A portable microfluidic-based biophotonic sensor for extracellular H_2O_2 measurements

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ABSTRACT

In this work a portable analytical biosensor for real-time extracellular monitoring of released hydrogen peroxide (H_2O_2) is presented. The biosensor is based on the optical detection of the cytochrome *c* (cyt *c*) oxidation state. The setup consists of an integrated microscope combined with a compact spectrometer. The light being absorbed by cyt *c* is enhanced via multiscattering produced by random aggregates of polystyrene beads in a cross-linked cyt *c* matrix. Using ink-jet printing technique, the sensing elements, namely cyt *c* loaded polystyrene aggregates, are fabricated with high reliability in terms of repeatability of size and sensitivity. Additionally, the sensing elements are enclosed in a microfluidic channel assuring a fast and efficient analytes delivery. As an example, the effect of trace concentrations of functionalized cadmium selenide/zinc sulfide (CdSe/ZnS) core shell quantum dots on the green algae *Chlamydomonas reinhardtii* is investigated, showing extracellular H_2O_2 release with different production rates over a period of 1 hour. In conclusion, the presented portable biosensor enables the highly sensitive and non-invasive real-time monitoring of the cell metabolism of *C. reinhardtii*.

Keywords: portable biosensor, ink-jet, microfluidics, oxidative stress, reactive oxygen species, quantum dots.

1. INTRODUCTION

Recent studies conclude that oxidative stress, an unbalanced generation of reactive oxidative species (ROS), is involved in ageing and diseases as well as defense agent in response to a pathogen invasion[1, 2]. In a healthy system ROS are counterbalanced by antioxidants maintaining a fragile equilibrium, however, misbalanced ROS levels may potentially lead to cell damage, cancer, neurological disorder or degenerative process of biological aging [3, 4]. Hydrogen peroxide (H_2O_2) is one of the most stable and, therefore, abundant ROS which is released through the cell membrane when an oxidative stress event occurs[5, 6].

Most of the current biochemical assays for detecting H_2O_2 are based on fluorescent dyes and cannot be used for prolonged measurements because of the limited stability of the dye [7]. Furthermore, dyes are toxic leading to interferences with the system under observation. In parallel, quantum dots have a huge potential as fluorescent labels but their limited biocompatibility is one of the major hindrance for biological application[8]. Alternatively, *Suarez et al.* reported a novel non-invasive real-time H_2O_2 biosensor based on the optical detection of the redox state of cytochrome *c* (cyt *c*). It was implemented using a spectrometer combined with an inverted microscope in dark-field configuration[9]. In this paper, we implement this detection principle in a portable setup containing integrated microscope, microfluidic platform and miniaturized spectrometer.

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As a first demonstration, we show the dynamics of H_2O_2 release by the alga *C. reinhardtii* exposed to low concentrations of functionalized CdSe/ZnS core shell quantum dots (Q-dots). Algae are of the most important environmental species since they lay at the bottom of the food chain in the aquatic environment and are exposed to pollution in the first place [10, 11]. On the other hand, oxidative stress can be related to toxicity leading to further knowledge on the toxicity of quantum dots to algae [12].

2. METHODOLOGY

The protein cyt *c* contains a heme group acting either as an electron acceptor or donor. Its ferrous group Fe^{II} can be oxidized in the ferric Fe^{III} state (the so called pseudo-peroxidase behavior) by the reduction of H_2O_2 to water [13]. The change in the redox state implies a change in the absorption properties of the protein. In this paper we use the fact that the absorption peak of cyt *c* at $\lambda = 550$ nm changes according to its redox state [14]. Therefore, changes in absorption of the cyt *c* at $\lambda = 550$ nm can be used as a fingerprint for the detection of H_2O_2.

Vandewalle et al. showed that the limit of detection for H_2O_2 using cyt *c* in solution lays in the micromolar range [15]. To be able to detect lower concentrations of H_2O_2, the amount of sensing cyt *c* has to be reduced. Due to the weak absorption cross-section, a small amount of cyt *c* exhibits only a poor absorption, limiting the sensitivity of the method. In order to increase cyt *c* absorption, *Suarez et al.* suggested a multiscattering approach enclosing the cyt *c* in a highly scattering media made of polystyrene beads (PS) (dielectric spherical particles with diameter of 500 nm) [9]. In this way the absorption of cyt *c* increases due to increased optical path length through the scatter.

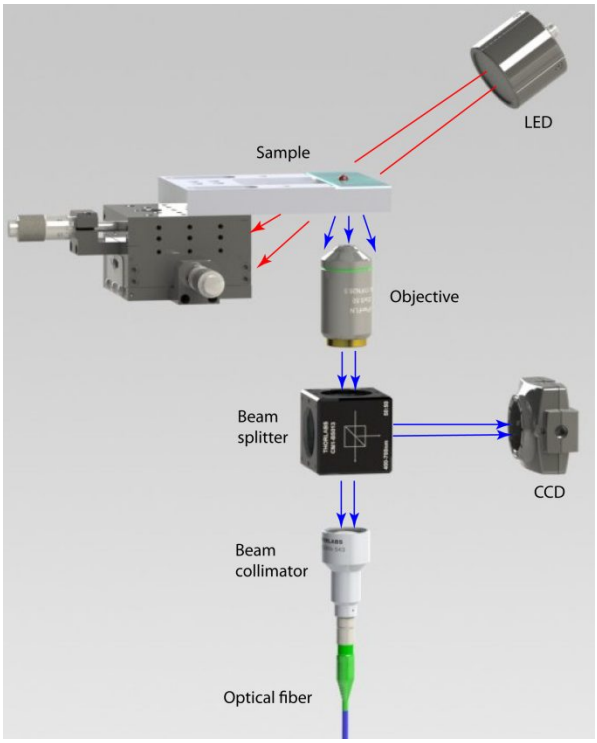

Figure 1. Schematic drawing of the portable setup. For clarity only the main elements are shown. The red arrows represent the direct light from the LED light source whereas the blue arrows indicate the light scattered by the sample.

To implement the above mentioned principle, we use a dark-field configuration and a white light LED as the light source (Fig. 1). Consequently, only the scattered light stemming from the sample and no direct light is detected. Since we aim to

work with small amounts of cyt *c*, directly transmitted light carrying no information with respect to cyt *c* has to be suppressed (in a bright-field configuration the absorption signal is completely hidden by the direct transmitted light). After the scattered light is collected by the objective the beam is split into an optical fiber connected to a spectrometer and a CCD camera serving for alignment of the aggregate.

The portable setup consists of a front and back section. All optical parts (Thorlabs) are fixed on the front side (Fig. 2a) of the mounting plate, whereas spectrometer, microfluidic pump and power supply (Fig. 2b) are mounted on the backside. To collect scattered light from the sample a 20x objective (UPlanFL 20x, Olympus, NA = 0.45) is used and mounted on a translation stage in order to adjust the focal distance to the sample. The spectrometer (HR4000+, Ocean Optics) is connected to a PC in order to save and visualize the recorded spectra. To visualize the sample we use a web camera (C270, Logitech) and, finally, the microfluidic pump (Xcalibur, Tecan) and LED (R11/D3/N/B, Relco Group Ltd) are powered by a 24 VDC switching power supply (TXH 120 124, Traco Power). The microfluidic pump is controlled using Labview. The complete setup has dimensions of 30×15×45 cm^3 and is, hence, compact and portable.

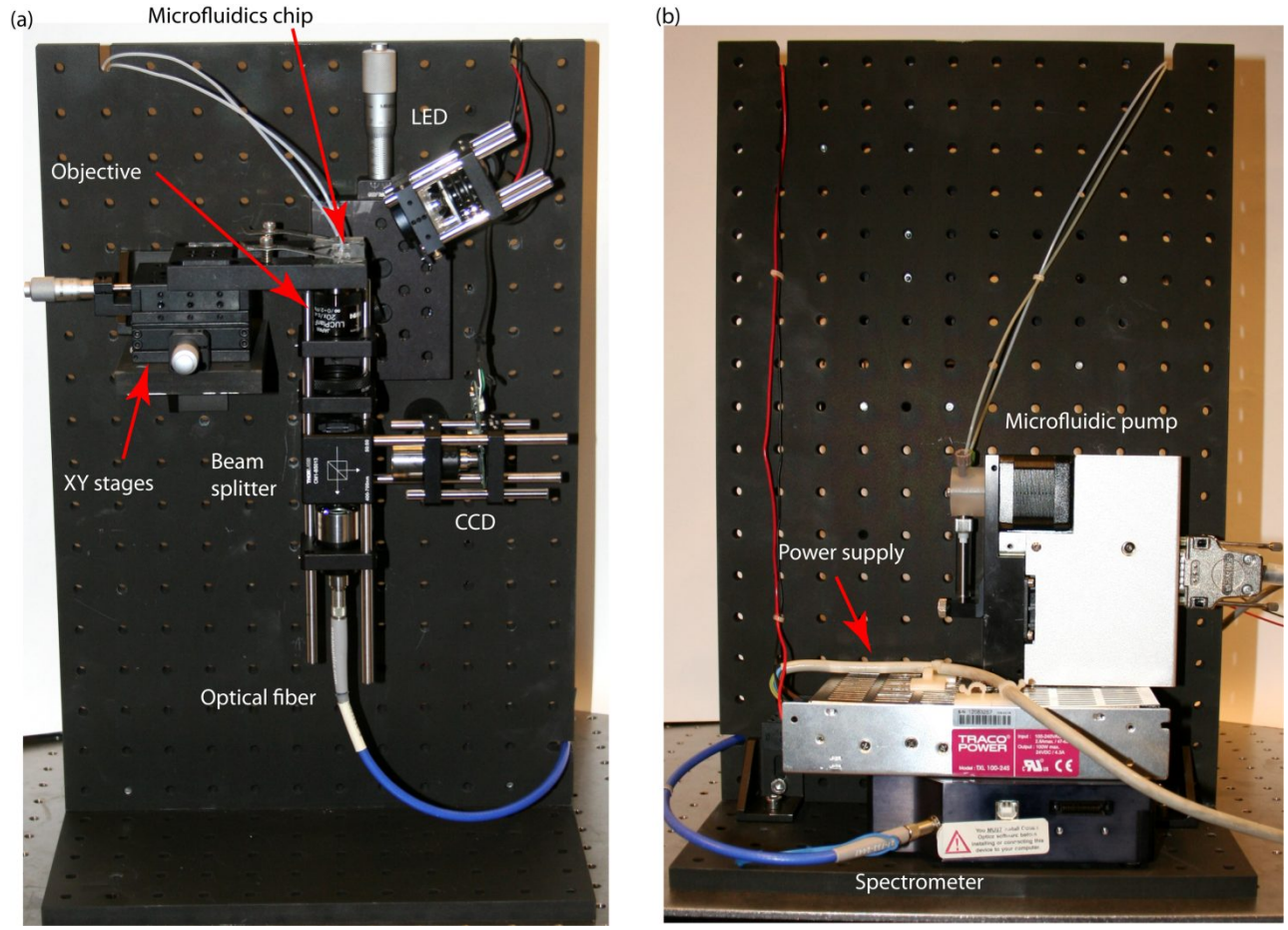

Figure 2. Pictures of the portable setup: (a) front view, (b) back view.

The sample including sensing spot and microfluidic chip is mounted on two translational stages enabling precise alignment with respect to the objective. The sensing spot, a PS/cyt *c* aggregate, is deposited using ink-jet technique (Fig. 3a). Ink-jet technique allows the fabrication of aggregates with high reproducibility, precise control of its position and very low amounts of material [16]. Briefly, the ink-jet setup consists of an ink-jet head with a nozzle of 50 µm hole diameter (Microdrop technologies, Germany) and a XY stage (Newport) on which the substrate is mounted. The ink-jet

nozzle, connected to the solution reservoir, generates drops by means of a piezoelectric system. After deposition the printed aggregates are crosslinked using glutaraldehyte in vapor phase. Finally, the produced PS/cyt *c* aggregates are stored at 4 °C in a closed container at 100 % relative humidity. The utilization of ink-jet technique enables the realization of sensing spots containing only 13 pmol of cyt *c*.

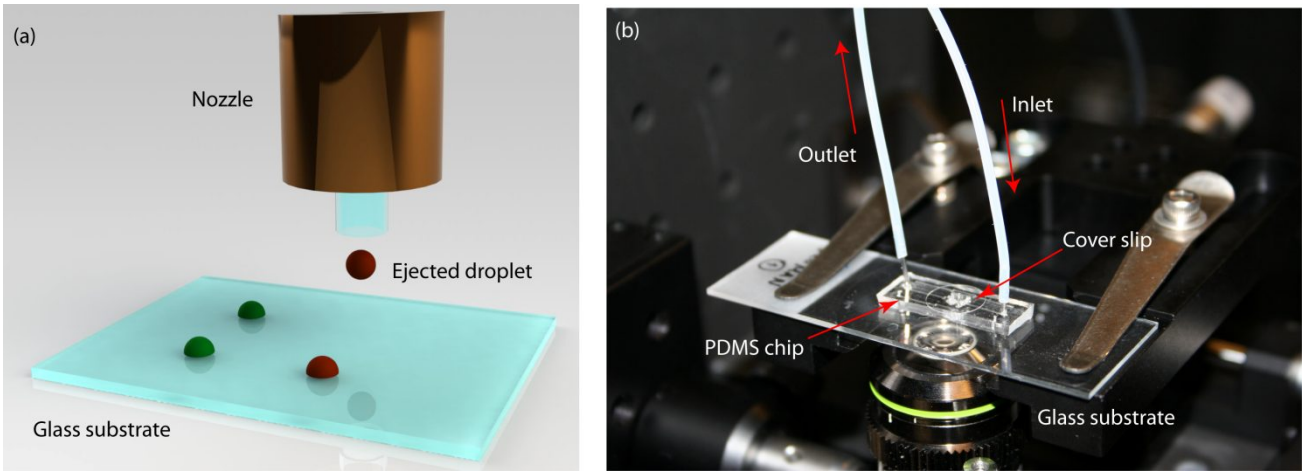

Figure 3. (a) Schematics of the ink-jet printing technique. (b) Picture of the realized microfluidic chip mounted on the portable set-up.

The crosslinked aggregates are enclosed in a microfluidic PDMS chip. The PDMS chip is attached to the glass substrate by baking the assembly (PDMS and glass substrate) at 80 °C [17]. Unfortunately, this procedure is incompatible with cyt *c* which tends to denature at temperatures higher than 40 °C. To overcome this obstacle, the ink-jet deposition of the cyt *c*/PS dispersion has been carried out subsequently to the baking step through an orifice in the PDMS. Finally the chamber is sealed with a glass cover slip (Fig. 3b).

3. RESULTS AND DISCUSSION

3.1. Hydrogen peroxide detection

As a proof of concept, we measure the scattering spectra of cyt *c* (13 pmol) in the presence of H_2O_2. The spot is enclosed in the microfluidic chip as described in the previous section. Prior to the measurements, the cyt *c* is reduced by adding ascorbic acid to the buffer solution. Figure 4a shows several consecutive spectra recorded every 15 min in the presence of 8 µM H_2O_2. The absorption peak, characteristic for cyt *c*, at $\lambda = 550$ nm, can clearly be identified. The gradual decrease of the characteristic dip with time is attributed to the oxidation of cyt *c*. The black curve below represents the reference signal through the bare glass substrate. The ratio between the reference signal and the cyt *c* scattering spectra serves as a proof that no direct light is reaching the spectrometer but only scattered one. Finally, these data show that we are able to detect the absorption of cyt *c* at amounts as low as 13 pmol and observe its consecutive oxidation.

To quantify the oxidation state of the cyt *c* spot we introduce an oxidation state coefficient as described elsewhere [9]. It is based on the difference between measured signals at $\lambda = 556$ nm, where the scattering intensity does not depend on the oxidation state, and $\lambda = 550$ nm, which corresponds to the center of the absorption peak. We use this coefficient to compare the response of the biosensor at different H_2O_2 concentrations (Fig. 4b). Control experiments in buffer solution show no changes of the oxidation state coefficient. On the other hand, in the presence of H_2O_2, the coefficient decreases indicating an oxidation of cyt *c*. As expected the oxidation rate of the cyt *c* depends on the concentration of H_2O_2 present in the solution.

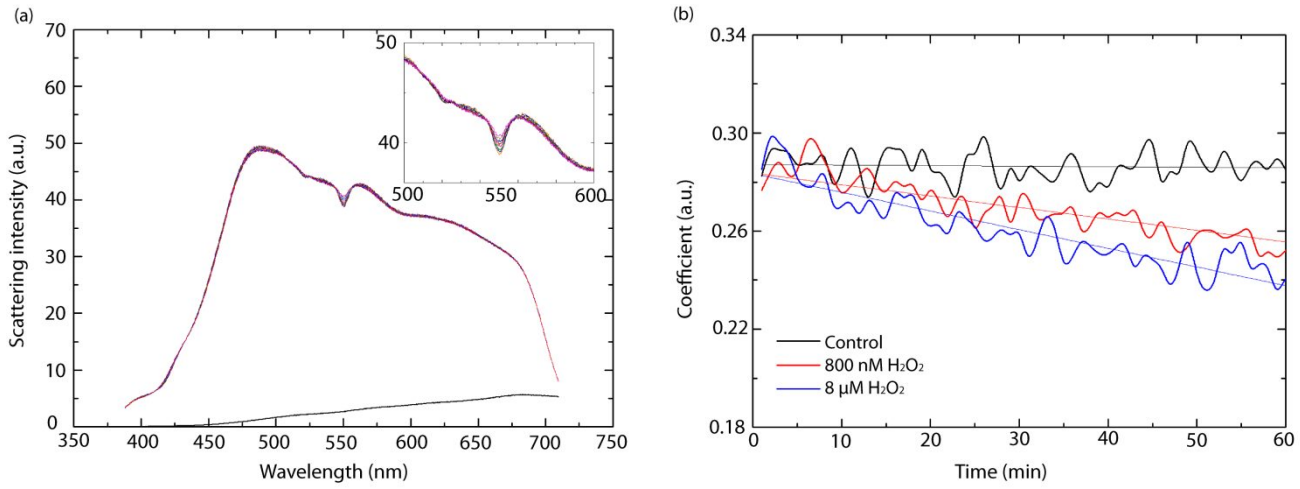

Figure 4. (a) Cyt *c* (13 pmol) scattering spectra recorded every 15 min. in the presence of 8 µM H_2O_2. The black curve corresponds to the reference signal through glass substrate. (b) Time evolution of the oxidation coefficient for different H_2O_2 concentrations.

3.2. Oxidative stress

As an example to demonstrate the performance of the biosensor, the dynamics of stress-related H_2O_2 released by the aquatic microorganism *C. reinhardtii* has been measured. To perform the experiments we fed the green alga *C. reinhardtii* suspended in PBS buffer solution ($5 \cdot 10^5$ cell/ml) in the channel of the microfluidic chip. The control experiments carried out in non-stress situations have not revealed a change of the oxidation state coefficient as shown in Fig. 5. This implies that no significant amount of H_2O_2 is released under those conditions. It is assumed that H_2O_2 is released during oxidative stress events through the cell membrane [18], hence, under non-stress conditions the oxidant and antioxidant species are balanced inside alga. In order to induce a stress situation functionalized cadmium selenide/zinc

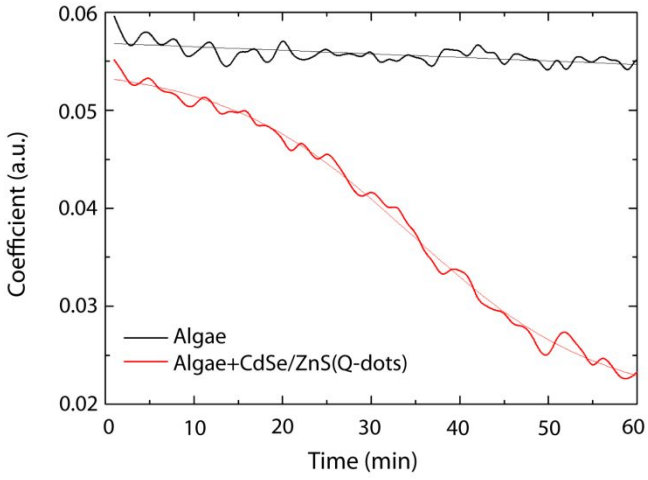

Figure 5. Time evolution of the oxidation state coefficient for algae suspended in PBS solution (black, control experiment) and alga exposed to 140 nM of CdSe/ZnS Q-dots (red).

sulfide (CdSe/ZnS) core shell quantum dots with a hydrodynamic diameter smaller than 10 nm have been added to the algae/PBS suspension. After addition of 140 nmol of quantum dots a clear and rapid drop of the oxidation state coefficient was observed, indicating a stress event triggered by the quantum dots present in the dispersion. The significant change in the oxidation state coefficient clearly demonstrates the performance of the described set-up to detect the overproduction of H_2O_2, related to a misbalanced oxidant/antioxidant budget or oxidative stress event. Furthermore, the extracellular detection scheme allows a completely non-invasive monitoring of H_2O_2 released by biological systems under stressed conditions.

4. CONCLUSION

A portable analytical tool for the sensitive detection of H_2O_2 is presented in this work. The biosensor is based on the optical detection of the oxidation state of the hemoprotein cyt *c* using a dark-field spectroscopy set-up combined with a microfluidic chip. Additionally, the utilization of ink-jet print technique enables the realization of reliable, reproducible and robust sensing elements. Moreover, it provides a simple yet elegant portable set-up for a very sensitive detection of H_2O_2. In order to improve the sensitivity of the biosensor the detection element, namely cyt *c*, is embedded in a multiscattering media made of polystyrene beads in a crosslinked cyt *c* matrix. First, we showed the detection of 800 nM H_2O_2 using a sensing spot containing only 13 pmol of cyt *c*. To illustrate the performance of the biosensor we successfully monitored the dynamics of the H_2O_2 released by the green alga *C. reinhardtii* under stress conditions induced by exposure to CdSe/ZnS Q-dots. In summary, the sensor provides a non-invasive way to monitor the cell metabolism in real time. Due to the non-invasive nature of the detection scheme the sensor provides an ideal tool for biological experiments and, moreover, its portability is of great interest for applications such as point-of-care diagnostics and field measurements.

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