In vivo assessment of cancerous tumors using boron doped diamond microelectrode

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The in vitro and in vivo electrochemical detection of the reduced form of glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) using boron doped diamond (BDD) microelectrode for potential application in the assessment of cancerous tumors is presented. Accurate calibration curve for the determination of GSH could be obtained by the in vitro electrochemical measurements. Additionally, it was shown that it was possible to separate the detection of GSH from the oxidized form of glutathione (GSSG) using chronoamperometry measurements. In vivo GSH detection measurements have been performed in human cancer cells inoculated in immunodeficient mice. These measurements have shown that the difference of GSH level between cancerous and normal tissues can be detected. Moreover, GSH detection measurements carried out before and after X-ray irradiation have proved that it is possible to assess in vivo the decrease in GSH concentration in the tumor after a specific treatment.

Glutathione is a tripeptide, which is found in high concentrations in many living cells and exists in reduced form (L-γ-glutamyl-L-cysteinyl-glycine, GSH) and oxidized form (disulfide form of GSH, GSSG)⁵⁻⁷. It is one of the strongest biological anti-oxidant, which means that under oxidative stress, GSH will be oxidized to GSSG, which in turn will be immediately reduced back to GSH by an enzyme (glutathione reductase)⁵⁻⁸. Because of this fast turnover, GSH concentrations in living cells are usually much higher compared to GSSG. For this reason, the ratio of GSH to GSSG often serves as a sensitive indicator of oxidative stress and is a key marker for the redox status of cells⁵⁻⁸.

Additionally, it was reported that GSH concentrations in cancerous cells are much higher when compared to healthy tissues⁵. It is thus believed that this high concentration of GSH is the principal reason of the high resistance of cancer stem cells against oxidative stress such as radiotherapy or chemotherapy⁶⁻⁹.

For this reason, the accurate in vivo detection of GSH becomes essential for the assessment of biological characteristics of cancer cells. Several techniques exist in order to measure GSH concentration and most of them involve liquid chromatography with different detection methods such as fluorescence or UV⁷⁻⁹. However, most of these methods are based on column derivatization followed by fluorimetric detection or on the conversion to their phenyl or pyridine derivatives followed by UV detection⁷⁻⁹. Therefore, these methods require expensive equipment and time-consuming procedures in order to measure GSH concentration. Additionally, these techniques are not suited for in vivo GSH detection and thus require tissue samples obtained through biopsy, which is an invasive procedure for the patient. An in vivo glutathione concentration measurement technique involving labeling with monochlorobimane before further detection using HPLC was reported¹⁰. However, this method was tested on plants only (Arabidopsis) and it was shown that the concentration measured includes both the reduced and oxidized form of glutathione¹⁰. Another in vivo method for GSH detection in human brain by means of double quantum coherence filtering was also reported¹¹. This analysis gave satisfactory results for in vivo GSH determination but this indirect detection method involves complicate spectra analysis and time consuming calibration procedures¹¹.

Electrochemical methods are a viable alternative due to their simplicity, rapidity and excellent sensitivity. Several electrochemical methods for glutathione detection have already been proposed using electrodes such as platinum, gold or gold/mercury¹²⁻¹⁴. These methods have shown to be quite efficient for in vitro glutathione detection but these techniques are not suited for in vivo applications. Therefore, the development of a new method for in vivo glutathione detection is needed.

The in vitro and in vivo electrochemical detection of the reduced form of glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) using boron doped diamond (BDD) microelectrode for potential application in the assessment of cancerous tumors is presented. Accurate calibration curve for the determination of GSH could be obtained by the in vitro electrochemical measurements. Additionally, it was shown that it was possible to separate the detection of GSH from the oxidized form of glutathione (GSSG) using chronoamperometry measurements. In vivo GSH detection measurements have been performed in human cancer cells inoculated in immunodeficient mice. These measurements have shown that the difference of GSH level between cancerous and normal tissues can be detected. Moreover, GSH detection measurements carried out before and after X-ray irradiation have proved that it is possible to assess in vivo the decrease in GSH concentration in the tumor after a specific treatment.
electrolyte: 0.1 M PBS. The reported from (A) at 2.3 V was plotted versus GSH concentration. Support electrode was 2.3 V vs. Ag/AgCl. (B) Calibration curve for GSH detection: the current density obtained in boron doped diamond (BDD) electrode15. This electrode material was selected for its exceptional characteristics of boron doped diamond, a new method is proposed for an accurate and rapid detection of GSH in normal and cancerous cells. Several in vivo measurements of GSH in tumors and normal tissues are presented and prove that it becomes feasible to perform an in vivo assessment of the biological features and malignancy of tumors using this method.

**Results**

**In vitro experiments.** Figure 1A displays cyclic voltammetry measurements recorded at 100 mV s⁻¹ on BDD for different concentrations of GSH (ranging from 0 to 10 mM) in 0.1 M PBS. Figure 1A shows that the on-set potential of GSH oxidation to GSSG (reaction 1) is situated approximately at 1.25 V.

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2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}^+ + 2\text{e}^-
\]

This figure shows also that the oxidation current recorded at a given potential increases with increasing GSH concentration. Using this feature, a calibration curve for GSH determination can be constructed and such curve for a potential of 2.3 V is presented in Figure 1B (the background current measured in 0.1 M PBS in absence of GSH was subtracted from each point and the linear regression was fitted to pass through the origin). This figure shows that accurate calibration (slope: \(3.03 \times 10^{-4} \pm 0.09 \times 10^{-4}, r^2: 0.988\)) curve can be obtained for GSH detection in this concentration range. From the data provided on Figure 1B, the lower detection limit using this technique was estimated at 0.3 mM taking three times the standard deviation.

However, as reported in18, the concentration levels of GSH measured in tumors can reach values around 60 mM. Therefore, the measurements presented in Figure 1 were repeated but for a higher GSH concentration range. Figure S1 displays the calibration curve for GSH detection and for a concentration range between 0 and 80 mM. This figure shows that the precision of such curve obtained from cyclic voltammetry measurements remains acceptable (slope: \(3.36 \times 10^{-4} \pm 0.06 \times 10^{-4}, r^2: 0.998\)) for higher GSH concentrations.

It is worthwhile to mention, that successive voltammetric scans performed on BDD for GSH solutions have shown that the activity of the electrode material decreases with successive measurements due probably to the formation of a polymeric film at the surface as already observed with other compounds such as dopamine16,19. Even for low concentrations of GSH (2 mM), a decrease in the oxidation current recorded at 2.3 V of approximately 8% was observed after 5 successive voltammetric scans. However, the activity of the BDD electrode can be recovered through cathodic treatment. In fact, Figure S2 shows the differences between cyclic voltammograms for 0.1 M PBS solution and recorded: (a) before GSH detection measurements, (b) after 100 voltammetric scans recorded in the presence of 10 mM GSH and (c) after 20 minutes of cathodic treatment at \(-3\) V performed after the GSH detection measurements. This Figure S2 shows clearly that GSH detection measurements induced a decrease of about 80% of the current recorded in PBS. However, the figure shows also that cathodic treatment can recover the activity of the BDD electrode.

In order to perform an accurate assessment of the cancerous tumor, it would be advantageous to be able to distinguish between the concentrations of the reduced form (GSH) and oxidized form (GSSG) of glutathione between healthy and cancerous cells. However, cyclic voltammetry measurements performed in the presence of GSSG have shown that if the chosen potential value for the calibration plot is 2.3 V, accurate detection of GSSG can be achieved. In fact, Figure 2A and 2B display such calibration curve for low (between 0 and 10 mM; slope: 1.8 \times 10^{-4} \pm 0.07 \times 10^{-4}, r^2: 0.988) and high (between 0 and 80 mM; slope: 1.12 \times 10^{-4} \pm 0.03 \times 10^{-4}, r^2: 0.996) concentrations of GSSG, respectively. These figures prove that GSSG can also be detected using the same technique previously used for GSH.
However, the on-set potentials of GSH and GSSG are slightly different as shown in the cyclic voltammogram comparison presented in Figure 3. Therefore, if one carries out the detection using chronoamperometric measurements at carefully chosen potentials (between 1.2 and 1.5 V in this case), it would be possible to detect only GSH and thus separate it from GSSG.

Figure 4A displays chronoamperometry measurements performed at 1.3 V and in 0.1 M PBS solutions containing between 0 and 10 mM GSH. This figure shows clearly that the anodic current recorded increases with increasing GSH concentrations. In fact, it is possible to construct highly accurate calibration plots for GSH detection (slope: $3.69 \times 10^{-6} \pm 0.09 \times 10^{-6}$, $r^2: 0.997$) using this technique as shown in Figure 4B. Moreover, in the same figure, it is also proven that the differences in current intensity between 0.1 M PBS in the presence and absence of 10 mM GSSG is almost negligible compared to the plot related to GSH. Therefore, it is possible to separate the detection of the reduced form from the oxidized form of glutathione using this technique.

Moreover, in the case of a solution containing both GSH and GSSH, it would still be possible to separate the detection of GSH from GSSG if the potential applied during the chronoamperometry measurement is carefully selected i.e. before the on-set potential of GSSG oxidation. However, it would not be possible to separate the detection of GSSG from GSH. In fact, if the on-set potential of GSSG oxidation was selected for the chronoamperometry measurement,
GSH oxidation would also occur and the current measured would be related to the oxidation of both GSH and GSSG.

In order to simulate in vivo experiments, the chronoamperometry measurements and corresponding calibration curves presented in Figure 4A and 4B have been repeated but at 37 °C. The results are displayed in Figure 5A and 5B and they show that an increase in temperature from 23 to 37 °C has no significant influence on GSH detection using this method.

It is worthwhile to notice that the construction of the calibration curves presented as well as the separation of the detections of GSH and GSSG was possible due to the unique properties of boron doped diamond (large potential window and low capacitive current). In the present work, in vitro and GSSG was possible due to the unique properties of boron doped diamond (large potential window and low capacitive current). The results are displayed in Figure 5A and 5B and they show that an increase in temperature from 23 to 37 °C has no significant influence on GSH detection using this method.

In vivo experiments. Figure 6 shows chronoamperometry measurements recorded at 1.3 V in subcutaneous normal and tumor tissues of nude mice. Measurements were performed in normal tissue and inside xenograft tumors derived from human squamous cell carcinoma cells (HSC-2 cell) that had been inoculated in three different mice for two weeks. This figure shows that the anodic current recorded increases when the measurement is performed inside the tumor when compared to the healthy tissue. Moreover, measurements performed in three distinct individuals but under the same conditions give similar results thus proving that the method is highly reproducible. If one considers that the difference between the different currents recorded is almost exclusively due to the difference in GSH concentration inside the tissue (due to its affinity for oxidation), this measurement proves that it is possible to perform an in vivo assessment of the tumor using this method.

It is worthwhile to notice though that biofouling of the BDD electrode occurs after one measurement, which induces a decrease in the current difference between the measurements performed in normal and tumors tissues. For this reason, it is necessary to perform a cathodic treatment (−3 V for 20 minutes) in 0.1 M PBS between each measurement. Additionally, if one wishes to obtain reliable results using this method, the in vivo current measurement should not last more than 5 seconds.

In order to detect a decrease in GSH concentration inside the tumor can be detected, among the three mice used for our investigation; two mice were X-ray irradiated for two minutes but at different levels (2 Gy and 6 Gy). X-ray irradiation has shown to decrease considerably the amount of GSH in living tissues as reported in 20.

Then, after three hours, GSH detection measurements were carried out in the HSC-2-derived xenograft tumors of the three mice (among which only two were X-ray irradiated) and compared with the results presented in Figure 6. Figure 7 displays this comparison and it shows that the current density recorded in the tumor decreased significantly after irradiation whereas the current density measured in the tumor remained unchanged for the mouse, which was not irradiated. Moreover, the decrease in current density increased with increasing intensity of the irradiation. This figure thus shows that it is possible using this technique to detect the variation of GSH concentrations in cancerous tumors before and after a specific treatment.

Discussion
In the present work, in vitro and in vivo detection of the reduced form of glutathione was performed on BDD microelectrodes using cyclic voltammetry and amperometry measurements for potential application in cancerous tumors assessment.

In summary, first, it is possible to build highly accurate calibration curves for the reduced form of glutathione (GSH) detection using BDD microelectrode and in 0.1 M PBS solution (pH7.4) using cyclic voltammetry and chronoamperometry measurements. Second, the separate detection of the reduced and oxidized forms of glutathione (GSSG) is difficult to achieve using cyclic voltammetry measurements due to the similar oxidation on-set potential of oxidation. However, when using chronoamperometry measurements at a
carefully selected potential (1.3 V vs Ag/AgCl), it is possible to measure only the concentration of the reduced form as its oxidation starts slightly before GSSG. Finally, GSH detection measurements carried out in subcutaneous xenograft tumors derived from human cancer cells in immunodeficient mice have shown that it is possible to measure in vivo the difference in concentration of GSH between cancerous and healthy tissues with a high reproducibility. Moreover, measurements performed before and after X-ray irradiation have shown that the variation of GSH concentration inside the tumor could be detected.

**Methods**

**Chemicals and materials.** Glutathione (GSH), glutathione disulfide (GSSG), disodium hydrogen phosphate dodecahydrate and disodium hydrogen phosphate dihydrate were purchased from Wako. Chemicals were used without further purification.

**Preparation of BDD microelectrodes.** BDD microelectrodes were prepared using a microwave plasma-assisted chemical vapor deposition (MPCVD) set-up (ASTeX Corp.). Acetone was used as a carbon source, and B(OCH₃)₃ as a source of boron. The tungsten needle was used as a cylinder, the working geometric area was about 6.3 × 10⁻⁶ cm². The electrochemical detection of glutathione was performed in 0.1 M phosphate buffer saline solution (PBS), which is prepared by mixing disodium hydrogen phosphate dodecahydrate and dehydrate until reaching a pH of 7.4. For the in vivo electrochemical experiments, the reference electrode was Ag/AgCl, the counter electrode was a silver wire and the working electrode was the BDD microelectrode. All potentials quoted in this work are with respect to the Ag/AgCl reference electrode (0.2 V vs SHE).

**Mouse xenograft tumor model.** Human oral squamous cell carcinoma cell line HSC-2 (1 × 10⁶) were implanted subcutaneously in the flank of nude mice. Two weeks after implantation, the GSH concentration in the tumor was measured using a BDD microelectrode. In order to measure in vivo the concentration of GSH, the BDD needle, the silver wire and the Ag/AgCl wire were inserted in the tissue to be analyzed to a depth between 2 and 3 mm. All animal experiments were performed in accordance with protocols approved by the Ethics Committee of Keio University.

**Electrochemical measurements.** In vitro electrochemical measurements were carried out in a single-compartment cell using an AUTOLAB PGSTAT potentiostat at room temperature (23°C). The reference electrode was Ag/AgCl, the counter electrode was a platinum wire and the working electrode was the BDD microelectrode. Considering the tungsten needle as a cylinder, the working geometric area was about 6.3 × 10⁻⁶ cm². The electrochemical detection of glutathione was performed in 0.1 M phosphate buffer saline solution (PBS), which is prepared by mixing disodium hydrogen phosphate dodecahydrate and dehydrate until reaching a pH of 7.4. For the in vivo electrochemical experiments, the reference electrode was Ag/AgCl, the counter electrode was a silver wire and the working electrode was the BDD microelectrode. All potentials quoted in this work are with respect to the Ag/AgCl reference electrode (0.2 V vs SHE).

Figure 7 | Assessment of the biological features of HSC-2-derived xenograft tumor before and after X-ray treatment. The results presented in figure 5 are compared with chronoamperometry measurements recorded on BDD microelectrode at 1.3 V vs Ag/AgCl in the HSC-2-derived xenograft tumor of mouse 1 (untreated; measurement performed three hours after the results presented in Figure 5), in the HSC-2-derived xenograft tumor of mouse 2 (three hours after the tumor had been X-ray irradiated for 2 minutes at 2 Gy) and in the HSC-2-derived xenograft tumor of mouse 3 (three hours after the tumor had been X-ray irradiated for 2 minutes at 6 Gy). T=37°C.

Figure 8 | SEM image of the BDD microelectrode. The image shows the tip of the needle shaped BDD microelectrode.
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**Author contributions**

Y.E., H.S. and S.F. conceived the project and designed the experiments. M.Y. and S.F. performed the experiments. K.Y. helped for the in vivo experiments. O.N. and S.F. analyzed the data. S.F. wrote the manuscript. All authors reviewed the manuscript.

**Additional information**

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Competing financial interests: The authors declare no competing financial interests.

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