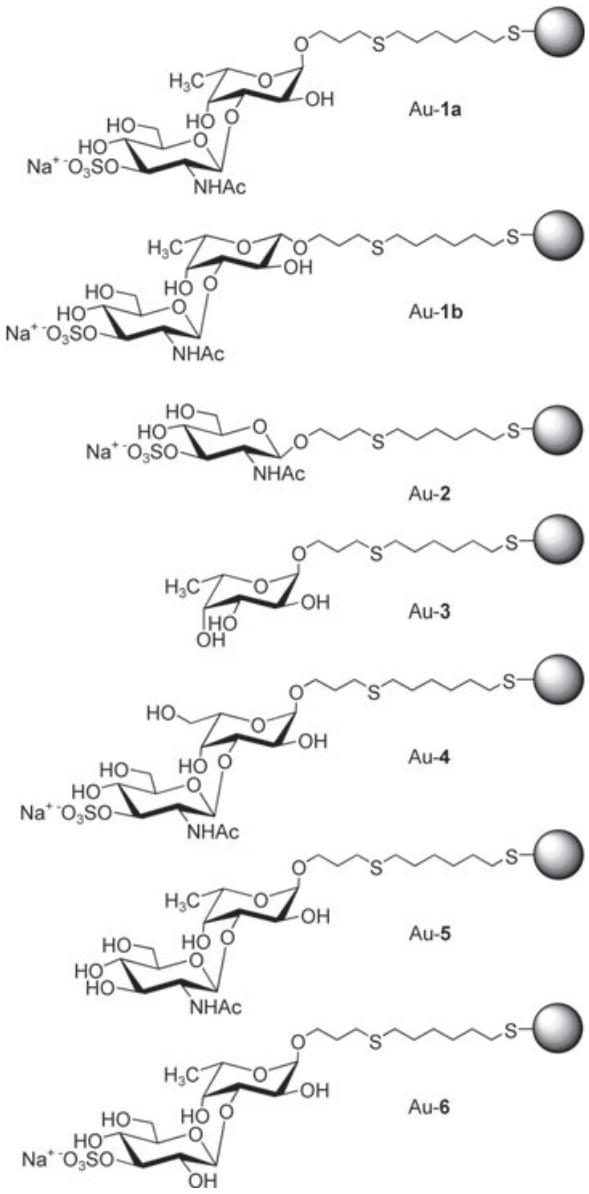
Gold Glyconanoparticles as Probes to Explore the Carbohydrate-Mediated Self-Recognition of Marine Sponge Cells

Adriana Carvalho de Souza,[a] Koen M. Halkes,[a] Johannes D. Meeldijk,[b] Arie J. Verkleij,[b] Johannes F. G. Vliegenthart,[a] and Johannis P. Kamerling*[a]

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 Glc*p*NAc3*S*(β1–3)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-**1a/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 glyconanoparticles Au-**2** and Au-**3**. The three gold glyconanoparticle systems Au-**4** (α-L-Fuc*p* replaced by α-L-Gal*p*), Au-**5** (β-D-Glc*p*NAc3*S* replaced by β-D-Glc*p*NAc), and Au-**6** (β-D-Glc*p*NAc3*S* replaced by β-D-Glc*p*3*S*) were used to evaluate the relevance of the modified sites in the self-recognition process.

[a] *Dr. A. Carvalho de Souza, Dr. K. M. Halkes, Prof. Dr. J. F. G. Vliegenthart, Prof. Dr. J. P. Kamerling*
Bijvoet Center, Department of Bio-Organic Chemistry
Section of Glycoscience and Biocatalysis, Utrecht University
Padualaan 8, 3584 CH Utrecht (The Netherlands)
Fax: (+31) 30-254-0980
E-mail: j.p.kamerling@chem.uu.nl

[b] *J. D. Meeldijk, Prof. Dr. A. J. Verkleij*
Institute of Biomembranes, Department of Cell Biology
Utrecht University
Padualaan 8, 3584 CH Utrecht (The Netherlands)

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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 diameter of 20 nm throughout the whole grid, while the Au-**1b** nanoparticles had formed only a few small aggregates of less then 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[2]).

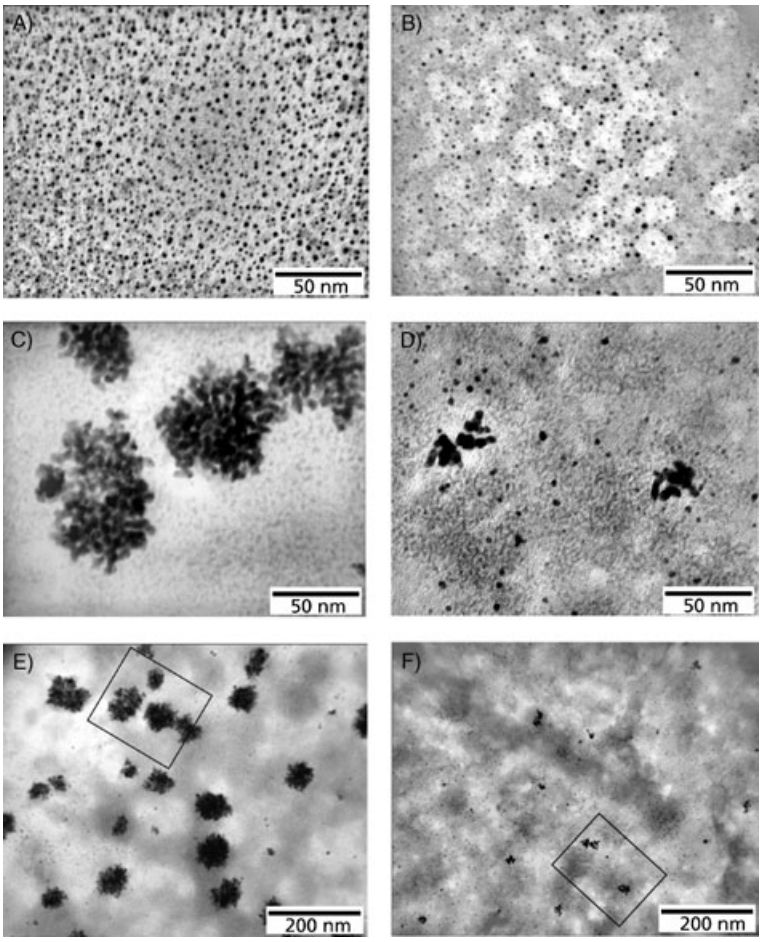

Figure 1. *TEM images of Au-**1a** and Au-**1b** (0.1 mg mL^{-1}) under different incubation conditions. A) Au-**1a** in water, B) Au-**1b** in water, C) 16 h incubation of Au-**1a** in 10 mM $CaCl_2$, D) 16 h incubation of Au-**1b** in 10 mM $CaCl_2$, E) 16 h incubation of Au-**1a** in 10 mM $CaCl_2$,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

The results for nanoparticles Au-**2** and Au-**3** established that the monosaccharide constituents of β-D-Glc*p*NAc3*S*-(1→3)-α-L-Fuc*p*- 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-Neu*p*Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→Cer) and Gg3 (β-D-Gal*p*-NAc-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→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

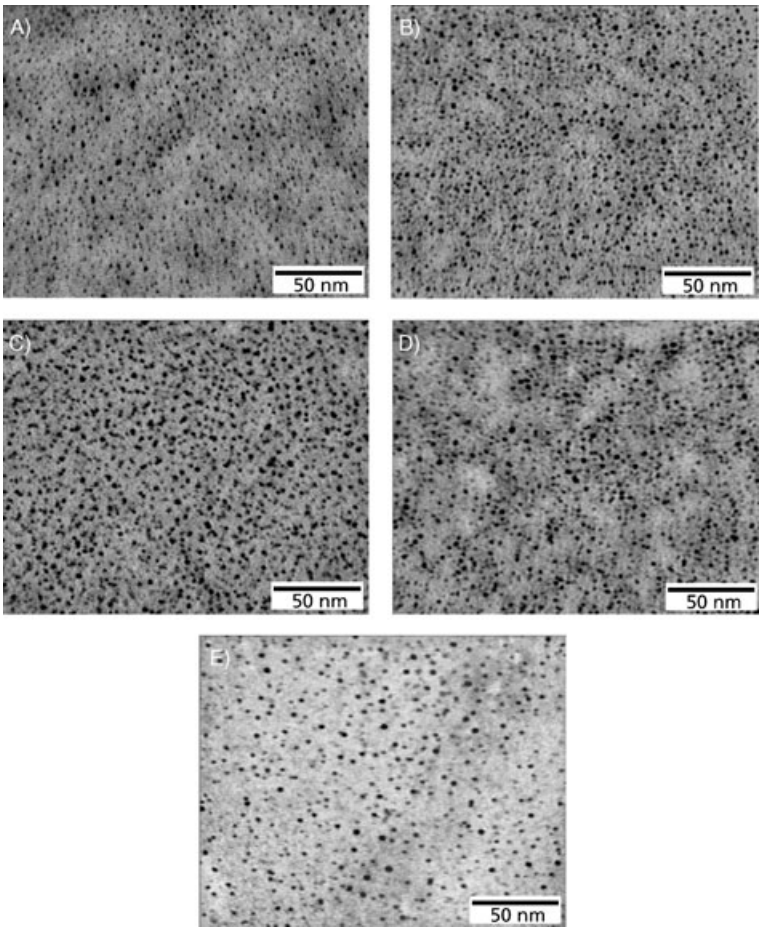

Figure 2. *TEM images of Au-**2** to Au-**6** (0.1 mg mL^{-1}) in 10 mM $CaCl_2$. A) Au-**2**, B) Au-**3**, C) Au-**4**, D) Au-**5**, E) Au-**6**.*

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-**1a**/**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-**1a**, 1.82 nm (36%); Au-**1b**, 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 μL) 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-**1a**/**b** to Au-**6** (0.1 mg mL^{-1}) were examined after a 16 h incubation period. Samples showing aggregation (i.e., Au-**1a** and Au-**1b**, 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-**1a**) 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-**1a** (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 (5×15 mL). The residue was dissolved in water (0.1 mg mL^{-1}), and an aliquot (1 μL) was examined by TEM. Gold glyconanoparticles Au-**1a** (0.1 mg mL^{-1}) in aqueous $MgCl_2$ (10 mM) were incubated for 16 h, and an aliquot (1 μL) was placed onto a carbon-coated copper grid and monitored.

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

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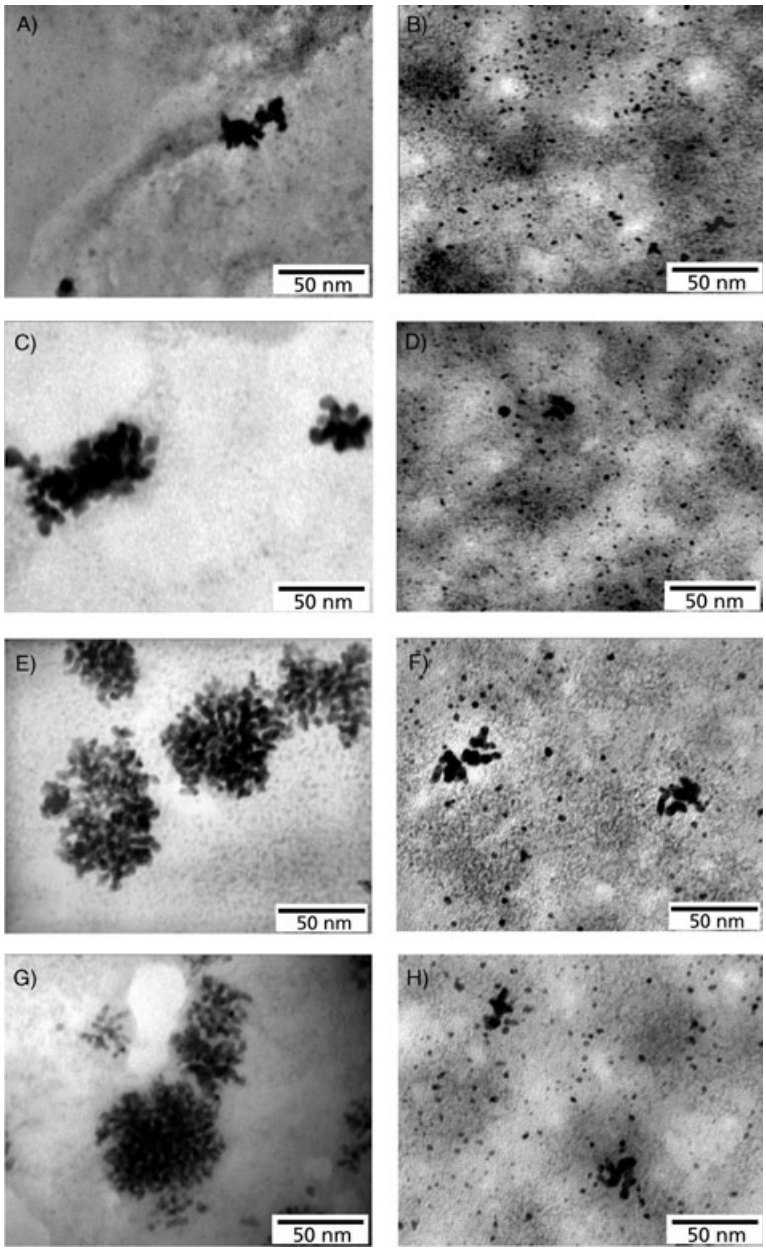

Figure 3. *TEM images of Au-**1a** and Au-**1b** in 10 mM $CaCl_2$ after different incubation times. A) Au-**1a** for 1.5 h, B) Au-**1b** for 1.5 h, C) Au-**1a** for 3 h, D) Au-**1b** for 3 h, E) Au-**1a** for 16 h, F) Au-**1b** for 16 h, G) Au-**1a** for 7 days, H) Au-**1b** for 7 days.*

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