

COMMENTARY

Properties of temporary adhesion systems of marine and freshwater organisms

Birgit Lengerer^{1,*} and Peter Ladurner²

ABSTRACT

Underwater adhesive secretions are a promising source of inspiration for biomedical and industrial applications. Although marine permanent adhesives have been extensively investigated, reversible adhesion, e.g. as used for locomotion and feeding, is still poorly understood. Here, we summarise the current knowledge on secretion-based, temporary adhesive systems in aquatic environments, with a special emphasis on the morphology and structure of adhesive organs and adhesive material. Many animals employing temporary adhesion to the substratum rely on so-called duo-gland adhesive organs, consisting of two secretory gland cells and one supportive cell. We give a detailed depiction of a basic duo-gland adhesive organ and variations thereof. Additionally, we discuss temporary adhesive systems with an alternative building plan. Next, the topography of secreted adhesive footprints is described based on examples. The limited data on the composition of temporary adhesives are summarised, separating known protein components and carbohydrate residues. There are still large gaps in our understanding of temporary adhesion. We discuss three proposed models for detachment, although the actual mechanism of voluntary detachment is still a matter for debate.

KEY WORDS: Duo-gland adhesive organ, Bioadhesion, Footprint, Biological glue

Introduction

Recent studies have proven the high potential of using bio-inspired adhesives for biomedical applications (Kim et al., 2016; Li et al., 2017; Zhao et al., 2017; Zhu et al., 2017). The requirements for medical adhesives are exacting. Such adhesives must be biocompatible and adhere strongly to various surfaces while staying elastic, and they should preferably work under wet conditions. All available commercial adhesives compromise one or more of these aspects (Vinters et al., 1985; Li et al., 2017). Synthetic adhesives are often toxic, carcinogenic, allergenic, elicit environmental concerns and/or fail to comply with legislative restrictions. In contrast, adhesives occurring in nature are biocompatible, non-toxic and capable of adhering to a variety of surfaces, including in dry, wet or underwater environments. They can also be permanent or temporary and do not provoke exothermic reactions. Therefore, there is a growing body of research focused on the characterisation and biomimetic utilisation of biological adhesive systems.

Biological attachment is a common feature among many species (von Byern and Grunwald, 2010; Peled-Bianco and Davidovich-Pinhas, 2015; Smith, 2016). As the conditions for adhesion (see

Glossary) in aquatic and terrestrial environments are substantially different (Ditsche and Summers, 2014), only underwater adhesion is discussed here. The wide variety of attachment systems can be ordered according to various features, such as the fundamental physical mechanisms underlying their operation, their biological function and the duration of their contact (Gorb, 2012). Attachment can be permanent, temporary or transitory (Whittington and Cribb, 2001). Temporary adhesion is defined as reversible adhesion allowing voluntary separation of the surfaces after a certain interval, whereas transitory adhesion enables simultaneous attachment and movement along a substrate, for example, as in gastropods (Flammang, 1996). However, it is difficult to clearly distinguish between temporary and transitory. Here, we use the term ‘temporary adhesion’ to refer to attachment that can be released voluntarily and in which the contact between adhesive organs and the attached surface is lost upon detachment.

Many attachment devices have evolved independently and cover diverse biological functions. For example, animals of the interstitial meiofauna (i.e. organisms that live between sand granules of marine or freshwater beaches) must secure themselves to the substrate to avoid displacement from their environment. At the same time, many of these species exhibit a highly mobile lifestyle. An attachment–release system allows them to cope with these requirements.

In recent decades, the research on adhesive secretions has mainly focused on marine, permanently attaching animals, such as mussels and barnacles (reviewed in Kamino, 2010; Maier and Butler, 2017; Waite, 2017). In contrast to the substantial advances in the understanding of permanent adhesives, the field of temporary adhesion is largely unexplored.

Here, we summarise current knowledge on underwater reversible adhesive systems. Because the focus is on temporary adhesion, the transitory adhesion (see above) present in gastropods is not discussed. Many aquatic reversibly attaching animals possess a duo-gland adhesive system, which allows them to rapidly attach and release from the substrate. However, not all reversibly attaching animals rely on this structure; therefore, alternative states of organization of the adhesive mechanism are discussed as well. We summarise the known protein and carbohydrate components of temporary adhesives. After voluntary detachment, the adhesive material stays behind on the substrate and is named the adhesive ‘footprint’ (see Glossary). One open question concerning temporary adhesive systems is how quickly voluntary detachment can be achieved. We describe three detachment models that have been proposed and discuss the likelihood that they play meaningful roles in different organisms.

Duo-gland adhesive organs

Duo-gland adhesive organs have been described in echinoderms (Hermans, 1983; Flammang et al., 1994), gastrotichs (Boaden, 1968; Tyler and Rieger, 1980), nematodes (Adams and Tyler, 1980), and free-living (Tyler, 1976, 1977) and parasitic flatworms (El-Naggar

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Glossary**Adhesion**

The connection between dissimilar particles or surfaces.

Annotated protein

Protein with described structure and function. By comparing newly found sequences with protein databases, assumptions as to their functional, structural and physiochemical properties can be made.

Antennule

Paired antennules that are present at the seventh larval stage of barnacles, the cyprid. The antennules are segmented and highly specialized for surface exploration and temporary attachment.

Cilia

Cellular protrusions with various functions. In contrast to microvilli, they contain microtubules for mechanical support and for motility.

Cohesion

The intermolecular attraction of similar or identical molecules.

Cyprid

The seventh and final larval stage in the development of barnacles.

Footprint

The adhesive material that is left behind on the substrate when a temporary adhering animal moves on.

Glycoconjugate

A carbohydrate that is covalently linked to a non-sugar moiety such as a protein, peptide or lipid.

Glycoprotein

Protein with covalently linked oligosaccharide chains on its amino acid chains.

Gregarious settlement

Barnacles are obligatory cross-fertilizing and therefore require potential mating partners in close proximity. As the adults are sessile, this proximity is achieved through gregarious settlement behaviour of the cyprid larvae.

Hemidesmosome

A multiprotein complex that connects epithelial cells to the extracellular matrix.

Intermediate filament

Cytoskeletal component that plays an essential role in the cell integrity of many tissues.

Lectin

A carbohydrate-binding protein that specifically binds different sugar moieties. Owing to their selectivity, lectins can be used for the identification of carbohydrate residues in tissues or protein extracts.

Microvilli

Cell protrusions with various functions, including absorption, secretion, cellular adhesion and mechanotransduction.

Next-generation sequencing

High-throughput sequencing method in which a large number of DNA sequences are processed in parallel. Bioinformatics is used to reconstruct the original DNA or RNA sequence.

Transmission electron microscopy (TEM)

A microscopy technique in which an electronic beam is transmitted through a section of a specimen to achieve high-resolution images of the internal structure.

Western blot

A method to detect proteins in a tissue homogenate or extract, using protein-specific antibodies.

and Kearn, 1983; Cribb et al., 1998). Most duo-gland adhesive organs consist of three cell types: adhesive (viscid) gland cells, releasing (de-adhesive) gland cells and supportive cells (also called anchor cells). It was proposed that the adhesive gland cells expel the proteinaceous glue, the releasing gland cells produce a de-adhesive substance, and the anchor cells provide mechanical support (Tyler, 1976; Hermans, 1983). Previously, Boaden (1968) had been the first to investigate the adhesive organs of the interstitial gastrotroch *Turbanella hyalina* at an ultrastructural level. Boaden (1968), based on an idea by Dr Erwin, described the presence of two secretory

glands and suggested that they might either both simultaneously secrete a polymerising agent or that one gland might secrete a material that polymerises on contact with seawater, whereas the other secretes a de-polymerising agent. Boaden (1968) was the first publication to propose a secretion-based temporary adhesive system in interstitial invertebrates. The term 'duo-gland adhesive organ' was later defined by Tyler during his intensive studies of adhesive organs in Turbellaria (Tyler, 1976). His detailed descriptions lay the foundation for the discovery of duo-gland adhesive organs in various other invertebrates (Tyler, 1977; Tyler and Rieger, 1980; El-Naggar and Kearn, 1983; Cribb et al., 1998). Based on Tyler's definition, Hermans (1983) proposed the presence of duo-gland adhesive organs in echinoderm tube feet. Prior to this, echinoderm tube feet were believed to attach using suckers, but Hermans' observations were confirmed in burrowing echinoids, which possess sensory-secretory complexes that share many features with the duo-gland adhesive organs in Turbellaria (Flammang et al., 1991).

The minimal unit of a duo-gland adhesive organ comprises one cell of each cell type and can be found in free-living flatworms of the order Macrostomida, such as *Macrostomum lignano* (Figs 1 and 2A) (Lengerer et al., 2014). In *M. lignano*, the adhesive organs are positioned at the tip of the tail plate (Fig. 1A,B). Both gland cells form long unbranched necks, which together penetrate one anchor cell (Fig. 1C–E). The anchor cell forms a collar of strengthened microvilli (see Glossary) surrounding the necks of both gland cell types (Fig. 1E,F). Both gland cells secrete their vesicles, containing the adhesive and the releasing material, at the tip of this modified microvilli collar. We presume that the tips of the microvilli become attached to the surface by adhesive secretions, and the tension during attachment is transmitted through the anchor cells. The anchor-cell-specific intermediate filaments (see Glossary) connect to hemidesmosomes (see Glossary) and disperse the tension to the extracellular matrix of the tail plate (Fig. 1G). Within the microvilli collar of the anchor cell, the adhesive gland cell is located ventrally to the releasing gland cell (Figs 1H and 2A) (Lengerer et al., 2014).

In the proseriate flatworm *Myozona* sp., the building plan is equally simple, but instead of one adhesive gland cell, two project through each anchor cell (Fig. 2B). The releasing gland cell neck is always located in between the two adhesive gland cell necks. A dense row of cilia (see Glossary) additionally encircles and probably supports the modified microvilli (Fig. 2B) (Tyler, 1976). Among the Macrostomida, *Paromalostomum* sp. has been described as one of the most adhesive species (Tyler, 1976). This is likely due to wide distribution of adhesive organs over the entire body length of this species. In addition, the topology of the adhesive area could play a role (Fig. 2C). The outer part of the adhesive organs, the adhesive papilla, is folded into longitudinal ridges, so that the papilla appears star-shaped. One single adhesive gland broadens to this star-shaped structure, while the releasing gland cell branches and forms five to seven necks located in the grooves between the adhesive gland cell folds (Fig. 2C) (Tyler, 1976). Presumably, this topography allows maximizing the attachment area without increasing the number of cells involved.

Whereas in basal flatworms both gland cells secrete through the microvilli collar, higher flatworms and echinoderms have separate openings for each gland cell neck (Rieger et al., 1991; Flammang, 1996). Species that live in habitats with strong water currents consequently exhibit exceptionally well-developed adhesive organs. One example is the proseriate *Otoplanid* sp., which in its natural environment is exposed to strong wave action. The adhesive organs appear as broad cushion-shaped papillae, with numerous adhesive and releasing gland necks protruding on the entire surface

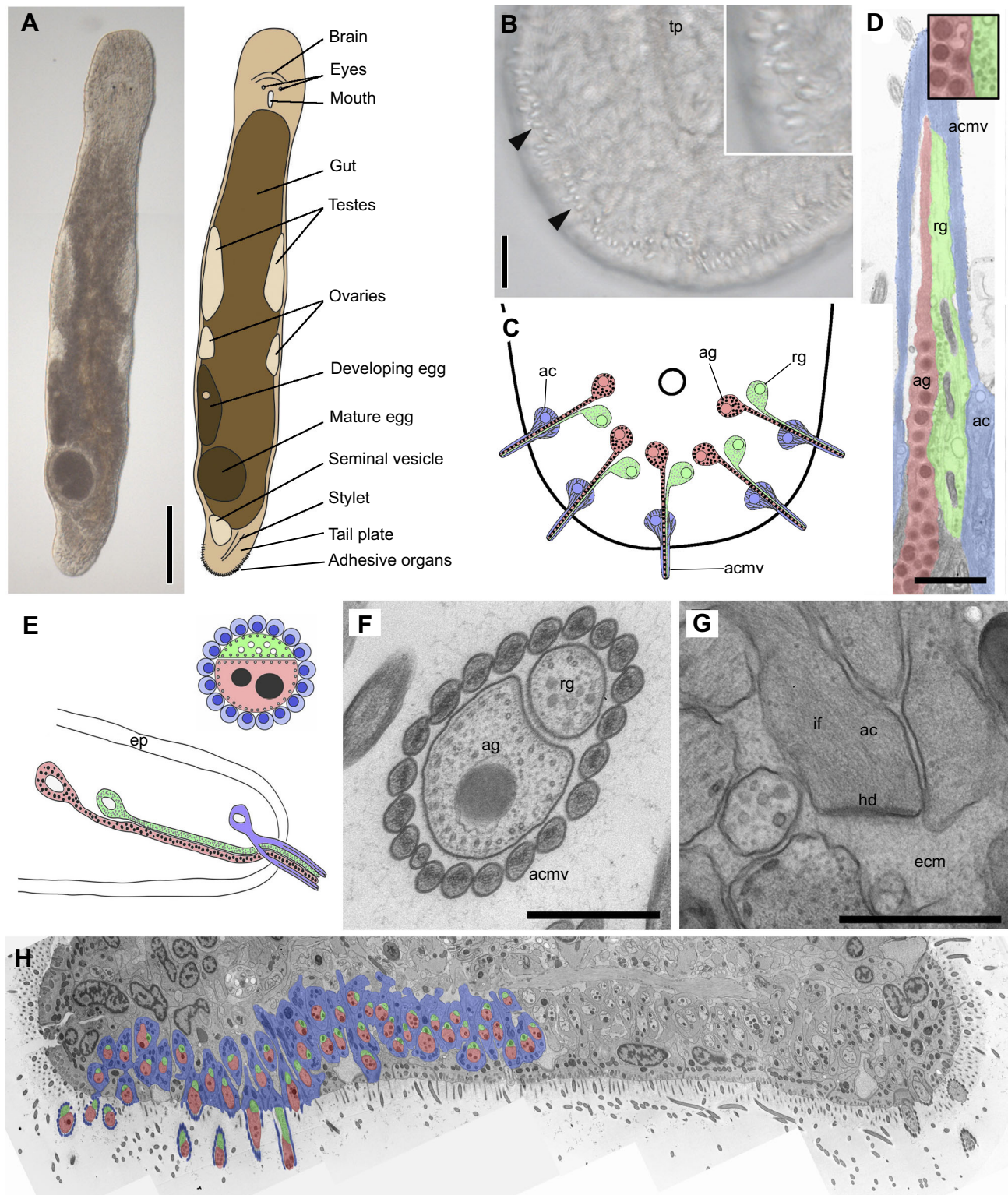


Fig. 1. Morphology of a simple duo-gland adhesive organ, illustrated using the marine flatworm *Macrostomum lignano* as an example. The presented adhesive system resembles the simplest possible duo-gland adhesive organ, consisting of only three interacting cells. (A) Interference contrast image and schematic drawing. (B) Interference contrast image of the ventral side of the tail plate, visible parts of the adhesive organs highlighted with arrowheads. (C,E) Schematic drawings of the location of the three main duo-gland cell types, anchor cell (blue), adhesive gland cell (red) and releasing gland cell (green). (D,F) Transmission electron microscopy (TEM) images of the adhesive organ at the level of the microvilli collar in (D) sagittal plane and (F) cross-section. (G) TEM image of basal cytoplasmic extension of an anchor cell (ac) with intermediate filaments (if); the cell is connected to the extracellular matrix (ecm) via a hemidesmosome (hd). (H) Cross-section TEM image through the tail plate. ac, anchor cell; acmv, anchor cell microvilli; ag, adhesive gland cell; ecm, extracellular matrix; ep, epidermis; hd, hemidesmosome; if, intermediate filaments; mt, microtubules; rg, releasing gland cell; tp, tail plate. Scale bars: (A) 200 μ m, (B) 10 μ m, (D) 1 μ m, (F,G) 0.5 μ m. Images modified after Lengerer et al. (2016, 2014).

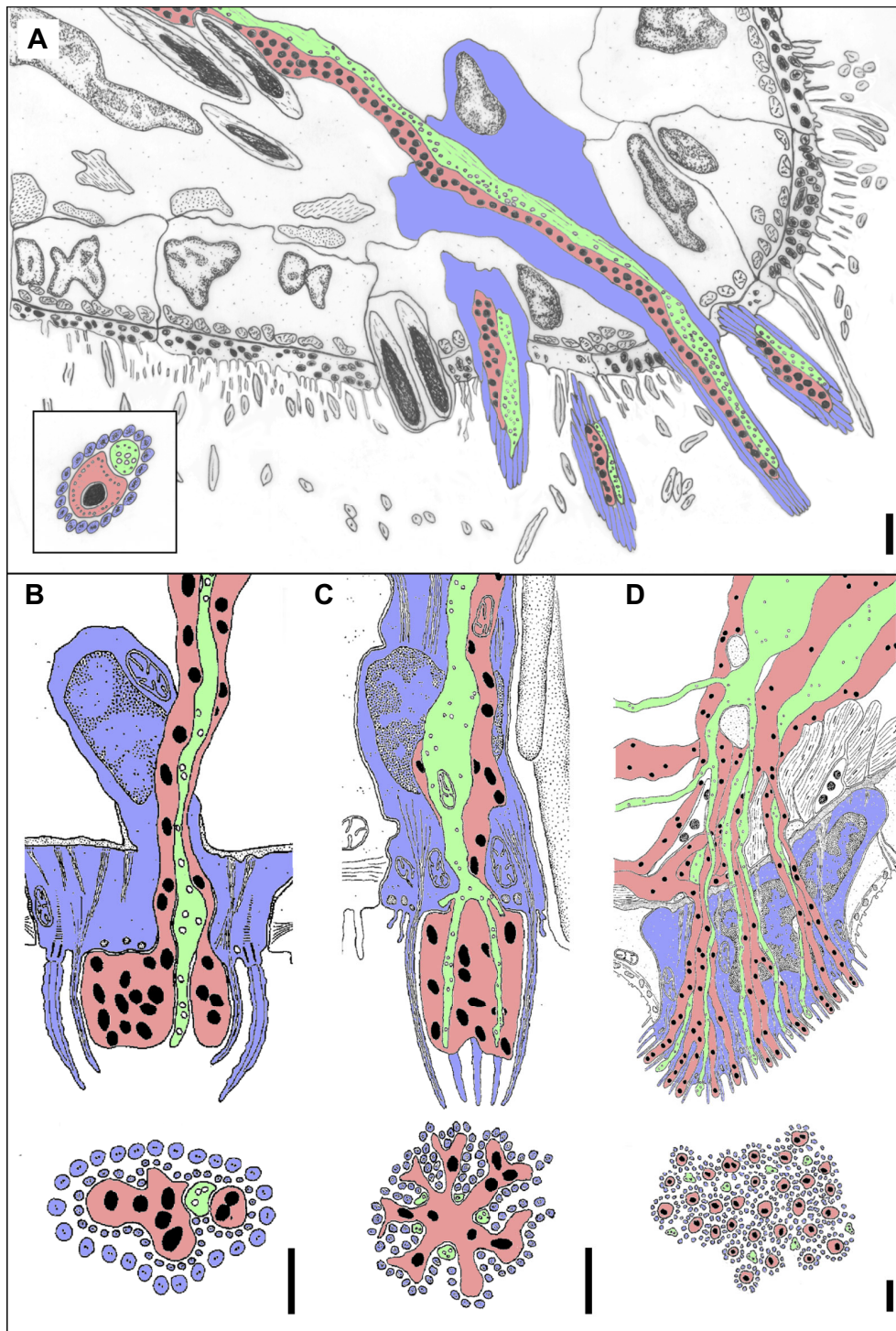


Fig. 2. Topology of flatworm duo-gland adhesive organs. The drawings resemble duo-gland adhesive organs of varying complexity in morphology and involved cell number. The variation in topology most likely reflects phylogenetic relationships and adaptations to different habitats. (A) *Macrostomum lignano*, (B) *Myozona* sp., (C) *Paromalostomum* sp. and (D) *Otoplanid* sp. Adhesive gland cells are indicated in red, releasing gland cells in green, and anchor cells in blue. Shown are drawings of longitudinal sections through adhesive organs at the level of the epidermis (on top) and transverse sections at the level of the adhesive papilla (below and inset in A). (A) Original drawing; (B–D) adapted by permission from Springer Nature: Springer Zoomorphologie, Comparative ultrastructure of adhesive systems in the Turbellaria, Tyler 1976. Scale bars: 1 µm.

of the anchor cells (Fig. 2D). Adhesive and releasing gland cells are highly branched and penetrate more than one anchor cell. The adhesive gland cell necks are more numerous and are surrounded by microvilli. By contrast, releasing gland cell necks simply project between the microvilli collars of the adhesive gland necks (Fig. 2D) (Tyler, 1976).

Although the diameter of the adhesive area alone in echinoderms surpasses the entire body size of the animals described above, the building plan of the echinoderm duo-gland system shares striking similarities with that of flatworms (Fig. 3) (Flammang et al., 1994;

Flammang, 1996). Echinoderms use numerous hydraulic tube feet for their locomotion (Fig. 3A). The area of attachment is the tube foot disc, which in sea stars is completely covered with microvilli and secretory pores of the adhesive system (Fig. 3B). In sea urchins, secretory pores are absent, and the adhesive and releasing granules are expelled through microvillar-like projections. Generally, the echinoderm duo-gland adhesive system consists of supportive cells with numerous microvilli and one (in sea urchins) or two (in most sea stars) adhesive gland cells and a releasing (de-adhesive) gland cell (Fig. 3C,D). Similar to higher flatworms, the adhesive gland necks of

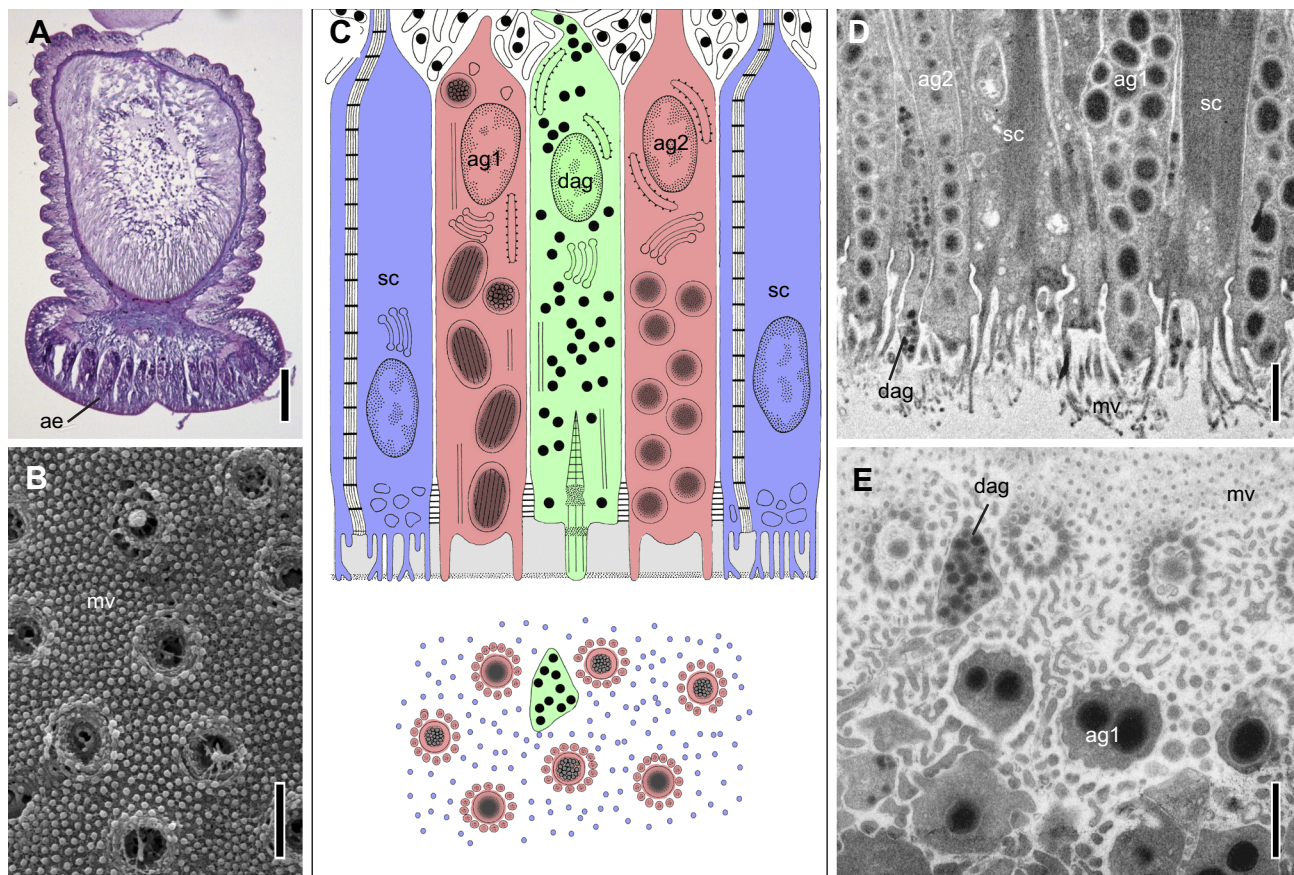


Fig. 3. Topology of an echinoderm adhesive epidermis, illustrated using the sea star *Asterina gibbosa* as an example. (A) Longitudinal histological section through an adhesive tube foot. (B) Scanning electron microscopy (SEM) image of the adhesive epidermis surface, showing the microvilli layer and secretory pores. (C) Schematic drawings of a longitudinal section of a sea star duo-gland adhesive organ (top) with supportive cells (blue), adhesive gland cells (red) and a releasing gland cell (green). Transverse section (bottom) of the distal region of the adhesive organ, demonstrating the arrangement of the adhesive gland necks with the microvilli collar (red), the releasing gland (green) and the microvilli (small blue circles). Drawings are not to scale. (D,E) TEM images of the adhesive epidermis in a (D) longitudinal and (E) transverse section at the level of the microvilli. ag, adhesive gland cell; ae, adhesive epidermis; dag, de-adhesive gland cell; mv, microvilli; sc, supportive cell. Original images. Scale bars: (A) 100 µm; (B,D,E) 1 µm.

sea stars are surrounded by a microvilli collar, while the releasing gland cell lack this supportive structure (Fig. 3E) (Flammang, 1996).

The role of secretory cells

In the sea star *Asterias rubens*, five cell types can be found in the adhesive dermis: two non-ciliated adhesive gland cell types, ciliated releasing gland cells, non-secretory ciliated cells and support (anchor) cells. The releasing gland cells exhibit neurosecretory-like features, with the basal end penetrating the nerve plexus and a subcuticular cilia. Therefore, the release of vesicles is likely triggered by the nervous system (Flammang et al., 1994). Evidence for the function of the different gland cell types has historically mainly been based on morphological (Tyler, 1976; Hermans, 1983; Flammang et al., 1994) and immunohistochemical studies (Flammang et al., 1998). Tyler (1976) fixed several free-living platyhelminths for transmission electron microscopy (TEM; see Glossary) at the moment of attachment and observed which vesicles had been secreted. Based on his observations, he classified cells as either adhesive or releasing gland cells. A similar approach was later followed in echinoderms, leading to the same conclusions (Flammang et al., 1994, 1998; Hennebert et al., 2008; Santos et al., 2009b). In *A. rubens*, the adhesive footprint was used for polyclonal antibody production (Flammang et al., 1998). The antibodies led to a strong staining within the two adhesive gland cells, confirming

that they are the main source of the adhesive material. In contrast, the releasing gland cells showed no immunoreactivity, indicating that they do not significantly contribute to footprint material (Flammang et al., 1998). Furthermore, when sea stars were allowed to voluntarily detach, the releasing gland cells of the corresponding tube feet appeared empty, indicating that they had secreted their vesicles (Flammang, 1996). The appearance of adhesive and releasing vesicles shares striking similarities among different taxa. In most investigated species, the adhesive vesicles contain at least two materials of different electron density, whereas the releasing vesicles appear homogeneous (Tyler, 1976; Flammang, 2006; Santos and Flammang, 2006; Lengerer et al., 2014). In addition, adhesive vesicles tend to be larger than releasing vesicles and more numerous over the adhesive area (Tyler, 1976; Flammang, 2006; Santos and Flammang, 2006). Although the function of the adhesive gland cells in glue production seems obvious today, the role of the releasing gland cells is still debated (see ‘How organisms effect detachment’, below).

The role of anchor cells

Another common feature of duo-gland adhesive systems is the presence of supportive anchor cells with an enforced filamentous network (Harris and Shaw, 1984; Silveira, 2006; Tyler, 1976, 1977). It was proposed that the filaments are required to bear the

tension forces during attachment (Tyler, 1976). In the free-living marine flatworm *M. lignano*, anchor-cell-specific intermediate filaments have been identified (Lengerer et al., 2014, 2016). Upon knockdown of these intermediate filaments, the morphology of the anchor cells and their modified microvilli was severely impaired and the animals failed to efficiently attach themselves to the substrate (Lengerer et al., 2014). The presence of anchor-cell-specific intermediate filaments has also been demonstrated in the freshwater flatworm *Dugesia japonica* (Tazaki et al., 2002), indicating that there is a conserved role for these filaments among marine and freshwater species. In *M. lignano*, a similar phenotype was achieved via the knockdown of an anchor-cell-specific formin-like gene (Lengerer et al., 2018). The knockdown resulted in anchor cells with drastically shortened microvilli that lacked their characteristic actin-dense inner core. The phenotype resembled that obtained by knockdown of intermediate filaments, leading to non-adhesive animals (Lengerer et al., 2018). In both knockdowns, the morphological aberrations were restricted to the anchor cells and the morphology of adhesive and releasing glands was not affected. These findings provide experimental evidence that the morphological integrity of supportive cells is essential for the adhesive process. In echinoderms, the supportive cells are the most abundant cells of the adhesive epidermis (Flammang, 1996). Similar to flatworms, they are densely filled with intermediate filaments and connect to collagen of the connective tissue. Together with the adhesive gland cells, supportive cells form numerous microvilli, which cover the surface of the adhesive disc (Flammang, 1996).

Nevertheless, not all species with a duo-gland adhesive system possess specialised anchor cells. For example, in gastrotrichs, a duo-gland adhesive system has been described, but the anchor cells were missing (Tyler and Rieger, 1980). Instead, the adhesive tension in these animals is thought to be supported by the prominent cuticle and cytoskeletal fibres in the gland cells themselves (Tyler and Rieger, 1980).

Other 'building plans'

Duo-gland adhesive systems are widespread, but they are not the only building plan that enables reversible adhesion. Even in the phylum Platyhelminthes, in which duo-gland organs are commonly present, alternative adhesive systems have been described (Tyler, 1976; Whittington and Cribb, 2001). The main difference to duo-gland adhesive systems is the mechanism of detachment, which in alternative adhesive systems relies on mechanical forces, rather than a releasing secretion (see 'How organisms effect detachment'). For example, the kalyptorhynch *Schizochilus caecus* possesses two, morphologically distinct adhesive glands (Ehlers, 1989). In the ectoparasite *Entobdella soleae*, contradicting observations about the function of the two glands were made (El-Naggar and Kearn, 1983; Kearn and Evans-Gowing, 1998). Initially described as a duo-gland adhesive system (El-Naggar and Kearn, 1983), later findings supported the theory that both glands contribute to the adhesive material (Kearn and Evans-Gowing, 1998). Assigning the function of the two glands based solely on morphological studies is challenging, and the results are often ambiguous. For example, the cephalopod *Euprymna scolopes* uses dermal secretions to coat itself completely with sand. When threatened, it instantaneously releases the sand to mislead potential predators (Singley, 1982). Singley (1982) described the presence of a duo-gland adhesive system, but in a recent study, it was proposed that the glands provide different components of the glue, and the rapid release is achieved through muscular movements (von Byern et al., 2017).

Barnacles are primarily known for their strong, permanent attachment, but before they settle, at their last larval stage, the cyprids (see Glossary) search for an optimum location to undergo metamorphosis. While testing suitable surfaces, the cyprids rapidly attach and detach using their paired antennules (see Glossary) (Walker, 1981; Aldred and Clare, 2008). The morphology of the cyprid adhesive system has been comprehensively described in various species, and it appears that the temporary glue is produced in different gland cells than the later secreted permanent cement (Nott and Foster, 1969; Walker, 1971; Yap et al., 2017). In the stalked barnacle *Octolasmis angulata*, the temporary adhesive glands of the cyprid are located within the mantle and form long, vesicle-filled necks to the adhesive area at the tip of the antennules. The second described gland type comprises the permanent cement and a releasing gland is apparently absent (Yap et al., 2017). Sometimes the presence of only one cell gland type immediately rules out the possibility of a duo-gland adhesive system. For example, the freshwater *Hydra magnipapillata* has only one gland cell type at the area of attachment (Rodrigues et al., 2016a). This animal is a predominately sessile polyp, but it can voluntarily detach in response to environmental changes. The adhesive material is produced by the basal disc cells; there is no releasing gland cell (Rodrigues et al., 2016a).

Footprint topography

After temporarily adhering, the organisms move on, and the adhesive is left behind on the substrate as a 'footprint'. In sea urchins, sea stars and sea cucumbers and in *Hydra*, the shape and diameter of the footprints correspond to those of the tube feet and basal disc, respectively (Thomas and Hermans, 1985; Santos et al., 2009b; Rodrigues et al., 2016a). The appearance of these footprints shares striking similarities among different species (Fig. 4). In echinoderms, the footprints have been described as a sponge-like meshwork on a thin homogeneous film covering the substratum (Flammang et al., 1994, 1998; Hennebert et al., 2008; Santos et al., 2009b). The meshwork size varies among taxa, with the sea star *A. rubens* forming wider meshes of approximately 1–5 µm (Fig. 4A) (Flammang et al., 1998; Hennebert et al., 2008), compared with the dense meshwork (<1 µm) of sea urchins (Fig. 4B) and sea cucumbers (Santos et al., 2009b). This difference might be explained by differing modes of adhesive secretion. In sea stars, the adhesive is secreted through secretory pores (Hennebert et al., 2008), whereas sea urchins use microvillar-like cell projections (Santos et al., 2009b). The footprints of the freshwater *H. magnipapillata* (Fig. 4C) resemble those of sea stars, and accordingly the basal disc is covered with secretory pores (Rodrigues et al., 2016a). Nevertheless, the origins of the two adhesive layers vary. *Hydra magnipapillata* has only one secretory gland cell type; therefore, the thin adhesive layer and the meshwork are formed by the same cell (Rodrigues et al., 2016a). In *A. rubens*, two adhesive gland cell types are present. The type 2 granules are secreted first; they then form the thin homogeneous film. The type 1 granules most likely have a bulk function and form the thick meshwork on top (Hennebert et al., 2008). In *A. rubens*, the microstructure of footprints is identical in the adhesive material before detachment (TEM sections through attached tube feet), after voluntary detachment and forced detachment through peeling. These findings indicated that the structure of footprints is not altered by the release of the de-adhesive substance. Additionally, the topography of footprints of *A. rubens* in a hydrated state and after drying appeared similar (Hennebert et al., 2008). However, when comparing footprints among species, one should not forget that the description of the footprint topography has mainly been obtained from fixed and/

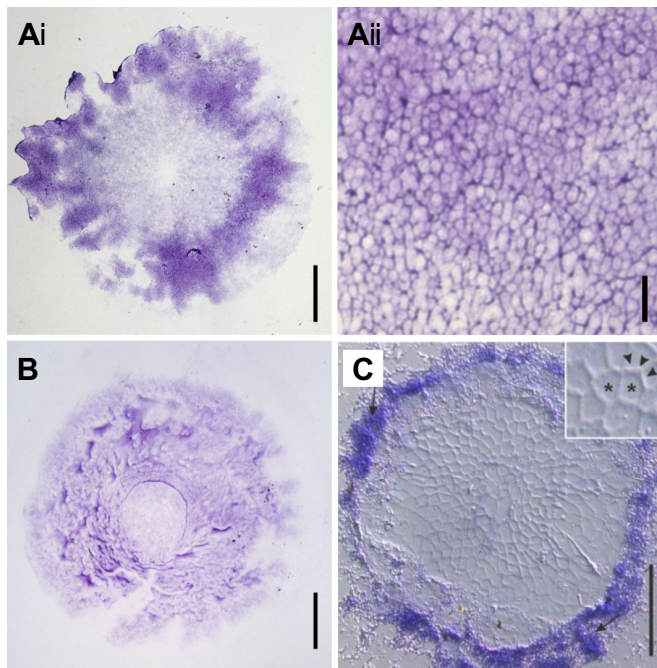


Fig. 4. Footprints, stained with Crystal Violet, of different species that use temporary adhesion mechanisms. Footprint of (Ai,ii) the sea star *Asterias rubens*, (B) the sea urchin *Paracentrotus lividus* and (C) the freshwater polyp *Hydra magnipapillata* after voluntary detachment. Inset in C is a magnification of the central area of the footprint. Asterisks indicate the thin layer and arrowheads the meshwork. Note that the meshwork size is larger in sea stars and *Hydra* footprints, which secrete their adhesive material through secretory pores, whereas the meshwork appears dense in sea urchins, which secrete through microvillar-like cell projections. (A,B) Original images; (C) modified after Rodrigues et al. (2016a). Scale bars: (Ai) 200 µm; (Aii) 10 µm; (B) 100 µm; (C) 50 µm.

or dehydrated materials. In its natural state underwater, the adhesive material is likely swollen and might appear differently. Accordingly, TEM sections of firmly attached tube feet have shown that the meshwork is filled by an electron-lucid substance, which is likely shrunk or lost during drying (Flammang et al., 1994).

Adhesive proteins

In echinoderms, the organic fraction of footprints is composed primarily of proteins and carbohydrates (Flammang et al., 1998; Santos et al., 2009a). The proteins are essential for adhesion and cohesion (see Glossary), as demonstrated by the removal of footprints after experimental treatment with the enzyme trypsin (Thomas and Hermans, 1985; Flammang, 1996). Accordingly, one common feature of identified and predicted temporary adhesive proteins is the prevalence of domains known to mediate protein–protein and protein–carbohydrate interactions (Santos et al., 2013; Hennebert et al., 2014, 2015a; Dreanno et al., 2006a; Rodrigues et al., 2016b). The prevalence of lectin-binding domains is particularly noteworthy (Hennebert et al., 2014; Lebesgue et al., 2016; Rodrigues et al., 2016b; Toubarro et al., 2016). The characterisation of adhesive proteins has been long hampered by their insoluble nature and small amounts of material (Rodrigues et al., 2014; Hennebert et al., 2015b). Recently, several temporary adhesive proteins and protein domains in different species have been identified, but unfortunately only three temporary adhesive proteins have been fully characterised (Table S1).

In *A. rubens*, the first full-length sequence of a protein known to be involved in temporary adhesion has been ascertained (Hennebert

et al., 2014). The sequence encodes a large protein of 3853 predicted amino acids named Sea star footprint protein 1 (Sfp-1). Immunohistochemistry has been used to localise Sfp-1 within type 1 adhesive vesicles, which form the fibrillary meshwork of the footprints. Western blots (see Glossary), mass spectrometry analysis and antibody staining have revealed that the large precursor protein is processed into four fragments before secretion. Each subunit consists of conserved domains, known to be involved in protein, carbohydrate and metal binding. Furthermore, 5% of the protein consists of the amino acid cysteine. This remarkably high ratio may be necessary to form intramolecular disulphide bonds (Hennebert et al., 2014). In addition to Sfp-1, 34 footprint-specific proteins have been identified in *A. rubens*. Another 41 proteins have been found in footprints as well as in the mucus secreted by the animals, which could be incorporated in the adhesive footprints (Hennebert et al., 2015a). Among these are both annotated and non-annotated proteins (see Glossary), which most likely represent novel temporary adhesive proteins (Hennebert et al., 2015a).

Along with the sea star *A. rubens*, the sea urchin *Paracentrotus lividus* is among the most thoroughly investigated echinoderm species in terms of temporary adhesion. The protein fraction of its footprints is strongly biased in its amino acid composition, with a predominance of glycine, alanine, valine, serine, threonine and asparagine. In addition, the levels of proline and half-cysteine are higher than in average eukaryotic proteins (Santos et al., 2009a). A disc-specific proteome revealed 328 non-redundant proteins, which was the first list of potential adhesive proteins in *P. lividus* (Santos et al., 2013). One protein, Nectin, was found to be secreted and further investigated as a potential adhesive component (Toubarro et al., 2016). At least three Nectin variants are present within the tube foot disc (Lebesgue et al., 2016; Toubarro et al., 2016), which are predicted to derive from the same gene (Toubarro et al., 2016).

Barnacles are considered as one of the most abundant bio-fouling organism and cause severe economic damage in shipping (Schultz et al., 2011). Therefore, there is growing interest to uncover their gregarious settlement behaviour (see Glossary). The barnacle cyprid larvae explore surfaces and select a suitable site for settlement (Walker, 1971). During this exploration phase they produce a temporary adhesive, before secreting permanent cement and undergoing metamorphosis to the sessile form (Nott and Foster, 1969). The temporary adhesive is proteinaceous and acts as a settlement pheromone (Clare and Matsumura, 2009). In the barnacle *Amphibalanus (Balanus) amphitrite*, the glycoprotein (see Glossary) ‘settlement-inducing protein complex’ (SIPC) was described as the cue to gregarious settlement (Dreanno et al., 2006a). Immunostaining with polyclonal antibodies revealed the presence of SIPC within the cyprid attachment disc and footprints, identifying it as a component of the temporary adhesive (Dreanno et al., 2006b). Furthermore, SIPC is able to absorb to various surfaces, highlighting its function as an adhesive protein (Petrone et al., 2015). In recent years, several transcriptomic and proteomic analyses revealed proteins expressed in the cyprids of the species *B. amphitrite* (Thiyagarajan and Qian, 2008; Chen et al., 2011, 2014; Chandramouli et al., 2015) and *Magabalanus volcano* (Yan et al., 2017). However, the involvement of these proteins in cyprid temporary adhesion still needs to be characterized.

In the freshwater polyp *H. magnipapillata*, adhesive proteins have been characterised using a combination of next-generation sequencing (see Glossary) and mass spectrometry (Rodrigues et al., 2014, 2016b). Using region-specific RNA sequencing, a list of transcripts predominantly expressed in the foot of the animals has

been created. For 40 of these transcripts, the expression within the basal disc was further confirmed with whole-mount *in situ* RNA hybridisation. From these, 21 proteins were validated with mass spectrometry of the adhesive footprints (Rodrigues et al., 2016b).

In the flatworm *M. lignano*, *in situ* hybridisation screening of tail-specific transcripts has revealed 20 transcripts expressed in adhesive organs in intact animals and during tail regeneration (Lengerer et al., 2018). Investigations of the nature of these transcripts are currently underway.

Carbohydrate composition of the adhesive

Carbohydrates are commonly detected in temporary adhesive glands, but their role in the adhesive process is currently unknown. In the flatworms *Schmidtea mediterranea* (Zayas et al., 2010) and *M. lignano* (Lengerer et al., 2016), in the sea star *A. rubens* (Hennebert et al., 2011), and in the cephalopods *Idiosepius* spp. (von Byern et al., 2008) and *E. scolopes* (von Byern et al., 2017), lectin (see Glossary) labelling has been used to characterise carbohydrates and indicated the presence of various sugar moieties within the secretory gland cells (Table S2). In *M. lignano*, high-resolution microscopy revealed that one lectin (PNA) specifically labelled the outer rim of the adhesive vesicles, indicating the presence of a galactosyl (b-1,3) *N*-acetylgalactosamine glycoconjugate (see Glossary) in parts of the adhesive vesicles (Lengerer et al., 2016). The reaction of lectins to secreted footprints and footprint-specific proteins was also tested in *A. rubens* (Hennebert et al., 2011). Surprisingly, the labelling of tube foot sections, footprints and footprint proteins led to different results. Of the 11 lectins labelling the tube feet at the area of the adhesive epidermis, only four (DBA, WGA, RCA and Con A) also reacted with secreted footprints. These lectins indicate the presence of

N-acetylgalactosamine, *N*-acetylglucosamine, galactose, mannose and glucose residues in the footprints. Eight lectins reacted with two footprint proteins, which were therefore classified as glycoproteins. Nevertheless, two of these lectins did not lead to a labelling of tube foot sections or footprints (Table S2). These discrepancies could be explained by dissimilar accessibility of the carbohydrate moieties and/or conformation changes (Hennebert et al., 2011). Based on these findings, it seems obvious that classical histological staining and lectin labelling of adhesive areas are not sufficient to predict the presence of carbohydrates within the adhesive material. For this reason, the direct investigation of footprint material in addition to the identification on histological sections should be favoured.

How organisms effect detachment

For animals with duo-gland systems, a secreted 'de-adhesive substance' was predicted (Tyler, 1976; Hermans, 1983). This secretion could either outcompete the binding between the adhesive layer and the adhesive organ surface (competition model) or enzymatically degrade the binding (enzymatic model). Animals lacking an additional secretion to detach themselves are predicted to use mechanical detachment through muscular contractions instead (Fig. 5).

Competition model

The adhesive area of temporary adhering animals is commonly covered with a prominent glycocalyx, called a 'fuzzy coat' by some authors (Ameye et al., 2000; Lengerer et al., 2016; Schröder and Bosch, 2016). In theory, the attachment to the substrate occurs through the thin homogeneous layer of the footprints, whereas the meshwork on top provides cohesive strength and connects the adhesive material to the glycocalyx of the animals. As the adhesive

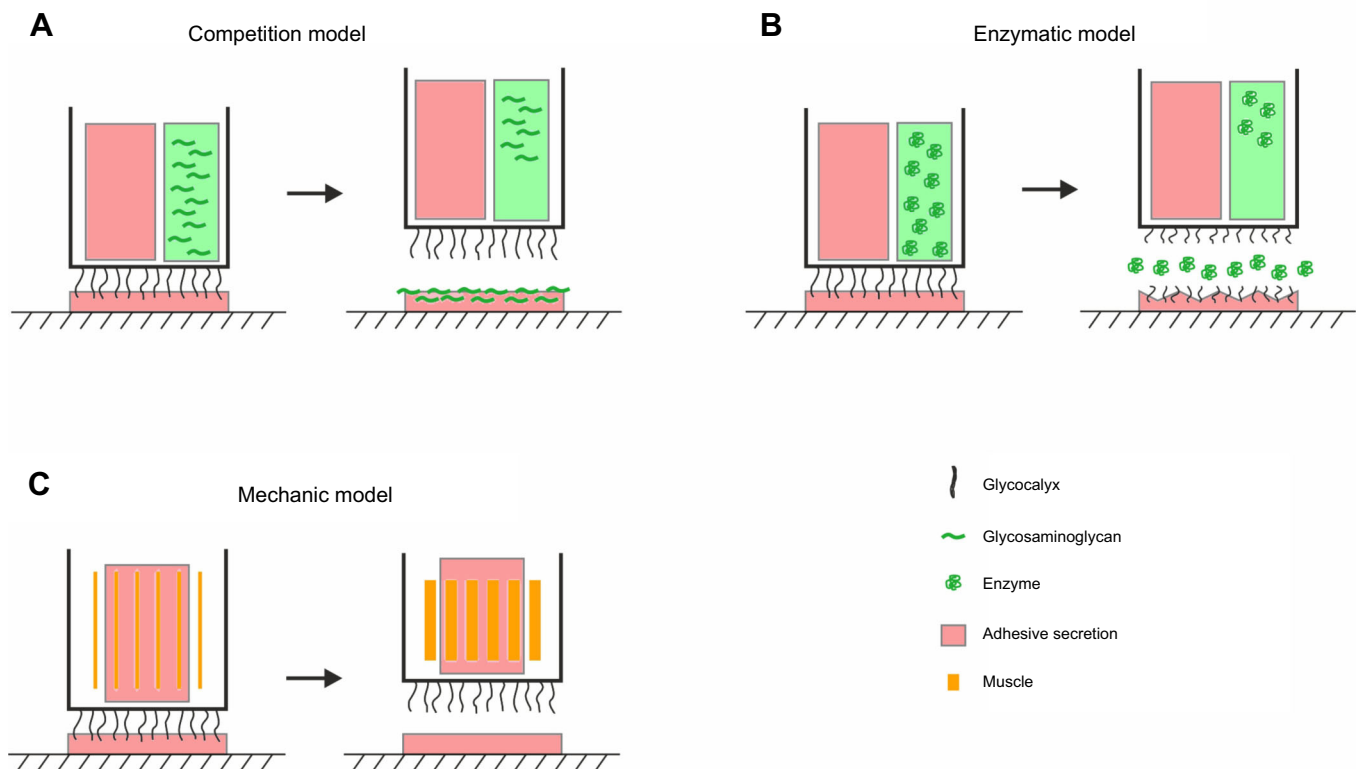


Fig. 5. Illustration of the different models proposed for detachment in temporary adhesive systems. Schematic adhesive organs during attachment (on left) and (A) detachment through glycosaminoglycans ('competition model') supplied by the releasing gland cell (green), (B) detachment through enzymes ('enzymatic model') also sourced from the releasing gland cell and (C) the action of muscular contractions ('mechanical model').

footprint stays attached to the substrate, the detachment must occur either between the adhesive material and the glycocalyx, or within the glycocalyx layer (Flammang et al., 1998). Hermans (1983) was one of the first to propose that the de-adhesive secretion competes with the glycocalyx for binding sites on the adhesive. He predicted that the de-adhesive material consists of glycosaminoglycans that strongly react with the adhesive and thereby release the animal from the substrate (Fig. 5A) (Hermans, 1983). In the sea star *Leptasterias hexactis*, the supplement of heparin, a well-described glycosaminoglycan, inhibited the attachment of the animals (Thomas and Hermans, 1985). The authors concluded that glycosaminoglycans similar to heparin are released during detachment (Thomas and Hermans, 1985). Although later studies hinted at an enzymatic release (Flammang, 1996; Flammang et al., 1998), the competition theory has never completely been ruled out. In addition, a combination of the different detachment modes is possible.

Enzymatic model

Several authors have proposed that the de-adhesive substance contains enzymes that cleave the bond between animal and adhesive material (Fig. 5B) (Flammang, 1996; Flammang et al., 1998; Kearn and Evans-Gowing, 1998; Hennebert et al., 2015a). If the de-adhesive secretion indeed competed for the binding of the adhesive material to the glycocalyx, the de-adhesive material should stay incorporated in the footprints (Fig. 5A). Yet, immunostaining directed against the collected footprint material led to no reaction in the releasing vesicles. In contrast, the glycocalyx was strongly immunoreactive, indicating that material of the glycocalyx is a substantial part of the footprint material (Fig. 5B) (Flammang et al., 1998). Furthermore, in voluntarily detached tube feet from *A. rubens*, the glycocalyx is no longer distinguishable in TEM preparations (Flammang, 1996). These observations support the hypothesis that soluble enzymes, cleaving at the area of the glycocalyx, are responsible for rapid detachment.

Recent studies have reinforced the proposed model for enzymatic properties of de-adhesive secretions. Hennebert et al. (2015a) showed the presence of two proteases in the footprint proteome of *A. rubens*. In this study, mass spectrometry of footprint material was performed, a method with much higher sensitivity than those used previously (Hennebert et al., 2015a). This difference in sensitivity could explain why, against the hypothesis that the de-adhesive enzymes are not part of the footprint, traces of them were indeed found. In addition, in sea urchins, the expression of several proteases and glycosylases has been authenticated. Significantly higher expression in the tube foot disc than in the tube foot stem was demonstrated, indicating that these enzymes might be expressed in the secretory gland cells (Lebesgue et al., 2016). However, it is unknown whether these enzymes are produced in the de-adhesive glands or whether they actually contribute to the de-adhesion process. In future studies, the potential role of enzymes in detachment might be tested by their functional knockdown through RNA interference or by the use of specific inhibitors.

Mechanical detachment

In reversibly attaching animals lacking a duo-gland system, the most common mode of detachment is release through mechanical forces (Fig. 5C). Besides morphological characterisation, behaviour observations can help determine whether detachment is achieved through muscular contractions (Aldred et al., 2013; Rodrigues et al., 2016a). In the freshwater polyp *H. magnipapillata*, video analyses have been used to demonstrate the detachment process (Rodrigues et al., 2016a). The video analyses, combined with the

characterisation of the actin filament distribution, led to the conclusion that release is induced by muscular contractions in the basal disc (Rodrigues et al., 2016a). A similar detachment mode has been described in barnacle cyprids (Aldred et al., 2013). To voluntarily detach, cyprids peel and twist their attached antennules with force (Aldred et al., 2013). In addition, in the cephalopods *Idiosepius pygmaeus*, *E. scolopes* and *Sepia tuberculata*, the presence of a dermal muscle layer and their very fast movements indicate muscular detachment (von Byern and Klepal, 2006). Although in animals with a duo-gland adhesive system, pure mechanical detachment seems unrealistic, muscular contraction might coincide with secretion of the de-adhesive substance and facilitate release.

Concluding remarks

Temporary adhesion is fundamental to the survival and basic functions of many marine and freshwater animals. The most common building plan for reversible adhesion is an adhesive duo-gland system, in which attachment and detachment are both triggered by secretions. For many organisms, the morphology of adhesive organs has been described, but information on the composition of the adhesive and releasing substances is still sparse.

In recent years, the potential of biomimetic glues has attracted several working groups to the field of bioadhesion. Although some advances in protein composition of temporary adhesives have been made, many open questions remain. Which domains or motifs make contact with the substrate? What is the role of carbohydrates? How is the connection to the glycocalyx achieved, and how is rapid voluntary release possible? Are the mechanisms allowing reversible adhesion conserved among different taxa? Is temporary and permanent adhesion substantially different or do permanent adhesives just lack the mechanisms to voluntarily detach? How are the adhesive and releasing vesicles trafficked and how is their secretion controlled?

It is our hope that temporary adhesive systems will gain the attention they deserve and that many of these questions will be answered. A better understanding of reversible adhesive systems will surely contribute to the development of novel glues and strategies for biomedical applications.

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Competing interests

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Supplementary information

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