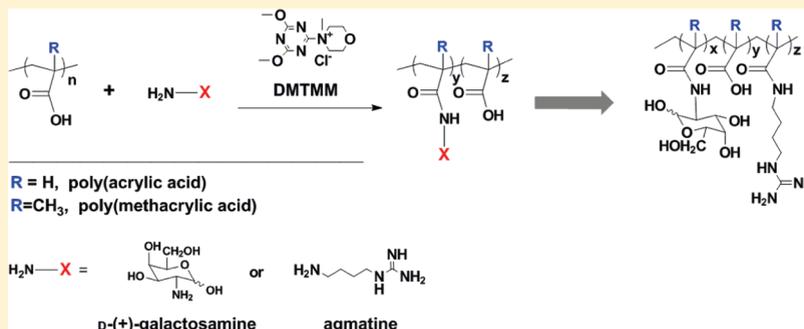


An In-Depth Analysis of Polymer-Analogous Conjugation using DMTMM

 Jeisa M. Pelet[‡] and David Putnam^{*,†,‡}
[†]Department of Biomedical Engineering and [‡]School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York 14853, United States

Supporting Information

ABSTRACT:



Combinatorial libraries have become increasingly popular in the field of functional biomaterials. One approach for creating diverse polymer libraries is polymer-analogous conjugation of functional groups to polymer scaffolds. In this study, we show that a water-soluble condensing agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), can be employed to conjugate two disparate model ligands, D-(+)-galactosamine (Gal) and agmatine (Agm), to the side chains of either poly(methacrylic acid) (pMAA) or poly(acrylic acid) (pAA) at various substitution ratios. The degree of substitution was found to be directly influenced by media pH, polymer concentration, structure of ligands, and polymer precursor. A nearly 2-fold increase in conjugation efficiencies for both ligands to pAA was achieved as compared to pMAA under identical conditions reaching up to 56% and 78% of Gal and Agm of total content, respectively. These two structurally similar polymers showed remarkably different performances, which reveals that the selection of a polymer precursor is crucial for the optimal design of polymeric libraries, particularly when complex structural ligands are involved. The approach employed provides a basis from which larger and more diverse combinatorial libraries of functionalized polymers with multiple moieties can be generated.

INTRODUCTION

Combinatorial libraries have become increasingly prominent in the field of functional biomaterials. For instance, combinatorial libraries of polymers¹ have been widely explored for biomedical applications including gene delivery^{2–8} and medical device development.^{9–11} Of particular importance to the advancement of the field is the development of new synthetic strategies to create increasingly larger and more diverse libraries. In particular, polymeric libraries provide insight into the structure–function relationships of biomaterials to identify favorable structural parameters and allow further optimization of their design.

One approach for creating diverse polymer libraries is polymer-analogous conjugation of functional groups to polymer scaffolds. For example, coupling between the carboxyl groups of a polymer and the amino groups or hydroxyl groups of different ligands can lead to polymer libraries with a range of pre-engineered functionalities. Over the past few years, numerous condensing agents

have been developed and extensively used to form amides and esters.^{12,13} Carbodiimides, such as dicyclohexylcarbodiimide (DCC) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC), are particularly popular condensing agents. While these condensing agents are widely used, their synthetic byproducts can be difficult to remove. Additionally, many of these reagents require the use of organic solvents and anhydrous conditions to avoid competitive hydrolysis by water, and are expensive.

Condensation of both poly(methacrylic acid) and poly(acrylic acid) with amine-containing antibodies has been previously reported with carbodiimides and *N*-hydroxysuccinimide (NHS).¹⁴ [1,3,5]-Triazine-based condensing agents have been exploited due to their great versatility.^{15,16} Recently, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was developed

Received: March 6, 2010

Revised: December 9, 2010

Published: February 10, 2011

Table 1. Amine-Containing Ligand (Gal or Agm) Conjugation to pMAA^a

entry	ligand	condensing agent	[pMAA] ₀ (mg/mL)	[COOH] ₀ : [condensing agent] ₀ (mol L ⁻¹)	actual substitution (AS) ^b (%)	conjugation efficiency (CE) ^c (%)
1	Gal ^d	DMTMM	3	1:0.4	12	30
2	Gal ^d	DMTMM	3	1:0.7	18	26
3	Gal ^d	DMTMM	3	1:1	20	20
4	Gal ^d	DMTMM	3	1:2	23	12
5	Gal ^d	DMTMM	1	1:1	16	16
6	Agm ^e	DMTMM	3	1:0.4	21	53
7	Agm ^e	DMTMM	3	1:0.7	30	43
8	Agm ^e	DMTMM	3	1:1	37	37
9	Agm ^e	DMTMM	3	1:2	53	27
10	Agm ^e	DMTMM	1	1:1	17	17
11	Gal ^f	EDC	2	1:1	26	26
12	Gal ^f	EDC	2	1:2	30	15
13	Agm ^f	EDC	2	1:1	25	25
14	Agm ^f	EDC	2	1:2	28	14

^a Reactions were conducted at room temperature for 24 h. pMAA $M_n = 8100$ (PDI = 1.24). ^b As determined by ¹H NMR (400 MHz) with D₂O as the solvent. ^c As calculated by CE = AS/([condensing agent]₀/([COOH]₀)). ^d Reactions were conducted in 0.1 M borate buffer, pH 6.7, with [NH₂]₀ = 2 · [DMTMM]₀. ^e Reactions were conducted in 0.1 M borate buffer, pH 9.1, with [NH₂]₀ = 2 · [DMTMM]₀. ^f Reactions were conducted in a final concentration of 0.042 M MES, 0.42 M NaCl, and 0.017 M phosphate buffer, pH 7.5 with [NH₂]₀ = 5 · [EDC]₀ and [NHS]₀ = 2.5 · [EDC]₀.

and shown to facilitate an efficient one-step condensation of both small molecules and polymers.^{17–19} Other favorable attributes of DMTMM are easy removal of excess reagent and byproducts from the reaction; compatibility with many solvents including water, alcohols, and diethyl ether, ethyl acetate, and tetrahydrofuran;¹⁶ and high reaction yields; and it is relatively inexpensive. DMTMM can adapt to a wide pH range, and in some cases, no rigorous pH control is necessary.^{20,21} DMTMM has been employed for the modification of a number of polymers including polysaccharides,^{21,22} poly(γ -D-glutamic acid)²³ and as a coupling agent for peptidomimetics.²⁴ Furthermore, DMTMM has been previously reported for direct conjugation of amine-containing ligands, including hydrophobic and hydrophilic groups, to poly(acrylic acid).²⁰ Although these groups were substituted to the side chains with high efficiency, successful conjugation is dependent on a manifold of parameters including the ligand properties, particularly when cationic species are involved. To our knowledge, the optimization for effective conjugation of such moieties to polymer precursors using DMTMM has yet to be reported. The characterization of postpolymerization modifications of polymers with structurally heterogeneous functional groups will be of significant benefit for the development of new materials.

Herein, we report the conditions for optimal polymer-analogous conjugation of two diverse model ligands, D-(+)-galactosamine (Gal) and agmatine (Agm), to the side chain carboxyl groups of both poly(methacrylic acid) (pMAA) and poly(acrylic acid) (pAA). The choice of these two amine-containing ligands is based on their unique structural characteristics, particularly their different size and net charge, which can influence the conjugation efficacy by steric hindrance or electrostatic effects. We report optimal conditions under which these model ligands conjugate to pMAA and pAA using DMTMM and create limited polymer libraries containing several single and binary substitution ratios of both ligands to each individual polymer. The goal of this work is to form the synthetic foundation upon which large, diverse polymer libraries can be generated to investigate the structure–function relationships of polymeric materials.

EXPERIMENTAL PROCEDURES

Materials. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride hydrate (+99%) and D-(+)-galactosamine hydrochloride (99%) were purchased from Acros Organics. Agmatine sulfate was purchased from Fluka ($\geq 99\%$) or Sigma-Aldrich ($\geq 97\%$) and was recrystallized from water/ethanol (1.4:1 v/v). *N*-Hydroxysuccinimide (98%) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride were purchased from Sigma-Aldrich. All other reagents were purchased from Fisher Scientific at the highest purity available. pMAA with average-number molecular weight (M_n) of 8100 and polydispersity index (PDI) of 1.24 was synthesized by reversible addition–fragmentation chain transfer (RAFT) polymerization as previously reported by our group.²⁵ pAA was synthesized analogous to pMAA by RAFT polymerization with the exception of [AA]₀ = 3.0 mol L⁻¹ and the purification process. Instead of precipitation in diethyl ether as for pMAA, pAA was purified by drying under vacuum for 10 min to remove ~50% of the methanol and diluting with deionized water, followed by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against deionized water for 3 days. Samples were subsequently lyophilized for 24 h. Initial reactant ratios were varied to obtain polymers with different M_n ; [AA]₀: [I]₀: [CTA]₀ = 110:0.25:1 corresponded to $M_n = 10\,400$ (PDI = 1.20), [AA]₀: [I]₀: [CTA]₀ = 350:0.25:1 corresponded to $M_n = 33\,200$ (PDI = 1.21), and [AA]₀: [I]₀: [CTA]₀ = 620:0.25:1 corresponded to $M_n = 55\,100$ (PDI = 1.23). M_n values for pAA were calculated based on pMAA standards.

Amine-Containing Ligand (Gal or Agm) Conjugation to pMAA or pAA Using DMTMM. A representative example for 40% targeted Gal conjugation to pMAA, which corresponds to [COOH]₀: [DMTMM]₀: [Gal]₀ = 1:0.4:0.8 (Table 1, entry 1), is as follows: In a 10 mL pear-shaped flask equipped with a magnetic stir bar, pMAA (24 mg, 0.28 mmol repeating units) and Gal (47 mg, 0.22 mmol) were dissolved with 6 mL of 0.1 M borate buffer, pH = 8.5. After 10 min under stirring, a DMTMM solution (30 mg, 0.11 mmol; in 2 mL of 0.1 M borate buffer,

Table 2. Amine-Containing Ligand (Gal or Agm) Conjugation to pAA^a

entry	ligand	[pAA] ₀ (mg/mL)	[COOH] ₀ : [DMTMM] ₀ :	actual substitution (AS) ^b (%)	conjugation efficiency (CE) ^c (%)
			[NH ₂] ₀ (mol L ⁻¹)		
1	Gal ^d	3	1:0.5:1	35	70
2	Gal ^d	3	1:0.7:1.4	41	59
3	Gal ^d	3	1:1:2	52	52
4	Gal ^d	3	1:2:4	56	28
5	Gal ^d	1	1:1:2	43	43
6	Agm ^e	3	1:0.5:1	30	60
7	Agm ^e	3	1:0.7:1.4	46	66
8	Agm ^e	3	1:1:2	57	57
9	Agm ^e	3	1:2:4	78	39
10	Agm ^e	1	1:1:2	32	32

^a Reactions were conducted at room temperature for 24 h using DMTMM as the condensing agent with $[\text{NH}_2]_0 = 2 \cdot [\text{DMTMM}]_0$, pAA $M_n = 10\,400$ (PDI = 1.20). ^b As determined by ¹H NMR (400 MHz) with D₂O as the solvent. ^c As calculated by $\text{CE} = \text{AS} / ([\text{DMTMM}]_0 / ([\text{COOH}]_0))$. ^d Reactions were conducted in 0.1 M borate buffer, pH 7.0. ^e Reactions were conducted in 0.1 M borate buffer, pH 8.0.

pH = 8.5) was transferred dropwise, and the pH was adjusted to 6.7 with additions of 1 N hydrochloric acid (HCl). The reaction flask was capped with a rubber septum and continuously stirred (600 rpm) for 24 h at room temperature. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M borate buffer (pH = 8.5) for 2 days and deionized water for 2 additional days and lyophilized for 24 h. The ligand content relative to the total repeating units (or side chains) in each polymer was determined by ¹H NMR and is referred to as a percentage value. For example, pMAA-12% Gal means 12% of the total side chains in pMAA are grafted with a pendant Gal. Agm conjugations to pMAA, Gal conjugations to pAA and Agm conjugations to pAA followed the same procedure as previously described with the pH adjusted to 9.1, 7.0, and 8.0, respectively, by additions of 1 N HCl or 1 N sodium hydroxide (NaOH). For Agm conjugations to pMAA and pAA, the product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M HCl (pH ~3) for 2 days and deionized water for 2 additional days and lyophilized for 24 h.

Amine-Containing Ligand (Gal or Agm) Conjugation to pMAA Using EDC/NHS. The conjugation of amine-containing ligands to pMAA using EDC/NHS was modified from previously established protocols.¹³ A representative example for 100% targeted Gal conjugation to pMAA which corresponds to $[\text{COOH}]_0 : [\text{EDC}]_0 : [\text{NHS}]_0 : [\text{Gal}]_0 = 1 : 1 : 2.5 : 5$ (Table 1, entry 11) is as follows: In a 25 mL pear-shaped flask equipped with a magnetic stir bar, pMAA (20 mg, 0.23 mmol repeating units) was dissolved with 6 mL of 0.05 M MES, 0.5 M NaCl buffer, pH 6.5. After complete dissolution, EDC (44 mg, 0.23 mmol; in 2 mL of 0.05 M MES, 0.5 M NaCl buffer, pH 6.5) and NHS (67 mg, 0.58 mmol; in 2 mL of 0.05 M MES, 0.5 M NaCl buffer, pH 6.5) solutions were sequentially added to the reaction, and the pH was adjusted to 6.5 with additions of 1 N NaOH. The reaction flask was capped with a rubber septum and continuously stirred (600 rpm) for 1 h at room temperature after which a Gal solution (248 mg, 1.15 mmol; in 2 mL of 0.1 M phosphate buffer, pH 7.5 was transferred

dropwise. The pH was readjusted to 7.5 with additions of 1 N NaOH, and the reaction was allowed to continuously stir overnight (600 rpm) at room temperature. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M phosphate buffer (pH = 7.5) for 2 days and deionized water for 2 additional days and lyophilized for 24 h. The ligand content relative to the total repeating units (or side-chains) in each polymer was determined by ¹H NMR and is referred to as a percentage value. Agm conjugations to pMAA were carried out under the same reaction conditions. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M HCl (pH ~3) for 2 days and deionized water for 2 additional days and lyophilized for 24 h.

Sequential Conjugation of Amine-Containing Ligand (Gal or Agm) to pMAA-graft-Gal, pAA-graft-Gal, or pAA-graft-Agm Using DMTMM. A representative example for 50% targeted Agm conjugation to available carboxyl groups in pMAA-12%Gal, which corresponds to $[\text{COOH}]_0 : [\text{DMTMM}]_0 : [\text{Agm}]_0 = 1 : 0.5 : 1$ (Table 3, entry 1) is as follows: In a 10 mL pear-shaped flask equipped with a magnetic stir bar, pMAA-12%Gal (24 mg, 0.20 mmol COOH) and Agm (46 mg, 0.20 mmol) were dissolved with 6 mL of 0.1 M borate buffer, pH = 8.5. After 10 min under stirring, a DMTMM solution (28 mg, 0.10 mmol; in 2 mL of 0.1 M borate buffer, pH = 8.5) was transferred dropwise, and the pH was adjusted to 9.1 with additions of 1 N NaOH. The reaction flask was capped with a rubber septum and continuously stirred (600 rpm) for 24 h at room temperature. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M HCl (pH ~3) for 2 days and deionized water for 2 additional days and lyophilized for 24 h. The ligand content relative to the total repeating units (or side chains) in each polymer was determined by ¹H NMR and is referred to as a percentage value. Agm conjugations to pAA-graft-Gal and Gal conjugations to pAA-graft-Agm followed the same procedure as previously described with the pH adjusted to 8.0 and 7.0, respectively, by additions of 1 N HCl or 1 N NaOH. For Gal conjugations to pAA-graft-Agm, the product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M borate buffer (pH = 8.5) for 2 days and deionized water for 2 additional days and lyophilized for 24 h.

Simultaneous Conjugation of Gal and Agm to pAA Using DMTMM. A representative example of 25% targeted Gal and 75% targeted Agm conjugations to pAA which corresponds to $[\text{COOH}]_0 : [\text{DMTMM}]_0 : [\text{Gal}]_0 : [\text{Agm}]_0 = 1 : 1 : 0.25 : 0.75$ (Table 4, entry 1) is as follows: In a 10 mL pear-shaped flask equipped with a magnetic stir bar, pAA (24 mg, 0.33 mmol repeating unit), Gal (18 mg, 0.083 mmol) and Agm (57 mg, 0.25 mmol) were dissolved with 6 mL of 0.1 M borate buffer, pH = 8.5. After 10 min under stirring, a DMTMM solution (91 mg, 0.33 mmol; in 2 mL of 0.1 M borate buffer, pH = 8.5) was transferred dropwise, and the pH was adjusted to 7.0 with additions of 1 N HCl. The reaction flask was capped with a rubber septum and continuously stirred (600 rpm) for 24 h at room temperature. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (3.5 kDa MWCO) against 0.001 M HCl (pH ~3) for 2 days and deionized water for 2 additional days and lyophilized for 24 h. The ligand content relative to the total repeating units (or side chains) in each polymer was determined by ¹H NMR and is referred to as a percentage value.

NMR Spectroscopy. ¹H NMR spectra of polymer conjugates were obtained using an Inova 400 MHz spectrometer. 2-D

Table 3. Sequential Conjugation of Agm to pMAA-graft-Gal and pAA-graft-Gal, and of Gal to pAA-graft-Agm^a

entry	polymer	[COOH] ₀ :[DMTMM] ₀ : [NH ₂] ₀ (mol L ⁻¹)	ligand	actual substitution (AS) ^b (%)	conjugation efficiency (CE) ^c (%)
1	pMAA-12%Gal ^d	1:0.5:1	Agm	13	30
2	pMAA-12%Gal ^d	1:0.8:1.6	Agm	21	30
3	pMAA-12%Gal ^d	1:1.1:2.2	Agm	24	25
4	pMAA-18%Gal ^d	1:0.5:1	Agm	15	37
5	pMAA-18%Gal ^d	1:0.8:1.6	Agm	20	30
6	pMAA-18%Gal ^d	1:1.2:2.4	Agm	24	24
7	pMAA-20%Gal ^d	1:0.5:1	Agm	16	40
8	pMAA-20%Gal ^d	1:0.8:1.6	Agm	20	31
9	pMAA-20%Gal ^d	1:1.2:2.4	Agm	24	25
10	pAA-35%Gal ^e	1:0.5:1	Agm	12	37
11	pAA-35%Gal ^e	1:1.1:2.2	Agm	24	34
12	pAA-35%Gal ^e	1:4.8:9.6	Agm	42	13
13	pAA-56%Gal ^e	1:0.5:1	Agm	6	27
14	pAA-56%Gal ^e	1:1.1:2.2	Agm	10	21
15	pAA-56%Gal ^e	1:5.7:11.4	Agm	33	13
16	pAA-30%Agm ^f	1:0.6:1.2	Gal	20	48
17	pAA-30%Agm ^f	1:1.3:2.6	Gal	26	29
18	pAA-30%Agm ^f	1:6.8:13.6	Gal	35	7
19	pAA-46%Agm ^f	1:2.7:5.4	Gal	14	10
20	pAA-46%Agm ^f	1:6.8:13.6	Gal	26	7
21	pAA-57%Agm ^f	1:1.1:2.2	Gal	4	8
22	pAA-57%Agm ^f	1:4.3:8.6	Gal	8	4
23	pAA-78%Agm ^f	1:5:10	Gal	3	3

^a Reactions were conducted at room temperature for 24 h using DMTMM as the condensing agent with [NH₂]₀ = 2 · [DMTMM]₀ and [polymer]₀ = 3 mg/mL. pMAA *M*_n = 8100 (PDI = 1.24); pAA *M*_n = 10 400 (PDI = 1.20). ^b As determined by ¹H NMR (400 MHz) with D₂O as the solvent. ^c As calculated by CE = AS / [((([DMTMM]₀) / ([COOH]₀)) · X_{COOH})], where X_{COOH} is the fraction of available carboxyl groups in a polymer chain. ^d Reactions were conducted in 0.1 M borate buffer, pH 9.1. ^e Reactions were conducted in 0.1 M borate buffer, pH 8.0. ^f Reactions were conducted in 0.1 M borate buffer, pH 7.0.

Table 4. Simultaneous Conjugation of Gal and Agm to pAA^a

entry	[COOH] ₀ : [DMTMM] ₀ : [Gal] ₀ : [Agm] ₀ (mol L ⁻¹)	Gal:Agm actual substitution (AS _{Gal} :AS _{Agm}) ^b (%)	Gal:Agm conjugation efficiency (CE _{Gal} :CE _{Agm}) ^c (%)	overall conjugation efficiency (CE _{overall}) ^d (%)
1	1:1:0.25:0.75	13:39	52:52	52
2	1:1:0.5:0.5	54:56	27:28	55
3	1:1:0.75:0.25	36:17	48:68	53
4	1:1:1:1	30:30	30:30	60
5	1:2:1:1	38:32	38:32	35

^a Reactions were conducted in 0.1 M borate buffer, pH 7.0, at room temperature for 24 h using DMTMM as the condensing agent with [polymer]₀ = 3 mg/mL. pAA *M*_n = 10 400 (PDI = 1.20). ^b As determined by ¹H NMR (400 MHz) with D₂O as the solvent. ^c As calculated CE_{Gal} = AS_{Gal} / (([Gal]₀) / ([COOH]₀)) and CE_{Agm} = AS_{Agm} / (([Agm]₀) / ([COOH]₀)). ^d As calculated by CE_{overall} = (AS_{Gal} + AS_{Agm}) / (([DMTMM]₀) / ([COOH]₀)).

DOSY NMR spectra of polymer conjugates were obtained using an Inova 600 MHz spectrometer. Deuterium oxide (D₂O) was used as the solvent. To the samples that were not readily soluble in D₂O, small additions of deuterium chloride or sodium deuterioxide (30 wt % in D₂O) were added. Resonances were referenced to HOD at 4.81 ppm.

RESULTS AND DISCUSSION

The effective design of polymeric libraries with a range of functionalities requires careful characterization of the conjugation process, which is greatly influenced by the polymer precursor and

selected ligands. In this study, we evaluate two polymer backbones, pMAA and pAA. Both polymers are readily soluble in water and can easily undergo chemical modifications. The high content of carboxyl groups from the side chains allows diverse combinations of multiple pendant groups grafted to the polymer backbone via amide bonds. However, these related but structurally different polymers showed significant differences in conjugation efficiencies of our two model ligands, Gal and Agm, which sparked our interest to investigate the trends distinguished for such conjugations under various conditions.

NMR Analyses for Gal and Agm Conjugation to pMAA or pAA. The side chains of both pMAA and pAA were modified by

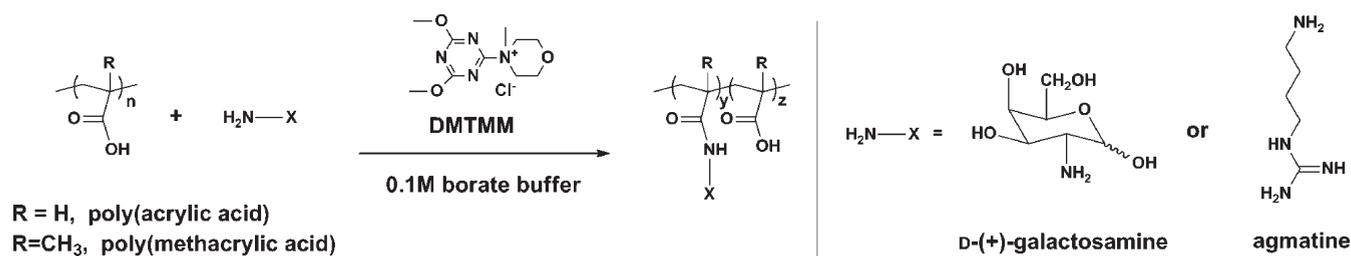


Figure 1. Synthesis schematic for condensation reaction of poly(methacrylic acid) or poly(acrylic acid) and amine-containing ligand (Gal and Agm) using DMTMM as the condensing agent.

the substitution of Gal and Agm using DMTMM as the condensing agent (Figure 1). DMTMM activates the carboxyl groups in the polymer by forming a 2-acyloxy-4,6-dimethoxy-1,3,5-triazine intermediate followed by the nucleophilic attack of the amino group resulting in an amide linkage between the polymer side chains and the ligand of interest.¹⁹ The degree of side chain substitution was determined from the peak intensity ratio of the polymer backbone to the side chain groups by ¹H NMR (Inova 400 MHz) spectroscopy with D₂O as the solvent, and is referred to as a percentage value. Figure 2A–C shows the ¹H NMR spectra for pMAA-20%Gal, pMAA-37%Agm, and pMAA-18%Gal-24%Agm, respectively. The chemical shift for the protons in the α -methyl group ($-\text{CH}_3$) in the polymer backbone of pMAA are distinguished at 0.6–1.4 ppm (peak a), while the protons of the methylene group ($-\text{CH}_2$) are at 1.4–2.3 ppm (peak b). Gal has distinctive chemical shifts at 3.5–4.7 ppm corresponding to 6H along the ring structure at C-2 to C-6 positions and at 5.2 ppm corresponding to 1H at C-1 position (peaks d). Similarly, Agm proton chemical shifts are located at 1.6 ppm for 4H (peak f) and at 3.2 ppm for 4H (peak e). Figure 2D–F illustrates the ¹H NMR spectra for pAA-56%Gal, pAA-30%Agm, and pAA-56%Gal-33%Agm, respectively. In the case of pAA and pAA conjugates, the pAA backbone protons have chemical shifts of 1.2–2.0 ppm (peak b) and 2.0–2.8 ppm (peak c) for the methylene ($-\text{CH}_2$) and methyne group ($-\text{CH}$), respectively. Grafted Gal and Agm to pAA have chemical shifts analogous to the pMAA conjugates. However, for pAA, an unexpected peak was observed at 3.7 ppm. Further analysis revealed that this peak was from methanol esterification of the carboxylic acid during RAFT polymerization. The peak was accounted for the quantitation of Gal substitution in pAA.

Because of the cationic nature of Agm, the removal of excess Agm from the reaction was performed through dialysis against an acidic environment (pH \sim 3). To corroborate the absence of unconjugated ligand in the final product, diffusion-ordered spectroscopy (DOSY) was carried out for both pMAA and pAA conjugates. DOSY is a pulsed field gradient NMR experiment in which species in a mixture are distinguished by their translational diffusion coefficients. Figure 3 shows a representative example of the 2-D DOSY NMR spectrum in D₂O for pAA-56%Gal-33%Agm that demonstrates that both ligands are covalently attached to the polymer side chains and that no residual ligands or byproducts remained in solution after dialysis. In the spectrum generated, the diffusion coefficients have arbitrary units; however, it is important to note that all species in the polymer have the same translational diffusion and that this diffusion is much slower than water (HOD peak), a much smaller molecule. Similar results were obtained for pMAA conjugates.

Effect of pH on Gal and Agm Conjugation to pMAA. The extent to which both Gal and Agm conjugate to pMAA is influenced by the pH of the selected buffer. It was previously established that, prior to the addition of DMTMM to the reaction, both the carboxylic acid and the amine should be premixed to form an ammonium carboxylate salt.¹⁹ Formation of this salt can also be enhanced by maintaining the pH of the aqueous solution between the acid dissociation constants (pK_a) of both the acid and the amine of interest. The pK_a for the amino group in Gal is 8.49 and 8.02 for the α - and β -configuration, respectively,²⁶ while for the amino group in Agm, it is 9.07.²⁷ The pK_a for weak polyacids varies depending on the solution conditions and polymer structure. A titration curve of pMAA with NaOH revealed a functional pK_a of 6.3. On the basis of this information, the pH range that is likely most favorable for Gal conjugation to pMAA is from 6.3 to 8 and for Agm conjugation to pMAA is from 6.3 to 9. In addition, at these pH values the polymer chain is in an extended conformation due to the charge repulsion from the carboxylate groups along the polymer backbone. Figure 4A shows the relationship of conjugation efficiency for both Gal and Agm as a function of pH. The conjugation efficiency was calculated from the molar ratio of actual ligand content in the polymer to targeted substitution. pMAA-Agm conjugates experienced solubility issues mostly in acidic environments, and the pH range at which these conjugates were soluble directly depended on the degree of Agm substitution. Moreover, reactions conducted at pH 8 formed conjugates that precipitated out of solution and were not able to be properly analyzed (represented by dashed line in Figure 4A). From this analysis, it was determined that the optimal pH in 0.1 M borate buffer for the highest conjugation efficiency of Gal and Agm to pMAA was 6.7 and 9.1, achieving a maximum conjugation efficiency of 20% and 37%, respectively.

Effect of pH on Gal and Agm Conjugation to pAA. The behavior of pAA in aqueous media is remarkably different from that of pMAA. A titration curve for pAA generated with NaOH reflected a functional pK_a of 5.9, which gives a slightly larger pH range for effective conjugation as compared to pMAA. The optimal pH values in 0.1 M borate buffer were found to be 7.0 and 8.0 corresponding to conjugation efficiencies of 52% and 57% for Gal and Agm conjugation to pAA, respectively (Figure 4B). Interestingly, although the pAA structure is comparable to that of pMAA, the conjugation efficiencies for pAA were significantly higher (2.6- and 1.5-fold for Gal and Agm, respectively) at their optimal pH values. The lack of an α -methyl group in the pAA backbone may have permitted higher conjugation efficiencies relative to pMAA.

During Agm and Gal conjugations to both pMAA and pAA, the solution pH was noticed to decrease to \sim 6.5–8.5 as the

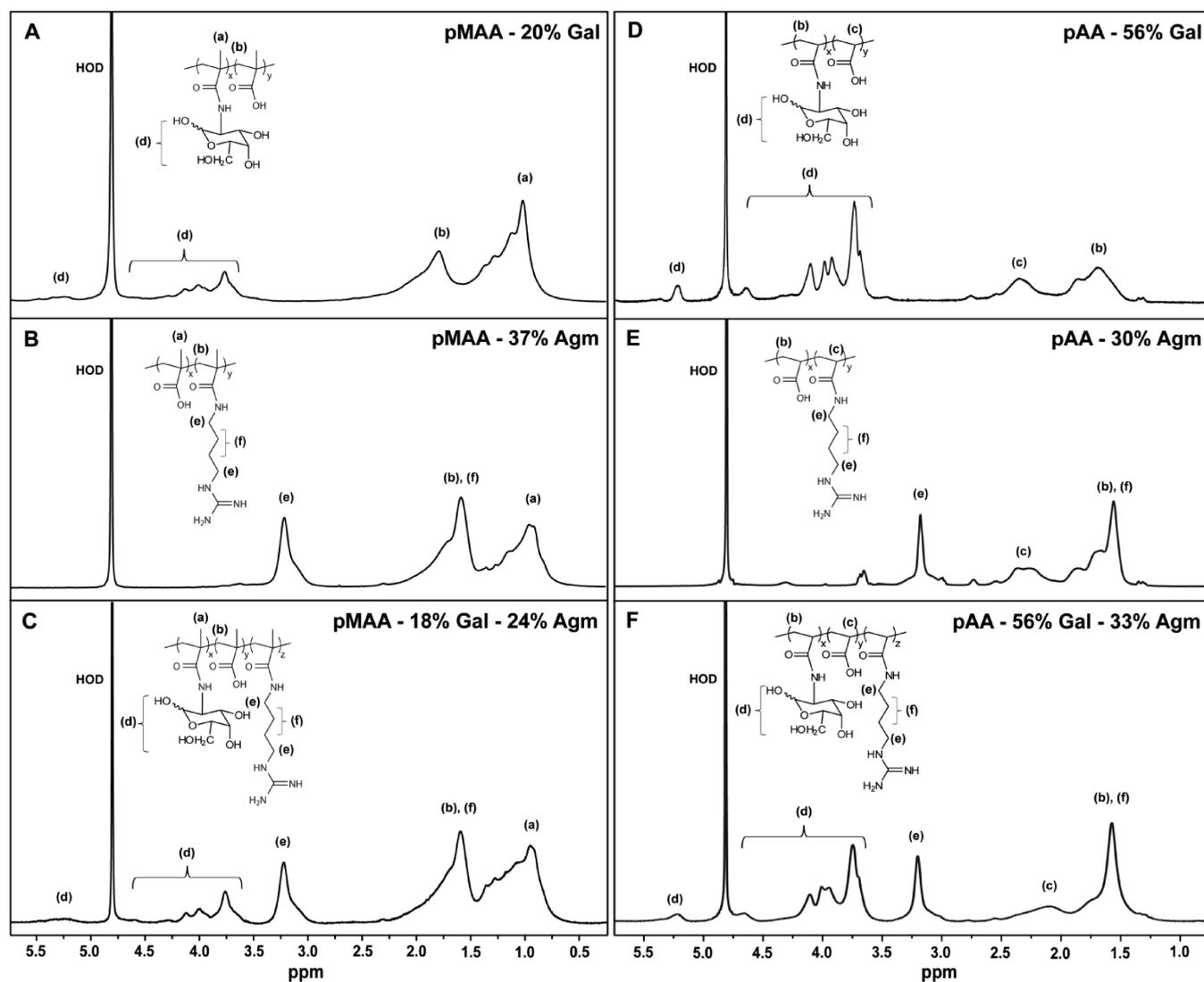


Figure 2. ^1H NMR spectra (400 MHz, D_2O) of (A) pMAA-20%Gal, (B) pMAA-37%Agm, (C) pMAA-18%Gal-24%Agm, (D) pAA-56%Gal, (E) pAA-30%Agm, and (F) pAA-56%Gal-33%Agm. Peaks: (a) and (b) pMAA backbone; (b) and (c) pAA backbone; (d) Gal; (e) and (f) Agm. pMAA $M_n = 8100$ (PDI = 1.24); pAA $M_n = 10400$ (PDI = 1.20).

reaction proceeded. At these pH values, amine groups will have a lower reactivity toward the ester intermediate. However, increasing the pH to 8 or 9 after 5 h of reaction to favor the deprotonation of the amine groups and enhance reactivity toward the ester intermediate did not have a positive impact on the conjugation efficiency leading to equal or lower Gal and Agm substitutions in both pMAA and pAA (Supporting Information Table S1). Table S1. It is possible that the ester intermediate begins to hydrolyze before the amidation reaction completely takes place, as also suggested by the lower conjugation efficiencies for reactions carried out at higher pH values (Figure 4).

Single Conjugation of Gal and Agm to pMAA or pAA.

Table 1 includes the actual ligand substitutions of Gal and Agm in pMAA and conjugation efficiencies at various $[\text{COOH}]_0$: $[\text{DMTMM}]_0$ molar ratios. The conjugation efficiency was calculated from the molar ratio of actual ligand substitution to targeted substitution, which is determined from the $[\text{DMTMM}]_0$ to $[\text{COOH}]_0$ molar ratio. From this data, a noticeable trend is distinguished in which the conjugation efficiency decreases as the

reactant ratios of $[\text{DMTMM}]_0$ and $[\text{NH}_2]_0$ relative to $[\text{COOH}]_0$ increases, likely due to steric hindrance. This behavior is observed for both Gal and Agm conjugations to pMAA. The highest conjugation efficiencies obtained with DMTMM were 30% for Gal and 53% for Agm; however, this was when only 40% of the carboxyl groups in pMAA were targeted. For a direct comparison to other common conjugation reagents, an analogous chemical modification using EDC/NHS was performed. Similar results were obtained at comparable targeted substitutions further suggesting that the polymer and/or ligand structure might be in part responsible for this relatively low conjugation efficiency.

Maximum total ligand substitutions (mol %) of 23% Gal and 53% Agm were achieved for pMAA. Decreasing the polymer concentration 3-fold had a negative impact on the conjugation efficiency particularly for Agm substitution, which was reduced by half its value. Potential reasons for such conjugation deficiencies might be the chemical characteristics of the polymer and/or ligands, or the short distance between the side chain carboxyl groups and polymer backbone along with the α -methyl group in

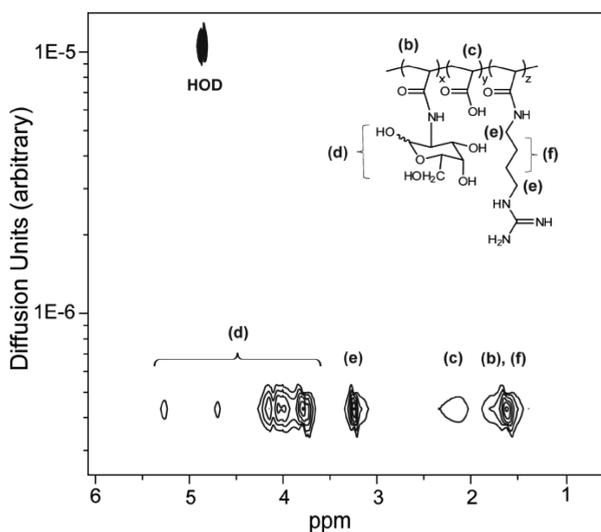


Figure 3. 2-D DOSY NMR spectrum (600 MHz, D_2O) of pAA-56% Gal-33%Agm. Chemical shifts and relative diffusion coefficients are shown along the horizontal and vertical axes, respectively. pAA $M_n = 10\,400$ (PDI = 1.20).

the polymer backbone which can generate steric hindrance. Previous work by Kazakov and collaborators^{14,28} demonstrated that pMAA has a local compact conformation at $pH < 5$ in part due to the hydrophobic methyl groups attached to the polymer chain. This suggests that the α -methyl group in the polymer backbone has an important role in the conformation of this polymer in solution, and this may influence its ability to interact with other moieties.

In contrast to pMAA, pAA achieved much higher conjugation efficiencies leading to higher total ligand contents under equivalent conditions. The highest total ligand substitutions (mol %) obtained for pAA were 56% Gal and 78% Agm (Table 2). Compared to pMAA, a similar decreasing trend of conjugation efficiency with increasing targeted substitution was observed for both Gal and Agm conjugations to pAA, likely due to steric hindrance. It is evident that, for both polymer precursors, Agm was more readily incorporated into the polymer side chains than Gal. The low Gal conjugation efficiency may be due to its relatively large, ring-like structure, which may cause steric hindrance as compared to the more elongated structure of Agm.

The solubility in aqueous solution of both pMAA-Agm and pAA-Agm conjugates was found to be directly affected by the degree of substitution of the ligand (Supporting Information Table S2). Agm substituted to less than half of the available carboxyl groups in the polymer will lead to solubility issues in more acidic environments. In contrast, Agm substituted to more than half of the available carboxyl groups in the polymer will experience solubility issues in more basic environments. For instance, by visual inspection, pMAA-25%Agm is insoluble at $3 < pH < 5.8$ and pMAA-37%Agm is insoluble at $3 < pH < 6.8$, while pMAA-53%Agm is insoluble at $pH > 4.9$. Similarly for pAA-Agm conjugates, pAA-30%Agm is insoluble at $3 < pH < 5.9$, while pAA-57%Agm is insoluble at $pH > 8$. pAA-78%Agm remains soluble at all pHs. These overall pH behaviors are likely due to the presence of both positive and negative charges in the polymer, which at specific pHs cause electrostatic interactions between the charged polymer chains, leading to aggregation and altered solubility.

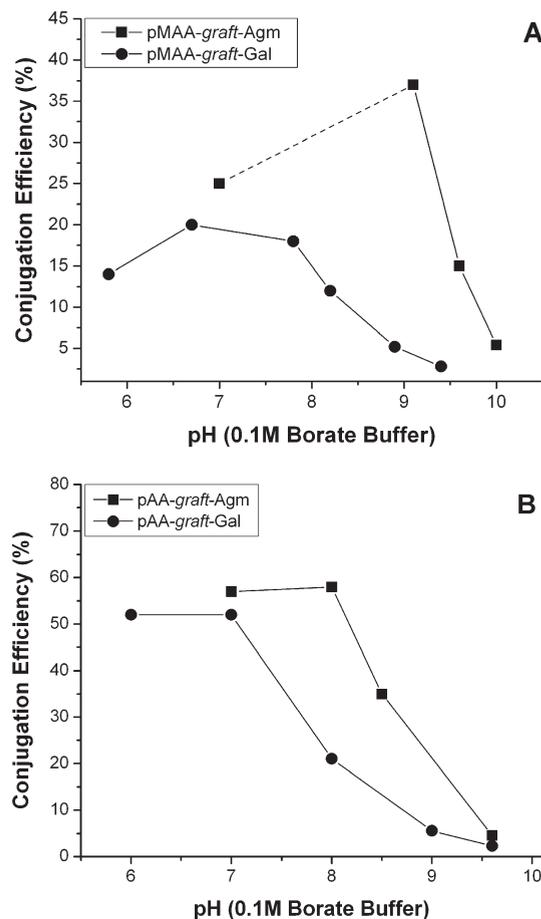


Figure 4. Relationship of conjugation efficiency with pH (adjusted at $t = 0$ h) in 0.1 M borate buffer using DMTMM as the condensing agent: (A) (■) pMAA-graft-Agm and (●) pMAA-graft-Gal; dashed lines represent the pH range that was not analyzed due to precipitation of the polymer conjugates. (B) (■) pAA-graft-Agm and (●) pAA-graft-Gal. Reactions were conducted at room temperature for 24 h with $[polymer]_0 = 3$ mg/mL and $[COOH]_0:[DMTMM]_0:[NH_2]_0 = 1:1:2$. Data were generated from an average of two repetitions with standard error of $\leq 6\%$. pMAA $M_n = 8100$ (PDI = 1.24); pAA $M_n = 10\,400$ (PDI = 1.20).

Polymers with lower Agm substitution ($< 50\%$) become soluble at higher pH values, as the available carboxyl groups undergo ionization providing a net negative charge which reduces electrostatic interactions between the polymer chains. On the contrary, polymers with higher Agm substitution ($> 50\%$) become soluble at lower pH values, as the available carboxyl groups become protonated generating a net positive charge that reduces electrostatic interaction between the polymer chains. The higher the Agm substitution, the more soluble these conjugates become due to a net positive charge in the polymer regardless of the degree of ionization of the available carboxyl groups, as distinguished for pAA-78%Agm. Aggregation of polymer conjugates at certain pHs was confirmed through dynamic light scattering (Supporting Information). Figure S1 shows the relationship of mean particle diameter (Z-average, nm) with pH for pMAA-37%Agm (A) and pAA-57%Agm (B). pMAA-37%Agm shows aggregation at $pH 3-7$, while pAA-57%Agm shows aggregation at $pH > 8$.

For pMAA-Gal conjugates, only relatively high Gal substitution ($\geq 20\%$) produced solubility issues. However, these were on a much smaller scale and were accompanied by a light opaqueness

of the polymer solution at all pH ranges. pAA-Gal conjugates did not show any solubility issues regarding the Gal total content, corroborating the role of the cationic charge produced by Agm in solubility issues and aggregation.

To evaluate the impact of the polymer molecular weight on the conjugation efficiency of the ligands, various Agm and Gal conjugations were carried out with pAA of higher M_n , specifically 33 100 Da and 55 200 Da. Solubility issues were more prominent with increasing polymer M_n for pAA-Agm conjugates. As the reaction progressed, these polymer conjugates precipitated out of solution, in which case they were acidified until fully dissolved prior to dialysis. Total ligand content and conjugation efficiencies were consistent with those values for lower M_n polymers (Supporting Information Table S3). To aid the solubility of these polymer conjugates, equivalent reactions were carried out at a lower initial pH (pH 6). However, conjugation efficiencies were \sim 40% lower than the reactions carried out at an initial pH of 8, and solubility issues were still present (Supporting Information Table S4).

In addition to the ease in preparation of single-step reactions and the mild reaction conditions, another significant advantage of postpolymerization modifications mediated by DMTMM is its reproducibility. For single conjugations of Gal or Agm to pMAA and pAA, 70% of the conditions were repeated at least twice and up to four times, all with standard errors of \leq 6%. This attribute provides a batch-to-batch consistency that is important to reduce variability of the final product.

Sequential Conjugation of Gal and Agm to pMAA or pAA. Owing to their different optimal conjugation characteristics, simultaneous conjugation of both Gal and Agm to pMAA was not viable in a one-step reaction. Following a single-step conjugation containing both ligands, Agm was the only ligand efficiently conjugated, limiting Gal substitution in the polymer side chains (Supporting Information Table S5). Moreover, Gal was unable to be subsequently incorporated in high amounts into pMAA-graft-Agm, making a two-step reaction, where Gal was first introduced to the polymer followed by Agm, the most favorable route to having both groups present. The corresponding substitutions and conjugation efficiencies of the two-step approach are shown in Table 3. In contrast to pMAA, both Gal and Agm were able to conjugate to pAA sequentially. For both polymers, similar to single conjugations, the conjugation efficiency decreased as higher substitutions were targeted, possibly due to an increase in steric hindrance produced by the neighboring pendant groups. Although lower conjugation efficiencies relative to single substitutions were obtained, the versatility of pAA to incorporate both moieties likely makes it a more suitable polymer for this type of conjugation, particularly when a high content of a positively charged moiety (i.e., Agm) is desired.

Simultaneous Conjugation of Gal and Agm to pAA. In contrast to pMAA, Agm and Gal were able to conjugate simultaneously to pAA with comparable overall efficiency to single conjugations (Table 4). A pH of 7 was selected to accommodate both Gal and Agm conjugations to pAA resulting in the effective substitution of both groups. The flexibility of simultaneous conjugation to pAA offers vast possibilities for dual-functionalization and perhaps multiple simultaneous conjugations of other ligands to these polymer precursors.

CONCLUSION

Combinatorial libraries are a practical approach to investigate the structure–function relationships of polymeric biomaterials. In particular, branched polymers with reactive side chain groups

are an approach to polymer precursors due to multiple sites available for modifications with different functional groups. With this in mind, our goal was to fully evaluate the utility of DMTMM as a condensing agent for the amidation reaction of carboxyl groups in the side-chains of both pMAA and pAA, using two distinct amine-containing ligands, Gal and Agm. The conjugation efficiency of these moieties was found to be influenced by the media pH, polymer concentration, ligand structure, and polymer structure. Under comparable conditions, the substitution of both ligands in pAA was significantly higher than in pMAA, reaching close to a 2-fold increase in conjugation efficiencies, presumably due to the absence of a hydrophobic α -methyl group that generated solution conformation issues in the pMAA reactions. In addition, Agm surpassed Gal total content in both polymers. Nevertheless, total Gal and Agm content in pMAA reached 23% and 53%, while total Gal and Agm content in pAA reached 56% and 78%, respectively. Single and sequential conjugations to pMAA and pAA as well as simultaneous conjugation to pAA of these two model ligands revealed a consistent trend of conjugation efficiencies with targeted substitutions. The distinguished trends provide useful information to develop a systematic approach for the synthesis of large combinatorial libraries with various moieties of interest.

ASSOCIATED CONTENT

S Supporting Information. Mean particle diameter (Z-average, nm) of polymer conjugates, Gal and Agm conjugation to pMAA or pAA conducted at other reaction conditions not included in the manuscript, solubility of pMAA and pAA conjugates (by visual inspection), Gal and Agm conjugation to pAA of different molecular weights, simultaneous conjugation of Gal and Agm to pMAA and Gal and Agm conjugation to pAA in water (without pH control). This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*To whom correspondence should be addressed. E-mail dap43@cornell.edu, Ph (607) 255-4352, Fax (607) 255-7330.

ACKNOWLEDGMENT

We thank the Cornell University NMR facility, Dr. Ivan Keresztes for his expertise and assistance with the NMR, and the Alfred P. Sloan Foundation Graduate Scholarship Programs and National Action Council for Minorities in Engineering (NACME), Inc. for a Sloan Fellowship (J.M.P.).

REFERENCES

- (1) Brocchini, S. (2001) Combinatorial chemistry and biomedical polymer development. *Adv. Drug Delivery Rev.* 53, 123–130.
- (2) Anderson, D. G., Akinc, A., Hossain, N., and Langer, R. (2005) Structure/property studies of polymeric gene delivery using a library of poly(β -amino esters). *Mol. Ther.* 11, 426–434.
- (3) Anderson, D. G., Lynn, D. M., and Langer, R. (2003) Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angew. Chem., Int. Ed.* 42, 3153–3158.
- (4) Green, J. J., Langer, R., and Anderson, D. G. (2007) A combinatorial polymer library approach yields insight into nonviral gene delivery. *Acc. Chem. Res.* 41, 749–759.

- (5) Lynn, D. M., Anderson, D. G., Putnam, D., and Langer, R. (2001) Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. *J. Am. Chem. Soc.* 123, 8155–8156.
- (6) Wong, S. Y., Sood, N., and Putnam, D. (2009) Combinatorial evaluation of cations, pH-sensitive and hydrophobic moieties for polymeric vector design. *Mol. Ther.* 17, 480–490.
- (7) Wong, S. Y., and Putnam, D. (2007) Overcoming limiting side reactions associated with an NHS-activated precursor of polymethacrylamide-based polymers. *Bioconjugate Chem.* 18, 970–982.
- (8) Thomas, M., Lu, J. J., Zhang, C., Chen, J., and Klivanov, A. M. (2007) Identification of novel superior polycationic vectors for gene delivery by high-throughput synthesis and screening of a combinatorial library. *Pharm. Res.* 24, 1564–1571.
- (9) Brocchini, S., James, K., Tangpasuthadol, V., and Kohn, J. (1998) Structure-property correlation in a combinatorial library of degradable biomaterials. *J. Biomed. Mater. Res.* 42, 66–75.
- (10) Jaffe, M., Pai, V., Ophir, Z., Wu, J., and Kohn, J. (2002) Relationships of erodable polymeric biomaterials, I: poly(desamino-tyrosyl arylates). *Polym. Adv. Technol.* 13, 926–937.
- (11) Weber, N., Bolikal, D., Bourke, S. L., and Kohn, J. (2004) Small changes in the polymer structure influence the adsorption behavior of fibrinogen on polymer surfaces: validation of a new rapid screening technique. *J. Biomed. Mater. Res.* 68A, 496–503.
- (12) Montalbetti, C. A. G. N., and Falque, V. (2005) Amine bond formation and peptide coupling. *Tetrahedron* 61, 10827–10852.
- (13) Hermanson, G. T. (2008) *Bioconjugate techniques*, 2nd ed. (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL) pp 219–223, Part II, Academic Press, London.
- (14) Kazakov, S. V., Muronetz, V. I., Dainiak, M. B., Izumrudov, V. A., Galaev, I. Y., and Mattiasson, B. (2001) Light scattering study of the antibody-poly(methacrylic acid) and antibody-poly(acrylic acid) conjugates in aqueous solutions. *Macromol. Biosci.* 1, 157–163.
- (15) Giacomelli, G., Porcheddu, A., and Luca, L. d. (2004) [1,3,5]-Triazine: a versatile heterocycle in current applications of organic chemistry. *Curr. Org. Chem.* 8, 1497–1519.
- (16) Kamiński, Z. J., Paneth, P., and Rudziński, J. (1998) A study on the activation of carboxylic acids by means of 2-chloro-4,6-dimethoxy-1,3,5-triazine and 2-chloro-4,6-diphenoxy-1,3,5-triazine. *J. Org. Chem.* 63, 4248–4255.
- (17) Kunishima, M., Kawachi, C., Iwasaki, F., Terao, K., and Tani, S. (1999) Synthesis and characterization of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride. *Tetrahedron* 40, 5327–5330.
- (18) Kunishima, M., Kawachi, C., Hioki, K., Terao, K., and Tani, S. (2001) Formation of carboxamides by direct condensation of carboxylic acids and amines in alcohols using a new alcohol- and water- soluble condensing agent: DMT-MM. *Tetrahedron* 57, 1551–1558.
- (19) Kunishima, M., Kawachi, C., Morita, J., Terao, K., Iwasaki, F., and Tani, S. (1999) 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride: an efficient condensing agent leading to the formation of amides and esters. *Tetrahedron* 55, 13159–13170.
- (20) Thompson, K., and Michielsen, S. (2006) Novel synthesis of N-substituted polyaxrylamides: derivatization of poly(acrylic acid) with amines using a triazine-based condensing reagent. *J. Polym. Sci., Part A: Polym. Chem.* 44, 126–136.
- (21) Farkaš, P., and Bystrický, S. (2007) Efficient activation of carboxyl polysaccharides for the preparation of conjugates. *Carbohydr. Polym.* 68, 187–190.
- (22) Shlottmann, S. A., Jain, N., Chirmule, N., and Esser, M. T. (2006) A novel chemistry for conjugating pneumococcal polysaccharides to luminex microspheres. *J. Immunol. Methods* 309, 75–85.
- (23) Joyce, J., Cook, J., Chabot, D., Hepler, R., Shoop, W., Xu, Q., Stambaugh, T., Aste-Amezaga, M., Wang, S., Indrawati, L., Bruner, M., Friedlander, A., Keller, P., and Caufield, M. (2006) Immunogenicity and protective efficacy of bacillus anthracis poly- γ -D-glutamic acid capsule covalently coupled to a protein carrier using a novel triazine-based conjugation strategy. *J. Biol. Chem.* 281, 4831–4843.
- (24) Shieh, W.-C., Chen, Z., Xue, S., McKenna, J., Wang, R.-M., Prasad, K., and Repič, O. (2008) Synthesis of sterically-hindered peptidomimetics using 4-(4,6-dimethoxy-1,3,4-triazine-2-yl)-4-methylmorpholinium chloride. *Tetrahedron Lett.* 49, 5359–5362.
- (25) Pelet, J. M., and Putnam, D. (2009) High molecular weight poly(methacrylic acid) with narrow polydispersity by RAFT polymerization. *Macromolecules* 42, 1494–1499.
- (26) Blaskó, A., Bunton, C. A., Bunel, S., Ibarra, C., and Moraga, E. (1997) Determination of acid dissociation constants of anomers of amino sugars by ^1H NMR spectroscopy. *Carbohydr. Res.* 298, 163–172.
- (27) Gründemann, D., Hahne, C., Berkels, R., and Schomig, E. (2003) Agmatine is efficiently transported by non-neuronal monoamine transporters extraneuronal monoamine transporter (EMT) and organic cation transporter 2 (OCT2). *J. Pharmacol. Exp. Ther.* 304, 810–817.
- (28) Kazakov, S. V., Galaev, I. Y., and Mattiasson, B. (2002) Characterization of macromolecular solutions by a combined static and dynamic light scattering technique. *Int. J. Thermophys.* 23, 161–173.