Insoluble electrospun membranes for analyte pre-concentration in saliva

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***Abstract*— Insoluble electrospun membrane based on poly acrylic acid (PAA) crosslinked with ethylene glycol (EG) was prepared. The surface of the crosslinked PAA fibers was then grafted with polyethylenimine (PEI), octyl amine (OA) and EG using carbodiimide or thermal crosslinking to obtain insoluble membranes having anionic, cationic, hydrophobic and neutral characteristics for filtration of biomolecules from saliva. The resulting surface chemistries were confirmed by their respective ATR-FTIR spectra. Differences in fiber morphology and diameters were observed between the membranes. All the membrane variants were tested for their efficacy as solid phase extraction (SPE) matrices in the pre-concentration of ethanol in synthetic saliva, using a microfluidic chip. Gas chromatography assays showed no statistical difference for ethanol in eluents through each of the tested insoluble electrospun membrane variants versus their corresponding controls. Further studies would include the testing of these membranes in the pre-concentration of clinical biomolecules, including drugs of abuse (e.g., cocaine, THC), nutrients (e.g., glucose), proteins (e.g., albumin, HbA1C) and nucleic acids for sample preparation within point of care devices.**

I. INTRODUCTION

Electrospinning produces nanoscale fibrous membranes having large specific surface area (1 to 35 m^2/g), excellent interconnectivity of pores, potential to incorporate active chemistry and functionality at nanoscale for use in multiple fields [1]. For example, they are used in air and water filters, electronics and sensors, drug therapy, tissue engineering and regenerative medicine [2-6]. However, a niche area for electrospun membranes that is still in its infancy is the fabrication of insoluble electrospun membranes that do not degrade within water and other solvent systems.

For electrospinning, usually, high molecular weight polymers that are soluble in some solvent (water or organic) is required. Essentially, their polymer solubility can limit the scope for applications. This is especially important in the case of filtration and solid-phase extraction/separation membranes, wherein the membranes are required to be insoluble and non-degradable. Electrospun membranes are made insoluble by crosslinking. They are usually prepared by electrospinning polymer solutions, and crosslinking affected after electrospinning. Protein-based polymers, polyamines and PVA are crosslinked by glutaraldehyde (GA), either by immersing them in GA solutions in their corresponding

non-solvents (e.g., acetone or ethanol for PVA), or by incubating them in GA vapor environment [7, 8]. Another approach is to spin polymers mixed with a crosslinker and affecting crosslinking by heat treatment [9], or ageing of the spinning solution [10].

The use of electrospun membranes for solid phase extraction is still underdeveloped and as such can provide novel advantages, one of which being incorporation within microfluidic chips for better point of care devices [11]. The insoluble electrospun membranes could be easily manufactured and cost-effective with the surface properties exhibited by the polymer itself or modified via surface treatments providing an ability to separate out analytes of interest or interference molecules from the biological media. The most common methods for SPE use simple binding interactions through the 'use of van der Waals forces, hydrogen bonding, dipole-dipole forces and cation-anion interactions' [12]. Therefore depending on the analyte of interest the polymer used for electrospinning or surface modification upon the membrane would make for an efficient pre-treatment tool.

In this study, we report an insoluble electrospun membrane based on polyacrylic acid crosslinked with ethylene glycol, similar to that reported by Meng et al. [9]. We further modified these membranes, by grafting the anionic PAA-EG fiber surface with different chemical moieties to obtain cationic, neutral and hydrophobic insoluble electrospun membranes. The membranes were characterized and tested for their efficacy as solid phase separation matrices for pre-concentration of ethanol in synthetic saliva as a model clinical analyte within a complex biological fluid in a microfluidic point-of-care platform for medical diagnostics.

II. Materials and Methods

All chemicals unless otherwise stated were purchased from Sigma Aldrich UK.

A. Electrospinning Polyacrylic acid - Ethylene glycol and their Crosslinking

Ethanolic solution of PAA (Mw 450,000) containing EG (for crosslinking) were electrospun, the method modified from that reported by Meng et al. [9]. PAA was dissolved in absolute ethanol (Fisher) and left stirring for 48 h. Then, the crosslinker ethylene glycol (EG) was added. The final solution had 4 wt% PAA, and 12 wt% of EG relative to PAA. Just before electrospinning, 50 µl of 1 M sulphuric acid, per ml of PAA-EG solution, was added to activate EG for crosslinking. It was important to maintain the polymer solution temperature below 10^{o}C throughout the electrospinning process. The solution was loaded into 5ml leur-lock syringe and electrospun using the parameters: 22 Gauge needle, flat plate grounded collector, 20 cm distance between needle and collector, 0.8ml/hr flow rate, 15-20.5kV applied voltage, humidity less than 10% and a low air flow was produced over the collector to remove residual charge of the PAA-EG fibres. The electrospun PAA-EG membranes were then heated at 130^{0}C for 30 minutes to complete the crosslinking.

B. Surface modification

The anionic surface on PAA-EG membranes was modified by covalently grafting octyl amine (OA), polyethylenimine (PEI, branched Mw. 2500), and EG to obtain hydrophobic, cationic and neutral insoluble electrospun membranes. For the grafting, carbodiimide crosslinking between –COOH on PAA and $–NH_2$ on OA and PEI groups was used. Stock

solutions of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide) (EDC) and N-hydroxysuccinimide (NHS) in 0.05M 2-morpholinoethane sulfonic acid (MES) solution were prepared and pH adjusted to 5.3 using 1N NaOH and/or 1N HCl. PAA membranes (8mm discs) were also equilibrated in MES buffer (pH 5.3) containing OA (0.1M) or PEI (0.1 wt%). All handling of EDC solutions was done on ice [13]. EDC and NHS were added to the PAA membranes in MES buffer such that the final solution concentration of each of EDC and NHS was 0.1M. The resulting solution was incubated on ice for 1 h before transfer to room temp, wherein they were left overnight for the crosslinking reaction to complete on a shaker at 150rpm. Following crosslinking the samples were washed in DI water, and stored at 4^0C until further use. For grafting EG on PAA-EG membranes, the membranes were saturated with 10% EG solution in DI water, and heated at 130^oC for 30 min to obtain neutral surfaced insoluble electrospun membranes.

C. Characterization of Membrane Properties

The surface chemistry of the different membrane variants was confirmed by recording their ATR-FTIR spectra using a Perkin Elmer Spectrum One Fourier Transform Infrared (FTIR) spectrometer equipped with a Specac Golden Gate ATR accessory. Each spectrum, acquired in transmittance mode, was an average of 128 scans at a resolution of 4 cm^{-1}.

The electrospun membranes were also sputter coated for 30 s with gold using an AGAR high resolution sputter-coater and observed using a JEOL JCM-6000 NeoScope Benchtop SEM for morphology.

The fiber diameters were measured on SEM images using Image J software. A minimum of 60 measurements were made on five different SEM images, each representing a non-overlapping random field of view for each electrospun membrane configuration.

D. Microfluidic Chip Fabrication

Microfluidic molds were designed using Solidworks CAD software and saved as STL files. The molds were created using 3D printing techniques with an Objet 30 Pro (Stratasys, US) using jetted photopolymer deposition. QSil 218 PDMS (ACC Silicones, UK) was mixed at a ratio of 1:10 for 2 minutes and then degassed using a centrifuge for about 4 minutes. The molds were cleaned by rinsing with iso-propyl alcohol and then DI water to remove contaminants; they were then dried using nitrogen. The mold was placed into a custom made stainless steel frame which was cleaned in the same manner. After degassing the PDMS it is poured gently into the mold and left in the oven at a set temperature of 45° C for 4 hours. The cured PDMS was removed from the mold; a handheld corona treater, BD20-AC (Electro-Technic Products Inc., US), was used to bond the PDMS to a 75x50mm glass slide (Sigma Aldrich, UK). The treated surfaces were then pressed together and placed in an oven at 54° C for 8 hours. The microfluidic device incorporates sample inlets 3mm long, 250µm high with 1mm diameter, 10mm entrance port, 500µm wide followed by a 8mm diameter collection outlet open chamber of 2mm depth and total volume 250µl.

E. Testing of Efficacy of the Insoluble Electrospun Membranes in the Pre-Concentration of Ethanol in Synthetic Saliva

The composition of synthetic saliva used in this study was (mg/L): potassium chloride: 1360, bovine mucin: 1300; potassium hydrogen phosphate: 950; sodium chloride: 860;

sodium azide: 500; sodium hydrogen carbonate: 440; potassium thiocyanate: 250; calcium chloride: 210; urea: 180; magnesium chloride: 60; Brij 30: 100; with ultrapure (>18MΩ) deionized water as the solvent.

Three 8 mm discs of each membrane variant were stacked in each chamber of the microfluidic cartridge and held down with a nitrile o-ring. The 2 ml of test solution (GC standard ethanol solution of concentrations 1, 0.5, 0.1, 0.05 or 0.01% in synthetic saliva or ultrapure DI water) was loaded into 10ml leur-lock syringe (Fisher, UK) and connected to the inlet of the membrane incorporated chamber on the microfluidic cartridge using PTFE tubing (RS Components, UK). Using a syringe pump, the test solution in the syringe was pumped at a flow rate of 10ml/hr and 1ml of eluent solution was collected from the open chamber outlet. The remaining test solution in the syringe was used as the control.

For the assay of ethanol in the different solutions was done using an Agilent 6890N gas chromatograph equipped with an FID and a TCH detector (in series). Manual injections of 1 µl were split 22:1 in the split injection port. Injector and detector temperatures were 250^{o}C. The column was a 30 m x 0.25 mm HP Innowax column with highly polar polyethylene glycol (PEG) stationary phase thickness of 0.25 µm (max temp: 260^{o}C). The oven program was 50^{o}C for 2 min, ramp 1 of 15^{o}C/min to 80^{o}C, ramp 2 of 40^{o}C/min to 120^{o}C, hold at 120^{o}C for 2.0 min; a total run time of 7 min. The hydrogen carrier gas has a flow rate of 1.6 ml/min through the column and a head pressure of 40 kPa and a total flow rate of 50 ml/min.

Ethanol calibration standards of concentrations of 0.0001, 0.00025, 0.0005, 0.00075, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.075, 0.1, 0.25, 0.5, 0.75, and 1 wt % were prepared using stock solutions of: 10, 1, 0.1, 0.01 and 0.001 wt % ethanol in DI water and in synthetic saliva. The calibration standards and test samples were assayed for ethanol concentrations using the above gas chromatography method.

F. Statistical Analysis

Statistical analyses were carried out using statistical software (SPSS v.20). Statistical variances between groups were determined by one-way analysis of variance (ANOVA). Tukey's test was used for post hoc evaluation of differences between groups. A p value of <0.05 was considered to be statistically significant. Unless otherwise mentioned, all data presented are expressed as mean ± standard error of mean.

III. Results and Discussion

Making electrospun membranes insoluble has significant potential for their application as solid phase separation matrices for filtration and chromatography. In this study, we report one such membrane and its tailorable surface chemistry.

A. Fabrication of insoluble electrospun membranes based on polyacyrlic acid and ethylene glycol

PAA and EG were dissolved in ethanol at 88:12 weight ratio, at a solution concentration of 4 wt%. This solution had a pH and conductivity of 3.26 and 1 µS respectively (Table 1). To make this solution electrospinnable, we added 50µl of 1M sulfuric acid, bringing its pH and conductivity to 1.28 and 950 µS respectively. At this pH the carboxylic acid

groups on PAA would be charged ($–COO^-$) and its 950 μS conductivity at 4wt% solution concentration was appropriate for polymer solution to overcome surface tension to form the tailor cone and the electrospinning jet as reported by Meng et al. [9]. However, to replicate Meng et al.'s method in our laboratory, we had to modify two key electrospinning parameters – one, a smaller spinneret needle gauze size of 22 and second, a much lower ambient temperature <10^{o}C compared to the room temperature. If spun at room temperature, the fibers did not deposit on the collector and instead formed cobweb like structure in the form of a cone extending from the tip of the spinneret needle to the collector in mid-air. This we think could be due to rapid evaporation of the solvent (ethanol) before the electrospinning jet reaches the collector plate. Electrospinning with a cold polymer solution helped in overcoming this problem [14].

The crosslinking of PAA and EG was effected after electrospinning by heating the as-spun membranes at 130^{o}C for 30 min. The completion of crosslinking was ascertained using ATR-FTIR. The FTIR spectra for the raw materials PAA and EG, and their crosslinked electrospun forms are shown in Fig. 1. EG showed its typical O–H stretching, C–H asymmetric and symmetric stretching peaks at 3294 cm^{-1}, 2936 cm^{-1} and 2874 cm^{-1} respectively. Further, the C–O stretching vibrations were observed as a doublet at 1083 cm^{-1} and 1033 cm^{-1}; while its CH_2 rocking and C–C stretching peaks were observed at 881 cm^{-1} and 860 cm^{-1} respectively. The FTIR spectrum for PAA was characterized by a strong carbonyl peak of its –COOH functional groups at 1695 cm^{-1}, broad –OH peak of the –COOH group from 2900 cm^{-1} to 3300 cm^{-1}, overlapping with the $–CH_2$ peak at 2934 cm^{-1}. The completion of the crosslinking reaction between PAA and its crosslinker EG, is evident from the complete absence of –OH peak and the prominent $–CH_2$ peaks of EG in the PAA-EG spectrum; the appearance of peaks at 872 and 1042 for the C-C and CH_2 bonds corresponding to EG; and the presence of the carbonyl –OH (2900 cm^{-1} to 3300 cm^{-1}) and C=O (1698 cm^{-1}), as well as the $–CH_2$ (2924 cm^{-1}) peaks of PAA.

B. Surface Modification of insoluble electrospun PAA-EG membranes

Both EDC-NHS and ester formation between –COOH and –OH groups, based crosslinking reactions are zero-length crosslinking mechanisms, wherein desired side chain moieties are covalently grafted on to the PAA-EG membranes. Reactions were carried out between –COOH groups on PAA-EG membranes and $–NH_2$ on OA and PEI using EDC-NHS crosslinking chemistry to obtain hydrophobic and cationic membranes. Similarly, ester reaction between –COOH PAA-EG and –OH groups on EG was used to obtain neutral surfaced membranes. Again, the completion of the crosslinking addition of side chains on PAA-EG membranes was ascertained using FTIR spectra (Fig. 2a-c).

TABLE 1: CONDUCTIVITY AND PH OF PAA SOLUTIONS FOR ELECTROSPINNING

	Conductivity (μS)	pH
4% PAA (wt/v) in Ethanol	1	3.05
4% PAA-EG in Ethanol	1	3.26
4% PAA-EG in Ethanol + H_2SO_4	950	1.28

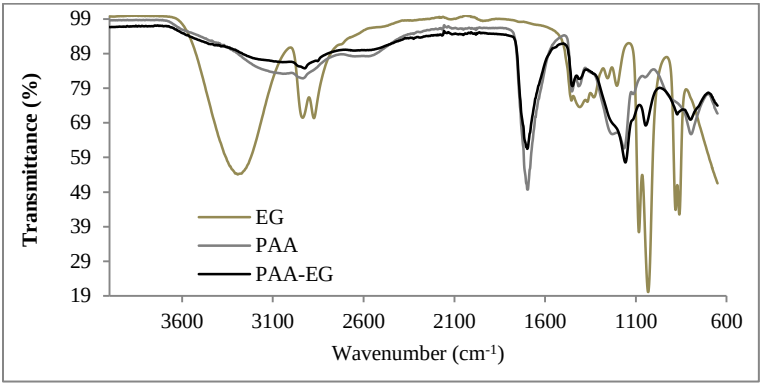


Fig. 1. ATR-FTIR spectra for PAA and EG (as supplied), and the insoluble electrospun PAA-EG membrane.

As illustrated in Fig. 2a. the grafting of OA on PAA-EG was evident from the absence of peaks for free –COOH groups, which were replaced by OA specific peaks. The peaks for $–CH_2–$ groups on the octyl residues are prominent (2955 cm^{-1}, 2923 cm^{-1}, and 2854 cm^{-1}). Ester carbonyl at 1732 cm^{-1}, amide I carbonyl at 1627 cm^{-1}, and amide II at 1557 cm^{-1} further confirm the formation of amide linkages join the octyl groups joining with the PAA backbone on the membrane. Similarly, the PEI specific prominent peaks, imide II at 1723 cm^{-1}, amide I carbonyl at 1634 cm^{-1} and amide II at 1553 cm^{-1} confirm the grafting of PEI on PAA-EG membranes (Fig. 2b). The decrease in the broad –OH peak and shift in carbonyl peak of the –COOH on PAA showed the covalent attachment of grafting of EG on PAA-EG (Fig. 2c).

C. Morphology of the different Insoluble Electrospun Membrane Variants

Morphology of the electrospun membrane variants was visualized under SEM. The as-spun and cross-linked PAA-EG membranes shown in Fig. 3a have thin and smooth fibers of average diameter 0.548 µm and ranged between from 0.371 to 1.307 µm (Fig. 3b). All surface modified membranes had larger fiber diameters compared to PAA-EG (Fig. 3c-h).

The hydrophobic PAA-EG-OA fibers had fiber diameters ranging from 0.589µm to 2.687µm and an average fiber diameter of 1.1µm (Fig. 3c&d.). However, the most frequent fiber diameter was around 0.842µm. The larger fiber diameters are as expected, owing to the additional OA layer grafted on PAA-EG. But, some of the fibers on the surface of the membrane were much thicker compared to the majority in core. This could be due to the aggregation of loose fibers on the surface through hydrophobic interactions between the octyl groups on adjacent fibers.

Similar, aggregation of fibers into larger fiber diameters on the surface was also observed with PAA-EG-PEI membranes (Fig. 3e&f). But, in this case, it could be due to the crosslinking between the neighboring fibers by the multi-amine group containing branched PEI (large) molecules acting as crosslinkers. The PAA-EG-PEI fiber diameters ranged between 0.525 and 2.11µm, with an average diameter size of 1.24µm and the most frequent fiber diameter around 0.658µm (Fig. 3f).

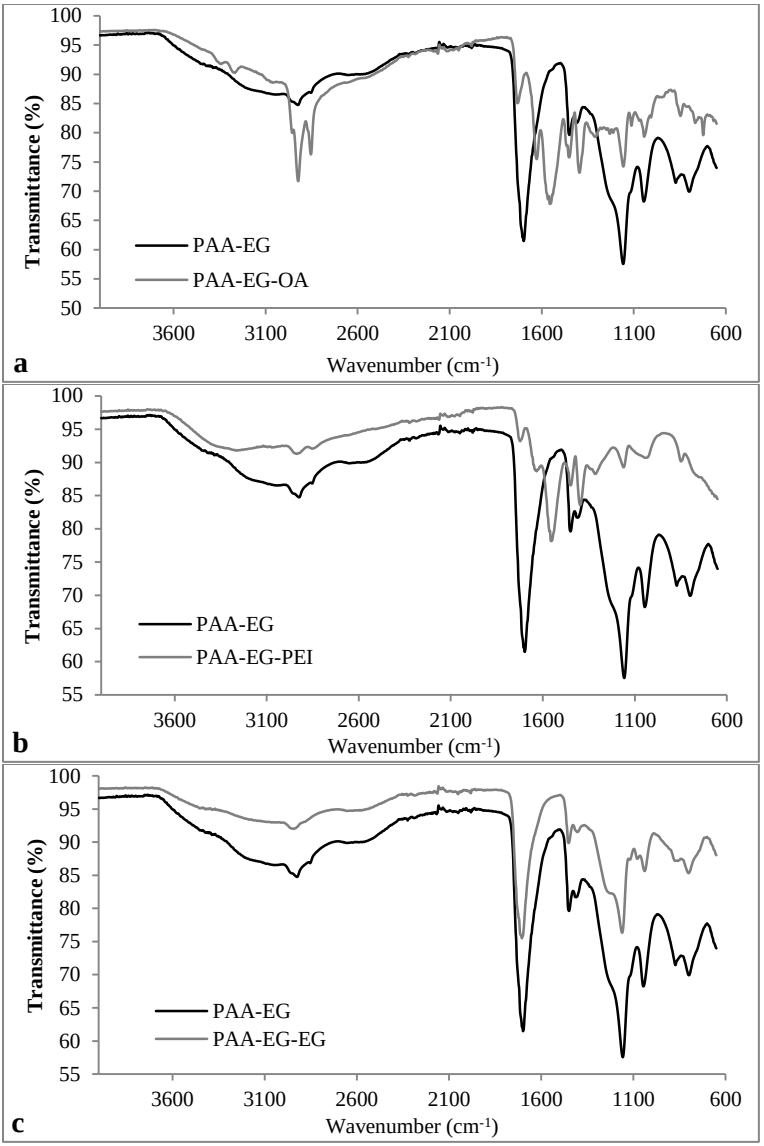
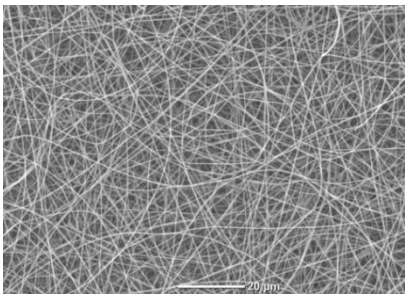


Fig. 2. ATR-FTIR spectra for a) PAA-EG-OA, b) PAA-EG-PEI and c) PAA-EG-EG insoluble electrospun membranes, in comparison with that of the base PAA-EG membrane.



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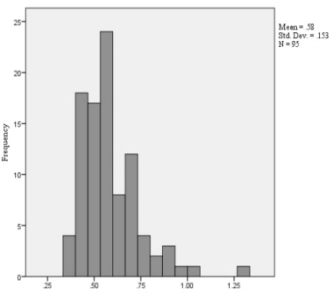

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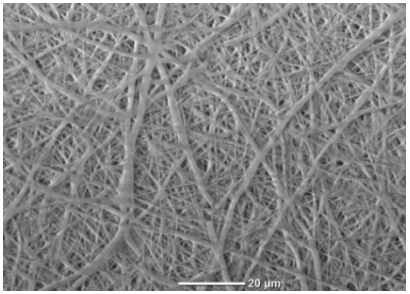

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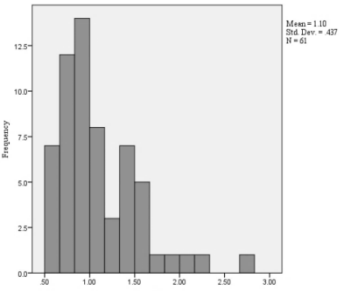

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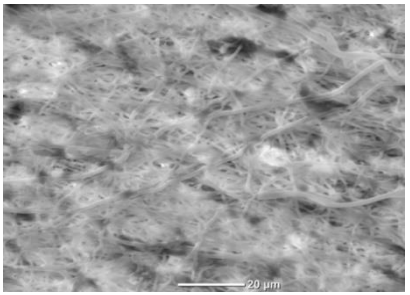

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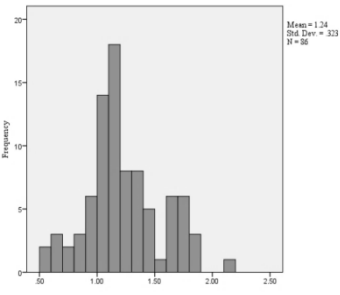

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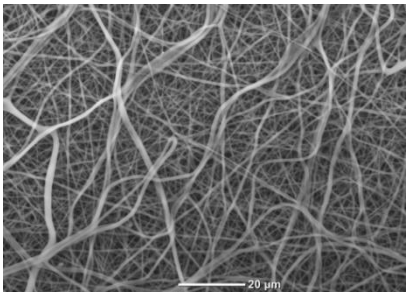

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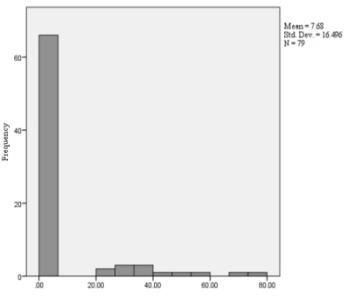

h

Fig 3. Morphology and fiber diameter distribution histograms respectively for the different electrospun membranes: a & b) PAA-EG, c & d) PAA-EG-OA, e & f) PAA-EG-PEI, and g & h) PAA-EG-EG.

The PAA-EG-EG fibers showed a different kind of fiber aggregation at the membrane surface compared to both PAA-EG-OA and PAA-EG-PEI. EG being a very short bifunctional crosslinker can only crosslink closely apposed fibers, as evident from the clear outline of the neighboring parallel fibers joined together (Fig. 3g). The larger size of branched PEI and longer chains of OA entangled through hydrophobic interactions, we

think obscures the outline of neighboring fibers making them appear as thicker single fibers (Fig 3c&e). The covalent grafting the small EG molecules on PAA-EG fibers had the smallest increase in fiber diameter among the three surface modifications as evidenced by the smallest increase in the minimum fiber diameter of 0.389µm compared to 0.525µm and 0.589µm respectively for PAA-EG-PEI and PAA-EG-OA membranes. The fiber diameters for PEI-EG-EG ranged from 0.389µm to 77.865µm, with average fiber diameter of 7.682µm and the most frequent fiber diameter around 1.201µm. The larger fiber diameters for PEI-EG-EG are due to the aggregated fibers skewing the results.

D. Solid-Phase Extraction testing using microfluidic chips

The electrospun membranes were stacked in piles of 3 within an open chamber microfluidic chip and held tightly using nitrile o-ring. A solution of saliva with a predetermined concentration of ethanol was eluted through the membranes. The eluents and original ethanol containing saliva controls were assayed for ethanol using GC and results presented in Fig. 4a-d.

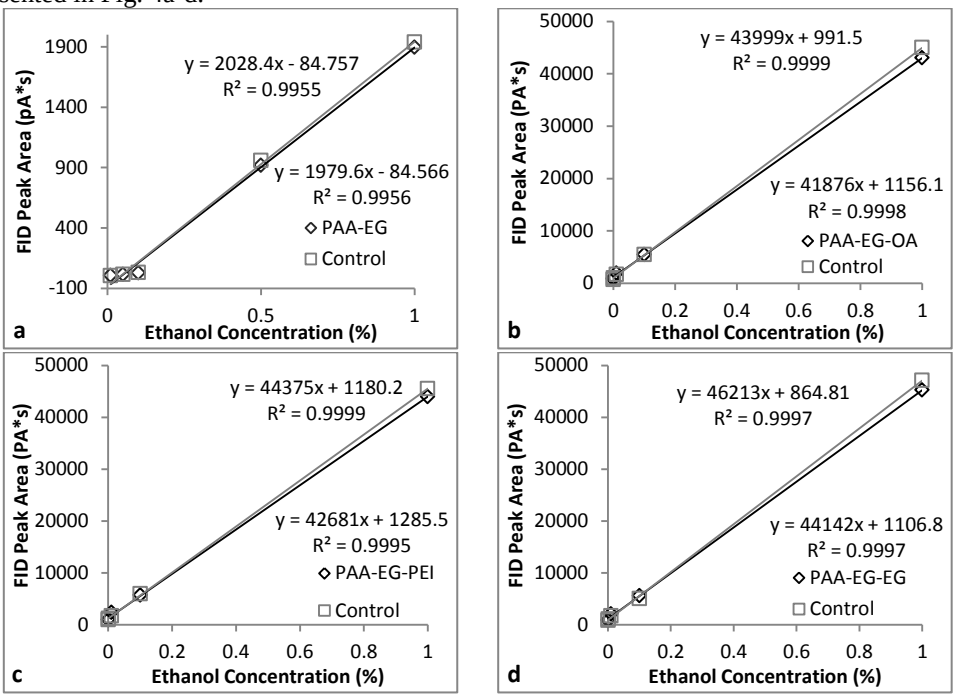

Fig. 4. Solid phase extraction of ethanol in synthetic saliva through the insoluble electrospun membranes, with the concentration of ethanol in the spiked synthetic saliva and their corresponding eluents assayed using gas chromatography. a) PAA-EG, b) PAA-EG-OA, c) PAA-EG-PEI and d) PAA-EG-EG.

In the solid phase extraction of ethanol in synthetic saliva, all membrane variants (anionic, cationic, hydrophobic and neutral) allowed elution of ethanol through them, which is evident from the little difference (R^2 values >0.99) between the ethanol concentrations in the eluent and their corresponding control samples. However, they failed to completely filter the mucin in the synthetic saliva. The eluent solution was cloudy, but less cloudy

compared to the controls. Increasing the quantity of the membrane used for the solid phase extraction could potentially increase their efficiency in filtering the mucin in saliva.

IV. Conclusion

Insoluble PAA-EG membranes were successfully electrospun and characterized to provide anionic, cationic, neutral and hydrophobic membrane surface properties. The membranes exhibited varying fibre morphologies with all post-treatment modifications effectively being incorporated onto the membrane as shown in the FTIR results. The membrane morphology also altered with addition of altering surface chemistries as shown in the SEM images as well as fibre diameter sizes and range, with the modifications causing an increase in range of fibre diameters as well as size. The microfluidic SPE extraction of ethanol from saliva also showed promising results with the GC results showing >0.99 R^2 linearity compared with the standard curve for ethanol in synthetic saliva. Overall, the results were promising, but can be improved upon to gain further insight into the relationship of the membrane and separation through increase in the quantity of the membrane used for the solid phase extraction to potentially increase their efficiency in filtering the mucin in saliva.

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