

GLYCODENDRIMERS: TOOLS TO STUDY MULTIVALENT GALECTIN-1
INTERACTIONS

by

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For my amazing wife and children.

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ABSTRACT

Galectin-1 is a carbohydrate binding protein that mediates cancer processes through multivalent interactions with glycoproteins expressed on the surface of cancer cells and in the extracellular matrix. A series of multivalent PAMAM dendrimers were functionalized with lactose and applied to the study and mediation of multivalent galectin-1 interactions. An ELISA was designed to study the interaction of galectin-1 with surface immobilized glycodendrimers. The results of the ELISAs indicate that galectin-1 binds well to the multivalent framework. DLS and fluorescence microscopy were used to study that interaction of galectin-1 with glycodendrimers in solution. These solution-based assays indicate that the glycodendrimers nucleate galectin-1 into nanoparticles. The ability of the glycodendrimers to organize the galectin-1 into biologically active arrays was investigated in cellular assays. A homotypic cellular aggregation assay using DU145 human prostate carcinoma cells, which express a putative galectin-1 ligand (Mucin1), was designed to study the influence of multivalent glycodendrimers on cellular aggregation/tumor formation. All generations of glycodendrimers were observed to inhibit cellular aggregation by diverting the galectin-1 from its native role in cellular cross-linking of cancer cells. To further probe multivalent interactions in cancer, the glycodendrimers were applied to a tube formation assay to study galectin-1 angiogenic processes. Galectin-1 was observed to accelerate neovascularization, and the impact of the galectin-1 was mildly inhibited by the glycodendrimers.

CHAPTER 1

OVERVIEW OF THE ROLE OF GALECTIN-1 IN CANCER PROGRESSION
AND SYNTHETIC MULTIVALENT SYSTEMS FOR THE STUDY OF GALECTIN-1Introduction

Each year, approximately 8 million people die from cancer and another 14 million people are diagnosed with the disease.¹ Current cancer treatment strategies generally employ a combination of therapies, including targeted therapies. Targeted therapies typically use small, monovalent molecules to inhibit a specific metastatic pathway. For example, several small molecule kinase inhibitors, which target specific transformative activities of cancer cells involved in proliferation and survival, have been approved by the U.S. Food and Drug Agency (FDA).² However, major drawbacks to the use of small, monovalent molecules as cancer therapeutics include weak binding constants and fast dissociation rates.⁶

Multivalency, the binding of multiple ligands to multiple receptor binding sites,^{7,5} is a powerful approach to treat cancer malignancies while also providing a platform to better understand cellular mechanisms that drive cancer metastasis. Multivalent protein-carbohydrate interactions mediate a myriad of malignant cellular processes, including cellular aggregation/tumor formation, metastasis, and angiogenesis.^{3,8,9} These multivalent protein-carbohydrate interactions generally rely on multiple points of attachment to enhance the individual binding interaction between one carbohydrate and its receptor, which is typically weak.^{7,10,11}

Proteins that mediate multivalent malignant cellular activities are intriguing molecular targets.⁸ Galectin-1, for example, is a multivalent carbohydrate binding protein that mediates the malignant cellular activities by cross-linking glycoproteins in the tumor microenvironment. Specifically, galectin-1 has been reported to be involved in multivalent mechanisms that cluster cell surface glycoproteins,^{12,13} cross-link receptors,^{14,15} and form lattices and larger aggregates.^{16,17,18}

Multivalent frameworks have proven to be powerful tools to modulate and study protein carbohydrate interactions. A variety of synthetic multivalent scaffolds including linear polymers,^{19,20,21} star^{22,23,24} and hyperbranched^{25,26,27} polymers, gold nanoparticles,^{28,29,30} dendrimers,³¹ proteins,³² beads³³ and surfaces^{34,35,36,37} have been functionalized with carbohydrates and then applied to the study and the mediation of multivalent protein-carbohydrate interactions.³⁸ For example, these carbohydrate functionalized scaffolds have been used to study biological processes such as cellular aggregation/tumor formation³⁹ viral cell attachment^{40,32} bacterial recognition,⁴¹ and signal transduction.⁴² Much of galectin-1 pathways remains unresolved, and there is a paucity of studies using multivalent frameworks to explore multivalent galectin-1 mechanisms. A better understanding of galectin-1 mechanisms can advance the overall understanding of malignant cellular activities and give insight into the rational design of multivalent therapeutics.

This chapter will cover galectin-1, its role in the tumor microenvironment, and multivalent mechanisms in cancer. First, the structure and function of galectin-1 will be discussed. The next sections will cover multivalent interactions involving galectin-1 in

cellular adhesion, mobility and invasion, tumor-induced angiogenesis, and apoptosis. Then, a background of multivalent interactions are discussed. Last, synthetic frameworks that have been used to modulate galectin-1 processes are reviewed.

Galectin-1 Structure and Function

The Galectin Family of Lectins

Galectin-1 is one of 15 members of the β -galactoside binding family of proteins called the galectins, which share a conserved amino acid sequence in the carbohydrate recognition domain (CRD).⁴³ The galectin family can be subdivided into three groups based on the structure of the protein: (i) monovalent galectins containing one CRD that are capable of homodimerizing to become functionally bivalent; (ii) bivalent galectins with two non-identical CRDs connected by a peptide linker; and (iii) chimeric galectins with one CRD and a unique N-terminus.^{44,45} Galectin-1, 2, 5, 7, 10, 11, 13, 14, and 15 have one CRD and are capable of forming homodimers. Those with two dissimilar CRDs connected by a short linker peptide include galectin-4, 6, 8, 9, and 12. Galectin-3 is the only chimera-type; this protein consists of a C-terminal CRD fused to a non-lectin N-terminal domain composed of tandem repeats of short amino-acid stretches that participates in oligomerization.^{44,46} Glycan-binding specificity, protein valency, and cross-linking properties of individual galectins differentiates their biological responses.^{15, 44,47,48,49,50} Of the 15 members of the lectin family, galectin-1 and galectin-3 appear to be the major players in cancer biology and, therefore, have stimulated significant research interest.⁵⁰⁻⁵² However, less is known about galectin-1 pathways compared to galectin-3.

Galectin-1

Galectin-1 is a homodimeric protein composed of 14.5 kDa subunits;⁴³ the dimer is maintained by hydrophobic interactions at the monomeric interface and by the well-defined hydrophobic core (**Figure 1**).⁵³ As shown in **Figure 1**, the monomeric units are anchored such that the two CRDs are located on opposing ends of the quaternary structure at a distance of approximately 5 nm.¹⁷ Each CRD is able to accommodate a tetrasaccharide.⁴⁶ The apposing CRDs initiates cellular recognition and signal transduction events by binding appropriately glycosylated ligands.⁴⁶

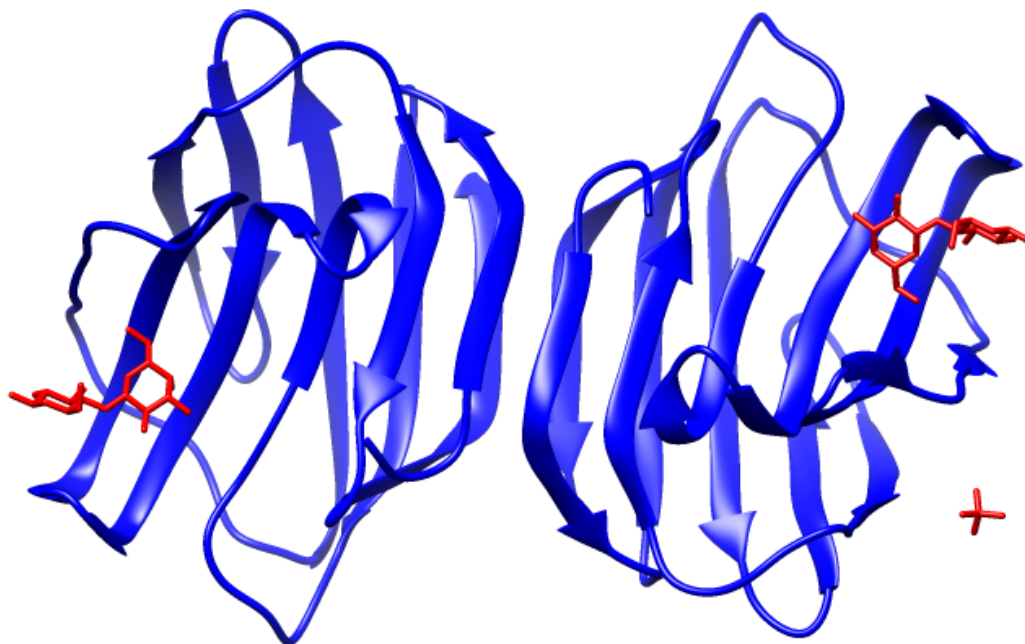


Figure 1. Dimeric structure of galectin-1. Galectin-1 (blue) with lactose (red) bound in the apposing carbohydrate recognition domains.

Galectin-1 Synthesis and Secretion

As is characteristic of cytoplasmic proteins, galectin-1 is synthesized on cytosolic ribosomes and possesses the archetypal acetylated N-termini and lack of signal peptides.⁴⁶

From the cytosol, galectin-1 can be targeted to cell nuclei, translocated to the intracellular side of cell membranes, or secreted.^{46, 53} Secretion occurs through an unorthodox mechanism that involves direct translocation across the plasma membrane, bypassing the classical vesicle-mediated endoplasmic reticulum/Golgi pathway for exocytosis.^{44, 53} This novel export machinery uses β -galactoside-containing surface molecules as export receptors for intracellular galectin-1.^{44, 53} By specifically targeting the β -galactoside binding site motif, this export machinery provides a quality control mechanism that recognizes only properly folded galectin-1.⁵³ Although galectin-1 lacks identifiable secretion signal sequences, it is found on the extracellular side of cell membranes and in the extracellular matrix (ECM) of various normal and neoplastic tissues.^{46, 53}

Galectin-1 in Cancer

The extracellular expression of galectin-1 is altered in a variety of cancer cell types,⁵⁴ including melanoma,^{55, 56} ovarian,⁵⁷ lung,⁵⁸ prostate,^{59, 60} bladder,⁶¹ thyroid,⁶² pancreatic,⁶³ head-neck,⁶⁴ cervical,⁶⁵ uterine,⁶⁶ and colorectal cancers.⁶⁷ In addition, galectin-1 is often overexpressed in the stroma surrounding tumor cells.^{68,69}

The increased expression of galectin-1 has been correlated with a variety of processes in cancer progression, including the cellular aggregation/tumor formation, metastatic spread of cancer, angiogenesis, and apoptosis.^{9,50,53} A brief summary of galectin-1 binding partners, cellular location, and associated activities in cancer is provided in **Table 1**. More detail regarding the binding partners and associated biological activities of galectin-1 are provided in recent reviews.^{9, 44, 47, 50, 53, 70}

Intracellular Galectin-1

Intracellular galectin-1 acts as a scaffold protein for intracellular signaling pathways in a carbohydrate independent manner. Relevant to cancer cell biology, intracellular galectin-1 participates in protein-protein interactions with H-Ras,⁷¹ protocadherin-24,⁷² and Gemin4⁷³ in a carbohydrate-independent manner.^{50, 53} These proteins are structurally unrelated and do not share any common domains or motifs.⁵³

Table 1. Galectin-1 binding partners and associated cancer processes.

Localization	Binding partner	Biological activities	Cell Type	Refs.
Intracellular	H-Ras	H-Ras/MEK/ERK cascade activation	Bladder cancer	71
	Pro-24	B-catenin signaling inhibition	Colon cancer	72
	Gemin4	Pre-RNA splicing modulation	Cervical cancer	73
Extracellular	90K/Mac-2BP	Homotypic cell adhesion	Melanoma	56
	Mucin-1	Cell adhesion	Prostate cancer	74
	Laminin	Cell-ECM adhesion	Endothelial	75
	Fibronectin	Cell-ECM adhesion	Endothelial	76
	Neuropilin-1	Proliferation, migration, and adhesion induction	Endothelial	77
	VEGFR	Neovascularization activation	Endothelial	13
	CD45	Membrane redistribution, and T-cell death induction	T-cell	78, 79
	CD43	Membrane redistribution, and T-cell death induction	T-cell	14, 78
	CD7	T-cell death induction	T-cell	78

Extracellular Galectin-1

Extracellular galectin-1 participates in the defining β -galactoside binding activity.^{44, 56, 60, 75,80,81} For example, the metastatic spread of cancer occurs in part through interactions between galectin-1 and glycoproteins in the ECM, such as laminin and fibronectin.⁵³ The binding of cell-surface glycoproteins, such 90K/Mac-2BP and Mucin-1, by galectin-1 mediates cellular aggregation/tumor formation.⁵³ Galectin-1 mechanisms in

cellular aggregation/tumor formation, migration and invasion, tumor-induced angiogenesis, and apoptosis of activated T cells are discussed in detail in the following sections of this chapter.

Galectin-1 Mechanisms of Action in Cancer

This section will address galectin-1 mediated mechanisms in cellular aggregation, adhesion to the ECM, migration and invasion, angiogenesis, and apoptosis.

Homotypic Cellular Aggregation

Galectin-1 has been reported to mediate homotypic aggregation of cancer cells through multivalent interactions with cell-surface glycoconjugates in a variety of cell types.^{50, 51} Tinari *et al.* demonstrated that galectin-1 induces homotypic aggregation in human melanoma cells (A375).⁵⁶ Galectin-1 was observed to bivalently bind the cell surface glycoprotein 90K/Mac-2BP on adjacent cells to promote aggregation.⁵⁶

Mucin-1 is another putative cell-surface receptor of galectin-1.^{53, 82} Mucin-1 is a large transmembrane protein that is overexpressed and aberrantly glycosylated with the Thompson-Freidenreich (TF) antigen (a Gal β 1-3GalNAc disaccharide).⁸³ Galectin-1 binding to the TF antigen was reported by Jeschke *et al.*⁷⁴ The TF antigen was conjugated on a polyacrylamide framework (TF-PAA), and galectin-1 binding to TF-PAA was monitored by following cellular aggregation using a chorionic carcinoma cell line (BeWo) known to express Mucin-1.⁷⁴ TF-PAA was observed to strongly diminish the interactions that drive cellular aggregation in the BeWo cell line, indicating that the binding of galectin-1 to the TF antigen expressed on Mucin-1 mediated cellular aggregation.⁷⁴

Providing further support for the galectin-1 TF antigen interaction as a significant pathway in cancer cell aggregation, Glinsky *et al.* observed the interaction of galectin-1 with the TF-antigen on MDA-MB-435 breast cancer cells.⁸⁴ Using confocal microscopy, galectin-1 accumulation was observed at the interface between MDA-MB-435 cells.⁸⁴ Aggregation was inhibited by a the TF-antigen specific peptide P-30, indicating the galectin-1 binding to the TF-antigen mediated homotypic aggregation.⁸⁴

These studies indicate that multivalent interactions of galectin-1 with cell-surface glycoconjugates (e.g., Mucin-1) mediate aggregation through cellular cross-linking (**Figure 2**).⁸¹ Multivalent binding of TF antigen by galectins has been reported to cluster Mucin 1 on the cell surface, which facilitates cellular aggregation by exposing adhesion molecules.^{82,85} Although the mechanism of galectin-1 Mucin-1 mediated cellular aggregation is not fully resolved, it is likely similar to that of galectin-3. Galectin-3 mediates cellular aggregation by binding the TF antigen expressed on Mucin-1.⁸² Galectin-3 binding to Mucin-1 in the human colon cancer cell line HT-29 was monitored by confocal microscopy, which revealed that the interaction of galectin-3 with Mucin-1 polarized the cell surface⁸². Mucin-1 becomes clustered on the surface of the cell, which exposes smaller adhesion molecules previously concealed by the large transmembrane protein.⁸² Exposed adhesion molecules interact with adhesion molecules on neighboring cells to aggregate cells. Because both galectin-1 and galectin-3 are known to bind to the TF antigen on Mucin-1,⁷⁴ it is therefore likely that galectin-1 mediates aggregates through similar a mechanistic pathway. Thus, it is likely that galectin-1 mediates cellular aggregation by cross-linking

adjacent cells and exposing adhesion molecules through the clustering of Mucin-1 (**Figure 2**).

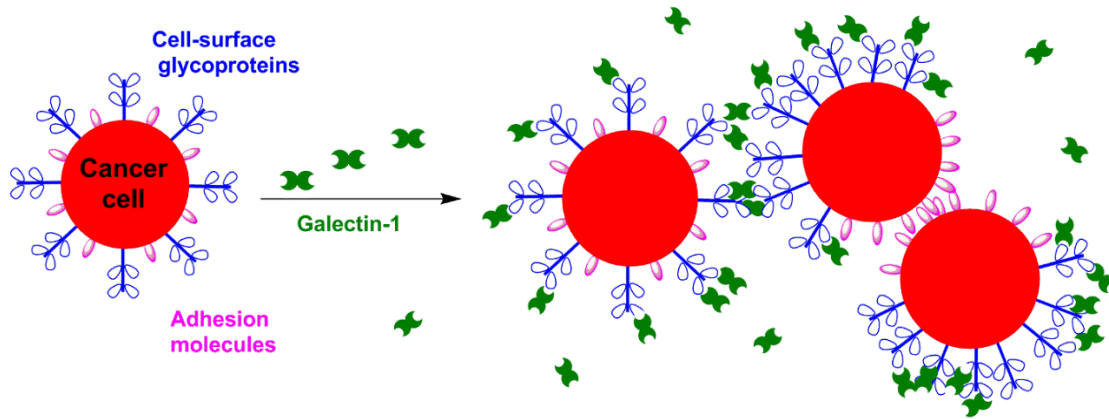


Figure 2. Galectin-1 mediation of homotypic cellular aggregation. Galectin-1 mediates homotypic aggregation of cancer cells through multivalent interactions with cell-surface glycoproteins on adjacent cells.

Heterotypic Cellular Aggregation

Galectin-1 is also known to mediate the adhesion of tumor cells to endothelial cells, thereby promoting dissemination of tumor cells in the human body.^{84,86} Tumor cell-endothelial cell adhesion proceeds through interactions with cell-surface adhesion molecules, such as integrins, ICAM-1, VCAM-1, and selectins.^{87,88} Clause *et al.* demonstrated enhanced adhesion of prostate carcinoma cells (PC-3 and DU145) to endothelial cells in the presence of galectin-1.⁸⁶ Furthermore, Glinsky *et al.* demonstrated that galectin-1 accumulates at tumor cell-endothelial cell contact points, which suggests that tumor associated endothelium expresses cognate galectin-1 receptors.⁸⁴

Cellular Adhesion to the ECM

Galectin-1 has been observed to arbitrate the adhesion of cancer cells to the ECM.^{60,}
⁷⁶ Laminin, fibronectin, and other glycoproteins presented in the basement membrane provide the necessary epitopes for galectin-1 cell-ECM cross-linking.^{70,89} A comprehensive list of basement membrane proteins and associated biological activities is provided in the referenced review.⁷⁰

Galectin-1 has been observed to biphasically arbitrate cell-ECM interactions. Dose-dependent binding of galectin-1 to laminin and fibronectin in cell-ECM adhesion has been reported in melanoma⁷⁵ and ovarian⁷⁶ cancer cell lines. As shown in **Figure 3a**, the pro-adhesive mechanism proceeds through galectin-1 mediated cross-linking of ECM proteins and cell-surface glycoconjugates. These studies suggest that one activity of galectin-1 is to modulate cancer cell adhesion during metastasis. Conversely, negative regulation of cell-ECM adhesion, in which galectin-1 inhibits cell-ECM interactions by competitively binding matrix glycoproteins or cell-surface glycoconjugates, has also been well documented (**Figure 3b**). In studies by the Barondes' group, differentiating C2C12 mouse myoblasts were adhered to a laminin-immobilized surface.⁹⁰ Exogenous galectin-1 was observed to inhibit adhesion to and migration on the laminin-coated plates.⁹⁰ Similar anti-adhesive properties have been observed with other cell types in the presence of exogenous galectin-1.^{91,92,93} These studies suggest another role for galectin-1 – the modulation of tumor cell detachment, which allows tumor cells to travel to a secondary site.

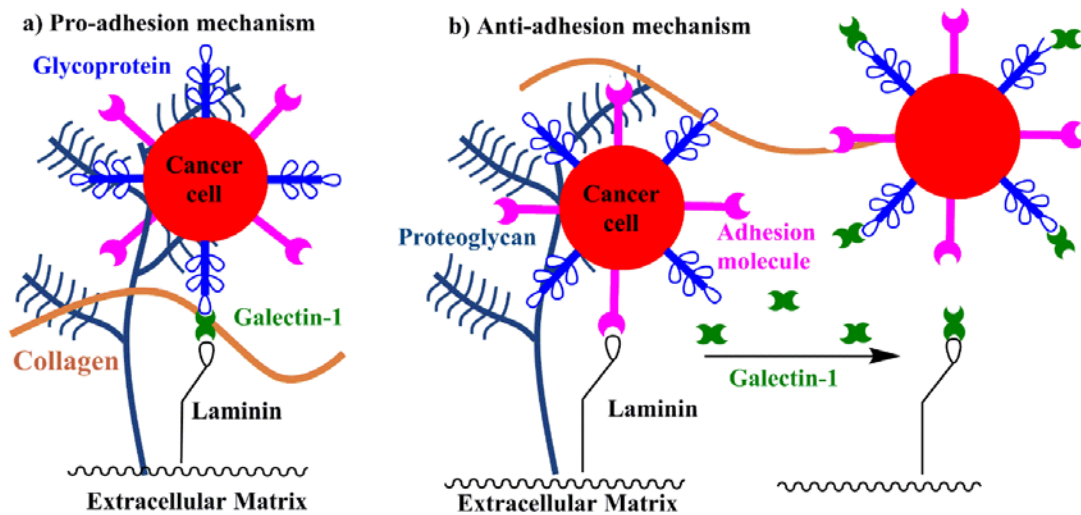


Figure 3. Biphasic arbitration of cell-ECM interactions by galectin-1. (a) Galectin-1 mediated cross-linking of cell-surface glycoconjugates and ECM glycoproteins promotes adhesion. (b) Competitive binding to ECM glycoproteins by galectin-1 inhibits adhesion and promotes dissemination of tumor cells.

Taken together, these studies demonstrate that galectin-1 binding to ECM glycoproteins arbitrates cancer cell adhesion and detachment. Factors that may account for the biphasic activity include: (i) expression of cell-type dependent glycoconjugate ligands for galectin-1; (ii) expression of cell-type dependent receptors for ECM proteins that influence galectin-1 binding; (iii) co-expression of multiple galectins exhibiting antagonistic properties; and (iv) changes in expression levels and/or oligomerization of galectin-1.⁷⁰

Metastasis: Cancer Cell Migration and Invasion

The hematogenous dissemination of cancer cells is essential to metastasis. During the metastatic spread of cancer, malignant cells detach from a primary site and migrate to and invade a secondary site.⁸⁹ Galectin-1 expression has been identified as a signature of cellular invasiveness of mammary carcinoma cell.⁹⁴ Furthermore, elevated levels of

galectin-1 have been measured in the tissue at the invasion front of glioblastoma tumors^{95,96,97} and oral squamous cell carcinomas.⁹⁸ A comprehensive list of cancer cell types in which galectin-1 has been observed to mediate migration and invasion is provided in a recent review.⁷⁰

Galectin-1 is involved in multiple metastatic processes: (1) adhesion of tumor cells to the ECM;^{70,89} (2) binding of ECM glycoproteins;^{9,70} and (3) enhancing proteolytic enzyme pathways.^{99,100} Initially, galectin-1 mediates tumor cell-ECM adhesion at the primary site by cross-linking cell surface glycoproteins, such as integrins,⁸⁹ and glycosylated proteins in the ECM,¹⁰¹ such as laminin and fibronectin.⁵¹ Galectin-1 then directly mediates migration and invasion by competitively binding receptors involved in cell-ECM interactions, which allows cancer cells to detach from the primary site (**Figure 4**).^{9,70} Pursuant to this mechanism, a correlation likely exists between a tumor cell's transformation to a migratory phenotype and galectin-1 expression levels: extracellular galectin-1 expression is likely up-regulated in order to competitively bind glycoconjugates involved in cell-ECM cross-linking interactions. Endothelial cell migration has been impaired by interfering with endogenous galectin-1 expression by, for example, siRNAs or antisense nucleotides, which supports the direct involvement of galectin-1 binding in tumor cell migration.^{77, 102, 103} Once the tumor cell migrates to and invades a secondary site, galectin-1 mediates adhesion by cross-linking tumor cells with the ECM.

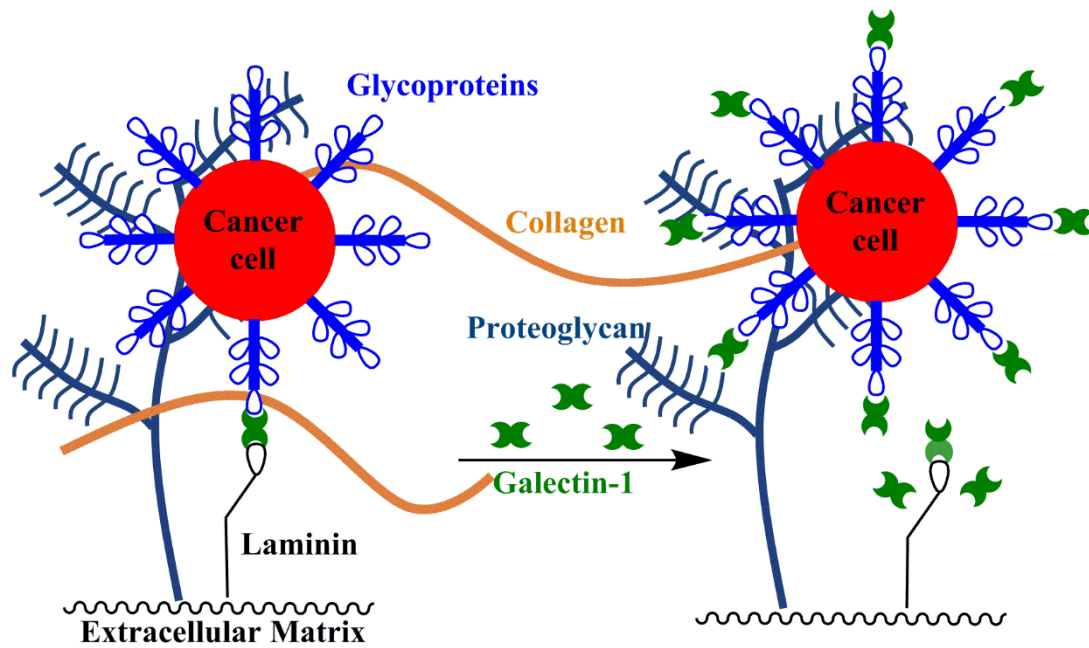


Figure 4. Galectin-1 competitively binds receptors involved in cell-ECM adhesion to promote migration and invasion.

Extracellular galectin-1 has also been observed to stimulate up-regulation and secretion of proteolytic enzymes, such as matrix metalloproteinases (MMP), that degrade ECM glycoproteins.⁹⁹ Activation of the signal transduction pathway that triggers secretion of proteolytic enzymes most likely occurs through galectin-1 mediated clustering of cell-surface receptors.

Tumor Induced Angiogenesis

This section will discuss galectin-1 mediation of proangiogenic pathways. Angiogenesis is a complex processes and has been reviewed in great detail.^{104,105,106} Here, a brief overview of angiogenesis will be provided followed by a discussion of galectin-1 mechanisms in angiogenesis.

Angiogenesis is the growth of new blood vessels out of preexisting capillaries. Blood vessels are fundamentally composed of endothelial cells, and once activated, these cells can extend the vascular network by interconnecting to form tubes that direct and maintain blood flow.¹⁰⁴ Tubule formation involves matrix degradation, proliferation, migration, tube formation, and matrix deposition and is depicted in Figure 5.³

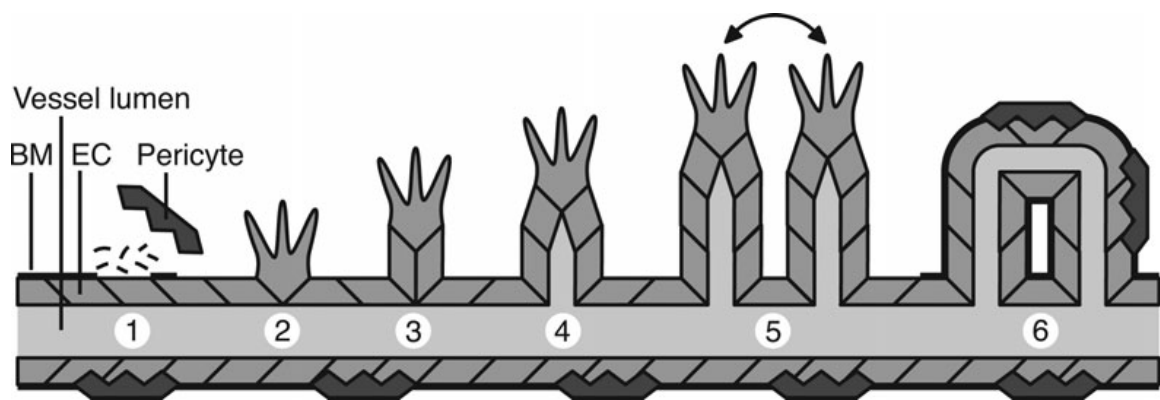


Figure 5. Angiogenesis. Illustration of the angiogenesis cascade that involves: (1) Pericyte detachment and basal membrane degradation in response to endothelial cell activation; (2) Migration of endothelial tip cells in the direction of the growth factor gradient; (3) Provision of support of endothelial tip cells by the underlying stalk cells; (4) Continuation of this process to form luminized vessel sprouts; (5) Fusion of sprouts; and (6) Formation of a functional vessel which is further stabilized by deposition of a basal membrane and attraction of pericytes for structural support.³ Figure reproduced with permission from 3.

Angiogenesis occurs naturally during ovulation and wound healing.¹⁰⁴ Tumor-induced angiogenesis, on the other hand, is a pathologic condition in which tumor cells secrete growth factors, such as vascular endothelial growth factors (VEGFs), to promote the growth of new blood vessels.^{105, 107} These growth factors activate quiescent vasculature in host tissue to stimulate the growth of new capillaries.¹⁰⁴ The growth of new capillaries is an indispensable process of metastasis – angiogenesis must occur for tumors to grow beyond a critical size of a few mm².^{104, 108} New blood vessels generated from existing

vasculature provide tumors with the necessary blood supply, oxygen, and nutrients for proliferation. Thus, the neovascular system provides a critical interface between cancer cells and host tissue.

During angiogenesis, endothelial cells are involved in different processes to form the neovasculature, and galectin-1 binding of glycoconjugates mediates many of these processes. Elevated levels of galectin-1 expression have been observed in the vasculature of many tumors, including prostate,^{86, 102} lung,¹⁰⁹ colon,¹¹⁰ and oral.⁷⁷ While galectin-1 has been observed to enhance tumor-induced angiogenic processes,^{107, 111, 112} the mechanisms of action, however, have not been fully resolved.

Endothelial cell activation is the first step in the angiogenic cascade, and this initiates pericyte detachment, basal membrane degradation, and endothelial cell migration.¹⁰⁵ Vascular endothelial growth factor receptors (VEGFR) expressed on the surface of endothelial cells are activated through binding of vascular endothelial growth factor (VEGF), which initiates pro-angiogenic cellular processes. Galectin-1 has been observed to participate in VEGFR activation,^{13,77,103} and there are two likely mechanistic pathways for galectin-1 involvement: (i) cross-linking of co-receptors (e.g. NRP-1) that initiates VEGFR activation (**Figure 6a**); or (ii) clustering of VEGFR, which renders these receptors more accessible to VEGF (**Figure 6b**).

Galectin-1 binding to neuropilin-1 (NRP-1), a membrane-bound co-receptor for VEGFs, has been observed to activate VEGFR2 signaling.^{13, 77} Ensuing from the galectin-1/NRP-1 interaction, enhanced adhesion and migration of endothelial cells was observed.⁷⁷ Binding of galectin-1 to VEGFR2, however, was not observed, indicating that galectin-1

binding to NRP-1 indirectly activates VEGFR2 signaling.⁷⁷ In light of the propensity of galectin-1 to cluster receptors, activation of the VEGFR2 signaling pathway likely proceeds through galectin-1 mediated clustering of NRP-1 (**Figure 6a**). Furthermore, galectin-1 has been reported to directly bind to and induce the clustering of VEGFRs (**Figure 6b**).¹³ Increased density of VEGFRs renders these receptors more accessible to VEGFs.

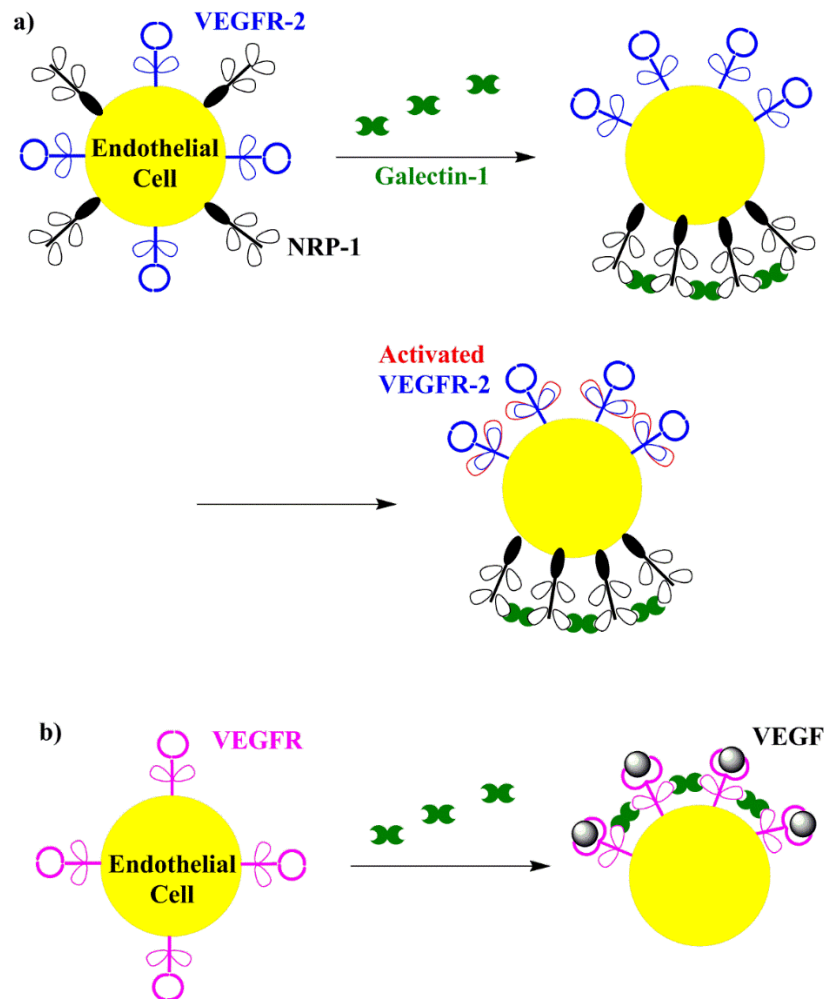


Figure 6. Galectin-1 mediated endothelial cell activation. (a) Binding of galectin-1 to NRP-1 initiates VEGFR activation signal; and (b) Clustering of VEGFRs so that the receptors are more accessible to VEGF.

It is likely that the glycosylation patterns of cell surface receptors (e.g., VEGFRs and NRP-1) discriminates galectin-1 binding. Croci *et al.* observed that glycan specific binding of galectin-1 to VEGFR-2 promoted EC signaling and preserved the angiogenic phenotype in the absence of the putative binding partner, VEGF-A.¹¹² Glycome remodeling of the EC surface facilitated binding of galectin-1 to *N*-glycans expressed on VEGFR-2 in anti-VEGF refractory tumors but inhibited galectin-1 binding in anti-VEGF sensitive tumors, which explains the proliferation of certain tumor types during anti-VEGF treatment.¹¹² In contrast to anti-VEGF-sensitive tumors, which display high levels of α 2-6-linked sialic acid glycans that inhibit galectin-1 binding, anti-VEGF refractory tumors exhibit vasculature glycosylation patterns (e.g. β 1-6GlcNAc) that facilitate interactions with galectin-1 and an increase in galectin-1 expression.¹¹²

Tumor vascularization, another critical component of the angiogenic cascade, has been correlated with elevated levels of galectin-1 in the endothelium.^{13,110,111,89} In addition to activating endothelial cells, it has been proposed that galectin-1 acts as a scaffold for neovasculature, supporting new capillary growth by cross-linking endothelial cells and in the ECM (**Figure 7**).⁷⁰ Vascular endothelial cells have highly glycosylated cell-surfaces,⁵² and the ECM in the tumor stroma contains significant levels of known galectin-1 receptors laminin and fibronectin.⁵² Activated endothelial cells, after invading the stroma, adhere to the ECM to form a lumen of a new capillary tube.¹⁰⁴ Capillary tubes then coalesce into loops, forming the new vasculature paramount for tumor growth and metastasis.¹⁰⁴ Galectin-1 likely acts as a scaffold for vessel growth and vascular network formation by establishing physical connections between vascular endothelial cells and the ECM in the

tumor microenvironment, which provide the necessary physical support for neovasculature.^{27,102,113} Vascularization of endothelial cells (EAhy926 cells and HUVECs) has been observed in tube formation assays.^{13, 114}

In galectin-1 knockout experiments using mice, impedance of tumor growth due to inadequate angiogenesis has been observed, which suggests that galectin-1 forms multivalent lattices to support neovasculature.^{102, 103} Furthermore, *in vivo* inhibition studies of galectin-1 activity in the chick chorioallantoic membrane (CAM) revealed tortuous and irregular neovessel growth in the absence of galectin-1, indicating defective vascular guidance.¹⁰² Interestingly, galectin-1 inhibition decreased neovascularization in a manner similar to anginex, a galectin-1 specific angiogenesis inhibitor.¹⁰²

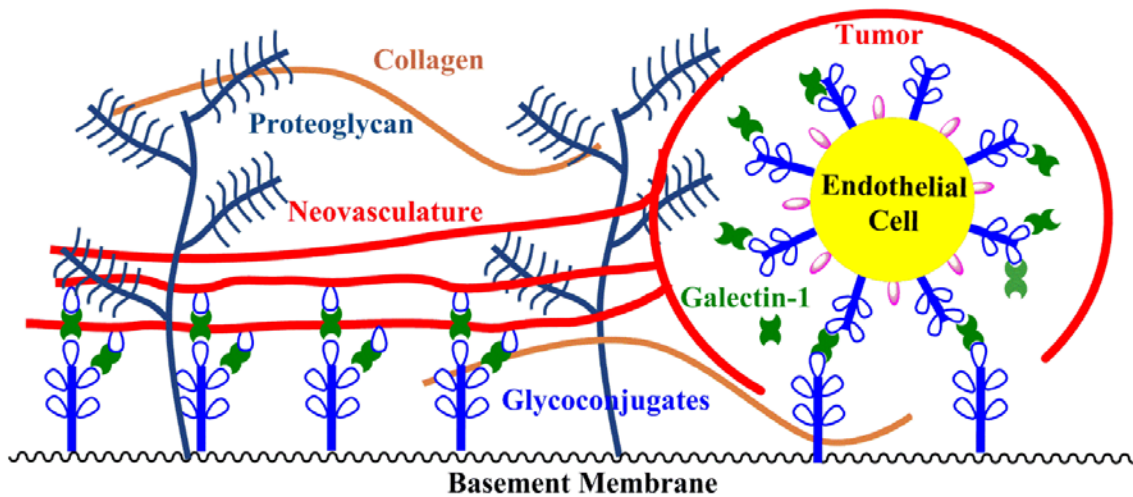


Figure 7. Provision of structural support for neovasculature by galectin-1.

These studies indicate that galectin-1 augments key angiogenic pathways by mediating the activation of endothelial cells and the formation of a neovasculature network through multivalent carbohydrate binding.^{77,102,103, 107,110, 113}

Galectin-1 Induced T-Cell Apoptosis

The poor prognosis associated with elevated galectin-1 expression is related to tumor evasion of the immune response.⁵³ Tumors evade the immune response by secreting galectin-1, which triggers apoptosis of infiltrating T-cells. This section will discuss the mechanism of galectin-1-induced apoptosis of activated T-cells that allows tumors to evade the immune response.

Galectin-1 regulates apoptotic signaling pathways through colocalization of receptors into signaling complexes.^{15,16,42, 115} CD45, CD43, and CD7 have been identified as specific apoptotic-related receptors for galectin-1.⁷⁸ As shown in **Figure 8**, galectin-1 cross-linking induces segregation and clustering of these receptors into discrete membrane microdomains, which are capable of transducing the cell death signal.⁷⁸ Pace *et al.* performed immunofluorescent localization studies using confocal microscopy to monitor the localization of the receptors before and after galectin-1 treatment.⁷⁸ Prior to galectin-1 treatment, receptors CD45 and CD43 were randomly distributed across the cell surface.⁷⁸ Galectin-1 binding resulted in redistribution of these receptors and localization of CD45 in apoptotic membrane blebs⁷⁸. Interestingly, CD43 was observed to segregate from CD45 and to colocalize with CD7 in distinct blebs on the cell-surface.^{14,78} These results suggest that CD43 may act as a galectin-1 concentrating agent, facilitating subsequent interactions between galectin-1 and CD7.¹⁴ Furthermore, only dimeric galectin-1 activates the apoptotic signaling pathway that triggers the death of activated T-cells, which indicates that bivalent cross-linking of cell-surface receptors mediates transduction of apoptotic signals.⁷⁹

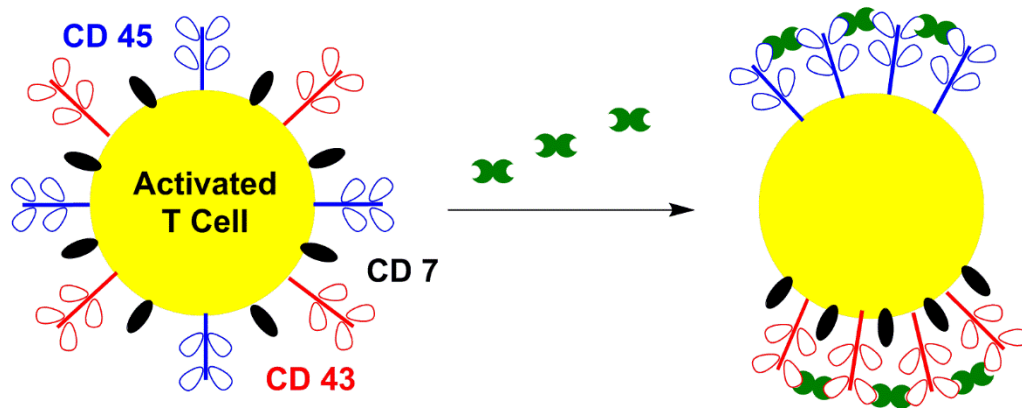


Figure 8. Galectin-1 mediated T-cell apoptosis. Galectin-1 induces segregation and clustering of CD45 in distinct microdomains from CD43/CD7 complexes.

Localization of apoptotic receptors into homogenous signaling complexes is critical to T-cell apoptosis: the signaling pathway that transmits the cell death signal is activated upon galectin-1 mediated segregation and localization.¹⁴ A remarkable characteristic of galectin-1 apoptosis mechanism is the ability of the protein to discriminate among and homogeneously cluster cell-surface receptors via selective cross-linking. In addition to the β -galactoside specificity provided by the hydrogen bonding amino acids in the CRD, the extended binding pocket of galectin-1 permits discrimination among receptors in terms of the galactosides composition of the receptor.⁴⁸

A distinct mechanism for galectin-1-mediated immunosuppression was proposed by Rubinstein *et al.* whereby tumor secreted galectin-1 impairs T-cell effector functions involved in eradicating cancer cells from a host.¹¹⁶ Effector impairment was suggested to occur either by activation of T-cell apoptotic signaling pathways or by elevation of the activation threshold of naive T cells.¹¹⁶ In light of the findings by Pace *et al.* discussed above, it is likely that galectin-1-mediated T-cell receptor clustering activates T-cell apoptotic signaling pathways involved in immunosuppression.

In support of the role of galectin-1 in tumor immunosuppression, *in vivo* blockage of galectin-1 expression in tumor cells of syngeneic mice promoted tumor rejection and stimulated tumor-specific T cell-mediated responses.¹¹⁶ Upon subsequent treatment with galectin-1 sufficient tumors, the syngeneic mice exhibited an enhanced tumor immune response by resisting tumor challenge.¹¹⁶ Together, these results demonstrate the importance of multivalent galectin-1 mechanisms in tumor immunosuppression.

Multivalent Mechanisms

From the above discussion of galectin-1 mediated cellular mechanisms in cancer, it should be understood that galectin-1 interacts multivalently with glycosylated receptors throughout the metastatic progression of cancer. Because a single interaction between a carbohydrate and its receptor (e.g., protein) are attenuated,¹¹ nature presents multiple copies of receptors to enhance individual binding interactions and elicit a biological response.^{5,7} To better understand multivalent protein/carbohydrate interactions in cancer, synthetic multivalent frameworks can be applied to modulate native multivalent cellular mechanisms.

Multivalent frameworks can augment binding interactions through several distinct mechanisms. Multivalent ligands present multiple copies of binding epitopes that can bind a receptor upon dissociation of a previous interaction (**Figure 9a**).¹¹⁷ Binding enhancement is a statistical effect resulting from the high localized concentration of epitopes that effectively increase the time that the binding site is engaged (i.e. decrease dissociation kinetics (k_{off})).¹¹⁸ Examples of binding affinity enhancement due to the statistical effect can

be found with high density receptor displays in which decreased dissociation rate constants are observed.^{119, 120,121,122} Additionally, as shown in **Figure 9b**, a multivalent ligand can bind an oligomeric receptor by simultaneously interacting with multiple binding sites.¹¹⁷ This mechanism is known as the chelate effect, and it is entropically favorable compared to the constituent monovalent interactions: translational and rotational entropic costs are paid with the first receptor-ligand contact, and subsequent intramolecular binding interactions proceed without paying these entropic costs (although, conformational entropy still factors in the free energy of binding).^{117, 118} The chelation binding mode has been elegantly exploited in the design of multivalent toxin inhibitors¹²³, such as Shiga-like toxins^{28,124,22,125,126} and cholera toxins.^{127,128,129,130,131} Furthermore, as shown in **Figure 9c**, the binding of a multivalent ligand to multiple cell-surface receptors can result in clustering of the receptors in a microdomain.¹¹⁷ Receptor clustering has been observed to activate cell-signaling pathways^{132,133} and modulate a myriad of biological activities from bacterial chemotaxis^{134,135,136} to immune system functions.^{137,138,139} As shown in **Figure 9d**, multivalent ligands can interact with auxiliary binding sites on a receptor to enhance binding.^{5,140,141} In addition to enhancing binding affinity, a synthetic multivalent ligand that is bound to multiple receptors on one entity (e.g., cell-surface receptors) can sterically inhibit interactions with natural ligands (**Figure 9e**). In this mechanism, known as steric shielding, the backbone of the multivalent ligand effectively forms a layer on the cell-surface that precludes attachment of the natural ligand to the bound receptor.^{7,118} Steric shielding is a unique mechanism for inhibition that is fundamentally inaccessible by monovalent molecules. Recently, Vonnemann *et al.* observed size-dependent steric

shielding with globular multivalent inhibitors.⁴⁰ Larger globular inhibitors, which had increased contact area with the ligand, were stronger inhibitors of ligand binding to L-selectin functionalized nanoparticles compared to smaller globular inhibitors.⁴⁰

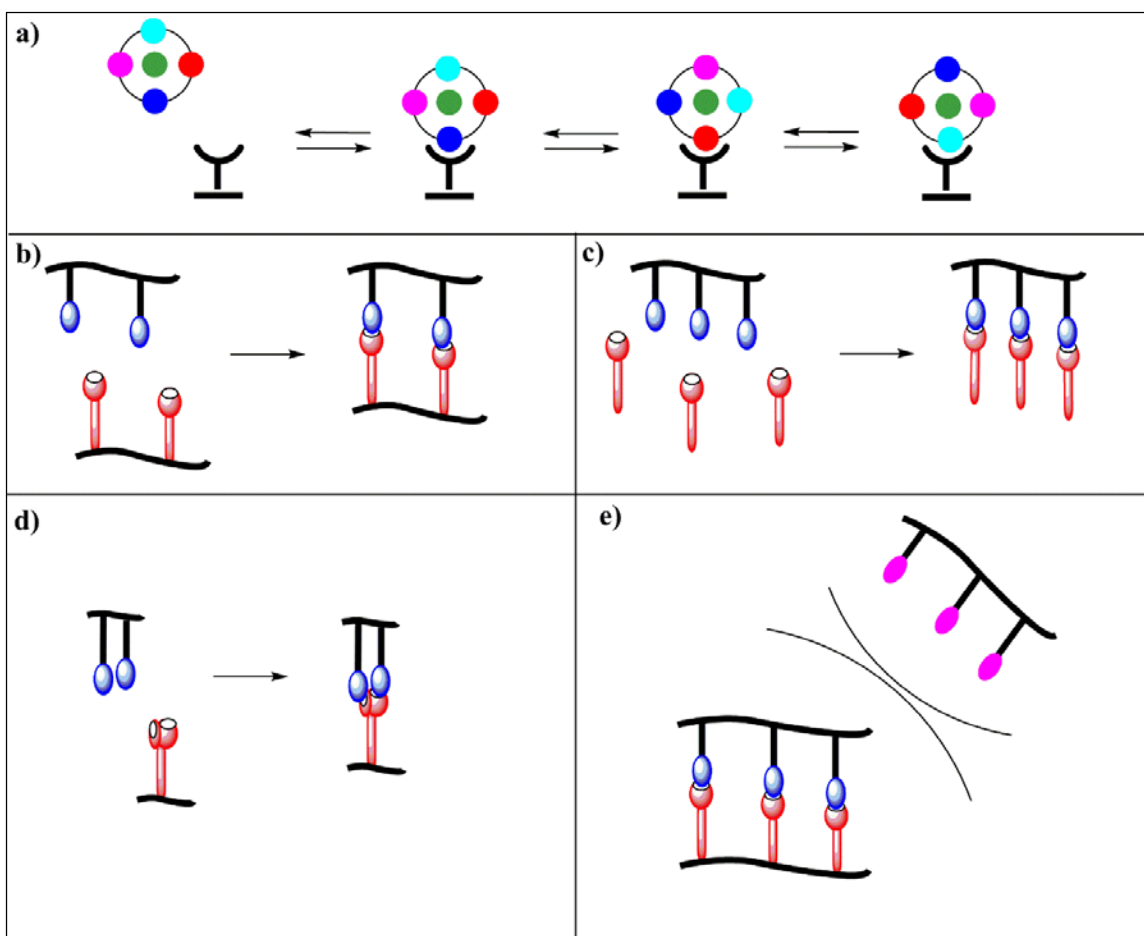


Figure 9. Multivalent Protein Carbohydrate Interaction Mechanisms. (a) Statistical effect, in which a high concentration of ligands on a multivalent scaffold allows for the receptor to be occupied over a longer period of time. (b) Chelate effect, in which a multivalent receptor coordinates with a multivalent ligand. (c) Receptor clustering, in which a multivalent ligand binds to and induces the clustering of independent receptors. (d) Secondary interactions, in which binding affinity is enhanced due to the ability of a multivalent ligand to occupy multiple binding sites on a receptor. (e) Steric inhibition, in which a multivalent ligand, once bound to a multivalent receptor, sterically prevents the approach of another ligand. Reprinted with permission from 5.

In designing multivalent ligands to target biological processes, more binding epitopes are not necessarily better. In fact, some studies indicate that there may be an optimal valency. Reina *et al.* observed a decrease in IC_{50} when the valency of a glycodendrofullerene was increased from 12 mannosides (IC_{50} 2 μ M) to 36 mannosides (IC_{50} 70 μ M).¹⁴² The decrease in inhibitory impact was attributed to congestion of sugars at the fullerene surface which obstructed interactions with the receptor (DC-SIGN).¹⁴² Interestingly, steric interference was abrogated when longer spacer arms were used, which drastically increased inhibitory activity (IC_{50} 0.3 μ M) by improving access to the mannose ligands.¹⁴²

Synthetic Multivalent Systems for Binding of Galectin-1

Several compounds that target galectin-1 interactions have been designed, and a few of these synthetic systems utilize multivalent ligand displays. This section will cover the synthetic multivalent ligands used to modulate galectin-1 activity.

Lactulose Amine Dimers

Rabinovich *et al.* synthesized lactulose amine dimers to target galectin-1 (**Figure 10**).¹⁴³ The lactulose amine dimers inhibited binding of galectin-1 to the protein 90K in solid-phase assays.¹⁴³ In addition, the dimers demonstrated selective regulatory effects in galectin-1 mediated tumor-cell apoptosis, homotypic cellular aggregation, and angiogenesis.¹⁴³ For example, the dimer connected by 8 carbons inhibited homotypic cellular aggregation and angiogenesis whereas the 12 carbon chain dimer did not.¹⁴³ However, apoptosis was enhanced when in the presence of either dimer.¹⁴³

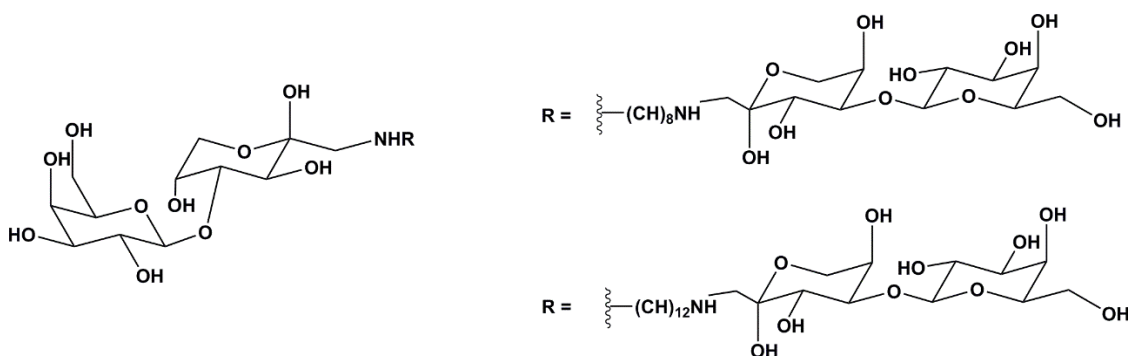


Figure 10. Synthetic lactulose amine dimers to target galectin-1.

Self-Assembled Pseudopolyrotaxanes

Belitsky *et al.* designed a self-assembled pseudopolyrotaxanes as a flexible and adaptable multivalent ligand for galectin-1 (**Figure 11**).⁴ Lactoside-displaying cyclodextrin units were non-covalently conjugated on a polymeric polyviologen backbone.⁴ Rotational and translational freedom along the polymeric backbone provides a mobile display of lactosides.⁴ Agglutination assays revealed that galectin-1 interacted with flexible multivalent ligands with higher affinity than compared to less dynamic ligand displays.⁴ Multivalent inhibition enhancement was attributed to multivalent lactosides successively binding the galectin-1 CRD, which effectively decreased k_{off} .⁴

Glycodendrimers

Dendrimers are a powerful framework to investigate multivalent protein-carbohydrate interactions and to mediate associated biological activities. The polymeric framework is symmetrical and highly branched. Dendrimers have sizes (4 nm to 7 nm used to synthesize **1-4**) that allow for biomimicry of receptors¹⁴⁴ and contain a variable number of end groups for synthetic modifications.¹⁴⁵ These characteristics contribute to the utility

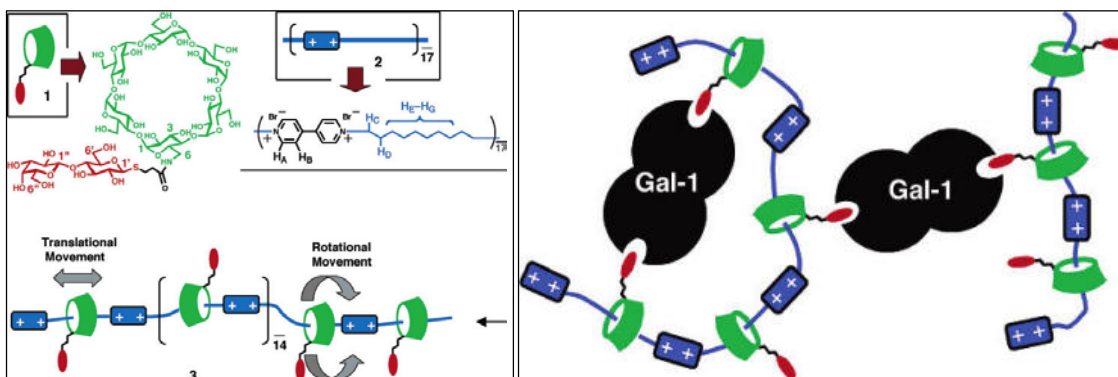


Figure 11. Self-Assembled Pseudopolyrotaxanes. Synthetic scheme shown on left wherein cyclodextrine units are functionalized with external lactose units, and the lactose-displaying cyclodextrins assemble along a polyviologen backbone. Statistical binding mechanism for Galectin-1 is shown on the right. Reprinted with permission from 4.

of dendrimers in biological studies and applications,¹⁴⁶ such as DNA- and drug-delivery,¹⁴⁷ tissue repair,¹⁴⁸ and cellular imaging.¹⁴⁹ Here, poly(amidoamine) (PAMAM) dendrimers were used, and this class of dendrimers have a spheroidal profile and bear terminal amines that can be synthetically modified (**Figure 12**). The number of amino end groups doubles with each increase in generation, starting with generation zero (G0) PAMAM dendrimer that presents a theoretical maximum of 4 terminal amines to G10 that presents a theoretical maximum of 4096 end groups.

Glycodendrimers, which are generated by functionalizing the PAMAM scaffold with carbohydrates, have been employed for the study of a variety of biological processes mediated by protein-carbohydrate interactions.³¹ By tethering multiple glycosides to the multivalent framework, glycodendrimers can enhance the binding affinity of a generally weak monovalent interaction between a lectin and a carbohydrate to study biological processes involving protein carbohydrate interactions.¹⁵⁰ For example, glycodendrimers have been used to study multivalent interactions mediated by carbohydrate binding proteins

known as lectins. Woller *et al.* used mannose functionalized dendrimer to control clustering of Concanavalin A (ConA), a plant lectin, in a generation-dependent and valency-dependent manner¹²⁰. Binding of ConA was observed to alter the binding activity in hemagglutination and precipitation assays¹²⁰. Goodman *et al.* used lactose functionalized dendrimer to study galectin-3 interactions, showing that aggregate formation was a function of the size of the multivalent framework and the number of nucleation sites¹⁵¹. Michel *et al.* then applied the lactose functionalized dendrimers to the study of galectin-3 mediated cellular aggregation, demonstrating inhibition of aggregation occurred when small glycodendrimers (i.e. G(2)) were used and larger glycodendrimers (i.e. G(6)) augmented aggregation.³⁹

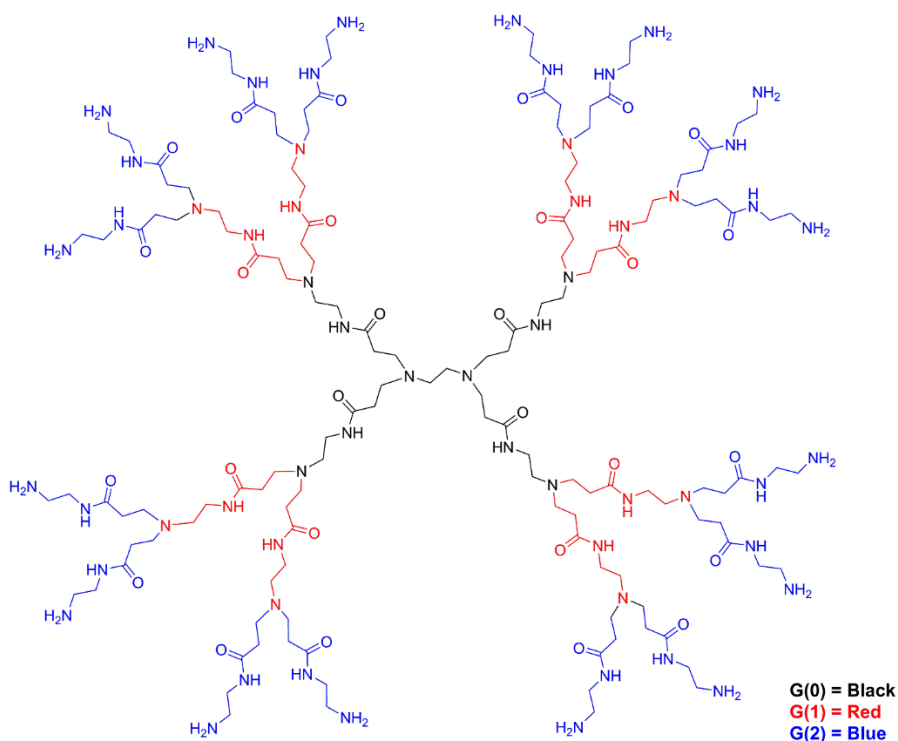


Figure 12. Poly(amidoamine) (PAMAM) Dendrimer.

Using Lactose-Functionalized Dendrimers to Study Multivalent Galectin-1 Binding

Multivalent binding of galectin-1 to β -galactosides is involved in multiple steps in tumor growth and metastasis. Single, monovalent interactions between galectin-1 and a glycoside occur with low affinities,¹⁵⁰ but ligands for galectin-1 typically possess an array of carbohydrates to enhance binding affinity.^{13,50, 91, 152} Presenting multiple glycosides on a multivalent framework enhances the strength and specificity of the interaction.^{153,154} Glycodendrimers were synthesized to mimic natural ligands for the study of multivalent biological processes. Here, generations 2, 3, 4, and 6 polyamidoamine (PAMAM) dendrimers were functionalized with lactose (a β -galactoside) to generate a series of glycodendrimers (**1**, **2**, **3**, and **4**) to better understand multivalent mechanisms in cancer process. Synthesis and characterization were performed according to reported procedures¹⁵¹ and representative spectra can be found in reference 151. **Figure 13** shows the four generations of lactose-functionalized dendrimers used for these studies and the lactose loading of each generation as determined using NMR and MALDI-ToF MS.

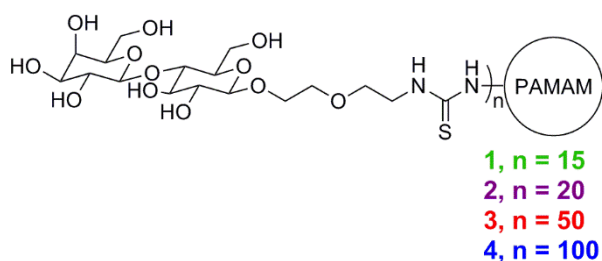


Figure 13. Lactose functionalized PAMAM dendrimers used. Color coding used to indicate the generation and the number of lactosides is used throughout this thesis.

It was hypothesized that the addition of multivalent lactose functionalized dendrimers **1-4** to galectin-1 mediated cellular processes in cancer would alter the

presentation of the galectin-1 to the cells. From the modulation of the biological activities, a better understanding of the galectin-1 mechanisms involved in these processes would emerge. To study the influence of synthetic multivalent ligands on galectin-1 mediated cellular processes, a variety of assays were performed.

First, the interaction of galectin-1 and surface adsorbed multivalent dendrimers **1-4** were investigated using an ELISA. The ELISA was used to determine if galectin-1 binds to the glycodendrimers. Chapter 2 covers the results of the ELISAs.

Second, the binding of the galectin-1 to **1-4** in solution was investigated using Dynamic Light Scattering (DLS) and Fluorescence Microscopy (FM). Different stoichiometries of galectin-1 to glycodendrimer were used. It was hypothesized that the glycodendrimers nucleate galectin-1 into nanoparticles, and the size of the nanoparticles could be altered by changing the stoichiometry. The results of the DLS and FM studies are discussed in Chapter 3.

From the surface-immobilized assay and the solution-based assay, it was hypothesized multivalent glycodendrimers **1-4** organized galectin-1 into biologically active arrays. These biologically active arrays could be applied to modulate and study multivalent biological processes. To investigate the influence of glycodendrimers on galectin-1 mediated cancer processes, compounds **1-4** were applied to a cellular aggregation assay and vascular tube formation assay. The cellular aggregation assay was used as a model to study tumor formation, and the tube formation assay provides a model to study angiogenesis. For the cellular aggregation assay, the DU145 cancer cell line was chosen because it is known to express Mucin-1, a cognate galectin-1 receptor.^{74,155} It was

hypothesized that multivalent glycodendrimers would alter the presentation of the galectin-1 to the cells, which would inhibit homotypic aggregation. The results of the cellular aggregation assay are discussed in Chapter 4. For the tube formation assay, glycodendrimers **1-4** were introduced to endothelial cells in the presence of galectin-1 in order to better understand the role of the galectin-1 in angiogenesis, and these results are covered in Chapter 5.

Conclusion

Cancer is a leading cause of death world-wide. The malignant spread of cancer is advanced in part through multivalent protein carbohydrate interactions. Galectin-1, which is commonly over expressed in malignant cancer, mediates a variety of cellular processes in cancer progression by interacting with glycoconjugates in the tumor microenvironment. For example, galectin-1 has been found to arbitrate cellular aggregation, migration and invasion, tumor-induced angiogenesis, and T-cell apoptosis. Studies using animal models support the critical role of galectin-1 in mediating tumor growth and metastasis.^{114, 116} Taken together, these studies indicate that galectin-1 inhibition is a promising therapeutic strategy against cancer. Because galectin-1 mediates metastatic processes via multivalent interactions with glycoconjugates, traditional monovalent therapeutics are fundamentally ineffective. Synthetic, multivalent systems, which can simultaneously target multiple interactions, offer an auspicious alternative. In order to develop robust multivalent galectin-1 therapeutics, a better understanding of galectin-1 mechanisms in cancer is paramount. In this thesis, lactose functionalized dendrimers were used to bind galectin-1 and to modulate

its biological activities. The results of the ELISAs, DLS, fluorescence microscopy, homotypic cellular aggregation assay, and an angiogenesis assay results are reported here.

CHAPTER 2

EVALUATION OF THE GALECTIN-1 INTERACTION WITH SURFACE-
ADSORBED GLYCODENDRIMERSIntroduction

Galectin-1 mediates the adhesion of cancer cells to the extracellular matrix (ECM) by multivalent cross-linking of cell-surface glycoconjugates with glycoconjugates in the ECM^{60,76} (see Chapter 1 for a list of glycoconjugates involved in cell-ECM adhesion and a discussion of the mechanisms involved). Several assays that mimic the cell surface have been designed and used to investigate multivalent protein carbohydrate interactions with synthetic multivalent scaffolds, and these assays typically involve surface immobilization of a glycoconjugate (either a natural glycan or a multivalent scaffold).

Surface plasmon resonance (SPR) is useful for studying binding interactions of lectins with surface-adhered glycans under flow.^{156,157} Measurements are based on mass-change as lectins are bound and eluted from the glycan-adhered surface. Stowell *et al.* used SPR to investigate glycan specificity of galectin-1, observing that galectin-1 binds with higher affinity to α 2-3-sialylated glycans (but does not bind α 2-6-sialylated glycans) and to poly-*N*-acetyllactosamine sequences over glycans expressing a single *N*-acetyllactosamine epitope.⁴⁹ Back scattering interferometry (BSI) is a sensitive technique for studying lectin/glycan binding interactions both in solution and with surface-immobilized glycans, and detects the act of complexation without regard to size of the species.¹⁵⁸ Fluorescent binding assays, such as fluorescence resonance energy transfer (FRET)^{159,160} using disparately labelled galectin and glycoconjugate, have been employed

to analyze the influence of glycan valency, structure, and presentation on lectin binding.^{17,159} Belardi *et al.* expanded FRET to a microarray format using well defined glycopolymers functionalized with glycans and observed that galectin-1 cellular cross linking was dependent on glycan structure and epitope spacing.¹⁷ Enzyme linked immunosorbent assays (ELISAs) are relatively simple, high throughput platforms to detect the occurrence of binding interactions between lectins and surface-adhered glycans.¹⁶¹

To study the interaction of galectin-1 with surface-adhered glycodendrimers, an enzyme-linked immunosorbent assay (ELISA) was designed. ELISAs have been used to study protein-carbohydrate interactions in a variety of systems because the assay is sensitive, rapid, and the fundamental design can be readily adapted to different systems.^{161, 162, 163, 164, 165, 166, 167, 168} Generally, a solid surface is functionalized to present a layer of carbohydrates.¹⁶⁹ The lectin of interest is then bound to the carbohydrate functionalized surface, and binding is detected using an antibody and a detection system such as horseradish peroxidase.^{169, 170} After lectin binding has been established, synthetic multivalent systems are introduced.¹⁷¹ The synthetic multivalent system is identified as a good lectin binding if inhibition of the lectin-carbohydrate interaction is observed. If, however, full inhibition is not observed (i.e. binding is only partially inhibited or not inhibited), then the synthetic multivalent system is determined to be only weakly bound or not bound by the lectin. Representative ELISA binding and inhibition experiments are presented in **Figures 14a and b.**

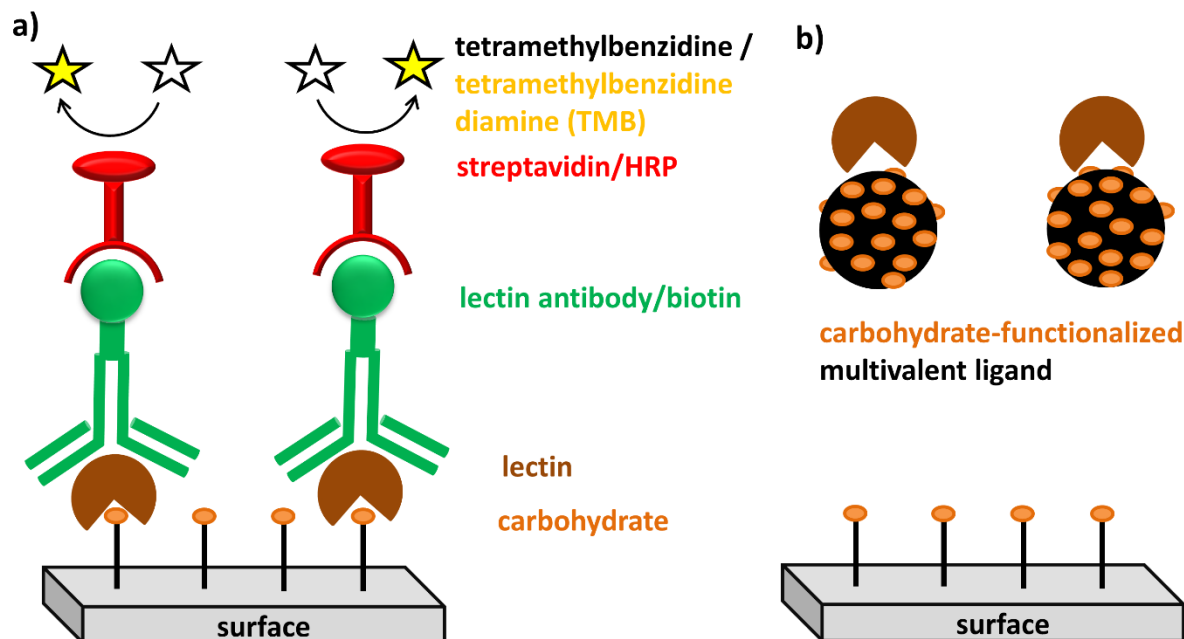


Figure 14. ELISA. (a) Traditional ELISA with carbohydrate functionalized surface. Lectin binding is indicated by enzymatic oxidation of TMB, which produces a measurable color change. (b) Inhibition of lectin binding by a synthetic multivalent system is indicated by the absence of TMB oxidation.

Traditional ELISAs work well for most lectins. For galectins, however, the ELISA and inhibition ELISA protocols have been modified for the study of multivalent binding. Andre *et al.* immobilized a series of glycodendrimers on the surface of a well plate to investigate the influence of glyconjugate valency on galectin-1 and galectin-3 binding affinity.¹⁷² Lactose was used to inhibit the glycodendrimers/galectin interaction, and the measured IC_{50} values were used to evaluate the effect of valency of binding affinity. The binding affinity of galectin-1 was observed to increase with increased ligand valency, while monomeric galectin-3 afforded only modest affinity enhancements with increasing valency.¹⁷² Wolfenden *et al.* adapted this modified ELISA protocol to study glycodendrimer binding interactions with galectin-3.^{173,174} Glycodendrimers were

immobilized on the surface of a 96-well plate, and galectin-3 was co-incubated with varying concentrations of monomeric carbohydrates to inhibit the galectin-3/glycodendrimer interaction. Binding was detected using a biotinylated galectin-3 antibody and a horseradish peroxidase/streptavidin conjugate. Based on previous studies by Andre *et al.*¹⁷² and Wolfenden *et al.*^{173,174} using glycodendronized surfaces, an ELISA was designed to measure relative binding affinity of galectin-1 for a series of lactose-functionalized dendrimers (**1-4**) immobilized on a surface. The results of the ELISA can be used to determine how well galectin-1 binds to the glycodendrimers, to determine if valency influences galectin-1 recruitment, and to suggest possible interaction mechanisms of galectin-1 with surface immobilized glycoconjugates. The assay design and results from the assay are reported here.

Results

To study the interaction of compounds **1-4** with galectin-1 using an ELISA protocol, the glycodendrimers were adsorbed to the surface of a 96-well plate, and galectin-1 was added with serially diluted lactose as shown in **Figure 15**. The ELISA to study galectin-1 binding was modified from existing ELISA protocols. Galectin-1 was biotinylated to interact with a streptavidin-horseradish peroxidase conjugate (SAv-HRP) detection system, which circumvented the use of an antibody to detect binding. SAv-HRP addition followed by tetramethylbenzidine (TMB) oxidation and quenching with H₃PO₄ produced a measurable color change at 450 nm corresponding to bound galectin-1.

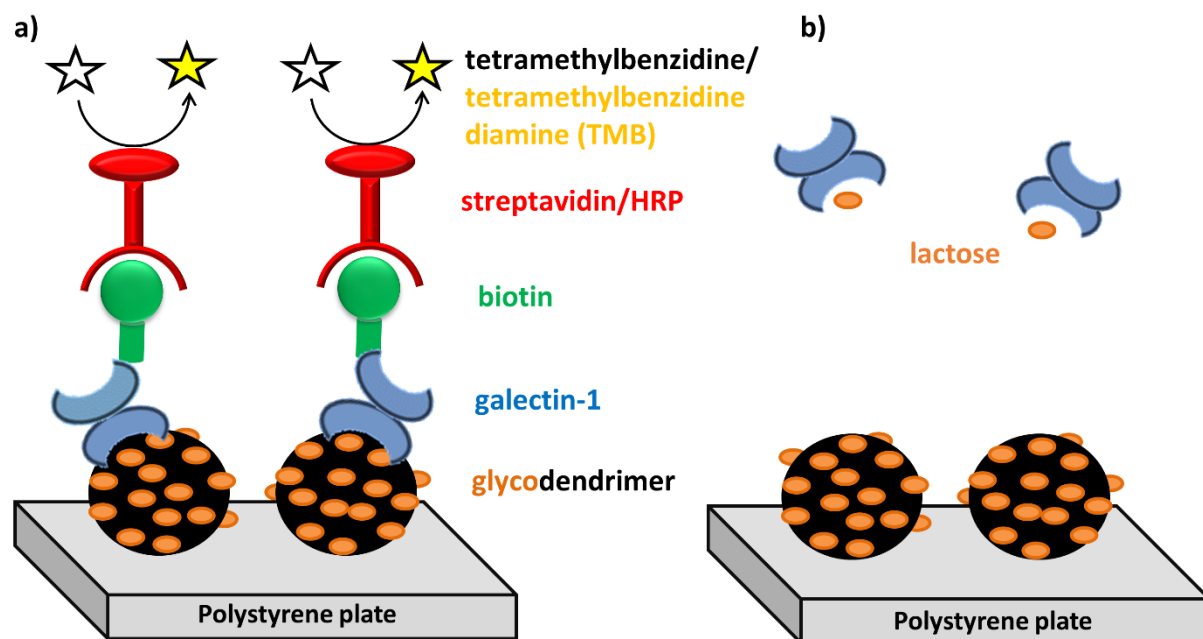


Figure 15. Modified ELISA to study galectin-1 binding interactions. (a) Glycodendrimers were adsorbed to the surface of a plate, and biotinylated galectin-1 was added to measure binding affinity. (b) Monomeric lactose was added to inhibit galectin-1 binding to the glycodendronized surface.

Initial experiments were performed to determine the amount of galectin-1 needed to generate a good signal. This was done by co-incubating serially diluted galectin-1 with the glycodendronized 96 well plate, washing unbound galectin-1, then sequentially adding TMB and H_3PO_4 . 50 μL of a 5.0 $\mu\text{g/mL}$ galectin-1 solution in PBS was chosen because the log phase of the standard curve indicated that this was the optimal concentration (**Figure 16**).

Galectin-1 binding to **2**, **3**, and **4** was observed and is shown in **Figure 17** (see Figure 62 in Appendix A for the ELISA binding curves with data points and error bars). In addition to showing that galectin-1 does bind well to the glycodendrimers, relative differences among glycodendrimer architectures were evaluated by comparing the maximum absorbance values and the IC_{50} values for the three generations of

glycodendrimers. The relative amount of galectin-1 recruited by a glycodendrimer generation can be determined from maximum absorbance values. The results indicate that larger glycodendrimers, which have more ligands, recruit more galectin-1: compound **4** recruited more galectin-1 than **3** or **2**.

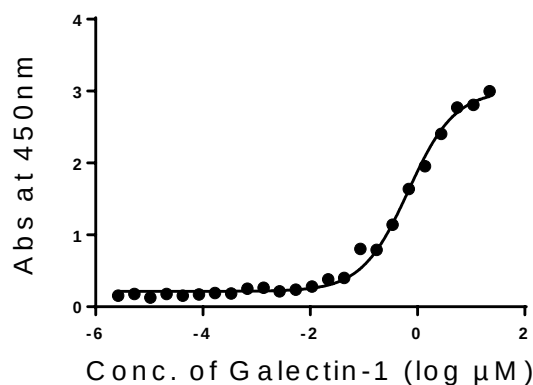


Figure 16. Saturation curve for the binding of galectin-1 to surface immobilized **2**.

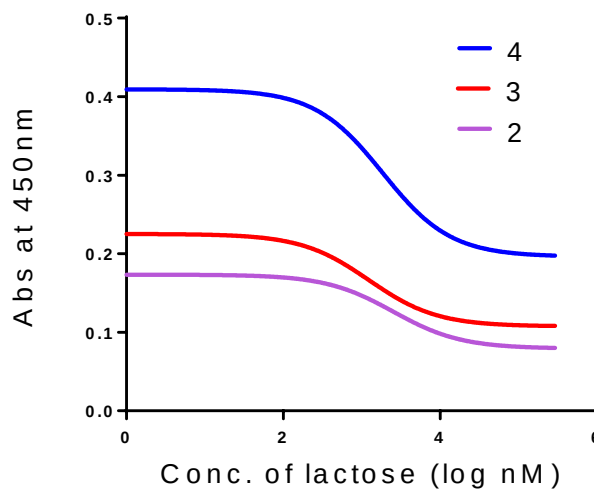


Figure 17. ELISA binding curve with **2**, **3**, and **4**.

IC_{50} values indicate the amount of lactose required to inhibit half of the galectin-1 binding to the glycodendronized surface, and can indicate different binding affinities

among the glycodendrimer series. IC₅₀ values calculated from the inhibition ELISA binding curves are shown in **Table 2**. The third generation lactose functionalized dendrimer **2** had the highest IC₅₀ value, which was about 30% higher than the IC₅₀ values for fourth and sixth generation glycodendrimers **3** and **4**. The narrow range in IC₅₀ values suggests that galectin-1 interacts similarly with each generation.

Table 2. ELISA results for interaction of **2**, **3**, and **4** with galectin-1.

Compound	PAMAM generation	Number of Lactosides	IC ₅₀ (nM) Lactose	Maximum absorbance
2	3	20	2.52	0.17
3	4	45	1.85	0.23
4	6	100	1.74	0.41

Discussion

Inhibition of the galectin carbohydrate interaction was used to assess the binding affinity of the synthetic multivalent system for galectin-1. Two properties of the galectin-1 binding interaction with glycodendrimers are evident from the surface binding assays. First, maximum absorbance values indicate that galectin-1 recruitment was valency-dependent, with more galectin-1 recruitment occurring for higher generation dendrimers (**4** > **2** and **3**). Second, inhibition of the galectin-1/glycodendrimer interaction by monomeric lactose was similar regardless of the generation of the glycodendrimer. The similarity in the IC₅₀ values observed for **2**, **3**, and **4** suggests that galectin-1 was bound to each generation with a similar affinity regardless of the fundamental architectural differences in size, valency, and ligand density among the dendrimer generations.

The similarity in IC_{50} values for galectin-1 binding to different glycodendrimer generations requires an explanation that goes beyond a monovalent interaction between galectin-1 and a lactoside. Brewer *et al.* has proposed a “bind and jump” mechanism for multivalent lectin binding to a multivalent glycoconjugate which could explain the similarity in IC_{50} values.^{175,176} As shown in **Figure 18**, in this multivalent mechanism, the initial binding event is followed by internal diffusion of the lectin where the lectin jumps to and binds an adjacent glycoside.^{175, 176} The initial binding of the galectin to the multivalent framework occurs with favorable free energy.^{175,176} Each subsequent binding event is entropically less favorable and occurs with less favorable free energy, resulting in a gradient of decreasing microaffinities.^{175, 176} Accordingly, similar concentrations of monomeric lactose would be required to inhibit the initial galectin-1 binding interaction, and this is reflected in similar IC_{50} values among the different generations of glycodendrimers.

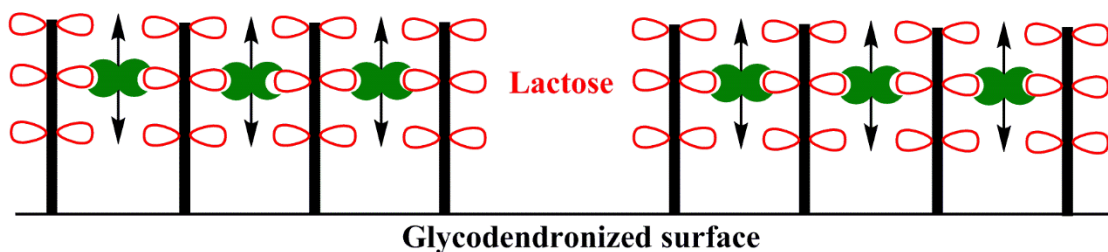


Figure 18. Bind and jump mechanism for galectin-1 interaction with surface adsorbed glycodendrimer.

If the interaction of the galectin-1 with the glycodendronized surface was a monovalent interaction, generations that bear more epitopes and recruit more galectin-1 would require more lactose to inhibit the interaction, which would be reflected in a gradient of IC_{50} values. The IC_{50} values, however, differed by a range of only about 30%; the third

generation lactose functionalized dendrimer 2, which had the fewest lactosides in the series and the lowest maximum absorbance, had the highest IC₅₀ value, whereas compounds 3 and 4 had similar IC₅₀ values. Thus, monovalent interactions cannot explain the IC₅₀ values.

The ELISAs reported here indicate that the galectin-1 binds well to the glycodendrimers, and these synthetic multivalent ligands can be used to study and modulate galectin-1 mediated cancer processes. However, it is likely that the interaction of galectin-1 with surface immobilized glycoconjugates is different than what would be observed in solution based assays. Computer simulations suggest dendrimers lay flat on a surface and that increased interaction strength results in dendrimers spreading out and flattening on the surface.¹⁷⁷ These simulations were supported by subsequent studies with transmission electron microscopy, which showed the surface adsorbed dendrimers are flat with a circular profile.¹⁷⁸ When glycosides are conjugated to the PAMAM backbone, the resulting glycodendrimers adopt a circular shape with a slight declivity in the center upon surface adsorption.¹²⁰ Ligands are likely located in a volume shell on the periphery of the donut-shaped, surface adsorbed glycodendrimers, and the lectins cluster around the exposed surface.¹⁷⁹ However, some of the ligands on the glycodendrimer interact with the surface. Wolfenden *et al.* characterized the glycodendronized surface using XPS, and this data demonstrates that the glycosides participate in surface adsorption: GalNAc epitopes showed increased adsorption relative to lactosides, and glycodendrimers with a greater number of epitopes exhibit increased surface adsorption relative to glycodendrimers with fewer epitopes.^{173,174} Thus, it is likely that a portion of glycosides that otherwise would be

available to bind the lectin in solution interact with the well plate surface. Because the epitopes conjugated on the dendrimer participate in surface adsorption, the binding interactions analyzed in the ELISA format are likely different than what would be observed in solution based assays.

The ELISA was not able to produce reliable data for the binding of galectin-1 to **1**. The abnormal behavior of the ELISA binding curve with **1** may be explained by the interaction of the glycodendrimers with the surface of the plate (see Figure 62 in appendix A). The generation 2 PAMAM dendrimer is relatively flat compared to higher generation glycodendrimers that tend to adopt a globular shape, and this suggests that a majority of the lactosides on the functionalized generation 2 PAMAM dendrimer **1** interact with the surface of the 96 well plate. Because these epitopes were likely engaged with the surface, they were not available for recruiting galectin-1, which would explain the unique shape of the ELISA inhibition curve (see appendix). XPS studies were performed to confirm that similar amounts of **1**, **2**, **3**, and **4** were adsorbed to the surface (see appendix).

Conclusion

The ELISA results reported here indicate that galectin-1 binds well to the lactose functionalized dendrimers, and these synthetic multivalent frameworks can be applied to the study and mediation of multivalent galectin-1 interactions, such as cellular aggregation/tumor formation and angiogenesis. Maximum absorbance values show that glycodendrimers with more lactosides recruit more galectin-1. Furthermore, the similarity in binding affinities (IC_{50} values) indicates a multivalent mechanism is at play, most likely

involving an interplay of different effects such as lectin clustering,^{120,180} bivalent interactions,¹⁷ and statistical concentration effects.^{119, 120,121,122}

Multivalent frameworks have been reported to cluster lectins, for example by presenting epitopes of differing affinities where higher affinity ligands bind and cluster lectins¹²⁰ or by presenting pre-organized epitopes at spacing sufficient to bind multiple lectins¹⁸⁰. Bivalent interactions occur as the lectin simultaneously binds two epitopes,¹¹⁷ and this type of interaction has been observed with galectin-1 mediated cross-linking of cellular receptors.¹⁷ Furthermore, the presentation of a high localized concentration of binding epitopes allows galectin-1 to bind a proximal lactoside as soon as a previous interaction dissipates.^{117,118} Examples of statistical concentration effects have been reported for high density receptor displays on multivalent ligands in which decreased dissociation rate constants were observed.^{119, 120,121,122}

Experimental Procedures

Galectin-1 was provided by Dr. Linda Baum and Mabel Pang of UCLA. ELISA reagents were purchased from BD Biosciences. 96 well plates were purchased from Corning.

Conjugation of Biotin to Galectin-1

Galectin-1 was labeled with a sulfonated N-Hydroxysuccinimide (NHS) ester of biotin (EZ LINK Sulfo-NCS-LC-Biotin, ThermoScientific), which reacts with primary amines on lysine and generate a stable biotin-protein conjugate via amide bond

formation.¹⁸¹ A schematic representation of the biotin labeling reaction is provided in **Figure 19**.

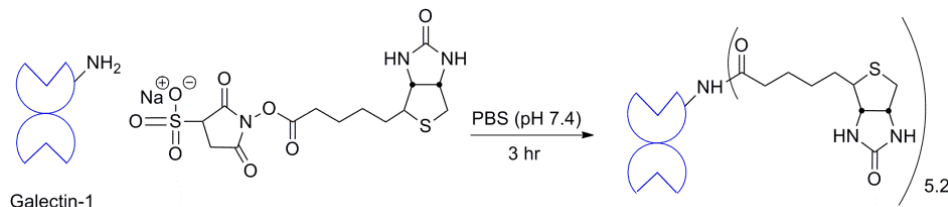


Figure 19. Schematic representation of galectin-1 biotinylation.

Biotinylation of galectin-1 was achieved with EZ-Link Sulfo-NHS-LC-Biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate) bearing a 22.4 Å spacer arm purchased from Thermo Scientific. A stock solution of Sulfo-NHS-LC-Biotin was prepared at 1 mg/mL in PBS (pH 7.4, 15 mM NaCl). To a solution of galectin-1 in PBS, the biotin reagent was added in an 8 molar excess, and the reaction was stirred at room temperature (19.3 °C) for 3 hours. After 3 hours, the reaction mixture was purified by dialysis against PBS (pH 7.4, 15 mM NaCl) with a 1 kD MWCO (Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane).

Characterization of biotin conjugation to galectin-1 was performed with a Pierce Biotin Quantitation Kit, and spectrometric measurements were made using a Molecular Devices SoftMax Pro 5 SPECTRA max Plus 3.84 (Serial No.: SMP500-14501-EARG). To a 1mL cuvette (PLASTIBRAND), 800 µL of PBS buffer (pH 7.4, 15 mM NaCl) was added and the spectrophotometer was zeroed at 500 nm. To a microtube of 4'-hydroxyazobenzene-2-carboxylic acid (HABA)/Avidin Premix, equilibrated to room temperature (19.3 °C), 100 µL of millipore H₂O was added and mixed with a pipette until

solubilized. The HABA/Avidin Premix solution was added to the 1 mL cuvette containing the PBS buffer and mixed. The absorbance of the HABA/Avidin solution was measured at 500 nm. To the cuvette, 100 μ L of biotinylated galectin-1 was then added and mixed thoroughly. The absorbance of the HABA/Avidin/biotinylated galectin-1 mixture was measured at 500 nm. From the change in absorbance at 500 nm, the number of moles of biotin per mole of galectin-1 was calculated using Beer's Law, to yield 5 moles biotin per galectin-1.

Preparation of Glycodendrimer Adsorbed 96 Well Plates

To each well of a Nunc MaxiSorp 96 well plate (Thermo Scientific), 50 μ L of a 0.025 mg/mL glycodendrimer solution was added. The well plate was covered and stored for 24 h at 4 °C. The solvent was removed from the well plate, and to each well 250 μ L of 3% BSA solution in PBS (pH 7.4, 15 mmol NaCl) was added to block non-specific interactions. The plate was covered and let stand for 2 hour at RT. After 2 hours, the plate was emptied, washed once with PBS (pH 7.4, 15 mmol NaCl), and dried. Dried plates were either used immediately or covered and stored at 4 °C for later use.

Saturation Curve for Galectin-1 Binding to 2

To a processed polystyrene (PPI) plate (Corning Inc., costar 96 well, V bottom), 60 μ L of 0.5% BSA in PBS (pH 7.4, 15 mM NaCl) were added to all wells, except wells A1, D1 and G1. To well A1, D1, and G1, 60 μ L of a stock biotinylated galectin-1 solution (1.28 mg/mL) were added. To wells A2, D2, and G2, 60 μ L of a stock biotinylated galectin-1 solution (1.28 mg/mL in PBS) were added. To generate 24

sequential concentrations of biotinylated galectin-1, serial dilutions were performed so that 60 μ L remained in each well. From each well on the PPI plate, 50 μ L were transferred to the corresponding well on the glycodendrimer coated Nunc MaxiSorp plate (Thermo Scientific). The plate was covered and placed on a shaker/agitator for 45 minutes.

After 45 minutes, the plate was emptied and washed twice with PBS-T (pH 7.4, 15 mM NaCl) and once with PBS (pH 7.4, 15 mM NaCl). To each well, 100 μ L of streptavidin-horseradish peroxidase (SAv-HRP) (1:1000 dilution of the stock purchased from BD Biosciences) were added. The plate was covered and placed on a shaker/agitator for 45 minutes.

After 45 minutes, the plate was emptied and washed twice with PBS-T (pH 7.4, 15 mM NaCl) and once with PBS (pH 7.4, 15 mM NaCl). To each well, 100 μ L of a 3, 3', 5, 5' tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) solution (mixed at a 1:1 ratio of solution from a kit purchased from BD Biosciences) were added. The plate was covered and incubated for 10 minutes. A blue color change was observed. After 10 minutes, to each well, 100 μ L of H_3PO_4 were added and a yellow color change was observed. Absorbance was measured at 450 nm, with a reference at 620 nm (Molecular Devices SoftMax Pro 5 SPECTRA max Plus 3.84 (Serial No.: SMP500-14501-EARG); SoftMax Pro 5.4.4 software).

ELISA to Study Galectin-1 Binding to Glycodendrimers

To a PPI plate, 60 μ L of 0.5% BSA in PBS (pH 7.4) were added to each well, except wells A1, C1, E1 and G1. To wells A1, C1, E1, and G1, 60 μ L of 100 mg/mL lactose

solution were added. To wells A2, C2, E2, and G2, 60 μ L of the 100 mg/mL lactose solution were added. To generate 24 sequential concentrations of lactose, serial dilutions were performed, starting with wells A2, D2, and G2, so that 60 μ L remained in each well.

From each well, 50 μ L were transferred to the corresponding well on the glycodendrimer coated prepared plate (preparation of the glycodendrimer coated plate is described above). To all wells, biotinylated galectin-1 (50 μ L of a 5.0 μ g/mL solution) was added, and the plate was covered and placed on a shaker/agitator for 45 minutes.

After 45 minutes, the plate was emptied and washed twice with PBS-T (pH 7.4, 15 mM NaCl) and once with PBS (pH 7.4, 15 mM NaCl). To each well, 100 μ L of streptavidin-horseradish peroxidase (SAv-HRP) (1:1000 dilution of the stock purchased from BD Biosciences) was added, and the plate was covered and placed on a shaker/agitator for 45 minutes.

After 45 minutes, the plate was emptied and washed twice with PBS-T (pH 7.4, 15 mM NaCl) and once with PBS (pH 7.4, 15 mM NaCl). To each well, 100 μ L of TMB: H_2O_2 (mixed in a 1:1 ratio from a kit purchased from BD Biosciences) was added. The plate was covered and incubated for 10 minutes. A blue color change was observed. After 10 minutes, 100 μ L of H_3PO_4 was added to each well and a yellow color change was observed. Absorbances were measured at 450 nm, with a reference at 620 nm.

Analysis of ELISA Binding Curves

Data collected from the ELISAs were analysed with GraphPad Prism software, which generated logarithmic binding curves as a function of the log of the concentration of lactose (mM).

Solution Preparation

Glycodendrimer was dissolved into PBS (pH 7.4, 15 mmol NaCl) at 2 mg/mL. Biotinylated galectin-1 was prepared in PBS (pH 7.4, 15 mmol NaCl) at 5.0 µg/mL.

For Streptavidin-Horseradish Peroxidase (SAv-HRP), the stock was purchased from BD Biosciences, Inc. To prepare the working assay solution, the stock was diluted 1:1000 in 0.5% BSA in PBS (pH = 7.4, 15 mM NaCl). For each plate, 10 mL of this SAv-HRP was required.

For the TMB:H₂O₂ solution, the 3, 3', 5, 5' tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) solutions were purchased from BD Biosciences, Inc. Immediately prior to use, equal volumes of each reagent were mixed thoroughly for the working assay solution. For each plate, 10 mL of the TMB:H₂O₂ solution were required.

CHAPTER 3

EVALUATION OF THE GALECTIN-1
INTERACTION WITH GLYCODENDRIMERS IN SOLUTIONIntroduction

Galectin-1 can interact multivalently with non-surface bound glycoconjugates to form multivalent nanoparticles that can be used to study and mediate biological activities. Multivalent scaffolds have been used to study non-surface bound multivalent assemblies of proteins with oligosaccharides in solution using a variety of assays. Fluorescence binding assays, such as fluorescence anisotropy and fluorescence lifetime (FL), can be used to study thermodynamic binding interactions between disparately labelled lectin and glycan.^{182,183} Salomonsson and co-workers used fluorescence anisotropy to investigate the thermodynamic parameters of galectin-1 binding with multivalent glycoproteins (fetuin, asialofetuin, and human serum glycoproteins).¹⁴⁰ Dynamic light scattering (DLS) is useful for studying the size of glycan/protein nanoparticles formed in solution.^{151, 184,185,186} This technique requires large amounts of sample and works best for relatively monodisperse nanoparticles. Wang *et al.* used DLS to measure the size of aggregates formed with carbohydrate functionalized gold nanoparticles and lectins, demonstrating that these aggregates increase in size as the concentration of the lectin increases.¹⁸⁵ Isothermal titration calorimetry (ITC) is commonly used to measure binding constants in solutions.^{187,188} ITC, however, requires large amounts of sample and is limited by the formation of insoluble protein/carbohydrate aggregates. Brewer used ITC to investigate the binding specificity of galectin-1 toward poly-N-acetyllactosamine (LacNAc) epitopes and

observed that galectin-1 preferentially binds LacNAc epitopes located on the terminus of oligosaccharides, whereas galectins -3 and -7 also bind internal LacNAc units.¹⁵⁰

Here, fluorescence microscopy and DLS were used to study solution-based interactions between galectin-1 and the glycodendrimers at different stoichiometries. The fluorescence microscopy assay was based on using standard fluorescent microspheres of various sizes to create a standard for size measurements that enable conversion of pixel counts to nanometers. Both the galectin-1 and the glycodendrimers were fluorescently labelled, and after incubation images were captured of the resulting fluorescent aggregates.

DLS correlates fluctuations of scattered light with time to calculate hydrodynamic radii and polydispersity (size distribution) of particles in solution. The autocorrelation function ($g(t)$) is exponential in nature and is fit assuming particles are spherical. The analysis algorithms of the DLS software fit the autocorrelation function by either: (1) applying Taylor expansion to the exponential correlation function and using a normal, linear least squares fit to determine cumulants; or (2) fitting the exponential directly using a non-linear least squares fitting algorithm to solve for Γ (time delay) and amplitude factors. The first method is referred to as the method of cumulants. Cumulants are coefficients to the Taylor series expansion and can be related to the intensity correlation decay rate, Γ , and distribution width. NNLS and CONTIN algorithms use of the second fitting procedure. In both cases, the resulting physical parameters (i.e. diameter and polydispersity) are calculated from the determined variables (cumulants or Γ and amplitude factors). Both fitting methods typically provide similar mean values, but the method of cumulants has increased sensitivity to background corrections¹⁸⁹. To measure the diameter of galectin-1

nanoparticles, the method of cumulants was used. If, however, the fit of the correlation function was poor, such as when samples were relatively polydisperse or a low signal was observed, either CONTIN or NNLS was used, depending on which analysis had the better fit.

The results from the fluorescence microscopy and DLS assays are reported here.

Results

The sizes of the galectin-1/glycodendrimer aggregates that were formed using multivalent lactose-functionalized PAMAM dendrimers **1-4** were determined by fluorescence microscopy (FM) and dynamic light scattering (DLS). Nanoparticle size was measured when a large, medium, or slight excess of galectin-1 was used relative to the concentration of the dendrimer (220:1, 9:1, or 3:1 ratio of galectin-1 to dendrimer, respectively). Galectin-1 and the glycodendrimers 1, 2, 3, and 4 were mixed and incubated at room temperature for 1 hour prior to analysis.

Fluorescence Microscopy (FM)

For fluorescence microscopy, the galectin-1 was labeled with AlexaFluor-555 and the glycodendrimers were labeled with AlexaFluor-488. Fluorescent labeling of both species was observed to generate brighter conjugates. The molar ratio used to label the glycodendrimers, however, did not allow for reproducible detection of the AlexaFluor-488 species due to low signal. DLS was used to confirm that fluorescent labeling of the galectin-1 and the glycodendrimers did not affect aggregation. (The average diameter (nm) of

nanoparticles formed with **4** in a 9 molar excess of galectin-1 measured using DLS were 1800 ± 35 and using FM were 1600 ± 750 .)

After 1 hour incubation, three 15 μ L aliquots were removed from the mixture, mounted, and approximately 20-30 images were captured using a fluorescent microscope (Olympus B61X microscope with a 100x oil immersion objective) equipped with a CCD camera. Aggregate diameter was quantified using image analysis software (Pixcavator) as performed in references^{39,151}.

Fluorescent microsphere standards (200 nm, 1000 nm, and 10000nm, Molecular Probes) were used to create a standard curve to convert pixel count to particle diameter, as shown in **Figure 20**. (Representative fluorescent micrographs are shown in **Figure 21a-c**.) The calibration curve equation is $y = 17.24x - 123.7$ with an R-squared value of 0.9539. DLS was used to confirm the reported diameters of the fluorescent microsphere standards (**Table 3**). As shown in **Figure 21b** and **Figure 21c**, some bleeding was observed in the images captured of larger red fluorescent microsphere standards (1000 nm and 10000 nm), but this was not significant because the influence of glycodendrimers concentration on the size of galectin-1 nanoparticles was still observable.

The results from the fluorescence microscopy studies using **2**, **3**, and **4** are summarized in **Figure 22** (tabulated data is shown in **Table 4**), and representative micrographs are shown in **Figure 23**. (Aggregates formed using **1** were below the detection limits of the technique.) In the presence of a large excess of galectin-1 (220:1), all of the glycodendrimers **2**, **3**, and **4** organized galectin-1 into relatively small, similarly

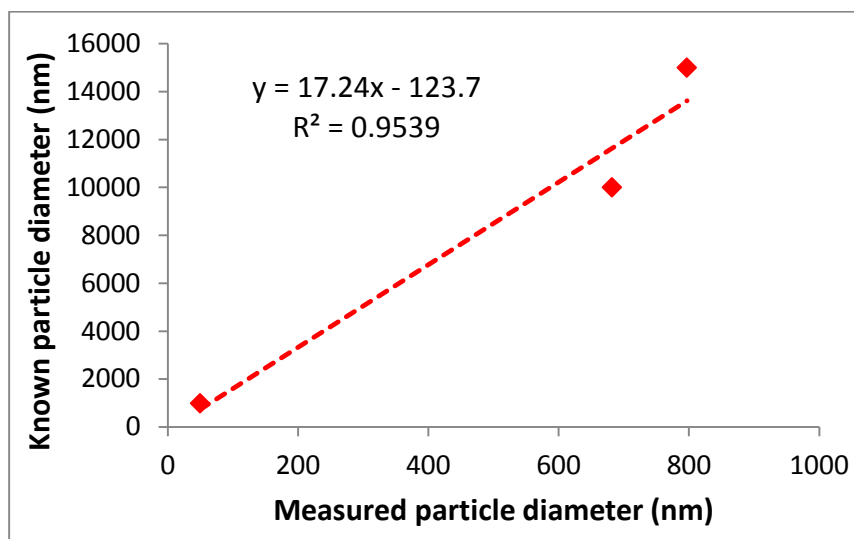


Figure 20. Calibration curve correlating measured pixels to reported particle diameter (nm).

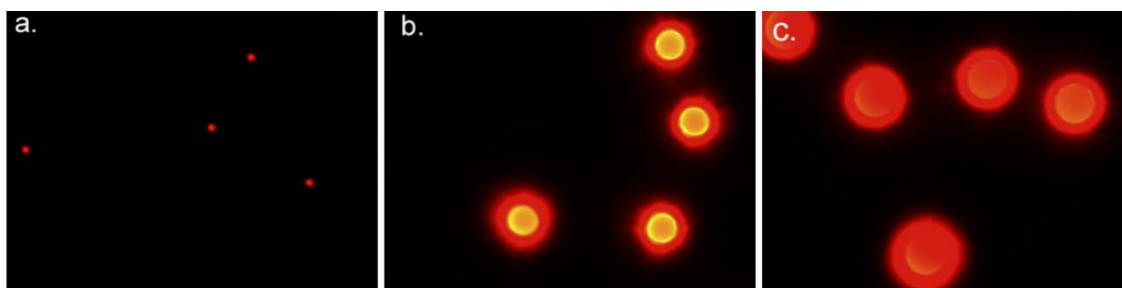


Figure 21. Representative fluorescent micrographs for fluorescent microsphere standards. (a) 200 nm fluorescent microsphere standard. (b) 1000 nm fluorescent microsphere standard. (c) 10000 nm fluorescent microsphere standard.

Table 3. Measured diameter (nm) of fluorescent microsphere standards using DLS.

Reported Diameter (nm)	Measured Diameter (DLS, nm)	Effective Relative Variance
200	230 ± 11	0.051 ± 0.047
1000	1100 ± 20	0.005 ± 0.0
10000	9800 ± 260^a	2.9 ± 0.57^a

^a Results from NNLS analysis

sized nanoparticles (**Figure 23a-c**). When a 9:1 or a 3:1 ratio of galectin-1 to glycodendrimer was used (**Figures 23d-f** and **23g-i**), the aggregates that formed were generally larger and more polydisperse than when a 220-fold excess of galectin-1 was used.

Only fourth generation dendrimer **3** forms comparable aggregates regardless of whether a slight excess of galectin-1 or a large excess of galectin-1 is added.

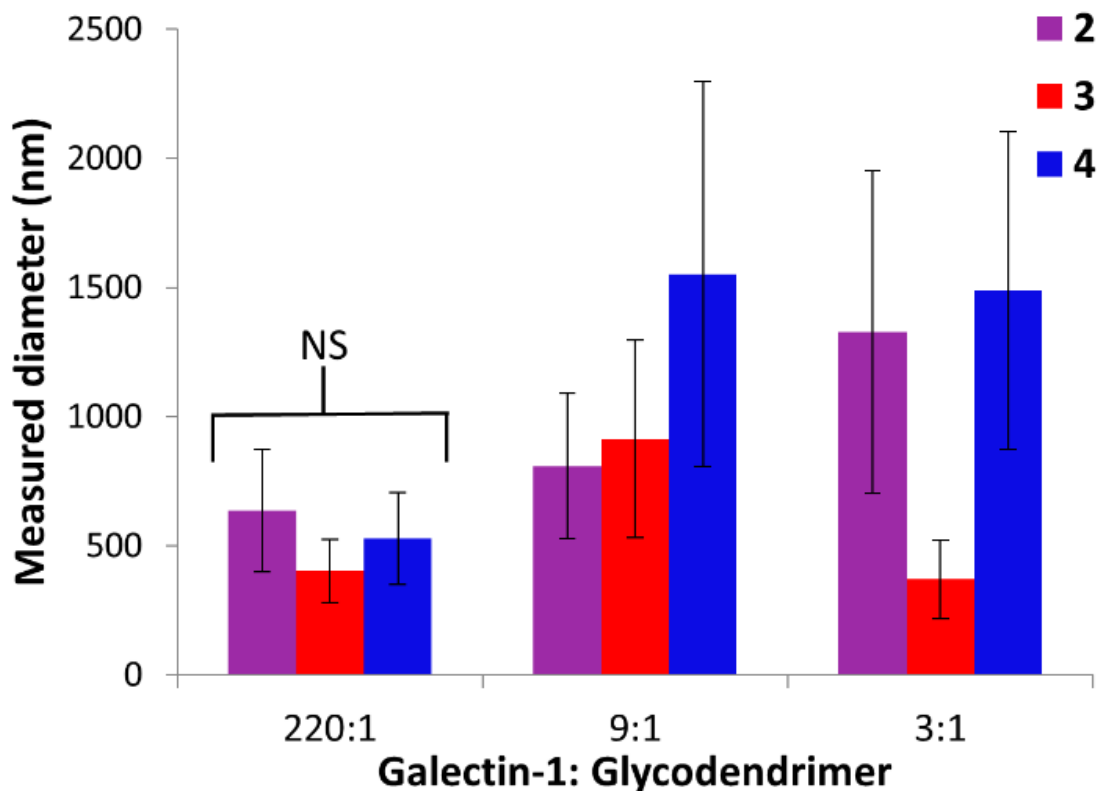


Figure 22. Average diameter (nm) of multivalent galectin-1 nanoparticles formed with multivalent glycodendrimers. For compounds **2** (purple), **3** (red), and **4** (blue), nanoparticle diameter (nm) was measured upon the addition of 0.18 μ M glycodendrimer for 220: 1, of 4.5 μ M glycodendrimer for 9: 1, and of 13 μ M glycodendrimer for 3:1 to 40 μ M galectin-1. Data are shown as mean $\pm$ one S.D. of measurements from at least three experiments ($n \geq 3$). NS represents non-significant difference in aggregate size measured for all generations determined by ANOVA.

Fluorescent nanoparticles were monitored over time, and the aggregates were observed to disaggregate after 4 hours (**Table 5**). While the size of the nanoparticles remained relatively similar (when detected), the number of measured nanoparticles decreased from 71 to 0 after 4 hours.

Table 4. Diameter (nm) and polydispersity of galectin-1/glycodendrimer aggregates measured using fluorescence microscopy.

Compound	Galectin-1 (μM)	Dendrimer (μM)	Galectin-1: Dendrimer	Average Dia. (nm)	Polydispersity
1	40	0.18	220:1	NDA ^a	
		4.5	9:1	NDA	
		13	3:1	NDA	
2	40	0.18	220:1	640 ± 340	1.2
		4.5	9:1	810 ± 280	1.1
		13	3:1	1300 ± 630	1.2
3	40	0.18	220:1	400 ± 140	1.1
		4.5	9:1	910 ± 380	1.2
		13	3:1	370 ± 160	1.2
4	40	0.18	220:1	530 ± 180	1.1
		4.5	9:1	1600 ± 750	1.2
		13	3:1	1500 ± 620	1.2

^aNDA: No detectable aggregates

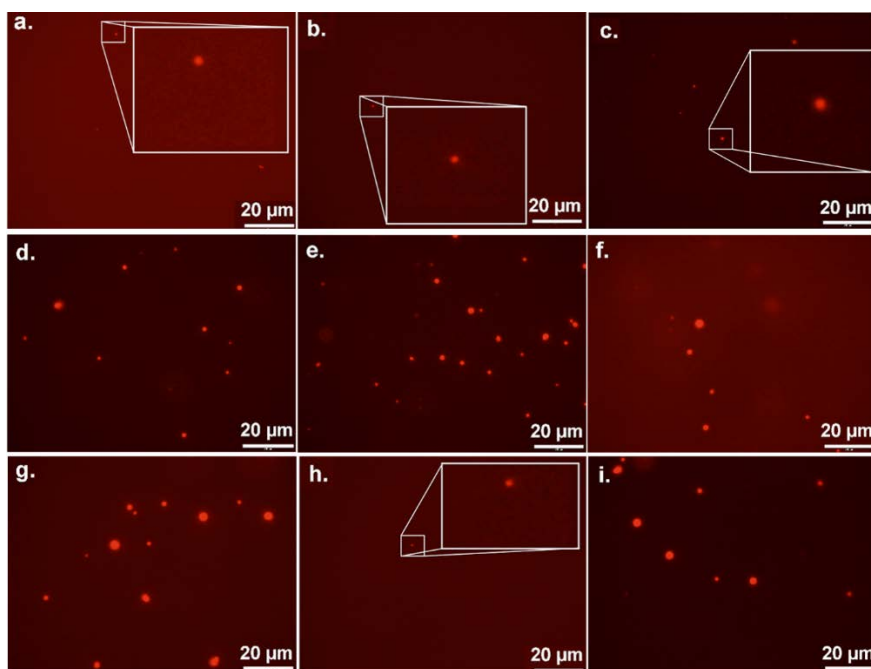


Figure 23. Representative fluorescent micrographs of glycodendrimer mediated galectin-1 nanoparticles. Nanoparticles formed with compounds (a) **2**, (b) **3**, and (c) **4** in a 220 molar excess of galectin-1 are shown in the top row and magnified by 4x for visualization. Nanoparticles formed with compounds (d) **2**, (e) **3**, and (f) **4** in a 9 molar excess of galectin-1 are shown in the middle row. In the bottom row, nanoparticles formed with compounds (g) **2**, (h) **3** (magnified by 4x for visualization), and (i) **4** in a 3 molar excess of galectin-1 are shown.

Table 5. Diameter (nm) and polydispersity of galectin-1/glycodendrimer aggregates measured using fluorescence microscopy over the course of 5 hours.

Galectin-1(μ M)	4 (μ M)	Galectin-1: Dendrimer	Time (hr)	Average Dia. (nm)	Particles (No.)	Polydis persity
40	4.5	9:1	1	1600 $\pm$ 750	71	1.2
			2	1700 $\pm$ 230	37	1.01
			3	1700 $\pm$ 350	6	1.06
			4	NDA ^a		
			5	NDA		

^aNDA: No detectable aggregates

Dynamic Light scattering (DLS)

DLS was used as a complementary technique to characterize galectin-1 nanoparticles formed using **4** by measuring hydrodynamic radii of the aggregates in solution. These results, shown in **Figure 24**, also indicate the formation of small, homogeneous nanoparticles when a large excess of galectin-1 (220:1) was used. In agreement with the results obtained from the fluorescence microscopy studies, the nanoparticle sizes that were determined by DLS were larger when smaller-fold excesses of galectin-1 were used. Polydispersity and the range of measured diameters were also larger in the fluorescence microscopy assays, due to both the capability to detect a broad range of nanoparticles and optimization of the DLS signal, which was bias toward the detection of larger particles. Aggregates formed with **1**, **2**, and **3** exceeded the detection limits of DLS.

Using DLS, the specificity of the interaction between galectin-1 and the lactosides on the multivalent glycodendrimers was assessed. Serially diluted solutions of monomeric lactose were co-incubated with galectin-1 and compound **4**, and inhibition of aggregation was achieved by monomeric lactose, with an IC₅₀ of 1.9 mM, as shown in **Figure 25** (tabulated data is provided in **Table 7**). This result indicates that a specific interaction

between the lactose endgroups on the dendrimers and the carbohydrate recognition site of galectin-1 occurs when nanoparticles are formed.

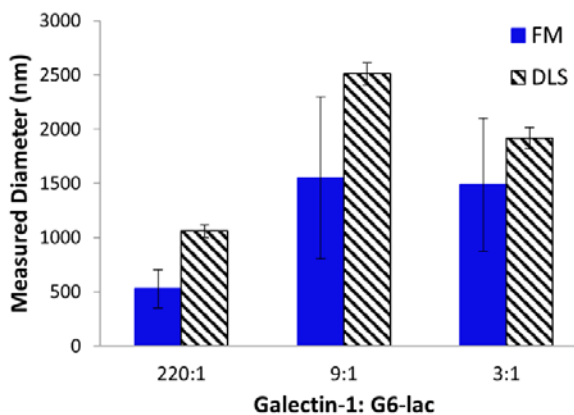


Figure 24. Comparison of average nanoparticle diameter (nm) formed with 4 measured by FM (blue) and DLS (diagonal stripes). Data are shown as mean $\pm$ one S.D. of measurements from at least three experiments ($n \geq 3$)

Table 6. DLS signal and measured effective diameter (nm) of galectin-1/glycodendrimers aggregates.

Compound	[Galectin-1] (μ M)	[Dendrimer] (μ M)	Galectin-1: Dendrimer	Effective Dia. (nm)	Relative Variance	Signal (kcps)
1	40	0.18	220:1	NDA ^a		
		4.5	9:1	NDA		
		13	3:1	NDA		
2	40	0.18	220:1	NDA		
		4.5	9:1	NDA		
		13	3:1	NDA		
3	40	0.18	220:1	NDA		
		4.5	9:1	NDA		
		13	3:1	NDA		
4	40	0.18	220:1	1100 $\pm$ 62 ^b	0.44 $\pm$ 0.46	56 $\pm$ 35
		4.5	9:1	2500 $\pm$ 110	0.16 $\pm$ 0.069	200 $\pm$ 65
		13	3:1	1900 $\pm$ 100	0.29 $\pm$ 0.11	40 $\pm$ 5.6

^aNDA: No detectable aggregates

^bResults from CONTIN analysis

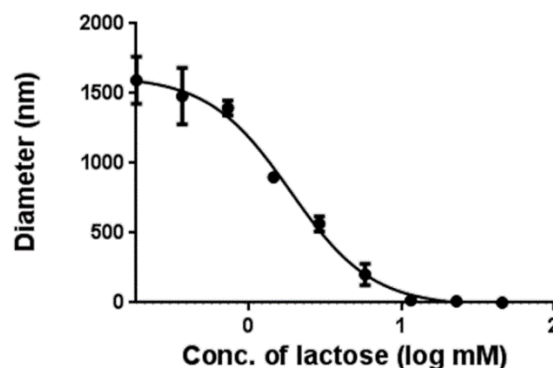


Figure 25. Lactose inhibition of galectin-1 nanoparticle formation with compound **4**.

Table 7. DLS signal and aggregate diameter (nm) measured for monomeric lactose inhibition of galectin-1/**4** aggregate formation.

Galectin-1 (μ M)	4 (μ M)	Lactose (mM)	Log [Lactose(mM)]	Effective Diam. (nm)	Polydispersity	Signal (kcps)
40	0.39	0.18	- 0.74	1600 $\pm$ 240	0.17 ± 0.089	43 $\pm$ 28
		0.36	- 0.44	1500 $\pm$ 290	0.14 ± 0.085	40.0 $\pm$ 18
		0.72	- 0.14	1400 $\pm$ 240	0.13 ± 0.083	62 $\pm$ 46
		1.4	0.16	910 $\pm$ 44	0.17 ± 0.052	16 $\pm$ 5.3
		2.9	0.46	600 $\pm$ 70	0.24 ± 0.094	4.9 $\pm$ 1.5
		5.8	0.76	200 $\pm$ 86	0.56 ± 0.30	4.8 $\pm$ 4.5
		12	1.1	29 $\pm$ 27	0.55 ± 0.20	2.7 $\pm$ 1.7
		24	1.4	20 $\pm$ 12	0.98 ± 1.1	1.5 $\pm$ 0.48
		48	1.7	NDA ^a		

^aNDA: No Detectable Aggregates

Control experiments were performed with different functional groups on the multivalent framework, and this data is shown in **Table 8**. No aggregates were detected upon the addition of a polyhydroxylated sixth generation dendrimer (G6-OH), indicating

that binding requires more than a mere array of hydrogen bonds. (Particles of a diameter 6.8 ± 0.38 nm as measured by NNLS analysis agree with the reported value for a polyhydroxylated generation 6 PAMAM dendrimer).¹⁹⁰ Small nanoparticles (340 ± 20 nm) were obtained when mannose functionalized G(6)-PAMAMs (G6-Man) were combined with galectin-1, and neither monomeric lactose nor monomeric mannose inhibited the formation of these aggregates (**Table 9**).

Table 8. Controls for aggregate formation with galectin-1 and multivalent framework with different functional groups.

Compound	[Galectin-1] (μ M)	[Dendrimer] (μ M)	Diameter (nm)
G6-OH	40	0.39	6.8 ± 0.38 ^{b,c}
		1.7	9.1 ± 1.2 ^{b,c}
		1.8	6.5 ± 1.9 ^{b,c}
G6-Man	40	0.39	NDA
		3.1	340 ± 21

^b Results from NNLS analysis

^c Measured average effective diameter for nanoparticles with G6-OH agrees with reported value for G6-OH aggregate diameter¹⁹⁰

Table 9. Measured diameter of galectin-1/G6-Man aggregates in the presence of monomeric lactose and monomeric mannose.

G6-Man (μ M)	Galectin-1 (μ M)	Carbohydrate	Carbohydrate (mM)	Diameter (nm)
3.1	40	X ^a	X	340 ± 21
3.1	40	Mannose	5.8	370 ± 35
			12	390 ± 7.3
			23	350 ± 15
3.1	40	Lactose	12	390 ± 110

^aX: no monomeric carbohydrate added

Discussion

The results of the fluorescence microscopy and DLS studies described above reveal that multivalent glycodendrimers organize galectin-1 into nanoparticles. In the presence of a large excess of galectin-1, multivalent glycodendrimers **2-4** organize galectin-1 into relatively small and remarkably homologous nanoparticles (**Figure 22** and **Figure 23a-c**). This is likely a result of the multivalent framework being saturated with galectin-1, providing few uncomplexed nucleation sites for incorporation into larger nanoparticles (**Figure 26**). Therefore, an increase in the concentration of the multivalent framework should correlate to an increase in aggregate size, as was observed for 9:1 and 3:1 ratios of galectin-1 to glycodendrimer (**Figures 22** and **26**). The exception to this is that small homogeneous nanoparticles were observed for compound **3** when a slight excess of galectin-1 was used (3:1). In this lactoside-rich environment, the presence of a large excess of lactoside residues apparently enabled increased nucleation at the expense of aggregation, but it is not clear why **3** is different from the other dendrimers in this regard. Overall, the results described here agree with mathematical modeling studies that identified scaffold concentration as a key determinant in maximizing scaffold-mediated nucleation.¹⁹¹

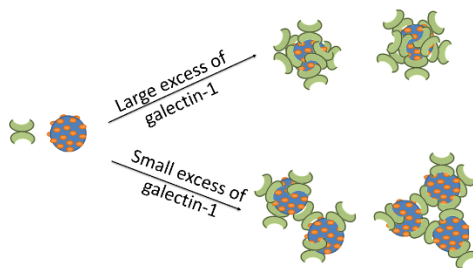


Figure 26. Schematic representation of Galectin-1/glycodendrimer aggregates at varying stoichiometries.

The size of the nanoparticles formed in the presence of a large excess of galectin-1 is fundamentally remarkable. In the presence of enough galectin-1 to saturate the multivalent framework, aggregates approximately 400 nm in diameter were measured (**Figure 22**). The distance between the galectin-1 CRDs is approximately 5 nm.¹⁹² The diameter of the G(3), G(4), and G(6)-PAMAM dendrimers used to synthesize compounds **2**, **3**, and **4**, respectively, range from approximately 4 nm to 7 nm.¹⁴⁴ Therefore, multiple galectin-1 and glycodendrimer particles must interact to form the 400 nm aggregates, and this is favorable even when the scaffold is ostensibly saturated with galectin-1.

The time-lapse measurements with fluorescence microscopy illustrate the binding affinity enhancements that can be achieved via multivalency (**Table 5**). Monovalent interactions between a lectin and a carbohydrate are weak, occurring with a K_d of approximately 10^{-3} M.^{193,194} The multivalent presentation of epitopes can effectively increase the time in which the lectin is bound by the ligand, and the high localized concentrations of lactosides presented on the multivalent dendrimer framework were observed to nucleate galectin-1 into nanoparticles that persisted for almost 4 hours.

The formation of nanoparticles reported herein was the result of two aggregation phenomena: nucleation and growth. Nucleation describes the processes where free monomeric units assemble to form nuclei.¹⁹⁵ Once the assembly reaches the critical size, the nuclei acts as an activated state for growth.¹⁹⁵ Particles smaller than the critical size, because of high surface-to-volume ratio, are unstable and dissipate into monomers, and these monomers can polymerize with the activated state.¹⁹⁵ The activated nanoparticle continues to grow until a thermodynamically equilibrated state is reached in which the total

interfacial area is minimized.¹⁹⁶ The free energy of the polymer is proportional to the surface area, and stabilization of the nanoparticle is achieved by adopting a shape that minimizes the surface for a given volume.^{195, 197} The stability of the larger polymer is due to molecules located in the volume on the periphery of the polymer being higher in energy than molecules located in the internal volume.¹⁹⁷ The surface area of a large particle, whose volume is equal to the sum of the volumes of several small particles, is smaller than the total surface areas of the small particles. Consequently, the interfacial curvature of the solid to liquid phase is smaller for large particles, and this characteristic lowers the stress and the energy of the system. Thus, the larger nanoparticle is energetically more favorable than the smaller ones. To form energetically favorable nanoparticles, interfacial area of smaller nanoparticles is reduced by diffusional mass transfer from areas of greater interfacial curvature to areas of smaller curvature through a process called Oswald Ripening.¹⁹⁶

Nucleation and growth are determined by the intermolecular attractive and repulsive forces of the system, such as charge-charge interactions, hydrogen bonding, dipole-dipole interactions, van der Waals interactions, and interactions due to the hydrophobic effect. Nanoparticle growth occurs when the attractive forces of the system compensate for and overpower the repulsive forces.

For heterogeneous polymers, nucleation of monomers can be initiated by a nucleating agent, and multiple copies of the nucleating agent can be incorporated into the nanoparticle to provide additional nucleation sites for growth¹⁹⁸. Dendrimers functionalized with lactosides provide nucleation sites to recruit galectin-1. Attractive forces between the lactosides on the functionalized dendrimers and galectin-1 mediate

nanoparticle assembly. Nucleated galectin-1, because of the dimeric structure, can interact multivalently with a second glycodendrimer during nanoparticle growth, and this processes likely occurs numerous times to form the nanoparticles reported herein.

Aggregates formed with compounds **1**, **2**, and **3** exceeded the detection limit of DLS. However, fluorescent microscopy images captured with **3** helps clarify the discrepancy with the DLS measurements. Shown in **Figure 27a** below, dynamic and amorphous nanoparticles were observed with **3**. Visual inspection of the fluorescent micrograph suggests that these particles are oligomers of smaller aggregates. These oligomers were observed to continuously reorganize and disperse (**Figure 27b**), and these dynamic interactions with galectin-1 nanoparticles formed with **2** and **3**, respectively, likely obfuscated the sensor. The large oligomers are likely thermodynamically less stable than the smaller aggregates as evidenced by the transitory formation.

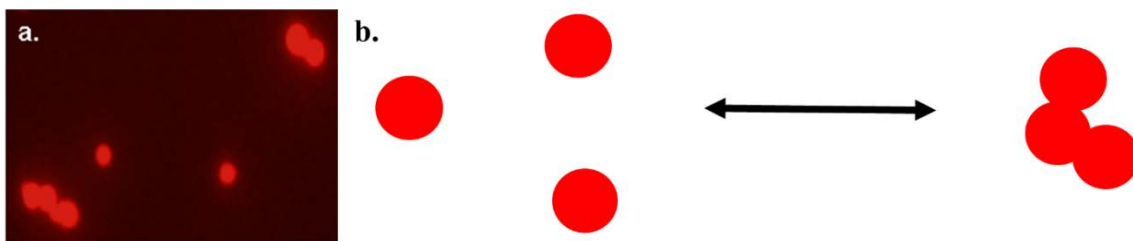


Figure 27. (a) Fluorescent micrograph of dynamic nanoparticles formed with galectin-1 and **2** and **3**. (b) schematic illustration of dynamic interaction.

Galectin-1/**1** nanoparticles were not detected by DLS or fluorescence microscopy. Because galectin-1 is known to bind these glycodendrimers, it is likely that aggregates formed but were below the detection limit of the fluorescence microscopy technique (which is about 200 nm). However, it is also possible that **1** does not bind galectin-1 very

well, and this may also explain the unreliable ELISA results for the binding of galectin-1 to **1**.

The lactose inhibition assay supports the specificity of the interaction, and the IC₅₀ value to inhibit galectin-1 aggregates formed with **4** as measured by DLS (1.9 mM) matches with the IC₅₀ value from the ELISA (1.7 mM). These similar IC₅₀ values may be explained by the “bind and jump” mechanism (see Chapter 2 for a discussion of this mechanism).^{175,176}

Controls run with other functional groups on the dendrimer also support the specificity of the interaction: no aggregates were formed with a polyhydroxylated PAMAM dendrimer, and the aggregates formed with the mannose functionalized generation 6 PAMAM dendrimer likely proceeded through non-specific interactions. The inability to inhibit galectin-1 aggregates formed with mannose functionalized generation 6 PAMAM dendrimer using lactose or mannose indicates these nanoparticles likely do not rely on interactions in the β -galactoside binding site on galectin-1. Non-specific glycodendrimer/galectin-1 interactions are most likely responsible for formation of these aggregates. The extracanal activity of galectin-1 has been reported in literature.¹⁹⁹ Miller *et al.* observed galectin-1 binding to α -galactomannan derivatives, and this interaction was characterized to occur at a site distinct from the canonical CRD using NMR.¹⁹⁹ It is likely that the interaction with the α -galactomannan derivatives and mannose functionalized generation 6 PAMAM dendrimer is a non-specific interaction that is accessible with multivalent frameworks. Thus, monomeric carbohydrates (mannose and

lactose) are not able to inhibit non-specific multivalent interactions between the backbone and the galectin-1.

Conclusion

In a large excess of galectin-1, nanoparticles formed with **2**, **3**, and **4** are remarkably homogenous in size, with a diameter of ~400 nm. Because the glycodendrimer initiates galectin-1 nucleation, and multivalent galectin-1 can cross-link glycodendrimers, the galectin-1/glycodendrimer nanoparticles reported here are likely formed through multiple nucleation and growth events.

These aggregates likely reflect protein-carbohydrate interactions occurring at the cell surface and other non-surface bound multivalent assemblies. For example, galectin-1 has been observed to activate apoptotic signaling pathways by clustering receptors through a network of multivalent interactions,⁷⁸ and the results above indicate that changes in ligand concentration influence galectin-1 nucleation. Furthermore, galectin-1 has been demonstrated to mediate cellular aggregation by cross-linking cell surface glycoproteins, and these results suggest that cellular aggregation can be modulated with a multivalent framework.⁵⁶ Overall, the galectin-1/glycodendrimer nanoparticles reported above may alter the presentation of galectin-1 to the cells to modulate the biological activities of the lectin, such as cellular aggregation and microtubule formation occurring during angiogenesis.

Experimental Procedures

Galectin-1 was provided by Dr. Linda Baum and Mabel Pang of UCLA

Fluorescence Microscopy

Reagents for fluorescence microscopy were purchased from Molecular Probes. Both the galectin-1 and the glycodendrimer were fluorescently labelled (discussed below) to measure galectin-1 nanoparticles using fluorescence microscopy. Fluorescent images were captured on an Olympus BX-61 motorized epifluorescent microscope with MicroSuite software with a 100x oil immersion objective at an exposure time of 2 ms.

Gimp 2.8 image manipulation software was used to combine images for subsequent analysis. Pixcavator 6.0 Image Analysis software (Intelligent Perception) was used to measure particle perimeter in terms of pixel count. Particles below a threshold of 80 “round” (as calculated by Pixcavator) were excluded from analysis.

Galectin-1 Glycodendrimer Nanoparticle Formation Assay. At a constant concentration of galectin-1 (40 μ M), aggregate size was measured at ratios of galectin-1 to glycodendrimer of 220:1, 9:1, and 3:1. Assays were run in triplicate and incubated for 1 hour at room temperature. From each sample, three 15 μ L aliquots were spotted on a microscope slide, and at least three pictures were captured from each spot.

Fluorescent Microsphere Standards. Fluorescent microsphere standards (200 nm, 1000 nm, and 10000 nm reported diameter) (FluoSpheres Fluorescent Microspheres, Molecular Probes) were used to establish a calibration curve to convert the measured

particle diameter (pixels) to diameter (nm) at an exposure time of 2ms (**Figure 22**). Stock solutions of the microsphere standards were diluted 100 fold in PBS. Images were captured with the epifluorescent microscope and analyzed with Pixcavator.

Alexa Fluor 488 Labeled Glycodendrimer. Alexa Fluor 488 hydrazide powder (1 mg) was dissolved in 500 mL millipore H₂O for a 2 mg/mL stock solution. Lyophilized glycodendrimers were dissolved in millipore H₂O for 2 mg/mL stock solutions. To the glycodendrimer solution, NaIO₄ was added (2 eq. per dendrimer), and the reaction mixture was stirred at RT (19.3 °C) for 2 hr. After 2 hr, Alexa-Fluor 488 hydrazide (1 equiv. per dendrimer) was added and allowed to react for 0.5 hr at RT. After 0.5 hr, the reaction mixture was stored at -78 °C until frozen. The frozen reaction mixture was thawed and purified by dialysis against 1 kD MWCO (Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane) in millipore H₂O. The purified reaction mixture was frozen at -78 °C, lyophilized to dryness, and reconstituted in PBS for a 2 mg/mL solution.

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 M⁻¹cm⁻¹, which measured a labeling ratio of 1 to 1 Alexa Fluor 488 to dendrimer.

Alexa Fluor 555 Labeled Galectin-1. Galectin-1 was labeled with Alexa Fluor 555 NHS Ester (succinimidyl ester) (Molecular Probes).²⁰⁰ At a 6 to 1 ratio of galectin-1 to Alexa Fluor 555, the reagents were allowed to react at room temperature (19.3 °C) for 1 hr in PBS. The reaction mixture was purified by dialysis using 1 kD MWCO dialysis tubing

(Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane) in PBS. Quantification of galectin-1 labeling was achieved using a UV-Vis Spectrometer (Molecular Devices, SpectraMax Plus, Softmax Pro 5). Absorbance was measured at 555 nm with a molar extinction coefficient of $150,000 \text{ M}^{-1}\text{cm}^{-1}$, yielding a labeling ratio of 5 to 1 Alexa Flour 555 to galectin-1.

Dynamic Light Scattering

Dynamic light scattering was performed using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corp.) to measure galectin-1/glycodendrimer aggregates at the same concentrations and ratios that were used in the fluorescence microscopy assays.

Galectin-1 Glycodendrimer Nanoparticle Formation Assay. To a final concentration of galectin-1 (40 μM), varying amounts of glycodendrimer (0.18, 4.5, and 13 μM ; final concentration) were added to achieve the following ratios of galectin-1 to glycodendrimer: (a) 220: 1; (b) 9: 1; and (c) 3: 1. PBS was added to the UV-cuvette (Plastibrand) to bring the total volume of the solution to 200 μL . Assays were incubated for 1 hour at room temperature (19.7 $^{\circ}\text{C}$). Each sample was scanned in triplicate, at intervals of approximately five minutes, and vortexed between scans.

Lactose Inhibition Assay. A lactose inhibition assay was performed in which monomeric lactose was co-incubated with galectin-1 and compound 4. Assay solutions were prepared at a final concentration of 40.0 μM galectin-1 and 0.35 μM 4 in a final volume of 200 μL . Separate samples were prepared for each concentration of monomeric

lactose tested. Three trials were prepared for each concentration, and triplicate measurements were performed for each sample at approximately five minute intervals.

Galectin-1 Binding Specificity Assay. Mannose-functionalized PAMAM dendrimer generation 6 (G6-Man) was synthesized according to reported procedure.¹²⁰ To galectin-1 (40 μ M final concentration), mannose functionalized generation six dendrimer (0.39 μ M and 3.1 μ M final concentrations, respectively) was added. Separate monomeric mannose (5.8 mM and 12 mM final concentrations) and monomeric lactose (12 mM) inhibition experiments were performed to inhibit the galectin-1 aggregate formed with the mannose functionalized dendrimer. Additionally, to a final concentration of galectin-1 of 40 μ M, a polyhydroxylated generation six dendrimer was added at different ratios (0.39 μ M, 1.7 μ M, and 1.8 μ M final concentrations, respectively) although no aggregate was detected.

Solution Preparation. Galectin-1 was prepared in filtered PBS buffer. Lactose functionalized dendrimers (compounds **1**, **2**, **3**, and **4**), mannose functionalized generation size mannose functionalized dendrimer, and a polyhydroxylated dendrimer generation six were prepared by dissolving a lyophilized sample in filtered Millipore water to final concentrations of 10 μ M and 100 μ M. Monomeric lactose and mannose were separately taken up in filtered PBS buffer for 100 μ M solutions. Prior to mixing the reagents, all solutions were filtered using a 0.22 μ m millipore filter (Millex® - GV 0.22 μ m Syringe-Driven Filter Unit) to eliminate dust particle interference. Assays were run at total volume

of 200 μL in the rinsed UV-cuvette (Eppendorf UVette®, 50 – 2000 μL , 220 – 1600 nm, center height 8.5 nm).

CHAPTER 4

EXAMINATION OF GALECTIN-1 MEDIATED
CELLULAR AGGREGATION USING GLYCODENDRIMERSIntroduction

Assays that incorporate cells have also been applied to further the understanding of multivalent protein-carbohydrate interactions in biological process. Fluorescence microscopy, through fluorescent labelling of the protein and/or the glycoconjugate, can be used to visualize the distribution of lectin or glycoconjugate during biological processes. For example, cell-surface glycans have been fluorescent tagged to investigate glycan distribution upon lectin binding, demonstrating that different galectin-1 receptors are segregated and clustered during T-cell apoptosis.⁷⁸ Similarly, clustering of Mucin-1 has been demonstrated to occur during galectin-3 mediated cellular aggregation.^{39, 82} A hemagglutination assay can be used as a model to study lectin-mediated cellular adhesion of red blood cells in a microtiter plate format, and this assay can be used to detect the presence of a lectin or measure the minimum concentration of lectin to induce agglutination.²⁰¹ Galectin-1 binding specificity and affinity has been valuated with a hemagglutination inhibition assay.^{200,202}

Cellular aggregation assays have been used to further the understanding of the biological activities mediated by multivalent galectin-1 binding. Tinari *et al.* observed inducement of homotypic cell aggregation in human melanoma cells (A375) by galectin-1 and demonstrated that bivalent binding of 90K/Mac-2BP, a cell surface glycoprotein, on apposing cells facilitated aggregation.⁵⁶ Using oligosaccharide derivatives and structural

analogs of methyl β -lactosaminide, Iurisci *et al.* was able to inhibit homotypic aggregation of the A375 cell line.⁸⁰ Furthermore, Clausse *et al.* observed that increased galectin-1 expression in prostate two carcinoma cell lines (PC-3 and DU145) promoted heterotypic aggregation of cancer cells to endothelial cells.⁸⁶ However, mechanistic elucidation of cell-surface interactions during galectin-1 mediated multivalent cellular recognition processes remains largely unresolved.

Here, a cell-based assay was used to study the influence of galectin-1 and glycodendrimers on cellular aggregation processes. After determining that lactose-functionalized dendrimers **1-4** reproducibly nucleate galectin-1 into aggregates, the multivalent glycodendrimers **1-4** were applied to study galectin-1 mediated cellular aggregation with DU145 human prostate cancer cells. The DU145 cell line was chosen because it expresses a putative galectin-1 ligand – the Thomsen Friedenreich (TF) antigen on Mucin-1.^{155, 74} The results of the cellular aggregates assay are reported below.

Results

Homotypic Cellular Aggregation Assay

The DU145 human prostate carcinoma cell line expresses elevated levels of galectin-1 relative to healthy cells,^{56, 86} which suggests that galectin-1 is important to the biological activities of these cancer cells. To investigate galectin-1 mediation of cellular aggregation/tumor formation, cellular aggregation assays were performed in the presence of exogenous galectin-1, and with galectin-1 and increasing concentrations of multivalent glycodendrimers **1, 2, 3, and 4**. The glycodendrimers at concentrations chosen so that

approximately equal concentrations of lactosides were presented. Controls without galectin-1 and with glycodendrimers were previously performed.³⁹ Cells were co-incubated with galectin-1 and glycodendrimers for 1 hour with gentle agitation. Assays were run in triplicate, and three spots from each replicate were viewed with a microscope and videos were captured. From the videos, four representative images were captured per spot for a total of 12 images per replicate, and the 12 images were stitched together as shown in **Figures 28-32**. Image analysis software (Pixcavator, Intelligent Perception) was used to quantify cellular aggregation by defining pixel counts corresponding to five or more cells as aggregates.

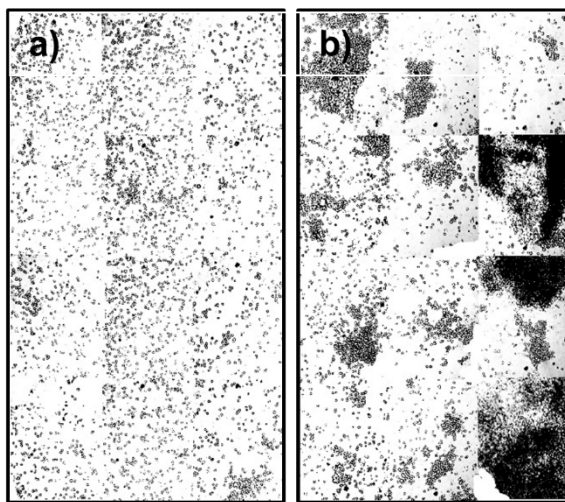


Figure 28. Representative images of cellular aggregation of DU145 cells. (a) Untreated DU145 cell standard. (b) Aggregated DU145 cells in the presence of galectin-1 (3.7 μ M).

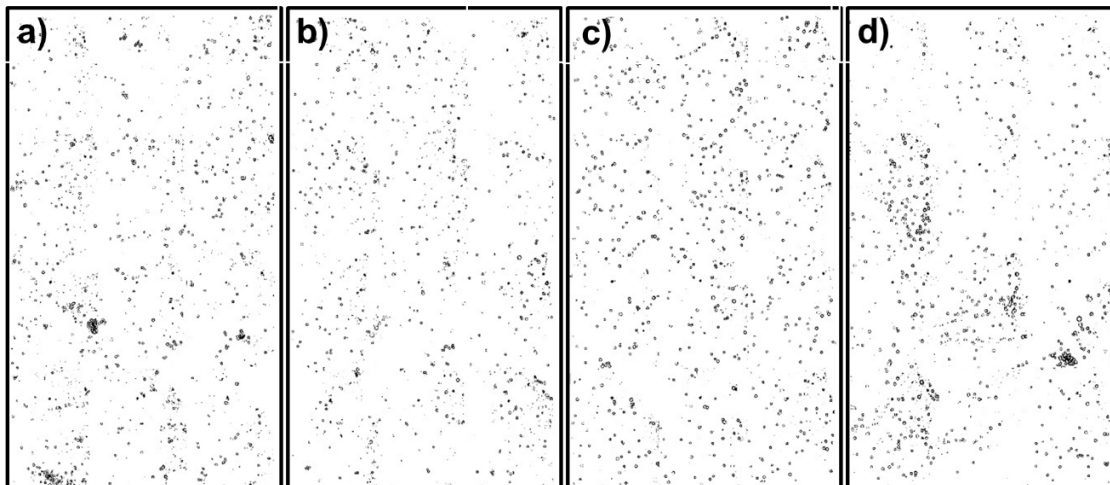


Figure 29. Representative images showing inhibition of galectin-1 (3.7 μ M) mediated homotypic aggregation of DU145 cells by (a) 33 μ M **1**, (b) 66 μ M **1**, (c) 99 μ M **1**, and (d) 132 μ M **1**.

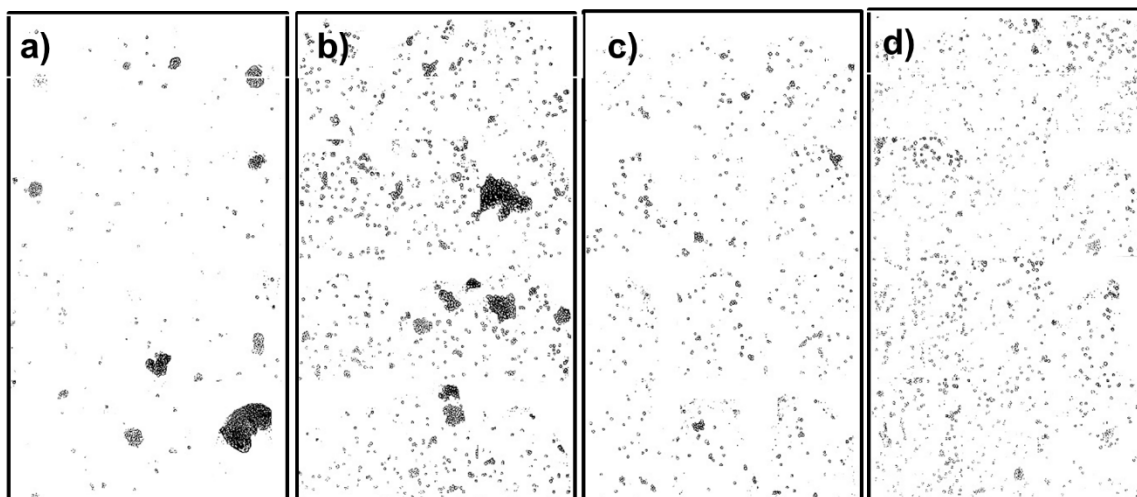


Figure 30. Representative images of homotypic aggregation of DU145 cells in the presence of galectin-1 (3.7 μ M) and (a) 17 μ M **2**, (b) 35 μ M **2**, (c) 52 μ M **2**, and (d) 69 μ M **2**.

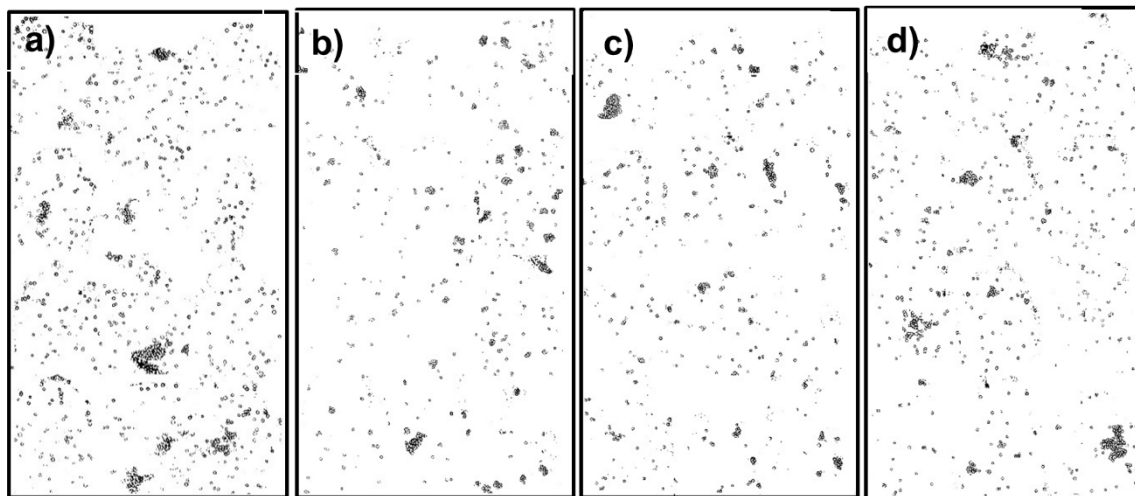


Figure 31. Representative images of homotypic aggregation of DU145 cells in the presence of galectin-1(3.7 μ M) and (a) 8 μ M **3**, (b) 16 μ M **3**, (c) 24 μ M **3**, and (d) 36 μ M **3**.

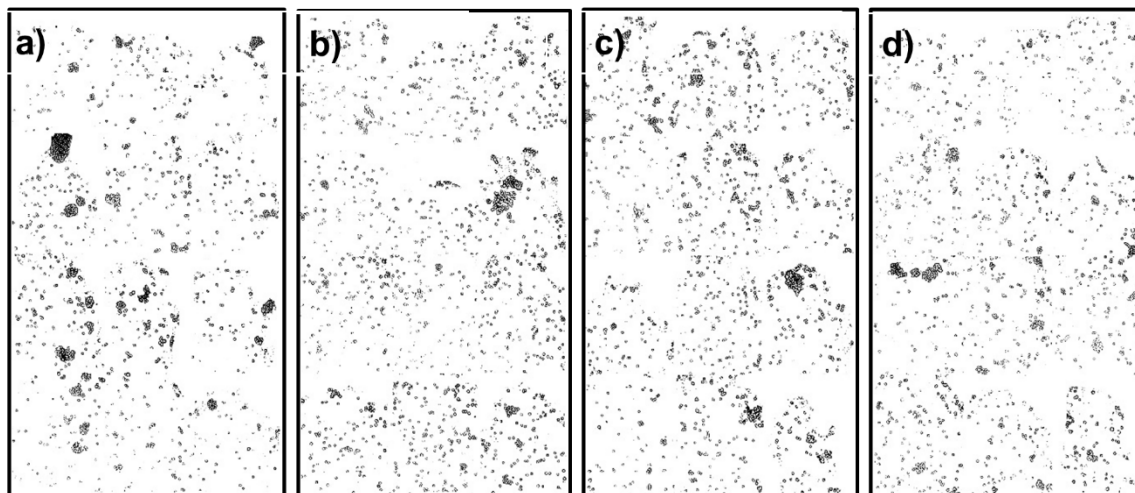


Figure 32. Representative images of homotypic aggregation of DU145 cells in the presence of galectin-1(3.7 μ M) and (a) 2 μ M **4**, (b) 4 μ M **4**, (c) 6 μ M **4**, and (d) 9 μ M **4**.

As shown in **Figures 28** and **33**, untreated DU145 cells were not aggregated (free cells) (**Figure 28a**); upon the addition of exogenous galectin-1, however, extensive aggregation was observed (**Figure 28b**), which comports with literature demonstrating galectin-1 inducement of cellular aggregation.^{51, 52, 58, 60, 75, 86, 203} The interaction between the galectin-1 and the DU145 cells generated large aggregates that exceeded the detection

limits of the image analysis software. Visual inspection of galectin-1 treated cells confirmed nearly complete aggregation of all cells; therefore, the percentage of free cells for the galectin-1 treated DU145 cells without glycodendrimer (galectin-1 std) was conservatively set at 20%.

As shown in **Figure 33a**, when lactose functionalized dendrimers **1-4** were added to the DU145 cells with galectin-1, cellular aggregation was inhibited. The smallest glycodendrimer, second generation compound **1**, most effectively inhibited cellular aggregation. Even at the lowest concentration of **1** shown in **Figure 33**, complete inhibition of cellular aggregation was observed (**Figure 33a**). Incomplete inhibition of aggregation was observed for compounds **3** and **4**. For fourth generation lactose functionalized dendrimer **3**, the percentage of free cells plateaued at 50% (**Figure 33c**). With sixth generation lactose functionalized dendrimer, **4**, only 30% of the cells remained clustered (**Figure 33d**). Although glycodendrimer concentrations were normalized to present a similar concentration of lactoside residues at each stage in the assay, dose-responsive inhibition of galectin-1 mediated cancer cell aggregation was only observed with lactose functionalized G(3)-dendrimer **2** at these concentrations (**Figure 33b**, and representative images **32e-h**). Nearly complete inhibition of cellular aggregation was observed with compound **2** at the highest concentration of **2**. The inhibition observed with compounds **1** and **2** indicates that the smaller glycodendrimers are the most effective inhibitors of galectin-1 induced cellular aggregation.

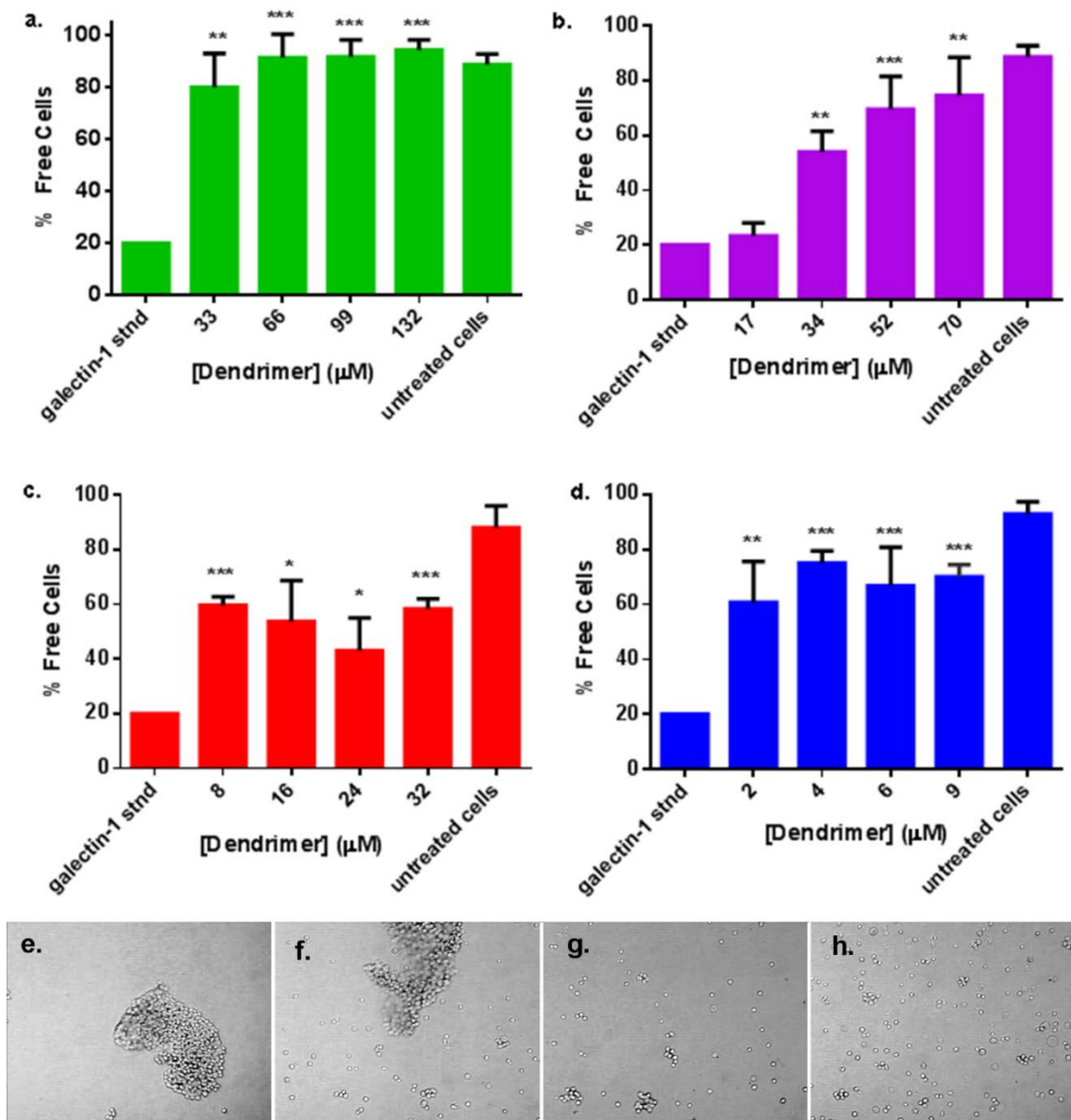


Figure 33. Cellular aggregation assays with DU145 human prostate carcinoma cells. Cancer cell aggregation assays with (a) **1**, (b) **2**, (c) **3**, and (d) **4** were performed in the presence of 3.7 μM galectin-1 and increasing glycodendrimer concentrations, with controls for galectin-1 treated cells and untreated cells. Glycodendrimer concentrations were normalized to present the same concentration of lactose residues. Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least three experiments ($n \geq 3$). Statistical analysis was performed using an unpaired two-tailed student's T-Test by comparing the % free cells to the galectin-1 standard and * indicates a p value < 0.05 , ** indicates a p value < 0.01 , and *** indicates a p value < 0.001 . Representative images of cellular aggregation are provided for DU145 with 3.7 μM galectin-1 and: (e) 17 μM **2**; (f) 34 μM **2**; (g) 52 μM **2**; and (h) 70 μM **2**.

Because **1** was observed to be the most potent inhibitor, additional experiments were performed to explore the dose-responsive interaction. As shown in **Figure 34**, lower concentrations of **1** (3.3 μ M and 16 μ M) were applied to the DU145 cells in the presence of galectin-1 (3.7 μ M), and the percent free cells decreased from free cells at 66 μ M, to 80% at 33 μ M, to 70% at 16 μ M, to 35% at 3.3 μ M. Black and white images of cellular aggregation at 3.3 μ M, 16 μ M, 33 μ M, and 66 μ M are provided in **Figure 35a–d**.

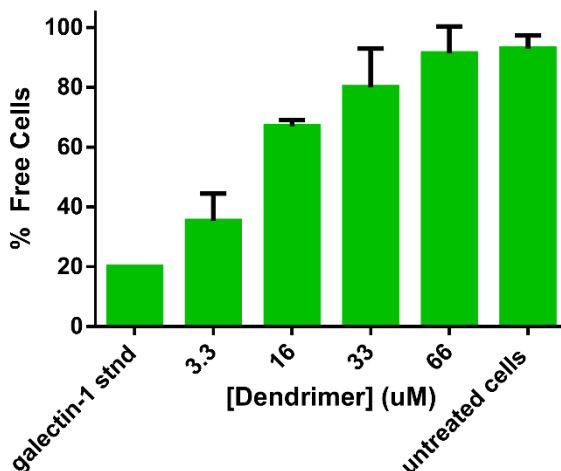


Figure 34. Dose responsive inhibition of galectin-1 mediated cellular aggregation of DU145 cells by **1**. Data is shown as mean $\pm$ one S.D. of triplicate measurements from at least three experiments ($n \geq 3$).

Monomeric Lactose Control

A control experiment was performed to measure the ability of monomeric lactose to inhibit aggregation of DU145 cells in the presence of 3.7 μ M exogenous galectin-1. The concentration of monomeric lactose required to inhibit cellular aggregation is 6 mM (**Figure 36**). On a per lactose basis, this concentration is 15-fold higher than the 66 μ M concentration of **1** that was required for complete inhibition of cellular aggregation.

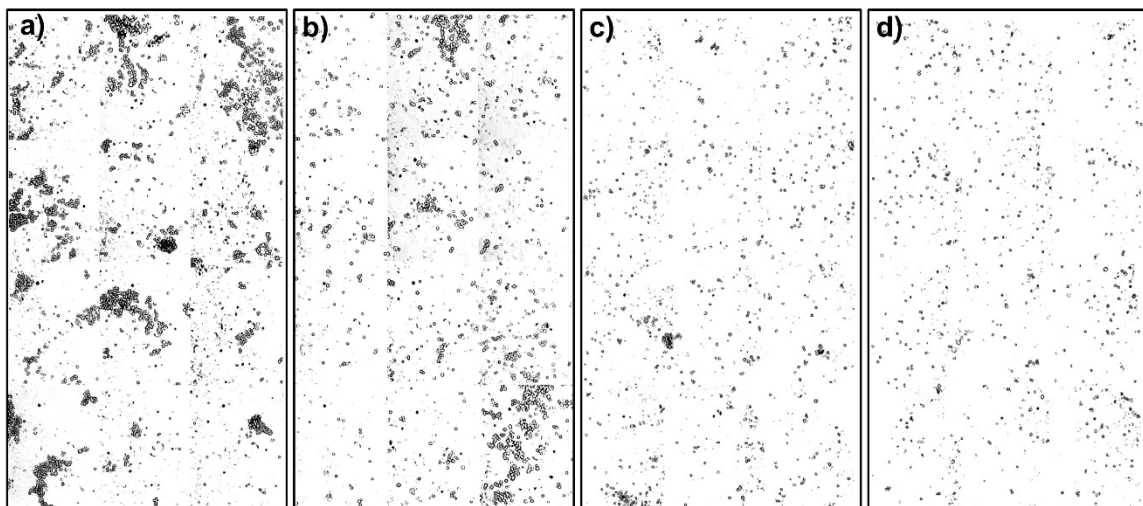


Figure 35. Representative images of dose responsive inhibition of galectin-1 mediated cellular aggregation of DU145 cells using **1**.

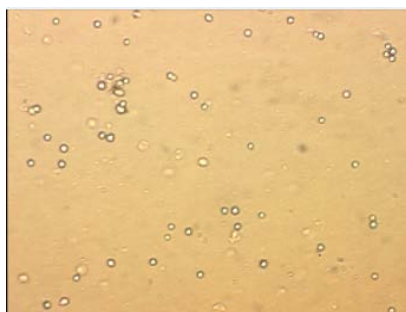


Figure 36. Inhibition of galectin-1 mediated cellular aggregation in the DU145 cell line by monomeric lactose.

Binding Specificity Control

To investigate the specificity of aggregation inhibition, mannose-functionalized generation 6 PAMAM dendrimer (**G6-Man**) was introduced to the DU145 system in the presence of galectin-1 (3.7 μM), and the assay was performed the same as reported for **1-4**. As shown in **Figures 37 and 38**, cellular aggregates persisted over the range of G6-Man concentrations (2 μM , 3 μM , 6 μM , and 10 μM), indicating that mannosides do not significantly impact galectin-1 mediated cellular aggregation.

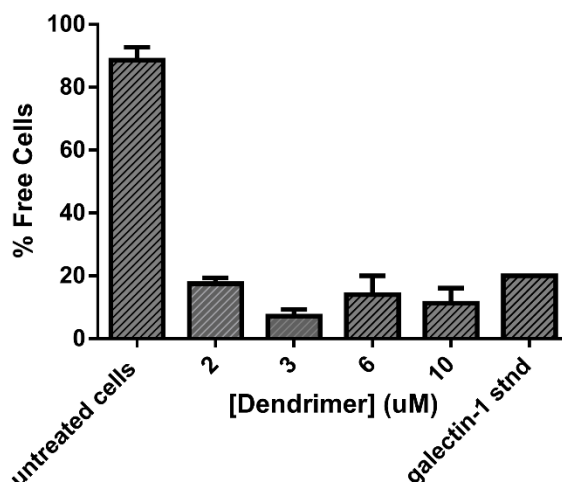


Figure 37. Cellular aggregation assay with DU145 human prostate carcinoma cells and mannose functionalized generation 6 PAMAM dendrimer. Cancer cell aggregation assays were performed in the presence of 3.7 μM galectin-1 and a final concentration of mannose functionalized G(6) of 2 μM , 3 μM , 6 μM , and 10 μM . Controls for galectin-1 treated cells and untreated cells were performed. Data are shown as mean $\pm$ one S.D. of triplicate measurements from two experiments ($n \geq 2$).

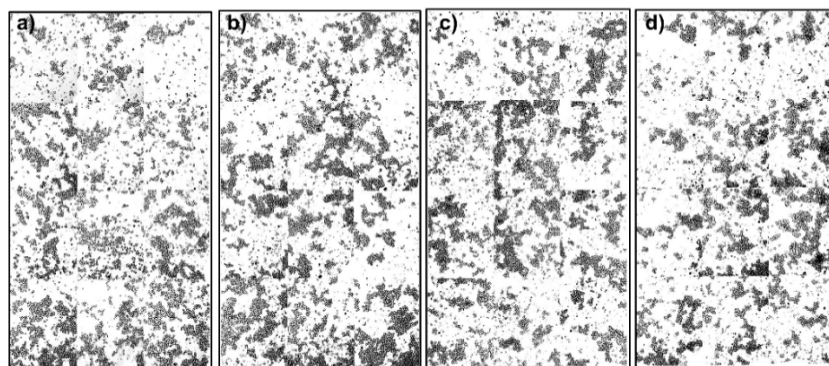


Figure 38. Representative images of galectin-1 mediated cellular aggregation of DU145 cells in the presence of (a) 2 μM , (b) 3 μM , (c) 6 μM , and (d) 10 μM mannose functionalized G(6) PAMAM dendrimer.

Fluorescence Microscopy to Determine Cell Surface Localization of Galectin-1 and Impact of Glycodendrimer

To determine the cell surface distribution of galectin-1, galectin-1 was labeled with a galectin-1 antibody and goat anti-rabbit A488. Compound **1**, which elicited the greatest

response to galectin-1 mediated homotypic aggregation, was chosen to demonstrate the impact of multivalent glycodendrimers on the surface localization of galectin-1. These microscopy experiments were performed the same as the homotypic aggregation assays previously described for **1**. Aliquotes were removed and incubated with a galectin-1 antibody. After incubation, the cells were fixed and a fluorescent anti-galectin-1 antibody was added. For the untreated cells, a faint green fluorescence was visible throughout the cell surface, indicating that galectin-1 was generally disbursed over the cell surface (**Figure 39a**). In the presence of exogenous galectin-1, visualization of galectin-1 distribution using the galectin-1 antibody-fluorescent conjugate showed punctated fluorescence, demonstrating that galectin-1 (when in higher concentrations) is localized at cell-cell interfaces (**Figure 39b**). The patterning of galectin-1 in the presence of **1** (132 μ M) and galectin-1 (3.7 μ M), and in the presence of **1** (132 μ M) and absence of galectin-1 (Figures 38c and 38d, respectively), were similar to the untreated standard.

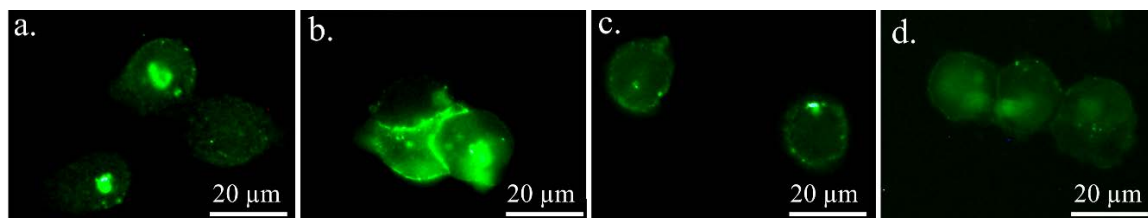


Figure 39. Fluorescent micrographs of DU145 cells in the absence and/or presence of galectin-1 and lactose-functionalized dendrimers investigated with galectin-1 antibody and a fluorescent conjugate. (a) Untreated cells. (b) Cells incubated with galectin-1 (3.7 μ M). (c) Cells incubated with galectin-1 (3.7 μ M) and 132 μ M **1**. (d) Cells incubated with 132 μ M **1** in the absence of galectin-1.

Discussion

The DU145 human prostate carcinoma cell line was chosen to demonstrate that multivalent interactions initiated by a synthetic multivalent system can be used for effectively controlling cellular processes. DU145 cells express elevated levels of both galectin-1⁸⁶ and its putative receptor Mucin-1,¹⁵⁵ which suggests that galectin-1 mediated β -galactoside binding is critical to cellular aggregation/tumor formation in this cell line. In the presence of exogenous galectin-1, extensive cellular aggregation was observed, which agrees with literature reporting galectin-1 enhancement of aggregation.^{51,52,86,203}

As shown in **Figure 39**, the fluorescence images indicate that galectin-1 reorganizes the cell surface to increase aggregation potential in the DU145 cell line, most likely by interacting with Mucin-1 on the cell surface. Comparing **Figure 39a** to **Figure 39b**, the presence of exogenous galectin-1 results in punctate fluorescence localized at cell-cell interfaces. When the cells were incubated in the presence of **1** and galectin-1 (**Figure 39c**), the cells looked similar to the untreated standard, indicating the glycodendrimers diverts galectin-1 from repatterning the cell surface. When the cells were incubated with **1** but no additional galectin-1 was added (**Figure 39d**), the cells again looked similar to the untreated cells.

There are two likely mechanisms for galectin-1 mediation of cellular aggregation: (1) as shown in **Figure 40a**, cross-linking of receptors (TF antigen Mucin-1) on adjacent cells can directly facilitate aggregation; and (2) alternatively, as shown in **Figure 40b**, clustering of receptors (TF antigen Mucin-1) can expose adhesion molecules that interact with adhesion molecules on neighboring cells to cause aggregation. The second method is

similar to the interaction of galectin-3 with the TF antigen on Mucin-1.³⁹ Because galectin-1 is also known to bind TF antigen Mucin-1,⁷⁴ the second mechanism, which involves receptor clustering, is the most likely mechanism for galectin-1 mediated cellular aggregation/tumor formation in the DU145 cell line.

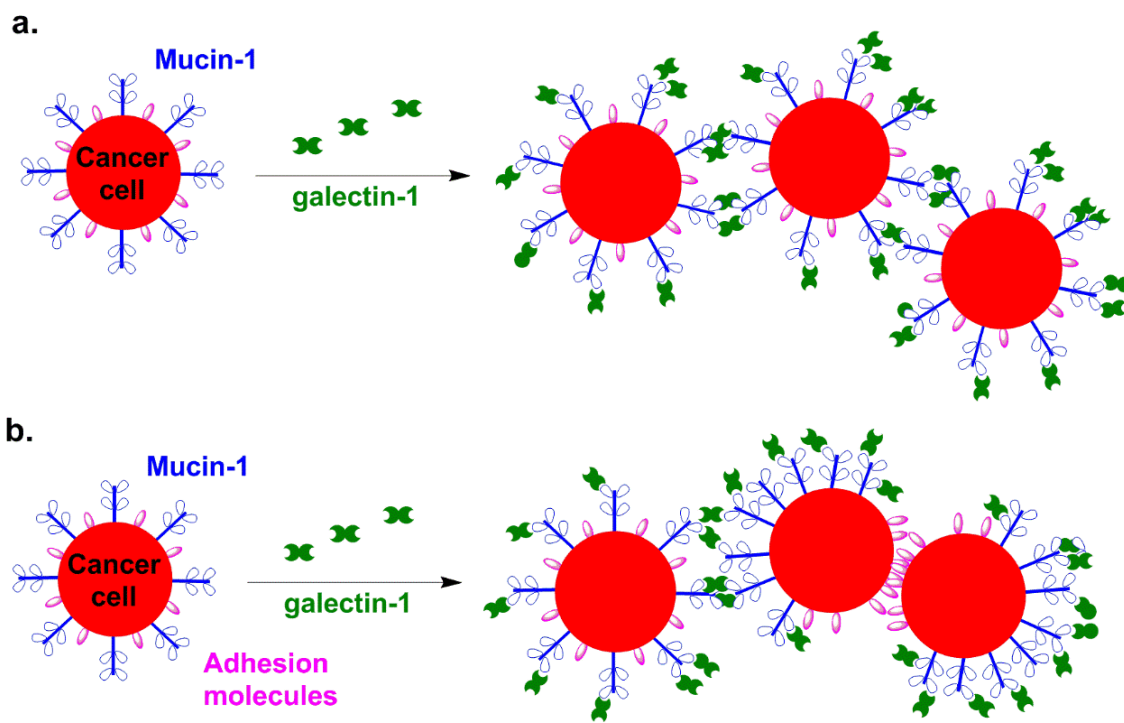


Figure 40. Scheme of galectin-1 mediated cellular aggregation. (a) Cross-linking of Mucin-1 on apposing cells induces cellular aggregation. (b) Mucin-1 is redistributed and clustered on the cell surface in response to galectin-1 binding, which exposes adhesion molecules that interact with other adhesion molecules to mediate cellular aggregation.

The addition of glycodendrimers had a pronounced impact on the galectin-1 cellular aggregation system. Lactose-functionalized dendrimers **1**, **2**, **3**, and **4**, which can nucleate galectin-1 into nanoparticles (see Chapter 3), divert the galectin-1 from its role in cellular aggregate/tumor formation. As shown schematically in **Figure 41**, the glycodendrimers

seem to be providing competitive binding sites for galectin-1, which alters the presentation of the galectin-1 to the cells.

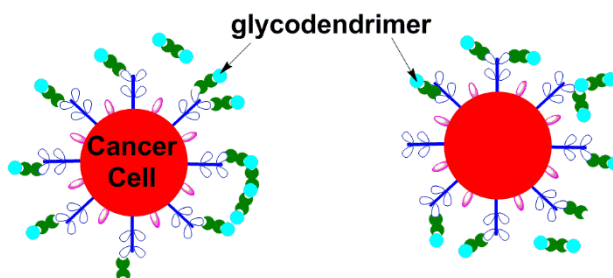


Figure 41. Scheme of glycodendrimer inhibition of galectin-1 mediated cellular aggregation.

Conclusion

The studies reported here indicate that the pattern of galectin-1 that is presented to the cells influences their behavior, thus advancing the understanding of the mechanism of action of galectin-1 mediated cellular aggregation processes and indicating that multivalent interactions can be very effectively used to organize proteins in biologically active arrays.

All four generations of the glycodendrimers inhibited galectin-1 mediated cellular aggregation of the DU145 cells, which indicates that glycodendrimers mediate inhibition of cellular aggregation by competitively binding galectin-1. The glycodendrimers' interaction with galectin-1 alters the presentation of the lectin to cells and thereby prevents cellular cross-linking. Lactose functionalized G(2)-PAMAM **1** was the most potent inhibitor of galectin-1 induced cellular aggregation, exhibiting complete inhibition of cancer cell adhesion at low dosage.

Inhibition by monomeric lactose illustrates the avidity enhancement afforded by multivalency. Monomeric lactose inhibited cell adhesion at a concentration of 6 mM, while

inhibition of cellular adhesion by **1** occurred at a lactose concentration of 0.4 mM. This is a 15 fold increase in the concentration of lactose required to disrupt galectin-1 mediated cancer cell adhesion compared to the multivalent counterpart. The pronounced inhibition suggests that multivalent glycodendrimers **1-4** have a strong influence on the native cellular aggregation mechanism.

Experimental Procedures

All reagents for cell-based media were purchased from Gibco by Life Technologies, except NaHCO₃, which was purchased from Fisher Scientific. Galectin-1 and galectin-1 antibody were provided by Linda Baum and Mabel Pang of UCLA.

Homotypic Cellular Aggregation Assay

Human prostate carcinoma cells (DU145, ATCC HTB-81) were purchased from ATCC, and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Increasing glycodendrimer concentrations were added to a constant concentration of galectin-1 (3.7 μ M) and cancer cells (~ 240,000/ependorf). Glycodendrimer concentrations were calculated to present approximately equal concentrations of lactosides at the same stage in the assays irrespective of PAMAM generation. Control assays for untreated cells (untreated standard) and the galectin-1 treated cells (galectin-1 standard) were performed. Control assays with the glycodendrimers and without galectin-1 were performed previously and can be found in reference.³⁹

Assay Preparation. Adhered cells were dissociated from growth dishes using 0.5 mM EDTA (0.02% EDTA/0.25% trypsin (Life Technologies) and collected in a 15 mL Falcon tube. After sufficient centrifugation to form a cell pellet, the EDTA solution was removed and the pellet was re-suspended in 1 mL serum free media (SFM). Cells were counted using a hemocytometer, following a 10x dilution in SFM of a 10 μ L aliquot of the 1 mL cell suspension and a subsequent 2x dilution in Trypan Blue (TPB) of 10 μ L aliquot of the previously diluted sample. Centrifugation and dilution of the cells in SFM were performed to achieve a concentration of 16×10^6 cells/mL. 15 μ L of the cells were added to each Eppendorf for a final concentration approximately 240,000 cells/70 μ L. For all assays, other than the untreated control, a final galectin-1 concentration of 3.7 μ M was used. 2 mg/mL stock solutions of glycodendrimers were prepared in PBS buffer. The volume glycodendrimer (compounds **1-4**) added was doubled at each stage in the assay, and SFM was added to bring the final volume to 70 μ L. **Table 10** below contains the volume of the reagents added for the untreated control (Tube 1), galectin-1 with increasing glycodendrimer concentrations (Tubes 2-5), and galectin-1 treated control. Assays were incubated at 37 °C and gently rotated for 1 hour.

Table 10. DU145 Cell-Based Assay. Tube 1: untreated DU145 control; Tubes 2-5: DU145 cells with galectin-1 and increasing glycodendrimer; and Tube 6: galectin-1 treated control.

Tube	1	2	3	4	5	6
Dendrimer (μ L)	0	10	20	30	40	0
Galectin-1 (μ L)	0	15	15	15	15	15
Cells (μ L)	15	15	15	15	15	15
SFM (μ L)	55	30	20	10	0	40

Saturation Curve for Compound 1. A saturation curve was prepared using compound **1**. DU145 cells (240,000 cells/70 μ L) were incubated in the presence of galectin-1 (3.7 μ M final concentration) and 3.3 μ M of **1**, 16 μ M of **1**, 33 μ M of **1**, and 66 μ M of **1**, respectively.

Monovalent Lactose Inhibition Assay. To assess the multivalent enhancement in inhibiting cancer cell adhesion achieved with the dendrimer scaffold, a control assay was performed using monomeric lactose. Assays were prepared (240,00 cells/70 μ L, 3.7 μ M galectin-1; final concentrations) with a series of lactose concentrations and incubated for 1 hour at 37 °C with gentle rotation.

Inhibition Specificity Assay. To investigate the specificity of aggregation inhibition, mannose-functionalized generation 6 PAMAM dendrimer (2 μ M, 3 μ M, 6 μ M, and 10 μ M, final concentrations) was incubated with DU145 cells (240,000 cells/70 μ L) in the presence of galectin-1 (3.7 μ M, final concentration).

Analysis. Assays were run in triplicate. From each Eppendorf tube, three 10 μ L aliquots were spotted on a microscope slide. At 4x magnification, 4 images were taken from each spot, for a total of 36 images. 12 images were stitched together and converted to black cells and white background for quantification by Pixcavator 5.0. Representative black and white cell images are provided in Figure **28-32**.

Images were captured on a Jenco microscope with 10x objective and a Canon Powershot A630 camera at 4x magnification. Color images were converted to black and white, where cells were represented by black coloring. Black and white images of cellular

aggregation were analyzed by Pixcavator 5.0. Analysis parameters were set as follows: Size (10); Max Contrast (10); Border Contrast (100); Average Contrast (0); Intensity Dark (0); and, Intensity Light (255). Unmark light was selected to exclude analysis of light color particles. To quantify cell aggregation, pixel counts corresponding to particles of 5 or more cells were defined as aggregates; pixel counts corresponding to particles containing fewer than 5 cells were defined as free cells. Percent free cells (% free) were determined by dividing the sum of the number of pixels corresponding to free cells by the total number of pixels.

Statistical analyses were performed using unpaired two-tailed student's T-Test by comparison to the galectin-1 standard. Statistically significant data is represented as * if $p < 0.05$, ** if $p < 0.01$, and *** if $p < 0.001$.

The interaction between the galectin-1 and the DU145 cells generated large aggregates that exceeded the detection limit of the technique. Visual inspection of galectin-1 treated cells confirmed nearly complete aggregation of all cells; therefore, the percentage of free cells for the galectin-1 treated DU145 cells without glycodendrimer (galectin-1 std) was conservatively set at 20%.

Fluorescence Microscopy Imaging

Homotypic cellular aggregation assays were performed as described above. For each reaction to be visualized, a solution containing a 1:50 dilution of galectin-1 antibody was prepared in whole media for a final volume of 200 μL . To 200 μL whole media anti-galectin-1 mixture, a 45 μL sample of a reaction mixture was added, and the resulting mixture was plated on an 8-chamber Lab-Tek II Chamber Coverglass System (Nunc) and

incubated at 37 °C overnight. The next day, the cells were washed twice with PBS-T and once with PBS and fixed with 4% PFA solution for 20 minutes at room temperature (20.6 °C). After 20 minutes, the cells were washed twice with PBS-T and once with PBS, and 50 μ L of a 100:1 dilution of Alexa488 goat anti-rabbit IgG (Molecular Probes). The cells were incubated at 37°C overnight and washed the following day (twice with PBS-T and once with PBS). A coverslip was then mounted on the microslide using Prolong Gold with DAPI (Invitrogen) and allowed to cure at room temperature (20.6 °C) while shielded from light. Slides were then analyzed using an Olympus BX-61 motorized epifluorescent microscope with MicroSuite software using a 60x oil immersion objective.

Solution Preparation

Galectin-1 was prepared at 0.5 mg/mL in PBS buffer (pH 7.4, 15 mM NaCl) from a 7.2 mg/mL stock solution. Glycodendrimer solutions were prepared in millipore H₂O from the lyophilized powder for the following concentrations: (1) 230 μ M of **1**; (2) 120 μ M of **2**; (3) 56 μ M of **3**; and (4) 15 μ M of **4**. These glycodendrimer concentrations presented approximately equal concentrations of lactosides at the same stages in the cell assay. SFM was prepared by dissolving the DMEM packet (Gibco) and approximately 1.65 g NaHCO₃ in 1000 mL of millipore H₂O. For the DU145 complete media with 10% fetal bovine serum (FBS), to SFM, 5 mL vitamin, 5 mL streptavidin/penicillin mixture, 10 mL essential amino acid, 5 mL non-essential amino acid, and 50 mL FBS were added and filled to 500 mL with SFM and sterilized using a sterile filter unit.

CHAPTER 5

INVESTIGATION OF GALECTIN-1 MEDIATED
ANGIOGENESIS USING GLYCODENDRIMERSIntroduction

Angiogenesis is the process of forming new blood vessels out of pre-existing capillaries. This process is carried out by endothelial cells. Endothelial cells compose the inner lining of blood vessels. Relevant to cancer cell activity, new vessel formation is essential to supply oxygen and nutrients to a tumor, which allows the tumor to grow and metastasize. Throughout the angiogenic cascade, endothelial cells undergo different processes which require interactions with other cells and with the extracellular environment.¹⁰⁵ These interactions frequently involve galectin-1 (see Chapter 1).

Elevated levels of galectin-1 expression have been observed in the vasculature of many tumors, including prostate,^{86, 102} lung,¹⁰⁹ colon,¹¹⁰ and oral cancers.⁷⁷ Galectin-1 has been observed to augment the formation of microtubules from endothelial cell.¹³ Recently, studies have demonstrated that galectin-1 indirectly mediates endothelial cell activation by binding cell surface receptors to organize the receptors into biologically active arrays.^{13, 77} While galectin-1 has been observed to augment tumor-induced angiogenesis,^{107, 111, 112} the mechanisms have not been fully resolved.

As shown in **Figure 42**, there are three likely pathways through which galectin-1 can mediate pro-angiogenic processes: (a) cross-linking endothelial cells and the ECM to provide structural support for neovascularization (**Figure 42a**); (b) activating cells to undergo new branching processes (**Figure 42b**); or (c) activating endothelial cells to

initiate angiogenesis (**Figure 42c**). The distribution of galectin-1 at cell-ECM contact points would suggest that the protein acts as a scaffold for neovasculature. The localization of galectin-1 at neovasculature branching points would support involvement of the protein in tubule differentiation. If, however, galectin-1 is distributed on the surface of endothelial cells that compose the neovasculature network, the protein is likely involved in activating endothelial cells to undergo capillary growth.

Human umbilical vein endothelial cells (HUV-EC-C [HUVEC] ATCC CRL-1730), which form microtubules in the presence of basement membrane²⁰⁴, were used to evaluate multivalent galectin-1 mediated angiogenic processes. To modulate and study multivalent galectin-1 interactions with endothelial cells during angiogenesis, glycodendrimers **1-4** were incubated with HUVECs in the presence of galectin-1. It was hypothesized that the addition of synthetic multivalent ligands to galectin-1 mediated angiogenic processes would divert the galectin-1 from its native function and inhibit neovascularization. The impact of the glycodendrimers will shed light on the role of the galectin-1 as either a scaffold for microtubule growth, an initiator of capillary differentiation, or an endothelial cell activator.

Phase contrast microscopy (Nikon Eclipse TS-100, 4x objective) was used to visualize microtubule formation. (Initial investigations using brightfield microscopy were unsuccessful because of the inability to visually distinguish the interface between microtubules and the basement membrane). Phase contrast microscopy is an optical technique that enhances the contrast of transparent specimens, such as living cells. This technique employs an optical mechanism (i.e. an annulus condenser and a phase plate) to

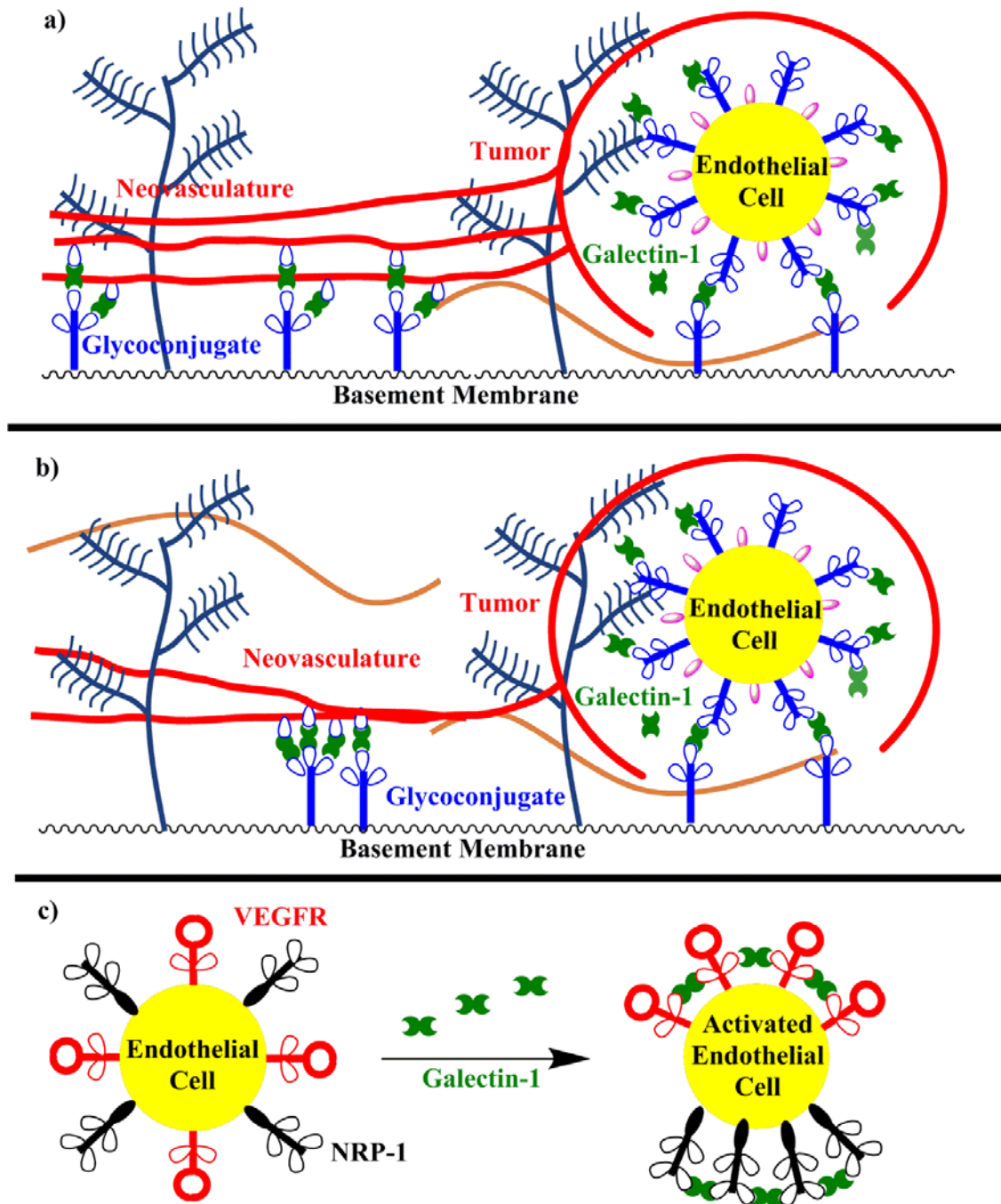


Figure 42. Galectin-1-mediated pro-angiogenic mechanisms. (a) Cross-linking of glycoconjugates in the ECM and in the neovasculature by galectin-1 can provide physical support for microtubule growth. (b) Localized concentrations of galectin-1 at microtubule branching points would suggest a role in tubule differentiation. (c) Galectin-1 binding can activate endothelial cells, causing the cells to form new capillaries.

translate small variations in the wavelength of transmitted light (phase) into changes in amplitude, which can be visualized as differences in image contrast.²⁰⁵ Wave fronts that illuminate the specimen either pass through undeviated or are diffracted and impeded in phase by structural components of the specimen.²⁰⁵ Light waves that are diffracted and shifted in phase by the specimen are transformed into amplitude differences that can be visualized.²⁰⁵

The results of the angiogenesis assay are reported here.

Results

Tube Formation Assay

The HUVEC line (HUVEC, CRL-1730) was used to assess the impact of glycodendrimers on microtubule formation and to further understand the role of galectin-1 in angiogenesis. For the assay, an angiogenesis chamber (μ Slide Angiogenesis, Ibidi) was coated with chilled Matrigel (10.6 μ L of an 8.6 mg/mL solution of the basement membrane in serum free media, Corning). The Matrigel coated chamber was incubated for 1 hour at 37°C so that the basement membrane layer hardened. HUVECs in the presence or absence of galectin-1 and/or **1-4** were seeded on the basement membrane and incubated at 37 °C. Images were captured using an inverted phase contrast microscope (Nikon Eclipse TS-100) at 4x magnification equipped with a digital camera and operating software (Nikon Elements F). Images were analyzed using ImageJ Angiogenesis Analyzer software (NIH).

As show in **Figure 43**, varying concentrations of a cell suspension (50,000 cells/mL, 100,000 cells/mL, 200,000 cells/mL, 300,000 cells/mL and 400,000 cells/mL in

whole media) were seeded on the Matrigel coated wells and monitored for neovascularization. After a four hour incubation period, a concentration of 50 μ L of 50,000 cells/mL (2,500 cells/well) was identified as the optimal concentration to monitor microtubule formation because individual microtubules could be visualized (**Figure 43a**).

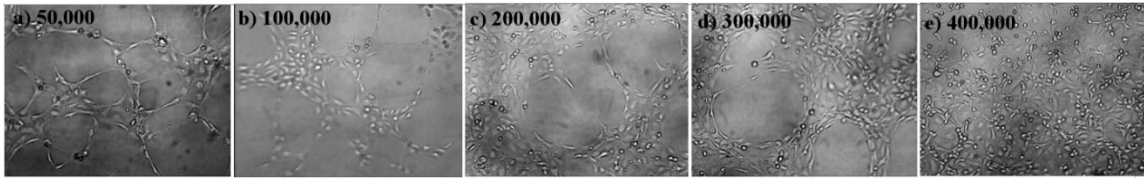


Figure 43. Microtubule formation of HUVECs at (a) 50,000 cells/mL. (b) 100,000 cells/mL. (c) 200,000 cells/mL. (d) 300,000 cells/mL. (e) 400,000 cells/mL.

As shown in **Figure 44**, assays were performed with varying concentrations of galectin-1 (0.034 μ M, 0.34 μ M, 0.68 μ M, and 1.0 μ M in PBS) and neovascularization was monitored. A galectin-1 concentration of 10 μ g/mL (0.34 μ M) was observed to augment microtubule formation of the HUVECs compared to the untreated standard. A similar concentration of galectin-1 has been reported to enhance HUVEC microtubule formation.¹³

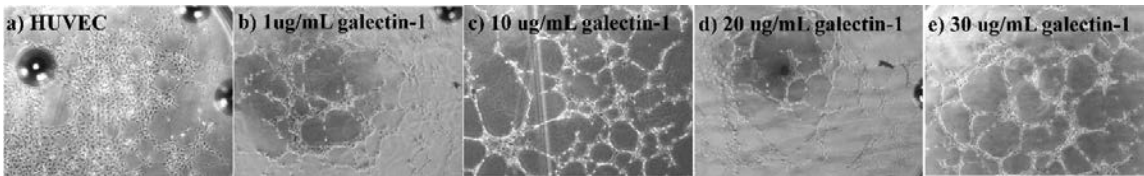


Figure 44. Impact of galectin-1 on microtubule formation with HUVECs. (a) HUVEC standard without galectin-1. (b) HUVECs with 1 μ g/mL galectin-1. (c) HUVECs with 10 μ g/mL galectin-1. (d) HUVECs with 20 μ g/mL galectin-1. (e) HUVECs with 30 μ g/mL galectin-1.

Image J software (NIH) was used to quantify capillary growth. Nodes (pixels with 3 neighbors), junctions (multiple nodes), and microtubule length were measured to assess

the impact of glycodendrimers on galectin-1 mediated angiogenesis. Shown in **Figure 45** is a representative image of a well seeded with HUVECs in the presence of galectin-1 and the corresponding analysis of the microtubule network (**Figure 45a** and **b**, respectively).

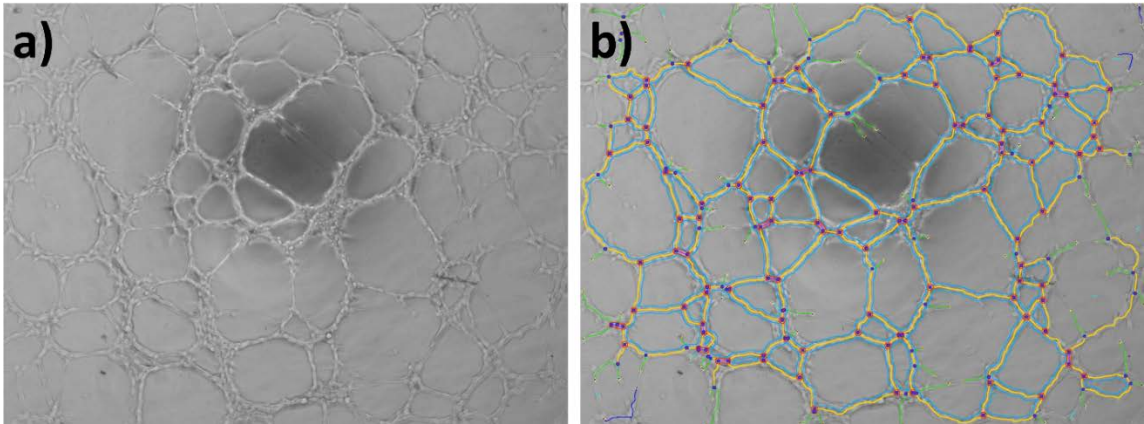


Figure 45. Representative images of the microtubule network for HUVECs in the presence of galectin-1. (a) Phase contrast image of the microtubule network. (b) Analysis of the network using ImageJ Angiogenesis Analyzer. Yellow indicates a tube, the blue circles are nodes, and red parameter around blue node indicates junctions.

Microtubule growth kinetics of untreated HUVECs (2,500 cells/well in whole media) were monitored over 7 hours to identify the optimal time for maximum tubule formation. Shown in **Figure 46** are the results of time course measurements of untreated HUVECs, demonstrating that maximum microtubule formation occurs in 5 hours.

At a HUVEC concentration of 2,500 cells/well in whole media, microtubule growth was monitored in the presence of galectin-1 (0.34 μ M in PBS, final concentration) over 6 hours to observe the impact of galectin-1 on microtubule growth. As shown in **Figure 47**, HUVECs in the presence of galectin-1 reach a maximum number of nodes and junctions and a maximum length in 4 hours.

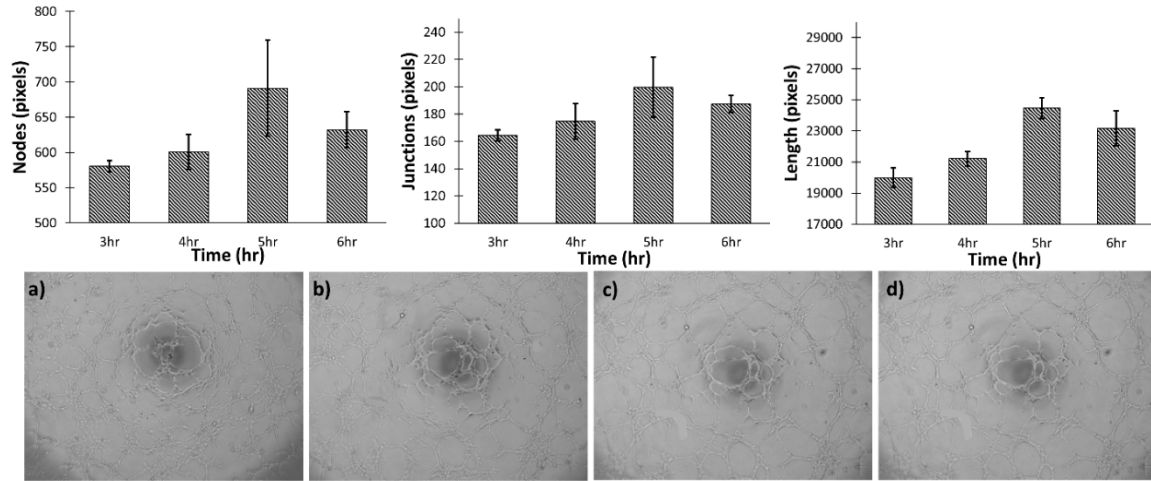


Figure 46. New capillary growth (nodes, junctions, and length) of untreated HUVECs monitored after 3 hours, 4 hours, 5 hours, and 6 hours of incubation. Representative images of the untreated HUVECs at (a) 3 hours, (b) 4 hours, (c) 5 hours, and (d) 6 hours.

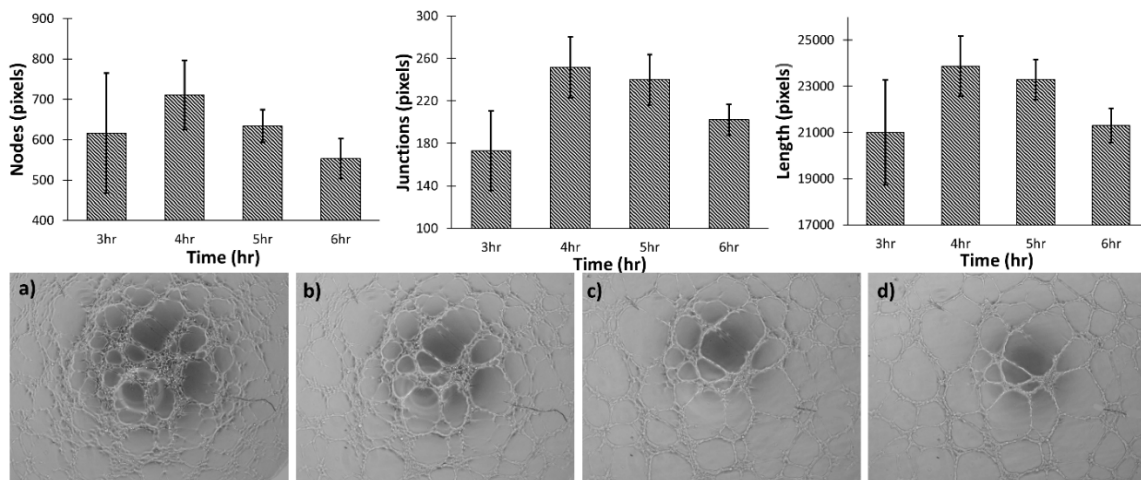


Figure 47. New capillary growth of HUVECs in the presence of galectin-1 after incubating for 3 hours, 4 hours, 5 hours, and 6 hours. Representative images of HUVEC cells in the presence of 0.34 μ M Galectin-1 are provided for (a) 3 hours, (b) 4 hours, (c) 5 hours, and (d) 6 hours of growth.

To evaluate the impact of multivalent glycodendrimers on galectin-1 mediated angiogenic processes, HUVECs (2,500 cells/well in whole media) were seeded on a Matrigel plug in the presence of galectin-1 (0.34 μ M in PBS) and increasing concentrations of glycodendrimers **1-4** (0.5 μ L, 4.6 μ L, and 46 μ L of 2 mg/mL solutions in whole media).

Controls were run for the untreated HUVECs and HUVECs in the presence of galectin-1 (0.34 μ M) and absence of glycodendrimers. To prepare reaction mixtures, the glycodendrimer (2mg/mL in PBS) was mixed with galectin-1 (0.34 μ M in PBS) in whole media. To the mixture, the cell suspension was added to achieve a final concentration of cells of 50,000 cells/mL. The resultant mixture was gently agitated. Chambers were incubated at 37 °C and one image was captured per well. Angiogenesis was observed each hour over the course of 6 hours (not reported), and the results at 4 hours are reported here. Data was normalized with respect to the untreated HUVECs.

Glycodendrimer **3** (0.13 μ M, 1.3 μ M, and 13 μ M) in the presence of galectin-1 (0.34 μ M) was assessed for impact on galectin-1 mediated angiogenesis of HUVECs (**Figure 48**). As shown **Figure 48**, significant inhibition of nodes and junctions (**Figure 48a** and **48b**, respectively) and microtubule length (**Figure 48c**) was observed using compound **3**. Controls run with **3** in the absence of galectin-1 show a slight increase in nodes and junctions (**Figures 49a-c**).

Glycodendrimer **2** (0.28 μ M, 2.8 μ M, and 28 μ M) was incubated with HUVECs in the presence of galectin-1 (0.34 μ M), and the impact on neovascularization is shown in **Figure 50**. As shown **Figure 50**, mild inhibition of the number of nodes and junctions was observed using compound **2** (**Figure 50a,b**). Microtubule length, however, was not impacted (**Figure 50c**). Controls run with **2** in the absence of exogenous galectin-1 demonstrate increase in nodes and junctions (**Figures 51a,b**).

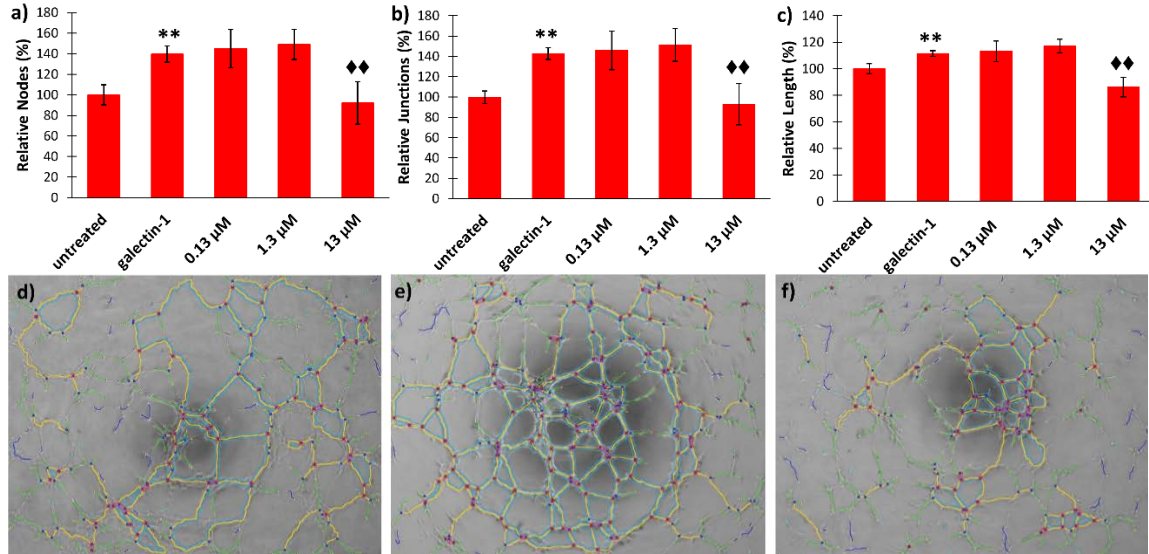


Figure 48. Microtubule growth of untreated HUVECs, HUVECs in the presence of galectin-1 (0.34 μ M), and HUVECs in the presence of galectin-1 (0.34 μ M) and increasing concentrations of **3** (0.13 μ M, 1.3 μ M, and 13 μ M) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length. Representative phase contrast images of the analyzed network are provided in for (d) untreated HUVECs, (e) HUVECs treated with galectin-1 (0.34 μ M), and (f) HUVECs in the presence of galectin-1 (0.34 μ M) and **3** (13 μ M). Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least two experiments ($n \geq 2$). Statistical analysis was performed using an unpaired two-tailed student's T-Test. Galectin-1 treated HUVECs were compared to the untreated cells and ** indicates a p value ≤ 0.01 . HUVECs treated with **3** in the presence of galectin-1 were compared to the galectin-1 standard and ♦♦ indicates a p value ≤ 0.01 .

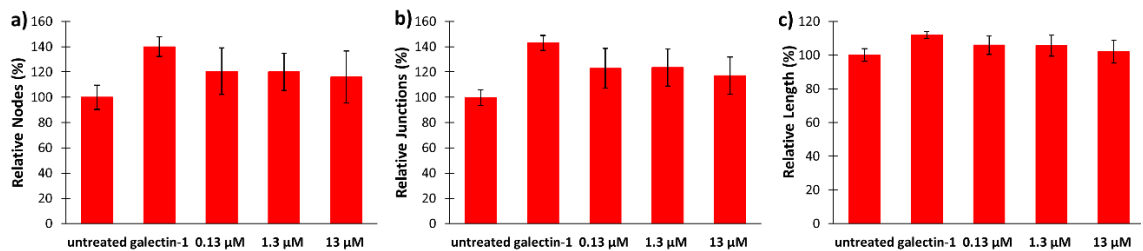


Figure 49. Microtubule growth of untreated HUVECs and HUVECs in the presence of increasing concentrations of **3** (0.13 μ M, 1.3 μ M, and 13 μ M) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length. Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least two experiments ($n \geq 2$).

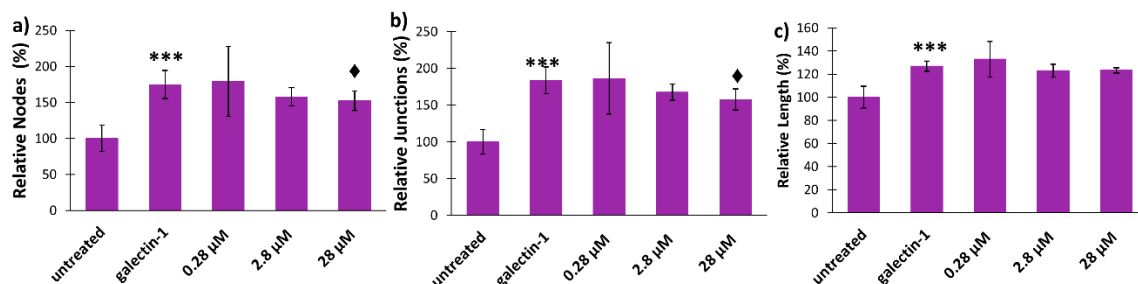


Figure 50. Microtubule growth of untreated HUVECs, HUVECs in the presence of galectin-1 (0.34 μ M), and HUVECs in the presence of galectin-1 (0.34 μ M) and increasing concentrations of **2** (0.28 μ M, 2.8 μ M, and 28 μ M) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length. Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least two experiments ($n \geq 2$). Galectin-1 treated HUVECs were compared to the untreated cells and *** indicates a p value ≤ 0.001 . HUVECs treated with **2** in the presence of galectin-1 were compared to the galectin-1 standard and ♦ indicates a p value ≤ 0.05 .

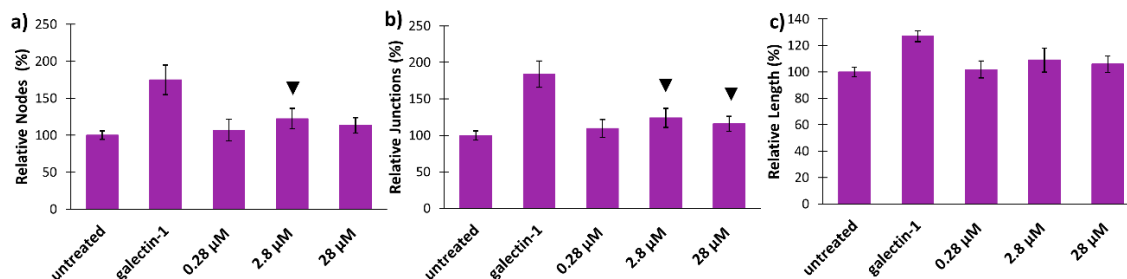


Figure 51. Microtubule growth of untreated HUVECs and HUVECs in the presence of increasing concentrations of **2** (0.28 μ M, 2.8 μ M, and 28 μ M) measured after 4 hours of incubation. Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least two experiments ($n \geq 2$). The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length. HUVECs in the presence of **2** were compared to the untreated standard and ▼ indicates a p value ≤ 0.05 .

As shown in **Figure 52**, glycodendrimer **4** (0.035 μ M, 0.35 μ M, and 3.5 μ M) in the presence of galectin-1 (0.34 μ M) showed no impact on the number of nodes and junctions and microtubule length of galectin-1 mediated angiogenesis (**Figure 52a-c**). Controls run with **1** in the absence of galectin-1 demonstrated an increase in nodes and junctions (**53a,b**) but not length (**53c**).

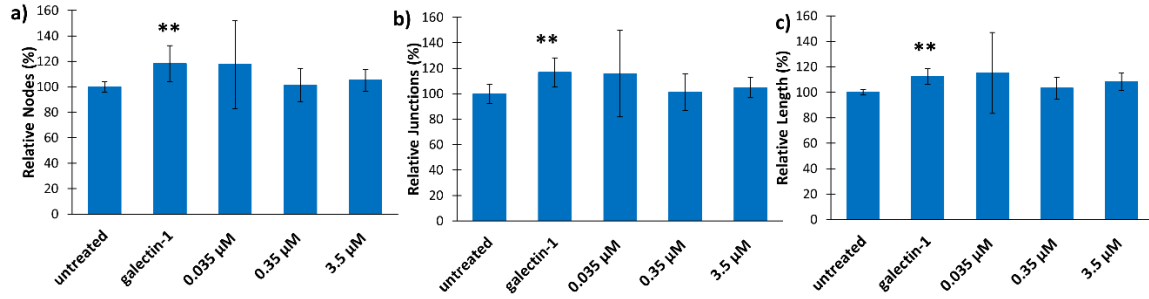


Figure 52. Microtubule growth of untreated HUVECs, HUVECs in the presence of galectin-1 (0.34 μ M), and HUVECs in the presence of galectin-1 (0.34 μ M) and increasing concentrations of **4** (0.035 μ M, 0.35 μ M, and 3.5 μ M) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length. Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least two experiments ($n \geq 2$). Galectin-1 treated HUVECs were compared to the untreated cells and ** indicates a p value ≤ 0.01 .

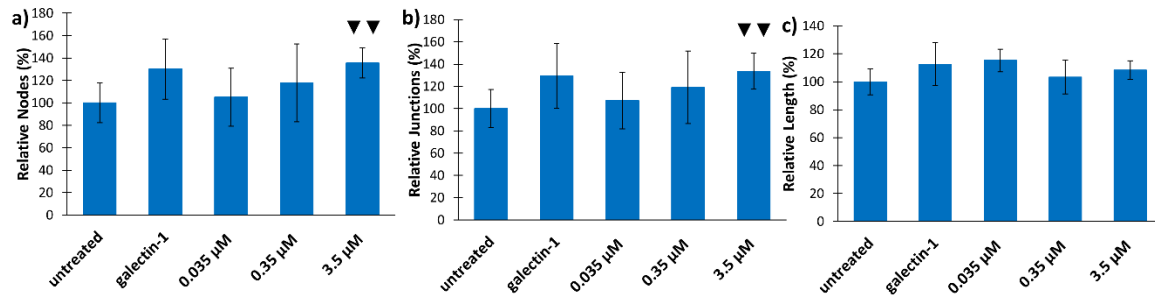


Figure 53. Microtubule growth of untreated HUVECs and HUVECs in the presence of increasing concentrations of **4** (0.035 μ M, 0.35 μ M, and 3.5 μ M) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length. Data are shown as mean $\pm$ one S.D. of triplicate measurements from at least two experiments ($n \geq 2$). HUVECs in the presence of **4** were compared to the untreated standard and ▼▼ indicates a p value ≤ 0.01 .

As shown in **Figure 54**, glycodendrimer **1** (0.53 μ M, 5.3 μ M, and 53 μ M) in the presence of galectin-1 (0.34 μ M) showed a mild impact on the number of nodes and junctions (**Figure 54a, b**) but not microtubule length (**Figure 54**). Controls run with **1** in the absence of galectin-1 showed no impact as expected (**Figures 55a-c**).

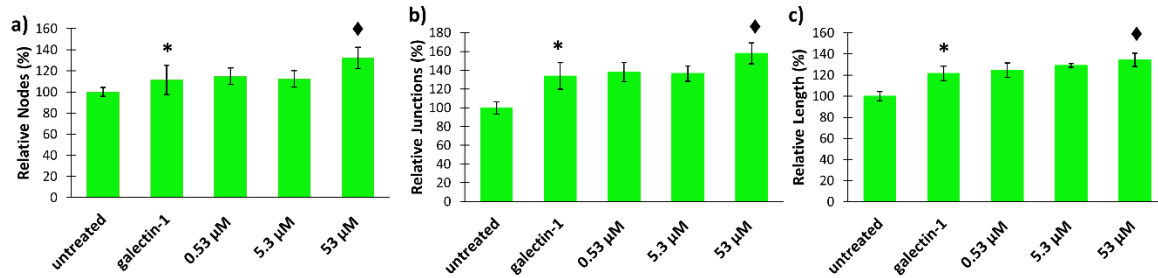


Figure 54. Microtubule growth of untreated HUVECs, HUVECs in the presence of galectin-1 (0.34 μM), and HUVECs in the presence of galectin-1 (0.34 μM) and increasing concentrations of **1** (0.53 μM , 5.3 μM , and 53 μM) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length.

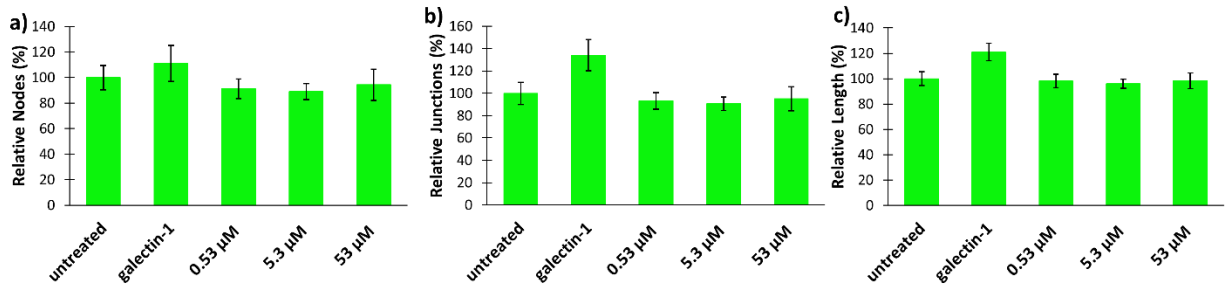


Figure 55. Microtubule growth of untreated HUVECs, and HUVECs in the presence of increasing concentrations of **1** (0.53 μM , 5.3 μM , and 53 μM) measured after 4 hours of incubation. The number of pixels in the network were measured to quantify (a) nodes, (b) junctions, and (c) length.

Microscopy Experiments to Determine Distribution of Galectin-1 in Microtubule Network

To determine the cell surface distribution of galectin-1, HUVEC cells in the presence and absence of galectin-1 were fluorescently labeled using anti-galectin-1 and goat anti-rabbit Alexa-488. These microscopy experiments were performed the same as the tube formation assays previously described. After tubule formation, the cells were fixed, and a galectin-1 antibody was added and incubated overnight. Then, goat anti-rabbit Alexa-488 was added to the cells and incubated. For the untreated cells, a faint green fluorescence was visible predominately at the surface of the capillary walls. (**Figure 56a**). In the

presence of exogenous galectin-1, visualization of galectin-1 distribution using the galectin-1 antibody fluorescent conjugate showed a more vivid fluorescence around the cell, demonstrating that galectin-1 (when in higher concentrations) is localized on cell surface (**Figure 56b**). The spreading of the excess galectin-1 on the cell surface, rather than lining the interface of the microtubules and the basement membrane or clustering at branching points, suggests that the galectin-1 (when presented in excess) is likely involved in activating the endothelial cells, such as shown in **Figure 42c**.

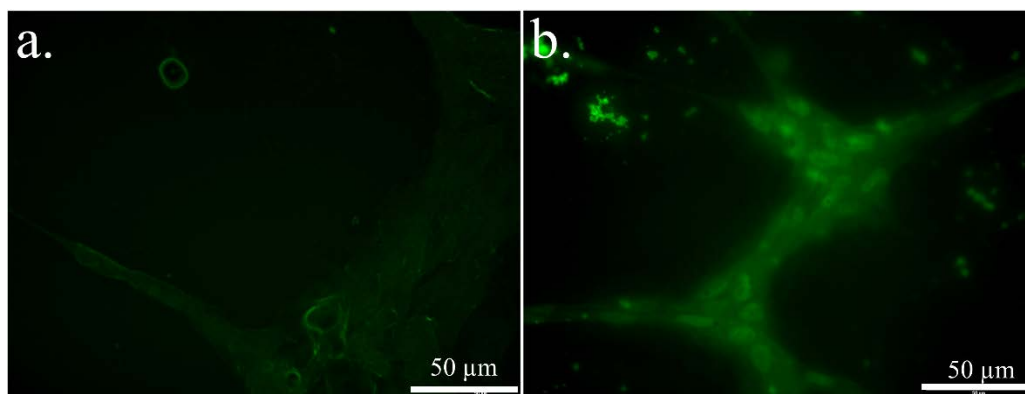


Figure 56. Representative fluorescent micrographs of microtubule network treated with an anti-galectin-1. (a) Untreated HUVECs. (b) HUVECs in the presence of galectin-1.

Discussion

To evaluate multivalent galectin-1 mediated angiogenesis, a microtubule formation assay was designed. In the assay, multivalent glycodendrimers were used to modulate the activity of the galectin-1. Cells in the presence and absence of exogenous galectin-1 and/or glycodendrimers were seeded on basement membrane and incubated. After incubation, the number nodes (pixels with 3 neighbors) and junctions (groups of nodes) and length of the microtubule network were measured using Image J software (NIH). These parameters were monitored because the branching (i.e. nodes and junctions) and length of the cellular

network is indicative of the angiogenic response and would likely help identify the role of multivalent galectin-1 interactions in angiogenesis.

Angiogenesis was initially observed every hour for at least 7 hours. From visual observation of microtubule formation, it took approximately 2 hours for the HUVECs to interact with the basement membrane. At 3 hours, the cells were observed to coalesce into capillaries and then continue to grow into a mature network. As shown in **Figure 46**, untreated HUVECs reached maximum nodes and junctions and tube length after 5 hours. In the presence of galectin-1 (0.34 μ M in PBS), the rate of neovascularization was enhanced, and the microtubule network was observed to reach maximum nodes, junctions and length in 4 hours (**Figure 47**). Not only was the rate of formation accelerated in the presence of exogenous galectin-1, but a statistically significant increase in nodes, junctions, and length was also observed compared to the untreated HUVECs (**Figures 48, 50, 52, and 54**).

The accelerated rate of formation suggests that the galectin-1 likely activates endothelial cells to carry out the angiogenic cascade. Several cell surface receptors, such as VEGFR¹³ and NRP-1⁷⁷, have been identified in signaling endothelial cell activation, and galectin-1 has been reported to bind to these receptors. Furthermore, the fluorescent micrograph shown in **Figure 61b** shows that exogenous galectin-1 spreads out across the surface of the HUVECs, which suggests that the galectin-1 (when presented in an excess) binds cell surface receptors.

Increasing concentrations of glycodendrimers **1-4** were incubated with HUVECs in the presence of galectin-1 (0.34 μ M) to modulate and study galectin-1 mediated

angiogenesis. The range of glycodendrimers concentration used in the angiogenesis assay were identified from the ratios of glycodendrimer to galectin-1 at which an impact was observed in the homotypic cellular aggregation assays previously described (See Chapter 4). These glycodendrimer concentrations presented a similar lactoside concentrations.

Glycodendrimers **3** and **2** both inhibited galectin-1 mediated neovascularization (**Figures 48** and **50**). In the presence of the highest concentration of **3** (13 μ M), nodes, junctions, and length were each significantly attenuated (34%, 35%, and 23%, respectively) (**Figure 48a-c**). This indicates that glycodendrimers diverted the excess galectin-1 from spreading out on the surface of the HUVECs. It is likely that **3** nucleates the excess galectin-1 into nanoparticles that prevent the galectin-1 from interacting with the receptors on the surface of the HUVECs (**Figure 57**).

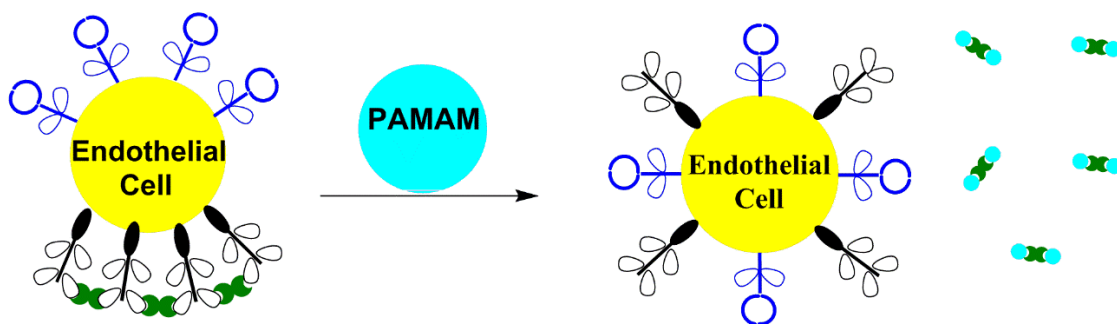


Figure 57. Schematic representation of inhibition of galectin-1 mediated endothelial cell activation by glycodendrimers **2** and **3**.

In the absence of excess galectin-1, however, a slight increase in nodes and junctions was observed at the highest concentration of **3** (**Figure 49a-c**). It is likely that in the absence of exogenous galectin-1, glycodendrimer **3** facilitates interactions of the

endogenous galectin-1 with the HUVECs to enhance angiogenesis. This control indicates that **3** interacts with exogenous galectin-1 to modulate galectin-1 mediated angiogenesis.

Inhibition of galectin-1 mediated angiogenesis was also observed with **2**. The most pronounced impact using **2** was observed at 28 μ M, where nodes were reduced by 13% and junctions were reduced by 14% (**Figure 50a, b** respectively). Unlike **3**, glycodendrimer **2** did not significantly inhibit microtubule length. The most likely explanation for the observed inhibition of nodes and junctions is that multivalent glycodendrimer **2** nucleates the excess galectin-1, preventing the protein from spreading out on the HUVECs (**Figure 57**). In the absence of galectin-1, **2** was observed to significantly increase the number of nodes and junctions (**Figure 51a, b**). This control experiment demonstrates that the interaction of glycodendrimer **2** with exogenous galectin-1 inhibits galectin-1 mediated angiogenesis.

Multivalent glycodendrimer **4** did not impact the number of nodes and junctions or the length of the microtubule network (**Figure 52**). However, it is possible that the impact of **4** on galectin-1 mediated angiogenesis was masked since a significant increase nodes and junctions were observed at 3.5 μ M in the absence of exogenous galectin-1 (**Figure 53a, b**).

Multivalent glycodendrimer **1** demonstrated a unique impact on galectin-1 mediated angiogenesis. Microtubule growth was promoted in the presence of **1** and exogenous galectin-1 (**Figure 54**), and no impact was observed with **1** in the absence of galectin-1. The interaction of galectin-1 with **1** in the absence of cells may explain the enhancement. Small aggregates formed with **1** (which were below the detection limits of

the fluorescence microscopy experiments previously discussed in Chapter 3) likely behave similar to a free lectin. The small galectin-1 aggregates formed with **1** do not undergo multiple nucleation and growth events like those required to form the large aggregates measured with **2-4**, which leaves much of the galectin-1 available. The large aggregates formed with glycodendrimer **2-4** likely sequester much of the galectin-1 inside the nanoparticle in order to grow to the observed sizes. Thus, the galectin-1 nucleated by **1** is likely more readily available to interact with the cells.

The concentration of **4** was increased and incubated with cells that were preserved from an early passage. It appears that the additional cryopreservation changed the rate of microtubule growth as the cells were not matured after 4 hours (data not shown). Furthermore, assays run with higher passage HUVECs did not behave in the same manner as earlier passaged cells. It is likely that HUVECs mutated as a result of both cryopreservation and long growth periods (i.e. higher passages).

Conclusion

In conclusion, the results of the tube formation assay indicate that galectin-1 accelerates angiogenesis. The fluorescence labeling studies indicate that the excess galectin-1 spreads out across the surface of endothelial cells. The most likely mechanism for galectin-1 mediation is that the protein interacts with cell-surface receptors, activating endothelial cells to augment angiogenic processes, rather than providing structural support or inducing branching.

Significant inhibition of nodes and junctions was observed with **2** and **3**. Only **3** showed a significant inhibition in the length of the microtubule network. The glycodendrimers are likely nucleating the galectin-1, and changing the presentation of the protein to the cells. No impact of galectin-1 mediated angiogenesis was observed with **4** in the presence of exogenous galectin-1; however, it may be that the impact of **4** on angiogenesis was masked because an enhancement was observed with **4** in the absence of galectin-1. Interestingly, only **1** was observed to enhance microtubule formation. It is likely that the small galectin-1 nucleated aggregates formed with **1** behave more like free lectin. Control assays run in the presence of glycodendrimers and the absence of exogenous galectin-1 indicate that the interaction of the glycodendrimers with the galectin-1 modulates angiogenesis.

Experimental Procedures

Galectin-1 and galectin-1 antibody were provided by Dr. Linda Baum and Mabel Pang. Chambers (μ -Slide Angiogenesis) for angiogenesis assay were purchased from Ibidi™. Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (ATCC® CRL-1730™). Fetal bovine serum (FBS) (ATCC® CRL-30-2020™) and serum free media (F-12K Medium, Kaighn's Modification of Ham's F-12 Medium, ATCC® CRL-30-2004™) were also purchased from ATCC. Endothelial cell growth supplement (ECGS) and heparin were purchased from Sigma. Matrigel® was purchased from BD Bioscience®.

Tube Formation Assay

μ -Slide Angiogenesis chambers (Ibidi™), pipette tips, and single use aliquots of Matrigel (BD Bioscience®) were stored at -20°C. Angiogenesis chambers were covered and stored in the incubator at 37°C overnight prior to use. The night before running an angiogenesis experiment, Matrigel was removed from the freezer, placed in a beaker filled with ice, and thawed overnight at 4°C. To each well of the angiogenesis chamber, 10.6 μ L of chilled Matrigel aliquot, was added, with care taken not to fully purge the pipette tip in order to avoid air bubbles in the basement membrane layer. HUVECs in the presence and absence of galectin-1 and glycodendrimers **1-4** were seeded on the basement membrane and incubated at 37°C. For each reaction mixture, 3 measurements were taken, and the assays were run in duplicate.

Images of microtubes were captured every hour. Inverted phase contrast microscope (Nikon Eclipse TS-100) equipped with digital camera (DS-2MBW) was used to capture images at 4x magnification and images acquisition software (NIS Elements-F). Images captured on the software were encoded as RGB and imported to ImageJ with Angiogenesis Analyzer plug in (NIH).

Assay Preparation. Adhered cells were dissociated from growth dishes using 0.5 mM EDTA (0.02% EDTA/0.25% trypsin (Life Technologies) and collected in a 15 mL Falcon tube. After sufficient centrifugation to form a cell pellet, the EDTA solution was removed and the pellet was re-suspended in 0.5 mL serum free media (SFM). Cells were counted using a hemocytometer, following a 10x dilution in SFM of a 10 μ L aliquot of the 1 mL cell suspension and a subsequent 2x dilution in Trypan Blue (TPB) of 10 μ L aliquot

of the previously diluted sample. A sufficient volume of the cell suspension was added to the reaction mixture to achieve a concentration of 50,000 cells/mL. To confirm that each well was seeded with 2,500 cells, the first well was visually inspected and adjustments to the volume of the cell suspension were performed. A galectin-1 concentration of 0.34 μ M or 0.034 μ M in whole media was used for all reaction mixtures except the untreated control. The concentrations of glycodendrimers **1-4** used were 0.5 μ L, 4.6 μ L, and 46 μ L of 2 mg/mL solutions in whole media. For **1** and **4**, the concentration was increased to 46 μ L, 92 μ L, and 160 μ L of 2 mg/mL solutions in whole media. To make the reaction mixtures, galectin-1 and the glycodendrimers were added to whole media and mixed. Then, the cells were added to the mixture and gently agitated. **Table 11** below shows the volume of the reagents added for the untreated control (Tube 1), galectin-1 (0.34 μ M) control (Tube 2), and galectin-1 with increasing glycodendrimer concentrations (Tubes 3-5). **Table 12** shows the volume of the reagents added when the glycodendrimers concentration was increased for the untreated control (Tube 1), galectin-1 (0.34 μ M) control (Tube 2), and galectin-1 with increasing glycodendrimer concentrations (Tubes 3-5). **Table 13** shows the volume of the reagents added when the galectin-1 concentration was reduced by 10 fold for the untreated control (Tube 1), galectin-1 (0.034 μ M) control (Tube 2), and galectin-1 with increasing glycodendrimer concentrations (Tubes 3-5). 50 μ L of the reaction mixture was seeded in a well of the angiogenesis chamber. Assays were incubated at 37 °C and over the course of at least 6 hours.

Table 11. HUVEC Tube Formation Assay in the Presence of Galectin-1 and Increasing Glycodendrimer Concentrations. Tube 1: HUVEC. Tube 2: Galectin-1. Tube 3-5: Galectin-1 and Glycodendrimer.

Tube	1	2	3	4	5
Dendrimer (μL)	0	0	0.5	4.6	46
Galectin-1 (μL)	0	10	10	10	10
Cells (μL)	20	20	20	20	20
Whole Media (μL)	180	170	169.5	163.5	125

HUVEC Optimization. Cell suspensions were prepared in whole media at concentrations of 50,000 cells/mL, 100,000 cells/mL, 200,000 cells/mL, 300,000 cells/mL, and 400,000 cells/mL. 50 μL of these cell suspensions were added to separate wells of the angiogenesis chamber and incubated at 37°C. Tube formation was monitored every hour for eight hours.

Galectin-1 Optimization. Different concentrations of galectin-1 in PBS were prepared with the optimal HUVEC concentration (50,000 cells/mL) in whole media to determine the optimal galectin-1 concentration. To separate wells of the angiogenesis chamber, 50 μL of a solution comprising 50,000 cells/mL and 1 $\mu\text{g/mL}$ (0.034 μM) galectin-1, 10 $\mu\text{g/mL}$ (0.34 μM) galectin-1, 20 $\mu\text{g/mL}$ (0.68 μM) galectin-1, and 30 $\mu\text{g/mL}$ (1.0 μM) galectin-1 were added and incubated.

Solution Preparation. HUVEC whole media was prepared from F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) by supplementing 0.05 mg/mL ECGS and 0.1mg/mL Heparin and adjusting to a final concentration of 10% FBS (ATCC® CRL-30-2020™) by volume. Cells were propagated in whole media using a cell culture dish

(Corning cell culture dish, 100mm x 20mm, treated polystyrene) and incubated at 37.5°C with 5% CO₂. The media was changed every 2-3 days.

Galectin-1 was prepared in stock solutions of 0.2 mg/mL in PBS. Glycoendrimer was taken up in whole media for to make a 2 mg/mL stock solution.

Matrigel (BD Bioscience®) was prepared in single use aliquots of 175 µL at 8.3 mg/mL basement membrane matrix.

Fluorescence Imaging Microscopy

Fluorescence microscopy experiments were performed the same as the tube formation assays previously described. After tubule formation, the wells were washed (3x) with serum free media. The cells were fixed with methanol for 20 min at room temperature. The galectin-1 antibody was diluted fifty fold in PBS, and 10µL of the antibody solution was added to each well and incubated at 37°C overnight. The next day, the cells were washed (3x) with serum free media. Then, 10µL of a 100:1 goat anti-rabbit Alexa-488 in PBS was added to the cells. Slides were then analyzed using an Olympus BX-61 motorized epifluorescence microscope with MicroSuite software using a 40x oil immersion objective.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Tumor formation, metastasis, and angiogenesis are driven by multivalent protein carbohydrate interactions and frequently involve galectin-1. For example, galectin-1 has been observed to augment cellular aggregation, cancer cell mobility and invasion, and the growth of new blood vessels from tumors. A better understanding of the multivalent processes involving galectin-1 will lead to a better understanding of the metastatic spread of cancer and, consequently, the development of cancer therapies. Here, four generations of PAMAM dendrimers (G(2), G(3), G(4), and G(6)) were functionalized with lactose to generate a series of multivalent frameworks (**1**, **2**, **3**, and **4**, respectively) and applied to the study and mediation of galectin-1 processes.

An ELISA was designed to simulate interactions with surface bound multivalent assemblies. Based on the ELISA results, multivalent frameworks **1-4** were identified as good binders of galectin-1. The interaction of glycodendrimers **1-4** with galectin-1 in solution was investigated using dynamic light scattering and fluorescence microscopy. These studies indicate that compounds **2-4** nucleate galectin-1 into nanoparticles and, in the presence of a large excess of galectin-1, the aggregates are relatively small and homogenous in size.

To study the impact of glycodendrimer nucleation of galectin-1 on multivalent biological processes, **1-4** were applied to galectin-1 cellular process. The results of these cellular assays demonstrate that that pattern of the galectin-1 presented to the cells has a pronounced impact on the biological activities.

A homotypic aggregation assay using cancer cells (DU145) was used as a platform to study tumor formation. The results of the homotypic cellular aggregation assay show that **1-4** were able to inhibit aggregation by binding galectin-1. This indicates that the presentation of the galectin-1 to the cancer cells is critical to cellular aggregation/tumor formation. The most likely explanation for the inhibition of homotypic cellular aggregation observed with all compounds is the interaction of the galectin-1 with the glycodendrimers in solution. The fluorescence microscopy assay indicates that smaller aggregates are formed in a large excess of galectin-1. Thus, smaller aggregates were likely formed in the presence of the DU145 cells (because of the endogenous galectin-1) which inhibited cross-linking of Mucin1.

To further probe the ability of the glycodendrimers modulate cancer process, a tube formation assay was designed as a model to study galectin-1 mediated tumor-induced angiogenesis. Galectin-1 was observed to accelerate the rate at which the cellular network reached maturity, as measured by the nodes, junctions, length. Furthermore, exogenous galectin-1 was observed to significantly increase nodes, junctions, and length compared to the untreated cells. The most likely explanation for the impact of galectin-1 is that the galectin-1 (when in presented in an excess) interacts with cell surface receptors to activate endothelial cells. Glycodendrimers **2** and **3** were observed to inhibit galectin-1 mediated angiogenesis by diverting the exogenous galectin-1 from spreading out on the HUVEC. Inhibition in nodes, junctions, and length (only **3** inhibited length) was likely caused by the glycodendrimers nucleating the excess galectin-1 into nanoparticles. In the absence of galectin-1, a slight enhancement in nodes and junctions was observed, indicating the

smaller aggregates formed in an excess of glycodendrimers behave more like free lectins and promote interactions with the cells. This is supported by the observation that **4** in the presence of excess galectin-1 had no impact of angiogenesis, but in the absence of galectin-1, enhancement was observed. Furthermore, compound **1**, which formed small aggregates below the detection limits of fluorescence microscopy, also enhanced neovascularization.

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APPENDICES

APPENDIX A

SUPPLAMENTAL DATA

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA Binding Curves for Galectin-1 Interaction with Glycodendrimers 2, 3, and 4.

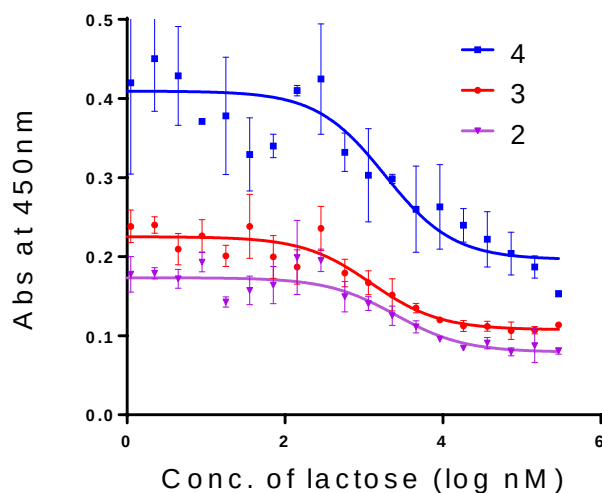


Figure 58. ELISA binding curves with 2, 3, and 4 showing data points and error bars.

ELISA to Study Non-specific Interactions with PAMAM Dendrimer Backbone

Control experiments were performed by adsorbing unfunctionalized generations 2, 3, 4, and 6 PAMAM dendrimers to the well plate surface. The remainder of the ELISA was performed as previously described. After sequential additions galectin-1 and the color generating reagents, washing between each layer, absorbance was measured as shown in **Figure 59**.

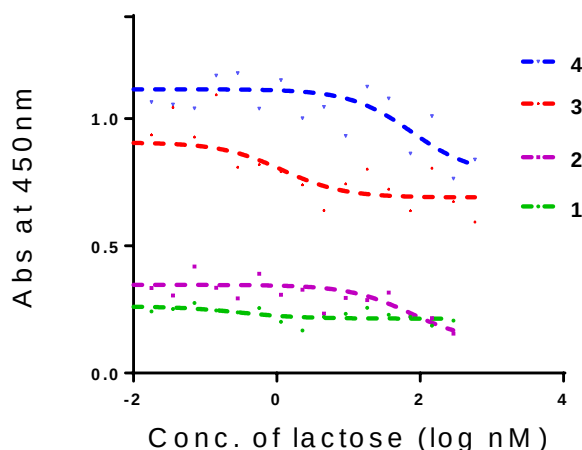


Figure 59. Non-specific ELISA binding curves with generation 2, 3, 4, and 6 PAMAM dendrimer.

Experimental Protocol

Nunc MaxiSorp Plate Preparation. Stock solutions for each generation of the PAMAM dendrimers (Dendritech) were prepared at 2 mg/mL in PBS (pH 7.4, 15 mM NaCl). The 2 mg/ml stock solution was diluted at 1:500 for a 4.0 μ g/mL solution and 50 μ L of this solution were added to each well of the Nunc MaxiSorp 96 well plate (Thermo Scientific). The well plate was covered, placed on an agitator/shaker for 10 minutes to ensure the bottoms of the wells were covered by the dendrimer solution, and stored for 24 hours at 4 °C. After 24 hours, the solvent was removed by emptying the well plate. To each well, 250 μ L of the 3% BSA solution in PBS (pH 7.4, 15 mM NaCl) was added to block non-specific interactions. The plate was covered and let stand for 2 hours at room temperature. After 2 hr, the plate was emptied, washed once with PBS (pH 7.4, 15 mM NaCl), dried. Dried plates were either used or covered and stored at 4 °C.

ELISA. To a processed polystyrene (PPI) plate, 60 μ L of 0.5% BSA in PBS (pH 7.4, 15 mM NaCl) were added to all wells, except A1, D1 and G1. To wells A1, D1, and G1, 60 μ L of the lactose solution (100 mg/mL in PBS) were added. To wells A2, D2, and G2, 60 μ L of lactose solution (100 mg/mL in PBS) were added. To generate 24 sequential lactose concentrations, serial dilutions were performed so that 60 μ L remained in each well. From each well on the PPI plate, 50 μ L were transferred to the corresponding well on the glycodendrimer coated Nunc MaxiSorp plate. Biotinylated galectin-1 (50 μ L of a 12.5 μ g/mL solution) was added to the wells. The plate was covered and placed on a shaker/agitator for 45 minutes.

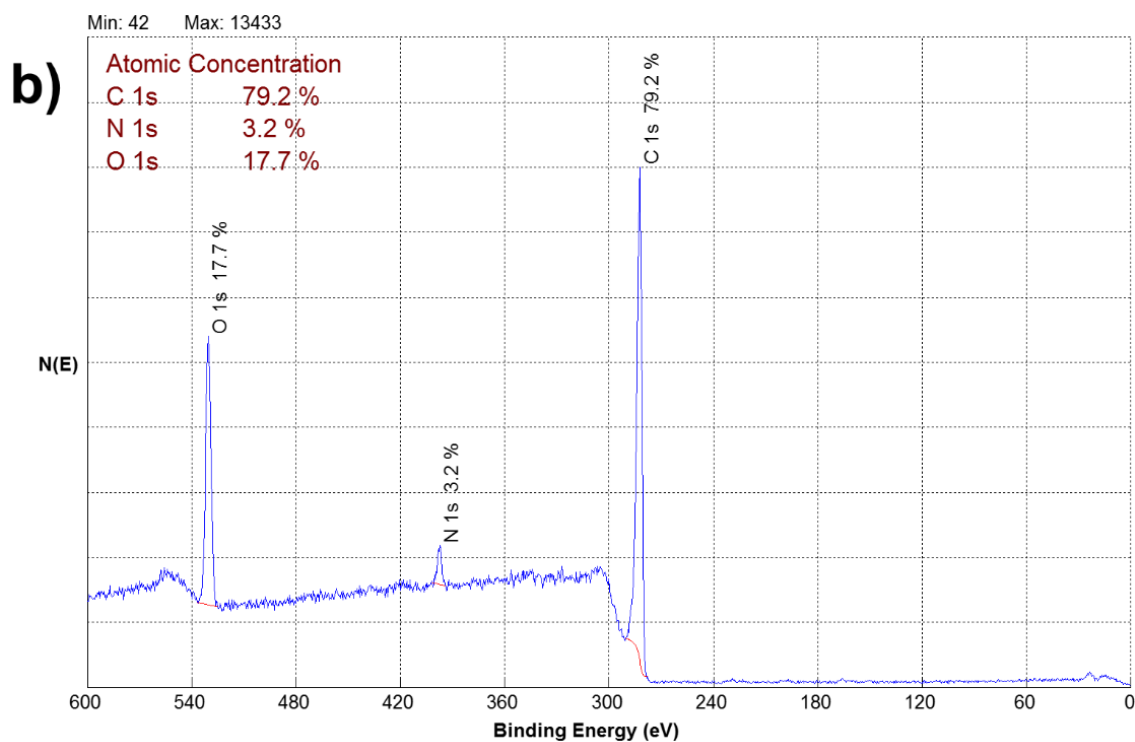
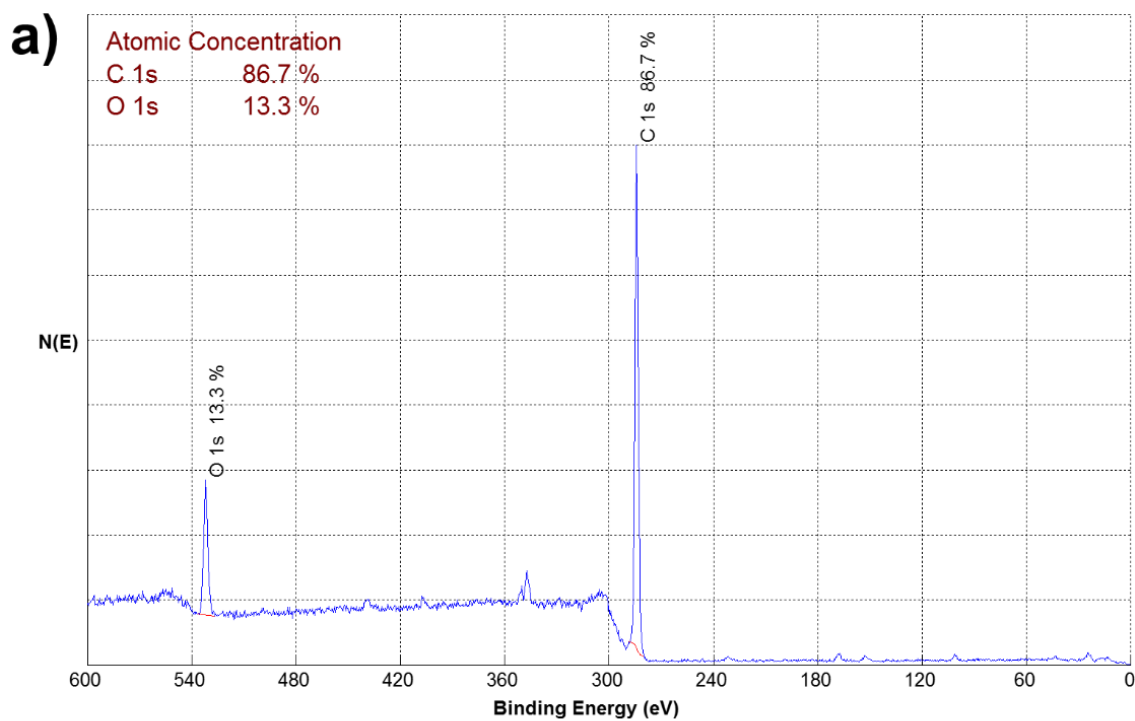
After 45 minutes, the plate was emptied and washed twice with PBS-T (pH 7.4, 15 mM NaCl) and once with PBS (pH 7.4, 15 mM NaCl). To each well, 100 μ L of streptavidin-horseradish peroxidase (SAv-HRP) (1:1000 dilution of the stock purchased from BD Biosciences) were added. The plate was covered and placed on a shaker/agitator for 45 minutes.

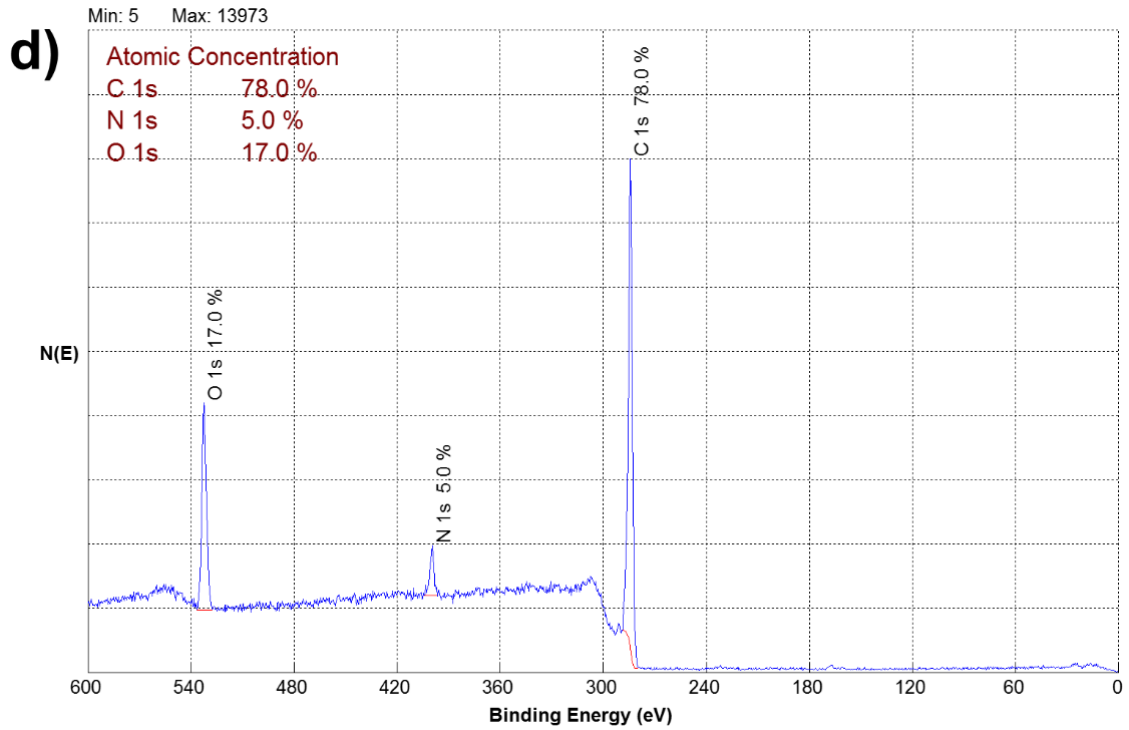
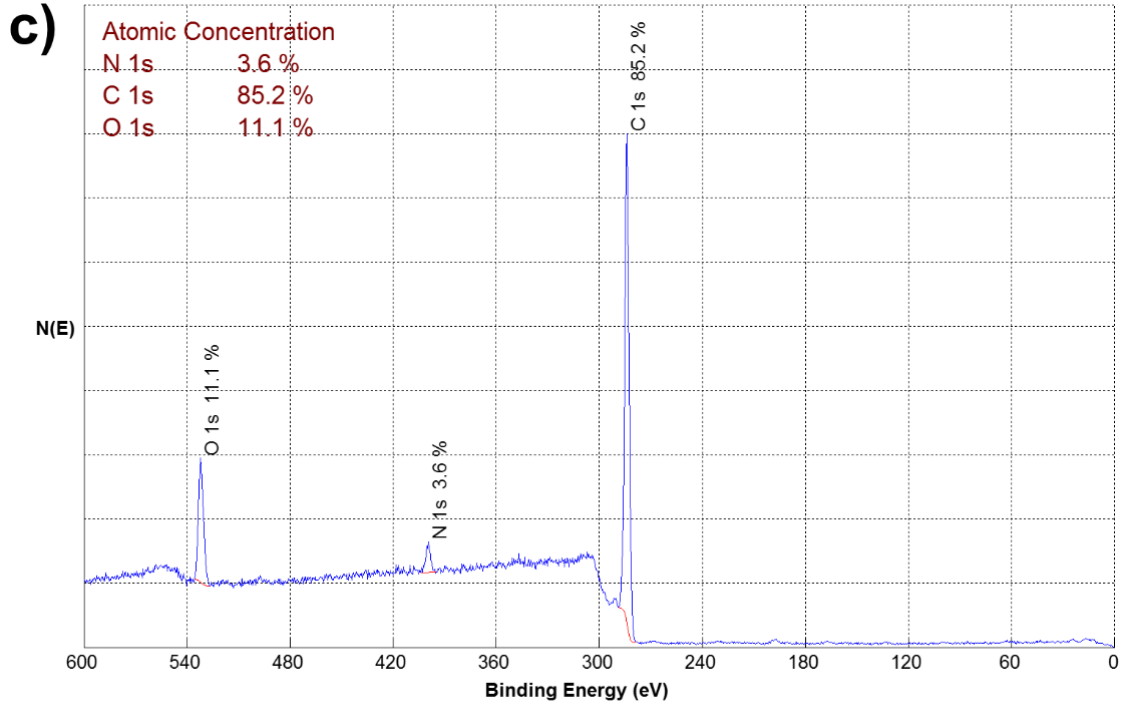
After 45 minutes, the plate was emptied and washed twice with PBS-T (pH 7.4, 15 mM NaCl) and once with PBS (pH 7.4, 15 mM NaCl). To each well, 100 μ L of TMB:H₂O₂ (mixed in a 1:1 ratio from a purchased from BD Biosciences) were added. The plate was covered and incubated for 10 minutes. A blue color change was observed. After 10 minutes, 100 μ L of H₃PO₄ was added to each well and a yellow color change was observed. Absorbances were measured at 450 nm, with a reference at 620 nm.

X-Ray Photoelectron Spectroscopy (XPS). XPS was used to characterize the percent nitrogen (%N) on the glycodendronized surface (**Table 12**). The nitrogen signal, which is attributed to the PAMAM dendrimer and thiourea linker, is absent without the surface adsorbed glycodendrimers (**Figure 60a-e**). Thus, the nitrogen signal can be used to determine if more/less glycodendrimer was adsorbed to the surface of the well than would be expected based on the % nitrogen of the compound measured from the molecular weight determined using MALDI. As shown in Table A1 for **1**, the percent nitrogen measured using XPS is similar to the nitrogen content expected for a monolayer of **1**. Therefore, the aberrant signal was likely the result of a poor interaction with surface adsorbed **1** because the lactosides on the relatively flat glycodendrimer interacted with the well.

Table 12. Comparison of Nitrogen content (%N) calculated from the molecular weight using MALDI and the nitrogen content (%N) as measured by XPS.

Compound	%N (MALDI-ToF)	%N (XPS)
1	5.2	5.0
2	6.3	3.6
3	6.1	5.1
4	7.6	7.9





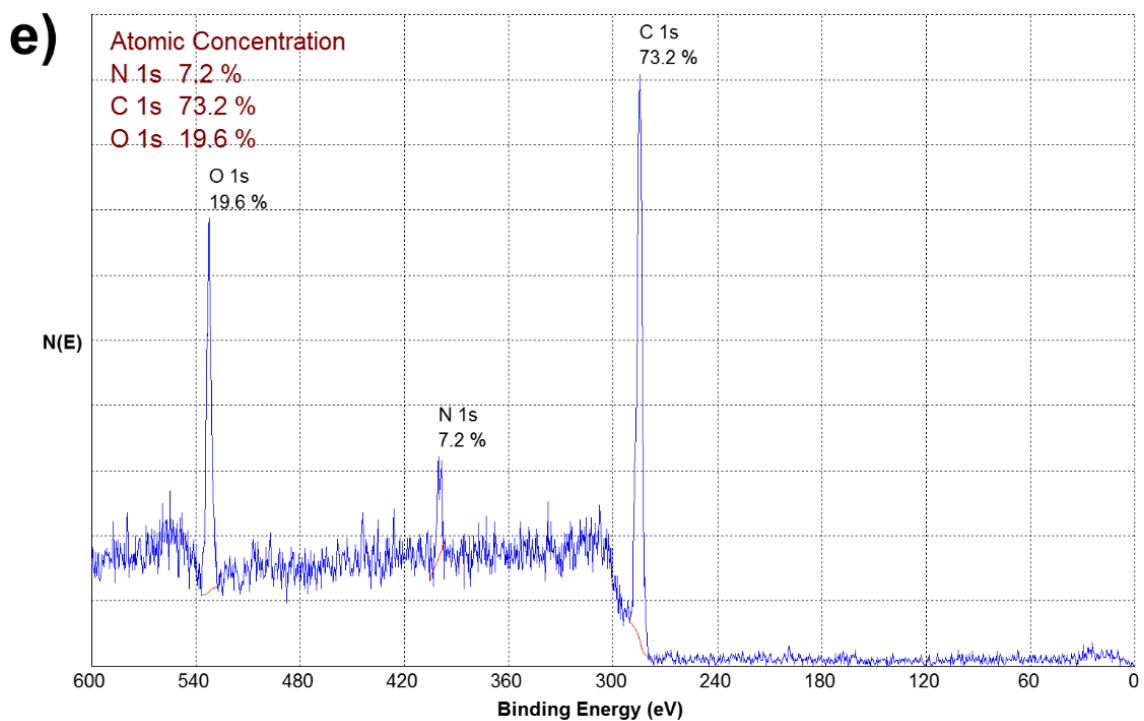


Figure 60. Representative XPS characterization of surface of well in the absence and presence of **1-4**. (a) Unfunctionalized surface of well. (b) Atomic signal from surface adsorbed **1**. (c) Atomic signal from surface adsorbed **2**. (d) Atomic signal from surface adsorbed **3**. (e) Atomic signal from surface adsorbed **4**.

Fluorescence Microscopy

Assay to Measure Size of Glycodendrimer Nucleated Galectin-1 Aggregates in Solution

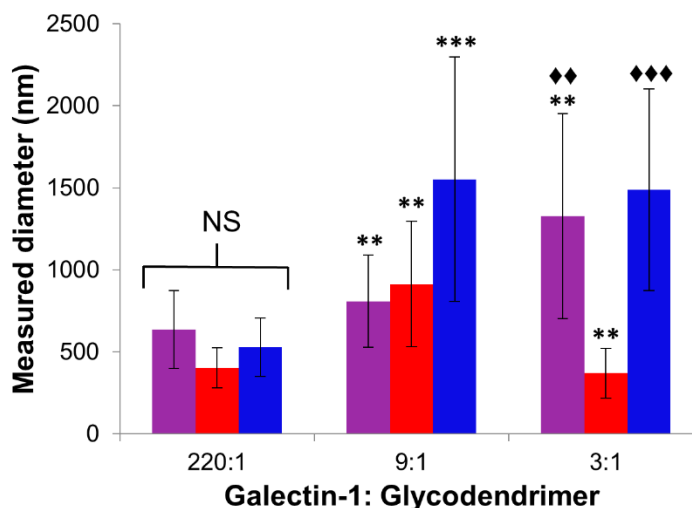


Figure 61. Average diameter (nm) of multivalent galectin-1 nanoparticles formed with multivalent glycodendrimers. Nanoparticle diameter (nm) was measured in the presence of galectin-1 at 220 molar excess, a 9 molar excess, and a 3 molar excess with compounds **2** (purple), **3** (red), and **4** (blue), respectively. Data are shown as mean $\pm$ one S.D. of triplicate measurements from three experiments ($n \geq 3$). Statistical analyses were performed using a paired two-tailed student's T-Test. Symbols (* and $\blacklozenge$) are used to indicate a statistically significant change in the size aggregates as the ratio of galectin-1 changes relative to a compound. The symbols *, **, and *** indicate a statistical significance of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, by comparing aggregates formed at 9:1 with respect to aggregates formed at 220:1, and by comparing aggregates formed at 3:1 with respect to those formed at 9:1. The symbols $\blacktriangle$, $\blacktriangle\blacktriangle$, and $\blacktriangle\blacktriangle\blacktriangle$ indicate a statistical significance of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, by comparing aggregates formed at 3:1 with respect to those formed at 220:1. NS represents non-significant difference in aggregate size measured at 220:1 for all generations determined by ANOVA.

APPENDIX B

MATRIX METALLOPROTEINASE SUBSTRATE-LINKED DENDRIMERS

Introduction

Cancer cells secrete matrix metalloproteinases (MMPs) which promote invasion and metastasis by degrading proteins in the ECM. Previous efforts were undertaken to link a matrix metalloproteinase (MMP) substrate to a dendritic framework for the study of prodrug release.²⁰⁶ The MMP substrate has a FRET signal that is quenched when the peptide is attached but gives off a fluorescence signal upon cleavage of the peptide by cancer cell secreted MMP. A fluorescence assay was designed in which the fluorescence intensity was monitored for four experimental conditions (in the presence and absence of cells) to determine the efficacy of the dendritic drug delivery system: (1) serum free media; (2) G2-Lac; (3) 580 MMP Fret Substrate I; and (4) G2-Lac-MMP substrate (580 MMP Fret Substrate I, Anaspec). The results of the dendritic drug design indicated that generation 2 lactose-functionalized dendrimer can improve delivery of the MMP substrate to the MMP substrate.

Results

To confirm that the second generation lactose functionalized dendrimer (G2-Lac) improves delivery of the MMP substrate to the MMP, the MMP substrate (580 MMP Fret Substrate I, Anaspec) linked G2-Lac (G2-Lac-MMP substrate) was used in a FRET assay with cancer cells. MMP substrate that incorporated a FRET labeled MMP substrate specific to cancer-associated MMP-2 and MMP-9 was linked to G2-Lac via amide bond formation with lactose-functionalized dendrimers.²⁰⁶ The G2-Lac-MMP substrate conjugate was incubated *in vitro* with A549 lung carcinoma cancer cells. Upon cleavage of the MMP

substrate, a fluorescence signal can be observed. Fluorescence was monitored over the course of 130 minutes for on-dendrimer MMP (G2-Lac-MMP) and free MMP-substrate, with controls for G2-Lac in the absence of MMP and cells alone. The results are summarized in **Figure 62**.

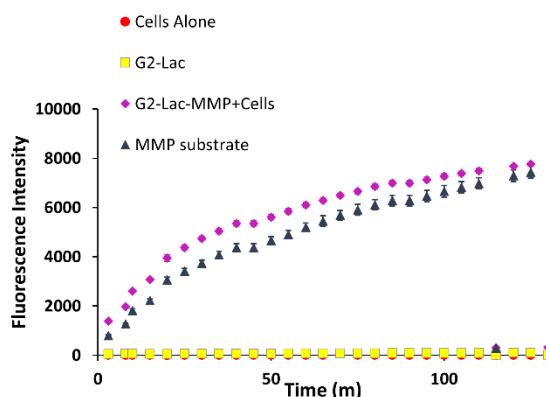


Figure 62. Fluorescence intensity of MMP-substrates incubated with A549 cells. Results represent background subtracted intensities (backgrounds consist of experimental conditions run the absence of A549 cancer cells). Results represent the averages of triplicate runs. Errors bars represent the standard deviation from triplicate runs. The concentration of MMP substrate was roughly held constant for all experimental conditions.

Over time, the fluorescence signal increased in the presence of the MMP-substrate, which indicated that more of the substrate was cleaved by cellular MMP-2 and MMP-9. The G2-Lac-MMP conjugate, however, generated a higher fluorescence signal than the free MMP substrate. This indicates that G2-Lac mediated delivery of the MMP substrate to the cellular MMP. As expected, no additional fluorescence was observed in the absence of MMP (cells alone and G2-Lac).

To determine if the higher loading of the MMP substrate on G2-Lac would further improve delivery of the MMP substrate, the G2-Lac-MMP conjugate was synthesized in the presence of excess MMP substrate (3:1 ratio of MMP substrate to G2-Lac). Loading of

the MMP substrate on the dendrimer was determined by UV-Vis to be 1 MMP substrate to 10 G2-Lac. G2-Lac-MMP was resubmitted to excess MMP substrate and the labeling ratio initially increased to 2 MMP to 10 G2-Lac. This indicates that a labeling ratio of MMP substrate to G2-Lac of 0.2 is the maximum loading as the sterics prevent a higher degree of functionalization. The signal for the MMP-G2-Lac conjugate was below that of the MMP off dendrimer control (data not shown). Furthermore, visual inspection of the MMP-G2-Lac conjugate indicated that the coloring changed from a deep purple to a light purple. To investigate this, the labeling ratio calculated again from the absorbance of the MMP-G2-Lac conjugate. The labeling ratio decreased from 0.2 to 0.07, indicating that some of the MMP had been quenched. The peptide linker appears to be labile and susceptible to degradation upon repeated freezing and lyophilization.

Experimental Procedures

General Experimental Methods

General reagents were purchased from Acros and Aldrich Chemical Companies. PAMAM dendrimers were purchased from Dendritech. Lactose-functionalized dendrimer obtained from Dr. Anna Michel.²⁰⁶

Addition of MMP Substrate on Lactose Functionalized Generation 2 PAMAM Dendrimer (G2-Lac)

580 MMP Substrate I (Anaspec, 0.7 mg, 396 nmol, 3 equiv.) was dissolved in 200 μ L DMSO and added to Dendrimer (G2-Lac) (0.84 mg, 129 nmol). NHS was dissolved in DMSO to a concentration of 20 mg/mL, 23 μ L (0.45 mg, 3.91 μ mol, 30 equiv.) of which

was added to the dendrimer/MMP solution. EDC was dissolved in Millipore water to a concentration of 20 mg/mL, of which 40 μ L (0.8 mg, 5.15 μ mol, 43 equiv.) was added to the dendrimer/MMP solution. The final dendrimer concentration in solution was 500nM. The reaction was shielded from light in 3500 Da MWCO dialysis membrane (Spectra/Por) tubing in 250 mL Millipore water for 6 hr, switching the water out every 2 hr. The purified solution was then frozen and lyophilized to yield a purple fluffy solid.

The degree of MMP loading was determined using a standard curve prepared with free 580 MMP Fret Substrate I. Serial dilutions of the purified product G2-Lac-MMP were prepared in terms of the known dendrimer concentration. The absorbance of samples was measured at 577 nm and read against the standard curve at 577 nm (the maximum absorbance of QXL-570, the quencher used in the MMP-substrate). Through this method, a labeling ratio of MMP to dendrimer was calculated to be 0.1.

Resubmission of MMP- and Lactose Functionalized Dendrimer to Excess MMP

Dendrimer was resubmitted to reaction conditions using excess MMP recovered from dialysis. Dialysis solution from each wash was saved, frozen, and lyophilized to yield a purple solid. The lyophilized MMP powder was dissolved in 50 μ L DMSO. MMP dendrimer (G2-Lac) (1.6 mg) was dissolved in 100 μ L DMSO, to which the MMP solution was added. NHS was dissolved in DMSO to a concentration of 20 mg/mL, 23 μ L (0.45 mg, 3.91 μ mol, 30 eq.) of which was added to the dendrimer/MMP solution. EDC was dissolved in Millipore water to a concentration of 20 mg/mL, of which 40 μ L (0.8 mg, 5.15 μ mol, 43 eq.) was added to the dendrimer/MMP solution. The final dendrimer

concentration in solution was 500nM. The reaction was shielded from light in 3500 Da MWCO dialysis membrane (Spectra/Por) tubing in 250 mL Millipore water for 12 hr, switching the water out every 2 hr.

The degree of MMP loading was determined using a standard curve prepared with free 580 MMP Fret Substrate I. Serial dilutions of the purified product G2-Lac-MMP were prepared in terms of the known dendrimer concentration. The absorbance of samples was measured at 577 nm and read against the standard curve at 577 nm (the maximum absorbance of QXL-570, the quencher used in the MMP-substrate). Through this method, a labeling ratio of MMP to dendrimer was calculated to be 0.2.

The product was frozen and lyophilized to dryness to remove an excess solvent and enable a more accurate measurement of mass. After lyophilization, the labeling ratio decreased to 0.07.

Fluorescence Assay

A549 cells were purchased from ATCC and cultured pursuant to the ATCC recommended protocol. Prior to use, cells were dissociated from growth dishes using 0.02% EDTA/0.25% Trypsin (Life Technologies) and diluted to 500,000 cells/mL in serum free F12K media (SFM, Gibco). The assay was run in a standard 96-well quartz plate (Hellma). Five experimental conditions were observed in the presence and absence of cells: (1) SFM; (2) G2-Lac; (3) 580 MMP Fret Substrate I; and (4) G2-Lac-MMP. Experiments run without cells were used as the appropriate background and subtracted from the fluorescence total. Experiments were run in triplicate using 100 μ L cell solution per well (50,000 cells per experiment). To wells (2) and (5), 30 μ L G2-Lac dendrimer was added

(2 mg/mL in Millipore H₂O). To well (4), 30 μ L G2-Lac-MMP dendrimer was added (2 mg/mL in Millipore H₂O). Samples were then monitored every 5 minutes for 130 min for fluorescent intensity and changes in lifetime using a NovaFluor Lifetime Microplate Reader at 532/556 ex/em with 1s signal averaging taking 3 readings per well per time point.

APPENDIX C

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