Immobilization of Trypsin onto Macroporous Monolithic Poly(epoxy-acrylamide) Cryogels

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ABSTRACT: immobilization of bovine pancreatic trypsin onto polyacrylamide monolithic cryogels with epoxy functionality (Epoxy-MPAAGs) was achieved through the incorporation of ethylenediamine-glutaraldehyde spacer arm on the cryogels’ surface. Immobilization yields (protein content) ranged from 39 to 73 mg per g monolithic cryogel. The immobilization yield appeared to be affected by the cryogels’ intricate pore structures. The values of immobilization yields (protein contents) obtained in this work for the Epoxy-MPAAGs cryogels immobilised trypsin concur to those reported in the literature for trypsin immobilised onto relatively similar support materials. Activity measurements from the native trypsin before immobilization and the residual free trypsin after immobilization by hydrolysis of low molecular mass N-α-benzoyl-D, L-arginine-p-nitroanilide (BAPNA) demonstrated activity recovery (activity based immobilization yield) of about 60 – 70% for poly(epoxy-acrylamide) cryogel immobilized trypsin preparations. Activity values determined for the poly(epoxy-acrylamide) cryogel immobilized trypsin by hydrolysis of the same standard substrate, BAPNA, indicated that the immobilized enzyme retained up to about 50% of the activity of the native free trypsin. These results show that poly(epoxy-acrylamide) monolithic cryogels are well suited for trypsin immobilization.

Key words: Enzyme trypsin, immobilized trypsin, polyacrylamide cryogels

INTRODUCTION

The term immobilized enzyme refers to an enzyme that is physically confined to or localized within a support with retention of its catalytic activity, thus can be used repeatedly and continuously [1, 2]. Desired properties of a good support vary depending on the type of enzyme, the immobilization method and the intended application. Important requirements for a support material are water insolubility, high enzyme binding capacity and chemical inertness towards substrates as well as microbes. Other requirements include thermal and mechanical stability [1, 2]. The enzyme binding capacity is determined by the available internal and external surface areas, the ease with which the support can be activated or derivatized to produce the required density of enzyme binding sites. Generally, the support should be highly macro-porous with pores sizes greater than 10 nm for accommodation of typical enzymes. Also the support should consist of very small particles to provide large specific surface area. [1]. Endeavours to this end have enabled the production of cryogels (from the Greek kryos - meaning frost or ice) in

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which polycrystals of frozen solvent act as porogens during gel formation [3, 4]. Typical monolithic cryogels are featured by interconnected systems of macropores and unique sponge-like morphology that can permit unhindered diffusion of solutes of practically any size [3]. Cryogel’s adequate osmotic, chemical and mechanical stabilities make them attractive matrices for biocatalysts and chromatography of large entities such as protein aggregates, membrane fragments and viruses. In general, the cryogels have found different applications in the medical, biotechnological and pharmaceutical areas [3, 5, 6]. Due to the often-conflicting requirements, there is no universally recommended support at present. This calls for more research focusing on developing suitable supports for some intended applications.

Recently macroporous poly(vinyl alcohol) (PVA) cryogels have been successfully used for chemical and physical immobilization of different enzymes such as papain, subtilisin, glucose oxidase, lipase and trypsin [7 - 11]. Also, Plieva and co-workers [5, 6, 12] have reported on the synthesis of hydrophilic macroporous polyacrylamide monolithic cryogels with epoxy functionality (Epoxy-MPAAGs). Generally, the PVA cryogels exhibit poor mechanical stability since they are non-spongy and have small pore size range of 0.1-1 µm; much less than the pore size of the Epoxy-MPAAGs cryogels (1-100 µm) [3, 6]. Apparently there is no report on the immobilization of trypsin onto Epoxy-MPAAGs cryogels. Consequently, in this work, trypsin coupling ability of macroporous epoxy-functionalised polyacrylamide monolithic cryogels is examined and the catalytic behaviour of the immobilized trypsin is investigated.

EXPERIMENTAL

Materials
Acrylamide (AAm) 99 % electrophoresis grade, \( N,N' \)-methylene-bis-acrylamide (MBAAm) 99 %, allyl glycidyl ether (AGE) 99% GC grade, ammonium persulphate (APS) 98 % and \( N,N,N',N' \)-tetramethylethylenediamine (TEMED) 99 %, Bovine serum albumin (BSA) ≥ 96 % electrophoresis grade, N-α-benzoyl-D, L-arginine-p-nitroanilide (BAPNA) ≥ 98 % and protein determination kit - [Reagent A, 1 ml of final pH 11.25 contained: Bicinchoninic acid (BCA) soln., sodium carbonate, sodium tartarate, sodium bicarbonate in 0.1 N sodium hydroxide. Reagent B: copper (II) sulphate pentahydrate 4 % (w/v)] were obtained from Sigma (St. Louis, USA). Bovine pancreatic trypsin (EC 3.4.21.4) salt free sample was from Novozenzymes Denmark. Ethylenediamine, glutaraldehyde (50 % v/v) and sodium borohydride were purchased from Merck (Darmstadt, Germany).

Equipment
For trypsin activity assays a computer assisted UV-Spectrophotometer, (Shimadzu UV-1650 PC) connected to a thermal control unit (Julabo 13) was used. A Watson-Marlow peristaltic pump (0-99 rpm) was used for flow-through activity assays.

Methods

Preparation of Monolithic Epoxy-functionalized Polyacrylamide (Epoxy-MPAAGs) Cryogels
Epoxy-MPAAGs monolithic cryogels (approx. 0.5 ml each) were synthesized at -12 °C by free radical polymerization of the co-monomers AAm, MBAAm and AGE using cryogelation technique as described by Plieva et al. [6]. In the reaction mixtures, total monomer concentrations of 5, 10 and 15 % (w/v) were used. The variation in monomer concentrations in the recipes afforded cryogels of varying pore sizes and structures. Cryogels’ preparation procedure and their characterization have been previously described in detail by the authors [13].

Immobilization of Trypsin onto the Monolithic Epoxy-MPAAGs Cryogels
24 monoliths were placed in a plastic bottle and washed with about 50 mL de-ionized water and then with about 70 mL of 0.2 M Na₂CO₃ soln. The monoliths were then soaked into about 70
mL of 0.5 M ethylenediamine (prepared by mixing 2.35 mL of ethylenediamine with 68 mL of 0.2 M aq. Na₂CO₃) and left overnight under gentle shaking at room temperature (22-24 °C). The monoliths were then washed 6 times with 20 mL portions of 0.1 M Na₃PO₄/HCl buffer, pH 7.2, under gentle shaking. The monoliths were then soaked into a 60 mL of 5 % v/v, glutaraldehyde soln. (prepared by mixing 6 mL of 50 % glutaraldehyde soln. with 54 mL of 0.1 M Na₃PO₄/HCl pH 7.2 buffer) and left under gentle shaking at room temperature overnight. The monoliths were then washed thoroughly with plenty of de-ionized water (8 portions, about 100 mL each) and then coupled with 0.2 g trypsin (50 mL solution of 4 mg trypsin/mL phosphate buffer) and left under gentle shaking for about 24 hrs at room temperature. The monoliths were then washed with de-ionized water (4 portions, 100 mL each) and then the Schiff base which is formed after coupling of trypsin with the aldehyde-activated monolith was reduced by several runs of freshly prepared 0.1 M NaBH₄ (in 0.1 M Na₂CO₃/HCl pH 9.2). The reduction was carried out either under flow-through conditions with the monoliths packed in glass columns or in stationary (batch) mode until a fairly persistent final colour was observed (after about 30 min). The monoliths were then washed with plenty (3 portions, about 100 mL each) of de-oxygenated de-ionized water and stored in refrigerator for further analyses.

**Determination of Protein Content by the Bicinchoninic Acid (BCA) Method**

For the determination of protein content, the BCA stock solution was prepared by mixing reagents A and B at a ratio of 50:1 (v/v) respectively. For the standard BSA curve, samples of diluted BSA solutions were obtained by successive dilutions of 1 mg/mL BSA soln. in 0.1 M Na₃PO₄/HCl pH 7.2 buffer. The trypsin samples of unknown concentrations taken before and after immobilization were diluted (dilution factor, n = 5) for the BCA analysis. Samples of unknown concentrations from three consecutive washings after immobilization were also taken for the BCA analysis. 10 µL aliquots from each of the samples or washings were immediately incubated at 38 °C into 200 µL BCA soln. for 25 min. A blank solution containing only the Na₃PO₄/HCl buffer was prepared. The analysis of each sample/washing was done in duplicate. UV absorbances were read against the blanks at 550 nm and an average value recorded. Thereafter, the amount of protein in the unknown samples was interpolated from the standard curve. The protein content of the respective preparation of cryogel immobilized trypsin was determined as the difference between the amount of protein in liquid before and after immobilization. The protein based immobilization yield was calculated as the percentage of the protein content of the cryogel immobilized trypsin preparation to the respective amount of trypsin utilized for immobilization.

**Determination of the Activity Based Immobilization Yield**

Fresh free trypsin solution (before immobilization) of approximately 4 mg/mL and residual free trypsin solution (after immobilization) from each of the immobilization preparation were diluted (n = 4) with the 0.1 M Na₃PO₄/HCl pH 7.2 buffer while 6.0 mg BAPNA were dissolved into 6.0 mL of 50 % aqueous ethanol. The reactant solutions were separately thermally equilibrated at 40 °C for about 25 minutes prior to reactions in thermally controlled cuvettes set at 40 °C for 3 min. The reaction mixture in the cuvette contained 0.28 mL BAPNA solution, 0.52 mL of 0.1 M Na₃PO₄/HCl pH 7.2 buffer and 0.2 mL of the diluted trypsin solution. Two blank solutions comprising of only the BAPNA solution and the 0.1 M Na₃PO₄/HCl buffer were also prepared. The absorbances were recorded at 400 nm using a Shimadzu UV-1650 PC spectrophotometer. Activity based immobilization yield (IY) was determined as the percentage of the activity units of the actually immobilized trypsin (AI) to the activity units for the fresh free trypsin utilised for the immobilization (TI),
i.e., \( IY = \frac{AI}{TI} \times 100\% \) where \( AI = TI - FI \) and \( FI \) is the activity units of the residual free trypsin after immobilization.

**Determination of the Activities of the Immobilized Trypsin**

The enzymatic activities of the immobilized trypsin were assessed under flow-through condition which involved passing the substrate, \( N-\alpha\)-benzoyl-D, L-arginine-\( p \)-nitroanilide (BAPNA) at pre-determined flow rates/residence times through the column of the cryogel-trypsin conjugates. The initial linear rate of hydrolysis of the substrate was used for the determination of trypsin activity.

**RESULTS AND DISCUSSION**

**Immobilization of Trypsin onto the Monolithic Epoxy-MPAAGs Cryogels**

The native Epoxy-MPAAGs cryogel monoliths produced were macroporous polymeric materials of interconnected pore structure (fig. 2), with pore size ranging from ~10 \( \mu \)m to 150 \( \mu \)m. They were white in colour but turned dark brown on treatment with glutaraldehyde and turned persistent pale-yellow at the end of the \( \text{NaBH}_4 \) reduction stage during the process of trypsin immobilization (fig. 3). The colour change could be attributed to covalent bond formation with each stage of trypsin immobilization reaction which could alter the cryogel’s chromophore structure and hence affect light absorptivity.

**Procedure for the Determination of the Activity of the Immobilized Trypsin**

Exactly 10 mg BAPNA was dissolved into 10 ml of 50 \% ethanol. The BAPNA solution was mixed with 0.3 M Tris-HCl/0.1 mM \( \text{CaCl}_2 \) pH 7.6 in a 1:2.5 ratio BAPNA to buffer respectively. The substrate BAPNA was pumped at pre-determined residence times (~0.1-8 min) through a column of the cryogel-trypsin conjugate that was equilibrated at 30 °C using a thermostated water-bath. For each residence time of choice, steady state condition (system equilibration) was ensured by allowing 5 minutes’ elution of BAPNA solution before taking samples for measurements. Thereafter the \( p \)-nitroaniline containing effluents were collected and diluted (in duplicates) with de-ionized water (\( n = 10 \)). Blank solutions from their counterpart native cryogels were similarly produced and diluted. The absorbances of the samples against their counterpart blanks were determined spectrophotometrically at 410 nm.
The enzyme trypsin was covalently attached onto the surface of the Epoxy-MPAAGs cryogels using ethylenediamine-glutaraldehyde spacer arm (reaction scheme in fig. 4). This Schiff base-glutaraldehyde covalent immobilization has been reported to be suitable for the immobilization of trypsin [14]. The reaction begins with a nucleophilic attack by amine groups of the ethylenediamine on epoxy groups of the cryogel monolith to form a stable secondary amine linkage. The amine-activated monolith then reacts with glutaraldehyde to form an aldehyde-activated monolith. Thereafter, coupling of protein (trypsin) with the aldehyde-activated monolith is done. After coupling of protein, the formed Schiff bases are reduced by adding sodium borohydride [15, 16]. Although the method involves many steps and hence time consuming, it results in a longer spacer placed between the support and ligand useful in avoiding steric hindrance effects with small ligands [17].
**Determination of Protein Contents by the Bicinchoninic Acid (BCA) Method**

The quantity of trypsin immobilized (protein content) by the Epoxy-MPAAGs of initial monomer concentrations 5, 10 and 15 % (w/v) and the respective immobilization yields were estimated by the standard BCA method. Figure 5 is a BSA standard curve for the determination of protein content. The graph shows linear variation of absorbance with concentration of standard BSA up to 1.0 mg/ml and forms the basis for the determination of protein content from absorbance measurements.

The results of protein contents and protein based immobilization yields are summarized in table 1. The results show that both the protein contents and the protein based immobilization yields of the cryogel-trypsin conjugates increased with increasing density of the monolithic cryogels – a consequence of increasing monomer concentration in the reaction mixtures. This increase in protein content and immobilization yield could be attributed to the apparent increase in specific surface area of the monolithic cryogels with the increase in the initial monomer concentration in the reaction mixtures. The variation of specific surface area of the cryogels associated with the increase in initial monomer concentration in the cryogelation system should lead to the variation in the number density of the available functional groups responsible for protein coupling, i.e., the interfacial epoxy functionalities. However due to technical difficulties, specific surface area of monolithic cryogels were not determined. The results in table 1 also show that the immobilization yield of trypsin onto the 5 % Epoxy-MPAAGs cryogels was about 20 %. This means that about 80 % of the initial amount of trypsin taken for immobilization was not immobilized; indicating that the initial amount of trypsin exceeded the coupling capacity of the cryogels. In the same way, about 60 and 50 % of the initial amount of trypsin were not immobilized onto the 10 and 15 % Epoxy-MPAAGs cryogels respectively. This suggests that the amount of free trypsin used for immobilization tests i.e., 0.2 g trypsin (50 mL solution of 4 mg trypsin/mL) was so high that the monoliths were saturated with the enzyme leading to low percentage immobilization yields. The percentage immobilization yields of the cryogels could therefore be improved by operating with relatively less amount of free trypsin in the immobilization recipes.
Fig. 5: Bovine Serum Albumin (BSA) Standard Curve for the Determination of Protein Contents by the Bicinchoninic Acid (BCA) Method

Table 1: Respective Protein Contents in the 5, 10 & 15 % Monolithic Cryogels as Deduced from the Bicinchoninic Acid (BCA) Method. (Protein Content Expressed as Milligrams of Protein Present in a Single Monolith of 0.5 ml and per Weight (g) of the Monolith)

<table>
<thead>
<tr>
<th>Cryogels’ Initial Total Monomer Concentration (%)</th>
<th>Protein Content per Single Monolith (mg/monolith)</th>
<th>Protein Content per Weight of Monolith (mg/g-monolith)</th>
<th>Immobilization Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.7</td>
<td>39</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>2.0</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>4.0</td>
<td>73</td>
<td>48</td>
</tr>
</tbody>
</table>

The values of protein content in mg/g-carrier obtained in this study are much higher than those reported for trypsin immobilized by polyvinyl alcohol cryogels (PVAC) activated by various dialdehydes (including glutaraldehyde as in our study) at comparable pH and temperature conditions [18]. The Epoxy-MPAAGs cryogels attached more trypsin (i.e., 39, 51 and 73-mg/g, for the 5 %, 10 % and 15 % Epoxy-MPAAGs cryogels, respectively) compared to the PVC cryogels (i.e., ~ 7 mg/g although obtained at a relatively low trypsin coupling concentration [18]). In this work ethylenediamine was chemically coupled between the surface groups of the cryogel and glutaraldehyde, resulting in a longer spacer arm that might have contributed to the easy accessibility and attachment of trypsin macromolecules. Moreover, the Epoxy-MPAAGs cryogels having multiple polar functionalities could be more hydrophilic than polyvinyl alcohol thus providing for a relatively more favourable condition for enzyme trypsin immobilization [19].

The value of protein content obtained in this work for trypsin immobilised onto the 15 % Epoxy-MPAAGs cryogels, i.e., 73 mg/g-monolith, is close to that reported elsewhere [20] for the immobilization of trypsin onto glutaraldehyde-activated magnetic nanoparticles at pH 8.3 and room temperature (82 mg/g-support). For the covalently immobilized trypsin by epoxy-monolithic silica at fairly different conditions, Calleri et al. [21] reported enzyme loading of 66.07 mg/monolith at pH 8 (the mass of the monolith was not disclosed). For the silica based immobilization of trypsin, Calleri et al. [21] maintained that improved immobilization yield could be achieved through optimization of coupling conditions, e.g., through increasing initial
trypsin concentration (for instance up to 10 mg/mL). The significant influences of the initial trypsin concentration and initial coupling pH on the amount of trypsin immobilized have been reported elsewhere [22]. It has also been reported that free bovine trypsin at neutral or slightly alkaline pH is not stable when temperature is higher than 40 °C due to autodigestion [23]. For that reason the coupling conditions engaged in this work (i.e., pH 7.2 at room temperature, 22-24 °C) stand to be more favourable. Nevertheless it is reported that because they are relatively highly macroporous, monolithic cryogels possess much lower surface areas compared to other chromatographic supports and in turn can result in small amounts of immobilized ligands [17], i.e., the available surface area is the major reason that the enzyme loading on cryogels is lower than what is found for other supports. Nevertheless, the our results are still very reasonable taking into account the small amounts of enzyme and the safe, moderate operating temperature conditions (22- 24 °C) employed for enzyme coupling in this work.

**Determination of the Activity Based Immobilization Yield**

Presented in figures 6 and 7 are graphs for the initial rates of hydrolysis of BAPNA for both free trypsin before immobilization and residual free trypsin (after immobilization by the 10 and 15 % Epoxy-MPAAGs cryogels). The slopes of these linear graphs represent the activities of free trypsin before immobilization and those of residual free trypsin after immobilization. In these figures the differences in activities (slopes of the linear graphs) before and after immobilization are equivalent to the loss of activity of the initial trypsin due to coupling. This activity loss is considered as the limiting activity that can be recovered from the cryogel-trypsin conjugates. The results suggest that a maximum of about 60 % of the original free trypsin activity that disappeared during the immobilization process could be recovered in the 10 % Epoxy-MPAAGs cryogel-trypsin conjugates while for the 15 % Epoxy-MPAAGs cryogel-trypsin conjugates a maximum of about 70 % of the original free trypsin activity could be recovered from the immobilized enzyme. Generally these values for the activity based yields concur with the protein-based yields evaluated by the BCA method.

![Graph](image)

**Fig. 6:** Absorbance v/s Time Graphs: Estimation of Initial Rates of Hydrolysis of BAPNA for the Determination of the Activity Based Immobilization Yield for the 10 % Epoxy-MPAAGs Cryogel-Trypsin Conjugates.
Fig. 7: Absorbance v/s Time Graphs: Estimation of Initial Rates of Hydrolysis of BAPNA for the Determination of the Activity Based Immobilization Yield for the 15 % Epoxy-MPAAGs Cryogel-Trypsin Conjugates.

Activities of the immobilized trypsin
The initial linear rates BAPNA hydrolysis (determined from the rate of change of absorbance with time, (fig. 8) were used to deduce the respective activities of immobilized trypsin. The activities of the trypsin immobilized onto the respective 10 and 15 % (w/v) cryogels were compared under the same flow-through conditions. Flow-through method was applied in determining the activity of the immobilized cryogels by hydrolysis of BAPNA. Cryogels, being highly porous, do not practically retard the flow of the eluent when packed in a chromatographic column. For that matter it was possible to calibrate the pump to give known flow rates/residence times for which the pumped substrate can be in contact with the immobilized trypsin. This suggests that up-scaling of the trypsin-epoxycryogels conjugates prepared in this work for on-line industrial flow injection analyses should be practical.

Fig. 8 Initial Linear Rate of Hydrolysis of BAPNA by Trypsin Immobilized onto 10 % poly(Epoxy-acrylamide) Cryogel (Left) and 15% poly(Epoxy-acrylamide) Cryogel (Right).
The values of enzymatic activities of the cryogel-immobilised trypsin are shown in table 2. Apparently the trypsin immobilized onto the 15 % Epoxy-MPAAGs cryogels could not display higher activities than the trypsin immobilized onto the 10 % Epoxy-MPAAGs cryogels despite possessing higher protein content (4 mg/monolith\_0.5 mL) than the latter. The specific activity of the 10% cryogel-trypsin conjugate (280 U/g-protein) was about 50% that of the free trypsin used in this work, i.e. 550 U/g-protein. Also the specific activity of the 10% cryogel-trypsin conjugate was over 2 fold higher than that of the 15% cryogel-trypsin conjugates. Probably the trypsin immobilized onto the 15 % Epoxy-MPAAGs cryogels has been bound relatively strongly with multiple bonds leading to less active conformations of the enzyme. The apparent observed dependence of experimental values of specific activity with cryogels initial monomer concentration could be attributed to the effect of the variation of micropore structure on substrate diffusion to the active enzyme sites. However the substrate BAPNA is a fairly small molecule (molecular mass about 0.4 kDalton) to render its hydrolysis by the cryogel-trypsin conjugate diffusion controlled thus minimal diffusional limitation is expected.

**CONCLUSION**

The hydrophilic macroporous poly(epoxy-acrylamide) monolithic cryogels make effective carriers for bovine pancreatic trypsin. The immobilization procedure involving the inclusion of a long ethylenediamine-glutaraldehyde spacer arm provides for both excellent coupling environment and better recovery of activity. Enzyme loading of up to 70 mg per g cryogel and activity recovery of about 60 to 70 % were obtained for bovine pancreatic trypsin immobilized onto the macroporous poly(epoxy-acrylamide) monolithic cryogels. The influence of cryogels’ intricate pore structures on immobilization yield is evident. The results suggest that upon optimization, the Epoxy-MPAAGs-trypsin conjugates could be up-scaled for cost effective industrial bio-catalytic applications.

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**Table 2:** Respective Activities for the Immobilized Trypsin onto Cryogels of Various Initial Total Monomer Concentrations

<table>
<thead>
<tr>
<th>Initial Monomer Concentration (% w/v)</th>
<th>Condition of Hydrolysis of BAPNA</th>
<th>Activity (U) x 10(^{-3})</th>
<th>Specific Activity (U/g-monolith)</th>
<th>Specific Activity (U/g-protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Flow-through</td>
<td>543</td>
<td>14.3</td>
<td>280</td>
</tr>
<tr>
<td>15</td>
<td>Flow-through</td>
<td>464</td>
<td>8.4</td>
<td>116</td>
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