Journal of Membrane Science 475 (2015) 71–79

Contents lists available at ScienceDirect

Journal of Membrane Science

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

# Plasminogen purification from serum through affinity membranes

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ARTICLE INFO

*Article history:*
Received 13 June 2014
Received in revised form
3 October 2014
Accepted 7 October 2014
Available online 16 October 2014

*Keywords:*
Plasminogen
L-lysine
Cellulose
Microporous membranes
Affinity chromatography

ABSTRACT

The use of plasmin in some eye treatment and, in particular, as a facilitator for eye surgery to prevent retinal detachment is beneficial for the patient and entails less surgical risks than conventional vitrectomy. Plasmin is obtained by activation of plasminogen using a variety of enzymes, including tissue plasminogen activator, urokinase and streptokinase. The isolation of plasminogen from blood is normally performed with bead-based affinity chromatography however affinity membranes are ideally suited for this application since they can be easily packed in small units providing a fast and economic process.

In this work, we prepared affinity membranes for plasminogen purification using L-lysine as affinity ligand. To this aim regenerated cellulose membranes were used as a support for ligand immobilization. The efficiency of L-lysine coupling was inspected by applying different binding protocols and the immobilization yield was studied as a function of the reaction conditions. The membranes have been characterized in batch and in complete chromatographic cycles using bovine and human serum as test probes. The results obtained indicate that the L-lysine affinity membranes are a promising alternative for the purification of plasminogen.



## 1. Introduction

A variety of macular diseases, inducing traction at the vitreoretinal interface, such as macular hole, macular edema, diabetic retinopathy, macular pukers, may benefit from vitrectomy [1,2]. Vitreoretinal surgery presents serious risks because mechanical peeling of the vitreous may not result in complete vitreous removal and may be traumatic to the retina [2]. Several studies [1–4] demonstrated that the limits associated with vitrectomy could be reduced by applying enzymatic vitrectomy, which allows an atraumatic vitreoretinal separation. For this reason, enzymatic vitrectomy is envisaged to augment or even replace conventional vitrectomy in view of the reduced surgical risks, surgeon time and costs [3,4]. Enzymatic vitreous disruption, that is the cleavage of the vitreoretinal interface by enzymes, is a non-surgical method of inducing a posterior vitreous detachment and liquefaction of the vitreous, allowing for a cleaner retinal surface in comparison to current mechanical procedures.

Several enzymes are suitable for the hydrolysis of the vitreous–retina interface [3] and among those plasmin is the most promising because it can be isolated from the patient's serum; its activity decreases to an undetectable level within 24 h and it has the property to hydrolyze a variety of glycoproteins, including lam inin and fibronectin, which are present at vitreoretinal interface and contribute to the adhesion of vitreous to the limiting membrane [1–4]. Recently, an ocriplasmin therapeutic preparation, also known as microplasmin, obtained from recombinant *Pichia pastoris*, has been approved by FDA and by several European countries. However, due to the high cost of an injectable dose it is important to search for feasible cheaper alternatives as, for instance, autologous plasmin [5,6].

Since plasmin is obtained by enzymatic conversion of plasminogen, a possible route for its production is the purification of plasminogen from the patient's blood and its subsequent activation to plasmin. The conventional methods for the purification of human plasminogen, like precipitation, extraction from Cohn's fraction III and gel filtration have been replaced by affinity chromatography using L-lysine affinity beads [7–9]. Exquisite selectivity and high binding capacity are the characteristics that contribute to the success of affinity chromatography; on the other hand, slow diffusive mass transport, high pressure drops and bead compressibility represent some of the drawbacks and limitations associated with packed-bed chromatography. Microporous membranes can be used as affinity supports to overcome the limitations of porous beads [10]: the convective flow of the solution through the membrane pores reduces mass transfer resistance; indeed the molecules flow very close to the active binding sites of the membrane and thus binding kinetics dominates the adsorption rate

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http://dx.doi.org/10.1016/j.memsci.2014.10.013
0376-7388/

[11]. Therefore, in comparison to affinity beads, membrane affinity chromatography can operate at higher flow rates and lower operating pressures; this translates into a faster and more cost-effective purification process [10,12,13]. Furthermore, a more rapid process decreases the probability of inactivation of the biomolecules [11].

The main disadvantage of membrane adsorbers for protein capture is the lower binding capacity with respect to chromatographic beads, but for larger molecules which cannot enter the bead pores like plasmids, nucleic acids and viruses, convective supports like membranes and monoliths are the optimal solution [13–18]. One of the most promising applications of membrane adsorbers is in polishing steps of bioprocess manufacturing and, in particular, of monoclonal antibody production [19–22]. For impurities removal, where capacity is not an issue, the high processing speed and the lower buffer consumption together with the disposable technology justify the success of membrane chromatography.

For the specific application under investigation, due to the small volume of an ocular injection, which is typically lower than 10 nL, affinity membranes are ideally suited for the development of a single use filter to be utilized directly by the surgeon in the operating theater, since they can be easily packed in small units providing a fast and economic process.

In this work, we prepared affinity membranes for plasminogen purification using L-lysine as affinity ligand. To this aim, L-lysine was coupled to epoxy activated regenerated cellulose membranes by applying two different binding protocols. In the first protocol, the membranes were modified adapting the procedure described by Young et al. [23], developed for microporous PVDF membranes, in which L-lysine was immobilized as a substrate for neuron culture; the second protocol was developed by modifying the procedure for the immobilization of L-lysine onto epoxy activated silica beads for plasminogen purification [24].

Optimization of the binding protocols was obtained by varying the operating conditions, namely L-lysine concentration, reaction time and temperature in order to achieve the higher immobilization yield. The yield was obtained with measures of ligand density by means of a colorimetric assay based on the use of acid Orange 7 as an indicator [25,26].

The affinity membranes were then tested in full chromatographic cycles using bovine and human serum, and the results were compared to the ones obtained with a commercial L-lysine affinity resin used as a benchmark.

## 2. Experimental

### 2.1. Affinity supports

Affinity membranes were prepared by coupling L-lysine to cellulosic microporous membrane supports, Sartobind Epoxy™ membranes, kindly provided by Sartorius Stedim Biotech GmbH (Göettingen, Germany). The membrane matrix, made of reinforced stabilized cellulose, has a porosity of about 73%, a nominal pore size of 0.45 μm and an average thickness of 230 μm as reported in Table 1 [27].

As a comparison, a commercial chromatographic affinity resin, Lysine HyperD™ (Pall, Milan, Italy), packed in a 1 mL column, was tested in parallel experiments.

**Table 1**
Properties of Sartobind Epoxy™ membranes.

| Thickness (μm) | Density (g/cm$^3$) | Area$_{BET}$ (m$^2$/g) | Pore size (μm) | Binding capacity (mg/mL) | Epoxy group density (μmol/cm$^3$) |
|---|---|---|---|---|---|
| 230 | 0.413 | 2.25 | 0.45 | 1.1–5.5 | 86.95–95.65 |

### 2.2. Chemicals and solutions

L-lysine, the levorotatory optical isomer of the amino acid lysine, that exhibits affinity towards plasminogen [28], has been coupled on the membrane supports considered. Due to the presence of the amino groups, L-lysine has the property to interact with proteins [29] and needs to be immobilized via its α-amino or α-carboxyl group in order to be used as ligand in chromatography. Two different forms of lysine were tested: L-lysine which has a molecular weight of 146.19 g/mol and L-lysine monohydrochloride, with a molecular weight of 182.65 g/mol.

Human and bovine sera were used as a plasminogen source, whereas pure plasminogen was used only as a protein standard for analytical purposes. The feed solutions were prepared by diluting the relevant serum in equilibration buffer and by filtering them prior to use through a Millex 0.22 μm low protein adsorption syringe filter (Millipore, Milan, Italy).

50 mM phosphate buffer pH 7.5 and 100 mM sodium phosphate buffer pH 8 were used as equilibration and washing buffers, while a solution of ε-aminocaproic acid was used for the elution step; all buffers were filtered prior to use through 0.45 μm cellulose nitrate membrane filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany). All experiments have been conducted with a layered stack of 5 membrane discs of 2.6 cm diameter and with chromatography beads packed in a 1 mL volume column. All proteins and chemicals have been purchased from Sigma-Aldrich (Milan, Italy) unless otherwise stated.

### 2.3. Equipment

A thermostatic shaker water bath, SW 23 Julabo (Labortechnik GmbH, Seebach, Germany), was used for membrane modification and ligand density measurements. A Shimadzu UV-1601 UV–visible spectrophotometer has been used for protein concentration measurements, at 280 nm for pure protein solution and at 485 nm for the colorimetric assay.

The purity of the eluted fractions was analyzed with both gel electrophoresis and HPLC. SDS-PAGE analysis of the protein solutions was performed with a Criterion electrophoresis system using precast gels and molecular mass markers Precision Plus Protein™ Standards All Blue. All electrophoresis materials and reagents were from Bio-Rad Laboratories (Segrate, Mi, Italy). An Alliance 2695 separation module with a 2487 dual-wavelength absorbance detector was used for HPLC analysis, using a size exclusion column, Waters BioSuite™ Ultra High Resolution SEC, all from Waters (Milan, Italy).

Flow experiments were performed using a FPLC (Fast Protein Liquid Chromatography) system, Äkta Purifier 100 controlled by Unicorn™ software, from GE Healthcare (Milan, Italy). Prior to use, the membranes were thoroughly washed with equilibration buffer and packed in a membrane holder constructed on purpose to accommodate membranes of 2.6 cm in diameter. The membrane holder made with stainless steel can allocate up to 15 membranes, layered one above the other. The inlet flow is distributed radially and made uniform by using Teflon porous frits which were included at the top and at the bottom of the membrane stack [30]. The chromatographic cycles were monitored on-line following the UV absorption at 280 nm, pH, conductivity and pressure profiles as a function of the volume fed to the column. All experiments were conducted at room temperature.

### 2.4. Ligand immobilization

L-lysine was immobilized on the membranes following two different binding protocols; in both cases, due to the protonation of the L-lysine ε-amino group at basic pH values [31], it is inferred