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Research Article

Development and Characterization of PEGylated Chromatographic Monoliths as a Novel Platform for the Separation of PEGylated RNase A Isomers

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PEGylated or polyethylene glycol-modified proteins have been used as therapeutic agents in different diseases. However, the major drawback in their procurement is the purification process to separate unreacted proteins and the PEGylated species. Several efforts have been done to separate PEGylation reactions by chromatography using different stationary phases and modified supports. In this context, this study presents the use of chromatographic monoliths modified with polyethylene glycol (PEG) to separate PEGylated Ribonuclease A (RNase A). To do this, Convective Interaction Media (CIM) Ethylenediamine (EDA) monolithic disks were PEGylated using three PEG molecular weights (1, 10, and 20 kDa). The PEGylated monoliths were used to separate PEGylated RNase A modified, as well, with three PEG molecular weights (5, 20, and 40 kDa) by hydrophobic interaction chromatography. Performance results showed that Bovine Serum Albumin (BSA) can bind to PEGylated monoliths and the amount of bound BSA increases when ammonium sulfate concentration and flow rate increase. Furthermore, when PEGylated RNase A was loaded into the PEGylated monoliths, PEG–PEG interactions predominated in the separation of the different PEGylated species (i.e., mono and di-PEGylated). It was also observed that the molecular weight of grafted PEG chains to the monolith impacts strongly in the operation resolution. Interestingly, it was possible to separate, for the first time, isomers of 40 kDa PEGylated RNase A by hydrophobic interaction chromatography. This technology, based on PEGylated monoliths, represents a new methodology to efficiently separate proteins and PEGylated proteins. Besides, it could be used to separate other PEGylated molecules of biopharmaceutical or biotechnological interest.

1. Introduction

During the last decades, several proteins have been used to treat different diseases where their high biological activity and high specificity have been demonstrated. However, some of these kinds of proteins have unfavorable properties such as low solubility, instability, and rapid clearance by the human body [1]. In this context, PEGylation has been one of the most successful strategies to overcome these drawbacks [2]. PEGylation is the covalent attachment of an activated polyethylene glycol (PEG) molecule to a protein [3]. This modification forms a steric barrier against proteolytic enzymes or antibodies, increases thermal stability, reduces immune response, and also increases the molecular size of the protein that enhances the circulation and clearance times in the human body [4, 5]. The first commercial PEGylated protein, for human consumption, was launched 30 years ago and since then several PEGylated drugs have been approved by the FDA [2].

In a common PEGylation reaction, several conjugates with different PEG chains attached to them are obtained. With this, the reaction mixture contains unreacted protein, mono-PEGylated and di-PEGylated proteins (sometimes even poly-PEGylated conjugates), and unreacted PEG [4, 6]. However, the PEGylated species have different effectiveness and activity levels and, the desired conjugate is usually the mono-PEGylated protein, which presents a higher biological activity and the best pharmacokinetic properties. The di-PEGylated protein or those conjugates with higher PEGylation degrees are less biologically active mainly because the active site becomes hindered by the PEG chains [6, 7]. For this reason, it is highly desirable to separate the mono-PEGylated protein from the other species. Nonetheless, its separation is a hard issue and an interesting

engineering problem because the physical and chemical properties are similar to those of the undesired products. In this context, chromatographic methods have represented the most attractive and effective alternative for their separation since it can exploit the different properties altered during the PEGylation reaction [4].

The chromatographic methods that have been used to separate mono-PEGylated proteins are hydrophobic interaction (HIC), anion exchange (AEX), size exclusion (SEC), and cation exchange (CEX) chromatographies [4, 6, 8]. Our research group has been working extensively in the separation of PEGylated proteins. In 2009 Cisneros-Ruiz et al. [9] separated PEGylated from unPEGylated Ribonuclease A (RNase A) using activated CH sepharose 4B media; in 2014 Hernández–Martínez and Aguilar [6] used PEG-modified Sepharose 6B supports with 550, 2000, and 5000 g mol^{-1} PEGs to separate the same PEGylated enzyme finding that the PEG–PEG interactions between the PEGylated support and the PEGylated proteins promoted their separation; in 2016 Mayolo-Deloisa et al. [4] analyzed the separation of PEGylated RNase A, β-lactoglobulin, and lysozyme by HIC between conventional (toyopearl butyl 650C and butyl sepharose) and monolithic (CIM C4 A) supports, they found that monolithic supports are a suitable alternative to traditional chromatographic media for the separation of PEGylated proteins.

The last two mentioned works present a guideline for a new idea: to combine the advantages of a chromatographic PEGylated support with the features of a monolithic one: PEGylated monoliths. Chromatographic monoliths, considered 4th generation chromatographic materials, consist of a single piece of highly open porous material. Monoliths can be used in all chromatographic modes. This kind of chromatographic supports can be operated at high convective flow rates since they possess small micropores and mesopores that allow low backpressures at high eluent flow rates [10]. These advantages have been used to purify oligonucleotides, proteins, PEGylated proteins, and other biotechnological products [11]. Another crucial advantage of monoliths is their tunable chemical, physical, and biological properties since they can present different chemical groups that can be functionalized with an extensive range of molecules to create supports with novel ligands [12]. In their work, Almedia et al. [13] modified monoliths with two ligands (lysine and cadaverine) for the recuperation of minicircle DNA. In this work, monoliths with ethylenediamine (EDA) groups were PEGylated using different PEG molecular weights. The PEGylated monoliths were used to separate PEGylated RNase A, a therapeutic protein with anticancer properties [14]. With this, it is possible to establish the basis of a new efficient platform to separate different PEGylated proteins for multiple applications.

2. Materials and Methods

2.1. Materials. Ribonuclease A, bovine serum albumin (BSA), sodium cyanoborohydride, hydrochloride acid, and Tris(hydroxymethyl)-aminomethane were bought from Sigma-Aldrich (St. Louis, MO, USA). Ammonium sulfate, dibasic potassium phosphate (K_2HPO_4), and monobasic potassium phosphate (KH_2PO_4) were purchased from J. T. Baker (Center Valley, PA, USA). Methoxy poly(ethylene glycol) propionaldehyde of nominal molecular masses of 1.0, 10.0, and 20.0 kDa came from JenKem Technologies (Allen, TX). CIM EDA disk monoliths were purchased from BIA Separations (Ajdovščina, Slovenia). Methanol of HPLC grade was purchased from Honeywell Burdick and Jackson (Morris Plains, NJ, USA). All other chemicals used were all at least of analytical grade.

2.2. Chromatographic Monolith PEGylation. CIM EDA disks (column volume, CV 0.34 mL) were PEGylated using three PEG molecular weights (1.0, 10.0, and 20.0 kDa) through reductive amination. To modify the monoliths, a syringe pump (Fusion 200, Chemyx Inc.) system was employed. The monoliths were washed with 10.0 mL of MilliQ (MQ) water at 1.0 mL/min, then equilibrated with 5.0 mL of a methanol solution at 10.0%, 20.0%, 50.0%, 75.0%, and 100.0% v/v at pH 6.0 using the same flow rate. Afterwards, 20.0 mL of methanol 100.0% v/v at pH 6.0 with 250.0 mM sodium cyanoborohydride and methoxy poly(ethylene glycol) propionaldehyde (mPEG) using a molar ratio 5 : 1 (amine : aldehyde active sites) [15] were passed through the disk at 0.1 mL/min during 24 hours. The monoliths were then washed with 5.0 mL of methanol pH 6.0 solutions at 100.0%, 75.0%, 50.0%, 20.0%, and 10.0 % v/v, 5.0 mL of water and 5.0 mL of ethanol 20.0% v/v at 1.0 mL/min consecutively. The experiments were carried out at room temperature, and the PEGylated monoliths were stored at 4°C until their use.

2.3. Monolith PEGylation Yield Calculation. The yield (or extent) of monolith PEGylation was determined by measuring free amine groups on the support using the same syringe pump system as mentioned before. Free amine groups were quantified using the method described by Noel et al., adapted to monoliths [16]. Briefly, the PEGylated monoliths were washed with 5.0 mL of MQ water and conditioned with 5.0 mL of MQ water at pH 3.0 at 1.0 mL/min. After this, 5.0 mL of a 50.0 mM orange II solution prepared with MQ water at pH 3.0 was pumped through the monoliths at 0.16 mL/min submerged in a water bath at 40°C. Then, the modified monoliths were air-dried. The unbound orange II molecules were removed, 25.0 mL of MQ water at pH 3.0 were pumped using a 1.0 mL/min flowrate. The bonded molecules were eluted using 90.0 mL of MQ water at pH 12.0 at 2.0 mL/min, collecting the fraction volume. The PEGylated monoliths were washed with 5.0 mL of MQ water and with an ethanol solution at 20.0% v/v. The pH of the collected volume was adjusted by the addition of 250.0 μL of 1.0 M HCl and the absorbance at 484 nm was measured using a microplate spectrophotometer (Biotek, VT, USA). The orange II concentration was determined with a calibration curve previously prepared (ε = 22.19 mM^{-1} cm^{-1}). The molar modification percentage was calculated according to the following equation:

$$\%\text{Modification} = \frac{\text{Total amino ligands } (\mu\text{mol}) - \text{Free amino ligands } (\mu\text{mol})}{\text{Total amino ligands } (\mu\text{mol})} * 100. \quad (1)$$

2.4. Adsorption Experiments. Because of the nature of the monolithic supports, the adsorption experiments on the PEGylated monoliths were performed only in dynamic mode. To do this, BSA was used as a protein model as used and preferred in similar experiments reported elsewhere [6, 17]. The breakthrough curves were obtained connecting the PEGylated monoliths to an Äkta Avant chromatography system (GE Healthcare, Uppsala, Sweden) and equilibrated with buffer B composed by 20.0 mM Tris–HCl pH 8.2 with 1.5 or 2.0 M of ammonium sulfate (AS). Protein solutions at different concentrations (1.0, 2.0, and 3.0 mg BSA/mL prepared en buffer B with 1.5 or 2.0 M AS) were loaded using a 50.0 mL superloop (GE Healthcare, Uppsala, Sweden) until the monoliths were saturated. The protein bonded was eluted with 20.0 mM Tris–HCl pH 8.2 buffer. The dynamic binding capacity (DBC) was obtained from the breakthrough curves for each PEGylated monolith as the amount of protein bonded per column volume when the outlet protein concentration reached 10% of the feed protein concentration. The 10% breakthrough ($DBC_{10\%}$) was calculated using the following equation:

$$DBC_{10\%} = \frac{1}{V_C}\int_0^{v_{10}} (C_F - C_{out})dv, \quad (2)$$

where V_C is the column volume in mL, V_{10} is the volume in mL applied at 10% breakthrough, C_F is the feed protein concentration in mg/mL and C_{out} is the outlet protein concentration in mg/mL. All experiments were carried out by triplicate and, the presented values correspond to the average of these results with their corresponding standard errors.

2.5. PEGylated RNase A Preparation. The PEGylation reactions were made according to the methodology reported by Mayolo-Deloisa et al. [4]. Briefly, 5.5 mL of an RNase A solution at a concentration of 3.0 mg/mL in a 100.0 mM sodium phosphate buffer at pH 5.1 + 100.0 mM of $NaBH_3CN$ were added to a vial containing 82.5 mg of mPEG with molecular weights of 5.0, 20.0, and 40.0 kDa. The reaction mixtures were stirred for 17 hours at 4°C, stopped by freezing at −20°C, and stored at this condition until their use.

2.6. Purification of PEGylated RNase A Conjugates by SEC. To obtain the PEGylated protein standards, the previously prepared PEGylation reactions were separated by SEC using an Äkta Prime Plus chromatography system (GE Healthcare, Uppsala, Sweden) equipped with a 5 mL injection loop, and an XK 2670 column packed with Superose 12 (70.0 × 2.6 cm, CV 320.0 mL). The mobile phase, a 10.0 mM sodium phosphate buffer at pH 7.2 + 150.0 mM potassium chloride was used to perform the isocratic elution at 1.0 mL/min. The fractions that presented absorbance at 280 nm were collected and concentrated using an Amicon chamber with a Diaflo ultrafiltration membrane of 3.0 kDa (Amicon Inc, MA, USA). The identity of each of the PEGylated and unreacted species has been previously confirmed and reported. Finally, the PEGylated protein was lyophilized and stored at −4°C [6]. These lyophilized PEGylated proteins were used as standards to carry out separation studies and peak identification in the PEGylated monoliths assays.

2.7. Separation of PEGylated RNase A through PEGylated Monoliths. The PEGylated monoliths were connected to an Äkta Avant System, equipped with a 0.1 mL injection loop. The experiments were performed at room temperature, using a flow rate equal to 1.0 mL/min. Buffer A, composed of 20.0 mM Tris–HCl pH 8.2 and phase B formed by phase A with 3.0 M of AS (due to low hydrophobicity of RNase A) were used in hydrophobic interaction mode.

The chromatographic runs were done using the following program: equilibration with phase B (10.0 CVs), injection (0.1 mL of PEGylation reaction without dilution), washing with phase B (3.0 CVs) and then immediately a step gradient was employed, 50% phase B (20.0 CVs), 20% phase B (17.5 CVs) and 0.0% phase B (20.0 CVs). The column eluent was monitored at 215 nm and also conductivity was measured throughout each run. All experiments were carried out by triplicate under the same conditions to allow performance comparison.

2.8. SDS–PAGE Analysis. As it will be mentioned later, two partially resolved peaks were observed for the 40.0 kDa PEGylated RNase A purification with the 20.0 kDa PEGylated monolith. To assess their identity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method reported by Mejia-Manzano et al. [18] using a 12.0% (w/v) Mini-PROTEAN precast gel (BIO-RAD, CA, USA). The desalted fractions containing the obtained peaks were mixed with 6x loading buffer and heated for 10 min at 99°C. The gel was silver-stained to detect proteins and a barium–iodine complex was used to visualize mPEG as it has been reported elsewhere [18].

2.9. A Combination of PEGylated Monoliths to Improve Separation. To improve the separation of PEGylated RNase A, two and three monoliths were placed consecutively into the Äkta Avant System in different orders. The use of consecutive monoliths is possible since the housing provided by BIA Separations can hold up to three monolithic disks. The PEGylated monolith configurations tried were: 10-20, 20-10, 1-10-20, and 20-10-1 kDa. The runs were performed using a flow rate of 2.0 mL/min and, the chromatographic program was the same as the previously described.

3. Results and Discussion

3.1. PEGylation Yield of Modified Monoliths. The separation and purification of PEGylated proteins has been a concern in different production schemes. In this work, monolithic disks were PEGylated through reductive amination to separate the different PEGylated RNase A species obtained after its conjugation reaction. Since EDA monoliths have primary amines and mPEG is activated with aldehyde groups, it is possible to graft the polymeric chains to the support using sodium cyanoborohydride as a reducing agent and methanol as protic solvent [19]. Besides, PEG is well soluble in methanol, even more than in water [20] and the solution viscosity is negligible at the PEG concentration used. The effectiveness of the PEGylation reaction on the monoliths for each PEG

Table 1: Molar modification percentage of EDA disk monoliths with PEG through reductive amination and dynamic binding capacity of the PEGylated monoliths.

PEG size (kDa)	Monolith molar modification (%)	Flow rate (mL/min)	$DBC_{10\%}$ (mg BSA/mL resin)			
			1		2	
			Ammonium sulfate (M)			
		BSA (mg/mL)	1.5	2	1.5	2
1	39.76	1	1.13 ± 0.05	2.67 ± 0.03	1.72 ± 0.05	3.21 ± 0.03
		2	1.68 ± 0.03	4.12 ± 0.14	2.71 ± 0.18	5.08 ± 0.05
		3	2.75 ± 0.02	5.09 ± 0.15	3.51 ± 0.18	6.85 ± 0.21
10	62.18	1	0.80 ± 0.02	2.72 ± 0.01	1.19 ± 0.04	2.92 ± 0.03
		2	1.33 ± 0.06	3.89 ± 0.04	1.92 ± 0.08	4.52 ± 0.04
		3	1.56 ± 0.03	5.07 ± 0.05	2.54 ± 0.07	5.75 ± 0.03
20	52.24	1	0.93 ± 0.01	3.18 ± 0.02	1.10 ± 0.02	3.02 ± 0.13
		2	1.90 ± 0.01	5.16 ± 0.01	2.20 ± 0.01	5.27 ± 0.03
		3	2.89 ± 0.05	6.26 ± 0.02	3.08 ± 0.19	6.88 ± 0.09

Data of $DBC_{10\%}$ presented represents the mean of three runs and their standard error.

molecular weight was determined indirectly by measuring the amount of unreacted primary amines using the orange II dye. This dye has the advantage to attach to primary amines via electrostatic charges under acidic conditions and can be released under alkaline conditions [16]. Table 1 shows the molar modification percentages of the PEGylated monoliths with different PEG sizes under the same experimental conditions. The amount of PEG molecules bound to the monoliths presents a maximum when using mPEG of 10.0 kDa and later decreases. This behavior shows the influence of PEG molecular weight in its linkage capability with the monolithic surface. At low molecular weights of mPEG (i.e., 1.0 kDa), the interactions between PEG chains and the amine groups on the monolithic surface are lower than those observed with the 10.0 kDa mPEG. On counterpart, at a high molecular weight of mPEG (i.e., 20.0 kDa), the polymeric chains attached to the support can probably change their structure to mushroom conformation [21] allowing steric effects and PEG–PEG interactions to hinder the contact between amine groups and free PEG chains thus avoiding the formation of new bonds between the polymer and the monolith. In this situation, the PEG–PEG interactions between the 20 kDa mPEG chains are more frequent due to its higher structural flexibility, allowing different spatial configurations that enhance the interactions between them [6]. Interestingly, the modification yield behavior obtained in this work differs from the data reported by Hernández–Martinez and Aguilar [6], where conclusions indicate that at higher PEG molecular weights, the modification percentage decreases on traditional resin supports. This different behavior could be associated with the different support structures. The high porous structure of monoliths allows better interactions between the mobile and stationary phases. Therefore, even using larger mPEG molecules (e.g., 10 kDa), the modification yield is not compromised. Lastly, since the modification percentage of PEGylated monoliths is molar, which means that the 20.0 kDa PEG chain has 20 times more glycol groups than the 1.0 kDa PEG chain, a difference in its chromatographic behavior is expected.

3.2. Adsorption Capacity of PEGylated Monoliths. A relevant parameter to determinate the performance of columns is their dynamic binding capacity (DBC). In this work, the DBC in HIC mode for each PEGylated monolith was determined using BSA as model protein under different operative conditions: flow rate, salt concentration, and feed protein concentration. Since the EDA monolithic disk is a weak anionic support [8], it was not possible to use it as a control in saturation curve experiments under the same conditions. The breakthrough curves, which show the rise of protein concentration in the effluent with time until this value reaches the same concentration of the feed [22], are presented in Figure 1. As seen, breakthrough curves present the same shape independently of the mPEG molecular weight attached to the monolith, this indicates that the protein adsorption process is the same in all three cases. However, at a high flow rate (i.e., 2.0 mL/min), the amount of BSA adsorbed by the PEGylated monoliths increases. This could be related to the transfer mechanism of the monolith: convective mass transfer. This behavior is opposite to that reported by Hernández–Martínez and Aguilar [6] since they used PEGylated Sepharose 6B, a porous bead material with a diffusion mass transfer.

On the other hand, when a 2.0 M concentration of AS was used, the PEGylated monoliths took more time to saturate than at the 1.5 M concentration, which means that the modified supports adsorb more BSA at higher AS concentrations. This behavior is observed because, at a high AS concentration, the hydrophobic regions of proteins become more exposed and promote more interactions with the grafted PEG chains in the monolith. When 1.5 M AS concentration is used, those hydrophobic regions are hindered even at high feed protein concentration, therefore a plateau-like curve is reached in a shorter time. Lastly, the polymer chain molecular weight attached to the monolith influences more the saturation times at 2.0 M SA. The hydrophobicity of PEG molecules increases with its molecular weight [23]. Therefore, at higher molecular weights, the hydrophobic interactions between the protein and the PEGylated monolith increase resulting in more proteins adsorbed into the support.

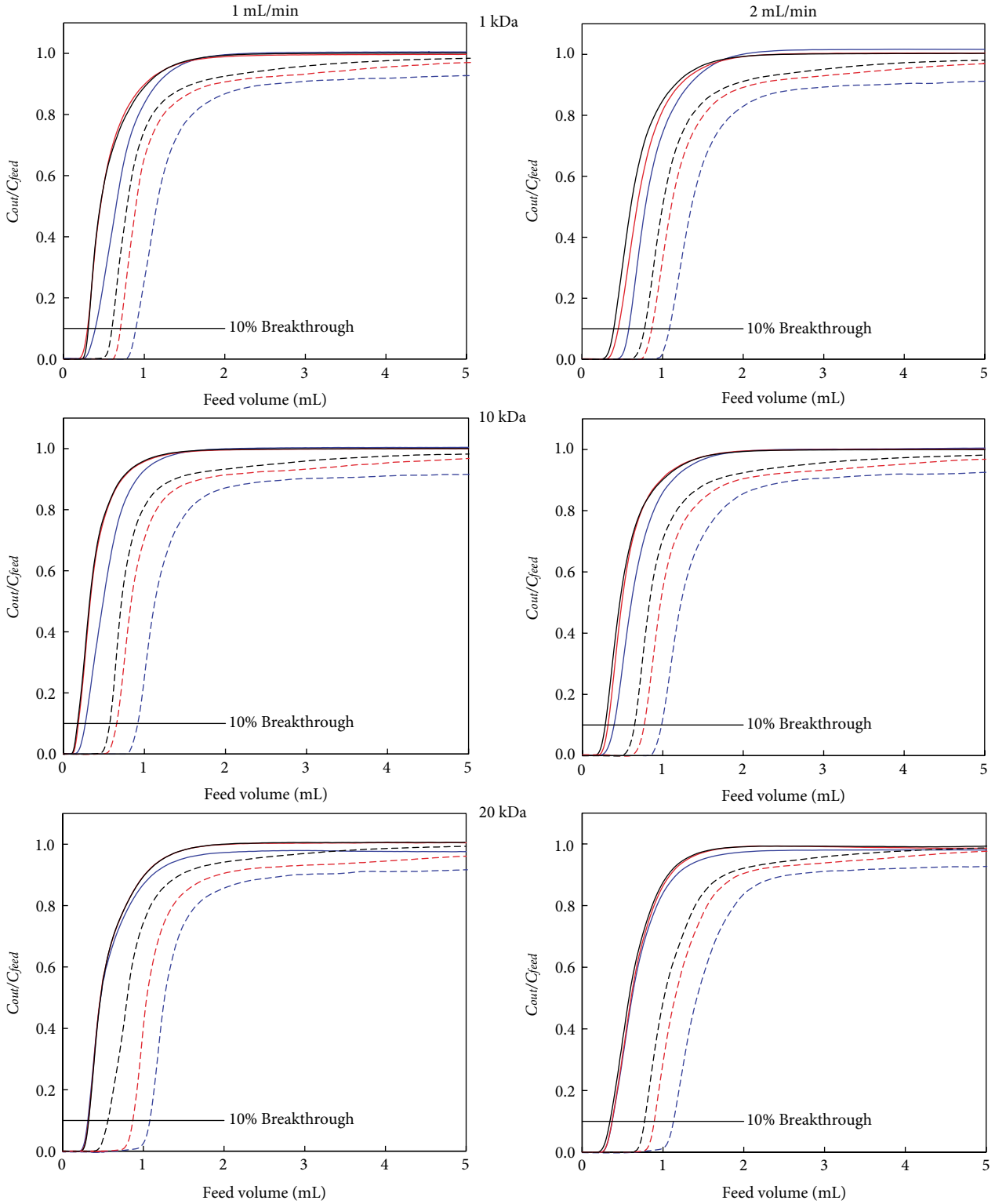

Figure 1: Breakthrough curves of PEGylated monoliths using BSA. The two ammonium sulfate concentrations used were 1.5 and 2.0 M and are represented by solid lines and dashed lines respectively. The color of the lines indicate the different BSA concentrations tested (blue lines, 1.0 mg/mL; red lines, 2.0 mg/mL and black lines, 3.0 mg/mL). Each column of graphs corresponds to each of the two Flow rates used (1.0 and 2.0 mL/min). All presented curves are the mean of three replicates.

In this same line, $DBC_{10\%}$ values for the PEGylated monoliths were determined (Table 1) for each one of the breakthrough curves shown in Figure 1. The $DBC_{10\%}$ of PEGylated monoliths increases with the rise of flow rate. This behavior is contrary to that reported by Hernánde–Martinez and Aguilar [6] due to the difference in mass transfer mechanism. The $DBC_{10\%}$ values in Table 1 are higher than those reported by Hernández–Martínez and Aguilar in their PEGylated Sepharose 6B. The convective mass transfer in monoliths plus the high available surface area from the monolithic structure and the hydrophobicity of PEG molecules allow better protein-support interactions and increase its adsorption capacity even at high flow rates. On the other hand, the higher DBC values were observed at high AS and fed protein concentration, which corresponds to the breakthrough curves behavior. Finally, the molecular weight of the PEG molecules grafted to the monolith showed a singular behavior. The PEGylated monolith with PEG of 10.0 kDa had the lowest DBC values while those modified with mPEG of 1.0 and 20.0 kDa showed, in general terms, similar values. This tendency could be attributed to the structural flexibility of the PEG chains since flexibility increases as the length does [24]. The PEG of 10.0 kDa attached to the monolith can modify its lineal structure into different spatial configurations. The different PEG configurations allow interactions hiding some hydrophobic regions that cannot bind to proteins or do so in a weaker way. A similar effect occurs with the 20.0 kDa PEG, but in spite of this phenomenon, its length allows adsorption of more proteins on its surface. For its part, the chain of 1.0 kDa PEG avoids other spatial configurations making its hydrophobic regions available for protein binding. The higher DBC values obtained were 6.85 ± 0.21 and 6.88 ± 0.09 mg/mL for the 1.0 kDa and 20.0 kDa PEGylated monoliths, respectively, at 2.0 mL/min, an AS concentration of 2.0 M and 3.0 mg/mL of BSA.

3.3. Capacity of PEGylated Monoliths to Separate PEGylated Proteins. The performance of the PEGylated monoliths to separate PEGylated proteins was evaluated using PEGylated RNase A. RNase A was conjugated with three PEG molecular weights to analyze the influence of the size of PEG chains attached to the protein in the separation of PEGylation reactions. As mentioned, DBC results indicated that at 2.0 mL/min the adsorption process has better performance; however, with PEGylated proteins, the amount of binding protein was lower at this particular flow rate (data not shown). Therefore, it was decided to use a volumetric flow rate of 1.0 mL/min in the subsequent experiments. Figure 2 shows the separation profiles of PEGylation reactions of RNase A. An important point to mention is that in previous reports, the PEGylation reaction is diluted in AS buffer before column injection [4, 6, 17] since proteins bind to HIC ligands at high salt concentrations and are eluted at low ones [25]. In this work, the reaction mixtures were not diluted in AS buffer before injection. Despite this, the PEGylated proteins were attached and separated using the PEGylated monoliths, showing that PEG–PEG interactions govern in this kind of chromatographic separation. In all cases, a small amount of

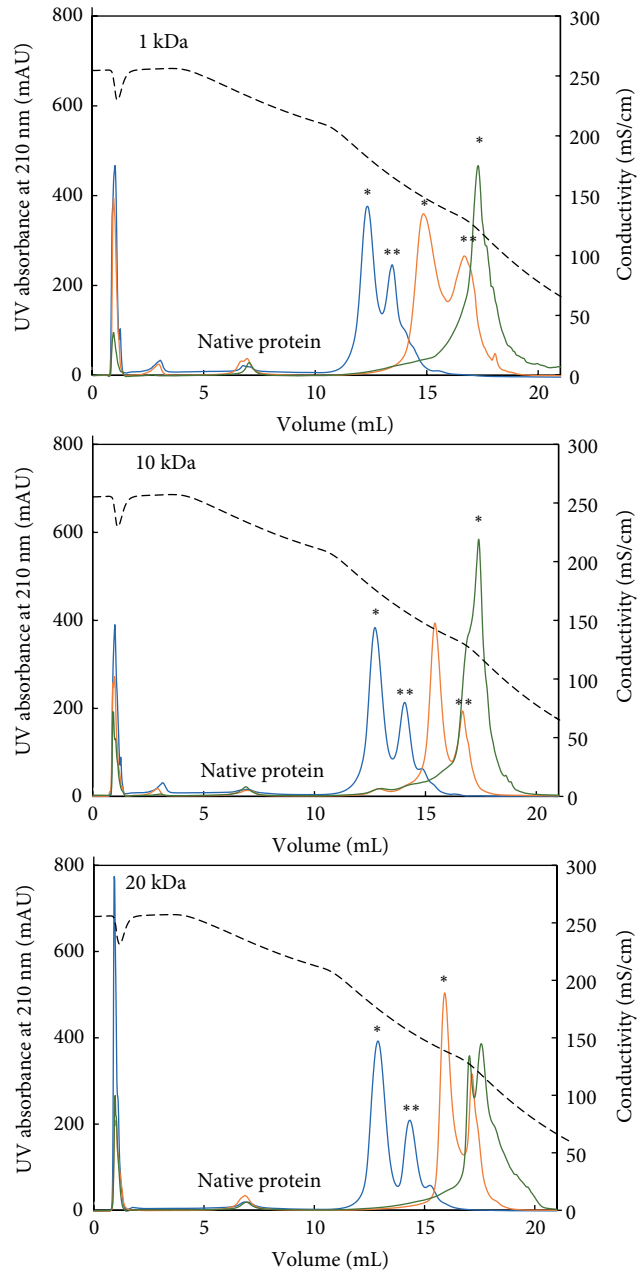

Figure 2: Separation of PEGylated Ribonuclease A using PEGylated monoliths. Chromatogram at 215 nm of Ribonuclease A PEGylation reactions with different PEG molecular weights 5.0 (blue line), 20.0 (orange line) and 40.0 kDa (green line) were separated using PEGylated monoliths modified with different PEG molecular weights 1.0, 10.0, and 20.0 kDa. Each experiment was carried out by triplicate. Conductivity was also measured and is presened in the chromatograms (black dashed line). *mono-PEGylated protein, **di-PEGylated protein.

the native protein binds weakly to the support, making its retention time different from that of the PEGylated species. On anion exchange chromatographic supports, protein PEGylation reduces their binding strength [26]. However, in PEGylated monoliths the effect is opposite because of the PEG–PEG interactions between the PEGylated protein

Table 2: Retention time and resolution factor (Rs) of PEGylated Ribonuclease A using PEGylated monoliths.

PEG MW in PEGylated monolith (kDa)	PEGylated Ribonuclease A								
	5 kDa			20 kDa			40 kDa		
	Retention time (mL)		Rs	Retention time (mL)		Rs	Retention time (mL)		Rs
	Mono-PEG	Di-PEG		Mono-PEG	Di-PEG		1st peak	2nd peak	
1	12.35	13.46	0.62 ± 0.03	14.95	16.7	0.94 ± 0.02	NA	NA	NA ± NA
10	12.83	14.19	0.95 ± 0.02	15.4	16.72	1.10 ± 0.04	NA	NA	NA ± NA
20	12.92	14.35	1.02 ± 0.01	15.94	17.17	1.04 ± 0.01	17.05	17.6	0.76 ± 0.01

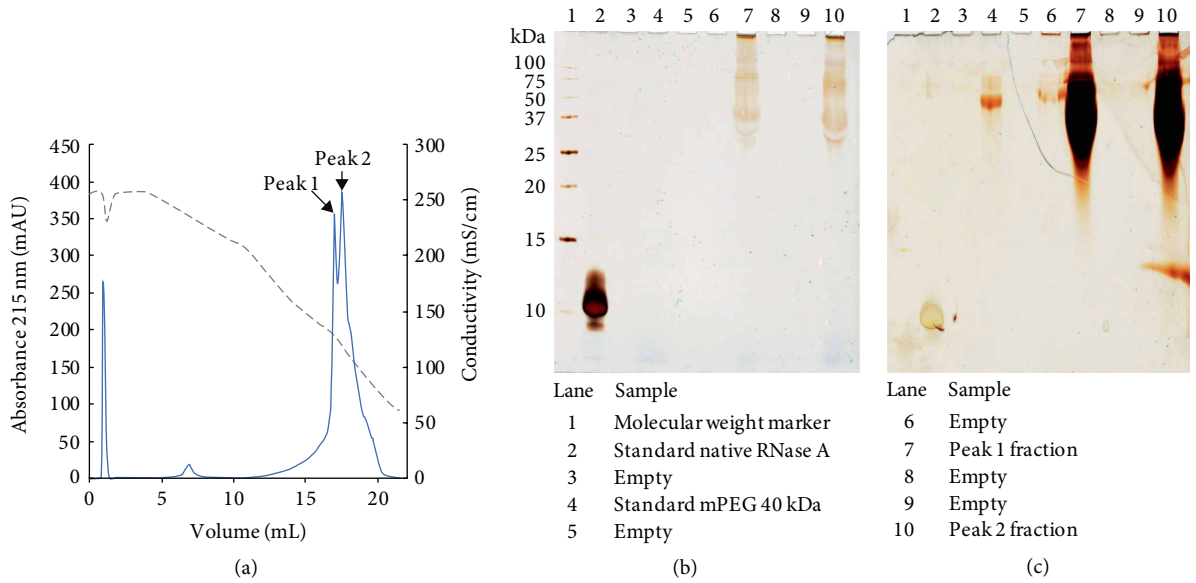

Figure 3: Separation and observation of 40.0 kDa PEGylated RNase A isomers using the 20.0 kDa PEGylated monolith. (a) Chromatogram at 215 nm of 40.0 kDa PEGylated RNase A in the 20.0 kDa PEGylated monolith using a step gradient elution. Buffer A: Tris–HCl 20.0 mM pH 8.2. Buffer B : Buffer A + 3.0 M ammonium sulphate. Loop 0.1 mL, Volumetric flow 1 mL min^{-1}. (b) Silver staining for protein detection of SDS–PAGE analysis of the chromatographic fractions. (c) I_2-$BaCl_2$ staining for mPEG detection of SDS–PAGE analysis of chromatographic fractions.

and the PEGylated monolith. In the separation process, the retention time (RT) of PEGylated RNase A depends strongly on the PEG molecular weight attached to the protein (Table 2), while the molecular weight of the PEG grafted to the monolith only shifts RT lightly. This behavior has an explanation in the fact that PEG molecules in the PEGylation mixture (i.e., free and protein-bound PEG) are more than the PEG molecules grafted to the monolith. Therefore, the strength of PEG–PEG interactions between the PEGylated species and the PEGylated monolith is very similar regardless of the PEG molecular weight attached to the support. On the other hand, when the molecular weight of the PEG attached to the protein increases, RT increases too (Table 2) since the amount of PEG–PEG interactions augments.

In terms of separation capacity, the resolution increases as the molecular weight of the polymer attached to the monolith increases (Figure 2 and Table 2). At high PEG molecular weights in the monolith, PEG–PEG interactions of the di-PEGylated species with the modified monolith are more extensive than the interactions of the mono-PEGylated species. Therefore, the di-PEGylated conjugate binds stronger in comparison with the other one, allowing a better separation between them.

Interestingly, when the 20.0 kDa PEGylated monolith was used to separate 40.0 kDa PEGylated RNase A, two peaks were obtained instead of one, which is usually expected (Figure 2). RNase A has a molecular weight of 13.68 kDa [27], therefore, the formation of a di-PEGylated conjugate is unlikely with a PEG of 40.0 kDa because of the produced steric hindrance. Once a PEG chain of this size is attached to the protein, it hinders other amino acid residues from reaction with other PEG molecules [1]. Hence, those two peaks could correspond to isoforms of the 40.0 kDa mono-PEGylated RNase A (i.e., conjugates with positional isomerism). To verify this point, silver and I_2-$BaCl_2$ staining of an SDS–PAGE gel of those peaks were analyzed. Figure 3 shows that those two peaks have the same profile and, due to their molecular weight, correspond to mono-PEGylated species and not to di-PEGylated species. The difference in retention times between the mono and the di-PEGylated species increases when the PEG MW attached to protein increases. For instance, with PEGylated RNase A of 5.0 and 20.0 kDa, the retention time difference changes from

Table 3: Retention time and resolution factor (Rs) of PEGylated Ribonuclease A using combined PEGylated monoliths in series.

PEGylated monoliths order (kDa)	PEGylated Ribonuclease A								
	5 kDa			20 kDa			40 kDa		
	Retention time (mL)		Rs	Retention time (mL)		Rs	Retention time (mL)		Rs
	Mono-PEG	Di-PEG		Mono-PEG	Di-PEG		1st peak	2nd peak	
10-20	13.41	14.79	1.06 ± 0.01	16.2	17.31	1.07 ± 0.02	17.30	17.73	0.67 ± 0.04
20-10	13.44	14.82	1.03 ± 0.01	16.22	17.33	0.97 ± 0.03	17.31	17.74	0.57 ± 0.05
1-10-20	13.76	15.08	1.06 ± 0.01	16.52	17.55	0.98 ± 0.04	17.53	17.97	0.52 ± 0.02
20-10-1	13.71	15.01	0.99 ± 0.01	16.46	17.53	0.85 ± 0.09	17.52	17.91	0.45 ± 0.00

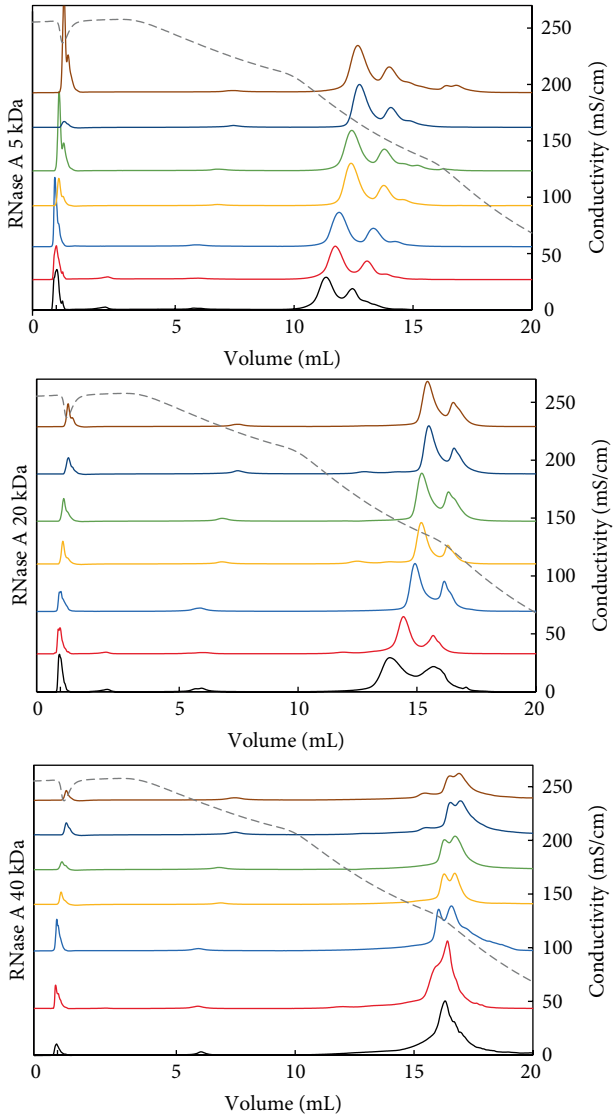

Figure 4: Comparison of PEGylated Ribonuclease A separation using different PEGylated monoliths series configurations. Ribonuclease A PEGylation reactions with PEG molecular weights of 5.0, 20.0, and 40.0 kDa were separated using PEGylated monoliths modified with PEGs of 1.0, 10.0, and 20.0 kDa in seven different configurations: 1.0 kDa (black line), 10.0 kDa (red line), 20.0 kDa (light blue line), 10.0-20.0 kDa (yellow line), 20.0-10.0 kDa (green line), 1.0-10.0-20.0 kDa (dark blue line), and 20.0-10.0-1.0 kDa (brown line). Each experiment was carried out by triplicate. Conductivity was also measured and is presented in the chromatograms (gray dashed line).

1.11 to 1.75 mL and, the retention time difference of both peaks in Figure 3 (PEGylated RNase A with 40 kDa) is only 0.55 mL. Therefore, it can be concluded that those peaks correspond to isomer species. Since proteins have several available reactive groups, the mPEG is probably binding to different residues on the protein [1]. The isomer separation of PEGylated proteins has been reported earlier using ion exchange chromatography [3, 8]. Nevertheless, to our knowledge, its separation by HIC has not been reported. PEGylation often results in a "charge-shielding effect" in which the PEG chains can reduce some of the electrostatic interactions between a PEGylated protein and a stationary phase due to steric hindrance [3]. In this work, a similar phenomenon occurs: "hydrophobicity-shielding effect" where depending on the PEGylation site, the hydrophobic interactions could change due to a masking effect of the hydrophobic or hydrophilic residues.

The separation of PEGylated RNase A from conjugation reactions has been studied previously. Mayolo-Deloisa et al. [4] separated PEGylated RNase A using a CIM C4 A monolithic disk, their best peak resolution was 1.16 and, in this work, the best resolution reached was of 1.1 ± 0.04. On the other hand, Hernández–Martínez and Aguilar [6] also achieved the separation of PEGylated RNase A conjugates using PEGylated sepharose 6B. However, their run-time and work volumes were at least two times larger than the used in this work.

3.4. Improving the PEGylated RNase A Separation. To achieve a better the separation between PEGylated species, two and three, of the previously described PEGylated monoliths, were placed serially in different configurations into the chromatographic system. Four combinations (10-20, 20-10, 1-10-20, and 20-10-1 kDa) were tested. Table 3 shows the peak resolution and retention times obtained for the different PEGylated species. The use of 2 or 3 sequential PEGylated monoliths allows the use of faster volumetric flow rates without affecting the retention time and resolution. Two flow rates (1.0 and 2.0 mL/min) were tested and, since this factor did not affect the chromatographic profiles (data not shown), a flow rate of 2.0 mL/min was used in the following experiments. In all cases, the use of more than one PEGylated monolith increased the retention time of the PEGylated species and this parameter was not affected by the order in which the disks were placed (Figure 4). When more than one modified support is used, the interactions between PEGylated proteins and the PEGylated supports are larger than those using a single disk.

Using more than one PEGylated monolith, the peak resolution was improved slightly only for the 5.0 kDa PEG-RNase A reaction. This is attributed to the nonuniformity of the PEG chain lengths due to the use of PEGylated monoliths with different PEG molecular weights. For the case of 20.0 and 40.0 kDa PEG-RNase A conjugates, the strength of the PEG–PEG interactions is not the same through the support and therefore, the proteins are eluted at different times inducing broader peaks and diminishing their resolution as it can be observed in Table 3 and Figure 4. Moreover, when the PEGylated monoliths are placed in decreasing PEG molecular weight order, the peak resolution falls. This phenomenon occurs because when the PEGylated protein is released from the 20.0 kDa PEGylated monolith, the salt concentration is not enough to bind the PEGylated protein to the 10.0 kDa PEGylated monolith and therefore, the PEG-RNase A species passes through and the peak resolution decreases.

These last experiments evidence the capability of using two or more monoliths at higher flow rates without observable changes in the separation behavior. In our particular case, to enhance the separation between the PEGylated RNase A species or other PEGylated proteins of biotechnological interest, it is necessary to use bigger PEGylated monoliths with the same PEG molecular weight.

4. Conclusions

As has been mentioned, the separation and purification of PEGylated proteins is a critical step in many production processes. For this reason, different chromatographic supports have been tested with varying results. In this work, a novel type of support was created and studied: PEGylated monoliths. Due to the chemistry of EDA disks, it was possible to PEGylate them using mPEG propionaldehyde via reductive amination showing that its modification degree depends on the length (molecular weight) of the mPEG to be attached. This reaction could be extrapolated to bigger monoliths with the same reactive groups to increase their workflow and processing capacity.

PEGylated monoliths are a suitable option to separate different proteins through HIC because: (1) it is possible to use them at high flow rates since their dynamic binding capacity does not decrease; (2) these modified supports take advantage of the hydrophobic PEG–PEG interactions between the PEGylated protein and the PEGylated monolith that predominate in the separation process; (3) sample preparation (dilution in a suitable buffer) is not necessary before injection, which means that it is possible to process larger sample volumes in less time and; (4) it is possible to separate PEGylated protein isoforms when long PEG chains are attached to both the monolith and the protein. On the other hand, in terms of resolution capacity, PEGylated monoliths with large PEG molecular weights allow a better separation of the PEGylated species.

This strategy based on PEGylated monoliths has a great potential to separate not only proteins or PEGylated proteins but also other PEGylated molecules of biopharmaceutical or biotechnological interest. Therefore, this represents a research opportunity area that requires the optimization of the monolith support PEGylation reactions to achieve better resolution capacities and to extrapolate its use to other molecules. In this way, a new and more efficient platform to separate and purify PEGylated proteins and other PEG conjugates can be established.

Data Availability

The experimental data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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