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Three immobilized enzymes acting in series in layer by layer assemblies: Exploiting the trehalase-glucose oxidase-horseradish peroxidase cascade reactions for the optical determination of trehalose

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ABSTRACT

A new optical biosensor for trehalose determination has been realized immobilizing three glycoenzymes on a transparent support. Trehalase, glucose oxidase and horseradish peroxidase have been alternated with layers of Concanavalin A by a "layer-by-layer" (LbL) deposition. The driving force of this assembly is the biospecific complexation between Concanavalin A and sugar residues in the glycoenzymes. As confirmed by UV–vis spectroscopy, the LbL deposition allowed a high ordinate architecture with high loading of enzymes. After the assembly, the functionality of immobilized enzymes was spectrophotometrically proven, demonstrating also that they can act in series catalyzing cascade reactions.

The prepared biosensor was used to optically detect trehalose, giving a LOD of 10 μM and a linear response up to 4 mM, and it showed also good time stability. The trehalose content in a real sample (eyewash) was successfully determined by the biosensor.

1. Introduction

Trehalose is a disaccharide found in a large variety of organisms that can survive conditions of extreme dehydration and high temperatures remaining in the absence of metabolic processes (anydrobiosis) for extended periods. In these organisms trehalose serves to preserve the cells in response to stress and extreme conditions. It is well assessed that, among saccharides, trehalose is the most effective in preserving biological molecules although the origin of its peculiarity is still under scrutiny [1].

Trehalose is currently used in several commercially available food, cosmetic and pharmaceutical products to maintain the integrity of biomolecules during the freezing or drying procedures (for review see [2]).

Besides HPLC [3], the analytical methods reported in the literature for trehalose determination are mainly based on enzymatic assays, in which the enzyme trehalase (TRE) specifically hydrolyses the trehalose into two glucose molecules. The newly produced glucose molecules are then quantified by bio-assays exploiting glucose selective enzymes. In the case of electrochemical biosensors the presence of at least a further enzyme (glucose oxidase) is required [4]. In the elicited paper, the trehalase is immobilized onto suitable solid supports (membranes) and the glucose oxidase (GOx) is linked to the electrode so that TRE and GOx are on separate locations of the instrumental setup. Recently, the co-immobilization of TRE and GOx on the same electrode has been proposed to produce electricity from trehalose in fuel cells [5]).

At variance, to assay spectrophotometrically the glucose produced by trehalose hydrolysis, two additional enzymes are required, for example hexokinase and glucose-6-phosphate dehydrogenase [6] or glucose oxidase and peroxidase [7], so that the whole optical determination of trehalose needs three enzymes acting in series.

While the use of pairs of immobilized enzymes is nowadays extensively exploited in sensing, the use of three or more immobilized enzymes remains still challenging and there are comparatively much less multienzyme than bienzyme sensors (for a

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recent example of a cascade of three enzymes see [8]). This is because, in the presence of enzymes acting in cascade, the requirements of optimal spatial arrangement become more and more stringent as the enzyme number increases.

In this respect, of crucial importance are the methods to immobilize enzymes, as they have to be assembled according to suitable architectures and their activity must be preserved for long periods of time, ensuring their facilitated recovery and reuse.

Among different immobilization approaches, the layer-by-layer (LbL) assembly presents several advantages as the control of the molecular architecture and the film thickness at a nanometer scale. Moreover it is an extremely mild procedure with a high versatility in the assembly (for reviews see [9–11]). The building blocks of LbL films were originally polyelectrolytes, but they have also been extended to enzymes, proteins, and other materials [12]. With regard to immobilization of enzymes, the LbL approach is very convenient. Upon immobilization the enzymes maintain their biological activity and are often stabilized, being thus less sensitive to denaturing agents.

The driving force for LbL deposition is usually the electrostatic interaction between oppositely charged materials, but also interactions such as hydrogen bonding, hydrophobic interaction, and biospecific affinity can be used in the LbL assembly [13,14]. Biospecific affinity between the biotin-(strept)avidin is a gold standard for enzyme immobilization [14–16], however its exploitation for multilayers preparation requires the presence of several biotin moieties at different sides of the enzymes.

In the case of glycosylated enzymes, the interaction between Concanavalin A (Con A) and sugars can be used to construct multilayer films. Con A is a plant lectin, extracted from the jack bean, which exists as a tetramer at neutral pH and has four binding sites for sugars such as glucose and mannose, forming a highly specific 1:4 Con A-sugar complex [17]. Therefore, the lectin-sugar interaction between Con A and sugar residues of glycoenzymes can be used to assemble LbL films [14].

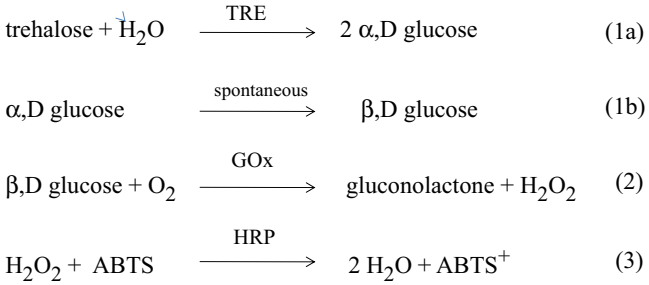
Electrostatic immobilization of multiple enzymes in a single LbL film was proposed for the first time by Lvov et al. [18]. Later on, thin films made of couples of enzymes acting in series like as the glycoenzymes glucose oxidase (GOx) and horseradish peroxidase (HRP) were constructed exploiting their sugar-lectin interactions with Con A. These assemblies were used mainly for electrochemical detection of glucose [19–21] and also of phenolic compounds [22].

In the present contribution, *three* glycoenzymes (viz. TRE, GOx and HRP) acting in series have been immobilized exploiting the concavalin-assisted LbL deposition. In the resulting enzyme assembly, the product of the first catalytic process is used as substrate for the second one, and so on. Specifically, TRE catalyzes trehalose hydrolysis into two glucose molecules which are in turn oxidized by GOx. The hydrogen peroxide produced in the latter reaction is the substrate of HRP and is used to oxidize a leuco dye (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid, ABTS) to a coloured (green) product, whose formation can be optically probed (see Scheme 1). After the assembly, the sequence of the cascade reactions has been exploited for the optical determination of trehalose.

2. Experimental

2.1. Reagents

Poly(dimethyldiallylammonium chloride) (PDDA), ABTS, Con A and the glycoenzymes GOx, HRP and TRE were purchased from Sigma–Aldrich. All other chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared with bidistilled water from Carlo Erba Reagents.



Scheme 1. Reaction sequence catalyzed from trehalase, glucose oxidase and horseradish peroxidase.

2.2. Apparatus

Absorption spectra were recorded by JASCO V-530 spectrophotometer. For measurements, the quartz plate supporting the multilayer was placed at 45° with respect to the measuring beam (in the spectrophotometer) by inserting the plate along the diagonal of a 1 cm path length cuvette, containing 2.5 ml of solution. Measurements were performed at (25 ± 1) °C.

2.3. Procedures

2.3.1. Preparation of multilayers

The assembly by LbL deposition of glycoenzymes and Con A was performed on an optical quartz support (1 × 5 cm slide, Hellma).

Before starting the adsorption steps, the quartz slide was cleaned by dipping it into a freshly prepared "piranha" solution (oleum H_2SO_4 and 30% H_2O_2, 3:1) for 15 min on ice (care should be taken in preparing and handling this solution, as the reaction is exothermic and the solution is highly corrosive) and subsequently left in water for 5 min, rinsed in acetone and dried under nitrogen flow. After this treatment the surface resulted negatively charged and a layer of the positively charged polycation PDDA was electrostatically adsorbed by dipping the slide in a 2 mg/ml PDDA solution in water for 30 min. Subsequently, it was washed in water for 2 min and immersed in a solution of 1 mg/ml Con A in 20 mM Tris–HCl buffer, pH = 7.4 for 15 min. At pH = 7.4 Con A is negatively charged and interacts electrostatically with PDDA polycation, accordingly a layer of this protein is adsorbed on the slide. Layers of glycoenzymes and Con A were alternatively adsorbed on the quartz slide trough biospecific interactions between Con A and sugar residues in the glycoenzymes. The adsorption steps were performed by incubating the slide at room temperature with a Con A or glycoenzyme solution for 15 min and then rinsing the specimen with distilled water. This time period was sufficient for substantial amounts of biomaterial to be adsorbed. A concentration of 1 mg/ml was found, in a systematic optimization procedure, to be optimum for each glycoenzyme. The above described adsorption steps were repeated in order to obtain the required number of layers, following the selected sequence. In this way, both sides of the slide are covered with the same number of layers. In the following, we will define the multilayer obtained by means of this procedure by the number of bi-layers grown on each side of the quartz slide. For example, the notation $(\text{Con A/enzyme1})_n + (\text{Con A/enzyme2})_m$ will denote a specimen on which have been deposited first n bilayers made of Con A and the enzyme1 and subsequently the LbL procedure continued through the deposition of m bilayers made of Con A and the enzyme2.

After immobilization, the quartz-supported multilayer was stored in buffer at 4 °C.