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Reactive Polyanions Based on Poly(4,4-dimethyl-2-vinyl-2-oxazoline-5-one-*co*-methacrylic acid)

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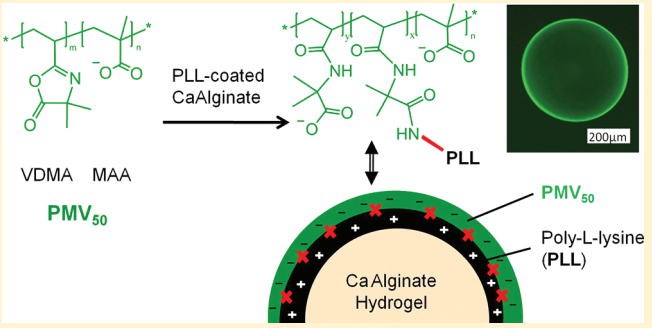
Supporting Information

ABSTRACT: The formation of reactive polyanions by semi-batch copolymerization of 4,4-dimethyl-2-vinyl-2-oxazoline-5-one (VDMA) and methacrylic acid (MAA) by both free radical and photoinduced radical polymerization is described. The reactivity ratios of these two monomers were determined to be 1.36 and 0.41 for r_1(VDMA) and r_2(MAA), respectively, using ^{1}H NMR spectroscopy. During the free radical copolymerization of a 50:50 VDMA:MAA copolymer (PMV_{50}) in anhydrous DMSO or THF at 60 °C, up to 40% of the azlactone groups in the copolymer are hydrolyzed by water formed largely by conversion of methacrylic acid diads into cyclic anhydride. Storage in organic solutions leads to further transhydration, while solid PMV_{50} is stable for at least 6 months at room temperature. Increasing the VDMA comonomer content reduces this transhydration, likely through decreasing the incidence of methacrylic acid diads in the backbone. Alternatively, conducting the copolymerization at 20 °C using photoinitiation is also effective at suppressing this transhydration. The resulting reactive polyanions bind under physiological conditions to poly-L-lysine-coated calcium alginate hydrogel beads and spontaneously cross-link with the polyamine to form covalent networks of interest for long-term therapeutic cell encapsulation. This represents the first such use of a VDMA-containing polyanion in aqueous environments. The copolymers were characterized by ^{1}H NMR, quantitative ^{13}C NMR, ^{13}C DEPT-135 NMR, and FT-IR spectroscopies as well as by elemental analysis.



INTRODUCTION

Polymers with electrophilic groups such as activated esters[1,2] anhydrides,[3] azlactones,[4] and isocyanates[5] are finding increased use in biomaterials, both for cross-linking and for attaching camouflaging molecules such as poly(ethylene glycol),[4] cell adhesion motifs such as RGD,[6] or immune-modulating molecules such as certain anti-inflammatory cytokines.[7]

Ideally, these reactive polymers should react spontaneously and quantitatively with polymeric nucleophiles under physiological conditions, without generating potentially cytotoxic small molecule side products. Our group develops water-soluble polyanions containing electrophilic groups, designed to electrostatically bind to polyamine-coated hydrogel beads, and then spontaneously form covalently cross-linked networks of interest for long-term immuno-protection of encapsulated cells. We recently described a copolymer of methacrylic acid with methacryloylethylacetoacetate (MOEAA) that is able to form both shell-cross-linked[8] and core-cross-linked[9] cell-compatible beads that are stable under physiological conditions for up to 4 weeks.[10] An analogous reactive polyanion formed by partial hydrolysis of poly(methyl vinyl ether-*alt*-maleic anhydride) forms more permanent networks upon cross-linking with poly-L-lysine-coated calcium alginate (AP) beads due to formation of amide cross-links.[3] Residual anhydride groups not used in cross-linking are rapidly converted into additional anionic carboxylates, considered to promote biocompatibility by shielding the cationic charges on the poly-L-lysine (PLL).

The present work aims to add control over copolymer composition and reduce the rate of residual electrophile hydrolysis to allow for postfunctionalization of the capsules with bioactive molecules, while retaining the advantages of stable amide cross-links. One electrophilic monomer that has recently attracted interest in this context is 4,4-dimethyl-2-vinyl-2-oxazoline-5-one (VDMA).[11] Azlactones react with amines, alcohols, and thiols in a ring-opening reaction under mild conditions to form amide, ester, and thioester bonds, respectively.[11,12] VDMA is readily polymerized by free radical means or living free radical polymerization (LFRP),[13] and copolymers incorporating azlactone groups have been used to prepare thin films[4,14,15] and to immobilize proteins and other biorelevant molecules onto polymer supports.[16−19]

Most of the published work, including the preparations of cross-linked thin films using a layer-by-layer approach,[14] has been carried out in organic media, as the VDMA homopolymer is not soluble in water. VDMA has been shown to incorporate preferentially during copolymerization with methyl methacrylate,[20] *N*,*N*-dimethylacrylamide,[21] vinylpyrrolidone,[22] butyl acrylate, and styrene[15] in organic solvents.[18] To date, VDMA has not

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been copolymerized with an acidic comonomer or used to form LBL assemblies with polycations in aqueous conditions.

The present work hence describes the preparation and study of VDMA copolymers with methacrylic acid, poly(MAA-*co*-VDMA), or PMV. The key use of these reactive polyanions will be to form covalently cross-linked hydrogels under physiological conditions, typically in the presence of cells.[23,24] Such hydrogel networks are finding use as long-term biocompatible scaffolds or tissue mimetics for applications including immuno-isolation of transplanted therapeutic cells such as islets of Langerhans and stem cell studies.

Copolymers of VDMA with MAA promise to be water-soluble and able to form polyelectrolyte complexes with polyamines. While the cross-linking reaction is expected to be rapid, hydrolysis of residual VDMA to carboxylic is expected to be slower[3] than for the analogous anhydride-based copolymers, promoting covalent cross-linking while still ensuring conversion of electrophilic groups prior to transplantation.

In principle, VDMA homopolymers may be used for this purpose, as they can be partially hydrolyzed in mixed organic/aqueous solution prior to use. This approach was described recently by Messman[22] and has been independently used by us to form analogous reactive polyanions from poly(methyl vinyl ether-*alt*-maleic anhydride).[3] The explicit use of an anionic comonomer, MAA, is thought to ensure random distribution of charges and electrophiles along the backbone and to minimize extraneous groups resulting from hydrolyzed VDMA, while being more cost-effective.

EXPERIMENTAL SECTION

Materials. 4,4-Dimethyl-2-vinyl-2-oxazoline-5-one (VDMA) was purchased from TCI America, Portland, OR. Methacrylic acid (MAA, 99%), 2,2-dimethoxy-2-phenylacetophenone, 5-aminofluorescein (AF), HEPES sodium salt, poly(L-lysine hydrobromide) (PLL, 40−60 kDa), anhydrous DMSO, anhydrous THF (inhibitor free), ethylene carbonate 98%, phenethylamine, and glutaric acid were purchased from Sigma-Aldrich, Oakville, ON, and used as received. Sodium alginate (Pronova UP MVG, batch no. FP-610-03) was purchased from Novamatrix, Norway. Sodium chloride (Caledon Laboratories Ltd., ON) and calcium chloride (minimum 96% powder, anhydrous, Sigma-Aldrich, ON) were used as received. Sodium hydroxide and hydrochloric acid solutions were prepared from concentrates (Anachemia Chemical, Rouses Point, NY) by diluting to 0.100 or 1.000 M with deionized water. Azobis-(isobutyronitrile) (AIBN) was obtained from Dupont, Mississauga. Dimethyl-d_6 sulfoxide (99.9 atom %, DMSO-d_6) and D_2O (99.9 atom %, D) were purchased from Cambridge Isotope Laboratories, Inc. Andover, MA. Poly(methacylic acid) (PMAA) was prepared by free radical polymerization of methacrylic acid as described by Mazumder et al.[25]

Methods. *Reactivity Ratio Determination during Free Radical Copolymerization.* Copolymerization was followed in a 5 mm NMR tube by 500 MHz ^{1}H NMR, involving a 50:50 mol % ratio of comonomers at 8% w/v total monomer loading, 2 mol % AIBN in DMSO-d_6, at 60 °C for 4 h, similar to the protocol described by Aguilar et al.[26] ^{1}H NMR spectra were taken at increasing time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 14, 19, 29, 39, 49, 59, 89, 119, 149, 179, 209, 239, and 249 min), and the monomer concentrations were measured by integration of the vinyl MAA peak at 5.55 pm and an average of the two vinyl VDMA peaks at 6.35 and 6.15 ppm. The reactivity ratios were then calculated using both eqs 2 and 3 in Aguilar's paper, based on fitting the instantaneous comonomer concentrations to the terminal model of the copolymerization equation.[26] The 95% confidence contour plot was determined using Matlab and the method described by Box et al.[27]

Semibatch Free Radical Copolymerization of 2-Vinyl-4,4-dimethylazlactone with Methacrylic Acid. PMV$_{50}$. Prior to reaction all glassware was heated in an oven at 70 °C for 2 days to remove moisture. A semibatch copolymerization was performed, starting with a total initial monomer loading of 8% w/v and an initial comonomer mole ratio of 35:65 VDMA:MAA, with gradual addition of more VDMA to approximately maintain this comonomer feed ratio. Specifically, in a 100 mL water jacketed round-bottom flask VDMA (1.87 g, 13.5 mmol), MAA (2.15 g, 25 mmol), AIBN (77 mg, 0.469 mmol, 1 mol %), and ethylene carbonate (45 mg, internal standard for ^{1}H NMR) were dissolved in 50 mL of anhydrous DMSO (or THF) to give an 8% w/v initial comonomer solution. A stirring bar was added, and the system purged with $N_{2(g)}$ for 1 h. The flask was heated to 60 °C using a circulating water bath, under $N_{2(g)}$ bubbling through a T-junction and an oil bubbler. After 5 min of heating, t = 0 min, continuous addition of a 0.003 mol/mL solution of VDMA in anhydrous DMSO (or THF) was started. The rate of addition was 3.75 mL/min until t = 30 min, and 1.875 mL/h from there until t = 60 min, using an automated syringe pump. Aliquots of 0.5 mL of the reaction mixture were taken at t = −5, 0, 10, 20, 30, 40, 50, and 60 min and added to 0.15 mL of DMSO-d_6 for subsequent ^{1}H NMR analysis at 600 MHz.

The reaction was stopped after 1 h by exposing the system to air and immediately cooling the water jacket with cold water. The reaction solution was then diluted with 50 mL of THF and precipitated into 600 mL of anhydrous diethyl ether under stirring. The resulting dispersion was centrifuged for 20 min at 3500 rpm, the supernatant was removed, and the solid precipitate washed with diethyl ether. The resulting product was dried in a vacuum desiccator at room temperature for 1 h, ground with a mortar and pestle, dried under vacuum for another hour, and then redissolved in 20 mL of THF. This solution was reprecipitated into 200 mL of anhydrous diethyl ether and treated as above, with final drying in the vacuum desiccator for 3 days, to give a white powder, with isolated yields of 43 mol % (44 wt %) in DMSO, and 22 mol % (23 wt %) in THF, with respect to the total amount of monomers added to the reaction (initial VDMA plus VDMA additions).

Synthesis of Fluorescently Labeled PMV$_{50}$ (PMV$_{50}$f). PMV$_{50}$f was synthesized as described above, except that 1 mol % of aminofluorescein (89.6 mg, 0.24 mmol) with respect to the total VDMA used in the reaction (3.37 g, 24.2 mmol) was added to the VDMA monomer before this mixture was divided into the initial VDMA reaction mixture and the VDMA feed solution.

Semibatch Synthesis of Other PMV Compositions. The syntheses of other PMVs with copolymer compositions of 55:45, 65:35, 70:30, and 80:20 VDMA:MAA were carried out in a similar manner as described above, using initial VDMA mole fractions of 0.40, 0.45, 0.60, and 0.72, respectively, as obtained from Figure 1b. The same concentration and rate of VDMA addition were used as described above.

Semibatch Photocopolymerization of 2-Vinyl-4,4-dimethylazlactone with Methacrylic Acid. Two semibatch photocopolymerization designed to obtain PMV$_{50}$ and PMV$_{70}$ were performed in a similar manner as described for the free radical polymerization, starting with a total initial monomer loading of 8% w/v and initial comonomer mole ratios of 35: 65 and 60:40 VDMA:MAA, respectively, with gradual addition of more VDMA to approximately maintain this comonomer feed ratio. The reactions were carried out in a photochamber containing three black-light bulbs (Microlites Scientific, 350 nm, 8 W). A cylindrical reaction vessel (2.5 cm in diameter and 12 cm in height) was used, fitted with a cold finger fed with 22 °C tap water to minimize heating. The photoinitiator used was 2,2-dimethoxy-2-phenylacetophenone (0.5 mol % with respect to the total monomer concentration). VDMA solution in DMSO (0.003 mol/mL) was added at 7.5 mL/min until t = 30 min and 3.75 mL/h from there until t = 60 min, to account for the higher rate of photopolymerization. As described above, the reaction was monitored by ^{1}H NMR and polymer was isolated.

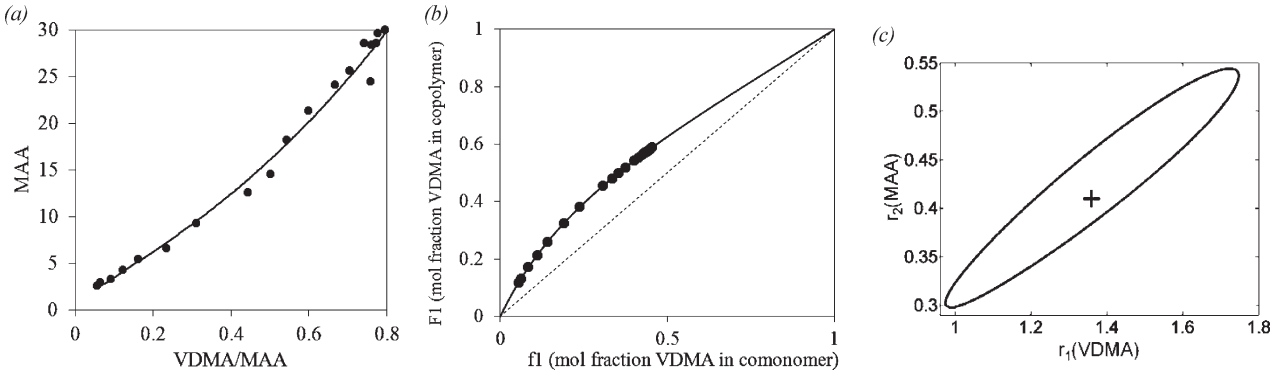


Figure 1. (a) [MAA] vs [VDMA]/[MAA] (arbitrary units) from a copolymerization of VDMA and MAA with an initial mole ratio of about 1:1. Experimental data from ^{1}H NMR: black diamonds; fitted black line calculated using eq 2 of Aguilar's paper.[26] (b) Instantaneous copolymer composition curve for VDMA and MAA in DMSO at 60 °C; black dots correspond to the experiment data points. (c) 95% joint confidence contour for calculated reactivity ratios.

Covalent LBL Assemblies around Calcium Alginate−PLL Capsules (AP−PMV$_{50}$ Capsules). Calcium alginate capsules were prepared as described previously.[3] Briefly, a 1 wt % solution of Novamatrix UP MVG sodium alginate in saline was filter sterilized (0.22 μm) and extruded through a flat-tipped 27-gauge needle with a concentric air flow (3.5 L/min) using a modified syringe pump, at a liquid flow rate of 0.5 mL/min, into a 1.1% w/v $CaCl_2$ gelling bath. The resulting calcium alginate beads were washed once with 1.1% w/v $CaCl_2$, coated with PLL (filter sterilized (0.22 μm), 0.1% in saline, for 6 min), and washed once each with 1.1% w/v $CaCl_2$ and saline, to give AP beads. AP−PMV$_{50}$ capsules were formed by coating AP beads with 0.2% filter sterilized PMV$_{50}$f solution in 35 mM HEPES buffered saline at pH 7.8 for 10 min, followed by two washes with saline. All washing and coating steps involved a ratio of 1 mL beads to 3.3 mL washing or coating solution.

To confirm covalent cross-linking, 1 M sodium citrate was added to 30 capsules on a microscope slide to extract calcium from the core, leaving only the alginate−PLL−PMV$_{50}$ shell. The supernatant was removed and replaced with 0.1 M NaOH to deprotonate the primary amines on PLL, thus eliminating all electrostatic interaction between PLL and both alginate and PMV$_{50}$, leaving only covalent cross-links to maintain shell integrity.

Characterization. *GPC Analysis.* Molecular weights were determined using PMV that was completely hydrolyzed by reaction in basic (pH ∼ 10) distilled deionized water for 3 days and an aqueous gel permeation chromatography (GPC) system consisting of a Waters 515 HPLC pump, a Waters 717 plus Autosampler, three Ultrahydrogel columns (0−3, 0−50, 2−300 kDa), and a Waters 2414 refractive index detector. A mobile phase consisting of 0.3 M $NaNO_3$ and 0.05 M phosphate buffer (pH 7) at a flow rate of 0.8 mL/min was used for all polymers, and the system was calibrated with narrow-disperse poly(ethylene glycol) standards (Waters, Mississauga, ON).

^{1}H NMR. i. Determination of Instantaneous Monomer Feed Ratio and the Final Copolymer Composition. During the free radical and photo-semibatch copolymerization of PMV$_{50}$, 0.5 mL aliquots were removed from the reaction mixture at −5, 0, 10, 20, 30, 40, 50, and 60 min and added to 0.15 mL of DMSO-d_6 for ^{1}H NMR analysis on a Bruker AV 600. The monomer peaks of VDMA and MAA were integrated at each time point to obtain the instantaneous comonomer feed ratios. The amounts of MAA reacted were determined by tracking the decreasing MAA concentration. The amount of VDMA reacted was determined by subtracting the actual amount of VDMA present in the reaction from the total (initial plus incremental) amount of VDMA, at each time point. The average VDMA content in the PMV$_{50}$ copolymer formed during this hour was determined, based on the amounts of VDMA and MAA consumed during the reaction.

ii. Analysis of DMSO and/or THF Remaining in Final Product. The final PMV copolymer was isolated and analyzed by ^{1}H NMR at 600 MHz in DMSO-d_6 to determine the amount of residual solvent (DMSO and/or THF) in the isolated product.

Quantitative ^{13}C NMR To Determine the Degree of Hydrolysis as Well as the VDMA:MAA Ratio in PMV Copolymers. The longitudinal relaxation times, T_1, of the ^{13}C nuclei in PMV$_{50}$ were determined using a saturation-recovery experiment (pulse program "satrect1.av_bb"). The T_1 times were then used to set up a quantitative ^{13}C NMR experiment using inverse-gated decoupling without NOE enhancement, a 30° flip angle, 3000 scans, and a recovery delay time D_1 of 10 s. Analyses were performed using 10 wt % solutions of as-formed, and of fully hydrolyzed, PMV$_{50}$f in DMSO-d_6 and D_2O, respectively, on a Bruker AV 600. ^{13}C NMR DEPT-135 experiments were performed to confirm the correct assignment of the carbon peaks.

Elemental Analysis To Determine the Ratio of VDMA:MAA in PMV Copolymers. Residual solvents and AF were removed from the polymer by dialysis: 200 mg of PMV$_{50}$(f) was dissolved in 20 mL of 35 mM of pH 7.8 HEPES buffered saline and diluted to 100 mL with distilled water after 2 h. This solution was then dialyzed against 4 L of distilled deionized water for 1 week, with daily water changes using cellulose dialysis tubing with a molecular weight cutoff of 14 kDa (Membra-cel, Viskase, Darien, IL). This process hydrolyzes all remaining azlactone groups, prior to elemental analysis. The dialyzed solutions were concentrated using a rotary evaporator, followed by precipitation with 1 M HCl. The resulting precipitate was dried in a vacuum oven at 25 °C for 3 days. Elemental analysis was performed on a Therm FlashEA 1112 elemental analyzer, using the carbon:nitrogen ratio to determine the VDMA to MAA ratio.

UV−vis Determination of AF Content in PMV$_{50}$f. The amount of AF bound to the dialyzed PMV$_{50}$f was determined by UV−vis spectroscopy on a Varian Cary 50 Bio, using the AF absorbance (λ_{max} ∼ 490 nm) of a 0.10% polymer solution, and the extinction coefficient of free AF of 86 000 M^{-1} cm^{-1} at pH 9.[28]

FT-IR Analysis. Powder samples of as-formed PMV$_{50}$ and of fully hydrolyzed PMV$_{50}$ were made into KBr pellets with spectrograde KBr and analyzed on a Thermo Scientific Nicolet 6700 FT-IR spectrometer equipped with DTGS detector, extended KBr beam splitter, and OMNIC v8 software. Resolution was set to 4 cm^{-1} with 32 scans.

Model Studies. To investigate the mechanism of azlactone hydrolysis, model studies were carried out where VDMA monomer alone (control experiment), or VDMA and either MAA, poly(methyacrylic acid) (PMAA), or 1,5-pentanedioic acid (glutaric acid) were dissolved in DMSO-d_6 at a 35:65 mole ratio of azlactone to carboxylic acid, and heated at 60 °C for 1 h, to mimic the polymerization conditions. The solutions were then

stored at room temperature, and the degree of hydrolysis of VDMA was monitored by ^{1}H and ^{13}C quantitative NMR over time.

Potentiometric Determination of the Rate of Hydrolysis of PMV_{50} in Aqueous Buffer. A Mandel Scientific PC-Titrate automated potentiometric titrator was used to monitor the pH of a 0.2% PMV_{50} solution in pH 8.1, 35 mM HEPES buffered saline. Photopolymerized PMV_{50} (30 mg) was first dissolved in 1 mL of DMSO-d_6, and a ^{13}C NMR was taken to determine the degree of hydrolysis. This solution was diluted with HEPES buffer to make a 0.2% aqueous PMV_{50} solution.

RESULTS AND DISCUSSION

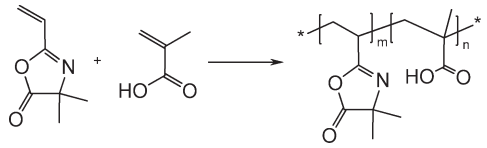
Synthesis of Poly(2-vinyl-4,4-dimethylazlactone-*co*-methacrylic acid), PMV_{50}, by Free Radical Polymerization. Copolymerization of methacrylic acid (MAA) and 2-vinyl-4,4-dimethylazlactone (VDMA) has not yet been reported in the literature. Heilmann et al.[20] reported the reactivity ratios of VDMA and methyl methacrylate in methyl ethyl ketone as 0.83 and 0.61, respectively, indicating a slightly preferential incorporation of VDMA. VDMA was expected to, and did, behave similarly in the present copolymerization. The calculated reactivity ratios extracted from a ballistic copolymerization of VDMA and MAA starting near a 1:1 molar ratio (Figure 1a) were found using the method described by Aguilar[26] and found to be 1.36 and 0.41 for r_1(VDMA) and r_2(MAA), respectively. The high sensitivity of the 500 MHz NMR instrument used allowed coverage of large [VDMA]/[MAA] range in a single NMR copolymerization experiment. Figure 1b shows the instantaneous copolymer composition graph calculated using these fitted r values as well as (full circles) the range of comonomer feed ratios and copolymer compositions covered in the NMR experiment. Figure 1c shows the 95% joint confidence contours for these r values.

Transhydration during Formation of PMV_{50}. The NMR data revealed the appearance of both monomeric and polymeric hydrolyzed azlactone groups during this copolymerization. Hydrolysis of VDMA monomer is shown by the sharp amide peak at 8.02 ppm as well as by the corresponding vinyl peaks at 6.26, 6.06, and 5.57 ppm (Figure 2a,b). The appearance of a broad amide peak at about 7.7 ppm confirms the presence of hydrolyzed azlactone units in the copolymer (Figure 2a), formed either by copolymerization of hydrolyzed monomer or by partial hydrolysis of the copolymer.

This complicates the determination of the reactivity ratios, as this system would more accurately be described as a terpolymerization of VDMA, hydrolyzed VDMA and MAA, characterized by six reactivity ratios.[29] A full analysis, though intriguing, is beyond the scope of this paper. We hence proceeded with the estimation of binary reactivity ratios, accepting that this approach ignores the additional small loss during the copolymerization of VDMA to form hydrolyzed VDMA and possibly even of MAA to form methacrylic anhydride.

Using the binary reactivity ratios, we first aimed for an equimolar VDMA:MMA copolymer in order to balance electrophilic groups with the anionic charges needed for solubility and complexation to polyamines. A semibatch copolymerization was hence carried out in anhydrous DMSO or THF, starting with an initial VDMA:MAA mole ratio of 35:65, as interpolated from the instantaneous copolymer composition graph calculated based on the measured reactivity ratios (Figure 1b), and adding sufficient VDMA throughout the reaction to maintain this comonomer ratio. ^{1}H NMR spectra were taken every 10 min to confirm that the molar comonomer feed ratio of VDMA to MAA remained at roughly 35:65 (Figure 3a) throughout the copolymerization. Figure 3b shows the amounts of MAA and VDMA reacted at each of these time points. The comonomer feed ratio drifts just slightly during the copolymerization (Figure 3a), with the calculated instantaneous VDMA content of the copolymer increasing from 58% to 63% over the course of the reaction in DMSO and from 57% to 65% in THF. Averaging over the course of the reaction predicts that about 60% VDMA is incorporated into PMV_{50} in both solvents.

Scheme 1. Free Radical Solution Copolymerization of VDMA with MAA



This VDMA percentage is slightly higher than the actual value determined by ^{13}C NMR and elemental analysis due to the fact that the VDMA/DMSO solution interfered with smooth piston movement, causing significant back-pressure in the automated syringe pump and decreased total injection volumes.

This empirical semibatch approach was considered sufficient to give near constant copolymer composition throughout the 1 h copolymerization, obviating the need for explicit analysis and programming of nonlinear semibatch additions.[30]

Copolymerizations carried out in DMSO and THF resulted in very similar compositions, though polymer yields were higher in DMSO at 44 wt % compared to 23 wt % in (inhibitor-free) THF. ^{1}H NMR analysis of the isolated PMV_{50} polymer revealed that all residual monomer was removed from the polymer. However, residual DMSO proved to be difficult to remove from the isolated polymer: after two precipitations (the second from THF) into diethyl ether and multiple washings, between 15 and 30 wt % of DMSO and 3 wt % THF (determined by ^{1}H NMR analysis taken 3 days after the second precipitation, Table 1) still remained with the isolated polymers. A third precipitation from THF reduced the amount of DMSO to 8 wt % and THF to 1 wt %, but at the cost of increased hydrolysis of azlactone groups in the polymer. When the analogous copolymerization was performed in THF, two precipitations from THF into diethyl ether led to 8 wt % of THF remaining. [The 8 wt % THF remaining is reflective of the polymer being dried under vacuum for 3 days after the finial precipitation. ^{1}H NMR spectra collected 2 weeks later (after being stored under vacuum) show a decrease in THF to 3 wt % (DMSO remains constant).]

Although small amounts of DMSO are tolerated by cells, THF is thought to be not very cytocompatible, and hence future work will look at removing all of the THF from the isolate polymer. However, recent cell viability studies[23,24] found no adverse affects arising after coating cell-containing AP capsules with 0.2 wt % PMV_{50}.

GPC analysis on fully hydrolyzed PMV_{50} revealed that PMV_{50} copolymers prepared in DMSO had a higher molecular weight, with average M_n values of 100 kDa and M_w values of 170, compared to the single PMV_{50} prepared in THF which had an M_n value of 40 kDa and M_w of 70 kDa (Table 1). The reason for the lower molecular weights of PMV_{50} prepared in THF is not clear but may at least partly be due to the lower viscosity and higher chain transfer activity of (inhibitor-free) THF.

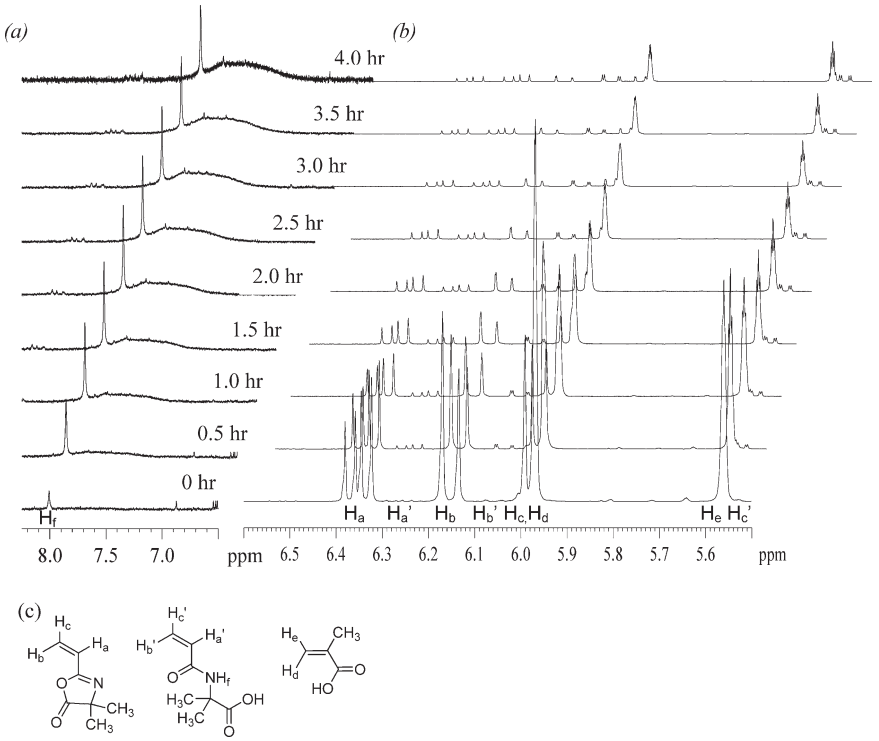


Figure 2. ^{1}H NMR (500 MHz) spectra over 4 h of PMV_{50} synthesis: (a) magnified amide region, showing both a sharp amide signal from hydrolyzed VDMA at 8.0 ppm and a broad amide signal from partly hydrolyzed PMV_{50} around 7.7 ppm; (b) alkene monomer region, with proton labels under 0 h referring to monomer structures shown in (c); (c) (i) VDMA, (ii) hydrolyzed VDMA, and (iii) MAA with reference to (b).

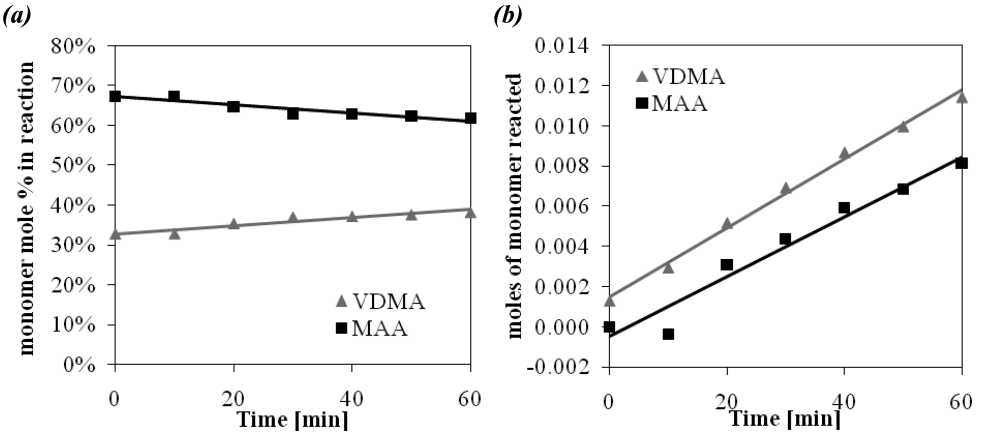


Figure 3. Semibatch copolymerization in DMSO: (a) monomer ratio during the reaction, determined by from ^{1}H NMR; (b) moles of monomers reacted, calculated by subtracting moles remaining from the total amount of monomer added. (This process ignores the minor loss of monomers to hydrolyzed VDMA and anhydride formation.)

Table 1. Summary of PMV_{50} Characterization

	VDMA:MAA			GPC		
solvent	^{1}H NMR (during reaction)	^{13}C NMR (quantitative)	elemental analysis	M_n (kDa)	M_w (kDa)	solvent remaining in isolated polymer (after 2 precipitations and 3 days drying under vacuum)
DMSO	60:40	50:50 ± 5	45:55	100	170	15−30 wt %
THF	60:40	50:50 ± 5	45:55	40	70	8 wt %

Determining Percentage of VDMA in PMV_{50}. ^{1}H NMR spectra of the isolated polymer showed broad, overlapping polymer peaks that could not be accurately quantified; hence quantitative ^{13}C NMR was employed. A ^{13}C DEPT-135 NMR

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experiment was performed on PMV_{50} to verify the assignment of peaks (Table 2). These assignments are in agreement with those of Messman et al.[22] Subsequently, quantitative ^{13}C NMR spectra were taken of PMV_{50} in DMSO-d_6 and of fully hydrolyzed PMV_{50} in D_2O, both showing that 50 ± 5% VDMA was incorporated into PMV_{50}. This copolymer composition was determined by comparing the area of peak C_4' (VDMA) to the average of the areas of the four peaks C_{7-10}, representing MAA. Averaging eqs 1−3 gave a 50:50 copolymer composition.

$$C_{10} = (C_6' + C_{10} + C_5') - 2C_4' \quad (1)$$

$$C_8 = [(C_8 + C_9 + C_1' + C_3') - 2C_4']/2 \quad (2)$$

$$C_7 = (2C_2' + C_7) - 2C_4' \quad (3)$$

Elemental analysis of fully hydrolyzed PMV_{50} gave a 45:55 VDMA:MAA copolymer based on the C:N ratio, in agreement with the ^{13}C NMR data (Table 1).

Determining the Amount of Hydrolysis in As-Formed PMV_{50}. Analyses of the quantitative ^{13}C NMR spectra of as-formed, and of fully hydrolyzed PMV_{50}, showed that 39 ± 6% of the VDMA groups in the as-formed PMV_{50} are already hydrolyzed. The amount of hydrolysis of as-formed PMV_{50} was measured in DMSO-d_6 using quantitative ^{13}C NMR, by comparing the integrations of the quaternary carbons C_4 (azlactone, 65 ppm) and C_4' (hydrolyzed azlactone, 55 ppm) (Figure 4b). Comparing peak areas for C_6 and C_6' and for C_5 and C_5' gave similar results.

Model Studies into PMV_{50} Hydrolysis. Further investigation showed that PMV_{50} continued to hydrolyze over time in DMSO-d_6, with the degree of hydrolysis, as measured by quantitative ^{13}C NMR, reaching 94% after 4 months at room temperature (Figure 5a). In contrast, a sample of the same PMV_{50} stored in

Table 2. ^{13}C Chemical Shifts for As-Formed and Fully Hydrolyzed PMV_{50}, Both Taken in DMSO-d_6[a]

carbon	as-formed PMV_{50} [ppm]	hydrolyzed PMV_{50} [ppm]
1 (CH_2) VDMA	32−37	32−37
2 (CH_3)$_2$ VDMA	15−25	15−25
3 (CH) VDMA	37−46	37−46
4 (C) VDMA	*65*	*55*
5 (O−C═N(C)) VDMA	*165*	*175−182*
6 (O−C═O) VDMA	*182*	*177−180*
7 (CH_3) MAA	15−25	15−25
8 (C) MAA	51−56	51−56
9 (CH_2) MAA	32−37	32−37
10 (COOH) MAA	170−176	170−176

[a] Peaks for carbons 4, 5, and 6 (italicized) moved significantly upon hydrolysis and were hence used to determine the degree of hydrolysis.

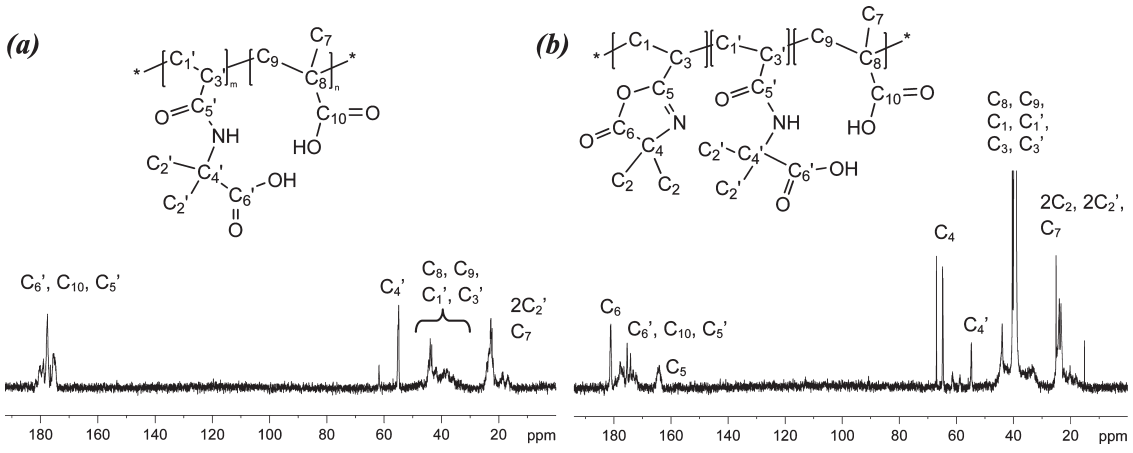

Figure 4. Inverse gated decoupling ^{13}C NMR of (a) dialyzed and hydrolyzed PMV_{50} in D_2O and (b) PMV_{50} (synthesized in DMSO) in DMSO-d_6.

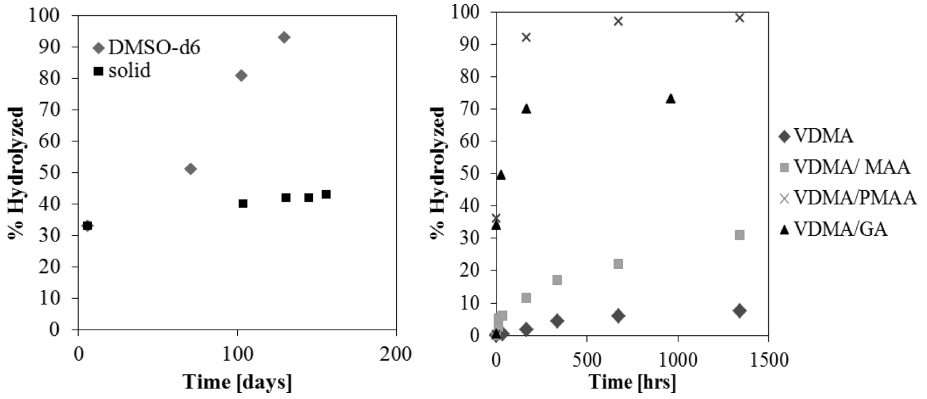

Figure 5. Hydrolysis over time of (a) PMV_{50} in DMSO-d_6 vs in solid form and (b) monomer model studies in DMSO-d_6.

solid form at room temperature proved more stable, with the amount of hydrolysis increasing from an initial level of 33% to reach 40% after 3.3 months and 43% after 6 months. These findings suggest that the VDMA groups may react with polymeric MAA units, either by directly reacting with single neighboring MAA units or by reacting with water liberated when MAA diads cyclize to form methacrylic anhydride units, and that this process is accelerated in solution.

To confirm this reaction, mixtures of VDMA and MAA monomers, and of VDMA monomer and PMAA homopolymer, were dissolved in DMSO-d_6 at the same concentration as used in the copolymerizations and heated at 60 °C for 1 h, to mimic the conditions that occur during synthesis, followed by storage at room temperature over time. The degree of hydrolysis of VDMA was monitored by ^{1}H and ^{13}C quantitative NMR and after 1 h of heating at 60 °C was found to be 6% for the MAA/VDMA mixture and 36% for the PMAA/VDMA mixture. After 1 month at room temperature these values had increased to 22% and 97%, respectively (Figure 5b). (A control experiment using just VDMA dissolved in DMSO-d_6 revealed only 0.5% hydrolysis after heating for 1 h and only 6% after 1 month at room temperature, indicating that the contributions to hydrolysis from ambient moisture or reaction with DMSO are minimal.) These findings suggest that hydrolysis occurs faster when the MAA units are present in polymeric form, presumably because they can readily form cyclic methacrylic anhydride units by intramolecular dehydration.[31] ^{13}C NMR spectra of PMAA and VDMA in DMSO-d_6 initially and after 1 week at room temperature (92% hydrolysis) shows a the decrease of the carboxylic acid peak at 179 ppm and the appearance of a broad anhydride peak at 172 ppm, as well as a broadening of the peaks for the PMAA methyl groups at 16 ppm, the quaternary carbon at 44 ppm, and the methylene carbon at 53 ppm (Figure 6).

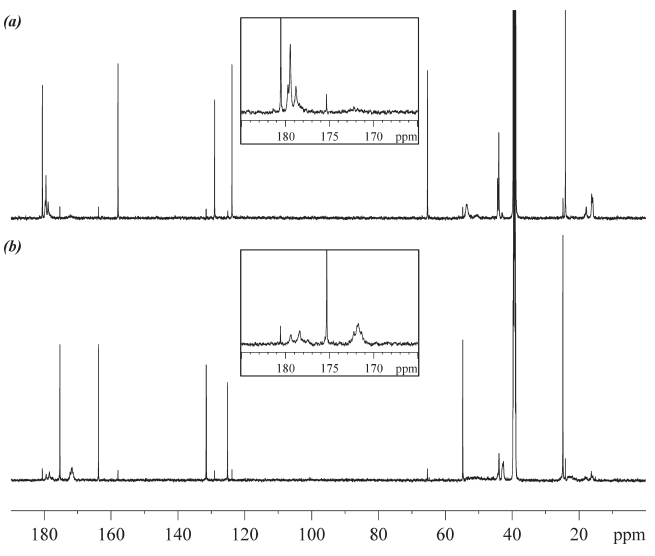

Figure 6. ^{13}C NMR spectra of a mixture of PMAA and VDMA in DMSO (a) initially and (b) after 1 week, with insets showing a blown-up spectra of the carboxylic acid/anhydride region.

It is also interesting to note that VDMA monomer in mixtures with PMAA hydrolyzes faster than the azlactone units in PMV_{50}. This may simply be due to stereoelectronic differences between VDMA monomer and azlactone units in PMV_{50} but may also be due to the fact that PMV_{50} has fewer MAA diads compared to PMAA.

Additional model studies were carried out using mixtures of VDMA with varying amounts of 1,5-pentanedioic acid (glutaric acid, GA) which is able to form a six-membered cyclic anhydride upon dehydration. The rate of VDMA hydrolysis in presence of GA was intermediate between those of VDMA/PMAA and VDMA/MAA mixtures and varied with the VDMA/GA ratio. Heating a mixture of VDMA and GA (35:65 azlactone:carboxylic acid ratio) in DMSO-d_6 at 60 °C for 1 h led to hydrolysis of 34% of the initial VDMA and formation of an equivalent amount of cyclic glutaric anhydride. After 1 month in DMSO-d_6 at room temperature, this transhydration had reached 70%.

Comparison of the FT-IR spectra of as-formed PMV_{50} and of fully hydrolyzed PMV_{50} (Figure 7) shows the disappearance of the C═O stretch absorption corresponding to both the azlactone and the six-membered ring anhydride[32] at 1824 cm^{-1} and the increase of the amide II band at 1539 cm^{-1}.[22] Note that the azlactone C═N band at 1668 cm^{-1} could not be used as it overlaps with the amide C═O band at 1661 cm^{-1} of the hydrolyzed azlactone.[15] Similarly, the carboxylic acid C═O band at 1733 cm^{-1} cannot be used as it represents both MAA and hydrolyzed azlactone.

Overcoming Transhydration. Two approaches were used to overcome the transhydration reaction. First, the mole ratio of VDMA in the copolymer was increased in order to decrease the probability of MAA diads. Table 3 shows how the increase of VDMA content in PMV decreases the amount of transhydration. The 70:30 VDMA:MAA PMV copolymer was the highest VDMA content polymer that would instantaneously dissolve in 35 mM HEPES buffered saline (pH 7.8). The 80:20 copolymer took 1−2 min to completely dissolve.

The second approach to minimize the transhydration reaction was to carry out the copolymerizion at lower temperatures, as it was observed in the model experiments that heating in DMSO accelerated the hydrolysis of azlactone groups. PMV_{50} was hence synthesized by a semibatch photocopolymerization at about

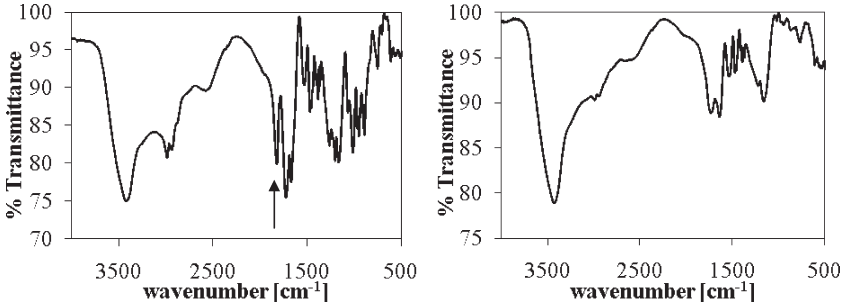

Figure 7. IR spectra (KBr pellets) of (a) as-formed PMV_{50} (arrow indicates azlactone/anhydride peak) and (b) fully hydrolyzed PMV_{50}.

20 °C, resulting in hydrolysis of only 7% of the azlactone groups in the isolated copolymer.

Rate of Hydrolysis of PMV_{50} in Aqueous Solution. Current literature on the rate of hydrolysis of the corresponding homopolymer poly(VDMA) is limited and only states that the polymer is hydrolytically stable in water, as its hydrophobic nature prohibits dissolution.[22] In contrast, the half-life of the VDMA analogue 4,4-dimethyl-2-phenyloxazolin-5-one in water has been reported[33] as 36 min at pH 8 and 25 °C, which is in agreement with the rates determined for our water-soluble VDMA copolymers. Potentiometric measurements of a 0.2 wt % solution of as-formed photopolymerized PMV_{50} (7% hydrolyzed) in 35 mM HEPES pH 7.8 buffered saline revealed that the azlactone hydrolysis is essentially complete after 3 h, with an approximate half-life of about 30 min (Figure 8). A rapid initial drop in pH from 8.1 to 7.5 is attributed to the protonation of the free carboxylic acid groups present and is followed by a more gradual decrease in pH to about 7.25, which is attributed to the hydrolysis of azlactone as well as any anhydride groups present. Similar rates of hydrolysis were observed for the partially hydrolyzed PMV_{50} prepared by copolymerization at 60 °C.

Table 3. Degree of Hydrolysis of Isolated PMV as a Function of Comonomer Ratio and Method of Copolymerization

VDMA:MAA (EA and ^{13}C NMR)	% hydrolysis (^{13}C NMR)
Free Radical Polymerization (60 °C)	
45:55	35−45
55:45	9
65:35	7
70:30	4
80:20	<1
Free Radical Photopolymerization (20 °C)	
45:55	7
70:30	4

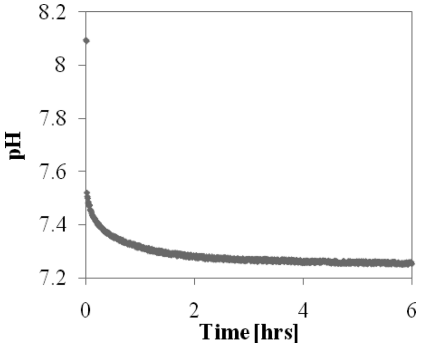

Figure 8. Change in pH over time of PMV_{50} in aqueous medium is measured by dissolving 20 mg of as-formed photopolymerized PMV_{50} (7% hydrolyzed after synthesis) in 20 mL of pH 8.1, 35 mM HEPES buffered saline at time 30 s.

AP-PMV_{50}f Capsules. Finally, calcium alginate−PLL (AP) capsules were coated with a 0.2% HEPES buffered PMV_{50}f solution for 10 min (Figure 9a). Although some immune-isolation research looks to replace PLL with more bio- and cyto-compatible components,[34] PLL was selected as the polycation for this study, so that subsequent *in vivo* and *in vitro* studies[23] would allow for a direct comparison to conventional APA capsules. These studies aim to test whether PMV_{50} is better able to mask the PLL, resulting in improved cell and host compatibility.[24]

The PLL and PMV_{50} coating conditions were optimized to allow for sufficient cross-linking between the PLL and $PMV_{50}f$ layers, while maintaining good cell viability.[23,24]

To prove that covalent cross-linking has occurred, AP-PMV_{50}f capsules were first treated with 1 M sodium citrate to liquefy the calcium alginate core, leaving only an alginate−PLL−PMV_{50}f shell (Figure 9b). This shell was then treated with 0.1 M sodium hydroxide (Figure 9c), which deprotonates the primary ammonium cations on PLL, eliminating its ionic interactions with the two polyanions. Thus, the only remaining interactions should be the amide cross-links between PLL and PMV_{50}f. If no covalent reaction occurred, the complex would dissolve completely after the addition of sodium hydroxide.

Future work will explore the effect of molecular weight and VDMA/acid ratio on the rate of hydrolysis in aqueous environments, on the polyelectrolyte complexation with polyamines, and on the biophysical properties of the covalent networks formed around AP capsules.

CONCLUSIONS

The reactivity ratios for VDMA and MAA were determined to be 1.36 and 0.41 for r_1(VDMA) and r_2(MAA), respectively. A copolymer of approximately 50:50 VDMA:MAA was synthesized by a semibatch copolymerization carried out at 60 °C under anhydrous conditions with a near-constant VDMA:MAA feed ratio of about 35:65 mol %. Quantitative ^{13}C NMR showed that about 40% of the azlactone groups in the isolated polymer were hydrolyzed during the copolymerization, with concomitant formation of cyclic anhydride from methacrylic acid diads. This transhydration side reaction could be largely suppressed by using higher VDMA:MAA mole ratios or by use of photopolymerization at 20 °C. The half-life of the azlactone groups on PMV_{50} was found to be about 30 min in HEPES buffer, sufficiently long to

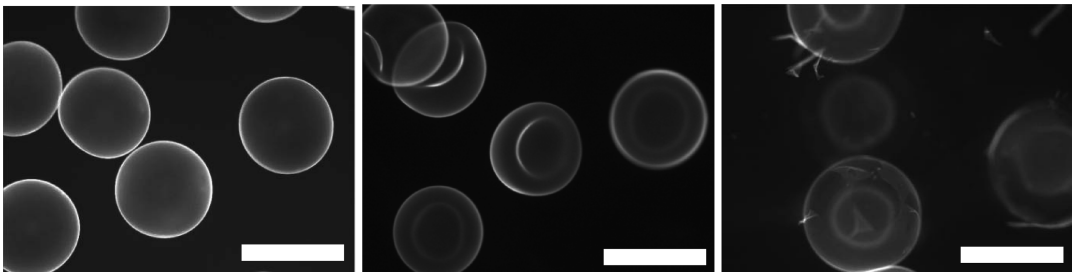

Figure 9. (a) A−PLL−PMV_{50}f as seen under the fluorescent optical microscope, (b) after the addition of 1 M sodium citrate, and (c) after the addition of 0.1 M sodium hydroxide, the complex remains. Scale bar denotes 500 μm.

allow formation of covalently cross-linked shells around AP capsules developed for cell encapsulation. Future work will explore the aqueous hydrolysis, polyelectrolyte complexation, and covalent cross-link formation of other VDMA:MAA copolymers, as described above.

■ ASSOCIATED CONTENT

Supporting Information. A more extensive investigation of the reactivity of the transhydrated PMV_{50} polymer with phenethylamine. This material is available free of charge via the Internet at http://pubs.acs.org.

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