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Infrared photo-initiated fabrication of monolithic porous layer open tubular (monoPLOT) capillary columns for chromatographic applications

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Abstract

Investigation into the development of a fabrication approach for capillary porous layer open tubular (PLOT) chromatographic columns via infrared (IR) photo-initiated polymerisation and the optimisation of the technique is presented in this work. Polyimide coatings on fused silica capillaries absorb strongly in the visible region of the light spectrum making commonly used ultra-violet and visible light photo-initiated polymerisation methods impossible inside this type of capillary. In addition, polystyrene-based materials, which are commonly used as reversed phases and hydrophobic substrates in both liquid (LC) and gas chromatography (GC) also absorb strongly in the ultra-violet (UV) region making them unsuitable for polymerisation via common photo-initiated methods. However, by using infrared light, photo-polymerisation in polyimide coated capillary was made possible herein. Crucially, selecting a suitable photo-initiator with a high extinction coefficient ensures that the penetration ability of the incident light is greatly reduced thus making the technique highly suited to PLOT column fabrication. The described procedure provides a straight forward method for the photoinitiated fabrication of monoPLOT columns in polyimide coated capillary.

1. Introduction

Over the past few decades growing interest in the areas of hyphenated liquid chromatographic techniques (particularly liquid chromatography-mass spectrometry – LC-MS), miniaturisation of separation equipment, and proteomics, has led to an increased interest in open tubular and porous layer open tubular capillary column formats. These types of stationary phase exhibit high flow through permeability, potentially high separation efficiencies and good peak capacity. Recently, there has been considerable interest in new fabrication procedures for OT and PLOT columns using porous polymer phases within fused silica capillaries [1]. The vast majority of these works have used approaches involving either ultraviolet (UV) light for larger internal diameter (ID) capillaries [2-5], or thermally initiated polymerisation for the formation of polymer layers in capillary with IDs less than 25 μm [4-11]. Thermally initiated techniques for the fabrication of PLOT columns are often considered to be preferable to photo-initiated methods since they can also be applied with standard polyimide coated capillaries, which are far more robust than their Teflon coated counterparts.

However, thermally initiated polymerisation of PLOT columns becomes highly problematic in capillary IDs larger than 10 μm [12]. Recently, a highly controllable thermally initiated method for monoPLOT column fabrication was developed, which makes use of the laminar flow properties of the polymerisation mixture as it flows through the capillary [13]. Although this technique demonstrated excellent reproducibility and was highly scalable, the formation of porous layers more than a few μm thick rapidly lead to turbulent flow within the capillary and thus increasing variability of layer thickness for thicker films.

Photo-initiated polymerisation using IR light is a highly promising alternative technique to thermal initiation for the fabrication of porous polymer layers in polyimide coated capillary, however there have been very few works reported on the fabrication of polymer monolithic phases at wavelengths > 400 nm. Dulay *et al.* [14] performed the first monolithic synthesis at wavelengths outside the UV region by forming a sol-gel stationary phase in polyimide coated capillary at 470 nm. More recently, Walsh *et al.*

[15] also produced methacrylate based polymer monoliths using light at 470 nm and also in polyimide coated media using LED sources in the visible region at 660 nm [16].

Significantly, to the authors' knowledge, there has been no published reports on the fabrication of porous monolithic substrates via IR initiated (> 700 nm) polymerisation, yet photoinitiation by IR has many significant benefits. Most notably, as eluded to above, polymerisation using IR light is hugely advantageous when attempting to form monolithic phases by photoinitiation within standard polyimide coated fused silica capillary, which is the standard format for the vast majority of capillary separation methods, including capillary-LC, capillary-gas chromatography (GC), and capillary zone electrophoresis (CZE). However, polyimide absorbs strongly in the UV region, and to a lesser extent within the visible region, up to approximately 600 nm, thus controlled photoinitiated polymerisation below this wavelength is far from trivial. Furthermore, compounds such as styrene, which form the backbone of many desirable monolithic polymers for use in both LC and GC, also absorb in the UV region, thus making photoinitiation of styrene based stationary phases impossible using UV light, regardless of the type of capillary used. Added to these fundamental problems, and on a more practical level, is the fact that exposure to UV light can be hazardous, making it far safer to work within the visible and near IR regions. Thus, there exists substantial interest in new controllable methods to form porous polystyrene based substrates within polyimide coated capillaries. Therefore, herein a new method to form monoPLOT capillary columns via photoinitiation in the near IR range (830 nm) was investigated. Using IR photopolymerisation the authors explored the fabrication of PLOT columns in polyimide coated capillaries of various capillary ID, and demonstrate the application of the resultant capillary columns in capillary chromatography.

2. Experimental

Reagents and Materials

All chemicals used within this study were of reagent or analytical grade purity. Ethylene dimethacrylate (EDMA), butyl methacrylate (BuMA), styrene, divinylbenzene, 1-decanol, toluene, 3-(trimethoxysilylpropyl) methacrylate, isopropanol, *N*-methoxy-4-phenylpyridinium tetrafluoroborate (MPPTFB), trifluoroacetic acid (TFA) and proteins

used for chromatographic evaluation of prepared column (Insulin (INS), Ribonuclease B (RNase B), Trypsin (TRY), Ribonuclease A (RNase A), Cytochrome C (Cyt C)_, Myoglobin (MB), Horseradish Peroxydase (HRP), Phosphatase B (PP2), Carbonic Anhydrase (CA), Concanavalin A (Con A)) were all purchased from Sigma-Aldrich (Gillingham, UK). *N*,*N* Dimethylacrylamide, 1*H*-benz[e]indolium, 2-[2-[2-chloro-3-[(1,3-dihydro-1,1,3-trimethyl-2*H*-benz[e]indol-2-ylidene)ethylidene]-1-cyclohexen-1-yl]ethenyl] -1,1,3-trimethyl-, butyltriphenylborate (H-Nu 815), and co-initiator, (butyryl choline butyltriphenylborate) Borate V, were purchased from Spectra Group Ltd., (Milllbury, Ohio, United States). All solvents which were used for the preparation, or for the synthesis and washing of prepared monoliths, namely, sodium hydroxide (NaOH), hydrochloric acid (HCl), acetonitrile (ACN), acetone, and methanol (MeOH), were purchased from Lab Scan (Gliwice, Poland). Deionised water was supplied from a Milli-Q system (Millipore, Bedford, MA, USA). Polyimide coated (15 µm coating thickness) fused silica capillary, 100 µm ID, 0.375 mm OD was purchased from Composite Metal Services Ltd., Charlestown, United Kingdom.

Instrumentation

Capillaries were filled with monomer mixture and washed using a KDS-100-CE syringe pump (KD Scientific, Inc., Holliston, MA, USA). Photoinitiated polymerisation at 830 nm was performed in two purpose built photoreactors; these are described in detail in the supplementary information provided. A Sputter Coater S150B (BOC Edwards, Sussex, UK) was used for coating capillary monolithic stationary phase samples with a 30 nm gold layer. Scanning electron microscopy (SEM) analysis was performed on an S-3400N instrument (Hitachi, Maidenhead, UK). Optical microscopy evaluation of samples was performed on a Meiji Techno EMZ-8TR stereomicroscope (Meiji Techno UK Ltd., Somerset, United Kingdom). Separation of proteins was carried out on a Dionex Ultimate 3000 RSLC*nano* System (Dionex, Sunnyvale, CA, USA) comprising of SRD-3400 degasser, NCS-3500 RS nano LC pump module, WPS-3000TBFC Analytica autosampler with 100 n*L* injector loop and VWD-3400RS detector with a 3 n*L* cell.

Procedures

Fused silica capillaries were initially pretreated through activation of the surface silanol groups of the inner walls by sequential flushing with 1 *M* NaOH, deionised water, 0.1 *M*

HCl, deionised water, and acetone. The pretreated capillary was silanised using a 50 %wt solution of 3-(trimethoxysilylpropyl) methacrylate in toluene at 80 °C for 24 hours.

The monomer mixture consisted of 24 %wt styrene, 16 %wt divinylbenzene; the composition and ratio of both the porogen mixture and initiator was varied during the experiment, however the final mixture was 24 %wt styrene, 15.5 %wt divinylbenzene, 18 %wt acetonitrile, and 39 %wt 1-decanol. In both cases the amount of initiator was the same, 0.5 %wt H-Nu 815, 0.5 %wt Borate V, and 2.5 %wt MPPTFB (with respect to monomers). The mixture was vortexed, centrifuged for 1 min at 13,000 RPM, the supernatant removed, and deoxygenated under a flow of nitrogen for 10 min. The desired length of silanised capillary was filled with the monomer mixture and the ends of the capillary were sealed with rubber septums. Initial columns were fabricated in the box type IR reactor and in these instances the capillary was coiled, loaded into the chamber, and exposed to 2 mW/cm^2 for 26 hours. However, later the feed through reactor was used in order to produce a more uniform layer. Polymerisation in the feed through reactor was achieved with a chamber power of 13 mW/cm^2 and exposure times of 4 hours. Post curing, the resultant monolithic column was washed with MeOH at 1 µL/min for 1 hour to remove residual porogen and unreacted monomers.

3. Results and discussion

A key advantage of IR initiated reactions over UV initiated, particularly with regard to the manufacture of monoPLOT columns, is the high extinction coefficient, ε > 250,000 L/mol/cm (and thus corresponding high absorptivity) of the photoinitiators used, in this case H-Nu 815. Light in the visible and IR region has a higher penetration ability compared to UV light [17], however, the use of an initiator with a high extinction coefficient leads to the absorbance of significantly more light, and so the amount of light penetrating deep into the cavity of the capillary is greatly reduced. A high percentage of the incident light is absorbed by the polymerisation mixture at the inner walls of the capillary, and so the growth of the polymer occurs almost exclusively from the capillary wall inwards. Using the Beer-Lambert Law an approximate comparison of the

percentage of light transmitted ($\%I_O$) through a 100 μm ID capillary for three different photoinitiators can be made – see Table 1.

Table 1 Comparison of percentage of light transmission for three photo initiators

Initiator	%wt	ε (L mol^{-1} cm^{-1})	Abs (AU) @1 μm / @100 μm	%I_O @1 μm / @100 μm
DAP*	0.4	1.2 x 10^3	1.873 x 10^{-4}	99.9%
λ = 342 nm			1.873 x 10^{-2}	95.7%
H-Nu 635†	0.4	2.3 x 10^5	1.242x 10^{-2}	97.1%
λ = 635 nm			1.242	5.7%
H-Nu 815	0.4	>2.5 x 10^5	1.324 x 10^{-2}	96.9%
λ = 815 nm			1.324	4.7%

** 2,2-Dimethoxy-2-phenylacetophenone (DAP)*

† *2,4,5,7-Tetraiodo-3-hydroxy-9-cyano-6-fluorone (H-Nu 635)*

From Table 1 it can be seen that for DAP used at a wavelength of 342 nm, the optical power of the light transmitted through to the opposite side of a 100 μm ID capillary is 95.7% of that of the incident light. This is actually problematic for the fabrication of a monoPLOT column, since the optical power of the light at any location within the capillary is almost the same as that which is incident at the boundary of the capillary ID. However, for H-Nu 815, the intensity of the light at the opposite capillary wall has fallen to just 4.7% that of the incident light. Therefore, compared with photoinitiation using DAP, this approach is much more favorable for the fabrication of open tubular formats. However, this method requires the use of a light source capable of providing a very even coverage of light on all areas of the capillary simultaneously, since the penetrating ability of the IR light in such polymerisation mixtures is low. Fig. 1 presents a typical profile for a PS-DVB polymer layer formed using the H-Nu 815 initiator with a non-homogenous light source. In this case the IR light was provided from above and below the capillary using the box type IR oven described in the supplementary information – the resultant negative effect on the homogeneity of the monolith growth can be clearly seen. Interestingly this was also observed, albeit to a lesser extent, by Eeltink *et al.* in Teflon coated capillary with methacrylate monomers and using 2,2-Dimethoxy-2-phenylacetophenone as the initiator[3].

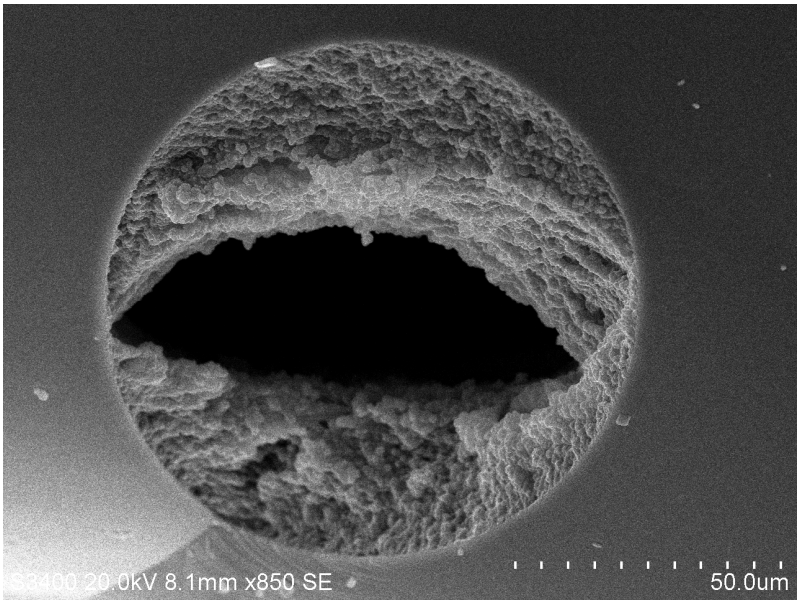
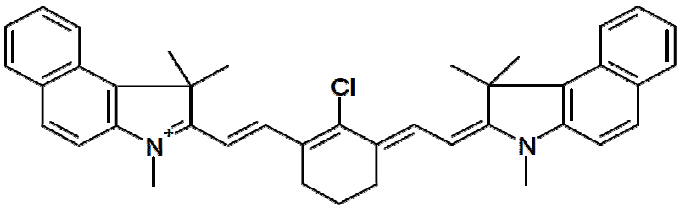


Fig. 1 SEM image of a non-uniform porous PS-DVB layer inside a 100 µm ID polyimide coated capillary due to non-homogenous light. In this case the light sources were situated above and below the capillary.

The initiator used in this work is a relatively new compound and so its reactive properties are predominantly undocumented. According to the manufacturer's information [18], in order to enhance initiator performance, H-Nu 815 should be used as a two-component mixture with co-initiator butyltriphenyl-borate (Borate V). Structures for these two compounds are shown in Fig. 2 (a) and (b).

(a)



(b)

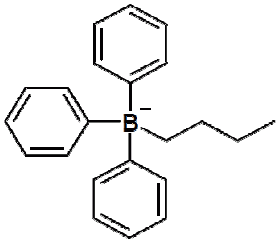


Fig. 2 Chemical structure of (a) H-Nu 815 and (b) Borate V.

The mechanism for the formation of free radicals from H-Nu 815 and Borate V can be given by:

$$Cy^{+}[RB(Ph)_3]^{-} \xrightarrow{hv} [Cy^{\bullet}] + R^{\bullet} + (Ph)_3B$$

eq. (1)[19]

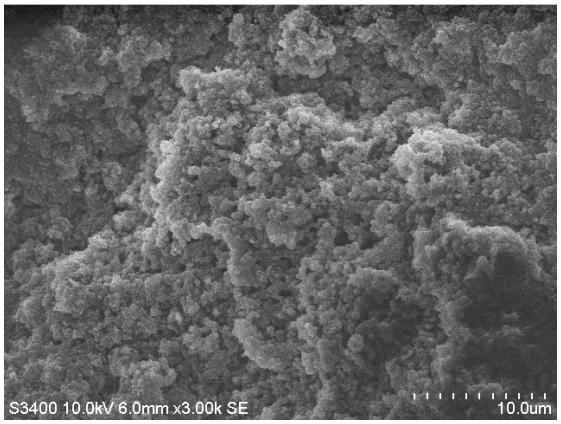
where *Cy* is H-Nu 815 and *RB(Ph)$_3$* is Borate V.

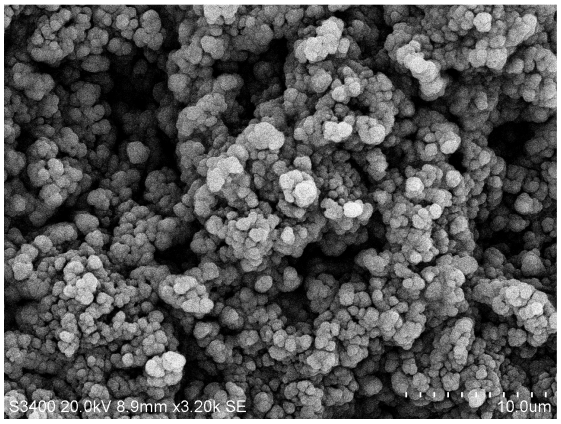
During initial photopolymerisation experiments, several problems with solubility of the initiator were discovered. According to the manufacturer's guidelines, it was expected that the initiator would be soluble in methacrylate type monomers and although the recommended solvents (1-decanol, 1,4-butanediol, 1-propanol, and *N*,*N* dimethylacrylamide) were used, a gold flake-like precipitate was observed. However, a colour change of the polymerisation mixture to dark green indicated that some initiator was getting into the solution. In early studies the supernatant of the solution was utilised for polymerisation, but it was found that results were not reproducible and in almost all cases no (or very little) polymerisation was observed. Additionally, when it was possible to fabricate a monolithic phase the porosity was extremely low and the phase itself was non-homogenous. Neither heating nor sonication of the polymerisation mixture improved solubility of the initiator.

Kabatc and Paczkowski [19] described the use of two- and three-component photoinitiator systems for visible light induced polymerisation. where five cyanine dyes were investigated as photosensitisers, in combination with one or two co-initiators. It is known that irradiation of cyanine organoborate salts with visible light results in efficient generation of free radicals [20]. However, for the systems containing dye and *N*-alkoxypyridinium salt, it was found that the latter also acts as a source of free radicals formed in the second photochemical reaction between the dye radical and the *N*-alkoxypyridinium salt. Kabatc and Paczkowski found that the use of three-component systems, comprising of cyanine dye, organoborate and *N*-alkoxypyridinium salts, were 4.05 to 8.25 times more efficient as photoinitiators compared to the systems consisting of the dye and organoborate only. Based on these findings, the same approach in the case of H-Nu 815 and Borate V system was applied herein, and 2.5 %wt *N*-methoxy-4-phenylpyridinium tetrafluoroborate (with respect to monomers) was added to the polymerisation mixture. However, unfortunately the solubility of the initiator and co-initiators remained poor, and as before little or no polymerisation was observed to occur even after exposure to 2 mW/cm^2 of IR light over a period of 26 hours.

Walsh *et al.* [21], in their work on fabrication of polymer monoliths photoinitiated at 660 nm using a sensitiser dye with a structure similar to H-Nu 815, suggested the use of a mixture of acetonitrile, isopropanol and 1-decanol as the porogen mixture for polymerisation. As no problems with solubility were reported, a similar porogen mixture was applied in this current work, comprising of 15 %wt acetonitrile, 20 %wt isopropanol, 22 %wt 1-decanol. After vortexing the mixture it was observed that the colour of solution turned clear deep emerald green, with no visible precipitate. This increased solubility is thus attributed to the increase in polarity of the porogen mixture.

The above approach resulted in complete polymerisation of the mixture, however, the resultant monolith was extremely dense. The use of polar solvents as porogens in styrene and methacrylate systems is well known to produce smaller pores, smaller globules, and denser monolith [20], as precisely observed in this case. In Fig. 3 (a) a section of monolith formed using this mixture and possessing extremely dense structure is shown. The porogen mixture was then experimentally optimised to ensure that solubility was maintained while monolith porosity was increased. The resultant optimal polymerisation mixture consisted of 24 %wt styrene, 15.5 %wt divinylbenzene, 18 %wt acetonitrile, and 39 %wt 1-decanol. In both cases the amount of initiator was the same, 0.5 %wt H-Nu 815, 0.5 %wt Borate V, and 2.5 %wt MPPTFB (with respect to monomers).

(a) 



(b)

Fig. 3 SEM images of porous PS-DVB layers formed inside a 100 µm ID polyimide coated capillary. Polymerisation mixture: 12 %wt styrene, 12 %wt vinylbenzene chloride, 15.5 %wt divinylbenzene, (a) 15 %wt acetonitrile, 20 %wt isopropanol, 22 %wt 1-decanol, and (b) 18 %wt acetonitrile, 39 %wt 1-decanol. In both cases the amount of initiator was the same, 0.5 %wt H-Nu 815, 0.5 %wt Borate V, and 2.5 %wt

MPPTFB (with respect to monomers). Polymerisation conditions: Exposed to 2 mW/cm^2 of IR light at 830 nm for 26 hrs.

From Fig. 3 (a) and (b) the difference in porosity can be very clearly seen; by removing isopropanol from the porogen mixture and optimising the acetonitrile/1-decanol ratio it was possible to produce a more desirable porous monolithic structure with larger pores and globules. From the work carried out by Kabatc *et al.* [19], it is clear that the amount of MPPTFB has a significant effect on the efficiency of the polymerisation. A further optimisation was carried out to observe the effects of the amount of MPPTFB present in the mixture, the aim being to further control the growth of the polymer layer by altering the amount of MPPTFB. The experiment was performed using between 0.5 and 2.5 %wt of MPPTFB in the same PS-DVB polymerisation mixture. Other than the percentage of MPPTFB, the polymerisation mixture remained the same, and the efficacy of the system was calculated by measuring the layer growth. Samples of capillary were filled with polymerisation mixtures containing 0.5, 1.0, 1.5, 2.0 and 2.5 %wt MPPTFB and were exposed to 13 mW/cm^2 of IR light at 830 nm for 4 hours. The samples were then washed and the layer thickness for each was measured from SEM images (n = 6 to 12). The relationship between %wt MPPTFB in the solution and layer thickness is shown in Fig. 4.

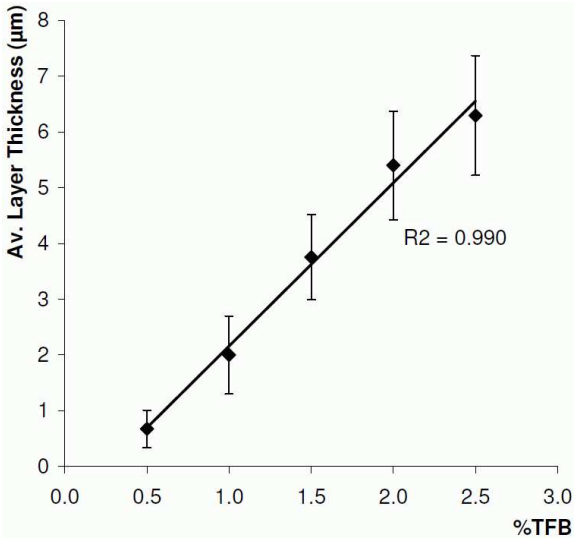
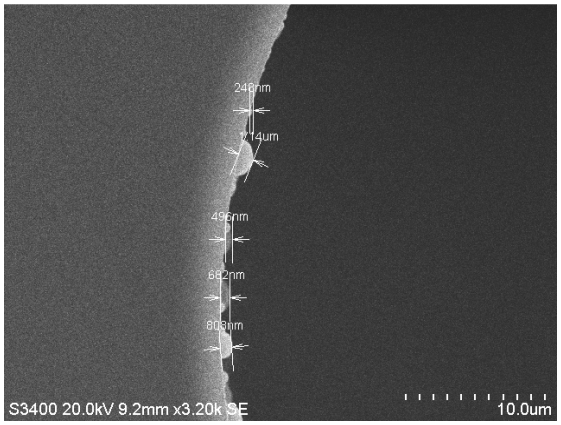


Fig. 4 Comparison of polymer layer thickness and %wt of MPPTFB in the polymerisation mixture. Polymerisation mixture: 24 %wt styrene, 15.5 %wt divinylbenzene, 18 %wt acetonitrile, and 39 %wt 1-decanol 0.5 %wt H-Nu 815, 0.5 %wt Borate V, and varying %wt MPPTFB (with respect to monomers). Polymerisation conditions: Exposed to 2 mW/cm^2 of IR light at 830 nm for 4 hrs.

As expected, with an increased amount of MPPTFB in the polymerisation mixture, the layer formation was observed to be faster, showing a linear relationship between the layer thickness and the percentage of MPPTFB. It is interesting to note that the percentage of MPPTFB in the polymerisation mixture had no noticeable effect on the morphology of the monolithic structure, with pore and globule sizes being comparable across the range of MPPTFB used, as the MPPTFB simply acts as a secondary source of free radicals [19]. Fig. 5 shows three sample SEM images of polymer layers formed using (a) 0.5 %wt, (b) 1.5 %wt, and (c) 2.5%wt MPPTFB, where it can be seen that the morphology of the monoliths was similar in each case.

(a)



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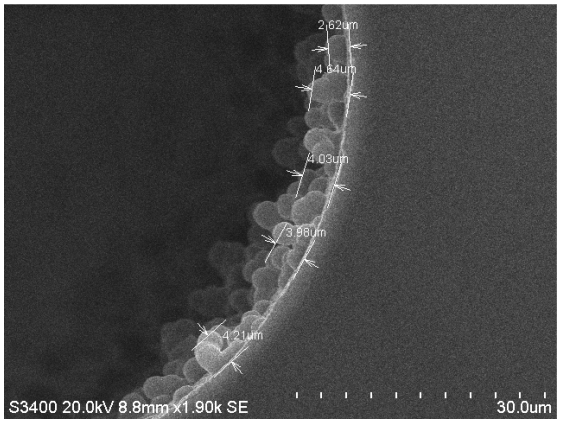

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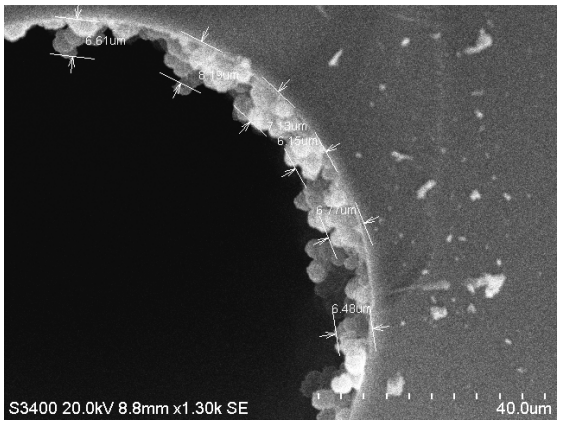

Fig. 5 *SEM images of PS-DVB polymer layer formed within a 100 µm ID capillary using (a) 0.5%$_{wt}$, (b) 1.5 %$_{wt}$, and (c) 2.5%$_{wt}$ TFB. Polymerisation mixture and conditions as per Fig. 4.*

The above experiments were considered extremely promising for obtaining controlled layer thickness in PS-DVB monoPLOT columns formed within polyimide coated capillaries, something which previous to this work could not readily be achieved.

Initial Chromatographic Performance Evaluation

To demonstrate the practical application of these new IR prepared monoPLOT columns, an initial (unoptimised) capillary LC separation was also carried out for a mixture of proteins (Insulin, Ribonuclease B, Trypsin, Ribonuclease A, Cytochrome C, Myoglobin, Horseradish Peroxydase, Phosphatase B, Carbonic Anhydrase, Concanavalin A). The column used was a reversed-phase PS-DVB monoPLOT capillary column, 100 µm ID x 30 cm long, with a layer thickness of approximately 2 µm. A 45 minute, acetonitrile-water (0.1% TFA constant) mobile phase gradient was applied from 1 to 90% acetonitrile. Measured back pressure was < 1.9 bar. The low pressure separation of these ten proteins on the column can be seen in Fig. 6.

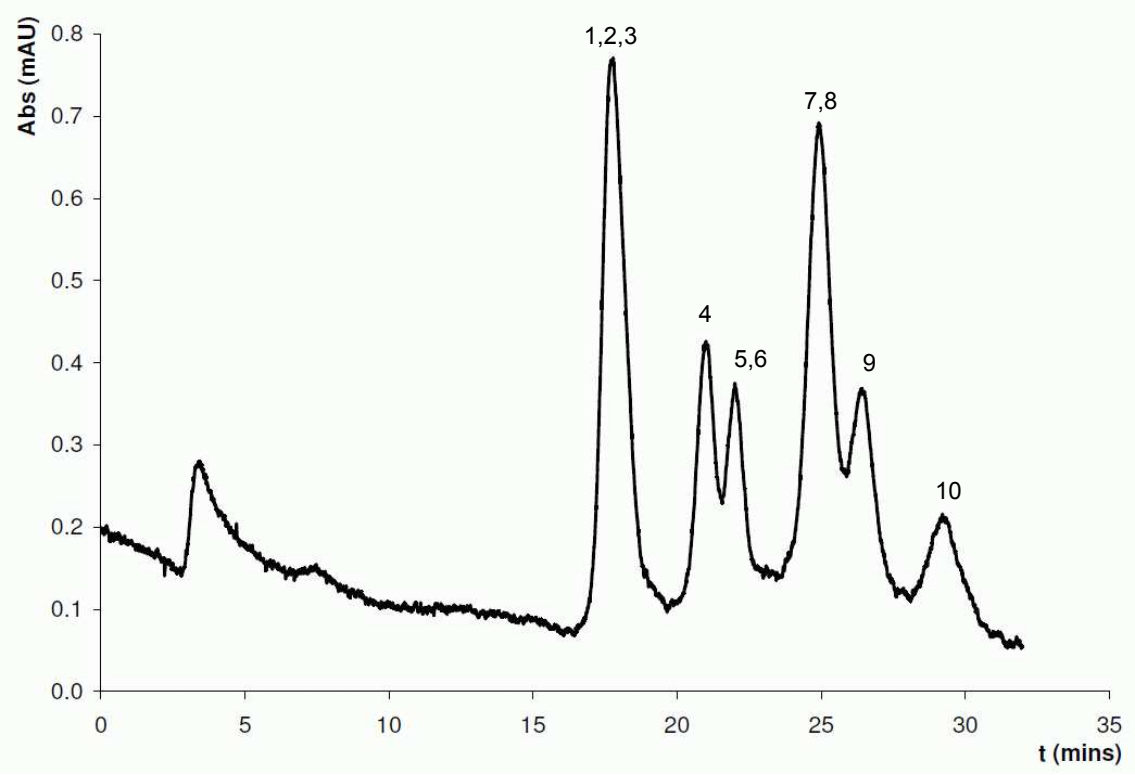

Fig. 6 Separation of ten proteins (1. INS, 2. RNase B, 3. TRY, 4. RNase A, 5. Cyt C, 6. MB, 7. HRP, 8. PP2, 9. CA, and 10. Con A) using a constant flow rate of 0.9 µL/min with a 45 min gradient from 1 to 90% acetonitrile. Column: 30 cm x 100 µm ID PS-DVB monoPLOT column, layer thickness ~2 µm. Mobile phase: ACN/H_2O gradient from 1 to 90% with 0.1% TFA at a flow rate of 0.9 µL/min. UV detection at 214 nm.

The purpose of this demonstration was not to optimise the separation, but instead to confirm that a viable reversed-phase layer existed in the capillary and that a separation

of a simple mixture was possible. A stability study was carried out for the fabricated column and it was found that the column was stable for over 30,000 column volumes. The retention time RSD% was measured to be ~0.9 % for the longest retained peak (ConA, *k*' = 6.61). Column-to column reproducibility was also investigated via measuring layer thickness by scanning C4D (sC4D). It was found that the RSD% of the layer thickness for each individual column was approximately 2 % (*n* = 30), while the RSD% between each column was 5 – 7 % (*n* = 6) depending on layer thickness; columns with thicker layers were observed to have higher layer thickness RSD%. The observed results were extremely promising, considering the relatively wide bore of this particular monoPLOT column and it's very short length compared to those usually used for LC separations [4-9]. Greater chromatographic evaluation of this new capillary column technology in both cap-LC and GC is currently underway.

4. Conclusions

The results presented herein demonstrate a new technology and approach for the fabrication of monoPLOT columns by photoinitiation, with precisely controlled layer thickness and length. The fine control of the monolith morphology and the formation of PS-DVB polymer layers within polyimide coated fused silica capillaries has been demonstrated using photoinitiated polymerisation from IR light at 830 nm. These novel procedures show great potential and open up many more possibilities for the fabrication of monoPLOT columns, whose application within various modes of capillary chromatography is sure to increase rapidly. The work also provides some interesting insights into the polymerisation process and the various user-controlled effects that can be employed during polymerisation.

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Notes: The authors declare no competing financial interest.

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