Compositional tuning of epoxide-polyetheramine “click” reaction toward cytocompatible, cationic hydrogel particles with antimicrobial and DNA binding activities

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Abstract:

The “click” characteristics of nucleophilic opening of epoxide have recently been exploited for the development of a functional hydrogel particle system based on commercially available bisepoxide and triamine polyetheramine monomers. Key features of these particles include high cationic charges and responsiveness to temperature, pH, and oxidation. Despite these advantages, the cytocompatibility of these particles must be considered prior to use in biomedical applications. Here we demonstrate that, by introducing a diamine polyetheramine as a comonomer in the “click” reaction, and tuning its molar ratio with the triamine monomer, cationic nanoparticles with improved cytocompatibility can be prepared. The reduced cytotoxicity is primarily due to the hydrophilic backbone of the diamine comonomer, which has polyethylene glycol as a primary component. The resulting nanoparticles formed from the diamine comonomer exhibited a lower surface charge, while maintaining a comparable size. In addition, the responsiveness of the nanoparticles to temperature, pH, and oxidation was conserved, while achieving greater colloidal stability at basic pH. Results from this study further demonstrated that the nanoparticles were able to encapsulate Nile red, a model for hydrophobic drug molecules, were effective against the bacteria *Staphylococcus aureus*, and were capable of binding DNA through ionic complexation. Based on the results from this work, the use of diamine comonomers significantly reduces the cytotoxicity of similarly developed hydrogel nanoparticles, allowing for numerous biomedical applications, including nanocarriers for therapeutic agents with poor water solubility, treatment of bacterial infection, and non-viral vectors for gene therapy.

Keywords: polymeric nanoparticles; stimuli-responsive nanogels; click chemistry; DNA delivery; antimicrobials

1. Introduction

The use of epoxy chemistry is ubiquitous in the polymer field, and is routinely used during curing to form thermoset epoxy polymers. With increasing attention on the “click” characteristics of epoxide reactions, which highlights the category of nucleophilic opening of spring-loaded rings in the broad “click chemistry” family [1], the use of epoxy chemistry has recently been expanded to the synthesis of other functional polymers. For example, epoxy-thiol click reactions have been utilized for synthesis of amphiphilic, alternating copolymers that can self-assemble into well-defined ultrathin nanotubes with tunable surface functionalities [2]. Similarly, such reaction allowed for direct preparation of organic monoliths with highly regular microstructure for capillary liquid chromatography [3]. In addition, a new family of hyperbranched polymers, formed from polymerization of epoxy and amine monomers, has been created, which respond to multiple stimuli [4, 5]. Further, comb-like, oligodimethylsiloxane-based polymers with alkyl side chains have been developed by click coupling of α,ω -glycidyl ether oligodimethylsiloxane with a primary alkylamine [6]. Of particular importance to this work, epoxy chemistry has been investigated for the development of a biomedically relevant hydrogel particle system that can be functionally tailored to specific applications [7, 8]. In this system, step-growth polymerization of commercially available bisepoxide and polyetheramine monomers, with temperature-induced phase separation, has been used to generate stable colloidal hydrogel particles in water through a simple, environmentally friendly, one-pot process (Scheme 1a). These hydrogel particles exhibit a number of properties, including a cationic surface charge, volumetric sensitivities to temperature and pH, and responsiveness to oxidants.

While the use of cationic polymers in biomedical applications has drawn significant attention [9-15], primarily due to their ability to interact electrostatically with negatively charged

1 macromolecules, the high positive surface charge of these polymers function as a double-edged
2 sword, often leading to charge-induced toxicity [16-19]. In regards to epoxy-amine “click”
3 hydrogel particles, a previous *in vitro* study demonstrated acute cytotoxicity, which could be
4 ameliorated by reducing the surface charge of the particles [8]. Unfortunately, modification of
5 reaction parameters to reduce the surface charge in these systems significantly increased the
6 particle size, causing problems for intravenous use of these particles for drug delivery. In
7 general, nanoparticles (< 500 nm) have prolonged circulation in the body, when introduced
8 intravenously, compared to larger particles [20], and further reduction in size to < 200 nm helps
9 to overcome rapid clearance due to splenic filtration [21-23]. Therefore, alternative strategies are
10 necessary to reduce the surface charge of the particle without drastically increasing particle size
11 beyond what is biomedically relevant. In addition, considering the prospects of multi-
12 responsiveness offered to biomedical applications where stimuli-triggered mechanisms are
13 beneficial, it is desirable to conserve such features in the particles.

14 Here, we show that by tuning the composition of the epoxy-amine hydrogel particles,
15 through rational selection of polyetheramine monomers, the cytocompatibility was significantly
16 improved while maintaining a particle size of < 500 nm. Furthermore, the nanoparticles
17 developed in this work remain responsive to temperature, pH, and oxidants. Utilizing these key
18 features of the cationic epoxy-amine hydrogel nanoparticles, we demonstrated encapsulation of a
19 relevant “drug” molecule, antimicrobial activity, and DNA binding capacity, supporting the
20 potential of these nanoparticles in biomedical applications.

2. Materials and Methods

2.1. Materials

Jeffamine T403 polyetheramine (T403) and Jeffamine ED900 polyetheramine (ED900) were kindly provided by Huntsman Chemical. Polyethylenimine (PEI, branched, $M_n = 20,000$ g/mol), 1, 3-butadiene diepoxide (BDE), and Nile red (NR) were purchased from Sigma-Aldrich. Hydrogen peroxide (30 vol.%) was obtained from Fisher Scientific. All chemicals were used as received.

2.2. Hydrogel nanoparticle synthesis

Preparation of the epoxide-polyetheramine hydrogel nanoparticles was carried out following procedures described previously [7] (Scheme 1). Briefly, the monomers of polyetheramines and BDE were dissolved in deionized (DI) water to a total monomer concentration of 15 wt%. The stoichiometric molar ratio of amino hydrogen of polyetheramines to epoxide group of BDE was kept at 2:1 to ensure particle formation [8]. The composition of the polyetheramines was varied with the molar ratio of the primary amine groups ($-NH_2$) of T403 to ED900 changing from 6:0 to 6:3, 6:4, and 6:6. The monomer solution was incubated statically in an isotherm water bath at 65 °C for 15 min to allow the formation of water soluble intermediate prepolymers. The solution was then diluted to a concentration of 0.5 wt% with DI water, and returned to the water bath and incubated for 30 min without stirring to allow particle formation. Afterwards, the nanoparticles were collected and purified by repeated centrifugation at the relative centrifugal force of 21,000 and dispersed in DI water.

2.3. Characterization

Hydrodynamic sizes and zeta (ζ) potentials of the synthesized epoxide-polyetheramine hydrogel nanoparticles were determined using a Zetasizer Nano-ZS system (Malvern Instruments, Malvern, UK) with a fixed angle of 173°. The nanoparticles were dispersed in water at a concentration of 0.1 mg/mL and measurements were performed at a default temperature of 25 °C in triplicate. Transmission electron microscopy (TEM) of the nanoparticles was performed using a Zeiss Libra 200 HT FE MC TEM with an acceleration voltage of 200 kV. TEM samples were prepared by placing droplets of nanoparticle suspensions onto carbon-coated copper grids for 2 min, blotting of excess solution with filter paper, staining with uranyl acetate, and air drying. Solution behaviors of the polyetheramine monomers of T403 and ED900, as well as the mixtures obtained after the initial 15 min reaction, were studied by measuring the solution cloud point, which was defined as the temperature at which the optical transmittance of the solution was at 50% of its initial value [24].

To characterize the nanoparticles' responsiveness to temperature and pH, the particle size was measured as a function of the stimuli by dynamic light scattering using a Malvern Zetasizer Nano-ZS. For the temperature study, samples were allowed to thermally equilibrate at respective testing temperature for 30 min prior to data collection. For the pH study, measurements were taken on samples that were subjected to 10 min sonication following each pH adjustment. A turbidity measurement was used to examine the response of nanoparticles to oxidation. Briefly, the optical density at 400 nm was recorded as a function of time for 1 vol.% H₂O₂ treated nanoparticle suspensions using a Biomate 5 UV-Vis spectrometer. Hydrophobicity of the nanoparticles was qualitatively examined by loading NR into nanoparticles and recording fluorescence emission spectra of NR using a Perkin Elmer LS55 luminescence spectrometer. NR loading was achieved following a classical infiltration method as previously reported [7].

2.4. *In vitro* macrophage culture and cytotoxicity study

A clone of the murine derived RAW264.7 macrophages was cultured as described previously [8]. Briefly, the cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C, in a 5% CO₂ humidified incubator. Confluent cells were detached mechanically and plated at a density of 1×10^5 cells/well in 100 µL of medium in a 96-well plate. The cells were allowed to adhere for 20 h followed by incubation with DMEM solutions of various epoxy-amine nanoparticles at concentrations (0.5 µg/mL, 10 µg/mL, 50 µg/mL, and 100 µg/mL) for 4 h. A water soluble tetrazolium salt (WST-1) assay (Roche Applied Science, USA) was then conducted to quantitatively examine the cytotoxicity of the nanoparticles. Before addition of the assay solution, medium containing particles was removed from each well to minimize potential interference to the absorbance reading by the nanoparticles. A BioTek Synergy 2 Multi-Mode Microplate Reader (BioTek, USA) was used to measure the absorbance at 440 nm against a blank medium control for background correction. Cells treated with the cationic polymer PEI were included as a negative control, while non-treated cells served as positive control. The viability of the treated cultures was presented as a percentage of absorbance obtained in the positive control.

In parallel, a lactate dehydrogenase (LDH) release assay (Pierce LDH Cytotoxicity Assay Kit, Thermoscientific, USA), which also provides quantitative evaluation of cytotoxicity, was performed on the supernatants collected prior to the WST-1 assay. Briefly, 50 µL of supernatant taken from each well was mixed with 50 µL of LDH substrate mixture and incubated at room temperature for 30 min in the dark. The reaction was then terminated using the LDH stop solution and the absorbance at 490 nm with a reference wavelength of 680 nm was measured

1 using the BioTek Microplate Reader. The relative LDH release was expressed as the ratio of
2 LDH release over the total intracellular LDH level obtained from the lysis control group.

3 Macrophage adhesion and viability were also investigated using a live/dead staining
4 assay (Life Technologies, USA). After a 4 h treatment with epoxy-amine nanoparticles, the cells
5 were gently rinsed with PBS and incubated in the staining solution prepared in PBS,
6 supplemented with 2 μ M calcein AM and 4 μ M EthD-1 for 30 min at 37 °C. Fluorescent images
7 were captured using a Zeiss Axio Observer A1 inverted fluorescent microscope (Zeiss,
8 Germany).

9 2.5. *Bacterial cultures and evaluation of antibacterial activities*

10 *Staphylococcus aureus* (*S. aureus*, ATCC 6538, VA, USA) was statically incubated in
11 brain heart infusion (BHI, Fisher Scientific, USA) broth at 37 °C to allow growth overnight. The
12 overnight culture was then diluted in tryptic soy broth (TSB, Fisher Scientific, USA)
13 supplemented with 1 vol.% glucose to an optical density (OD₆₀₀) of 0.01 and was incubated for 3
14 h at 37 °C to an OD₆₀₀ of approximately 0.3 (mid-log phase). The culture was then centrifuged at
15 15,000 rpm for 10 min at 4 °C to pellet the bacteria. The supernatant was displaced with 0.264 M
16 D-glucose and the bacterial pellet was resuspended in TSB. The centrifugation and resuspension
17 procedures were repeated twice to remove any residual broth. Subsequently, the bacterial
18 suspension was adjusted to an OD₆₀₀ of approximately 0.1.

19 To measure the antimicrobial properties of the epoxy-amine nanoparticles, 900 μ l of the
20 bacterial suspension and 100 μ l of nanoparticles in 0.264 M D-glucose were added to each well
21 of a 24-well plate to achieve different particle concentrations (0, 10, 50, 100, 150 μ g/ml). The
22 bacteria and nanoparticles were allowed to interact at room temperature (RT) for 0, 2, 4, and 6 h

on a shaker operating at 100 rpm. The OD₆₀₀ for each sample was recorded at each time point in triplicate for statistical analyses. The OD₆₀₀ for nanoparticles at testing concentrations were also measured and marked as blank samples in order to take into consideration their contribution to the overall OD readings. In addition to the spectroscopic measurements, serial dilutions of the bacteria/nanoparticle suspensions from 10⁻⁴ up to 1 were plated on BHI agar plates at the end of 6 h contact. Colony forming units (CFUs) were visualized after overnight incubation at 37 °C.

2.6. Formation of epoxy-amine nanoparticle/DNA complexes

To test the DNA binding capacity of the TED nanoparticles, complexation with both a random DNA oligonucleotide and plasmid were tested. To test binding of a DNA oligonucleotide, a randomly generated 26 bp DNA oligonucleotide was dissolved in ultrapure water at a concentration of 55.5 µg/mL. For testing plasmid complexation, the pANIC10A-GFP stuffer plasmid (16 kb) containing the *pporRFP* orange fluorescent reporter gene (OFP) under the control of the PvUbi1+3 constitutive promoter, and the *mGFP5-ER* gene inserted in reverse orientation to remove the *ccdB* cassette, was used [25]. This plasmid was propagated in *E. coli* and purified using a ZymoPURE Giga Prep kit (Zymo Research, Irvine, CA), prior to dilution to 55.5 µg/mL in water. For a 20 µL complexation reaction, 9 µL DNA solution (500 ng of DNA oligo or plasmid) was mixed with 1-4 µL of various nanoparticle solutions with final concentrations from 0.2 mg/mL to 0.00391 mg/mL, generated using a 2-fold dilution. The final volume was then adjusted to 20 µL with PBS (pH 7.4). This design allowed comparison of nanoparticle:DNA mass ratios from 8 to 0.25. Following a 20 min incubation at room temperature, gel retardation assays (0.8-1.0% agarose containing ethidium bromide, 90-100 milliAmps, 45 min) were performed on the mixtures and compared to a Bionexus Hi-Lo™ DNA

Marker to determine the association between DNA and epoxy-amine nanoparticles. For comparison, branched PEI ($M_n = 20,000$ g/mol) was included in the DNA binding study.

2.7. Statistical analysis

Data were reported in the form of mean $\pm$ standard deviation (STDEV). Results from the WST-1 assay, LDH release assay and the bacterial optical density readings were statistically analyzed using a one-way ANOVA with a Tukey-Kramer HSD (Honestly Significant Difference) post-hoc test through the use of JMP software (SAS Institute Inc., USA). In all cases, a p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of monomer composition on the epoxy-amine nanoparticle formation and physicochemical properties

The influence of monomers was studied by including ED900 as a comonomer of T403 and varying the molar ratio of these two polyetheramines during the “click” reaction with bisepoxide. Particle formation was evident for TED 6:0, TED 6:3 and TED 6:4, corresponding to the molar ratio of $-NH_2$ groups from T403 to those from ED900 being 6:0, 6:3 and 6:4, respectively. Further increasing the amount of ED900 to a ratio of 6:6 was found to disrupt any particle formation. As shown in Figure 1, the average hydrodynamic diameter of TED 6:0 particles was 270 nm, while the addition of ED900 changed the average diameter to 240 nm (TED 6:3) and 460 nm (TED 6:4). Regardless of the composition, these epoxy-amine nanoparticles were cationic and maintained good colloidal stability in water, as shown by the photomicrograph insets in Figure 1. Zeta potential measurements of the nanoparticles revealed a reduction in surface charge as the amount of ED900 increased, with TED 6:0 exhibiting the

highest average ζ -potential of +65 mV, to +45 mV of TED 6:3 and TED 6:4 showing the lowest ζ -potential of +30 mV (Fig. 1). The morphology of the nanoparticles was investigated by TEM. As inferred from the TEM micrographs (Fig. 1), the use of comonomer ED900 and its concentration in the initial reactant formulation had little effect on the spherical morphology of the nanoparticles.

3.2. Effect of monomer composition on the stimuli responsiveness of the epoxy-amine nanoparticles

Previous work indicated that nanoparticles prepared from T403 and BDE (i.e., TED 6:0) were responsive to temperature, pH and oxidant stimulation [7]. Given the benefits of stimuli-responsiveness for biomedical applications of nanoparticles, the ability to retain these traits was studied for the nanoparticles developed in this work. Similar to TED 6:0 nanoparticles, TED 6:3 nanoparticles were thermally responsive over a temperature range from 0 °C to 45 °C, with a reduction in volume as temperature increased (Fig. 2a). The swelling ratio, defined as particle volume at 0 °C to volume at 45 °C, was comparable between TED 6:3 and TED 6:0. For TED 6:4, the particle volume fluctuated randomly as temperature increased (Fig. S1a), indicating that a greater amount of ED900 diminished the temperature responsiveness of the resulting nanoparticles.

In terms of pH responsiveness, TED 6:0, 6:3, and 6:4 nanoparticles all exhibited volume reduction as the solution pH shifted from acidic to basic (Fig. 2b and S1b). The pH responsive behavior of these epoxy-amine nanoparticles was mainly attributed to the presence of amino groups in their structure. As pH increases, deprotonation of the amine groups becomes more significant, thus weakening electrostatic repulsion between the polymer chains within the

nanoparticles resulting in shrinkage. Specifically, the volume change ratio represented by $V_{\text{pH}2}$: $V_{\text{pH}10}$ was 350 for TED 6:3 (Fig. 2b) and 6 for TED 6:4 (Fig. S1b). This parameter was not calculated for TED 6:0, as significant agglomeration developed at pH 10 (Fig. S2a) causing large variation in the volume measured by DLS (Fig. 2b). The observed aggregation phenomenon can be explained by the hydrophobic interaction between the deprotonated nanoparticles. In contrast, the TED 6:3 nanoparticles showed good colloidal stability at pH 10 (Fig. S2b), likely due to the hydrophilic oligo(ethylene glycol) segment of ED900 weakening the hydrophobic association between nanoparticles.

Furthermore, the oxidation responsiveness of the TED 6:3 nanoparticles was analyzed in the turbidity study. A rapid decay in the optical density of the particle suspension, upon treatment with H_2O_2 , implied oxidation-induced breakage of the nanoparticles [7] (Fig. 2c). Comparison of the turbidity profile of TED 6:3 to that of TED 6:0 revealed that, under the same concentration of H_2O_2 , the decrease of optical density was much faster for TED 6:3 than for TED 6:0 (Fig. 2c).

3.3. *Polarity of the epoxy-amine nanoparticles*

Nile red (NR) dye was used as a polarity indicator. Both TED 6:0 and 6:3 nanoparticles were able to encapsulate NR molecules (Fig. 3 inset), indicating that these epoxy-amine nanoparticles maintained a hydrophobic environment conducive to capture of lipophilic reagents. Although the color of the NR-loaded particle suspensions was identical, a difference was detected in the NR fluorescence spectra (Fig. 3). The fluorescence emission maxima for NR in TED 6:0 appeared at ~633 nm, whereas a 4 nm red shift to ~637 nm was observed for NR in TED 6:3. Such a shift suggested that TED 6:3 was slightly more hydrophilic than TED 6:0, as

NR is a well-known solvatochromic dye exhibiting high sensitivity to changes in the polarity of its surrounding [7, 26].

3.4. Cytotoxicity of the epoxy-amine nanoparticles

Cytotoxicity of TED 6:0 and 6:3 nanoparticles was examined using RAW264.7 macrophages. Figure 4a shows the cell viability, as measured by the WST-1 assay, as a function of nanoparticle concentration. With the TED 6:0 treated culture, cell viability was significantly reduced as compared to the control at all tested concentrations except 0.5 $\mu\text{g/mL}$, with 50% cell viability achieved at particle concentration of 10 $\mu\text{g/mL}$. For the TED 6:3 group, 90% cells remained viable at 10 and 50 $\mu\text{g/mL}$, with a lethal dosage to induce 50% cell death (LD_{50}) at 100 $\mu\text{g/mL}$ where cell viability was significantly lower than the control. This 10-fold higher LD_{50} indicates that TED 6:3 was less cytotoxic than TED 6:0. Nevertheless, both types of nanoparticles were more cytocompatible than the negative control cationic polymer PEI, where 10 $\mu\text{g/mL}$ treatment drastically reduced cell viability to ~20%.

The LDH release assay, carried out in parallel, offered another measure of nanoparticle toxicity by analyzing the integrity of the cell membrane. As shown in Figure 4b, the release of cytosolic LDH as a function of nanoparticle concentration differed between TED 6:0 and 6:3 treated cultures. A significant increase from the basal level LDH leakage of 3% to 15% was observed at 10 $\mu\text{g/mL}$ of TED 6:0, while a similar effect was achieved with 50 $\mu\text{g/mL}$ of TED 6:3 (Fig. 4b). This result further confirmed that TED 6:3 was more cytocompatible than TED 6:0. The apparent difference between the LD_{50} of TED 6:3 and the results from LDH leakage indicate that, from a mechanistic perspective, membrane damage by these cationic nanoparticles only partially accounts for the observed cell death.

Live/dead staining of the macrophage cultures provided a qualitative view of the effects of the nanoparticles on cell viability, where live cells were stained green and nonviable cells stained red. As shown in Figure 4c, for each experimental group, the increase in nanoparticle concentration led to fewer live (green) and more dead (red) cells. In addition, at 100 $\mu\text{g/mL}$, TED 6:3 nanoparticle treated cultures showed a higher number of cells staining green compared to the TED 6:0 treated culture, suggesting TED 6:3 was more cytocompatible than TED 6:0 nanoparticles. Staining of the untreated control revealed high cell viability (Fig. S3).

3.5. Antimicrobial activity of the epoxy-amine nanoparticles

The OD_{600} of the bacteria/TED 6:3 nanoparticle mixture as a function of TED 6:3 concentration and time was plotted in Figure 5. The relative values of the OD_{600} represented the viability of the *S. aureus*. As demonstrated in Figure 5a, the OD_{600} of the *S. aureus* suspension without nanoparticles (0 $\mu\text{g/mL}$) remained constant over time, indicating that they generally stayed metabolically stable with no growth or death in the D-glucose solution up to 6 h. In contrast, cell death was found for the *S. aureus* after exposure to TED 6:3 nanoparticles. A potent microbial killing effect was observed at all tested TED 6:3 nanoparticle concentrations ranging from 10 to 150 $\mu\text{g/mL}$, which was evidenced by the decrease in OD_{600} of the suspension after incubation for 2 h, 4 h, and 6 h, respectively as compared to the untreated control at those time points. In addition, comparison of a constant nanoparticle concentration over time demonstrated that prolonged exposure yielded more pronounced toxicity to *S. aureus*. The effect of extending incubation time beyond 2 h was significant ($p < 0.05$) for the concentrations of 10 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$. It is worth noting that the presence of TED 6:3 nanoparticles alone had a measurable absorbance reading at 50-150 $\mu\text{g/mL}$ (Blanks in Figure 5a). Therefore, the overall OD readings contained contributions from nanoparticles. This explained the generally higher

OD₆₀₀ for the bacterial suspension and TED 6:3 mixture, which increased with increasing nanoparticle concentrations.

The interference of nanoparticles to the viability assay was diminished using serial dilution and a plating technique was also used to visually observe the number of viable CFUs after contact with TED 6:3 nanoparticles. As illustrated in Figure 5b, the 6 h exposure of *S. aureus* to 10 and 50 µg/mL TED 6:3 reduced the numbers of CFUs. This is in agreement with the observations based on the OD₆₀₀ measurements. In comparison with the 0 µg/mL group, decreases in bacterial populations of approximately four orders of magnitude were observed after interacting with TED 6:3 nanoparticles at cytocompatible concentrations (10 and 50 µg/mL). When the TED 6:3 nanoparticle concentration was further increased to 100 or 150 µg/mL, less than 15 CFUs were detected growing on the BHI plates, showing the bactericidal effect of the TED 6:3 nanoparticles at relatively higher concentrations.

3.6. *Complexation of the epoxy-amine nanoparticles with DNA*

The interaction between TED 6:3 nanoparticles and DNA was verified using a gel retardation assay. Figure 6a-b shows the electrophoretic mobility of the DNA oligo with various concentrations of TED 6:3 nanoparticles and PEI. It should be noted that in the gel retardation assay, ethidium bromide is quenched when the DNA is bound to the polymer, leading to the apparent “disappearance” of the DNA as it is retained in the well with the polymer. Complete retardation of 500 ng of the DNA oligo was observed with 1.0 µg of TED 6:3 and 0.25 µg of PEI (Fig. 6a-b). This result demonstrates that the PEI was able to bind 4 times more DNA oligo than the TED 6:3 nanoparticles, at equivalent weights; however, the PEI used in this study was linear, thus the comparison was not direct. Similar to the results from the DNA oligo, PEI was able to

bind 4 times more plasmid DNA than the TED 6:3 nanoparticles (Fig. 6c-d). In both cases, a 2-fold increase in polymer concentration was required to bind an equivalent amount of plasmid DNA compared to DNA oligo. These results demonstrate that TED 6:3 nanoparticles were capable of forming complexes with single stranded and plasmid DNA through electrostatic interactions.

4. Discussion

The “click” characteristics of nucleophilic ring opening of epoxide have allowed this strategy to be employed for the construction of hydrogel colloids [7, 8]. Through the reaction of bisepoxide and a triamine in water, cationic epoxy-amine hydrogel particles with thermal, pH, and oxidant responsiveness were obtained. As with other cationic materials, cytotoxicity of this family of epoxy-amine particles was found to be greatly affected by surface charge density, with a reduction in charge density decreasing particle cytotoxicity [8]. While surface charge density is an important component in biomedical applications, particle size is equally important. The objective of this work was to engineer epoxy-amine hydrogel nanoparticles with diameters < 500 nm, and demonstrate their potential in biomedical applications. The strategy that was employed used a secondary monomer of polyetheramine, along with Jeffamine T403, to reduce surface charge and maintain small particle sizes. Several criteria were applied to guide the process of monomer selection from commercially available polyetheramines. First, the monomer should be soluble in water, which was the preferred solvent in our protocol for particle preparation. Second, the monomer must contain two or more amine groups ($-NH_2$) to ensure polymerization. Finally, the number of ether spacing units between any adjacent amine groups should be higher than that in Jeffamine T403, in order to lower the charge density of the particles. These criteria were met by the Jeffamine ED series of monomers, which are aliphatic polyether diamines with backbones

1 predominated by polyethylene glycol (PEG). In the present work, Jeffamine ED900 was selected
2 as the comonomer to incorporate since, on average, each ED900 molecule contained 18.5 ether
3 units between the two terminal primary amine groups, among which 12.5 are ethylene oxide. In
4 contrast, no more than 6 propylene oxide units were present between any two terminal amines in
5 a Jeffamine T403 triamine molecule.

6 Hydrogel particle formation was readily achieved until the amount of ED900 was
7 increased to a -NH₂ molar ratio of T403:ED900 of 6:6. At this composition, phase separation
8 failed to occur during the reaction. Since the mechanism of particle formation for this epoxy-
9 amine system was aggregation polymerization of precursor particles generated *in situ* due to the
10 intermediate reaction products being thermally sensitive [7], the lack of temperature-induced
11 phase separation negates the use of the ED900 at this high concentration. An increase in the
12 amount of ED900 in the initial reactant mixture enhanced the hydrophilicity of the products, due
13 to the increased hydrophilicity of ED900 compared to T403, and reduced their thermal
14 sensitivity. This effect was demonstrated through a cloud point study of the solutions during the
15 reactions. As shown in Figure 7, a sharp transition in optical transmittance around 27 °C was
16 observed for the mixture from the TED 6:0 preparation; whereas for TED 6:3 and 6:4, such
17 transitions were shifted to higher temperatures, and the transition profiles appeared gradual and
18 more diffusive. In principle, the influence of hydrophilicity on phase transition noted in this
19 study was in agreement with that reported for the well-known family of thermal responsive block
20 copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) [27].

21 Hydrodynamic sizes and ζ -potentials of the hydrogel particles clearly demonstrated the
22 applicability of ED900 as a comonomer to produce nanoparticles with lower ζ -potentials. Taking
23 TED 6:0 and 6:3 as examples, over comparable size ranges (i.e. ~ 250 nm), the ζ -potential of

TED 6:3 nanoparticles was 30% less than that of TED 6:0. This effect was attributed to the structural differences between Jeffamine T403 and ED900 (Scheme 1). As the terminal reactive -NH₂ groups in ED900 are separated by a much longer and more hydrophilic PEG spacer, upon reaction with diepoxide, the derived secondary/tertiary amine groups in the nanoparticles are further apart than those generated with T403. Since these amine groups account for the cationic nature of the epoxy-amine nanoparticles, it is reasonable to observe TED 6:3 bearing lower positive charges than TED 6:0. A similar effect of spacer length on charge was recently reported, where low molecular weight PEI ($M_w = 600$ Da) reacted with various length, short PEG-like diol glycidyl ethers, and the resultant cationic polymer prepared from shorter bridges showed a higher positive zeta potential [28]. It is worth noting that with the molar ratio of T403 to ED900 increased from 6:0, 6:3 to 6:4, the average hydrodynamic diameter of the resulting nanoparticles decreased first then increased. This phenomenon likely resulted from the complex effects of the comonomer ED900 on the reaction. On one hand, its hydrophilic nature increased the stability of the intermediate precursor particles, thus reducing the number of particles aggregating to form the final product. On the other hand, with ED900 being diamine terminated and T403 triamine terminated, a greater amount of ED900 in the reaction mixture reduced the formed epoxy-amine network density, allowing greater swelling and larger hydrodynamic size. These actions have collectively led to the observed size change as the molar ratio of the two Jeffamine monomers increased.

The influence of ED900 was not limited to the physicochemical characteristics of the nanoparticles. In terms of thermal response, minute differences were observed between TED 6:0 and 6:3, both showing gradual volume reduction as temperature increased. However, their behaviors toward pH and oxidation were highly varied. The most notable difference was the

improved colloidal stability at basic pH when ED900 was used as the comonomer for particle synthesis. This effect primarily arose from the hydrophilic PEG backbone of ED900. The ability of PEG to interact favorably with water molecules allowed TED 6:3 nanoparticles to remain well-dispersed, despite losing the majority of charge due to deprotonation of amine groups under basic pH with their average ζ -potential at pH 10 reduced to +18 mV. Such an outcome was analogous to the improvement of aqueous stability [29] of nanomaterials via the widely adopted strategy of pegylation. Even though the TED 6:3 nanoparticles were more hydrophilic than TED 6:0, they were able to encapsulate the hydrophobic dye Nile red similarly to TED 6:0. This was an important feature, as the inclusion of ED900 did not diminish the potential of these epoxy-amine particles as nanocarriers for hydrophobic drug molecules. Additionally, TED 6:3 nanoparticles were more sensitive to oxidation than TED 6:0 nanoparticles. As assessed by the turbidity experiments, TED 6:3 nanoparticles were more prone to oxidation induced fragmentation due to the incorporation of ED900, a diamine monomer, in contrast to T403, a triamine monomer. Furthermore, the hydrophilic nature of ED900 facilitated the particles to imbibe more water upon oxidative loosening of the polymeric network, thus bringing the refractive index of the hydrated particles closer to that of water. Indeed, when the oxidation process was monitored using DLS, TED 6:3 nanoparticles swelled more rapidly and to a greater extent than TED 6:0 nanoparticles within the same timeframe (Fig. S4).

Specifically with regards to biomedical applications, the ability to develop nanomaterials that are responsive to multiple stimuli present in the surrounding environment is a crucial feature that may enable more specific release of therapeutics. One example of this can be observed with diseases like cancer, where the microenvironment surrounding cancerous lesions often exhibit higher temperature [30], lower pH [31], and high levels of oxidative species [32]. Another

1 example of tuning a nanocarrier for delivery to a specific microenvironment can be observed in
2 the case of orally delivered therapeutics. In the case of these compounds, there is a distinct pH
3 change when moving through the stomach to the intestine. For treatment of intestinal pathogens,
4 such as *Giardia lamblia*, tuning a nanocarrier to release its drug cargo only once it reaches the
5 intestine will prevent premature release and failure of therapy. Similar strategies can also be used
6 to target specific organelles of pathogenic organisms or host cells, in order to more effectively
7 tune a therapeutic strategy [33]. As such, the ability to retain the stimuli-responsiveness of the
8 TED nanoparticles demonstrates the tunability of these nanoparticles for the development of
9 targeted therapeutics.

10 In order to demonstrate that the cytocompatibility of the epoxy-amine nanoparticles was
11 improved with ED900 as a comonomer, *in vitro* macrophage cultures were carried out and the
12 effects of nanoparticles on cell viability were characterized via several common methods. The
13 first method, the WST-1 assay, was used to assess cell metabolism and provide quantitative
14 analysis of cell viability. The results showed that the LD₅₀ was 10 times higher for TED 6:3
15 nanoparticles compared to TED 6:0 nanoparticles (Fig. 4a), indicating TED 6:3 was less
16 cytotoxic than TED 6:0. The improvement in cytocompatibility was attributed to the reduction in
17 ζ -potential of the particles from +65 mV (TED 6:0) to +45 mV (TED 6:3) at similar particle
18 sizes. The reduced cytotoxicity of cationic polymers with a decrease in surface charge has been
19 observed in numerous studies, and is not unique to this work [8, 18, 34-36]. In addition to the
20 WST-1 metabolic assay of cell viability, the LDH assay was used to determine the effect of the
21 nanoparticles on cell permeability. In this assay, a high level of LDH release was indicative of
22 compromised plasma membrane integrity, a feature commonly observed in various cell death
23 mechanisms [37] and often suggested as a key event underlying cationic material-mediated

1 cytotoxicity [38]. Again, TED 6:3 was found to be less disruptive to cell membranes than TED
2 6:0, as a significant increase in LDH release was observed with a higher concentration of TED
3 6:3 (Fig. 4b). Collectively, results obtained from the *in vitro* cell studies demonstrated the
4 effectiveness of comonomer ED900 in improving nanoparticle cytocompatibility. It is important
5 to note that immortalized mouse macrophage cells were used here for cytotoxicity study. The
6 responses of other relevant cell types, especially those derived primarily, should be carefully
7 examined in the future for further biomedical development of these nanoparticles.

8 Among a wide range of biomedical applications being explored for cationic materials, a
9 great deal of attention has been placed on drug delivery for treatment of microbial infections and
10 gene therapy. Thus, to examine the biomedical potential of the epoxy-amine cationic hydrogel
11 particles, TED 6:3 nanoparticles were analyzed to characterize their interactions with bacteria
12 and DNA, respectively. Cationic polymers have been reported to exert antimicrobial actions on
13 various bacterial strains [9, 39, 40], and are considered promising candidates for new
14 antimicrobial agents with a reduced chance of developing resistance [9, 41, 42]. In addition, the
15 propensity of macromolecules to readily complex with cationic polymers allows for biomedical
16 researchers to test numerous DNA and RNA therapies, without the need for complicated
17 conjugation methods. Although the exact antimicrobial mechanisms of cationic polymers are not
18 fully understood, one of the commonly acknowledged mechanisms involves alteration of the
19 membrane permeability of cells due to electrostatic interactions of the cationic polymers with the
20 negatively charged cell surface [9, 43]. In the present work, the antibacterial activity of TED 6:3
21 nanoparticles was tested using the Gram-positive bacterial strain *Staphylococcus aureus*, which
22 has potent pathogenicity, is prominent in biomedical implant-related infections, and rapidly
23 develops drug resistance [44]. Both suspension culture and agar plating revealed the antibacterial

1 effect of TED 6:3 nanoparticles toward *S. aureus*. Together with the results from the macrophage
2 study, this demonstrated that at 10 µg/mL of TED 6:3 nanoparticle concentrations, cytotoxicity
3 against Gram-positive bacteria but not mammalian cells was attainable. When treated at 5
4 µg/mL, a concentration one order of magnitude lower than the 50 µg/mL cytotoxic dosage shown
5 from the LDH assay of macrophage study, the particles were ineffective against *S. aureus* (data
6 not shown), highlighting the concentration-dependent effect. While the initial findings are
7 encouraging, in-depth studies are needed in order to gain a mechanistic understanding of the
8 observed antimicrobial effect. Furthermore, it is worthwhile to continue investigating the effect
9 of TED 6:3 nanoparticles over a broad spectrum of bacteria to gauge the potency, as well as,
10 specificity of their antibacterial activity.

11 In addition to antibacterial activity, the ability of cationic materials to be used as nonviral
12 vectors for gene therapy has received significant attention in recent years with the growth of the
13 nanotechnology field. In this work, the ability of TED 6:3 nanoparticles to bind to DNA
14 oligonucleotides was examined, which were used as a model for single stranded gene silencing,
15 such as ssRNA and miRNA [45, 46]. When compared with the control polymer, PEI, one of the
16 most widely studied nonviral cationic vectors [47-49], four times more (gram weight) TED 6:3
17 nanoparticles were needed to complex an equivalent amount of DNA. Considering the high
18 amino density in PEI, this was not surprising; however, the LD₅₀ of TED 6:3 nanoparticles was
19 more than ten times higher than PEI (Fig. 4a), showing the increased potential of TED 6:3
20 nanoparticles over PEI for gene therapy applications. Besides reduced cytotoxicity, TED 6:3
21 nanoparticles are inherently rich in hydrophilic ethylene oxide repeating unit and
22 functionalizable hydroxyl groups. Both attributes are highly beneficial for gene delivery as they
23 could prolong circulation time of the nanoparticles by improving their biological inertness and

serum tolerance *in vivo*. Furthermore, the TED 6:3 nanoparticles demonstrated binding affinity for plasmid DNA. In effect the results from this study demonstrate the first step in evaluation of TED 6:3 nanoparticles as a functional nanocarrier for gene therapy. However, complexation of nucleic acids is only one aspect of gene therapy, and the ability to protect these macromolecules from enzymatic degradation is crucial for long-term delivery in the cellular environment. Future studies will take these considerations into effect and focus on modifying the nanoparticles to provide protection against nuclease-mediated degradation, facilitate efficient uptake by cells of interest, allow proper intracellular decomplexation, and achieve organellar targeting [48, 49].

5. Conclusions

In this work, the successful preparation of an extended family of epoxy-amine based, cationic hydrogel particles has been achieved by rationally introducing Jeffamine diamine ED900 as a comonomer of Jeffamine triamine T403, and tuning their molar ratios for the “click” reaction with bisepoxide in water. The major influence of ED900 on the physicochemical properties of the hydrogel nanoparticles formed from this reaction was reflected in the reduction of surface charge. Among the ED900-containing formulations investigated herein, TED 6:3 yielded nanoparticles with responsiveness to temperature, pH, and oxidation. Further, the reduction in surface charge has contributed to the improved cytocompatibility of TED 6:3 nanoparticles as compared to TED 6:0, which were of similar size but structurally lacked PEG. The potential of TED 6:3 nanoparticles in biomedical applications was evaluated for: (1) delivery of hydrophobic drug molecules; (2) treatment of microbial infections; and (3) nanocarriers for gene therapy. The results from these experiments demonstrated that TED 6:3 nanoparticles were able to encapsulate the hydrophobic dye Nile red, a test-case for hydrophobic drug encapsulation; were effective at inhibiting the growth of *Staphylococcus aureus*; and were able to

complex DNA oligonucleotides and plasmid. These results demonstrate the potential of TED 6:3 nanoparticles in biomedical applications, with future research aimed at refining the characteristics of the nanoparticles for specific applications.

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Scheme and Figure Captions

Scheme 1 Schematic illustration of the two-step preparation of polyetheramine-based cationic hydrogel particles *via* epoxy-amine “click” reaction, without (a) or with (b) the comonomer Jeffamine ED900.

Figure 1 DLS measurements of hydrodynamic sizes, TEM images, and zeta potential data of epoxy-amine hydrogel particles with varying polyetheramine monomer compositions.

Figure 2 Responsiveness of TED 6:0 and TED 6:3 nanoparticles to temperature, pH, and oxidation. (a) Thermal responsiveness was characterized via monitoring the hydrodynamic sizes as a function of temperature. For comparison, volumes were normalized to the volume at 45 °C. (b) The normalized volumetric swelling vs. pH. (c) Response to hydrogen peroxide determined by recording turbidity profile of nanoparticle suspensions as a function of time using a UV-vis spectrophotometer at 400 nm. The inset photomicrographs show the suspensions before and after 1 vol.% H₂O₂ treatment.

Figure 3 Fluorescence emission spectra ($\lambda_{\text{exc}} = 550$ nm) of Nile red (NR) encapsulated in TED 6:0 and TED 6:3 nanoparticles. The photomicrographs show the appearance of the Nile red loaded nanoparticle suspensions.

Figure 4 The cytotoxicity of TED 6:0 and TED 6:3 nanoparticles at different concentrations to RAW264.7 macrophages after 4 h exposure. (a) Cell viability normalized to the

untreated culture as determined by WST-1 assay measuring cell metabolic activity. (b) Lactate dehydrogenase (LDH) release normalized over the total intracellular LDH level obtained from the lysis control group. (c) Representative fluorescent images of the cultures after live/dead staining (green: viable cells; red: nonviable cells). Same letters above bars indicate no significant difference ($p > 0.05$) according to one-way ANOVA and the post-hoc Tukey-Kramer HSD test.

Figure 5 *S. aureus* viability after exposure to TED 6:3 nanoparticles. (a) Optical density reading of the bacterial suspension at 600 nm (OD_{600}) as a function of exposure time to various concentrations of TED 6:3 nanoparticles (Mean $\pm$ STDEV). Within each concentration, same letters above bars indicate no significant difference ($p > 0.05$) according to one-way ANOVA and the post-hoc Tukey-Kramer HSD test. The OD_{600} for nanoparticles at each testing concentration was measured and marked as blank samples. (b) Representative images of colony forming units plated from the serial dilutions of cell suspension after contact with TED 6:3 nanoparticles at indicated concentrations for 6 h. The initial array arrangement of various bacterial suspensions inoculated at the indicated dilutions was also shown schematically.

Figure 6 Agarose gel retardation assays of the complexes of DNA oligonucleotides and plasmids. (a) Complexation of TED 6:3 nanoparticles and DNA oligo, showing complete complexation at a mass ratio of 2. (b) Complexation of PEI and DNA oligo, showing complete complexation at a mass ratio of 0.5. (c) Complexation of TED 6:3 nanoparticles and pANIC10A-GFPstuffer, showing complete complexation at a mass ratio of 4. (d) Complexation of PEI and pANIC10A-GFPstuffer, showing complete complexation at a mass ratio of 1. The complexes were prepared by varying the carrier to DNA mass ratio from 0 to 8.

1

2 Figure 7 Phase transition profiles of 5.0 mg/mL solution diluted from the reaction mixtures
3 obtained after various molar ratios of T403:ED900 reacting with bisepoxide at 65 °C for 15 min.

4

5

(a) Without comonomer ED900

(b) With comonomer ED900

1,3-butadiene
diepoxide

Jeffamine T403

Jeffamine ED900

Figure(s)

FIG 1

FIG 2

FIG 3

FIG 4

FIG 5

FIG 6

FIG 7