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# Glucose-Based Poly(ester amines): Synthesis, Degradation, and Biological Delivery

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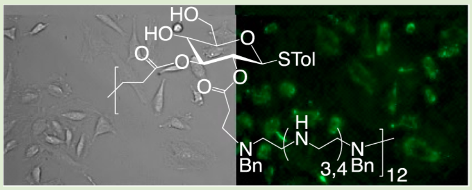
Supporting Information

**ABSTRACT:** Herein, the synthesis and characterization of two glucose-based degradable poly(ester amines), **GluN3** and **GluN4**, are described. Data from gel shift assays, PicoGreen dye exclusion, and dynamic light scattering studies reveals that these polycations can form polyplexes with plasmid DNA at a relatively low N/P ratio and that the polymers degrade rapidly at physiological pH conditions. Assessment of in vitro data via flow cytometry and live cell fluorescence microscopy indicated that **GluN3** and **GluN4** polyplexes can be internalized by HeLa cells in a highly effective manner with low cytotoxicity profiles.



The delivery of nucleic acids has shown promise for the treatment of acquired and inherited diseases; yet, the vehicle structure has proven to be an essential variable in improving efficacy.[1] Cationic polymers are a common and versatile class of delivery vehicles as they can be readily synthesized, tailored, and examined for their ability to bind and compact many sizes and types of polynucleotides into colloidal complexes (termed polyplexes).[1] The design of new vehicles for plasmid DNA (pDNA) delivery is typically driven by several objectives: (i) to achieve effective cellular uptake and intracellular trafficking, (ii) to promote high transgene gene expression, (iii) to provide colloidal stability for in vivo systemic circulation, and (iv) to prevent toxicity and immunogenicity over multiple administrations. While some polycations are very effective for nucleic acid transfection, many of the most effective polymer vehicles are often also associated with a considerable degree of toxicity.[2] Also, while the exact mechanisms of toxicity for polymer-based vehicles are still unclear, there appears to be a direct correlation linking increased toxicity with increased polymer length.[3] In vivo, polymers with high molecular weight can accumulate in organs such as the liver or kidney, which can cause toxic side effects and tissue damage.[4] To this end, the development of carriers that degrade into small molecules to lower toxicity and organ accumulation is of great interest to the field of biomaterials development.

Studies have shown that polyplexes can enter cells through the endosomal pathway. After endocytosis, polyplexes traffic within cells via known pathways such as clathrin-coated vesicles and caveolae, which can lead to trafficking to lysosomes or other organelles. Indeed, polyplexes need to either escape these pathways or be rerouted within the cell to release their payload at its site of action (i.e., cytoplasm for siRNA or inside the nucleus for pDNA). Thus, the delivery vehicle is essential for protecting polynucleotides from nuclease degradation during extracellular and intracellular transport.[5] The polymeric vehicle should dissociate from the polynucleotide cargo to achieve the desired effect within cells. Dissociation of the polyplexes can occur via several means, for example, by association with high salt concentrations, charged proteins and other biomacromolecules that exist within the cell, as well as through degradation of the polymer vehicle.[6]

Degradable polymers have the possibility to be tuned to alter their cargo release kinetics within the cell based on the polymer chemistry, facilitating nucleic acid release upon polymer degradation over a desired time period. Various biodegradable cationic polymers, such as linear poly(β-amino ester)s[3a,7] and poly(ester amine)s (PEAs),[8] have been studied as nonviral gene delivery vehicles. For example, Langer et al. have reported a library of poly(β-amino ester)s and their transfection efficiencies in vitro.[3a,7,9] Numerous other types of biodegradable polymers have also been developed for nucleic acid delivery, such as poly(4-hydroxy-L-proline ester),[10] cross-linked low molecular weight PEI,[11] and poly[α-(4-aminobutyl)-L-glycolic acid].[12] Many degradable carriers are polyester in nature due to the tunable hydrolysis kinetics of the ester bond. Esters are known to rapidly hydrolyze usually under acidic or alkaline conditions to form small molecules consisting of terminal carboxylic acid and alcohol groups. For example, poly(β-amino ester)-based carriers hydrolyze during the pH drop (from ~7 to ~5) that occurs during endosomal/lysosomal trafficking, which, in turn, facilitates nucleic acid release.[7,13]

Previously, our group has synthesized several glycopolycation-based polynucleotide carrier structures, which incorporate various carbohydrate units and oligoamine functionalities linked via amide bonds.[3c,14] The results of our studies in this field have

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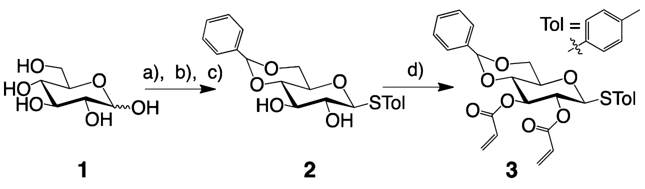
shown that the incorporation of repeated carbohydrate moieties within the polymer can successfully serve to enhance biological delivery. Additionally, various oligoamine functionalities along the polymer backbone have served to promote pDNA or siRNA polyplex formation and protection of nucleic acids from enzymatic degradation.[14b]

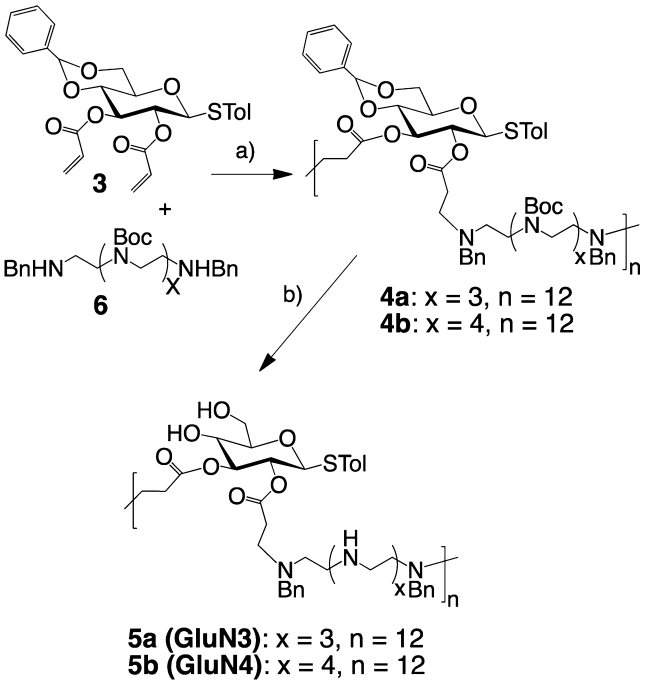
Herein, we extend our design of glycopolycation carriers to include a polyester-based design scaffold to facilitate full degradation of the polymer carrier, decrease toxic side effects, and enhance nucleic acid release. The polymers were created by first synthesizing a diacylate-glucose monomer (Scheme 1) and then polymerizing via Michael-addition with two oligoethyleneamine monomers containing terminal secondary amines and either three or four internal Boc-protected secondary amines (Scheme 2). As shown in Scheme 1, the diacylate glucose monomer was synthesized in several steps. Compound **1** was reacted according to previously published procedures to yield the precursor, structure **2**.[15] Next, **2** was reacted with acryloyl chloride in the presence of triethylamine (TEA) and 4-dimethylaminopyridine (DMAP), yielding the diacrylate compound, structure **3**. The biodegradable polyester was then synthesized by step-growth polymerization via Michael-addition (Scheme 2).[13] In brief, the benzyl-Boc-protected oligoamine (**6**) in THF was added to a THF solution of containing **3** at 50 °C and stirred for 48 h. The products (**4a** and **4b**) were precipitated in cold hexane, washed with hexane several times, redissolved in $CH_2Cl_2$, and dialyzed against $CH_2Cl_2$.

**Scheme 1. Synthesis of the Diacrylate Glucose Monomer (3)[a]**



[a] Reagents and conditions: (a) $Ac_2O$, Pyr.; (b) *p*-thiolcrestol, $BF_3{\cdot}OEt_2$, $CH_2Cl_2$; (c) NaOMe, MeOH; (d) acryloyl chloride, TEA, DMAP, $CH_2Cl_2$.

**Scheme 2. Polymerization of the Diacrylate Glucose and Benzyl-Boc-Protected Oligoamine Monomers via Michael Addition[b]**



[b] Reagents and conditions: (a) THF, 50 °C; (b) 4N HCl in dioxane.

The protected polyester structures (polymers **4a** and **4b**) were characterized by GPC containing a triple detection system (static light scattering, viscometry, and refractive index) in DMF as the mobile phase. The synthesis of polymers **4a** and **4b** were optimized to yield structures with similar degrees of polymerization ($n$ = 12: **4a** $M_\mathrm{w}$ = 13.5 kDa, *Đ* = 1.9; **4b** $M_\mathrm{w}$ = 15.3 kDa; *Đ* = 1.7) to compare the effect of the polymer chemistry on the biological properties. It should be noted here that the deprotected polymer structures (**5a**/**GluN3** and **5b**/**GluN4**) were not able to be analyzed for molecular weight via GPC because the acidic aqueous mobile phase needed to analyze our glycopolycations (and elute these cationic structures from the SEC column)[3c] caused rapid polymer degradation and peaks in the GPC for the deprotected polymers were not observed. The Boc (on the amines) and benzilidene (on the glucose) protecting groups were removed via an acidic dioxane treatment in anhydrous conditions to yield the final water-soluble polyester structures (**5a**/**GluN3** and **5b**/**GluN4**).

The **GluN3** and **GluN4** polymers were then combined with pDNA in water to form nanocomplexes (polyplexes) at N/P ratios from 2 to 10 (Figure 1). These polyesters can neutralize pDNA and completely prevent it from migrating through a gel when electrophoresed at N/P ratios of 3 and above (Figure 1), indicating polyplex formation at N/P ratios above 3 for both structures. Dynamic light scattering results reveal that these degradable polyesters can form polyplexes with pDNA (**GluN3** polyplex size = 65 nm and **GluN4** polyplex size = 55 nm) that have highly positive Zeta potentials (**GluN3** polyplexes = +37 mV and **GluN4** polyplexes = +29 mV). The high Zeta potential of polyplexes likely facilitates endocytosis in vitro via nonspecific interactions with negatively charged cell membrane glycosaminoglycans.

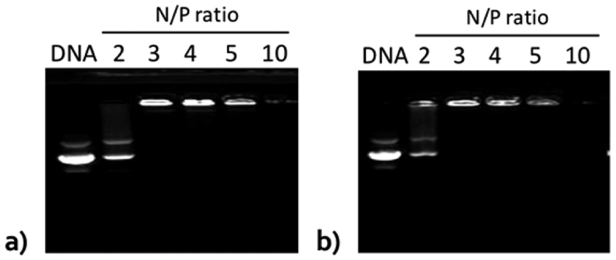


**Figure 1.** Gel electrophoresis assay of (a) **GluN3**; (b) **GluN4** polyplexes.

Both polyesters, **GluN3** and **GluN4**, contain pH-sensitive ester bonds in their polymer backbone that are degradable in aqueous conditions. As previously mentioned, the final deprotected polymer structures (**GluN3** and **GluN4**) degraded too rapidly during GPC analysis and thus we were not able to directly measure degradation kinetics by this technique. Also, NMR analysis of polyester degradation was also not successful due to deuterium exchange with the functional end groups (hydroxyl and carboxylic acid). To this end, we monitored polymer degradation with two indirect methods, via a PicoGreen pDNA binding assay and monitoring solution pH over time. Solutions containing polymers **GluN3** or **GluN4** were incubated in $H_2O$, PBS buffer (pH = 7.4), and acetate buffer (pH = 5.0) at 37 °C for a number of time points between 0 and 168 h. After each designated time point, the solutions containing the polymers (or degraded polymers) were then combined with pDNA and polyplexes were formed (or not formed depending on the status of polymer degradation). The binding of pDNA to the polymers (or lack of binding due to degraded polymer) was then observed as a function of time by monitoring the percent fluorescence intensity of PicoGreen. The PicoGreen fluorescence intensity increases as the dye is able to intercalate into pDNA; thus free pDNA has the highest fluorescence intensity (the 100% fluorescence point, Figure 2a,b).[14b] Solutions with 100% fluorescence intensity indicate a lack of polyplex formation due to fully degraded polymer and solutions with a lower percentage indicate partially degraded polymer.

In PBS (pH 7.4) and acetate (pH 5.0) buffers, both polymers appeared to bind pDNA fully (fluorescence at zero) and proceed to degrade as indicated by the lack of polyplex formation (and increase in PicoGreen fluorescence). Interestingly, the polymers appeared to degrade faster at pH 7.4 than at pH 5.0. Yet, in water, the polymers did not seem to degrade rapidly, as indicated by the lack of PicoGreen intercalation, indicating the buffer could play a role in degradation. When comparing both polymers, **GluN3** appeared to degrade faster than **GluN4**, and this difference is particularly noticed at pH 5.0. Because this assay is indirect, we are not sure if this observation indicates that **GluN3** is degrading faster or that this polymer just binds pDNA weaker due to the lower amine content in the monomer. At pH 7.4 both polymers degrade rapidly; **GluN3** appears to degrade very fast as more than 50% of PicoGreen fluorescence was observed within the first hour and complete degradation appears to be possible after 24 h of incubation in PBS. It is possible that the hydroxyl and thiol-toluene groups restrict movement of the glucose and aromatic rings, which could stabilize both acid- and alkaline-catalyzed transition states, thereby increasing the rate of hydrolysis.[16]

If degradation occurs, the hydrolysis products would likely be a glucose derivative and a carboxylic acid derivative. Upon polymer degradation, while the glucose derivative would not be expected to alter the solution pH, the carboxylic acid derivative should decrease the pH of the solution. Indeed, as shown in Figure 2c, a decrease in the pH of **GluN3** and **GluN4** solutions has been observed in PBS. This drop in pH as a function of time suggests that these carboxylic acid derivatives are forming and supports the hypothesis that these polyesters are degrading.

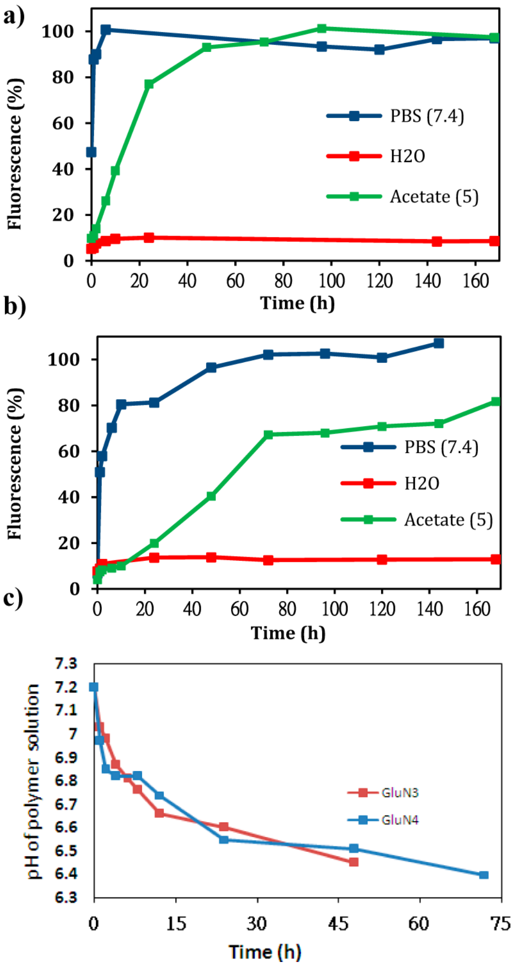


**Figure 2.** PicoGreen intercalation assay monitoring polymer degradation. Polymer solutions were combined with pDNA after the indicated time point and PicoGreen intercalation was monitored as a function of time (reported as fluorescence %, where 100% is pDNA only). The fluorescence is quenched as intact polymer binds to pDNA, which excludes PicoGreen intercalation. High PicoGreen intercalation (and fluorescence) is found in the absence of bound polymer (indicating degraded polymer). (a) **GluN3** binding to pDNA after the indicated time points of incubation (N/P = 20) and (b) **GluN4** binding to pDNA after the indicated time points of incubation (N/P = 20) in different conditions, PBS (pH = 7.4), $H_2O$, and acetate buffer (pH = 5.0). (c) Polyester solution pH as a function of time in PBS. The pH decreases with time, indicating polyester degradation (forming a dicarboxylic acid as a byproduct).

Next, the ability of these polyesters to protect pDNA against nuclease degradation was examined via gel electrophoresis. After polyplex incubation with FBS (fetal bovine serum), which contains various nucleases that degrade nucleic acids, at 37 °C for 0, 1, 2, 4, 6, or 8 h, SDS (sodium dodecyl sulfate) was added to release the pDNA from the polymer in the polyplexes. The integrity of the pDNA after electrophoresis was used to indicate the capacity of the polyesters to protect pDNA from nuclease degradation. As shown in the Supporting Information, Figure S1, pDNA complexed with **GluN4** showed no sign of degradation (band **e**) even after incubation with FBS for 8 h at 37 °C. It is interesting to note that the polymers appear to be more stable from degradation when they are in polyplexes than when they are free in solution. The strong binding of the polyesters to pDNA could potentially be enhanced by the benzyl groups and could stabilize polyplex formation and protection of both the polymer and pDNA from degradation. Intercalation is well-known to enhance pDNA binding properties.[17]

The size and stability of polyplexes formed with pDNA and either **GluN3** and **GluN4** upon incubation with culture media containing serum (DMEM containing FBS) was studied by dynamic light scattering. After the polyplexes were formed in nuclease-free water, DMEM supplemented with 10% v/v FBS was added, and the size of the polyplexes were measured at designated time points (0, 20, 40, and 60 min, Supporting Information, Figure S2). The **GluN3** and **GluN4** polyplexes had similar particle sizes over the incubation time in serum-containing media. When the polyplexes were initially formed in water, the size was around 60 nm. Upon addition of DMEM, the particles immediately increased to about 100−130 nm, which could be due to the swelling of the polyplexes through interaction with serum proteins. After 20 to 60 min of incubation time, the polyplexes did not appear to further grow in size.

Flow cytometry was utilized to investigate the ability of the polyplexes to be internalized into HeLa cells. Both the number of cells positive for polyplexes containing Cy5-labeled pDNA and the average intensity of Cy5 fluorescence in the cell population were assessed. JetPEI, a commercially available polycation transfection reagent, was used as a positive control at an N/P ratio of 5 (manufacturer's recommended formulation for optimal transfection). The transfection experiment was performed in serum-containing medium (DMEM with 10% FBS) with polyplexes formed at an N/P ratio of 15, where we were able to observe polyplex internalization in cultured cells. HeLa cells were incubated with each polyplex solution for 4 h to allow internalization. After removing the cell surface-bound polyplexes and further incubating for 30 min, the cells were trypsinized and isolated for cellular uptake assays. As shown in Figure 3a, **GluN3** and **GluN4** can both successfully promote cellular uptake of Cy5-labeled pDNA within serum-containing medium. These polyesters facilitate greater pDNA uptake than JetPEI, as demonstrated by the higher average intracellular Cy5 fluorescence intensities. These data, that the **GluN3** and **GluN4** vehicles facilitate effective pDNA cellular uptake, is further supported by the enhanced FITC intensity inside cells transfected with FITC-pDNA, as assessed by fluorescence microscopy (Figure 3b). It is interesting to note that **GluN4** promoted higher cellular internalization than **GluN3**, which is likely due to the higher amine content of **GluN4**. These results are similar to results we have obtained previously with our polyamide systems (polyglycoamidoamines), where we observed higher binding affinity and transfection efficiency of the analogues containing four ethyleneamines (N4) over three ethyleneamines (N3).[14] Interestingly, while highly effective polyplex internalization was found, transgene expression was low in HeLa cells. As shown in Figure 3b, the fluorescence appears to be mostly localized to the cytoplasm of these cells (particularly for **GluN4**), which could be related to polymer degradation and potential endosomal release. This property could be important for cytoplasmic delivery of nucleic acids, which is a subject of future study for these systems.

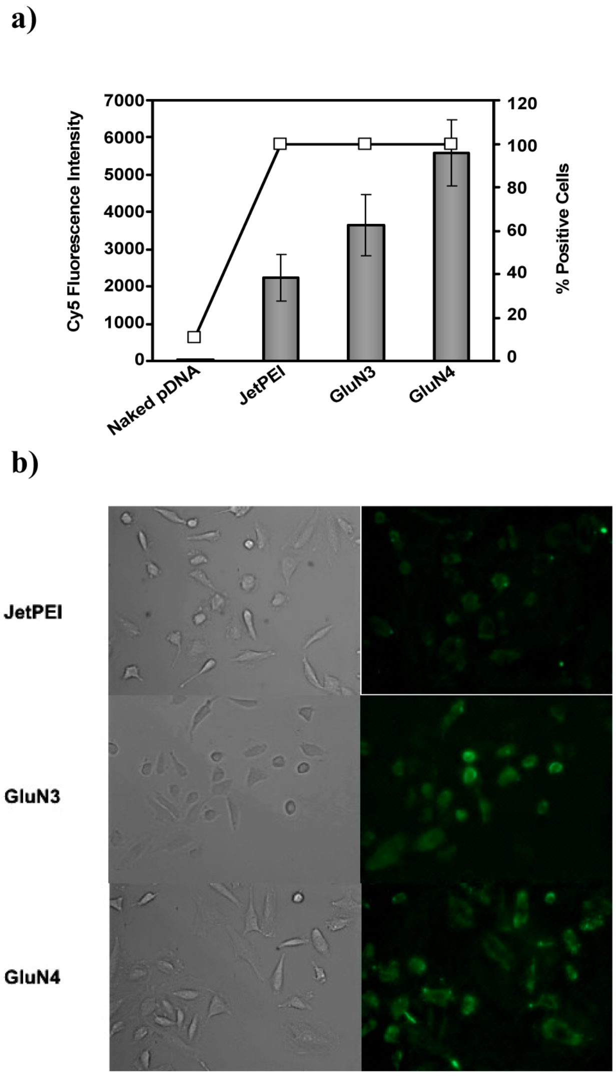


**Figure 3.** (a) Flow cytometry assays of polyplex internalization in HeLa cells in cell culture media containing serum. Polyplexes were formulated with Cy5-labeled pDNA complexed with JetPEI, **GluN3**, or **GluN4** at N/P ratios of 5, 15, and 15, respectively. (b) HeLa cells transfected with FITC-labeled pDNA complexed with JetPEI, **GluN3**, and **GluN4** in serum-containing media at N/P = 15, as imaged using a Nikon inverted microscope TE2000-U.

In conclusion, two new glucose-based degradable polyesters with variation in amine stoichiometry (**GluN3** and **GluN4**) were synthesized, characterized, and examined for their ability to deliver pDNA with HeLa cells. These polymer vehicles were created to have an ester bond between the sugar and the ethyleneamine moieties to facilitate rapid polymer hydrolysis and nucleic acid release, which also aids in decreasing toxic side effects of the vehicle. The polyester degradation rate was monitored with a PicoGreen dye exclusion assay, indicating rapid degradation of these materials in both PBS (pH 7.4) and acetate (pH 5.0) buffers. The internalization of polyplexes formulated with pDNA and polymers in HeLa cells was assessed by flow cytometry and indicated that the polyesters can successfully enhance cellular uptake of pDNA and, at a constant N/P ratio of 15, do so to a greater extent than JetPEI in serum-containing medium. The glucose-based polyesters structures created and examined herein are interesting charged macromolecules with potential to be used in other biomaterials formulations. Indeed, these structures degrade rapidly yet offer a scaffold able to be tailored for sustainable and controlled-release applications.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Synthetic methods, monomer and polymer characterization, polyplex formation and characterization, biological methods and evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare the following competing financial interest(s): T.M.R. is a consultant to and has stock options in Techulon, Inc.

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