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A novel electron beam-based method for the immobilization of trypsin on poly(ethersulfone) and poly(vinylidene fluoride) membranes

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ABSTRACT

A novel technique for the covalent immobilization of trypsin in a one-step reaction using low-energy electron beam is described. The enzyme immobilization was applied on poly(ethersulfone) and poly(vinylidene fluoride) microfiltration membranes. For this purpose, the membranes were dipped in an aqueous solution of trypsin followed by electron beam treatment.

The effect of irradiation conditions on the immobilization was investigated, as well as the resulting membrane properties with respect to enzymatic activity, immobilized enzyme concentration, pure water flux, scanning electron microscopy, and porosimetry. This technique shall provide a simple, inexpensive method for enzyme immobilization on various polymer membranes and offer a tool for the application in enzymatic membrane reactors.

1. Introduction

The use of enzymes such as trypsin has proven to be of great importance, particularly for industrial and biochemical applications [1,2]. However, the use of free enzymes is limited due to the labile nature of native enzymes which makes a reuse therefore difficult. In contrast, immobilized enzymes exhibit several advantages, e.g. they offer improved thermal and operational stability [3]. Furthermore, immobilized enzymes can be easily removed from the reaction mixture, and thus enable the reuse preventing a contamination of the product at the same time [4]. Enzyme-membrane reactors take advantage of the membrane having the simultaneous task of supporting the biocatalyst as well as acting as a selective barrier for the products to be separated. Many approaches for the immobilization of enzymes on polymer supports including membranes have been described in the literature including adsorption, covalent coupling, cross-linking and incorporation in the polymer substrate [5–8].

Based on these studies it was shown that surface properties of the matrix such as hydrophobicity, mechanical stability, charge and chemical structure can have a major effect on the immobilization, influencing the resulting enzyme activity [9]. Furthermore, the immobilization techniques can also affect the stability and activity of the enzyme due to chemical modifications which are often necessary in the coupling procedure. The selection of an appropriate matrix and immobilization method plays therefore an important role for the linkage of the enzyme.

We have reported that electron beam (EB) technology can be efficiently used for the hydrophilization of polymer membranes by the directed immobilization of functional organic molecules on the membrane surface [10,11]. The approach combines surface activation of the matrix polymer and the simultaneous immobilization of small molecules by use of low-energy EB in an aqueous system. The procedure neither requires any preceding surface functionalization nor the use of catalysts or other toxic reagents. In addition, it avoids the synthesis of monomers/polymers, thus avoiding additional synthetic and purification steps as well as the use of organic solvents. Since EB activation allows for operating independently from specific functionalities present at the matrix polymer's surface, the method can be adopted to a vast variety of membrane polymers and ensures also the formation of cross-links within the membrane polymer [12–15]. Furthermore, in contrast to UV irradiation, EB is capable of interpenetrating polymer membranes. This way, also the inner surface of the membrane is activated for the desired modification reactions.

In this publication, a novel technique for the covalent immobilization of trypsin on polymer membranes in a one-step reaction using EB irradiation is described. The resulting membranes exhibit the desired enzymatic activity, while performance characteristics such as water permeability, porosity or mechanical stability were not altered by the modification procedure. The EB-based immobilization technique, therefore, provides a directed, fast, and environmentally friendly method for the coupling of trypsin onto polymer membranes.

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2. Experimental

2.1. Materials

Poly(ethersulfone) membranes (Millipore, Express® PLUS, 0,22 μm) and two types of poly(vinylidene fluoride) membranes (Millipore, Durapore®, hydrophilic, 0.22 μm and 0.45 μm) were purchased from Carl Roth GmbH & Co. Glycyl–glycine (Gly–Gly), 2-amino-2-methyl-1,3-propanediol (AMPD), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), dimethyl sulfoxide (DMSO), p-nitroaniline (p-NA) as well as calcium chloride dihydrate, hydrochloric acid (HCl, reagent grade), sodium dihydrogen phosphate were obtained from Sigma Aldrich. Deionised (DI) water was used for preparing all buffer solutions. Albumin from bovine serum (67 kDa) and trypsin from bovine pancreas (24 kDa) were also purchased from Sigma Aldrich. Bicinchoninic acid (BCA) protein assay reagent A + B was provided by Pierce. All chemicals were used without further purifications.

2.2. Membrane modification

Membrane samples (∅ 10 mm) were placed in a 48 well microtiter plate. The modifications were performed by immersing the membranes for 5 min into an aqueous buffer solution (HEPES pH 8.0) of trypsin at room temperature followed by electron beam irradiation. The control experiment for enzyme adsorption on the membrane surface was performed by similar treatment (immersing for 5 min in the trypsin solution) without subsequent irradiation. Concentrations of the trypsin were varied within a range of 2–10 mg/ml for PES membranes or rather 4–10 mg/ml for the PVDF membranes and irradiation does in the range of 50–200 kGy were applied. Irradiation was performed in an N_2 atmosphere with O_2 quantities <10 ppm using a home-made electron accelerator. The voltage and the current were set to 160 kV and 10 mA, respectively. The absorbed dose was adjusted by the speed of the sample transporter. The modified membrane was rinsed three times per 10 min with buffer solution and afterwards quantified with the BAPNA assay and accordingly, the BCA assay.

2.3. Membrane characterization

2.3.1. Determination of the trypsin activity (BAPNA assay)

The residual activity of the immobilized trypsin was determined at room temperature using Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) as substrate [16]. The procedure for testing the activity of immobilized trypsin was performed according to the method of Oliveira et al. [17].

The substrate solution was prepared by mixing 0.3 mM BAPNA, 1 ml DMSO and 9 ml 50 mM TRIS–HCl buffer containing 10 mM $CaCl_2 \cdot 2\ H_2O$, pH 8.5. Then, 1 ml of the substrate solution was added to the modified and washed membranes. The plate with the samples was shaken for 5 min at room temperature and 200 μl of the supernatant were transferred to a 96 well microtiter plate. Then the optical absorption of the solution at 405 nm was monitored over a period of 130 min using a microtiter plate reader (Infinite M200, Tecan, Germany). After every sampling 200 μl of fresh substrate solution were added to the samples. The resulting extinctions from the BAPNA assay were converted (nmol) and displayed in concentrations vs. time diagrams. For calibration, seven standards based on a 0.3 mM p-nitroaniline stock solution with concentrations of 83.00, 41.50, 20.75, 10.38, 5.19, 2.59 and 0.00 μg/ml were used.

2.3.2. Trypsin concentration (BCA assay)

Trypsin concentrations on the membranes were investigated using the bicinchoninic acid kit [18]. The beforehand modified membranes, stored in the trypsin-buffer-solution were shaken for 1 h at room temperature. Afterwards, the samples were washed three times with 1 ml of sodium dihydrogen phosphate buffer solution (pH 7.0). Then, the BCA reagent was added to the membrane samples and the plate was incubated for 25 min at 37 °C. The plate was then shaken for 5 min at room temperature, the solution was transferred to a new microtiter plate and light adsorption at 562 nm was measured using a microtiter plate reader (Infinite M200, Tecan, Germany). For calibration, seven trypsin concentrations of 1000, 500, 250, 125, 62.50, 31.25 and 0.00 μg/ml were used.

2.3.3. Water permeability

For the investigation of the water permeability a stainless steel pressure filter holder (16249, Sartorius, Germany) for dead end filtration was used. A volume of 200 ml of deionised water was pressed through the membrane sample (active area: 13.2 cm^2) at 1 bar and the time of flow-through was recorded.

2.3.4. SEM

The surface and the cross-sectional area morphologies of modified and unmodified membranes were investigated using an Ultra 55 SEM (Carl Zeiss Ltd., Göttingen, Germany) under magnifications ranging from 10,000 to 25,000. The samples were manually cut and subsequently coated with a thin (30 nm) chromium film using the Z400 sputter system from Leyold, Hanau.

2.3.5. Pore size determination

For investigation of pore size distribution and bulk porosity of the membranes the mercury porosimeter PoreMaster PM-60-15 (Quantachrome GmbH & Co. KG, Odelzhausen, Germany) was used.

3. Results and discussion

3.1. Electron beam modification

The EB treatment results in the generation of a mixture of ions, excited molecules and free radicals as described for the radiolysis of water [19] ensuring the activation of both the dissolved trypsin [20] as well as of the membranes [10–15]. The formed radicals/activated species can undergo various reactions, such as cross-linking or recombination reactions. This way, links between the polymer matrix and the enzyme can be formed (Fig. 1). It is worth emphasizing that the use of an aqueous system is a crucial requirement since different results were obtained in the dry state as also reported in the literature [21].

3.2. Enzymatic performance

The activity of trypsin which was immobilized on different PES and PVDF membranes was characterized by hydrolysis of BAPNA as described in the experimental section. First, the enzyme immobilization was investigated as a function of the irradiation dose to determine the optimal EB dose for trypsin immobilization on the different membranes (Fig. 2). To be able to distinguish between simple enzyme adsorption on the membrane surface and the effect of irradiation-based immobilization, an adsorption experiment was always performed parallel to the irradiation procedure (label: 0 kGy). The dose range 50–200 kGy was chosen according to prior investigations [10–12] that revealed this optimum dose range for the membrane polymer activation. For both membrane types, PES and PVDF, the optimal dose for trypsin immobilization was