Colloids and Surfaces A 529 (2017) 508–512

Contents lists available at ScienceDirect

Colloids and Surfaces A

journal homepage: www.elsevier.com/locate/colsurfa

Enzymatically active free standing membranes based on an easy two step preparation from alginate catechol and glucose oxidase/peroxidase

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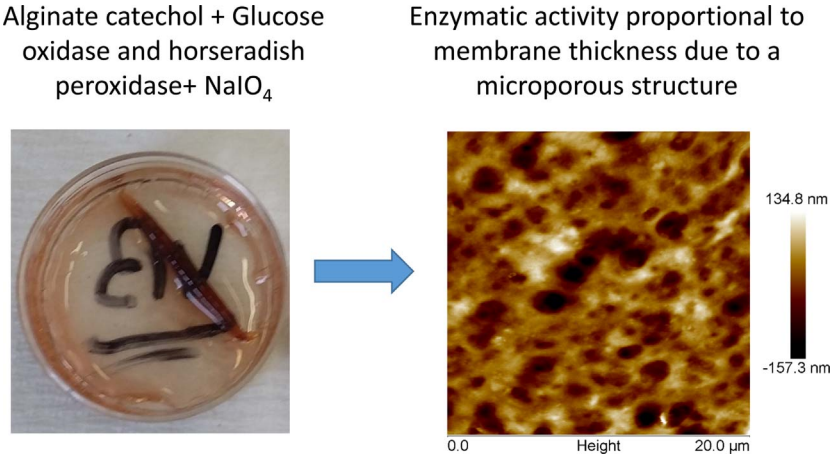
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Catalytic membranes
Catechol oxidation
Glucose oxidase
Peroxidase
Macroporous structure

ABSTRACT

Thick and robust free standing membranes were prepared by blending alginate modified with catechol groups and a mixture of two enzymes: glucose oxidase and peroxidase. The dried composite paste was crosslinked with sodium periodate, allowing to oxidize the catechol groups in quinones and to obtain thick free standing membranes. The enzymatic activity of those membranes was proportional to the mass of the used reactants, and hence to the membrane thickness, in the 100 µm range. This was possible owing to the microporous structure of the Alg-Cat@enzyme membrane.

1. Introduction

Even if many trends show that next-generation enzymes will be designed synthetically using concepts from materials science [1], the functionalisation of materials with natural enzymes to afford them with some catalytic activity is of major interest for the design of bioreactors or biosensors. This is not an easy task owing to the intrinsic low conformational stability of most of these biomolecules. Hence many strategies have been used to immobilize enzymes at solid-/liquid or liquid/gas interfaces, going from passive adsorption, to covalent grafting [2], to immobilization in sol gel matrixes [3] and to layer-by-layer (LBL) deposition [4,5]. In the latter case the adsorption of the target enzyme

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http://dx.doi.org/10.1016/j.colsurfa.2017.06.045
Received 7 April 2017; Received in revised form 13 June 2017; Accepted 16 June 2017
Available online 19 June 2017
0927-7757/

is alternated with the adsorption of a polyelectrolyte carrying opposite charges to that of the selected enzyme [6,7]. In most cases, the enzymatic activity is proportional to the number of deposition steps, hence to the amount of enzyme deposited, because in LBL deposition each deposition step leads to an identical amount of deposited material, with some exceptions however when the film growth displays an exponential growth regime [8]. However there are some examples where the measured enzymatic activity corresponds to the enzyme deposited in the last adsorption step even if the multilayer film was made from many more enzyme layers [9]. This originates most probably from a low porosity of these LBL films impeding the substrates to diffuse in the deepest parts of the films. LBL films can also be post functionalized with enzymes to yield self-standing enzymatically active membranes [10].

The major drawback of the enzymatic functionalization of LBL films is the long and repetitive processing (which can nevertheless be automatized by the use of dipping robots) A prototypal example has been recently described where alkaline phosphatase was immobilized with alginate catechol (Alg-Cat) in a layer-by-layer manner to yield a film which activity was proportional to the film thickness [11]. However the film was less than 10 nm thick after 10 deposition cycles requiring about 4 h of deposition ! Fortunately this drawback was compensated by a high degree of enzymatic stability as a function of the storage time. The film stability was due to its post deposition treatment with sodium periodate. $NaIO_4$ oxidizes the catechol groups of alginate catechol into reactive quinones, which allow to crosslink the film and to graft the enzyme through nucleophilic attack or Schiff base reaction of amines to the reactive quinones of the polymer [11].

Herein, we wish to develop enzyme active materials based on such reactive polymers but without the drawback of long processing times. Therefore we wish to rely on the possibility to obtain coatings having the same composition of LBL films but obtained by coacervation of the two polymers used in the LBL processing [12].

It is the aim of the present article to show that stable and extremely thick, porous and enzymatically active self-standing membranes can be obtained by coarcervation-drying and post crosslinking from a blend of alginate catechol and a mixture of two enzyme, namely glucose oxidase (GOX) and horseradish peroxidase (POX). The porosity of these membranes is in the macroporous regime and allows to obtain membranes those glucose sensing activity is proportional to their thickness.

2. Materials and Methods

2.1. Chemicals

All the solutions were prepared form doubly distilled and deionized water (Milli Q+ system from Millipore, $\rho = 18.2\ M\Omega\ cm$). The used enzymes, glucose oxidase from Aspergillus Niger (GOX, ref. G7141) and Horseradish peroxidase (ref. 8375) were purchased from Sigma Aldrich and used without purification. The pH of the sodium acetate buffer was adjusted with concentrated hydrochloric acid and checked with a Hi221 pH meter calibrated in pH range between 4.0 and 9.0.

Sodium periodate (ref. 311448), and guaiacol (2-methoxyphenol, ref. 253200) were also purchased from Sigma Aldrich. Hydrogen peroxide was furnished as a 35% (v/v) solution in water by Across Organics.

The alginate–catechol (Alg-Cat) polymer has been synthesized and characterized as described elsewhere [11]. Briefly, dopamine is covalently coupled to sodium alginate (medium viscosity) through carbodiimide chemistry leading to Alg-Cat in a single synthetic step. Then AlgCat was purified by dialysis and displayed that 15% of disaccharide units constituting the polysaccharide is modified by a dopamine entity. The determination of the grafting ratio was determined by ^1H NMR spectroscopy.

2.2. synthesis of the composite Alg-Cat@enzyme membranes and evaluation of their enzymatic activity

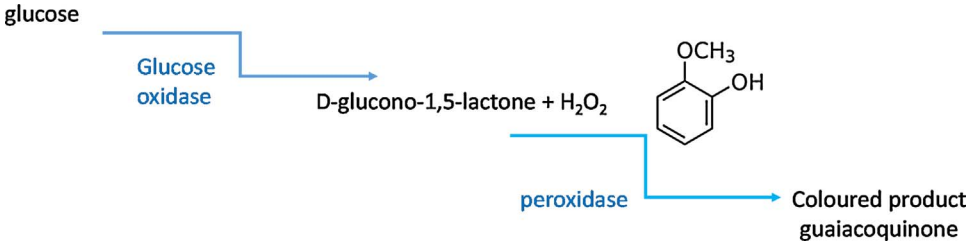
5,5 mg of Alg-Cat were dissolved in 2 mL of acetate buffer, 50 mM, pH = 5,06. 8 mg of a mixture of both enzymes GOX and POX (4 mg POX + 4 mg GOX) was dissolved in 2 mL sodium acetate buffer, 50 mM, pH 5,06. The polymer and mixture of enzymes were then mixed during 30 minutes, under slight magnetic stirring. This mixture (4 mL) was poured in a circular polystyrene Petri dish and allowed to dry under the laminar air flow in a hood during 1 night to obtain a uniform dry film. The next day, 1 mL of a 10 mM $NaIO_4$ solution dissolved in the sodium acetate buffer, was added to the dry film, under agitation, during 30 min. The mixture was placed 1 hour under the hood, for evaporation. The obtained crosslinked membrane was washed several times with sodium acetate buffer and kept in the refrigerator (at 4–5 °C) in 3 mL sodium acetate buffer. This stage defined time t = 0 for the enzymatic assays.

In some experiments all the amounts of Alg-Cat and of enzymes were multiplied by 2, 3 or 4 and poured in Petri dished of the same area to yield membranes containing 2, 3 or 4 times more active material, and hence displaying nominal thickness 2, 3 or 4 higher than the membranes produce from 5.5 mg of Alg-Cat and 8 mg of enzyme. These experiments were performed in order to asses if the enzymatic activity is proportional to the amount of used enzyme and hence if all the enzyme initially added to produce a composite Alg-Cat@enzyme membrane is accessible to the glucose + guaiacol substrates.

The day at which the enzymatic activity of the membrane was measured, the sodium acetate buffer was removed and replaced by the Tris buffer buffer, 50 mM, pH 8.5. The membrane still contained in the Petri dish but floating freely, was washed several times with the Tris buffer. Then, the whole buffer was removed but taking care not to break the thin Alg-Cat@enzyme membrane. Then, 2 mL of a glucose solution (1 mg mL^{-1} in Tris buffer) was added simultaneously with a 200 µL of concentrated guaiacol (Sigma). 100 µL of this reaction mixture were collected after 5 min, 30 min, 60 min, 90 min and 180 min of reaction. The oxidation of guaiacol by H_2O_2 obtained following the reaction between glucose and enzymes were read in the 96 wells plates using a Xenius spectrophotometer (SAFAS, Monaco), at a wavelength of 470 nm.

The reactions implied are represented in Scheme 1.

At the end of this assay the glucose-guaiacol reaction mixture was removed, the membrane was carefully washed with the sodium acetate buffer and stored again at 4 °C before a new enzymatic assay (performed in Tris buffer).



Scheme 1. Reactions implied when glucose and guaicol are put in contact with a membrane containing glucose oxidase and horseradish peroxidase.