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Original Article

Deciphering the molecular mechanisms underlying sea urchin reversible adhesion: A quantitative proteomics approach

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ABSTRACT

Marine bioadhesives have unmatched performances in wet environments, being an inspiration for biomedical applications. In sea urchins specialized adhesive organs, tube feet, mediate reversible adhesion, being composed by a disc, producing adhesive and de-adhesive secretions, and a motile stem. After tube foot detachment, the secreted adhesive remains bound to the substratum as a footprint. Sea urchin adhesive is composed by proteins and sugars, but so far only one protein, *Nectin*, was shown to be over-expressed as a transcript in tube feet discs, suggesting its involvement in sea urchin adhesion. Here we use high-resolution quantitative mass-spectrometry to perform the first study combining the analysis of the differential proteome of an adhesive organ, with the proteome of its secreted adhesive. This strategy allowed us to identify 163 highly over-expressed disc proteins, specifically involved in sea urchin reversible adhesion; to find that 70% of the secreted adhesive components fall within five protein groups, involved in exocytosis and microbial protection; and to provide evidences that *Nectin* is not only highly expressed in tube feet discs but is an actual component of the adhesive. These results give an unprecedented insight into the molecular mechanisms underlying sea urchin adhesion, and opening new doors to develop wet-reliable, reversible, and ecological biomimetic adhesives.

Significance: Sea urchins attach strongly but in a reversible manner to substratum, being a valuable source of inspiration for industrial and biomedical applications. Yet, the molecular mechanisms governing reversible adhesion are still poorly studied delaying the engineering of biomimetic adhesives. We used the latest mass spectrometry techniques to analyze the differential proteome of an adhesive organ and the proteome of its secreted adhesive, allowing us to uncover the key players in sea urchin reversible adhesion. We demonstrate, that *Nectin*, a protein previously pointed out as potentially involved in sea urchin adhesion, is not only highly expressed in tube feet discs, but is a genuine component of the secreted adhesive.

1. Introduction

Adhesives found in nature perform in ways that man-made products simply cannot match. Some are reversible, others are very effective underwater and many are universal in their performance to substrata of varying composition and structure. Yet, only a very limited number of model systems have inspired novel biomimetic adhesives, including the well-known gecko foot for dry adhesion and mussel glue for wet adhesion [1]. In order to speed up the engineering of innovative adhesives, it is essential to understand better how biological adhesives function, including their mode of action, their basic components, building principles and function-specific adaptations selected by evolution. Sea urchin reversible adhesion is no exception. Although in the last decade a significant effort was made to answer many questions regarding morphology and biomechanical properties of sea urchin adhesive organs, and the

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molecular mechanisms underlying sea urchin adhesion reversibility remain largely a mystery [2]. Nevertheless, being water-resistant, effective on natural and man-made substrata and reversible, sea urchin adhesives have great potential to inspire the development of new biomimetic surgical and dental adhesives, cell/tissue immobilizing agents but also cell/molecule displacers and antifouling coatings.

In sea urchins, adhesion takes place at the level of a multitude of small appendages, adoral tube feet, and involves the secretion of an adhesive between these specialized organs and the substratum (Fig. 1A). Tube feet are used in locomotion and attachment, being extremely well designed for reversible adhesion. They are composed of an enlarged and flattened apical disc that makes contact with and adheres to the substratum, and an extensible tether, the stem, allowing the tube foot to lengthen, flex and retract (Fig. 1B) [3]. Morphological studies have shown that when the substratum is suitable, the disc reorients its apical surface to be parallel to the substratum, makes contact and releases the content of adhesive granules contained inside specialized secretory cells, thus initiating the attachment process. Detachment of tube foot is accomplished by the release of de-adhesive granules content by a second type of secretory cells, breaking the bonds established between the adhesive and the disc, thus leaving a thin layer of adhesive material (footprint) strongly attached to the substratum (Fig. 1D) [2]. Yet, sea urchin tube feet do not contain exclusively these secretory cells, but present a complex histological structure related with their adhesive function, being composed of an inner myomesothelium, a connective tissue layer, a nerve plexus and an outer epidermis covered externally by a well-developed, multilayered glycocalyx, the cuticle (Fig. 1C) [3].

Although numerous individuals are necessary to obtain a sufficient amount of sea urchin adhesive material, earlier proteomic studies successfully identified proteins extracted from the sea urchin *Paracentrotus lividus* secreted adhesive [4]. The water content of the obtained adhesive was not measured but, in terms of dry weight, it is mainly made up of proteins (6.4%), lipids (2.5%), carbohydrates (1.2%) and a large inorganic fraction (45.5%) [4]. The protein fraction of *P. lividus* adhesive was further characterized in terms of amino acid composition, highlighting a bias towards 6 amino acids (glycine, alanine, valine, serine, threonine, and asparagine/aspartic acid), together with higher levels of proline (6.8%) and half-cysteine (2.6%) than the average eukaryotic proteins [4]. These traits are common to many marine adhesives and are pointed out as key factors for their high adhesive strength, cohesion and insolubility. *P. lividus* adhesive insolubility was partially overcome using strong denaturing and reducing conditions, from which 13 proteins could be extracted, and 6 were identified by mass spectrometry as alpha and beta tubulin, actin, and the histones H2A, H2B, H3 and H4 [4].

To bypass the challenge of solubilizing the secreted adhesive, a subsequent study performed protein extraction on dissected adhesive tube feet discs, a source of soluble adhesive and de-adhesive precursors [5]. The adhesive disc proteome was shown to contain 328 non-redundant proteins, of which only 2% were putative adhesive proteins [5]. Among these was *Nectin*, a *P. lividus* cell adhesion protein secreted by eggs and embryos [6,7], never before reported in the adult adhesive organs. Recent research showed that adult tube feet express two mRNA *Nectin* variants (GenBank AJ578435 and KT351732) that are over-expressed (2.5-fold) in the tube feet disc relatively to the stem, their expression being localized in the disc adhesive secretory cells and cuticle, thus suggesting an involvement in sea urchin adhesion [8]. Besides *Nectin*, only one more *P. lividus* protein was pointed out as putatively adhesive — *Toposome* [5], which is a modified calcium-binding iron-less transferrin also secreted in eggs and embryos [9,10]. In terms of putative de-adhesive proteins, surprisingly, no proteases or glycosylases that could trigger sea urchin tube foot de-adhesion by degradation of the secreted adhesive components were identified until now [5].

Sea stars are sea urchin's close relatives, being also echinoderms and attaching with a temporary adhesive secretion produced by their tube feet. Both adhesives have a similar biochemical composition; contain high amounts of small side-chain and charged/polar amino acids, probably for high cohesive strength and interactions with the substratum, respectively [4,11]; and high amounts of cysteine most certainly responsible for their insolubility [4,11]. Although putative adhesive and de-adhesive proteins have been extracted from sea star and sea urchin adhesive organs and adhesive secretions [4,5,12,13], up to now only one protein, sea star footprint protein 1 (Sfp1), was unequivocally assigned as a constituent of the sea star adhesive [14]. However, upon secretion, Sfp1 forms a structural scaffold and thus appears to provide sea star adhesive with cohesiveness rather than adhesive properties [14].

Therefore, although promising, the available molecular information on echinoderms reversible adhesion remains scarce, indicating that other approaches are needed. In this study we used a label-free quantitative proteomic approach coupled with high-resolution mass-spectrometry to perform the first differential proteome of an adhesive organ, comparing protein expression levels in tube foot adhesive part (disc) *versus* non-adhesive part (stem), revealing the key proteins involved in sea urchin reversible adhesion. We also profiled the proteome of *P. lividus* secreted adhesive disclosing its major components, strongly supported by western-blot and immunohistochemistry evidences of the obtained expression patterns and protein identifications.

2. Material and methods

2.1. Sample preparation

Sea urchins from the species *P. lividus* (Lamark 1816) were collected at low tide on the west coast of Portugal (Estoril, Portugal). After collection, the animals were transported to “Vasco da Gama Aquarium” (Dafundo, Portugal) and kept in open-circuit tanks at a temperature of 15 °C and salinity of 33‰. Then, sea urchins were placed in small plastic aquariums (3 L) filled with seawater, covered internally with removable glass plates to which animals were allowed to attach and then forced to

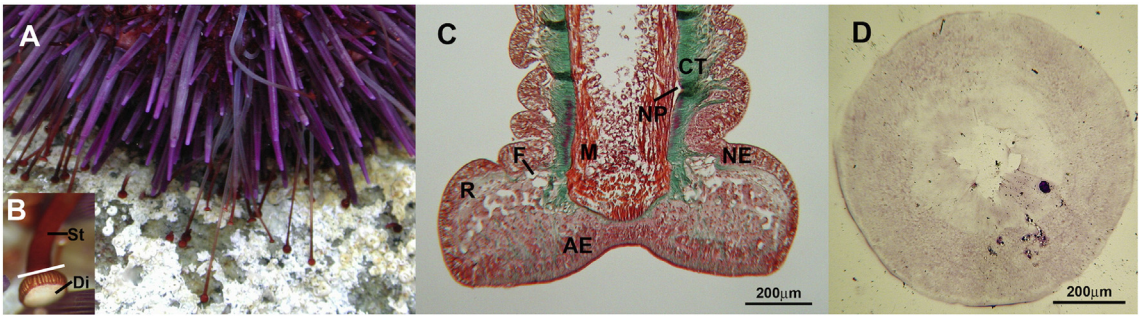


Fig. 1. Sea urchin *Paracentrotus lividus* attaching to a rock with its adoral tube feet. (A) Enlarged view of a tube foot (B) composed by a disc (Di) and a stem (St). Histological structure of an adoral tube foot (C) stained with Masson's trichrome showing the disc adhesive epidermis (AE) with its ossicles, the frame (F) and the rosette (R), the stem non-adhesive epidermis (NE), the nerve plexus (NP), the connective tissue (CT) and the muscle (M). Adhesive footprint left on the substratum after tube foot detachment (D) stained with 0.1% Crystal Violet.

detach. Broken tube feet were collected either attached to the glass plates or detached floating in the aquarium water, pooled and dissected to separate the discs from the stems (see white line in Fig. 1B). Glass plates were abundantly rinsed with Milli-Q water and air-dried followed by scrapping of the adhesive footprints using disposable scalpels. Hundreds of tube feet and adhesive footprints belonging to 60 different sea urchins were collected to minimize individual variability and to obtain enough material for subsequent protein extracts.

2.2. Protein extraction and digestion

To achieve the best protein extraction yield and reproducibility, tube feet disc and stem proteins were extracted by automated frozen disruption methodology as previously described [5]. About 250 μL (in volume) of both tissues was placed in a previously chilled teflon sample chamber containing 8 stainless steel beads (5 mm diameter) and deep frozen in liquid nitrogen. The chamber was then placed in a Mikro-Dismembrator (Retsch) and set to 3000 rpm for 5 min. To avoid sample loss, the resulting powder (still in a deep frozen state) was resuspended for 5 min with vigorous shaking in solubilization buffer (6 M urea 50 mM ammonium bicarbonate and 1:10,000 protease inhibitors) still inside the teflon chamber. The resulting homogenate was then centrifuged at 11,500 rpm for 10 min and the obtained supernatant was stored at −20 °C until further use. The tube feet disc and stem protein extracts were submitted to reduction with DTT at a final concentration of 4 mM at 56 °C for 25 min; subsequently samples were alkylated with iodoacetamide at a final concentration of 8 mM at room temperature for 30 min in the dark. Proteins were then digested with Lys-C in 6 M urea and incubated for 4 h at 37 °C. The solution was then diluted to a final urea concentration of 2 M with 50 mm ammonium bicarbonate, and the solution was incubated at 37 °C overnight with trypsin. The digestion was quenched by acidification to 5% formic acid and peptides were desalted using Sep-Pak C18 cartridges, dried in vacuum, and stored at −80 °C for further use. As for the adhesive, it was processed as previously described [4]. Briefly, the adhesive was washed five times with 1 mL milliQ H_20 and between washes centrifuged for 20 min at 13,400 rpm at 4 °C. Then, it was suspended in 1.5 mL of a protein precipitating solution to eliminate the non-protein components (10% trichloroacetic acid, 0.07% β-mercaptoethanol (*w*/*v*)) for 1 h 30 min at 4 °C, and washed three times with 1.5 mL chilled 0.07% β-mercaptoethanol in acetone (*v*/*v*), centrifuging 20 min at 13,400 rpm at 4 °C between washes. The obtained pellet was vacuum dried and solubilized in 50 μL of 2% sodium dodecyl sulfate, 0.5 M dithiothreitol in 63.2 mM Tris–HCl pH 6.8. The homogenized suspension was heated for 3 h at 60 °C and 800 rpm. After cooling, it was centrifuged at 13,400 rpm for 20 min at RT. Adhesive protein extract was then loaded on a 12.5% SDS-PAGE gel and run for 10 min to purify the proteins from detergents and buffer components, followed by Coomassie blue R-250 staining. The obtained concentrated protein bands were in-gel digested with Lys-C and trypsin according to standard procedure.

2.3. Fractionation by strong cation exchange (SCX)

The digests from tube feet discs and stems were fractioned using strong cation exchange (SCX). The SCX system consisted of an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) with one C18 Opti-Lynx (Optimized Technologies, OR) trapping cartridges and a Zorbax BioSCX-Series II column (0.8-mm inner diameter, 50-mm length, 3.5 μm particle size). The peptides were dissolved in 10% formic acid (FA) and loaded onto the trap columns at 100 μL min − 1 and subsequently eluted onto the SCX column with 80% acetonitrile (ACN) and 0.05% FA. SCX solvent A consists of 0.05% formic acid in 20% ACN, while solvent B was 0.05% formic acid, 0.5 M NaCl in 20% ACN. The SCX salt gradient is as follows: 0–0.01 min (0–2% B); 0.01–8.01 min (2–3% B); 8.01–14.01 min (3–8% B); 14.01–28 min (8–20% B); 28–38 min (20–40% B); 38–48 min (40–90% B); 48–54 min (90% B); 54–60 min (0% B). A total of 50 SCX fractions (1 min each, that is, 40 μL elution volume) were collected and the 25 most peptide-rich fractions were used for subsequent LC–MS/MS analysis.

2.4. Analysis by liquid chromatography coupled to mass spectrometry

Nanoscale liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was performed for each SCX fractions on a reversed-phase easy nano-LC 1000 (Thermo Fisher Scientific, Odense, Denmark) coupled to an Orbitrap Q-exactive mass spectrometer (Thermo Scientific, Bremen, Germany) using higher-energy collisional dissociation (HCD) fragmentation. Briefly, peptides were loaded on a double-fritted trap column (100 μm inner diameter × 2 cm, packed with 5 μm C18 resin; ReproSil-Pur AQ; Dr. Maisch, Ammerbuch, Germany) at a flow rate of 5 μL/min in 100% buffer A (0.1% formic acid in HPLC grade water). Peptides were transferred to an analytical column (50 μm inner diameter × 50 cm, packed with 2.7 μm C18 particles, Poroshell 120 EC-C18; Agilent Technologies, Waldbronn, Germany) and separated using a 180 min gradient from 7 to 30% buffer B (0.1% formic acid in 100% acetonitrile) at a flow rate of 100 nL/min. For mass spectrometric analysis, the survey scans were acquired at 35,000 resolution to a scan range from 350 to 1500 m/z. The 20 most intense precursors were submitted to HCD fragmentation using an MS/MS resolution set to 17,500, a precursor automatic gain control (AGC) target set to 5 × 104, a precursor isolation width set to 1.5 Da, and a maximum injection time set to 120 ms. RAW output files were submitted to Mascot (version 2.5.1) via Proteome Discoverer (version 1.3, Thermo Fisher Scientific) and searched against UniProt/SwissProt database for sea urchin [Taxonomic Identifier: 7656; 10/2014 database containing 32,238 entries: 285 reviewed (SwissProt) + 32,554 unreviewed (trembl)]. The protein sequence encoded by the recently discovered *Nectin* mRNA variant [8] (GenBank KT351732) was manually added to the previous database. Enzyme specificity was set to trypsin, maximum missed cleavages = 2. Carbamidomethylcysteine was set as fixed and methionine oxidation was set as variable modifications. The following peptide filters were set: minimal mascot ion score 20; peptide length between 6 and 45 amino acids; peptide confidence high; search engine rank 1; peptide rank 1 and a peptide false discovery rate (FDR) was set to 1%. Concerning the adhesive, in-gel digested proteins were analyzed using only the RP-LC–MS/MS files. The mass spectrometry proteomics raw data as well as search results have been deposited to the ProteomeXchange Consortium [15] via the PRIDE partner repository with the dataset identifier PXD003122.

2.5. Data analysis

Tube feet disc and stem differential proteome PSMs were normalized using the cytoplasmic actin of *Lytechinus variegatus* (O18548; Σ coverage: 85, 19%) as a reference protein. The adhesive proteome PSMs were normalized using the MW of each identified protein. Since a high percentage of the identified proteins were uncharacterized, Blast2GO 3.0.7 was used for functional analysis of the identified proteins, which consists of three main steps: blast to find homologous sequences, mapping to collect GO-terms associated with blast hits and annotation to assign functional terms to query sequences from the pool of GO terms collected in the mapping step [16]. Functional assignment was based on GO database b2g_jan15 containing 42,466 GOs and 4101 enzymes. Sequence data of identified proteins was uploaded as a multiple FASTA file for batch analysis by Blast2GO software. The blast step was performed against the public SwissProt database using blastp. Other parameters were kept at default values: e-value threshold of 1e-3 and a recovery of 20 hits per sequence. Furthermore, minimal alignment length (hsp filter) was set to 33 to avoid hits with matching regions smaller than 100 nucleotides. QBlast-NCBI was set as blast mode. An

annotation configuration with an e-value-hit-filter of 1.0E-6, Annotation CutOff of 55 and GO weight of 5 have been selected. GO-Slim, a reduced version of the Gene Ontology, that contains a selected number of relevant nodes, was also used in order to provide a broader view of the ontology. To group all identified proteins in selected subgroups of GO categories (biological process, molecular function and cellular compartment) the analysis tool of combined graph was used. To obtain a compact representation of the information, a sequence filter of 20 was selected. In addition Venn charts were produced using the original accessions.

2.6. SDS-PAGE and western blotting

To validate our protein expression results and protein identifications, tube feet disc and stem protein extracts were separated by SDS-PAGE, followed by western blot analysis [17]. To achieve the best protein extraction yield and reproducibility, tube feet disc and stem proteins were extracted by automated frozen disruption methodology as described above, followed by solubilization in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). Tube feet disc and stem protein extracts were separated by SDS-PAGE (12%), in mini-gel format (7 × 7 cm Tetra system from Bio-Rad). Twenty micrograms of protein sample were loaded per lane. Protein concentration was determined by the Bradford protein assay, using bovine serum albumin (BSA) as a standard (Bio-Rad). Samples were diluted 5-fold in milliQ water and mixed with reduction buffer (62.5 mM Tris–HCl, pH 6.8, 20% (*v*/*v*) Glycerol, 2% (*w*/*v*) SDS, 5% (*v*/*v*) β-mercaptoethanol). Prior to electrophoresis, samples were heated at 100 °C for 5 min. Protein bands were stained with Coomassie brilliant blue R-250. Then, proteins were transferred from the gel to PVDF membranes (Millipore) and stained with Ponceau S to monitor protein transfer. Membranes were blocked overnight at 4 °C with TBS (10 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 5% skimmed milk. Afterwards, the membranes were incubated overnight at 4 °C with the primary antibody used in TBS-T (TBS Buffer with 0.1% Tween 20) containing 1% skimmed milk. Antibodies used were: anti-*β Actin* polyclonal antibody (Sigma) at a dilution of 1:5000, anti-*α Actinin* polyclonal antibody (Sigma) at a dilution of 1:2000, anti-*α Tubulin* polyclonal antibody (Sigma) at a dilution of 1:5000, anti-*Nectin* monoclonal antibody at a dilution of 1:1000 [18] and anti-*Toposome* monoclonal antibody at a dilution of 1:10,000 [19]. Membranes were washed three times for 10 min each with TBS-T and incubated for 1 h at room temperature with anti-rabbit IgG (Roche) (at 1:5000 dilution) or anti-mouse IgG (Roche) (at 1:5000 dilution), according to the primary antibody. Immunoreactivity was detected with Pierce ECL western blotting substrate, following the manufacturer's instructions (Pierce).

2.7. Immunohistochemistry

Some of the identified proteins were also imunolocalized in *P. lividus* adhesive footprint and adoral tube feet, as previously described [20] with slight modifications. Briefly, sea urchins were placed inside small aquaria filled with artificial seawater and turned upside-down to induce attachment to clean microscope slides. Animals were repeatedly detached to induce novel attachments and thus obtain more footprints. Then, slides were thoroughly washed with milliQ water and used immediately. In addition, unattached adoral tube feet were dissected and fixed for 24 h in non-acetic Bouin's fluid at 4 °C. They were then dehydrated in graded ethanol and embedded in paraffin wax (Paraplast, Sigma, Steinhem, Germany). The tube feet were sectioned longitudinally at a thickness of 7 μm with a Reichert Autocut 2040 microtome (Leica Microsystems, Groot-Bijgaarden, Belgium), and the sections were mounted on clean silane-coated glass slides. Then tube foot sections were processed for an indirect immunohistochemical staining method according to the following protocol: they were first treated with 50 mM NH4Cl for 15 min, followed by permeabilization in PBS with 0.25% Triton-X-100 for 1 h, and by pre-incubation for 30 min with 10% fetal bovine serum (Sigma) in PBS to block nonspecific-binding sites. The primary antisera, diluted 1:200 in PBS-Tween-BSA1, were applied on the sections overnight at 4 °C. After several washes in PBS, the sections were incubated for 1 h with Alexa Fluor488-conjugated anti-rabbit IgG (Invitrogen) at a dilution of 1:1000 in PBS-Tween-BSA1 and To-Pro 3 (Invitrogen) for nuclei counterstaining. After the final rinses in PBS, the sections were mounted with Vectashield (Vector Laboratories) and were observed by using a Leica SPE confocal microscope equipped with 488 nm and 635 nm laser lines, spectral detection and 10 × 0.3NA and 20 × 0.7NA dry lenses. Adhesive footprints were observed using the Alexa Fluor488-conjugated anti-rabbit IgG on an Olympus BX60 fluorescent microscope, equipped with a 10 × 0.4NA lens and a Hamamatsu Orca R2 monochrome camera. Negative controls were carried out replacing the primary antiserum by the buffer alone.

2.8. Proteolytic activity

To evaluate the proteolytic activity of tube feet discs versus stems, a Pierce Fluorescent Protease Assay Kit was used [21]. Briefly, the fluorescence measures were carried out with a Fluorolog-3 (Horiba Jobin Yvon) in a quartz cuvette with 0.5 cm optical path, with standard fluorescein excitation/emission filters (485/538 nm) and for the calibration trypsin was the general protease chose. Tube feet disc and stem samples were prepared as described above for western-blots, and trypsin standards and casein solution were also prepared in the same RIPA buffer. All samples and standards were incubated with the substrate at room temperature for 20 min. The estimate of protease concentration in the samples was calculated by a linear regression with the trypsin standards and then divided by the total protein amount used on the assay (μg protease/μg protein). Protein concentration was determined by the Bradford protein assay, using BSA as standard (Bio-Rad).

3. Results and discussion

To perform the first differential proteome of the sea urchin *P. lividus* adhesive organs, dissected discs and stems (Fig. 1B) were digested subsequently with two proteases (trypsin and Lys-C). The resulting peptides were pre-fractionated by strong cation exchange chromatography and the resulting fractions analyzed by LC–MS/MS, followed by database searching (UniProt/SwissProt for sea urchins) using the Mascot search algorithm (see Figs. 2A and 3A and Supplementary information S1 in [22]).

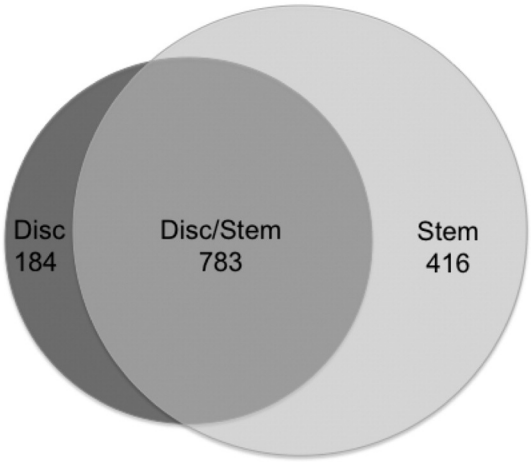


Fig. 2. *Paracentrotus lividus* tube feet differential proteome. Venn diagram showing the number of proteins identified exclusively in tube feet adhesive part - the disc, exclusively in the non-adhesive part - the stem and common to both tissues.

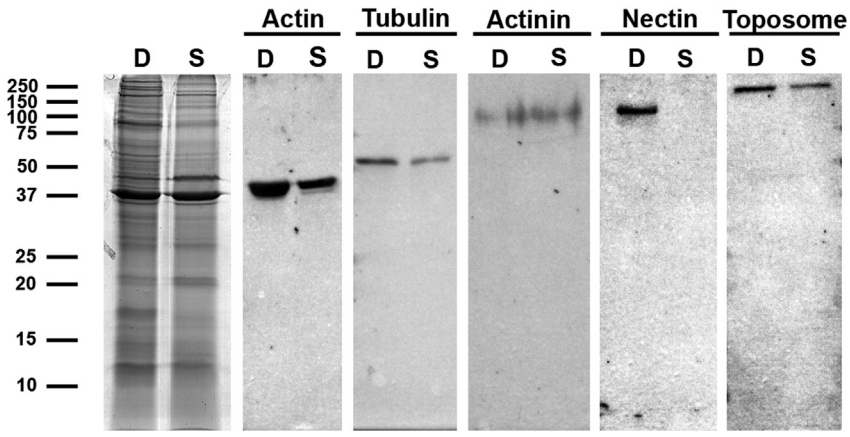


Fig. 3. *Paracentrotus lividus* adoral tube feet disc and stems protein profiles and expression levels of some selected proteins. SDS-PAGE and Western blots of tube feet disc (D) and stem (S) protein extracts, showing expression levels of *β-Actin*, *α-Tubulin*, *α-Actinin*, *Nectin* and *Toposome*. Molecular weight markers are represented on the left hand of the figure.

The analysis of the tube feet differential proteome resulted in the identification of 1384 non-redundant proteins of which 82% belong to sea urchins, but still annotated as uncharacterized (see Supplementary information S1 in [22]). Therefore, GO annotation with Blast2Go Pro was used to have a look at the functional analysis of the identified proteins, enabling us to successfully annotate 91.9% of the proteins (see Fig. 4A and Supplementary information S1 in [22]).

The tube foot disc and stem proteomes presented, respectively, a total of 968 and 1199 non-redundant proteins, of which 783 proteins were common to both tube foot parts (Fig. 2) (see also Supplementary information S1 in [22]). Of these, only the proteins presenting at least a 3-fold differential change in abundance were considered as being significantly over-expressed in the tube foot adhesive part — the disc, likely representing the most relevant proteins for sea urchin reversible adhesion (see Fig. 5A and Supplementary information S1 in [22]). We will next discuss several of these proteins and their potential role in reversible adhesion processes.

The biosynthesis, packaging and release of the adhesive and de-adhesive secretions by the tube feet discs follows the so-called regulated secretory pathway [15]. This means that in the nucleus, the genes encoding adhesive and de-adhesive proteins are transcribed into mRNAs, which are then matured and exported to the cytosol where they are translated at the level of ribosomes [24]. Indeed, in sea urchin adhesive discs we observed an over-expression of transcription- and translation-related proteins, such as *60s ribosomal proteins* (*I3*, *I7*, *L15*, *L23*; 4 to 8-fold), *40S ribosomal proteins* (*S5*, *S6*, *S8*, *SA*; 4 to 7-fold), *Eukaryotic translation initiation factor 4 gamma* (4-fold) and *Protein Churchill* (3-fold) (see Supplementary information S1 in [22]). This can be related with the reversible nature of sea urchin adhesion (attachment and detachment happening in just a few seconds) [3], probably requiring constant copying of the genes coding for adhesive and de-adhesive proteins, in order to express sufficient amounts of both secretions, for repeated and quick adhesion and de-adhesion. The same has been suggested for the mussel *Mytilus edulis* foot protein 3 (Mefp-3), for which multiple copies of a gene are necessary to express sufficient amounts of Mefp-3 for repeated and quick thread formation (less than 5 min), particularly during periods of high water flow [25].

After being translated, the rough endoplasmic reticulum (RER) captures the adhesive and de-adhesive protein precursors from the cytosol as they are being synthesized, since they possess a polypeptide signal sequence that directs the engaged ribosome to the endoplasmic reticulum membrane. So far all the adhesive protein precursors described present such a signal peptide, which will be cleaved off later in the RER [24]. This intensification in protein biosynthesis probably leads to the observed disc over-expression of proteins related with protein oligomerization [*Btb poz domain-containing protein* (4 and 5-fold)], protein folding and stability [*Peptidyl-prolyl cis-trans isomerase* (5-fold), *T-complex protein 1 subunit theta* (5-fold), *Heat shock protein 90* (4-fold), *Chaperonin containing subunit 7* (4-fold), *Protein disulfide-isomerase A5* (3-fold)] and protein sorting [*Sorting nexin* (3 to 6-fold), *Calumenin*, 3.5-fold)] (see Supplementary information S1 in [22]). In accordance, sea urchin *P. lividus* and sea star *Asterias rubens* adhesive footprints have been reported as highly insoluble, requiring strong reducing and denaturing solubilizing agents and being composed by

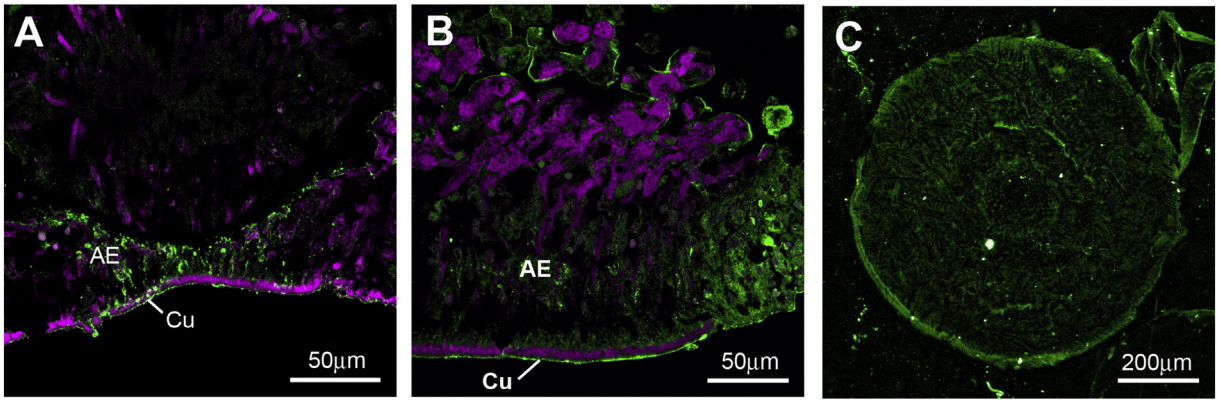


Fig. 4. Immunolabeling of *Nectin* in *Paracentrotus lividus* adoral tube foot disc and adhesive material. Tube foot longitudinal section labeled with antibodies directed against *Nectin* (Q70JA0) and observed with a confocal microscopy (immunolabeling in green and nuclei in purple). Immunoreactivity was detected in the central (A) and peripheral adhesive epidermis (AE) (B), in the disc cuticle (Cu) (A, B), and in the adhesive footprint left in the substratum after detachment (C), demonstrating that *Nectin* is secreted by the disc and is an actual component of the adhesive.

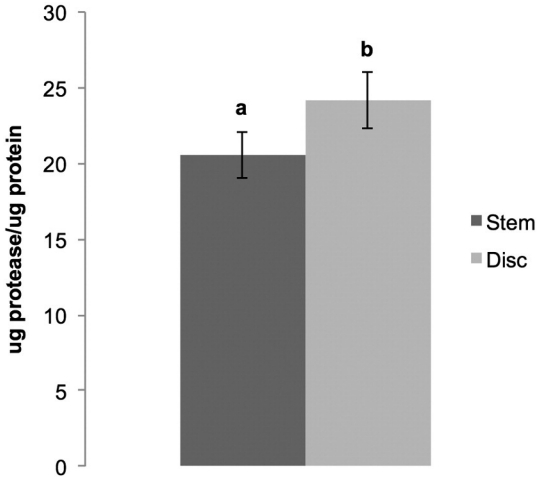


Fig. 5. Proteolytic activity of *Paracentrotus lividus* tube feet discs and stems. Proteolytic activity of tube feet discs and stems protein extracts measured by a fluorescent protease assay kit. Significant differences between the means are indicated by different letters ($p_{t\text{-}test} < 0.05$).

proteins with considerable amounts of cysteine (2.6 and 3.2%, respectively) [4,11,12]. In sea urchins, this was further corroborated by the presence of three proteins in the footprint material presenting a shift in mobility in 2D diagonal SDS-PAGE, attributed to the presence of intra- or inter-molecular disulfide bonds [4].

Then, the newly formed adhesive and de-adhesive proteins are transferred to the Golgi apparatus. During their transfer from one compartment to the next, these proteins can undergo post-translational modifications (PTMs), which are a common feature of many adhesive proteins [24]. PTMs such as N-glycosylation will take place in the endoplasmic reticulum, with subsequent oligosaccharide processing in the Golgi apparatus, while O-glycosylation or phosphorylation will occur in the Golgi apparatus [23]. In sea urchin adhesive disc, we observed an over-expression of transferases like *Dual specificity tyrosine-phosphorylation-regulated kinase* (4-fold) and *Phosphoacetylglucosamine mutase* (3-fold) (see Supplementary information S1 in [22]), involved, respectively, in phosphorylation of proteins and amino sugars. So far there are no reports of the presence of phosphoproteins in sea urchin or sea star reversible adhesives, despite the observed bias of the adhesive components towards serine and threonine residues (approximately 7–8% per residue), which makes their adhesive proteins prone to phosphorylation [4,11]. However, in other marine adhesive proteins, there are several reports of protein phosphorylation in serine residues (mussels, tube-worms and sea cucumbers) [26–29], a PTM that is believed to impart a potential for both cohesive (by Ca^{2+} bridging) and adhesive contributions (mediate adhesion to calcareous substrata, play a role in the condensation of the adhesive proteins in the adhesive cell secretory granules and/or be involved in protein–protein cross-linking) [27,29–32]. Additionally, one glycosyltransferase involved in protein glycosylation, was also over-expressed in the sea urchin adhesive disc — *Protein O-fucosyltransferase 1* (3-fold) (see Supplementary information S1 in [22]). This enzyme adds O-fucose through an O-glycosidic linkage to serine or threonine residues in a number of cell surfaces and secreted proteins. In sea urchins, the carbohydrate moiety of the adhesive material is poorly characterized, and so far all is known is that *P. lividus* adhesive contains 1.2% of neutral sugars [4]. However, in sea stars, the adhesive carbohydrate moiety was fully characterized, containing 3% of neutral sugars, 1.5% amino sugars and 3.5% uronic acids [11]. In addition, the sea star adhesive has been reported to contain at least two glycoproteins with galactose, N-acetylgalactosamine, fucose, and sialic acid residues in their side chains, as well as, sialylated proteoglycans, that together are believed to provide both cohesion and adhesion through electrostatic interactions by polar and hydrogen-bonding functional groups of their glycan chains [33]. In freshwater mussel adhesive proteins, extensive serine and threonine O-glycosylation and tryptophan C-linked mannosylation have been reported [34–36], as well as N-glycosylation in one barnacle cement protein [37]. The roles of the sugar residues in marine adhesives are still unknown, but have been proposed to increase conformational stability, enhance protein binding ability [38] and add resistance to enzymatic degradation [36].

From the Golgi apparatus, the formed adhesive and de-adhesive proteins are transferred to immature secretory granules by means of transport vesicles. As the granules mature, their contents become concentrated, probably as the result of both the continuous retrieval of membrane and the progressive acidification of the granule lumen [24]. Concordantly, sea urchin tube feet discs showed over-expression of proteins involved in transporting membrane-bounded vesicles like *Vacuolar protein sorting-associated protein* (3.5 and 4-fold), *Clathrin heavy chain* (3-fold) and *Lymphoid-restricted membrane protein* (3-fold), as well as, proteins involved in signal transduction as *Ras-related protein Rab* (3.3 and 5-fold), GTPase SUrab10p (4.5-fold), *Guanine nucleotide-binding protein subunit beta-2* (4.5-fold), *Glutamate receptor 4* (3-fold) and *Signal transducing adapter molecule 1* (3-fold) (see Supplementary information S1 in [22]). Although, many of these proteins can also be key players in sea urchin nervous system, but regulated secretory granule exocytosis shares many common aspects with synaptic vesicle exocytosis and most likely uses the same basic protein components [5,12]. Furthermore, given that mature secretory granules are so densely filled with contents, adhesive and de-adhesive cells can release large amounts of material promptly by exocytosis when triggered to do so [24]. Since sea urchin adhesive cells are non-ciliated, they are thought to interact with the nearby sensory cells via the disc nerve plexus (Fig. 1C), whereas de-adhesive cells are believed to be controlled by direct stimulation of their apical cilia [39,40].

Still, not much is known about the mechanisms regulating the exocytosis of sea urchin tube foot adhesive and de-adhesive secretions. However, sea urchin eggs are known to undergo a regulated, calcium-dependent exocytosis of their cortical secretory granules at fertilization [41]. Plus, in most secretory granules, calcium content is several orders of magnitude higher than the concentrations of free calcium ions found in the cytosol, being trapped inside the granules by calcium-sequestering organic molecules, such as acidic glycoproteins, glycosaminoglycans or proteoglycans [42]. Strikingly, in sea urchin tube feet disc two calcium-binding protein, *Calmodulin* (9-fold) and *Epidermal growth factor receptor substrate 15* (6-fold) (see Supplementary information S1 in [22]), were significantly over-expressed. Both have the ability to bind calcium via their EF-hand domains, and therefore could regulate tube foot adhesive and de-adhesive secretions exocytosis through calcium, similarly to the reported calcium-calmodulin-dependent triggering of the acrosome reaction of sea urchin spermatozoa [43]. Nevertheless, sea urchin tube feet discs also present high levels of proteins with serine/threonine kinase and phosphorylase activity (*Dual specificity tyrosine-phosphorylation-regulated kinase*, 4-fold; *Serine/threonine-protein phosphatase 1a*, 3.2-fold; *Protein phosphatase 1E*, 3-fold) (see Supplementary information S1 in [22]), suggesting a regulation of adhesive and de-adhesive granules exocytosis through calcium, in combination with a modulation of exocytosis by protein phosphorylation and dephosphorylation. In sea urchin eggs, an inhibitory phosphoprotein has been shown to obstruct calcium-stimulated exocytosis; but there is still no evidence that a protein phosphatase is an essential component of the mechanism controlling exocytosis [44].

In *P. lividus* sea urchins, adhesive secretions are produced by two types of secretory cells, and then delivered through the disc cuticle onto the surface where they form a thin film of adhesive material (Fig. 1D) that binds the tube foot disc to the substratum. One type of secretory adhesive cell is restricted to the central area of the disc and contains larger granules with a homogenous core of medium electron density surrounded by a thin clear space, whereas the second type of secretory adhesive cells is distributed in all the rest of

the disc epidermis, enclosing smaller granules with an electron dense small core, surrounded by a large electron lucent rim [3]. In terms of proteins with known adhesive properties, *Nectin* has been previously pointed out as a likely candidate, since purified egg *Nectin* was shown to significantly increase the binding of dissociated embryonic cells to the substratum [6] and therefore could also be involved in adult substratum attachment [5]. In addition, *Nectin* contains six galactose-binding discoidin-like domains (DS) that can bind molecules bearing galactose and N-acetylglucosamine carbohydrate moieties [7] and therefore could have also a cohesive role by connecting the adhesive components [5]. In the present study we found that *Nectin* is indeed highly expressed in the disc (5.4 to 13-fold), being the second most over-expressed disc protein (see Supplementary information S1 in [22]). In addition, we discovered that not only the egg/embryonic *Nectin* (Uniprot Q70JA0) [6,7] is over-expressed in the disc, but also the newly reported *Nectin* variant (GenBank KT351732; differing in 15 predicted amino acids [8]) and a third uncharacterized *Nectin*-like protein (Uniprot W4Z4Y0). Among the three *Nectin*-like proteins highly over-expressed in the disc, W4Z4Y0 presented the highest fold difference (13-fold; see Supplementary information S1 in [22]), shares 63.3% similarity with the egg/embryonic *Nectin* (Q70JA0) (see Supplementary information S2 in [22]), and was identified based on 1 unique peptide and 2 peptides in common with the two other variants (see Supplementary information S1 and S2 in [22]). As for the variants Q70JA0 and KT351732, they were much more alike in terms of sequence (98.5% similarity; see Supplementary information S2 in [22]) [8] and fold difference (respectively 5.9- and 5.4-fold; see Supplementary information S1 in [22]), being identified based on 28 common peptides, but also 2 unique peptides for the variant Q70JA0 and 6 unique peptides for the variant KT351732 (see Supplementary information S1 and S2 in [22]). The existence of protein variants among marine adhesive proteins is not new and in the mussel *M. edulis* there are several known adhesive foot protein variants namely from mfp-3 (>25 known variants), mfp-5 (2 known variants), and mfp-6 (5 known variants) [45]. It has been proposed that having adhesive protein variants with diverse isoelectric points and post-translational modifications could increase the variety of interactions that these adhesives can undergo and thus provide flexibility to match distinct underwater surface features [46,47]. Taken together, our results seem to indicate that *P. lividus* adhesive discs express at least three *Nectin* variants, which sequences differ in only a few amino acid substitutions, and probably derive from a single gene [8]. This hypothesis is in accordance with multiple *Nectin* isoforms previously observed in tube feet disc 2DE gels, presenting different degrees of phosphorylation and glycosylation [5]. In addition, this study demonstrates that the two previously reported over-expressed *P. lividus* tube feet disc *Nectin* transcripts (GenBank AJ578435 and KT351732) [8], are indeed translated into two protein variants that are also significantly over-expressed in the adhesive disc, thus strengthening the relevance of *Nectin* for sea urchin reversible adhesion. The observed protein expression levels were further validated by western-blot using an anti-*Nectin* monoclonal antibody (anti-variant Q70JA0) [18], clearly demonstrating its over-expression in the disc (Fig. 3), in agreement with our quantitative proteomics data. The same antibody was used to immunolocalize *Nectin* in the tube feet disc resulting in strong labeling in the central and peripheral epidermis mirroring the location of adhesive secretory cells, as well as, in the cuticle (Fig. 4A–B). Concerning the second putatively adhesive *P. lividus* protein, *Toposome* [5,9,10], our study demonstrates, both by quantitative proteomics (1.3 to 1.9-fold) (data not shown) and western-blot (Fig. 3) that this protein is not significantly over-expressed in the adhesive disc, thus refuting previous hypothesis of its involvement in sea urchin reversible adhesion [5]. Interestingly, a new *Aggregan core protein* was found to be over-expressed (4-fold) in the (see Supplementary information S1 in [22]), suggesting the presence of proteoglycans that can either derive from chondroitin sulfate proteoglycans from the tube feet disc cuticle [11] or be secreted into the adhesives of sea urchins, similarly to sea star adhesives that contain sialylated proteoglycans [33].

Once released, marine adhesive proteins spread readily on the substratum where they auto-assemble to form the adhesive joint, usually accompanied by a gelation or curing process of the adhesive. In aquatic organisms, this process could result from a pH or ionic strength differential between the secretory granules and water [24]. As mentioned above, many marine adhesive proteins are post-translationally modified with different chemical groups, and together with phosphates, sulfates have been pointed out as potentially involved in non-covalent adhesive and/or cohesive interactions, possibly through Ca^{2+} or Mg^{2+} bridging [24]. Concordantly, two sulfatases, *Arylsulfatase* (6 to 9-fold) and *N-acetylglucosamine-6-sulfatase* (3-fold) were highly over-expressed in the disc (see Supplementary information S1 in [22]). These enzymes catalyze the hydrolysis of sulfate esters and can be involved in remodeling of sulfated glycosaminoglycans in the extracellular space. In sea urchin embryos, *Arylsulfatase* strongly binds to sulfated polysaccharides, its deposition being dependent on the crosslinking of proteins such as collagen-like molecules, thus suggesting that it functions by binding to components of the extracellular matrix [48]. In addition, in the sea star *A. rubens* both the adhesive secretory granules and the outer most layer of the cuticle (the fuzzy coat) were shown to consist of proteins but also carboxylated and sulfated acid glycosaminoglycans [49]. Thus the identified over-expressed sulfatases could be involved in the binding of sea urchin secreted adhesive to the disc cuticle or the binding of the adhesive components increasing the cohesion of the adhesive film.

Additionally, we found that the sea urchin tube feet discs presented four over-expressed proteins with peroxidase activity such as *Tryparedoxin* (10-fold), *Dual oxidase 1* and *2* (8 to 10-fold), and *Peroxiredoxin 4* (3-fold) (see Supplementary information S1 in [22]). In sea urchin eggs an *Ovoperoxidase* is known to catalyze the formation of di-tyrosine residues between polypeptides of the fertilization envelope in order to harden it [50]. Like *Ovoperoxidase*, *Dual oxidase 1* and *2* are heme-dependant peroxidases, which favor tyrosine as a substrate [51]. Recently, an *Ovoperoxidase*-like protein was found in sea star adhesive material [13], which together with our results strengthens the potential role of heme-dependent peroxidases in the polymerization of echinoderm temporary adhesives. In other marine attaching organisms such as mussels and tubeworms, adhesive curing is achieved by the action of another enzyme, a tyrosinase that converts tyrosine into 3,4-dihydroxyphenylalanine (DOPA) [52,53]. As for the remaining oxidoreductases over-expressed in sea urchin tube feet discs, such as *Tryparedoxin* and *Peroxiredoxin 4*, they can also be involved in curing or act as antioxidants, metabolizing hydrogen peroxide into water molecules. It is known that marine species are exposed to continuous environmental changes, such as solar radiation, pollution, microorganisms, pathogens, salinity and temperature, leading to the activation of inner defense responses, including the production of reactive oxygen species (ROS) [54]. Many organisms have both enzymatic and non-enzymatic antioxidant defense mechanisms to minimize such injuries, and both types of antioxidants have been recently identified in the sea star integument mucus [13].

As for the de-adhesive secretion, it is believed to be composed by enzymes whose catalytic activity promotes breaking of the bonds established between the adhesive and the disc, explaining the recurrent presence of an adhesive footprint on the substratum after detachment [11]. Since sea urchin reversible adhesives are made of a mixture of proteins and carbohydrates (free or conjugated) it is likely that de-adhesive enzymes such as proteases and glycosylases could trigger de-adhesion by degradation of the secreted adhesive, but none of these enzymes were found [5], until now. In the present study, several proteases and glycosylases were significantly over-expressed, being potential components of the de-adhesive secretions. These comprise hydrolases acting on peptide bonds such as *Aminopeptidases* (3.7 and 6-fold), *Dipeptidases*

(3 to 4.5-fold), *Bleomycin hydrolase*-like (4 and 6-fold) and *Cathepsin z* (3-fold) (see Supplementary information S1 in [22]). These results were further confirmed using a fluorescent proteolytic assay to measure the proteolysis of casein using protein extracts from tube feet discs and stems. As shown in Fig. 5, the disc has a significantly higher proteolytic activity than the stem (p-value = 0.004), which is in agreement with the above-mentioned identification and over-expression of several proteases in *P. lividus* tube feet discs. Interestingly, other hydrolases acting on glycosyl groups such as *N-(beta-n-acetylglucosaminyl)-l-asparaginase* (4-fold), *Carbohydrate-binding family 9*-like (3.3-fold) and *Sialidases* (3-fold) were also over-expressed in the disc (see Supplementary information S1 in [22]). Although, the carbohydrate fraction of *P. lividus* adhesive is still poorly characterized, this might be an indication that it contains free or conjugated asparagine-oligosaccharides and sialic acids similarly to sea star adhesives [33].

The 163 pool of over-expressed disc proteins also puts in evidence that sea urchin reversible adhesion is an energy-consuming process, since proteins involved in the citric acid cycle [*Phosphoenolpyruvate* (5.5-fold), *Malate dehydrogenase* (5-fold), *Pyruvate carboxylase* (4-fold), *Succinyl ligase* (4-fold), *Fumarate hydratase* (3-fold)], sugars metabolism [*Glucose-6-phosphate 1-dehydrogenase* (5 fold), *UDP-glucose 4-epimerase* (4-fold), *Glucosamine-6-phosphate isomerase* (3.5-fold), *6-phosphogluconate dehydrogenase* (3.3-fold), *Triosephosphate isomerase* (3-fold)], and fatty acid metabolism [*Acyl-coenzyme A oxidase* (4-fold)] were also significantly over-expressed in the disc (see Supplementary information S1 in [22]). Likewise, in barnacle cyprid larvae, approximately 30% of the over-expressed proteins were energy-related proteins indicating that as in sea urchins, larval temporary attachment is an extremely energy-consuming process [55].

Finally, sea urchin tube feet discs also present a group of over-expressed proteins that are probably involved in post-detachment tube foot disc repair and regeneration. Sea urchin reversible adhesion is a traumatic process and tube feet discs can be injured superficially (at the cuticle level) or even amputated [5]. Therefore it is not surprising to identify over-expressed extracellular matrix (ECM) components [*Fibrillar alpha collagens* (4 to 10-fold), *Arylsulfatase* (6 to 9-fold), *Fras1-related extracellular matrix protein 2* (5-fold), *Fibrosurfin* (4-fold)], proteins related with ECM-cell interactions and cytoskeleton organization [*Microtubule-actin cross-linking factor 1* (3 to 4.5-fold), *Gelsolin-like protein 2* (4-fold); *Actin-related protein 2/3 complex subunit 2* and *4* (3 to 4-fold), *Nidogen-2* (3.5-fold), *Fascin* (3-fold); *Wiskott-aldrich syndrome protein* (3-fold), *Beta-parvin* (3-fold), *Dynein light chain* (3-fold), and *Stathmin* (3-fold)] and proteins involved in neuronal differentiation [*Dihydropyrimidinase* (5.3-fold)] (see Supplementary information S1 in [22]). In addition, a protein implicated in biomineralization — *P19 protein* was the one with the highest fold difference (20-fold) (see Supplementary information S1 in [22]), which is expected since tube feet discs are internally supported by a calcified skeleton (composed of a distal rosette and a proximal frame) that is absent in stems [3]. Indeed, it has been demonstrated that in sea urchin embryos *P19* mRNA expression is restricted to skeletogenic cells throughout embryogenesis [56].

To the best of our knowledge this study provides the first quantitative proteomic analysis of a marine adhesive organ, comparing the protein expression levels in the adhesive (the disc) versus the non-adhesive (stem) part of the tube feet, putting in evidence the expression levels of 163 key proteins specifically involved in sea urchin reversible adhesion.

This knowledge was further improved by identifying the secreted adhesive proteome as well, using the latest mass-spectrometry technology. Although, *P. lividus* adhesive material has been previously analyzed, only 6 proteins could be identified [4]. For this, adhesive footprints from several sea urchins were collected, followed by protein purification by SDS-PAGE, in-gel digestion of the obtained concentrated protein bands, analysis by LC–MS/MS and database searching as above explained (see Supplementary information S1 in [22]). The analysis of the adhesive proteome resulted in the identification of 611 non-redundant proteins of which 75% were uncharacterized, 99% of which (606 proteins) were successfully annotated (see Figs. 4B and 5B and Supplementary information S1 in [22]). If we analyze the annotated proteins in terms of relative abundance, it becomes clear that the main adhesive components are actins (27.9%), histones (24.4%), tubulins (11.9%), ribosomal proteins (7.9%) and myosins (1.4%), totalizing 73.5% (Fig. 6; see also Supplementary information S1 in [22]). The three most abundant protein groups were the same previously identified in sea urchin adhesives [4] and recently, these same five protein groups were reported as being present in the sea star *A. rubens* adhesive and integument mucus [13]. It has been hypothesized that these proteins are not components of adhesive but are remains of cellular epidermal material [13]. In addition, the presence of cytoskeletal components, such as actin, tubulin and myosin, can also be related with secretory granules mobility, since these proteins are known to play an active role granule translocation and attachment to the plasma membrane during exocytosis [57]. Nevertheless, the possibility that these proteins actually belong to sea urchin adhesive should not be discarded. Structural proteins such as tubulin and actin have a flexible arrangement, being usually associated with tension-bearing functions, making them candidate proteins as cohesive elements in the adhesive matrix [4,58]. As for histones and ribosomal proteins, nowadays, there are evidences that they also possess antimicrobial activity, possibly protecting the adhesive against microbial degradation [59,60]. In addition, histones are also natural polyelectrolytes that could provide polycations (basic proteins) for complex coacervation (i.e. combination of two or more polyelectrolytes such as proteins in solution to form soluble aggregates) of the sea urchin adhesive as reported for other marine adhesives [5,61]. In sandcastle worms, given the presence of both polyanions (acidic proteins) and polycations (basic proteins) in the granules, coacervation is driven by the electrostatic attraction and neutralization of these oppositely charged polyelectrolytes [27,62]. In mussels, polyanions are not known to be involved in adhesion but it has been demonstrated that a zwitterionic variant of the protein fp-3 (Mfp-3S) can coacervate with itself through both electrostatic and hydrophobic interactions [63,64]. Thus, complex coacervation would be adaptively beneficial for sea urchins adhesives as well, because it allows accommodating very high protein concentrations in a fluidic adhesive that can be positioned and spread over a surface without loss to the surrounding seawater, and then be cured or transformed into a more solid material [64,65].

In terms of proteins with known adhesive properties, *Nectin* is the most abundant protein in the adhesive. Four variants of *Nectin* are

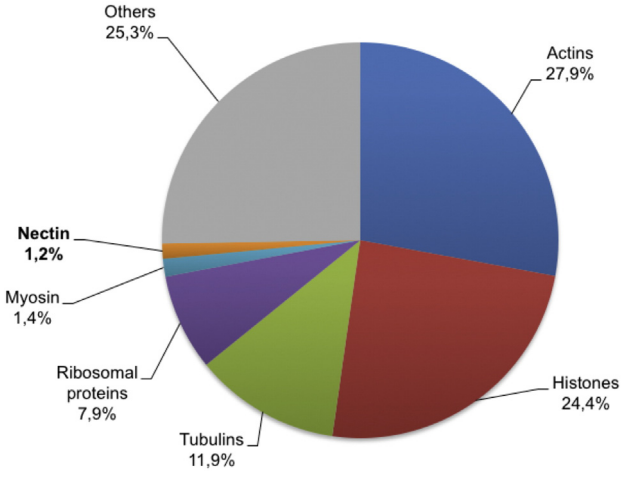
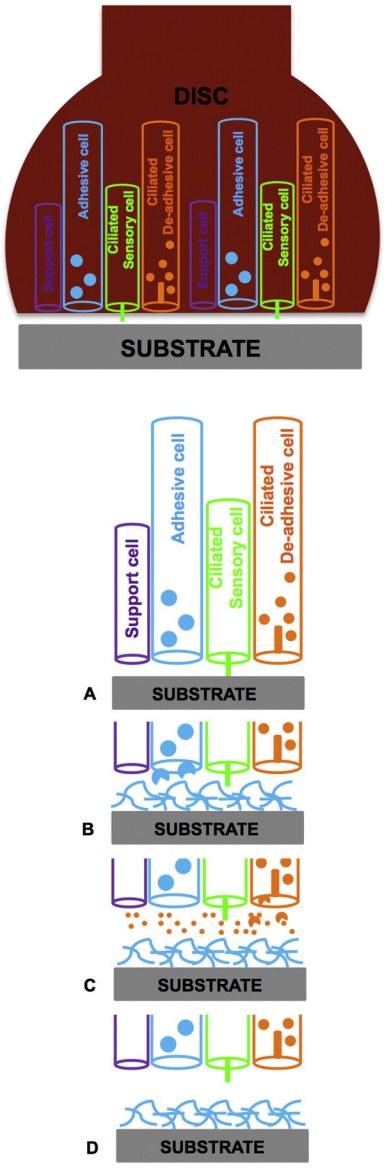


Fig. 6. *Paracentrotus lividus* tube feet secreted adhesive material proteome. Relative abundance of the proteins identified in the secreted adhesive proteome after annotation with Blast2GO.



Increased energy consumption due to secretion of adhesive and de-adhesive proteins	Citric acid cycle	*Phosphoenolpyruvate; Malate dehydrogenase; Pyruvate carboxylase; Succinyl ligase; Fumarate hydratase*
	Sugars metabolism	*Glucose-6-phosphate 1-dehydrogenase; UDP-glucose 4-epimerase; Glucosamine-6-phosphate isomerase; 6-phosphogluconate dehydrogenase; Triosephosphate isomerase*
	Fatty acid metabolism	*Acyl-coenzyme A oxidase*
Tissue repair and regeneration after detachment	Extracellular matrix components	*Fibrillar alpha collagen; Arylsulfatase; Fras1-related extracellular matrix protein 2; Fibrosurfin*
	ECM-cell interactions and cytoskeleton organization	*Microtubule-actin cross-linking factor 1; Gelsolin-like protein 2; Actin-related protein 2/3 complex subunit 2 and 4; Nidogen-2; Fascin; Wiskott-aldrich syndrome protein; Beta-parvin; Dynein light chain; Stathmin*
	Neuronal differentiation	*Dihydropyrimidinase*
	Biomineralization	*P19 protein*
Constant copying of genes coding for adhesive and de-adhesive proteins (A)	Transcription and translation	*60s ribosomal protein | 40s ribosomal protein | Eukaryotic translation initiation factor | Protein Churchill*
Intensification of for adhesive and de-adhesive proteins biosynthesis (A)	Oligomerization	*Btb poz domain-containing protein*
	Folding and stability	*Peptidyl-prolyl cis-trans isomerase | T-complex protein | Heat shock protein | Chaperonin | Protein disulfide-isomerase*
	Sorting	*Sorting nexin | Calumenin*
Adhesive protein post-translational modifications (A)	Phosphorylation	*Dual specificity tyrosine-phosphorylation-regulated kinase | Phosphoacetylglucosamine mutase*
	Glycosylation	*Protein O-fucosyltransferase*
Transfer of adhesive and de-adhesive proteins to secretory granules (A)	Transport of membrane-bounded vesicles	*Vacuolar protein sorting-associated protein | Clathrin heavy chain | Lymphoid-restricted membrane protein*
Exocytosis of adhesive and de-adhesive proteins to secretory granules (A,B,C)	Signal transduction	*Ras-related protein Rab | GTPase SUrab10p | Guanine nucleotide-binding protein subunit beta | Glutamate receptor | Signal transducing adapter molecule*
	Regulation through calcium and phosphorylation / dephosphorylation	*Calmodulin | Epidermal growth factor receptor substrate | Dual specificity tyrosine-phosphorylation-regulated kinase | Serine/threonine-protein phosphatase | Protein phosphatase*
Adhesive secretion (glue) components (B,D)	Adhesive proteins	*Nectin*
	Cohesive proteins	*Actin | Tubulin | Myosin*
	Proteoglycans	*Aggregan core protein*
	Curing	*Arylsulfatase | N-acetylglucosamine-6-sulfatase | Tryparedoxin | Dual oxidase | Peroxiredoxin*
	Antimicrobial	*Histone | 60s ribosomal protein | 40s ribosomal protein*
De-adhesive secretion components (C)	Proteases	*Aminopeptidase | Dipeptidases | Bleomycin hydrolase | Cathepsin z*
	Glycosylases	*N-(beta-n-acetylglucosaminyl)-l-asparaginase | Carbohydrate-binding family | Sialidase*

Fig. 7. Proposed molecular model of the key players in sea urchin reversible adhesion. The most relevant over-expressed proteins for the disc secretory function are proposed as well as the main components of the adhesive and de-adhesive secretions. Proteins have been divided according to their known function and putative involvement in the several steps of reversible adhesion: before attachment (A), during attachment (B), during detachment (C) and after detachment (D).

present in the adhesive (Q70JA0, KT351732, W4Z4Y0, W4ZF96) totalizing 1.2% in terms of relative abundance (Fig. 6, see also Supplementary information S1 in [22]). Q70JA0 and KT351732 are the most abundant variants, and due to their sequence similarity were identified based on 28 common peptides and 4 and 2 unique peptides, respectively (see Supplementary information S1 and S2 in [22]). The remaining *Nectin*-like variants, W4Z4Y0 and W4ZF96, share around 65% of sequence similarity with Q70JA0 (see Supplementary information S2 in [22]), and were identified with only 1 unique peptide, except for W4Z4Y0 that shared 1 more peptide in common with Q70JA0 and KT351732 (see Fig. 6, Supplementary information S1 and S2 in [22]). The recently discovered *Nectin* variant 2 (KT351732) was shown to differ from the first reported *Nectin* (Q70JA0) in 33 single nucleotide substitutions, resulting in 15 amino acid changes [8]. Likewise, the two newly identified *Nectin*-like proteins (W4Z4Y0 and W4ZF96) were both identified with only 1 unique peptide presenting only two amino acid changes relatively to the equivalent peptide in Q70JA0 and KT351732 (Supplementary information S1 and S2 in [22]). These results further support our hypothesis that several *Nectin* variants, differing only by a few amino acids, are being over-expressed in the disc and then secreted for substratum attachment. The presence of *Nectin* in the secreted *P. lividus* adhesive was also confirmed by immunohistochemistry using a monoclonal antibody raised against the egg/embryonic *Nectin* (Q70JA0) [18]. The entire adhesive footprint was strongly labeled, proving that *Nectin* is not only over-expressed in the disc, but is an actual component of *P. lividus* tube feet adhesive secretion (Fig. 4C).

4. Conclusions

To our knowledge this is the first study that combines the analysis of the differential proteome of an adhesive organ, with the proteome of its secreted adhesive. This strategic option in combination with the usage of the latest proteomics and mass spectrometry technologies, allowed us to obtain an unprecedented look on the molecular mechanisms underlying sea urchins reversible adhesion. We expanded the proteome of the sea urchin tube foot disc from the previously identified 328 to 968 non-redundant proteins. Most importantly, by using quantitative

proteomics to compare protein expression levels in the adhesive part (the disc) versus the non-adhesive part (the stem) of sea urchin tube feet, we provide the first list of 163 highly over-expressed disc proteins (>3-fold), bringing new knowledge on the key elements involved in sea urchin reversible adhesion. Plus, the analysis of the sea urchin adhesive proteome allowed us to expand the list of components from 6 to 611 non-redundant proteins. Of these, we demonstrate that more than 70% of the adhesive components fall within five protein groups probably involved in adhesive exocytosis and its protection against microbes. Finally, we present evidences that the putatively adhesive protein *Nectin*, is not only highly expressed in tube feet discs (5.4 to 13-fold), but is an actual component of the secreted adhesive, constituting the first report on a sea urchin footprint adhesive protein. These findings allowed us to propose the first molecular model for sea urchin reversible adhesion (Fig. 7), making a strong contribute towards a deeper knowledge on the functional properties and associated molecular mechanisms of marine adhesives, crucial to the desired engineering of innovative biomimetic adhesives for industrial and medical applications.

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