

Nanoparticles To Study Lectins in *Caenorhabditis elegans*: Multivalent Galactose β 1-4 Fucose-Functionalized Dendrimers Provide Protection from Oxidative Stress

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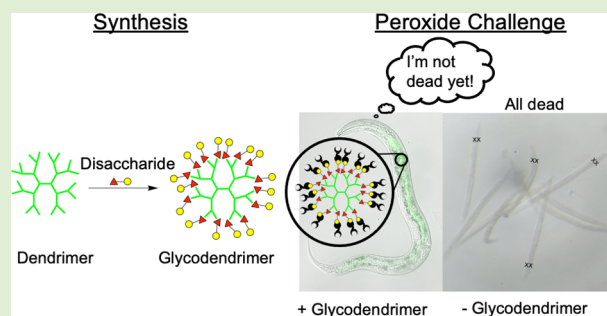


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ABSTRACT: Galectins are galactoside-binding lectins that are functional dimers or higher-order oligomers. Multivalent binding has been shown to augment the relatively low affinity of the galectins for their galactoside-binding partners, enabling the galectins to play an important role in the global remodeling of cells that occurs during the stress conditions of disease states, including heart disease and cancer. The presence of galectins in the nematode *Caenorhabditis elegans* and their galactoside-binding properties have been demonstrated, but the role of multivalent interactions for *C. elegans* galectins is unknown. Here, we describe the synthesis of Gal β 1-4Fuc-functionalized poly(amidoamine) dendrimers and their utility in studies using *C. elegans* during oxidative stress. *C. elegans* were fed Gal β 1-4Fuc-functionalized dendrimers and RNA interference to knock down lectins *lec-1* and *lec-10* while undergoing oxidative stress. *C. elegans* that were pretreated with the glycodendrimers were less susceptible to oxidative stress than untreated controls. Worms that were fed fluorescently tagged glycodendrimers and imaged indicated that the dendrimers are primarily present in the digestive tract of the worms, and uptake into the vulva and proximal gonads could also be observed in some instances. This study suggests that multivalently presented Gal β 1-4Fuc can protect *C. elegans* from oxidative stress.



INTRODUCTION

Galectins are beta-galactoside-binding proteins, and all galectins have a conserved eight-amino acid sequence in their binding pocket.^{1,2} Galectins are classified into three subfamilies, as shown in Figure 1.³ Prototype galectins have one carbohydrate recognition domain (CRD) which can noncovalently associate into dimers or multimers (mammalian galectin-1, 2, 5, 7, 10, 11, 13, 14, and 15, Figure 1a). Tandem-repeat galectins have two linked CRDs (mammalian galectin-4, 6, 8, 9, and 12, Figure 1b), and the only mammalian member of the third subfamily is galectin-3, which has one CRD and a nonlectin N-terminal domain and is called the chimera galectin (Figure 1c). The N-terminal domain is disordered in solution and is thought to induce aggregation into a multivalent display of CRDs.^{1,4–6}

The shallow carbohydrate-binding pocket of the galectins' CRD binds beta-galactosides with weak monovalent affinity,⁷ and *N*-acetylglucosamine (Gal β 1-4GlcNAc) is thought to be the primary endogenous ligand for vertebrate galectins.^{8–10} The Thomsen–Friedenreich antigen (Gal β 1,3GalNAc α 1-O-Ser/Thr) is the putative ligand for interactions such as the cancer cell surface association of Thomsen–Friedenreich-modified Mucin-1 with extracellular galectin-3.¹¹

The multivalent format in which all of the galectins in all three subfamilies display their CRDs induces the formation of

multivalent protein–carbohydrate interactions.^{12–15} Multivalency enables a series of weak monovalent protein–carbohydrate interactions to act synergistically so that these interactions can become physiologically relevant.^{16,17} In humans, multivalent interactions between galectins and their carbohydrate-binding partners have been implicated in many biological phenomena.^{18–20}

Although nematodes are the most prevalent animals on Earth, the function of galectins in nematodes is still not well understood. Most research on nematode galectins has focused on the model organism *Caenorhabditis elegans*²¹ in which 12 members of the *C. elegans* galectin protein family have been isolated and purified.^{1,22} LEC-6 and LEC-12 are the prototype galectins in *C. elegans* (Figure 1a); LEC-1, LEC-2, LEC-3, LEC-4, and LEC-5 are the tandem-repeat galectins in *C. elegans* (Figure 1b); and LEC-7, LEC-8, LEC-9, LEC-10, and LEC-11 are the chimera galectins in this species (Figure 1c).²³

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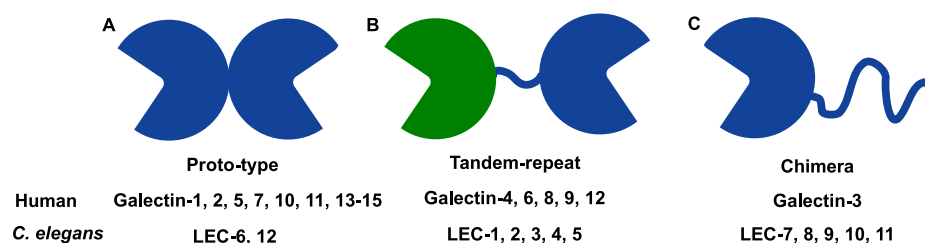


Figure 1. Schematic representation of galectins: (A) prototype, (B) tandem-repeat, and (C) chimera.

Although *N*-acetylglucosamine (Gal β 1-4GlcNAc) binds to the *C. elegans*' galectins, endogenously they lack this disaccharide unit.²⁴ Instead, Gal β 1-4Fuc, which has only been found on the N-glycans of Protosomia, has been identified as the endogenous ligand for LEC-6 and LEC-10.^{24–27} Binding of Gal β 1-4Fuc has also been confirmed for LEC-1, -2, -4, -9, and -12 and has been proposed as a nonhost target for binding to human galectins-1 and -3.^{24,28,29} LEC-8 has been reported to bind to glycolipids (which do not have the Gal β 1-4Fuc ligand),³⁰ and LEC-1 may preferentially bind to O-glycans or glycosaminoglycans.³¹ The relatively low reported monovalent binding affinities (K_D values for LEC-1 and LEC-6 with Gal β 1-4Fuc are 57 and 18 μ M, respectively)²⁸ strongly suggest that, as is the case for human galectins, multivalent effects are important for galectin–carbohydrate interactions in *C. elegans*.

Because of the prevalence and the widespread usefulness of *C. elegans* as an animal model system,^{32,33} a more in-depth study of the role of multivalency for galectins of *C. elegans* is highly warranted. We hypothesized that a multivalent presentation of the Gal β 1-4Fuc ligand would take advantage of multivalency when binding to galectins in *C. elegans*.

Four different generations of Gal β 1-4Fuc-functionalized poly(amidoamine) dendrimers (PAMAM dendrimers, generations 2, 3, 4, and 6) were synthesized to determine whether the size of the framework or the number of carbohydrate ligands impacts the multivalent protein–carbohydrate interaction in *C. elegans*. The generation two, or G(2)-PAMAM, has 16 amino endgroups, and the number of endgroups doubles with each subsequent generation. The main reason why the study of more than one generation is desirable is because the dendrimer size and shape may influence the multivalent interactions of interest. The smaller generation PAMAMs are much more amorphous in shape than the larger generation dendrimers; G(2)-PAMAM dendrimers are highly asymmetrical.³⁴ As the generation number increases, the dendrimers become more spherical in shape. G(4) marks the transition from asymmetrical to spherical, and its shape can best be described as globular.³⁴ The larger generations including G(6) are spherical in shape.³⁴

Previously, *C. elegans* lacking galectins have been reported to be hypersusceptible to oxidative stress but otherwise develop normally under standard laboratory conditions.²² For example, *lec-1* and *lec-10* mutants are more susceptible to stress conditions such as infection and oxidation.^{31,35} These results suggest that galectins are important to the host defense system, although the mechanisms of action are not known.^{22,31,35} Similarly, in humans, galectin-3 has recently been proposed to play an important role in inflammation caused by oxidative stress during disease states such as diabetes.³⁶ To study the *in vivo* effects of multivalency of galectins, Gal β 1-4Fuc-functionalized dendrimers were synthesized and subsequently admin-

istered to *C. elegans* during oxidative stress. Survival assays were performed in the presence of hydrogen peroxide on animals treated with RNA interference (RNAi) to knockdown *lec-1* (referred to as *lec-1(RNAi)*) or *lec-10* (referred to as *lec-10(RNAi)*). Differences in the response to oxidative stress in the presence of Gal β 1-4Fuc-functionalized dendrimers suggest that *C. elegans* lectins may act via different mechanisms to promote survival during oxidative stress. Additionally, using fluorescently labeled Gal β 1-4Fuc-functionalized dendrimers we show that all four generations of glycodendrimers were consumed and collected in the pharynx and gut *C. elegans*.

EXPERIMENTAL SECTION

Worm Strains and Maintenance. All *C. elegans* strains used were Bristol N2 provided by the CGC. Worm strains were maintained at 20 °C on high-growth media (HG: 3 g/L NaCl, 20 g/L Bacto-peptone, 30 g/L Bacto-agar in distilled water, with 4 mL/L cholesterol (5 mg/mL in ethanol), 1 mL/L 1 M CaCl₂, 1 mL/L 1 M MgSO₄, and 25 mL/L 1 M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) plates on *E. coli* OP50 using standard methods.

RNAi Strains. RNAi interference experiments were conducted using the standard RNAi feeding method. Control (pL4440), *lec-1(RNAi)*, and *lec-10(RNAi)* were obtained from the Ahringer library³⁷ and were all sequence-verified prior to use.

Bacteria and Nematode Growth. RNAi bacteria were grown from glycerol stocks overnight shaking (250 rpm) at 37 °C in Luria Broth supplemented with 12.5 mg/L of tetracycline and 100 mg/L of carbenicillin. Overnight culture (1 mL) was used to fully cover a NGM plate that had been supplemented with 1 M IPTG (isopropyl thiogalactopyranoside) and 100 mg/L of carbenicillin and left to dry overnight at room temperature. (NGM: 3 g/L NaCl, 2.5 g/L Bacto-peptone, and 17 g/L Bacto-agar in distilled water, with 1 mL/L cholesterol solution (5 mg/mL in ethanol), 1 mL/L 1 M CaCl₂, 1 mL/L 1 M MgSO₄, and 25 mL/L 1 M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving.) One hour prior to the use of RNAi-seeded plates, 200 μ L of 0.1 M IPTG was spotted on top of plates and left at room temperature to fully dry. Synchronized embryos from adult hermaphrodites were obtained by bleaching (alkaline-bleach solution: 41.5 mL water, 2.5 mL 5 N KOH, and 6.0 mL sodium hypochlorite) followed by repeated washing of collected eggs in M9 buffer (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 5 g/L NaCl, and 1 mL/L 1 M MgSO₄ in distilled water)³⁸ and were placed onto NGM RNAi-seeded plates and the temperature was raised at 20 °C until Day 1 of adulthood (3 days).

Fluorescence Microscopy Procedure. Forty Day 1 adult *C. elegans* were picked into 20 μ L of an 8 mg/mL solution of Alexafluor-488 labeled 10, 11, 12, or 13 in M9 in a 96-well plate for 1 h at 20 °C. Prior to imaging, worms were transferred to a 4% agarose pad before being anesthetized (Levamisole, 7 μ L of a 12.5 mM solution in M9) and imaged. Using a Nikon Ti-E, worms were imaged at 20 $\times$ and 40 $\times$ magnification using both FITC/DITC channels. Overlay images were prepared using FIJI.³⁹

Oxidative Stress Assay. Day 1 adult worms (20 worms/group) were transferred to a 96-well plate containing 40 μ L of a 3 mM or a 6 mM hydrogen peroxide solution prepared in M9 buffer. Worms were

incubated at 20 °C, and survivability was assessed every hour. Worms that did not respond to repeated touching with a platinum wire were considered dead.

Oxidative Stress Assay with Glycodendrimers. Day 1 adult hermaphrodite worms (30 worms/group) were transferred to a 96-well plate containing 20 μ L of an 8 mg/mL solution of glycodendrimers that had been prepared in M9 buffer. The worms were shielded from light and incubated at 20 °C for 1 h before a solution of hydrogen peroxide was added such that the final concentration of peroxide was 3 mM and the final total volume in the well was 40 μ L. Every hour, the number of animals that had survived was assessed. Worms that did not respond to repeated touching with a platinum wire were considered dead. Three independent experiments were performed.

Statistics. Statistical analyses were performed using a two-way analysis of variance (ANOVA) with Tukey's multiple comparison test comparing *C. elegans* treated with and without the dendrimer across each time point. Survival assays were assessed using Kaplan–Meier log rank analysis. Error bars correspond to + standard deviation. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

General Synthetic Materials and Methods. All standard chemicals and reagents were purchased from Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Alfa Aesar (Johnson Matthey Company, Ward Hill, MA, USA), Fisher (Fisher Scientific, Hampton, New Hampshire, USA), or Chem-Impex (Chem-Impex International, Wood Dale, IL, USA) and were used without further purification. PAMAM dendrimers were purchased from Dendritech (Dendritech, Midland, MI, USA). Compounds 1 through 6 were synthesized using previously established procedures.²⁷

Reactions were monitored via thin-layer chromatography (TLC). TLC was performed on silica gel glass plates containing 60 G F – 254, and visualization was achieved with a UV light and a cerium ammonium molybdate or ninhydrin stain. Column chromatography was performed on Silicycle 230–400 mesh silica gel. ¹H NMR and ¹³C NMR were performed on a Bruker DRX500 (500 MHz) or Bruker DRX600 (600 MHz). Chemical shifts (δ) were reported in ppm downfield from an internal TMS standard. Matrix-assisted laser desorption/ionization (MALDI) spectra were recorded on a Bruker III Biflex with a 337 nm nitrogen laser using freshly recrystallized trans-3-indolacrylic acid (IAA) or 2,5-dihydroxybenzoic acid (DHB) as a matrix.

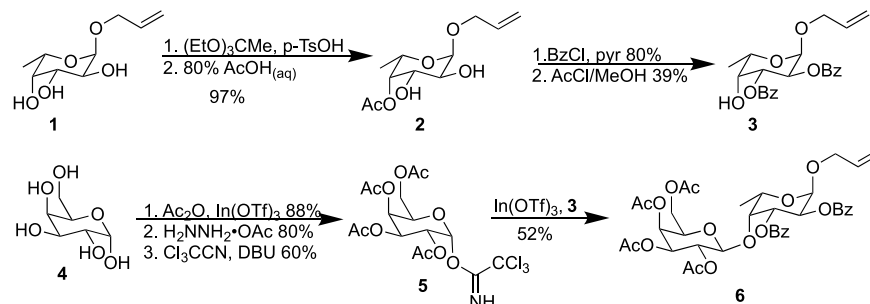
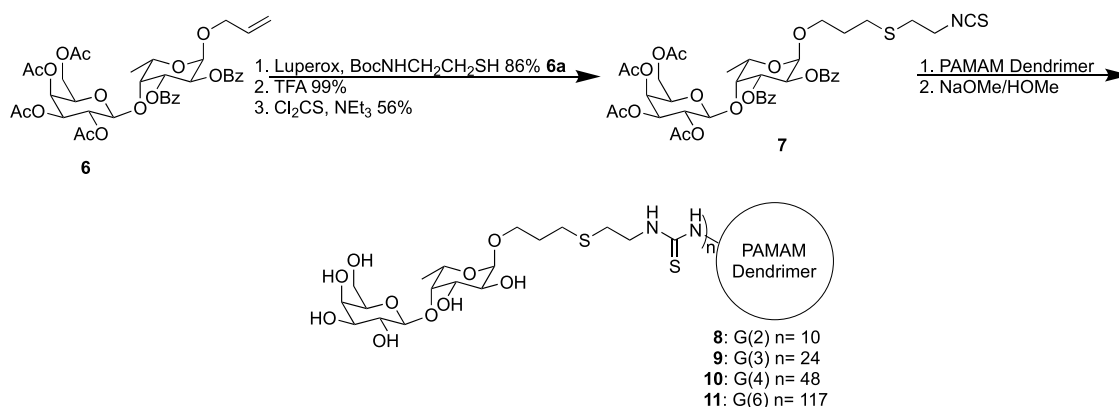
1-O-(6-t-Butylcarbamate-4-thiohexyl)-2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3-di-O-benzoyl- α -L-fucopyranoside (7a). Product 6 (243 mg and 0.330 mmol) and Boc-protected aminoethanethiol (292 mg and 1.65 mmol) were transferred to a 25 mL flask, and 3 $\times$ 2.5 mL aliquots of toluene were used to azeotrope off excess water. Disaccharide 6 was dissolved in 5 mL of toluene, and the solution was degassed with argon for 30 min. Toluene was brought to reflux for 10 min before Luperox (1,1-bis(tert-butylperoxy)cyclohexane, 64.4 mg, 0.25 mmol) was added. The reaction was allowed to reflux for 5 h or until the TLC indication reaction had completed. The crude reaction mixture was concentrated *in vacuo* and was subjected to column chromatography (1:1 hexanes to ethyl acetate). Product 7a was obtained in 86% yield (261.2 mg). ¹H NMR (500 MHz, CDCl₃) δ 1.30 (d, J = 6.6 Hz, 3H), 1.44 (s, 9H), 1.82 (p, J = 6.9 Hz, 2H), 1.93 (s, 3H), 1.96 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.48 (t, J = 6.5 Hz, 2H), 2.55 (ddd, J = 19.0, 12.4, 7.2 Hz, 1H), 3.09–3.31 (m, 4H), 3.48 (dt, J = 10.0, 6.1 Hz, 1H), 3.63 (td, J = 6.9, 1.2 Hz, 1H), 3.80 (dt, J = 10.1, 5.8 Hz, 1H), 4.16 (dd, J = 13.9, 7.2 Hz, 1H), 4.25 (d, J = 1.4 Hz, 1H), 4.43 (d, J = 7.9 Hz, 1H), 4.83 (s, 1H), 4.94 (dd, J = 10.5, 3.5 Hz, 1H), 5.17 (dd, J = 3.5, 1.2 Hz, 1H), 5.20 (d, J = 2.7 Hz, 1H), 5.27 (dd, J = 10.5, 7.9 Hz, 1H), 5.50–5.55 (m, 2H), 7.33–7.41 (m, 4H), 7.46–7.55 (m, 2H), 7.97 (dd, J = 8.4, 1.4 Hz, 2H), 8.03–8.07 (m, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 16.00, 20.56, 20.58, 20.60, 20.67, 20.82, 28.35, 28.41, 29.34, 32.28, 39.60, 60.40, 65.30, 66.43, 66.82, 68.79, 69.35, 69.88, 70.52, 70.85, 76.50, 76.78, 77.03, 77.23, 77.28, 79.40, 96.42, 101.76, 128.14, 128.41, 128.59, 129.71, 129.75, 130.03, 130.08, 133.09, 133.17, 155.74, 165.80, 166.18, 169.18, 170.06, 170.21. HRMS (ESI) m/z : 920.3373 [M + H]⁺, Calcd for C₄₄H₅₇NO₁₈S 920.3369 [M + H]⁺.

1-O-(6-Isothiocyanato-4-thiohexyl)-2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3-di-O-benzoyl- α -L-fucopyranoside (7). Carbamate 7a (156.4 mg and 0.17 mmol) was transferred to 3 mL of methylene chloride and was chilled to 0 °C before (164 μ L and 174 mg) trifluoroacetic acid was added, and the solution was allowed to stir overnight. Once TLC indicated that the reaction had completed, the product was concentrated *in vacuo*. Removal of the carbamate was nearly quantitative. Free amine (134 mg and 0.17 mmol) was dissolved in 2.0 mL of dry methylene chloride along with 1.40 mL (0.37 mmol) of triethylamine. This solution was cooled to 0 °C and placed under argon. Separately, thiophosgene (18 μ L and 0.2 mmol) was dissolved in dry methylene chloride (5.6 mL). The thiophosgene solution was added dropwise over the course of 2 h. The solution was allowed to stir for an additional hour while it warmed to RT. After TLC indicated that the reaction had completed, 1 mL of ultrapure water was added to quench any remaining thiophosgene. After 30 min, the mixture was poured into DI water and extracted (3 $\times$ 10 mL) with methylene chloride, washed with brine (10 mL), dried over MgSO₄, and concentrated *in vacuo*. Column chromatography was performed using 1:1 hexanes and ethyl acetate to afford 82.8 mg (0.10 mmol) of pure product 7 (56%). ¹H NMR (500 MHz, CDCl₃) δ 1.31 (d, J = 6.7 Hz, 3H), 1.77–1.90 (m, 2H), 1.93 (s, 3H), 1.97 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.50–2.65 (m, 3H), 3.18 (dd, J = 11.3, 6.6 Hz, 1H), 3.23 (dd, J = 11.3, 7.2 Hz, 1H), 3.49 (t, J = 6.9 Hz, 2H), 3.63 (t, J = 6.8 Hz, 1H), 3.77–3.85 (m, 1H), 4.16 (q, J = 6.7 Hz, 1H), 4.26 (d, J = 1.2 Hz, 1H), 4.44 (d, J = 7.9 Hz, 1H), 4.94 (dd, J = 10.5, 3.4 Hz, 1H), 5.17 (dd, J = 3.5, 1.1 Hz, 1H), 5.23 (d, J = 2.6 Hz, 1H), 5.28 (dd, J = 10.5, 7.9 Hz, 1H), 5.48–5.57 (m, 2H), 7.35–7.42 (m, 4H), 7.49–7.56 (m, 2H), 7.96–7.99 (m, 2H), 8.04–8.09 (m, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 14.12, 16.02, 20.56, 20.60, 20.82, 22.70, 29.06, 29.28, 29.37, 29.71, 31.94, 32.25, 45.01, 60.42, 65.33, 66.18, 66.82, 68.89, 69.34, 69.64, 69.82, 70.53, 70.56, 70.84, 76.47, 76.76, 77.02, 77.22, 77.27, 96.36, 101.77, 128.15, 128.18, 128.47, 128.62, 129.68, 129.74, 130.02, 130.09, 133.11, 133.20, 133.27, 165.76, 166.18, 169.18, 170.03, 170.05, 170.20. HRMS (ESI) m/z : 884.2136 (M + Na), Calcd 884.2234 (M + Na). IR (CH₂Cl₂) ν max: 2112 (NCS) cm^{–1}.

Gal β 1-4Fuc-Functionalized Dendrimers 8–11. Approximately 40 mg of PAMAM dendrimers was taken from the stock and lyophilized to remove any water. Once the dendrimers were dried to a constant mass, a 100 mg/mL solution was prepared using DMSO as the solvent, and 20.7 mg of dendrimers in DMSO was transferred to a conical vial charged with isothiocyanate 7. The reaction volume was adjusted to 2 mL, and the reaction mixture was left standing for 3 days. The crude reaction mixture was transferred to dialysis tubing (1 kDa MWCO) and dialyzed against DMSO (3 $\times$ 2 h solvent change and 1 $\times$ overnight). Lyophilization afforded a yellow oil. This oil was suspended in 1:1 MeOH:H₂O, and 0.8 M NaOMe prepared in methanol was added dropwise with the pH taken at 1 h intervals. When the pH remained basic, the solution was neutralized with 0.5 M AcOH. MeOH and AcOH were removed *in vacuo*, and the remaining aqueous solution was dialyzed against Millipore water (3 $\times$ 2 h solvent change and 1 $\times$ overnight) and lyophilized to yield a product as a tan fluffy material.

G2 8: ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.19 (d, J = 6.4 Hz, 3H), 1.70–1.84 (m, 2H), 2.23 (s, 6H), 2.46 (s, 2H), 2.65 (d, J = 43.2 Hz, 10H), 2.87 (s, 1H), 3.03–3.20 (m, 7H), 3.25–3.78 (m, 25H), 3.87 (q, J = 6.7 Hz, 1H), 4.13 (d, J = 7.7 Hz, 1H), 4.20 (s, 1H), 4.54 (s, 1H), 4.60 (d, J = 3.6 Hz, 3H), 5.07 (s, 1H), 7.71 (s, 2H), 7.83–8.19 (m, 4H), 8.26 (s, 1H). ¹³C NMR (151 MHz, DMSO) δ 14.43, 16.64, 17.16, 18.88, 18.94, 23.13, 28.13, 29.77, 30.54, 33.53, 36.90, 37.25, 38.64, 38.82, 38.88, 39.19, 39.55, 39.69, 39.83, 39.97, 40.11, 40.24, 40.38, 40.50, 40.88, 43.70, 49.83, 49.98, 52.56, 60.46, 66.11, 66.59, 68.34, 69.00, 69.56, 71.06, 73.86, 75.83, 82.71, 99.53, 105.14, 170.01, 171.44, 171.79, 172.06, 172.22, 172.72, 183.20. MALDI-TOF MS M_w = 7200 m/z .

G3 9: ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.15 (d, J = 6.3 Hz, 3H), 1.62–1.84 (m, 2H), 2.16 (s, 4H), 2.25–2.48 (m, 4H), 2.52–2.83 (m, 8H), 3.04 (s, 3H), 3.13 (s, 3H), 3.27 (d, J = 8.7 Hz, 2H), 3.30–3.44 (m, 6H), 3.44–3.55 (m, 4H), 3.57 (dd, J = 10.3, 4.9 Hz, 1H), 3.59–

Scheme 1. Synthesis of Protected Gal β 1-4FucScheme 2. Synthesis of Gal β 1-4Fuc-Functionalized Dendrimers

3.71 (m, 3H), 3.83 (q, $J = 6.6$ Hz, 1H), 4.09 (d, $J = 7.5$ Hz, 1H), 4.56 (d, $J = 3.6$ Hz, 2H), 7.46–7.94 (m, 3H), 8.00 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 16.64, 28.16, 29.79, 30.56, 33.70, 37.37, 38.66, 39.45, 39.62, 39.78, 39.95, 40.04, 40.12, 40.21, 40.28, 40.37, 40.45, 40.86, 43.82, 50.04, 52.61, 60.55, 66.15, 66.60, 68.42, 69.03, 69.59, 71.05, 73.85, 75.84, 82.67, 99.53, 105.13, 171.93, 172.40. MALDI-TOF MS $M_w = 16,900$ m/z .

G4 10: ^1H NMR (500 MHz, DMSO- d_6) δ 1.15 (d, $J = 6.3$ Hz, 3H), 1.65–1.83 (m, 2H), 2.17 (s, 4H), 2.30–2.44 (m, 3H), 2.43–2.48 (m, 3H), 2.53–2.72 (m, 7H), 3.05 (s, 3H), 3.14 (s, 3H), 3.19–3.44 (m, 28H), 3.45–3.56 (m, 4H), 3.56–3.64 (m, 2H), 3.67 (s, 1H), 3.84 (d, $J = 6.7$ Hz, 1H), 4.09 (d, $J = 7.4$ Hz, 1H), 4.16 (s, 1H), 4.46 (s, 1H), 4.57 (d, $J = 3.6$ Hz, 2H), 4.71 (s, 1H), 4.81 (s, 0.29H), 5.02 (s, 1H), 7.59 (s, 2H), 7.76 (s, 1H), 7.97 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 16.65, 28.16, 29.79, 30.60, 33.70, 37.41, 38.67, 39.50, 39.66, 39.83, 40.00, 40.09, 40.16, 40.26, 40.33, 40.42, 40.50, 40.91, 43.66, 50.03, 52.67, 60.56, 66.17, 66.61, 68.44, 69.05, 69.60, 71.06, 73.86, 75.85, 82.73, 99.55, 105.17, 141.23. MALDI-TOF MS $M_w = 37,900$ m/z .

G6 11: ^1H NMR (600 MHz, DMSO- d_6) δ 1.15 (d, $J = 5.7$ Hz, 3H), 1.67–1.84 (m, 2H), 2.44–2.48 (m, 5H), 2.52–2.87 (m, 11H), 2.94–3.21 (m, 7H), 3.33–3.74 (m, 19H), 3.84 (d, $J = 6.6$ Hz, 1H), 4.10 (s, 1H), 4.18 (s, 1H), 4.50 (s, 2H), 4.57 (s, 2H), 4.75 (s, 1H), 5.05 (s, 1H), 7.41–7.89 (m, 4H), 7.99 (s, 2H). ^{13}C NMR (151 MHz, DMSO) δ 16.19, 27.68, 29.31, 30.12, 33.10, 36.72, 39.10, 39.24, 39.38, 39.52, 39.66, 39.80, 39.94, 40.06, 40.43, 43.29, 44.30, 49.48, 60.09, 65.67, 66.14, 67.97, 68.57, 69.13, 70.57, 73.38, 75.37, 82.30, 99.05, 104.69, 171.30. MALDI-TOF MS $M_w = 98,900$ m/z .

Alexa-Fluor 488-Labeled Glycodendrimer. The following procedure is adapted from Cousin.⁴⁰ Alexa-Fluor 488 hydrazide powder (1 mg) was dissolved in 500 μL Millipore H_2O to form a 2 mg/mL stock solution. Lyophilized Gal β 1-4Fuc-functionalized glycodendrimers were dissolved in Millipore H_2O to make 2 mg/mL stock solutions. To the glycodendrimer solution, NaIO_4 was added (2 equiv per dendrimer), and the reaction mixture was stirred at RT for 2 h. After 2 h, Alexa-Fluor 488 hydrazide (1 equiv per dendrimer) was added and allowed to react for 0.5 h at RT. After 0.5

h, the reaction mixture was purified by dialysis against 1 kDa MWCO (Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane) in Millipore H_2O . The purified reaction mixture was frozen and lyophilized to dryness. Characterization of dendrimer labeling was determined using a UV–Vis spectrometer (Molecular Devices, SpectraMax Plus, Softmax Pro 5). Absorbance was measured at 494 nm using an extinction coefficient of $71,000 \text{ M}^{-1} \text{ cm}^{-1}$, indicating that a labeling ratio of 1 to 1 Alexa Fluor 488 to dendrimer was obtained.

MALDI Procedure. Sample preparation is critical to obtain successful MALDI data. Samples were dissolved in 5 mL of ultrapure water, frozen, and lyophilized. The material went from a yellowish oil to an off white fluffy solid which is ideal for MALDI sample preparation. A 1 mg/mL aqueous solution of dendrimers was prepared. This is the dendrimer stock solution. Either a 2.25 mg/mL solution (4:1, acetonitrile/water) of IAA or a 20 mg/mL aqueous solution of DHB was used to prepare the samples on the plate. For IAA, the dendrimer was mixed with the matrix and then plated. For DHB samples, the dry drop method from Bruker was used.

RESULTS AND DISCUSSION

Synthesis of Gal β 1-4Fuc-Functionalized Dendrimers.

The synthesis of the fully protected disaccharide is shown in Scheme 1. α -L-Allyl fucoside (1)⁴¹ was treated with trimethylorthoacetate to generate an orthoester intermediate, which was treated with 80% aqueous acetic acid to afford 4-acetylallyl fucoside (2) using a previously established procedure.⁴² The remaining hydroxyl groups were protected using benzoyl chloride under standard conditions. To form the 1–4 linkage, the acetyl group needed to be selectively cleaved. This was achieved using dilute acidic conditions following an established procedure to afford product 3.²⁷

Synthesis of trichloroacetimidate 5 began with the peracetylation of galactose 4 using $\text{In}(\text{OTf})_3$ as the Lewis-acid mediator⁴³ followed by selective removal of the acetyl group at the anomeric position with hydrazine acetate. Using

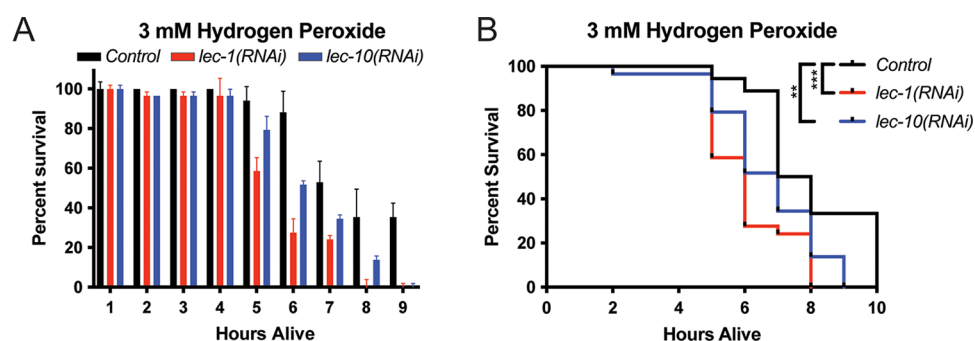


Figure 2. Galectins afford resistance to oxidative stress in *C. elegans*. (A and B) Knockdown of *lec-1(RNAi)* (−7%) or *lec-10(RNAi)* (−20%) increases susceptibility to 3 mM hydrogen peroxide. $n = 30$. (B) Kaplan–Meir log rank analysis. ** $p < 0.01$ and *** $p < 0.001$.

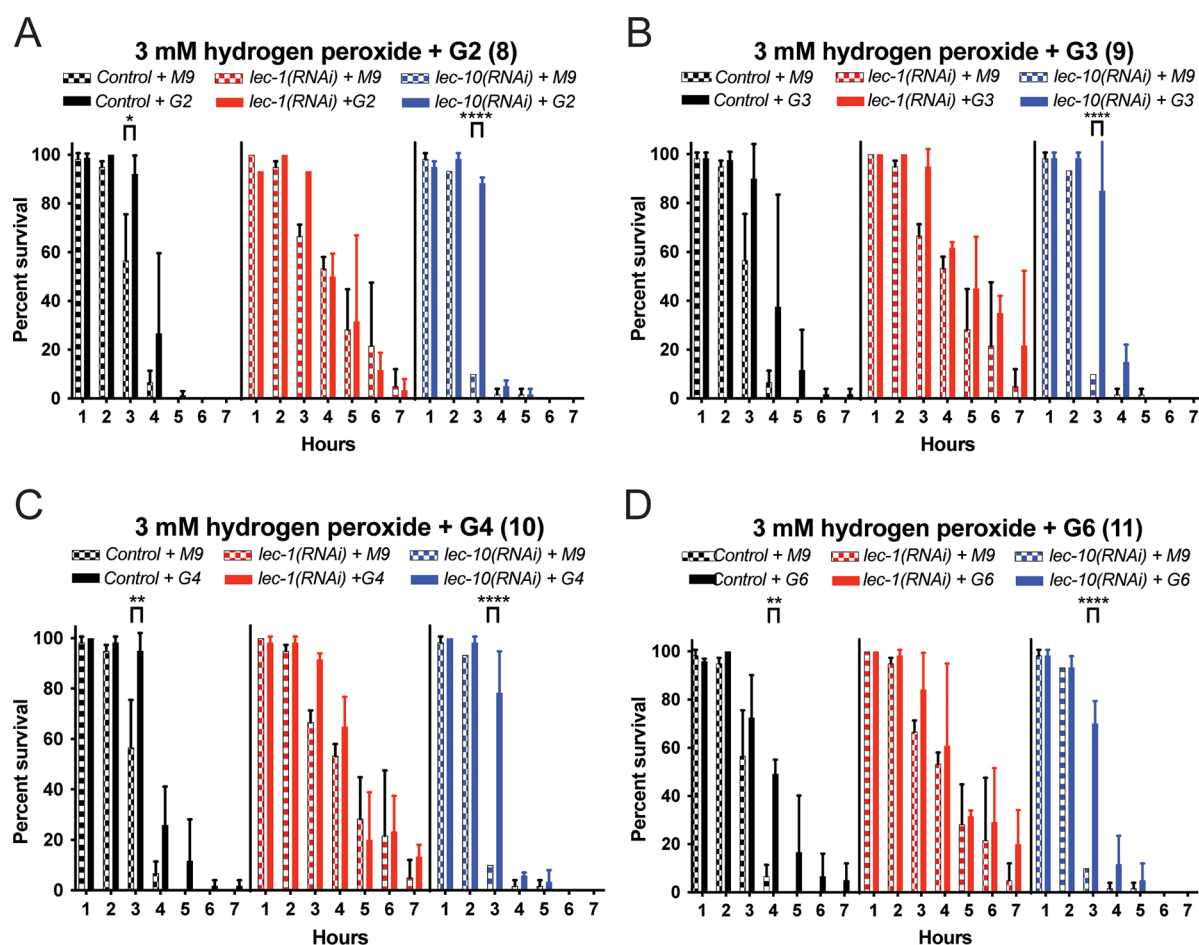


Figure 3. Gal β 1-4Fuc-functionalized multivalent glycodendrimers extend *C. elegans* survival during hydrogen peroxide challenge (A) 8 (0.56 mM), (B) 9 (0.24 mM), (C) 10 (0.11 mM), and (D) 11 (0.04 mM) improve survival of control and *lec-10(RNAi)* *C. elegans* when challenged with 3 mM hydrogen peroxide. Two-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

DBU as a base, treatment with trichloroacetonitrile formed the trichloroacetimidate **5**. The 1–4 linkage of disaccharide **6** was formed by reacting **3** and **5** with indium triflate.⁴⁴

With disaccharide **6** in hand, the allyl group was elaborated using Boc-protected aminoethanethiol as shown in Scheme 2 to form **6a** (not shown). Once liberated, the free amine was converted to isothiocyanate **7** with thiophosgene. The (2-isothiocyanatoethylthio)propyl linker was chosen because it affords flexibility between the disaccharide and the dendrimer such that the endgroups can occupy a large possibility of locations on the dendrimer surface. Moreover, this tethering

group has been used with good success in many prior protein–carbohydrate binding experiments.⁴⁵

The formation of a thiourea linked the disaccharide to the dendrimer. Protecting groups were removed using methanolic sodium methoxide (Zemplén conditions). Gal β 1-4Fuc-functionalized generation 2, 3, 4, and 6 dendrimers **8–11** were synthesized in this manner. The degree of Gal β 1-4Fuc functionalization of the substrate dendrimer was determined using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and ¹H NMR with both the Gal β 1-4Fuc-functionalized dendrimers and their precursor, ester-protected Gal β 1-4Fuc-functionalized den-

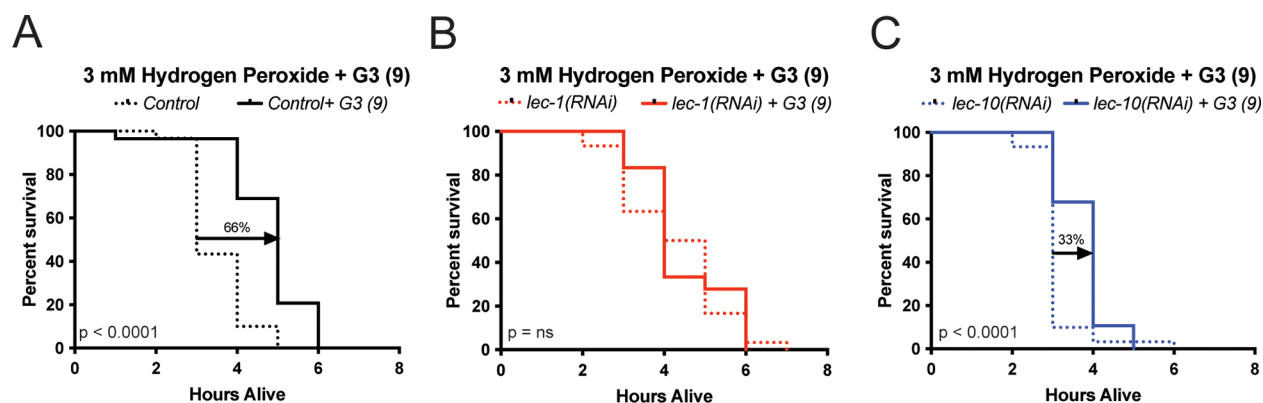


Figure 4. Gal β 1-4Fuc-functionalized **9** improves survival of *C. elegans* during oxidative stress. (A, C) Control (A) or *lec-10(RNAi)* *C. elegans* pre-exposed to Gal β 1-4Fuc-functionalized dendrimers **9** (0.24 mM) increase survival (+66%, or 33%, respectively) compared to unexposed worms. (B) *lec-1(RNAi)* worms pretreated with Gal β 1-4Fuc-functionalized dendrimers **9** (0.24 mM) do not improve survival compared to untreated worms. All worms were challenged with 3 mM hydrogen peroxide. Kaplan–Mier log rank statistical analysis. Percent change in survival is indicated. *** $p < 0.001$ and **** $p < 0.0001$.

drimers to be on average 10, 24, 48, and 117 disaccharides for G(2), G(3), G(4), and G(6), respectively. The average number of Gal β 1-4Fuc was determined using the weight-average molecular weight (M_w) as determined by MALDI-TOF MS. Chemical transformations were confirmed with ^1H and ^{13}C NMR, and mass spectrometry spectra are provided in the Supporting Information.

***C. elegans* Galectins Are Required for Resistance to Oxidative Stress.** It has previously been reported that *C. elegans* *lec-1* knockout mutants are hypersusceptible to both hydrogen peroxide and paraquat, two oxidizing agents. *lec-10* knockout mutants have also been reported to have increased susceptibility to hydrogen peroxide.³⁵ Thus, to study galectin/glycan interactions in *C. elegans*, survival assays in the presence of 3 mM hydrogen peroxide were performed on animals treated with RNAi to knockdown *lec-1* (referred to as *lec-1(RNAi)*) or *lec-10* (referred to as *lec-10(RNAi)*). As shown in Figure 2, *lec-1(RNAi)* and *lec-10(RNAi)* worms are more susceptible to oxidative stress than controls (referred to as *L4440(RNAi)*), which is consistent with prior results reported with *lec-1* or *lec-10* knockout mutants.^{31,35} A seven percent reduction in median survival for *lec-10(RNAi)* compared *L4440(RNAi)* and a 20 percent reduction for *lec-1(RNAi)* were observed. A survival assay was also performed in the presence of 6 mM hydrogen peroxide, and the survival results were comparable to those obtained under the 3 mM conditions (data not shown).

Glycodendrimers Provide Protection to *C. elegans* during Oxidative Stress. Multivalency is important for galectin/galactoside binding in mammalian systems. We therefore hypothesized that *C. elegans* incubated with Gal β 1-4Fuc-functionalized dendrimers prior to oxidative stress should display increased survival rates. To test this hypothesis, *C. elegans* were treated with Gal β 1-4Fuc-functionalized dendrimers **8**, **9**, **10**, or **11** for 1 h prior to the addition of 3 mM hydrogen peroxide, and survival was determined every hour until all animals died.

During the first two hours of oxidative stress, control, *lec-1(RNAi)*, and *lec-10(RNAi)* worms had indistinguishable survival with or without treatment of Gal β 1-4Fuc-functionalized dendrimers **8**, **9**, **10**, or **11**. However, 3 h postoxidative stress, control and *lec-10(RNAi)*-treated animals pretreated with the Gal β 1-4Fuc-functionalized dendrimers had increased

survival compared to untreated animals (Figures 3 and 4). *lec-1(RNAi)* and *lec-10(RNAi)*-treated worms were hypersusceptible to 3 mM hydrogen peroxide (Figure 2), and pretreatment with Gal β 1-4Fuc functionalized dendrimers increased survival during oxidative stress challenge (Figures 3). Overall survival was increased by ~25 to 35% among control and *lec-10(RNAi)* worms. This suggests that Gal β 1-4Fuc-functionalized dendrimers offer protection to *C. elegans* during oxidative stress.

Overall, a statistically relevant increase in survival was not observed for *lec-1(RNAi)* relative to control worms following pre-treatment with any of the Gal β 1-4Fuc functionalized dendrimers **8**, **9**, **10**, or **11** (Figures 3, 4B, and S23B,E,H in the Supporting Information). Although LEC-1 is important for survival to oxidative stress, as suggested by decreased survival relative to the control during oxidative stress (Figure 2B), the results using glycodendrimers **8**–**11** suggest that there is potential redundancy among galectins in *C. elegans*.

Our results suggest that LEC-10 is not an essential multivalent binding partner for glycodendrimers **8**–**11** in this assay. The results upon addition of **8**–**11** were comparable for *L4440(RNAi)* and *lec-10(RNAi)* worms, indicating that one of the remaining galectins (and not LEC-10) is binding to the glycodendrimers. However, LEC-1 could be an important binding partner for multivalent carbohydrate interactions because *lec-1(RNAi)* worms showed no difference in survival rates for worms pre-exposed to M9 and glycodendrimers. When the amount of LEC-1 was reduced, the protective effect of glycodendrimers **8**–**11** was lost. This is especially interesting because the Gal β 1-4Fuc disaccharide endgroups of **8**–**11** have been reported to be the native binding partner for LEC-10 and not for LEC-1,³¹ meaning that the system was prebiased to afford exactly the opposite result of what was observed. Multivalent binding to an exogenous source of multivalent Gal β 1-4Fuc could reasonably be expected to overcome the monovalent preference of LEC-1 for Gal β 1-4GalNAc, explaining why the glycodendrimers reported here are active with LEC-1 even if monovalent binding affinities for Gal β 1-4Fuc with LEC-1 are relatively low.

Although we anticipated that the different generations of Gal β 1-4Fuc-functionalized dendrimers would have different effects—or different levels of effects—in the oxidative stress assay, this was not the observed result. No generational

dependence was observed, and all four generations afforded similar results. This could be because the inherent flexibility of the linking group compensates for the shape differences between the generations, thereby nullifying the distinctions between the frameworks in terms of disaccharide endgroup spacing. Alternatively, the concentrations of the glycodendrimers that were used may have been above the concentration at which generational differences could be observed. For human galectin-3, the ratio of galectin-3/glycodendrimer influences the size of the polymer–protein aggregate.⁴⁶ This could also be true for *C. elegans* galectins in which case the concentration at which the assay was performed may not have been optimized for identifying differences between the generations. Finally, it is possible that, for *C. elegans* galectins, the maximum observable effect was achieved by Gal β 1-4Fuc-functionalized G(2)-PAMAM dendrimers in which case the additional disaccharides provided on larger frameworks did not have a significantly different impact on the worm survival assays. Additional studies using concentration gradients of the glycodendrimers are expected to add clarity regarding this point.

AlexaFluor 488-Tagged Gal β 1-4Fuc Dendrimers Are Seen in the *C. elegans* Digestive Tract in Confocal Microscopy Experiments. To better ascertain the site of action of the dendrimers within the worm, a fluorescent tag (AlexaFluor 488) was added to all generations of dendrimers. Approximately one tag was added per dendrimer, as described in the Materials and Methods section. To determine where the dendrimers were functioning, worms were incubated in the fluorescently tagged glycodendrimer for 1 h before being anesthetized (Levamisole, 7 μ L of a 1 mM solution in M9 buffer) and imaged. As shown in Figure 5, the fluorescent signal was observed in the pharynx, gut, vulva, and sometimes in the neurons (fluorescein derivatives are known to collect in amphid and phasmid neurons).⁴⁷ However, G6 dendrimer 11 was not observed in the neurons. This could be due to the overwhelmingly strong signal in the gut masking any smaller signal in a neuron or because G6 glycodendrimer 11 may be too large to pass readily out of the gut or into a neuron.

LEC-1 is primarily expressed in the cuticle and pharynx, and LEC-10 is mainly expressed in the cytoplasm of intestinal cells.^{48,49} Differences in the overall localization of dendrimers in the three different RNAi knockdowns used were small, but in control *L4440(RNAi)* worms there was an observed larger physical area of fluorescence compared to the knockdowns. This is presumably because galectins are not suppressed in control animals, enabling more binding of the fluorescent glycodendrimers. Embryos, oocytes, and somatic gonads of more mature worms (i.e., Day 1 adults with fertilized embryos) were illuminated by the dye, as shown in Figure 6. Less fluorescence was observed in the neurons of *lec-1(RNAi)* worms. In addition, *lec-10(RNAi)* worms treated with dendrimers 10 or 11 had decreased dye uptake in the vulva relative to *lec-1(RNAi)* and control worms. An overall decrease of the signal in the vulva was also observed using dendrimers 8 and 9 in *lec-1(RNAi)* and *lec-10(RNAi)* knockdowns compared to control worms. A fluorescent signal was also seen in the proximal gonads. More studies are needed to fully rationalize the differences in observed distribution patterns for the different generations of fluorescently labeled dendrimers. Additional images are provided in the Supporting Information.

RNAi knockdown of *lec-1(RNAi)* or *lec-10(RNAi)* did not cause any obvious phenotypical abnormalities, which is

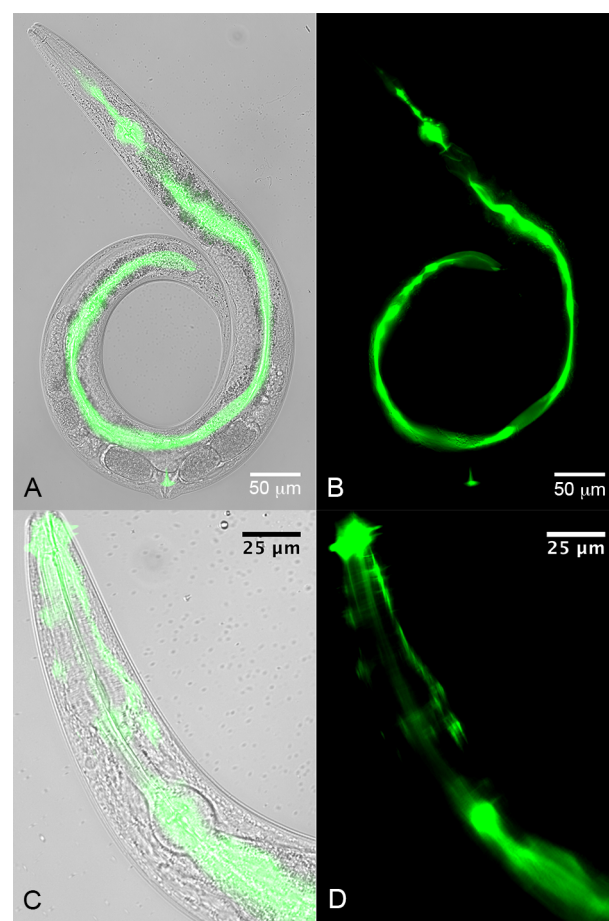


Figure 5. Gal β 1-4Fuc-functionalized G2 dendrimer 8 localizes throughout the body of Day 1 adult control *C. elegans*. (A and B) Fluorescently labeled 8 throughout the lumen of the intestine and the vulva, and (C and D) in the head and pharynx. (Green DIC exposure time: 327 ms and FITC exposure time: 1 s).

consistent with previously reported observations for the mutants.^{31,35} Although LEC-1 is expressed mainly in the cuticle and pharynx,⁴⁸ and LEC-10 is primarily expressed in the intestine,⁴⁹ only small differences were observed between the fluorescence images of *lec-1(RNAi)* and *lec-10(RNAi)* worms. This may be due to other lectins also binding to the glycodendrimers: for example, previous studies have reported that LEC-10 and LEC-6 share similar carbohydrate-binding partners,⁴⁹ and LEC-1 and LEC-6 can be coisolated.⁵⁰ More studies are needed to fully elucidate the binding of the glycodendrimers to the galectins in *C. elegans*.

CONCLUSIONS

Taken together, the results of these oxidative stress assays and imaging experiments in *C. elegans* suggest that the Gal β 1-4Fuc-functionalized dendrimers 8–11 are able to bind to worm galectins and protect them from oxidative stress. At the concentrations of glycodendrimers that were used in these studies, no generational dependence was observed for the dendrimers in the oxidative stress assays, with all four generations affording similar results. *C. elegans* *L4440(RNAi)* and *lec-10(RNAi)* that were pre-incubated with Gal β 1-4Fuc-functionalized dendrimers 8–11 before being exposed to oxidative stress were afforded a significant survival advantage, but *lec-1(RNAi)* was not protected by the glycodendrimers.

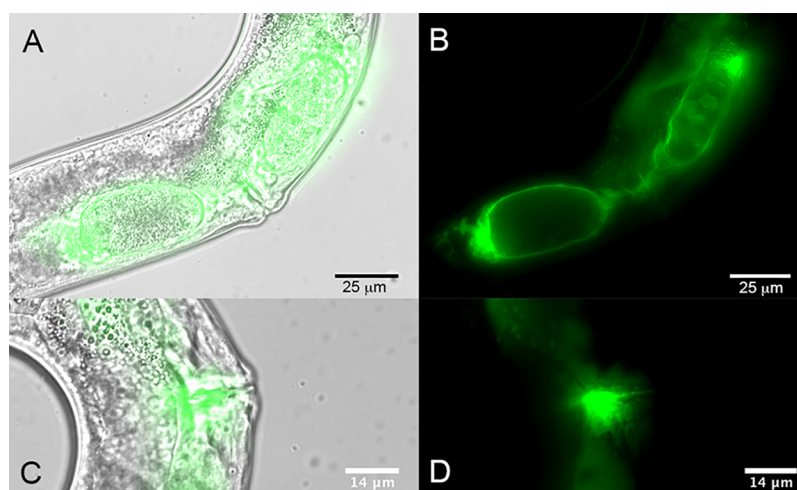


Figure 6. Galβ1-4Fuc-functionalized G2 dendrimer **8** shown localizes to the vulva and somatic gonad in Day 1 adult *C. elegans*. (A and B) **8** localizes throughout reproductive organs including the eggs in control worms, and (C and D) the vulva *lec-10(RNAi)* worms. (Green DIC exposure time: 327 ms and FITC exposure time: 1 s (B) and 865 ms(D)).

These results suggest that multivalently presented Galβ1-4Fuc can protect *C. elegans* from oxidative stress by binding to galectins including LEC-1. Future knock down experiments with worms lacking other galectins and also lacking combinations of galectins will enable us to determine whether LEC-1 is the only galectin in *C. elegans* that is impacted by exposure to glycodendrimers or whether other galectins can also be studied in oxidative stress assays using carbohydrate-functionalized dendrimers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biomac.1c01001>.

¹H NMR and ¹³C NMR spectra of **6a**, **6b**, and **7–11**; MALDI-TOF MS spectra of **8–11** and their protected precursors; Kaplan–Meir log rank analysis of **8–11** with *lec-1(RNAi)*, *lec-10(RNAi)*, and *L4440(RNAi)*; confocal images of **8–11** with *lec-1(RNAi)*, *lec-10(RNAi)*, and *L4440(RNAi)* (PDF)

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Author Contributions

H.V.K. synthesized and characterized the glycodendrimers. R.S.M. prepared the *C. elegans* and supervised all experiments involving *C. elegans*, which were performed with the help of H.V.K. and M.J.C. M.J.C. supervised all experimental work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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