pH-Responsive Poly(styrene-*alt*-maleic anhydride) Alkylamide Copolymers for Intracellular Drug Delivery

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Many macromolecular therapeutics such as peptides, proteins, antisense oligodeoxynucleotides (ASODN), and short interfering RNA (siRNA) are active only in the cytoplasm or nucleus of targeted cells. Endocytosis is the primary route for cellular uptake of these molecules, which results in their accumulation in the endosomal−lysosomal trafficking pathway and loss of therapeutic activity. In this article, we describe the synthesis and pH-dependent membrane-destabilizing activity of a new "smart" polymer family that can be utilized to enhance the intracellular delivery of therapeutic macromolecules through the endosomal membrane barrier into the cytoplasm of targeted cells. These polymers are propylamine, butylamine, and pentylamine derivatives of poly(styrene-*alt*-maleic anhydride) (PSMA) copolymers. The PSMA−alkylamide derivatives are hydrophilic and membrane-inactive at physiological pH; however, they become hydrophobic and membrane-disruptive in response to endosomal pH values as measured by their hemolytic activity. Results show that the pH-dependent membrane-destabilizing activity of PSMA derivatives can be controlled by varying the length of the alkylamine group, the degree of modification of the copolymer, and the molecular weight of the PSMA copolymer backbone. Butylamine and pentylamine derivatives of PSMA copolymers exhibited more than 80% hemolysis at endosomal pH values, which suggests their potential as a platform of "smart" polymeric carriers for enhanced cytoplasmic delivery of a variety of therapeutic macromolecules.

1. Introduction

Cytoplasmic delivery of promising biomolecular drugs such as therapeutic peptides, proteins, genes, antisense oligodeoxynucleotides, and short interfering RNA (siRNA) molecules remains a significant challenge.[1−5] These biomolecules are typically taken up by targeted cells via endocytosis and trafficked through the endosomal−lysosomal pathway, which leads to degradation by lysosomal enzymes and loss of therapeutic activity.[6,7] Endosomal escape remains a significant challenge to intracellular delivery of biomolecular therapeutics. Consequently, several research groups have focused on the development of agents that can enhance the cytoplasmic delivery of large therapeutic molecules into target cells.

Viral vectors haven proven efficient for cytoplasmic delivery of nucleic acids,[8] as a result of the pH-dependent membrane-destabilizing activity of fusogenic peptide sequences in the viral protein coat.[9] Hemagglutinin, from the influenza virus, is an example of such a fusogenic protein.[9] Hemagglutinin has fusogenic peptide sequences that exist in ionized, hydrophilic conformations at physiologic pH values, but convert to hydrophobic helices in response to the acidic pH of the endosome.[9−12] These hydrophobic helices fuse with the endosomal membrane, leading to its destabilization and escape of the endosomal contents into the cytoplasm[13] of infected cells. Several synthetic peptides have been designed to mimic fusogenic protein sequences and have been shown to increase cytoplasmic gene delivery.[13] Despite the endosomolytic activity of such peptides, potential toxicity and immunogenicity could limit their clinical utility.

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Another approach has been the use of cationic, typically amino-based, polymers which facilitate endosomal release through the "proton-sponge" effect.[14−19] These polymers contain numerous tertiary amino groups that become protonated at endosomal pH. It is thought that these polymers buffer the endosome against the drop in pH, leading to an increased flux of protons and their counterions into the endosome.[20,21] The influx of ions is presumed to cause an increase in osmotic pressure, leading to the disruption of the endosome. Such cationic polymers have shown activity for endosomal release of drugs both in vitro and in vivo.[14−19]

Our group has focused on the design and synthesis of "smart" pH-responsive, membrane-destabilizing polymers to enhance the cytoplasmic delivery of therapeutic macromolecules.[22−28] These are amphiphilic polymers that contain a critical balance of acidic carboxyl groups and hydrophobic alkyl or aromatic groups.[23−28] The carboxylate ions of these polymers become protonated at endosomal pH values, and the polymers undergo a change from a hydrophilic, biologically inert state to a hydrophobic and endosomal membrane-destabilizing one. These "smart" pH-responsive polymeric carriers have been shown to enhance the cytoplasmic delivery of therapeutic biomolecules both in vitro[24,25,29] and in vivo.[26] The work reported here extends our previous research with pH-sensitive acrylic acid polymers and copolymers to a new class of pH-responsive and membrane-destabilizing polymeric carriers based on poly(styrene-*alt*-maleic anhydride) copolymers (PSMA).

Styrene and maleic anhydride are known to produce alternating copolymers[31−33] that have been used in a variety of applications.[32] Maeda and co-workers used low molecular weight PSMA copolymers (<6 kDa) clinically to deliver the antitumor protein neocarzinostatin (NCS).[30,34−36] The polymer−protein conjugate, known as SMANCS, is formed using "partial

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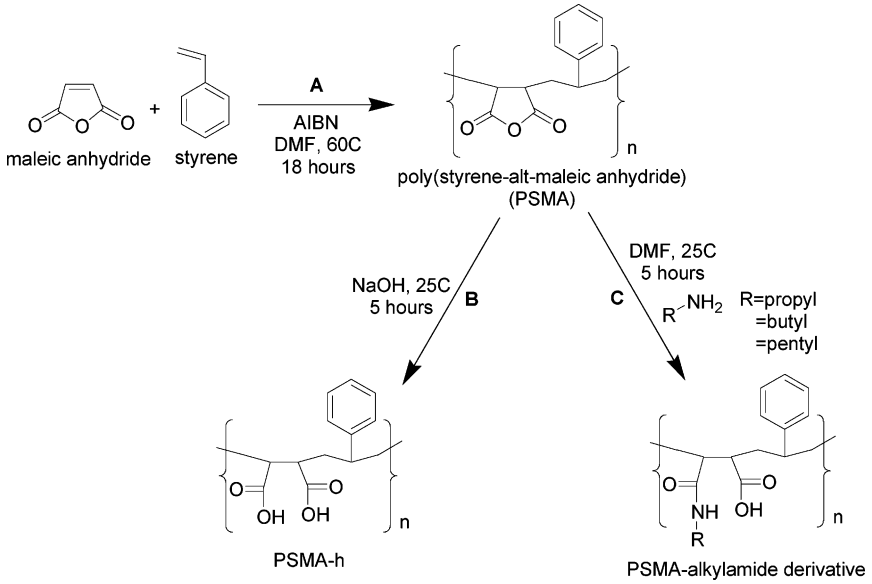

Figure 1. General synthetic schemes. (A) Synthesis of PSMA alternating copolymer. (B) Hydrolysis of the anhydride groups to form PSMA-h. (C) Modification by alkylamines to form PSMA alkylamine derivatives.

half-esters" of SMA, in which 70% of the maleic anhydride groups were opened using butanol.[30,34] SMANCS significantly improved the pharmacological properties of NCS by increasing both its circulatory half-life and its lipid solubility, and it has been clinically effective in treating liver cancer.[30,34−36] The SMANCS conjugate is also known to accumulate in tumor tissue and the lymphatic system through the EPR effect (enhanced permeability and retention).[30,36] SMA copolymers have also been shown to noncovalently bind with albumin during systemic circulation, thereby reducing polymer clearance from the body, and SMA has been conjugated with other anti-cancer agents to exploit this property.[37] In an investigation of macrophage activation by SMANCS, Maeda and co-workers found that contact with SMA produced changes in cell membrane fluidity and noted SMA could lyse red blood cells at physiological pH above certain concentrations.[38] Additional research by Maeda and co-workers indicated that the SMA butylester enhanced uptake of SMANCS in cells due to increased cell binding and speculated that translocation across the endosomal membrane was enhanced by increased membrane solubility as a result of increased hydrophobicity of NCS and SMA at acidic pH.[39]

In the research reported here, we extend these previous findings to show that alkylamine derivatives of PSMA are capable of destabilizing biological membranes at acidic pH values and show how this activity can be modulated for use in intracellular drug delivery applications. Specifically, we have synthesized several alkylamine derivatives of PSMA copolymers that become membrane-disruptive at endosomal pH values. The membrane-destabilizing activity of these copolymers can be controlled by (a) the chain length of the hydrophobic alkyl moiety reacted with the PSMA backbone, (b) the extent of alkylamine modification of the PSMA backbone, and (c) the molecular weight of the parent PSMA backbone. The membrane-destabilizing activities of these new copolymer compositions were examined using a standard red blood cell hemolysis assay, which has been shown to correlate with efficacy of intracellular drug delivery.[13] Our results suggest that these new copolymer compositions can be utilized as carriers to enhance the cytoplasmic delivery of a variety of biomolecular drugs.

2. Materials and Methods

2.1. Materials. All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) and used without further purification unless otherwise noted. The free-radical initiator 2,2′-azo-bis(isobutyronitirle) (AIBN) was recrystallized from methanol prior to use. Triton X-100 detergent was obtained from Rohm and Haas (Philadelphia, PA). Solvents used were ACS grade and obtained from Sigma-Aldrich with the exception of ethyl ether (EMD, Gibbstown, NJ). HPLC-grade dimethylformamide (DMF) for GPC was obtained from Acros Organics (Fairlawn, NJ). Deuterated solvents were obtained from Cambridge Isotope Labs (Andover, MA) with the exception of deuterated sodium hydroxide, obtained from Aldrich.

2.2. Methods. *2.2.1. Synthesis of Poly(styrene-alt-maleic anhydride).* Poly(styrene-*alt*-maleic anhydride) (PSMA) was prepared through a thermally initiated free-radical polymerization of styrene and maleic anhydride (Figure 1A). Equimolar amounts (0.005 mol) of styrene (573 μL) and maleic anhydride (0.49 g) were combined in a 5 mL round-bottom flask with AIBN and dimethylformamide (DMF). The amounts of DMF and AIBN used were varied to control the molecular weight and polydispersity of the PSMA product. Typically, the amount of AIBN used ranged from 0.5 to 2 mol % relative to total monomer; between 0.2 and 8 mL DMF was used as a solvent (16 to 88 wt %). Flasks were sealed prior to polymerization with a vacuum adaptor and subjected to three rounds of freeze−vacuum−thaw to remove oxygen from the reaction vessel. Polymerizations were carried out overnight (18 h) by immersing the flasks in a 60 °C oil bath. The polymerization product was either a viscous liquid or a solid mass at the conclusion of 18 h, depending on the amount of DMF present during the reaction. The polymerization product was dissolved or diluted in acetone followed by dropwise addition into a 100-fold excess (v/v) of cold diethyl ether to precipitate pure PSMA polymer, which was then filtered and dried under vacuum at room temperature.

A small portion (∼0.1 g) of the dried PSMA copolymer was then subject to base-catalyzed hydrolysis (Figure 1B) prior to NMR analysis. Briefly, 0.1 g of PSMA was added to 2 mL of 2 N NaOH solution and stirred for 5 h at room temperature. The copolymer was recovered from NaOH solution by acid precipitation using 1 N HCl. The solution was then centrifuged, and the recovered copolymer was redissolved in deionized water (DI) (∼3 mL). The resulting copolymer solution was then extensively dialyzed against DI using SpectraPor Slide-a-Lyzer cassettes (MWCO = 3500; Pierce, Rockford, IL) to remove sodium salts and neutralize the solution prior to lyophilization.

The composition of the hydrolyzed PSMA (PSMA-h) was determined by ^{1}H NMR spectroscopy in a deuterated sodium hydroxide solution (1 N). All NMR spectroscopy was done on a Bruker DRX499 system. The characteristic aromatic peaks of the styrene subunits (δ = 6−7.5 ppm, 5H) and the peaks of the backbone hydrogens from styrene and maleic anhydride (δ = 0−3 ppm, 2H from maleic anhydride R−CH−COO-, 3H from styrene R−CH_2−R′, R−CH−Ar) were used to determine copolymer composition by solving a set of simultaneous equations. NMR analysis showed the copolymers are composed of 50% styrene and 50% maleic anhydride, as expected. The PSMA-h was also used to determine the pH-sensitive membrane activity of unmodified PSMA copolymers.

The molecular weight of the unhydrolyzed copolymers was determined by size exclusion chromatography using Tosoh TSK-GEL α-3000 and α-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to a Viscotek GPCmax VE2001 and refractometer VE3580 (Viscotek, Houston, TX). HPLC-grade DMF containing 0.1 wt % LiBr was used as the mobile phase. The molecular weights of the synthesized copolymers were determined using a series of poly(methyl methacrylate) standards.

2.2.2. Preparation of Alkylamine Derivatives. Alkylamine derivatives of PSMA were synthesized by reacting primary alkylamines with the anhydride groups in the PSMA backbone (Figure 1C). In a typical reaction, 100 mg of PSMA was dissolved in 2 mL of anhydrous DMF in a 5 mL round-bottom flask. The copolymer solution was sparged with nitrogen before adding a predetermined amount of propylamine, butylamine, or pentylamine modifier. The reaction was carried out at room temperature for 5 h with stirring. The copolymer was recovered by precipitation in cold diethyl ether followed by filtration and dried under vacuum at room temperature. The propylamine, butylamine, and pentylamine derivatives of the PSMA backbone are referred to as $PSMA_{mw}$-propylXX, $PSMA_{mw}$-butylXX, and $PSMA_{mw}$-pentylXX, respectively. The mw subscript refers to the molecular weight of the PSMA backbone, while the XX refers to the percentage of maleic anhydride groups modified by the alkylamine relative to the total number of maleic anhydride groups in the PSMA backbone.

The degree of alkylation achieved was determined by ^{1}H NMR spectroscopy in 1 N NaOD solution. The extent of modification was determined by comparison to the spectrum of the unmodified PSMA; an increase in the peak area between δ = 0−3 ppm was attributed to the protons of the alkylamine modifier. The degree of modification is reported relative to the number of maleic anhydride groups in the copolymer backbone.

2.2.3. Potentiometric Titration. A Fisher Scientific Accumet 950 pH/ion meter was used to conduct potentiometric titrations of selected alkylamine derivatives of a 38 kDa PSMA (PDI = 2.24) copolymer. The molecular weight and polydispersity characteristics of this polymer are similar to those used in the hemolysis experiments described below. Polymer solutions (33 mg/mL) were prepared in 3 mL of 0.33 M NaOH and stirred for 1.5 h to hydrolyze any remaining anhydrides. A total of 12 mL of deionized water was added, and the solution was titrated with 0.03 M HCl from pH = 11.00 to pH = 2.00 with constant stirring. The degree of ionization, α, is defined as $\alpha = \alpha_n + [H^+]/C_p$ where α_n is the degree of neutralization, $[H^+]$ is the free proton concentration determined from the pH of the solution, and C_p is the concentration of carboxylic acid groups on the polymer. The Henderson−Hasselbalch equation was used to relate the apparent dissociation constant, pK_a, pH, and α. All titrations were performed at room temperature.

2.2.4. Hemolysis Assay. Membrane-destabilizing polymers cause hemolysis of red blood cells at pH values similar to those found in the endosome,[23,27,40,41] and endosomal release has been correlated to red blood cell (RBC) hemolysis.[13] The capacity of the PSMA copolymers to induce pH-dependent membrane destabilization was determined using an RBC hemolysis assay described elsewhere.[27] Briefly, whole human blood was collected from volunteer donors using protocols in accordance with University of Washington and NIH guidelines. RBCs were isolated by blood centrifugation and washed three times with a 0.15 M saline solution. After the final wash, the RBCs were diluted 1:10 in 0.1 M phosphate buffer solution (PBS) at the desired pH values (5.8, 6.6, or 7.4) to yield a final RBC concentration of 10^8 RBCs per 200 μL. In eppendorf tubes, 800 μL of PBS at a particular pH value was mixed with 200 μL of RBC solution followed by addition of polymer solution. For hemolysis experiments, PSMA copolymers were modified with alkylamines and tested for hemolytic activity without specifically hydrolyzing residual anhydrides. However, it is expected that most of the highly labile anhydride groups will be lost during preparation of the aqueous polymer stock solutions, as the initial polymer dissolution is performed under basic conditions. The PSMA copolymer and RBC mixtures were incubated in a 37 °C water bath for 1 h. During this incubation time, membrane-destabilizing polymers interact with the RBC membranes, releasing hemoglobin (Hb) into solution. The tubes were centrifuged for 5 min at 13 500 g to separate intact RBCs and disrupted membranes from the solution. The supernatant, containing the released Hb, was collected and transferred to 96-well plates, and the absorbance was measured on a Saphire 2 plate reader (Tecan, Austria) at 541 nm, which is the characteristic wavelength for Hb. The observed hemolysis of RBCs in PBS solutions and in a Triton X-100 detergent solution (1% v/v) were used as negative and positive controls, respectively. The observed hemolytic activity of a given copolymer composition at a given concentration and pH value was normalized to that of the positive control, Triton X-100 solution. All hemolysis assays were done in triplicate, and data is reported as the average ± the standard error of the mean. Statistical analysis of the hemolytic activity of different polymer compositions was done using Student *t*-tests with a 95% confidence interval as the threshold for significance.

2.2.5. LDH Cytotoxicity Assay. Lactate dehydrogenase (LDH) is a cytosolic enzyme that is not normally secreted outside the cell but is released into the culture medium following damage to cell membranes. An LDH assay was used to measure the cytotoxicity of the alkylamine derivatives of PSMA copolymers as a function of polymer concentration. NIH3T3 fibroblasts were grown in tissue-culture polystyrene 96-well plates to a final concentration of 1000 cells/well. The culture medium was composed of 100 μL of Delbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Adherent fibroblasts were incubated with PSMA copolymer and PSMA propyl-, butyl-, and pentylamine derivatives at concentrations of 40, 80, and 160 μg/mL for 24 h. Untreated fibroblasts and fibroblasts treated with a 10% Triton X-100 detergent solution were used as positive and negative controls, respectively. Following 24 h of incubation, the incubation solutions were aspirated, and the cells were treated with 100 μL of 1% Triton X-100 to release LDH present in the surviving fibroblasts. The LDH content of each well was measured using a standard LDH assay kit according to manufacturer's specifications (Roche, Indianapolis, IN). Results are reported as a percentage of surviving cells relative to the positive control, untreated fibroblasts. All copolymer treatments and controls were evaluated in triplicate, and average results are presented ± the standard error of the mean. One-way ANOVA analysis was used to compare cell survival between the polymer treatment groups and positive controls using a 95% confidence interval as the threshold for significance.

3. Results and Discussion

3.1. PSMA Copolymer Synthesis. The free-radical polymerization of PSMA is facile, and the copolymer is readily recovered. ^{1}H NMR analysis of all the PSMA copolymers prepared indicates a 50:50 styrene/maleic anhydride composition, as expected from the tendency of these monomers to polymerize together as one unit (Figure 2A). The molecular weight and polydispersity of the PSMA copolymers was controlled by varying the molar ratio of free-radical initiator and volume of added solvent. Table 1 shows the compositions,

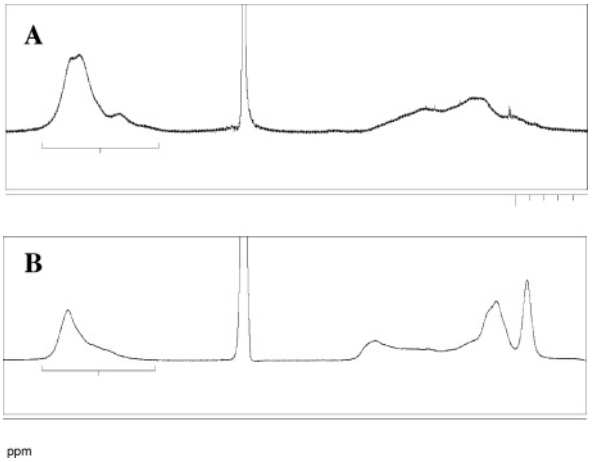

Figure 2. The ^{1}H NMR spectra of unmodified $PSMA_{32k}$ copolymer is presented in panel A. The composition of the copolymer was determined by comparison of the aromatic and alkyl proton peak areas. Panel B shows the ^{1}H NMR spectra of the same copolymer following modification with butylamine ($PSMA_{32k}$-b80). The increase in the peak area below 3.0 ppm relative to the aromatic region was attributed to the alkylamine modifier.

Table 1. Polymer Characteristics of the PSMA Copolymers Used in This Study

polymer no.	M_w[a] (Da)	PI	mol % S[b]	mol % MA[b]
$PSMA_{32k}$	31 600	3.07	50	50
$PSMA_{63k}$	63 300	2.03	50	50
$PSMA_{83k}$	82 500	6.15	50	50

[a] Determined from size exclusion chromatography. [b] As determined by ^{1}H NMR analysis.

molecular weights, and polydispersity indices of three PSMA copolymers prepared for this study.

3.2. Alkylamine Derivatives. Alkylamine modification of the PSMA backbone is used to control its pH-solubility response and resultant membrane-disruption activity. Each alkylamine chain reacts with one maleic anhydride group in the PSMA backbone to form an alkylamide linkage and one carboxylic acid group, which confers pH sensitivity to the modified copolymer. The ^{1}H NMR analyses of PSMA copolymer derivatives indicate that the reactions of propylamine, butylamine, and pentylamine with PSMA go to completion, allowing stoichiometric control of the degree of alkyl chain modification. Table 2 shows the degree of alkylamine modification of three PSMA copolymers used in this study and the nomenclature used to identify these PSMA derivatives. Figure 2B shows a representative ^{1}H NMR spectrum for a PSMA butylamine derivative. In some cases, the degree of alkyl modification is higher than expected on the basis of stoichiometry; this can be attributed to small errors in measurement of the alkylamine solutions used for the reaction.

As the hydrophobicity of the PSMA backbone is increased by modification with alkylamine groups, the pK_a of the copolymer is shifted upward. Increases in pK_a with hydrophobicity have been previously observed with "smart" copolymers of 2-ethylacrylic acid and methacrylic acid.[42] In the case of the PSMA copolymers we have prepared, hydrophobicity is increased by increasing the degree of modification with a given alkylamine modifier or by modification with a longer alkylamine chain. For butylamine derivatives of a 38 kDa PSMA copolymer, modification at 40%, 60%, and 80% produced a shift in the pK_a of the PSMA copolymer to 5.1 $\pm$ 0.15, 5.9 $\pm$ 0.05, and 6.6 $\pm$ 0.09, respectively. PSMA derivatives prepared using pentylamine are more hydrophobic than those prepared from butylamine at a given degree of modification; the 80% pentylamine derivative has a pK_a of approximately 7.1 $\pm$ 0.06, compared to a pK_a of 6.6 for the butylamine derivative at the same degree of modification (Figure 3). Increased polymer hydrophobicity results in increased membrane disruption and can shift the pH response of the polymer, which is further discussed with the results of the hemolysis assays.

In previous work, Maeda and co-workers prepared butyl "partial half-esters" of PSMA in which 70% of the maleic anhydride groups in the copolymer were opened using butanol.[30,34] The purpose of this modification was to improve the pharmacological properties of the anti-cancer drug NCS.[30,35] Though the butylester chemistry is well-characterized,[34] it was not used here because primary alkylamines are more nucleophilic than primary alcohols, and their reaction with anhydrides is faster. Further, amide bonds are significantly more resistant to hydrolysis than esters, leading to greater in vitro and in vivo stability of the resulting PSMA copolymer derivative.

Following modification with alkylamines, a number of anhydride groups remain in the PSMA copolymer backbone. These anhydrides may be further modified to introduce additional functionality into the copolymer, e.g., by conjugating drugs or cell targeting ligands. For example, we have grafted cysteamine onto the PSMA backbone using the same reaction chemistry used to prepare the alkylamine derivatives. Both NMR analysis and Ellman's assay for free thiol groups indicated the stoichiometric completion of this reaction. Cysteamine conjugation to the PSMA backbone provides a free sulfhydryl group that can be used for conjugation of different biomolecules to the polymer via disulfide linkages. Our group has demonstrated the feasibility of disulfide chemistry for drug conjugation,[27] which allows the release of the incorporated drug molecules into the cytoplasm following endosomal release by glutathione-mediated reduction of the disulfide bond. Other classes of biomolecules can be grafted onto the PSMA copolymer using the anhydride groups through a variety of synthetic methods described elsewhere.[43]

3.3. Hemolysis Results. The alkylamine derivatives of PSMA copolymers are amphiphilic molecules that contain both hydrophilic carboxylic acid domains and hydrophobic aromatic and alkyl domains. As the environmental pH is lowered to and below the pK_a of the carboxylic acid groups on the polymer backbone, they become more protonated, and the polymer becomes more hydrophobic. In this hydrophobic state, PSMA derivatives are capable of interacting with biological membranes such as the red blood cell (RBC) membrane, causing disruption. The hemolytic activity of the PSMA−alkylamine copolymers can be readily controlled in several ways. Specifically, increased hemolytic activity can be obtained by the following methods: (a) increasing the chain length of the alkylamine modifier, (b) increasing the extent of alkylamine modification, (c) increasing the molecular weight of the PSMA backbone, or (d) by increasing the concentration of PSMA copolymer present in solution.

We have found that modifying the PSMA backbone with longer (more hydrophobic) alkyl side chains will lead to greater hemolysis at any pH value. In general, propylamine derivatives and unmodified PSMA copolymers have insignificant hemolytic activity at all pH values. Butylamine derivatives exhibit pH-sensitive membrane disruption but are less hemolytic than the pentylamine derivatives. These trends are readily apparent in the RBC membrane-destabilizing activity of PSMA derivatives prepared from a 32 kDa PSMA backbone (Figure 4). At a copolymer concentration of 20 μg/mL, neither the unmodified

Table 2. Alkylamine Derivatives of PSMA Copolymers and the Corresponding Nomenclature[a]

	$PSMA_{32k}$ derivatives			$PSMA_{63k}$ derivatives			$PSMA_{83k}$ derivatives		
modifier	target %	actual %[b]	name	target %	actual %[b]	name	target %	actual %[b]	name
propylamine	40	40	$PSMA_{82}$-p40	40	50	$PSMA_{63}$-p40			
	60	64	$PSMA_{82}$-p60	60	64	$PSMA_{63}$-p60			
	80	88	$PSMA_{82}$-p80	80	82	$PSMA_{63}$-p80			
butylamine	40	44	$PSMA_{82}$-b40	40	44	$PSMA_{63}$-b40			
	60	64	$PSMA_{82}$-b60	60	62	$PSMA_{63}$-b60			
	80	84	$PSMA_{82}$-b80	80	88	$PSMA_{63}$-b80			
pentylamine	40	44	$PSMA_{82}$-pn40	40	46	$PSMA_{63}$-pn40	40	35	$PSMA_{83}$-pn40
	60	72	$PSMA_{82}$-pn60	60	67	$PSMA_{63}$-pn60	60	53	$PSMA_{83}$-pn60
	80	88	$PSMA_{82}$-pn80	80	88	$PSMA_{63}$-pn80	80	65	$PSMA_{83}$-pn80

[a] Percent alkylamine modification is reported relative to the total number of maleic anhydride groups present in the PSMA polymer backbone. [b] Determined by ^{1}H NMR analysis.

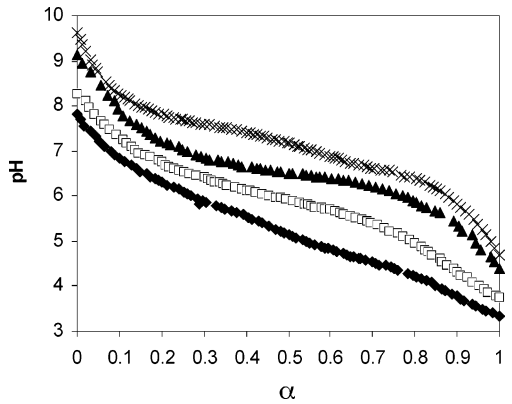

Figure 3. Representative pH vs α (extent of ionization) curves for alkylamine derivatives of $PSMA_{38k}$ copolymer. The derivatives tested are 40% butylamine (◆), 60% butylamine (□), 80% butylamine (▲), and 80% pentylamine (×). As polymer hydrophobicity increases as a result of alkylamine modification, the pK_a increases.

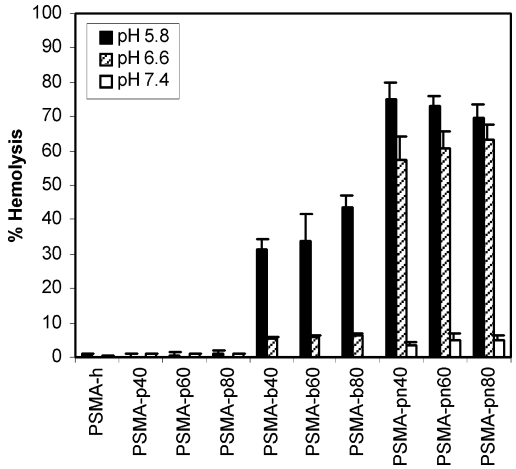

Figure 4. Hemolysis of alkylamine derivatives of $PSMA_{32k}$ copolymer (M_w = 32 kDa, PI = 3.0) at a polymer concentration of 20 μg/mL. Hemolytic activity is normalized to that of the positive control, 1% v/v Triton X-100. Hemolysis results are averages of three independent studies, each conducted in triplicate ± standard error of the mean (SEM).

$PSMA_{32k}$-h (in which all the anhydride groups have been hydrolyzed) nor the propylamine derivatives of $PSMA_{32k}$ exhibit hemolytic activity at any pH value. The butylamine derivatives of $PSMA_{32k}$ exhibited moderate levels of hemolysis at pH 5.8 (30−40%), very little hemolysis at pH 6.6 (<10%), and negligible hemolysis at pH 7.4. The pentylamine derivatives of $PSMA_{32k}$ also showed pH-dependent membrane disruption, with

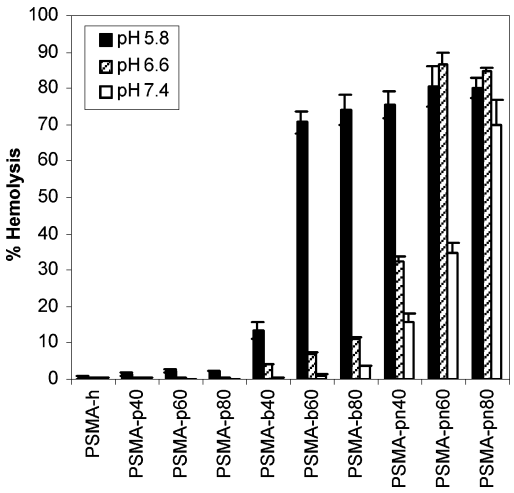

Figure 5. Hemolysis of alkylamine derivatives of $PSMA_{63k}$ copolymer (M_w = 63 kDa, PI = 2.0) at a polymer concentration of 20 μg/mL. Hemolytic activity is normalized relative to that observed from the positive control, 1% v/v Triton X-100. Hemolysis results are representative data from a single experiment conducted in triplicate ± standard error of the mean (SEM).

a favorable 70−75% hemolytic activity at pH 5.8. These derivatives also produced 57−65% hemolytic activity at pH 6.6 but remained inactive at pH 7.4. This activity profile is highly favorable for intracellular delivery applications and will allow for rapid endosomal escape of the therapeutic cargo at early points (less acidic) along the endocytotic pathway. It is important to note the effect of chain length of the alkylamine group on the hemolytic activity of the PSMA derivative. At equal degrees of modification, the pentylamine derivatives of $PSMA_{32k}$ exhibited greater hemolytic activity than butylamine derivatives at a given pH value ($p < 0.0002$ for all comparisons). These results indicate the feasibility of tailoring the pH-dependent membrane-destabilizing activity of the PSMA polymer backbone on the basis of the desired drug delivery application.

The relationship between hemolytic activity and the type of alkylamine modification is also apparent in the derivatives of $PSMA_{63k}$ as shown in Figure 5. Neither the hydrolyzed copolymer ($PSMA_{63k}$-h) nor the propylamine derivatives exhibit hemolytic activity at any pH. The butylamine derivatives of $PSMA_{63k}$ are highly hemolytic, reaching 74% hemolysis at pH 5.8, while remaining relatively inactive at pH 6.6 and 7.4. The pentylamine derivatives of $PSMA_{63k}$ displayed 76−80% hemolysis at pH 5.8, 32−70% hemolysis at pH 6.6, and 20−70% hemolysis at pH 7.4. The high levels of hemolytic activity observed for these derivatives at pH 7.4 is expected to be

substantially decreased upon conjugation to therapeutic drug molecules, which are typically hydrophilic in nature. However, the therapeutic conjugates would be expected to retain the desirable high levels of hemolytic activity at pH 6.6 and 5.8.[28] Similar to the results observed with the $PSMA_{32k}$ polymer, the pentylamine derivatives of $PSMA_{63k}$ exhibited higher hemolytic activity compared to the butylamine derivative at equal degrees of modification across the range of pH values tested ($p < 0.04$). The single exception is the $PSMA_{63k}$-pn80 derivative (80% hemolysis) which is not statistically more hemolytic than the butyl derivative (74% hemolysis) at the same degree of modification ($p = 0.06$).

Another important variable controlling hemolytic activity in the PSMA system is the degree of alkylamine modification. For a given type of alkylamine chain, increasing the degree of modification on the PSMA backbone leads to increased hemolytic activity. This can be seen in the butylamine derivatives of $PSMA_{32k}$ (Figure 4). Increasing the butylamine modification from 40% to 80% significantly increases the amount of hemolysis that is observed at pH 5.8 from 31% to 44% ($p = 0.01$), while maintaining low hemolytic activity at higher pH. The same trend was observed with the butylamine derivatives of $PSMA_{63k}$, where hemolysis increases from 13% to 74% as modification increases from 40% to 80% ($p = 0.0001$) (Figure 5). As for the pentylamine derivatives of PSMA copolymers, the observed hemolytic activity at pH 5.8 remains almost constant regardless of the degree of modification in the range 40−80% (Figures 4 and 5). However, their hemolytic activity increases substantially at pH 6.6 and 7.4 with an increase in the degree of alkylamine modification (Figures 4 and 5). This can be attributed to the significant increase in the hydrophobicity of the PSMA polymer due to the increased pentylamine modification, which causes a shift in the pK_a values of the carboxylic acid groups toward higher, more physiologic pH values.

We also examined the effect of increasing the molecular weight of the PSMA backbone by comparing the hemolysis profiles of the alkylamine derivatives of $PSMA_{32k}$ and $PSMA_{63k}$ polymers (Figures 4 and 5). For the butylamine and pentylamine derivatives of these copolymers, the hemolytic activity of the $PSMA_{63k}$ derivatives is greater than that of those prepared from $PSMA_{32k}$ at the same degree of modification. As an example, the 80% butylamine derivative of the 32 kDA PSMA copolymer produced 44% hemolysis at pH 5.8 compared to 74% hemolysis produced by the same derivative of the 63 kDa PSMA copolymer ($p < 0.0001$).

Copolymer solubility may also play a role in hemolytic behavior as the molecular weight of the PSMA backbone increases. When we examined the hemolytic activity of pentylamine derivatives prepared from an 83 kDa PSMA copolymer, the hemolytic activity at pH 5.8 unexpectedly decreased with increasing pentylamine modification, and it was completely lost at the highest degree of modification (80%) (Figure 6). In addition, the pentylamine derivatives of the 83 kDa PSMA copolymer retained significant hemolytic activity at pH 6.6 and 7.4 throughout the modification range (40−80%). This hemolysis profile suggests that the pentylamine derivatives of $PSMA_{83k}$ are sufficiently hydrophobic to phase separate or precipitate at highly acidic conditions, limiting their interaction with the RBC membrane.

Our data also show that membrane disruption is a function of copolymer concentration. The hemolytic activity of the 60% butylamine derivatives of $PSMA_{63k}$ increased from 5% to 75% at pH 5.8 as polymer concentration increased from 5 to 40 μg/mL (Figure 7). It is worth mentioning that the hemolytic

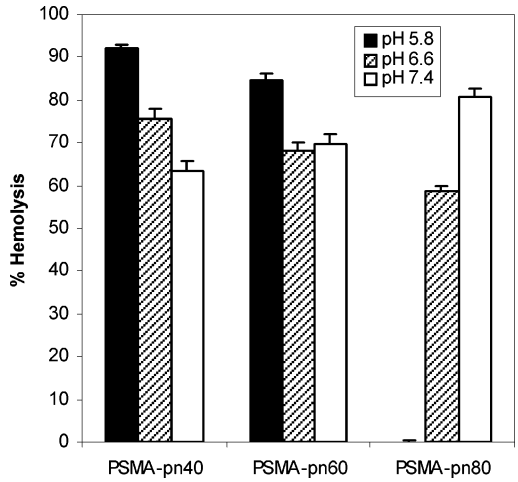

Figure 6. Hemolysis of pentylamine derivatives of $PSMA_{83k}$ copolymer ($M_w = 83$ kDa, PI =6.2) at a concentration of 20 μg/mL. Hemolytic activity is normalized relative to that observed from the positive control, 1% v/v Triton X-100. Hemolysis results are averages of three independent studies, each performed in triplicate ± standard error of the mean (SEM).

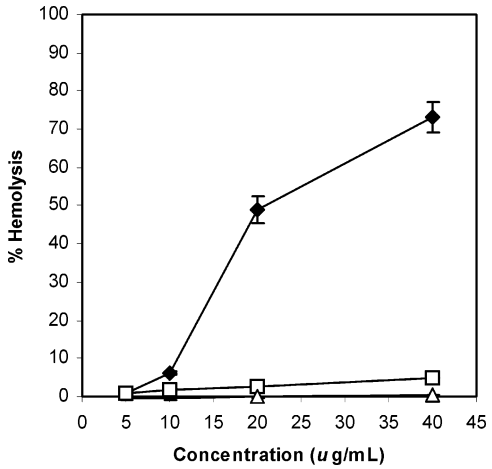

Figure 7. The concentration-dependent hemolytic activity of the 60% butylamine derivative of $PSMA_{63k}$ polymer backbone normalized to that of the positive control, 1% v/v Triton X-100. Hemolysis was tested at pH = 5.8 (◆), pH = 6.6 (□), and pH = 7.4 (△). Hemolysis results show representative data from a single experiment conducted in triplicate, ± standard error of the mean (SEM).

activity of this derivative at pH 6.6 and 7.4 did not increase with the increase in polymer concentration (Figure 7). At pH 7.4 and 6.6, these copolymers are not sufficiently protonated to become membrane disruptive regardless of copolymer concentration.

The composition of the alkylamide PSMA derivatives we describe can be tuned to achieve endosomal release and intracellular delivery. At lower molecular weights, the pentylamine derivatives may be best-suited for intracellular delivery applications, as they are capable of significant membrane disruption at the endosomal pH of 5.8 without destabilization of cell membranes at the physiological pH of 7.4. At these lower molecular weights, the butylamine derivatives are not as attractive for intracellular delivery, due to lower membrane activity. For in vivo delivery applications, copolymers of lower molecular weight (~30 kDa) may be preferred, as they may be excreted via the kidneys. However, higher dosing concentrations of these polymers may be needed due to competition between uptake by target cells and renal excretion. As polymer molecular weight increases, butylamine derivatives become potential

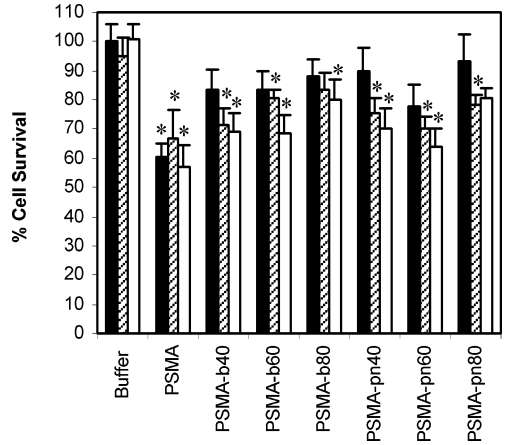

Figure 8. Survival of NIH-3T3 cells when incubated with $PSMA_{63k}$ polymer and its butylamine and pentylamine derivatives, normalized to cell survival incubated in buffer (negative control). The effects of polymers and derivatives were tested at concentrations of 40 (solid bars), 80 (hashed bars), and 160 (white bars) μg/mL. Results show the average of three independent studies, each conducted in triplicate $\pm$ standard error of the mean (SEM). Asterisks denote polymer treatments with statistically significant decreases in cell viability as determined by ANOVA analysis with a 95% confidence interval.

delivery vectors, as they are highly hemolytic at endosomal pH but remain inert at intermediate and physiological pH. At these higher molecular weights, the pentylamine derivatives are also very hemolytic at endosomal pH but have significant hemolytic character at intermediate and physiological pH. It is possible that this could lead to undesirable lysis of cells during circulation. On the other hand, previous work in our group has shown that conjugating a hydrophilic drug molecule, such as an antisense oligonucleotide or siRNA, to a polymer can significantly increase its hydrophilicity and thereby reduce its membrane activity, particularly at pH 7.4 and 6.6.[27,40] Thus, if the therapeutic cargo is extremely hydrophilic, pentylamine derivatives of high molecular weight PSMA copolymers may prove to be useful vectors. Clearly, there will be a need to optimize the various factors in PSMA drug carrier system, including the composition of the alkylamine, the molecular weight and degree of amide modification of the backbone PSMA, the level of drug conjugation, and any other conjugations such as targeting ligands.

3.4. Cytotoxicity Results. The cytoxicity of the 63 kDa PSMA and its butyl and pentylamine derivatives was examined using the LDH assay. The extent of cell survival was determined after 24 h of incubation with copolymer solutions at concentrations of 40, 80, and 160 μg/mL. As a negative control, cells were also treated with solutions of equivalent volume containing no copolymer. The percentage of cell survival is reported relative to untreated cells. Statistical differences in cell survival between copolymer treatment groups and the negative control were examined using one-way ANOVA. Statistically significant differences between polymer treatment groups and the negative controls are denoted with * symbols in Figure 8.

Of the polymers tested, unmodified PSMA copolymers produced the most cytotoxicity relative to the buffer controls. The modified PSMA copolymers typically produced less toxicity than the unmodified PSMA backbone at all three concentrations. At a concentration of 40 μg/mL, none of the butylamine or pentylamine derivatives were statistically toxic compared to the controls, with the single exception of 60% pentylamine derivative. At 80 μg/mL and 160 μg/mL the polymer compositions generally decreased cell survival by relatively small levels compared to untreated controls (Figure 8). The high percentage of cell survival in our experiments with PSMA copolymer derivatives is encouraging given the high polymer concentration and the extended contact time with the cells. The observed low toxicity profile of the PSMA−alkylamine derivates at 40 μg/mL (cell survival rate 82−90%) compares favorably to poly(β-amino esters) and acid-degradable and low molecular weight derivatives of poly(ethylenimine) (PEI).[15,16,44,45] The low toxicity of the PSMA derivatives is also apparent when compared to the toxicity of high molecular weight PEI (>25 kDa), a common and highly toxic polymeric transfection agent.[44−46] These results collectively indicate that PSMA−alkylamine derivatives have low cellular toxicity in cultured cell assays.

4. Conclusions

Cytoplasmic delivery remains a fundamental obstacle to many biomolecular therapies. In this study, we have developed a new class of pH-sensitive, membrane-destabilizing polymers based on poly(styrene-*alt*-maleic anhydride) copolymers. These polymers are intended to function as a platform technology for the delivery of many types of biomolecules. PSMA is readily synthesized from inexpensive monomers and has been previously used clinically as the anti-cancer drug SMANCS. We modified the polymers by reacting the maleic anhydride group with primary alkylamines, to obtain alkylamide/carboxylic acid derivatives capable of membrane disruption at endosomal pHs. By adjusting the degree and type of alkylamine modification, as well as the molecular weight of the PSMA backbone, we have been able to "molecularly engineer" the membrane-disrupting activity of these polymers to act only within specific pH ranges. Anhydride moieties that remain in the polymer backbone after alkylamine modification can be readily used for further functionalization, such as conjugation of cell-targeting ligands. Therapeutic biomolecules could also be conjugated via disulfide bonds, and as an example of this conjugation chemistry, we have bound cysteamine to the anhydride groups with high efficiency. Our results suggest that these new copolymer compositions can be utilized as carriers to enhance the cytoplasmic delivery of a variety of biomolecular drugs.

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