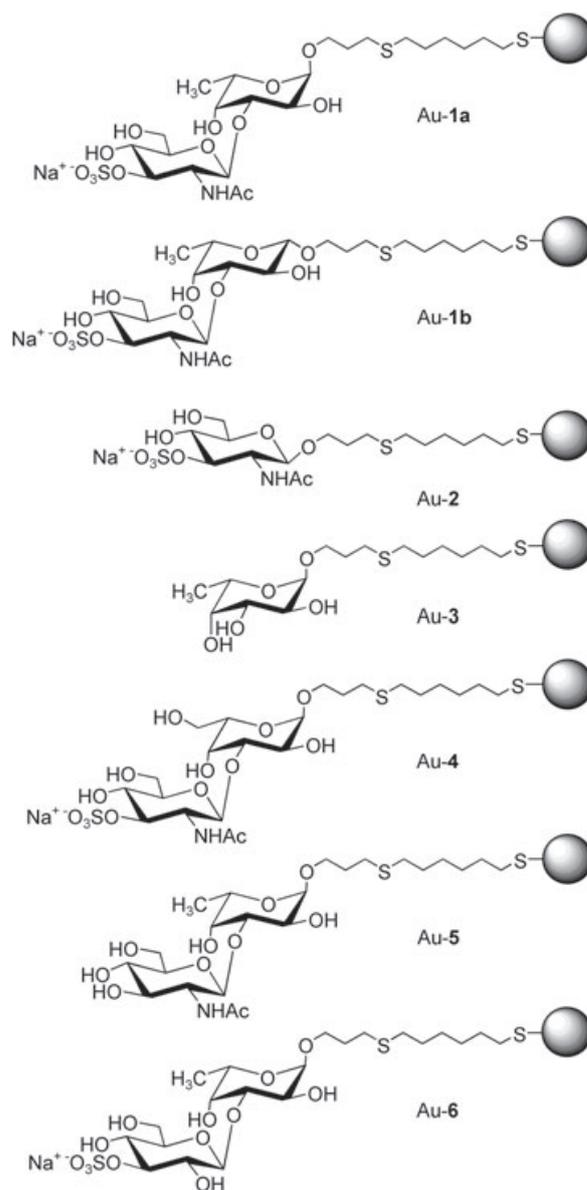


Gold Glyconanoparticles as Probes to Explore the Carbohydrate-Mediated Self-Recognition of Marine Sponge Cells

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Cell aggregation in the red-beard marine sponge *Microciona prolifera* is mediated by a 2×10^4 kDa proteoglycan-like macromolecular aggregation factor (MAF), and is based on two highly polyvalent functional properties; a Ca^{2+} -dependent proteoglycan self-interaction and a Ca^{2+} -independent cell-binding activity.^[1–3] MAF, the first circular proteoglycan described, is composed of two *N*-glycosylated proteins, MAFp3 and MAFp4, with twenty units of each glycoprotein forming the central ring and the radiating arms, respectively. Each MAFp3 carries one or two copies of a 200 kDa acidic glycan, g-200, whereas each MAFp4 carries about 50 copies of a 6 kDa glycan, g-6.^[3] The MAFp4 arms of the sunburst-like proteoglycan are linked to cell-surface binding receptors, while the MAFp3 ring exposes the g-200 glycans so that they can engage in the Ca^{2+} -dependent self-association (for a detailed review, see ref. [4]). By making use of MAF-specific monoclonal antibodies, it could be demonstrated that the self-association of MAF occurs through highly repetitive epitopes on the g-200 glycan.^[5,6] One of these epitopes was shown to be the sulfated disaccharide $\text{Glc}p\text{NAC}3S(\beta 1-3)\text{Fuc}p$.^[7] To gain insight into the role of carbohydrate interactions in MAF self-aggregation, we designed a challenging system for mimicking the g-200 self-association.^[8] By using the synthetic sulfated disaccharide, multivalently presented as a bovine serum albumin conjugate, and surface plasmon resonance spectroscopy, it was shown that Ca^{2+} -dependent carbohydrate self-recognition is a major force in the g-200 association phenomenon.

Gold glyconanoparticles have been successfully used as inert multivalent systems to explore either carbohydrate self-interactions or carbohydrate binding to proteins.^[9–14] In the present study, water-soluble gold glyconanoparticles coated with synthetic carbohydrates related to the sulfated disaccharide fragment (Scheme 1) were used as multivalent systems to investigate the g-200 glycan–glycan interaction by transmission electron microscopy (TEM). Very recently, an NMR study of intact



Scheme 1. Gold glyconanoparticles Au-1 a/b to Au-6, related to the MAF sulfated disaccharide epitope.

MAF glycans suggested the presence of α -Fuc residues.^[15] However, earlier structural analysis of oligosaccharide fragments obtained from a partial acid hydrolysate of the g-200 glycan could not identify the anomeric configuration of the fucose residue in these fragments.^[7,16] Therefore, gold glyconanoparticles coated with the α - or the β -anomer (Au-1a and Au-1b) of the native sulfated disaccharide epitope were used in the aggregation experiments. The importance of each of the two monosaccharide units for the self-recognition process of the disaccharide epitope was determined by studying the gold glyconanoparticle systems Au-2 and Au-3. The three gold glyconanoparticle systems Au-4 (α -L-Fucp replaced by α -L-Galp), Au-5 (β -D-GlcNAC3S replaced by β -D-GlcNAC), and Au-6 (β -D-GlcNAC3S replaced by β -D-Glc3S) were used to evaluate the relevance of the modified sites in the self-recognition process.

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The aggregation behavior of the different nanoclusters, Au-1a/b to Au-6, was analyzed in the presence or absence of Ca^{2+} ions. Initially, aliquots of each gold glyconanoparticle system in aqueous solution (0.1 mg mL^{-1}) were placed onto a copper grid and then observed under the transmission electron microscope. TEM micrographs of Au-1a/b to Au-6 in water showed, in all cases, uniformly dispersed nanodots throughout the grid surface.^[17] Subsequently, the experiments were repeated in aqueous 10 mM CaCl_2 —this being the calcium concentration commonly found in sea water.^[18] After 16 h of incubation of Au-1a/b to Au-6 in CaCl_2 (0.1 mg mL^{-1}), only the Au-1a and Au-1b nanoparticles presented aggregates (Figures 1 and 2).

As is evident from Figure 1, the Au-1a and Au-1b aggregates had a striking difference in size. To investigate the aggregation profiles of these two gold glyconanoparticle systems over time, incubations in 10 mM CaCl_2 (0.1 mg mL^{-1}) were carried out for 1.5 h, 3 h, 16 h, and 7 days. The TEM micrographs of the Au-1a and Au-1b time series revealed clear differences in their aggregation pattern (Figure 3). After 1.5 h incubation, the Au-1a nanoparticles presented aggregates with a mean di-

ameter of 20 nm throughout the whole grid, while the Au-1b nanoparticles had formed only a few small aggregates of less than 10 nm diameter (Figure 3A and B). For both nanoparticle series, further aggregation was observed during the extended incubation times (3 and 16 h; Figure 3C–F). However, no significant differences in mean diameter of the aggregates were observed on comparing the 16 h and 7 days incubation times (Figure 3E–H). The Au-1a aggregates reached a mean diameter of approximately 100 nm, while the Au-1b nanoparticles presented small groups of aggregates with a maximum diameter of 15 nm.

These findings indicate that the α -anomeric product exhibits stronger self-recognition; this supports the idea that α -L-fucose is the anomeric form of fucose found in MAF. Addition of EDTA (final concentration 50 mM) to Au-1a aggregates dispersed the aggregates completely; this confirmed the dependence of this carbohydrate–carbohydrate interaction on Ca^{2+} ions. The specificity for Ca^{2+} ions of the Au-1a self-recognition is supported by the fact that incubation of Au-1a in 10 mM MgCl_2 (0.1 mg mL^{-1}) did not result in aggregate formation (in agreement with studies performed with purified MAF^[22]).

The results for nanoparticles Au-2 and Au-3 established that the monosaccharide constituents of β -D-GlcpNAc3S-(1 \rightarrow 3)- α -L-Fucp- were not able to reproduce the interactions observed in the disaccharide. Therefore, the sulfated disaccharide as a whole is essential in the self-recognition process. Interestingly, the C6 methyl group of L-fucose was found to have an irreplaceable function in the self-recognition phenomenon, as the Au-4 nanoparticles did not form aggregates. A possible explanation for this observation is that the change of the hydrophobicity at the C6 position, from a methyl in Au-1a to a hydroxymethylene group in Au-4, disturbed the possible hydrophobic interactions between disaccharide epitopes on different particles. It is widely believed that protein folding,^[19] drug binding to proteins,^[20] and carbohydrate binding to lectins^[21] are largely driven by hydrophobic interactions. Nanoparticles Au-5 and Au-6, lacking the sulfate or the *N*-acetyl group, respectively, did not aggregate in the presence of Ca^{2+} ions. Our results demonstrate the involvement of these functional groups in the carbohydrate self-recognition—possibly through the coordination of Ca^{2+} ions, which may be the driving force for homotopic-carbohydrate interactions.^[8,22] SPR measurements have also revealed an important role of the *N*-acetyl group in the heterotopic-carbohydrate interaction between GM3 (α -NeupAc-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow Cer) and Gg3 (β -D-GalpNAc-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow Cer)).^[23]

Although the role of Ca^{2+} ions in homotopic-carbohydrate interactions is not yet understood at the molecular level, it can be inferred from our studies that Ca^{2+} ions have an essential role in the approach and organization of the sugar moieties of

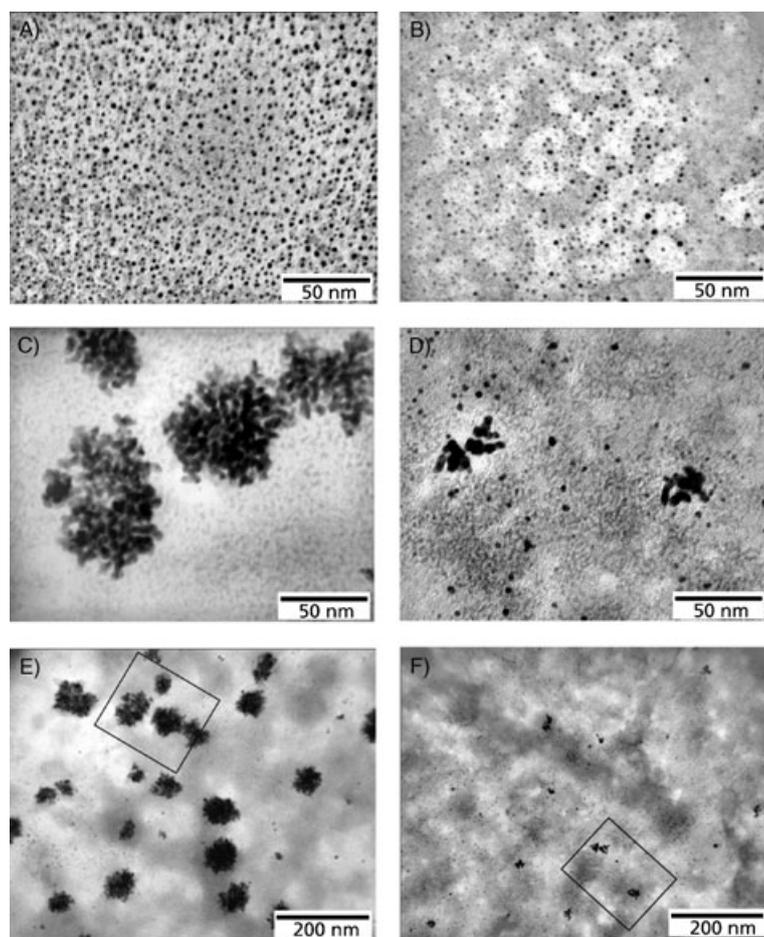


Figure 1. TEM images of Au-1a and Au-1b (0.1 mg mL^{-1}) under different incubation conditions. A) Au-1a in water, B) Au-1b in water, C) 16 h incubation of Au-1a in 10 mM CaCl_2 , D) 16 h incubation of Au-1b in 10 mM CaCl_2 , E) 16 h incubation of Au-1a in 10 mM CaCl_2 , frame corresponds to Figure 2C, F) 16 h incubation of Au-1b in 10 mM CaCl_2 , frame corresponds to Figure 2D.

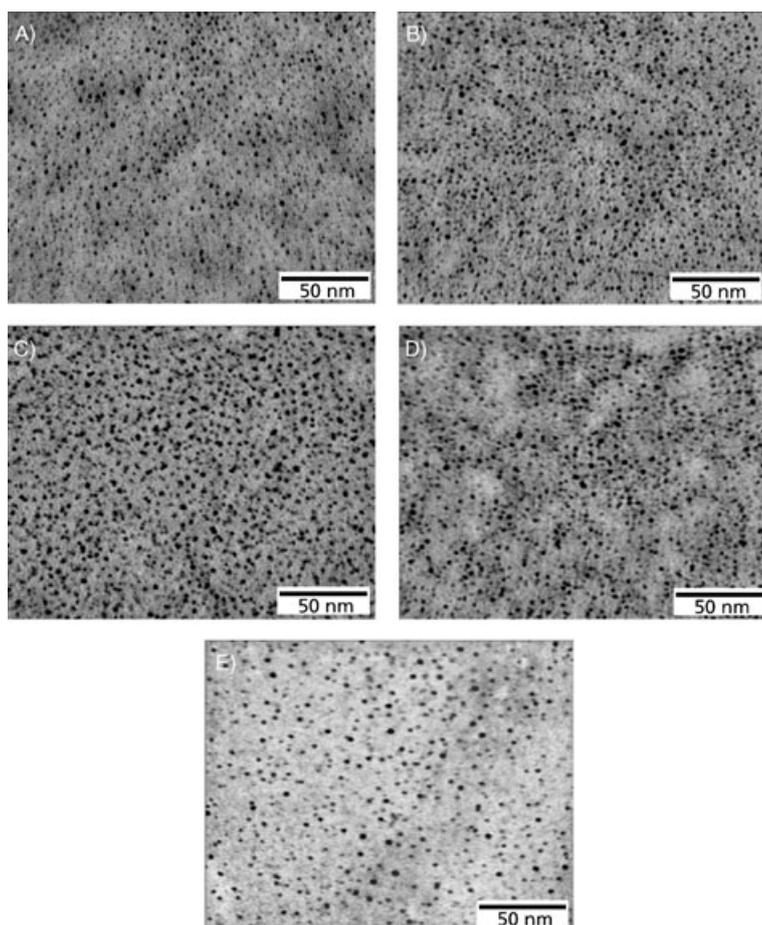


Figure 2. TEM images of Au-2 to Au-6 (0.1 mg mL^{-1}) in 10 mM CaCl_2 . A) Au-2, B) Au-3, C) Au-4, D) Au-5, E) Au-6.

the g-200 sulfated disaccharide. After coordination with Ca^{2+} , the sulfated disaccharide reaches an adequate conformation, wherein other interactions, such as hydrophobic contacts, can stabilize the whole complex. This proposed mechanism would explain the observed specificity for Ca^{2+} ions and the essential role of the hydrophobic methyl group in the L-fucose residue of the disaccharide epitope. This reasoning is in agreement with suggestions in the literature on the role of Ca^{2+} ions.^[22,24–27] Moreover, studies on the self-recognition of the Lewis X trisaccharide have shown that hydrophobic contacts are needed to stabilize the neighboring polar Ca^{2+} -coordinating site in this trisaccharide.^[22] The influence of the anomeric configuration of the L-fucose moiety can be rationalized by the possibility that, in contrast to the α -form, the β -form of the sulfated disaccharide would not generate enough/appropriate hydrophobic contacts to stabilize large aggregates.

It is proposed that carbohydrate–carbohydrate interactions, such as those taking place during sponge-cell adhesion, also occur in the cell-recognition and adhesion machinery of more complex, multicellular organisms.^[28–30] Therefore, it is imperative to further investigate which elements of carbohydrate structures are essential for the discrimination between self and nonself. Currently, additional interaction assays with atomic

force microscopy and crystal-structure determination are being performed with synthetic carbohydrates related to the g-200 disaccharide epitope.

Experimental Section

The preparation of the gold glyconanoparticles Au-1 a/b to Au-6 (Scheme 1) has been reported earlier.^[17] The mean diameter and the carbohydrate-weight percentage of the particles are as follows: Au-1 a, 1.82 nm (36%); Au-1 b, 1.71 nm (40%); Au-2, 1.51 nm (23%); Au-3, 1.80 nm (22%); Au-4, 1.80 nm (39%); Au-5, 1.63 nm (41%); Au-6, 1.55 nm (37%).^[17] TEM images were obtained with a Philips Tecnai 12 microscope at 120 kV accelerating voltage. For a typical experiment in water, a single drop ($1 \mu\text{L}$) of an aqueous gold glyconanoparticle solution (0.1 mg mL^{-1}) was deposited onto a carbon-coated copper grid (QUANTIFOIL on 200 square mesh copper grid, hole shape R 2/2), and allowed to dry at room temperature for several hours. Experiments in which pure water was replaced by an aqueous solution of either CaCl_2 (10 mM) or MgCl_2 (10 mM) were performed by following the same procedure. With CaCl_2 (10 mM) as medium, aliquots of glyconanoparticles Au-1 a/b to Au-6 (0.1 mg mL^{-1}) were examined after a 16 h incubation period. Samples showing aggregation (i.e., Au-1 a and Au-1 b, 0.1 mg mL^{-1}) were incubated with CaCl_2 (10 mM) for 1.5 h, 3 h, 16 h, or 7 days, then examined. The sample showing the largest aggregates (Au-1 a) was tested for its specificity for Ca^{2+} . A solution of EDTA (final concentration, 50 mM) in water was added to a sample of Au-1 a (0.1 mg mL^{-1}) in aqueous CaCl_2 (10 mM) and incubated for 16 h, then the mixture was loaded on a 30 kDa Nalgene centrifugal filter and washed with water ($5 \times 15 \text{ mL}$). The residue was dissolved in water (0.1 mg mL^{-1}), and an aliquot ($1 \mu\text{L}$) was examined by TEM. Gold glyconanoparticles Au-1 a (0.1 mg mL^{-1}) in aqueous MgCl_2 (10 mM) were incubated for 16 h, and an aliquot ($1 \mu\text{L}$) was placed onto a carbon-coated copper grid and monitored.

Keywords: aggregation · carbohydrates · cell recognition · gold · marine sponges · nanoparticles

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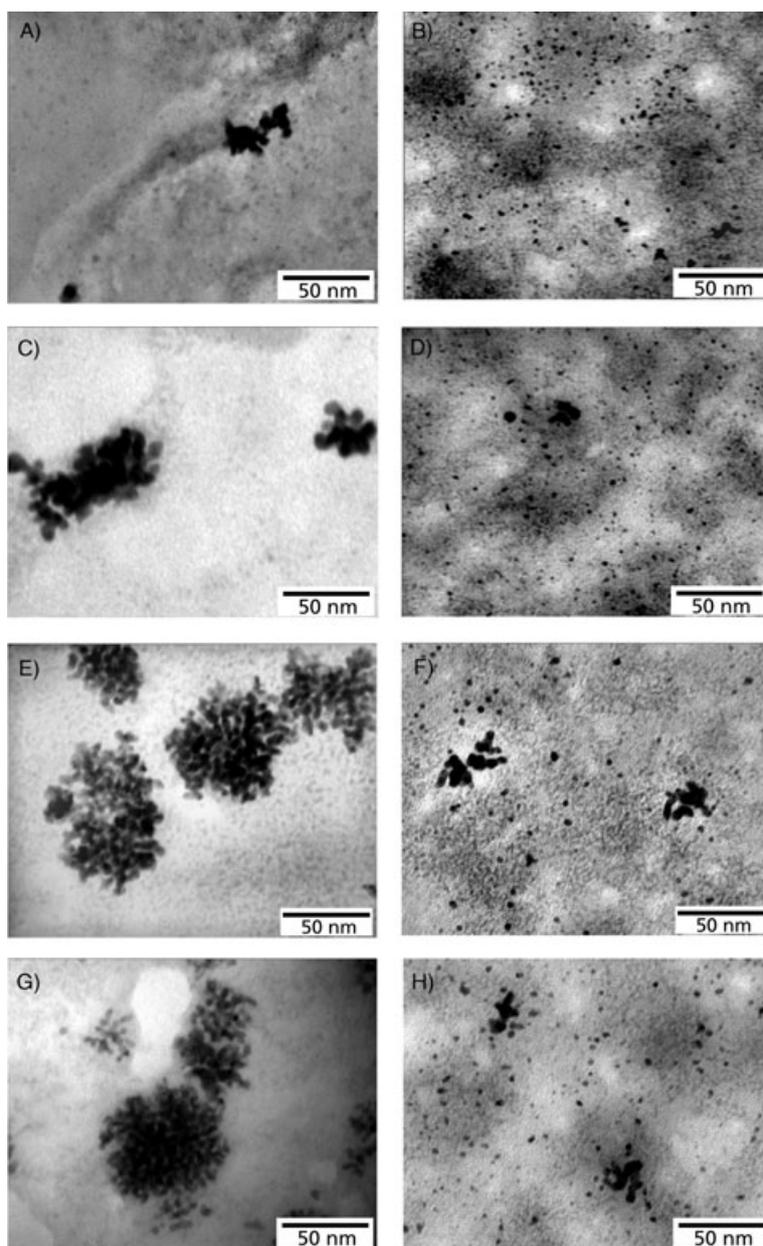


Figure 3. TEM images of Au-1 **a** and Au-1 **b** in 10 mM CaCl₂ after different incubation times. A) Au-1 **a** for 1.5 h, B) Au-1 **b** for 1.5 h, C) Au-1 **a** for 3 h, D) Au-1 **b** for 3 h, E) Au-1 **a** for 16 h, F) Au-1 **b** for 16 h, G) Au-1 **a** for 7 days, H) Au-1 **b** for 7 days.

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