**Microwave-Assisted Grafting Polymerization Modification of Nylon 6
Capillary-Channeled Polymer Fibers for Enhanced Weak Cation
Exchange Protein Separations**

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ABSTRACT

A weak cation exchange liquid chromatography stationary phase (nylon-COOH) was prepared by grafting polyacrylic acid on to native nylon 6 capillary-channeled polymer (C-CP) fibers via a microwave-assisted radical polymerization. To the best of our knowledge, this is the first study of applying microwave-assisted grafting polymerization to affect nylon material for protein separation. The C-CP fiber surfaces were characterized by attenuated total reflection (ATR) infrared spectroscopy and scanning electron microscope (SEM). The anticipated carbonyl peak at 1722.9 cm^{-1} was found on the nylon-COOH fibers, but was not found on the native fiber, indicating the presence of the polyacrylic acid on nylon fibers after grafting. The nylon-COOH phase showed a ~12X increase in lysozyme dynamic binding capacity ($\sim 12\text{ mg mL}^{-1}$) when compared to the native fiber phase ($\sim 1\text{ mg mL}^{-1}$). The loading capacity of the nylon-COOH phase is nearly independent of the lysozyme loading concentration ($0.05 - 1\text{ mg mL}^{-1}$) and the mobile phase linear velocity ($7.3 - 73\text{ mm s}^{-1}$). The reproducibility of the lysozyme recovery from the nylon-COOH (RSD = 0.3 %, $n = 10$) and the batch-to-batch variability in the functionalization (RSD = 3%, $n = 5$) were also investigated, revealing very high levels of consistency. Fast baseline separations of myoglobin, α -chymotrypsinogen A, cytochrome c and lysozyme were achieved using the nylon-COOH column. It was found that a 5X increase in the mobile phase linear velocity (7.3 - 36.5 mm s^{-1}) had little effect on the separation resolution. The microwave-assisted grafting polymerization has great potential as a generalized surface modification methodology across the applications of C-CP fibers.

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- 1 **Keywords:** Capillary-channeled polymer, fiber, surface modification, microwave-
- 2 assisted polymerization, ion exchange, chromatography

1. Introduction

In recent years, macromolecular therapeutics have played increasingly important roles in the pharmaceutical industry [1, 2]. Protein-based drugs are the most common of these macromolecular drugs. The manufacture of protein therapeutics involves two major operations, upstream processing (i.e., production via cell culturing/fermentation) and downstream processing (i.e., purification/recovery). Upstream processing has been largely improved over the last two decades [3, 4], while the downstream processing continues to be the rate limiting step in protein drug production [3, 5, 6]. Chromatographic separation is the bottle-neck of the downstream processing due to its high costs and time consumption. Thus there is a continuous interest in the development of stationary phases that can provide high-throughput, cost-effective protein separations [7, 8].

Traditional liquid chromatography (LC) stationary phases are composed of highly porous, micro-sphere packed-bed support phases. While the majority of supports are silica-based, polymeric materials find application due to their chemical robustness [9]. Currently, use of sub-2 μm diameter silica supports dominates the realm of “small molecule” separations [10, 11]. However, the application of such supports in protein separations suffers from the slow solute diffusion into and out the pores, limitations of flow rate and backpressure, and the pore clogging due to the complexity of the production mixtures. To this end, there has been a resurgence of interest in the use superficially porous silica support phases [12, 13]. Additionally, alternative support phases, such as monoliths, membranes and fibers, have been developed for protein separations [14-19]. A recent review discussed the advantage of the using fiber based

stationary phase for preparative-scale protein separations. The preparation of preparative monolithic stationary phases remains challenging due to the low-homogeneity of in situ polymerization, while fiber based stationary phases may be prepared outside of the column and packed into large columns [19]. Capillary-channeled polymer (C-CP) fibers have been under study in this laboratory towards applications in protein analytics and downstream processing [20-25]. The C-CP fiber-packed bed presents a number of physical advantages over porous microsphere-packed beds. The combination of a virtually non-porous fiber structure and high permeability allows for analytical separations that can be run at high mobile phase linear velocity (100 mm s^{-1}) while maintaining low column backpressure ($< 2000 \text{ psi}$) [26, 27]. Short inter-fiber diffusion distances combine with the lack of porosity to minimize the mass transfer resistance of proteins, allowing for very high processing throughput [28]. C-CP fibers are melt-extruded from commodity polymers such as polyamide (nylon 6), polyester (PET) and polypropylene (PP). The low-cost of the C-CP fibers makes them an attractive candidate in terms of implementation on the industrial-scale.

The different surface chemistries provided by the base C-CP fiber phases allows for protein separations under reversed-phase (RP), hydrophobic interaction (HIC), and ion-exchange (IEX) modes [21, 22, 29, 30]. Just as in the case of poly(styrene-divinylbenzene) (PS-DVB) bead phases [9, 31, 32], there exists a rich tool box of surface modification chemistries to effect greater chromatographic efficiency and selectivity. Several surface modification strategies have been pursued on C-CP fibers to affect greater efficiency/selectivity, with the caveat that retention of the basic hydrodynamic properties was essential. For example, simple amination of the PET

surface has allowed creation of a biotinylated surface for affinity separations [33] and the creation of polymeric polyethyleneimine (PEI) phases for weak anion exchange protein separations [34, 35]. A new class of surface functionalizing agent, ligand tethered lipids (LTL), was developed for surface functionalization of PP C-CP fibers for protein extractions and separations [36, 37]. These modification strategies introduce different types of surface chemistries on C-CP fiber phase and expand the potential applications of C-CP fiber phase.

In terms of the ability to mitigate any decomposition/deactivation of product proteins, ion exchange-based separations are extremely attractive [8]. Unfortunately, hydrophobic interactions between proteins and non-polar support phases lead to separations that are actually mixed-mode in nature [9, 38, 39]. Hydrophobic interactions can cause proteins to de-nature during separations, lead to low protein recoveries, and peak tailing in chromatograms. Therefore, hydrophilic supports such as acrylates are common in IEX protein separations [40]. Among the types of C-CP fiber materials, nylon 6 is the most hydrophilic. Based on the acid/base character depicted below for the natural carboxylic acid and primary amine end groups,

native nylon 6 C-CP fibers have been used in IEX protein separations [30, 41]. However, the low density of these ion-exchange ligands limits the performance of the material in protein separations [35]. Thus additional surface modifications on the native nylon 6 C-CP fibers are needed to improve the chromatography properties of this phase.

Presented here is a proof-of-concept in the modification of nylon 6 C-CP fibers used in weak cation exchange (WCX) protein separations, without sacrificing the nature of the hydrophilic surface or the column hydrodynamics. As-extruded fibers were functionalized via a microwave-assisted grafting polymerization. Acrylic acid was polymerized and grafted onto the native nylon 6 C-CP fibers as the functional ligands. The modified fiber (nylon-COOH) packed columns exhibit significantly-increased protein dynamic binding capacity. Protein recoveries and the reproducibility of the modification were investigated on the nylon-COOH fibers. Finally, fast protein separations were demonstrated on the nylon-COOH fiber phase. To the best of our knowledge, this is the first study of applying microwave-assisted grafting polymerization to affect nylon material for protein separation. The microwave-assisted modification is a versatile method to introduce various functional ligands onto the nylon C-CP fiber phase, thus it greatly expands and improves the application of nylon 6 C-CP fiber phase for analytical and, eventually preparative, protein separations.

2. Materials and methods

2.1 Chemicals and instrumentation

Unless otherwise specified, chemicals were purchased from commercially available sources and used without further purification. Nylon 6 C-CP fibers were obtained from the Material Science and Engineering department, Clemson University. The denier per filament (DPF) of the fibers employed here is 2.67 g per 9000 m, with each fiber having a cross-sectional perimeter of 208 μm . Acrylic acid (99.5%) and potassium persulfate (KPS) (99%) were purchased from Alfa Aesar (Haverhill, MA). Activated alumina powder was purchased from Polysciences, Inc. (Warrington, PA). All

1 HPLC solvents were purchased from EMD (Billerica, MA). All other chemicals and all
2 proteins were purchased from Sigma-Aldrich (St. Louis, MO). Deionized water (DI-H₂O)
3 was secured from a Milli-Q water system. The protein loading/elution characterization
4 and chromatographic protein separations were performed on a Dionex Ultimate 3000
5 HPLC system, LPG-3400SD Quaternary pump, and MWD-3000 UV-vis absorbance
6 detector (Thermo Fisher Scientific Inc., Sunnyvale, CA). A Rheodyne model 8125 low
7 dispersion injector with a 5 μ L injection loop was used for protein sample injections. The
8 microwave-assisted polymerizations were performed in a Sunbeam SBM7700W
9 microwave oven, without any modifications of the commercial unit.

2.2 Microwave-assisted nylon C-CP fiber surface modification

10 The surface grafting polymerization method was modified from an earlier-
11 reported method [42]. All DI-H₂O used in the modification reactions was purged with
12 nitrogen for 30 min to remove oxygen prior to use. The acrylic acid was filtered through
13 an activated alumina bed to remove remnant stabilizing agents before use. The
14 modification solution was prepared by dissolving acrylic acid (2.0 mL, 29 mmol) and
15 potassium persulfate (100 mg, 0.37 mmol) in 20 mL DI-H₂O. The native nylon 6 C-CP
16 fibers were removed from the fiber spool and placed on a dying fork. For each
17 modification reaction, 720 fibers of ~35 cm length were used. The fibers were rinsed
18 with excess amounts of DI-H₂O, methanol, and then DI-H₂O again, to remove any
19 chemical residues left from the fiber extrusion process. The fibers were immersed into
20 the modification solution in a 50 mL beaker and the beaker placed in the microwave
21 oven. Due to the small scale of the reaction, another beaker containing 30 mL water
22 was also placed in the microwave oven as a heat sink for better control and

1 reproducibility of the experiments. The microwave reaction was run at 100 W for 10 min.
2 After reaction, the fibers were removed from the beaker immediately and washed with
3 excess amounts of DI-H₂O until no homopolymer parciptates were visible on the fibers.
4 A control experiment was run with the fibers submerged in neat DI-H₂O instead of in the
5 modification solution to assess any thermal or microwave-induced decomposition.

2.3 Preparation of the C-CP fiber columns

7 The C-CP fiber columns were packed following the previously-reported
8 procedure [27]. In this study, 720 native or modified nylon 6 fibers were pulled through
9 polyether ether ketone tubing (PEEK, 0.762 mm i.d., IDEX Health & Science LLC, Oak
10 Harbor, WA). After packing, columns were mounted on the HPLC system and washed
11 with DI-H₂O at 0.2 mL min⁻¹ for 12 h to remove any non-covalently bound chemicals
12 (initiators, homopolymers) from the fibers. Once cleaned, the fiber-packed columns
13 could be cut to appropriate length (20 cm, in this study) and stored in ambient
14 conditions.

2.4 Characterization of the modified nylon 6 C-CP fibers

16 Scanning electron microscope (SEM) images were taken at the Clemson
17 University Electron Microscopy Laboratory, using a Hitachi SU6600 system operating in
18 the variable pressure mode, with a 20 kV accelerating voltage. Attenuated total
19 reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was performed on a
20 Thermo-Nicolet Magna 550 FITR in the Analytical Testing Lab of the Material Science &
21 Engineering Department, Clemson University. The ligand densities of native and
22 modified nylon C-CP fibers were determined by acid-base titration. Native and modified
23 fibers were washed in 100 mM HCl solution for 1 min, and then large amounts of DI-

1 H₂O to remove residual HCl from the fibers. The fibers were then washed with acetone
2 to remove water, and dried under mild mechanical vacuum for 20 hours at room
3 temperature to remove residual solvent. The dried fibers, were weighed, placed in DI-
4 H₂O, and were titrated with standardized 0.1142 M NaOH solution and phenolphthalein
5 used as the end-point indicator.

2.5 Liquid chromatography

6 All experiments were performed on a Dionex Ultimate 3000 HPLC system. The
7 dynamic loading capacity (DLC) of the columns (column length: 200 mm, i.d.: 0.762 mm)
8 was determined through breakthrough (frontal) analysis as described previously [23],
9 using lysozyme as the test protein. After the column was cleaned and equilibrated with
10 20 mM phosphate buffer (pH=6.5, designated as buffer A), lysozyme at the chosen
11 concentration in the buffer was introduced to the column. UV absorbance at 280 nm
12 was monitored as a means of detecting breakthrough and quantifying the amount of
13 protein retained on-column. When the UV absorbance of the eluting solution reached a
14 plateau, indicating column saturation, buffer A was then applied on the column to
15 remove non-bound protein. When the absorbance returned to the original baseline,
16 buffer B (1.0 M NaCl in buffer A) was introduced to the column to affect protein elution.
17 The amount of lysozyme retained was determined by integration of the breakthrough
18 curve (equal area method).[43] A blank experiment was performed using an empty
19 PEEK tubing to determine the system hold-up volume/time. The DLC at 10%
20 breakthrough was calculated at the point that the absorbance of the eluting solution
21 reached 10% of its maximum absorbance (plateau). While there was very little
22 evidence that it was necessary, columns were cleaned/regenerated between

1 experiments by passing a 100 mM NaOH solution for 10 min to remove residual,
2 strongly-bound proteins.

3 The analytical quality of the protein separations was determined using gradient
4 separations (from 100% buffer A to 50% buffer B) of a four-protein mixture. For each
5 separation experiment, 5 μ L of the protein mixture containing myoglobin, α -
6 chymotrypsinogen A, cytochrome C and lysozyme (0.25 mg mL⁻¹ each) was injected
7 and the chromatogram was recorded at 216 nm. The gradient baseline absorbance was
8 obtained by running the gradient with no protein injected. The absorbance baseline was
9 subtracted from protein separation chromatograms.

10 **3. Results and discussions**

11 **3.1 Microwave-assisted nylon surface polymerization modification**

12 Different methods have been reported in the literature for the modification nylon
13 materials. Some of the modification methods target activation of the amide groups [44,
14 45], unfortunately resulting in cleavage of the amide bond and causing inevitable
15 physical damage to the nylon bulk structure. Such a decrease of mechanical strength
16 to the C-CP fibers would result in breakage in the column packing process. The other
17 principal type of modification introduces the target species onto the nylon surface via
18 polymerization of a monomer containing the desired functional groups [42, 46]. Radical
19 polymerization reactions are usually induced by UV light, conventional heating, plasma
20 or microwave energy [42, 46, 47]. UV light treatment is a straight forward, easily
21 controlled approach, but is not applicable on large quantities of material or on materials
22 that have non-uniform shapes, such as C-CP fibers. The conventional heating method
23 requires mixing or stirring of the materials in the reactive solution. Such an approach on

C-CP fibers would results in folding and twisting, greatly reducing the homogeneity of the fiber column packing if not prohibiting it all together. Plasma beam treatments are difficult to impart on non-uniform surfaces and cause severe degradation of the polymers and decreased their mechanical properties due to oxidative damage [46, 48]. Finally, microwave-irradiation is a well-established technique and its application to surface polymerization has been widely investigated [49-52]. Microwaves consist of electromagnetic radiation that has frequencies of 300 MHz to 300 GHz, corresponding to wavelengths of ~1 m to 1 mm, which can penetrate through many materials. The majority of microwave sources that are used for industrial and domestic purposes operate at a frequency of 2.45 GHz, corresponding to the wavelength of ~12 cm. In comparison to conventional heating methods, microwave irradiation offers many advantages including non-contact heating, rapid heating, high levels of temperature homogeneity, selective heating (some materials absorb more microwave radiation than others) and low energy cost. Non-thermal “microwave effects” that enhance the reactions may exist, but the exact mechanisms are still under discussion [53-55].

In this study, native nylon 6 C-CP fiber surfaces were functionalized by radical grafting polymerization as depicted below, with KPS used as the radical initiator. The mechanisms of KPS decomposition and KPS-initiated grafting polymerization have been discussed [42, 56].

1 The power of the domestic microwave oven used for the modification reaction was set
2 at 100 W, the lowest power option on that particular unit. The scale of the reaction (20
3 mL) in this study is quite small in comparison to the heating capacity of the domestic
4 microwave oven. As such, an additional beaker containing 30 mL of water was placed
5 in the microwave oven as a “heat sink”. Without the “heat sink”, the reaction mixture
6 heats too quickly. In such cases, large amounts of air bubbles and homopolymer
7 precipitants are formed after 5 min of microwave reaction and the modifications lack
8 reproducibility. With the heat sink, the reaction is run for 10 min with greatly improved
9 reproducibility. It is believed that using a more controllable microwave reactor, such as
10 employed in the organic synthesis community would improve the modifications.
11 However, the less expensive domestic microwave oven is sufficient for the proof-of-
12 concept study of using the nylon-COOH C-CP fiber phase for protein separations.

13 The acrylic acid-functionalized nylon fibers (nylon-COOH) were characterized
14 using ATR-FTIR and compared to the native nylon 6 starting material (Fig. 1). Both
15 spectra show virtually identical peaks from the nylon bulk structure: 3294 cm^{-1} (amide A,
16 N-H stretch), 3062 cm^{-1} (NH stretch), 2931 cm^{-1} and 2862 cm^{-1} (anti-symmetric and
17 symmetric $-\text{CH}_2-$ stretch), 1636 cm^{-1} (amide I), 1545 cm^{-1} (amide II), 1457 cm^{-1} (CH_2
18 scissors), 1369 cm^{-1} (amide III and CH_2 wagging), 1260 cm^{-1} (NH bending and C-H
19 stretch), 1197 cm^{-1} (CH_2 twist-wagging), 1168 cm^{-1} (CO-NH skeletal motion), 685 cm^{-1}
20 (out of plane bends of NH), 579 cm^{-1} (out of plane bends of C=O). The results are
21 consistent with reported values [57]. The peak at 1722.9 cm^{-1} (C=O stretch) in the
22 Nylon-COOH case is consistent with literature reported carbonyl C=O stretch in poly
23 acrylic acid [58], indicating the presence of those new groups on the nylon-COOH fiber

surfaces, which were not apparent in the spectrum of the native nylon 6. The fact that the other spectral features are not changed reflects the fact that the polymer backbone is not perturbed by the modification process.

As noted previously, the driving force for these efforts was the desire to attain high densities of carboxylic acid WCX groups on the already-hydrophilic nylon 6 support. Earlier efforts in assessing the end group densities on nylon 6 were based on the classic ninhydrin reaction, which actually measures primary and secondary amine densities, which should equate nominally with the carboxylic acid values as there are no 2° amines in the nylon system. Here, a simple acid/base neutralization titration has been applied to specifically quantify the –COOH densities. Those determinations yield values of $28 \pm 9 \mu\text{mol g}^{-1}$ of fiber for the native nylon 6 and $575 \pm 7 \mu\text{mol g}^{-1}$ for the nylon-COOH, an ~20X increase following the microwave-enhanced grafting method. The –COOH value here is a factor of ~2.5X less than obtained for the same fiber using the ninhydrin test, which is not out of line given the uncertainties of that method. In comparison to other polymer-based WCX phases, density of the nylon-COOH is higher than carboxylic acid functionalized poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads (–COOH density $400 \mu\text{mol g}^{-1}$) [59] and chloroacetic acid modified polymeric monolith (–COOH density $351 \mu\text{mol g}^{-1}$) [60]. To better classify the nylon-COOH ligand density versus other WCX phases, it is better to express that value of $\sim 820 \pm 10 \mu\text{mol m}^{-2}$, based on native fiber specific surface area of $\sim 0.7 \text{ m}^2 \text{ g}^{-1}$. In terms of a surface area basis, this is a very high density of surface ligand, in comparison to a surface functionalized silica gel developed for protein separations, having a carboxylic acid density of $0.568 \mu\text{mol m}^{-2}$ [61]. It must be emphasized that,

1 based on the virtual non-porosity of the C-CP fibers, all of the surface ligand are
2 accessible, versus those that might lie within porous structures of other phases. While it
3 may be questioned, we have no direct evidence as to the primary “directionality” of the
4 polymerization process, normal to or laterally on the surface.

5 As stated previously, it is imperative that any modification process not lead to
6 sacrifices in the hydrodynamic efficiency of the C-CP fiber columns. SEM imaging of
7 the fibers provides some evidence of potential macroscopic changes as seen in Fig. 2.
8 The images of the column cross sections of the native nylon 6 and nylon-COOH (Figs.,
9 2a and b, respectively) suggest that the modified fibers are packed more tightly (i.e.,
10 their fiber diameters have increased), even though the number of the fibers is the same
11 in both columns (720 fibers). This point is more readily seen in the magnified views of
12 Figs. 2c and d. In the chromatography experiments, the back-pressure of using nylon-
13 COOH column is ~2× higher than using native nylon column (though still comparatively
14 low). While not determined explicitly, this would suggest that the interstitial fraction of
15 the columns packed with nylon-COOH fibers has decreased from a value of $\varepsilon_i = 0.63$ to
16 ~ 0.45 [9]. One might question whether the increased thickness is simply the result of
17 swelling in the hot water, microwave modification step. In fact, micrographs of control
18 samples of the nylon 6, microwaved in DI-H₂O instead of the modification solution,
19 showed no difference in SEM images in comparison to the native fibers. Hence, the
20 increase in fiber thickness following modification appears to be due to the added
21 polyacrylic acid layer from the grafting polymerization. As a final assessment of
22 potential damage/change during the microwave modification process, side-on SEM
23 images (Figs. 2e and f) indicate no macro-damage to the fiber channel structures.

1 These findings support the desired outcome that the grafting polymerization is a non-
2 destructive surface modification method.

3.2 Dynamic loading capacity

4 The DLC of the native and modified nylon 6 C-CP fiber phases were determined
5 by breakthrough experiments using lysozyme as the model protein. Preliminary
6 assessment of the throughput and yield characteristics of the C-CP fiber phases were
7 done with the same lysozyme/nylon 6 system [23]. Under buffer conditions of pH 6.5,
8 the carboxylic acid on the native nylon (as the end groups) and nylon-COOH fiber
9 phase carry negative charges. Lysozyme has the isoelectric point of ~ 11.3 and so has
10 a net positive charge in the buffer, thus it interacts with the native and modified nylon
11 fiber phases via electrostatic interactions. Various concentrations ($0.05 - 1.0 \text{ mg mL}^{-1}$)
12 of lysozyme solutions were loaded onto the fiber columns at a flow rate of 0.4 mL min^{-1}
13 ($U_o \cong 29.2 \text{ mm s}^{-1}$). Increasing the ionic strength of the buffer (1.0 M NaCl) elutes the
14 lysozyme from the fiber phases following each loading. Representative breakthrough
15 curves (UV absorbance at 280 nm) are shown in Fig. 3. Plotting on a time axis depicts
16 the expected temporal response; as the concentration is increased, breakthrough
17 occurs at shorter times/volumes. Frontal analysis of the breakthrough data in terms of
18 the absolute amounts of protein allows calculation of the dynamic binding capacities
19 presented in Table 1, in terms of the mass of lysozyme per unit fiber mass (mg g^{-1}) and
20 bed volume (mg mL^{-1}). The DLC of nylon fiber phase varies from about $0.4 - 1 \text{ mg mL}^{-1}$
21 on the native nylon to about $10 - 12 \text{ mg mL}^{-1}$ on the nylon-COOH. There is a good bit
22 of scatter in the native nylon 6 data as the absolute difference between the
23 breakthrough volumes in those cases is very close to the actual column breakthrough

(hold-up) volume. On the other hand, the precision of replicate loadings is much better for the nylon-COOH phase as the absolute values are much higher. There is a very slight (positive) dependence on the binding capacity for the modified surfaces versus the load concentrations, conforming to the upper-concentration regions of what would be expected to be a Langmuir or Langmuir-linear isotherm [24, 34, 43]. Fitting of the isotherm data results in a maximum dynamic binding capacity (Q_m) of 12.4 mg mL^{-1} bed volume, which is very comparable to WAX C-CP fiber phases [34]. It is easy to conclude that the 12X increase in DLC is attributed to the added acrylic acid (-COOH) ligands following modification.

Any situation wherein large increases in capacity are realized brings along natural questions as to potential perturbations of the loading/elution kinetics. One useful metric towards this regard is the ratio between the 10% and 50% breakthrough (BT) volumes obtained through frontal analysis. These values reflect the amount of bound lysozyme at the point when the lysozyme concentration of the eluent reaches 10% and 50% of the maximum (solution) concentration. The ratio of the DLC at 10% to the DLC at 50% reflects the sharpness of the breakthrough curve. In these lysozyme loading experiments, the ratios on the nylon-COOH column range from 0.82 – 0.86, indicative of the high efficiency of mass transfer on the nylon-COOH phase. There is no significant change in the 10%/50% BT ratio across the protein feed concentration changes, which would be expected in a diffusion-limited loading situation. This reflects the convective-diffusion driven solute transport that takes place in the C-CP fiber beds [28]. Overall, the protein DLC of a separation media at low protein feeding concentrations (e.g., 0.05 mg mL^{-1}) is especially important for industrial applications,

1 since many of the desired proteins can be present at low concentrations in cell lysate
2 media. This high loading capacity is complemented by the efficient adsorption kinetics,
3 suggesting that the nylon-COOH phase holds great potential for use in large-scale
4 protein extraction and purification.

5 As described previously, downstream processing continues to be a limiting factor
6 in the production of bio-therapeutics. The advantages of operating a separation under
7 conditions which affect high throughput (T) and yield (Y) are ultimately desirable, these
8 are some of the most outstanding attributes of C-CP fiber phases [23, 24]. Figure 4
9 presents representative lysozyme breakthrough curves on the nylon-COOH column at
10 various flow rates/linear velocities. In this experiment, the lysozyme load concentration
11 was kept at a constant value of 1 mg mL^{-1} . The breakthrough curves are plotted on the
12 basis of the load solution volume to better-reveal any kinetic limitations. As seen in the
13 scale expansion, there is a very slight bias in the volume equating to the 50% load, with
14 the slowest application yielding an ~4% higher binding capacity than the highest velocity,
15 though the latter occurred at a 10× higher velocity/shorter time scale. Thus, a vast
16 improvement in T is observed. It is also interesting to note that the volume
17 displacement at the 10% breakthrough between the different velocities and the 50%
18 level are virtually the same, thus the mass transfer/adsorption kinetics are not sacrificed
19 at the higher linear velocities. Only in the case of the highest linear velocity (73 mm s^{-1})
20 does it appear that mass transfer limitations are occurring, as the slope of the total
21 breakthrough curve begins to decrease. The negligible difference on DLC at various
22 linear velocities indicates the low mass transfer resistance of the nylon-COOH phase at

1 high flow rates, thus fast protein loading/elution can be realized to improve the
2 throughput of protein separations.

3 Table 2 compares figures of merit for the nylon-COOH fiber phase developed
4 here with other commercially-available or literature-reported high permeability/high
5 throughput weak cation exchange phases. The lysozyme loading capacity of nylon-
6 COOH fiber (12 mg mL⁻¹) is higher than polyacrylate-grafted polyacrylamide cryogel
7 monolith (6 mg mL⁻¹) [62] and methacrylic acid- polyethylene glycol diacrylate monolith
8 (0.065 mg mL⁻¹) [63]. The lysozyme loading capacity of nylon-COOH fiber is
9 comparable to CIMacTM CM monolith (9 – 11 mg mL⁻¹, <http://www.biaseparations.com>)
10 but lower than ProSwift WCX-1S monolith (23 mg mL⁻¹, www.thermofisher.com) and
11 Sartobind® C membrane (21 mg mL⁻¹, www.sartorius.com). However, it needs to be
12 pointed out that the linear velocities of the protein feed on nylon-COOH fiber with 200
13 mm bed height is significantly higher than the flow linear velocity allowed on other
14 phases even with much shorter bed height (4 – 70 mm). Nylon-COOH fiber phase is
15 believed real advantages as an IEX phase capable of high throughput protein
16 separations.

3.3 Consistency of the protein loading and elution

17 The ability to consistently recycle columns after saturation is a key attribute in
18 terms of practical column lifetime. In many instances, some form of clean-in-place (CIP)
19 step is required, both to remove undesirable contaminants such as host cell proteins (in
20 downstream processing) as well as “unrecovered” target proteins in analytical
21 separations. More so than in analytical-scale separations, the ability to maintain
22 consistent, high efficiency is challenging in preparative-scale applications where
23

complete column saturation may occur. This is of course the case in the dynamic loading studies employed here, and so an evaluation of column stability/reproducibility was performed. After an initial cleaning using a 100 mM NaOH solution, 10 complete load/elute cycles were executed without any CIP performed in between. The subsequent load/elute transients are presented in Fig. 5, stacked from first-to-last from the bottom-to-top. As seen in each case, the nylon-COOH fiber bed is saturated. The consistency shown here is quite impressive in terms of both the load and elution steps, with the load masses (via breakthrough volumes) differing by only 0.2 %RSD ($n = 10$) and the recoveries (via the integrated areas under the curve) by only 0.3 %RSD ($n = 10$). As an extension, though not needed here, the same experiment was repeated 6 additional times, with 10 min. 100 mM NaOH CIP exposures between each. Here again, there was no loss in binding capacity or recovery efficiency. This bares testament to the chemical robustness of the acrylic acid overlayer and the physical robustness of the base nylon fibers towards use for downstream processing applications.

3.4 Batch-to-batch reproducibility of the modification process

It is a natural concern, due to the fact that the microwave modification reactions were not well controlled, that the batch-to-batch reproducibility would be suspect when using a simple domestic microwave oven. Different from commercial units designed for organic synthesis, the operational aspects of these units are not held to high rigor or precise control. To test the reproducibility of the polymerization process, columns were made once a week for 5 consecutive weeks. Shown in Fig. 6 are the load/elute transients for those 5 columns (1.0 mg mL^{-1} lysozyme loading concentration at 0.5 mL min^{-1}), directly overlaid to emphasize the quality of the processing. The consistency

shown here is very impressive in terms of both the load and elution steps, with the differences in load masses differing by only 3 %RSD ($n = 5$) and the recoveries by only 2 %RSD ($n = 5$). The overall performance of these columns is equivalent, though there are very slight differences seen in the transition regions of the breakthrough curves as seen in the insert of the figure. Since all of the columns were manually assembled following the microwave modifications, the variation due to the column packing is likely a contributing factor in the shapes of breakthrough curves. It is believed that these variations can be overcome by optimizing the column packing method and using better, controllable microwave reactors.

3.5 Rapid protein separation

While the load/elute studies readily demonstrate the enhanced loading capacity of the nylon-COOH phase versus native nylon 6, it would be equally desirable to have that phase be viable for analytical protein separations. The primary metrics here are the capacity to adequately separate the mixture components (an enthalpic quality) while also providing very high throughput (an entropic quality). Protein separations were evaluated at linear velocities of 7.3 and 36.5 mm s⁻¹ for a four-protein (myoglobin, α -chymotrypsinogen A, cytochrome C and lysozyme) mixture using a more or less generic NaCl gradient. The chromatograms of the separations are showed in Fig. 7. The top set of chromatograms (Fig. 7a) reflects the protein separations on the native nylon 6 fiber phase. The native nylon has carboxylic acid end groups, however their low density leads to poor retention and broad elution peaks.[35] The cytochrome C component (2) is split into two peaks, the first peak co-eluted with the non-retained myoglobin (1) and the second co-eluting with chymotrypsinogen-A (3) as confirmed by single component

1 injections. Meanwhile, the lysozyme peak (4) exhibits severe tailing and poor recovery.
2 Use of the lower linear velocity (dashed line) provides little significant improvement.
3 The same gradient separations were performed on the nylon-COOH column, as in the
4 case of the nylon 6. The four proteins were well separated on the nylon-COOH column
5 (Fig. 7b), with the hydrophilic myoglobin still remaining un-retained. The other proteins
6 show well-behaved responses, with high recoveries. Tuning the gradient results in
7 baseline separation of four proteins (Fig. 7c). The increase on the mobile phase linear
8 velocity (5X) did not significantly impair the separation in both fig 7b and 7c. Consistent
9 with previous protein separations on C-CP fiber phases [21, 22], increases in linear
10 velocity do not diminish the resolution, with a tendency for the proteins to elute at a
11 slightly lower solvent strength [21]. In these instances, the apparent loss of recovery
12 (based on lower absorbance signals) is due to solute dilution per unit time and some
13 time constant bias in the optical detection system. In the best case of the high velocity
14 separation depicted in Fig. 7c, the protein processing rate is 1 mg protein on a 0.36 mL
15 bed in a time of less than 6 mins. Indeed, this throughput is limited primarily from the
16 (needless) 1.5 min solvent hold time before the initiation of the gradient. Clearly the
17 nylon-COOH fiber phase provides a much-improved platform for high-throughput WCX
18 protein separations.

19 **4. Conclusions**

20 This proof-of-concept study demonstrates the feasibility and practical advantages
21 of the nylon-COOH C-CP fiber phase for protein separations. The native nylon C-CP
22 fibers were functionalized with acrylic acid via a microwave-assisted grafting
23 polymerization. Acid/base titrations reveal increased ligand density (-COOH, 575 ± 7

1 $\mu\text{mol g}^{-1}$) on nylon C-CP fibers after functionalization. The resulting nylon-COOH fiber
2 packed column showed a 12X increase in protein dynamic binding capacity versus
3 native nylon fibers, with values that are nearly independent to the mobile phase linear
4 velocity. Excellent recovery of lysozyme was demonstrated on the nylon-COOH fiber
5 phase under ion-exchange elution conditions. The intra- and inter-batch reproducibility
6 was found to be 0.3 % and 3%. Fast protein separations (6 min gradients employed
7 here, but much faster are reasonable²²) were demonstrated on the nylon-COOH fiber
8 phase, and the separation resolution was not affected by increasing the mobile phase
9 linear velocity.

10 Acrylics are a very versatile family of monomers. A large number of acrylic
11 monomers that contain different functional ligands is industrially available.
12 Implementation of a similar modification route would allow the functionalization of the
13 nylon 6 C-CP fibers with a variety of acrylic monomers such as (3-
14 acrylamidopropyl)trimethylammonium chloride for strong anion exchange, N-[3-
15 (dimethylamino)propyl]acrylamide for weak anion exchange, 3-allyloxy-2-hydroxy-1-
16 propanesulfonic acid for strong cation exchange and allyl glycidyl ether for epoxide
17 coupling chemistry. Although more study of the modification conditions and the protein
18 separations on nylon C-CP fiber stationary phase is still needed, the results from this
19 study suggest that the nylon C-CP fiber modified by microwave-assisted grafting
20 polymerization has a great potential for protein separations on the analytical and
21 preparative scales.

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Figure captions

Figure 1. ATR-FTIR spectrum of the native and modified nylon C-CP fibers.

Figure 2. SEM images of the (left) native nylon C-CP fiber and (right) nylon-COOH C-CP fiber and column cross-sections.

Figure 3. Lysozyme loading breakthrough curves on the nylon-COOH C-CP fiber column at constant mobile phase linear velocity and various protein loading concentrations. The breakthrough curves are plotted on the time basis. (Column length: 200 mm, I.D.: 0.762 mm. Loading buffer: lysozyme in 20 mM phosphate buffer, pH 6.5. Flow rate: 0.4 mL min⁻¹. Detection: 280 nm)

Figure 4. Lysozyme loading breakthrough curves on the nylon-COOH C-CP fiber column at constant protein loading concentration and various mobile phase linear velocities. The breakthrough curves are plotted on the volume basis. (Column length: 200 mm, I.D.: 0.762 mm. Loading buffer: 1 mg mL⁻¹ lysozyme in 20 mM phosphate buffer, pH 6.5. Detection: 280 nm)

Figure 5. Chromatograms of 10 continuous lysozyme loading/elution cycles on the nylon-COOH C-CP fiber column without column regeneration in between. (Column length: 200 mm, I.D.: 0.762 mm. 1 mg mL⁻¹ of lysozyme in buffer A was loaded at 0.5 mL min⁻¹ for 8 min at room temperature (~ 20 °C), column washed with buffer A at 0.5 mL min⁻¹ for 15 min, and elution with buffer B at 0.5 mL min⁻¹ for 7 min. Detection: 280 nm.)

Figure 6. Chromatograms of lysozyme loading/elution on 5 nylon-COOH C-CP fiber columns prepared in different weeks. (Column length: 200 mm, I.D.: 0.762 mm. 1 mg mL⁻¹ of lysozyme in buffer A was load at 0.5 mL min⁻¹ for 8 min at room temperature (~20 °C), columns washed with buffer A at 0.5 mL min⁻¹ for 15 min, and elution with buffer B at 0.5 mL min⁻¹ for 7 min. Detection: 280 nm.)

Figure 7. Separations of (1) myoglobin, (2) α-chymotrypsinogen A, (3) cytochrome C and (4) lysozyme on the native and nylon-COOH C-CP fiber columns at linear velocities of 44 and 219 cm min⁻¹ (0.1 and 0.5 mL min⁻¹) reported on a volume basis. Blue dashed lines depict the gradient composition of buffer B used in the separation. (Injection volume: 5 μL, sample concentration: 0.25 mg mL⁻¹ of each protein, temperature: ~20 °C, detection: 280 nm)

Table 1. Dynamic loading capacity (DLC) of lysozyme on nylon-COOH column at different protein loading concentrations.

Loading Concentration (mg mL ⁻¹)	Native nylon DLC @50% BT (mg g ⁻¹)	Native Nylon DLC @50% BT (mg mL ⁻¹)	Nylon-COOH DLC @50% BT (mg g ⁻¹)	Nylon-COOH DLC (mg mL ⁻¹)		
				@10% BT	@50% BT	10%/50% Ratio
0.05	0.63±0.44	0.37±0.26	19.1±0.2	9.21±0.08	10.7±0.1	0.86
0.1	1.81±0.02	0.7±0.01	20.1±0.02	9.18±0.02	11.2±0.01	0.82
0.2	0.87±0.19	0.52±0.11	20.6±0.01	9.44±0.01	11.4±0.01	0.82
0.4	0.81±0.27	0.48±0.16	21.1±0.4	9.71±0.21	11.7±0.2	0.83
0.6	1.24±0.47	0.74±0.28	22.2±0.03	10.4±0.02	12.4±0.02	0.84
0.8	0.94±0.67	0.56±0.40	22.3±0.04	10.4±0.03	12.4±0.02	0.84
1.0	1.66±0.10	0.99±0.06	22.6±0.9	10.5±0.5	12.6±0.5	0.84

Table 2 Comparison of nylon-COOH with other WCX phases

Name	Protein binding capacity (mg mL ⁻¹)	Linear velocity (cm min ⁻¹)	Bed height (mm)	Ref
Nylon-COOH C-CP fibers	12	44 - 440	200	
Polyacrylate-grafted polyacrylamide cryogel monolith	6	N/A		[62]
Methacrylic acid-polyethylene glycol diacrylate monolith	0.065	<19	70	[63]
CIMac TM CM monolith	9 – 11	Recommended 1-10 Maximum 15	5	BIA
ProSwift WCX-1S monolith	23	Recommended 6.4 – 25.6 Maximum 32	50	Thermo
Sartobind® C membrane	21	Recommended 2 – 6 Maximum 233	4	Sartorius

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5

Figure 6

Fig. 6

Figure 7

Fig. 7