Affinity membrane adsorbers for binding arginine-rich proteins

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ABSTRACT
Delivering protein chemotherapeutics into cancer cells is a challenge. Fusing the protein to an arginine-rich cell-penetrating peptide offers a possible solution. The goal of this work was to develop an affinity membrane for the purification of Arg-rich fusion proteins via capture chromatography. Membranes were prepared by grafting polymers bearing diethyl-4-aminobenzyl phosphonate (D4ABP) ligands from macroporous membrane supports. Incorporation of D4ABP was studied by infrared spectroscopy and energy dispersive spectroscopy. Protein-binding capacities of 3 mg lysozyme/mL were measured. While further studies are required to evaluate binding kinetics and Arg-selectivity, achieving higher protein-binding capacity is needed before investment in such studies.

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Introduction
Advances in anticancer drugs have led to protein chemotherapeutics that work by targeting abnormal proteins expressed within cancer cells, rather than by killing rapidly dividing cells, some of which might be healthy. A challenge in the development of new protein therapeutics is delivering them successfully into cells.[1,2] One approach is to make use of cell-penetrating or cell-permeable peptides (CPPs) by fusing the peptide to the protein therapeutic, enabling both to enter the cell.[3,4] One CPP is the TAT peptide (GRKKRRQRRRPQ), which is rich in arginine (R) and is a well-studied fragment of the HIV transactivator protein. The TAT peptide has been conjugated with silica nanoparticles,[5] fused to drug biopolymer carriers[6] and fused directly to antitumor peptides[7] all for the purpose of enhanced intracellular drug delivery. A large number of arginine units in the TAT peptide that facilitate transmembrane transport of the fusion chemotherapeutic also serve as a target for developing a purification strategy for CPPs, particularly for those that lack an Fc binding domain that cannot be purified by Protein A chromatography.

The purification process for protein therapeutics prepared by cell culture involves centrifugation and depth filtration to remove intact cells and cell debris, followed by chromatography operations that typically include one or more affinity chromatography, ion-exchange (IEX) chromatography and hydrophobic interaction chromatography (HIC) steps to isolate the target protein, and ends with ultrafiltration/diafiltration for concentration and formulation of the product.[8] Affinity chromatography, which is the reversible binding of a target protein to an immobilized ligand for which the target protein has high affinity, is a powerful technique because it can obtain a high degree of purification in a single step. An important example is Protein A chromatography, which is used for monoclonal antibody (mAb) purification. One drawback to Protein A affinity chromatography is that it is an order of magnitude more expensive than other chromatography steps, as the ligand which binds the product is a protein itself.[9] A second, more limiting drawback is that it is not effective for purification of proteins that lack an Fc binding domain or those that lose activity at the low pH needed for elution from the Protein A column.

Alternative affinity chromatography techniques for protein purification include (1) immobilized metal ion affinity chromatography (IMAC), in which histidine-rich regions within the protein or His-tags of fusion proteins have an affinity for metal ions[10]; (2) implementation of less expensive, orthogonal IEX and HIC steps in series[11,12]; and (3) use of synthetic ligands.[13–15]
Depending on the target protein, other affinity ligands may be used to bind to specific chemical characteristics, such as is done with glycoproteins, dehydrogenases and endotoxins. Ayyar et al. provide an in-depth review of affinity chromatography that describes these and other Protein A alternatives, including their commercial availability.

This work explored the potential for a new class of affinity chromatography materials, which targets the Arg-rich region or Arg-tag of a protein or fusion protein, with an important class being CPPs such as the TAT peptide. Beyond the utility for obtaining CPPs, using chromatography media that bind to an Arg-tag may be preferable to IMAC media that bind the widely used His-tag, a metal ion-based affinity group. IMAC employs chelated Ni(II), Zn(II) or Co(II) ions that can leach metal ions into solution, causing protein loss and lower protein yields, as well as posing a health risk, as Ni(II) and Co(II) are known carcinogens. Additionally, Arg residues generally are located on the outer surfaces of proteins, making them more accessible for binding than His. One chemistry that has been shown to have a strong affinity for Arg-rich proteins is the “Arginine tweezer” formed by polymeric bisphosphonates. Covalently attaching similar functionality to the surface of a chromatographic stationary phase may yield new media that can effectively purify proteins bearing Arg-tags or Arg-rich regions.

A column packed with a bed of resin beads is the traditional stationary phase used in the purification of therapeutic proteins. An important advancement in chromatography for protein separations has been the emergence of membrane chromatography, in which a stack of porous membranes replaces the resin bed. In this platform, ligand-binding sites are attached to the internal surfaces of the membranes, and fluid flows through the open pore network of the membrane bed. Literature reviews of membrane processes in biotechnology articulate that one advantage membranes offer is the convective transport of the protein to the binding site due to their open pore structure. Thus, lower residence times and faster flow rates can be implemented to increase productivity (i.e., the throughput of purified protein per column volume per time). Increased productivity is becoming more important in commercial-scale manufacturing, and is one of the key motivations for innovative solutions like continuous downstream bioprocessing. This work is motivated by the prospect of using the aforementioned chemistry in conjunction with this high-throughput membrane platform to contribute to the development of a new chromatographic separation medium for the isolation and purification of Arg-rich proteins and peptides.

This study employed surface-activated atom transfer radical polymerization (ATRP) to graft polymers with functional epoxide groups capable of further modification with affinity ligands. This approach has been shown to yield membranes with protein-binding capacities that are competitive, and in some cases superior relative to commercial resins. Because bisphosphonate monomers are not available from commercial vendors, diethyl-4-aminobenzyl phosphonate, a molecule containing a single phosphonate group on a disubstituted benzyl amine, was selected as the affinity ligand. The hypothesis was that the proximity of phosphonate groups within the polymer after ligand incorporation may achieve some of the characteristic “tweezer” shapes like that of the trisubstituted bisphosphonate.

Experimental section

Materials

The following chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO) with purities reported in weight percent where available: 2,2′-azobis (2-methylpropionitrile) (AIBN, 98%); α-bromoisobutyryl bromide (BIB, 98%); 2-butanone (MEK, ACS Reagent, ≥99.5%); copper(I) chloride (Cu(I)Cl, ≥99.995%); dichloromethane (DCM, ≥99.5%, contains 50 ppm amylene); glycidyl methacrylate (GMA, 97%, contains 100 ppm monomethyl ether hydroquinone as inhibitor); hydrochloric acid (HCl (aq), 37%, ACS grade); inhibitor removers for removing hydroquinone and monomethyl ether hydroquinone; lysozyme from chicken egg white (lyophilized powder, protein ≥90%); sodium chloride (NaCl, ≥99.0%); tetrahydrofuran (THF, anhydrous, ≥99.9%, inhibitor-free); zinc tetrafluoroborate hydrate, (Zn(BF$_4$)$_2$·xH$_2$O).

2-Bromo-2-methylpropionic acid (BPA, 98%) and 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA, 98 +%) were purchased from Acros Organics (Geel, Belgium).

The following chemicals were purchased from Fisher Scientific (Fairlawn, NJ): hydrogen peroxide (H$_2$O$_2$ (aq), 30%); methanol (HPLC grade, 99.9%); tetrahydrofuran (THF, certified, contains about 0.025% butylated hydroxytoluene as a preservative); phosphate buffered saline (PBS); sulfuric acid (H$_2$SO$_4$, 95.0–98.0%).

Diethyl 4-aminobenzyl phosphonate (D4ABP, 99%), methanol (anhydrous, 99.9%) and sodium hydroxide (NaOH, 97.0%) were purchased from Alfa Aesar (Ward Hill, MA).

Poly(glycidyl methacrylate) (PGMA, MW = 290,000 g/mol, polydispersity index (PDI) = 1.7 (gel-permeation chromatography (GPC)) was prepared by radical
polymerization of glycidyl methacrylate in MEK at 60°C with AIBN as an initiator. Purified PGMA was obtained from four precipitations from the MEK in diethyl ether and then dried under vacuum. The polymer was dissolved in THF to make a 0.5 wt% solution for dip-coating a PGMA anchoring layer onto silicon wafers.

Buffers used in this study include a binding buffer (B) composed of 10 mM PBS buffer modified to pH 7.4 with NaOH, and elution buffer (E) composed of B with 3 M NaCl. The protein solutions for this study comprised lysozyme at various concentrations in the range 0.1–1.0 mg/mL in buffer B. All protein solutions were filtered through a 0.2 μm cellulose acetate syringe filter (VWR, Radnor, PA) prior to use. Distilled deionized water was obtained using a Milli-Q water purification system (EMD Millipore, Bedford, MA) and was used to prepare all buffer solutions. All buffers and protein solutions were used at room temperature.

Regenerated cellulose membranes with a reported nominal pore size of 1.0 μm, thickness of 70 μm and diameter of 47 mm were purchased from Whatman, Inc. (Dassel, Germany). Single-side polished prime grade silicon wafers (1 cm × 3 cm) were purchased from Nova Electronic Materials (Flower Mound, TX).

**Silicon wafer preparation and surface modification**

Silicon wafers were cleaned, dip-coated and functionalized according to methods described in the literature with one difference: the solvent for PGMA deposition in this study was THF. Initiator groups for ATRP were incorporated by reaction with 2-bromo-2-methyl propionic acid as described in Bhut et al.

Volumes and masses are reported on a basis of one 1 cm × 3 cm silicon wafer. PGMA was grown from the surface of the functionalized wafers by surface-initiated ATRP of glycidyl methacrylate (GMA) (3.6 M, 5.2 g) in THF (5.0 mL) with a catalyst complex of Cu(I)Cl (9 mM, 0.019 mL) and PMDETA (9 mM, 0.019 mL) for 21 h. Details about this procedure were reported earlier by Wang et al.

Wafers were stored in THF and dried with N₂ before thicknesses were measured.

The epoxide-ring opening reaction was carried out in 22 mL glass test tubes sealed with rubber stoppers containing D4ABP (0.730 g, 0.25 M) in CHCl₃ (12 mL) and catalyst Zn(BF₄)₂ × H₂O (0.025 M, 0.104 g) under ambient conditions for times of 30 min, 1 h and 2 h on a shaker table at 40 rpm. After the reaction, wafers were rinsed three times in 10 mL CHCl₃ and stored in CHCl₃ until being dried with N₂ for thickness measurements.

**Membrane surface modification**

**Membrane surface activation and polymer grafting**

Membranes were activated by reacting with BIB (18 μM, 111 μL) in THF (49.9 mL) for 2 h under constant stirring at 35°C in a glovebox (MBRAUN UNILab) with a nitrogen environment in the same fashion as was reported earlier.

Membranes were modified further using surface-initiated ATRP of GMA (3.6 M, 17 g) in THF (16.35 mL) catalysed with a complex of CuCl (9 mM, 29.7 mg) and PMDETA (9 mM, 0.063 mL) for 21 h.

**Incorporation of D4ABP ligand**

Membranes were reacted with D4ABP under multiple conditions, based on the Zn(BF₄)₂ × H₂O-catalysed ring-opening reaction in solution at room temperature described in the literature. The reaction conditions listed in Table 1 were used for the heterogeneous surface modification reaction illustrated in Scheme 1. Formulation conditions varied solvent type, concentrations, epoxide:ligand:catalyst ratio, agitation rate, reaction times and headspace gas. Additional notes on the dissolution of D4ABP in these and other solvents are provided in Supplemental Material.

**Surface characterization**

**ATR-FTIR spectroscopy**

Membrane surfaces were characterized with attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) using a Thermo Scientific Nicolet 550 Magna-IR Spectrometer with a diamond crystal. An ATR correction was applied to each spectrum and the baseline was corrected manually within the Omnic ESP version 6.1a software. A background spectrum was collected prior to each measurement of a dried membrane sample, taken with 16 scans and a resolution of 4.0 cm⁻¹.

**Ellipsometry**

The surface dry layer thicknesses of the polymer layers before and after reaction with D4ABP were measured using a multi-angle ellipsometer (Beaglehole Instruments Picometer™, He–Ne laser, λ = 632.8 nm). Measurements were taken at three locations on each surface and averaged. Refractive indices of 3.875, 1.455, 1.525, 1.451 and 1.525 were used for silicon, silicon dioxide, PGMA, BPA and ATRP-grafted PGMA, respectively, within the model. Igor Pro 4.0.9.1 (Wavemetrics, Oswego, OR) software program was used to apply the Cauchy model to obtain the dry layer thickness from the measured values of Ψ and Δ and the specified refractive indices (RI).
**Scanning electron microscopy/energy dispersive X-ray spectroscopy**

The surface composition of the membrane was analysed using a Hitachi S3400 SEM via EDX. Aztec Version 3 Software for SEM (Oxford Instruments) was used for image analysis. Membrane samples were soaked in THF for 24 h to remove unbound reaction compounds. From SEM images, spectra were gathered from regions that visually represented the whole of the membrane surface. Additionally, spectra of unique surface features were collected to understand the nature of the artifacts but are not included in the data reflecting changes in the membrane surface composition over time. For each spectrum within each sample, elemental composition is reported on the basis of weight per cent.

**Membrane performance**

Membranes were equilibrated in buffer B before being introduced into 40 mL of lysozyme protein solution. Solutions were placed in a shaker bath for 22 h (50 rpm, 22°C). The initial and final protein concentrations were measured using a calibration curve relating absorbance (280 nm) to concentration with a Cary UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA). Static binding capacity was calculated using a mass balance, taking into account the small volume of buffer carried over after equilibration of the membrane in buffer B, to determine the mass of protein bound to the membrane per unit volume.

**Results and discussion**

**Surface characterization**

**D4ABP incorporation into PGMA-modified membranes**

ATR-FTIR spectroscopy was used to characterize the membrane surface chemistry before and after each surface modification step. Figure 1 shows the spectra of a

### Table 1. Conditions for catalyst-assisted ring-opening of PGMA epoxide groups for reaction with D4ABP.

| Solvent | Solvent volume per membrane (mL) | D4ABP (M) | Zn(BF\(_4\))\(_2\)H\(_2\)O (M) | Agitation (rpm) | Reaction time (h) | Gas in head-space |
|---------|----------------------------------|-----------|-------------------------------|-----------------|-----------------|-----------------|
| A       | MeOH 45                          | 0.25      | 0.025                         | None            | 2.5             | Air             |
| B\(^a\) | MeOH 45                          | 0.25      | 0.025                         | 40              | 7               | N\(_2\)         |
| C       | MeOH 45                          | 0.25      | 0.025                         | 40              | 19              | N\(_2\)         |
| D       | MeOH 45                          | 0.25      | 0.025                         | 40              | >400            | N\(_2\)         |
| D\(_2\) | CHCl\(_3\) 45                    | 0.25      | 0.025                         | 40              | 2, 4, 9, 43     | Air             |
| D\(_3\) | CHCl\(_3\) 12\(^d\)             | 0.25      | 0.025                         | 40              | 0.5, 1, 2       | Air             |
| E       | MeOH 45                          | 0.25      | 0.025                         | 40              | 7               | N\(_2\)         |
| F       | MeOH 45                          | 0.25      | 0.025                         | 40              | 7               | N\(_2\)         |
| G\(^b\) | MeOH 100                         | 0.011     | 0.012                         | 40              | 19              | Air             |
| H\(^b\) | CHCl\(_3\) 100                   | 0.011     | 0.012                         | 40              | 19              | Air             |
| H\(_2\) | CHCl\(_3\) 100                   | 0.011     | 0.012                         | 40              | 2, 4, 9, 43     | Air             |
| H\(_4\) | CHCl\(_3\) 100                   | 0.011     | 0.012                         | 40              | 20              | Air             |
| I\(^b\) | DCM 100                          | 0.011     | 0.012                         | 40              | 27              | Air             |
| J       | THF 12.6                         | 1.23      | 0.025                         | None            | 4               | Air             |
| J\(_2\) | THF 12.6                         | 1.23      | 0.025                         | None            | 4, 8, 16, 24, 48, 123 | Air |

\(^a\)Membrane rinsed with DCM prior to reaction.

\(^b\)Added: (1) catalyst, (2) membrane, (3) D4ABP in 3 batches over 5 min to solvent.

\(^c\)Added: (1) catalyst, (2) membrane, (3) D4ABP in 2 batches over 5 min to solvent.

\(^d\)Volume is reported on the basis of one Si wafer chip.

![Scheme 1. Reaction of D4ABP with ATRP-grafted PGMA.](image)
bare membrane and one after ATRP of GMA. Figure 2 shows the spectra for membranes modified by D4ABP under different conditions. Spectra A, B, C and D in Fig. 2 were compared to see the effect of reaction time. Spectra B, E and F were compared to determine the effect of the reaction environment (agitation, headspace gas, rinse condition) with the same solvent, MeOH. The peak near 1500 cm$^{-1}$ is attributed to the 1,4-disubstituted aromatic ring of D4ABP. No significant difference is observed among conditions A, B, E and F in Fig. 2; so subsequent runs used an agitation speed of 40 rpm, headspace of air and no DCM rinse. Longer reaction times yielded higher relative absorbance values for the peak at 1518 cm$^{-1}$, which we hypothesized was due to higher conversion of epoxide groups with D4ABP. However, the time associated with condition D was determined to be unreasonably long.

The guiding reaction described in the literature used homogenous reaction conditions. In our case, the reaction was heterogeneous, so we employed the suggestion that a higher catalyst-to-ligand ratio may increase the rate of reaction. Figure 3 shows condition G from Table 1 (1.1:1.0 catalyst-to-ligand). A comparison with condition C (0.1:1.0) shows no increase in peak absorbance at 1518 cm$^{-1}$. Solvent selection was evaluated, as some solvents may interact/coordinate with the catalyst. It was reported that DCM was an effective solvent; however, D4ABP was found to not dissolve well in DCM. Chloroform also was tested as a solvent similar in structure, but containing an acidic proton capable of hydrogen bonding. The reaction with higher catalyst-to-ligand ratios was carried out in MeOH, CHCl$_3$ and DCM (conditions G, H, I). An effort was made to increase the concentration of D4ABP through the use of THF (condition J). Figure 3 shows that the relative absorbance for the peak near 1500 cm$^{-1}$ is largest in condition H, with CHCl$_3$. Additionally, the peak near 1450 cm$^{-1}$ observed for condition H may be attributed...
to the vibrations of P-CH$_2$- bonds. The small peaks near 850 cm$^{-1}$ in spectra H, I and J may be attributed to the C-H out-of-plane bending vibrations associated with a para-substituted benzyl group, as well as the stretching of the P-O-C aliphatic arms, both of which are present within the D4ABP compound but not on the membrane surface before the reaction.

In addition to being a hydrogen bond donor, CHCl$_3$ has a larger dipole moment than DCM, which leads to better dissolution of the catalyst and D4ABP in the solvent, and thus yields a faster rate of reaction. However, MeOH has a higher relative polarity than CHCl$_3$, and so polarity alone is not the only solvent factor impacting the rate of this heterogeneous reaction. Because it is known that PGMA is soluble in CHCl$_3$ and precipitates in cold methanol, a higher extent of reaction in CHCl$_3$ may be due to a greater accessibility to epoxy groups for the well-solvated PGMA in this solvent.

To better understand the relationship between peak height at 1500 cm$^{-1}$ and reaction time, additional time studies were conducted for conditions D (but in CHCl$_3$) and H, noted D2 and H2, respectively. A longer time study, with reaction times ranging from 4 to 123 h, was performed with reaction condition J in conjunction with EDX surface analysis of membrane samples. This study is noted as J2 and the accompanying EDX results are presented in a later section.

Figure 4 shows the FTIR spectroscopy results of reaction conditions D2, H2 and J2. In addition to the changes occurring near 1500 cm$^{-1}$ the peaks in the fingerprint region (1000–1150 cm$^{-1}$) of cellulose change with longer reaction times, and appear to be more characteristic of rayon. It appears that the acidic conditions introduced by the catalyst may be reorganizing some of the hydrogen bonds within the cellulose structure. In the application of membrane chromatography, structural integrity is important, and so this

![Figure 4. ATR-FTIR spectra of membranes modified under specified conditions. Part (a) presents conditions H2 and D2 for 2 and 43 h, an insert (b) is given for better view. Part (c) presents condition J2 for times 4–123 h and shows entire scan, an insert (d) is given for better view.](Image)
change to the substrate may be undesirable. While these changes were not as pronounced in the spectra for the membranes under the reaction conditions with THF, long reaction times were required to observe a peak height near 1500 cm\(^{-1}\) that was comparable to the heights for shorter reaction times using CHCl\(_3\). For this reason, and also because the peak at 1500 cm\(^{-1}\) decreases as this membrane restructuring occurs, condition H2 for a time of 2 h was selected as the preferred condition.

These spectra were compared with spectral libraries after subtracting the spectrum for PGMA-21 h. The compound diethyl 4-(bis(2-hydroxyethyl)amino)benzylphosphonate shows a match of 78%. This finding indicates that D4ABP may be cross-linking with two epoxy groups found on neighboring or adjacent chains of PGMA. This outcome is reasonable considering the increased nucleophilicity of the substituted amine on D4ABP. The following sections discuss efforts made to estimate the amount of D4APB incorporated into the PGMA nanolayer chains using both a model Si wafer substrate and the PGMA-modified regenerated cellulose membrane substrate.

**Estimating D4ABP incorporation into PGMA-modified Si Wafer**

**Thickness and RI measurements.** Dry layer thickness measurements were performed after each subsequent reaction, using data on the previously measured layers in the model fit. All of the wafers underwent the same treatment up to the final reaction with D4ABP. The reaction with D4ABP was done for 30 min with wafers 1 and 3, 1 h with wafers 4 and 5 and 2 h with wafers 2 and 6. Figure 5 presents the dry layer thickness measurements. While a difference in the polymer layer thickness before and after incorporation of D4ABP was anticipated, it was not observable within the standard deviation of the measurements. In the combined layer model, the RI of ATRP PGMA and D4ABP-PGMA were obtained by the model fit in addition to the layer thickness. Figure 6 shows the RI of the ATRP PGMA layer and the D4ABP-PGMA layer for each wafer. There was a decrease in the RI after the incorporation of D4ABP, which is supported by the accepted literature values for the individual species: PGMA (1.525) and D4ABP (1.497). Wafer 2 exhibited a refractive index higher than expected (1.570) for reasons that are unknown.

**Fractional conversion analysis.** To estimate the fractional conversion, we considered the D4ABP-PGMA polymer film to be a mixture of two species. The reaction of PGMA with D4ABP was assumed to occur uniformly throughout the layer, and it was assumed that PGMA and D4ABP-PGMA form an ideal mixture. Equation (1) was used as the framework to compute fractional conversion, where \(v\) is the molar density and \(x\) is the mole fraction:

\[
\frac{1}{v_{\text{PGMA} - \text{D4ABP}}} = \frac{x_{\text{PGMA}}}{v_{\text{PGMA}}} + \left(1 - x_{\text{PGMA}}\right) \frac{1}{v_{\text{D4ABP}}}
\]  

(1)
For a binary system, the mole fraction of the product \((1 - x_{PGMA})\) is also the fractional conversion, \(f\). To make use of this relationship, we obtained the molecular volume, \(N\), of the individual and the mixed species with the Lorentz–Lorenz equation (Eq. 2), a common expression used for refractive index analysis.\(^{[41]}\)

\[
\Theta = \frac{n^2 - 1}{n^2 + 2} = \frac{4\pi}{3} N \alpha
\]

(2)

The refractive index of one species is represented by \(n\) and the molecular polarizability is represented by \(\alpha\). For this analysis the molecular polarizabilities of the ATRP PGMA layer and the PGMA-D4ABP layer were assumed to be the same, making \(N\) proportional to \(\Theta\). Equation (1) can be rearranged to take the form of Eq. (3):

\[
x_{PGMA} = \left(\frac{V_{PGMA} - V_{D4ABP}}{V_{PGMA}}\right)\left(\frac{1}{V_{D4ABP} - V_{PGMA}}\right)
\]

(3)

and through substitution, can be combined with Eq. (2) to obtain \(x_{PGMA}\) in terms of \(\Theta\) as shown in Eq. (4):

\[
x_{PGMA} = \left(\Theta_{PGMA} - \Theta_{D4ABP}\right)/\left(\Theta_{PGMA} - \Theta_{D4ABP}\right)\left(\frac{1}{\Theta_{D4ABP} - \Theta_{PGMA}}\right)
\]

(4)

Because the RI for wafers 1, 3 and 5 did not change significantly after the reaction (Fig. 6), only wafers 2, 4 and 6 are reported in Table 2. The variation in \(f\) is the standard deviation of three measurements on the wafer.

The fractional conversion varied widely between the two 2 h wafer samples due to the difference in the RI values measured for the PGMA-D4ABP layer obtained from each wafer. Although the data were not consistent enough to quantify the amount of D4ABP incorporated, it confirmed a chemical change in the polymer film. It may be possible that the polymer contained a gradient of converted epoxy groups, as some are more or less accessible within the dense polymer layer. This means the RI would not be uniform throughout the polymer film, as the model assumes. This may be one reason why large variation among wafers was observed.

| Time (h) | Wafer | Fractional conversion, \(f\) |
|---------|-------|-----------------------------|
| 0.5     | 1     | –                           |
| 1       | 3     | 0.57 ± 0.08                 |
|         | 4     | –                           |
| 2       | 2     | 0.24 ± 0.20                 |
|         | 6     | 1.17 ± 0.22                 |

### Measuring D4ABP incorporation using elemental analysis of the membrane surface

Ellipsometry cannot be used to quantify the amount of D4ABP reacted to the surface of the PGMA nanolayers grafted from the surface of the regenerated cellulose membrane. Instead, EDX was performed on select regions of the membrane surface. Figure 7 shows images of the membrane surface prepared using various reaction conditions. The SEM images show that the membrane pore morphology changes as a result of reaction with D4ABP. This finding complements the findings from FTIR that showed changes in the cellulose structure with longer reaction times that appear to be a result of the acidic conditions introduced by the catalyst. Table 3 shows the composition of carbon, oxygen, phosphorous and zinc (contaminant) present in PGMA-modified membranes reacted under condition J2 over a range of times. Included in this table are the theoretical compositions of regenerated cellulose, PGMA and fully reacted D4ABP-PGMA. The results of EDX indicate an increase in carbon and a decrease in oxygen as the reaction proceeds. X-rays in EDX are generated from within a region of about 2 µm from the surface. Thus, the underlying RC support contributes significantly to the measured composition, especially at low reaction times where the polymer coating is thinnest. The decrease in weight percentage oxygen aligns with the reaction proposed in Scheme 1. The source of the increase in weight percentage carbon is unclear. Phosphorous content is observed in all membranes that underwent the reaction, and is noticeably higher (3.6%) for the condition with the reaction time of 123 h. Fractional conversion was calculated based on weight percentage of phosphorous in the samples compared with a theoretical weight percentage for the fully reacted polymer. This calculation indicates that the reaction was about 40% complete after a time of 123 h.

An increase in phosphorous content is also supported with ATR-FTIR analysis of these samples, as shown in Fig. 4d. Over time, a steadily increasing peak height near 1500 cm\(^{-1}\) (attributed to the 1,4-disubstituted aromatic ring of D4ABP) is observed with a more pronounced peak at 123 h.

### Static binding capacity

Maximum binding capacities of only 3 mg/mL lysozyme were measured for membranes modified following condition H2, which was identified to be the preferred condition among those studied. This value was lower than desired and lower than membranes prepared by other groups that used bisphosphonate ligands. He et al.\(^{[25]}\) photo graft polymerized copolymers of bisphosphonate-m-xylylene methacrylamide...
and methacryloylamino-2-hydroxypropane from low porosity PET track-etched membranes and measured lysozyme capacities of 2 mg/mL. Liu et al. used surface-initiated ATRP to graft this same copolymer from the surface of regenerated cellulose membrane and measured static binding capacities in the range of 10–12 mg/mL lysozyme per volume membrane. They indicated that incorporation of the spacer monomer was important for achieving these capacities.

Estimating the number of epoxy groups per membrane volume by measuring changes in dry mass before and after ATRP indicated that the molar ratio of adsorbed lysozyme to potential binding sites (assuming 100% conversion of epoxy groups) is less than 1:1000. This indicates either relatively little D4ABP was incorporated into the membrane or sufficient D4ABP was incorporated, but was not in a favourable orientation to facilitate multivalent affinity adsorption, as observed with bisphosphonate-based polymers. Liu et al. offer another explanation for the difference in affinity between bisphosphonate and monophosphonate ligands based on molecular dynamics simulations. They demonstrated that hydrogen bonds form and break at picosecond time scales. The presence of two phosphate arms in bisphosphonate ligands ensures the formation of at least one hydrogen bond between each repeat group and Arg groups on lysozyme at all times.

Table 3. Elemental analysis of regenerated cellulose membrane surface after modification with PGMA for 21 h and D4ABP in THF for various reaction times.

| Composition wt% | Fractional conversion |
|-----------------|-----------------------|
| Time (h)        | C   | O   | P   | Zn   |                     |
| 0               | 59  | 41  | 0.4 | 0.00 |                     |
| 4               | 57  | 40  | 0.9 | 3    | 0.1                 |
| 8               | 72  | 27  | 0.4 | 1    | <0.1                |
| 16              | 71  | 26  | 0.6 | 1.6  | <0.1                |
| 24              | 72  | 26  | 0.8 | 1.2  | 0.1                 |
| 48              | 72  | 27  | 0.9 | 0.5  | 0.1                 |
| 123             | 70  | 24  | 3.6 | 1.8  | 0.4                 |
| RC              | 47  | 53  | 0   | 0    | 0.0                 |
| PGMA            | 60  | 40  | 0   | 0    | 0.0                 |
| PGMA+D4ABP     | 59  | 28  | 9   | 0    | 1.0                 |

Conclusions

ATR-FTIR spectroscopy, RI analysis and EDX measurements support incorporation of the benzyl phosphonate ligand into ATRP-grafted PGMA through a catalyst-assisted ring-opening reaction. Formulation conditions that yielded large IR peak heights associated with the ligand were used to construct affinity membranes that bound lysozyme, a model protein with an Arg-rich region. The lysozyme-binding capacity using D4ABP was low compared to bisphosphonate ligands used in prior studies, but it is not clear if this outcome is due to low fractional conversion of epoxide groups on the membrane to ligand or if the orientation of the ligands is unfavourable for multivalent binding to occur. Because there is the potential that two epoxy groups reacted with the primary amine of D4ABP, there is potential to increase the ligand incorporation through other reaction approaches, such as coupling the D4ABP with the GMA monomer before performing ATRP. However, the approach taken here to couple the ligand after grafting PGMA circumvents challenges associated with ATRP of complex monomers and allows the use of commercial reactants. Further studies would need to be performed to understand important concerns including binding kinetics and Arg-selectivity. However, achieving higher static binding capacities is needed before investment in these studies should be undertaken.

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