Evolution of multicellularity in Porifera via self-assembly of glyconectin carbohydrates

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This work is dedicated to the memory of Maurice Demarty

Abstract: Research done in the last century on Porifera has provided insights into the molecular mechanism of the biological processes of cell adhesion, innate immunity, and self-recognition. Evidence that this mechanism is based on glyconectin self-assembly is shown by the structure to function relationships deduced from studies of carbohydrates isolated from three different sponge species. The structural studies were performed on purified glyconectin carbohydrates from *Microciona prolifera*, *Halichondria panicea* and *Cliona celata* using nuclear magnetic resonance and mass spectrometry. Seventeen novel, species-specific carbohydrate sequences were revealed that belong to the Porifera glyconectin family. The functional, cell recognition analyses of carbohydrate self-association were performed by measuring binding forces between individual glycan molecules under physiological conditions; the results show that the association strength between homotypic pairs of glycans (400 pN) are higher than those between heterotypic pairs (20 pN). This difference is sufficient to explain the species-specific separation of glycan-coated beads *in vitro* and the sorting of cells in nature. We propose that the glyconectin carbohydrates, which are the constituents on the cell surface that are the most exposed to the environment, were responsible for the molecular recognition processes that underpinned the emergence of multi-cellularity.

Keywords: evolution of multicellularity, Porifera, cell recognition and adhesion, glyconectin carbohydrates and proteoglycans, atomic force microscopy

An approach to study the evolution of multicellularity

Why?

Changing patterns of matter and energy achieved in multicellular life their most sophisticated form so far known. During biological evolution, two essential steps occurred: the first was the emergence of cells and the second subsequent one was the development of multicellular assemblies. In an effort to understand the emergence and evolution of the complex and versatile multicellular life forms present today on earth, as well as to satisfy our curiosity of whether other kinds of similarly hierarchically organized life could exist in the universe, whether it be in the past, present or future, two common, related questions may be formulated in the following way: what were the molecular mechanisms that enabled the creation and persistence of multicellular (or multi unit) life? And, can the similar or alternative mechanisms be predictable in both time and space? In order to devise experiments that could generate data to provide, in part, the answer to these questions, we begin with the following logical statement: the evolution of multicellular forms of life required the emergence of cellular self-non-self discrimination and adhesion, where the sensor molecules guiding such recognition and adhesion should be present at the outermost cell surface (although many processes based on novel as yet imaginary physico-

chemical principles might be invoked in extraterrestrial life forms). By gaining recognition and adhesion properties, the primordial multicellular organisms could preserve functional and morphological identity throughout their life cycles.

How?

In our experimental study of the molecular bases guiding the evolution of multicellularity, it was imperative to select the most appropriate model systems and organisms. We have chosen xenogeneic cell recognition and adhesion of several sponge species from the phylum Porifera because they represent today the simplest multicellular life forms, closest in terms of evolution to primordial multicellular life. In consequence, the molecular mechanism of self-non-self discrimination and adhesion in sponges should be most similar to the mechanism that operated during the emergence of multicellularity. At the beginning of the last century, fundamental phenomenological experiments showed that the xenogeneic re-aggregation of dissociated sponge cells is promoted by extracts made from the surfaces of their cells (Wilson 1907, Galstoff 1925, Curtis 1962, Moscona 1968). Numerous repetitions and variations of these experiments and characterization of aggregation-promoting extracts, called at the time aggregation factors, have been done (Humphreys 1963, Cauldwell *et al.* 1973, Muller and Zahn 1973, Jumblatt *et al.* 1980). More sophisticated purification and characterization of aggregation-promoting extracts have shown that they contain a new class of large cell surface proteoglycan-like molecules, heavily covered by long glycan chains, named by G.N. Misevic glyconectins (GNs, derived from glyco connecting, connecting cells via glycans; Papakonstantinou and Misevic 1993, Dammer *et al.* 1995, Misevic and Popescu 1995, Guerardel *et al.* 2004, Misevic *et al.* 2004). Large GN glycans are the outermost macromolecules on the cell surface and display an enormous variability despite their similar structures (Fig. 1). These glycans should therefore be considered as the primary candidates for sensing the environment and performing the self/non-self recognition and adhesion essential for the evolution of the multicellularity. In the first part of this report we describe the key experiments performed to identify, isolate and sequence functional glyconectin glycan molecules. In the second part we explain our novel experimental approach and show quantitative measurements of recognition and adhesion phenomenon on the molecular and cellular level. Finally, in the last part we present a few thoughts and facts about the value of the Porifera model system to basic research.

Emergence of complexity

The emergence of more complex multicellular organisms was based on the appearance of higher degrees of complexity and the multistep nature of cell recognition and adhesion systems. These can be related to 1) allogeneic self-non-self discrimination in the divergence of species and 2) syngeneic organ and tissue specificity during morphogenesis.

Structural analyses of glyconectin glycan self-non-self recognition molecules

Structural analyses of glyconectins (GNs) isolated from three sponge species *Microciona prolifera* (GN1), *Halichondria panicea* (GN2) and *Cliona celata* (GN3), used as a experimental model system to study evolution of the multicellularity, will be reviewed here. General approach, depicted in Fig. 1, consists of four steps ranging from the release of glycans of their protein core towards glycan fragmentation, fingerprinting and sequencing using nuclear magnetic resonance (NMR) and mass spectrometry (MS). In the first step protein free polysaccharide chains from purified GNs were prepared by extensive pronase digestion of the protein part (Misevic *et al.* 1982, Misevic and Burger 1993). This was followed by separation of intact glycans from free amino acids by gel filtration and ion-exchange chromatography. In the second step glycans were separated and isolated by column chromatography and/or gel electrophoresis (Fig 1). GN1 was found to have two major glycans with molar masses 200×10^3 and 6×10^3, as previously reported (Misevic *et al.* 1982, Misevic and Burger 1986, 1990a, 1990b, 1993, Spillmann *et al.* 1993, Spillmann *et al.* 1995), GN2 had one major glycan with molar mass of 180×10^3 kD, representing more then 60% of the total carbohydrate content, and GN3 contained also one major glycan species (50% of total carbohydrates) with molar mass of 110×10^3 kD (Fig. 1). The third step in our analyses was chemical and enzymatic fragmentation of GN glycans. The results obtained revealed that each species has its own fingerprint signature. The last step was sequencing of each GN glycan fragment by combination of two dimensional COSY90 high resolution NMR and three types of MS: EI-MS - Electronic Impact Mass Spectrometry, CI-MS - Chemical Ionization Mass Spectrometry and MALDI-TOF MS - Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (Fig 1). Complex analyzes of NMR and MS fingerprinting data revealed that four large glycans g200 and g6, g180, and g110 of GN1, GN2 and GN3 respectively, are built by novel repetitive units (Misevic *et al.* 1982, 1987, Misevic 1989, Spillmann *et al.* 1993, 1995, Misevic and Burger 1986, 1990a, 1990b, 1993, Popescu and Misevic 1997) (Fig. 2). As shown in Table 1 four short sulfated and one pyruvilated unit in GN1, eight larger and branched pyruvilated oligosaccharides in GN2 which represent heterogeneous but related family of structures, and four sulfated units in GN3 were sequenced (Guerardel *et al.* 2004, Misevic *et al.* 2004).

We propose two possible models of organization for GN1 and GN3 carbohydrate moieties within the glycan chain (Fig. 2A and B). The first model represents a high molecular weight, linear, acid sensitive polysaccharide connected to an acid resistant domain. This polysaccharide may be composed of either heterogeneous short repetitive units or a large homogeneous repetitive unit comprising acidic labile glycosidic bonds (Fuc/Ara). The actual size of a homogeneous repetitive unit is difficult to assess since most Fuc- and Ara-glycosidic linkages are cleaved in mild acidic conditions. The second model represents a mixed ramified polysaccharide composed of an acid resistant core connected through Fuc/

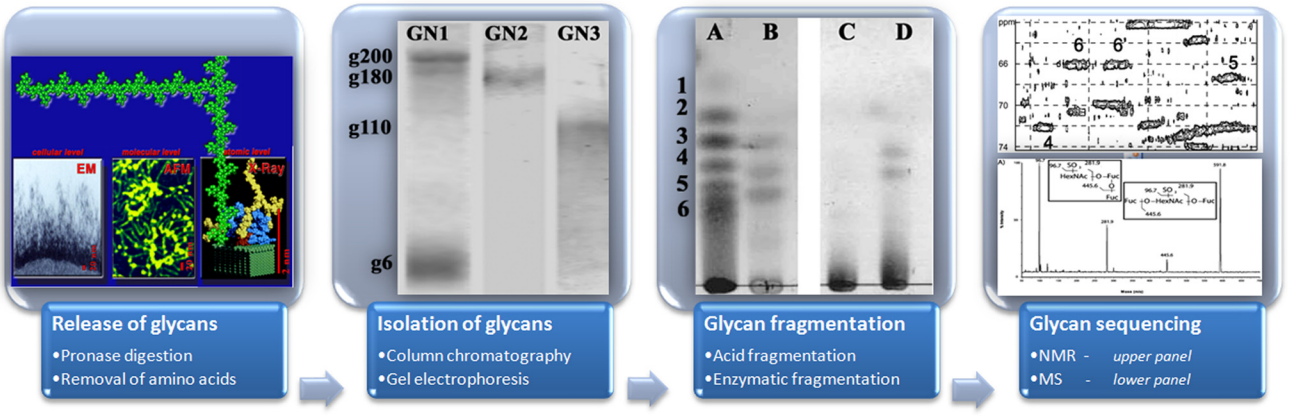

Fig. 1: The fist panel shows EM, AFM and X-ray images of glycans dimensions at cellular, molecular and atomic level. EM; the Electron Microscope image of cells stained for acidic polysaccharides. These glycans are the most peripheral molecules (over 200 nm) from the cell surface with very high density and abundance. AFM; Atomic Force microscope image of GN1 with g200 glycan arms of 180 nm. X-ray; model of protein on plasma membrane in blue with small glycans in yellow and large glycan in green which is an order of magnitude longer then presented if the real length of g200 glycan would be taken in account. In the second panel as the example of the second step of structural analyses, a polyacrylamide gel electrophoresis of purified glyconectin glycan fraction is presented. Electrophoresis of glyconectin glycans was performed on a polyacrylamide gradient gel (7.5-15%). Gels were stained with 0.3% alcian blue in 3% acetic acid in aqueous 25% isopropanol. Lane a, 20 µg of GN1 glycans; lane b, 20 µg of GN2 glycans; lane c, 20 µg of GN3 glycans. The third panel shows the third step of structural analyses of glycans by fingerprinting with trifluoroacetic acid hydrolyses. TLC analysis of hydrolyzed fractions of GN1 and GN2 stained by sulfuric orcinol. Lane 1, standard Glc degrees of polymerization (DP); lane 2, 0.1 M trifluoroacetic acid hydrolysis of GN1; lane 3, 0.1 M trifluoroacetic acid hydrolysis of GN2; lane 4, 1 M hydrolysis of GN2. The forth step in structural analyses using NMR and MS sequencing are shown in the forth panel. Complex sequencing procedure in combined NMR and MS complementary approach requires sophisticated instrumentation and high skills.

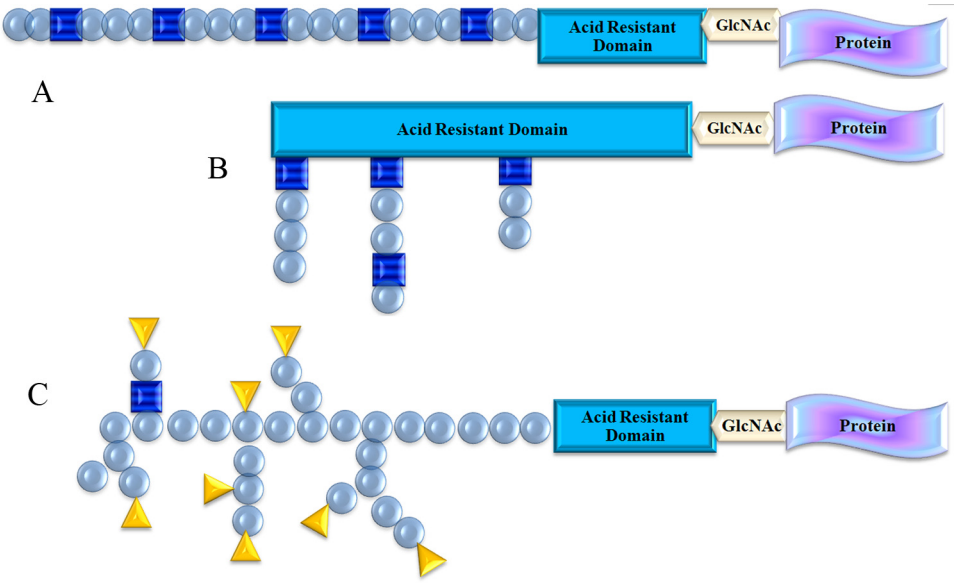

Fig. 2: A and **B.** Two putative models of ultrastructural organization of GN1 and GN3 glycans with linear and/or ramified repetitive units (blue circles symbolize Hexose and/or GlcNAc, blue striped squares Fucose). **C.** Model of GN2 highly ramified repetitive glycan structure (blue circle symbolize Hexose and/or GlcNAc, yellow triangles Py(4,6)Gal), blue striped squares Fucose.

Ara residues to small oligosaccharides that are released by mild acidic hydrolysis.

In contrast with GN1 and GN3, mild hydrolysis of GN2 released large oligosaccharides that were further fragmented in smaller units using stronger acidic conditions. Analysis of both fractions revealed that the acid labile carbohydrate moiety of GN2 comprised a highly ramified polysaccharide backbone. It is constituted by an extremely heterogeneous mixture of hexose (mannose and galactose) oligomeres all terminated by Py(4,6)Gal residues and randomly interrupted

Table 1: Glycan structures obtained by NMR and MS after mild hydrolyses of isolated GN1, GN2 and GN3 polysaccharides**.**

GN1	GN2	GN3
GlcNAc-Fuc-(SO_3)GlcNAc-Fuc	Py(4,6)Gal-$(Hex)_{0-1}$-Fuc	HexNAc-(SO_3)Ara/Fuc-Fuc
GlcNAc-(SO_3)Gal-Fuc	Py(4,6)Gal-$(Hex)_{0-3}$-GlcNAc	Hex-HexNAc-(SO_3)Ara/Fuc-HexNAc-Fuc
Gal-(SO_3)Gal-GlcNAc-Fuc	Py(4,6)Gal-$(Hex)_{1-5}$	
Py(4,6)Gal-GlcNAc-Fuc	Py(4,6)Gal-$(Hex)_{0-2}$[Hex]Hex	
(SO_3)GlcNAc-[Fuc]Fuc	Py(4,6)Gal-[Hex]Hex-Hex	
	Py(4,6)Gal-Hex-[Py(4,6)Gal]Hex	
	$(Hex)_4$-[Py(4,6)Gal]Hex	
	PyGal-Hex-[Hex]HexNAc	

by Fuc and GlcNAc residues. The observed heterogeneity of released oligosaccharides did not permit definitive conclusions about the ultrastructural organization of repetitive motifs.

In conclusion, structural analyses of GN1, GN 2 and GN3 isolated from three different sponge species revealed that their carbohydrate content ranges between 40–60% of their total mass thus characterized them as a heavily glycosilated macromolecules. The physico-chemical properties of each of four major GN glycan (GM1 g200 and g6, GN2 g189 and GN3 g110) such as size, composition, high content of anionic groups (carboxyl and/or pyruvate and/or sulfate), resistance to most glycosaminoglycan degrading enzymes, monoclonal antibodies mapping and their highly repetitive new type of sequences characterized them as novel class of acidic glyconectin type of glycans. Using the above interdisciplinary approach and technologies we have found that also higher invertebrates like sea urchins as well as vertebrates like mammals (rodents and humans) have similar type of glyconectin glycan structures (Papakonstantinou and Misevic 1993, Misevic and Popescu 1995). Therefore, glyconectin carbohydrates can be considered as a new family of species-specific glycans containing different classes of molecules present in Metazoans.

Functional measurements of glyconectin glycans self-non-self recognition properties by Atomic Force Microscopy and color coded cell-bead adhesion

For complete understanding of molecular mechanisms guiding evolution of multicellularity, it is necessary to complement structural studies on glyconectin glycans with quantitative functional measurements of cell adhesion and recognition. Such structure to function relationship in Porifera experimental model system was established by taking two complementary approaches. The first one was Atomic Force Microscopy (AFM) measurements of intermolecular binding strength between individual glyconectin glycans under physiological conditions (Dammer *et al.* 1995). The second one was quantification of color coded cell-cell, bead-bead and bead-cell recognition and adhesion mediated by glyconectin glycans (Popescu and Misevic 1997, Misevic *et al.* 2004).

AFM measurements of intermolecular binding strength

Intermolecular binding forces between cell surface molecules are keeping cells together in multicellular organisms. To provide direct and quantitative evidence that glyconectin carbohydrates can indeed support cell adhesion, in 1993 we have developed a novel technology based on AFM measurements of binding strength between glyconectin carbohydrates under the physiological conditions (Dammer *et al.* 1995). Interactions between individual adhesion molecules (immunoglobulin, selectin, cadherin, integrins and extracellular matrix adhesions) were usually investigated by kinetic binding studies, calorimetric methods, x-ray diffraction, nuclear magnetic resonance and other spectroscopic analyses. These methods do not provide direct measurement of the intermolecular binding forces, which are fundamental for ligan-receptor association related to cell adhesion and recognition. To measure glyconectin to glyconectin interaction forces, we covalently attached glyconectins via their protein part to an AFM sensor tip and a flat mica surface (Fig. 3). The attachment process involved only glyconectin proteins but did not modify functional carbohydrate adhesion sites. As shown in the schematic presentation in Fig. 3 the cantilever tip having attached glyconectin molecules was carefully moved toward the substrate surface and a series of approach-and-retract cycles were collected in physiological liquid medium. GN-GN binding was characterized by measuring both the force necessary to separate the GN-functionalized sensor tip from the GN surface (final jump-off) and the percentage of interaction events under different ionic conditions (Dammer *et al.* 1995). These two indicators of GN activity varied reversibly with the Ca^{2+} concentration, in agreement with GN-promoted cell adhesion and GN-coated bead aggregation (shown in the following section of functional analyses). At a Ca^{2+} concentration of 10 mM, the average force between GNs was 125 pN, ranging up to 400 pN, with high probability of binding (60 ± 10%). At a Ca^{2+} concentration of 2 mM, cell adhesion and GN bead aggregation were sharply reduced, and the force (40 ± 15 pN) and probability of binding (12 ± 5%) were also reduced (Dammer *et al.* 1995). The interaction between GNs is Ca^{2+}-selective, as reported with a cell aggregation assay. Indeed, 10 mM Mg^{2+} could not replace Ca^{2+} in AFM experiments or in adhesion of glyconectin-coated beads (Dammer *et al.* 1995).

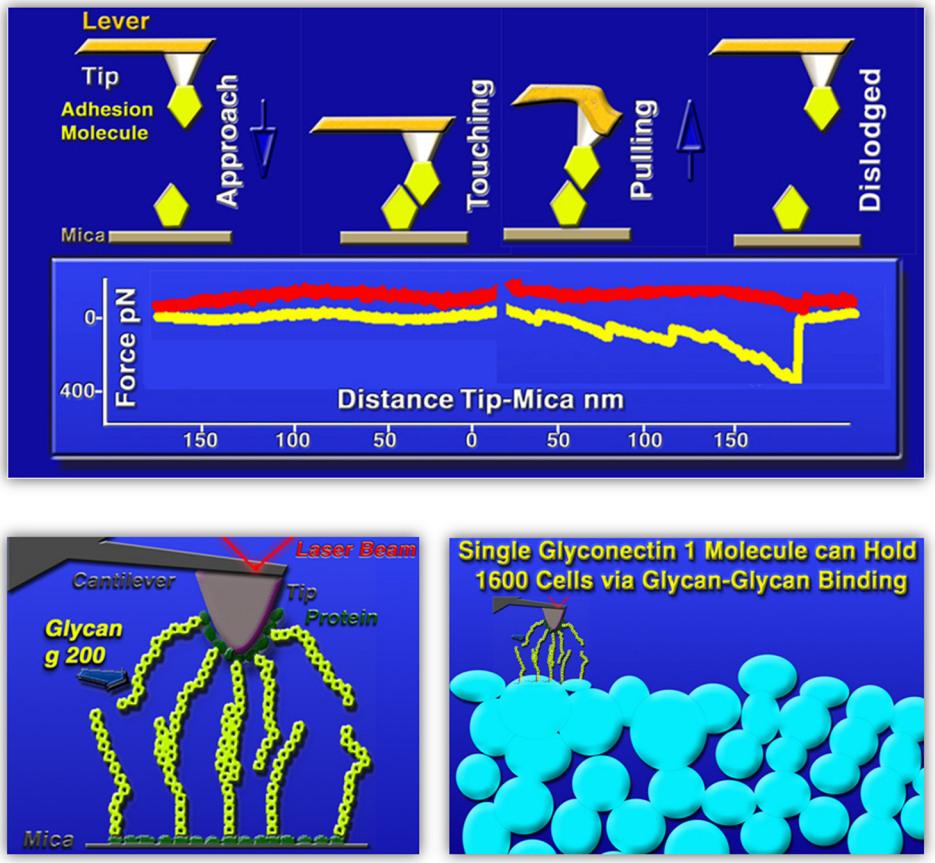

Fig. 3: Schematic presentation of AFM measurements of intermolecular binding strength between glyconectin 1 carbohydrates.

Use of the monoclonal antibody (mAb) block 2 provided a third line of evidence that the AFM-measured interactions originate from binding between glyconectin glycans. This antibody recognizes a carbohydrate epitope of GN1 (see Table 1) and specifically inhibits GN1-promoted cell adhesion and GN1-coated bead aggregation (Dammer *et al.* 1995). In AFM experiments, block 2 Fab fragments in 10 mM Ca^{2+} SWT (Sea Water buffered with 20 mM Tris pH 7.4) reduced the interactive force to approximately the level measured at 2 mM Ca^{2+}. A control mAb did not prevent GN1-GN1 binding under equivalent conditions. Thus, during AFM measurements in all tested experimental conditions, GN1-GN1 interactions resemble cell-cell adhesion events observed *in vivo*.

The shape of the approach-and-retract cycles shows that string-like structures were responsible for GN1-GN1 interactions. These strings are likely to be the GN1 arms composed of glycans with a relative molecular weight of 200 x 10^3 (g200), which have been shown to mediate polyvalent GN1-GN1 binding (Fig. 3). This possibility is further supported by the fact that the length (180 nm) and the number (20 copies) of the g200 glycan per GN1 molecule are similar to the length and number of GN1 arms as measured by AFM and electron microscopy (Dammer *et al.* 1995). Finally, the inhibitory mAb block 2 is directed against a self-association epitope located on the g200 glycan. The shape of the approach-and-retract curves between glyconectins suggested the presence of long-range interactions, interpreted as the lifting and extension of string-like glyconectin glycans, followed by further stretching until the elastic force of the cantilever equals the strength of the binding and the lever "jumps off". At a physiological Ca^{2+} concentration of 10 mM in seawater multiple jump-offs were frequently observed, indicating polyvalent binding with an average adhesive force of 40 ± 15 pN per release (Dammer *et al.* 1995).

AFM measurements of intermolecular binding between homotypic pairs of GN2 and GN3 showed that intermolecular binding forces per pair of molecules are, as in GN1, in the range of 400 pN. Heterotypic combination of GNs revealed intermolecular binding strength of 20 pN (detailed results to be published). Similar results were obtained with purified GN glycans confirming the results of intact GNs where only carbohydrate moieties were available for interaction while the protein part was used for immobilization to surfaces (see Fig. 3). Therefore, carbohydrate to carbohydrate interaction is responsible for GN-GN self-non-self recognition and adhesion. In conclusion, measurement of binding forces intrinsic to adhesion molecules is necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. Our atomic force microscopy results of the binding strength between cell adhesion glyconectin glycans under physiological conditions showed that homotypic adhesive force of 400 piconewtons per molecular

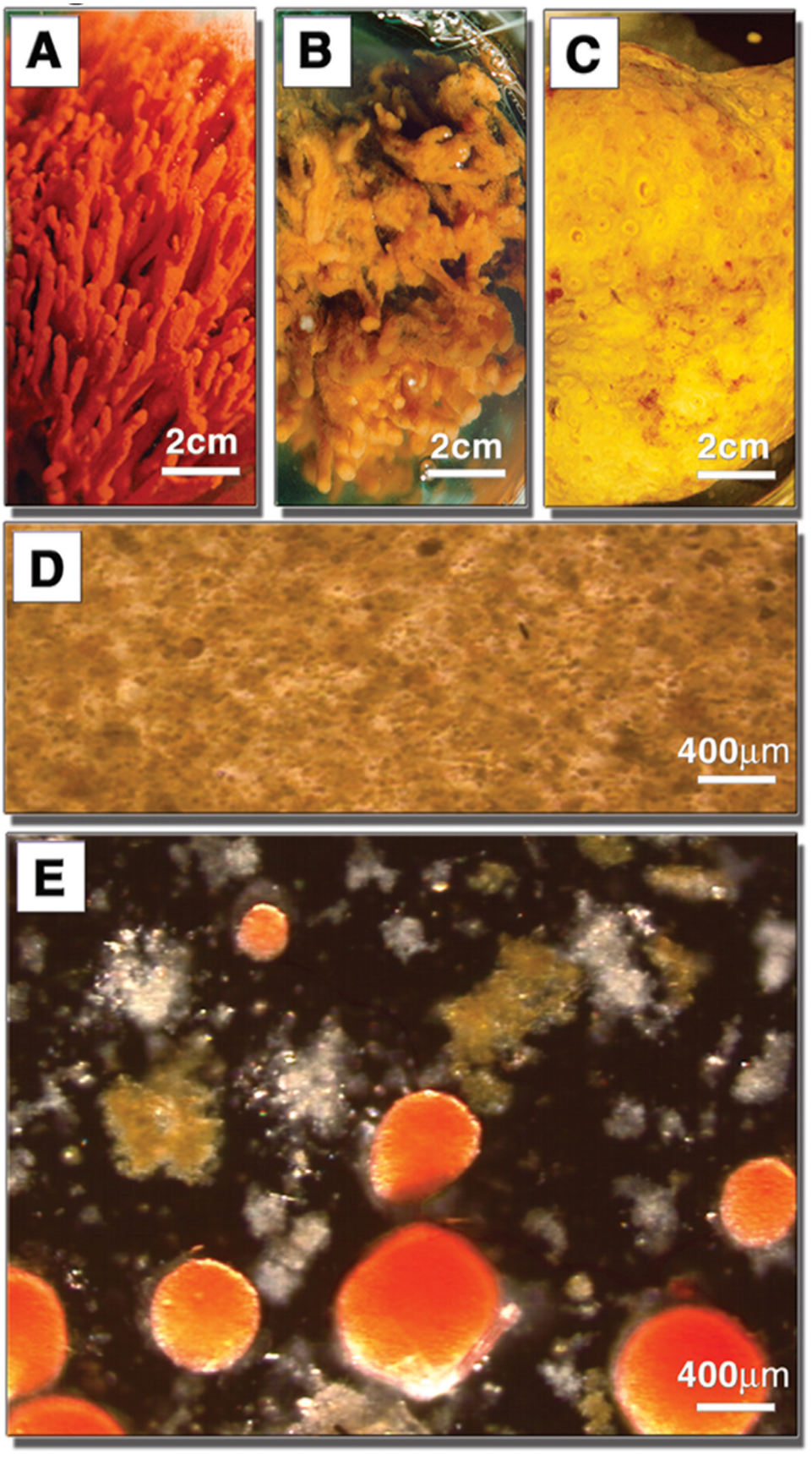

Fig. 4: Glyconectin glycoconjugates are cell adhesion and recognition molecules. Ca^{2+}-dependent glyconectin to glyconectin interactions mediate species-specific cell-cell recognition and adhesion. **A.** *M. prolifera*, **B.** *H. panicea*, and **C**. *C. celata* living sponges. Shown are self- and non-self-discrimination and adhesion in the suspension of mixed *M. prolifera* (orange), *H. panicea* (white), and *C. celata* (brown) live cells bearing glyconectins. **D** and **E**) ACMFSW (Artificial Calcium and Magnesium Free Sea Water) without 10 mM Ca^{2+} (D) and ACMFSW with 10 mM Ca^{2+} at 0°C after 20 min of rotation (**E**). The microscopically observed color of the cells is somewhat different from that of the whole sponge. Early cell sorting experiments were usually done with binary sponge combinations at room temperature without rotation. The sorting is thus dependent on the presence of recognition molecules at the cell surface, cell motility, and speed of new synthesis and/or secretion of additional recognition molecules. Our rotary assays using either metabolically attenuated or fixed cells reduce the number of variable parameters.

pair could hold the weight of 1600 cells assuring the integrity of the multicellular sponge organism (Dammer *et al.* 1995). Interaction forces between heterotypic molecules were 20 times lower and are thus not sufficient to sustain existence of heterotypic aggregates under physiological hydrodynamic conditions of natural sea environment. Furthermore, this data also explain why small and loose unspecific aggregation was sometimes observed during the initial stage of heterotypic mixing under mild agitation.

Glyconectin glycans mediate color coded cell and bead adhesion

The cell adhesive function of three sponge glyconectins purified from *Microciona prolifera* (GN1), *Halichondria panicea* (GN2) and *Cliona celata* (GN3) was tested in a rotary reaggregation assay with live metabolically attenuated and/or fixed cells depleted of endogenous GNs. All three glyconectins, at concentrations mimicking *in vivo* conditions, mediated cell adhesion in the presence of physiological sea water with 10 mM $CaCl_2$, and not below 1 mM $CaCl_2$ (Guerardel *et al*. 2004, Misevic *et al.* 2004). In the absence of GNs, independently of $CaCl_2$ concentration, no aggregation could be observed. Magnesium ions could not replace Ca^2 titration experiments of Ca^{2+} concentration dependence of sponge glyconectin self-interactions revealed a transition at 5mM and 100% interactions at physiological 10 mM $CaCl_2$, identical to that of Ca^{2+} dependent glyconectin promoted cell adhesion (Jumblatt *et al.* 1980, Dammer *et al.* 1995). These experiments indicated that a Ca^{2+} dependent glyconectin to glyconectin interactions play a pivotal role in cell adhesion of the three selected marine sponge species. The specificity of adhesion of GNs bearing cells was tested in a trinary species combination (*Microciona prolifera, Halichondria panicea* and *Cliona celata*) with living dissociated and metabolically attenuated cells in artificial sea water at 0°C in the presence, and absence of 10 mM Ca^{2+} (physiological concentration in seawater). In a rotary assay, species-specific recognition and adhesion occurred only with 10 mM Ca^{2+} within 5-15 min. (Fig. 4). Upon removal of GNs from cell surface by repetitive washing none of the three species displayed aggregation in the presence of 10 mM Ca^{2+}. Adding back the purified GNs to the same live cells at 0°C completely restored species-selective cohesion. Similar results were obtained with non-living fixed cells. These experiments indicated that glyconectins and Ca^{2+} mediate the initial steps of xenogeneic cell recognition and adhesion of the three selected sponge species and were extending previously reported phenomenological and biochemical studies about the role of proteoglycan-like glycoconjugates in binary assays of dissociated sponge cells (Wilson 1907, Galstoff 1925 , Curtis 1962, Humphreys 1963, Moscona 1968, Cauldwell *et al.* 1973 , Müller and Zahn 1973, Jumblatt *et al.* 1980).

In the second type of recognition assay, we reconstituted the observed cell recognition by using artificial system of glyconectin color coated beads. Glyconectin 1 was attached via its protein part to fluorescent pink, glyconectin 2 to fluorescent green, and glyconectin 3 to fluorescent blue latex-amidine beads leaving glycan molecules free for interactions. Unlabeled glyconectins were immobilized on a nitrocellulose membrane in such a manner that the three molecules were used to draw the subsequent letters of the words GLYCONECTIN RECOGNITION. The three bead types were mixed and added to the coated membrane in the presence of 10 mM $CaCl_2$ or absence of calcium ions. As shown in Fig. 5, within 5-15 min of constant rotation species-specific bead-bead aggregation and homophilic recognition between membrane-bound and bead-bound glyconectins were observed through three separate colored aggregates and selective staining of each letter only with 10 mM $CaCl_2$. Both processes occurred at apparently similar rates for each of the three glyconectins. In control experiments with glyconectin 1 separately attached to pink, yellow and white beads, as expected, mixed color aggregates were formed upon addition of 10 mM $CaCl_2$. In the absence of 10 mM $CaCl_2$, bead aggregation did not occur either in the mixture of three glyconectins or of one glyconectin coated to three color beads. *Ex vivo* color coded cell-bead experiments

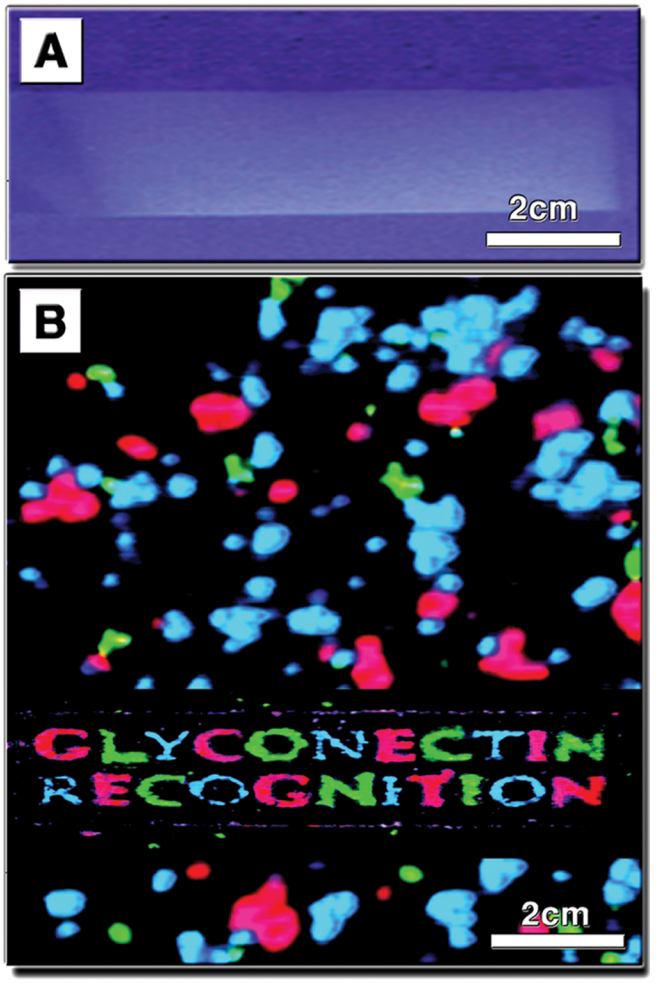

Fig. 5: Simultaneous species-specific glyconectin to glyconectin recognition in suspension and blotting assay. Letters were drawn using 4 µl of 1.5 mg/ml glyconectins on a Hybond-C extra nitrocellulose membrane (Amersham Biosciences) and probed in SWT with pink, green, and blue fluorescent beads coated with glyconectin 1, 2, and 3, respectively. **A.** SWT without 10 mM Ca^{2+}. **B.** SWT with 10 mM Ca^{2+}. All photographs were taken after 30 min of mixing.

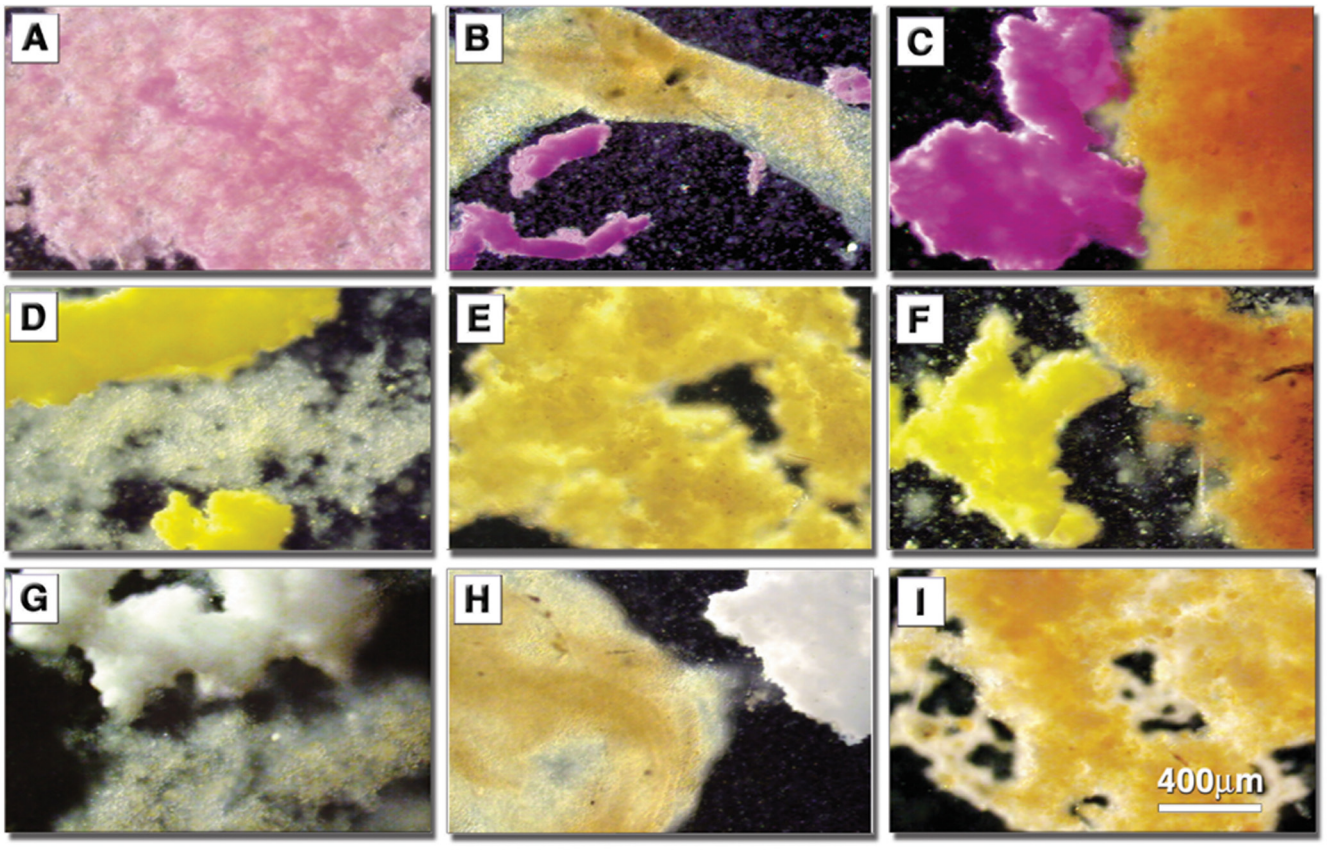

Fig. 6: Species-specific glyconectin to glyconectin interactions mediate bead-cell recognition and adhesion. Xenogeneic glyconectin self-recognition in a mixture of glutaraldehyde-fixed cells and glyconectin-coated beads in SWT in the presence of 10 mM Ca^{2+}. *M. prolifera* cells bearing glyconectin 1 were incubated with: glyconectin 1 (pink beads) (**A**), glyconectin 2 (yellow beads) (**D**), and glyconectin 3 (white beads) (**G**). *H. panicea* cells bearing glyconectin 2 were incubated with: glyconectin 1 (**B**), glyconectin 2 (**E**), and glyconectin 3 (**H**) color-coded beads. *C. celata* cells bearing glyconectin 3 were incubated with: glyconectin 1 (**C**), glyconectin 2 (F), and glyconectin 3 (**I**) color-coded beads (glutaraldehyde fixation changes cell colors, i.e. *M. prolifera*, orange to yellowish white; *H. panicea*, white to yellowish brown; and *C. celata*, brown to brownish orange. We did not observe differences in adhesion properties between fixed and live metabolically attenuated cells in a rotary assay.

showed that artificial beads covered with glyconectin glycans will co-aggregating in the species-specific manner only with homotypic cells having same glyconectin glycans (Fig. 6). Similar types of experiments were also done with purified glycans from all three species. Results obtained confirmed that self-non-self discrimination of GNs is based on selective carbohydrate to carbohydrate self-assembly (Misevic *et al.* 1987, Misevic and Burger 1993) which represents a novel mechanism complementary to well studied protein to protein and protein to carbohydrate interactions of adhesion and recognition molecules.

Color coded bead experiment was also performed by overlaying agarose gel containing electrophoretically separated three glyconectins with color coated glyconectin beads. As shown in Fig. 7, after overnight incubation at room temperature, in the presence of 10 mM $CaCl_2$ under gentle agitation, species specific staining of gel glyconectin bands identical to ones stained with Toluidine blue and Amido black showed that glyconectin to glyconectin interactions are highly species-specific (Guerardel *et al.* 2004, Misevic *et al.* 2004).

The combinations of the above described experiments demonstrate species-specific molecular self-recognition of glyconectins in an elementary reconstituted bead adhesion system which fully resembles glyconectin mediated cell-cell recognition and adhesion. Thus, glyconectin glycans mediate self and non-self discrimination via selective glycan to glycan assembly in the initial step of sponge cell adhesion and xenogeneic recognition.

"Evolution" of the Porifera model system in research

In the second part of the past century, zoology and ecology research on Porifera was highly considered. Unfortunately, the same sponge model system was often neglected in the field of biochemistry and molecular biology. This research was put to the bottom of the list of priory and was classified as risky, marginal and not serious (e.g. comments that "if possible this research should be avoided for the sake of the scientists and institutions involved"). Fortunately, and in

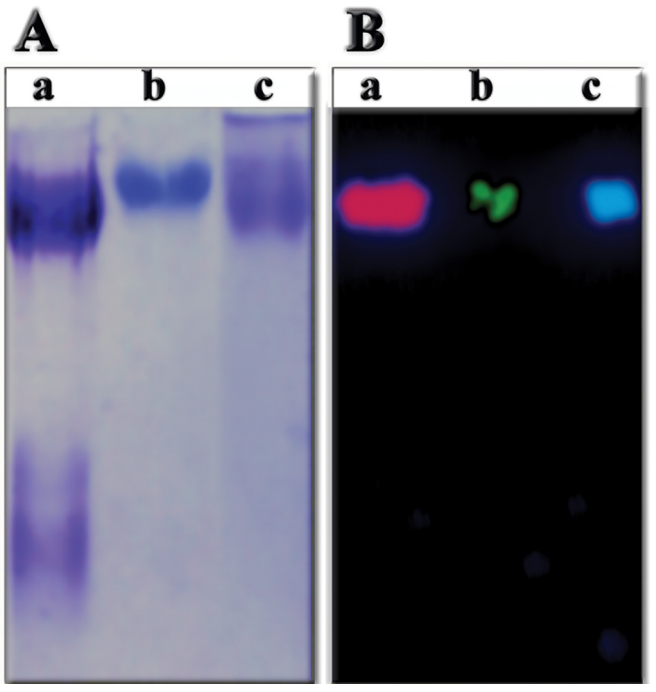

Fig. 7: Electrophoretic separation of sponge glyconectins. **A.** 0.75% agarose gel stained with 0.02% toluidine blue followed by 0.1% Amido Black 10B. a-c, GNs from *M. prolifera* GN1, *H. panicea* GN2, and *C. celata* GN3, respectively (10 µg each). **B.** 0.75% agarose gel stained with color-coded fluorescent beads coated with GN1 (pink) (a), GN2 (green) (b), and GN3 (blue) (c) in the presence of SWT with 10 mM $CaCl_2$.

contrast to the expectations of the official representatives of the scientific community, molecular- and cellular-oriented fundamental research on sponges - exemplified in this article by evolution of multicellularity, as well as by other reports in this book - have generated a vast body of knowledge of new structures, novel molecular mechanisms and new nanotechnologies. Consequently this interdisciplinary research on sponges, which integrates biology, physics, chemistry and mathematics, starts to gain deserved respect as measured by the appearance of publications in journals with high impact factors and the citations of these papers, and the level of attendance at the international conferences in the now clearly established interdisciplinary sponge field. In conclusion we are arguing that any model system is valuable if competent scientists can use it to develop and test original ideas to help solve fundamental scientific questions.

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