Directing Neuronal Signaling through Cell-Surface Glycan Engineering

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S *Supporting Information*

ABSTRACT: The ability to tailor plasma membranes with specific glycans may enable the control of signaling events that are critical for proper development and function. We report a method to modify cell surfaces with specific sulfated chondroitin sulfate (CS) glycosaminoglycans using chemically modified liposomes. Neurons engineered to display CS-E-enriched polysaccharides exhibited increased activation of neurotrophin-mediated signaling pathways and enhanced axonal growth. This approach provides a facile, general route to tailor cell membranes with biologically active glycans and demonstrates the potential to direct important cellular events through cell-surface glycan engineering.

Glycosaminoglycans (GAGs) are sulfated, linear polysaccharides that participate in many fundamental physiological processes, including cell division, cancer metastasis, angiogenesis, and neuronal development.[1−3] Increasing evidence suggests that GAGs interact with diverse proteins in a sulfation-dependent manner and modulate cellular signaling events.[3a,b,4] For example, a specific sulfation motif found on chondroitin sulfate GAGs (CS-E, Figure 1A) engages neurotrophin growth factors and their receptors, thereby stimulating downstream signaling pathways to enhance neurite outgrowth.[3a,4a] Indeed, distinct sulfation patterns have been shown to modulate pathways involved in development,[3b,c,4c] normal physiological function,[4a,b] and disease.[5] Thus, the ability

to present specific GAG structures on cell surfaces could provide a novel approach to control key cellular events. Such a method may also accelerate structure−function studies, as the complex, heterogeneous nature of GAGs on cell surfaces has hampered efforts to understand the roles of specific sulfation motifs *in vivo*.

Although a few strategies for engineering cell-surface glycans have been reported,[6] the remodeling of cell membranes with complex polysaccharides such as GAGs has not been demonstrated. In addition, such strategies cannot be readily adapted to GAGs, and application of these methodologies has been largely limited to imaging[6b−d] or studying cell-surface phenomena, such as receptor clustering.[6e] Here we develop a method to display specific sulfated GAG structures on cell surfaces using a liposomal fusion strategy. We show that tailoring membranes with the CS-E structure activates growth factor-mediated signaling pathways and enables the fine-tuned modulation of neuronal growth. These findings demonstrate that chemically controlling the presentation of exogenous glycans on cell surfaces can induce sustained effects on cellular signaling and function. Our studies also highlight the potential for glycan engineering to modulate complex cellular events, and they provide a powerful, new tool for remodeling cell membranes with a wide variety of important biomolecules.

We chose to utilize liposomes as glycan carriers due to their biocompatibility, ease of preparation, low cytotoxicity, and tunable biophysical properties.[7] Elegant studies have used glycan-presenting vesicles for intracellular antigen delivery[8] or sugar-encapsulated vesicles with folate receptors for cell-specific metabolic labeling.[6d] However, only two liposomal methods have been developed to our knowledge for the cell-surface display of exogenous molecules, namely fluorophores.[9] We expanded on these methods in an effort to incorporate large, sulfated GAGs into cell membranes (Figure 1B). To promote membrane fusion and surface presentation of the glycans rather than intracellular uptake, we used cationic 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) and neutral 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) as our primary lipids. Phosphatidylethanolamine lipids are an abundant, natural component of the neuronal cell membrane, and DOPE-based liposomes containing a lipid-functionalized fluorophore have been used to label a variety of cell types, including primary neurons.[9b] Here we also incorporated 2-
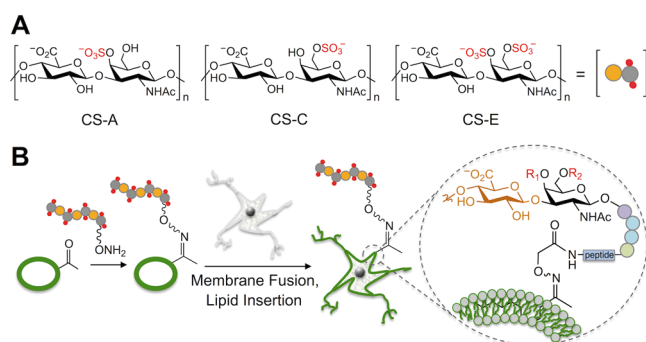

Figure 1. (A) CS polysaccharide structures used in this study. $n \approx 110$. (B) Strategy to remodel cell surfaces with CS GAGs and control signaling pathways.

dodecanone into the liposomes to add a ketone handle for appending the glycans via oxime chemistry.

CS polysaccharides containing a peptide fragment with an N-terminal amine were readily derivatized with an aminooxy group by coupling them to phthaloyl-protected aminooxyacetic acid, followed by cleavage of the phthaloyl group with hydrazine monohydrate (Scheme S1). The polysaccharides were then incubated with dodecanone-containing liposomes at 25 °C for 3 h to produce GAG-displaying vesicles. The synthetic ease and versatility of this approach represent advantages compared to existing methods, which require the radical-mediated synthesis of polymers end-functionalized with lipids and glycans with derivatizable reducing ends.[6e,f] With our approach, the lipid reagents are commercially available, and many biomolecules can be derivatized with aminooxy groups, including various glycans, peptides, lipids, nucleic acids, and proteins,[10] thus providing a general strategy for displaying a diverse range of bioactive molecules.

Preliminary optimization of liposomal membrane fusion was performed on rat pheochromocytoma (PC12) cells using liposomes functionalized with a hydrazide-conjugated fluorophore (AF488-hyd). We found that a 2:1 ratio of DOPE:DOTAP was optimal for membrane fusion, as visualized by fluorescence microscopy (Figure S1). To approximate the relative levels of fluorophore incorporation at the cell surface, we incubated liposomes containing varying concentrations of AF488-hyd with PC12 cells on ice for 30 min. Cells labeled with liposomes containing 10 mol % AF488-hyd displayed similar fluorescence signal profiles by fluorescence-assisted cell sorting (FACS) analysis as cells labeled with an anti-CS-E monoclonal antibody[4d] that detected endogenous CS-E levels (Figure 2A). These results suggest that this liposomal strategy can incorporate exogenous molecules into cell membranes at levels roughly similar to those of endogenous CS polysaccharides.

We next examined whether this approach could be used to display large GAG polysaccharides on cell surfaces. Liposomes containing 2:1 DOPE:DOTAP and 10% w/w dodecanone were functionalized with CS-E-enriched polysaccharides (~70 kDa).
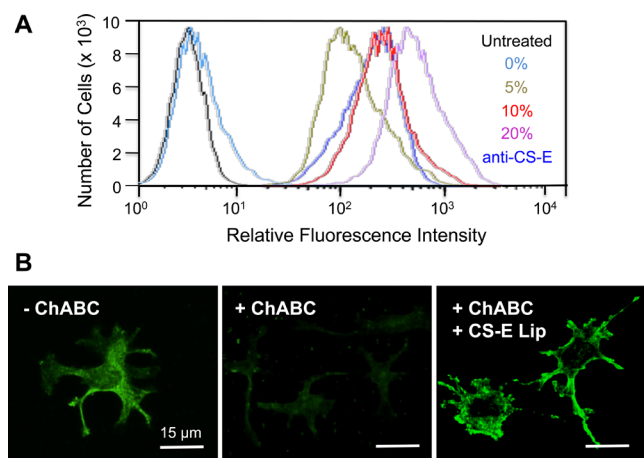

Figure 2. Controlled cell-surface display of CS polysaccharides and fluorophores. (A) FACS analysis of PC12 cells treated with liposomes presenting varying amounts (0−20%) of AF488-hyd. (B) Immunofluorescence detection of CS-E (green) on PC12 cells treated with or without chondroitinase (ChABC) and CS-E-functionalized liposomes as indicated.

To characterize their biophysical properties, we used transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential measurements. The liposomes exhibited parameters predicted to favor membrane fusion, including spherical morphologies, average diameters ranging between 132.6 and 159.6 nm, and good stabilities (zeta potentials of ±46−69 mV; Figure S2). The change from positive to negative electrokinetic potential (69 to −46 mV; Figure S2C) confirmed successful conjugation of the sulfated polysaccharides. Furthermore, energy dispersive spectroscopy (EDS) verified the presence of sulfur on CS-functionalized liposomes after CS conjugation (Figure S2D). To test for membrane fusion, PC12 cells were treated with chondroitinase to remove endogenous CS, incubated with CS-E-modified liposomes, and immunostained with an anti-CS-E antibody. Importantly, strong immunostaining for CS-E was observed on the surfaces of cells treated with CS-E-functionalized liposomes compared to chondroitinase-treated cells without liposome addition, indicating efficient incorporation of the polysaccharides (Figure 2B).

Having validated the method, we investigated whether the approach could be used to control cellular signaling pathways. Previous studies from our laboratory have demonstrated that CS-E polysaccharides can recruit nerve growth factor (NGF) to the cell surface and promote the assembly of NGF-tropomyosin receptor kinase (Trk) complexes.[4a] Complex formation, in turn, activates protein kinase B (Akt) and phosphatidylinositol 3-kinase (PI3K) signaling pathways and thereby enhances neurite outgrowth.[11] Thus, we reasoned that cell-surface presentation of exogenous CS-E polysaccharides might recruit NGF to the membrane and assist in the formation of activated signaling complexes to induce these biological responses. Embryonic day 18 (E18) rat cortical neurons were cultured for 7 days and then treated with liposomes displaying CS-E- or CS-C-enriched polysaccharides (30 min, 37 °C). Neurons were stimulated with the neurotrophin NGF for 0, 10, 30, or 60 min, and Akt activation was monitored using a phospho-Ser473 Akt antibody. Remarkably, neurons remodeled with CS-E polysaccharides showed approximately a 3-fold increase in Akt activation relative to untreated neurons at each time point (Figure 3), consistent with increased recruitment of NGF to the cell surface and robust activation of Trk receptors. In contrast, neurons remodeled with CS-C polysaccharides showed phospho-Akt levels similar to those of untreated neurons. These results support the importance of the CS-E motif in NGF-stimulated Trk activation.[4a] Moreover, they show that liposomal-mediated presentation of specific, sulfated CS polysaccharides on cell surfaces can activate important neuronal signaling pathways.

We next examined whether the exogenous CS-E-mediated activation of Trk pathways could direct neuronal growth. Rat E18 hippocampal neurons were cultured for 2 days and subsequently treated for 30 min with liposomes prefunctionalized with CS-A-, CS-C-, or CS-E-enriched polysaccharides. Neurons were then incubated in media lacking liposomes for an additional 24 h and immunostained with an anti-α-tubulin antibody to image the processes using confocal fluorescence microscopy. Initial studies revealed no appreciable difference in neurite outgrowth between neurons displaying the different sulfation motifs. We postulated that the membrane lifetime of the exogenous CS GAGs might be too short to elicit functional responses such as neurite outgrowth, which requires *de novo* protein and lipid biosynthesis. Therefore, we assayed the
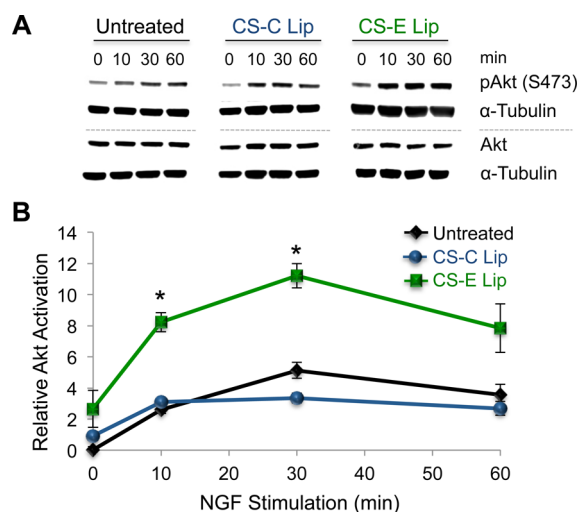
Figure 3. Presentation of CS-E polysaccharides on neuronal cell surfaces enhances NGF-mediated Akt activation. (A) Representative Western blots and (B) quantitation of Akt activation in neurons displaying CS-C- or CS-E-enriched polysaccharides. Akt activation was normalized against total Akt levels at each time point and compared to untreated neurons. Data represent mean ± SEM (*$P < 0.05$) from at least three experiments.

Figure 4. Presentation of CS-E polysaccharides on neuronal cell surfaces stimulates neurite outgrowth. (A) Representative images and (B) quantification of neurite outgrowth for neurons treated with unmodified liposomes or liposomes displaying CS-A-, CS-C-, or CS-E-enriched polysaccharides. Neurite outgrowth was normalized and plotted relative to untreated neurons. Data represent mean ± SEM (*$P < 0.05$) from at least three experiments. See SI for details.

membrane lifetime of the exogenous lipids by treating PC12 cells with liposomes bearing fluorophore-conjugated CS-E polysaccharides and monitoring the fluorescence signal over 24 h (Figure S3). A decrease in signal was observed within 6 h, and loss of the signal progressed over the course of 10 h. After 16 h, weak fluorescence was detected around the cell periphery, suggesting that the CS-conjugated lipids had been internalized or had diffused into the medium.

To circumvent this problem, we repeated the outgrowth assays with multiple additions of liposomes every 8 h over a 24-h period. Under these conditions, we found that cell-surface presentation of CS-E polysaccharides significantly enhanced neurite outgrowth by 36.3 ± 3.3% relative to untreated neurons (Figures 4 and S4, 10% dodecanone). As expected, neurons displaying CS-A or CS-C polysaccharides showed minimal neurite outgrowth when compared to untreated neurons (10.8 ± 4.8% and 1.3 ± 1.8%, respectively). As a further control, dodecanone-containing liposomes were reacted with CS-E polysaccharides lacking the aminooxy functionality and then incubated with the cells. No difference in neurite outgrowth was observed relative to untreated neurons.

Remarkably, the extent of neurite outgrowth could be finely tuned by controlling the concentration of CS-E polysaccharides at the cell surface. Liposomes containing 0% to 10% dodecanone were conjugated with aminooxy-functionalized CS-E and then incubated with E18 rat hippocampal neurons as above. Notably, we observed a dose-dependent increase in neurite outgrowth from 4.0% to 36.3% as the dodecanone concentration was increased from 2.5% to 10% (Figures 4B and S4). Together, these studies demonstrate that this approach for engineering glycans on cell surfaces can be used to finely modulate both the signaling and functional responses of neurons.

In conclusion, we have developed a powerful, synthetically facile method to display large, complex CS polysaccharides on cell surfaces using chemically modified liposomes. The surface density, as well as the specific glycan structure, can be precisely controlled to activate key intracellular signaling pathways and
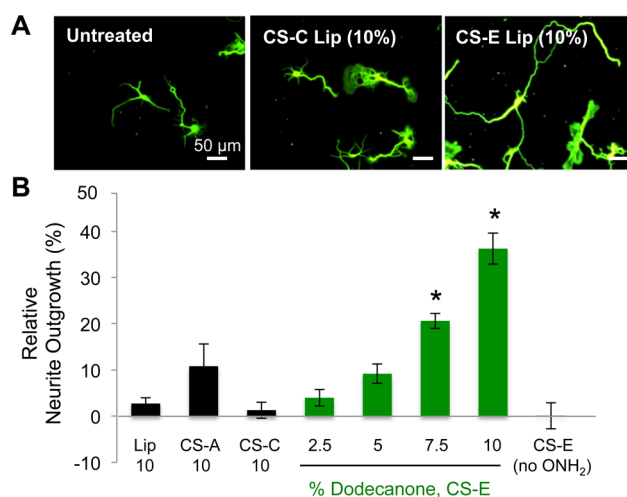
induce functional responses in a dose-dependent manner. We show that the presentation of CS polysaccharides enriched in a specific sulfation motif can trigger Akt signaling pathways and exert sustained effects on neuronal growth. In the future, we envision that this general strategy can be extended to perform comparative analyses of the effects of various glycans on cellular signaling and function. Moreover, the use of targeted liposomal delivery strategies both *in vitro* and *in vivo* will expand application of this approach to many important biological contexts, such as neurodegeneration, stem cell differentiation, and cancer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, compound and liposome characterization, supporting figures and schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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