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Phosphonium vs. Ammonium Compact Polyelectrolyte Complex Networks with Alginate – Comparing Their Properties and Cargo Encapsulation

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Abstract

Phosphonium and ammonium polymers can be combined with polyanions to form polyelectrolyte complex (PEC) networks, with potential applications in self-healing materials and drug delivery vehicles. While various structures and compositions have been explored, to the best of our knowledge, analogous ammonium and phosphonium networks have not been directly compared

to evaluate the effects of phosphorus versus nitrogen cations on the network properties. In this study we prepared PECs from sodium alginate and poly[triethyl(4-vinylbenzyl)phosphonium chloride], poly[triethyl(4-vinylbenzyl)ammonium chloride], poly[tri(*n*-butyl)(4-vinylbenzyl)phosphonium chloride], poly[tri(butyl)(4-vinylbenzyl)ammonium chloride] and poly[tris(hydroxypropyl)(4-vinylbenzyl)phosphonium chloride]. These networks were ultracentrifuged to form compact PECs (CoPECs) and their physical properties, chemical composition and self-healing abilities were studied. In phosphate buffered saline, the phosphonium polymer networks swelled to a higher degree than their ammonium salt containing counterparts. However, the viscous and elastic moduli, along with their relaxation times, were quite similar for analogous phosphoniums and ammoniums. The CoPEC networks were loaded with anions including fluorescein, etodolac, and methotrexate, resulting in loading capacities ranging from 5-14 w/w% and encapsulation efficiencies from 29-93%. Anion release occurred over a period of several days to weeks, with the rate depending greatly on the anion structure and polycation substituent groups. Whether the cation was an ammonium or phosphonium had a smaller effect on the release rates. The cytotoxicities of the networks and polycations were investigated and found to depend on both the network and polycation structure.

Keywords: phosphonium, ammonium, ionic network, polyelectrolyte complex, drug delivery, self-healing, sodium alginate

Introduction

Many traditional therapeutics suffer from limitations such as low patient compliance, side effects due to poor targeting, and difficulty controlling dosage.¹ For example, despite its convenience, the

oral administration of drugs often results in poor targeting to the disease site and a short circulation time of less than 12 hours.² To address these issues, controlled drug delivery systems based on nanoparticles³ micelles,⁴ liposomes⁵ and hydrogels⁶ have been of interest over the past few decades. These systems have been tuned to controllably release their payload to specific areas of the body over long periods of time. In particular, hydrogel networks are of interest for drug delivery as they are composed of large amounts of water, which can provide physical similarities to tissues and exhibit excellent capabilities to encapsulate hydrophobic drugs.⁶ Although hydrogels have found many uses in medicine, some disadvantages arise from their tendency to swell under biological conditions, and the delivery of therapeutics can be too rapid if the pore size is too large or there is no means to entrap and slow the release of molecules.⁷ To address these issues, pore sizes can be tuned⁸ and drugs can be entrapped in networks by covalent^{9,10} or ionic^{11–13} interactions.

While covalent bonding has been extensively employed for the preparation of networks, they can also be held together through physical entanglements or ionic interactions.¹⁴ The mixture of two oppositely charged polymers can create polyelectrolyte complexes (PECs) where the main driving force for network formation is the entropic release of bound counterions^{15,16} and water molecules¹⁷ (Equation 1).

Many different polycation/polyanion pairs have been explored for the creation of ionic networks.^{18–20} Processes such as ultracentrifugation or extrusion were shown to transform the initial weak, fluid-like complexes into compacted polyelectrolyte complex (CoPEC) networks with higher elasticity and tensile strength.¹⁴ These properties depend on network hydration and the presence of salt was shown to result in a dynamic exchange between the ions, resulting in self-healing properties.^{21–23} In polyion complex networks, ammonium-based polycations such as

poly(allylamine hydrochloride)^{23–25} and poly(diallyldimethylammonium chloride)^{26–28} have been the most thoroughly studied, while their analogous phosphonium polymers have been less explored. Ionically crosslinked polymer networks prepared from poly(acrylic acid) and poly(triethyl(4-vinylbenzyl)phosphonium chloride) were recently reported that were able to self-heal in physiologically relevant salt concentrations.²⁹ Ionic networks composed of sodium hyaluronate (**HA**) and four different phosphonium polymers were also explored for drug encapsulation and release.¹³ These networks exhibited the ability to self-heal in less than 24 hours and were able to retain and release drug molecules over 60 days, making them promising candidates for drug delivery.

While ammonium and phosphonium polymers have both been investigated for polyelectrolyte complex network formation, the properties of analogous ammonium and phosphonium networks have not been directly compared to the best of our knowledge. Ammonium and phosphonium polymers were compared in the context of polyelectrolyte multilayers³⁰ but they were not compared in terms of drug encapsulation and release or self-healing in this context. There are differences between ammonium and phosphonium salts that could have an effect on ionic network formation and the resulting material properties. For example ammonium salts are smaller than their phosphonium analogues and also have shorter C-N bonds (~ 1.53 Å) when compared to C-P (~ 1.81 Å).³¹ Furthermore, the cationic charge distribution is more localized on phosphorus, while more delocalized to the carbons adjacent to nitrogen.^{32,33}

For biomedical applications, naturally occurring anionic polysaccharides are of significant interest for network formation. Alginates, for example, are unbranched polysaccharides which contain varying proportions of 1,4'-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues.³⁴ The residues vary in sequence and can be arranged in consecutive M residues (MMMM)

or G residues (GGGG) or alternating M and G residues (MGMGMG) (Figure 1). Divalent Ca^{2+} ions are typically employed as the crosslinking agent to create alginate hydrogels and the mechanical properties of the hydrogels can be tuned by changing the amount of Ca^{2+} added.³⁴ The divalent cations are believed to bind only to the guluronate blocks of the alginate chains as the structure of these blocks allows for a high degree of coordination of the divalent ions. The guluronate blocks of one polymer can then interact with adjacent guluronate chains in an egg-box model of crosslinking.³⁵ Alginate gels have been used for many different applications such as wound healing³⁶ and drug delivery, although as drug delivery vehicles these systems have shown relatively fast release of drugs and proteins in aqueous media.^{37–39}

Figure 1. Sodium alginate polymer containing blocks of (1,4)-linked β -D-mannuronate (M) and α -L-guluronate (G) residues.

Here we report CoPEC networks based on 5 different poly(oniums) and sodium alginate. First, commercially available triethylphosphine, triethylamine, tri-*n*-butylphosphine, tributylamine and tris(hydroxypropyl)phosphine were reacted with 4-vinylbenzyl chloride to generate the corresponding polymerizable phosphonium and ammonium salts. Each monomer was then polymerized by free-radical polymerization to generate high molar mass polymers. These polyelectrolytes were then dissolved in water, mixed with sodium alginate, and then compacted to create CoPECs. The thermal properties, swelling, rheology, and self-healing properties of the

CoPECs were studied and compared to gain insight into the effects of the polycation on the network properties. CoPECs were loaded with a dye molecule and two anionic drugs to determine whether the release rates would differ based on the central atom (i.e. P or N), substituent groups around the cation, or if the properties of the loaded drug were dominant.

Experimental

General materials. Triethyl(4-vinylbenzyl)phosphonium chloride (**Et-P**), Triethyl(4-vinylbenzyl)ammonium chloride (**Et-N**), tri(*n*-butyl)(4-vinylbenzyl)phosphonium chloride (**Bu-P**), tri(*n*-butyl)(4-vinylbenzyl)ammonium chloride (**Bu-N**) and tris(hydroxypropyl)(4-vinylbenzyl)phosphonium chloride (**Hp-P**) were synthesized as previously reported.^{40,41} Poly[triethyl(4-vinylbenzyl)phosphonium chloride] (**P-Et-P**), poly[tri(*n*-butyl)(4-vinylbenzyl)phosphonium chloride] (**P-Bu-P**) and poly[tris(hydroxypropyl)(4-vinylbenzyl)phosphonium chloride] (**P-Hp-P**) were also synthesized as previously reported.¹³ Triethylamine, tributylamine, 4-vinylbenzyl chloride, fluorescein sodium salt and D₂O were purchased from Sigma Aldrich and used as received. Sodium alginate (**Alg**) – low viscosity was purchased from Carbosynth (Compton, UK) and used as received. Phosphines were donated by Solvay (Niagara Falls, Canada) and used as received. Etodolac and methotrexate were purchased from Ontario Chemicals Inc. (Guelph, Canada) and used as received. Phosphate buffered saline (PBS) had a pH of 7.4 and consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄.

General methods. Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AvIII HD 400 MHz Spectrometer (¹H 400.08 MHz). Spectra were referenced relative to the residual solvent peak (H₂O: ¹H δ = 4.79). Size exclusion chromatography (SEC) of the polymers was performed in 0.4 M tetrabutylammonium triflate in *N,N*-dimethylformamide (DMF)

using a Malvern Viscotek GPCmax instrument equipped with a Viscotek VE 3580 RI detector and two Inert series columns (P101609 and Q10183), operating at 50 °C. The flow rate was 1 mL/min. Number average molar mass (M_n), weight average molar mass (M_w), and dispersity ($\mathcal{D}$) were determined relative to poly(methyl methacrylate) (PMMA) standards. Differential scanning calorimetry (DSC) was performed at a temperature ramp rate of 10 °C/min on a DSC Q20 from TA Instruments (Waters, New Castle, DE) under an N₂ atmosphere. Aluminum T_{zero} pans containing 5–10 mg of sample were used. Thermogravimetric analysis (TGA) was completed on a Q600 SDT TA Instruments at a ramp rate of 10 °C/min up to 800 °C using a ceramic pan with 5–10 mg of sample. The onset degradation temperature (T_o) was determined as the temperature where 3% mass loss had occurred. UV-visible spectra were obtained on a Tecan Infinite M1000 Pro plate reader using Costar 96 well UV plates (#3635) with UV transparent flat bottoms. The cell toxicity assays were performed as previously reported¹³ and the full procedure is provided in the supporting information.

General method for the synthesis of ammonium polymers P-Et-N and P-Bu-N.

Monomers **Et-N**, **Bu-N** were dissolved separately in H₂O in round bottom flasks and initiator 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) was added to each. The flask was sealed with a septum, wrapped in Teflon tape, stirred, and degassed by bubbling N₂ using a needle at room temperature for 30 minutes. The reaction flasks were then placed into oil baths at 60 °C for 16 h.

Poly[triethyl(4-vinylbenzyl)ammonium chloride] (P-Et-N). Et-N (10.2 g, 40.2 mmol); 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) (65 mg, 0.20 mmol); deionized water (205 mL). No monomer peaks were visible in the resulting ¹H NMR spectrum, so no further purification was performed. Yield = 9.9 g, 97 %. ¹H NMR (400.08 MHz, D₂O) δ : 1.18 (broad s,

12 H, N-(CH₂)-CH₃, backbone CH₂ and CH), 3.03 (broad s, 6 H, N-CH₂-CH₃), 4.24 (broad s, 2H, Ar-CH₂-N), 6.55 (broad s, 2H, Ar-H), 7.20 (broad s, 2H, Ar-H). T_g = none detected up to 160 °C, T_o = 184 °C; SEC: M_n = 136 kg/mol, M_w = 315 kg/mol, $\bar{D}$ = 2.3.

Poly[tri(*n*-butyl)(4-vinylbenzyl)ammonium chloride] (P-Bu-N). Bu-N (9.60 g, 28.3 mmol); VA-044 (46 mg, 0.14 mmol); deionized water (190 mL). The polymer was purified by dialysis using 3.5 kg/mol molar mass cutoff (MWCO) membrane against 1 L of DI water for 48 h, changing the DI water once at 24 h. Yield = 9.0 g, 94 %. ¹H NMR (400.08 MHz, D₂O) δ : 0.79 (broad s, 11 H, N-(CH₂)₃-CH₃ and backbone CH₂), 1.19 (broad s, 7 H, N-(CH₂)₂-CH₂-CH₃ and backbone CH), 1.57 (broad s, 6 H, N-CH₂-CH₂-CH₂-CH₃), 2.94 (broad s, 6 H, N-CH₂-(CH₂)₂-CH₃), 4.35 (broad s, 2H, Ar-CH₂-N), 6.55 (broad s, Ar-H), 7.24 (broad s, Ar-H). T_g = none detected up to 250 °C, T_o = 275 °C; SEC: M_n = 63 kg/mol, M_w = 218 kg/mol, $\bar{D}$ = 3.5.

CoPEC network preparation. Phosphonium or ammonium polymer and sodium alginate were separately dissolved in deionized (DI) water at concentrations of 0.1 M in terms of the ions. After stirring, equal volumes of each of the solutions (typically 250 mL) were combined in a beaker and left to stir for 30 min. The polymer network was collected was then loaded into 15 mL ultracentrifuge tubes and centrifuged at 187,000 *g* for 1 h. The networks were removed from the tubes, cut into disks, and dried in a vacuum oven.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX). Samples were soaked in PBS for 24 h, rinsed with DI water, frozen, then lyophilized. They were then mounted on stubs covered in carbon tape and coated in 5 nm osmium using a SPI Supplies, OC-60A plasma coater. Imaging was performed using a LEO 1540XB instrument, operating at 1.0 kV and a working distance of ~ 4 mm. For samples **P-Et-P-Alg**, **P-Bu-P-Alg** and **P-Hp-P-Alg**, EDX was performed on the same instrument, with samples measured at an accelerating voltage of

20 kV and analyzed using an INCA EDAX system and software. Samples **P-Et-N-Alg** and **P-Bu-N-Alg**, were coated in 5 nm chromium and EDX was performed on a Hitachi SU3500 at an accelerating voltage of 20 kV and analyzed using the INCA EDAX system and software. The procedure for calculation of the carboxylate:onium ratio from the atomic percentage of N or P is provided in the supporting information.

Swelling measurement. The networks (about 150 mg) were dried and accurately weighed to determine the initial dry mass m_i . The networks were put into water (25 mL) at 37 °C with NaCl concentrations of 0 M (DI water), 0.1 M, 0.15 M, 0.25 M or into pH 7.4 PBS. They were then removed at various time points, blotted dry with paper towel, and weighed. The swelling of each network was then calculated as:

$$\text{Swelling \%} = \left(\frac{m_s - m_i}{m_i} \right) \times 100 \quad \text{Equation 2}$$

where m_s was the swollen mass and m_i was to the dried mass. The experiments were carried out in triplicate.

Rheology. The networks were prepared as described above, except that after ultracentrifugation they were pressed in between two Teflon® sheets to a thickness ~ 1 mm using a Carver 3851-0C melt press. The material was then punched into 5 cm diameter disks. The disks were soaked in PBS at 37 °C for 24 h prior to testing. Rheological measurements were then performed at 37 °C on an Anton Paar MCR 302 shear rheometer with a 50 mm diameter parallel plate tool (PP50). To prevent slip, fine grit sandpaper was affixed to the top and bottom plates. Small-amplitude oscillatory shear measurements were performed over the frequency range of 0.01 – 100 rad/s with a stress amplitude of 100 dyn/cm² for all samples. It was confirmed that these stress amplitudes were in the linear viscoelastic region (Figure S23). Frequency sweeps from 0.01 – 100 rad/s were

each performed in duplicate, and the results were highly reproducible. Crossover frequencies were taken as the intersection of the viscous (G'') and elastic (G') moduli (from the graph), and were the averages between the two runs. Relaxation times were determined as $1/\text{crossover frequency}$ with the frequency in units of rad/s.

Healing experiments. For the puncture tests, CoPECs were pierced with an 18-gauge needle to create 0.5 mm diameter holes. The networks were imaged immediately (Zeiss StereoLumar V12 microscope, 35x magnification) and then soaked in PBS and imaged at 0, 24 and 48 h.

Dye and drug loading. Both etodolac and methotrexate were purchased in their acidic forms, so each of the loading solutions were brought to a pH of 8 by the addition of NaOH prior to loading. Dried CoPEC disks with ~ 0.5 cm diameters (~ 150 mg) were placed in 0.1 M NaCl solutions containing 0.05 wt% fluorescein sodium salt, etodolac or methotrexate at 5.8 mg of network/mL, for 7 days to allow time for anion exchange to occur. After this time period, further uptake was not observed based on UV-visible absorbance analysis of the drug solution. The CoPECs were then taken out of the solution and quickly rinsed with DI water to remove any unbound surface molecules. The amount of dye or drug loaded into the CoPEC was determined by measuring the UV-visible absorbance (fluorescein 490 nm; etodolac 274 nm; methotrexate 372 nm) of the dye/drug solution before and after loading and calculating the change in concentration based on the calibration curve for the corresponding drug (Figures S28-S30). Loading capacities (LC) were then calculated as:

$$LC = \frac{M_D}{M_N} \times 100 \% \quad \text{Equation 3}$$

where M_D was the mass of drug encapsulated in the network and M_N was the dry mass of the CoPEC disk.

Encapsulation efficiency was calculated as:

$$EE = \frac{M_E}{M_I} \times 100\% \quad \text{Equation 4}$$

Where M_E was the mass of drug encapsulated in the CoPEC and M_I is the mass of drug in the solution before loading. Each experiment was performed in triplicate.

Release studies. The hydrated drug-loaded CoPECs prepared as described above were each incubated at 37 °C in 25 mL of PBS. 150 μ L aliquots of the release medium were then taken at various time points. The absorbances of these aliquots were measured on a plate reader at the appropriate wavelength for each drug (see dye and drug loading procedure above) to determine the concentration of released drug by comparison with its calibration curve in PBS (Figures S28-S30). The PBS release medium was changed at each time point. Cumulative percent release was calculated at each time point by dividing the mass of released drug by the total mass of encapsulated drug in the network. Each experiment was performed in triplicate.

Results and Discussion

Monomer and polymer synthesis. Monomers were synthesized by reacting triethylphosphine, triethylamine, tri(*n*-butyl)phosphine, tributylamine, tris(hydroxypropyl)phosphine, with 4-vinylbenzyl chloride to generate the corresponding salts (**Et-P**, **Et-N**, **Bu-P**, **Bu-N**, **Hp-P**; Scheme 1) as previously reported.⁴⁰⁻⁴² Tris(hydroxypropyl)amine was also reacted with 4-vinylbenzyl chloride, but the reaction did not proceed to completion and upon work-up the products swelled extensively and could not be purified. Polymerization of the remaining monomers was performed in water at 60 °C using the thermal initiator VA-044 for 16 h. Conventional free radical polymerization was chosen as it could provide high molar mass polymers.¹³ As previously

reported, **Et-P**, **Bu-P**, and **Hp-P** were polymerized to provide M_w values of 588 kg/mol for **P-Et-P** (measured by light scattering), 442 kg/mol for **P-Bu-P** ($\bar{D} = 2.6$, measured by SEC), and 666 kg/mol for **P-Hp-P** (measured by light scattering).¹³ The polymerization of **Et-N** proceeded with complete conversion (Figure S4). **Bu-N** polymerized but required purification by dialysis to remove residual monomer and afford pure polymer (Figure S7). The molar masses of **P-Et-N** ($M_w = 315$ kg/mol, $\bar{D} = 2.3$) and **P-Bu-N** ($M_w = 220$ kg/mol, $\bar{D} = 3.4$) were characterized by SEC in DMF containing 0.4 M tetrabutylammonium triflate (Figure S10).

Scheme 1. Syntheses of monomers, polymers and ionic networks. The specific structure of each polymer is included in Figure S1.

Based on TGA, T_o for the phosphonium polymers ranged from 290 – 344 °C (Figures S11A, 12A, 13A), while T_o for **Alg** was 220 °C (Figure S13B). Onset decomposition temperatures were much lower for the ammonium polymers, ranging from 184 – 275 °C (Figures S11B, 12B). The lower thermal stability of polyammonium salts has been well documented and can be explained by nucleophilic substitution or Hoffman elimination occurring at much lower temperatures (Scheme 2).⁴¹ For polymers **P-Et-N** and **P-Bu-N**, initial mass losses of ~25 and ~50% were observed at T_o , indicating a reverse nucleophilic substitution degradation pathway.

Scheme 2. Thermal degradation of ammonium polymers through Hoffman elimination or nucleophilic substitution.

DSC was also performed on all polymers to determine their glass transition temperatures (T_g). T_g values of **P-Et-P**, **P-Bu-P** and **P-Hp-P** were 230, 180 and 115 °C, respectively (Figures S14A, 15A, 16A). **P-Et-P** had the highest T_g which could be attributed to the better packing of the polymer chains and decreased segmental motion in comparison to **P-Bu-P** and **P-Hp-P**, which have more bulky pendent groups. No T_g values of **P-Et-N** and **P-Bu-N** were observed, as they degraded thermally before reaching the T_g (Figure S14B, 15B), in agreement with previous results for similar polymers.⁴¹ Similarly, no T_g for sodium alginate was observed below its degradation temperature (Figure S16B).

Network Synthesis and Characterization. Network preparation involved dissolving P or N-based polyelectrolyte and **Alg**, separately in DI water, at a concentration of 0.1 M in terms of the ions (onium or carboxylate). Equal volumes of solution at a 1:1 ratio of onium to carboxylate were then mixed and left to stir for 30 min. The resulting materials were then collected and ultracentrifuged to compact the networks⁴³ and form CoPECs **P-Et-P-Alg**, **P-Et-N-Alg**, **P-Bu-P-Alg**, **P-Bu-N-Alg**, and **P-Hp-P-Alg**.

TGA was performed on the dried CoPEC networks (Table 1) which revealed that all CoPECs followed a two-step decomposition pathway, with the initial decomposition due to alginate at ~220 °C (Figures S17, 18, 19). No T_g values were observed for **P-Et-P-Alg**, **P-Et-N-Alg**, **P-Bu-N-Alg**, or **P-Hp-P-Alg**. These results suggest that the polymers were well mixed in the **P-Et-P-Alg** and **P-Hp-P-Alg** networks as there was no detectable phase separation, which would be indicated by a T_g corresponding to the phosphonium polymer (Table 1; Figures S20A, 22). In contrast, the T_g of **P-Bu-P-Alg** (Table 1; Figure S21A) was observed at 145 °C and can likely be attributed to some degree of phase separation of the phosphonium and **Alg** given the bulky substituents around phosphorus that can hinder binding. Due to lack of measurable T_g for the analogous ammonium polymers, it is not possible to make conclusions regarding phase separation of the polymers in the analogous ammonium networks with **Alg**.

Table 1. T_o , T_g , atomic weight percent of P or N, carboxylate:onium ratios, swelling percent, crossover frequency and relaxation time values of polymer networks. Errors on the measurements correspond to the standard deviations on triplicate samples. ND = not detected.

Networks	T_o (°C)	T_g (°C)	Atomic w/w% of P or N (relative to C)	Carboxylate: onium Ratio	Swelling % in PBS (at 6 d)	Crossover Frequency; Relaxation Time
P-Et-P-Alg	232	ND	4.1 ± 0.2	(1.4 ± 0.2):1	300 ± 40	0.06; 17 s
P-Et-N-Alg	209	ND	3.5 ± 0.6	(2.2 ± 0.8):1	230 ± 10	0.2; 5 s
P-Bu-P-Alg	245	145	3.1 ± 0.1	(1.7 ± 0.8):1	320 ± 30	0.5; 2 s
P-Bu-N-Alg	218	ND	2.2 ± 0.2	(3.6 ± 0.8):1	230 ± 5	0.3; 3 s
P-Hp-P-Alg	246	ND	3.6 ± 0.1	(1.5 ± 0.1):1	160 ± 10	ND; > 100 s

Dry CoPEC networks were also analyzed by SEM-EDX to determine the atomic weight percentage of phosphorus or nitrogen relative to carbon, and thus the ratio of carboxylate (from **Alg**) to ammonium or phosphonium (Table 1). From these values, it is clear that there is excess carboxylate in all of the networks, even though stoichiometric amounts of carboxylate and onium ions were added to the formulations simultaneously. It is possible that the orientation of the carboxylates in the **Alg** chains makes it impossible for all carboxylates to effectively complex with phosphonium cation. Upon closer comparison, **P-Et-P-Alg** and **P-Et-N-Alg** did not have statistically different carboxylate:onium ratios. On the other hand, **P-Bu-N-Alg** had more than two-fold higher carboxylate:onium than **P-Bu-P-Alg**. This difference may result from more delocalization of the charge on nitrogen to surrounding carbon atoms, allowing more carboxylates to bind.^{33,44}

CoPEC Network Swelling. Physical properties of CoPECs can be changed by the addition of salt to break ion pairs, resulting in disruption of the network.⁴⁵ Therefore, it was of interest to determine the stability of the CoPECs in solutions containing different concentrations of NaCl and in PBS. Dried CoPECs were placed into either 0, 0.1, 0.15, 0.25 M NaCl or pH 7.4 PBS at 37 °C and the swelling was monitored for 16 days (Figure 2). Swelling varied based on the concentration of salt and the network composition. In DI water, most CoPECs swelled rapidly, turned white, and fragmented within a few hours. This phenomenon has been reported previously and can be attributed to the pores growing rapidly in water to a point where they become so large, they destroy the integrity of the CoPEC.⁴⁶ The growth of pores in the networks is consistent with their observed opacification, as larger pores would lead to increased scattering of light. On the other hand, our previously reported networks of **P-Hp-P**, **P-Bu-P**, or **P-Et-P** with **HA** underwent minimal

swelling in DI water and did not undergo fragmentation.¹³ All of the CoPECs, except for **P-Hp-P-Alg**, that were swelled in 0.25 M NaCl disintegrated over the first few hours and could not be measured further. High salt concentrations also led to the disintegration of previous phosphonium polymer networks with **HA**.¹³ The additional hydrogen bonding in CoPEC **P-Hp-P-Alg**, likely helped to stabilize the network, allowing it to survive for 6 days in 0.25 M NaCl before disintegrating. Interestingly, the network of **P-Hp-P** with **Alg** was more stable than the corresponding network with **HA**, which disintegrated in less than 2 days. These results in DI water and 0.25 M NaCl suggest that whereas the **HA** networks were destabilized by increasing salt concentrations, salt had a stabilizing effect on the **Alg** networks at low concentrations and a less destabilizing effect at higher concentrations.

At other salt concentrations, the networks composed of phosphonium polymers tended to swell to higher degrees than the analogous ammonium networks. For example, in 0.1 M NaCl, **P-Et-P-Alg** swelled to 250% and **P-Et-N-Alg** swelled to 230% after 24 h. Also at 0.1 M NaCl, **P-Bu-P-Alg** swelled to 220% and **P-Bu-N-Alg** only swelled to 170%. In 0.15 M NaCl and in PBS, **P-Et-P-Alg** swelled to 300% whereas **P-Et-N-Alg** swelled to only 230%. This trend was also observed for **P-Bu-P-Alg** and **P-Bu-N-Alg** in 0.15 M NaCl and in PBS where **P-Bu-P-Alg** swelled to 320% in comparison to **P-Bu-N-Alg** only swelled to 230%. Based on the swelling data, the equilibrium water content of these networks ranged from about 63 – 76% in PBS after 24 h. Higher swelling of the phosphonium polymer networks compared to the ammonium polymer networks may arise from weaker binding between the phosphonium and **Alg** carboxylates compared to ammonium cations with the **Alg** carboxylates. **P-Hp-P-Alg** swelled the least at all salt concentrations, which was attributed to hydrogen bonding interactions leading to a more densely packed network. With the exception of **P-Bu-P-Alg**, which began to exhibit increasing swelling in PBS after about 10

days, swelling of the CoPECs in 0.1 and 0.15 M NaCl and in PBS remained relatively stable over 16 days.

Figure 2. Percent swelling of CoPECs in solutions with different salt concentrations (relative to the dry state): A) **P-Et-P-Alg**; B) **P-Et-N-Alg**; C) **P-Bu-P-Alg**; D) **P-Bu-N-Alg**; and E) **P-Hp-P-Alg**. Swelling depended on the salt concentration and network composition. **P-Et-P-Alg**, **P-Et-N-Alg**, **P-Bu-P-Alg** and **P-Bu-N-Alg** disintegrated in 0.25 M NaCl after ~6 h and **P-Hp-P-Alg** disintegrated after 6 days. Error bars correspond to standard deviations (N = 3).

The structures of the materials were also probed by SEM. They were imaged after swelling in PBS for 24 h and then drying by lyophilization (Figure 3). Micrometer-sized pores were observed for all of the networks. Generally, the porosities were highest for **P-Bu-P-Alg** and **P-Bu-N-Alg**, followed by **P-Hp-P-Alg** then **P-Et-P-Alg** and **P-Et-N-Alg**. However, caution must be taken in interpreting these results as changes in morphology and artifacts can occur as a result of the drying process.

Figure 3. SEM images of A) **P-Et-P-Alg**; B) **P-Et-N-Alg**; C) **P-Bu-P-Alg**; D) **P-Bu-N-Alg**; E) **P-Hp-P-Alg** (the scale bar in A applies to all images).

Rheology. Rheology can provide information on the viscoelastic behavior of materials and the time scale of network relaxation.⁴⁷ Depending on the degree of crosslinking, as well as other aspects of a network, the material's viscous and elastic response to an applied stress can vary

greatly. The viscous (G'') and elastic (G') moduli of the CoPECs, swelled in PBS, were characterized under small-amplitude oscillatory shear over a frequency range of 0.01 to 100 rad/s with an applied stress amplitude of 100 dyn/cm (Figure 4, S24). All of the CoPECs had moduli that varied with frequency. **P-Et-P-Alg** and **P-Et-N-Alg** had relatively similar moduli, with G' ranging from about 0.2 to 10 kPa and G'' ranging from about 0.3 to 2 kPa over the studied frequency range. The crossover frequencies of the phosphonium and ammonium networks, at which $G' = G''$, were about 0.06 and 0.2 rad/s, respectively, corresponding to relaxation times of 17 and 5 s. As the longer relaxation time of the phosphonium network appears to conflict with its higher degree of swelling, and thus less dense network structure, it may relate to the higher molar mass of the phosphonium polymer. **P-Bu-P-Alg** and **P-Bu-N-Alg** also had similar moduli, with G' ranging from ~0.05 to 5 kPa and G'' ranging from about 0.1 to 1 kPa. The crossover frequencies were also very similar at about 0.5 rad/s for **P-Bu-P-Alg** and 0.3 rad/s for **P-Bu-N-Alg**, corresponding to a relaxation times of 2 and 3 s respectively. The short relaxation time of both of these networks could be due to the more hindered binding of the onium and carboxylate as both oniums contain bulky butyl groups. This steric hindrance may make the resulting ionic crosslinks more dynamic and easily broken than for the more strongly bound ion pairs. The effects of steric hindrance were also previously observed for polyamine-DNA CoPECs, where complexes formed from quaternary ammonium ions were less stable than those from primary ammonium ions.⁴⁸ **P-Hp-P-Alg** was predominantly elastic over the frequency range studied, with G' ranging from about 3 to 20 kPa and G'' from about 1 to 4 kPa. There was no crossover of G' and G'' in the measured frequency range, suggesting a relaxation time of longer than 100 s. Consistent with the low swelling of this network, these data suggest stronger and less dynamic noncovalent bonding in **P-Hp-P-Alg** than in the other networks. It was also noted that the rheology of the **Alg** networks was

different from that of **HA** networks formed with **P-Hp-P**, **P-Bu-P**, and **P-Et-P**. In general, the **Alg** networks had higher moduli. **P-Et-P** and **P-Bu-P** networks formed with **Alg** had shorter relaxation times than the corresponding **HA** networks. However, the **P-Hp-P-Alg** network had a longer relaxation time. Overall, the results indicate that the structure of the anionic polysaccharide is critical in determining interactions between the polyanion and polycation.¹³

Figure 4. Rheology frequency sweeps for (A) **P-Et-P-Alg**, **P-Et-N-Alg**; (B) Zoom of **P-Et-P-Alg**,

P-Et-N-Alg showing the crossover frequencies; (C) **P-Bu-P-Alg**, **P-Bu-N-Alg**; (D) Zoom of **P-Bu-P-Alg**, **P-Bu-N-Alg** showing the crossover frequencies; and (E) **P-Hp-P-Alg** in PBS. Replicate curves are in Figure S24.

Self-healing. The ability of CoPEC networks to self-heal, particularly under physiologically-relevant conditions, may be beneficial for drug delivery to tissues such as joints or skin, where mechanical stress can lead to network damage, but repair could occur upon the removal of the stress. To qualitatively evaluate the ability of the CoPECs to self-heal after damage, 0.5 mm holes were bored in the networks, which were then incubated in PBS at 37 °C for 48 h (Figure 5). After 24 h, the only networks to fully heal were **P-Et-P-Alg** and **P-Bu-P-Alg**. After 48 h, **P-Bu-N-Alg** had fully healed but **P-Et-N-Alg** and **P-Hp-P-Alg** had not. **P-Hp-P-Alg** networks had the longest healing time, with significant damage still detectable after 48 h. This result may correlate with the dominant elastic behavior and high viscous modulus of the **P-Hp-P-Alg** network as indicated by rheology, as these properties would slow the flow of polymer chains. **P-Hp-P** networks with **HA** also exhibited slow healing in our previous work.¹³ It is unclear why the **P-Et-N-Alg** network did not heal. However, the ability of most of the networks to heal indicated that the ionic bonding between the polyanions and polycations in the networks was generally dynamic, as previously reported for other CoPECs under certain conditions.^{21, 23}

Figure 5. Images of CoPECs following damage with a hole of 0.5 mm diameter (black circles), then healing over 48 h in PBS at 37 °C: (A) **P-Et-P-Alg**, (B) **P-Et-N-Alg**, (C) **P-Bu-P-Alg**, (D) **P-Bu-N-Alg**, (E) **P-Hp-P-Alg**.

Loading and release of anionic cargo. The presence of cationic and anionic charges throughout the polymer networks affords them with the capacity to ionically bind charged drug molecules. While the binding of cationic drugs is also likely possible, here we investigated the loading and release of the anionic dye fluorescein and two anionic drugs - etodolac or methotrexate (Figure 6). These molecules were loaded by immersing the networks in solutions 0.05 wt% solutions of dye/drug in 0.1 M NaCl. The loading capacity (LC, weight % relative to polymer) and encapsulation efficiency (EE) were calculated (Table 2).

Figure 6. Molecules chosen for loading: (a) fluorescein sodium salt; (b) etodolac sodium salt and (c) methotrexate disodium salt

Table 2. LC and EE of fluorescein, etodolac and methotrexate in **P-Et-P-Alg**, **P-Et-N-Alg**, **P-Bu-P-Alg**, **P-Bu-N-Alg** and **P-Hp-P-Alg** CoPEC networks. The mean values $\pm$ standard deviations are presented (N = 3).

	Fluorescein		Etodolac		Methotrexate	
	LC (%)	EE (%)	LC (%)	EE (%)	LC (%)	EE (%)
P-Et-P-Alg	12.1 $\pm$ 0.1	93.3 $\pm$ 0.1	14.3 $\pm$ 0.1	79.5 $\pm$ 0.8	11.7 $\pm$ 0.2	66.7 $\pm$ 0.9
P-Et-N-Alg	11.7 $\pm$ 0.1	90.3 $\pm$ 0.5	13.3 $\pm$ 0.1	74.1 $\pm$ 0.7	12.4 $\pm$ 0.1	70.6 $\pm$ 0.6
P-Bu-P-Alg	11.2 $\pm$ 0.4	84.1 $\pm$ 0.5	14.6 $\pm$ 0.1	81.2 $\pm$ 0.3	7.1 $\pm$ 0.2	40.4 $\pm$ 1.1
P-Bu-N-Alg	10.9 $\pm$ 0.1	84.2 $\pm$ 0.9	14.0 $\pm$ 0.1	78.0 $\pm$ 0.3	8.5 $\pm$ 0.2	48.3 $\pm$ 1.2
P-Hp-P-Alg	11.1 $\pm$ 0.3	85.9 $\pm$ 2.4	8.0 $\pm$ 0.8	44.5 $\pm$ 4.7	5.1 $\pm$ 0.2	29.0 $\pm$ 1.3

The loadings of the anions into the different networks were compared. The LCs of fluorescein in all CoPECs were similar, indicating that they did not depend greatly on the onium structure. The LCs of etodolac were also similar across most of the networks, and were generally higher than those of fluorescein, perhaps due to its more favorable interactions with the polymers in the

network. However, the loading of **P-Hp-P-Alg** with etodolac was lower, likely due to the higher polarity of **P-Hp-P**, resulting in a lower degree of hydrophobic interaction. For methotrexate, the LC varied depending on the structure of the onium polymer. **P-Et-N-Alg** and **P-Et-P-Alg** had the highest LCs and were similar to one another. The LC of **P-Bu-N-Alg** was also similar to that of **P-Bu-P-Alg**. However, these methotrexate LCs were significantly lower than for **P-Et-N-Alg** and **P-Et-P-Alg**, which could be due to the steric bulk of the butyl chains inhibiting drug binding. Finally, the LC of **P-Hp-P-Alg** was very low, indicating again that the increased steric bulk and hydrophilicity of this network could cause lower loading. The EEs correlated with the LCs and at the concentrations used in the loading experiments were greater than 70%, with the exception of for etodolac in **P-Hp-P-Alg**, and for methotrexate in **P-Bu-N-Alg**, **P-Bu-P-Alg**, and **P-Hp-P-Alg**. Overall for drug loading, the properties of the substituents on the phosphonium or ammonium ion dominated the drug loading properties compared to whether the cation was a phosphonium or ammonium.

Release of the molecules from the networks was studied in PBS at 37 °C. The release of fluorescein ranged from 30 – 100% in the first 8 days (Figure 7A, S25A). The fastest release occurred from **P-Bu-P-Alg** (~100 %) and **P-Bu-N-Alg** (~90 %), followed by **P-Hp-P-Alg** (~50 %), **P-Et-P-Alg** (~50 %), and **P-Et-N-Alg** (~30 %). The release of the etodolac (Figure 7B, S25B) was much faster than that of fluorescein from all of the networks. 100% release from **P-Bu-P-Alg** and **P-Bu-N-Alg** networks was reached in 4 days at very similar rates. 80% release of etodolac was reached in 8 days from **P-Hp-P-Alg**, **P-Et-P-Alg** and **P-Et-N-Alg**. The release of methotrexate disodium salt (Figure 7C, S25C) followed the same trends as those of etodolac, with the fastest from **P-Bu-P-Alg** and **P-Bu-N-Alg**, followed by **P-Hp-P-Alg** and **P-Et-P-Alg**, and finally **P-Et-N-Alg**.

Figure 7. Release of A) fluorescein, B) etodolac, and C) methotrexate from different CoPEC networks in PBS at 37 °C. Error bars correspond to standard deviations (N = 3). Complete release curves are given in Figure S25.

For each anion, the release rates from the networks followed the substituent trend butyl > hydroxypropyl > ethyl. More rapid release of anions from the networks containing the butyl substituents could be due to the steric bulk of the butyl substituents hindering the strong binding of the anion. It may also arise from the lower viscous and elastic moduli of these networks, as well as their dynamic properties indicated by the self-healing results, as the drug could more easily diffuse out of a more dynamic network than a static one. Like the butyl substituents, the hydroxypropyl substituents may also sterically hinder the strong ionic binding of drugs, favoring more rapid release. However, the lower swelling and rheological properties of the **P-Hp-P-Alg** may slow their release of drugs relative to the **P-Bu-P-Alg** and **P-Bu-N-Alg** networks. The ethyl

substituents were the least bulky, and led to the slowest release rate of each anion. Thus, the anion release rate can be tuned by changing the substituent on the onium and that atom. It also varied depending on the structure of the anion, as fluorescein was released more slowly than methotrexate or etodolac from each network. Different release rates for the different anions may relate to the different strengths of their ionic and hydrophobic interactions with the networks. In contrast, whether the cation was an ammonium or phosphonium did not have a large impact on the anion release rates, despite the differences in swelling of the phosphonium versus ammonium networks. In comparison to our previous systems involving **P-Hp-P**, **P-Bu-P**, and **P-Et-P** with **HA** complexed with hyaluronate¹³ the rate of anion release from the **Alg** networks was more rapid. In the phosphonium/**HA** systems, only 40 – 60% of fluorescein was released over 60 days, whereas in the **Alg** system, 50% release of fluorescein required only 2 – 12 days. Therefore, the choice of polysaccharide can affect network properties and release rates. In comparing the carboxylate:onium ratios, swelling, and rheology of the **Alg** versus **HA** networks, the only property that would be expected to contribute to a more rapid release of the anionic drugs is the higher carboxylate:onium ratios (1.4 – 3.6) for the **Alg** compared to the **HA** networks (0.5 – 1.4) as this excess of carboxylates may contribute to a more dynamic binding of the drug.

***In vitro* toxicity.** To probe the toxicity of the CoPECs, MTT metabolic assays were performed using C2C12 mouse myoblast cells. Varying concentrations of CoPEC leachate were added to the cells to assess the cytotoxicity of molecules that could diffuse out of the networks. After 24 h of incubation with the leachate, high cell metabolic activity (> 75 %) was retained for **P-Bu-P-Alg**, **P-Bu-N-Alg** and **P-Hp-P-Alg**, indicating these networks did not leach toxic species (Figure 8A). **P-Et-P-Alg** and **P-Et-N-Alg** led to metabolic activities of 56 and 27% respectively at 100%

leachate, indicating that some cationic polymers were likely leached into the culture medium, leading to toxicity.

Figure 8. Metabolic activities of C2C12 cells, as measured by MTT assays following 24 h of incubation in culture media containing: A) Leachate from phosphonium and ammonium CoPEC networks with **Alg**; B) Varying concentrations of polymers dissolved in culture medium. The metabolic activities of C2C12 cells incubated with **P-Et-P**, **P-Bu-P** and **P-Hp-P** were reported

previously¹³ but are included here for comparison. For both experiments, controls (100% metabolic activity) were cells incubated in media that was not exposed to CoPEC or polymer. Error bars correspond to the standard error (N = 6).

Cytotoxicities of the polymers alone were studied by incubating them with C2C12 cells in the cell culture media for 24 h. Then, MTT assays were performed to assess metabolic activity. **P-Et-P**, **P-Bu-P** and **P-Hp-P** were previously reported to result in low metabolic activities above 0.06 mg/mL and are shown here for comparison (Figure 8B).¹³ **P-Bu-N** also led to low cell metabolic activities above 0.06 mg/mL, similar to the phosphoniums. **P-Et-N** was more toxic than the other polymers, leading to low cell metabolic activities above 0.03 mg/mL. The high toxicity of **P-Et-N** likely explains the high toxicity of the leachate from **P-Et-N-Alg**, as very low concentrations of released **P-Et-N** would lead to toxicity. Given that **P-Et-P** had a similar toxicity profile to that of the remaining polymers, the lower metabolic activity of cells exposed to leachate from the **P-Et-P-Alg** network may arise from this network containing the lowest ratio of carboxylate:phosphonium (1.44:1), indicating there were higher amounts of cationic polymer in these networks compared to the other networks. Comparing the toxicities of **P-Bu-P**, **P-Bu-N** and **P-Hp-P** to their respective networks, the polymers were much less toxic than their **Alg** networks, in agreement with previous studies where the toxicities of cationic ammonium⁴⁹ and phosphonium¹³ polyelectrolytes were reduced upon complexation with polyanions.

Conclusions

CoPEC networks were prepared by mixing phosphonium and ammonium polymers with sodium alginate, and then were ultracentrifuged to create compact networks. **P-Hp-P-Alg** networks swelled the least, had the highest viscous and elastic moduli, and had the longest relaxation times,

most likely due to more hydrogen bonding in these networks than in the other networks. **P-Bu-P-Alg** and **P-Bu-N-Alg** networks had the shortest relaxation times, which could be attributed to weaker ionic interactions with alginate resulting from the bulky butyl substituents, compared to **P-Et-P-Alg** and **P-Et-N-Alg**, which both had longer times. Qualitative self-healing tests showed that **P-Hp-P-Alg** and **P-Et-N-Alg** did not fully heal over 48 h, but **P-Et-P-Alg** and **P-Bu-P-Alg** healed after 24 h, most likely due to their shorter viscoelastic relaxation times. The loading contents of the anionic dye molecule fluorescein in all of the networks were very similar, indicating that it did not depend heavily on the onium structure. The loadings for etodolac were very similar for all networks except for **P-Hp-P-Alg**, which could be attributed to the more hydrophilic nature of **P-Hp-P** and hydrophobic nature of etodolac. The loadings for methotrexate were highest for **P-Et-P-Alg** and **P-Et-N-Alg** and lower for **P-Bu-P-Alg**, **P-Bu-N-Alg** and **P-Hp-P-Alg** indicating that the more sterically bulky butyl substituents may interfere with methotrexate binding. Thus, the choice of polycation and drug both impact the drug loading content. More than 75% of etodolac and methotrexate was released from all of the networks over a period of 8 days. Fluorescein was released more slowly, but the release rate was significantly faster than from analogous networks prepared from the same phosphonium polymers with **HA**. Overall, it was concluded from this study that the structure of the drug, anionic polysaccharide, and the substituents on the onium ion all significantly impact the anion release rate. However, the use of nitrogen versus phosphorus cations did not greatly impact the anion release rates. *In vitro* cytotoxicity studies suggested that networks **P-Bu-P-Alg**, **P-Bu-N-Alg** and **P-Et-P-Alg** did not leach high concentrations of toxic species into the cell culture media, whereas **P-Et-P-Alg** and **P-Et-N-Alg** did, which could be attributed to the higher toxicity of **P-Et-N** in comparison to all other polymers and also to the higher ratio of **P-Et-P** to **Alg** in **P-Et-P-Alg**. Overall, these networks warrant further study for

drug encapsulation and controlled release, with further work focusing on the loading of cationic drugs and introducing biodegradable polycations. In addition, it will be of interest to explore the loading and release of cationic drugs from these networks.

Associated Content

Supporting Information. NMR spectra, SEC traces, TGA and DSC data, calculations of network composition, additional rheology data, procedure for MTT assay and additional MTT assay data. The Supporting Information is available free of charge on the ACS Publications website at DOI:

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Notes

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