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Fluorescence monitoring of trypsin adsorption in layer-by-layer membrane systems

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ABSTRACT

A combined fluorescence analysis, involving the use of steady-state fluorescence and fluorescence anisotropy was used, allowing eliciting information about the structural changes induced on trypsin after exposure to membrane surfaces with diverse chemistry, designed through a layer-by-layer methodology. Using this monitoring strategy it was possible to understand the influence of the surface chemistry on the structural characteristics of the attached proteins and how they relate to changes of their activity resulting from the adsorption process. This knowledge may be used to direct the development of surfaces with suitable chemistry, leading enzymatic-based processes with improved performance. The results obtained show clearly that trypsin exposed to different membrane surfaces, changes its conformation, either if it adsorbs to the membrane or if it remains in solution. A significant loss of enzymatic activity was observed upon the adsorption process, for the adsorbed and non-adsorbed protein. This loss of the trypsin activity was correlated with the presence of molecular unfolding events that mediate trypsin–membrane surface interactions and the decrease of the molecular mobility of the adsorbed trypsin, which was shown to be dependent on the chemical characteristics of the membrane surface. Changes on the selectivity of the adsorbed trypsin were also observed, and may be ruled by the strength of the enzyme–surface interactions established.

1. Introduction

The performance of an enzymatic process is crucially dependent from the control of the physico-chemical conditions of the enzyme local environment. In the case of immobilized enzymes, it is necessary to assure optimal accommodation and stability of the biocatalyst within the support, in order to render maximum enzyme activity and long term process efficiency.

The use of solids [1–6] (e.g. membranes) or liquid [7,8] (e.g. ionic liquids) membranes as enzymatic supports aim to assure a high structural stability of the enzyme leading to enzymatic processes with durable efficiency [9], although often in detriment of the enzymatic activity [10,11]. Indeed, this effect has been reported by different researchers, such as Koutsopoulos et al. [10] who attributed the reduction of trypsin activity, upon adsorption onto silica and polysulfone supports, to structural alterations undergone by this enzyme during the adsorption process.

Loss of enzymatic activity may be either explained by enzyme structural changes through the increase of the structural stiffness [12] followed by a loss of molecular mobility/flexibility, or by substrate/product transport constrains due to immobilization. Changes of protein conformation and structural stiffness [8] alter the ability of the enzyme to accommodate the substrate, resulting in a change of enzymatic selectivity. Furthermore, the decrease of the activity of the immobilized enzymes may be also due to a closer contact of the enzyme with the surface, which renders lower accessible active centres, leading to limited transport of substrates and products to and from the protein active site and a deficient control of the physico-chemical conditions of the active centre involving media (e.g. water activity, ionic concentrations).

The structural and functional status of immobilized enzymes depends widely on the nature and intensity of the protein–surface interactions established. The structural changes induced by the adsorption process in a protein [13–15] are closely related with the affinity of the protein towards the surface, the properties of the solvent (e.g. polarity and interfacial tension), and the physico-chemical characteristics of the support surface. This dependence highlights the importance of protein–membrane surface interactions to the performance of the enzymatic processes and the relevance of a suitable selection/design of the membrane surface chemistry, while emphasizing the need for a better understanding of the molecular events underlying protein attachment to membrane supports [10,11,13–16].

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From our knowledge most studies performed to infer about the influence of surface chemistry and morphology on the structural and functional properties of proteins involved the combination of several distinct techniques, such as AFM, FTIR-ATR and circular dichroism, thus implying laborious and time-consuming analysis [10,11,16]. Moreover, surface specific analytic techniques such as dual polarization interferometry (DPI) [17] or total reflection based measurements (e.g. TIRF, TIRE) [18] are also powerful techniques and particularly suitable to assess the presence of structural molecular events occurring at surfaces or very close to them. Actually, the ability of total reflection based techniques, such as TIRF to monitor non-adsorbed molecules is limited, since it depends on the penetration depth of the created evanescence wave. These techniques also require the use of surfaces or the presence of interfaces meeting specific characteristics, which may guarantee the presence of adequate conditions (e.g. interfaces with suitable refractive index differences) for optimum measuring performances, which may limit the variability of applicable surfaces or surface materials.

The present work proposes the use of a monitoring approach based uniquely on the use of steady-state fluorescence, which allows for expedite, non-invasive and real time identification of the structural changes undergone either by the adsorbed proteins, inspecting directly the surface, and by proteins in bulk solution upon contacting with the support surface. This monitoring approach combines the information provided simultaneously by fluorescence emission and fluorescence anisotropy. Besides these advantages, this information can be elicited without addition of external fluorescence probes. Instead, it is based on the high sensitivity of the intrinsic fluorescence emission of tryptophan residues present in the protein chain – a structural probe –, to changes of their local microenvironment and to alterations of their rotational ability.

This study was performed with a model enzyme, trypsin, which integrates the group of serine proteases, able to hydrolyse peptide bonds at the carboxylic groups of arginine and lysine [19]. Trypsin is one of the most unstable proteins in solution being highly susceptible to autolysis. In this study, trypsin is immobilized at diverse polyacrylonitrile based membrane (AN69 membrane) surfaces, distinctly modified with differently charged polyelectrolytes using a layer-by-layer technique [20–25]. The increasing interest on the use of supported enzymatic systems, in particular supported trypsin microsystems, in biosensors, in devices for medical diagnosis and for proteome analysis [26,27] justifies the selection of polyacrylonitrile based membranes, such as AN69 membranes. These membranes have good biocompatible properties and have been extensively used in biomedical applications [27–30].

The ultimate goal of this study is to evaluate how the information provided by fluorescence techniques can be used for selection and design of surface characteristics and process conditions, in order to achieve maximum enzyme activity, selectivity and stability.

2. Materials and methods

2.1. Preparation of trypsin solutions and modified membranes

Trypsin solutions were prepared at a concentration of 1 g/L, using trypsin from bovine pancreas (EC. 3.4.21.4, Ref. T9935 from Sigma–Aldrich), having an isoelectric point (IP) of 10.1, in 0.1 M Trizma buffer at pH 8 containing 0.02 M of $CaCl_2$, added to prevent trypsin autolysis. Trizma buffer was prepared using Trizma hydrochloride (Sigma–Aldrich) and Trizma base (Sigma–Aldrich).

2.2. Description of the immobilization procedure of trypsin at AN69 membranes

Trypsin was adsorbed at polyacrylonitrile (AN69) flat membranes, with a total area of 9.6 cm^2. The AN69 membranes with a nominal molecular weight cut-off of 20 kDa and a 20 μm thickness were kindly provided by Gambro-Hospal Company (Meyzieu, France). Trypsin was adsorbed at non-modified AN69 membrane surfaces and AN69 membrane surfaces modified with two distinct polyelectrolytes: the polycation polyethyleneimide (PEI), and the polyanion sodium alginate (ALG). AN69 membranes were modified using a layer-by-layer self-assembly of the above mentioned polyelectrolytes, as described by Guedidi et al. [22], rendering the preparation of the following membranes: AN69 modified with a single polyelectrolyte layer of PEI (AN69-PEI) or sodium alginate (AN69-ALG), and AN69 modified with a double polyelectrolyte layer: PEI followed by sodium alginate (AN69-PEI-ALG).

AN69 membranes were modified by successive immersion in independent 1 g L^{-1} of PEI or sodium alginate solutions at pH 6 (depending from the intended membrane modification) for 1 h, without applied pressure. Each immersion stage was followed by a washing step in order to remove the loosely adsorbed polyelectrolyte. The adsorption of trypsin was carried out by immersion of the above mentioned membranes in a solution containing 1 g L^{-1} of trypsin for 3 h, corresponding to the time needed to reach an adsorption/desorption equilibrium [22].

The amount of trypsin adsorbed at different membrane surfaces was calculated based on the difference between the amount of enzyme in the trypsin solution before and after the adsorption process. Trypsin was quantified by UV absorbance at λ of 280 nm.

These membrane modification procedures led to the preparation of the following membranes: AN69-TRY, AN69-PEI-TRY, AN69-ALG-TRY and AN69-PEI-ALG-TRY.

The adsorption of trypsin onto AN69-ALG and AN69-PEI-ALG membranes was followed by the deposition of an additional layer of sodium alginate, using the experimental procedure described above, resulting in the preparation of AN69-ALG-TRY-ALG and AN69-PEI-ALG-TRY-ALG membranes. The adsorption of the last alginate layer was performed in order to confer a higher stability to the adsorbed trypsin [22].

Although ALG and AN69 are both negatively charged, adsorption of ALG at the surface of AN69 membranes was monitored by fluorescence (the fluorescence analysis methodology is described below), and expressed by a narrowing of the emission band of the membrane fluorescence spectra (results not shown). Identically, fluorescence evidenced the adsorption of trypsin (positively charged), at a membrane surface with identical charge type, AN69-PEI. For these reasons experiments using these membrane-trypsin systems, i.e. AN69-ALG-TRY and AN69-PEI-TRY, were also performed. The presence of the adsorbed trypsin was easily detected by the fade of the AN69 membrane characteristic emission band ($\lambda_{em,max}$ = 420 nm for λ_{exc} = 290 nm) by the simultaneous appearance of an emission band in the characteristic protein fluorescence region ($\lambda_{em,max}$ = 352 nm for λ_{exc} = 290 nm) and the decrease of membrane fluorescence anisotropy (Fig. 1 (a) and (b)).

2.3. Monitoring structural changes of trypsin induced upon interaction with the membrane surface

The structural changes undergone by trypsin upon interaction with the membrane surface were inferred based on a fluorescence analysis methodology, which combined the information provided by steady-state fluorescence emission and steady-state fluorescence anisotropy [31,32]. This methodology was applied to assess the structural changes of adsorbed trypsin, by direct inspection of the surfaces, and the possible alterations induced on the non-adsorbed trypsin molecules that have contacted with the membrane surface during the adsorption process.

2.3.1. Steady-state fluorescence analysis

The fluorescence scans were acquired at λ_{exc} of 290 nm (to allow the exclusive excitation of tryptophan) and at a λ_{em} ranging from 300 to 550 nm, for the initial solution of trypsin, for the solution of trypsin after contact with the membrane surface and for the membrane with the adsorbed enzyme, once removed from the protein solution. The fluorescence spectra were obtained at the magic angle (54.7°), using excitation and emission slits set of 5 nm of bandpass.

Protein structural changes were inferred based on the sensitivity of fluorescence emission of tryptophan to its surrounding environment. Changes of the fluorescence emission of tryptophans may be triggered by protein folding/unfolding processes, leading to shifts of the maximum emission and changes of emission intensity. The dislocation of the maximum emission to higher emission wavelengths (maximum emission red shift) [31–33] may be ascribed to protein unfolding events. In contrast, an additional protein folding (in the absence of further molecular events) induces the dislocation of the maximum emission to lower wavelengths (maximum emission blue shift) [34–37]. An exhaustive description of this methodology may be found in previous publications from Portugal et al. [31,32].

2.3.2. Steady-state fluorescence anisotropy analysis

Fluorescence anisotropy provides information regarding the mobility of the fluorophores [38,39], present in the protein chain. Therefore, anisotropy data may be related with the structural characteristics of proteins. A protein unfolding process is generally followed by an increased mobility of fluorophores (in this case the tryptophans) at the protein matrix [31,32]. Oppositely, a protein folding process may result in a higher imprisonment of the fluorophores within the protein matrix and to a decrease of their mobility [31,38,39].

The fluorescence anisotropy was acquired with a Spex Fluorolog Spectrofluorimeter equipped with excitation and emission polarizers, using excitation and emission slits set at 5 nm of bandpass. Fluorescence anisotropy was acquired at a λ_{exc} of 290 nm, in a range of λ_{em} from 300 to 550 nm. Further information regarding fluorescence anisotropy analysis was included as Supplementary Material.