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Preliminary investigation into the use of porous polysquaraine particles for adsorption chromatography

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Abstract

Previous batch and column adsorption studies on poly(1-methylpyrrol-2-ylsquaraine), or PMPS, particles have shown that these particles have adsorptive preference for basic over acidic and aromatic over aliphatic in terms of their uptake of organic molecules. These preferences of acidity have been exploited in this study in simple adsorption chromatography experiments through which the separation of mixed solutions of phenol, acetophenone, and pyridine, elution in that order, from chromatographic columns of PMPS particles has been achieved. Furthermore, elution of lysine followed by phenylalanine, from chromatographic columns of PMPS particles, confirms the difference in interaction in PMPS particles for aromatic molecules over aliphatic molecules and that it has the potential to be exploited for molecular separation. Although PMPS particles are spherical in shape and 1.3 – 4 μm in diameter, they are prepared in reasonable yield in highly aggregated clusters and for the purposes of both adsorption capacity and mobile phase flow it was found that their usage in chromatographic columns in these clusters is preferred over their monodisperse (or de-aggregated) form.

1. Introduction

Chromatography is a separation technique based on compounds having different affinities towards a stationary phase and the mobile phase and can be classified according to the type of equilibrium process that is involved. This type of process, i.e. adsorption, partition, ion-exchange or size exclusion, is governed by both the chemical and physical nature of the stationary phase [1]. Adsorption Chromatography is one of the oldest types of chromatography. It utilises a mobile phase, either a liquid or gas, that is adsorbed onto the surface of a stationary phase. The components distribute between the two phases through a combination of adsorption and desorption processes. The equilibration that occurs between the mobile and stationary phases accounts for the separation of the different solutes. Size exclusion chromatography lacks an attractive interaction between the stationary phase and solute. The stationary phase is porous and the mobile phase passes through this, which separates the molecules according to their size. The pores are normally small and hence exclude the larger solute molecules; smaller molecules can enter the stationary phase matrix. The molecules that enter the matrix must flow

through a larger volume to exit the column. This causes the larger molecules to pass through the column at a faster rate than the smaller ones [2].

Commercially, stationary phases are silica based because of their high chromatographic efficiency (due to high surface areas) and high mechanical strength [3]. Silica based stationary phases that have octyl and octadecyl groups attached are widely used in liquid chromatography [4]. The octyl and octadecyl groups provide good retention, good resolution and good reproducibility of results for a wide range of analytes [5]. In addition to this, modified silicas that contain alkyl chains with polar groups embedded in them have been developed [6]. In recent years there has been a focus on the development of so-called 'smart materials' for stationary phases [7]. These include, among other things, hybrid organic / inorganic stationary phases formed from the mixed condensation of two high purity monomers, or stationary phases with TWIN (Two-In-One) technology. During the final stage of silica manufacturing a silica-organic layer is embedded to create a completely composite core-shell particle. This provides a column that has excellent efficiency [8], and the silica-organic shell protects the particle from chemical attack. Such core-shell smart materials also include pH responsive stationary phase materials based on polymer modified silica spheres [9]. Stationary phases that are used to separate chiral compounds are often based on proteins, cellulose derivatives, and macrocyclic antibiotics [10, 11]. These stationary phases contain said ligands immobilized onto a silica core material.

Nanostructured materials, particularly carbon-based nanomaterials, have also been under investigation for application in adsorption chromatography [12 – 15], including those that are in the form of stationary phase monoliths [16 – 18], consisting of a single porous structure instead of separate particles. These monolithic columns have been found to have advantageous characteristics such as ease of fabrication, versatility for a variety of surface chemistries, and good permeability. Thus far carbon-based nanomaterials have primarily included fullerenes and carbon nanotubes [12 – 14] although other types of porous carbon-based media for column chromatography involve expanded carbohydrates [19] and poly(methyl acrylate) [20] polymers, for example. Polymeric chemistries have the potential to give rise to new and exciting possibilities in the development of adsorption and separation technologies.

Poly(1-methylpyrrol-2-ylsquaraine), or PMPS (**Figure 1**), particles are blue-black colored, spherical, 1.3 – 4 μm diameter, porous nanostructured organic-based polymer materials [21, 22] whose porosity and adsorption characteristics have only recently been reported [23, 24], despite their first synthesis being recorded in 1965 [25]. Observation of their spherical particulate nature was also only recently reported in 2005 [21] and a detailed study of their particle size control has only just been published [26]. In terms of their adsorption characteristics, a detailed study of the adsorption of metal ion species into PMPS particles [23] was followed up by a study investigating the adsorption of four organic molecules, phenol, maleic acid, pyridine, and paraquat, into PMPS particles [24]. PMPS particles have also recently been reported for their adsorption of water [27], in terms of a humidity sensor, and estradiol-type endocrine disruptors from aqueous solution [28]. In each of these cases it was the adsorption characteristics of PMPS particles that were under investigation; thus far these particles have not been investigated for any chromatography, or molecular separation, applications even though column experiments were used in these previous studies by which the adsorption capacity and loading profile could be examined in a continuous flow system. Here we report a preliminary investigation into the potential for nanostructured PMPS particles to be used in chromatographic separation techniques by applying mixed solutions of phenol, acetophenone, and pyridine (**Figure 1**), as well as lysine and phenylalanine (**Figure 1**) to a packed column of PMPS particles. An investigation into the use of monodisperse verses aggregated PMPS particles for the column stationary phase is also reported as a

preliminary investigation into determining whether or not to pursue the preparation of a PMPS monolith stationary phase.

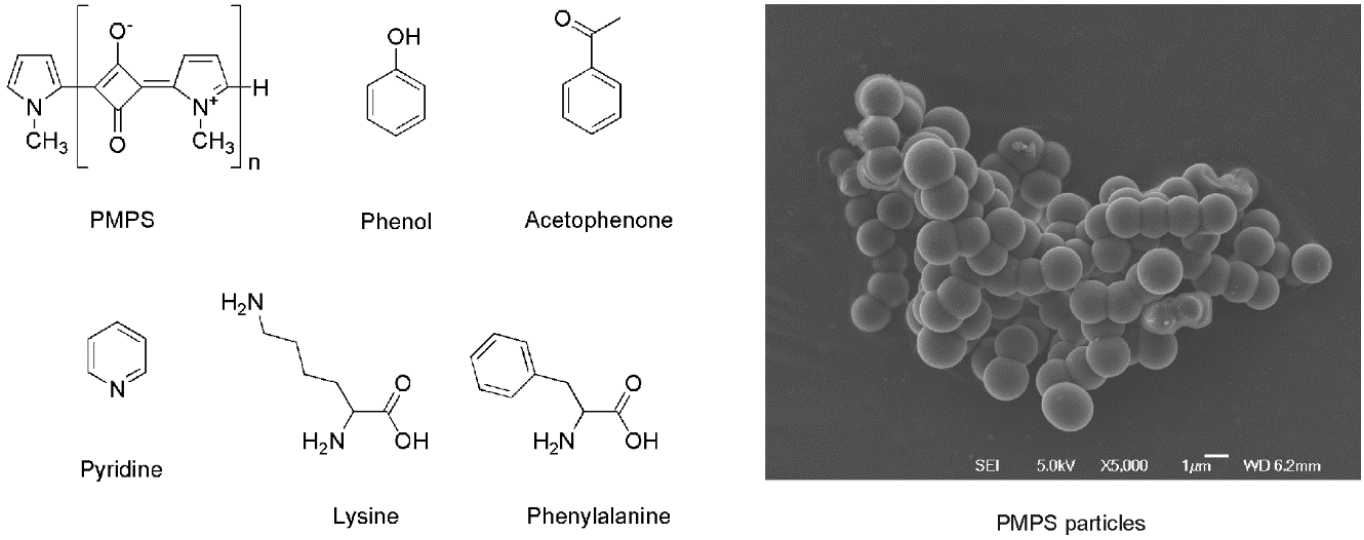

Figure 1. Chemical diagrams and an SEM image of the PMPS particles

2. Methodology

2.1 Materials

All chemicals were purchased from Sigma-Aldrich and were used as-received. All water used in this study was reverse osmosis water. Poly(1-methylpyrrol-2-ylsquaraine) particles were prepared according to the literature procedure [26] by refluxing equimolar amounts of squaric acid and 1-methylpyrrole at a concentration of 175 mmol.L^{-1} in 2.5 L butanol for 6 h, filtering, and then thrice repeated washings, firstly with 3 L of 45 mmol/L aqueous ammonia solution followed twice by 3 L water, before drying in a heated cabinet (60°C).

2.2 Mobile Phase

The mobile phase used for all column experiments was a water / acetonitrile (ACN) gradient. The gradient profile for the column experiments with phenol, acetophenone, and pyridine is tabulated in **Table 1**, whereas the gradient profile for the column experiments with lysine and phenylalanine is tabulated in **Table 2**. A water / ACN gradient was chosen as it is suitable for reverse phase chromatography and is commonly used throughout the pharmaceutical industry. The flow rate throughout the phenol, acetophenone, and pyridine column experiments was 6.5 mL.min^{-1}, whereas the flow rate for the lysine and phenylalanine column experiments was reduced to 5 mL.min^{-1}.

Table 1. Water / ACN gradient for the phenol, acetophenone, and pyridine column experiments

Time (minutes)	% ACN
0.00	2
8.00	99
13.00	99
13.10	2
15.00 (end)	2

Table 2. Water / ACN gradient for the lysine and phenylalanine column experiments

Time (minutes)	% ACN
5.00	0
10.00	2
15.00	4
20.00	6
25.00	8
40.00	10
45.00	8
50.00	6
55.00	4
60.00	2
65.00 (end)	0

2.3 Equipment

The equipment used was a Bio-Rad BioLogic LP System. This is a low-pressure system that is primarily designed for the separation of proteins. The instrument set-up consisted of a peristaltic pump, with a mixing valve, a single wavelength UV detector and LP Data View software. The UV detector was a single beam fixed wavelength absorbance detector specifically designed for protein chromatography. The optics module consisted of a low-pressure mercury lamp with 254 nm and 280 nm filters, a flow cell with a 2 mm path length, 80 µL internal volume and an illuminated volume of 3 µL. All tubing used other than peristaltic pump tubing was Tygon with an internal diameter of 1.6 mm. The peristaltic pump tubing was silicone with an internal diameter of 1.5 mm.

2.4 Column preparation

Columns were prepared by forming a slurry of PMPS particles in water, which was then poured into a Bio-Rad glass econo-column tube with an internal diameter of 1.0 cm and a porous support at the bottom of the column. Columns were then secured upright in a clamp and allowed to settle overnight (16 h) with a final column height of 10 - 11cm. Water was then pumped through the LP system and column for several hours until the baseline was stable. Once this was achieved with water, ACN was then pumped through to ensure each column was stable under both water and ACN environments. No change in column height was observed, for any column, following settling and baseline stabilization. Two examples of a stable baseline recorded over the 15 min solvent gradient profile for two separate columns are shown in the Electronic supplementary material.

2.5 Sample preparation

Samples for the phenol, acetophenone, and pyridine column experiments were prepared as ~10 $mg.mL^{-1}$ aqueous solutions and introduced onto a column using a 1 mL injection loop, with the UV detector set at 254 nm. An aqueous solution of DL-lysine and ninhydrin was prepared at 1 $mg.mL^{-1}$ concentration for each component while an aqueous solution of DL-phenylalanine and ninhydrin was prepared at 4 $mg.mL^{-1}$ concentration for each component. These solutions plus an equi-volume mixture of the two were introduced onto a column using a 1 mL injection loop, with the UV detector set at 254 nm.

3. Results and Discussion

3.1 Monodisperse verses aggregated PMPS particles

One of the advantages of using silica-based materials for the stationary phase in chromatography columns is that the types of silica particles utilized have mechanical hardness, and although they can close-pack within a column there are still defined interstices through which the mobile phase can flow. PMPS particles are not hard and display at least 65% mean deformation at rupture under compression (refer **Supplementary material**). Also, PMPS particles prepared at the concentration stated in section *2.1* are aggregated (refer **Figure 1**) with an approximate d_{50} of 60 – 70μm (refer **Supplementary material**). Previous column adsorption studies on PMPS particles [24, 28] were undertaken without any pre-treatment to disrupt the PMPS aggregates and there were no reported issues with solvent flow through these columns. However, as a pre-cursor to investigating chromatographic separation columns there is a requirement to investigate adsorption into monodisperse verses aggregated particles as well as solvent flow through columns made from monodisperse particles.

PMPS particles with minimal aggregation can be prepared, with decreased size and yield of product, at concentrations at least ten times lower than that reported in this paper but for consistency it was decided to de-aggregate PMPS particles made for this study using a ball mill. The resultant powder was very fine and not too dissimilar to activated carbon powder thus care was taken to contain the dust. A comparison between the adsorption capacities of both types of PMPS particles was undertaken using aqueous ammonium phosphate (prepared by adding ammonia solution to phosphoric acid solution, as detailed in the **Supplementary material**) because ammonium phosphate has one of the highest affinities for adsorption into PMPS particles thus far recorded [23]. This experiment found that the adsorption of ammonium phosphate into monodisperse (milled) PMPS particles was ~22% less than the levels of adsorption measured in the aggregated particles. It is possible that milling the particles closes up / collapses a percentage of pores in the particles although the flexibility of these particles should counteract that action. Furthermore, columns made of the monodisperse particles did not allow any solvent flow at low pressure once the powder had settled. This may be due to an artefact of PMPS production where aggregated particles grow into each other and are not perfectly spherical, thus allowing for a tighter packing arrangement. Columns prepared using the aggregated PMPS particles maintained a constant flow rate, of either solvent, without any significant back pressure. Overall, there was no apparent advantage to de-aggregating the PMPS particles as produced from the reaction described in section *2.1*.

3.2 Phenol, acetophenone, and pyridine column experiments

In a recent paper investigating both the batch and column adsorption of phenol, maleic acid, pyridine, and paraquat into PMPS particles the results showed that the particles adsorb little amount of maleic acid, roughly equal amounts of phenol and pyridine, and high amounts of paraquat [24]. These results gave rise to a preliminary model of adsorption where not only basicity of the analyte is favoured as well as aromaticity and charge. Little different between the adsorption of phenol and pyridine indicates that aromaticity has a stronger influence than acidity. For this reason, acetophenone was added to this current study as a neutral aromatic molecule to offer, with phenol and pyridine, three small aromatic molecules of differing acidity to target the possibility of separation down a PMPS column on this basis. Individual runs of solutions of the three molecules gave consistent retention times of ~5 mins for phenol, ~7 mins for acetophenone, and ~8.5 mins for pyridine so a mixed solution of all three (comprising ~10 $mg.mL^{-1}$ of each analyte) was run through a column and a representative trace is shown

in **Figure 2**. This elution order, although expected in order of acidity, needs to be coupled with the ACN concentration in the mobile phase. In other words, pyridine (having a higher affinity towards the PMPS particles) required an increased ACN concentration to elute, whereas the phenol was more easily eluted under an aqueous mobile phase; with the acetophenone somewhere in between the two.

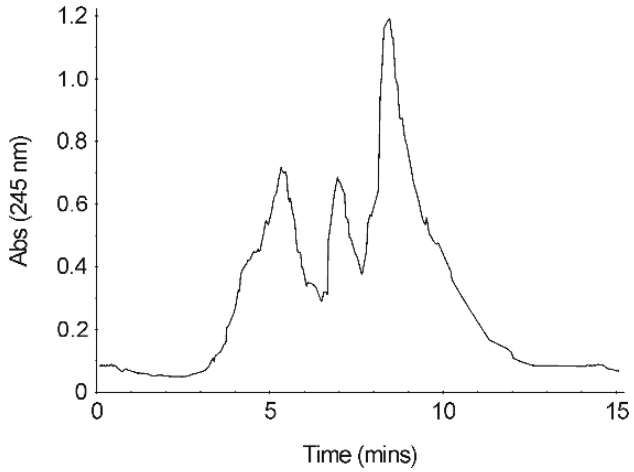

Figure 2. Chromatographic trace of phenol, acetophenone and pyridine (respectively) from a column of PMPS particles

The chromatogram in **Figure 2** is not baseline resolved but three peaks can be observed. Although all three components were added in approximately the same weight concentration there was not molar equivalence with pyridine having an increased molar presence due to its lower molecular weight. Pyridine was also used as received and was not re-distilled beforehand and would therefore have an increased molar absorptivity increasing the size of the pyridine peak. Interestingly, the pyridine peak shows some peak tailing, which is a common trait for basic compounds eluted through silica-based columns. Complete baseline resolution could not be obtained with simple alteration of the gradient profile, but the results shown in **Figure 2** were repeatable.

3.3 Lysine and phenylalanine column experiments

Racemic mixtures of both D and L enantiomers for both lysine and phenylalanine were used because the PMPS particles were not expected to separate chiral enantiomers. However, considering that lysine is a basic aliphatic amino acid (containing a side chain lysyl ($(CH_2)_4NH_2$) group) and phenylalanine is a neutral amino acid containing a benzyl group then separation by PMPS particles on the grounds of aromaticity was considered possible (even though both molecules had been pre-reacted with ninhydrin). Both amino acids were examined as simple aqueous solutions with the addition of ninhydrin to enhance detection, particularly for the lysine. The percentage of ACN in the mobile phase was also lowered to keep more emphasis on water as the eluent but this ultimately led to an increase in cycle time to 65 minutes. Sample concentrations were also decreased to keep the absorbance (at 245 nm) below 2.0. Multiple runs for lysine showed consistency of elution ~5 mins with a relatively sharp peak. Phenylalanine was also consistent in elution time (over multiple runs) but was stretched over 15 – 25 mins. A representative trace of the mixed solution is shown in **Figure 3**, which was also repeatable. Again, baseline resolution between the eluents was not obtained. Interestingly, solutions of DL-histidine (mixed with ninhydrin) eluted similarly to phenylalanine.

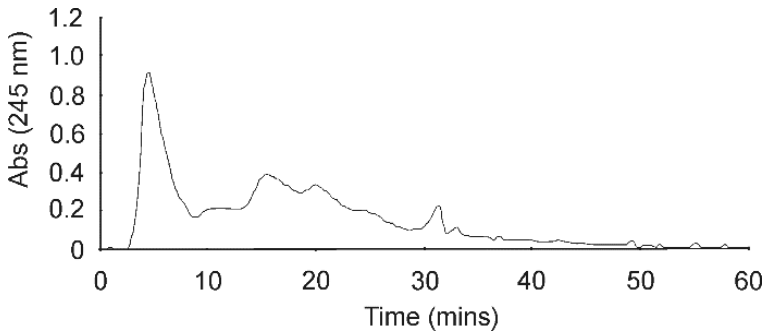

Figure 3. Chromatographic trace of lysine and phenylalanine (respectively) from a column of PMPS particles

These experiments confirm the difference in interaction in PMPS particles for aromatic (and potentially heterocyclic) molecules over aliphatic molecules, as evidenced in reference [24]. However, the specific nature of interaction between PMPS particles and aromatic compounds is still not fully understood. The peak tailing in **Figure 2** for pyridine was not unexpected whereas the extension of phenylalanine elution observed in **Figure 3** cannot be easily explained. Obviously, more work is needed to improve overall peak resolution in PMPS columns but the attraction of phenylalanine to the PMPS particles is like that observed for paraquat in reference [24]. In equilibrium adsorption studies for paraquat in PMPS particles it was found that although the majority of adsorption occurred instantaneously there was also a continued adsorption occurring at a slower rate over several hours. It was suggested that this was caused by the paraquat migrating deeper into the PMPS particles. Such behaviour also for phenylalanine could account for the prolonged elution. Unequal adsorption characteristics may indicate certain limitations to the use of PMPS particles as the solid phase in adsorption chromatography although a deeper understanding of these limitations may lead to other opportunities for their use.

Conclusion

Examination of the adsorptive capacity of PMPS particles in either their post-reaction aggregated state or monodisperse (ball-milled de-aggregated) form through conducting adsorption experiments of ammonium phosphate from aqueous solution gave results indicating that there was no advantage (in terms of adsorptive capacity) to de-aggregating the particles. This preliminary result suggests that there is potential in working towards creating a PMPS monolith stationary phase. However, chromatographic columns of the monodisperse particles were completely blocked and did not allow for the flow of an aqueous mobile phase whereas similar columns made of the aggregated material allowed flow without the build-up of any significant back pressure. Chromatographic columns of aggregated PMPS particles could produce a stable baseline upon aqueous mobile phase flow cycling through to a full acetonitrile flow and then returning back to water. Individual loadings of phenol, acetophenone, and pyridine in aqueous solutions onto the columns gave reproducible elution times of increasing value (respectively) that were then reflected in chromatograms of mixed solutions, although full baseline resolution between eluents could not be achieved. Furthermore, individual loadings of lysine and phenylalanine in aqueous solutions containing ninhydrin onto the columns gave reproducible elution times that were also reflected in chromatograms of mixed solutions to the two amino acids. These results indicate the potential of PMPS particles for use in the molecular separation of compounds with specific chemical differences and that further studies in this area are worth undertaking.

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