

Chromatographic matrix based on hydrogel-coated reticulated polyurethane foams, prepared by gamma irradiation



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ABSTRACT

Novel chromatographic materials for protein purification with high adsorption capacity and fouling resistance are highly demanded to improve downstream processes. Here, we describe a novel adsorptive material based on reticulated polyurethane foam (rPUF) coated with a functional hydrogel layer. rPUF provides physical rigidity through its macroscopic structure, whereas the hydrogel layer provides capacity to adsorb proteins by specific interactions. The hydrogel coating process was performed by the dip-coating method, using a polyvinyl alcohol (PVA) solution. The PVA hydrogel was linked to the rPUF material by using a radiation-induced crosslinking process in aqueous ethanol solution. The ethanol in the solvent mixture allowed a balance between PVA swelling and PVA dissolution during the irradiation step. The resulting material showed higher thermal stability than the non-irradiated one. In addition, a simultaneous radiation-induced grafting polymerization (SRIGP) was done by simple addition of glycidyl methacrylate monomer into the irradiation solution. In a further step, sulfonic ligands were included specifically in the hydrogel layer, which contained around 200% of PVA respect to the original rPUF. Materials were characterized by FT-IR, thermogravimetric analysis, SEM microscopy and EDX analysis. The cation-exchange rPUF material was functionally characterized by the Langmuir isotherm and a dynamic adsorption experiment to analyze the chromatographic properties for protein purification processes.

1. Introduction

Modern bioprocesses depend heavily on the availability of materials that allow obtaining competitive products in terms of quality and costs. Historically, hydrogels have been very important materials in the field of biochemistry. Chromatographic matrices and electrophoresis gels are currently the most successful materials used in protein analysis and purification. In addition, hydrogels have expanded their applications into various biological areas, such as contact lens materials, orthopedic applications, and devices for controlled release of drugs (Hyon et al., 1994; Hoffman et al., 2002; Casolaro et al., 2006; Bae et al., 2006).

Different types of hydrogels can be prepared from natural polymers, as well as from semisynthetic and synthetic polymers, most of which are obtained by crosslinking of their aqueous solutions. As an example, hydrogels have been obtained from polysaccharides and polyacrylamides (Porath and Flodin, 1959; Hjertén and Mosbach, 1962) and chemical crosslinking has been performed to improve their mechanical properties, which are still the main limitation.

From the early days of the radiation processing, radiation-induced crosslinking has been identified as useful to prepare hydrogels (Alexander and Charlesby, 1957; Danno, 1958). At well-defined irradiation doses, PVA hydrogels can be obtained from a PVA solution (Peppas and Merrill, 1976). To introduce some functionality, PVA hydrogels have been grafted by radiation methods with different ligands to prepare adsorptive materials to recover different ions. An example of this is the application of hydrogels in decontamination (Güven et al., 1999).

In the last two decades, one-piece porous polymeric materials (also called monoliths) have been applied to separate macromolecules (Krajnc et al., 2005). These materials add two advantages respect to hydrogels: (i) much higher mechanical resistance and (ii) an internal structure of interconnected channels which facilitate mass transport. Therefore, monolithic columns with these unique structures allow high flow rates at low pressures without losing column efficiency; as a result, a quick separation is achieved (Cabrera et al., 2000).

Porous monolithic materials can be prepared by means of a wide

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variety of techniques (reviewed by Svec (2010)), including the use of radiation-induced polymerization (Grasselli et al., 2001). As mentioned above, solid porous monoliths have good mechanical and hydrodynamic properties. However, they exhibit a very low adsorption capacity. This is associated with the fact that chromatographic ligands are linked to a low specific surface area of these non-hydrogel-based materials.

One particular case is the porous monolithic material called cryogel. This material is prepared by a polymerization reaction of water soluble monomers in freezing state, where the pore structure is obtained by the phase separation of water in frozen state during the polymerization process (Mattiasson et al., 2010). Therefore, the cryogel material is formed by a hydrophilic crosslinked polymer; however, this material has also low adsorption capacity for protein purification (Mattiasson et al., 2010). A recent publication has described an improvement of the adsorptive capacity of this material by simultaneous radiation-induced grafting polymerization (SRIGP) (Bibi et al., 2011). This improvement is due to the grafting modification that occurs in the bulk of the cryogel material. SRIGP is a modification method based on ionizing radiations, which generate radicals onto the base polymer that are responsible for chemical reactions. In this way, the grafting polymerization process occurs in the presence of methacrylate monomers onto a base polymer.

Here, we describe a novel approach to prepare porous monolithic adsorptive materials, based on radiation-crosslinking and functionalization of hydrophilic coating onto reticulated polyurethane foams (rPUFs). These materials were analyzed for chromatographic applications to recover macromolecules such as proteins.

2. Materials and methods

2.1. Materials

rPUF from Eurofoam Deutschland GmbH was kindly donated by Prof. Marcelo Fernandez Lahore (Jacobs University). rPUF type Filtren TM 60, with a pore size of about 250 μm , was used as base material without purification. The material was cut into small cylinders of 0.7 cm in diameter and 2.8 cm in height.

PVA Mowiol 10–98, M.W. 61,000 (Sigma-Aldrich, catalog number 10852) hydrolysis grade 98–98.8%, Hydroxyethyl cellulose (HEC), M.W. 720,000 (Sigma-Aldrich code number 9004-62-0) and Agarose (Biodynamics, Molecular biology, low electroendosmosis grade) were used. Glycidyl methacrylate (GMA), dimethyl acrylamide (DMAA) and Lysozyme were from Sigma Chemical Co. (USA). Acetone, sodium sulphite anhydrous, isopropanol, ethanol, and other chemical reagents were purchased from Anedra (Buenos Aires, Argentina). All chemicals used were analytical grade.

2.2. Hydrogel coating preparation

The preparation of the material was divided in four main steps: (i) foam coating; (ii) crosslinking and (iii) grafting procedure and (iv) ligand functionalization reaction.

2.2.1. Foam coating

rPUF was coated with different water-soluble polymer solutions. Each polymer solution was prepared taking into account the maximum dissolution of each polymer in water. The concentrations of each polymer solution are listed in Table 1.

rPUF cylinders were coated with hydrophilic polymer solutions by the dip-immersion method. Pieces of the porous material were submerged in a selected polymer solution at a given temperature for ten seconds and further squeezed to remove the polymer in excess. Immediately, they were immersed in isopropanol at 85 °C for ten seconds, squeezed and then dried in an oven at 55 °C until constant weight. The coating degree (CD%) was calculated as the percentage of increase in dried weight, as follows (Eq. (1)):

Table 1
Polymer solutions and conditions of the coating process.

Polymer	Concentration (w/v)	pH	Temperature (°C)
Agarose	8	7	85
HEC (90 kDa)	1.4	7	R.T.
PVA (61 kDa)	10	7	85

$$CD\% = 100 \cdot \left[\frac{W_1 - W_0}{W_0} \right] \quad (1)$$

where W_0 and W_1 are the initial and final weights of the material, respectively.

2.2.2. Grafting/crosslinking process

Radiation-induced crosslinking and grafting polymerization was performed by irradiation of polymer samples with a gamma source. Coated rPUF materials, soaked in an irradiation solution, were sealed in a sample container. Then, 0.4 g of coated and dried material (10 cylinders) was soaked in 100 mL of ethanol:water 1:1 v:v. To perform simultaneous crosslinking and SRIGP, GMA or GMA and DMAA monomers were added to the irradiation solution. The following conditions were studied: 2%v/v, 4%v/v, and 6%v/v of GMA, 5%v/v of DMAA, 5%v/v of DMAA + 1%v/v of GMA, and 5%v/v of DMAA + 4% v/v of GMA. Solvents were previously degassed for 15 min by nitrogen gas bubbling.

The samples in the containers were irradiated in a ^{60}Co irradiation source (PISI semi-industrial source, Comisión Nacional de Energía Atómica, Ezeiza, Argentina) with a 10 kGy irradiation dose at a dose rate of 1 kGy h^{-1} (if not specified) and room temperature. Dosimetry was performed with Red Perspex type dosimeters. After irradiation, materials were washed with ethanol:water 1:1 v:v and subsequently with 96% ethanol several times until all the homopolymer trapped inside the porous structure was washed out. Materials were dried at 55 °C in an oven until constant weight. The grafting degree (GD%) was calculated as the percentage of increase in weight, as follows (Eq. (2)):

$$GD\% = 100 \cdot \left[\frac{W_2 - W_1}{W_1} \right] \quad (2)$$

where W_1 and W_2 are the weights of the coated and the grafted material, respectively.

2.2.3. Hydrogel functionalization

Strong cation-exchange ligand was immobilized onto the hydrogel based on the ring-opening reaction of the epoxy groups of polyGMA with sodium sulphite (Camperi et al., 1999). Then, 0.2 g of grafted rPUF was incubated with 20 mL of a mixture of sodium sulphite/isopropanol/water (10:15:75 w/w/w) at 37 °C, overnight under agitation.

After extensive washing with water, the remaining epoxy groups to diol groups were hydrolyzed by incubation with a H_2SO_4 solution 0.5 M at 80 °C for 2 h (Camperi et al., 1999). After extensive washing with water, the cation-exchange rPUF (SP-rPUFs) were dried at 55 °C in an oven overnight.

The sulfonic groups were quantified by Inductively Coupled Plasma Spectroscopy with Atomic Emission Spectroscopy (ICP-AES) (INCAPE-CENACA, Universidad Nacional de la Plata, La Plata, Buenos Aires, Argentina).

2.3. Material characterization

2.3.1. Swelling degree

Swelling experiments were performed by placing 0.04 g of the material in distilled water overnight. After the incubation, the material was removed from the water, quickly and carefully dried with a filter paper, and its weight gain measured. The swelling degree was

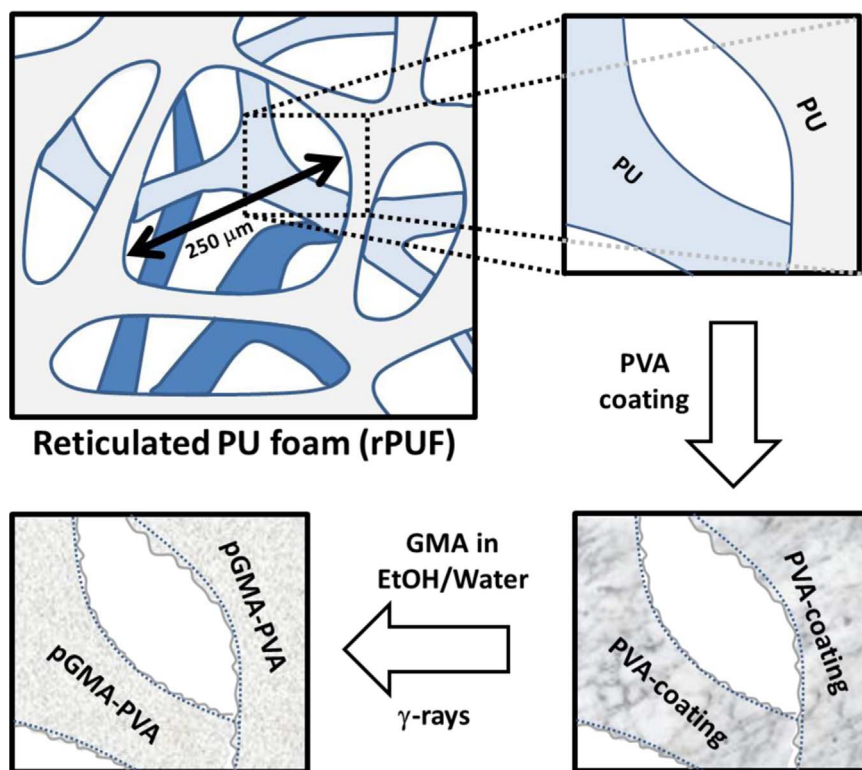


Fig. 1. (a) Scheme of the coating process onto reticulated-polyurethane foam (rPUF).

calculated as the percentage of solvent absorption per dry material weight after wetting the sample.

2.3.2. Scanning electron microscopy (SEM)

SEM pictures were captured with SEM-Carl Zeiss NTS-SUPRA40 (Centro de Microscopías Avanzadas, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina). All the samples were equilibrated in phosphate buffer with 3 M KCl, rinsed with distilled water, dried in an oven at 55 °C up to constant weight, and examined using SEM at different magnifications. The samples were no more than 5–8 mm thick. The samples were mounted on a circular metallic sample holder and coated with 20–50 nm thick of gold by sputtering before being analyzed.

2.3.3. Energy dispersive X-Ray (EDX) analysis

EDX spectra were captured with a microanalysis system Oxford Instruments INCA Energy 250 including a Si(Li) detector of 10 mm². The system is coupled to Carl Zeiss SMT Model Supra 40. Data were processed with INCA Energy and Thin Film ID of Oxford Instruments programs (Centro de Microscopías Avanzadas, UBA).

All the requirements mentioned for SEM are also valid for EDX analysis. However, for accurate quantitative analysis of the elements in the sample, the samples should be flat. For qualitative analysis, any shape of the sample is workable. Samples were coated with 20–50 nm thick of carbon.

2.3.4. Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR)

Dried samples were measured directly using an FTIR IR-Affinity instrument (Shimadzu Corporation, Japan), equipped with attenuated total reflectance accessory, GladiATR diamond single reflection (PIKE Technologies, USA). Spectra were acquired by scanning the specimens for 64 times in the wavenumber range from 4000 to 550 cm⁻¹ with a resolution of 4 cm⁻¹. ATR and atmosphere correction were applied for all spectra.

2.3.5. Thermal gravimetric analysis (TGA)

Thermal analyses were performed with TA Instruments Q500 thermobalance (TA Instruments, Delaware, USA) in rPUF samples. Approximately 5 mg of each sample was heated using a linear heating ramp (20 °C min⁻¹) from 30 °C up to 600 °C with a continuous N₂ flow at 60 mL min⁻¹ to guarantee an inert atmosphere. Weight loss was measured as a function of temperature, and derivative of weight change with respect to temperature (DTG) was calculated from the original signal. The temperature necessary to reach 5% of weight loss (T₉₅) was considered as the initial degradation point.

2.3.6. Protein adsorption equilibrium under static conditions

The adsorptive static capacity was determined in batch mode by a single experiment, where 0.16 g of modified material was equilibrated with 10 mL Phosphate Buffer 20 mM pH 7 (Buffer A) for 2 h under agitation. After equilibration, rPUFs were saturated with 10 mL of Lysozyme solution 2 mg mL⁻¹ in Buffer A for 3 h under agitation. The decrease in the optical density at 280 nm of the supernatants was recorded, and the equilibrium concentration and the amount of protein adsorbed to the material were calculated from absorbance measurements. A washing step with Buffer A was done. Desorption experiments were performed in a similar way by changing the elution Buffer A to Buffer A + 1 M NaCl (Buffer B).

2.3.7. Protein adsorption equilibrium under dynamic conditions

Protein adsorption and elution under dynamic conditions were performed using an ÄktaPrime (GE Healthcare Life Science). A column of 1 mL was prepared with 0.15 g of SP-rPUF. Before loading the column with the protein solution, it was equilibrated using Buffer A until the absorbance of the outlet stream had reached that of the inlet stream. Lysozyme 2 mg mL⁻¹ in Buffer A was pumped onto the column at a defined flow rate and the outlet absorbance at 280 nm measured. After loading the sample, the column was washed with 10 column volumes of Buffer A. The column was then eluted with Buffer B. The flow rate was 1420 cm/h for all the steps.

3. Results and discussion

In the last decade, the application of porous monolith materials to affinity chromatography has been expanded to analytical applications (Pfaunmiller et al., 2013). These materials have interconnected channels which facilitate mass transport and reduce the diffusion process. However, the inner surface of these materials is very low, which reduces their binding capacity and consequently the industrial applications. Therefore, the development of materials which can increase this property are welcome to expand the applications of these materials.

Here, we described the preparation of a novel protein adsorptive material based on a hydrogel-coated porous polymer. In particular, a commercial rPUF polymer was used as base material and a polymethacrylate-grafted PVA hydrogel was prepared in situ by radiation-induced process. Fig. 1 shows the scheme of the work proposed.

rPUF is a chemically inert industrial material with three-dimensional structure, excellent mechanical properties (high strength and elasticity), commercial availability and low cost. It has a high porosity (about 97%) and an open highly structured macrostructure (Fig. 1). Being an elastic material, the flexibility of rPUF also provides good stability and resistance to compression. However, rPUF is generally hydrophobic, with low compatibility with water soluble polymers.

On the other hand, as mentioned in the introduction section, hydrogels are important materials to prepare chromatographic matrices for protein purification and there are many very well established commercial products in this field.

Ionizing radiation is a powerful technique to prepare hydrogels in a single step without using initiators or crosslinkers, and reactions can be initiated at room temperature or sub-ambient temperature (Peppas and Merrill, 1976). Also, in the last decade, efforts have been made to study super-absorbent hydrogels by radiation-induced crosslinking (Ibrahim et al., 2007). One of the key factors for the success to prepare these hydrogels is the intermolecular radiation-induced crosslinking under low water concentration during the irradiation processes. As an example, PVA is degraded by ionizing radiation in dry state (Bhat et al., 2005) and may result in a cross-linked appearance when it is irradiated under a specific aqueous phase condition (Wang et al., 2000). Therefore, the irradiation conditions and sample preparation are critical issues to obtain the desired material. To our knowledge, water is the only solvent so far reported used to prepare PVA hydrogels by irradiation crosslinking methods of aqueous solutions of this polymer.

In addition to the crosslinking process, the radiation-induced grafting process can be used to functionalize the material. Grafting copolymerization methods are able to generate surface and/or bulk polymer modifications. There are several grafting techniques by applying radiation technology (Uyama et al., 1998). A comprehensive review has been published by Nasef and Güven (2012).

The SRIGP procedure has been long applied for the preparation of adsorptive membranes (Grasselli et al., 1999). It has been used to immobilize different kinds of ligands onto microfiltration membranes and applied to purify proteins by specific recognition. The following ligands have been used: iminodiacetic acid (Grasselli et al., 1999), sulfonic groups (Camperi et al., 1999), reactive dyes (Wolman et al., 2005), amino groups (Trelles et al., 2010) and arsenic ligands (Carbajal et al., 2009). In all these cases, the base materials were hydrophobic polymers such as polyethylene or polysulfone, the irradiation solvent was an ethanol/water mixture, and the main grafted monomer was GMA. Also, in all cases, the grafted polymethacrylate polymer was localized onto the surface of the base material.

In this work, we describe a similar procedure based on the SRIGP method applied onto a water-soluble polymer coated in hydrogel-state onto rPUF material. The epoxy reactive rings of the grafted polyGMA will be further used to introduce cation exchange properties to the material.

3.1. Preparation of the hydrogel-coated porous material

As mentioned in the Materials and Methods section, the preparation of the material was divided into different steps (Fig. 1): (i) physical coating of a water-soluble polymer on the open-foam polymer; (ii) crosslinking of both polymers; (iii) grafting of a reactive polymer; and (iv) specific chemical functionalization of the hydrogel. Steps (ii) and (iii) were done simultaneously.

3.1.1. Foam coating

The coating process was accomplished by the dip-coating technique by using rPUF material as base polymer. Three different hydrophilic polymer solutions were analyzed for the coating process: agarose, HEC and PVA.

Agarose was selected because it is the base polymer of the commercial chromatography matrices used for industrial protein purification. This polymer is a neutral polysaccharide which forms a highly hydrophilic hydrogel with a porous structure (Hjertén, 1964). HEC is a water-soluble polymer derived from cellulose. It is a nonionic polymer which has been used for membrane coating (Beeskow et al., 1995). Finally, PVA is a widely used synthetic hydrophilic polymer with excellent film-forming properties and good flexibility. In addition, it is water-soluble and inexpensive (Hallensleben and Hallensleben, 2000).

The coating process was done onto ten rPUF pieces (cylinder shaped) by immersion in solutions of the hydrophilic polymers as described in Section 2.2.1. After drying the material at 55 °C to constant weight, the coating degree (CD %) was determined and plotted in Fig. 2.

The coating process using agarose and HEC showed a CD% lower than 20%w/v and 50%w/v respectively, whereas that using PVA yielded the highest performance, with a CD% about 100%, two-fold higher than that obtained with the HEC coating.

The coating process with PVA was performed at 85 °C to reduce the viscosity of polymer solutions and improve the yield. However, some of the polymer leached during the drying process. To overcome this problem, a coagulation step was added before the drying step to increase the coating yield. Therefore, 2-propanol was selected to coagulate the coating layer of PVA, because it is well known that PVA has poor solubility in this solvent. In this way, after the immersion step in the polymer bath, samples were soaked in a solvent bath for 10 s and after its removal, they were dried in an oven at 55 °C.

We also studied the influence of a lag time between the immersion step in the polymer bath and that in the coagulation bath. For these experiments, the coating condition used was that corresponding to the best condition previously achieved (PVA 10%w/v, pH 7 at 85 °C). Each experimental condition was prepared using a set of ten cylindrical rPUF pieces. Fig. 3 shows the results of the CD% of samples prepared with different lag times. A lag time of less than 2 h was able to improve the coating process by around 20%.

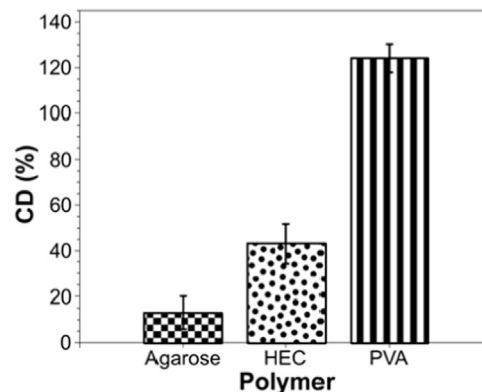


Fig. 2. Coating Degree (CD %) for the different water-soluble polymer formulations described in Table 1.

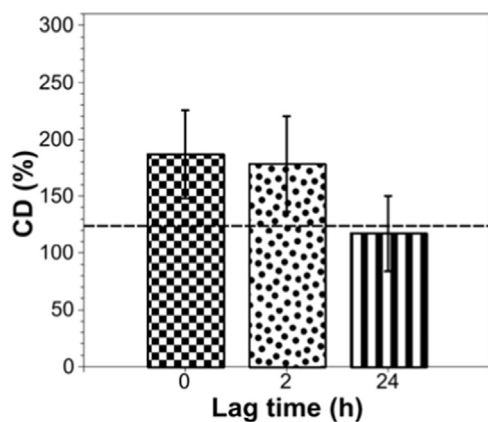


Fig. 3. Coating Degree (CD %) of rPUF coated with polyvinyl alcohol (PVA) 10%w/v followed by 2-propanol treatment. The X axis represents the lag times between both treatments. The broken line shows the CD % without 2-propanol treatment.

3.1.1.1. Infrared spectroscopy analysis. Infrared spectroscopy is a powerful tool to study the chemical composition of polymers. In addition, the ATR module adds advantages that simplify the polymer analysis (direct analysis of opaque sample) and allow obtaining additional information such as spatial resolution in case of flat surfaces.

As a consequence of the lower scale penetration of the IR signal using the ATR accessory, only the first microns of the samples in close contact with the crystal were analyzed. Therefore, FTIR-ATR is a powerful technique to identify different functional groups onto coated samples; however, the intensity of the absorptive band is not indicative of the proportion of components (Theophanides, 1984). To analyze the coating process, rPUFs coated with different polymers were pressed onto the diamond crystal and the FTIR spectra recorded.

All coated rPFU samples showed a CD% higher than 10%; however, no additional signals in the spectra in comparison to the base rPFU material were observed in samples coated with agarose or HEC (data not shown). This could be due to the poor stability of these coated polymers, where the pressure of the arm onto the ATR crystal sweeps the coating layer from the rPUF. PVA-coated rPUF clearly showed the presence of the hydrophilic polymer (Fig. 4c). For comparison, PUF and PVA film spectra were plotted in the same Figure (Fig. 4a and b respectively). Samples containing PVA showed an increase at 3300 cm^{-1} and 2900 cm^{-1} peaks, which correspond to the stretching of the -OH and -CH₂ groups respectively.

The rPUF material coated with PVA 10%w/v, pH 7.5, 85 °C (PVA-coated rPUF) was selected for the following modifications and further

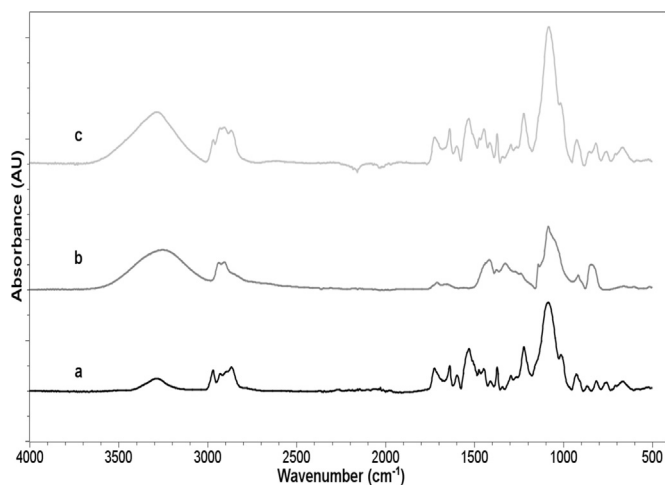


Fig. 4. FTIR-ATR spectra of (a) base material rPUF; (b) PVA film (non-irradiated) (c) and PVA-coated rPUF respectively.

analysis.

3.1.2. Cross-linking processes

Since the PVA coating without any further treatment is non-stable in aqueous media, a procedure is required to elude its leaching from the PUF material. Ionizing radiation is a very powerful technique to modify polymers. Polymer modification can be achieved by industrial sources such as gamma rays and accelerated electrons. The straightforward preparation of hydrogels by irradiation of aqueous solutions of PVA and other polymers has long been demonstrated (Alexander and Charlesby, 1957; Peppas and Merrill, 1976). In this particular case, i.e. the irradiation with a gamma ray source, the high penetrability is capable of generating reactive radicals through all the porous material and the surrounding solvent inside its porous structure (considering rPUF samples are irradiated in a solvent).

The poor solubility of PVA in alcohols was used to choose ethanol:water 1:1 v: v as irradiation solvent. Under this condition, the PVA is not dissolved and, at the same time, swells up to 300% (corresponding to a PVA concentration of approximately 25% w/v).

To study the effect of the irradiation process onto the PVA-coated rPUF, this material was irradiated with 10 kGy in a ⁶⁰Co source in aqueous/ethanol 1:1 v:v. This solvent condition keeps the PVA as a hydrogel in close contact with the rPUF surface. In addition, the solvent generates a number of different radicals by radiolysis in accordance to the type of solvents (see below).

After irradiation, the samples were washed and dried as described in Section 2.2.2 and their thermal stability and decomposition behavior analyzed by TGA.

Fig. 5 and Table 2 show the TGA curves and thermal properties of rPUF, PVA-coated rPUF and the same samples irradiated and immersed in ethanol:water mixture. The irradiated rPUF sample under the same experimental conditions (irradiation solvent, dose and dose rate) is shown as a comparison of the effects of ionizing radiation onto the base material.

PUFs are block copolymers that owe their elastic properties to the phase separation of hard and soft blocks. Hard blocks are rigid structures that are physically cross-linked, whereas soft blocks are stretchable chains which provide the polymer with elasticity. The thermal properties of these materials are governed by the weakest bond of the polymer chain, whereas the most frequent chain and the environment of the given groups are the dominant factors for overall thermal stability. In this way, two different decomposition zones were determined in rPUF materials in agreement with previous observations (Mohammadi et al., 2014). Soft and hard segments were formed during the forming reaction of PU and are related to different functional groups (Mequanint et al., 2002). While soft segments come from polyols, hard ones are formed by the reaction between diisocyanate and diol or diamine. Because each segment has different bond energy, they are broken at different temperatures and can be differentiated in TGA curves. A first zone in our rPUF sample extended from 210 °C to 330 °C, whereas a second zone, next to the first, extended until 410 °C.

The thermal properties of the non-irradiated PVA-coated rPUF sample also showed two degradation zones, but the second zone had a maximum at a temperature lower than that of the rPUF sample (Table 2 entries 1 and 3). PVA degradation causes an intensive signal of weight change (Strawhecker and Manias, 2000), which comes earlier and overlaps with the degradation of both segments of rPUF (Mohammadi et al., 2014).

The T₉₅ value for the base material (rPUF) was determined at 251 °C (Table 2), whereas, for the coated rPUF without any irradiation treatment, this temperature decreased to 212 °C. This temperature of decomposition could be related to the non-crosslinked PVA (Strawhecker and Manias, 2000).

When the PVA-coated rPUF was irradiated under these experimental conditions, the remaining weight after 400 °C was still higher than 20% (Fig. 5) and a new third maximum was observed at 411 °C (Table 2,

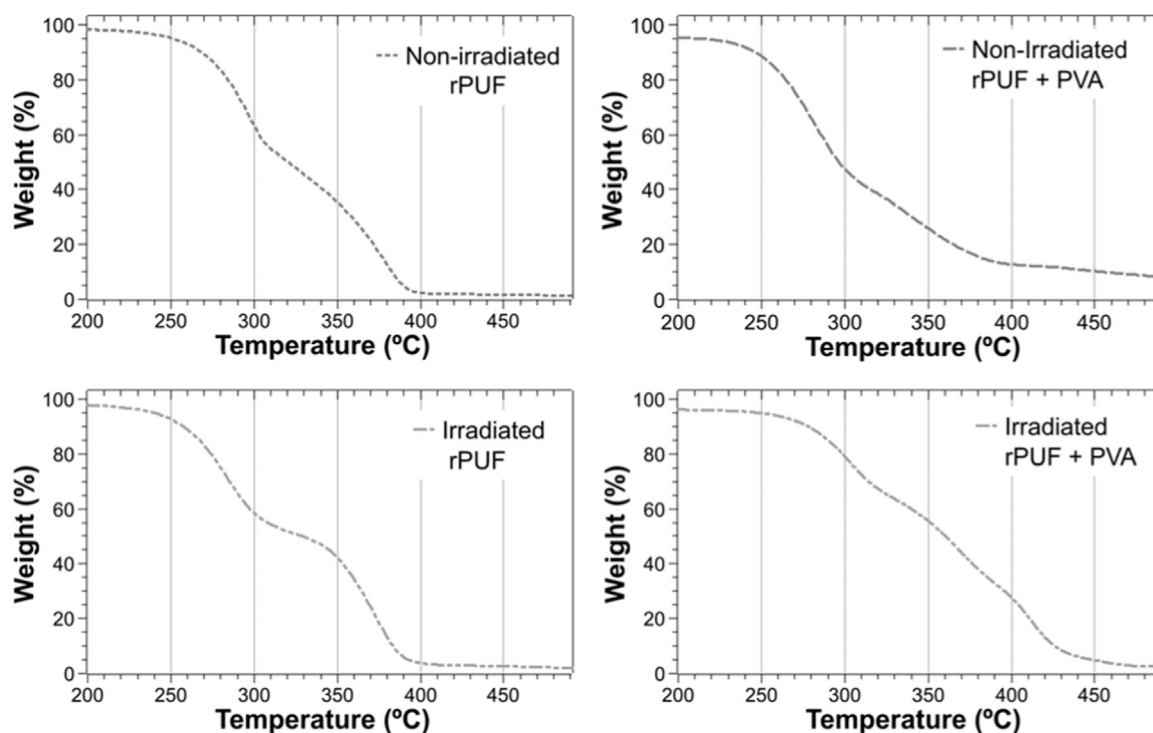


Fig. 5. TGA curves of non-irradiated rPUF material (upper left), non-irradiated PVA-coated rPUF material (upper right) and irradiated rPUF material (lower left) and irradiated PVA-coated rPUF material respectively.

entry 4). This value is compatible with the presence of crosslinked fractions.

To observe the effect of irradiation onto single materials, a PVA film and rPUF were compared with the irradiated ones under these experimental conditions. Fig. 6 shows the great difference in the derivative of TGA for both polymers irradiated in ethanol:water mixture. The curves of the derivative of TGA of rPUF material showed minor changes in both degradation zones, as described in the TGA curve (Fig. 5). Meanwhile, a shift of more than 130 °C was found in the degradation zone of the PVA film. As reported in Table 2, the T_{95} in PVA-coated crosslinked rPUF increased to 248 °C, and this increase, in comparison with the non-irradiated one, could be attributed to the PVA crosslinking (entries 3 and 4).

The creation of covalent bonds by crosslinking requires an additional amount of energy which shifts to higher degradation temperatures and could be correlated with an increase in its thermal stability. Although it cannot be conclusive from TGA data, a crosslinking process involving both polymers is highly possible.

Chemical modification onto PVA hydrogel irradiated under oxygen-free atmosphere was analyzed by FTIR. PVA films were irradiated at 10 kGy immersed in water and ethanol:water 1:1 v:v.

The FTIR spectra of the dried PVA films are shown in Fig. 7 and

compared with the spectrum of non-irradiated PVA. The characteristic absorption peaks agree with previous reports (Bhat et al., 2005; Sugiura et al., 2001). The spectrum of non-irradiated PVA showed a small signal in the carbonyl region ($1700\text{--}1740\text{ cm}^{-1}$ range), in agreement with that previously described (Bhat et al., 2005) (Fig. 7a). No additional increment in the carbonyl signal was found in irradiated samples, indicating that the degradation process by scission of ketone moieties generated by the keto-enol tautomerization of α -PVA radicals (Wang et al., 2000) is not a relevant process under these experimental conditions. In addition, no signals differences have been found between PVA and PVA hydrogels irradiated in water and ethanol:water mixture conditions (Fig. 7). Therefore, if PVA crosslinking occurs it will not involve the hydroxyl groups, as it is proposed for other crosslinked PVA hydrogels (Akhter et al., 1988; Mansur et al., 2008).

The effect of the PVA concentration on the yield of radiation-induced crosslinking deserves to be discussed. In this system, the PVA hydrogel is irradiated at concentrations in the range of 30%w/v (see above); therefore, according to the data reported by Wang et al. (2000) for PVA hydrogels irradiated in water, the G value for intermolecular crosslinking is the maximum. However, this discussion does not include the presence of ethanol in the medium. The presence of high amount of ethanol in the irradiation solvent will produce at least two phenomena:

Table 2

TGA parameters corresponding to rPUF materials.

Sample	T_{95}	1st Decomp. zone	Max. Derivative (1st)	Integration 1st zone	2nd Decomp. zone	Max. derivative	Integration 2nd zone	3rd Decomp. zone	Max. Derivative (3rd)	Integration 3rd zone
Unit	(°C)	(°C)	(% °C ⁻¹)	(%)	(°C)	(% °C ⁻¹)	(%)	(°C)	(% °C ⁻¹)	(%)
rPUF	251.2	297.4	1.149	52.2	379.2	0.907	44.5	No	–	–
rPUF/irradiated	240.0	284.4	0.928	46.8	371.5	1.119	47.8	No	–	–
PVA-coated rPUF	212.4	282.5	1.022	39.5	349.8	0.444	54.7	No	–	–
PVA coated rPUF/Irradiated	248.4	303.0	0.663	32.5	368.4	0.626	30.9	411.39	0.782	30.3

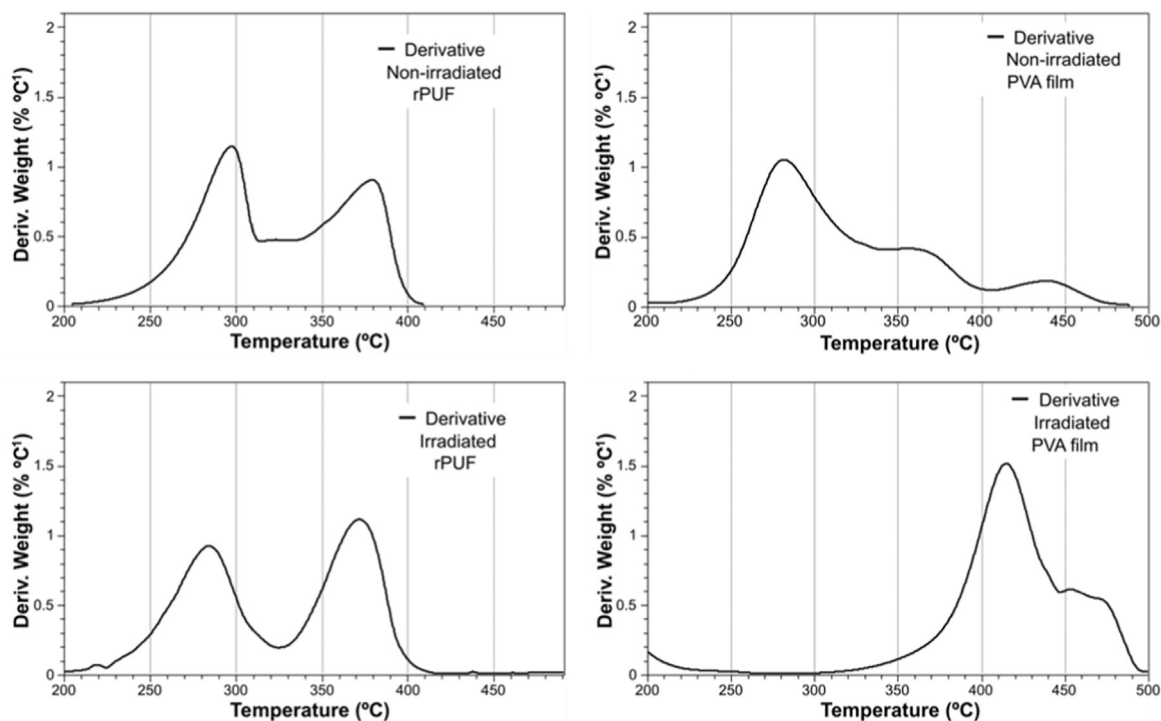


Fig. 6. Derivative of TGA curves of non-irradiated rPUF material (upper left), non-irradiated PVA film (upper right) and irradiated rPUF material (lower left) and irradiated PVA film (lower right) respectively.

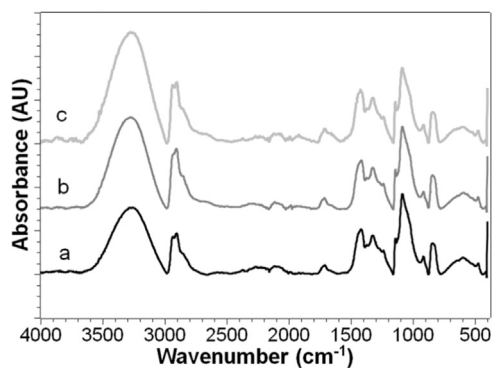


Fig. 7. FTIR-ATR spectra of (a) non-irradiated PVA and (b) PVA film irradiated in water and (c) irradiated in water:ethanol 1:1 mixture (Dose: 10 kGy).

(a) a solvent exchange in the PVA hydrogel (Takigawa et al., 1992) and (b) different kinds of radicals generated by solvent radiolysis (Roux et al., 1973). The water exchange by ethanol causes a phase separation as a consequence of the decrease in solvent power. This brings a heterogeneity within the PVA gel structure where the PVA-rich phase has the polymer chains very close to each other (Takigawa et al., 1992). Therefore, a high yield in the radiation-induced crosslinking process is expected. Considering the second phenomenon, water radiolysis yields a high number of hydroxyl radicals and hydrated electrons. However, the presence of a high concentration of ethanol (> 8 M) reduced the amount of HO \cdot radicals and H $_2$ O $_2$ to negligible values (Štefanić and LaVerne, 2002). Hydrogen- and carbon-based radicals, such as ethoxy or hydroxyethyl radicals, are the main reactive species generated in the solvent (Roux et al., 1973) and are able to react with the PVA hydrogel, in all its volume and at the surface of the insoluble polymer (rPUF), generating macro-radicals. Subsequently, these radicals will evolve mainly in crosslinking chemical reactions according to the solvent composition and the heterogeneity of the sample in different regions of the material. Considering the lack of carbonyl moieties, according to the FTIR data, and the increase in the thermal stability in the irradiated

PVA-coated rPUF, the hypothesis of PVA-PVA intermolecular crosslinking and intermolecular PVA-PU reactions is strongly supported.

3.1.3. SRIGP of PVA-coated rPUF

To add chemical functionality to the PVA-coated layer, a reactive polyGMA was simultaneously grafted. This grafted polymer allowed introducing different chemical ligands, such as sulfonic groups, to customize the hydrophilic layer.

SRIGP is a versatile tool to provide tailor-made properties to a polymer (Carbajal et al., 2003). This tool has been studied for decades and still maintains an industrial interest due to its great flexibility to prepare a complex variety of materials (Clough, 2001).

Ionizing radiation technology allows performing crosslinking and SRIGP simultaneously in one step onto PVA hydrogels (Bodugöz et al., 1999). This second process can be done by including methacrylic monomers in the irradiation solvent. Monomers dissolved in the solvent yield homopolymers and/or grafted polymers onto the base material. In the latter case, polymethacrylate will be linked to the swelled PVA hydrogel coated to the rPUF material.

After the irradiation process including different monomers (GMA and DMAA) and monomer concentrations (from 1% to 6%), the material was washed to remove the homopolymer. Then, it was dried and weighed to calculate the grafting degree (GD%), which corresponds to the percentage of increase in weight compared to the non-irradiated sample.

Fig. 8 shows a correlation between GD% and GMA concentration in the irradiation solution considering the grafting of one or two monomers. When a simultaneous grafting is performed using low monomer concentration, the SRIGP continues until consuming all the monomer available; therefore, a linear correlation between these variables is expected, as shown in Fig. 8. A similar behavior has been reported in previous studies for polyethylene and polysulfone membranes grafted under the same experimental conditions (Grasselli et al., 1999; Carbajal et al., 2003).

In a previous study, the use of the second monomer DMAA onto SRIGP of polysulfone membranes allowed obtaining an adsorptive

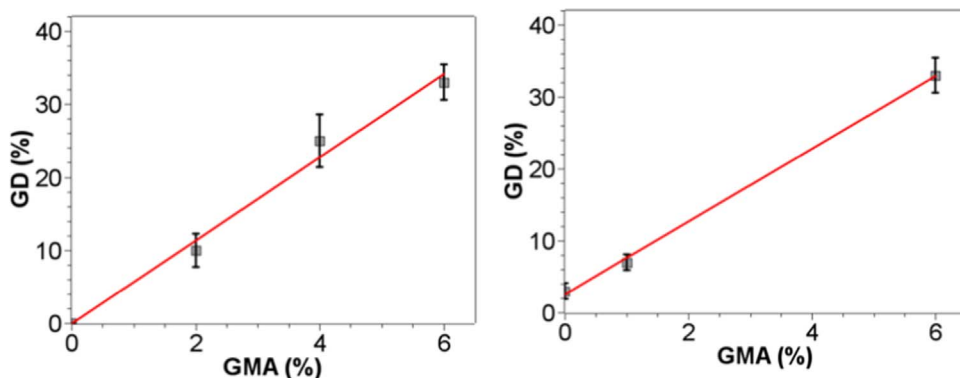


Fig. 8. Grafting Degree (GD %) of rPUF coated with PVA 10% w/v after being irradiated in the presence of (left) GMA in ethanol:water solution (1:1 v:v) and (right) GMA with the addition of DMAA 5% in ethanol:water solution (1:1 v:v).

membrane containing a surface grafted polymer with improved adsorptive properties. The presence of the hydrophilic monomer DMAA enhanced the adsorptive capacity as a consequence of improved hydrophilic properties of the grafted polymethacrylate layer (Wolman et al., 2005). Therefore, in the present work, we also studied the grafting procedure onto the PVA-coated rPUF using GMA/DMAA.

Considering the SRIGP process using GMA, GD% increased proportionally to the monomer concentration. Also, the amount of homopolymer increased proportionally (data not shown); therefore, the sample containing the initial monomer concentration of 6% showed a high yield of homopolymerization, which was difficult to remove from the foam. The best experimental condition to prepare grafted foam was the sample prepared using the initial monomer concentration of 4% in the sample.

Considering the copolymerization with DMAA, it showed a GD% similar to that without DMAA but higher amount of homopolymer was developed. These results are different from those previously reported (Wolman et al., 2005); however, the base polymers used in both works were different (polysulfone versus PVA-coated rPUF) and thus different grafting localization was reached (see below). Therefore, the SRIGP process using only GMA monomer was selected for further functionalization of the PVA-coated rPUF.

3.1.3.1. Solvents of the SRIGP process. In SRIGP processes, solvents have an important role in bringing about the swelling of the polymer to be grafted and hence enhancing the monomer accessibility to radical sites (Nasef, 2001). Accordingly, the solvent is one of the essential elements towards the success of SRIGP (Nasef, 2001). Generally, there are two reasons for the specific effect of solvents on SRIGP: (i) the radiolytic products from the solvent and its chain transfer constant, and (ii) the redistribution of the absorbed dose between the components of the monomer/solvent system. It has been established that the nature of the solvent not only determines the degree of grafting but also the location of grafting (i.e. surface or bulk). If a poor-swelling solvent is used, surface grafting most likely takes place due to the slowdown in monomer diffusion. This is due to the low concentration of absorbed monomer and low viscosity of poorly swollen polymer matrix. However, if a good-swelling solvent is used, bulk grafting is highly favored and homogenous grafting is most likely obtained.

In the present work, isopropanol, ethanol and ethanol:water (1:1 v:v) were used as solvents for the SRIGP process. Fig. 8 shows the GD% obtained using 4% GMA dissolved these solvents.

As shown in Fig. 9, the maximum GD% was reached using the ethanol:water mixture as a solvent. In addition, we also noticed that the SRIGP process carried out with isopropanol and absolute ethanol changed the integrity of the rPUF, reducing the flexibility of the material. In addition, alcohols prevent PVA swelling. Therefore, the high molar proportion of water in the solvent increased the swelling of PVA and reduced the swelling properties of PU, yielding higher GD%.

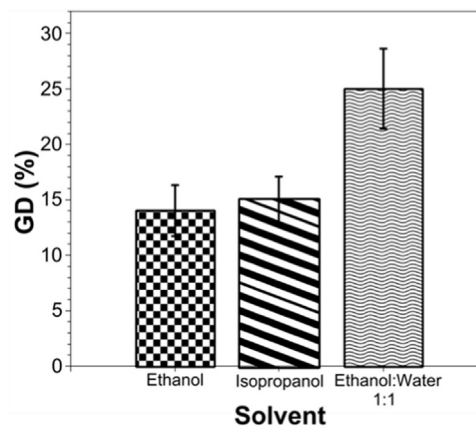


Fig. 9. GD % for PVA-coated rPUF samples irradiated in the presence of GMA 4% in different solvents.

3.1.3.2. Irradiation dose and dose rate of the SRIGP process. The irradiation dose and dose rate are important parameters of SRIGP, which could strongly affect the GD%. The former influences the amount of radicals, while the latter has a strong impact on the survival of such radicals and the termination of the formed graft growing chains. Generally, higher irradiation doses lead to higher GD% values. This is due to the enhancement of free radical formation in the grafting system and the involvement of more radicals in the grafting reaction. Considering the low monomer concentration and the total dose of 10 kGy used in this work, the SRIGP process evolves until it consumes all the monomer available (Grasselli et al., 1999).

Considering the dose rate, higher rates yield equal or lower GD% values as a consequence of the low diffusion rates of monomers. In this work, dose rates of 1 and 10 kGy h⁻¹ were used with a total dose of 10 kGy (Fig. 10). It is important to mention here that when the simultaneous irradiation technique is applied, the irradiation dose is a function of the grafting time, whereas when the pre-irradiation technique is applied, the irradiation dose and the grafting time are two independent parameters. Moreover, relatively lower doses and dose rates are required for SRIGP to achieve high GD% values (Nasef, 2001).

As expected, GD% was higher at the lower dose rate. This may be explained by the fact that at higher dose rates, the kinetics of free radical formation is much higher than the diffusion of the monomer, favoring the production of homopolymer in the solvent. Thus, 1 kGy h⁻¹ was the dose rate selected for the SRIGP process.

3.1.3.3. Dependence between CD% and GD%. Considering that the grafting process occurs in the PVA-coated hydrogel layer, GD% is expected to be directly correlated with CD%. Therefore, three PVA-coated rPUF samples containing different PVA amounts were modified by SRIGP using 4% GMA. As shown in Fig. 11, the amount of grafting had a direct correlation with CD % in the range studied.

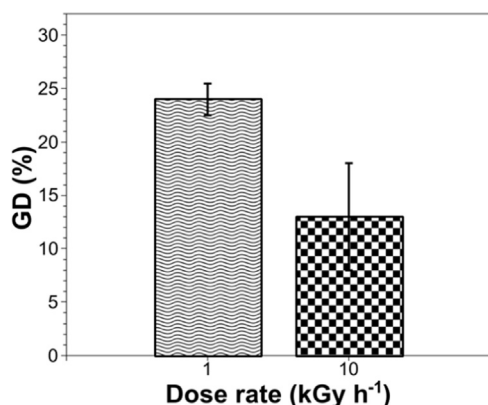


Fig. 10. GD % for PVA-coated rPUF samples irradiated in the presence of GMA 4% in ethanol:water solution (1:1 v:v). Total irradiation dose: 10 kGy.

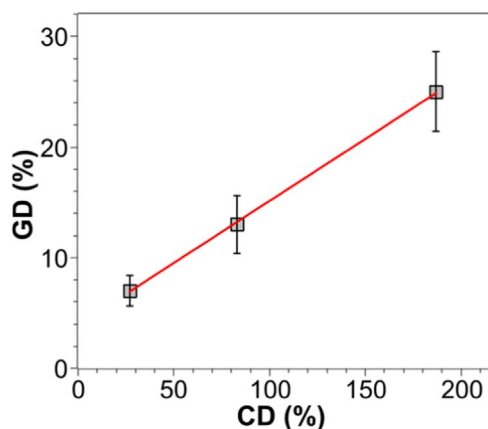


Fig. 11. GD % as a function of CD % for PVA-coated rPUF samples irradiated in the presence of GMA 4% in ethanol:water solution (1:1 v:v) at 10 kGy and 1 kGy/h.

Previous studies have shown that a homogenous grafting can be achieved in polymers that are able to swell because grafting proceeds by front mechanism, where it starts in layers close to the surface, and then proceeds inward towards the core of the film by progressive monomer diffusion through the swollen layers (Nasef, 2001). In this research, the swelling occurred onto the coated PVA-hydrogel. Therefore, grafting was expected to occur in the hydrogel layer supported on the rPUF. Section 3.2.3 shows the grafting localization by EDX (see below).

The swelling property of the GMA-grafted PVA-hydrogel coated rPUF was measured using distilled water at room temperature. The swelling degree was 80% considering the whole material, which means approximately 150–200% swelling of the PVA hydrogel.

3.1.4. Chemical functionalization of PVA-coated rPUF

The GMA-grafted PVA-hydrogel coated rPUF material was further functionalized with sulfonic groups according to very well described add-on chemical reactions (Camperi et al., 1999). Ligand capacity was calculated by quantification of sulfonic groups by elemental analysis, corresponding to 0.13 meq g^{-1} rPUF.

3.2. Material characterization

3.2.1. Infrared spectroscopy analysis

The FTIR-ATR spectrum corresponding to the GMA-grafted material showed comparatively higher carbonyl signal (1720 cm^{-1}) than the PVA-coated one, which was assigned to polyGMA. The characteristic epoxy ring signal at 900 cm^{-1} was overlapped with the signals of the base material (data not shown).

3.2.2. Scanning electron microscopy (SEM)

rPUF modifications were analyzed by observing scanning electron micrographs. SEM images of untreated and modified rPUF (coated/grafted rPUF) are shown in Fig. 12a/b and c/d, respectively.

A rough structure, which can be assigned to the modification process, was recognized onto the foam surface. Also, the modified rPUF (c/d) indicated no bulk deterioration compared to the untreated sample and a thicker structure was also noticed. Similarly, no pore blockage was detected.

The b/d images show the cross-section of the polymer of rPUF in addition to the surface. It is possible to differentiate the morphology of the internal body of rPUF in both cases. However, a contrast can be seen between the smooth surface of the untreated rPUF and the rough surface of the modified rPUF.

3.2.3. Energy-dispersive X-ray spectroscopy (EDX)

Grafting polymerization can be performed on the coating layer or in the polymer bulk according to the solubility of the monomers and solvents within the polymeric matrix. Using the same monomer (GMA) and solvent mixture (ethanol/water), the grafting location could be different according to the base material to be grafted. For example, when using polyethylene and polysulfone membranes as base materials, surface grafting is reached (Grasselli et al., 1999; Carbajal et al., 2003). However, when using acrylamide-based cryogel as base material, the polyGMA grafting occurs in the bulk of the polymer structure (Bibi et al., 2011). In both cases, the SRIGP technique was performed under the same experimental conditions.

To study the localization of the grafted polymer in the sample, an elemental in situ X-ray microanalysis was performed (SEM-EDX). Copper ions have characteristic energy values for the *s* electron different from other elements available in the sample. Therefore, a grafted PVA-coated rPUF sample was labeled with copper ions. This was performed by functionalization of polyGMA with iminodiacetic ligand, and copper ions were chelated on it, as previously described (Grasselli et al., 1999). Two different regions of the SEM picture in Fig. 13 were selected to perform the EDX analysis: one in the bulk of the rPUF material and the other on the outer region (PVA coating).

It can be clearly seen that the presence of copper is delimited to the surface region of the material, indicating that the PVA-hydrogel layer was grafted with polyGMA.

3.3. Adsorptive properties

The functional rPUF material containing sulfonic ligands (SP-rPUF) was characterized by standard adsorption experiments. A protein adsorption isotherm was performed under static conditions (Static Binding Capacity, SBC) and a protein adsorption experiment was performed under dynamic conditions. In both cases, we used Lysozyme as a model protein. SBC was tested in batch as described in Section 2.3.6, reaching $475 \pm 30 \text{ mg protein g}^{-1}$ matrix (Fig. 14).

The PVA hydrogel reduced the pore diameter in a small fraction. Therefore, considering the adsorptive process under dynamic conditions, no measurable backpressure was detected when the liquid flow through the foam filled the column.

Dynamic adsorption/desorption assays were tested onto a 1-mL column by injection of a protein sample and further elution, changing to the elution buffer (Fig. 15), to test the potentiality of the SP-rPUF to be used as an ion-exchange chromatographic matrix. It is important to remark that this adsorptive analysis was made under plug-flow conditions.

4. Conclusion

A novel functional hydrogel-coated porous monolith material was prepared based on commercial rPUF material. From three different hydrophilic polymers analyzed for the coating process, PVA was the

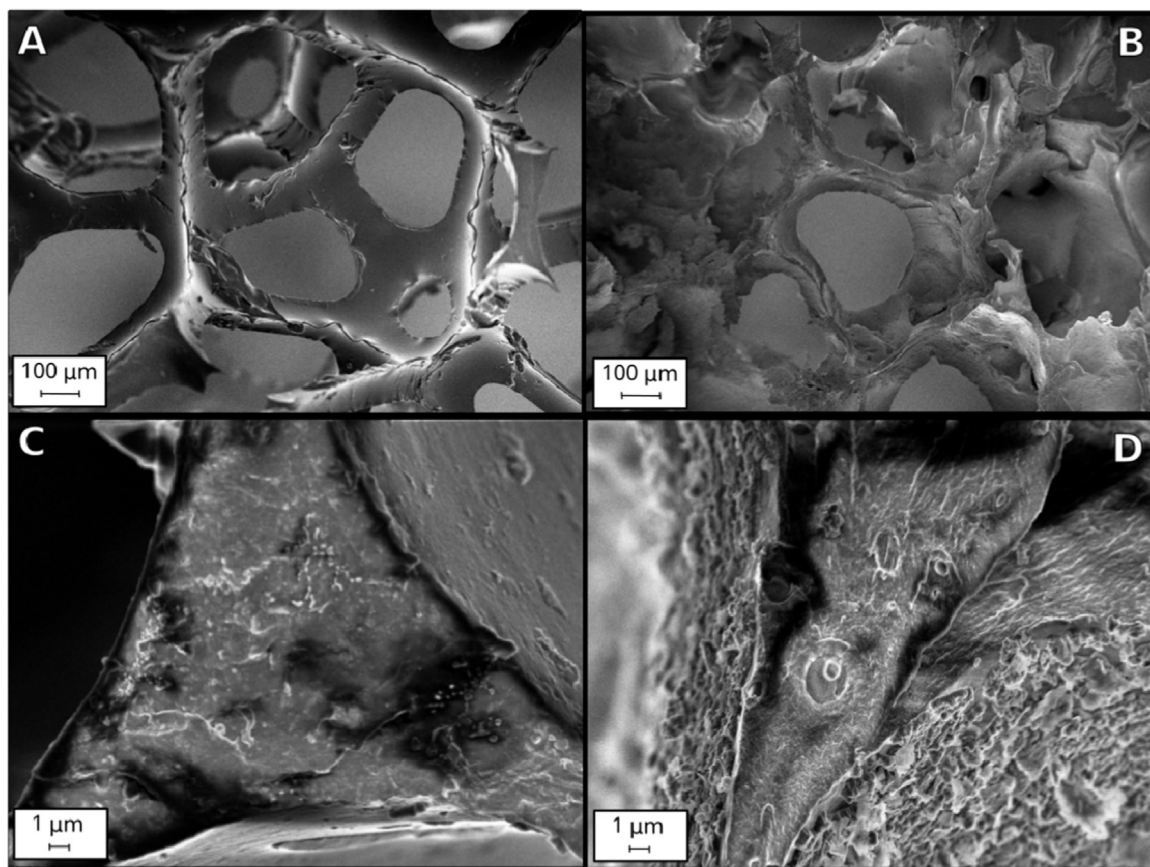


Fig. 12. SEM images of (a/b) virgin rPUF and (c/d) coated and grafted rPUF material, respectively.

best to obtain a high coating degree. An optimized coating method was also described considering the polymer concentration, temperature and coagulating conditions.

PVA coating was stabilized by crosslinking by irradiation with a

⁶⁰Co source. The irradiation conditions involved the use of a solvent mixture which balanced the PVA swelling, preventing the dissolution of PVA during the irradiation step. In addition, ethanol acts as a scavenger of the hydroxyl radicals generated by water radiolysis.

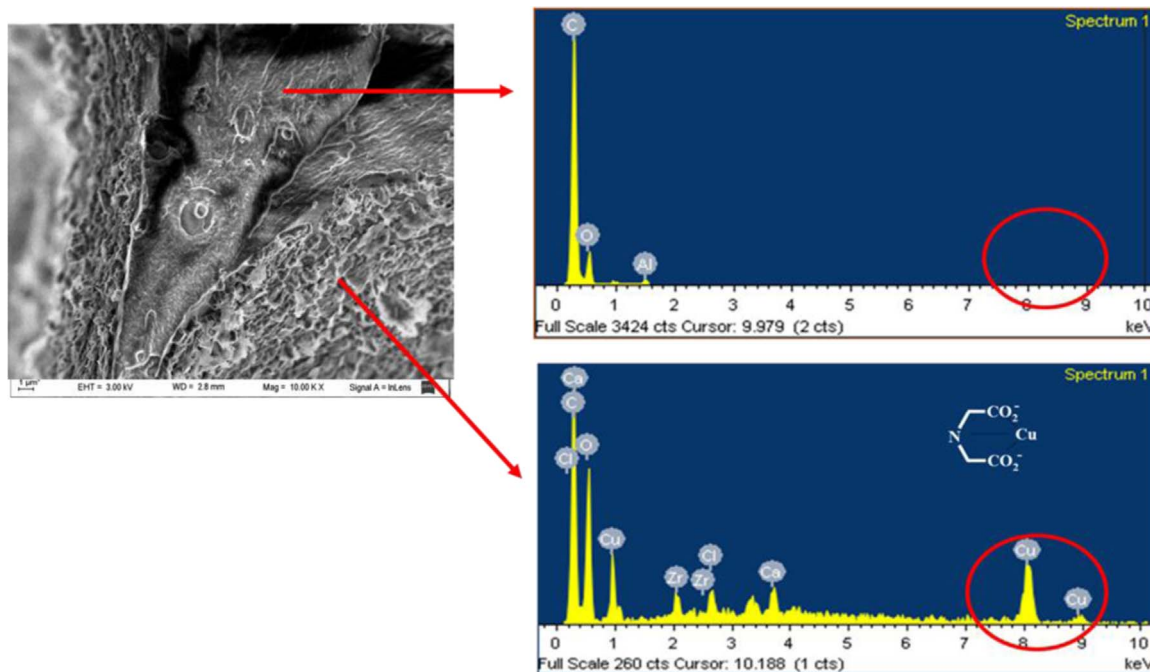


Fig. 13. SEM image of cross-section of adsorptive rPUF material (inner and coated zone). Spectra of elemental composition of the retro-dispersed electrons of rPUF-PVA-pGMA-IDA-Cu²⁺. Spectrum 1: inner zone of the material. Spectrum 2: coated zone of the material.

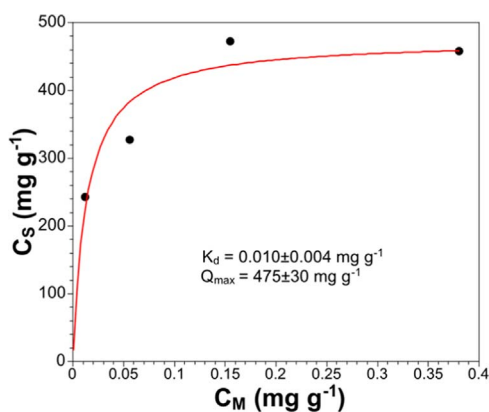


Fig. 14. Langmuir Isotherm for cation-exchange rPUF material and Lysozyme as model protein.

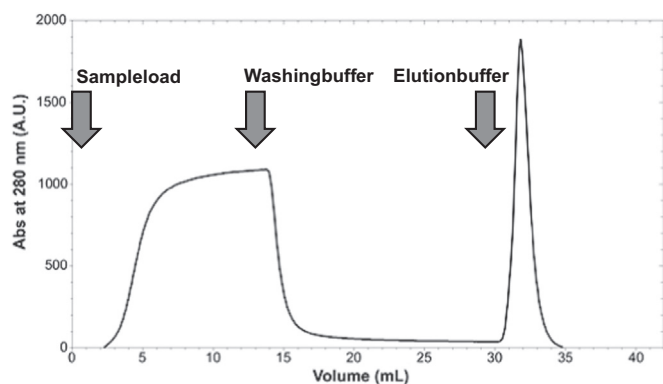


Fig. 15. Adsorptive and desorptive steps using rPUF column and Lysozyme as protein model. Chromatographic conditions: 1420 cm h⁻¹ flow rate (3 mL min⁻¹).

A functional hydrogel coating onto rPUF was successfully prepared by simultaneous radiation-induced crosslinking and SRIGP process. The material was characterized by FTIR spectroscopy and thermogravimetric analysis. The experimental data from these techniques suggested that a crosslinking process between polymers had taken place. The polymethacrylate grafting was localized in the PVA-hydrogel layer and the rPUF backbone remained unmodified, preserving the initial mechanical properties.

This novel procedure allowed preparing bi-structured materials by a single irradiation step of polymers coated with PVA-hydrogels in an ethanol:water mixture. In addition, the hydrogel layer of the bi-structured material was simultaneously grafted with polymethacrylate during the irradiation process.

The functional material showed remarkable adsorptive/desorptive properties, using the Lysozyme as model protein. Further studies in this field should be performed to compare this material with cutting-edge chromatographic adsorbents.

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