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Macrocyclic proteoglycan mimics. Potent inhibition of cell adhesion by a bundle of chondroitin sulfate chains assembled on the calix[4]resorcarene platform

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Abstract—Tailed calix[4]resorcarene macrocycle (tail = undecyl) can be used as a platform to assemble four glycosaminoglycan polysaccharide chains to give a new type of proteoglycan mimics. A tetra(chondroitin sulfate) derivative thus obtained from the reaction of macrocyclic octaamine and chondroitin sulfate lactone is readily immobilized on a tissue culture plastic (polystyrene) plate and inhibits fibronectin-mediated adhesion of BHK (baby hamster kidney) cells thereon remarkably strongly with 50% inhibition occurring at a 10 ng/mL or 40 pM concentration range.

Cell-matrix adhesion is triggered by the interaction of extracellular matrix (ECM) proteins such as fibronectin with their cell-surface receptor integrin, followed by clustering of the latter together with actin filaments connected thereto in the cell interior, ultimately leading to formation of focal adhesions (focal contacts) and stress fibers.[1] A group of ECM components known as anti-adhesive molecules inhibit cell adhesion.[1,2] Chondroitin sulfate (CS) proteoglycans such as versican (Fig. 1) are a member of this group.[3–7] While the detailed mechanisms of inhibition by CS proteoglycans have not yet been settled, there is good evidence that they are bound on the cell surface with annexin 6 as a putative receptor, thereby blocking the signal transduction to promote the formation of focal adhesions.[8] Cell adhesion plays important roles in regulating such diverse cell activities as signaling, differentiation, growth, translocation, and angiogenesis and transfer of tumor cells.[9–11] This also explains the cell-physiological significance and therapeutic application of inhibition of fibronectin/integrin-mediated cell adhesion.

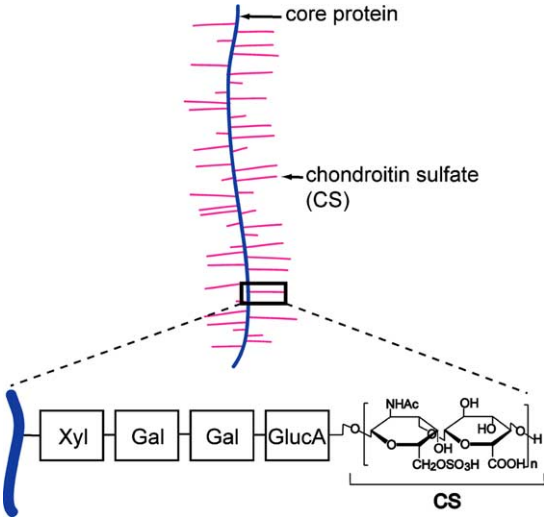

Figure 1. Schematic structure of versican as an example of chondroitin sulfate (CS) proteoglycans.

The present work is concerned about synthetic (artificial) anti-adhesive molecules in the context of proteoglycan mimics. Proteoglycans are complex architectures having many (in most cases) polyanionic glycosaminoglycan (GAG) polysaccharide chains attached to a common polypeptide core.

In Figure 1 is shown, in a schematic form, the structure of versican as an example of chondroitin sulfate (CS)

Keywords: Inhibition of cell adhesion; Proteoglycan mimic; Chondroitin sulfate; Fibronectin; Calix[4]resorcarene.
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proteoglycans, which possess CS, an alternate copolymer of sulfated *N*-acetylgalactosamine, and glucronic acid, as GAG chains. While CS-conjugated protein (serum albumin) and lipid (dipalmitoylphosphatidylethanolamine) derivatives are shown to inhibit cell adhesion,[5,12] the significance of clustering CS chains in native proteoglycans, and their mimics still remains to be uncovered. We have been working with saccharide cluster compounds having eight oligosaccharide chains and four long alkyl tails ($R=(CH_2)_{10}CH_3$) on the opposite sides of the macrocyclic calix[4]resorcarene framework (structure **1b** in a schematic form in Fig. 2).[13] Most characteristically, they are capable of unimolecularly forming a glycocluster motif in a well-defined geometry.[14] We report here that the present macrocycle can be used as a platform to assemble CS chains and that the resulting CS cluster compound as a new type of CS proteoglycan mimics exhibits a remarkably potent cell-adhesion inhibition activity at a ng/mL concentration range.

We utilize the facile amine-lactone reaction between macrocyclic octaamine **1a** (Fig. 2) and an oligosaccharide whose reducing terminus has been oxidized into a lactone moiety; the latter undergoes ring opening and is anchored on the macrocyclic platform via amide linkage with the rest of the oligosaccharide chain intact.[13f] As far as short-chain (di- to hepta-) oligosaccharides are concerned, the reactions occur almost on a stoichiometric basis and readily afford fully-, that is octa-substituted saccharide cluster compounds (**1b** in case where lactonolactone is used). The reaction proceeds smoothly also when the lactone derivative[12] of polysaccharide chondroitin sulfate (CS, mw 40,000–80,000) is used. An equimolar reaction with a slight lactone excess (lactone/**1a** = 1.2) in DMF affords mono-substituted CS derivative **1c** (23%) as judged by ^{1}H NMR integration.[15] On the other hand, a stoichiometric (with respect to the amine functionalities) reaction with a 1.6-fold lactone excess (lactone/**1a** = 12.5 or lactone/amine = 1.6) results in multiple substitution, giving rise to tetra-substituted derivative (**1d**, 16%).[16] This is also the case at higher lactone excess, suggesting that, probably for steric reasons, only one of two amino groups in proximity on adjacent benzene rings at sites A–D (Fig. 2) undergoes substitution; the bulky polysaccharide chain introduced at one amino group may block the other, which, however, still serves as the site of fluorescence labeling. Treatment of **1d** with a fluorescein reagent yields a fluorescein-labeled compound **1e**.[16]

The growth of adherent cells is adhesion-triggered. Upon adhesion, they often become expanded. Thus, baby hamster kidney (BHK) cells grow well with characteristic expansion when incubated[17] in a fibronectin-coated plastic (polystyrene) plate ($d = 6$ cm) (Fig. 3a). The adhesion-triggered cell growth is completely inhibited when the plate (only the left half with an area of 14 cm^2) is pretreated[17] with 1 mL (0.07 mL/cm^2) of a dilute aqueous solution (15 μg/mL) of the CS-bundle

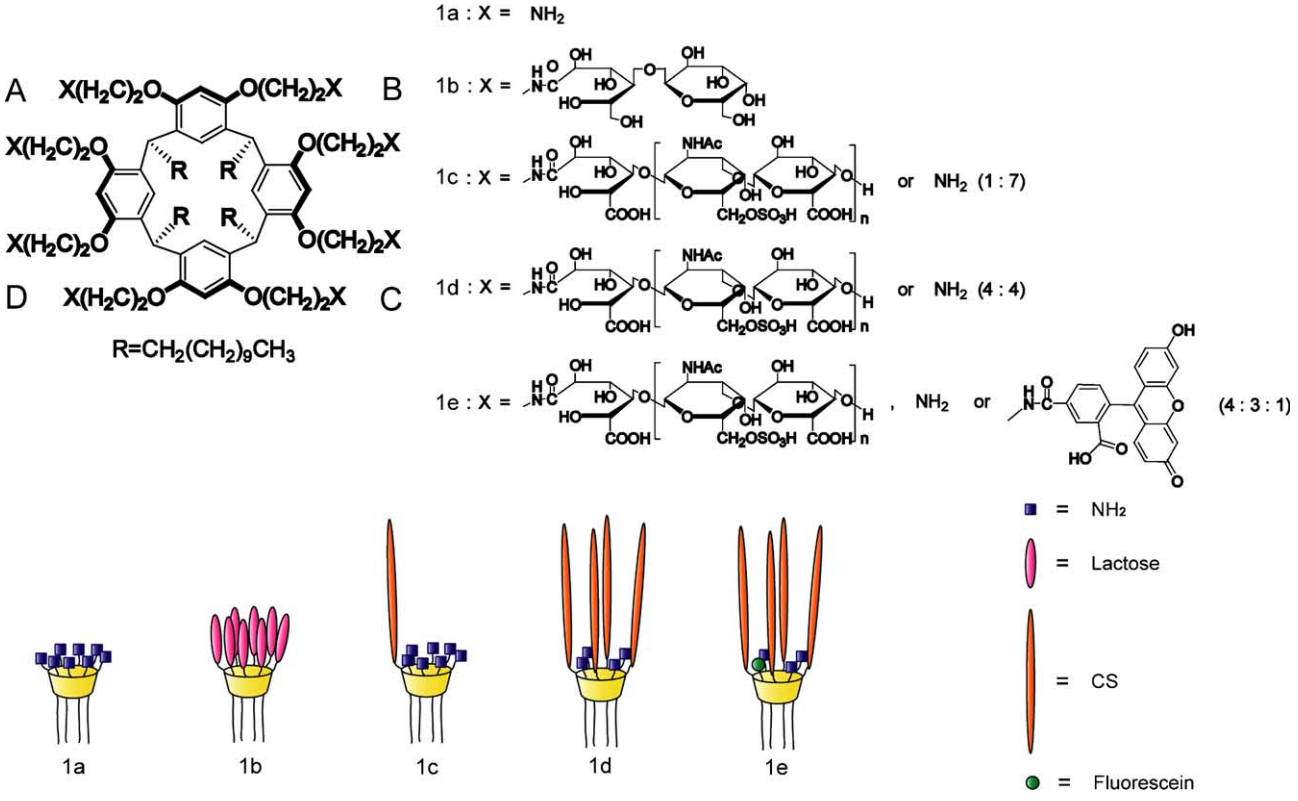

Figure 2. Structures of macrocyclic (calix[4]resorcarene) octaamine (**1a**), octa(lactose) (**1b**), mono(CS) (**1c**), and tetra(CS) derivatives with (**1e**) or without (**1d**) a fluorescence (fluorescein) label and their schematic representations.

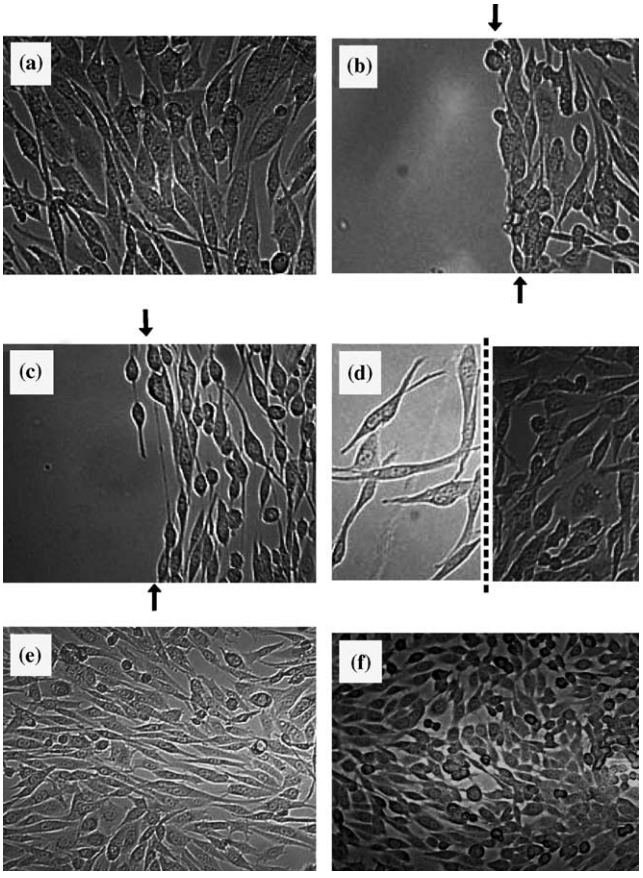

Figure 3. Optical micrographs of BHK-21 cells cultivated (see Ref. 17) on a fibronectin-coated plastic plate ($d = 6$ cm): (a) without any pretreatment of the plate; (b)–(d), with pretreatment of the left half (as illustrated by arrows or dotted line) of the plate with 1 mL of an aqueous solution of **1d** (15 μg/mL (b), 0.15 μg/mL (c), or 0.01 μg/mL (d)); (e) and (f), with pretreatment of the whole area of the plate with 1 mL of an aqueous solution of **1c** (1 μg/mL) or **1b** (2.5 mg/mL).

compound **1d** (3b). The anti-adhesion activity of **1d** remains the same upon 100-fold dilution of the solution down to 0.15 μg/mL (3c) and is still significant, resulting in >50% inhibition (3d), at 0.01 μg/mL (10 ng/mL or 40 pM assuming an average molecular weight of 60,000 for a CS chain). This may be compared with the corresponding values of 8,[5] 400—500,[5] and 0.6–2.2 μg/mL[12] for versican (a natural CS proteoglycan), CS-derivatized serum albumins, and CS-functionalized dipalmitoylphosphatidylethanolamines, respectively, at which they 50% inhibit the adhesion of BHK cells on a fibronectin-coated 96-well plate.

Control experiments indicate that neither CS or CS lactone nor mono(CS) compound **1c** (Fig. 3e) is active in inhibiting cell adhesion. A fully-substituted but simple (lactose-derived) saccharide cluster compound **1b**, even at a great excess, is not active, either (3f). Fluorescence monitoring using a microplate reader shows that fluorescence probe **1e** is strongly adsorbed on the plate practically in an irreversible manner. These results leave little doubt that the four alkyl chains ($R = (CH_2)_{10}CH_3$) in **1d** serve as an anchor to immobilize the molecule on the hydrophobic surface of the plate[18] and the clustering CS chains interfere with cell-fibronectin interactions.[19]

Preliminary SPR (surface plasmon resonance) analyses indicate that compound **1d** is hardly bound to a fibronectin-immobilized sensor chip. Strong fibronectin-**1d** interaction on the plate is thus unlikely.

Current knowledge on CS proteoglycans suggests that they do not necessarily inhibit fibronectin-integrin interactions but do inhibit, via interactions with relevant cell-surface receptors, the clustering of actin-linked integrins to form focal adhesions (focal contacts) and stress fibers as a strong cell-matrix adhesion junction.[8] In this context, the big difference in the inhibition activities of tetra(CS) and mono(CS) compounds **1d** and **1c** suggests that multivalency plays an important role in the CS-receptor interaction responsible for intracellular signal transduction.[19]

Multivalency is a common aspect of biological saccharide recognition events. So far, we assembled short-chain oligosaccharides on the framework **1a** to give what we called macrocyclic glycoclusters,[14] which exhibited unique adhesion,[13a,c,f] recognition,[13b,d] and transfection[13e,g] properties. The present work reveals that the same strategy is also applicable to glycosaminoglycan (GAG) polysaccharides to allow a new entry into artificial or 'neo' proteoglycans. The remarkably strong anti-adhesion activity of compound **1d** as a macrocyclic CS proteoglycan mimic may arise from a cooperation of four long alkyl tails to endow with an irreversible matrix-immobilizability and four CS chains, which collaborate with each other to manipulate intracellular signaling.[19] Further work now under way in this laboratory includes immobilization of various GAGs, function-characterization of GAG monolayers, and mechanistic elucidation of the present inhibition of cell adhesion.

Acknowledgements

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15. A solution of octaamine **1a** (mw 1450; 100 mg, 0.069 mmol) and CS lactone obtained by I_2 oxidation of CS (Seikagaku Kogyo, mw 40,000–80,000, repetition number *n* (referring to Fig. 1) 90–135; 5.0 g, 0.083 mmol assuming an average mw of 60,000) in DMF (100 mL) was stirred at room temperature for 72 h. After the reaction was over, the solvent was removed. The residue was taken in water (100 mL) and extracted with chloroform to remove unreacted octaamine (40 mg, 40%). Into the water phase was added an appropriate amount of hydrophobic resin powders (TOYOPEARL Phenyl-650M) and the mixture was vigorously shaken to allow adsorption of any hydrophobic ($R = (CH_2)_{10}CH_3$) macrocyclic derivatives on the resin. The resin was recovered by filtration and treated with methanol/water (85/15) to promote desorption of adsorbates. Evaporation of the solvent left 232 mg (23%) of CS derivative **1c**, whose ^1H NMR showed characteristic resonances for both tailed macrocycle and CS. Integration of the CH_3 signal at δ 2.0 for the acetamide moieties of CS relative to the CH_2 resonance at δ 1.25 for the undecyl moieties of the macrocycle indicated an averaged 1:1 (macrocycle to CS) formulation of the product (**1c**). There was no indication of in-solution aggregation of **1c**, whose GPC was hardly distinguishable from that of CS and DLS showed no notable big particles in a similar manner as CS.
16. A solution of octaamine **1a** (2.5 mg, 0.00172 mmol) and CS lactone (obtained from CS (1.29 g, 0.0215 mmol) in DMF (50 mL) was stirred at room temperature for 3 days as above. The solvent was removed and the residue was dialyzed against water for 3 days using a dialysis membrane (Float-A-Lyzer) with a cut-off mw of 100,000 (Spectrum) to remove unreacted CS lactone (mw 40,000–80,000) and low molecular-weight products if any. Repeated dialysis (three times) left 65 mg of high molecular-weight product, whose ^1H NMR integration ($NCOCH_3/CH_2$, as referred to above) corresponded to tetrasubstituted derivative **1d** (yield, 16%). Treatment of **1d** with 5-carboxyfluorescein succinimidyl ester (Molecular Probes) following the maker's protocol afforded a fluorescein-labeled compound **1e**. In GPC elution, **1d** appeared as a high molecular weight material (>800,000 based on pullulane standards). DLS showed the presence of 80-nm sized particles. Thus, compound **1d** is possibly aggregated in water.
17. BHK-21 cells (Riken Cell Bank) were seeded in a plate (BD Biosciences) at a density of $\sim 6 \times 10^5$ cells/plate and cultivated in 3 mL of DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum) and antibiotics (50 μg/mL streptomycin) at 37 °C for 48 h. After removing the medium, the plate was washed with PBS (2 mL × 2) and analyzed with an optical microscope for adherent cells. When pretreating the plate with compound **1d**, 1 mL of an aqueous solution thereof was applied on one of two half areas of the plate for 24 h. The solution was removed and the plate was washed with PBS (2 mL × 3) before being subjected to cell culturing described above.
18. Compound **1b** and analogues are readily immobilized on a hydrophobized sensor chip of SPR. See, Ref. 13f and Hayashida, O.; Shimizu, T.; Fujimoto, T.; Aoyama, Y. *Chem. Lett.* **1998**, 13–14.
19. If it is assumed that all of the 0.01 μg (4×10^{-14} mols, assuming a molecular weight of 24×10^4) of **1d** molecules under the lowest-concentration conditions (Fig. 3d) are adsorbed on the plate (14 cm^2), one molecule would have an occupation area of 6×10^4 nm^2 on the plate with a separation of 200–300 nm for nearest neighbors as the lower limit. Thus, it is likely that compound **1d** works unimolecularly as an intramolecular CS cluster. At this separation, however, molecules of **1d** having long polyanionic CS chains in an extended conformation might still be networked on the plate, leaving a possibility of intermolecular collaboration of the CS chains. Glycosaminoglycans are known to form hydrogels. It is also interesting in this regard that tetra(CS) compound **1d** is possibly aggregated in solution while mono(CS) counterpart **1c** is not (Ref. 15 and 16).