Colloids and Surfaces B: Biointerfaces 143 (2016) 309–317

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Influence of protein bulk properties on membrane surface coverage during immobilization

Francesca Militano, Teresa Poerio*, Rosalinda Mazzei*, Emma Piacentini, Annarosa Gugliuzza, Lidietta Giorno

Institute on Membrane Technology, ITM-CNR, c/o University of Calabria via Pietro Bucci, 17/C, 87036 Rende (CS), Italy

ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form 16 March 2016
Accepted 17 March 2016
Available online 19 March 2016

Keywords:
Biomolecules covalent immobilization
Regenerated cellulose
Immobilization rate
AFM topography

ABSTRACT

Biomolecules immobilization is a key factor for many biotechnological applications. For this purpose, the covalent immobilization of bovine serum albumin (BSA), lipase from *Candida rugosa* and protein G on differently functionalized regenerated cellulose membranes was investigated.

Dynamic light scattering and electrophoresis measurements carried out on biomolecules in solution indicated the presence of monomers, dimers and trimers for both BSA and protein G, while large aggregates were observed for lipase.

The immobilization rate and the surface coverage on functionalized regenerated cellulose membranes were studied as a function of biomolecule concentration. Results indicated that the saturation coverage of BSA and protein G was concentration independent (immobilized protein amount of 2.40 ± 0.03 mg/g and 2.65 ± 0.07 mg/g, respectively). Otherwise, a different immobilization kinetics trend was obtained for lipase, for which the immobilized amount increases as a function of time without reaching a saturation value.

Atomic force microscopy (AFM) micrographs showed the formation of monolayers for both BSA and protein G on the membrane surface, while a multilayer structure is found for lipase, in agreement with the trends observed in the related immobilization kinetics. As a result, the morphology of the proteins layer on the membrane surface seems to be strictly dependent on the proteins behavior in solution.

Besides, the surface coverage has been described for BSA and protein G by the pseudo second order models, the results indicating the surface reaction as the controlling step of immobilization kinetics. Finally, enzyme activity and binding capacity studies indicated the preservation of the biomolecule functional properties.

1. Introduction

The immobilization of biomolecules on solid surfaces is an essential technique in several biotechnological applications [1]. Among different surfaces, membranes are receiving more and more attention in biotechnology. Bio-functional membranes are applied in biocatalysis [2,3], separations [4], and biosensors [5,6].

A key requirement is to immobilize proteins without losing their bioactivity. It is well known that the physical and chemical properties, activity and stability of a protein depend on both the immobilization method and the type of polymeric matrix. Then, the choice of the appropriate support, in terms of materials, reactive groups as well as the immobilization conditions is of primary importance in the production of bio-hybrid systems. An ideal support must include physical resistance, hydrophilicity, inertness toward proteins, resistance to microbial attack, and availability at low cost [7].

Apparently, most of the commonly used synthetic membranes for various bio-applications, such as nylon, polysulfone, poly(methyl-methacrylate), polyethylene, polyamide, polypropylene and polyaniline membranes [8–12], are less suitable for biomolecules immobilization. Indeed, they exhibit undesirable surface properties responsible for strong non-specific adsorption of proteins and increased probability of protein denaturation. On the contrary, supports based on natural materials exhibit high compatibility with biological molecules. Among them, cellulose is a promising candidate as it satisfies all the required surface properties [13].

* Corresponding authors.
E-mail addresses: t.poerio@itm.cnr.it (T. Poerio), r.mazzei@itm.cnr.it (R. Mazzei).

http://dx.doi.org/10.1016/j.colsurfb.2016.03.055
0927-7765/

Biomaterials based on cellulose and its derivatives have been used as ultra, microfiltration and chromatographic supports for protein purification [14–16] and membrane carriers for enzyme immobilization in different applications [17,18].

Different strategies are available for proteins immobilization: adsorption, covalent binding, entrapment and cross-linking. When the long-term stability of the bounded molecules is required, covalent immobilization is more advantageous with respect to the other techniques. Covalent immobilization has the advantage of forming strong and stable linkages between the proteins and the carrier, which may also result in better biomolecular activity, eliminating the loss of activity by protein leakage from the support [19].

While the end-use of the bio-functional membrane varies with each application, the chemical activation of the surface is often required to introduce reactive functional groups before protein covalent coupling. Cellulose contains many hydroxyl groups on its backbone, thus offering many reactive sites for surface functionalization. One of the most common and efficient method for cellulose activation is the conversion of dihydroxyl group to dialdehyde by periodate oxidation [20,21]. This reactive system benefits from high selectivity and ease work-up procedure. The primary amine group of the lysine residues in proteins, which are usually located on the proteins surface, reacts with aldehydes forming Shiff base. Therefore, an in-depth understanding of the membrane-protein interactions is required in order to optimize surface immobilization techniques and improve bioactivity of immobilized biomolecules.

The surface-protein binding can occur *via* the active groups present on the membrane surface, directly or by introducing a spacer [22]. In particular, cellulose membranes activated by periodate oxidation, with or without subsequent spacer introduction, were successfully applied for the development of membrane bioreactors and affinity membranes [23–25].

In spite of the plethora of studies dedicated to the immobilization of enzymes on polymeric surfaces, to date there is still a considerable lack of knowledge on how to design biomembranes with large active surface area without affecting bioreactivity. To this aim, it is necessary to in-depth explore the boundary conditions for the control of protein coverage, distribution and orientation and, then, related bioreactivity. Indeed, the properties of the final protein layer are influenced not only by protein-membrane surface interactions, but also by protein–protein interactions. Therefore, the protein structural properties in relation to the bulk conditions must be considered.

The objective of this study was to understand the effect of proteins concentration, size and aggregation phenomena on the kinetics of immobilization, surface coverage and local distribution onto commercial regenerated cellulose membrane. Bovine serum albumin (BSA), enzyme lipase from *Candida rugosa* and recombinant Protein G have been used as model biomolecules. The three biomolecules used in this study were chosen because of their importance in biotechnology and for their different properties and behaviors.

The immobilization behavior was investigated as a function of the initial protein concentration and it was correlated to pure protein properties in solution, including molecular size, charge, and aggregation. Dynamic Light Scattering (DLS) and Native polyacrylamide gel electrophoresis (NativePAGE) were used to study protein size and aggregation phenomena, by simulating the operating parameters used in the immobilization step.

The covalent immobilization of biomolecules on the membrane surface was carried out on different functionalized cellulose membranes. An ethylenediamine–glutaraldehyde spacer was also introduced between membrane surface and proteins.

The effect of protein concentration on the immobilization kinetics was evaluated with and without spacer. Protein surface coverage and arrangement were evaluated by AFM and described by pseudo second order model. Finally, the bio-reactivity of immobilized lipase and Protein G was also tested.

2. Materials and chemicals

Lipase (from *C. rugosa*, Type VII, ≥700 units/mg solid), bovine serum albumin (BSA), human IgG, sodium periodate ($NaIO_4$, ≥99.8%), ethylendiamine (EDA), glutaraldehyde (GA), ninhydrin, ethanol and glycine were obtained from the Sigma-Aldrich Chemical Co., Recombinant Protein G >98% was purchased from Biovision. Sodium borohydride >98%, Pierce™ BCA Protein Assay Kit and NativePAGE™ Novex® Bis-Tris Gel System were obtained from Termo Fischer Scientific.

The physicochemical properties of the biomolecules used in this study are summarized in Table 1.

All other chemicals were of analytical grade and used without further purification.

Flat sheet ultrafiltration regenerated cellulose (RC) membranes with MWCO (molecular weight cut off) of 100 kDa were purchased from Millipore.

3. Methods

3.1. Membrane surface functionalization

Regenerated cellulose (RC) membranes, with a very low protein adsorption capacity, were functionalized in order to introduce reactive functional groups on the surface to bind proteins covalently according to Scheme S1.

RC membrane was cut into 1 cm diameter discs and washed with distilled water to remove glycerin used as the preservative. Then, the membranes were immersed into $NaIO_4$ aqueous solutions at different concentration (0.02, 0.2, 2.0 wt.%) to obtain aldehyde groups. The oxidization reaction was carried out at room temperature in darkness for 7 h, and then the oxidized membranes (RC-OX) were thoroughly washed with distilled water.

For spacer (EDA) introduction, the activated membranes were placed into an aqueous solution containing 5 wt.% EDA for 15 h. The membranes were then rinsed several times using distilled water to remove any loosely bound EDA, and further reacted with 5 wt.% GA aqueous solution for 2 h. The obtained membrane was named RC-OX-EDA-GA.

3.1.1. Infrared analysis

Chemical functionality changes were evaluated collecting infrared spectra of the membrane surface in Attenuated Total Reflection Infrared (ATR-IR) mode. Spectra have been collected on pristine, RC-OX and RC-OX-EDA membranes by using an infrared spectrometer Thermo-Scientific Nicolet iS10.

3.1.2. Ninhydrin test

The ninhydrin method was used to check the presence of amino groups after the introduction of the EDA spacer. A small piece of membrane (1 cm^2) was put in a tube containing 1 mL of ninhydrin reagent and heated in boiling water for 3 min. The solution was diluted with 5 mL of EtOH. Ninhydrin reacted with amino groups to form a purple-colored solution. The amine concentration was determined by measuring the UV absorbance at 570 nm. The ninhydrin test (repeated twice) was also carried out on the RC-OX-EDA-GA membrane in order to evaluate the eventual presence of unreacted amino groups.