); cell survival (left panel) and metabolism (right panel) were assessed by Neutral Red and MTT assays, resp. The 100% corresponds to the cell culture medium (alpha-MEM + 10% FCS); the results are presented as percentages of this reference, with a mean  $\pm$  s.d. According to the standard, a value of 70% (dotted line) determines the cytotoxicity.

coefficient and the concentration of EtO gas are very low in liquid compared with air. Therefore, during the timescale of these experiments, the effects of EtO treatment in water should be minimized, whereas its effect on CDH immobilized onto the electrodes was maximized because of the higher diffusion of the gas in the air and its direct chemical reaction with the enzyme.

Despite unmodified conformation and no change in the Heme/CDH ratio of CDH treated with EtO, a 40% loss of activity was observed (Figure 4a,b). We suggest that this is related to chemical modifications of amino acids without conformational change. Indeed, it has long been known that EtO can react with histidine, methionine, valine, and cysteine.<sup>[21,44]</sup> Even residual EtO remaining in sterilized devices can induce EtO adducts on proteins.<sup>[45]</sup> In the present work, such an adverse effect, responsible for the 70% loss of CDH activity when associated with the electrode, is limited by the presence of the MPC coating (Figure 2g).

Then, the effect of gamma irradiation of the solution containing CDH showed a conformational change of CDH which could explain the decrease in the Heme/protein ratio and the loss of enzyme activity (Figure 4a,b). In contrast, the conformation of GOx was unaffected, whereas a loss of enzyme activity and a decrease in the FAD/protein ratio were observed. This might be explained with UV-vis spectroscopy, which showed a shoulder at 320 nm after gamma irradiation (Figure 4e). We hypothesize that this shoulder is due to aggregate formation, as demonstrated by Hu et al<sup>[46]</sup> using human serum albumin exposed to gamma irradiation.

A slightly higher stability of GOx (when in solution) toward gamma irradiation was observed compared with CDH, which is probably due to a more stable protein folding, but this did not correspond to a significant benefit to indicate a preference for the use of GOx in the context of functionalized electrodes for glucose biosensing applications.

## 4. Conclusion

The aim of this study was to investigate the effect of the two most commonly used methods for sterilizing IMDs—gamma ir-

radiation and ethylene oxide exposure—and to attempt to explain the loss of sensor activity when it occurs. Using a low irradiation rate, no loss of enzyme activity was observed. However, the same irradiation procedure with enzymes in solution, where radiation damage is greater, showed conformational changes (CDH) or protein aggregation (GOx). This suggests that under standard irradiation conditions (25 kGy, 3–4 kGy h<sup>-1</sup>), this phenomenon could be responsible for the loss of sensor activity. In contrast, such conformational changes were not observed after EtO sterilization; we suggest that chemical modifications of the enzyme amino acids may be the main reason for the loss of activity.

Based on our results, gamma irradiation with a low radiation rate is a good procedure to preserve electrode activity in a terminal sterilization procedure of implantable glucose biosensor electrodes, with both GOx and CDH used as glucose oxidizing enzymes. In addition, no cytotoxic by-products were produced. To progress in this development, future research should assess the linearity of glucose sensing.

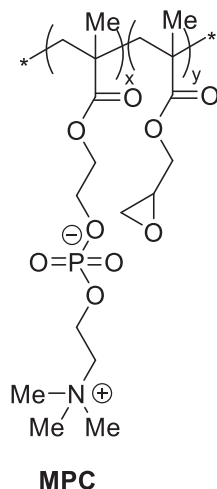
However, efforts to improve enzyme activity after sterilization with EtO should not be discouraged: indeed, EtO can be used for the sterilization of electronics, while gamma rays affect them. For full integration of bioelectrochemical sensors and electronics in one single-use implantable device, we suggest that although we observed a higher decay with EtO, it still could be beneficial to use this method with sensors containing electronics.

## 5. Experimental section

**Reagents—Enzymes:** Glucose Oxidase (GOx) and Cellobiose Dehydrogenase were produced, and purified, their activity tested and their ratio Heme/CDH or FAD/GOx calculated as previously reported.<sup>[8,47,48]</sup> GOx activity was 455 U mg<sup>-1</sup> and CDH 7.85 U mg<sup>-1</sup>.

**Reagents—MPC Polymer:** MPC polymer was synthesized as previously reported.<sup>[17]</sup> Briefly, 2-methacryloyloxyethyl phosphorylcholine (200 mg, 0.68 mmol, 70%) was dissolved in water (1 mL) and mixed with glycidyl methacrylate (40  $\mu$ L, 41.37 mg, 0.29 mmol, 30%) dissolved in isopropanol (0.6 mL). The mixture was deaerated by argon bubbling, and then AIBN (0.5 mg, 0.003 mmol) was added. The reaction mixture was stirred overnight at 70 °C. After cooling down, the polymer was precipitated with THF, separated by centrifugation, and dried in a vacuum.





**Electrode Fabrication and Functionalization—Electrode:** Microsquare carbon array electrode systems were fabricated on three-inch Si wafers with twenty devices on each wafer. These electrode devices have 1764 micro squares all with the same square edge length (30  $\mu\text{m}$ ), square electrode arrangement, and square to square row and column spacing (90  $\mu\text{m}$ ), following the previously reported design for Pt array electrode.<sup>[49]</sup> The total geometric footprint of the array is  $1.588 \times 10^{-2} \text{ cm}^2$ . Electrodes were fabricated on a  $\text{SiO}_2$  layer grown on polished three-inch Si wafers. The application of the carbon electrode precursor (SPR-350 photoresist) was by spin-coating to achieve a homogeneous coating on the surface. After patterning this photoresist layer using UV photolithography, pyrolysis in a forming gas atmosphere gave smooth conducting carbon micro square electrodes. Following sheet resistance measurements to confirm the desired electrical conductivity of the resulting carbon film, each wafer then was diced into  $5 \times 25 \text{ mm}$  electrodes.

To define the array pattern, the insulator layer (poly-(para-xylylene), a hydrophobic polymer with the trade name “Parylene C”) was first coated on the entire surface using chemical vapor deposition (CVD). A pattern and etch process was then employed to mask all areas on the electrode except the electrode array and contact pads; subsequent etching of the underlying parylene with an oxygen plasma then produced the final array.

**Electrode Fabrication and Functionalization—Functionalization:** The redox polymer [poly(1-vinylimidazole)Os(bpy)<sub>2</sub>Cl]<sup>+/2+</sup> (Os(bpy)<sub>2</sub>PVI) was synthesized with modification of published procedures.<sup>[7,50]</sup> Enzyme electrodes were prepared through drop-casting onto the carbon microarray of 30  $\mu\text{L}$  of a 5  $\text{mg mL}^{-1}$  redox polymer aqueous solution, 16  $\mu\text{L}$  of a 10  $\text{mg mL}^{-1}$  aqueous enzyme solution, and 7.4  $\mu\text{L}$  of a 15  $\text{mg mL}^{-1}$  PEGDGE (poly(ethylene glycol) diglycidyl ether) aqueous solution. Electrodes were allowed to stand for 24 h prior to testing to ensure the film had cured.

**Cyclic Voltammetry:** All electrochemical testing was carried out using a PalmSens4 potentiostat. Electrochemical testing was performed in PBS (50  $\text{mM}$  phosphate buffer containing 150  $\text{mM}$  NaCl, pH 7.4) at 37  $^\circ\text{C}$ . Testing was performed using a three-electrode setup, with an Ag/AgCl (3  $\text{M}$ ) reference electrode and a platinum mesh counter electrode. The electrodes were characterized using slow scan cyclic voltammetry (5  $\text{mV s}^{-1}$ ) in PBS and 100  $\text{mM}$  glucose to compare the performance of the electrode with and without substrate present. 100  $\text{mM}$  glucose concentrations were chosen to show the maximum difference between different sterilization methods and the control group. For quantitative evaluation, the current values at 0.4 V are compared.

**Sterilization:** Dry functionalized electrodes were packaged in radiation-resistant and permeable ethylene oxide sealed bags (Safe-Seal, Medicom) just after cyclic voltammetry assessment of the time point “before PBS/glucose.” For gamma sterilization, the bags were put in a <sup>137</sup>Cs irradiator. Samples received a low rate irradiation of 25 kGy in 96 h (260  $\text{Gy h}^{-1}$ ) at 18  $^\circ\text{C}$ . For EtO sterilization, the bags were exposed to EtO under vacuum in a humid atmosphere for 24 h at 18  $^\circ\text{C}$  followed

by 72 h of aeration at the same temperature (Anprolene AN74i, Andersen Sterilizers). The control electrodes were processed in parallel and kept at 18  $^\circ\text{C}$  for 96 h. Cyclic voltammetry was then performed immediately after sterilization.

**Circular Dichroism and Enzyme Characterization:** Circular dichroism measurements were performed at room temperature on a MOS-450 spectrometer (BioLogic, France) with increments of 1  $\text{nm}$ , an acquisition time of 0.5 s, and 10 consecutive scans. CD spectra were recorded between 190 and 300  $\text{nm}$  with a protein concentration of 0.9  $\text{mg mL}^{-1}$  in a 50  $\text{mM}$  sodium phosphate buffer pH 5.1. The path length for the quartz cell was 0.1  $\text{mm}$ . All measurements were performed in triplicate. The data were baseline corrected using the CD spectra for the buffer alone with or without methanol. Data were processed using Excel 2007.

**Assessment of Cytotoxicity:** Cytotoxicity of the electrodes (Control and submitted to sterilization procedures) was assessed according to the international standard ISO10993-5. Extracts were prepared by incubation of electrodes in the culture medium (alpha-MEM (Gibco, Ref A10490-01, France) at 37  $^\circ\text{C}$  for 24 h, providing the “Day 1” extract. A new medium was added to electrodes for a second and a third incubation of 24 h at 37  $^\circ\text{C}$  (“Day 2” and “Day 3” extracts). In the end, the extracts were supplemented with 10% (v/v) fetal calf serum (“FCS,” Lonza, France) before addition to the cell line. The L929 cell line was grown in alpha-MEM containing 10% (v/v) FCS. Cells were then seeded at 10 000 cells  $\text{cm}^{-2}$  in 24-well plates (Nunc, Denmark) and cultured for 72 h. At subconfluency, 1  $\text{mL}$  of the extracts was added to the cells. Wells containing only alpha-MEM + 10% FCS were used as a reference, providing the 100% value. Culture plates were then kept in the incubator at 37  $^\circ\text{C}$  for 24 h. After incubation, cell viability was assessed by the Neutral Red assay, and cell metabolic activity by an MTT assay.<sup>[51]</sup> The mean values of survival and metabolism measurements obtained from colorimetric tests were calculated from 5 to 7 repeats for each electrode. The results are presented in histograms as percentages of the reference (alpha-MEM + 10% FCS), with a mean  $\pm$  s.d. for four electrodes in each group.

**Statistical Analyses:** The data are presented either as arithmetic means  $\pm$  s.d. or arithmetic means with all the obtained data of the group. The differences between the groups were assessed with non-parametric tests using GraphPad Prism 10: Kruskal–Wallis for more than two independent samples, and Mann–Whitney test for two independent samples. A two-tailed  $p$ -value less than 0.05 is considered significant.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

R.B. did an investigation, wrote, reviewed, and edited. A.R. and K.J. did an investigation. S.G. did investigation, visualization, wrote, reviewed, and edited. A.L. did an investigation, and polymer synthesis, wrote, reviewed, and edited. T.M.B.R. did resources, investigated, wrote, reviewed, and edited. R.L., D.L., and W.S. did funding acquisition, resources, wrote, reviewed, and edited. A.K.G.F. did resources, wrote, reviewed, and edited. A.M. did funding acquisition, and resources, wrote the original draft, wrote, reviewed, and edited. N.M. did funding acquisition, methodology, resources, formal analysis, validation, visualization, wrote the original

draft, wrote, reviewed, and edited. C.B. did conceptualization, supervision, project administration, funding acquisition, methodology, resources, formal analysis, validation, and visualization, wrote the original draft, and wrote, reviewed and edited.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Keywords

ethylene oxide, gamma irradiation, glucose biosensors, protein conformation, terminal sterilization

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