REGULAR ARTICLE

Adhesion of freshwater sponge cells mediated by carbohydrate-carbohydrate interactions requires low environmental calcium

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Abstract

Marine ancestors of freshwater sponges had to undergo a series of physiological adaptations to colonize harsh and heterogeneous limnic environments. Besides diminished salinity, river-lake systems also have calcium contents far lower than seawater. Cell adhesion in sponges is mediated by calcium-dependent multivalent self-interactions of sulfated polysaccharides components of membrane-bound proteoglycans named aggregation factors. Cells of marine sponges have already been shown to require seawater average calcium concentration (10 mM) to sustain adhesion promoted by aggregation factors. We demonstrate here that the freshwater sponge *Spongilla alba* can thrive in a calcium-poor aquatic environment and that their cells are able to aggregate and to form primmorphs at calcium concentrations 40-fold lower than that required by cells of marine sponges. We also find that their specialized resistant reproductive bodies named gemmules need calcium and other micronutrients to hatch and generate new sponges. The sulfated polysaccharide composed of glucose and mannose units found in *S. alba* has molecular size notably lower than those present in aggregation factors of marine sponges. Assessments with atomic force microscopy/single-molecule force spectroscopy revealed that the low-molecular-size sulfated polysaccharide from *S. alba* self-interacts more efficiently at low calcium concentrations (1 mM) than that from the marine sponge *Desmapsamma anchorata*. Such an ability to retain multi-cellular morphology with low exogenous calcium contents must have been a crucial evolutionary step for freshwater sponges to successfully colonize inland waters.

Keywords

Porifera; proteoglycans; glycosaminoglycans; carbohydrate interactions; evolutionary adaptation.

Introduction

Sponges (Phylum Porifera) likely emerged in Precambriam 800 Mya, which makes these sessile filter-feeding animals the oldest extant metazoans (Fernàndez-Busquets 2010). Although marine sponge body fossils are found in shales dated between 660 and 635 Ma (Cryogenian Period), most of freshwater sponges´ fossil records (> 99.5%) are much more recent, dating back up to the Miocene epoch (~23 Ma) (Maloof et al. 2010; Pronzato et al. 2017). The remarkable gap seen between emergence of marine and freshwater sponges possibly relates to the extensive set of evolutionary adaptations that were necessary for such simple marine animals, in which the metabolism operates basically at cellular level, to succeed in colonizing freshwater habitats such as lakes and rivers (*e.g.* osmotic regulation and micronutrients requirement) (Erpenbeck et al. 2011). Besides physiological adaptations, most freshwater sponges have also developed gemmules, which are specialized resting bodies crucial for their surviving in harsh limnic environments (Marconi and Pronzato 2008).

Gemmules are resistant asexual reproductive bodies composed of modified archeocytes named thesocytes, in which the cytoplasm is full of reserve material stored into vitelline compartments, which are enveloped by a non-cellular outer layer constructed by specialized spicules termed gemmulescleres (Funayama 2013). When the environmental conditions are favorable for the sponge to thrive, gemmules stay dormant inside their bodies; however, in the advent of drought or winter freeze, sponge tissues are degraded but gemmules can endure desiccation and freezing for long periods in a cryptobiotic state (Marconi and Pronzato 2016). As soon the environmental conditions become propitious again, the gemmules hatch and then their thesocytes undergo mitosis, becoming either totipotent archeocytes or histoblasts, which migrate outwards, proliferate and differentiate into all the cell-types necessary to generate a fully functional sponge (Bart et al. 2019). Besides gemmules, freshwater sponges also

produce primmorphs commonly seen in marine sponges, which are less resistant resting bodies built of non-modified archeocytes surrounded by an epithelial outer layer reinforced with spongin (Vilanova et al. 2010).

In most animals, cell-cell adhesion is promoted by proteins such as cadherins, occludins and connexins (Abedin and King 2010); otherwise, intercellular adhesion and recognition in sponges are mediated by proteoglycan-like molecules named aggregation factors (AFs) (Jarchow et al. 2000). The supramolecular structure of the AFs resembles that of mammalians´ aggrecans: large, extracellular membrane bound, aggregating and modular proteoglycans (Fernàndez-Busquets and Burger 2003). The AF from the marine sponge *Clathria prolifera* is composed by a circular protein core attached to two different carbohydrate units: a small 6-kDa glycan that mediates the interaction of the AF with putative receptors present in the cell membrane and a larger 200-kDa sulfated polysaccharide (SP), which binds homophilically with identical units on the AFs of adjacent cells (Blunbach et al. 1998; Garcia-Manyes et al. 2006).

SPs components of AFs of different marine sponges have been shown to have notably complex chemical structures, being often composed by an intricate set of different sugar units (*e.g.* glucose, fucose and arabinose), bearing a variety of sulfation patterns, as well as by other acidic sugars such as hexuronic acids and pyruvated galactose (Vilanova et al. 2008). A good example of such complexity can be seen in the SP component of the AF of the marine sponge *Desmapsamma anchorata* (DaSP); the comprehensive set of nuclear magnetic resonance analyses employed to investigate its structure revealed the presence of a central core composed of non-sulfated and 2,4-disulfated glucose which is decorated with branches of 4,6-pyruvated galactose→2-sulfated fucose→4-sulfated glucose→non-sulfated glucose→ (Vilanova et al. 2016). Besides intrinsic complexity, SPs from marine sponges have also shown to have species-specific chemical compositions (Vilanova et al. 2008).

Previous reports based on cellular and non-cellular assays have shown that the multivalent self-interactions between the AF SPs that mediate sponge cell adhesion are calcium-dependent and species-specific (*e.g.* Bucior et al. 2004; Misevic et al. 2004; Vilanova et al. 2009). Assessments based on atomic force microscopy/single-molecule force spectroscopy assays (AFM/SMFS) demonstrated that the homophilic bindings of DaSP component of *D. anchorata* AF are mediated by "calcium bridges" between their sulfate groups (Vilanova et al. 2016). Moreover, simulations based on AFM/SMFS data obtained from the AF of *C. prolifera* have already indicated that the sponge cell mean disaggregation time is far shorter in calcium-deficient (0 mM) than in calcium-rich (10 mM) environments (Fernàndez-Busquets et al. 2009).

Seawater chemical composition is remarkably homogenous; except for minor variations in estuary and coral areas, calcium concentration in seawater is around 400 mg/L (~10 mM) (Millero et al. 2008). On the other hand, chemical composition of inland waters may vary at extreme levels. River-lake systems often contain calcium concentrations far lower (1–2 mg/L) than seawater; however, some freshwater bodies in limestone areas may present calcium contents up to 100 mg/L (Potasznik and Szymczyk 2015). In the present study, we demonstrate that the freshwater sponge *Spongilla alba* can thrive in a calcium-poor aquatic environment and that their cells are able to aggregate and produce primmorphs at calcium concentrations far lower than that required by cells of marine sponges; besides, we find that their gemmules demand calcium and other micronutrients to hatch and generate new sponges. We also show with AFM/SMFS assays that the SP with low molecular size purified from *S. alba* self-binds more efficiently at low calcium concentrations than the SP component of the AF of the marine sponge *D. anchorata.* Considering that subtle variations in the calcium content seem to be a pivotal environmental parameter for the viability of different species of freshwater sponges, the micro-chemical features of continental waters might be a milestone to increase the understanding on their biogeographical distributions.

Material and Methods

Sponge samples and water analyses

Specimens of the freshwater sponge *S. alba* were collected by SCUBA dive at Lake Carapebus, located in Rio de Janeiro State (SE Brazil). Sponge samples for *in vitro* assays were transported to the laboratory immersed in lake´s water and kept in an aquarium at 18 °C until processing and samples for extraction of SP´s were fixed in 70% ethanol immediately after collection. Analysis of the chemical composition of Lake Carapebus water was performed by the contract research organization (CRO) Eurofins-Innolab (Rio de Janeiro, Brazil). Water samples were collected by following the CRO instructions and the chemical parameters quantified with standard assays (see: https://www.astm.org/Standards/water-testing-standards.html). All reagents and standards were from Sigma-Aldrich unless otherwise stated.

Cell aggregation assays

Cells from *S. alba* were chemically dissociated as follows: sponge tissues were cut into small pieces (2–3 mm^3) and incubated in calcium- and magnesium-free artificial freshwater (CMFFW) composed of 2 mM NaCl, 0.2 mM Na_2SO_4, 0.1 mM KCl and 10 mM HEPES (pH 8.0), supplemented with 2.5 mM EDTA (CMFFW+E), for 1 h at room temperature, and then filtered (40 µm mesh), harvested by centrifugation (80 *g*, 10 min), resuspended in CMFFW and stored at 4 °C until further utilization. Aggregation assays were performed in triplicates by incubating 10^9 cells in 5 mL CMFFW supplemented with different concentrations of $CaCl_2$ (0.1→0.5 mM) or CMFFW+E for 1 h at room temperature. Number and size of cell aggregates (10 random fields per plate) formed at different calcium concentrations were quantified using a Leica DC 300 microscope (Leica) and compared by analysis of variance (ANOVA) with Tukey post hoc test by using the software Origin 8.0 (OriginLab).

Primmorph formation

Primmorphs were formed (in triplicate) from approximately 10^9 cells of *S. alba*, which were kept in culture for five days in 5 mL CMFFW supplemented with 0.25 or 0.5 mM $CaCl_2$ and tylosin+kanamycin antibiotics (0.01 mg/mL of each) at 18 °C, by following procedures previously described for marine sponges. Cultures were monitored with a Leica DC 300 microscope. Mature primmorphs formed at 0.25 mM $CaCl_2$ were fixed in 10% glutaraldehyde for 24 h at 4 °C, sectioned on a microtome (10 µm) and stained with 4% Toluidin Blue for histological examinations using a Axio Imager A1 microscope (Zeiss). Three primmorphs formed at 0.25 mM $CaCl_2$ were dissociated with CMFFW+E and then their cells were fixed in 10% glutaraldehyde for 24 h at 4 °C, quantified with an Axio Imager A1 microscope (10 fields per primmorph) and compared by ANOVA with Tukey post hoc test.

Gemmules hatching

Gemmules (five per plate, in triplicate) from *S. alba* (5 specimens) were sterilized with 1% H_2O_2 in modified M-medium (0.2 mM Na_2SO_4, 0.5 mM $NaHCO_3$, 0.5 mM KCl and 0.25 mM Na_2SiO_3; pH 8.0) (Rasmont 1961), supplemented with 0.5 mM $CaCl_2$,for 5 min at 4 °C, kept for 48 h in M-medium + 0.5 mM $CaCl_2$ or filtered (0.22 µm mesh) Lake Carapebus water (LCW) and then incubated at 18 °C in sterile conditions in 5 mL of either (i) M-medium supplemented with different concentrations of $CaCl_2$ (0.1→0.5 mM) or with 2.5 mM EDTA, (ii) LCW, (iii) LCW supplemented with 2.5 mM EDTA or (iv) LCW supplemented with 2.5 mM EGTA. After hatching, initial development of the sponges was followed with a Leica DC 300 microscope.

Extraction and purification of sulfated polysaccharides

Tissues of *S. alba* were cut into small pieces, immersed in acetone (three times) and dried at 60 °C. SPs were extracted from the dried tissues (~ 50 g) through proteolytic

digestion with papain and then partially purified with cethylpyridinium and ethanol precipitations (Stelling et al. 2019). Crude polysaccharide extracts (~ 50 mg) were applied into a Q-Sepharose XL column (GE healthcare), linked to a HPLC system (Shimadzu), equilibrated with 20 mM Tris-HCl and 1 mM EDTA and then eluted through a linear gradient of 0→3 M NaCl. Fractions of 0.5 mL were collected, checked for metachromasy and then pooled as two distinct fractions named G8 and G20, dialyzed against distillated water, lyophilized and stored at -20 °C for further utilization. SP from *D. anchorata* (DaSP) was extracted and purified as described elsewhere (Vilanova et al. 2016).

Agarose gel electrophoresis

S. alba SP´s isolated with anion-exchange chromatography were analyzed by agarose gel electrophoresis (Stelling et al. 2019). G8, G20 and the glycosaminoglycan standards heparin (HEP), dermatan sulate (DS) and chondroitin 4-sulfate (C4S) were applied (5 µg of each) into a 0.5% agarose gel in 0.05 M 1,3-diaminopropane acetate (pH 9.0). Electrophoresis was performed at 100 V for approximately 1 h and then the gel was fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide for 12 h at room temperature, dried and stained with 0.1% Toluidine Blue in 0.1:5:5 (v/v) acetic acid:ethanol:water.

Polyacrylamide gel electrophoresis

The molecular sizes of *S. alba* SPs were estimated by polyacrylamide gel electrophoresis (Stelling et al. 2019). Fractions G8 and G20 and the standards dextran (M_r ~8,000; DEX-8), chondroitin 6-sulfate (C6S), C4S and DS (10 µg of each) were applied into a 1-mm-thick 7.5% polyacrylamide gel in 60 mM Tris-HCl (pH 8.6). Electrophoresis was performed at 100 V for approximately 40 min at room temperature and then the gel was stained with 0.1% Toluidine Blue in 1% acetic acid.

Chemical analysis

Chemical analyses were performed as described elsewhere (Vilanova et al. 2009). Total hexose contents of *S. alba* SPs were estimated by phenol-H_2SO_4 reaction. Presence of hexuronic acid or pyruvated sugars was evaluated with carbazole reaction. After acid hydrolysis of G8 and G20 with 6.0 M trifluoroacetic acid for 5 h at 100 °C, their sulfate contents were measured by $BaCl_2$-gelatin method. Monosaccharide compositions were determined via gas-chromatography/mass-spectroscopy (GC/MS; Shimadzu) by analyzing alditol acetate derivatives produced by each fraction.

Single-molecule force spectroscopy assays

Self-binding forces of G20 from *S. alba* and the DaSP from *D. anchorata* at different concentrations of calcium were measured with a MFP-3D atomic force microscope (Asylum Research). SPs were immobilized on mica substrates and silicon nitride tips (NP-S) cantilevers (Bruker) by using a N-hydroxysuccinimide-poly-(ethylene glycol)-maleimide (NHS-PEG-MAL) linker of 4,750 Da (Iris Biotech GmbH), as described elsewhere (Vilanova et al. 2016). SMFS assays were performed at room temperature by approaching and separating the cantilevers and substrates functionalized with G20 or DaSP in CMFFW-T (2 mM NaCl, 0.2 mM Na_2SO_4, 0.1 mM KCl and 20 mM Tris, pH 8.0) or CMFSW-T (490 mM NaCl, 7 mM Na_2SO_4, 11 mM KCl, 2 mM $NaHCO_3$ and 20 mM Tris, pH 8.0), respectively, supplemented with 5 mM EDTA or different concentrations of $CaCl_2$ (0.1→10 mM). Spring constants of the cantilevers were determined by the thermal noise method (Lévy and Maaloum 2002). Approximately 2000 force curves were recorded with constant approach and retract velocities (2000 nm/s) and afterwards analyzed with custom-made MATLAB-based software (Math Works) in order to calculate molecular adhesion forces and elasticity. Force histograms were fitted as normal curves to obtain the average dissociation forces (Fmax) by using the software Origin 8.0.

Comentado [J1]: This is the first time this abbreviation appears in the manuscript. It should be explained what it stands for (calcium and magnesium free sea water).

Results and Discussion

Freshwater sponges are able to thrive in calcium-poor aquatic environments

Considering that the freshwater sponge *S. alba* (Fig. 1a), which was employed as a model in our study, was collected in a costal lake that is separated from the sea by a narrow sand bank (Fig. 1b), we analyzed the lake´s water to evaluate whether that proximity to seawater could influence its chemical composition. Seawater has average salinity of 35 ppt (mg/ton) whereas the salinity of Lake Carapebus was trace when expressed as ppt (Table 1) and, therefore, it is a genuine freshwater lake. Such a reduced salinity was due to low concentrations of major constituents of seawater, including chloride, sulfate, sodium, magnesium, calcium and potassium (Table 1). Its calcium content (~7 mg/L) was approximately 50-fold lower than that of seawater (~400 mg/L) and thus the Lake Carapebus can be considered as a calcium-poor freshwater environment. Chemical parameters presented in Table 1 were used as a basis to prepare the media (CMFFW, M-medium and CMFSW) employed in our *in vitro* and AFM/SMFS assays.

Although data on the calcium content of rivers and lakes harboring freshwater sponges are scarce and uncertain, partial information available in different reports allowed us to trace some correlations. Nine species of freshwater sponges, including *Metania reticulate, Trochospongilla paulula and Oncosclera navicella,* were found inhabiting waters with very low calcium content (~0.25 mg/L) at the River Negro (Brazil) (Küchlera et al 2000; Volkmer-Ribeiro 2012). Similarly, the freshwater sponge *Lubomirskia baikalensis*, along with other 14 species that comprise the family Lubomirskidae, which are endemic of the Lake Baikal (Russia), can also thrive in calcium-deficient (15–18 mg/L) waters (Rahmi et al. 2008; Khanaev et al. 2018). Besides the examples outlined above, the reduced calcium content (1–2 mg/L), which is commonly seen in inland waters (Potasznik and Szymczyk 2015), allows us to

speculate that most species of freshwater sponges should be also adapted to colonize calcium-poor aquatic environments.

S. alba cells aggregate at low calcium concentrations

Once the water chemistry of *S. alba´*s habitat was determined, we were able to prepare media (CMFFWs) suitable to evaluate the calcium content required to promote *in vitro* cell aggregation. As expected, *S. alba* cells kept in CMFFW supplemented with EDTA did not aggregate (Fig. 2a); otherwise, cells incubated in CMFFW containing different concentrations of calcium yielded aggregates with distinct features. Cells subjected to 0.1 mM $CaCl_2$ formed friable aggregates significantly smaller and more abundant ($p < 0.05$) than those formed at 0.25 and 0.5 mM $CaCl_2$ (Fig. 2b-f). Despite the noticeable but non-significant ($p > 0.05$) difference observed in their sizes, aggregates formed either at 0.25 or 0.5 mM $CaCl_2$ were quite resilient, being able to resist mechanical stress promoted by vigorous agitation of the incubation media.

Several reports available in the literature have shown that cells of marine sponges demand 10 mM calcium to undergo physiological aggregation (*e.g.* Misevic et al. 2004; Vilanova et al. 2009). Moreover, *in vitro* assays performed with micro-beads coated with SP components of AFs of different marine sponges (*e.g. D. anchorata, C. prolifera* and *Halicondria panicea*) have already demonstrated that the SP-SP interactions responsible for sponge cell adhesion operate at physiological (10 mM) calcium concentration (*e.g.* Bucior et al. 2004; Vilanova et al. 2016). The results outlined above show that the cells of the freshwater sponge *S. alba* require approximately 40-fold less calcium than cells of marine sponges to aggregate in an effective manner. We also found that the minimum calcium concentration (0.25 mM $CaCl_2$) that was necessary to yield consistent aggregates in our *in vitro* assays is in strict accordance with the calcium content (~0.2 mM) measured in the water of Lake Carapebus.

***S. alba* cells form primmorphs at low calcium concentrations**

After ascertaining the calcium requirements to promote cell aggregation, we assessed the capability of *S. alba* cells kept in culture at different calcium concentrations to produce primmorphs. Aggregates formed in CMFFW supplemented with 0.1 mM $CaCl_2$ disintegrated when detached from the substrate and thus were unable to generate primmorphs. On the other hand, cultures performed either at 0.25 or 0.5 mM $CaCl_2$ yielded primmorphs. On the first day, dissociated cells (Fig. 3a) incubated in CMFFW containing 0.25 mM $CaCl_2$ bound actively, forming small and irregular aggregates (Fig. 3b). During the second and third days, the aggregates grew irregularly by connecting one to each other (Fig. 3c-d). On the fourth and fifth days the aggregates ceased to interconnect, increased in size and acquired the round shape and smooth surface characteristic of mature primmorphs (Fig. 3e-f). Cultures conducted at 0.5 mM $CaCl_2$ produced primmorphs in a similar manner (data not shown).

Our histological analyses showed that *S. alba* primmorphs are constituted by an inner mass of archeocytes and lophocytes surrounded by an outer layer built of pinacocytes (Fig. 3g)**.** Totipotent archeocytes were significantly more abundant ($p < 0.001$) than spongin-secreting lophocytes and peripheral pinacocytes (Fig. 3f). In addition, staining of the sections with Toluidin Blue revealed an intense metachromasy (purple stain) in the primmorphs cells, especially in archeocytes, which indicates presence of intracellular and/or pericellular SPs**.**

Previous studies have already reported formation of primmorphs from cells of different marine sponges, such as *Suberites dumuncula, Hymeniacidon heliophila* and *Dysidea avara*, by using modified seawater-based media; however, all of them employed media with standard calcium concentrations (10 mM) (Müller et al. 2000; Natalio et al. 2010; Vilanova et al. 2010). Despite the lack of direct evidence, formation of primmorphs from cells of marine sponges at substantially lower calcium

concentrations is unlikely considering that they have been shown to require 10 mM calcium to undergo physiological aggregation (*e.g.* Misevic et al. 2004; Bucior et al. 2004; Vilanova et al. 2009). A recent study on spiculogenesis of the freshwater sponge *L. baikalensis* was performed with primmorphs kept in culture for up to 9 months in media prepared using Lake Baikal water, thus containing reduced calcium content (~0.4 mM) (Annenkov and Danilovtseva 2016). The primmorphs from *S. alba* cells produced at low calcium concentrations (up to ~0.2 mM) in our assays presented formation dynamics, morphological traits and cell composition alike to those produced by cells from the marine sponge *H. heliophila* at 10 mM calcium (Vilanova et al. 2010), thus suggesting that in spite of the contrasting calcium requirements, primmorphs should be generated in a similar manner by both freshwater and marine sponges.

***S. alba* gemmules require calcium to hatch**

Considering that freshwater sponges depend on their gemmules to endure seasonal drought and freeze commonly seen in rivers and lakes, we investigated with *in vitro* assays whether calcium participates in their hatching and development as new sponges. *S. alba* gemmules incubated for up to 30 days in M-medium supplemented with EDTA or distinct $CaCl_2$ concentrations (0.1→0.5 mM) encompassing the calcium content measured in the water of Lake Carapebus (~0.2 mM) did not hatch. On the other hand, 60% of the primmorphs (9 out of 15) kept in natural water from Lake Carapebus (LCW) hatched after 8 days (Fig. 4a). Accordingly, we proceeded with the evaluation of the role of calcium in the hatching of these gemmules by incubating them in LCW supplemented with EDTA or EGTA. As seen in the assays conducted with M-medium, gemmules kept in LCW containing either unspecific (EDTA) or specific (EGTA) calcium-chelators did not hatch after 30 days incubation. These results indicate that hatching of *S. alba* gemmules requires calcium as well as other micronutrients present in LCW and not in M-medium.

A study published in 1978 has shown that calcium is essential for hatching of gemmules of the freshwater sponge *Spongilla lacustris* and that it is able to overcome inhibitory effects of some divalent cations (Zn^{++}, Mn^{++}, Ba^{++} and Sr^{++}) during hatching and initial development (Ostrom and Simpsom 1978). Information available in this early study along with the results presented here are the only reports to date on the role of calcium in the development of new sponges from gemmules. Although we have failed in hatching gemmules of *S. alba* by using M-medium, previous studies have already shown that it is possible to hatch gemmules of different species of freshwater sponges with this artificial medium (*e.g.* Bart et al. 2019). In addition to minor differences in the concentration of some constituents, our modified M-medium does not contain magnesium, which is present in standard M-medium. Considering that there are no evidences on its physiological relevance for siliceous sponges, the hatch failure in M-medium should not be due to the lack of magnesium. The dark water of Lake Carapebus is loaded of dissolved organic carbon and other elements such as phosphates and nitrates (Marotta et al. 2010), which could be required to trigger hatching, thus allowing us to conclude that gemmules of *S. alba* require a complex set of micronutrients to hatch and produce new sponges.

Then, we followed the initial development of the sponges generated by *S. alba* gemmules. On the first 2 days after hatching (Fig. 4a), archeocytes and histocytes migrated outwards and proliferated actively on the substrate around the gemmules (Fig. 4b). Over the next four days (Fig. 4c-d), histocytes became basopinacocytes and archeocytes differentiated into a variety of cell types (*e.g.* choanocytes and sclerocytes), which began the formation of the basal pinacoderm (Fig. 4e), aquiferous system (Fig. 4f-g) and siliceous skeleton (spiculogenesis) (Fig. 4h-i). Afterwards, the choanosome and skeleton underwent further organization and an ectosome lining the outer surface (Fig. 4j) and an osculum (Fig. 4k) formed, giving origin to fully functional miniature sponges 6 days after the hatching. We also observed that the sponges in

formation released a great number of motile archeocytes toward the adjacent substrate (Fig. 4l), which in turn must act as phagocytes in the defense against pathogens and foreign body invasion during their development (Johnston and Hildemann 1982).

Our results revealed that the development of new sponges from gemmules of *S. alba* is similar to that described for other species of freshwater sponges such as *E. fluviatilis* and *S. lacustris* (Schill et al 2006; Bart et al. 2019). Assays with gemmules have already been used in molecular and physiological investigations. A study based on *Ephydatia mulleri* gemmules demonstrated that the homeobox gene *EmH-3* is overexpressed in totipotent archeocytes but not in somatic sponge cells (Richelle-Maurer and Van de Vyver 1999). Moreover, assessments of microRNAs expression showed that cells of quiescent gemmules of *S. lacustris* contain increased amounts of the stress protein Hsp-70 (Schill et al 2006). In addition to molecular studies, gemmules from *E. fluviatilis* were also used in spiculogenesis and innate immunity investigations (Funayama et al. 2005; Bart et al. 2019). These findings showed that hatching of gemmules is a valuable model for studying the developmental biology of sponges and to trace molecular correlations between these ancestral animals and other metazoans.

Sulfated polysaccharides from *S. alba* have low molecular size

As stated above, cell-cell adhesion in sponges is mediated by calcium-dependent self-binding between SPs (*e.g.* Bucior et al. 2004; Misevic et al. 2004; Vilanova et al. 2009); therefore, we investigated in further detail the role of calcium in the adhesion of *S. alba* cells by evaluating the self-interactions of its purified SP. Crude polysaccharide extracts from *S. alba* were applied into a Q-Sepharose column and then eluted through a linear NaCl gradient, yielding chromatograms with two peaks, which were collected as distinct fractions identified as G8 and G20 (Fig. 5a). Agarose gel electrophoresis of G8 revealed a polydisperse polysaccharide with low metachromasy whereas G20 is

homogeneous and strongly stained by Toluidin Blue (Fig. 5b). Molecular size estimations using polyacrylamide gel electrophoresis confirmed that G20 has a mass of approximately 20 kDa and a homogenous molecular weight distribution; on the other hand, G8 presented a highly polydisperse distribution and molecular weight around 8 kDa (Fig. 5c). Chemical analysis showed that both fractions are composed of glucose and mannose units and that G20 is more sulfated than G8 (Table 2). Its high polydispersity, small size and low sulfate content suggested that G8 is a degraded product of G20 rather than an intact and functional SP expressed in the cells of the sponge; for this reason, we designated G20 as the putative SP component of *S. alba* AF.

Besides containing only sulfated glucose and/or mannose units, G20 does not contain hexuronic acid or pyruvated sugars and thus it has a less complex composition than SPs from marine sponges such as the highly branched DaSP component of *D. anchorata* AF (Vilanova et al. 2008; Vilanova et al. 2016). G20 has a sulfate content slightly higher than DaSP (molar ratio ~0.80 and ~0.66, respectively); however, SPs from other marine sponges, such as that found in *Chondrilla nucula*, were already shown to be more sulfated (molar ratio ~1.5) (Vilanova et al. 2008). On the other hand, G20 has a molecular size significantly smaller than the SPs of most marine sponges. Both DaSP and the SP component of *C. prolifera* AF, which has already been used in several cell adhesion studies, have molecular masses *ca*. 10-fold higher than G20 (Garcia-Manyes et al. 2009; Vilanova et al. 2016). Therefore, our results demonstrate that the sulfated glucomannan deprived of carboxylated sugars from *S. alba* has comparable sulfate content but lower molecular size than SPs components of AFs of marine sponges. Although the presence of AFs in *E. fluviatilis* has been described almost 50 years ago, the chemical structures of their constituents have never been analyzed in detail, and thus it is the first report of the composition of a freshwater sponge SP (Fernàndez-Busquets and Burger 1999).

Sulfated polysaccharides from *S. alba* self-interact efficiently at low calcium concentration

Then, we performed AFM/SMFS assays to compare the calcium requirements needed to promote self-interactions of G20 from *S. alba* and the DaSP from the marine sponge *D. anchorata*. Assessments of self-binding forces of these SPs at different calcium concentrations (1→10 mM $CaCl_2$) were performed by using a well-established method for the immobilization of biomolecules with a heterobifunctional PEG linker (Fig. 6a), which adds distance and steric flexibility for the binding partners and reduces unspecific interactions (Hinterdorfer et al. 1996). Assays conducted with G20 and DaSP in media supplemented with calcium yielded comparable force curves (Fig. 6b). DaSP had average dissociation force (F_{max}) of 133 piconewtons (pN) and 37% binding probability in CMFSW-T+EDTA, whereas G20 yielded fewer (2%) and significantly weaker ($p < 0.05$) force curves in assays performed with CMFFW-T+EDTA (Fig. 6c-e). However, G20 reached highest F_{max} (174 pN) and binding probability (58%) when subjected to CMFFW-T supplemented with 1 mM $CaCl_2$, while DaSP only achieved maximum self-binding strength (183 pN) and probability (55%) in assays conducted with CMFSW-T containing 10 mM $CaCl_2$~~NaCl~~ (Fig. 6c-e). The slightly decreased F_{max} achieved by DaSP at 1 and 5 mM $CaCl_2$~~NaCl~~ were due to increased proportions of weaker binding forces (< 100 pN) alike to those seen in the assays performed with EDTA (Fig. 6c-e).

Previous reports based on AFM/SMFS have already demonstrated that the AF from *C. prolifera* achieves self-binding forces far higher in calcium-rich (10 ~~Mm~~mM) than in calcium-deficient (2 mM) assays (Popescu et al. 2003). Self-interactions of SPs isolated from AFs of different marine sponges have shown average forces between 180 and 310 pN at physiological calcium concentration (10 mM) (Bucior et al. 2004). Although DaSP has presented dissociation force at 10 mM $CaCl_2$ in accordance to that reported in the literature (Vilanova et al. 2016), it achieved weaker average forces in

our assays conducted in lower calcium (1 and 5 mM $CaCl_2$) and, therefore, requires seawater average calcium concentration for self-interacting in an efficient manner. On the other hand, G20 from *S. alba* already reached its maximum dissociation force at 1 mM $CaCl_2$ and thus has shown to be able to self-interact efficiently with reduced contents of exogenous calcium.

Despite "calcium bridges" between the SP component of AFs are the major forces driving cell-cell adhesion in sponges, self-interactions in the absence of calcium alike to those observed for DaSP had been reported (Fernàndez-Busquets et al. 2009; Vilanova et al. 2016). These weaker interactions have been attributed to hydrogen bonds between hydroxyl groups on the SPs, while the repulsive forces promoted by their anionic sulfate and/or carboxyl epitopes are neutralized by the high content of Na^+~~Na^{++}~~ ions present in seawater (Spillmann and Burger 2000). Considering that the CMFFW-T employed to assess self-interactions of G20 from *S. alba* has a reduced Na^+~~Na^{++}~~ content, the small binding forces revealed in our AFM/SMFS assays in the presence of EDTA was likely due to the repulsion between the highly anionic sulfate groups on their glucose and/or mannose units. Nevertheless, such calcium-free interactions mediated by hydrogen bonds are unable to promote physiological adhesion of sponge cells, as has been demonstrated for more than 100 years by dissociating tissues of marine sponges with artificial seawater deprived of calcium (Wilson 1907). The increased proportion of weak self-interactions observed for DaSP at calcium concentrations lower than 10 mM suggests that they are mediated by hydrogen bonds and not by "calcium bridges" and thus might not be able to sustain cell adhesion. On the other hand, self-interactions between the low-molecular-size SPs from the freshwater sponge *S. alba* at reduced calcium concentrations are strong enough to promote cell-cell adhesion.

Comentado [J2]: The discussion on the origin of the binding forces between SPs of DaSP at $CaCl_2$ concentrations below 10 mM is valid. The formation of hydrogen bonds when the electrostatic repulsion between sulfate groups is screened by the high NaCl concentration could explain the binding forces observed. However, it seems difficult to explain why the hydrogen-bond forces are not able to sustain cell adhesion like calcium-bridge forces, because they both have similar magnitudes (NS in Fig. 6e means not significant difference, right?). In our previous manuscript (Vilanova et al. 2016) we observed that, although the magnitude of the binding forces could be similar, the mean lifetime of the bonds could be very different, and that seemed to make the difference for the adhesion of the sponge cells in 10 mM $CaCl_2$ and not in calcium-free medium. Maybe we can include a comment about that here, referring to the paper Vilanova et al. 2016.

Conclusions

Although paleobiogeography of freshwater sponges is relatively well documented (*e.g.* Pronzato et al. 2017), studies correlating distribution of modern species and water chemistry of their habitats are scarce and fragmented. The comprehensive set of in vitro results presented here indicates that the freshwater sponge *S. alba* is able to thrive with 0.2 mM but not with 0.1 mM environmental calcium contents. However, freshwater sponges were already found inhabiting water bodies with far lower (< 0.01 mM) calcium concentrations (Küchlera et al 2000; Volkmer-Ribeiro 2012), which indicates that different species require distinct and specific calcium contents to survive, and thus it must be an environmental factor intrinsically related to their biogeographical distributions. On the other hand, marine sponges have been shown to be unable to sustain cell adhesion promoted by SPs of their AFs at calcium concentrations lower than 10 mM (*e.g.* Bucior et al. 2004; Misevic et al. 2004; Vilanova et al. 2009). Such a capability of freshwater sponges in retaining multi-cellular morphology in aquatic environments bearing reduced calcium contents must have been a crucial evolutionary step to colonize inland waters.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. Chemical compositions of Carapebus lake´s water and seawater.

Element	Carapebus Lake	Seawater[a]
	(mg/L)	
Chloride (Cl^-)	84.4	18,759
Sulfate (SO_4^{--})	55.1	831
Sodium (Na^+)	42.5	9,585
Calcium (Ca^{++})	7.4	389
Magnesium (Mg^{++})	5.7	1,323
Potassium (K^+)	3.6	329
Salinity[b]	*ND*[c]	~35

[a] Average values (Millero et al. 2008).

[b] Expressed as ppt (mg/ton)

[c] *ND* = not detected.

Table 2. Chemical composition of sulfated polysaccharides from *S. alba*.

	Glucose[a] (%)	Mannose[a] (%)	Sulfate/hexose[b] (molar ratio)
G8	70.2	29.8	0.53
G20	84.6	15.4	0.79

[a] Sugar contents determined with GC/MS.

[b] Total hexose and sulfate content measured with phenol-H_2SO_4 and $BaCl_2$-gelatin methods, respectively.

Legends for Figures

Fig. 1 Freshwater sponge *Spongilla alba* and its collection site. (**a**) *In situ* photograph of a *S. alba* specimen; scale bar = 3 cm. (**b**) Satellite Image of Lake Carapebus indicating the collection site of biological and water samples (yellow dot); scale bar = 500 m.

Fig. 2 Aggregation of *Spongilla alba* cells at different calcium concentrations. Dissociated cells were incubated for 1 hour in CMFFW supplemented with 2.5 mM EDTA (**a**) or 0.10 (**b**), 0.25 (**c**) and 0.50 (~~c~~**d**) mM $CaCl_2$; scale bars = 100 µM. Number (~~d~~**e**) and size (~~e~~**f**) of the aggregates (mean ± S.D.) formed by cells incubated at different calcium concentrations (triplicates, 10 random fields by plate) were compared by ANOVA; * ($p < 0.05$) and *NS* ($p > 0.05$).

Fig. 3 Primmorphs formed by *Spongilla alba* cells. Dissociated cells (**a**) were kept in culture in CMFFW supplemented with 0.25 mM $CaCl_2$ during five days. On the first day (**b**) cells aggregated actively, on the second (**c**) and third (**d**) days aggregates ~~grown~~ grew by connecting one to each and on the fourth (**e**) and fifth (**f**) days they increased in size and acquired the round shape and smooth surface characteristic of primmorphs; scale bars = 500 µm. (**g**) Section of *Spongilla alba* primmorph stained with toluidin blue; the solid arrow points out an archeocyte, the arrowhead a lophocyte and the pointed arrow a pinacocyte, scale bar = 50 µm. (**h**) Cell types (%) were quantified (mean ± S.D.) by dissociating primmorphs with CMFFW supplemented with 2.5 mM EDTA (10 fields from 3 primmorphs) and compared by ANOVA; * ($p < 0.05$).

Fig. 4 Development of new sponges from *Spongilla alba* gemmules. (**a**) Gemmules incubated with natural water from Carapebus Lake hatched after eight days (**a**) and pass through further development (**b-c**) for the next 6 days until they became fully functional miniature sponges (**d**). (**e**) Formation of basopinacoderm (bp). (**f**) Choanocyte chambers (cc) component of aquiferous system. (**g**) Clump of archeocytes

undergoing differentiation to give origin to choanocyte chambers. (**H**) Formation of siliceous skeleton (skt). (**i**) Detail of spiculogenesis showing a sclerocyte (sc) secreting a megasclere (ms). (**j-k**) Formation of exopinacoderm (ep) and osculum (os). (**l**) archeocytes (arc) released by sponges in formation. Scale bars = 1 mm (panels **a-d**), 200 µm (panels **e, f, h, j, k**), 100 µm (panel **l**) and 20 µm (panels **g, i**).

Fig. 5 Purification of sulfated polysaccharides from *Spongilla alba*. (**a**) Crude polysaccharide extracts from *S. alba* applied into a Q-Sepharose column and then eluted through a linear gradient of 0→3 M NaCl (pointed line) monitored by metachromasy (Abs_{525nm}) yielded two fractions (G8 and G20). (**b**) Agarose gel electrophoresis of GAG standards (HEP, DS and CSA) and G8 and G20. (**c**) Polyacrylamide gel electrophoresis of G8 and G20 and the standards C6S (~54 KDa), C4S (~36 KDa), DS (~25 Kda) and DEX8 (~8 KDa).

Fig. 6 Single molecule force spectroscopy analyses of sulfated polysaccharides from *Spongilla alba*. (**a**) Schematic view of ~~the expected topography of~~ calcium-mediated interactions between G20 polysaccharides from *S. alba* (double arrow, in yellow) immobilized onto atomic force microscopy cantilevers and substrates with a heterobifunctional PEG-Mal linker. (**b**) Typical self-binding force curves of the SP from the marine sponge *Desmapsamma anchorata* (DaSP) and G20 (represented in blue and gray, respectively, in all the panels) acquired in the presence (Ca^{++}) or absence (EDTA) of calcium with constant approach and retract velocities (2000 nm/s). (**c**) Force histograms of self-interactions of G20 and DaSP in the presence (1, 5 and 10 mM $CaCl_2$) or absence (EDTA) of calcium. (**d**) Binding probability (% of assays yielding valid force curves) and (**e**) Dissociation forces (F_{max}) of self-interactions of G20 and DaSP at different calcium concentrations. F_{max} (pN, mean ± S.E.) values were compared by ANOVA; * ($p < 0.05$) and *NS* ($p > 0.05$).

Comentado [J3]: I'm more used to see Ca^{2+} instead of Ca^{++}, but may the terminology is just slightly different in different research fields.

Figure 1

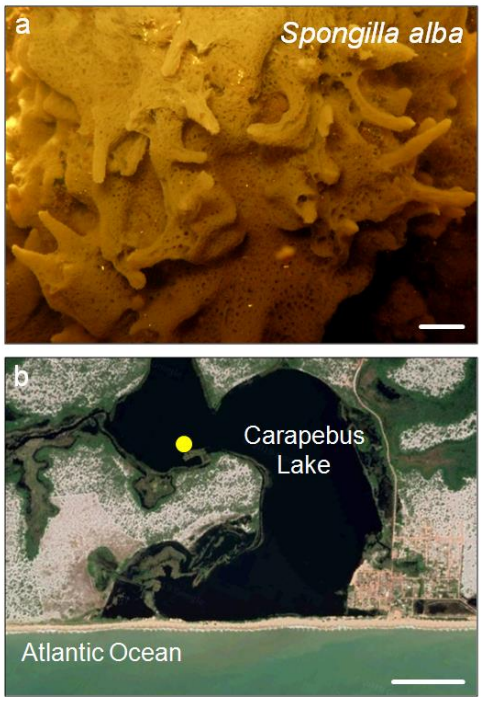
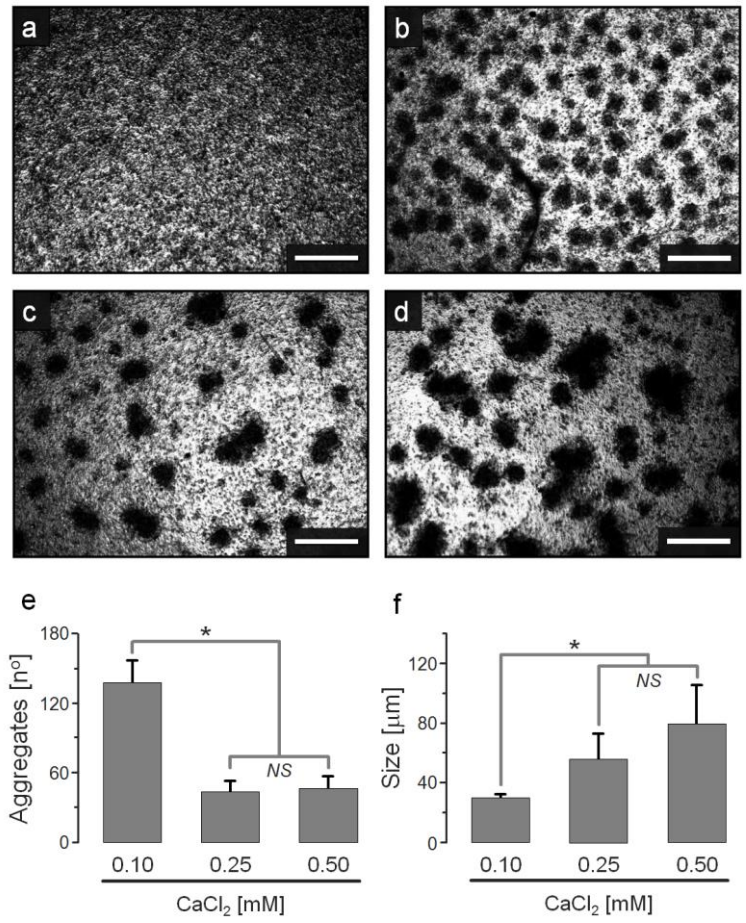


Figure 2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b
c
d
e
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120
60
0
*
NS
0.10
0.25
0.50
CaCl2 [mM]
f
Size [µm]
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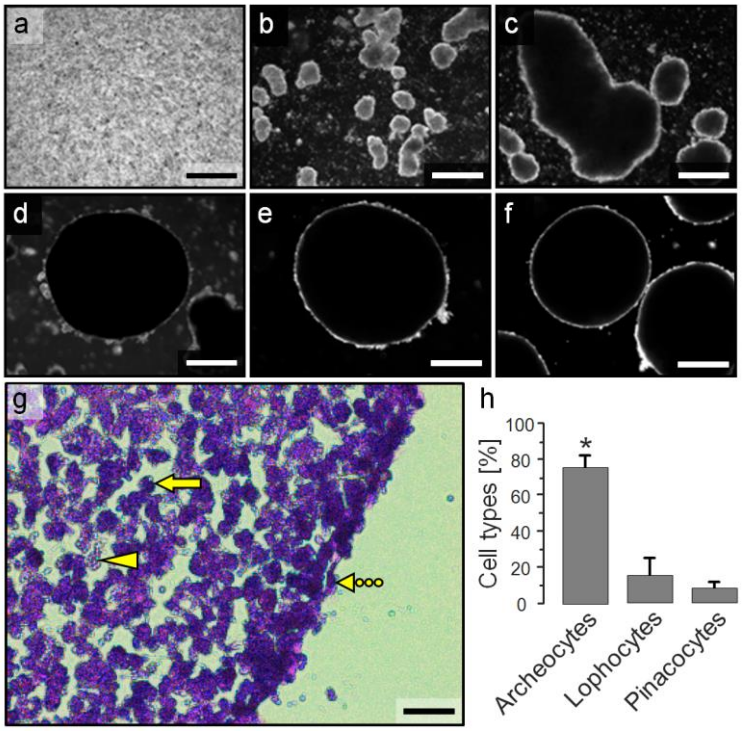
80
40
0
*
NS
0.10
0.25
0.50
CaCl2 [mM]

Figure 3


a
b
c
d
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g
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Cell types [%]
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20
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*
Archeocytes
Lophocytes
Pinacocytes

Figure 4

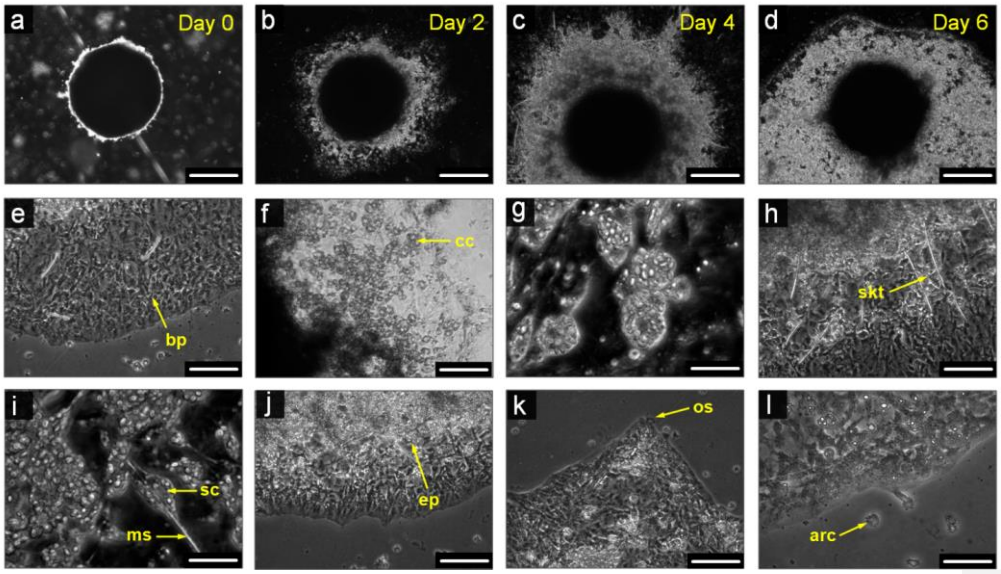
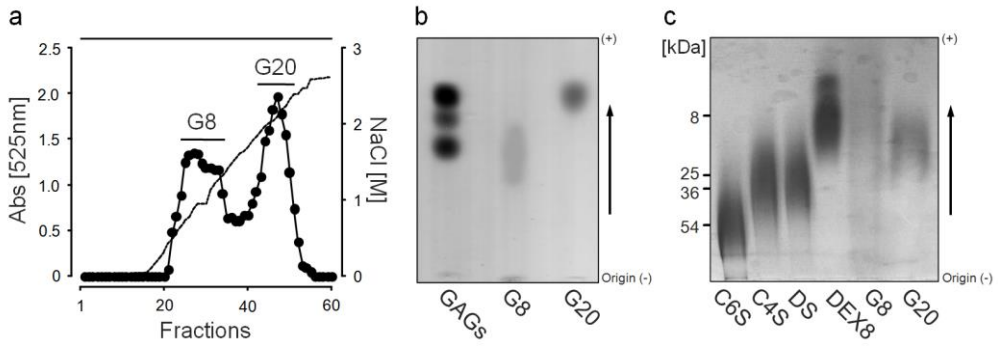


Figure 5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b
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Fractions
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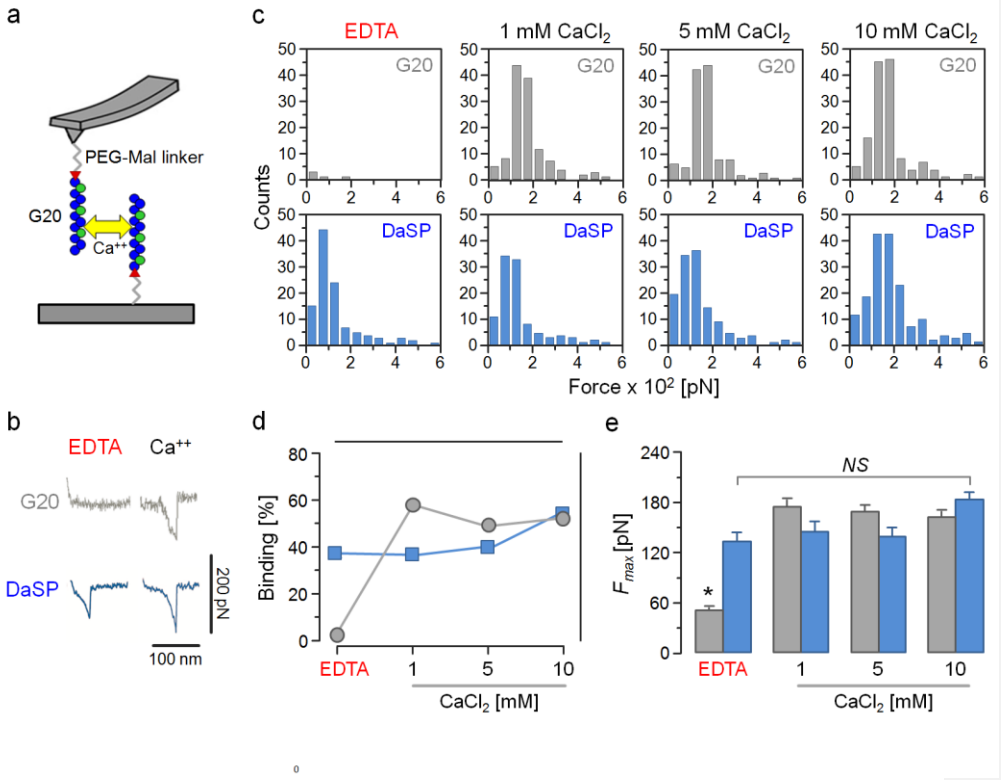
C6S
C4S
DS
DEX8
G8
G20

Figure 6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c
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10 mM CaCl2
G20
DaSP
Counts
Force x 10² [pN]
d
Binding [%]
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CaCl2 [mM]
e
NS
Fmax [pN]
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CaCl2 [mM]