Synthesis and characterization of rapidly-degrading polyanhydrides as vaccine adjuvants

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Abstract
There is a currently a need to develop adjuvants that are best suited to simultaneously enhance immune responses, induce immunologic memory, improve patient compliance (i.e., reduce doses and inflammation), and provide vaccine shelf stability for stockpiling and global deployment to challenging environments. Biodegradable polyanhydrides have been investigated extensively to overcome such challenges. It has been shown that controlling copolymer composition can result in chemistry-dependent immunomodulatory capabilities. These studies have revealed that copolymers rich in sebacic acid (SA) are highly internalized by antigen presenting cells and confer improved shelf stability of encapsulated proteins, while copolymers rich in 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) also exhibit enhanced internalization by and activation of antigen presenting cells (APCs), in addition to providing superior retention of protein stability following encapsulation and release. However, to date CPTEG:SA copolymers have not been synthesized and described. In this work, we hypothesized that new copolymers composed of CPTEG and SA would combine the advantages of both monomers in terms of enhanced thermal properties, maintaining antigenicity of encapsulated proteins following nanoparticle synthesis, and superior cellular internalization and activation by APCs, demonstrated by the upregulation of costimulatory markers CD80, CD86, and CD40, as well as the secretion of proinflammatory cytokines IL-6, IL-1β, and TNF-α. Herein, we describe the synthesis and design of novel CPTEG:SA nanoparticles with improved thermal properties, payload stability, and internalization by antigen presenting cells for applications in vaccine delivery. The performance of these new CPTEG:SA formulations was compared to that of traditional polyanhydride copolymers.

Keywords
polyanhydride, nanovaccine, rational design, vaccine

Disciplines
Biochemical and Biomolecular Engineering | Biology and Biomimetic Materials | Nanoscience and Nanotechnology | Pharmacology, Toxicology and Environmental Health | Polymer and Organic Materials

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Synthesis and characterization of rapidly-degrading polyanhydrides as vaccine adjuvants

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Keywords: polyanhydride; nanovaccine; rational design; vaccine;
ABSTRACT

There is a currently a need to develop adjuvants that are best suited to simultaneously enhance immune responses, induce immunologic memory, improve patient compliance (i.e., reduce doses and inflammation), and provide vaccine shelf stability for stockpiling and global deployment to challenging environments. Biodegradable polyanhydrides have been investigated extensively to overcome such challenges. It has been shown that controlling copolymer composition can result in chemistry-dependent immunomodulatory capabilities. These studies have revealed that copolymers rich in sebacic acid (SA) are highly internalized by antigen presenting cells and confer improved shelf stability of encapsulated proteins, while copolymers rich in 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) also exhibit enhanced internalization by and activation of antigen presenting cells (APCs), in addition to providing superior retention of protein stability following encapsulation and release. However, to date CPTEG:SA copolymers have not been synthesized and described. In this work, we hypothesized that new copolymers composed of CPTEG and SA would combine the advantages of both monomers in terms of enhanced thermal properties, maintaining antigenicity of encapsulated proteins following nanoparticle synthesis, and superior cellular internalization and activation by APCs, demonstrated by the upregulation of costimulatory markers CD80, CD86, and CD40, as well as the secretion of proinflammatory cytokines IL-6, IL-1β, and TNF-α. Herein, we describe the synthesis and design of novel CPTEG:SA nanoparticles with improved thermal properties, payload stability, and internalization by antigen presenting cells for applications in vaccine delivery. The performance of these new CPTEG:SA formulations was compared to that of traditional polyanhydride copolymers.
INTRODUCTION

There are many challenges associated with developing vaccination strategies to prevent pathogens from establishing infection in the host. With the advent of recombinant subunit protein-based vaccines, the need has increased to develop adjuvants that are best suited to enhance immune responses, induce immunologic memory, improve patient compliance (i.e., reduce doses and inflammation), and provide vaccine shelf stability for stockpiling and deployment to harsh environments. While traditional adjuvants enhance immune responses, some of them (e.g., MPLA, aluminum-based salts) induce inflammation and adverse reactions at the injection site and must be refrigerated for extended storage, limiting their use for long term storage or in harsh environments.

Nanoparticle-based technologies have long been investigated as a delivery vehicle/adjuvant system for vaccines. It has been shown that nanoparticle characteristics, such as size, shape, charge, and chemistry can be used to control cellular uptake, and ligation of the polymer can aid in targeting draining lymph nodes, thus enabling targeted delivery of the payload. These beneficial properties have enabled the design of nanoparticle-based systems that are being widely investigated as vaccine adjuvant platforms against infectious disease and for targeted delivery of tumor antigens and immune therapies against cancer.

Biodegradable polymer-based nanoparticle systems are promising for vaccine delivery due to their facile synthesis, ability to protect encapsulated proteins from enzymatic degradation, and erosion-controlled sustained release or pulsatile release mechanisms. Poly(lactic-co-glycolic acid) (PLGA) is the most widely investigated polymer in this category and is FDA-approved for human use, showing promise for vaccine delivery. However, PLGA exhibits bulk erosion...
which, along with the relatively high acidity of its degradation products (pKa glycolic acid: 3.83\textsuperscript{25}; lactic acid: 3.08\textsuperscript{26}), can cause denaturation of encapsulated proteins\textsuperscript{27}.

Biodegradable polyanhydrides have also been investigated extensively to overcome such challenges and meet the abovementioned vaccine requirements\textsuperscript{28}. Comprised of 1,6-bis(\textit{p}-carboxyphenoxy) hexane (CPH), 1,8-bis(\textit{p}-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), and sebacic acid (SA), these materials are safe, biodegradable, exhibit mild inflammation, and are currently FDA-approved to treat malignant glioblastomas\textsuperscript{29–31}. Controlling copolymer composition has been shown to enhance cellular uptake\textsuperscript{32,33}, ultimately resulting in chemistry-dependent immunomodulatory capabilities\textsuperscript{34,35}. These materials represent an efficacious vaccine adjuvant platform, as evidenced by sustained antibody responses to numerous antigens\textsuperscript{10,11,15,16,36}, enhanced germinal center formation\textsuperscript{37}, increased cytotoxic T cell responses\textsuperscript{38}, and protection against multiple bacterial and viral infections\textsuperscript{10,13,17}.

Previous work has shown that SA-rich chemistries are highly internalized by antigen presenting cells\textsuperscript{32} (APCs) and confer improved shelf stability of encapsulated proteins\textsuperscript{39}. CPTEG-rich chemistries also exhibit enhanced internalization by and activation of APCs\textsuperscript{32}, in addition to providing superior retention of protein stability following encapsulation and release\textsuperscript{39}. In this work, we hypothesized that new copolymers composed of CPTEG and SA would combine the advantages of both monomers in terms of enhanced thermal properties, maintaining antigenicity of encapsulated proteins following nanoparticle synthesis, and superior activation of APCs. Here, we describe the synthesis and design of novel CPTEG:SA nanoparticles with improved thermal properties, payload stability, and APC activation for applications in vaccine delivery. The performance of these novel nanoparticles was compared to that of the more commonly used 20:80 CPTEG:CPH and 20:80 CPH:SA nanoparticles.
MATERIALS AND METHODS

Materials

Sebacic acid (99%), bovine serum albumin, and chemicals used for CPTEG and CPH diacid and polymer synthesis, including 1,6-dibromohexane, triethylene 4-<i>p</i>-hydroxybenzoic acid, and 1-methyl-2-pyrrolidinone were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform, petroleum ether, ethyl ether, hexanes, sodium hydroxide, toluene, sulfuric acid, acetonitrile, dimethyl formamide, acetic anhydride, methylene chloride, pentane, and potassium carbonate were purchased from Fisher Scientific (Fairlawn, NJ). 4-<i>p</i>-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK). Deuterated chloroform used for <sup>1</sup>H NMR analysis was purchased from Cambridge Isotope Laboratories (Andover, MA). RAW 264.7 and J774 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and were used for assays.

Animals

Female BALB/c mice (6–7-week-old) were purchased from Charles River Laboratories (Wilmington, MA). The Institutional Animal Care and Use Committee (IACUC) at Iowa State University approved all protocols involving animals.

Copolymer Synthesis and Characterization

CPTEG and CPH diacids were synthesized as previously described<sup>35,40</sup>. CPTEG:CPH and CPH:SA copolymer synthesis was performed using melt polycondensation<sup>40</sup>. For synthesis of the novel CPTEG:SA copolymer, 2 g of total monomer (the mass of each monomer varied depending on copolymer composition) and 90 mL of acetic anhydride were added to a 100 mL round bottom flask and reacted for 30 min at 125 °C in an oil bath. The acetic anhydride was removed using a rotary evaporator, and the dried product was then reacted for 1 h at 140 °C in an
oil bath under vacuum (0.2 torr). The solid product was dissolved in approximately 20 mL of methylene chloride overnight and precipitated in 1 L hexanes to isolate the copolymer. At least three batches of each copolymer chemistry were synthesized in this work. Copolymer composition and molecular weight of each batch of copolymer synthesized were estimated using end group analysis of $^1$H NMR (DXR 500, Bruker) spectra. Copolymer thermal properties (glass transition temperature and melting point) were measured using differential scanning calorimetry (Q2000, TA Instruments, New Castle, DE), using a heating rate of 10 °C/min. The relative crystallinity of CPTEG:SA copolymers was determined using wide angle X-ray diffraction (Siemens D500, Siemens/Bruker).

The number average sequence length, degree of randomness, and reactivity ratios of CPTEG:SA copolymers was determined from $^1$H NMR spectra, using methods described previously$^{41}$. Films were prepared for contact angle measurements by dissolving copolymer in methylene chloride at 50 mg/mL, 300 μL was pipetted on a 6 cm glass slide, heated to 80 °C for 10 s and spin coated (WS-650Mz-23NPPB, Laurell Technologies, North Wales, UK) for 30 s at 2000 rpm with a 1500 rpm/s acceleration rate. An average of eight contact angle measurements of each chemistry of CPTEG:SA films was performed using a goniometer (NRL 100, ramé-hart, Succasunna, NJ) with 2 μL droplets of nanopure water.

**Erosion Kinetics**

Films were prepared by dissolving copolymers in methylene chloride at a concentration of 150 mg/mL and allowed to dry overnight. The scintillation vials containing dried copolymer were then heated to their respective melting points for one minute and then allowed to cool to room temperature. 15 mL of nanopure water was pipetted into each vial and 15 mL was removed and replaced daily over 45 days of study. Samples were lyophilized, the dry mass was weighed, and
the cumulative mass was determined over the course of the study. In separate vials, samples of
the film were collected on days 15, 30, and 45, the film samples lyophilized, dissolved in
deuterated DMSO, and characterized for copolymer composition by $^1$H NMR.

Nanoparticle Synthesis and Characterization

For protein stability characterization, CPTEG:SA nanoparticles, either empty or encapsulating
the model antigen ovalbumin (OVA, at 5% w/w), were synthesized via flash nanoprecipitation,
as previously described$^{15}$. For release kinetics experiments, CPTEG:SA nanoparticles
encapsulating the model protein bovine serum albumin (BSA, at 2% w/w) were spray dried using
a Büchi B90 HP spray dryer (Büchi, Flawil, Switzerland). Briefly, for spray dried nanoparticle
synthesis, polymer was dissolved in chloroform at 10 mg/mL with 5 mM sodium dioctyl
sulfosuccinate as an emulsifier. BSA was dissolved in nanopure water at 4 mg/mL, pipetted at a
1:20 (water:solvent) ratio into the solvent, and emulsified with the polymer via sonication for 30
s. The emulsion was spray dried at room temperature (pump rate 10%, spray rate 100%, gas flow
rate 100 L/min, inlet temperature ~30 ºC). CPTEG:SA nanoparticles were imaged using
scanning election microscopy (SEM; JEOL 840 A, JEOL Ltd., Tokyo, Japan), and nanoparticle
mean size and size distribution were determined using ImageJ (National Institutes of Health,
Bethesda, MD). Nanoparticle zeta potential was measured using Zetasizer Nano (Malvern
Instruments, Worcester, UK).

Protein Characterization

For characterization of OVA released from 5% (w/w) CPTEG:SA nanoparticles, 5 mg of each
nanoparticle formulation was suspended in 250 µL of nanopure water, sonicated for
approximately 15 seconds, and the samples were allowed to release overnight (~18 h), after
which the samples were centrifuged at 15,000 rcf for 5 min and 200 µL supernatant was
removed. The protein released was quantified via micro bichinchoninic acid assay, and the samples were diluted to 40 µg/mL for Native PAGE gel analysis (see below). To determine changes in the tertiary structure of the protein, the samples (40 µg/mL) were excited at a wavelength of 280 nm and the emission was measured over the range of 300 – 450 nm using a SpectraMax M3 fluorescent spectrometer (Molecular Devices, Sunnyvale, CA). To determine changes in the secondary structure of the protein, circular dichroism spectra were acquired using a Jasco J-715 spectrophotometer (Jasco Analytical Instruments, Easton, MD) of the samples (20 µg/mL) over a range of 195 – 260 nm.

**Native PAGE Gel Analysis**

Native PAGE gel analysis was performed using release supernatants from flash nanoprecipitated 5% OVA-loaded CPTEG:SA nanoparticles. Sample concentrations were adjusted to 20 µg/mL, then diluted with an equal volume of native sample buffer (Bio-Rad, catalog # 161-0738), and 25 µL of each diluted sample was added to the respective wells of a polyacrylamide gel (4-20% MP TGX Gel 10W 30 µL, Bio-Rad, catalog #4561093). The gel was subjected to 150 V for 60 min using 25 mM Tris, 192 mM glycine running buffer, placed in fixative solution (40% (v/v) ethanol, 10% (v/v) acetic acid) for three hours, and then stained overnight using flamingo fluorescent gel stain (Bio-Rad, catalog # 161-0491). The stained gel was imaged using a Typhoon 9400 flatbed scanner (GE Healthcare, Pittsburgh, PA).

**ELISA**

ELISA analysis of OVA released from CPTEG:SA nanoparticles was performed as previously described\(^\text{10}\). Briefly, high-binding Costar 590 EIA/RIA microtiter plates (Corning) were coated overnight with 100 µL of a 0.5 µg/mL solution of OVA released from the nanoparticles at 4 °C. The microtiter plates were then dumped and blocked for two hours with 2.5% (w/v) powdered
skim milk dissolved in PBS-Tween with 0.05% Tween 20, pH 7.4, that had been incubated for two hours at 56 °C to inactivate any endogenous phosphatase activity. Following block, microtiter plates were washed thrice with PBS-T. Pooled serum obtained from OVA-immunized mice was added at a dilution of 1:200 and serially diluted in PBS-T containing 1% (v/v) goat serum. Each sample was tested in duplicate. Following incubation overnight at 4°C, plates were washed thrice with PBS-T, after which the secondary antibody alkaline phosphatase-conjugated goat anti-mouse IgG heavy and light chain (Jackson ImmunoResearch) was added at a dilution of 1 µg/mL. Plates were incubated for two hours at room temperature and then washed thrice with PBS-T. 100 µL of alkaline phosphatase substrate (Fisher Scientific, Pittsburgh, PA) was added to each well at a concentration of 1 mg/mL dissolved in 50 mM sodium carbonate, 2 mM magnesium chloride buffer at pH 9.3 for colorimetric development. Plates were analyzed after 30 min using a SpectraMax M3 microplate reader at a wavelength of 405 nm. Titer is reported as the reciprocal of serum dilution at which the optical density (OD) value was at most 0.2, a conservative endpoint greater than the average OD of saline-mouse serum, at a 1:200 dilution, plus two standard deviations.

Protein Release Kinetics

For protein release characterization, approximately 5 mg of 2% (w/w) BSA-loaded CPTEG:SA nanoparticles was suspended in 250 µL of PBS, sonicated for 30 s, and placed on a shaker incubator at 37 °C. 200 µL were removed and replaced with fresh PBS at indicated time points, and released protein was measured via micro bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). Following 30 days of release, the buffer was changed to 40 mM sodium hydroxide to catalyze the release of any remaining protein and this information was utilized to determine the encapsulation efficiency, which was calculated as the total amount of protein
released divided by the theoretical amount of protein used for nanoparticle synthesis. The encapsulation efficiency of BSA in the various CPTEG:SA nanoparticle formulations ranged between 33-40%.

**Differentiation of Bone Marrow-derived Dendritic Cells (BMDCs)**

Bone marrow was harvested from femurs and tibia of BALB/c mice and differentiated into dendritic cells using a protocol previously described. Briefly, bone marrow isolates were harvested by flushing the tibia or femur with 10% FBS RPMI. Cells were counted and subsequently plated on petri dishes at 4 x 10^6 cells/plate in 10 mL media. 10 ng/mL of granulocyte macrophage colony stimulating factor (GM-CSF) was added to the plates on day 0 and fresh GM-CSF and media was added on day 3. On days 6 and 8 of culture, 10 mL of media was replaced with fresh GM-CSF and media, and the cells harvested on day 10 for nanoparticle stimulation assays.

**Biocompatibility and Cellular Internalization of CPTEG:SA Nanoparticles**

For biocompatibility assays, the murine RAW 264.7 monocyte/macrophage cell line was seeded in flat bottom 96 well tissue culture plates at a density of 100,000 cells/well (100 µL) and allowed to adhere overnight. The following morning, 100 µL was added to the wells containing empty CPTEG:SA nanoparticles at concentrations ranging from 500 µg/mL serially diluted two-fold down to 7.8 µg/mL for 24 hours, after which 25 µL of MTT reagent (2.5 mg/mL) was added to the wells and allowed to react for two hours. The entire volume was carefully pipetted off and 300 µL DMSO was added to the wells and absorbance measurements were made at 540 nm, using 690 nm as a background subtraction. The cell viability was calculated as the background corrected absorbance of the samples divided by the background corrected absorbance of unstimulated cells.
For cell internalization, the murine J774 monocyte/macrophage cell line, human THP-1 cell line, and murine BMDCs were seeded in flat bottom 24 well tissue culture plates at cell density of 500,000 cells/well (500 µL cell culture media) and allowed to adhere overnight. The following morning, 500 µL culture media was added to the wells containing CPTEG:SA nanoparticles encapsulating CdxSe1-x/ZnScore/shell fluorescent nanocrystals (1% w/w; excitation 405 nm; emission 450 nm; Cytodiagnostics, Burlington, Ontario, Canada) at a concentration of 125 µg/mL for 2.5 h, after which the cells were scraped, fixed, and the samples assessed for nanoparticle-positive cells via flow cytometry. In order to properly differentiate nanoparticles and nanocrystals from cells, control tubes were also analyzed containing solely the nanocrystal-loaded nanoparticles in addition to a tube containing nanocrystals alone suspended in buffer.

**APC Activation by CPTEG:SA Nanoparticles**

For cell activation studies, RAW 264.7 cells were seeded in flat bottom 24 well tissue culture plates at a cell density of 500,000 cells/well (500 µL cell culture media) and allowed to adhere overnight. The following morning, 500 µL cell culture media was added to the wells containing CPTEG:SA nanoparticles at a final concentration of 62.5 µg/mL for 24 h, after which 500 µL of cell culture supernatant was collected to evaluate the levels of cytokine secretions using a BioRad BioPlex 200 system (Hercules, CA). The cytokines IL-6, IL-1β, IL-17, TNF-α, IL-12, IL-10, IFN-γ, IL-5, IL-2, and IL-4 were assayed for.

The cells were scraped and transferred to polystyrene FACS tubes. Prior to labeling the cells, the Fc receptors were blocked with 1 mg/mL rat IgG (Sigma-Aldrich, St. Louis, MO) and 10 µg/mL anti-CD16/32 (eBioscience). Following 10 min of block, the cells were stained with fluorescently conjugated antibodies for CD80 (Biolegend, PerCP-Cy5.5, clone 16-10A1), CD86 (eBioscience, FITC, clone GL1), and CD40 (eBioscience, APC, clone 1C10) diluted at a 1:100
v/v in FACS buffer. Following 30 min of staining, the cells were washed in FACS buffer to remove excess dye and fixed using BD stabilizing fixative (BD Bioscience, Franklin Lakes, NJ). Flow cytometry analysis was performed using FlowJo (FlowJo, LLC, Ashland, OR) following data collection on a FACSCanto II (BD Bioscience, Franklin Lakes, NJ).

RESULTS

CPTEG:SA copolymers were synthesized with the following criteria in mind: 1) thermal properties of the copolymers must be above body temperature (37 °C) to maintain discreteness of the nanoparticles and encapsulated protein stability at in vivo temperatures, 2) synthesized nanoparticles must be discrete to maintain suspension quality for ease of administration, and 3) nanoparticles must maintain antigenicity of protein during encapsulation and release. Once these criteria were met, the nanoparticles were evaluated for their biocompatibility and APC internalization and cellular activation.

CPTEG:SA copolymer structural characterization

CPTEG:SA copolymers were synthesized by varying CPTEG molar composition from 0 – 25 mol%. Batches of the CPTEG:SA copolymers in molecular weight ranges of 10,000-20,000 Da resulted in an oily substance (data not shown) that was unsuitable for further processing; however, increasing the reaction time to maintain the molecular weight of each batch of CPTEG:SA copolymer between 20,000-30,000 Da resulted in a solid mass for all copolymers synthesized (Table 1). It should be noted that CPTEG:SA copolymers with >25 mol% CPTEG were not studied further because the copolymers were less solid, sticky, and failed to result in discrete nanoparticles (data not shown).

<table>
<thead>
<tr>
<th>Table 1. CPTEG:SA copolymer characterization</th>
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<tr>
<td>Chemistry</td>
</tr>
<tr>
<td>poly(SA)</td>
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1H NMR spectra of these copolymers revealed characteristic peaks associated with CPTEG and SA, with aromatic CPTEG proton peaks located in the δ = 6.8-8.1 ppm range, and inner chain proton peaks located between 3.6-4.4 ppm (Figure 1A). Characteristic SA proton peaks were located between 1.35-2.65 ppm. As expected, increasing the molar ratio of CPTEG in the copolymer backbone resulted in an increase in the proton peaks associated with CPTEG and the copolymer composition matched that of the feed composition, consistent with previous studies with CPH:SA and CPTEG:CPH copolymers. Further analysis of 1H NMR spectra of CPTEG:SA copolymers revealed a decrease in average sequence length with increasing CPTEG content, in line with previous reports for other polyanhydride copolymers (Figure 1B). All copolymers studied exhibited alternating or random copolymer formation, evidenced by degree of randomness values of approximately 2.0 (Table 1). This was further confirmed by calculating the reactivity ratios r₁ and r₂, which were determined to be r₁ = 1.0 – 1.8 and r₂ = 1.0 – 1.8 using the Mayo-Lewis method, or r₁ = 1.46 and r₂ = 1.01 using the Fineman-Ross method, indicating that either monomer can be added to the copolymer backbone at comparable rates, as is the case for random copolymers.
Figure 1. Characterization of CPTEG:SA copolymers using $^1$H NMR spectroscopy. (A) Molecular structure of CPTEG:SA copolymer. (B) $^1$H NMR spectra of CPTEG:SA copolymers. (C) Description of hydrogens in copolymer backbone for $^1$H NMR spectra. (D) Number average sequence lengths of repeating CPTEG or SA units determined from $^1$H NMR spectra.

Thermal analysis of the copolymers demonstrated increased melting temperatures with increasing SA molar composition, ranging from 52 °C for 25:75 CPTEG:SA to 78 °C for poly(SA) (Figure 2A and Table 1). A glass transition temperature was not detected in any of the copolymer chemistries in the temperature ranges studied. Wide angle X ray diffraction analysis of powders revealed semi-crystalline properties of all copolymers analyzed, with an increase in relative crystallinity with increasing SA molar composition, ranging from 26% for 25:75 CPTEG:SA to 59% for poly(SA) (Figure 2B and Table 1). The relative crystallinity of 10:90 CPTEG:SA closely matched that of the 20:80 CPH:SA copolymer control, whose relative crystallinity has previously been reported to be ca. 47%. 20:80 CPTEG:CPH is an amorphous
polymer and therefore does not contain any crystals. Therefore, CPTEG:SA copolymers demonstrate characteristic properties consistent with those previously observed in other polyanhydride copolymers and the investigated copolymer chemistries exhibited desirable thermal properties that may be useful for vaccine stability in vivo and long-term shelf storage.

Figure 2. Thermal properties and crystallinity of CPTEG:SA copolymers using DSC and WAXD. (A) Heat flow vs. temperature plot of CPTEG:SA copolymer powders measured with differential scanning calorimetry (DSC). The melting point was determined by the minima of the endotherm of each chemistry. (B) Wide angle X ray diffraction (WAXD) spectrogram of CPTEG:SA copolymer powders.

Erosion Kinetics of Polyanhydride Films

To assess the relative erosion kinetics of CPTEG:SA copolymers compared to traditional polyanhydride copolymers, copolymer films were prepared for contact angle measurements to
determine their hydrophobicity, followed by characterization of their erosion kinetics. Interestingly, contact angle measurements of CPTEG:SA films of various copolymer compositions revealed that hydrophobicities were relatively similar across copolymer composition, with 10:90 CPTEG:SA having the highest contact angle (76°) and 80:20 CPTEG:SA having the lowest (57°) (Figure 3A). The erosion kinetics data demonstrated a more rapid erosion profile for all CPTEG:SA copolymers studied compared to that of 20:80 CPTEG:CPH and 20:80 CPH:SA films (Figure 3B). The erosion rate of CPTEG:SA films increased with increasing SA molar composition (approximately 150-200 mg eroded over 45 days), compared to 20:80 CPTEG:CPH and 20:80 CPH:SA films, which had approximately 100 and 50 mg eroded over 45 days, respectively. This trend was also observed with the release of BSA from spray dried 2% (w/w) CPTEG:SA nanoparticles, in which increasing SA content resulted in a more rapid release of encapsulated protein (Figure 3C). ¹H NMR analysis of copolymer film samples performed at 15, 30, and 45 days revealed a decrease in SA composition in the films over time, indicating that SA-SA bonds are more water labile than CPTEG-CPTEG or CPTEG-SA bonds (Table 2). Therefore, CPTEG:SA copolymers exhibit rapid erosion rates compared to conventional 20:80 CPTEG:CPH and 20:80 CPH:SA copolymers and the rate of erosion can be tuned by modulating copolymer composition.
Figure 3. CPTEG:SA copolymers demonstrate chemistry-dependent erosion kinetics. (A) Contact angle measurements of CPTEG:SA copolymer films prepared via spin coating on glass slides. (B) Mass of CPTEG:SA copolymer films eroded as a function of time. 15 mL supernatants were collected daily and the entire volume was replaced with fresh nanopure water. (C) Cumulative mass % released from spray dried 2% (w/w) BSA-loaded CPTEG:SA nanoparticles. Statistical significance for contact angle measurements was determined via an ordinary one-way ANOVA with a Tukey’s multiple comparison test. Differences in letters above each copolymer chemistry indicate significant differences (p ≤ 0.05) in contact angle measurements between the various copolymers.

Table 2. CPTEG:SA copolymer film composition over time

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Time point</th>
<th>Mole Fraction CPTEG</th>
<th>Mole Fraction SA</th>
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<tbody>
<tr>
<td>5:95 CPTEG:SA</td>
<td>Day 15</td>
<td>0.05</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Day 30</td>
<td>0.06</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Day 45</td>
<td>0.06</td>
<td>0.94</td>
</tr>
<tr>
<td>15:85 CPTEG:SA</td>
<td>Day 15</td>
<td>0.28</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Day 30</td>
<td>0.28</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Day 45</td>
<td>0.38</td>
<td>0.62</td>
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Nanoparticles based on CPTEG:SA copolymers were synthesized in order to assess the size, discreteness, and ability to encapsulate and release antigenically stable protein (hen egg ovalbumin was used as a model antigen in these studies). Empty particles synthesized using flash nanoprecipitation method were discrete, with sizes ranging from 575 – 1054 nm, and with narrow size distributions (Figure 4 and Table 3). These diameters are slightly larger than those typically reported for CPTEG:CPH (~200 nm) and CPH:SA (~400 nm) nanoparticles\textsuperscript{11,46}. Zeta potential measurements of the nanoparticles showed zeta potentials ranging from -28.7 to -35.3 mV, consistent with previous reports for other polyanhydride nanoparticles (Table 3)\textsuperscript{11}.

CPTEG:SA nanoparticles were investigated for their ability to encapsulate and release stable antigen. Native PAGE gel analysis of hen egg ovalbumin (OVA) released from 5% (w/w) OVA-loaded CPTEG:SA nanoparticles demonstrated that all CPTEG:SA chemistries maintained the native conformational stability of the encapsulated antigen upon release (Figure 5A). Further analysis of OVA using circular dichroism revealed that the secondary structure of the released antigen was preserved (Figure 5B). In addition, fluorescence spectroscopy analysis of OVA revealed that the tertiary structure of released antigen was maintained (Figure 5C). Interestingly, there was a trend in increasing fluorescence signal from samples with higher CPTEG molar composition, matching that of the OVA standard. These studies indicated that CPTEG:SA nanoparticles can be synthesized into discrete nanoparticles that maintain protein stability following encapsulation and release.
Figure 4. CPTEG:SA nanoparticle characterization. SEM images of empty CPTEG:SA nanoparticles synthesized via flash nanoprecipitation. Briefly, copolymer was dissolved in methylene chloride at a concentration of 20 mg/mL. The solution was poured into a bath of pentane at a 1:250 volumetric ratio (methylene chloride: pentane), and the resulting suspension was vacuum filtered to collect the nanoparticles. The mean diameter of the nanoparticles were as follows: poly(SA): 655.6 ± 197.3 nm; 5:95 CPTEG:SA: 605.7 ± 284.2 nm; 10:90 CPTEG:SA: 575.3 ± 252.0 nm; 15:85 CPTEG:SA: 619.6 ± 279.5 nm; 20:80 CPTEG:SA: 1054.3 ± 477.2 nm; 25:75 CPTEG:SA: 798.5 ± 292.7 nm.
Figure 5. CPTEG:SA nanoparticles maintain protein conformational stability following encapsulation and release. (A) Native PAGE gel analysis of OVA released from 5% (w/w) OVA-loaded CPTEG:SA nanoparticles. Released OVA concentration was measured via microBCA and diluted to 40 µg/mL for analysis. (B) Circular dichroism spectra of OVA released from 5% (w/w) OVA-loaded CPTEG:SA nanoparticles. Released OVA concentration was measured via microBCA and diluted to 20 µg/mL for analysis. (C) Fluorescence spectra of OVA released from 5% (w/w) OVA-loaded CPTEG:SA nanoparticles. Released OVA concentration was measured via microBCA and diluted to 40 µg/mL for analysis. Samples were excited at 280 nm and fluorescence was measured from 300-450 nm.

Table 3. Characterization of CPTEG:SA nanoparticles

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Diameter (nm)</th>
<th>Polydispersity Index</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(SA)</td>
<td>655.6 ± 197.3</td>
<td>0.09</td>
<td>-31.7 ± 6.7</td>
</tr>
<tr>
<td>5:95 CPTEG:SA</td>
<td>605.7 ± 284.2</td>
<td>0.22</td>
<td>-33 ± 9.3</td>
</tr>
<tr>
<td>10:90 CPTEG:SA</td>
<td>575.3 ± 252.0</td>
<td>0.19</td>
<td>-33.7 ± 8.9</td>
</tr>
<tr>
<td>15:85 CPTEG:SA</td>
<td>619.6 ± 279.5</td>
<td>0.20</td>
<td>-35.3 ± 9.0</td>
</tr>
<tr>
<td>20:80 CPTEG:SA</td>
<td>1054.3 ± 477.2</td>
<td>0.20</td>
<td>-29.6 ± 12.7</td>
</tr>
<tr>
<td>25:75 CPTEG:SA</td>
<td>798.5 ± 292.7</td>
<td>0.13</td>
<td>-28.7 ± 8.8</td>
</tr>
</tbody>
</table>
CPTEG:SA Nanoparticles Are Biocompatible and Enhance Internalization and Activation of APCs

CPTEG:SA nanoparticles were evaluated for their biocompatibility as well as degree of internalization by APCs. An MTT assay performed on CPTEG:SA nanoparticles incubated with RAW 264.7 cells demonstrated that cells were >75% viable when incubated with nanoparticle concentrations of up to 62.5 µg/mL (Figure 6A). Interestingly, there was a trend in increasing cell viability with increasing CPTEG molar composition for concentrations >62.5 µg/mL. In comparison, both 20:80 CPTEG:CPH and 20:80 CPH:SA nanoparticles demonstrated similar biocompatibility, however these particles maintained higher cell viability at concentrations >62.5 µg/mL.

When CPTEG:SA nanoparticles were incubated with three different APC types, murine J774 monocyte/macrophage cell line, human THP-1 monocyte/macrophage cell line, and murine BMDCs, the data showed a high degree of internalization of these particles by all three cell types. Formulations rich in SA displayed the highest degree of uptake across all three cell types, with a trend of increasing rates of internalization with increasing SA molar composition (Figure 6B). Interestingly, depending on the cell type, 5:95 CPTEG:SA, 10:90 CPTEG:SA, and 15:85 CPTEG:SA copolymers had rates of internalization greater than or equal to that of poly(SA) and the 20:80 CPH:SA controls, while 20:80 CPTEG:SA nanoparticles were internalized to the same degree of as poly(SA) in J774 and THP-1 cells, but not in BMDCs. The 25:75 CPTEG:SA nanoparticles had the lowest degree of internalization, significantly lower than that of all other CPTEG:SA formulations, with rates of internalization similar to that of the 20:80 CPTEG:CPH control. These results demonstrate the biocompatibility of CPTEG:SA nanoparticles and show that copolymer chemistries with molar composition ≤ 20 mol% CPTEG are highly internalized.
by APCs, with certain chemistries having enhanced internalization rates compared to poly(SA) and traditional polyanhydride nanoparticles.

Figure 6. CPTEG:SA nanoparticles are biocompatible and are internalized by antigen presenting cells. (A) MTT assay of CPTEG:SA nanoparticles incubated with the murine RAW 264.7 monocyte/macrophage cell line. Cells were incubated with varying concentrations of the empty nanoparticles for 24 h, after which MTT reagent was added to the wells to determine cell viability. The results shown are the average of two independent experiments for all chemistries except 20:80 CPH:SA, which is demonstrative of one independent experiment. (B) Cell internalization assay of CPTEG:SA nanoparticles incubated with the murine J774 monocyte/macrophage cell line, human THP-1 monocyte/macrophage cell line, and murine bone-marrow-derived dendritic cells. CPTEG:SA nanoparticles encapsulating fluorescent nanocrystals (1% w/w) were incubated with cells for 2.5 h at 125 µg/mL and flow cytometry analysis was performed to determine the percentage of nanoparticle-positive cells. Statistical significance was determined via an ordinary one-way ANOVA with a Tukey’s multiple comparison test. Differences in letters above each copolymer chemistry indicate significant differences (p ≤ 0.05) between the various treatment groups.
As the CPTEG:SA nanoparticles were capable of being highly internalized by three various antigen presenting cell types, we next investigated their ability to activate RAW 264.7 cells in vitro. Following a 24 h stimulation of cells with CPTEG:SA nanoparticles, it was observed that all copolymer chemistries induced activation of RAW 264.7 cells, as evidenced by upregulation of the costimulatory cell surface markers CD40, CD80, and CD86 (Figure 7A). The levels of expression of these markers followed a similar trend to that observed with the internalization data (Figure 6B), where CPTEG:SA copolymers with 5-20% molar composition of CPTEG had greater than or equal levels of expression of these surface markers compared to poly(SA), 20:80 CPTEG:CPH, and 20:80 CPH:SA nanoparticles. In addition, following 24 h stimulation, the proinflammatory cytokines TNF-α, IL-6, and IL-1β were detected in the cell culture supernatants, further indicating that these nanoparticle formulations are able to activate APCs. A similar trend to both internalization and costimulatory molecule expression was observed, with decreasing cytokine secretion levels detected from cells stimulated with nanoparticles of increasing CPTEG molar composition (Figure 7B). Overall, CPTEG:SA nanoparticles with 5-20% CPTEG molar composition were able to induce secretion levels of all three cytokines greater than or equal to that of poly(SA) and the traditional polyanhydride nanoparticle controls. Interestingly, 5:95 CPTEG:SA nanoparticles were best able to induce IL-6 and IL-1β expression compared to poly(SA), as well as 20:80 CPTEG:CPH and 20:80 CPH:SA nanoparticles, which correlates with the internalization data presented in Figure 6B. We did not detect levels of secreted cytokines IFN-γ, IL-17, IL-12, IL-5, IL-2, IL-10, or IL-4. Overall, these data indicate the ability of CPTEG:SA nanoparticles to be internalized by and activate APCs, resulting in the expression of proinflammatory cytokines that can enhance immune responses.
Figure 7. CPTEG:SA nanoparticles activate antigen presenting cells and induce proinflammatory cytokine expression. RAW 264.7 cells were seeded at density of 500,000 cells/well in a 24 well cell culture plate and stimulated with CPTEG:SA nanoparticles at a concentration of 62.5 µg/mL for 24 h. Lipopolysaccharide (LPS) was used as a positive control at a concentration of 1 µg/mL. (A) Mean fluorescence intensity (MFI) of cell surface markers following nanoparticle stimulation for 24 h, determined via flow cytometry. (B) Concentration of cytokines detected in cell culture supernatants following 24 h stimulation. Data are presented as a mean with standard deviation. Statistical significance was determined via an ordinary one-way ANOVA with a Tukey’s multiple comparison test. Differences in letters above each copolymer chemistry indicate significant differences (p ≤ 0.05) between the various treatment groups.

DISCUSSION

There is a growing body of literature on the efficacy of nanoparticles for protein therapeutics as well as novel adjuvants that enhance immune responses to vaccines, in particular subunit vaccines. Polymeric nanoparticles are a promising class of carriers/adjuvants due to their facile synthesis and their ability to stabilize encapsulated payloads, ligate the polymer with various targeting ligands for targeted delivery, and tune release kinetics through
copolymerization. Polyanhydrides have been investigated as a protein and vaccine delivery platform due to their surface erosion characteristics, which enhances stability of encapsulated payloads\textsuperscript{39,51–53}. In addition, their degradation products are less acidic than that of PLGA, with pK\textsubscript{as} of 5.8 & 8.4 (CPTEG)\textsuperscript{40}, 3.7 & 6.7 (CPH)\textsuperscript{54}, and 4.8 & 5.6 (SA)\textsuperscript{54}. In this work, a new class of polyanhydride copolymers comprising of CPTEG and SA were synthesized in order to confer enhanced thermal properties for long term storage, ease of administration (discrete particles that suspend well), retention of protein stability following encapsulation and release, and improved uptake by and activation of APCs.

In addition to chemical properties of polymers being critical for maintaining protein conformational stability, maintaining thermal stability of both the antigen and the nanoparticle formulation is critical in the design of vaccines for long term storage\textsuperscript{39,55}. Maintaining protein conformational stability is critical for developing robust immune responses to vaccines because linked recognition plays a role in the affinity maturation process of B cells in germinal centers\textsuperscript{56}. Having the protein sequestered within a rigid polymer matrix with high thermal properties will allow for maintenance of protein stability during encapsulation and antigenicity of the payload upon release. In addition, high thermal properties can also help retain particle discreteness and morphology for long term storage, which would enable stockpiling vaccines for mass vaccination against highly lethal biodefense pathogens, or for deployment to harsh environments. We have previously shown that CPH:SA copolymers rich in SA were best able to maintain the activity of \textit{Bacillus anthracis} protective antigen (PA) for at least four months, despite the inability of the copolymer to release antigenic protein compared to CPTEG:CPH copolymers\textsuperscript{39}. This was attributed in part to the high glass transition temperature and melting points of this copolymer, though the exact mechanism has not been investigated. Hence, it was hypothesized
that copolymers of CPTEG and SA would have high thermal properties due to the high melting point (78 °C) of poly(SA), and copolymerization with CPTEG would improve antigenicity of the released protein. In our studies, CPTEG molar compositions upwards of 25% yielded melting points of at least 52 °C. These melting points are similar to those observed with CPH:SA copolymers (melting points of 20:80 CPH:SA and 50:50 CPH:SA are 66 °C and 50 °C, respectively 45).

Copolymers based on CPTEG:CPH or CPH:SA have demonstrated sustained release of encapsulated protein payloads over extended time periods, on the order of months 57. In this work, it was observed that CPTEG:SA copolymers had faster erosion kinetics (on the order of weeks) than 20:80 CPTEG:CPH and 20:80 CPH:SA copolymers. The ability to tune the erosion rate was dictated by the copolymer composition, consistent with previous reports for CPTEG:CPH copolymers 40. Despite having similar contact angles, chemistries rich in SA had the fastest rates of erosion and this was corroborated by 1H NMR spectroscopy measurements of the films over time, which showed that SA molar composition of the films decreased over time. This phenomena of rapid erosion of the CPTEG:SA copolymers compared to the traditional 20:80 CPTEG:CPH and 20:80 CPH:SA copolymers is likely due to absence of CPH in the copolymer backbone, whose hydrophobicity is greater than that of SA and CPTEG 22,40. Despite the fact that CPTEG is amorphous, which usually results in faster erosion kinetics in CPTEG:CPH copolymers 54, and the similar contact angles measured between the various CPTEG:SA copolymers, increasing CPTEG molar composition resulted in a slower rate of erosion. This is likely due to the presence of aromatic rings present in CPTEG, which tends to slow the degradation rates of these copolymers 58. These rapid erosion characteristics may be desirable for both drug and vaccine delivery, in order to have greater control over protein release.
kinetics, or by cocktailing these nanoparticle chemistries with slower releasing copolymer formulations such as 20:80 CPTEG:CPH.

The hydrophobicity of polyanhydrides results in surface erosion characteristics, which in addition to higher pKa values, allows for protection of the structural stability of encapsulated proteins\textsuperscript{39,53,59}. Copolymers containing CPTEG have previously demonstrated to maintain the stability of encapsulated proteins, with 50:50 CPTEG:CPH copolymers typically ideal for maintaining protein structure and antigenicity following release, presumably due to the lower acidity of degradative dicarboxylic acid products\textsuperscript{39,51–53}. Despite the enhanced thermal properties of SA-rich chemistries (poly(SA) and 20:80 CPH:SA), it was previously shown that copolymers rich in SA resulted in loss of stability of \textit{B. anthracis} protective antigen, and subsequently its antigenicity, which was attributed to the greater acidity of SA degradation products compared to CPTEG and CPH diacids\textsuperscript{39}. Therefore, it was hypothesized that copolymerizing the more protein-favorable CPTEG with SA might result in an amphiphilic copolymer that can maintain protein structure and antigenicity following encapsulation and release. Our data showed that CPTEG:SA nanoparticles encapsulating OVA maintained protein stability via native PAGE gel analysis and circular dichroism, however it was observed that copolymers rich in CPTEG successfully maintained the tertiary structure of the OVA, which is likely attributable to the lower acidity of CPTEG. Therefore, the favorable high thermal properties of CPTEG:SA copolymers and inclusion of the less acidic CPTEG into the copolymer backbone make these copolymers an attractive candidate for protein encapsulation, as well as long term shelf storage.

Polyanhydride nanovaccines display chemistry-dependent cellular internalization and intracellular persistence by APCs which can aid in adjuvanting poorly immunogenic proteins. This has been observed to be enhanced in polymer chemistries rich in CPTEG molar
composition\textsuperscript{32}, which had the highest degree of internalization and persistence in RAW 264.7 macrophages\textsuperscript{8}. In BMDCs, copolymer chemistries rich in SA had the highest percentage of cellular internalization, even greater than CPTEG-rich chemistries\textsuperscript{32}. In this work, CPTEG:SA nanoparticles exhibited high rates of internalization by APCs. Interestingly, although previously poly(SA) nanoparticles had been shown to be the most highly internalized formulation\textsuperscript{32}, multiple CPTEG:SA compositions (5:95 CPTEG:SA, 10:90 CPTEG:SA, and 15:85 CPTEG:SA) showed rates of internalization greater than or equal to that of poly(SA), depending on the cell type. The rates of internalization of the various nanoparticle chemistries appeared to correlate with the levels of costimulatory marker expression as well as proinflammatory cytokine secretion (Figure 7). By simultaneously examining cellular internalization, costimulatory cell surface marker expression, and cytokine secretion, it appears that there may be an optimal CPTEG:SA copolymer composition in the 5-15\% CPTEG molar composition range that was best able to be internalized by and activate APCs. This observation is hypothesized to occur because of the synergy between the cellular internalization and activation mechanism(s) of CPTEG- and SA-rich copolymers\textsuperscript{32,60}, though the underlying mechanism by which this is occurring is not fully understood. Previous work has demonstrated actin-dependent internalization of various CPTEG:CPH and CPH:SA polyanhydride nanoparticle formulations\textsuperscript{61}. Interestingly, varying copolymer composition had pronounced effects on whether cellular internalization was dependent on phagocytosis, micropinocytosis, clathrin- or caveolin-mediated endocytosis, or a combination thereof. This suggests that the mechanism of uptake of the CPTEG:SA copolymers may be finely dependent upon the copolymer composition, the cell type being targeted, and may likely be a combination of multiple cellular uptake mechanisms, with the 5-15\% CPTEG molar composition range in the copolymer backbone being optimally internalized by such routes.
Further investigation into the mechanism by which APCs internalize these nanoparticles could dramatically improve targeted delivery of payloads to drive robust immune responses.

CONCLUSIONS

In summary, this work described the synthesis and characterization of novel copolymers based on CPTEG and SA with favorable thermal properties for \textit{in vivo} protein/vaccine delivery and long-term shelf storage. The CPTEG:SA nanoparticles maintain protein stability and antigenicity upon release, are biocompatible, and are highly internalized by APCs, resulting in increased expression of costimulatory cell surface marker expression and proinflammatory cytokine secretion, making them an attractive candidate for further study as a vaccine adjuvant/delivery system.

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Synthesis and characterization of rapidly-degrading polyanhydrides as vaccine adjuvants

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