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Development of photocrosslinked sialic acid containing polymers for use in Aβ toxicity attenuation

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ABSTRACT

β-Amyloid peptide (Aβ), the primary protein component in senile plaques associated with Alzheimer's disease (AD), has been implicated in neurotoxicity associated with AD. Previous studies have shown that the Aβ-neuronal membrane interaction plays a crucial role in Aβ toxicity. More specifically, it is thought that Aβ interacts with ganglioside rich and sialic acid rich regions of cell surfaces. In light of such evidence, we have hypothesized that the Aβ-membrane sialic acid interaction could be inhibited through use of a biomimic multivalent sialic acid compound that would compete with the cell surface for Aβ binding. To explore this hypothesis, we synthesized a series of photocrosslinked sialic acid containing oligosaccharides and tested their ability to bind Aβ and attenuate Aβ toxicity in cell culture assays. We show that a polymer prepared via the photocrosslinking of disialyllacto-*N*-tetraose (DSLNT) was able to attenuate Aβ toxicity at low micromolar concentrations without adversely affecting the cell viability. Polymers prepared from mono-sialyl-oligosaccharides were less effective at Aβ toxicity attenuation. These results demonstrate the feasibility of using photocrosslinked sialyl-oligosaccharides for prevention of Aβ toxicity *in vitro* and may provide insight into the design of new materials for use in attenuation of Aβ toxicity associated with AD.

1. Introduction

Alzheimer's disease (AD), is the most prevalent cause of dementia in the aging population [1,2], is characterized by the presence of neurofibrillary tangles and amyloid plaques [3]. The main protein component of the plaques is beta-amyloid peptide (Aβ) [4–6]. It has been hypothesized by many that Aβ plays a causative role in the neurodegeneration associated with AD [3,4,7–10].

The mechanism by which Aβ causes neurotoxicity is the subject of much debate. Most believe that Aβ toxicity is linked to the formation of aggregated species, with the most toxic species being an intermediate between monomer and fibrils [11–14]. Some believe that Aβ acts via association with the cell membrane [15], to cause membrane depolarization, changes in membrane capacitance [16], membrane destabilization [17], pore formation [18,19] or free radical generation [20,21]. Consistent with these observations is that the first step in Aβ toxicity is Aβ binding to a membrane component.

Increasing evidence indicates that sialic acid containing gangliosides are pivotal in the mechanism of Aβ binding to cells [7,22–27]. Aβ has higher affinity for gangliosides which are clustered and gangliosides containing multiple sialic acids, than it does for other glycolipids [9,28–30]. Based on these findings, we hypothesized that we could develop a membrane mimic that was multivalent in sialic acids, that would bind Aβ, thus sequestering it, making the Aβ unavailable for cell binding, thereby reducing its neurotoxicity.

We chose to prepare the multivalent sialic acid materials via the photopolymerization of a series of sialic acid containing oligosaccharides. The three oligosaccharides were 3′-sialyl-*N*-acetyllactosamine (3′SLN), sialyllacto-*N*-tetraose b (LST b), and disialyllacto-*N*-tetraose (DSLNT). Each of the selected compounds contained the same or similar carbohydrate backbone of lacto-*N*-tetraose (LNT), but varied in number and orientation of sialic acid in the oligosaccharide. We tested the intrinsic toxicity of base oligosaccharides and their relative abilities to prevent Aβ toxicity in an N2A mouse neuroblastoma cell line. We developed a procedure to photopolymerize the oligosaccharides, then purified and characterized the resulting multivalent sialic acid polymers. Finally we examined the Aβ binding and toxicity attenuation properties of the novel photocrosslinked sialic acid containing polymers. This work could contribute to the development of a new class of therapeutics aimed at preventing Aβ toxicity in AD.

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2. Materials and methods

2.1. Materials

Sialic acid oligosaccharides were purchased from V-Labs, Inc (Covington, LA). The three oligosaccharides used all had either a lactosamine or lacto-*N*-tetraose backbone with one or two sialic acids. DSLNT (Neu5Acα2-3Galβ1-3(Neu5Acα2-6)-GlucNAcβ1-3Galβ1-4Glc), LST b (Galβ1-3(Neu5Acα2-6)GlucNAcβ1-3Galβ1-4Glc), and 3′SLN (Neu5Acα2-3Galβ1-3GlucNAc) were used. Aβ1-40 (>98% purity by HPLC and mass spectrometry) was purchased from Biosource International (Camarillo, CA). Mouse neuroblastoma N2A cells were purchased from ATCC (Manassas, VA). Cell culture reagents were purchased from Gibco-Invitrogen (Grand Island, NY). Propidium iodide (PI) was purchased from BD Biosciences (San Jose, CA). Iodo-Beads, G-5 desalting columns, Bolton hunter reagent (sulfo-SHPP) and CarboLink Coupling Gel were purchased from Pierce Biotechnology (Rockford, IL). ^{125}I was purchased from Amersham Biosciences (Piscataway, NJ). Triethylamine, tetrabutylammonium bromide, *N*-vinyl pyrrolidinone, and glycidyl methacrylate were purchased from Arcos Organics (Geel, Belgium). Irgacure 2959 was purchased from Ciba (Tarrytown, NJ). Periodic acid and resorcinol were purchased from Fisher (Fair Lawn, NJ). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Development of photocrosslinked sialic acid containing oligosaccharides

The development of the biocompatible photocrosslinked sialic acid containing polymers was adapted from a procedure presented by Leach and coworkers [31] for the generation of hyaluronic acid hydrogels. The first step in the reaction was to derivatize the oligosaccharide with the photopolymerizable cross-linker, glycidyl methacrylate. The epoxide on the glycidyl methacrylate was non-specifically reacted to an alcohol on the oligosaccharide as follows. One milligram of oligosaccharide (DSLNT, 3′SLN, or LST b) was added to 100 μl of dH$_2$O. Triethylamine (2.2 μl) was added to the oligosaccharide to act as a strong nucleophile. Glycidyl methacrylate (2.2 μl) and tetrabutylammonium bromide (a surfactant, 2.2 mg) were added to the mixture, which was then allowed to react overnight at room temperature. At this point, various methods were attempted to remove excess reagents including excess glycidyl methacrylate from the methacrylate modified oligosaccharides, however, all resulted in extremely high losses of the methacrylate derivatized oligosaccharides, thus, the ensuing steps of the polymerization were carried out without purification. However, excess epoxide from the glycidyl methacrylate was deactivated by heating the reaction mixture at 60 °C for 1 h. *N*-vinyl pyrrolidinone (3 μl) and Irgacure 2959 (photoinitiator, 2 mg) were dissolved in the methacrylate derivatized oligosaccharide solution, at which time the reaction mixture was placed under a 365 nm lamp for 11 min, completing the polymerization procedure. The power delivered to the oligosaccharide during polymerization was approximately 70 mW/cm^2.

2.3. Polymerized dot assay

To estimate the time of light exposure needed for crosslinking such that a slightly viscous solution was obtained, representing a photocrosslinked oligosaccharide of moderate molecular weight, a simple polymerized dot assay was developed. Approximately 100 μl of glycidyl methacrylate derivatized oligosaccharide was placed on a glass slide, to which *N*-vinyl pyrrolidinone and Irgacure were added. The mixture was irradiated for various lengths of time and the viscosity of the resulting polymer was qualitatively examined. At less than 10 min irradiation, the viscosity of the resulting mixture was indistinguishable from that of water. After 14 min of irradiation a soft gel was formed, while after 21 min of irradiation a firm gel was formed. At between 11 and 12 min of irradiation, noticeable changes in viscosity of the reaction mixture were observed, however, no gelation was detected.

2.4. Purification of sialic acid polymer

Once the polymerization was completed, the polymer solution was purified and fractionated using ultrafiltration. Unreacted monomer, glycidyl methacrylate, and other reagents were removed from the polymer via separation using a 3000 Da molecular weight cutoff ultrafiltration membrane. Polymer that was retained after ultrafiltration was further fractionated using a 10,000 Da molecular weight cutoff filter. The fraction of polymer that passed through the 10,000 Da membrane but was retained by the 3000 Da membrane was used in the binding and toxicity studies. Using this fractionation strategy, we expected to collect crosslinked oligosaccharides with a degree of polymerization between 2 and 8.

2.5. Proof of synthesis (FTIR)

Throughout the procedure of developing the photocrosslinked sialic acids, the chemical structures of the intermediates in reactions were checked using Fourier Transform Infrared Spectroscopy (Perkin Elmer System 2000 FTIR, Boston, MA).

2.6. Periodate–resorcinol assay for sialic acid determination

The periodate–resorcinol assay was utilized to colorimetrically determine the amount of sialic acid groups in a photocrosslinked polymer solution. In the assay, glycosidically bound sialic acids are oxidized with periodate, the product of which is reacted with the recorsinol reagent to give an easily detectable chromophore [32]. A brief description of the microplate version of the assay is given. Forty microlitres of a sialic acid containing sample were placed into a 96-well microplate. Ten microlitres of 0.032 M periodic acid solution was added to each well and mixed by shaking the plate for 5 min. The plate was then placed in an ice bath for 35 min to oxidize the sialic acids. Hundred microlitres of resorcinol reagent (0.6 g resorcinol in 60 ml of 28% HCl, 40 ml of dH$_2$O, and 25 μmol of CuSO$_4$) was added to each well upon completion of the oxidation step and mixed on the shaker for another 5 min. The microplate was then heated at 80 °C for 60 min in order to develop the chromophore. Upon completion, the microplate was removed from the oven, mixed for 1–2 min, and absorbance at 650 nm was immediately read using a microplate reader. Alternatively, the chromophore could be stabilized by addition of 95% *t*-butyl alcohol. Sialic acid concentration was linearly related to absorbance at 650 nm. Concentrations were estimated from a calibration using sialic acid standards.

2.7. Cell culture

N2A cells were grown in Minimal Essential Media (MEM) with the following added: 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (fungizone), and 2.2 mg/ml NaHCO$_3$. The cells were grown in a humidified 5% CO$_2$/air incubator. All cells used during experimentation were under the 15th passage.

2.8. Aβ solution

Lyophilized Aβ was prepared by dissolving 1 mg of the peptide in 100 μl DMSO. The stock was then diluted to 20 μM in minimal essential media (MEM) and aggregated with gentle mixing at 34 rpm for 24 h. This method of aggregation consistently produced toxic aggregated species in our hands as determined by a number of viability assays in different cell populations including the propidium iodide viability assay used with N2A cells.

2.9. Propidium iodide assay

Propidium iodide (PI) was used to measure the viability of the cells in all experiments. PI penetrates damaged membranes in dead and dying cells, and fluoresces when bound to double-stranded DNA [33]. Live cells are membrane impermeant, resulting in low fluorescence emission for this cell population. N2A cells were harvested and seeded in a 96-well flat bottom plate at a density of 50,000 cells/well. Cells were incubated for 24 h in MEM to allow cell adhesion, at which time medium was removed and replaced with either fresh medium, oligosaccharide or photocrosslined oligosaccharide, Aβ, or Aβ plus oligosaccharide or Aβ plus photocrosslined oligosaccharide. Cells were incubated for an additional 24 h in a CO$_2$ incubator, at which time cells were stained with PI for the viability assay. Supernatant from the wells was collected and placed in another 96-well conical bottom plate to make sure detached dead cells were counted in the viability assay. To the original plate with attached live cells, 100 μl of 0.5 g/L trypsin was added to detach cells. Cells in both the supernatant containing plate and the trypsin treated plate were stained with 12 μl of 33 μM PI for 30 min. After staining, the cells from the supernatant containing plate were then added back to the original plate, and the suspension was gently mixed, such that the total cell population from the original culture could be analyzed. Fluorescence staining of cells was assessed using flow cytometry (FACSArray Bioanalyzer, Becton–Dickonson, Bedford, MA). Cells were excited with a 532 nm laser and fluorescence was detected using a 564–606 nm filter. Gating was done so as to obtain percentages of the total cell population that were viable. Normalized viability values were obtained by dividing the percentage of viable cells in the sample by that in the control samples with no Aβ or other agent. Significance of the results was determined using a t test with $p < 0.05$, unless otherwise indicated. Normalized viability of cells with or without Aβ varied between experiments and batches of Aβ used. Typical variations between experiments performed on different days were on the order of 10%. This variability is likely due to both biological differences in cells along with differences in aggregation of Aβ. When ever possible, viability data were always compared to data collected on the same day to minimize these additional sources of variability.

Toxicity inhibition constants, K_I, were estimated from normalized viability of cells, V, treated with Aβ at different concentrations of photocrosslinked oligosaccharide, C_P, by fitting data from an inhibition curve (Eq. (1)) using a non-linear least squares regression. V_{max} is the maximum viability measured at high concentrations of photocrosslinked oligosaccharide.

$$V = \frac{V_{max} C_P}{K_I + C_P} \quad (1)$$

2.10. Radiochemical binding assay

Binding of Aβ to sialic acid polymers was determined using radiochemical techniques as described previously [1]. In brief, Aβ1-40 was radioiodinated via a modified Bolton Hunter method. Sulfo-SHPP (100 nmol) was iodinated with 200 μCi of ^{125}I using the IodoBead catalyst. The reaction was carried out for 15 min in

pH 8.0 borate buffer in a volume of 140 μl. The catalyst was removed and 10 nmol (100 μl) of freshly prepared Aβ1-40 was added to the iodinated sulfo-SHPP. The reaction was allowed to proceed for 3 h at 4 °C. The iodinated peptide was separated from free ^{125}I using a G-5 desalting column. The peptide was eluted from the column with phosphate buffer and stored at 4 °C until use. The free activity associated with radiolabeled Aβ was determined by precipitation of the peptide with 20 wt% trichloroacetic acid in the presence of 1 mg/ml bovine serum albumin. Precipitate activity was greater than 80%.

The sialic acid polymer was immobilized to a CarboLink agarose coupling gel according to manufacturer's directions. Sialic acid polymer (1 mg) was added to 1 mL of 0.1 M sodium phosphate buffer at pH 7.0 and dissolved. Polymer containing buffer (1 mL) was added to $NaIO_4$ giving a final concentration of 23 mM. This solution was reacted for 30 min at room temperature in order to oxidize aldehydes on sugars. Oxidized sialic acid polymer was then added to 2 mL CarboLink gel and allowed to react overnight with mixing. The gel was then centrifuged, supernatant decanted, then washed 3 times with phosphate buffered saline to remove polymer unbound to gel. Gels were stored at 4 °C until use.

Binding assays were carried out by incubating ^{125}I-labeled Aβ of different concentrations with 10 μl of the immobilized sialic acid polymer at room temperature for 2 h. Bovine serum albumin was added at a final concentration of 1 mg/ml to block non-specific binding. After 2 h, sufficient time for binding to come to equilibrium, free Aβ was removed from that bound to the immobilized gel by centrifugation at 12,000 *g* for 5 min. The activities of both bound and unbound Aβ were counted using a scintillation counter (Wallac microBeta, Perkin Elmer). To estimate non-specific binding of Aβ to the CarboLink gel without sialic acid polymer, different concentrations of Aβ were added to the unmodified gel. Free and bound Aβ activity was determined as described for the sialic acid modified gel. At the highest concentrations tested, non-specific binding of Aβ to the CarboLink gel accounts for approximately 25% of the total binding.

The equilibrium dissociation constant for Aβ to the sialic acid polymer was determined from the fit of a Langmuir isotherm (Eq. (2)) to the binding data using a non-linear least squares regression algorithm.

$$B = \frac{B_{\max} C_{A\beta}}{K_{A\beta} + C_{A\beta}} \tag{2}$$

where B is the amount of bound Aβ to the sialic acid polymer-gel corrected for the amount of Aβ bound to the gel alone, $C_{A\beta}$ is the concentration of free Aβ, $B_{\max}$ is the total Aβ binding sites on the surface of the gel, and $K_{A\beta}$ is the equilibrium dissociation constant.

3. Results

3.1. Synthesis of polymer

FTIR was used to confirm addition of glycidyl methacrylate to oligosaccharides and provide an estimate of unreacted glycidyl methacrylate remaining in the reaction mixture. Fig. 1 shows representative spectra of glycidyl methacrylate, DSLNT, and the product of the reaction. Glycidyl methacrylate has three notable peaks from the FTIR, a peak located at 1722 cm^{-1} associated with the ester, a peak located at 1300 cm^{-1} which corresponds to an epoxide, and a peak located at 1152 cm^{-1} that is associated with the aliphatic ether. The DSLNT spectrum is characterized by a broad peak (or peaks) in the region of 3200–2900 cm^{-1} associated with amines and hydroxyl groups on the oligosaccharide. When glycidyl methacrylate was reacted with DSLNT, the epoxide peak in the FTIR spectrum diminished in intensity while the ester group remained. Prominent peaks in the DSLNT spectra in the region between 1700 and 800 cm^{-1} could also be seen in the spectrum taken of the glycidyl methacrylate derivatized DSLNT.

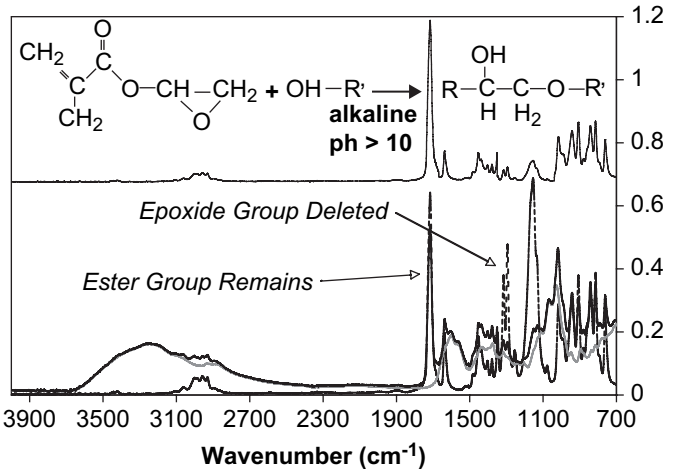

Fig. 1. Representative FTIR spectra of reactants, DSLNT (grey line), glycidyl methacrylate (dotted line) and product of glycidyl methacrylate reaction with DSLNT (bold line). Difference spectrum of DSLNT reaction product minus DSLNT is shown offset (fine line). Upon reaction, the peak corresponding to the epoxide functional group on the glycidyl methacrylate is depleted, while the peak corresponding to the ester functionality is undiminished, indicating that the desired reaction had occurred.

After photopolymerization, the absence of residual unreacted epoxide was confirmed by examining FTIR spectra for the epoxide peak at 1152 cm^{-1}. The degree of polymerization was estimated both from fractionation results and MALDI-TOF MS. The presence and concentration of sialic acid in the resulting polymer were determined via the resorcinol assay. Typical degrees of polymerization were estimated to be between 2 and 8.

3.2. Intrinsic toxicity and Aβ toxicity attenuation properties of oligosaccharide monomers

One of the goals of this research was to develop a compound that was not only able to inhibit Aβ toxicity but that was biocompatible as well. To that end, the intrinsic toxicity of the sialic acid containing oligosaccharide monomers and polymers were tested before examining the Aβ toxicity attenuation properties of the materials. The intrinsic toxicity of sialic acid containing oligosaccharide monomers is shown in Fig. 2. The results show that at concentrations of oligosaccharide 1 mM or below, minimal toxicity associated with the compounds was observed. Above the 1 mM concentration, DSLNT exhibited small but significant toxicity to N2A cells after 24 h exposure.

In Fig. 3 the viability of cells treated with 20 μM Aβ and sialic acid containing oligomeric monomers is shown. The normalized viability of N2A cells treated with aggregated Aβ was 70%. The only oligosaccharide monomer that even marginally attenuated Aβ toxicity was DSLNT, the oligosaccharide containing two sialic acids per monomer.

3.3. Intrinsic toxicity and Aβ toxicity attenuation properties of photocrosslinked oligosaccharides

The photocrosslinked oligosaccharide polymers were examined for both intrinsic toxicity and Aβ toxicity attenuation properties

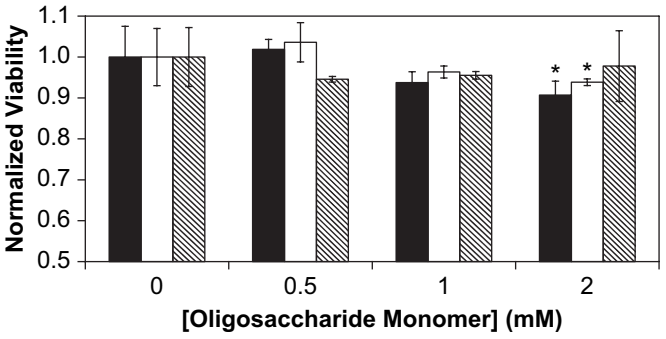

Fig. 2. Viability of N2A neuroblastoma cells treated with sialic acid containing oligosaccharide monomers for 24 h. DSLNT (solid bars), 3′SLN (open bars), and LST b (striped bars) were tested. Viability was assessed using a PI assay. Results were normalized by viability of cells untreated with oligosaccharides. Positive controls, cells treated with 1.5% H_2O_2 for 4 h, were included in the assay. Viability of positive controls was typically less than 20%. $n = 3$ or more independent measurements. Error bars represent standard error of the mean. Asterisk (*) indicate viability was significantly different than the negative control at $p < 0.05$.

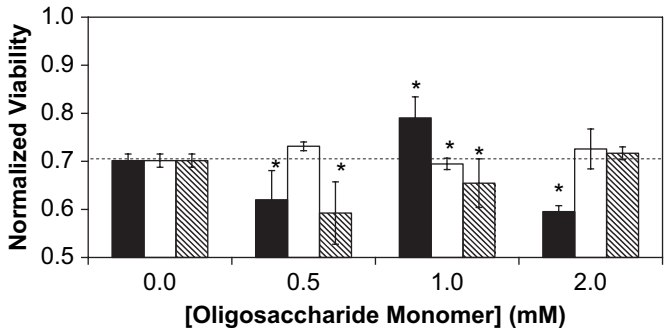

Fig. 3. Toxicity attenuation of 20 μM Aβ using the sialic acid containing oligosaccharide monomers. All cells were treated with 20 μM aggregated Aβ for 24 h. At the same time of Aβ addition, different concentrations of oligosaccharides, DSLNT (solid bars), 3′SLN (open bars), and LST b (striped bars), were added to cells. The dashed line represents viability of cells treated with Aβ alone. The uncertainty in viability measurements of cell treated with Aβ alone for the experiment shown is with bars at 0 concentration of oligosaccharides. Viability was assessed in N2A cells using the PI Assay. $n = 3$ or more independent measurements. Error bars represent standard error. Asterisk (*) indicate viability was significantly different than the Aβ treated cells at $p < 0.05$.

using PI assays (Fig. 4). At concentrations below 1 mM, neither photocrosslinked DSLNT or photocrosslinked 3′SLN exhibited any significant toxicity to cells. Data for photocrosslinked LST b are not shown, because at all concentrations tested, this polymer led to loss of viability in cells at levels comparable to positive controls (cells treated with 1.5% H_2O_2). We noted that without careful purification of polymers by ultrafiltration with a 3000 Da molecular weight cutoff filter, all polymers were toxic at a level comparable to positive controls (data not shown), indicating that unreacted reagents were harmful to cells. We cannot conclude definitively if photopolymerized LST b was toxic to cells or if we observed toxicity associated with the photocrosslinked LST b as a result of incomplete purification of unreacted reagents.

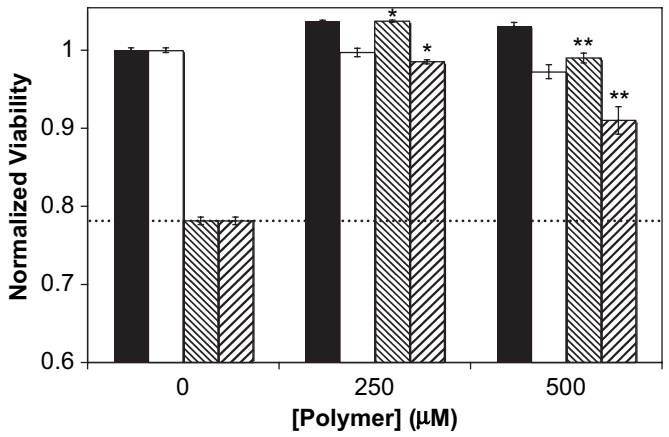

Fig. 4. Viability of N2A cells treated with sialic acid containing photocrosslinked oligosaccharides (polymers) in the presence or absence of 20 μM Aβ. Viability of cells treated with photopolymerized DSLNT without Aβ (solid bars), photopolymerized 3′SLN without Aβ (open bars), and photopolymerized oligosaccharides with 20 μM Aβ (DSLNT: fine stripes; 3′SLN: bold stripes). Photocrosslinked oligosaccharides were added at the same time as aggregated Aβ. The dotted line represented viability of cells treated with Aβ alone. Cells were treated for 24 h prior to viability assessment using a PI assay. Polymer concentrations are reported per sialic acid residue. $n = 3$ or more independent measurements. Error bars represent standard error. All cells treated with photopolymerized polymer and Aβ had significantly increased viability compared to cells treated with Aβ alone. Asterisk (*) indicate that viability of cells treated with 250 μM photopolymerized DSLNT and Aβ was significantly greater than viability of cells treated with the same concentration of photopolymerized 3′SLN and Aβ. Asterisks (**) indicate that viability of cells treated with 500 μM photopolymerized DSLNT and Aβ was significantly greater than viability of cells treated with the same concentration of photopolymerized 3′SLN and Aβ. In all cases $p < 0.05$.

We examined the ability of photopolymerized DSLNT and 3′SLN to attenuate the toxicity observed when cells were treated with 20 μM Aβ. As seen in Fig. 4, cells treated with Aβ were only 78% viable, however, cells treated with high concentrations of either photocrosslinked oligosaccharide and Aβ, had viabilities significantly higher than that cells treated with Aβ alone ($p < 0.05$). At 250 μM photocrosslinked sialic acid containing oligosaccharides completely attenuated Aβ toxicity. Photopolymerized DSLNT performed modestly better than photopolymerized 3′SLN, $p > 0.05$, thus photopolymerized DSLNT was used in further studies.

3.4. Relationship between Aβ binding and toxicity attenuation of photopolymerized DSLNT

We hypothesized that multivalent sialic acid polymers such as photopolymerized DSLNT would attenuate Aβ toxicity by binding to Aβ, thus preventing Aβ binding to the multivalent sialic acid regions on the cell surface. To test this hypothesis, we first examined if Aβ bound to photopolymerized DSLNT, and then examined if concentrations of polymer necessary for Aβ binding and toxicity inhibition were in the same range. As seen in Fig. 5, Aβ binds to photocrosslinked DSLNT. The equilibrium dissociation constant for the Aβ binding to photopolymerized DSLNT, $K_{A\beta}$, was estimated to be 0.22 ± 0.06 μM.

We measured Aβ toxicity attenuation by photopolymerized DSLNT at different concentrations of polymer (Fig. 6). We estimated the inhibition constants, K_I, for photocrosslinked DSLNT at two different concentrations of Aβ, 20 and 40 μM, and found K_I values of 3.6 ± 1.3 μM and 2.2 ± 1.1 μM, respectively. The toxicity inhibition constants are not significantly different from each other and only approximately an order of magnitude greater than binding affinity of Aβ to the photocrosslinked DSLNT.

While toxicity inhibition constants at the different concentrations of Aβ were the same, the maximum viability achieved or fractional increase in viability achieved in Aβ treated cells upon treatment with photopolymerized DSLNT, varied with different concentrations of Aβ. At 40 μM Aβ, photopolymerized DSLNT was less effective at attenuating Aβ toxicity than at 20 μM Aβ.

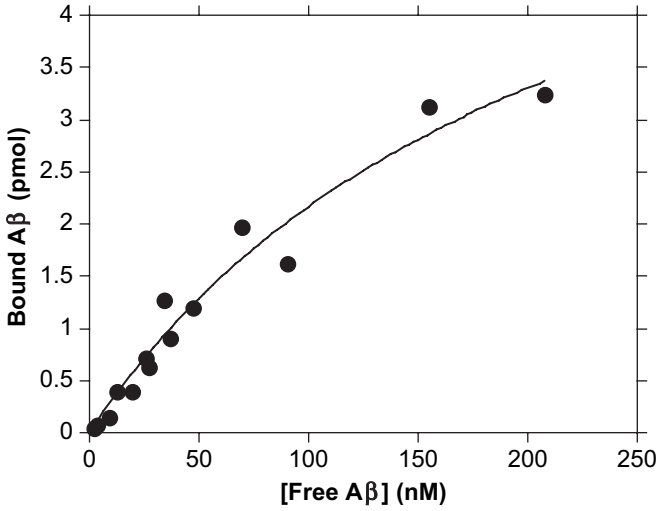

Fig. 5. Representative equilibrium binding isotherm of Aβ bound to photocrosslinked DSLNT. Filled circles represent experimental data. The line represents the fit of the model to the data. Isotherms were collected at room temperature. Aβ was allowed to come to equilibrium with 2.5 μg of immobilized polymer for 2 h before free and bound Aβ were measured. Bound Aβ was estimated as Aβ bound to polymer immobilized on the CarboLink gel minus Aβ bound to gel alone.

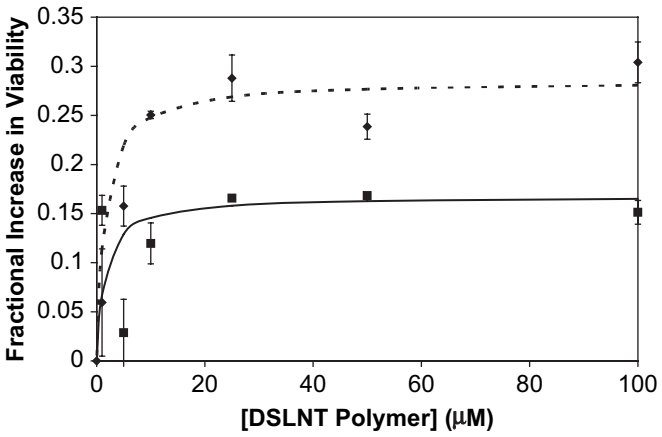

Fig. 6. Toxicity attenuation of Aβ by photocrosslinked DSLNT as a function of polymer concentration. Polymer concentrations are reported per sialic acid residue. N2A cells were either treated with 20 μM (solid line) or 40 μM (dashed line) aggregated Aβ. Different concentrations of polymer were added to cells at the same time as Aβ. Cells were treated for 24 h, after which viability was measured using a PI assay. Fractional increase in viability, defined as the viability of cells treated with Aβ and photocrosslinked DSLNT divided by the viability of cells treated with Aβ alone, is plotted. $n = 3$ or more independent measurements.

4. Discussion

In this work, we examined the feasibility of developing multivalent sialic acid polymers that were both biocompatible and able to attenuate Aβ toxicity. Others have developed multivalent sialic acid polymers by conjugating sialic acid to a dendrimer backbone for prevention of viral adhesion and infection that were effective both *in vitro* and *in vivo* [34,35]. However, when we have previously used sialic acid modified dendrimers to attenuate Aβ toxicity in cell culture models using neuroblastoma cells, dendrimer backbone toxicity was evident at concentrations between 3 and 80 μM dendrimer, depending upon the dendrimer concentration [1,36]. Therefore, we explored the development of multivalent sialic acid containing materials through different synthesis routes. Many other investigators have used glycidyl methacrylate derivatized polysaccharides for preparing biocompatible hydrogels [37–42], thus we explored the feasibility of using glycidyl methacrylate sialic acid oligosaccharides for preparing biocompatible soluble (low molecular weight) polymers for use in Aβ toxicity attenuation applications that might be useful in Alzheimer's disease.

We first tested the biocompatibility of both sialic acid containing oligosaccharide monomers and photocrosslinked polymers in a neuroblastoma cell line susceptible to Aβ toxicity (Figs. 2 and 4). Below 1 mM, no significant loss of cell viability was seen upon treatment of cells with sialic acid containing oligosaccharide monomers, and for two of the sialic acid containing polymers made from photocrosslinked DSLNT and 3′SLN, no significant loss of viability was seen at concentrations of 500 μM, the highest concentrations tested. Photocrosslinked LST b was toxic to cells (leading to 20% or below viability in N2A cells, data not shown), however, we cannot rule out that toxicity of the polymer was due to residual reagents from the polymerization that were not completely purified from the polymer.

We examined the ability of sialic acid containing monomers and polymers to attenuate Aβ toxicity (Figs. 3 and 4). At concentrations of 500 and 1 mM, monomer DSLNT and 3′SLN modestly attenuated Aβ toxicity, while LST b did not attenuate toxicity under the conditions tested (Fig. 3). In previous work, we have reported that monomeric sialic acid attenuated Aβ toxicity at sialic acid concentrations above 200 μM, but only at low (5 μM) concentrations of Aβ [1]. Thus, modest toxicity attenuation seen with DSLNT and 3′SLN are in line with results observed with the simpler saccharide, sialic acid. That LST b did not attenuate Aβ toxicity under the conditions tested may be attributed to the different orientation of the sialic acid on the oligosaccharide (linked via a α2-6 linkage to *N*-acetylglucosamine compared to the α2-3 linkage to galactose on both DSLNT and 3′SLN). Most virions and siglecs (sialic acid binding proteins) have specificity in the sialic acid linkage (α2-3 or α2-6) that they recognize [43,44]. During cancer metastasis, sialylation of cell receptors is also orientation specific [45–47]. Thus, it would not be surprising if Aβ was specific for sialic acid linkage, possibly an α2-3 linkage.

Photocrosslinked DSLNT and 3′SLN were both able to almost completely attenuate Aβ toxicity at high concentrations of polymer (Fig. 4). Photocrosslinked DSLNT and 3′SLN had increased Aβ toxicity attenuation properties compared to monomeric DSLNT and 3′SLN. We attribute the enhanced toxicity attenuation properties of the sialic acid containing polymers to the increased sialic acid valency in these materials. There are many examples of the role of valency in adhesion of ligands to multivalent materials [48–53]. More specific to Aβ, others have shown for biological membranes that Aβ binding affinity to sialic acid containing gangliosides increased in clustered or multivalent regions of the membrane compared to membrane regions where sialic acids or gangliosides were unclustered [25,30].

We hypothesized that multivalent sialic acid polymers made from photopolymerized DSLNT attenuated Aβ toxicity by binding Aβ and sequestering it, making it unable to bind to cell membranes. We demonstrate in Fig. 5 that Aβ does bind to photopolymerized DSLNT with a binding affinity (equilibrium dissociation constant) of 220 ± 60 nM. Aβ affinity to gangliosides in biological membranes, a potential binding site for Aβ on the cell membrane, has been reported to be on the order of 10^{-6} M[7,28]. The greater affinity of Aβ for photopolymerized DSLNT than ganglioside rich regions of the cell surface might indicate that photopolymerized DSLNT could effectively compete with cell surface for Aβ binding. Others have shown that inhibitor affinity for Aβ was correlated with the ability of such inhibitors to attenuate Aβ toxicity [54], supporting our speculation that Aβ affinity for photopolymerized DSLNT would be related to toxicity attenuation properties of the DSLNT polymer.

Aβ binding to DSLNT polymers does not appear to completely saturate at the concentrations tested. Given the propensity of Aβ to aggregate as a function of concentration [5,55], the shape of the Aβ binding isotherms may indicate non-specific binding to DSLNT polymers or may be reflective of different aggregation states of Aβ interacting with the DSLNT polymer. The method used to evaluate Aβ binding the DSLNT polymers cannot be used to discriminate between these possibilities.

When we measured the Aβ toxicity attenuation as a function of photocrosslinked DSLNT, we found that photocrosslinked DSLNT inhibited toxicity with an inhibition constant of 2–3 μM, regardless of the Aβ concentration. This inhibition constant is similar to that found when using sialic acid dendrimers of similar valency to attenuate Aβ toxicity [1]. DSLNT polymers attenuated Aβ toxicity as well as sialic acid dendrimers without the associated cytotoxicity of the dendrimeric materials. The DSLNT polymers attenuated Aβ toxicity with an inhibition constant that was not a function of Aβ concentration, but with a maximum increase in viability that was a function of Aβ concentration suggests that the mechanism of toxicity attenuation of Aβ might not be direct competition for Aβ binding. Analogous to what one might see in the presence of an inhibitor when examining the rate of enzymatic reactions, we would have expected that if inhibition was competitive, the apparent inhibition constant would change as a function of Aβ, but maximum viability (just like the maximum rate of reaction) would remain unchanged. One explanation of our results is that Aβ binds to polymeric DSLNT, and the polymer-DSLNT-Aβ complex kills cells, but at a much lower rate and/or with much less efficacy that

unbound Aβ. Regardless of the mechanism by which photocrosslinked DSLNT attenuates Aβ toxicity, we have shown that photopolymerized DSLNT is able to effectively attenuate Aβ toxicity without the associated toxicity we had previously seen with multivalent sialic acid dendrimers [1,36].

We observed Aβ toxicity attenuation at concentrations of photocrosslinked DSLNT in the 2–3 μM concentration range, which is likely too high a concentration for an *in vivo* application as a therapeutic for Alzheimer's disease treatment. In previous work, we showed that multivalent sialic acid dendrimers were able to attenuate Aβ toxicity at lower concentrations (in the high nanomolar range) and that toxicity attenuation was a function of sialic acid valency, along with other parameters [1,36]. We believe Aβ affinity for DSLNT polymers should increase with valency. Increasing the degree of polymerization of the DSLNT photopolymers should yield polymers that are able to attenuate Aβ toxicity at lower concentrations. However, delivery of soluble DSLNT polymers *in vivo* either in the blood, in the CSF, or across the blood brain barrier, will become increasingly more difficult as polymer size increases. Another approach to improve toxicity attenuation properties of sialic acid polymers without significantly increasing the size of the polymer would be to alter the size of the crosslinking molecules such that the spacing of sialic acid residues in the polymer more closely matched suspected binding sites of sialic acid on the Aβ oligomer or fibril. Delivery of multivalent sialic acid polymers in the blood should be feasible, however, intracerebral delivery may be considerably more problematic and highly dependent upon the lipophilicity of the polymer backbone. Recent studies suggest while some Aβ can be cleared from the cerebral cortex by agents delivered in the blood [56], more effective clearance is seen when antibodies or other agents which sequester Aβ are delivered to the brain [57].

5. Conclusions

We demonstrated the feasibility of synthesizing photocrosslinked multivalent sialic acid materials from sialic acid containing oligosaccharides and glycidyl methacrylate. The photocrosslinked sialic acid containing polymers were able to attenuate Aβ toxicity in a cell culture model without any notable cytotoxicity at concentrations up to 500 μM. We show that photopolymerized DSLNT binds to Aβ with an equilibrium dissociation constant on the order of 200 nM, suggesting that these materials bind Aβ with greater affinity than cell surface gangliosides. The photopolymerized DSLNT attenuated Aβ toxicity at concentrations as low as 2 μM. We believe with further optimization, both binding affinity and toxicity attenuation properties of photocrosslinked sialic acid polymers could be improved. These materials provide a useful tool in the examination of the mechanism of Aβ mediated cell toxicity and the development of new strategies to prevent Aβ toxicity associated with Alzheimer's disease.

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