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Short communication

Absorbance enhancement in microplate wells for improved-sensitivity biosensors

Guillaume Suárez [a,*], Christian Santschi [b], Gregory Plateel [a], Olivier J.F. Martin [b], Michael Riediker [a]

[a] Institute for Work and Health, Route de la Corniche 2, CH-1066 Epalinges-Lausanne, Switzerland
[b] Nanophotonics and Metrology Laboratory, Swiss Federal Institute of Technology Lausanne (EPFL), EPFL-STI-NAM, Station 11, CH-1015 Lausanne, Switzerland

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ABSTRACT

A generic optical biosensing strategy was developed that relies on the absorbance enhancement phenomenon occurring in a multiple scattering matrix. Experimentally, inserts made of glass fiber membrane were placed into microplate wells in order to significantly lengthen the trajectory of the incident light through the sample and therefore increase the corresponding absorbance. Enhancement factor was calculated by comparing the absorbance values measured for a given amount of dye with and without the absorbance-enhancing inserts in the wells. Moreover, the dilution of dye in solutions with different refractive indices (*RI*) clearly revealed that the enhancement factor increased with the ΔRI between the membrane and the surrounding medium, reaching a maximum value ($EF > 25$) when the membranes were dried. On this basis, two H_2O_2-biosensing systems were developed based on the biofunctionalization of the glass fiber inserts either with cytochrome *c* or horseradish peroxidase (HRP) and the analytical performances were systematically compared with the corresponding bioassay in solution. The efficiency of the absorbance-enhancement approach was particularly clear in the case of the cytochrome *c*-based biosensor with a sensitivity gain of 40 folds and wider dynamic range. Therefore, the developed strategy represents a promising way to convert standard colorimetric bioassays into optical biosensors with improved sensitivity.

1. Introduction

Colorimetric detection technologies provide to the analyst a valuable set of tools suitable in performing routine tests for biomedical diagnosis or environmental monitoring purposes. Despite their diversity in terms of format – ranging from standard cuvettes (Kuang et al., 2007) to multiwell microplates (Gillespie and Ainsworth, 2007), optical fibers (Malcik et al., 2005) or dipsticks (Liu et al., 2006) – the detection principle indistinctively bares on measuring changes in absorbance, as the physical response to a specific chemical reaction or biological recognition event. The most popular representative of colorimetric tests is the so-called enzyme-linked immunosorbent assay, referred to as ELISA, which combines high throughput and high sensitivity for quantitative or semi-quantitative detection of a large number of analytes (Martinez, 2011; Plested et al., 2003). Here, the measured absorbance change relies on the enzymatic conversion of a substrate into a chromogenic compound. Recently, the integration of novel materials in colorimetric detection systems has enlarged their capabilities thanks to their conformation-dependent absorption properties or pseudo-enzymatic catalytic behavior (Song et al., 2011). Typically, for a given system the absorbance change consecutive to the assembly (or disassembly) of functionalized gold nanoparticles (NPs) in a colloidal solution provides a sensitive indication on the presence of chemical (e.g. metal ions (Liu and Lu, 2003) or pH (Chen et al., 2008)) or biological (e.g. ssDNA (Mao et al., 2009; Sato et al., 2003), adenosine (Liu et al., 2006)) factors that control this mechanism. On the other side, some metal oxide NPs such as iron oxide (Fe_3O_4) have been shown to exhibit an intrinsic mimetic peroxidase activity (Gao et al., 2007) which enabled their use as detection labels in ELISA or in H_2O_2-based melamine sensors (Ding et al., 2010). In order to improve the analytical performances of colorimetric detection systems the main effort has been focused at the reaction/recognition level by seeking catalysts with higher reaction rates and recognition biomolecules with higher affinity constants. However, another track for sensitivity-enhancement strategies is clearly indicated by the physical nature of absorbance, governed by the Beer–Lambert law

$$A = \varepsilon l C \quad (1)$$

where being the molar absorptivity is ε, inherent to the species which is found at the molar concentration C in the sample, while the path length of light, l, remains an adaptable parameter.

Therefore, an interesting perspective to develop colorimetric assays with improved sensitivity resides in the ability to lengthen the trajectory of light through the sample. Such an approach is manifested in the detection systems based on either long path

* Corresponding author. Tel.: +41 21 314 58 97; fax: +41 21 314 74 21.
E-mail address: guillaume.suarez@chuv.ch (G. Suárez).

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flow cell where the light is guided through a few meters long capillary filled with the assay mixture (Zhang, 2006) or on-chip lateral beam guidance device particularly suitable for low-aspect-ratio sensing chambers (Grumann et al., 2006). However, in these strategies the gain in sensitivity is due to the fact that a larger volume of the sample – that is a higher number of absorbing molecules – is getting probed by the light. In other words, in such approaches the intrinsic sensitivity understood as the amplitude of signal generated per mole of absorber remains theoretically unchanged. In contrast, we have recently reported that for the absorption spectrum of a given biomolecule loaded into a random medium, such as cytochrome *c* embedded into an aggregate of polystyrene beads, absorbance values are enhanced due to the elongation of the light optical path (Suárez et al., 2012, 2013). In those preliminary studies the cytochrome *c* absorbance enhancement was efficiently used to develop a sensitive photonic biosensor able to detect in real-time the release of H_2O_2 by cells under oxidative stress. In the present work, we further extend the biodetection approach to the use of protein-functionalized glass fiber membranes as absorbance enhancing inserts adapted to microplate wells and which exhibit improved sensitivity compared to their corresponding bioassays in solution. In addition, this study experimentally proves the dependence of such an absorbance enhancement on the refractive index difference, ΔRI, between the scattering matrix and the surrounding medium.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (HRP type VI, 250–320 U mg_{solid}^{-1}), cytochrome *c* from bovine heart ($\geq$95%), glutaraldehyde solution 25%, hydrogen peroxide (30%), 3-amino-9-ethylcarbazole (AEC), bromocresol green sodium salt (BCG), ethanol, sodium ascorbate, phosphate disodic, phosphate monosodic, sodium chloride, calcium chloride and sodium acetate were all purchased from Sigma-Aldrich (Buchs, Switzerland).

2.2. Materials

Clear polystyrene flat bottom 96-well microplates were provided by Corning (Corning Inc., NY, USA). For the insert-based biosensor approach a circular aperture was drilled at the bottom of the wells bottom using a 2 mm diameter drill bit. Absorbance-enhancer inserts were cut out from glass fiber membranes GF/B (1 mm thickness) purchased from Membrane Solutions (North Bend, USA).

2.3. Glass fiber membrane loaded with BCG

In the experiments involving BCG, clean membrane inserts are placed in standard microplate wells before loading with 25 μL of BCG solution (120 μM) containing different amounts of glucose from 0% to 80%.

2.4. Biofunctionalization glass fiber membranes

Enhancer inserts made from glass fiber membrane are placed in the wells of the modified microplate (with bottom aperture) and are subsequently biofunctionalized with either cytochrome *c* or HRP via the following protocol: 25 μL of the protein solution (1 mg mL^{-1} HRP or 25 μM of cytochrome *c*) containing 0.5% of glutaraldehyde is added immediately after preparation onto each clean membrane insert and left to react for 30 min at room temperature. Then, the protein-modified membrane inserts are rinsed intensively by flowing several times PBS pH 7.2 or acetate buffer pH 5.0, for cytochrome *c* and HRP, respectively. In the case of cytochrome *c*-based biosensor, the heme-group was getting reduced prior to measurements by flowing several times 100 μL of ascorbic acid (1 mM in PBS) through the modified membrane. The cytochrome *c*-membranes are finally rinsed intensively with PBS solution.

2.5. Absorbance measurements

All the absorbance measurements are performed on a Infinite® 200 PRO multimode microplate reader from Tecan Group Ltd.

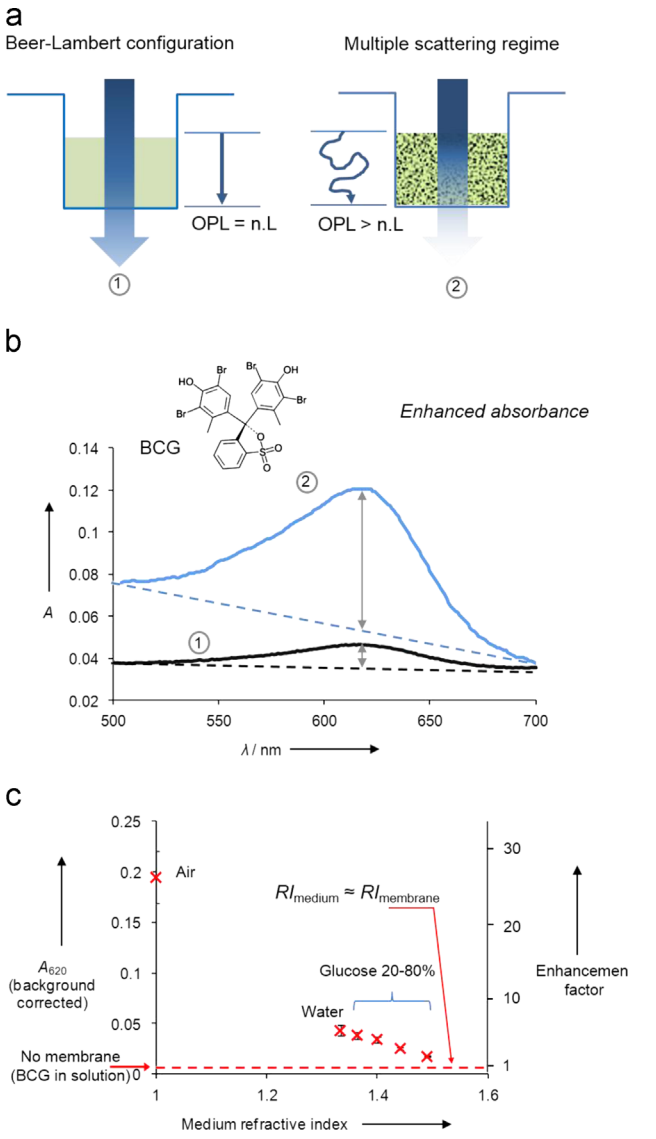

Fig. 1. (a) Detection principle based on the enhancement of the absorbance for a given molecule loaded in a random medium due to the elongation of the optical path length under multiple scattering regime. In comparison to the standard Beer–Lambert configuration, where the absorbance measurements are performed in solution (1), a significant enhancement is observed when the molecule is loaded into a strongly scattering medium (2). (b) Experimentally, the absorbance spectra of bromocresol green (3 nmol BCG) were measured in aqueous solution (1) and loaded into a glass fiber membrane (2). The height of BCG absorption peak (621 nm) was calculated (baseline between 500 and 700 nm) for varying values of $\Delta RI = Ri_{membrane} - RI_{medium}$. (c) The corresponding plot clearly indicates that the absorbance enhancement factor increases with ΔRI (maximum enhancement for membrane/air configuration). Ideally, there is no absorbance enhancement ($EF = 1$) when RI_{medium} is close to the value corresponding to the membrane material (i.e. borosilicate, $RI = 1.54$).