Synthetic polycations with controlled charge density and molecular weight as building blocks for biomaterials

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ABSTRACT
A series of polycations prepared by RAFT copolymerization of N-(3-aminopropyl)methacrylamide hydrochloride (APM) and N-(2-hydroxypropyl)methacrylamide, with molecular weights of 15 and 40 kDa, and APM content of 10–75 mol%, were tested as building blocks for electrostatically assembled hydrogels such as those used for cell encapsulation. Complexation and distribution of these copolymers within anionic calcium alginate gels, as well as cytotoxicity, cell attachment, and cell proliferation on surfaces grafted with the copolymers were found to depend on composition and molecular weight. Copolymers with lower cationic charge density and lower molecular weight showed less cytotoxicity and cell adhesion, and were more mobile within alginate gels. These findings aid in designing improved polyelectrolyte complexes for use as biomaterials.

KEYWORDS
Polycation; RAFT; polyelectrolyte complexes; anti-fouling; biocompatibility; encapsulation

1. Introduction

Cell encapsulation may in future enable cell-based treatments of enzyme and hormone deficiency disorders such as insulin-dependent diabetes,[1] Parkinson’s disease[2], and lysosomal storage disorders.[3] This approach involves enclosing cells that express a therapeutic peptide such as insulin, in an immunoisolating hydrogel matrix prior to transplantation into patients, avoiding the need for immunosuppressing agents. The best-known capsule types are based on a calcium alginate gel core coated with poly-L-lysine (PLL) and a final layer of alginate (referred to as Alginate/PLL/Alginate or APA capsules).[4] The resulting alginate/PLL polyelectrolyte complex in the shell reduces the permeability of the capsule to immune system components[4,5] such as immunoglobulins or antibodies, and prolongs cell and capsule survival beyond the loss of calcium to the host.[6–8]

Recent studies show instances of poor host compatibility in these hydrogel capsules that are mainly attributed to the PLL. Exposed PLL can stimulate macrophages to produce TNF-α,[9,10] induce inflammation[11], and bind more immunoglobulins.[12,13] Hallé showed that some of the PLL is exposed on the surfaces of APA capsules,[14] enhancing...
host cell adhesion. As well, some types of encapsulated cells are sensitive to PLL in the coating solution.

Our group recently showed that reactive synthetic polyanions can be used to decrease the undesired net cationic charge density remaining on such capsule surfaces.

A more fundamental approach to mitigate concerns with the use of polycations such as PLL is to replace PLL with lower charge density polycations. Sawhney and Hubbell grafted polyethyleneglycol (PEG) onto PLL in order to reduce its net charge density. They observed reduced protein and complement binding, and less fibrotic overgrowth; however, the membrane required an additional, initial PLL layer for reduced permeability and stability. Chaikof and co-workers also developed PLL-g-PEG copolymers that tried to balance electrostatic binding ability with cell cytotoxicity. De Vos and co-workers recently prepared calcium alginate capsules coated with PLL-b-PEG diblock polymers that showed less fibrotic overgrowth compared to APA capsules, though requiring 50 h incubation with PLL-b-PEG to form an effective anti-fouling layer.

While PEG serves well in mitigating host reactions, there is interest in alternate hydrophilic polymers. Poly(N-(2-hydroxypropyl)methacrylamide) (HPM) and analogs have shown good biocompatibility and have been suggested as a replacement for PEG.

N-(3-aminopropyl)methacrylamide hydrochloride (APM) was selected as the cationic monomer in this study due to its similarity in structure to PLL, and to its ability to copolymerize with HPM using RAFT conditions, similar to those recently reported. Polymers containing APM have been previously used in numerous applications including layer-by-layer and bulk hydrogel cellular scaffolds, antimicrobial polymers, and bioconjugation.

We describe here synthetic, controlled charge density copolymers of HPM and APM. These synthetic copolymers are modeled in part after chitosan, a natural, low-charge density cationic polysaccharide, with HPM serving as a neutral hydrophilic co-monomer to provide anti-fouling properties.

As cationic charge density and molecular weight (MW) strongly affect cytotoxicity as well as polycation binding to polyanions such as calcium alginate, we used reversible addition-fragmentation chain transfer (RAFT) copolymerization, a controlled radical polymerization, to prepare APM/HPM copolymers with four co-monomer ratios and two molecular weights, balancing electrostatic binding ability against cytotoxicity (Scheme 1). In particular, polycations with high MW and reduced charge density (lower APM content) were expected to form alginate complexes with better cytocompatibility compared to PLL.

The polycations are identified as pAPMX–Y, where X represents the mol% APM and Y is the MW in kDa. The interaction of these polycations with calcium alginate gel beads and with mammalian cells was examined to assess their suitability as replacements for PLL in alginate-type capsules, and possibly other hydrogel films and matrices.

2. Experimental

2.1. Materials

Sodium alginate (Pronova UP MVG, batch #: BP-0908–01) was purchased from Novamatrix (Sandvika, Norway). Poly-L-lysine hydrobromide (PLL, $M_n$ 15–30 and 40–60 kDa),
Poly(methyl vinyl ether-alt-maleic anhydride) (PMM, $M_n \approx 80,000$ g/mol), rhodamine B isothiocyanate (RbITC), HEPES sodium salt, 4-cyanopentanoic acid dithiobenzoate (CTP), and 4,4’-azobis(4-cyanopentanoic acid) (V-501) and 3-aminopropyltriethoxysilane (APTES, ≥98%) were purchased from Sigma–Aldrich (Oakville, ON Canada), and HPLC grade water, acetonitrile, reagent-grade dioxane, sodium chloride, and calcium chloride from Caledon Laboratories (reagent grade, Georgetown, ON) were used as received. APM hydrochloride and HPM were purchased from Polysciences (Warrington, PA) and were used as received. Dulbecco’s modified Eagle’s medium (DMEM, high glucose, pyruvate), Fetal bovine serum (qualified, Canada origin), alamarBlue® Cell Viability Reagent, Penicillin–Streptomycin (10,000 U/mL), 0.25% Tryspin–EDTA (1X) phenol red, and TryPLETE™ Express Enzyme (1X) were obtained from Invitrogen (Burlington, ON), and Bovine calf serum (USA origin) was obtained from Sigma–Aldrich. C2C12 Mus musculus myoblasts (CRL-1772) and NIH/3T3 Mus musculus fibroblasts (CRL-1658) were obtained from ATCC.

2.2. General RAFT polymerization procedure

RAFT co-polymerizations of APM and HPM with 4-cyanopentanoic acid dithiobenzoate (CTP) as RAFT agent and 4,4’-azobis(4-cyanopentanoic acid) (V-501) as initiator were carried out in a 2:1 water/dioxane mixture. The ratio of $[\text{CTP}]_0:[\text{V-501}]_0$ was kept at 1:0.33, and $[\text{M}]_0:[\text{CTP}]_0$ ratios of 130:1 or 330:1 were used to target the low and high MW polymers, respectively. The monomer concentration was 2 or 4 M, respectively. A typical reaction is as follows:

APM (1.18 g, 6.60 mmol), HPM (0.320 g, 2.23 mmol), CTP (18.9 mg, 0.0676 mmol), and V-501 (6.20 mg, 0.0221 mmol) were dissolved in 4.5 mL of 2:1 (v/v) water:dioxane. The reaction mixture was purged with $N_2$ for 45 min at room temperature and then immersed
in an oil bath at 70 °C for 5 h. The polymerization proceeded under positive \( N_2 \) pressure, and aliquots of the reaction mixture were removed periodically with a \( N_2 \)-purged needle and syringe. The crude reaction mixture was analyzed by GPC and \( ^1H \) NMR (600 MHz) to determine the MW, polydispersity index (PDI), and conversion. The conversion of both monomers was determined by comparing the integrations of the vinylic \( ^1H \) NMR peaks at 5.7 and 5.5 ppm (APM and HPM) to the combined CH peak of monomeric and polymeric HPM (4.0 ppm). The remaining polymer was isolated from the reaction mixture by precipitation in acetone and collected by centrifugation. The polymer was dried under vacuum overnight, yielding a pink powder. The conversion for this reaction was 66%, with 656 mg polymer isolated. Typical reaction conversions and polymer yields were 65–80%.

Copolymer composition was determined by \( ^1H \) NMR analysis of the isolated polymers dissolved in \( D_2O \). The area of the peak at 4.0 ppm (1H from HPM) was compared with the area of the signals at 3.0–3.4 ppm (4H from APM + 2H from HPM).

The MW (\( M_n \)) of the copolymer after precipitation was additionally determined by \( ^1H \) NMR (Bruker 600 MHz, 1024 scans) by comparing the end-group signal at 7.9 ppm (2H) with the polymeric HPM signal at 4.0 ppm (1H). The theoretical MW (\( M_n^{\text{theo}} \)) was calculated using Equation (1).[37]

\[
M_n^{\text{theo}} = \frac{[M]_0}{[\text{CTA}]_0} \times MW_m \times \text{conversion} + MW_{\text{CTA}}
\]

\([M]_0 \) and \([\text{CTA}]_0 \) are the initial concentrations of the monomer (APM + HPM) and chain transfer agent, \( MW_m \) and \( MW_{\text{CTA}} \) are the molecular weights of the monomers (weighted average) and chain transfer agent, and conversion is obtained from \( ^1H \) NMR analysis of the reaction mixture at the end of the polymerization.

### 2.3. Removal of sulfur-containing chain ends

The dithiobenzoate chain ends were removed by reacting pAPM\(_X\) copolymers (0.574 g, 0.0387 mmol chain end) with a 20:1 M excess of V-501 (0.219 g, 0.781 mmol) in 5.4 mL of 2:1 water:dioxane for 24 h at 75 °C after purging with \( N_2 \) for 45 min. The polymers were precipitated in acetone and isolated by centrifugation. \( ^1H \)-NMR spectra (600 MHz, 1024 scans) confirmed the removal of the end-groups by disappearance of the signal for the aromatic protons at 7.9 ppm. The polymers were then dialyzed against de-ionized water and isolated by freeze-drying to yield 0.371 g (65%). This reaction was performed similarly for all compositions and molecular weights with typical recoveries of the copolymer following end-group removal of 65–85%.

### 2.4. GPC

The MW of pAPM\(_X\) copolymers were determined by gel permeation chromatography consisting of a Waters 515 HPLC pump, Waters 717 plus Autosampler, three columns (Waters Ultrahydrogel-120, -250, -500; 30 cm × 7.8 mm; 6 μm particles), and a Waters 2414 refractive index detector. The columns were maintained at 35 °C. Samples were eluted at a flow rate of 0.8 mL/min in a 0.5 M acetic acid/0.5 M sodium acetate buffer pH = 4.8 mobile phase. The system was calibrated using narrow-dispersed PEG standards (Waters, Mississauga, ON).
2.5. Alginate–polycation complexation

Model complexation experiments were performed at 154 and 377 mM NaCl concentrations. Polycations were dissolved at 0.5 wt% in saline with pH adjusted at 7.0–7.5. Sodium alginate solution at 0.5 wt% was prepared in saline. All solutions were filtered through 0.45-μm filters. Polycation solutions (0.5 mL) were placed in vials and varying volumes of sodium alginate solution were added to give a 1:1 ratio of COO− to NH$_3^+$. For experiments at 377 mM NaCl, a 1.49 M NaCl stock solution was added to 0.5 mL of polycation in saline such that the final NaCl concentration would be 377 mM upon addition of the correct volume of sodium alginate solution to achieve a 1:1 ratio of COO− to NH$_3^+$. Samples were observed by optical microscopy.

2.6. Fluorescent labeling of PLL and pAPM$_x$

PLL and pAPM$_x$ copolymers were fluorescently labeled with RbITC. For example, pAPM$_{75-15}$ (HCl form, 0.12 g, 0.71 mmol monomer units) was dissolved in 12 mL of 0.20 M NaHCO$_3$ buffer (pH 9) before adding 1.9 mg (0.0035 mmol) RbITC dissolved in 0.76 mL N,N-dimethylformamide. The solution was stirred overnight and then dialyzed in 3.5 kDa MW cut-off cellulose dialysis tubing (Spectrum Laboratories) for four days with at least daily changes of 4 L of de-ionized water, until no rhodamine or DMF could be detected in the dialysate by UV/Vis spectroscopy, using a Cary 50 Bio UV/Vis spectrometer. The sample was isolated by freeze-drying to yield 0.10 g pAPM$_{75-15}$ RbITC (76% yield). The extinction coefficient of this labeled polymer was 0.32 mL cm$^{-1}$ mg$^{-1}$, measured at 560 nm in 21 mM HEPES buffer (pH 7.5), corresponding to a labeling degree of 0.070 mol%, and a labeling efficiency of 14% based on the absorption coefficient of free RbITC of $8.7 \times 10^4$ M$^{-1}$ cm$^{-1}$ at 560 nm. The labeling reaction was performed on all pAPM$_x$ copolymers and PLL (15–30 and 40–60 kDa), resulting in extinction coefficients of 0.31–0.45 mL cm$^{-1}$ mg$^{-1}$, labeling degrees of 0.062–0.089 mol%, and labeling efficiencies of 13–19%.

2.7. Preparation of calcium alginate beads

Calcium alginate beads (CaAlg beads) were prepared as previously described.[38] Briefly, a solution of sodium alginate (5–10 mL, 1 wt%) in saline was filtered (0.2 μm) and then extruded through a flat-tipped 27 G needle into 60 mL of aqueous gelling bath solution containing 1.1 wt% CaCl$_2$ (100 mM) and 0.45 wt% NaCl (77 mM), at a rate of 0.5 mL/min. An annular coaxial airflow of 3–4 L/min was adjusted to control CaAlg bead diameter (approx. 0.5 ± 0.05 mm). The beads were kept in the gelling bath for another 10 min after extrusion, then transferred into a fresh gelling bath solution for additional 10 min (using a 3:10 volume ratio of settled bead suspension to wash solution). All solutions were pre-cooled to 4 °C and the gelling bath was placed in an ice bath during bead formation to mimic conditions used during actual cell encapsulations.

2.8. Coating alginate beads

CaAlg beads were coated with 0.1 wt% PLL or APM/HPM copolymers in saline (pH 7.0–7.5) for 6 min. The beads were washed with either saline or gelling bath for 2 min followed by
a final saline wash. The beads were coated or washed in a 3:10 ratio of beads to coating or washing solutions and stored in the final saline wash at 4 °C.

### 2.9. Confocal microscopy

Confocal microscopy of fluorescently labeled capsules was carried out on a Zeiss LSM510 confocal laser scanning microscope equipped with argon and HeNe lasers, operated with Zeiss LSM510 software. The extent of pAPM Preservation Attraction Membrane (pAPM) diffusion into the capsules, or membrane thickness, was determined by the full width at half height of 10-pixel-wide (36 μm) line profiles across equatorial confocal sections of three capsules, generated using ImageJ software. Confocal microscopy was performed within 1–4 days of capsule formation.

### 2.10. Cell viability tests using alamarBlue assay

C2C12 myoblasts were cultured in DMEM supplemented with 10% FBS in the presence of 5% CO₂, 95% air with 100% humidity at 37 °C in a water-jacketed incubator. Cells were plated into tissue culture-treated 24 well polystyrene plates at 50,000 cells/well in 1 mL of media. Cells were incubated overnight to allow for attachment. The media was then removed and cells were washed with PBS. Cells were incubated with 250 μL of polymer solutions in phosphate buffered saline pH = 7.4 (PBS) added to cells with 300 μL of serum free media for total polymer concentrations of 0.01, 0.1, and 1 mg/mL for 20 h at 37 °C with experiments done in triplicate. alamarBlue (55 μL) was added to each of the wells and incubated for another 3 h. The fluorescence of the reduced alamarBlue was measured by exciting at 560 nm and measuring the emission at 590 nm on a Synergy 4 Plate reader from BioTek. Cell viability was determined from the relative alamarBlue conversion of a positive control treated without polycation.

### 2.11. Cell attachment and proliferation tests

Glass bottom black-walled 96 well plates (In vitro Scientific) were washed with 95% ethanol and dried under an air stream prior to coating with a 2 v/v% solution of APTES in 95% ethanol at pH 4.5 for 2 min, washed with 95% ethanol, dried under air, and cured overnight. The wells were coated with 0.1 w/v% poly(methyl vinyl ether-alt-maleic anhydride) (PMM) in acetonitrile (ACN) for 5 min and washed with ACN. Polycation solution in de-ionized water (pH 8–9) was added to each well and allowed to react overnight before washing with de-ionized water and drying. Some well bottoms were further coated with PMM as above, then hydrolyzed overnight in 35 mM HEPES buffer pH 7.8 overnight. The plates were then soaked in PBS and stored in a refrigerator for 3–11 days before use. All aqueous solutions were filtered with 0.2-μm membrane prior to addition to well. Prior to use, the plates were sterilized with 70% ethanol for 30 min and washed with PBS. Certain wells were then coated with 0.03 wt% alginate in saline for 5 min and washed twice with saline. The resulting plate contains surfaces of (a) polycation, (b) polycation/alginate, and (c) polycation/PMM for each polycation composition and MW. The control wells do not have polycation, but only the layer of hydrolyzed PMM bound to the APTES-functionalized glass. NIH/3T3 fibroblasts were seeded with 2500 cells/well in DMEM supplemented with 10% BCS for three days before observing cell morphology with optical microscopy and
cell viability with alamarBlue. The alamarBlue assay was conducted by replacing the media with 100 μL of media containing 10% of the alamarBlue reagent and then incubating the samples for 4.5 h. The results represent three individual experiments, each done in triplicate.

2.12. Statistics

All values are reported and graphed as mean ± standard deviation, with significant differences determined by one-way ANOVA with Games-Howell post hoc analysis. Significance was determined for $p < 0.05$.

3. Results and discussion

Our aim was to prepare polycations with reduced charge density, but similar MWs compared to the 15–30 and 40–60 kDa PLL commonly used with alginate beads.[4,5,36] Hence, APM/HPM copolymers with 10, 25, 50, and 75 mol% APM, and $M_n$ of 15 and 40 kDa (DP of 90 and 240) were targeted by RAFT copolymerization.

RAFT polymerization using CTP as chain transfer agent and V-501 as initiator in aqueous acetate buffer at pH 5.0–5.2 has been reported for HPM [27] and APM,[28] and for HPM with 5–10 mol% APM.[29] Narain [39,40] and McCormick [41] have used a water/dioxane mixture to polymerize APM by RAFT, also using CTP and V-501. Aqueous and mixed aqueous/organic solutions are useful as APM is not soluble in many organic solvents in its acid form.

In the current work, attempts to use aqueous acetate buffer (pH = 4.8–5.2) showed poor CTP solubility at higher CTP or APM concentrations. It appears that HPM, but not APM, helps solubilize CTP in purely aqueous media, and as a result, a 2:1 (v/v) water:dioxane mixture was used in all reactions.

Although a buffer was not used in the current work, the water/dioxane mixtures were acidic because APM was used as the hydrochloride salt. This slightly acidic medium helps minimize both aminolysis and hydrolysis of the dithioester group of CTP.[42]

RAFT copolymerization of APM and HPM to form pAPM75–15 using a 2:1 water:dioxane solvent mixture was followed by $^1$H NMR (Figure S1) and GPC. It showed a linear first-order kinetic plot up to 75–80% conversion (Figure 1), as well as linear growth of $M_n$ with conversion and low PDI (Figure 2). Similar results were seen with other monomer compositions (Figures S2) except that the observed $M_n$ values fell further from the expected values as the HPM content of the copolymer increased. This is likely because the GPC mobile phase, 1 M aq. acetate buffer, pH 4.8,[40] chosen for its compatibility with APM homopolymer and high APM copolymers, was not an ideal solvent for copolymers with high HPM content.

The initial pH of the polymerization mixtures was between 2 and 3, which Liu et al. showed was suitable for RAFT polymerization of monomers like APM using CTP because it ensures complete protonation of the amine and CTP hydrolysis is slow.[43] The relative consumption of the two monomers, and hence the composition of the copolymer formed, was monitored throughout the polymerization by $^1$H NMR, and showed only marginal drift (Figure S3).

Preparative copolymerizations containing 10, 25, 50, and 75 mol% APM were carried out using monomer:CTP ratios of 130:1 and 330:1 which would result in DPs of about 90 and 240, corresponding to 15 and 40 kDa, respectively, at ~70% conversion. The properties
of these PAPMx copolymers prepared by RAFT are described in Table S1. The MWs were determined by both NMR end-group analysis (Figure S4) and aqueous GPC (Figures S5 and S6). End-group analysis showed MWs consistently above the expected values, which is attributed to some hydrolysis of the dithiobenzoate end-group during polymerization/purification. Aqueous GPC tended to show values lower than expected, especially for HPM-rich copolymers as mentioned above. NMR analysis indicated that the final copolymers had compositions very close to the feed ratios, in agreement with the relative rates of comonomer incorporation described earlier (Figure S3).

Thus, RAFT copolymerization was successfully used to prepare two sets of APM/HPM copolymers having \( M_n \) of about 15 and 40 kDa and dispersities of 1.2–1.3, each with 10, 25, 50, and 75 mol% APM.

### 3.1. End-group removal by reaction with free radicals

The hydrophobic dithiobenzoate end-group in as-formed RAFT polymers can impact solubility, is prone to hydrolyze to an undesirable thiol end-group, and increases the cytotoxicity.

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**Figure 1.** Pseudo-first-order kinetic plot for RAFT polymerization of pAPM\(_{75}\) copolymers with monomer:CTP ratio of (♦) 130:1 and (O) 330:1.

**Figure 2.** (♦) Experimental \( M_n \) and (▲) PDI vs. Conversion plots for RAFT copolymerization of pAPM\(_{75}\) with a M:CTP ratio of (A) 130:1 and (B) 330:1 in 2:1 water:dioxane. The dotted line represents the theoretical \( M_n \). Experimental \( M_n \) and PDI were estimated by aqueous GPC and conversion was determined by \(^1\)H NMR (600 MHz).
We thus replaced the dithioester end-groups with 4-cyanopentanoic acid groups, by reaction with excess radical initiator. Complete exchange of the end-groups was confirmed by disappearance of the characteristic dithiobenzoate peaks in the 7.5–8.0 ppm region of the NMR spectrum (Figures S7). GPC analysis (Figures 3 and S6) showed a slight shoulder on the high MW side of many chromatograms after end-group removal, indicating that there had been some polymer–polymer termination, reflected in a marginal increase in dispersity (Table S1).

The change of end-group decreased the elution time (increased apparent MW) of the polymers (Table S1, Figures S5, and S6). The effect was most pronounced for the polymer with lowest MW and APM content (pAPM10–15), where, after end-group replacement, the apparent $M_n$ increased from 4.55 to 10.8 kDa, a MW much more in line with expected values. This is in agreement with the GPC mobile phase (1 M acetate, pH 4.8) not being an ideal solvent for copolymers with low APM content, a problem exacerbated by the presence of the hydrophobic dithiobenzoate group.

### 3.2. Interaction of polycations with sodium alginate and with CaAlg beads

Polycation/alginate complexation was explored using optical microscopy (Figure 4), by mixing alginate and 40 kDa polycations at a 1:1 charge ratio at pH 7 in either isotonic saline (154 mM NaCl) or 377 mM NaCl, which has the same ionic strength as the gelling bath used in forming CaAlg beads (100 mM CaCl$_2$, 77 mM NaCl). The lower the MW, 15 kDa polycations behaved similarly (images not shown).

No macroscopic phase separation was seen when combining the lowest charge density pAPM$_{10-40}$ with sodium alginate in either 377 mM NaCl or in isotonic saline. Similarly, pAPM$_{25-40}$ did not show macroscopic phase separation in 377 mM NaCl, but did form a mixture of transparent gels and liquid complex coacervate droplets with alginate in isotonic saline.

PLL (40–60 kDa) and pAPM$_{50-40}$ gave marginally translucent films and precipitates that became more transparent with increase in ionic strength.
PAPM$_{75-40}$ gave a solid precipitate with high contrast to the surrounding solution in both saline and 377 mM NaCl, consistent with low water contents and strong binding. These observations confirm the expected trend toward weaker polyelectrolyte complexation and higher water content, with increase in ionic strength or lower APM content.

The specific distribution of the polycations used in, e.g., capsule membranes requires careful consideration: exposure of polycation at the capsule surface can trigger an immune response,[14,15] while polycations penetrating deeply into cell-containing gels may harm the encapsulated cells while not enhancing capsule integrity or controlling permeability.

Accordingly, CaAlg beads were coated with fluorescently labeled versions of the polycations, and both the extent of binding and the distribution of the polycations within the capsules were assessed by confocal fluorescence microscopy. In addition, the effect of two commonly used capsule-washing protocols on polycation binding and distribution was examined.

Confocal microscopy images of capsules coated with the polycations and washed twice with isotonic saline are shown in Figure 5, while capsules washed once with gelling bath (100 mM CaCl$_2$; 77 mM NaCl) and then once with saline are shown in Figure 6. Figures 5 and 6 were reproduced in the supplementary information showing multiple capsules (refer to Figures S8 and S9). Line profiles, normalized to the same detector gain, are shown in Figure S10, and were used to determine the membrane thickness as defined by the full width at half height. The areas under the line profiles gave an indication of the relative amounts of polycation bound.

Polycations with higher MW and charge density were largely restricted to the capsule surface, reflecting strong electrostatic binding to alginate. The lower MW polycations diffused more deeply into the calcium alginate bead, with the low MW pAPM$_{25-15}$ becoming
Calcium and sodium ion concentrations are also known to affect the binding of polycations to CaAlg beads, affecting gel porosity, polycation hydrodynamic volume, and weakening polyelectrolyte complexation.\[4,38,45–47\] We similarly observed here that washing with the calcium chloride-based gelling bath promoted in-diffusion of the polycation (see Figure 6 and S10). Washing with Ca\(^{2+}\) after polycation coating can thus help redistribute polycations with high charge density/MW further into the alginate beads, creating thicker

distributed evenly throughout the bead (Figure 5(d)). The two polycations containing 10 mol% APM content were not bound at all.

Figure 5. Confocal cross-sectional images showing the distribution of RbITC-labeled polycations in CaAlg beads coated with: (a) PLL 15–30 kDa, (b) pAPM\(_{25-15}\), (c) pAPM\(_{50-15}\), (d) pAPM\(_{25-15}\), (e) pAPM\(_{10-15}\), (f) PLL 40–60 kDa, (g) pAPM\(_{75-40}\), (h) pAPM\(_{50-40}\), (i) pAPM\(_{25-40}\), and (j) pAPM\(_{10-40}\). Beads were washed twice with saline after polycation coating. The shell thickness as determined from full widths at half height of line profiles (Figure S10) is shown below each image. Confocal images were taken at different detector gains.

Figure 6. Confocal cross-sectional images showing the distribution of RbITC-labeled polycations in CaAlg beads: (a) PLL 15–30 kDa, (b) pAPM\(_{25-15}\), (c) pAPM\(_{50-15}\), (d) pAPM\(_{25-15}\), (e) pAPM\(_{10-15}\), (f) PLL 40–60 kDa, (g) pAPM\(_{75-40}\), (h) pAPM\(_{50-40}\), (i) pAPM\(_{25-40}\), and (j) pAPM\(_{10-40}\). Beads were washed once with gelling bath and once with saline. The shell thickness as determined from full widths at half height of line profiles (Figure S10) is shown below each image. Confocal images were taken at different detector gains.
membranes and potentially less undesirable polycation exposure at the bead surface (Figure S10).

PLL 15–30 and 40–60 kDa have the highest charge density of the polymers studied, but in terms of in-diffusion, behave more like pAPM_{50–15} and pAPM_{50–40}, respectively (Figure 5(a) and (f), Figure 5(c) and (h), and Figure S11). This is in agreement with other studies that showed higher degrees of in-diffusion of PLL compared to other polycations, when coating CaAlg beads [47,48] or other anionic hydrogels.[49] For example, PLL was found to diffuse more deeply into polyacrylate hydrogels than polyhistidine and polyarginine of the same MW, which was attributed to differences in chain conformation, the nature of the cationic group, and hydrophobicity.

Based on the polycation distribution in the CaAlg beads studied here, the most promising polycations for membrane formation include pAPM_{50–15}, pAPM_{50–40}, or pAPM_{25–40} (saline washes), as well as pAPM_{75–15} with a gelling bath wash. In the next section, we explore the compatibility of these polycations with model cells.

3.3. Polycation cell and host compatibility

Surgical implantations always trigger a local immune response to the injury, which is exacerbated if the implant binds proteins and host cells.

Although the general cytotoxicity of polycations including PLL is reduced in the presence of alginate,[9] other polyanions [33], and serum proteins,[50] some cell types may still be affected by the polycations used in the coating process. [9] Polycation cytotoxicity is attributed to the disruption of cell membrane, releasing cellular components [33,34,51] and causing necrosis,[9] which can lead to chronic inflammation.[52]

Chaikof examined the effect of PLL-g-PEG on the viability of islet clusters using Live-Dead staining after exposure to 80 μM (~1 mg/mL) polycation solutions for 40 min. Unmodified PLL was shown to disrupt the cell membranes leading to internalization of the polycation and 20% cell survival. Conversely, grafting 40% of the lysine units with 200 Da PEG gave a polycation that remained on the cell surface and showed 100% cell viability.[19]

Our approach involves diluting the charge density on the polycation by copolymerization of the amine-functional APM with an uncharged hydroxy-functional comonomer, HPM. Factors that will increase polycation cell membrane binding and rupture, include high charge density, high MW, branched structure, and chain flexibility.[33,34,51]

We used an alamarBlue assay to test viability of C2C12 myoblasts exposed to polycations in solution. In addition, we studied the binding of 3T3 fibroblasts to model surfaces grafted with the different polycations, as a proxy test for undesirable attachments of host cells to transplants.

3.4. Polycation cytotoxicity as measured by alamarBlue cell viability assay

C2C12 myoblasts were chosen for this assay because they stop dividing at confluence, a feature that has made them popular for cell encapsulation.[53] The cells were treated with polycations in serum-free media for 20 h at concentrations of 0.01, 0.1, and 1 mg/mL. Cell viabilities, defined as alamarBlue conversion relative to a control (Figure 7), show that cytotoxicity increased with polycation concentration, charge density, and MW.[33,34,51,54] Both high and low molecular weight PLL and pAPM_{75} resulted in cessation of metabolic activity.
at all concentrations, except the lowest concentration. Viability improved with pAPM50–15, and none of the low charge density polycations pAPM$_{25}$ and pAPM$_{10}$ show significant cytotoxicity, at all concentrations used. Pissuwan and co-workers have seen similar cytocompatibility with pHPM prepared by RAFT (CTP end-group removed), showing 100% viability for three different cell lines at 1000 μM (~0.14 mg/mL) after 24 h incubation.[44] The higher cytotoxicity of polycations with higher MW [34] is attributed to greater binding affinity for cell membranes [54] leading to increased membrane damage.[33,51]

The cytotoxicity of polycations can also be assessed through their effects on cell morphology. For example, Fischer et al. saw that increased polycation toxicity for L929 mouse fibroblasts was accompanied by an increase in cell debris due to lysis, and loss of spindle shape due to detachment from the cell culture dish.[51] The effect of the dissolved polycations on the C2C12 cell morphology was also examined by optical microscopy (Figure S12). As an adherent cell line, healthy C2C12 cells spread and remain attached to the tissue culture plate as seen in the control wells (Figure S12d). However, cells that were exposed to polycations of increasing charge density became less likely to be attached to the culture plate and more likely to show a spherical rather than elongated morphology (Figure S12).

3.5. Cell attachment and proliferation on polymer-modified substrates

High cationic charge densities (from pAPM or PLL) promote protein and cell binding, which can be useful in certain cell culture applications [31,55] but can lead to undesirable immune responses in transplanted biomaterials.

Hubbell found reduced binding of BSA, fibrinogen, complement, and cells on calcium alginate surfaces coated with PLL-g-PEO compared to PLL.[18] Similarly, Fairbanks found that pHPM gels were hydrophilic, non-cytotoxic, and had anti-fouling properties showing reduced fibronectin adsorption compared to tissue culture-treated polystyrene, and this prevented L929 mouse fibroblast attachment.[56]
We hence assessed the attachment and growth of NIH/3T3 cells over three days, on surfaces bearing different pAPM\textsubscript{x} copolymers. The polycations were covalently attached to a glass surface modified with APTES and an anhydride-containing polymer, poly(methyl vinyl ether-alt-maleic anhydride) (PMM). In some cases, the polycation layer was then coated with alginate or PMM. Cells were incubated in complete medium for three days with no change of medium to ensure that no unattached cells were lost. Attachment to the substrates was assessed by microscopy (PLL, pAPM\textsubscript{50–15} and pAPM\textsubscript{75–10} are shown in Figures 8 and pAPM\textsubscript{75} and pAPM\textsubscript{10} are shown in Figure S13).

Greater cell attachment is seen for higher charge density polycations (PLL, pAPM\textsubscript{75}) used as the top layer, for both MWs. Substrates coated with pAPM\textsubscript{50–15} show a mixture of well-spread attached cells (right half of image) and unattached spherical and clustered cells (left half of image). Adding an alginate layer on top of the polycation led to less cell attachment for both MWs of PLL- and pAPM\textsubscript{75} coated substrates (Figure S14), but had no observable effect on cell morphology for polycations with 50 or less mol% APM.

Cells on PMM surfaces were well attached and spread-out. Slides coated with either MW pAPM\textsubscript{10} followed by PMM gave varied results, attributed to poor PMM binding to the pAPM\textsubscript{10}.

NIH/3T3 fibroblasts are anchorage dependent,[57] and their attachment and proliferation on these surfaces was assessed by alamarBlue assay in multi-well plates (Figure 9). alamarBlue conversion was found to increase with the charge density of the polycations bound to the substrate (Figure S15). A statistically significant positive linear trend was observed for both the 40 and 15 kDa series of polymers, (\(p < 0.001\) and \(p < 0.001\) respectively), showing a dependence of adherent cell activity on charge density after three days.

Figure 8. Representative optical microscopy images of NIH/3T3 cells on polycation-grafted glass after three days of incubation. The PMM-on-polycation image shows cell attachment on a pAPM\textsubscript{25–15} surface that was coated with PMM (anhydride form) and then hydrolyzed.
of proliferation. In addition, pAPM 50–15 and pAPM 75–15 showed minor, not statistically significant, increases of cell activity with MW.

The effect of an alginate final layer on cell growth was found to be minimal, in agreement with previous studies of APA capsules showing little ability of the final alginate layer to either bind effectively to or to hide the PLL.[14]

PMM (anhydride form in acetonitrile) was coated onto the polycation layer and then hydrolyzed in order to assess cell attachment to a covalently bound polyanion. Cells showed significant attachment to all the PMM-coated polycation surfaces; however, they showed lower cell densities leading to lower alamarBlue conversion compared to control (PMM alone). This may result from incomplete binding of PMM to polycation surfaces from acetonitrile, in particular for pAPM10 (15 and 40 kDa) substrates which showed the lowest cell attachment of the PMM-coated substrates.

The attachment and proliferation of cells on PMM surfaces was somewhat surprising since calcium alginate capsules with anionic surfaces, including PMM, often show reduced fibrotic overgrowth compared to capsules with a cationic surface.[17] However, Ishihara and co-workers found that HeLa cells attached well onto polymer brushes that were highly anionic or cationic but not those that were zwitterionic (low net charge) because they fostered the greatest amount of fibronectin adhesion.[58] In the case of PLL (15–30 and 40–60 kDa) or pAPM75 (15 and 40 kDa), adding a layer of PMM likely causes a switch from a highly cationic to highly anionic surface, which was still able to promote cell attachment. With low charge density polycations, covalent binding of PMM may result in a more highly charged, albeit anionic, surface that improves in vitro cell attachment for NIH/3T3.

Brafman et al. screened a variety of commercial polymers and found that PMM was a promising support for long-term self-renewal of human embryonic stem cells.[59] In their study, the growth media did not contain serum proteins, but they found that the production

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**Figure 9.** alamarBlue conversion relative to control of NIH/3T3 cells after three days of proliferation on polycation-modified glass, and on polycation-modified glass additionally coated with alginate or PMM. Statistically significant difference to the control is marked by an ***(p < 0.05 determined by ANOVA using Games-Howell post hoc analysis).** The data points in the graph represent three individual experiments, each in triplicate (n = 9 for all, except pAPM75–15, pAPM75–15 with alginate on top, and pAPM75–40* with PMM on top where n = 8).
of extracellular proteins was higher than seen for cells grown on Matrigel, suggesting that the cells increased their rate of ECM production in response to the highly charged support. The above experiments suggest pAPM$_{50-15}$ may be a suitable candidate for capsule membranes used in cells encapsulation, whereas copolymers with more than 50 mol% APM would be useful in cell scaffolds and those with less than 50 mol% APM would be useful for anti-fouling matrices. Further studies explore the physicochemical and mechanical properties of the polyelectrolyte complex membranes based on pAPMx, explore covalent cross-linking to reinforce the polyelectrolyte complex membranes, and test the compatibility of pAPM$_x$-coated capsules with encapsulated cells and hosts.

4. Conclusions

A series of polycations with well-defined charge densities and MWs were prepared by RAFT copolymerization of APM with HPM, and investigated for use in alginate–polycation complexes. Polycations with higher charge density (pAPM$_{75-15}$, pAPM$_{75-40}$) formed stronger complexes with alginate and stronger cell attachment than those with lower charge density. The intermediate charge density copolymer pAPM$_{50-15}$ showed reduced fouling and cytotoxicity compared to high and low MW PLL and pAPM$_{75}$, yet still showed good membrane formation on calcium alginate capsules. The lowest charge density polycations (high and low MW pAPM$_{10}$ and pAPM$_{25}$) showed the best cytocompatibility, but bound only weakly to polyanions. Thus, the pAPM$_{50-15}$ copolymer emerged as a promising reduced cationic charge density alternative to PLL. If required, the mechanical robustness of such capsules may be increased through covalent cross-linking with reactive polyanions, and this approach is currently being explored.

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