Preparation of organic monolithic columns in polytetrafluoroethylene tubes for reversed-phase liquid chromatography

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Abstract

In this work, a method for the preparation and anchoring of polymeric monoliths in a polytetrafluoroethylene (PTFE) tubing as a column housing for microbore HPLC is described. In order to assure a covalent attachment of the monolith to the inner wall of the PTFE tube, a two-step procedure was developed. Two surface etching reagents, a commercial sodium naphthalene solution (Fluoroetch®), or mixtures of H_2O_2 and H_2SO_4, were tried and compared. Then, the obtained hydroxyl groups on the PTFE surface were modified by methacryloylation. Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy and scanning electron microscopy (SEM) confirmed the successful modification of the tubing wall and the stable anchorage of monolith to the wall, respectively. Special emphasis was also put on the reduction of the unwanted effects of shrinking of monolith during polymerization, by using an external proper mold and by selecting the adequate monomers in order to increase the flexibility of the polymer. Poly(glycidyl methacrylate-co-divinylbenzene) monoliths were in situ synthesized by thermal polymerization within the confines of surface-vinylized PTFE tubes. The modified PTFE tubing tightly held the monolith, and the monolithic column exhibited good pressure resistance up to 20 MPa. The column performance was also evaluated via the isocratic separation of a series of alkylbenzenes in the reversed-phase mode. The optimized monolithic columns gave plate heights ranged between 70 and 80 μm. The resulting monoliths were also satisfactorily applied to the separation of proteins.

Keywords: Polymer monolith; polytetrafluoroethylene; surface modification; monolith attachment; microbore column; reversed-phase liquid chromatography

1. Introduction

Monolithic columns are becoming very attractive stationary phases due to their advantageous hydrodynamic features and their easy flexible preparation and versatility. Thus, numerous reports on both silica- [1] and polymer-based monolithic columns [2, 3] and their application to sample preparation and separation have been described. Polymeric monoliths are prepared from a bulk polymerization mixture and their structure is defined by the monomer composition and polymerization temperature

without further processing [3]. Up to now, most of the related literature has been focused on the monolithic structure for enhancing column performance and on new column chemistries for tailoring selectivity [4], while research in the extension of these materials on column size (within inner diameters higher than 500 μm) and housing materials has been reduced [5-7]. On the other side, fused-silica capillaries have been traditionally employed as physical supports in the preparation of monolithic columns due to their easy covalent attachment to the wall after its vinylization. These capillary-scale columns have demonstrated to be of interest in miniaturized techniques (capillary/nano-LC and electrochromatography) and its application to the growing field of life-sciences. However, capillaries are too narrow for the flow-rate ranges employed in conventional HPLC.

In general, the fabrication of large monolithic columns with sizes larger than 500 μm involves some difficulties in preparation. An increase in the column tubing diameter (from capillary format to internal diameter above 1 mm) can produce heterogeneous monoliths with radial gradients of properties due to the slow dissipation of heat during the exothermic polymerization process [8]. Other problems are caused by the monolith shrinkage during polymerization. The forces put into play by longitudinal and radial shrinkage of the polymer are strong enough to extensively breakdown the monolith-tube anchorage. The unwanted effects of shrinking are negligible in capillaries, although very important in larger diameter tubes. A few approaches to reduce the shrinking effects have been suggested, including the use of solvents that keep the stationary phase in the swollen-state [9], polymerization under high pressures [10], and the employ of a titanium scaffold [11].

On the other hand, there is an increasing interest in developing monolithic columns confined in tubes made of materials different from silica, such as stainless steel, polyether ether ketone (PEEK) or polytetrafluoroethylene (PTFE) tubes of at least 0.5 mm i.d. Above this diameter, a column can be used in HPLC and other systems using conventional flow-rate ranges, such as in flow injection analysis and solid-phase extraction (SPE) devices for on-line sample preparation. However, few contributions have been reported for the preparation of micro-bore scale monolithic columns in non-silica molds [5-7, 12-13].

PTFE has been widely used in the electronics, chemical, and medical industries, due to its excellent properties such as high chemical inertness and thermal stability, low dielectric constant and transparency to UV radiation [14]. The properties of PTFE have

led to many successful applications, such as lining for reactors and electrical cables, substrate for printed circuit boards, anti-sticking coating for kitchen utensils, and adhesive tapes, etc. Moreover, PTFE has been extensively used as recommended material in several analytical applications, such as trace metal studies, automation methods, and in sample preparation devices (reactors, filters, membranes, etc.). Due to its UV transparent properties, PTFE capillaries have been also employed as separation media in capillary electrophoresis [15]. However, its poor adhesion properties and poor wettability have caused considerable problems in particular application fields such as microelectronic devices, adhesive and protective coatings and biomaterials [16-17]. In order to enhance its adhesiveness to other materials, or to improve its biocompatibility, several surface modification methods have been proposed to introduce polar groups such as hydroxyls or carboxylates onto PTFE surfaces. Along with wet chemical treatments [18-19], plasma [20] and ion beam [21-22] etching treatments are considered the most efficient techniques for PTFE surface modification. However, plasma and ion beam techniques cannot be used to modify the inner surface of capillaries, and they are not easy to use in narrow tubes either.

In this study, the use of PTFE tubing of 1/16'' o.d. × 0.8 mm i.d. as a column housing for microbore HPLC using polymeric monoliths is investigated. In order to assure covalent attachment of the monolith to the inner wall of the PTFE tube, a chemical modification of this material was first performed. For this purpose, two etchant reagents, a sodium naphthalene solution (Fluoroetch®) or mixtures of H_2O_2 and H_2SO_4, were tried and compared. The objective of this etching step was to create reactive hydroxyl groups on the PTFE surface. Then, these groups were subsequently modified by methacryloylation to obtain a vinylized surface. Special emphasis was also put on to reduce the unwanted effects of shrinking by using an external polymerization mold for the subsequent thermal polymerization stage; in addition, success was achieved by also selecting a proper selection of monomers to increase the flexibility of the resulting polymers.

The resulting monolithic columns were connected to a conventional HPLC system and its chromatographic properties were evaluated using a mixture of alkylbenzenes. After optimization of the polymerization conditions (composition and polymerization time) of the monolithic columns, a satisfactory chromatographic performance of probes was achieved. Furthermore, the capability of using the synthesized columns in the prepared PTFE supports for the separation of proteins was

also investigated. To our knowledge, this is the first report that demonstrates the use of PTFE tubing as supporting material for monoliths and its application to the separation of small molecules and proteins by reversed-phase LC.

2. Materials and methods

2.1. Chemicals and reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), tetrahydrofuran (THF) and triethylamine (TEA) were from Sigma-Aldrich (Steinheim, Germany). Divinyl benzene (technical grade, 80% mixture of isomers, 20% mainly ethylstyrene, DVB), 1-decanol and lauroyl peroxide (LPO) were supplied by Alfa Aesar (Karlsruhe, Germany). Azobisisobutyronitrile (AIBN) was from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from Merck (Darmstadt, Germany). Uracil, alkyl benzenes from Riedel de Haën (Seelze, Germany) and proteins such as ribonuclease A (bovine heart), cytochrome C (bovine pancreas) from Alfa Aesar, and myoglobin (horse skeletal muscle) from Sigma, were used as probes. Acetone, sulfuric acid and hydrogen peroxide (37%) were supplied by Panreac (Barcelona, Spain). Ultra-pure water was obtained with a Milli-Q water purification system from Millipore (Bedford, MA, USA). Unless otherwise stated, other chemicals used were of analytical grade. Polytetrafluoroethylene (PTFE) tubing of 1/16'' (1.6 mm) o.d. × 0.8 mm i.d. from Omnifit (Fisher Scientific, Loughborough, UK) was used.

Stock solutions of alkyl benzenes were prepared in ACN at 1.0 mg mL^{-1} each and kept at 4ºC until use. Working standard solutions were freshly prepared by dilution to the desired concentration with the mobile phase. Proteins were dissolved in water at concentration of 1.0 mg mL^{-1} each.

2.2. Instrumentation

Chromatographic analysis was carried out in an HPLC equipment from Jasco Analytica (Madrid, Spain), composed of a PU-2089 quaternary gradient pump, an AS-2055 autosampler with a 100 μL injection loop and MD-2018 photodiode array detector. The system was controlled using the LC-NETII/AFC interface also supplied by Jasco. Acquisition and data treatment was performed using the ChromNAV software (version 1.17.01). SEM photographs of PTFE surfaces and monolithic materials were performed with a scanning electron microscope (S-4100, Hitachi, Ibaraki, Japan)

provided by a field emission gun, an EMIP 3.0 image data acquisition system, and a microanalysis system (Rontec, Normanton, UK). FT-IR spectra of PTFE surfaces were obtained with a Nicolet Magna FT-IR 750 spectrometer (Madison, WI, USA) fitted with a single reflection attenuated total reflectance (ATR) accessory. Spectra were recorded at room temperature between 4000 and 550 cm^{-1} with 8 cm^{-1} nominal resolution at 50 scans per spectrum. Nitrogen adsorption surface area analysis of monolithic materials was performed on a Micromeritics ASAP2010 automated sorption analyzer (Rutherford, Germany). Gas chromatography–mass spectrometry (GC-MS) analysis was performed on a Focus DSQ II gas chromatograph provided with an AI 3000 autosampler and single quadrupole MS detector from Thermo Fisher Scientific (Austin, TX, USA).

2.3. Modification of inner wall surface of PTFE tubing

To modify the inner wall surface of PTFE tubing (Fig. 1A), the following two wet chemical procedures were adopted. The first one employed sodium-naphthalene based solution (Fluoroetch®, Acton Co., Limerick, Ireland) as etchant reagent. The detailed chemical and processing information of Fluoroetch® treatment can be found elsewhere [23]. Briefly, the PTFE tubing was flushed with the Fluoroetch® solution at 55-65ºC for 60 s. Then, the tubes were washed with MeOH for 20 s, followed by rinsing with water at 70ºC for 30 s, and acidified water (containing 5% acetic acid) at 70ºC for 60 s. Next, the tubing was dried by flushing air at 70ºC. The second inner surface PTFE modification procedure was adapted from Löhbach *et al.* [18]. Thus, the tubing was filled with a H_2O_2/H_2SO_4 (1:1) solution, sealed with caps, and left at 70ºC for 60 min. Next, the tubing was flushed with water followed by acetone, and then dried with nitrogen. Both protocols produced hydroxyl reactive groups on the PTFE surface (Fig. 1B). Afterwards, the PTFE activated surface (obtained with each protocol) was reacted with 2 M GMA in acetone containing 5 mM TEA at pH 8.0 for 5 min using a home microwave oven (output power: 800 W). This step allowed the introduction of double bonds (methacryloyl groups) onto the treated PTFE inner wall surface (Fig. 1C). The methacryloylated PTFE tubing was rinsed with acetone and then dried with nitrogen.

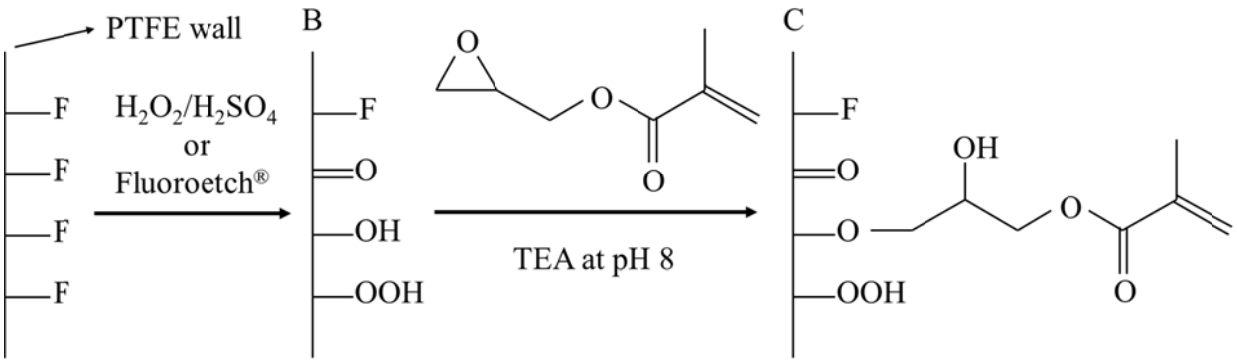

Fig. 1. Reaction scheme of the chemical modification of PTFE tubes: **U**nmodified (A); oxidized PTFE where reactive groups have been formed (B); binding of GMA to the wall (C).

2.4. Synthesis and characterization of polymeric monoliths

The treated (methacryloylated) **P**TFE tubing was cut to the desired length (*ca*. 12 cm) and filled with polymerization mixture containing a bulk monomer (GMA), a cross-linker (DVB), and a binary pore-forming solvent (1-decanol and THF) and LPO as initiator. After mixing, and to obtain **a** clear solution, sonication for 10 min followed by purging with nitrogen for 10 more **m**in was applied. Shrinking of the polymer during curing led to the detachment of the monolith from the wall. This was avoided by submerging the whole PTFE tube segment into an external polypropylene mold. This later was constructed, as depicted in Fig. 2, with a 225 mm height polypropylene Pasteur pipette, by thermally sealing the lower end and by cutting off the top of the upper bulb. Then, the PTFE tube was placed in the Pasteur pipette and filled up with the polymerization mixture using a syringe, in such a way that excess of **t**he mixture filled up the Pasteur pipette until entirely covering the tube. The mold with the tube inside was then vertically placed in an oven, and polymerization was carried out at 70°C. After the required polymerization time, the sealed lower tip of the Pasteur pipette was cut off in order to release the PTFE tubing. **T**hen, a pair of tweezers was used to slide the PTFE tube off from the bulk monolithic material. To avoid possible detachment of material at the other end during the displacement of the tube, about 1 cm of each **e**nd of the PTFE tube was cut off to obtain a 10-cm monolithic column. The morphology of the polymer at the ends of prepared columns was examined under microscope and **t**here were found to be free of visual defects, and the column ends were perfectly even. **T**he column was immediately connected to an HPLC pump and flushed for 30 min with ACN to remove the pore-forming solvents and unreacted monomers.

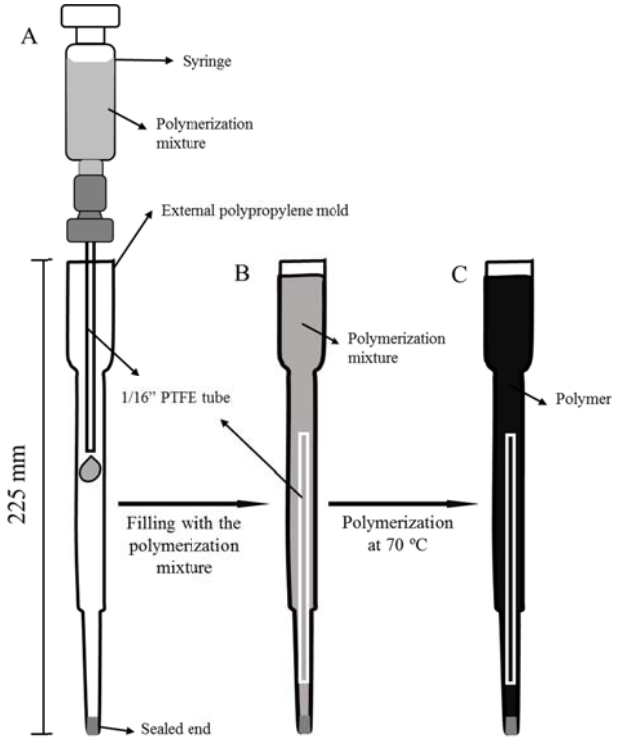

Fig. 2. Experimental set-up used for the preparation of molded polymeric monoliths in PTFE tubing.

To obtain enough amounts of monolithic material for nitrogen-adsorption experiments, bulk material was synthesized in glass vials under the same conditions as the PTFE tubing. After polymerization, the monolithic material was removed from the vial, cut into small pieces with a razor blade, and Soxhlet extraction was carried out with MeOH for 24 h, followed by drying at 50 ºC for 4 h.

The evaluation of unpolymerized monomer (GMA) and cross-linker (DVB) contents was carried out as follows. PTFE monolithic columns of 10 cm length were rinsed with 1 mL ACN immediately after polymerization, and the eluate was collected in sealed vials. The effluents obtained from different polymerization times were diluted to a fixed volume with ACN and compared to a filled, but not polymerized PTFE column (which represents 100 % of the monomer and cross-linker content) with the same length and inner diameter by using GC-MS. The procedure was applied to monoliths obtained at several polymerization times. The chromatographic conditions were: DB5-MS column (30 m, 0.25 mm i.d., 0.25 µm film thickness, Agilent); GC oven temperature program was as follows: 70°C; 8°C/min to 230°C; 20°C/min to 300°C and holding for 2 min; injection volume, 1 µL (splitless mode); 1 mL min^{-1} helium constant flow rate; injector, transfer line and ion source temperature, 280°C.

3. Results and discussion

3.1. Modification of inner wall surface of PTFE tubing

The surface of PTFE-based material was modified by wet chemical treatments to introduce polar groups such as hydroxyl groups into PTFE structure, thus enhancing its adhesiveness to other materials and molecules. For this purpose, two etchants were studied: a commercial sodium naphthalene solution (Fluoroetch®) or a mixture of H_2O_2/H_2SO_4. The treatment using Fluoroetch® is known to be a very effective method in industry for PTFE modification [18], whereas the second approach provides a milder modification of PTFE surface [18]. Thus, the internal surface of PTFE tubing was modified according to the procedures described in Section 2.3. In any case, both reactions produced hydroxyl groups (see Fig. 1A, first reaction step) onto the inner wall, which could be due to defluorination of PTFE molecular structure by the attack of complex radicals, generated from the interaction between the sodium metal and naphthalene (Fluoroetch® reagent) or from reactive atomic oxygen species from "piranha" solution (H_2O_2/H_2SO_4 mixture). As a result, the surface C–F bonds were transformed into C–H, CH_2OH and carboxyl (–COOH) bonds [18], and consequently, the hydrophobicity of the PTFE material decreases. The effectiveness of each chemical etching can be also established by evaluating the change in surface wettability in PTFE material. This parameter was measured though the contact angle of a droplet of water on the surface of the material. Thus, the water contact angle of the untreated PTFE decreased from 109º to 50º and 88º after treatment with Fluoroetch® [24] or H_2O_2/H_2SO_4 mixture [18], respectively.

To investigate the surface modification of PTFE tubing before and after each treatment, FT-IR measurements were obtained (see Fig. S1). The unmodified PTFE tubing (Fig. S1, trace A) showed the typical absorption bands of C-F bonds (from 1100 to 1300 cm^{-1}), whereas the FT-IR spectra of modified PTFE surface treated with either Fluoroetch® (Fig. S1, trace B) or H_2O_2/H_2SO_4 mixture (Fig. S1, trace C) showed a new broad absorption band at 3200 cm^{-1} (attributable to the OH group) [25]. This band was more intense in the case of the Fluoroetch® treatment. In addition, in this case, a small new band at 1730 cm^{-1} (attributed to stretching of the ester carbonyl group) was observed (Fig. S1, trace B).

The morphology of the PTFE tubing was also investigated by SEM. Fig. 3 shows the SEM micrographs of PTFE tubing before and after both chemical treatments. In comparison with the untreated hydrophobic PTFE tubing (Fig. 3A), the chemically etched PTFE with Fluoroetch® (Fig. 3B) showed randomly distributed deep cracks, being these results consistent with previous studies [24]. In addition, this chemical treatment turned the PTFE tubing from white to dark brown (Fig. 3B, inset). In the case of chemical etching using "piranha" solution (H_2O_2/H_2SO_4 mixture) small grooves were formed (Fig. 3C) and no color change in the PTFE tubing was evidenced (Fig. 3C, inset). In fact, as we shown later, this treatment provided sufficient number of free functional (hydroxyl) groups for chemical anchoring of monolith without damaging the mechanical strength of PTFE. However, the Fluoroetch®-modified supports containing polymeric monoliths led to the presence of artifacts and double peaks when these columns were tested in LC (data not shown). Besides, the use of this sodium-based product implied several reaction steps and some environmental and safety concerns. For all these reasons, the treatment with a H_2O_2/H_2SO_4 mixture was selected for further studies.

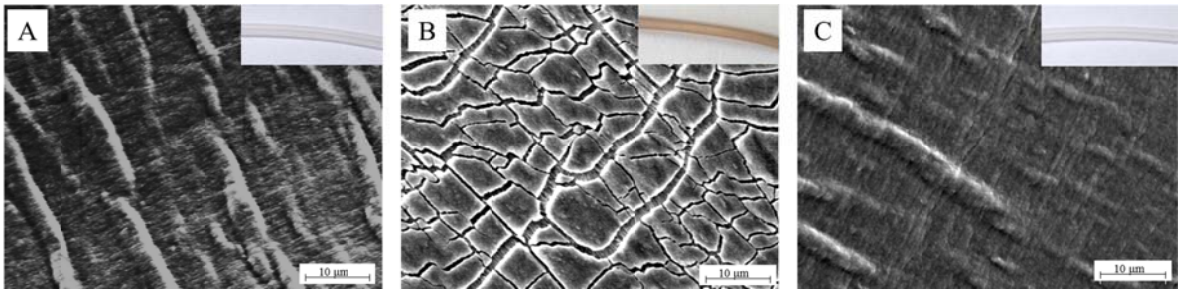

Fig. 3. SEM micrographs of surface of PTFE tubing untreated (A) and treated with Fluoroetch® (B) and with a H_2O_2/H_2SO_4 mixture (C).

Next, methacryloylation of the hydroxylated PTFE surface with GMA was accomplished. This chemical modification approach was selected over the traditional approach of using (trimethoxysilyl)propyl methacrylate as ~~common~~ organosilane reagent. In particular, the resulting C-O-Si bonding can be broken (especially by alkaline hydrolysis) more readily than typical C-O-C bonds obtained after methacryloylation step. Thus, prior published studies have demonstrated that GMA derivatives of natural polysaccharides (like dextran, hyaluronic acid) and related fibers could be prepared [26-28]. Thus, the epoxy group of GMA can react with hydroxyl groups of these polymers in the presence of several amines (*e,g,* trimethylamine, TEA) as catalyst in a mild basic environment (pH 8.0-9) [29-31]. The experimental results

showed that the best etching solution to introduce methacryloyl groups could be achieved by exposing the hydroxylated PTFE surface to 2 M GMA in acetone containing 5 mM TEA (pH 8.0) (for more details, see Experimental section). This reaction step is crucial to attach successfully the posterior monolith to the PTFE wall (as demonstrated below). In fact, when this step was skipped, and only a polymerization mixture containing GMA as bulk monomer was used, the resulting monolith was not bound to the PTFE wall.

As previously mentioned, shrinkage is an unavoidable process in any vinyl polymerization procedure and leads to longitudinal and radial contraction of the so-formed polymer. Besides, these phenomena could be strong enough to breakdown the monolith-tube anchorage, being particularly important in large diameter tubes. Thus, in order to avoid the undesirable effect of longitudinal shrinkage, an adequate polymerization set-up was designed (see Fig. 2). The details of preparation of monoliths using this system are given in Section 2.4. Using this system, the longitudinal shrinkage took place outside the confines of the polypropylene mold (a Pasteur pipette), and a perfectly filled PTFE tubing with monolith was obtained.

Regarding to the radial shrinkage, Svec *et al.* [32] prepared large-scale monoliths (8 mm i.d. in a stainless steel mold) in absence of this effect. The authors suggested that it was the result of both the absence of interfacial tension (compressing the polymer during its formation) and the lack of mixing along polymerization. However, these monoliths were not used as HPLC columns since probably a monolith detachment from the confining wall will be occurred. Other studies focused on the preparation of large-scale monolithic columns in housing materials [13, 33] have indicated that the radial shrinkage could be also significantly eliminated by the presence of enough anchoring groups as well as by the use of a proper mixture of monomers. In our case, it is likely that a combination of all these factors avoided the existence of radial compression effects. Several reports [34-36] have demonstrated that the elasticity and other morphological properties of polymeric monoliths are attributed to the type of crosslinker within polymer chains. In this work, two crosslinkers (EDMA and DVB) in the presence of GMA as bulk monomer were investigated. In addition, the preparation of GMA-based monolith relies on that this polymer is chemically and mechanically very stable and contains epoxy groups that can be further modified to prepare stationary phases suitable for ion exchange, hydrophobic interaction, reversed-phase or affinity separation. The initial conditions to prepare the polymerization mixture were adapted

from Wieder *et al.* [37]. The results showed that synthesized poly(GMA-co-DVB) monoliths were quite flexible and elastic to the bending stress of PTFE tubing and no losses in the monolithic material were evidenced. However, the resulting poly(GMA-co-EDMA) monoliths showed a reduced elastic behavior due to the dense three-dimensional structures found in these polymers (data not shown). This behavior could be due to the variation in reactivity of different crosslinkers as well as to their different solubilities (compared to the GMA monomer) and the corresponding growing polymers in the selected porogenic solvent (1-decanol and THF) [38-39].

Next, in order to demonstrate the successful covalent attachment of the monolith to the PTFE tubing wall, SEM micrographs of the cross-sections of a poly(GMA-co-DVB) monolithic column in a PTFE tube were taken. As shown in Fig. 4, no significant gaps between the inner wall of the tube and the polymeric monolith were found, which confirmed that the monolith was tightly attached to the PTFE inner wall. Besides, the monolith had the typical globular structure of polymeric monoliths and there was no optical evidence of an unwanted radial gradient of monolith density from the wall through the center of the monolith [7].

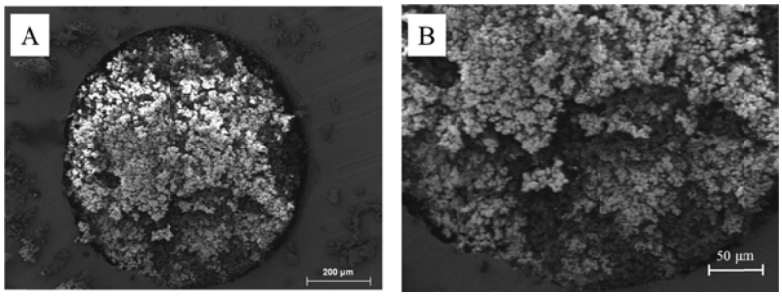

Fig. 4. SEM micrographs of GMA-based monoliths synthesized in PTFE tubing showing assured attachment of monolith to the inner PTFE wall.

An additional proof of the anchoring system uniformity was also obtained by evaluating the mechanical stability of the produced monolith. For this purpose, the relationship between flow rate and the backpressure drop of the monolith was measured to assess its adhesion to the wall. Good linear relationships (r = 0.998) between backpressure and flow rate were obtained (data not shown). Thus, the column (10 cm) could undergo a backpressure close to 20 MPa (at a flow rate 0.5 mL min^{-1}) indicating a satisfactory attachment between the monolith and the tubing wall and absence of compression of the stationary phase.

3.2. Preparation and characterization of polymeric monolithic columns in PTFE supports

Once described the method for chemical anchoring of the monolith to the inner wall surface of PTFE tubing, the optimization process of GMA-co-DVB monoliths for the separation of small molecules was accomplished. For this purpose, a combination of different variables (monomers/porogens, GMA/DVB and 1-decanol/THF ratios) in the polymerization mixture was studied to achieve a suitable fraction of mesopores, that is essential for the separation of low molecular weight compounds and a high mechanical stability of the separation columns (see Table S1). As a result of this study, the column A12 was selected, since it provided the best compromise between permeability and separation performance. Fig. 5A shows the chromatogram of a mixture of alkyl benzenes obtained using this column. As it can be seen, a poor separation of these analytes was achieved. This can be explained by the presence of large globules found in SEM micrographs (Fig. 5, part B) of this monolithic bed, which resulted in a low surface area (*ca.* 23.5 m^2 g^{-1}), and consequently, in a low presence or even in the absence of an adequate mesoporous structure.

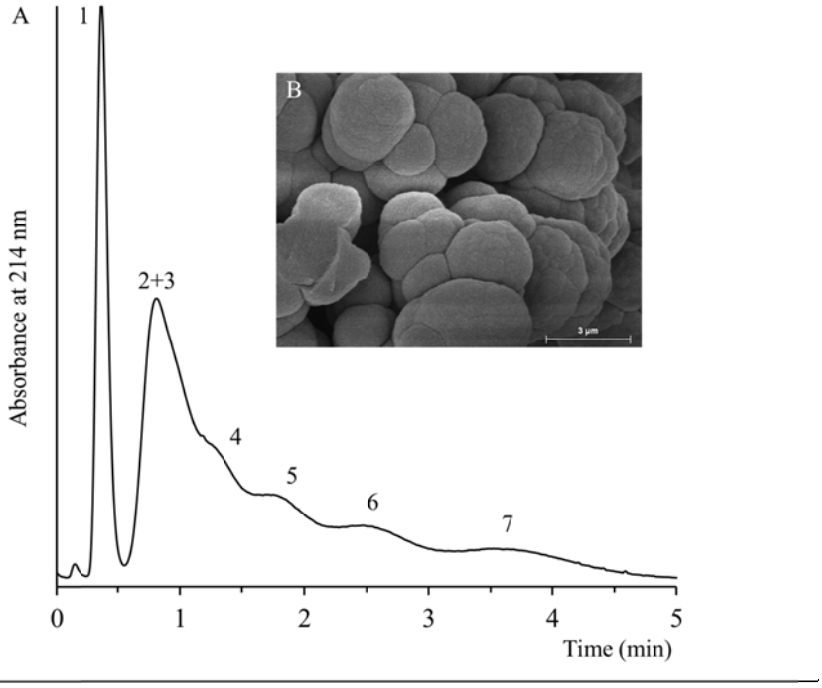

Fig. 5. Separation of alkyl benzenes (A) and SEM micrograph (B) of GMA-co-DVB monolithic column (column A12) in PTFE tubing. Working LC conditions: PTFE tubing, 100 mm × 0.8 mm i.d.; mobile phase, 50:50 (v:v) ACN:H_2O; flow rate, 0.5 mL min^{-1}; injection volume, 0.5 μL; UV at 214 nm. Peak identification: uracil (1), toluene (2), ethylbenzene (3), propylbenzene (4), butylbenzene (5), pentylbenzene (6) and hexylbenzene (7). Other details about the composition of the column A12 are given in Table S1.

In order to overcome this limitation, several authors [36-38] have studied the variation of the polymerization time in order to control both porous properties (mesoporous structure) and related efficiency of polymer monoliths. Thus, the influence of different polymerization times (from 3 to 20 h) on the separation performance of synthesized monoliths was studied. As shown in Fig. 6, an increase in polymerization time from 4 to 8 h led to a slight increase in the retention time of hexylbenzene; however, efficiency and resolution slightly worsened. When the polymerization time was increased to 12 and 16 h, the quality of separation obtained was even worse. SEM micrographs of these monolithic columns were taken (Fig. S2). As it can be seen, when the polymerization time was increased from 4 to 16 h (see Figs. S2A and S2B, respectively), an increase in the globule size was evidenced, with a probable reduction of the fraction of mesopores. Thus, these results were consistent with the surface area of the monolithic stationary phases, where 70.9 m^2 g^{-1} was achieved for a polymerization time of 4 h, whereas 33.2 m^2 g^{-1} was found when polymerization took 16 h. These results were in agreement with those previously found in several works by Svec's [8, 32, 34] and Bonn's groups [38, 39] and with other researchers in recent early-termination polymerization studies [40, 41].

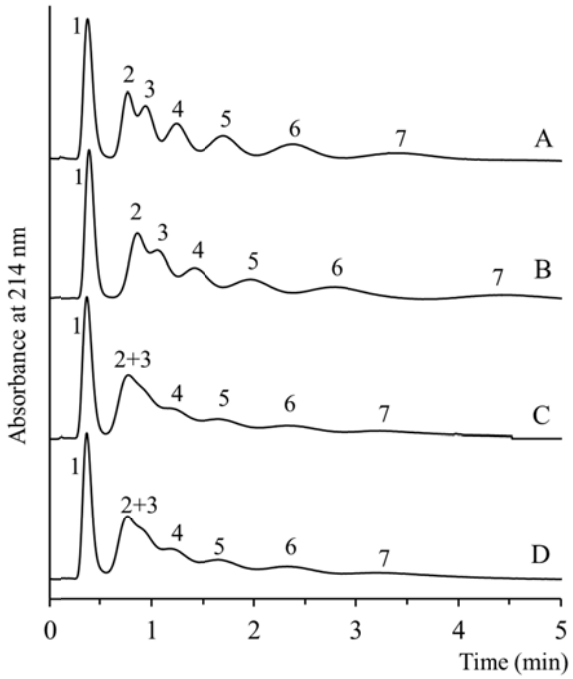

Fig. 6. Separation of alkylbenzenes in GMA-co-DVB monoliths in a PTFE tubing obtained at different polymerization times: A) 4, B) 8, C) 12 and D) 16 h. Composition of polymerization mixture and working LC conditions as in Fig. 5.

The determination of monomer and cross-linker conversion yields is an important issue in these studies to obtain both the estimation of the reaction kinetics and the evaluation of the polymerization efficiency. In this sense, the effect of the polymerization time on the monomer (GMA) and cross-linker (DVB) conversions was also studied. As shown in Fig. S3, GMA had a conversion yield close to 75% at 8 h, whereas the cross-linker was rapidly polymerized over 95% at this time. On the other hand, at a polymerization time of 4 h, the conversions of GMA and DVB were slightly above 50%. However, the monolithic column obtained at this polymerization time gave better separation efficiency (see Fig. 6A), and consequently, this time was selected for further studies.

Next, the chromatographic efficiency of this monolithic column was evaluated under isocratic conditions. Fig. 7 shows the plate height curves of several alkylbenzenes. As it can be seen, steep slopes were obtained in these curves for retained alkyl benzenes, and minimum plate heights values comprised between 70 and 80 μm were found. These efficiency values were better than those reported (100-125 μm) by Shu *et al.* [12] using poly(LMA-co-EDMA) monoliths in PEEK tubing with similar column dimensions (10 cm × 1.0 mm i.d.). However, the plate heights were higher than those recently published (32-38 μm) for poly(St-co-DVB) monolithic columns prepared in PEEK tubes [13]. Fig. 8 shows the gradient elution of alkyl benzenes using the recommended monolithic column. The flow rate was 0.5 mL min^{-1} and the gradient was 20-80% ACN in 10 min. As it can be seen, all analyte peaks were reasonably separated with a peak capacity of *ca.* 18.

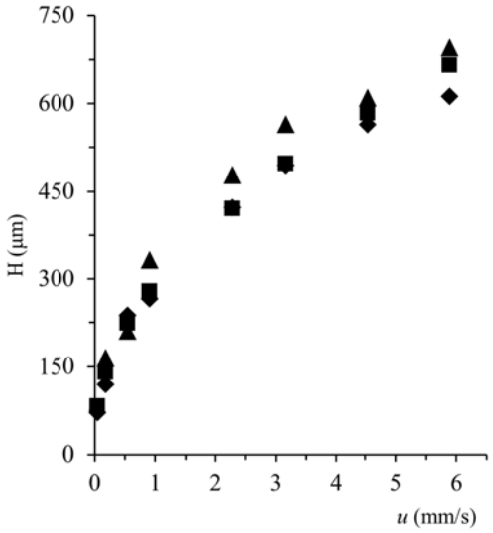

Fig. 7. Plate height curves for retained alkyl benzenes. Compounds: (◆) toluene, (■) propyl benzene, and (▲) pentyl benzene. Working LC conditions as in Fig. 5.

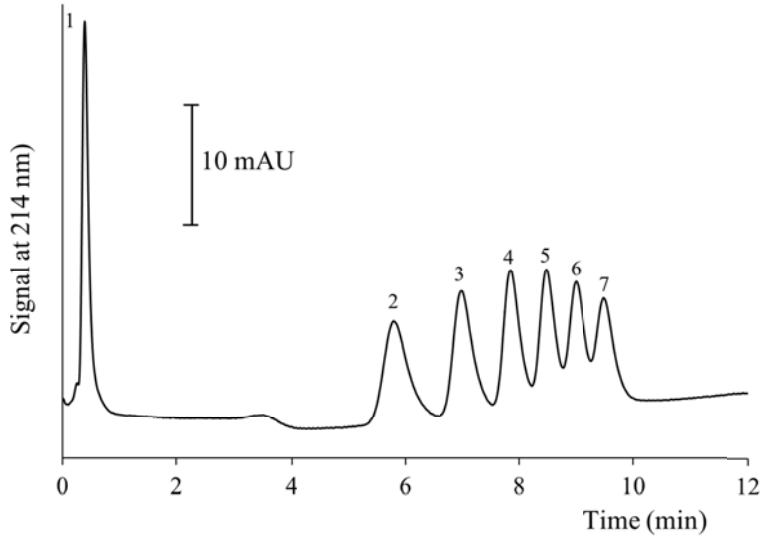

Fig. 8. Gradient separation of alkyl benzenes using a GMA-co-DVB monolithic column (polymerized for 4 h) in PTFE tubing. Working LC conditions: linear gradient from 20 to 80% ACN in 10 min; flow rate, 0.5 mL min^{-1}; UV at 214 nm. Peak identification and other details as in Fig. 5.

The optimized polymeric GMA-co-DVB column in PTFE tubing was also applied to the separation of large biological macromolecules. Fig. 9 shows the separation of a test mixture of three proteins on the 10 cm-long GMA-based monolithic column. As it can be seen, the proteins were well separated using gradient elution.

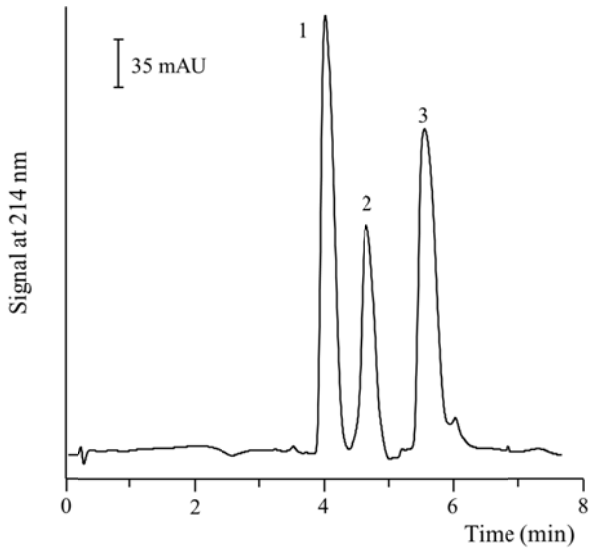

Fig. 9. Separation of proteins on GMA-co-DVB monolith in PTFE tubing. Working LC conditions: PTFE tubing, 100 mm × 0.8 mm i.d.; mobile phase, A = 0.1% aqueous TFA, B = 0.1% TFA in ACN; gradient from 5 to 70% B in A in 7.5 min; flow rate, 0.5 mL min^{-1}; injection volume, 0.5 μL; UV at 214 nm. Peak identification: (1) ribonuclease A, (2) cytochrome C and (3) myoglobin.

3.3. Repeatability studies of fabrication process

The repeatability of the fabrication process of polymeric monoliths in PTFE tubing was also evaluated by analyzing several chromatographic parameters, including run-to-run, day-to-day column (made from polymerization mixture) as well as column-to-column (prepared from different polymerization mixtures). The run-to-run repeatability was evaluated from series of six injections of the alkyl benzene test mixture at 0.5 mL min^{-1} performed on the GMA-based monolith, while the column-to-column repeatability was estimated by preparing five monoliths (which were subjected to the optimal modification protocol of PTFE tubing). As observed in Table 1, for the tested parameters, satisfactory RSD values (below 7%) were obtained in all cases.

Table 1

Repeatability and reproducibility of several chromatographic properties (expressed as RSD%) of GMA-co-DVB monoliths prepared in PTFE tubing[1].

Parameter	Repeatability	Reproducibility	
	Run-to-run column (n = 6)	Day-to-day column (n = 6, 3 days)	Column-to-column (n = 5)
t_0 (min)	0.3	0.5	1.0
$k_{pentylbenzene}$	0.4	0.7	1.1
$H_{pentylbenzene}$ (μm)	2.4	3.0	7.0

[1] Working LC conditions as in Fig. 5.

4. Conclusions

A method for chemical modification of the inner wall surface of a PTFE tubing to assure a covalent attachment of polymeric monolith has been developed. The success of binding of the monolith was demonstrated by using FTIR and SEM measurements, adhesion tests and chromatographic separations. The use of a proper mold and an adequate polymerization mixture composition was established to reduce undesirable shrinking phenomena. This study led to flexible polymers tightly attached to the PTFE surface. The resulting monolithic columns (with 1/16" o.d. and 0.8 mm i.d.) were flexible and resilient to bonding stress of PTFE tubing, and these can be easily connected to conventional HPLC systems. The monolithic columns also exhibited acceptable column efficiency, satisfactory pressure resistance (up to 20 MPa) and reproducibility. Additionally, the developed microbore columns in this work could be also employed in flow methods and other analytical methodologies for purification/preconcentration/separation purposes, which undoubtedly expand the application field of the organic monolithic stationary phases.

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