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Micro- and nanostructure of the adhesive material secreted by the tube feet of the sea star *Asterias rubens*

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ABSTRACT

To attach to underwater surfaces, sea stars rely on adhesive secretions produced by specialised organs, the tube feet. Adhesion is temporary and tube feet can also voluntarily become detached. The adhesive material is produced by two types of adhesive secretory cells located in the epidermis of the tube foot disc, and is deposited between the disc surface and the substratum. After detachment, this material remains on the substratum as a footprint. Using LM, SEM, and AFM, we described the fine structure of footprints deposited on various substrata by individuals of *Asterias rubens*. Ultrastructure of the adhesive layer of attached tube feet was also investigated using TEM. Whatever the method used, the adhesive material appeared as made up of globular nanostructures forming a meshwork deposited on a thin homogeneous film. This appearance did not differ according to whether the footprints were fixed or not, and whether they were observed hydrated or dry. TEM observations suggest that type 2 adhesive cells would be responsible for the release of the material constituting the homogeneous film whereas type 1 adhesive cells would produce the material forming the meshwork. This reticulated pattern would originate from the arrangement of the adhesive cell secretory pores on the disc surface.

1. Introduction

Contrary to man-made adhesives which form thin homogeneous films, biological adhesives usually possess complex hierarchical structures from the micrometric to the nanometric scale. For instance, the dry adhesives used by organisms like insects, spiders and lizards rely on their micro- and nano-patterned surface structure for robust and releasable adhesion (Artz et al., 2003; Creton and Gorb, 2007). Geckos, in particular, have developed the most complex adhesive structures, their toes typically bearing a series of scansors covered with uniform microarrays of setae, with each seta branching to form a nanoarray of hundreds of spatulae (Autumn, 2006). One may think that biological glues (adhesive secretions) would be much less complex but a look at the literature shows this is far from being the case. The permanent adhesives of mussels and tubeworms are deposited as solid micro-porous foams (Stewart et al., 2004; Waite et al., 2005). Many other adhesives, produced by organisms ranging from diatoms to frogs, form micro-porous reticulated networks (Naldrett, 1993; Hamwood et al., 2002; Graham et al., 2005; Chiovitti et al., 2006). At the nanoscale level, all adhesives appear to consist of fibrillar and/or globular structures (Higgins et al., 2000; Berglin and Gatenholm, 2003; Graham et al., 2005; Stevens et al., 2007; Phang et al., 2008). However, the ultrastructural characterization of adhesive secretions may be prone to artefacts. In the green alga *Ulva* for example, the adhesive produced by the settling spores appears as a reticulated network of fibrils in conventional SEM while it looks like a homogeneous gel-like pad in ESEM and AFM (Callow et al., 2003). This stresses the importance of using different techniques and integrating their results for a complete description of the micro- and nanostructure of biological adhesives. Moreover, in general, the processes—physical, chemical, or biological—that lead to the formation of such complex structures are still very speculative (see, e.g., Stevens et al., 2007).

Among marine adhesives, interest has centred mostly on permanent adhesives like those of mussels, barnacles and tubeworms, whereas non-permanent adhesives have received little attention. Temporary adhesives for example have been examined only in two taxa, monogenean flatworms and sea stars, in terms of their ultrastructure (Hamwood et al., 2002; Flammang, 2006). In the latter, the adhesive material was described using light microscopy (LM) (Thomas and Hermans, 1985), SEM (Flammang et al., 1994, 1998) and TEM (Flammang et al., 1994), and always appeared as a heterogeneous, sponge-like material. This material is secreted by specialized hydraulic organs, the tube feet, which consist of a proximal extensible stem and a distal flattened disc (see Flammang, 1996, for review). The disc is the part of the tube foot

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which mediates adhesion. Its epidermis encloses two types of adhesive secretory cells releasing, through individual pores, the adhesive secretion onto the foot surface (Flammang et al., 1994). These two cell types can be distinguished on the basis of the ultrastructure of their secretory granules. In type 1 adhesive cells, most of the granule volume is occupied by a bundle of parallel rods, whereas in type 2 adhesive cells, the granules contain a finely granular material surrounded by a clear cortex (Flammang et al., 1994; Flammang, 2006). Tube feet are also able to detach easily and voluntarily from the substratum through the release of the content of a third secretory cell type present in the disc epidermis, the so-called de-adhesive cells (Flammang, 1996; Flammang et al., 1994). After detachment, the adhesive secretion remains on the substratum as a footprint (Flammang et al., 1998).

All these investigations were done in different species and with different techniques, precluding the integration of the observations done on the structure of the adhesive with those made on the disc epidermis. The only TEM study available indicates that the adhesive is composed of electron-dense fibres derived from the rods constituting type 1 secretory granules (Flammang et al., 1994). Questions remain therefore about the mode of formation of the adhesive material. For instance, the significance of the presence of two types of adhesive cells in the disc epidermis has not been elucidated so far, nor has the influence of the de-adhesive secretion on the adhesive fine structure. Finally, the structure of sea star adhesive material at the nanometre scale has never been investigated. The aim of the present work was to address these different points in the sea star *Asterias rubens*. For this purpose, the fine structure of the adhesive material secreted by the tube feet in this species was investigated using LM, SEM, TEM, and AFM under various conditions. We also investigated the formation of the adhesive material by examining attached tube feet in SEM and TEM.

2. Materials and methods

2.1. Collection and maintenance of sea stars

Individuals of *A. rubens* Linné, 1758 were collected intertidally in Audresselles (Pas-de-Calais, France). They were kept in a marine aquarium with closed circulation (13 °C, 33‰ salinity) and fed mussels (*Mytilus edulis*, Linné, 1758.). Sea star adhesive material was obtained by allowing individual tube feet to adhere firmly to different substrata for a few minutes, and then to detach voluntarily, leaving the adhesive secretion on the substrata as a footprint.

2.2. Light microscopy

Fresh footprints deposited on clean microscope glass slides were stained with a 0.05% solution (in seawater) of Crystal Violet, rinsed in seawater and observed immediately with a Leitz Orthoplan light microscope equipped with a Leica DC 300F digital camera.

2.3. Transmission electron microscopy (TEM)

Resin blocks (Spurr) were used as substrata. Because tube feet do not adhere to clean Spurr blocks, these were placed in seawater for 2–3 days before the experiment to allow the formation of a primary film (see Flammang et al., 1994). Tube feet were chemically fixed while they were firmly attached to these blocks. The fixative consisted of a mixture of 1% glutaraldehyde and 1% osmium tetroxide in 0.1 M cacodylate buffer, containing or not 0.05% ruthenium red. After a maximum of 30 min of fixation at room temperature, the tube feet were rinsed in cacodylate buffer and then post-fixed for 3 h in 3% glutaraldehyde in the same buffer. This fixation method, combining glutaraldehyde and osmium tetroxide in the same solution, allows a rapid immobilization of the tissues (Cribb et al., 2004). The attached tube feet were dehydrated in graded ethanol and embedded in Spurr resin. Semi-thin sections (1 μm) were performed with a Reichert Om U2 ultramicrotome equipped with a glass knife. The adhesive material was sectioned either longitudinally (i.e., perpendicularly to the substratum surface) and transversally (i.e., parallely to the substratum surface). The sections were then stained with a 1:1 mixture of 1% aqueous solution of methylene blue in 1% sodium tetraborate and 1% aqueous solution of azur II. Ultrathin sections (40–70 nm) were cut with a Leica Ultracut UCT ultramicrotome equipped with a diamond knife. They were stained with uranyl acetate and lead citrate and observed with a Zeiss LEO 906E transmission electron microscope.

2.4. Scanning electron microscopy (SEM)

Footprints were collected on clean pieces of glass coverslips, pieces of mica or pieces of Teflon. The substrata with their footprints were chemically fixed at room temperature either in Bouin's fluid for 24 h or according to the protocol used for TEM. Some tube feet were cut off while they were firmly attached to pieces of glass coverslip and the feet still attached to their substratum were soaked in Bouin's fluid. After fixation, these tube feet were peeled away from the pieces of glass coverslip with forceps and both the foot and the substratum were processed in order to be able to compare the adhesive material present on the disc surface and on the substratum. All the samples (i.e., the footprints on the different substrata and the tube feet) were then dehydrated in graded ethanol and dried by the critical-point method. They were mounted on aluminium stubs, coated with gold in a sputter-coater and observed with a JEOL JSM-6100 scanning electron microscope.

2.5. Atomic force microscopy (AFM)

The adhesive material was observed either dry or still partially hydrated. In the first case, footprints deposited on glass pieces were rinsed with distilled water to prevent imaging artefacts due to salt crystallization during seawater drying. They were then allowed to dry in ambient air and were observed after several days. In the second case, fresh footprints deposited on pieces of glass or mica were rinsed with distilled water and observed in ambient air as rapidly as possible after their deposition. Some of these footprints were stained with a 0.05% solution (in distilled water) of Crystal Violet to facilitate their localization. A few footprints initially scanned in ambient air were then re-hydrated and observed under distilled water in a fluid cell.

The AFM microscope was operated in tapping mode (TM), which is known to minimize sample distortion due to mechanical interactions between the AFM tip and the surface. To further optimise imaging, the ratio between the set point amplitude and the amplitude of the free-oscillating cantilever was adjusted to avoid an excessive loading force applied to the sample and possible squeezing of the material between the tip and the substratum. Under these conditions, TM-AFM can provide three-dimensional imaging of the surface morphology with very high lateral and vertical resolution without damaging the surface. TM-AFM images were recorded at room temperature, either in a fluid-environment consisting of seawater or distilled water or in ambient atmosphere with a Nanoscope IIIa microscope (Veeco, Santa Barbara, CA). The probes were commercially available silicon cantilevers with a spring constant of 24–52 N/m, a resonance frequency lying in the 264–339 kHz range, and a typical radius of curvature in the 10–15 nm range. A better control of the oscillator quality factor was achieved by using a Q control box. This additional setup allows amplification of the signal-to-noise ratio of the resonance peak of

the cantilever in order to compensate the damping effect due to the liquid environment, and therefore provides better resolution of surface features.

3. Results

3.1. Scanning electron microscopy

Footprints left by tube feet on pieces of glass coverslips, pieces of mica, and pieces of Teflon were investigated by SEM. Whatever the substratum tested, the footprints are all more or less circular (Fig. 1A), having roughly the same diameter as the tube foot discs. However, the amount of adhesive material deposited varied according to the substratum used: tube feet secreted less material on Teflon than on glass or mica. The aspect of this material does not differ according to the fixative solution (i.e., Bouin's fluid Fig. 1A, C, and G, or the mixture of glutaraldehyde and osmium tetroxide Fig. 1B). On each substratum, a thin homogeneous film covering the surface can be distinguished (Fig. 1B, F, and G). At high magnification, this film is made up of globular structures in the nanometre range (Fig. 1F). However, because this film does not have a high relief, it was difficult to image clearly. Therefore, it was not possible to measure accurately the size of the globular structures. An additional material, also globular in appearance, is deposited on the thin film. The diameter of the globular structures constituting this material ranges between 50 and 100 nm (Fig. 1F). On glass and on mica, the thickness of material deposited is different from one area to another in a same footprint (see e.g., Fig. 1C). In thin areas, the material takes the form of a meshwork whose mesh components are about 1 to 5 μm in diameter (Fig. 1B, C, and F). In thick areas, the accumulation of material makes the mesh components no longer distinguishable (Fig. 1C and D). On Teflon, the material has the same appearance as in the thin areas of footprints observed on glass and on mica; i.e., only the homogeneous film and a meshwork are present (Fig. 1G). In the footprints deposited on glass and on mica, spheroidal structures ranging from 0.9 to 2.3 μm in diameter are scattered in both thin and thick areas (Fig. 1D and E). These structures are composed of granules about

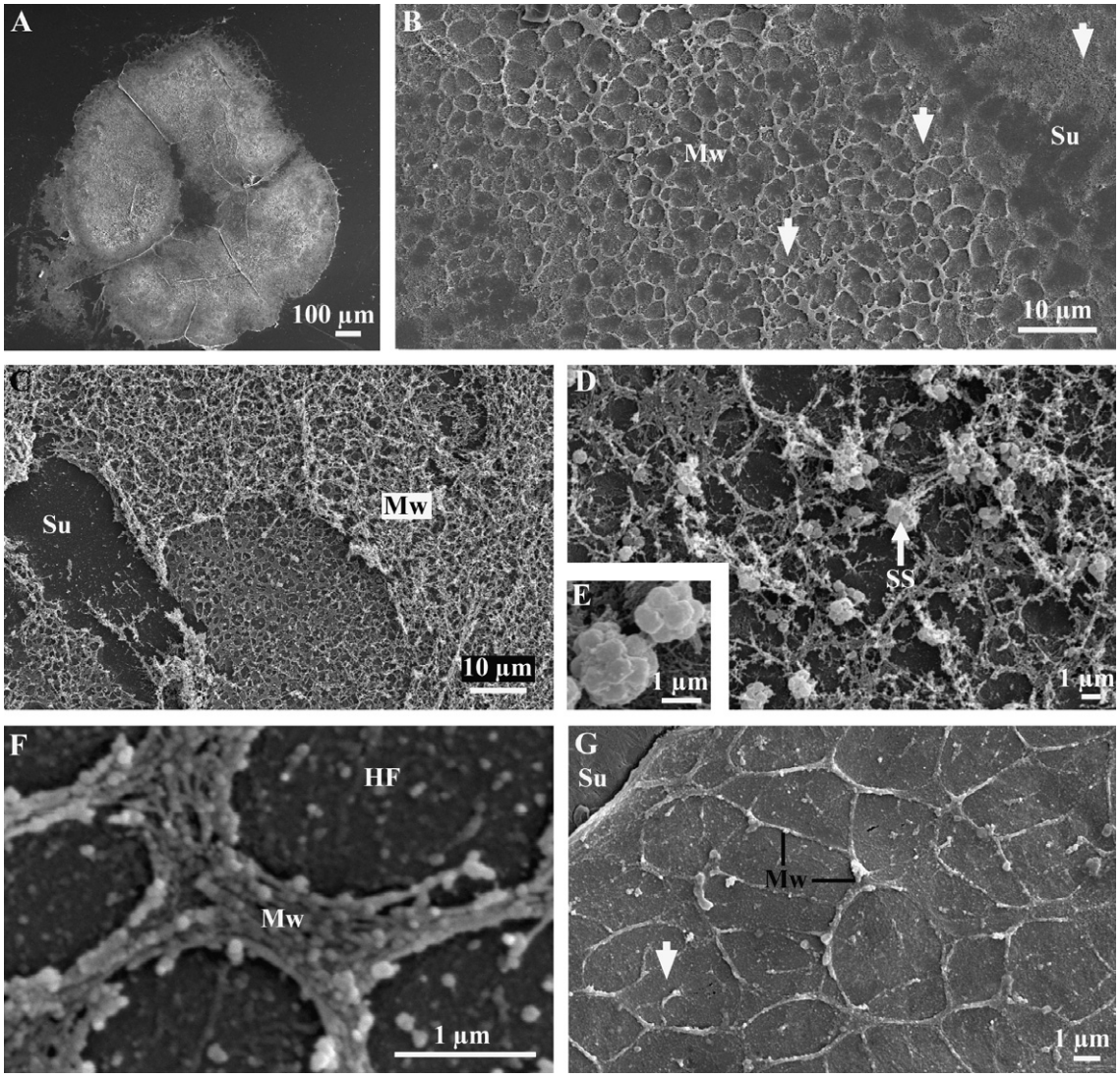

Fig. 1. SEM photographs of footprints deposited by the tube feet of *Asterias rubens* on pieces of glass (A, B, and E), mica (C, D, and F), and Teflon (G). (A) Low magnification view of a complete footprint. (B and G) Views of footprint thin areas where the adhesive material forms a meshwork deposited on a thin homogeneous film. In (B), arrows indicate the thin homogeneous film covering the substratum. (C) Footprint zone presenting different thicknesses of the adhesive material. (D) View of a footprint thick area with spheroidal structures scattered within the adhesive material. (E) Detail of two spheroidal structures. (F) Detailed view of a mesh component deposited on the thin homogeneous film illustrating the globular appearance of the adhesive material. All footprints were fixed in Bouin's fluid except the one illustrated in (B) which was fixed in the glutaraldehyde–osmium tetroxide mixture. *Abbreviations:* Mw, meshwork; Su, substratum; SS, spheroidal structures.

0.5 to 0.8 μm in diameter which, in turn, appear to be made up of little globular structures. They are not observed within the material deposited on Teflon. However, on this substratum, elongated structures of about 2 μm in length are observed, which are in continuity with the homogeneous film by one of their extremities (not illustrated). Like the film, these structures consist of little globules whose diameters range between 50 and 100 nm.

The adhesive material at the surface of the disc of tube feet chemically fixed while they were attached and subsequently peeled away from the substratum after this chemical fixation was also investigated, and compared to the material that these tube feet left on the substratum. The disc surface in these tube feet is flattened and the adhesive secretion remaining on this surface is the complementary image of that left on the substratum (Fig. 2A and B). This characteristic allowed us to compare the ultrastructure of the adhesive material present at a particular spot on the disc surface to that of the material present at the corresponding spot on the footprint. Moreover, it allowed the observation of the mode of fracture occurring when the tube foot was peeled away from the substratum. As noticed for footprints deposited on glass and mica, the amount of adhesive material deposited varies from one area to another on both the disc surface and the glass substratum. In areas where only the thin homogeneous film is present, two types of adhesive fractures were observed. In the first case, the film remained on the disc surface, covering all its surface features, and an area without any material is observed on the substratum

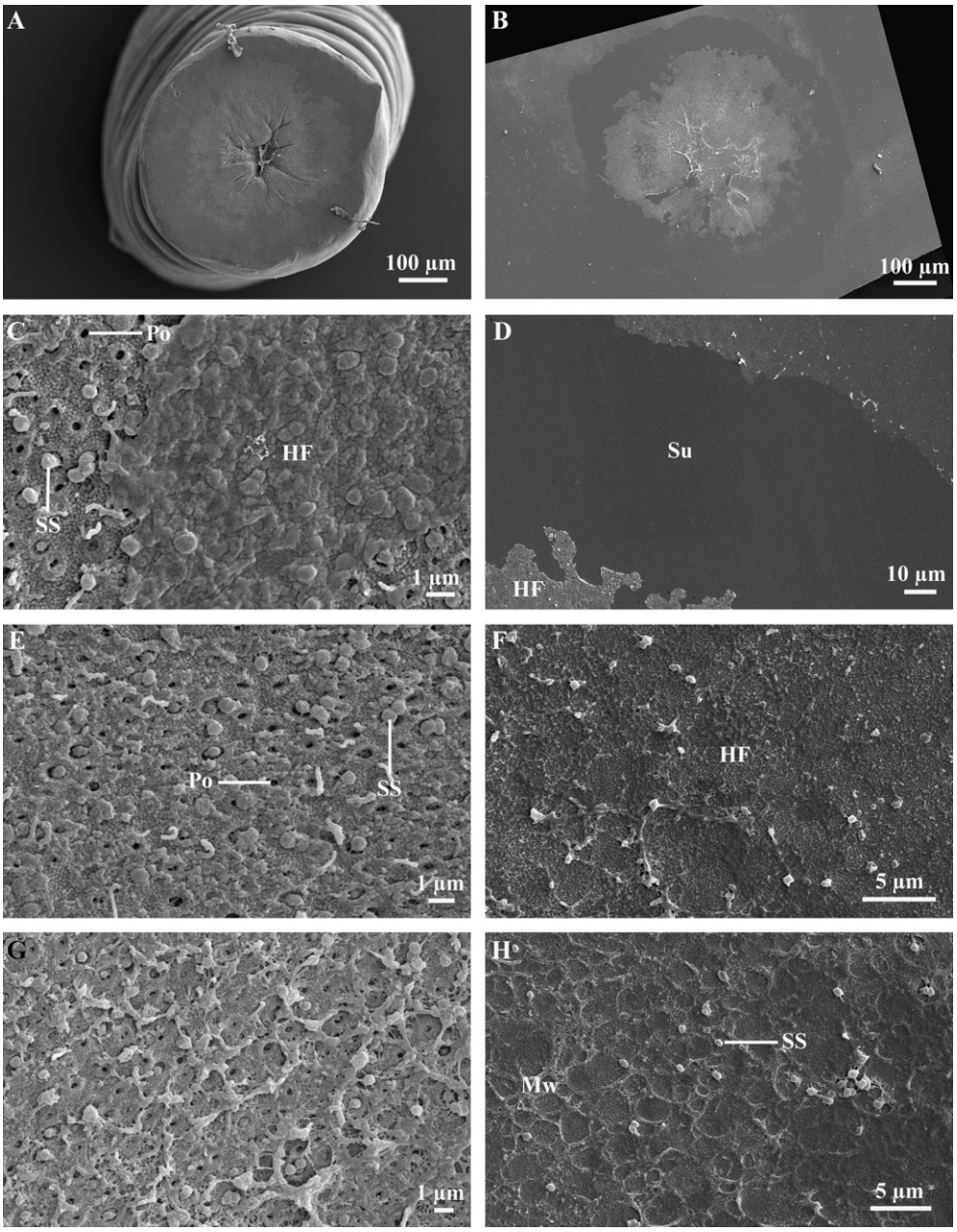

Fig. 2. SEM photographs of the disc surface of an attached tube foot of *Asterias rubens* peeled away from the substratum after its chemical fixation (A, C, E, and G) and of the material left on the substratum by this tube foot after peeling (B, D, F, and H). (A and B) Low magnification views of the peeled tube foot and of the corresponding substratum. (C and D) Adhesive fracture in which the homogeneous film is detached from the substratum and remains on the disc surface. (E and F) Adhesive fracture in which the homogeneous film is detached from the disc surface and is left on the substratum. (G and H) Cohesive fracture through the adhesive meshwork. *Abbreviations:* HF, homogeneous film; Mw, meshwork; Po, pore; Su, substratum; SS, spheroidal structure.

(Fig. 2C and D). This suggests that the adhesive fracture occurred just between the substratum and the homogeneous film. In the second case, the fracture took place just on the disc surface, leaving the homogeneous film on the substratum (Fig. 2E and F). In that kind of fracture, we notice spherical structures about 0.5 to 0.7 μm in diameter extruding from the pores of adhesive cells on the disc surface. In thick areas, where both the thin homogeneous film and the meshwork are present, fractures were always cohesive and took place through the latter. On the disc surface, at places where the meshwork is thin, some spherical granules extruding from the pores are still visible, while others are elongated and seem to fuse one with another, initiating the formation of mesh components (Fig. 2G). Identical structures are also visible within the material left on the substratum (Fig. 2H). At places where the meshwork is thicker, these granules are no longer visible (results not illustrated).

3.2. Atomic force microscopy

Because AFM does not require any fixation or preparation of the sample, it should have been possible to resolve the topographical structure of the adhesive material under seawater, without the possible artefacts associated with fixation and drying. However, despite repeated attempts, we were not successful in scanning footprints under native conditions. There were several reasons for this: the difficulty of localizing the transparent footprints, problems of stability of the cantilever, and artefacts caused by the crystallization of the salts present in the seawater. Footprints freshly deposited on glass or mica pieces were stained, rinsed with distilled water and rapidly investigated by AFM while still wet. Only areas containing a low amount of material were investigated because areas containing a lot of material were difficult to image due to the very high roughness. Again with this technique, the adhesive material appears as consisting of globular nano-structures forming a meshwork deposited on a homogeneous film (Fig. 3). The mesh components delineated by the adhesive material range from 1 to 5 μm in diameter (Fig. 3A). AFM allowed measurement of the height of the mesh component walls, which ranges from 70 to 200 nm. The diameter of the globular structures varies according to the conditions under which the adhesive material is observed, ranging from 80 to 200 nm in freshly prepared footprints and from 50 to 90 nm in completely air-dried footprints (Fig. 3B and C, respectively). Finally, footprints prepared similarly were re-equilibrated with distilled water in the fluid cell and observed underwater. The corresponding images, although presenting an important smearing effect, show that the meshwork is still visible in the re-hydrated material (Fig. 3D).

3.3. Light microscopy

Using LM, it was possible to examine the adhesive under seawater. However, a staining procedure with Crystal Violet had to be used to increase contrast. Once more, the quantity of adhesive material varies from one area to another in a same footprint. In thin areas, e.g., in the periphery of the footprint, the adhesive secretion presents a meshwork aspect, in which only the walls of the mesh components are stained by Crystal Violet whereas their inner part is not (Fig. 4A). In thick areas, the mesh components are more difficult to distinguish clearly.

Transverse (i.e., parallel to the substratum) semi-thin sections through the adhesive material of attached tube feet reveal an adhesive material structure similar to that observed with the other techniques (Fig. 4B). As the sections are usually slightly oblique, it is possible to distinguish on a same section the thin homogeneous film directly in contact with the substratum, the meshwork zone (constituting the major part of the adhesive layer), and the apex of the epidermis. Both the homogeneous film and the walls of the mesh components are strongly stained in deep blue by the methylene blue–azur II mixture while their inner part is slightly stained in purple (Fig. 4B). In some areas, an additional zone, in which the meshwork pattern is less conspicuous and the meshes are unstained, is present between the meshwork and the epidermis.

3.4. Transmission electron microscopy

The ultrastructure of the adhesive material was investigated using both longitudinal and transverse sections made through tube feet which were chemically fixed while firmly attached to Spurr resin blocks. In terms of ultrastructure of both the disc epidermis and the adhesive material, no obvious difference was found between samples treated with either of the two fixative solutions tested (i.e., with or without ruthenium red). Longitudinal sections show secretory material forming an adhesive layer between the disc epidermis and the substratum or, in some cases, accumulating in small folds of the epidermis (Figs. 5 and 6). The images obtained from attached tube feet present a certain heterogeneity regarding the ultrastructure of both the secretory granules and the adhesive layer. This heterogeneity is observed between different tube feet but also between different areas in the same foot. Three general patterns, representing the most frequently observed cases, can be recognized, although several intermediate situations exist. These patterns could reflect different stages in the secretion of the adhesive material; however, the order in which they are described may not be their actual chronological order of succession. Altogether, the thickness of the adhesive layer varies between 1.4 and 10.3 μm, independently of the pattern described. Moreover, in all three patterns, a thin homogeneous film made up of a granular material of medium electron-density is observed covering the primary film adsorbed on the substratum (Figs. 5 and 6). This layer, whose thickness varies between 0.3 and 3 μm, is able to fill out the smallest irregularities of the substratum. Bacteria are sometimes found embedded in this layer (Figs. 5B and 6A).

In the first pattern, three materials are deposited between the homogeneous film and the tube foot surface: a heterogeneous electron-dense granular material, a homogeneous granular material of medium electron-density and a loose electron-lucent material (Fig. 5A–E). The first two materials derive clearly from the granules contained in type 1 and type 2 adhesive cells, respectively, each material being secreted in front of its respective cell type (Fig. 5A and D). The alternation of the two types of adhesive cells in the disc epidermis is therefore translated in the adhesive material by the alternation of these two materials, which is clearly visible in both longitudinal and transverse sections (Fig. 5B and C). In most type 1 adhesive cells, the granules have lost their membrane and have begun to swell (compare with intact granules present in the basal part of some cells; Fig. 5A). The rods that originally made up the core of the type 1 granules loose their regular structure and their constituting material gathers into a hollow spheroid of electron-dense granular material, leaving an electron-lucent material inside and around the spheroids (Fig. 5D and E). In some cells, the shape of the granules is no longer distinguishable and the entire apical process of the cell is filled with a homogeneous granular material of medium electron-density (Fig. 5E). This material possesses the same appearance as the homogeneous granular material secreted by type 2 adhesive cells and as the material constituting the homogeneous layer covering the substratum (compare Fig. 5B, D, and E). Within type 2 adhesive cells, the secretory granules contain a finely granular material surrounded by a clear cortex (Fig. 5D). It is noteworthy that type 2 cells are much less conspicuous in attached tube feet than in tube feet which did not contact the substratum (see, e.g., Flammang et al., 1994), maybe because their

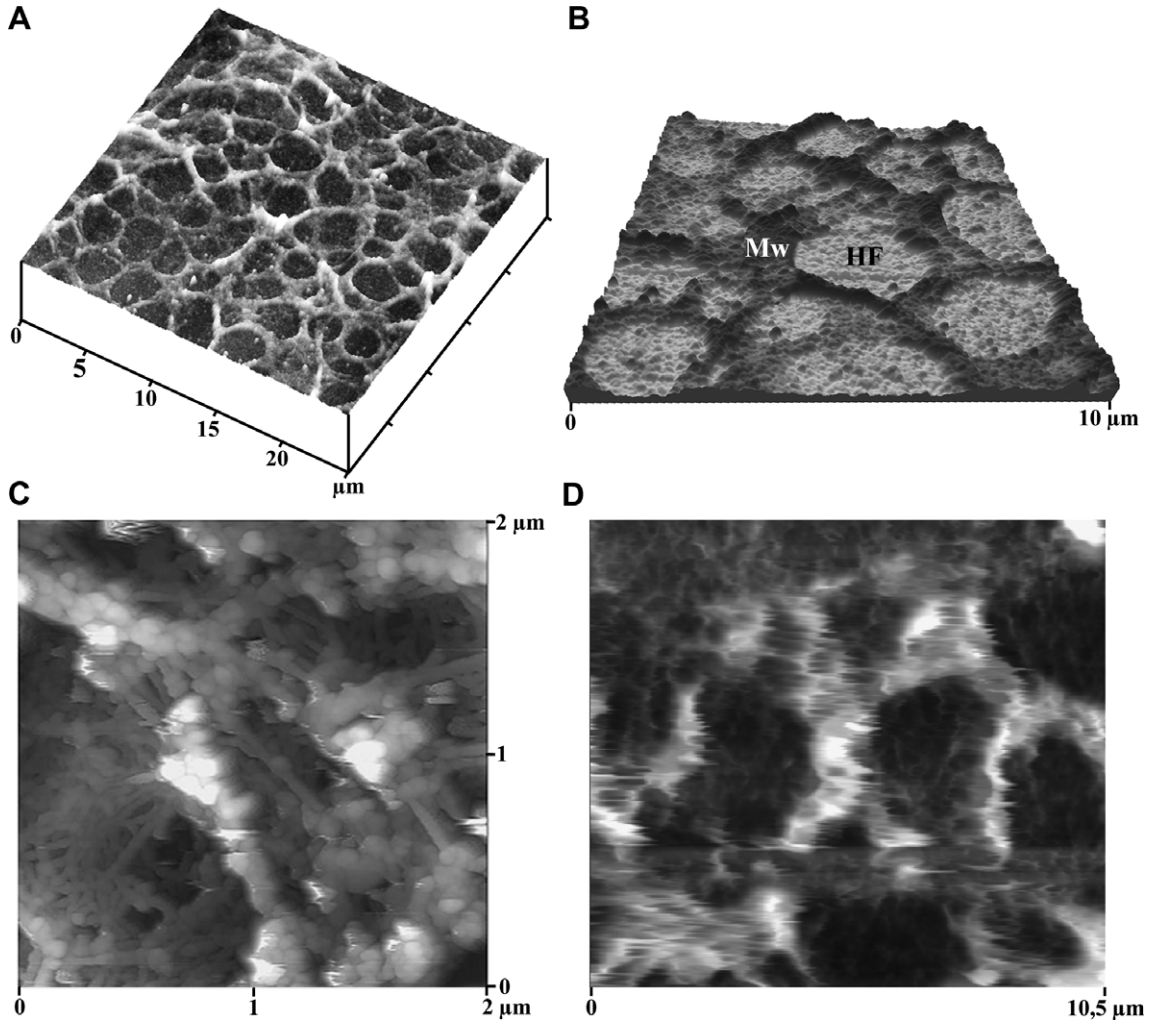

Fig. 3. TM-AFM photographs of the adhesive material deposited by the tube feet of *Asterias rubens* on mica (A and B) and glass (C and D) pieces. (A) Three-dimensional view of wet adhesive material observed in ambient air (*Z* scale = 400 nm). (B) Higher magnification of the same area showing the globular appearance of both the meshwork and the homogeneous film (*Z* scale = 370 nm). (C) Detailed topography view of a dry sample showing the succession of globular nanostructures. (D) Adhesive material observed under distilled water after it had been localised in air (*Z* scale = 800 nm). *Abbreviations:* HF, homogeneous film; Mw, meshwork.

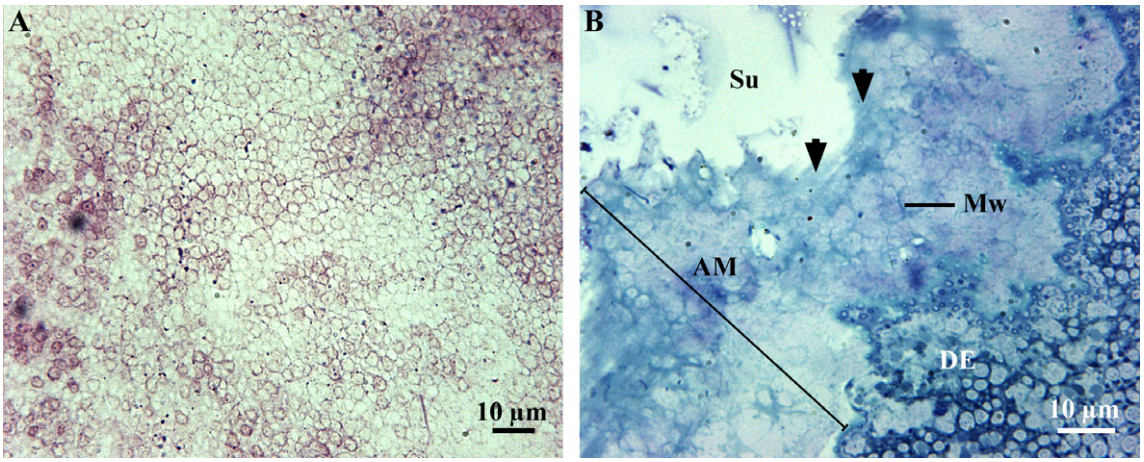

Fig. 4. LM photographs of the adhesive material secreted by the tube feet of *Asterias rubens*. (A) Fresh adhesive material stained with a solution of Crystal Violet in seawater. (B) Oblique semi-thin section through adhesive material of an attached tube foot stained with a mixture of methylene blue–azur II. Arrows indicate the thin homogeneous film covering the substratum. *Abbreviations:* AM, adhesive material; DE, disc epidermis; Mw, meshwork; Su, substratum.

apical process is depleted of secretory granules. As for the loose electron-lucent material, which is the third constituent of the adhesive material, it fills the spaces between the other two materials (Fig. 5B–E). Its appearance is similar to that of the electron-lu-

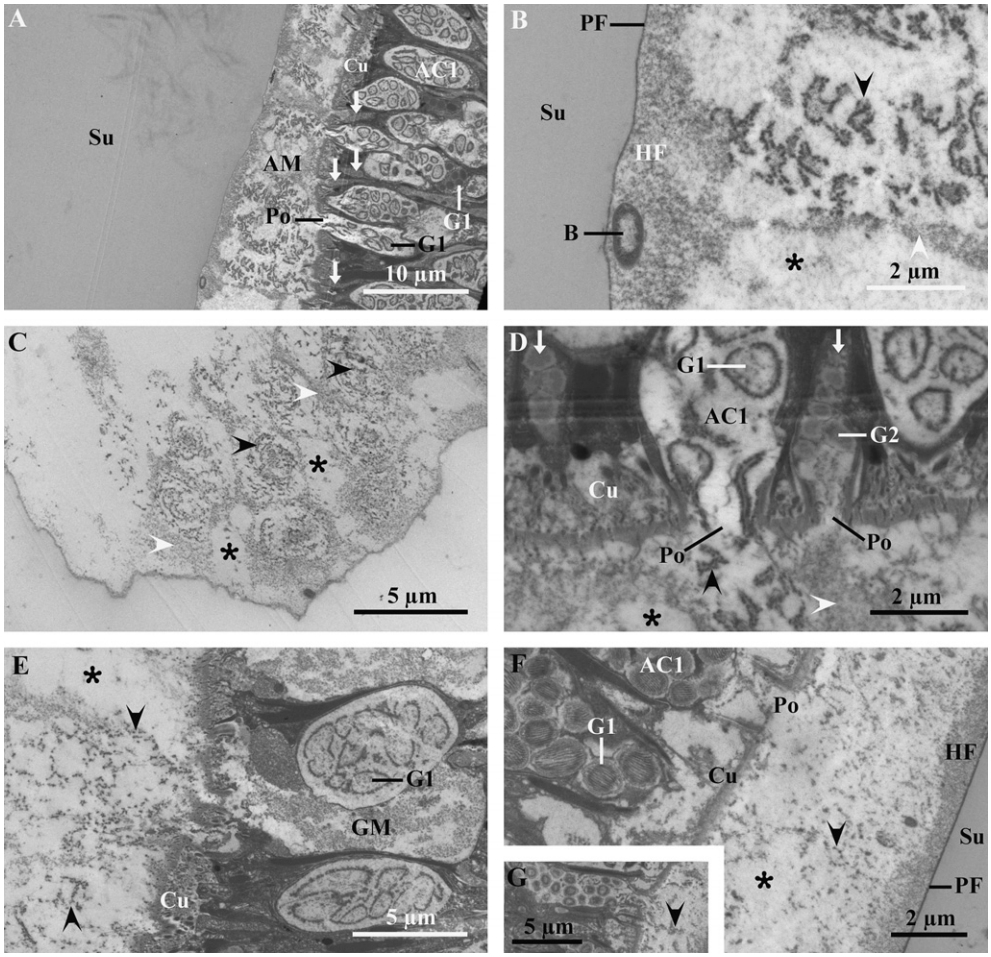

Fig. 5. TEM photographs of attached tube feet in *Asterias rubens*. (A and F) General views of longitudinal sections through the disc epidermis attached to the epoxy resin substratum by adhesive material. (B and C) Longitudinal and transverse sections through the adhesive material showing its different components. (D, E, and G) Details of the apical processes from the two types of adhesive cells and of the material they secrete. White arrows indicate type 2 adhesive cells. White and black arrowheads indicate the homogeneous granular material of medium electron-density and the heterogeneous electron-dense granular material present in the adhesive layer, respectively. Asterisks indicate the loose electron-lucent material. *Abbreviations:* AC1, type 1 adhesive cell; AM, adhesive material; B, bacterium; Cu, cuticle; G1, type 1 secretory granule; G2, type 2 secretory granule; GM, homogeneous granular material of medium electron density present in some type 1 adhesive cells; HF, homogeneous film; PF, primary film; Po, pore.

cent material observed inside and around modified type 1 granules (spheroids).

In the second pattern, small electron-dense granular particles are scattered within a layer made up of loose electron-lucent material and sandwiched between the thin homogeneous film and the disc surface (Fig. 5F and G). In the disc epidermis, most type 1 adhesive cells contain weakly swollen granules looking quite intact. In these granules, the rods seem on the verge of separating from each other (Fig. 5F). In some cells, swollen spheroids can be observed, as in the first pattern (Fig. 5G). A heterogeneous electron-dense granular material is released from these cells, and seems to spread and diffuse into the adhesive layer to form the small scattered electron-dense granular particles. As for type 2 adhesive cells, their appearance is similar to that described in the first pattern, except that they are not secreting any material.

The third pattern is always observed in samples in which the second pattern is also encountered. As in the second pattern, the homogeneous film is covered by a loose electron-lucent material enclosing electron-dense specks (Fig. 6A–C). In transverse section, these specks are not homogeneously distributed but appear to delineate mesh components (Fig. 6B). Several spheroid-like structures are found in the layer of adhesive material (Fig. 6C). They are filled with electron-lucent material and their periphery is made up of a compact electron-dense material forming accumulations at some places. In type 1 adhesive cells, different ultrastructures of the granules are observed. Some cells contain spheroids similar to those observed in the adhesive layer (Fig. 6A), as well as others in which the electron-dense material is not only compacted at the periphery but also forms several central structures. In several cells, a succession is observed in the ultrastructure of the secretory granules: granules located basally are just slightly swollen; progressing towards the apex of the cell, their rods start to gather at the periphery; and apically, they turn into spheroids (Fig. 6D). In some places, some empty apical processes from type 1 cells are noticed (Fig. 6A). In this third pattern, only one or two inconspicuous type 2 cells are observed.

In every tube foot, de-adhesive cells are scattered in the disc epidermis. These cells are not easily distinguishable in the first and the second patterns. In the third pattern, on the other hand, de-adhesive cells are conspicuous at the apex of the epidermis, presumably because the apical process of adhesives cells appears almost empty (results not illustrated).

4. Discussion

Sea stars have developed adhesion mechanisms to perform activities correlated with a benthic existence such as fixation, loco-

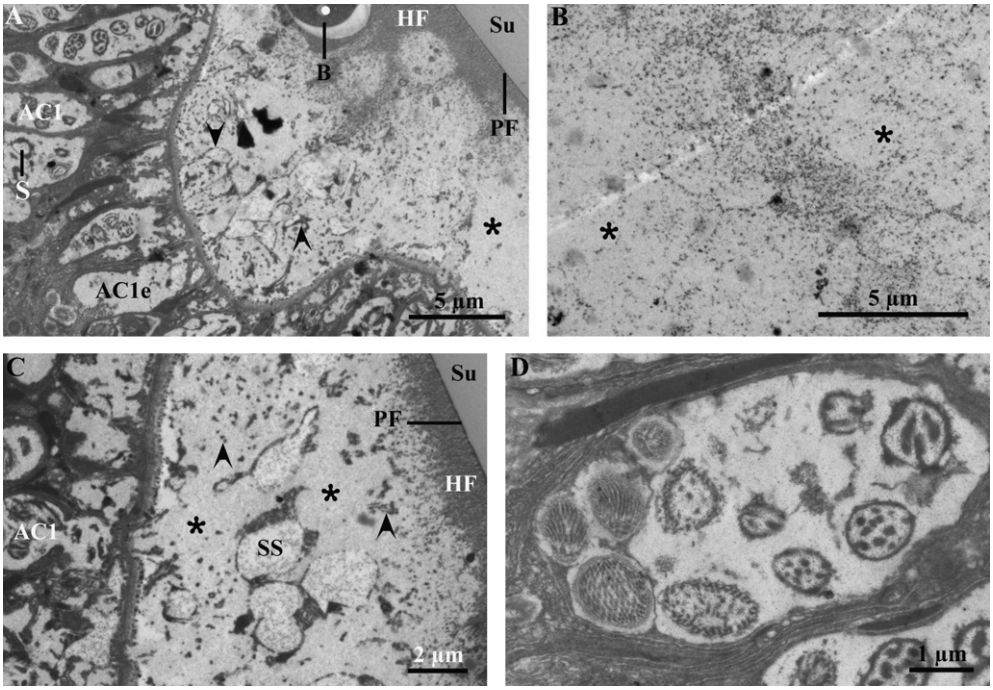

Fig. 6. TEM photographs of attached tube feet in *Asterias rubens*. (A) Longitudinal section showing adhesive material accumulating in a fold of the disc epidermis. (B) Transverse section through the adhesive material showing a meshwork pattern. (C) Spheroid-like structures in the layer of adhesive material. (D) Apical process of a type 1 adhesive cell in which a succession in the ultrastructure of the secretory granules is visible (the apex of the cell is on the right). Black arrowheads indicate the heterogeneous electron-dense granular material present in the adhesive layer. Asterisks indicate the loose electron-lucent material. *Abbreviations:* AC1, type 1 adhesive cell; AC1e, empty type 1 adhesive cell; B, bacterium; HF, homogeneous film; PF, primary film; S, spheroid; SS, spheroidal structure; Su, substratum.

motion and handling of food. In these organisms, attachment involves the release of an adhesive secretion between the tube feet and the substratum (Flammang, 2006). When tube feet voluntarily become detached, the adhesive material remains on the substratum as a footprint (Thomas and Hermans, 1985; Flammang, 2006).

The fine structure of the adhesive material deposited on various substrata by individuals of *A. rubens* was observed using LM, SEM, TEM, and AFM. Whatever the method used, it always appears as a sponge-like meshwork deposited on a thin homogeneous film. The thickness of the adhesive layer varies between different areas in a same footprint, giving different aspects to the adhesive material. In thin areas, meshes ranging between 1 and 5 µm in diameter are clearly distinguishable; whereas in thick areas, these meshes are obscured because of the accumulation of material. This microstructure of the adhesive material is identical before detachment (sections through attached tube feet) and after natural detachment (SEM of footprints) or forced peeling (SEM of adhesive material remaining on the substratum after chemical fixation and peeling). This demonstrates that the structure of the adhesive is neither altered by release of the de-adhesive secretion which occurs at tube foot detachment (Flammang et al., 1998, 2005) nor by separation instabilities such as fingering instabilities or cavitation (Gay, 2002).

The appearance of the adhesive material does not differ according to whether the footprints are fixed or not, and whether they are observed partially hydrated or dry. This is clearly different from what is observed for the adhesive released by the spores of the green alga Ulva. Under SEM, this adhesive appears as a reticulated network. On the other hand, AFM imaging underwater reveals a swollen gel-like pad around the spore, without any reticulated structure (Stanley et al., 1999; Callow et al., 2000). The latter was thus interpreted as an artefact of drying. In the case of sea star footprints, images obtained for hydrated samples are similar to those obtained for dried samples, confirming that the meshwork structure of the adhesive material is not an artefact of drying. In TEM, however, the meshes do not appear empty as in SEM but filled with a loose electron-lucent material. The presence of this loose material could be the reason why it is difficult to image sea star adhesive underwater with AFM (smearing effect). Indeed, AFM images of soft mucilage in diatoms show similar smear marks (Higgins et al., 2002). Comparison of the images obtained with the different techniques therefore suggests that, during drying (as for instance for sample preparation for SEM), the loose material would collapse, leaving empty mesh components on the thin homogeneous film.

Complex structures consisting of a micro-porous material (foam-like or sponge-like) deposited on the top of a homogeneous priming film seem to be common in adhesives from marine invertebrates. They have already been reported in several permanent adhesives such as the attachment plaques of the mussel byssus (Benedict and Waite, 1986; Waite, 1986; Waite et al., 2005), the basal cement of barnacles (Naldrett, 1993; Berglin and Gatenholm, 2003; Wiegemann and Watermann, 2003; Kamino, 2008), and the building cement of tubicolous polychaetes (Stewart et al., 2004; Stevens et al., 2007). Micro-porous reticulated structures have also been described in non-permanent adhesives—e.g., in diatoms, monogenean flatworms or barnacle larvae (Hamwood et al., 2002; Higgins et al., 2003; Phang et al., 2008)—but, to the best of our knowledge, this is the first time that a continuous priming film underlying the reticulated network is described in these non-permanent adhesives.

The thickness of the footprint is influenced by the substratum on which it is deposited. Indeed, although footprints left on glass, mica and Teflon have the same shape and diameter, the quantity of material deposited by the tube feet varies according to the substratum tested. Tube feet produce more adhesive material on glass and mica (two high-energy surfaces) than on Teflon (a low-energy surface). Variations in the quantity of material deposited according to the substratum have also been reported in barnacles, although in an opposite way. In these permanently-attached organisms,

cement laid down on a low-energy surface is thicker than cement deposited on a high-energy surface (Berglin and Gatenholm, 2003; Wiegemann and Watermann, 2003; Sun et al., 2004). This peculiar feature is thought to be a result of downward growth of the parietal plates and subsequent detachment of the weakly adhered base area, leading to the formation of a thick, multi-layered adhesive plaque. In sea stars, on the other hand, adhesion is temporary. If the substratum does not allow the formation of a firm holdfast (like Teflon), the sea star presumably detaches voluntarily at an early stage, leaving only a low amount of adhesive material on the substratum.

In structurally complex adhesives, the different components usually fulfil different functions (Waite et al., 2005; Kamino, 2008). Most marine adhesives, including sea star adhesive, are protein complexes, sometimes including polysaccharides too (Smith and Callow, 2006). The proteins constituting the adhesive have been characterized in the attachment plaque of mussels and in the cement of barnacles (see Kamino, 2008 and Silverman and Roberto, 2007 for reviews). Those constituting the priming film, rich in polar and charged residues, displace water from the substratum, form strong adhesive bonds with the surface, and provide cohesive coupling with the other components of the adhesive material (Waite and Qin, 2001; Lin et al., 2007; Urushida et al., 2007). Analogous components are probably involved in the formation of the homogeneous film within the adhesive of *A. rubens*. In SEM, this film has an identical morphology on Teflon, glass and mica. Similarly, in barnacles, Urushida et al. (2007) demonstrated that the surface-coupling protein (Mrcp-19 k) adsorbs readily to surfaces with various characteristics, including negatively charged, positively charged, and hydrophobic surfaces, in water. Naturally occurring surfaces may be heterogeneous in terms of surface characteristics. Adhesive materials able to adsorb to various surfaces are thus advantageous for marine invertebrates. In mussels and barnacles, another set of proteins make up the bulk of the adhesive (Kamino, 2008; Silverman and Roberto, 2007). These proteins self-organize into a porous layer whose advantages include economy of material, compliance, and crack-stopping behaviour (Wiegemann and Watermann, 2003; Waite et al., 2005). Once again, the components constituting the meshwork observed in the adhesive of *A. rubens* may provide similar benefits for sea star adhesion.

At the nanometre scale, both the homogeneous film and the material forming the meshwork are composed of a succession of globular nanostructures. The diameter of these structures is almost the same for dry footprints observed either with SEM or AFM (50 to 100 and 50 to 90 nm, respectively). On the other hand, AFM investigations on partially hydrated footprints reveal a globule diameter ranging between 80 and 200 nm. This slight difference in the size of the globular structures between dry and hydrated footprints suggests once again that the adhesive material may shrink upon drying. Interestingly, the same kind of globular structures have already been reported in other marine adhesive secretions, such as the cements of barnacles and tubicolous polychaetes (Berglin and Gatenholm, 2003; Wiegemann and Watermann, 2003; Stevens et al., 2007). In these organisms, the size of the globular structures

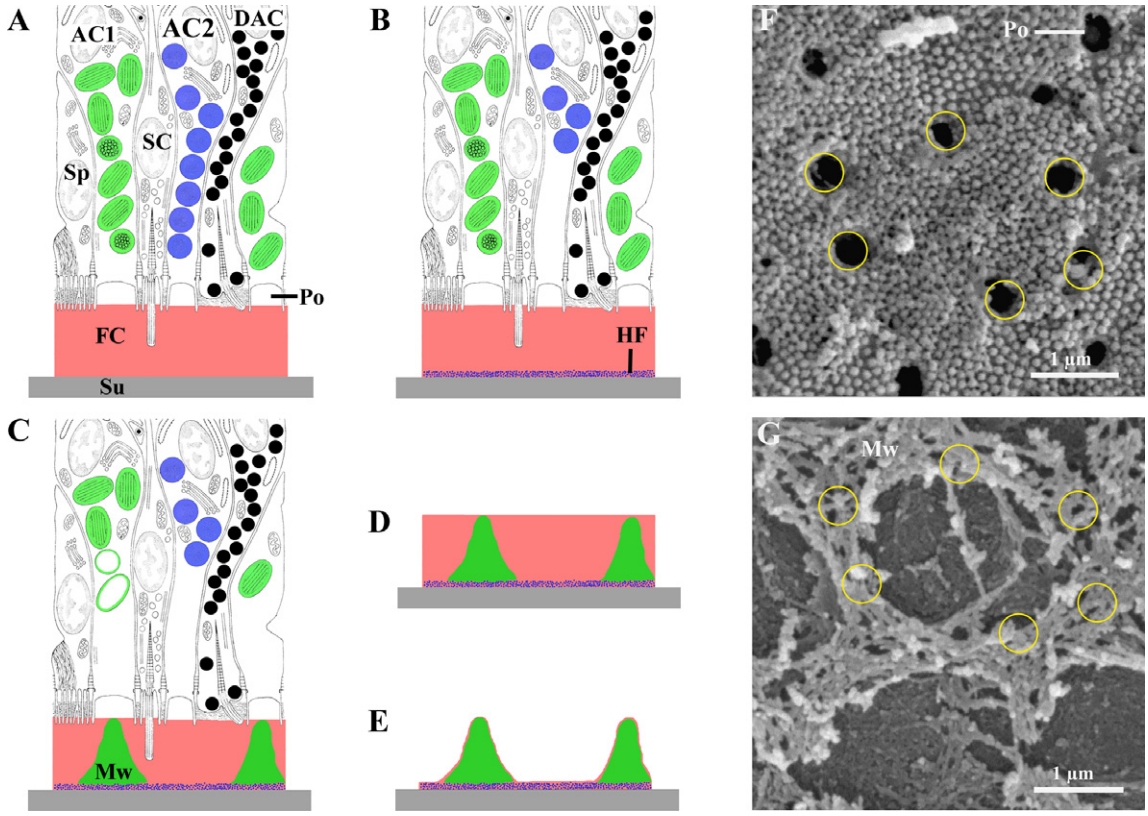

Fig. 7. Hypothesis about the mode of formation of the adhesive material micro-structure in the footprints of *Asterias rubens*. (A–E) Diagrammatic representations of longitudinal sections through the disc epidermis of a tube foot illustrating the proposed events in the secretion of the adhesive material (adapted from Flammang et al., 1994; not to scale). When a tube foot attaches to the substratum, it first contacts the surface with its thick fuzzy coat (A). Upon contact, type 2 adhesive cells release their contents, presumably one or several surface active molecules, which form a homogeneous film covering the substratum (B). Almost simultaneously, type 1 adhesive cells start to release their contents, molecules with a bulk function, which expand and form a thick meshwork structure (C). When the tube foot detaches itself, the adhesive material and the fuzzy coat are left on the substratum as a footprint (D). Upon drying, the fuzzy coat collapses giving the footprint its characteristic aspect. (F–G) SEM photographs taken at the same magnification and showing the distal surface of a tube foot (F) and a mesh component of a footprint (G). Six pores of the disc surface have been highlighted by circles in (F) and the form obtained has been superimposed upon the mesh component in (G). The match clearly indicates that the size and shape of the mesh components are the direct result of the repartition of the type 1 adhesive cells in the disc epidermis. *Abbreviations:* AC1, type 1 adhesive cell; AC2, type 2 adhesive cell; DAC, de-adhesive cell; FC, fuzzy coat; HF, homogeneous film; Mw, meshwork; Po, pore; SC, sensory cell; Sp, support cell; Su, substratum.

is in the same range as that of their equivalents described in dry sea star adhesive footprints (about 80 nm for barnacles and 50 to 100 nm for polychaetes). In the polychaete adhesive, contrary to what occurs in sea star footprints, the size of the globular structures does not vary according to whether the cement is dry or wet (Stevens et al., 2007).

Previous investigations have clearly demonstrated that the adhesive material in sea stars is released by the two types of adhesive secretory cells occurring in the disc epidermis of the tube feet (Flammang et al., 1994, 1998). However, how these secretions form the complex micro-structure of the footprint is not known. Our observations made in SEM and TEM on attached tube feet provide some indications about footprint formation (Fig. 7). In the disc epidermis, the apparent proportion between the two types of adhesive cells, as well as their apex ultrastructure, differ from what Flammang et al. (1994) described for tube feet which did not contact any substratum. The morphology of type 1 adhesive cells varies from cells with an apical process packed with intact granules to cells with an empty process. In all cases, however, these cells are conspicuous and easily distinguishable on the sections. On the other hand, type 2 adhesive cells are always much less conspicuous than in unattached tube feet, their apical processes being always smaller and containing fewer granules. This suggests that type 2 cells would be the first ones to release their contents, being therefore responsible for the formation of the homogeneous film covering the substratum. Indeed, the material released by these cells has the same appearance as the one constituting this film. As discussed previously, this priming film could displace water and bind strongly to the surface. Meanwhile, type 1 cells start to release their contents, a heterogeneous electron-dense material. At the beginning of this stage, the two constituents, secreted by their respective cells, alternate in the adhesive secretion (first pattern). The material from type 1 adhesive cells, which undoubtedly derives directly from the rods constituting the granules, seems to expand gradually either inside the cells or after its release. Indeed, in some cases, it is secreted as spheroidal structures, i.e., clusters of granules released together, visible in both SEM and TEM. In some areas on the disc surface of the detached tube feet, the secreted material expands and fuses with the one released from other cells, initiating the formation of a meshwork. Type 1 cells would be therefore at the origin of the meshwork pattern and the arrangement of their secretory pores on the disc surface could act as a template for the formation of this pattern (Fig. 7F and G). The third constituent of the adhesive layer, the loose electron-luscent material, is always observed in-between the different materials secreted by the two types of adhesive cells. This material could originate directly from the adhesive cells, in which a similar material is observed inside and around modified type 1 granules; or, alternatively, it could correspond to the outermost layer of the cuticle covering the disc epidermis, the so-called "fuzzy coat". Indeed, McKenzie and Grundy (1999) proposed that in natural hydrated conditions, the fuzzy coat could form a rather thick gel over the disc surface. This was corroborated by observations made on cryofixed tube feet in TEM (Ameye et al., 2000) as well as in cryo-SEM (Gorb and Flammang, unpubl. obs.). It is that gelatinous fuzzy coat, presumably made up of proteoglycans (Ameye et al., 2000), that could collapse when footprints are dried. According to the model proposed by Flammang et al. (1998), this layer is indeed detached from the disc surface and incorporated into the footprints through the release of the de-adhesive secretion.

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