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Recombinant Proteins: A Molecular Tool to Understand Marine Adhesion and to Advance Biomaterials

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Inspiration for innovation in healthcare regularly comes from observing the natural environment. Secreted adhesives are important for marine invertebrate attachment to submerged surfaces, and these systems have inspired investigations for better performing surgical adhesives. Natural marine adhesives are fundamentally proteins, therefore, most materials research has focused on the structure and function of proteinaceous components. Omics technologies have been used to identify proteins, but these candidates require further exploration to resolve function. Functional characterization begins by producing one specific protein in larger quantities with recombinant DNA technology. Recombinant proteins (RPs) are generally seen as mimics of individual marine adhesive proteins, representing a fundamental step in the development of bio-inspired glues. The literature details production of RPs from mussels, scallops, barnacles, tubeworms, ascidians, sea anemones, and sea stars, using bacteria, yeast, or insect and mammalian cells. Whole proteins, or components thereof, have been produced comprising the relevant amino acid sequences required for adhesion and have been investigated for use in healthcare via the production of materials that push the current limits of bio-inspired design. This is a thorough review of invertebrate marine adhesives investigated using biomimetic RPs, and a comprehensive overview of the innovative biomaterials designed utilizing knowledge from biological systems.

1. Introduction

In the turbulent wave-swept intertidal zone, adhesion is an important and widespread phenomenon. Across marine phyla, adhesion is the means of attaching firmly to submerged surfaces, allowing organisms to parry currents, resist their own buoyancy, defend themselves against predation, and immobilize prey.[1,2] Interest in secreted marine adhesives has increased over recent decades due to the potential for their development into biocompatible adhesives that can function within the wet environments of the human body.[3–5]

Marine invertebrates rely upon a particularly diverse array of adhesive strategies, which can be broadly grouped into four categories: permanent, instantaneous, transitory, and temporary adhesion.[6] Permanent adhesion is used by organisms such as mussels, oysters, barnacles, and ascidians during sessile phases of their life cycle.[7] Instantaneous adhesion involves single-use organs or cells, which allow for very fast adhesion, a technique commandeered by sea cucumbers for self-defense,[8] and by ctenophores for the swift capture of passing food.[9] Transitory adhesion, exemplified by limpets, enables simultaneous attachment and movement along a substrate.[10] And temporary adhesion allows sea stars, sea urchins, and flatworms to attach strongly but reversibly to the substrate, allowing for movement as necessary.[11] The sea, therefore, provides a plethora of inspiration for new materials, and particularly demonstrates ingenuity in the design and implementation of robust underwater adhesive strategies.

To mimic these adhesive strategies, we must first observe. In this case, because marine adhesives consist primarily of proteins, observation means unravelling the genetic information that is the dogma of molecular biology; the proteins within these adhesives are translated from the RNAs expressed within specialized glands, encoded by the genomic DNA that defines the species (**Figure 1**).[12] In the past decade, the time-consuming isolation and analysis of single genes or proteins has been either replaced or augmented by the use of omics, the large-scale study of biological molecules.[13] For instance, genomics, transcriptomics and proteomics can generate massive data sets that describe the organism's genes, mRNAs and proteins, respectively (Figure 1).

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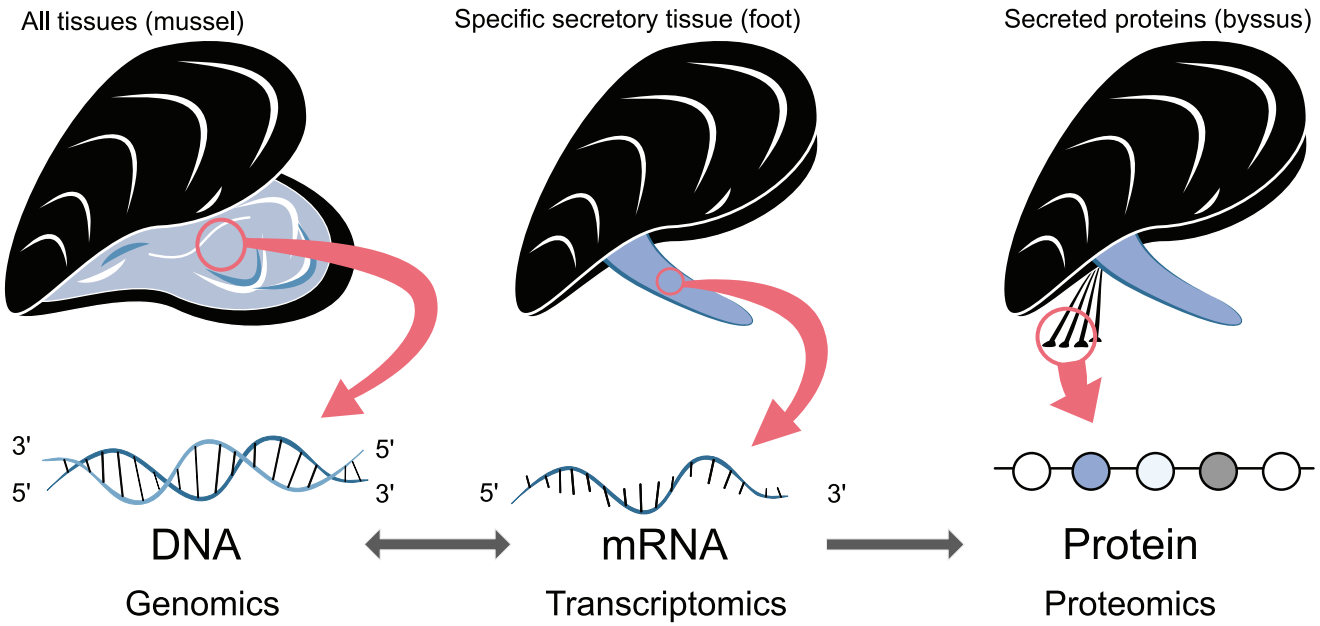

Figure 1. The adhesive protein synthesis pathway in a typical marine invertebrate, the mussel, and the molecular tools used to characterize protein-based adhesives. The genes encoding adhesive proteins are transcribed into mRNAs, which are then translated into protein precursors. These, in turn, can be post-translationally modified to give mature adhesive proteins. Nucleic acids (DNA and mRNA) can be extracted from the adhesive organ(s) (here, the mussel's foot). Both can be submitted to next-generation sequencing to obtain, respectively, the genome of the animal or the transcriptome of the adhesive organ(s). Proteins, on the other hand, can be extracted from the secreted material (here, the byssus) or the adhesive organ(s) and submitted to peptide sequencing by tandem mass spectrometry (MS/MS). The obtained peptide sequences can be used for a basic local alignment search tool (BLAST) search in the genome or transcriptome, allowing the recovery of the full-length sequence (if available) of the cDNA coding for the investigated protein.

In bioadhesion research, the combination of transcriptomics and proteomics (also known as proteotranscriptomics) has accelerated the identification of primary amino acid sequences of many adhesive proteins.[13,14] These methods, however, are often limited by available information for non-model species and rely on the use of computer algorithms to assign putative functions based on aligned amino acid sequences.[15] Unfortunately, in many cases this information is contextually meaningless as local sequence similarity can be a poor indicator of secondary and tertiary structure.[16] Consequently, identified proteins need further exploration to resolve their potential function. And the first step in this characterization often begins by producing an individual purified protein in larger quantities with recombinant DNA (rDNA) technology.

Previous reviews have mostly focused on recombinant mussel adhesives,[17,18] or briefly delved into recombinant production of marine invertebrate adhesives as part of broader reviews.[19] Yet, literature on the subject is growing rapidly as new model organisms are described (**Figure 2A**). Herein, we have endeavored to collate and examine an up-to-date catalogue of the adhesive and cohesive proteins of marine invertebrates explored via biomimetic recombinant proteins (Table S1, Supporting Information). Due to space constraints, the mechanical characterization of marine adhesive proteins is not within the scope of this review (for an overview of tested adhesion properties, we refer the reader to the recent publication of Heinritz et al.[20]). Thus far, recombinant adhesive and cohesive proteins have been produced from a half a dozen or so marine invertebrate taxa, although some attracted more attention than others (Figure 2B). This review begins by briefly summarizing the pursuit of adhesives inspired by marine invertebrates, followed by more in-depth descriptions of the species, the proteins studied, and the recombinant mimics investigated. Then it delves into the concept of hybrid proteins. The hybridization of proteins makes it possible to rearrange and combine protein sequences by genetic fusion to create new protein constructs that offer combinations of properties not available in the natural adhesives. The association of these proteins with other components such as ions, proteins, polysaccharides, or synthetic materials allows the self-assembly of hydrogels, fibers or films equipped with these desirable properties. These materials can be further processed or combined to fabricate complex biomaterials, pushing the current limits of bio-inspired design. To add functionalities, these biomaterials can be loaded with drugs, growth factors or even stem cells. The last part of the review therefore focuses on the use of recombinant adhesive proteins for the fabrication of complex biomaterials with added functionalities enabling the effective delivery of treatments for difficult-to-target tissues.

2. The Pursuit of Marine Bioinspired Adhesives

As natural marine adhesives are fundamentally proteins, with smaller percentages of carbohydrates and lipids, nearly all research has focused on the structure and function of proteinaceous components.[21] These components include the bulk proteins involved in cohesion; proteins that interact proximally with the cells of the organism; and proteins that adsorb distally to the varied, and often fouled, marine substrata. The characteristics of

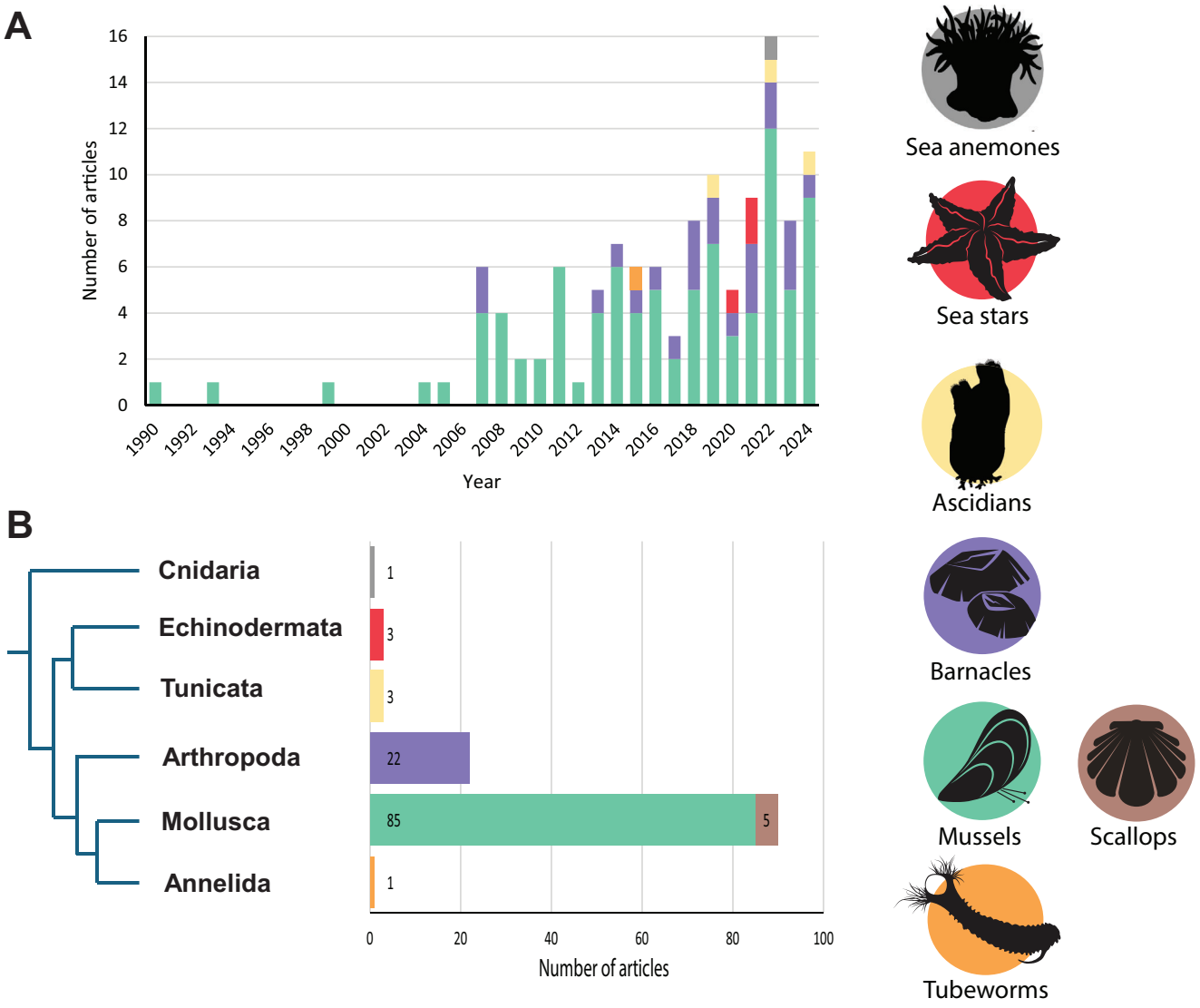

Figure 2. Scientific publications reporting the production of marine adhesive proteins. (A) With increasingly more adhesive proteins being described in various marine invertebrates and additional biomedical applications being sought, the number of articles comprising recombinant protein production has risen steadily in the last 20 years. (B) In this review, we catalogued around 120 publications. Up to the end of 2024, 6 phyla of marine invertebrates have inspired the production of recombinant adhesive proteins, albeit with varying degrees of effort, as reflected by the number of publications. The phylogenetic relationships between these organisms are shown on the left (based on).[2]

marine adhesives are linked to the physico-chemical properties inherent to the underlying proteins.[12,22] For proteins involved in the permanent adhesion of mussels and tubeworms, extensive post-translational modifications (PTMs) are required for adhesion. This includes PTMs such as the hydroxylation of tyrosine and the phosphorylation of serine residues.[23] The exemplary DOPA (3,4-dihydroxy-L-phenylalanine), which is formed by the PTM of tyrosine, plays important interfacial adhesive and bulk cross-linking roles (see Section 3.1.1).[24,25] By comparison, the proteins involved in the temporary or transitory adhesion of flatworms, gastropods, and echinoderms lack the latter PTM and instead exhibit multi-domain structures reminiscent of extracellular matrix proteins.[2,26]

For most applications, harvesting the natural adhesive is an unrealistic solution due to poor yields from direct extractions.[27] To overcome this, research into the production of biomimetic adhesives has developed in two different directions: the chemical synthesis of bio-inspired polymers and the production of recombinant adhesive proteins.[28]

Chemical synthesis of marine adhesives requires an understanding of the natural adhesion mechanism. Important adhesive components can be synthesized by the functionalization

of polymers with reactive groups or by peptide synthesis. Although they are highly simplified versions of biological adhesives, these chemically synthesized molecules usually retain the desired properties of their natural counterparts and can be produced on a large scale.[28,29] These materials can be based on a huge diversity of polymer backbones and have been reviewed extensively in the last decade.[30,31] Due to the early discovery of the importance of DOPA residues in mussel adhesion,[32] the most common method to engineer bio-inspired adhesives is to incorporate DOPA or another catechol functionality into the material, and these studies dominate the literature.[27,30,33] However, electrostatic and hydrophobic interactions also play an important role in the processing and performance within mussel, tubeworm, and barnacle adhesives.[31] This has led to more recent studies incorporating charged groups, such as amines or phosphates, as well as hydrophobic groups in polymeric adhesive materials.[24,31,34] The strength of electrostatic interactions can be controlled by varying the ionic strength or pH and can thus be used to tune the mechanical and adhesive properties of the material.[31]

The lower molecular weight and structural complexity of peptides are associated with easier chemical synthesis. Therefore, many studies have reported the design of peptides inspired by mussel, barnacle and tubeworm adhesive proteins and have screened them for adhesive properties and the ability to self-assemble.[35–37] One of the advantages of synthetic peptides compared with full-length proteins is that sequences can be easily modified to test the function and importance of individual amino acids; this can allude to their overall significance within the material. Yet, the small size of peptides precludes the study of the multiple and complex interactions that occur between proteins within natural adhesives, particularly as the 3D shape of a protein informs its function more than fragments of a primary sequence.[16]

Recombinant proteins are generally seen as the closest mimics of marine adhesive proteins and represent an intermediate stage in the development of bio-inspired glues.[4,38] Recombinant DNA technology comes with the advantage of reducing biological variability while preserving the important characteristics of natural adhesives. In such an approach, a whole protein (or parts of it) can be produced that comprises the relevant adhesive and cohesive amino acid sequences required for its function within an adhesive material (**Figure 3**). The first rDNA molecule was produced five decades ago in 1972 and since, this technology has made it possible to synthesize many proteins of interest.[39] For this, living host cells or organisms are harnessed as factories to build and construct proteins based on supplied genetic templates. DNA vectors are simple to construct synthetically or in vitro using well-established rDNA techniques. Therefore, DNA sequences of specific genes can be transferred to host cells for subsequent recombinant protein expression. Common hosts include bacteria, yeasts, insect or mammalian cells, or whole organisms such as plants.[40] Using rDNA technology to produce adhesive proteins is not devoid of hurdles, however. In addition to the need to choose an appropriate host for expression, a good understanding of the protein of interest and its coding gene is often required. Some considerations which rely on this knowledge include whether there are preferential codons used in the host compared to the species from which the gene is originating (known as codon bias);[41] the presence of PTMs on the natural protein and the ability of the host to perform them; the propensity of the protein to form insoluble aggregates termed "inclusion bodies"; the ability to reproduce the native structure of the protein upon refolding; and, ultimately, the possibility to provide high yields of the recombinant protein by up-scaling production processes.[42,43] Although some of these hurdles can be addressed through the choice of specialized host strains or optimized expression conditions (e.g., use of *Escherishia coli* BL21-CodonPlus bacterial strains to avoid codon bias; see Table S1, Supporting Information), they are often optimized by trial and error.

In terms of marine bio-inspired recombinant adhesives, the literature provides examples of production of adhesive and cohesive proteins from mussels, scallops, barnacles, tubeworms, ascidians, sea anemones, and sea stars; in bacteria, yeast, or insect and mammalian cells (Figure 2B; Table S1, Supporting Information). The first goal of recombinant adhesive protein production is usually to synthesize sufficient quantities of a relatively pure protein for the employment of downstream characterization methods. Recombinant adhesive proteins can also be combined or used in hybrid systems, such as those based on polysaccharides (Section 5), providing models in which to study secreted protein interactomes: a field still very much in its infancy and fundamental to all biological understanding.[44] Additionally, it is possible to alter and rearrange protein sequences by genetic engineering to create new constructs based on our understanding of functional components of previously studied proteinaceous materials (Figure 3). These truncated or chimeric proteins are potentially easier to produce and offer scope for the improvement of mechanical attributes based on creating or combining functionalized components.[45] Thus, this molecular toolbox has the potential to facilitate the development of novel biopolymeric material systems that offer combinations of properties not currently available in existing materials.[46,47] The significance of this is that biomimetic adhesive research offers an opportunity, not just to replicate a natural adhesive with all its inherent traits, but to redefine the material and push the current limits of design. The following sections provide an overview of the marine invertebrate adhesive systems for which the recombinant production of adhesive proteins has been reported. Details related to specific aspects of protein production, characterization, or applications are given for each group of organisms.

3. Recombinant Adhesive Proteins From Marine Invertebrates

Recently, Delroisse et al. (2023) reported that 29 of the 34 metazoan phyla contain species that attach to substrates using adhesive secretions.[2] Adhesive systems have been thoroughly described for only a few of these species. Recombinant proteins have been produced either to help characterize the adhesive system or to produce bio-inspired materials. To the best of our knowledge, rDNA technology has been used to produce marine biomimetic proteins from 6 phyla: Cnidaria, Echinodermata, Tunicata, Annelida, Arthropoda, and Mollusca (Figures 2B and 4; **Table 1**). It should be noted, however, that in some taxa, the adhesion mechanism has been well-characterized but no recombinant proteins have been produced (e.g., flatworms from the phylum Plathyhelminthes).[48]

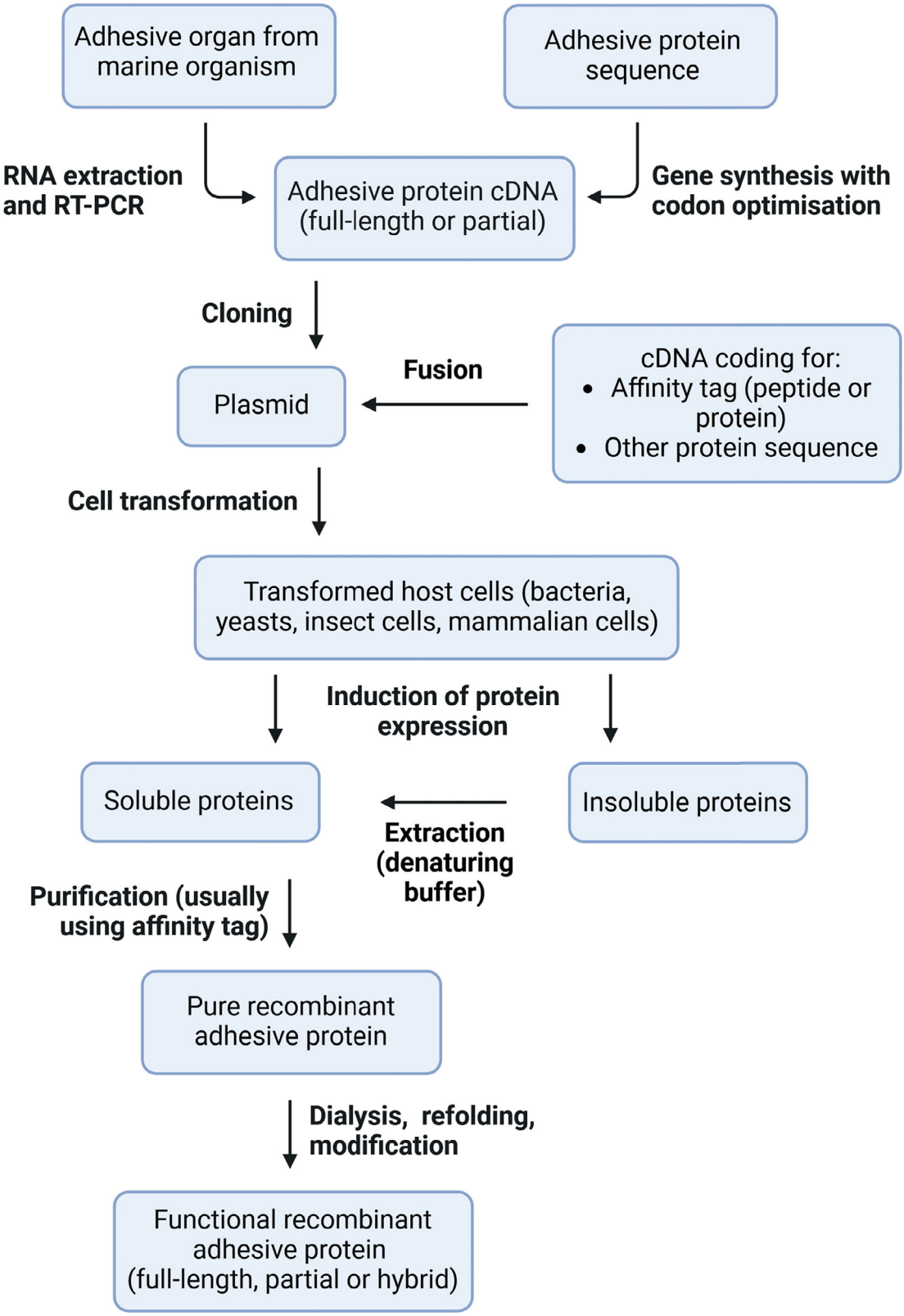

Figure 3. General outline of major steps for recombinant protein production and purification. First, the sequence coding for the protein of interest is obtained either by PCR amplification from mRNA extracted from the adhesive organ or through gene synthesis by a specialized company. The latter approach allows for codon optimization, overcoming limitations associated with inter-species differences in codon usage, thereby enhancing protein production yield. The sequence is then cloned into an expression vector, in frame with a sequence coding for a tag (to facilitate protein purification and

detection or to improve protein expression and solubility), or for another protein (e.g., to bring new functionalities). After transferring the vector into host cells (transformation/transfection), protein expression is induced. Proteins can sometimes be recovered from the culture medium, however, most of the time, the cells are collected and lysed to recover the proteins. The proteins can be found in the soluble fraction or as insoluble aggregates, known as inclusion bodies. In the latter case, they need to be extracted with denaturing buffers. Solubilized proteins are then purified using one or several purification methods to obtain pure proteins. Finally, the proteins are concentrated and refolded (usually through progressive buffer exchange), and may undergo additional processes, such as enzymatic treatment, to restore native function.

Table 1. List of marine adhesive proteins that have been produced recombinantly, partially, or in full-length, in various heterologous host cells.

Organism	Species	Adhesive protein	Recombinant protein	MW (kDa)	Host for expression
Mussels	*Mytilus edulis*	Mfp1	Partial	24-96	Y
		Mfp1	Full-length	130	Y
		Mfp1	Partial	7-25	B
		Mfp2	Full-length	42-47	Y
		Mfp3	Full-length	24	B
	Mytilus galloprovincialis	Mfp3	Full-length	7-27	B
		Mfp5	Full-length	10-18	B
		PreCol-D	Full-length	75-100	Y
		PreCol-NG	Partial	7	B
		TMP	Partial	21	B
		PTMP1	Full-length	49	B
	Mytilus californianus	Mfp3	Full-length	35	Y
		Mfp3	Full-length	6	B
		Mfp6	Full-length	13-14	B
		PreCol-D	Partial	37	B
	Mytilus coruscus	Mfp3	Full-length	9	B
		Mfp20	Full-length	14	B
	Perna viridis	Mfp5	Full-length	13-23	B
Scallops	*Chlamys farreri*]	Sbp5-2	Partial	21	B
		Sbp8-1	Full-length	19	B
		Sbp9	Partial	nr	B
Barnacles	Megab*alanus rosa*	Cp19k	Full-length	17-19	B
		Cp19k	Full-length	20	Y
		Cp20k	Full-length	20-22	B
		Icp3_36k	Full-length	36	B
		Icp2_57k	Full-length	57	B
	Fistulobalanus albicostatus	Cp19k	Full-length	18-37	B
		Cp20k	Partial	35	B
	Amphibalanus amphitrite	Cp20k	Full-length	nr	B
		Cp43k	Full-length	43	B
		Cp100k	Partial	nr	B
		SIPC	Full-length	200	IC
	Pollicipes pollicipes	Cp19k	Full-length	19	B
Ascidians	*Ciona robusta*	ASP1	Full-length	61	IC
		AAP1	Full-length	59	IC
		APAP1	Full-length	47	MC
		APAP2	Full-length	65	MC
Tubeworms	*Sabellaria alveolata*	Sa1	Full-length	27	B
Sea anemones	*Diadumene lineata*	TSRL	Full-length	25	B
Sea stars	*Asterias rubens*	Sfp1	Partial	56-68	B

Abbreviations: B, bacterium; IC, insect cells; MC, mammalian cell; MW, molecular weight; Y, yeast.

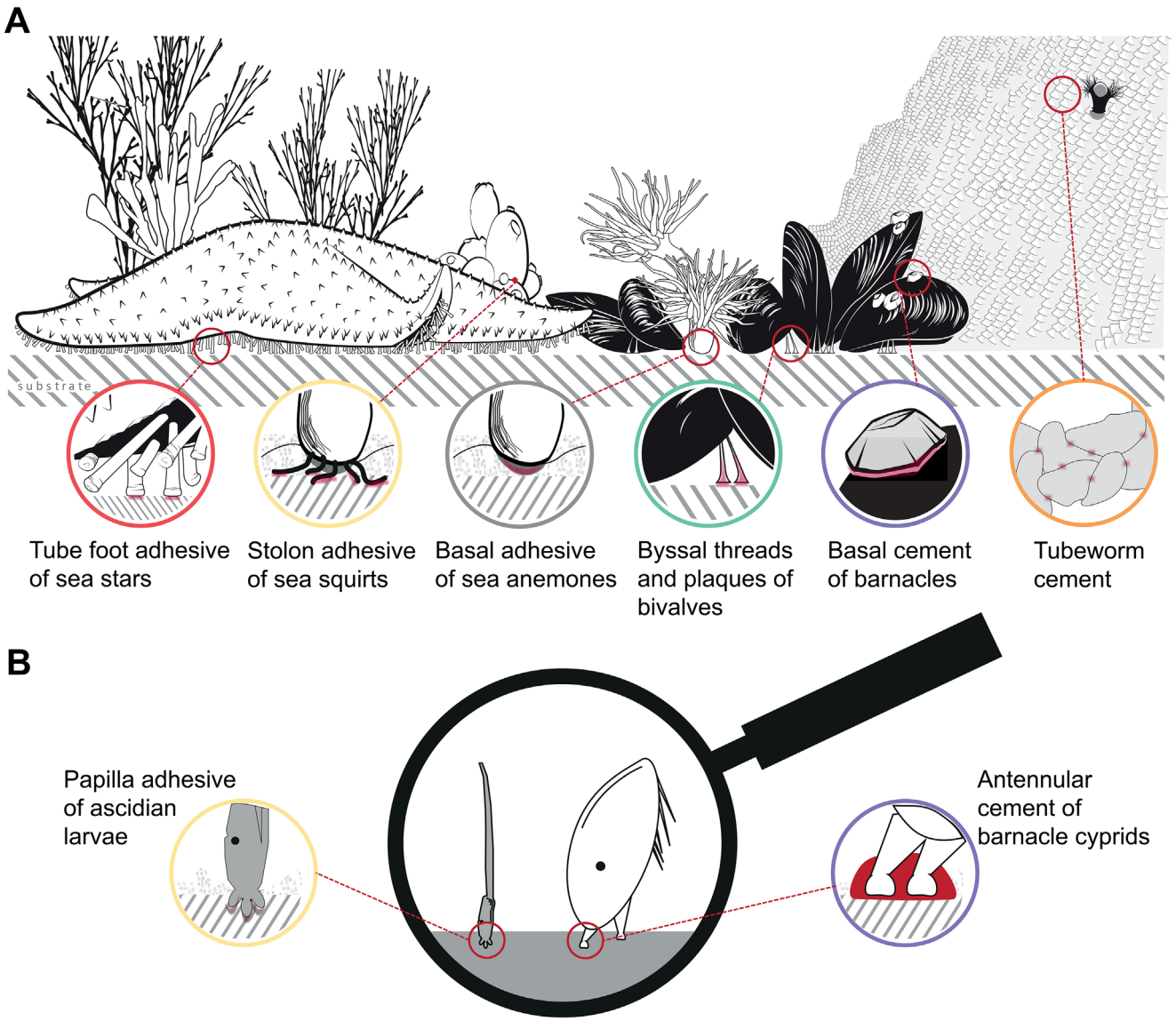

Figure 4. Marine invertebrate adhesive systems that were used as models for the production of recombinant adhesive proteins. (A) Example of an intertidal community illustrating the six metazoan taxa in which adult individuals rely on different types of adhesion to attach to the substrate: the permanent adhesion of bivalves, barnacles, tubeworms and ascidians; the transitory adhesion of sea anemones; and the temporary adhesion of sea stars (modified from[2]). (B) In two of these groups, the larval stages attaching to the substratum during settlement and metamorphosis (the ascidian tadpole larva and the barnacle cyprid larva) also inspired the production of recombinant adhesive proteins. Insets in colored circles (same color code as in Figure 2) show details of the adhesive organs with the adhesive secretions highlighted in red. In the case of tubeworms, the inset shows the structure of the tube consisting of sand grains glued together by cement spots.

3.1. Bivalve Adhesion

Bivalves are a class in the phylum Mollusca of approximately 10,000 extant species, including mussels, oysters, and scallops.[49] General defining features of this group include filter feeding and encasement in a calcium carbonate shell consisting of bilaterally symmetrical hinged valves. Many bivalve molluscs produce acellular proteinaceous fibers for underwater attachment, individually called byssal threads, or collectively, byssus (**Figure 4A**).[50] Byssus is produced by exocrine glands located within the foot of the bivalve mollusc.[51] The details of byssus production along with the macroscopic appearance, differ between bivalve genera, even within the same family. Many mussels produce a byssus comprising a proximal stem which is embedded in their soft tissue, other bivalves attach with a stem-free byssus in which individual threads embed directly.[52,53] Byssal production is further influenced by nutrition, habitat, the strength of water currents, agitation, ocean acidification, and available oxygen.[54,55] Some bivalves produce byssal threads at only one stage of life, while others produce byssi across their lifespan.[56] For example, the silverlip pearl oyster, *Pinctada maxima* (Jameson, 1901), produces a byssus until around 3 years of age, or when the oyster's weight becomes sufficient anchorage,[57] and the edible flat oyster, *Ostrea edulis* (Linnaeus,

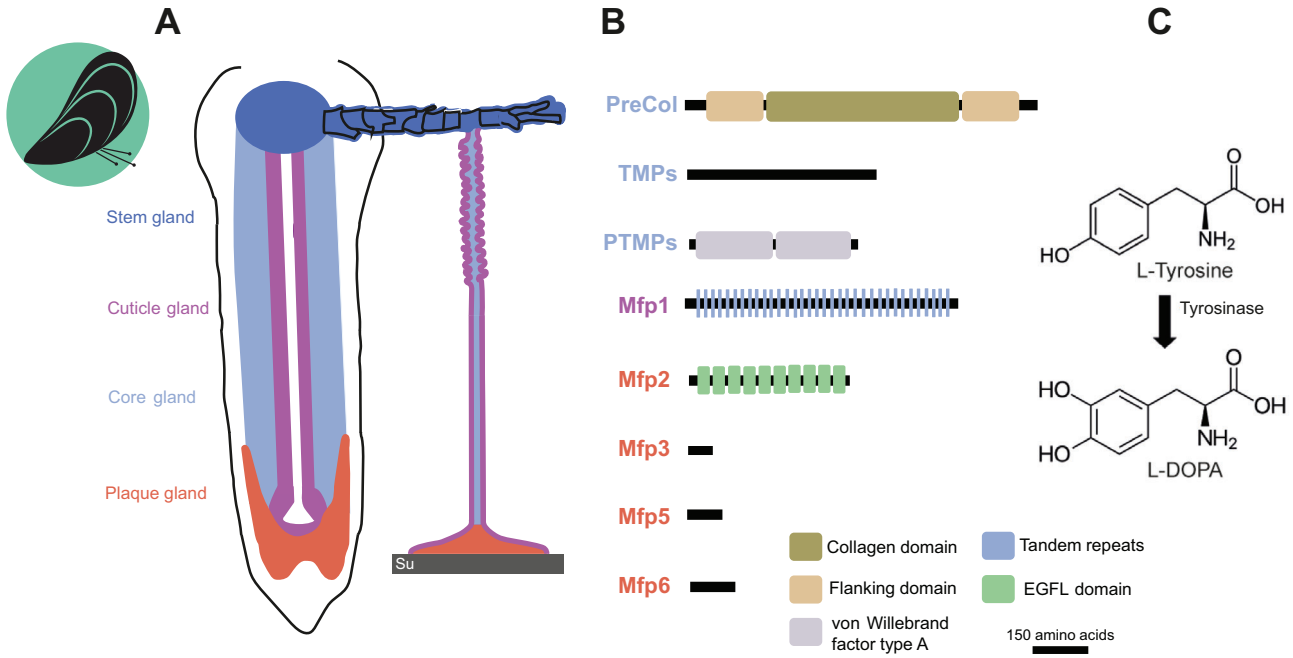

Figure 5. Mussels attach to surfaces using an acellular extracorporeal structure known as the byssus. (A) Each byssal thread is formed by proteins produced by four glands within the foot (modified from[22]). The stem gland produces the attachment stem (dark blue) which originates in the soft tissue; the core gland provides the proteins within the thread core (light blue); the plaque gland provides the bulk and interfacial proteins (orange) for attachment to any underwater substrate (Su); and the cuticle gland produces the cuticle covering both the thread and the plaque (purple). (B) Proteins constituting the byssus (only proteins whose sequence has been used to produce recombinant proteins are displayed. Prepepsinized Collagens (PreCol's), thread matrix proteins (TMPs) and proximal thread matrix proteins (PTMPs) are made by the core gland and found within the core. The 3 PreCol proteins consist of 3 different domains flanking an atypical collagen domain, while PTMP contains von Willebrand Factor A domains. Mussel foot protein-1 (mfp-1) contains many tandem repeats and provides the protective coating. Mfp-2-6 are examples of the proteins found within the adhesive plaque. Notably, only mfp-2 is distinguished with domains; it contains multiple EGF-like domains which form the porous bulk of the adhesive plaque. (C) Most mussel byssal proteins have been shown to contain DOPA, which is formed by post-translational conversion of tyrosine resides by tyrosinase. The catechol group of DOPA residues can contribute to both adhesive adsorption on the substrate and byssus cohesion.

1758), produces a byssal thread during the pediveliger larval stage and a cement at later stages.[58] While byssal attachment can be considered a permanent form of marine adhesion, some mussels can voluntarily eject their whole byssus at the stem.[59,60] The rayed pearl oyster, *Pinctada radiata* (Leach, 1814), which does not have a byssal stem region, can jettison individual threads and can use this technique to support locomotion.[61] And free-living scallops can also decouple from their stem-free byssus and reattach by forming a new byssus.[62] While most research has been conducted on distal attachment to substrates, the anchoring of the byssus to its own soft tissues is an emerging area of biointerface material research.[53,60] Further, understanding the cues of byssal detachment would be of great interest to those seeking to inhibit fouling from bivalves on submerged substrates.

3.1.1. Mytilid Adhesion

The byssus of mytilids (primarily *Mytilus* and *Perna* spp.) is the best-characterized marine bioadhesive, and it is from these organisms that most of the recombinant adhesive proteins have been produced (Figure 2B; Table 1; Table S1, Supporting Information). Recombinant mussel adhesive proteins were last extensively reviewed in 2018;[18,63] this overview will therefore include the more recent studies and we have endeavored to provide the complete catalogue of recombinant proteins to date.

For survival, the mussel byssus must be strong enough to keep the animal stably anchored in its habitat in the intertidal zone.[59] The byssus from species of the genus *Mytilus* has been the most frequently studied.[64–66] In *Mytilus edulis* (Linnaeus, 1758), the byssus is made up of approximately 20 – 100 threads, typically 4 – 5 cm in length.[67] The multiple attachment points via individual, radially dispersed threads and the multi-component nature of each thread allow force to be redistributed to new parts of the byssus/thread structure while time-dependent recovery occurs in deformed portions.[68] Longitudinally, the mussel byssus can be divided into distinct regions based on morphology and mechanical functionality; the stem, the proximal thread, the distal thread, and the attachment plaque.[24,68–70] In cross-section, each byssal thread consists of two distinct layers; a relatively thick collagenous core and a thinner protective cuticle coating (**Figure 5A**).[71] Four mussel glands have been identified and associated with the formation of byssus. Over the years, researchers have named these the white/collagen/core gland, the accessory/enzyme/cuticle gland, the phenol/purple/plaque gland, and the byssal/stem gland/stem generator.[60,72–75] This review will refer to the underlined gland names for consistency.

The first described "mussel adhesive protein"[32] was later named mussel foot protein-1 (mfp1) (Figure 5B) and found to

be expressed in the cuticle gland (Figure 5A).[66,76,77] In *M. edulis*, mfp1 consists of 86 tandem repeats of decapeptides (n = 72) and hexapeptides (n = 14), and contains 10–15 mol % DOPA.[78] After secretion, it is located within the micron-sized spherical granules that form the cuticle.[77,79] As the first marine adhesive protein to be identified and characterized, it was only natural that initial attempts at producing a recombinant mussel byssal protein were focused on mfp1. Filpula and colleagues (1990) designed a recombinant DNA construct (called 14-1) which, when expressed, would include 19 decapeptide repeats and one hexapeptide. To express a recombinant protein similar to the native size, plasmids were designed with one to four copies of 14-1 with the engineered proteins (ranging between 24 and 96 kDa) successfully expressed in the yeast *Saccharomyces cerevisiae*. The recombinant proteins accounted for 2 to 5% of the total cell protein extract.[78] Later, a pilot study effectively produced mfp1 protein analogues containing 20 decapeptide repeats in the bacterium *Escherishia coli* at high yields (up to 60% of total cell proteins) by using a gene that accounts for *E. coli* codon bias (Figure 3).[80] The recombinant proteins were present in soluble and insoluble (i.e., inclusion bodies) fractions. In 1999, Kitamura and coauthors also produced the same decapeptide repeats (n = 6) of mfp1 in *E. coli*, also at high yields. This time the recombinant proteins formed inclusion bodies.[81] In 2008, Lee et al. fused a mfp1 polypeptide (7 decapeptides) sequence with a truncated OmpA signal peptide for the successful expression of a soluble recombinant mfp1 by *E. coli*.[82] OmpA is an outer membrane protein of *E. coli* that delivers expressed proteins to the periplasm. In yet another study, Pilakka et al. (2023) fused the highly hydrophilic C-terminal domain of ice-nucleation protein K (InaKC) to 12 mfp1 decapeptide repeats as a novel solubility tag.[83] The authors utilized a protease to cleave the InaKC and trigger aggregation of the mfp1 decapeptides. However, in all these recombinant mfp1 proteins important native PTMs were missing, such as the hydroxylation of tyrosine residues into DOPA (Figure 5C). Protocols were then developed for the in vitro conversion of tyrosine residues to DOPA utilizing a bacterially derived tyrosinase[78] or a mushroom tyrosinase.[81,82] After modification, recombinant mfp1 usually showed better coating ability. More recently, a recombinant mfp1 consisting of 12 tandem repeats of the consensus decapeptide and modified with mushroom tyrosinase was used to demonstrate that reversible DOPA–metal cross-links contribute to the hardness and flexibility of the outer cuticle.[84–86]

Mussel byssus plaques are flattened microporous structures found at the terminal end of byssal threads, adhering to almost any underwater surface.[87] Plaques are formed by several DOPA–rich mussel foot proteins, mfp2 to 6, secreted by the plaque gland (Figure 5A and B). These proteins all possess specific localization within the attachment plaque in relation to their presumed functions. Specifically, mfp3, mfp5, and mfp6 have been related to the interfacial adhesion of the plaque to submerged substrata, with the first two playing an active role in surface attachment, whereas the latter is proposed to act as a reducing agent preventing the oxidation of DOPA residues to the non-adhesive dopaquinone.[32,88,89] The epidermal growth factor-like (EGFL) repeat-containing protein mfp2, on the other hand, is a cohesive protein forming the porous bulk of the attachment plaque,[90] while the histidine-rich mfp4 makes the connection between the plaque and the thread.[91] Further mfps have been elucidated in *Mytilus californianus* (Conrad, 1837) by transcriptomic analysis of the plaque gland but their roles are still unknown.[75] As mfp3 and mfp5 have been identified as key components for adhesion, most of the efforts in producing recombinant proteins have focused on them, while mfp6 has been studied to understand how dopaquinone conversion is avoided.

The first recombinant versions of plaque proteins were the mfp3 variant, mfp3A, and mfp5 from *Mytilus galloprovincialis* Lamarck, 1819. Full-length genes coding for these proteins, fused with a hexahistidine-tag coding sequence (His_6-tag) to allow purification, were produced in *E. coli*, without prior codon optimization.[92–94] Hereupon, immobilized metal affinity chromatography (IMAC) was used as the preferred method of recombinant protein purification in all subsequent studies (Table S1, Supporting Information). Production yield of both proteins, as well as of mfp3 from *Mytilus coruscus* A. Gould, 1861, was increased after adjusting for the codon usage preference of *E. coli*.[95–97] Platko et al. (2008) used another tag, a 9 amino acid sequence from human-influenza-virus hemagglutinin (HA), to facilitate the identification and purification of mfp3 from *M. californianus* after expression in the yeast *Kluyveromyces lactis*. The HA tag also inhibited the self-assembly of mfp3 into high molecular weight complexes, which could then be triggered by tag cleavage.[98]

Different mfp5 variants from the Asian green mussel, *Perna viridis* (Linnaeus, 1758) were also recombinantly produced in *E. coli*.[99,100] After modification with mushroom tyrosinase, recombinant mfp3 and 5 showed high adhesive abilities and adsorption capacities on various surfaces.[92–94,96,101] However, the in vitro modification of tyrosine into DOPA exhibits a low (<15%) modification yield which can limit underwater adhesion. More recently, Zwies et al. were able to increase this modification yield by a factor of about four using a recombinant bacterial tyrosinase from *Verrucomicrobium spinosum*.[102] In their study, they produced soluble mfp3 using a cleavable small ubiquitin-like modifier (SUMO) solubility tag and modified it in vitro. A similar modification yield was obtained in vivo when the tyrosinase from *V. spinosum* was co-expressed with mfp3.[103] However, the best DOPA levels were obtained when mfp3 and 5 were produced using an in vivo residue-specific DOPA incorporation strategy. A tyrosine auxotrophic strain of *E. coli* with an endogenous tyrosyl-tRNA synthetase (TyrRS) was used to express proteins while DOPA was supplemented in the growth media. With this method, a high DOPA incorporation level (> 90%) at each Tyr site was obtained.[104,105]

The plaque protein mfp6 has been produced recombinantly in E. coli by different lab groups from sequences obtained from M. californianus (Table S1, Supporting Information). The first, in 2013, showed that a recombinant mfp6 (rmfp6.1) was able to rescue mfp3 adhesion at pH 3.[106] In 2022, Shin et al., showed that rmfp6 can stabilize tautomer equilibrium in an oxidized DOPA-incorporated recombinant mfp3 at the pH of seawater.[88] However, the tyrosine residues in rmfp6 were not modified to DOPA, which may enhance the reducing power of native mfp6 according to another study.[107] Recently, a novel cysteine-rich protein similar to mfp6, mfp20, was identified in M. coruscus and its antioxidant function was demonstrated after production in E. coli.[108]

The byssal thread core in mytilids consists primarily of unique precursor collagens named preCols and thread matrix proteins (TMPs) (Figure 5A,B).[109,110] PreCol-P and -D are present in the thread core in a complementary graded dispersion; preCol-P is more abundant in the proximal thread part and preCol-D is more abundant in the distal region. PreCol-NG is present in an ungraded fashion throughout the thread core. Possessing different flanking domains, PreCol-P and PreCol-D have differing mechanical properties, offering either extensibility or stiffness and strength, respectively.[109] Full-length preCol-D has been recombinantly produced in the yeast *Pichia pastoris*.[111] The recombinant protein exhibited the ability to form fibrils with a correctly folded collagen triple helix, even in the absence of post-translational hydroxylation of proline residues. The flanking domains of preCols, on the other hand, have been expressed in *E. coli*, in fusion with special protein tags, such as SUMO or baculoviral polyhedrin protein, to increase or decrease their solubility, respectively.[112,113]

Also secreted by the core gland and contributing to the thread are the non-collagenous TMPs (Figure 5A,B).[114–116] As the name suggests, TMPs provide a viscoelastic matrix bridging the collagen fibers; however, unlike the preCols, they are ungraded along the thread. The C-terminus of TMP, which is approximately one third of the full-length TMP, was recombinantly expressed in *E. coli*. In this protein, asparagine residues can undergo spontaneous, non-enzymatic deamidation, which may contribute to the maturation of the mytilid byssus.[114] Another matrix protein containing two von Willebrand factor type A (vWA) domains, PTMP1, has been produced in *E. coli*, either fused with a SUMO tag or not.[116,117]

3.1.2. Non-Mytilid Bivalve Adhesion

Recently, Waite and Harrington (2022) called for more research on the byssus of species other than mussels given the great diversity of byssal structures among Bivalvia.[33] Indeed, not all byssi have a collagenous fiber core structure, distinct stem regions (see stem in Figure 5A), threads that contain mechanical gradients, or even multiple fibers like mytilids.[52,53] *Anomia* (Linnaeus, 1758) oysters utilize a single, thick, calcified byssus for substrate attachment.[118] *Atrina pectinata* (Linnaeus, 1767) and *Pinna nobilis* (Linnaeus, 1758) byssal threads do not exhibit mechanical gradients.[119] And while the cross-sectional cuticle/core structure of byssal threads is common among the species studied thus far, the ultrastructure can be vastly different.

In the oyster *Pinctada fucata* (Gould, 1850), while the existence of mechanical gradients is untested, the macrostructure suggests the distal portion of the thread, which represents 80% of the overall length is the extensible region.[120] The *P. fucata* byssal cuticle consists of compacted fibrils and does not contain nanoscale granules like in mussels. It surrounds core fibers interspersed with nanocavities. Around fifteen proteins have been identified in the byssus of *P. fucata* but none of them has been produced recombinantly.[121] Among the fifteen, a thrombospondin-1 (TSP1) and vWA domain-containing protein, and another vWA domain-containing protein they have named P-UF1 (or PU-F1 on the NCBI protein database) are potentially key molecular components of the byssus.[122] TSP1- domain-containing proteins were not only found to be key components in the byssus of the closely-related *Pinctada maxima* (Jameson, 1901);[123] but also within proteins of the adhesive of the flatworm *Macrostomum lignano* Ladurner, Schärer, Salvenmoser & Rieger, 2005;[48] the stolon proteins of the ascidian *Ciona robusta* (Section 3.3);[124] and within adhesives of the sea anemones, *Exaiptasia diaphana* (Rapp, 1829) and *Diadumene lineata* (Verrill, 1869) (Section 3.5).[125,126] In a study on the byssus of *P. maxima*, another highly expressed byssal thread protein was found to be similar to a DOPA-containing byssal protein from *A. pectinata*.[53,123] This foot protein (named apfp1 and Pmfp1 in *A. pectinata* and *P. maxima*, respectively) shares homology, via the presence of a C-type lectin domain, with a protein originally identified as perlucin in a marine gastropod, the greenlip abalone *Haliotis laevigata* Donovan, 1808.[127,128] Perlucin-like adhesive proteins have also been identified by in silico analysis of larval transcriptomes of the Pacific oyster, *Magallana gigas* (Thunberg, 1793),[129,130] and as one of the top differentially expressed foot-specific genes in the Zhikong scallop *Chlamys farreri* (K.H. Jones & Preston, 1904).[131,132] A perlucin cDNA sequence from *Haliotis discus discus* Reeve, 1846, corresponding to the mature full-length protein, was inserted into a vector that allowed for recombinant protein expression in the cytoplasm of *E. coli* due to fusion with maltose-binding protein (MBP) tag.[133] The authors investigated perlucin's ability to nucleate the growth of calcium carbonate crystals in relation to biomineralisation. However, the authors did not investigate perlucin expression within the abalone foot tissue. Investigation of perlucin-like recombinant proteins as they specifically relate to adhesion would be an intriguing area for future work, particularly as biomineralisation-associated proteins have also been described in barnacle adhesion (Section 3.2).

Scallops diverged from the oyster lineage around 425 MYA.[134] Most, if not all, scallops produce a byssus during early life, although like some oysters, many adults lose the ability as they reach full-size.[135] A novel adaptation for some members of this bivalve family is their ability to swim, thus relinquishing the permanence of attachment observed in mussels; scallops can detach, swim, and reattach their byssus voluntarily.[136]

Recent studies on scallop byssal proteins (sbps) have begun identifying attachment proteins in the species *C. farreri*. From robust scallop tissue transcriptome analyses, 75 foot-specific genes were identified.[131] Then, in-gel trypsin-digested byssal thread peptides were matched to seven transcripts, four of which encoded novel proteins. Despite some similarities to mussel byssus, homology searching against a database of 88 relevant mussel proteins generally failed to show molecular similarity. In 2017, the *C. farreri* genome confirmed an expanded family of foot-specific tyrosinases and linked it with evidence for DOPA within the byssus.[132] Among the different byssal proteins identified, three sbp sequences were used for the production of recombinant proteins (**Figure 6A**; Table 1; Table S1, Supporting Information): Sbp8-1, sbp9, and sbp5-2, named after the numbered sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein bands they were excised from. These recombinant proteins were used to decipher their function within the byssus but also, more recently, to produce bio-inspired materials (Section 5).

Sbp8-1 is a metalloproteinase inhibitor-like protein with an atypical arrangement of cysteines at the C-terminus (Figure 6A).[137] It is present in both the stem and the plaque and

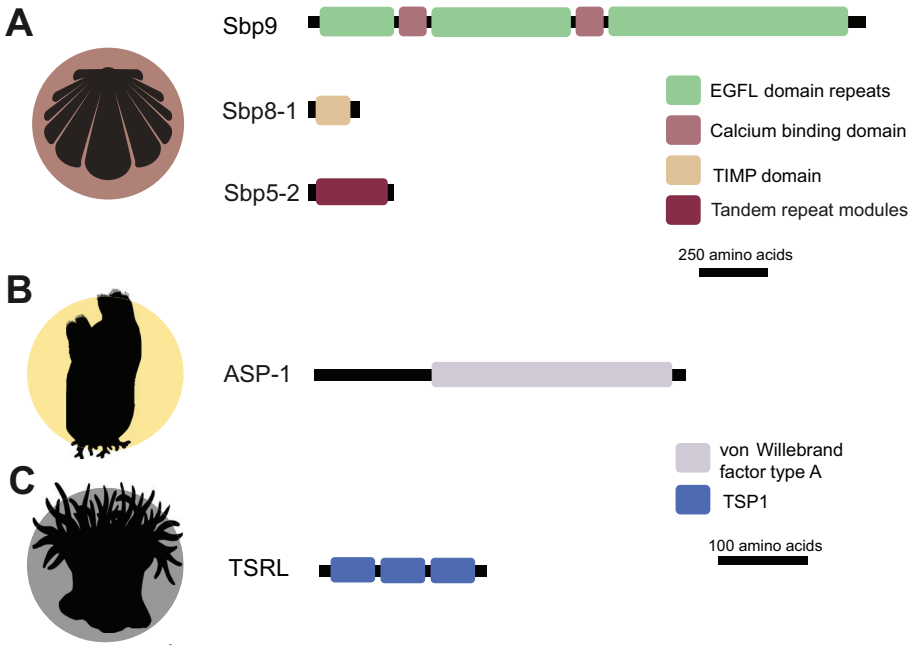

Figure 6. The adhesive proteins of scallops, adult tunicates, and sea anemones that have been produced recombinantly. In these organisms, the specific glands or secretory cells producing these proteins have not been identified. (A) Three scallop byssus proteins have been identified and recombinantly produced: sbp5-2, sbp8-1, and sbp9. Although sbp8 has been synthesized full-length, only fragments of sbp5-2 and sbp9 have been produced. (B) The sea squirt protein ASP-1, characterized by one von Willebrand type A domain, has been produced recombinantly in insect cells. (C) In sea anemones, only one adhesive protein, TSRL, which is comprised of three repeats of thrombospondin type 1, has been produced recombinantly.

is hypothesized to play a role in intermolecular cross-linking of the byssus. The full-length gene coding for this protein, fused with a thioredoxin (Trx) tag coding sequence to enhance solubility, was produced in *E. coli*, without prior codon optimization. Sbp9, on the other hand, was identified as a major structural protein from the byssal root structure.[138] This protein contains two calcium binding domains (CBD) flanked by 49 tandem EGFL domain repeats (Figure 6A). Repeated EGFL domains appear to be a feature of many polymeric holdfasts from various marine invertebrates, including the mussel protein mfp2 (Section 3.1.1), two sea star cohesion proteins (Section 3.6), and octovafibrin which is the major component of octopus egg tethers.[26,139,140] The features of sbp9 (CBDs and EGF domains) were incorporated into recombinant proteins. Codon-optimized genes were synthesized and used to produce three fragments of sbp-9 in *E. coli*. These truncated proteins consisted of the first CBD only (CBD1), the 4 EGFL domains following this CDB ($EGFL_4$), or the whole sequence CBD1-$EGFL_4$.[141] Finally, sbp5-2 is the most abundant protein of the byssal threads and according to Zhang et al., (2022), it consists of 14 tandem repeat modules (Figure 6A).[142] From the alignments provided by the authors, however, there does not appear to be tandem repeat motifs in this byssal protein, but rather 13 imperfect repeat motifs based on semi-regularly spaced cysteines.[143] The DNA sequence coding for the last 7 of these imperfect repeats was incorporated into truncated constructs, without prior codon optimization, for expression in *E. coli*. After the protein was extracted from inclusion bodies and purified, highly extensible fibers could be formed by drawing lyophilized expressed proteins dissolved in hexafluoro-2-propanol from $CaCl_2$ buffer with forceps (see also Section 5).[142]

3.2. Barnacle Adhesion

Barnacles (Arthropoda, Thecostraca) are crustaceans of over 2000 described species that can be divided into two groups, acorn or stalked barnacles (known as sessilia or pedunculate barnacles, respectively).[144] They are arguably best known for their ability to foul marine substrates and are a particular bane to the shipping industry where barnacle fouling causes excessive drag leading to increased fuel consumption and high maintenance costs.[145] Although barnacles can live attached to all manner of inanimate marine surfaces, relatively few species are major biofoulers. Some barnacle species also live epibiotically with a range of living organisms including corals, echinoderms, reptiles, and whales.[144,146] In the adult barnacle, the cement is derived from large unicellular glands and is secreted periodically under the base to maintain attachment (**Figure 7**A).[147] Both barnacle types have a similar pathway for secretion of the adhesive cement, however, differences in secretory product packaging and delivery have been noted between species.[148] Generally, cement secretions travel through a series of ducts lined with epithelial cells before delivery on the substrate. Finer details of this process are still being examined; however, it appears that cement leaves the cells in vacuoles or other vesicles and may be modified further in the collecting ducts.[148]

The adult cement, like other marine adhesives, is primarily proteinaceous. It is difficult to ascertain the exact number of barnacle cement proteins identified to date due to the adopted naming convention. Naldrett (1997) discussed the first major cement constituents based on apparent molecular weights from SDS-PAGE migration.[149] While problematic, subsequent studies continued with this nomenclature, also adding names based

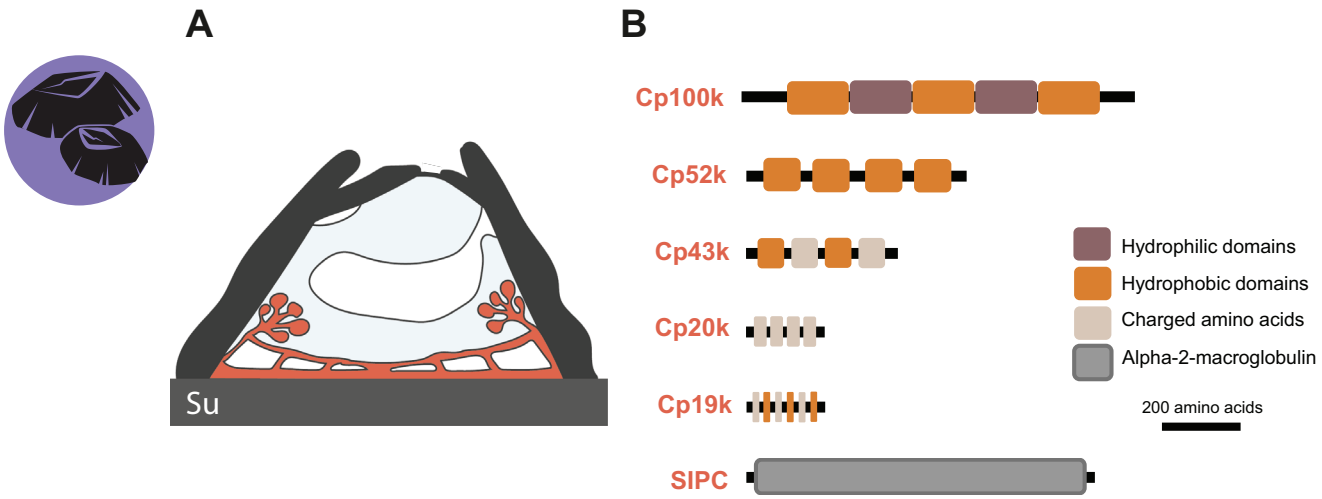

Figure 7. The adhesive system of adult barnacles. (A) Section through an acorn barnacle showing the organization of the cement apparatus (red) which consists of clusters of cement cells located in the basal part of the organism (modified from[147]). After synthesis, cement proteins are delivered to the interface between the barnacle's base and the substrate (Su) through a series of ducts. (B) Among the different proteins constituting the adult cement, six have been produced recombinantly. Most cement proteins are made up of combinations of hydrophilic, hydrophobic, or charged regions except for SICP which is an alpha-2-macroglobulin-like protein.

upon calculated molecular weights predicted from sequenced transcripts. Thus far, the key adhesion proteins reported are cp19k, cp20k, cp43k, cp52k, cp68k, and cp100k.[150,151] However, owing to peculiarities in PAGE migration behavior, versus predicted molecular weight based on sequencing cp43k and cp68k may be the same protein named differently.[29,151–153] Cp100k, cp52k, and cp43/68k are the three major components in terms of amount, making up the bulk of the cement (94%), while cp19k and cp20k are less abundant and thought to be located at the interfaces.[147,150]

Most studies using recombinant technology to characterize barnacle cement proteins have focused on the minor interfacial constituents cp19k and cp20k (Figure 7B; Table 1; Table S1, Supporting Information). The first cement protein to be produced recombinantly was cp19k from the acorn barnacle *Megabalanus rosa* Pilsbry, 1916, named rMrcp19k. Expressed in *E. coli* in soluble form under physiological conditions, rMrcp19k irreversibly adsorbed to a variety of surfaces underwater, including hydrophobic and charged substrates.[154] In the same study, homologous genes were also identified from *Amphibalanus improvisus* (Darwin, 1854) and *Fistulobalanus albicostatus* (Pilsbry, 1916) using the primers designed for *M. rosa*. The protein from *F. albicostatus* (named Balcp19k because the species was formerly known as *Balanus albicostatus*) was produced in fusion with a Trx tag to promote intramolecular disulphide bond formation and solubility, and was successfully expressed in *E. coli* with a yield of 5–10 mg per litre of culture.[155] The resulting recombinant protein, Trx-Balcp19k, self-aggregated into a gel-like substance when dialyzed against pure water. This gel was considered of particular interest because it had a high adhesive strength that rivalled commercial craft glues.[155] Later, it was shown that rBalcp19k, fused or not to Trx, self-assembles into typical amyloid fibrils in seawater.[156,157] The expression of cp19k protein from the stalked barnacle *Pollicipes pollicipes* (Gmelin, 1791), rPpolcp19k, in *E. coli* resulted in low yields of soluble protein under standard conditions.[158] To improve the solubility, a panel of *E. coli* molecular chaperones was co-overproduced with rPpolcp19k, resulting in increased amounts of rPpolcp19k, reaching 1–2 mg of protein per litre of *E. coli* culture. rPpolcp19k demonstrated high adsorption on both hydrophilic and hydrophobic surfaces.[158] Contrary to rBalcp19k, rPpolcp19k does not self-assemble into amyloid fibrils under seawater conditions, although it forms such fibrils under gland-like conditions (lower pH and ionic strength).[159]

The Kamino laboratory, also in 2007, published the characterization of a recombinant 20 kDa protein from *M. rosa* with a preference for adsorption to calcite in artificial seawater.[160] Described in their prior work, cp20k is a protein with low complexity cysteine-rich repeated cassettes; a pattern similar to sp-185 protein (also known as Balbiani ring-3 protein, BR3) from the midge *Chironomus tentans* Fabricius 1805.[161] Proteins with homology to the cysteine-rich cassettes of sp-185 can also be found in the proteotranscriptomic analysis of the byssus of the pearl oyster *Pinctada maxima*[123] and the footprints of the common sea star *Asterias rubens* Linnaeus, 1758 (sfp15; pers. obs; see ref. [162] and Section 3.6). It was shown recently that a recombinant protein expressed in *E. coli* and made up of four repeats of one of the cassettes of cp20k with a Trx tag possesses the same structure and properties as the native protein.[163] In the gregarious *M. rosa*, cp20K appears to be responsible for adhesion to the peripheral calcite shells of other barnacles or possibly to itself, as it has a calcareous base.[160] The latter function was debated (see refs. [147,164,165]) but rMrcp20k was able to affect the kinetics of $CaCO_3$ crystallization in vitro and could thus regulate biomineralization of the base plate in vivo.[166] However, cp20k is also present in pedunculate species without a calcareous base.[167,168] This protein might therefore have alternative roles. As proposed for the sp-185 protein of *C. tentans*,[169,170] cp20k could act intracellularly and in the cement duct to prevent the curing of the adhesive until it reaches the substrate. Further work with recombinant cp20k showed the cysteine-rich protein induced and accel-

erated steel corrosion yet could be utilized as a protective coating when reduced.[171]

Other recombinant cement proteins have been produced but their adhesive properties were not characterized and reported (Table 1; Table S1, Supporting Information). So et al. (2016) discovered most of the peptides excised from a 63 kDa band matched to a transcript with a predicted molecular weight of 43 kDa (Figure 7B). To understand the discrepancy, the authors engineered *E. coli* to express the full-length coding region and found the protein indeed migrated to approximately 60kDa.[151] Further examination revealed the shift was not due to heavy glycosylation and protein complexation, and the amino acid composition is consistent with the 58 and 68 kDa cement proteins reported for the species *Amphibalanus eburneus* (Gould, 1841) and *M. rosa*, respectively.[149,150] A 2015 study by the same research group did not find a potential 68 kDa protein candidate in their transcriptomes of *A. amphitrite*.[172] These results suggest a re-evaluation of the nomenclature may be necessary to avoid further confusion; an effort that has been partially initiated recently.[173] There has been a recombinant cp52k protein produced, however, the details remain unpublished (Zeng 2016, Master's thesis cited in ref. [29]). A recombinant cp100k fragment (from amino acid 10 – 142) and full-length cp20k-1 and cp20k-2 were produced to induce antibody production against the protein in rabbits for immunofluorescence studies.[174,175]

In addition to the previously described adult adhesion, the barnacle cyprid larva also uses adhesion mechanisms during settlement (Figure 4B). The cyprid larva first employs a temporary method of attachment while it searches for an appropriate substrate for settlement, followed by cementation prior to metamorphosis.[147] A pair of sensory-secretory antennules appraise surfaces for conspecific cues for settlement and contribute to temporary adhesion. Antennular gland secretions are used for attachment while mechanical forces allow detachment, thus creating a movement akin to walking.[176,177] The only putative temporary adhesive protein that has been characterized from cyprid footprints is settlement-inducing protein complex (SIPC), which is also known as MULTIFUNCin.[178,179] The SIPC is a glycoprotein complex that is expressed by larval, juvenile, and adult barnacles.[180] It is composed of 3 protein subunits with apparent molecular weights of 76, 88, and 98 kDa. Multiple roles have been proposed for SIPC including adhesion, acting as a conspecific biochemical cue aiding settlement, and potentially playing a role in biomineralization upon metamorphosis. The latter would make it yet another adhesive protein with links to biomineralization (Section 3.1.2). To elucidate the role of SIPC, a full length recombinant SIPC was expressed in insect cells using a baculovirus expression system (Figure 7B; Table 1; Table S1, Supporting Information).[181] Insect cell expression systems are naturally capable of the in vivo modification of amino acids to form DOPA, hydroxyproline, or phosphoserine.[182] This expression system allowed the production of a post-translationally modified rSIPC which was shown to be glycosylated. This rSIPC protein bound to chitin and induced the precipitation of $CaCO_3$, but it could not induce barnacle larval settlement. Of further interest, alpha-2-macroglobulin (α2m)-like proteins similar to SIPC are also found within the adhesives of ascidians and echinoderms (Sections 3.3 and 3.6). The potential role of these proteins in adhesion is yet to be uncovered.

After positioning for settlement, the cyprid then employs a permanent adhesive, the cyprid cement (Figure 4B). The glands utilized for this purpose differ from the unicellular glands used for temporary adhesion, both in shape and location. In acorn barnacles, the cyprid cement glands are located behind the compound eyes and consist of two cell types: α- and β- cells. In *Amphibalanus amphitrite* (Darwin, 1854), the cement is composed of chitin and lipids secreted from β-cells followed by a proteinaceous phase delivered by α-cells.[183] The latter include proteins with homology to adult cement proteins such as cp20k, cp52k, and cp100k but also some cyprid-specific proteins.[174,175,184] In *M. rosa*, Cleverley and colleagues (2021) identified two larval cement proteins (lcp): the arginine rich lcp3-36k and the lysine and arginine rich lcp2-57k. These proteins were produced recombinantly in *E. coli* and used to investigate the curing of the cyprid cement (Table 1; Table S1, Supporting Information). In this model, lcp2-57k would be cross-linked by a lysyl oxidase, while lcp3-36k would inhibit this reaction.[185]

3.3. Ascidian Adhesion

Ascidians (Tunicata, Ascidiacea) are model organisms in developmental biology due to their phylogenomic placement as a sister group to vertebrates.[186] They are significant macrofouling marine invertebrates.[187] Colloquially known as sea squirts, ascidians are a class within Tunicata identified by their characteristic water-filled, sac-shaped body wall constructed from a cellulose exoskeleton, or "tunic". In adult ascidians, the tunic also mediates adhesion to the substratum, a process long thought to involve peptides (tunichromes) containing DOPA and/or TOPA (3,4,5-trihydroxyphenylalanine) residues.[188] In some species, attachment occurs at specialized aboral holdfasts called stolons (Figure 4A).[124,189] Unfortunately, detailed studies on the secretory cells of these adhesive holdfasts are lacking. At the larval "tadpole" stage, ascidians disperse and colonize hard substrates before metamorphosis into the adult form (Figure 4B). In ascidian larvae, the papillae, or palps, are the sensory adhesive organs that aid initial attachment. Collocytes synthesize and deliver adhesive proteins to the anterior tip of the papillae.[190,191] Despite the prominence of numerous ascidian species in aquacultural fouling, recombinant proteins have only been described for one species, *Ciona robusta* Hoshino & Tokioka, 1967. Therefore, the descriptions here are limited to the *Ciona* spp., the vase tunicate, *Ciona intestinalis* (Linneaus, 1767), and *C. robusta*.[192]

Multiple proteins have been identified in both adult and larval stages.[124,193] A 2019 study identified 26 proteins involved in adult adhesion. Among them, 6 were novel proteins that were found highly expressed in the stolon transcriptome versus tissue transcriptomes from other body regions.[124] Like the other marine adhesives discussed thus far, the authors found EGFL and von Willebrand Factor (vWF)-like domains among the novel proteins. Interestingly, they also found proteins with TSP1-like domains, similar to the TSP1-like domains found in the foot proteins from *Pinctada* spp.[120,123] and in the thrombospondin-1 type 1 repeat-like (TSRL) adhesive protein identified in the striped sea anemone (Section 3.5).[125] Similar protein domains were also identified among the proteins encoded by the differentially expressed transcripts of ascidian larval papillae.

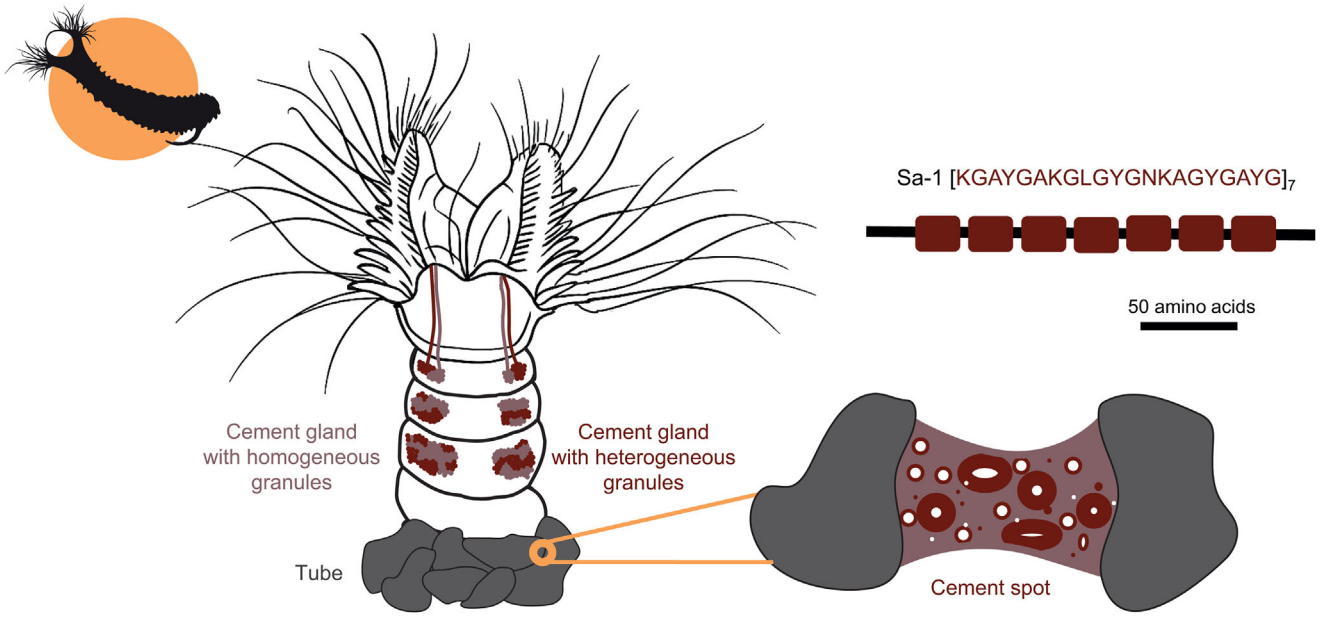

Figure 8. Schematic drawing of the adhesive system in tubeworms of the family Sabellariidae. Different adhesive proteins are packaged in one of two types of cement cells (containing either homogeneous granules or heterogeneous granules) located in three anterior segments of the worm. Granules from both types of cement cells travel through long cell processes and are secreted by the building organ, a structure located near the mouth. Once secreted, their contents coalesce to form the porous adhesive spots cementing sand grains together to build the tube in which the worm is living. To date, only one adhesive protein, Sa-1, has been produced recombinantly. It is produced by cement cells with heterogeneous granules and comprises seven glycine- and tyrosine-rich repeats.

The first recombinant adult ascidian adhesion protein was named ascidian stolon protein-1 (ASP-1; Figure 6B).[124] This novel protein contains one vWA domain, which is thought to contribute to its adhesive ability. The recombinant ASP-1 was further characterized by surface coating and quartz crystal microbalance analyses to test coating ability and adhesion (Table 1; Table S1, Supporting Information). Adhesion was greatly improved by the in vitro modification of tyrosine residues by mushroom tyrosinase; however, it is unclear whether the native protein is post-translationally modified in this way. Moreover, ASP-1 was produced in insect cells, a heterologous system which is known to provide the in vivo conditions necessary for the formation of DOPA residues.[182] This highlights the need to confirm the presence of DOPA in the native protein as, presumably, there would be no need for the use of an additional enzyme if the tyrosine residues within the ASP-1 are meant to be modified. The recombinant constructions that followed were from the sequences identified from larval papillae, and their corresponding proteins are the only recombinant marine adhesives to be produced from a mammalian cell expression system (HEK293) (Table 1; Table S1, Supporting Information). The ascidian papilla adhesive protein-1 (APAP-1) and APAP-2 contribute to cohesion and adhesion, respectively.[193] Both proteins contain functional domains: multiple EGFL domains for APAP-1 and three VWC-like domains for APAP-2. Furthermore, the APAP-2 is rich in serine residues, similar to the barnacle cement proteins, cp19k and cp68k; and the mussel foot proteins mfp5 and mfp6.[106,150,154,194] Phosphorylation is a possible modification at serine sites in APAP-2 which could contribute to mineral binding at the substrate interface.[193] The abundant serine residues in APAP-2 could also be sites of *O*-glycosylation, which would be consistent with the results of lectin labelling of collocytes and glue prints left by larvae on the substratum.[195] Again, whether or not PTMs occur within the native and/or the recombinant proteins produced within HEK293 cells needs confirmation.

3.4. Tubeworm Adhesion

Tubeworms (Annelida, Polychaeta) of the family Sabellariidae are annelid worms that live in self-built tubes attached to hard substrates from the intertidal zone down to the abyssopelagic zone at around 6000 m.[196] Adults, which reach 20 – 75 mm in length, use tentacles to fastidiously collect sand and other fine minerals for building individual tubular housings.[196] Particles are delivered to the building organ which is associated with the cement glands located within the parathorax. The building organ sorts and provides selected particles with a small amount of cement, a permanent proteinaceous adhesive, for the extension of the tube (**Figure 8**). Many sabellariid species are gregarious and their individual efforts in tubular home building have created large reefs, in some cases kilometres wide.[197,198]

Studies have described the cement of several gregarious Sabellariidae species, such as the sandcastle worm, *Phragmatopoma californica* (Fewkes, 1889); the West Atlantic sandcastle worm, *Phragmatopoma caudata* Krøyer in Mörch, 1863; and the honeycomb worm, *Sabellaria alveolata* (Linnaeus, 1767).[197,199] The cement consists primarily of proteins with highly repetitive block structures and also incorporates Mg^{2+} and Ca^{2+} ions.[200,201] In *P. californica*, several studies have described putative cement se-

quences, named Pc-1, Pc-2, etc.[197,201–204] By designing primers based on several sequences of *P. californica*, three sequences have also been retrieved from *S. alveolata*; coding for two DOPA-containing, basic (Sa-1 and Sa-2) and two phosphoserine containing, acidic proteins (Sa-3a/b).[205] The Sa-1 cement protein is glycine- and tyrosine-rich (Figure 8), while Sa-2 is rich in histidine residues. The cement protein Sa-1, which appears to be a homologue of Pc-1, has been produced in *E. coli* after codon optimization of its coding sequence and the addition of a his_6-tag for purification via metal affinity chromatography (Table 1; Table S1, Supporting Information). Although production was within the insoluble fraction, yields of 50 mg of pure protein per litre of culture were obtained. However, post-translational hydroxylation of tyrosine residues into DOPA by using a tyrosinase was not attempted, and further results remain unpublished (Lejeune and Van de Weerdt, unpublished, cited in ref. [200]).

3.5. Sea Anemone Adhesion

Sea anemones (Cnidaria, Hexacorallia) are polyps, usually sessile, that have a basic body plan consisting of a cylindrical column topped with a crown of tentacles surrounding the mouth, and a base attached either to a substrate or capable of burrowing into soft sediments. Sea anemones that attach to solid substrates do so via adhesives secreted by the ectoderm of the pedal disc (Figure 4A).[206] They are capable of transitory adhesion, moving slowly using muscular contractions.[126,207] Moreover, several species of sea anemone can detach from substrates, and at least one species is known to swim.[208]

Within the ectoderm of the pedal disc of the glass anemone, *E. diaphana*, previously named *E. pallida*, are two types of potential adhesive secretory cells containing distinct vesicles.[209] Histological staining of pedal disc and adhesive footprints revealed that DOPA-containing proteins and glycosylated proteins are possibly included in the adhesive material of *E. diaphana*.[209] Transcriptomics (unconfirmed by proteomics or qRT-PCR) revealed, again, that multi-domain extracellular matrix-like proteins are involved in adhesion of sea anemones and include putative proteins with EGFL, TSP1, and C-type lectin domains.[126] In a subsequent report, 13 of these proteins were identified by proteomics in the secreted adhesive, comprising both modular ECM-type proteins and enzymes, including a tyrosinase.[13] Differential gene expression and proteomics methods were also utilized to find putative adhesive proteins in the striped sea anemone, *D. lineata* (formerly *Haliplanella luciae*). Thirty-two proteins similar to those of *E. diaphana* were identified, including the cysteine-rich TSRL protein.[125]

The TSRL protein was produced recombinantly in *E. coli* (Figure 6C; Table 1; Table S1, Supporting Information). The recombinant protein self-assembled into hydrogels, absorbed onto diverse surfaces in the presence of calcium ions, was biocompatible and improved the survival of cells undergoing oxidative stress.[125]

3.6. Sea Star Adhesion

Found globally and throughout diverse marine ecosystems from the intertidal to the abyssopelagic zone, sea stars (Echinodermata, Asteroidea) are emblematic marine organisms of approximately 2000 species.[210] Sea stars utilize temporary adhesion which allows movement around their various environments.[211] In a repetitive attachment/detachment mechanism, they can firmly adhere and then release via chemical secretions from their numerous sensory-secretory tube feet (podia) (Figure 4A).[212] These secretions are produced, respectively, by unicellular adhesive and deadhesive glands that together form a so-called duo-gland adhesive system (**Figure 9A**).[11,213]

While mytilids have become the model species for permanent adhesion, the common sea star, *Asterias rubens*, has become the model of temporary adhesion. Early research began with studies on adhesive mechanics and ultrastructure and has culminated recently in the reporting of a comprehensive list of proteins involved in adhesion.[162,211,214] The study of sea star temporary adhesion has been aided by the rather permanent footprint which remains after tube foot detachment.[212] These footprints, analyzed by TEM and mass spectrometry, consist of fibrous elements delivered by type 1 adhesive cells layered over a homogenous film delivered to the substrate by type 2 adhesive cells (named AC1 and AC2, respectively). A third element delivered by de-adhesive cells (DAC) is thought to provide a means of detachment via an enzymatic process (Figure 9A).[162]

To date, a catalogue of 16 specific sea star footprint proteins (sfps) have been identified, most of which are multimodular proteins comprising several different functional domains.[162,215] This catalogue includes 15 proteins localized to AC1 and AC2, and one astacin-like enzyme localized to the cells predicted to be involved in de-adhesion of the tube foot. In situ hybridization localized sfp1-6 to AC1 cells, contributing to the cohesive fibrillar meshwork, and sfp7 and 8 to AC2, contributing to the substrate primer layer. The sfp9 to 15 are found within the vesicles of both cell types. Of interest, two sfps (sfp1 and 2) appear to consist of subunits linked by disulphide bonds and sfp3 and 4 contain repeating EGFL domains similar to those found in the adhesive proteins mfp2 (mussel), sbp9 (scallop) and APAP-1 (sea squirt).[216] The sfp9 contains α2m-like domains like the SIPC in barnacles. Furthermore, sfp15 is cysteine-rich in a similar way to barnacle cp20k and the BR3/sp-185-like proteins found in oyster byssus.[162]

One of the most abundant sfps, sfp1, has been more comprehensively characterized.[216] The sfp1 protein sequence includes a predicted signal peptide followed by a sequence of 3833 amino acids. Sfp1 is translated from a single mRNA and then cleaved into four subunits (sfp1α, -β, -γ, -δ) linked together by disulfide bridges in tube foot adhesive cells (Figure 9B). This protein is characterized by a high cysteine content (5%) and regions of homology to multiple conserved domains which may facilitate cohesive and adhesive interactions. Sfp1 has been immunolocalized to the secretory granules of AC1 within the adhesive epidermis and in the cohesive meshwork of the footprint material. Because it displays many specific protein-, carbohydrate-, and metal-binding domains, sfp1 may have a cohesive function, forming the structural scaffold of the footprint and interacting with itself or with other sfps.[216]

Anticipated difficulties in expressing the high molecular weight sfp1 (426 kDa) precluded the expression of the entire protein. Therefore, fragments of sfp1 were recombinantly produced in *E. coli* (Table 1; Table S1, Supporting Information). The recom-

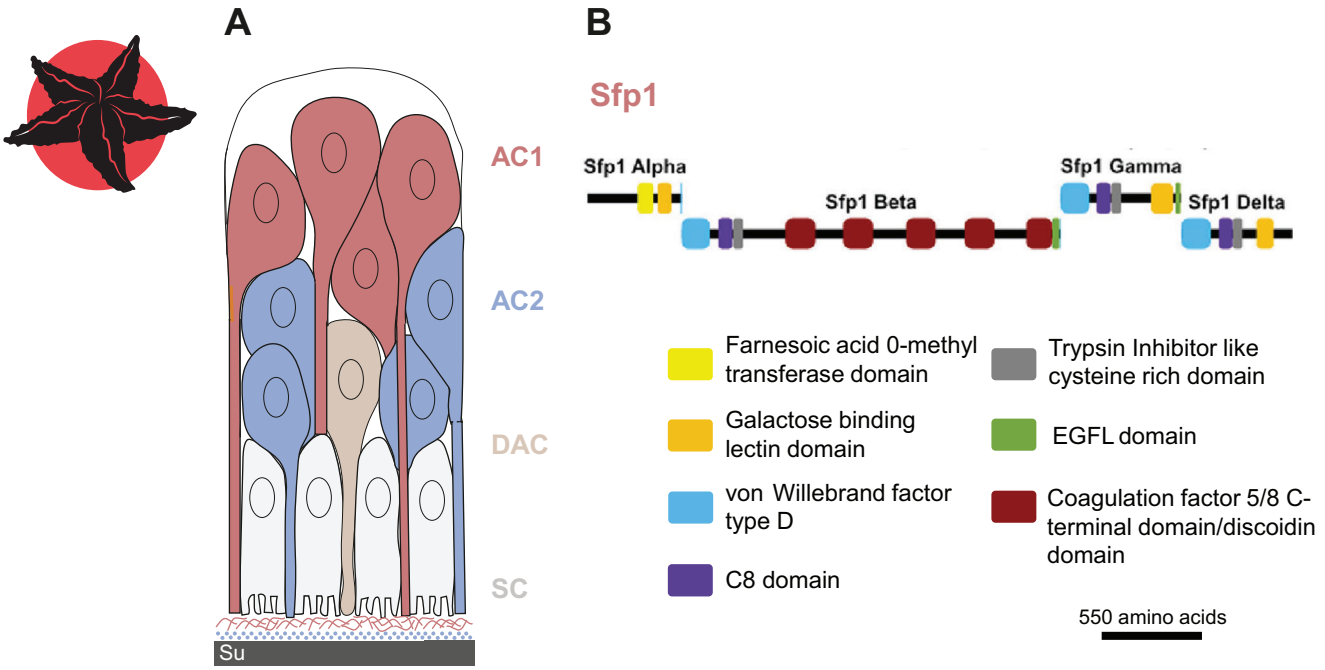

Figure 9. The duo-gland adhesive system of sea stars. (A) At the tip of sea star tube feet, the epidermis encloses three types of secretory cells -i.e., type 1 and 2 adhesive cells (AC1 and AC2) and de-adhesive cells (DAC)- interspersed with support cells (SC) (modified from[162]). When a tube foot attaches, type 2 adhesive cells release their contents, surface active proteins, which form a homogeneous film covering the substrate (Su). Concomitantly, type 1 adhesive cells release proteins with a bulk function, which form a thick cohesive meshwork structure. (B) Sfp1 is a large multimodular adhesive protein produced by type 1 adhesive cells. Thanks to its different functional domains, it can presumably interact with multiple partners and makes up the structural scaffold of the adhesive footprint that remains on the substrate after tube foot detachment. Two fragments of sfp1 have been produced recombinantly; the C-terminal part of the β subunit and the δ subunit.

binant fragments, the C-terminal end of the β-subunit (rsfp1β C-term), and the δ-subunit (rsfp1δ), include representatives of most of the sfp1 domains. The rsfp1β C-term fragment includes 2 FA58C domains and 1 EGFL domain. The complete rsfpδ fragment also has an EGFL domain, as well as vWF type D, D-gal lectin, C8 and TILa domains. In the native sfp1, sfp1δ shares similarity of sequence and domains with sfp1γ, and to a lesser extent both sfp1β and sfp1δ share similarity with the other subunit AC1 protein, sfp2. Consequently, the recombinant sfp1 proteins provided an opportunity to explore the most significant properties of the bulk portion of the adhesive. The sfp1 protein fragments fused with his_6-tags, were expressed in inclusion bodies, as is common for bacterial expression of recombinant adhesive/cohesive proteins reviewed thus far (Table S1, Supporting Information). Proteins forming inclusion bodies were denatured and reduced which allowed for further purification by immobilized metal affinity chromatography.[217] The experiment yielded 12 mg and 4 mg of rsfp1β and rsfp1δ per litre of culture, respectively. The rsfp1 protein fragments, particularly rsfp1β C-term, were found to self-assemble and adsorb to surfaces in the presence of cations and formed homogenous cytocompatible coatings.[217,218] In a follow-up study, smaller fragments of rsfp1β C-term were produced.[219] The analysis of their adsorption capacities on glass showed that two mechanisms are involved in rsfp1β C-term adsorption: one mediated by the EGFL domain and involving divalent cations, and one mediated by a sequence with no homology to known functional domains, in the presence of both monovalent and divalent cations. Using this approach, which has also been used in scallops and barnacles (Table 1; Table S1, Supporting Information), helps disentangle the roles of each functional domain or region within an adhesive protein.

For all species investigated above, adhesive secretions are usually composed of a variety of different proteins (see Figures 5–9). According to their sequence and structure, these proteins may achieve various subfunctions within the secreted adhesive (e.g., interfacial adhesive or bulk cohesive interactions). And although much emphasis has been placed on the recombinant production of short interfacial adhesive proteins (mussel mfp3, mfp5, barnacle cp19k, etc), many recent studies have also described the production of fragments of larger multimodular proteins (sea star sfp1, scallop sbp9, sea anemone TSRL, etc). These truncated proteins can not only help us interpret the role of their constituent domains but also retain the properties that make them interesting components of new biomaterials (Section 5).

4. Hybrid Recombinant Adhesive Proteins

Hybrid proteins result from the joining of two dissimilar target proteins in a single polypeptide chain (**Figure 10A**).[220] This is achieved by the in-frame fusion of their coding DNA in a similar way to the methods used to add purification or solubility tags to recombinant proteins (Figure 3). While inspired by the characteristics of the individual native proteins, the fused proteins may display different properties compared to the originals (**Table 2**). Furthermore, the strategic addition of a disparate protein may aid the increased production of a desired recombinant adhesive.

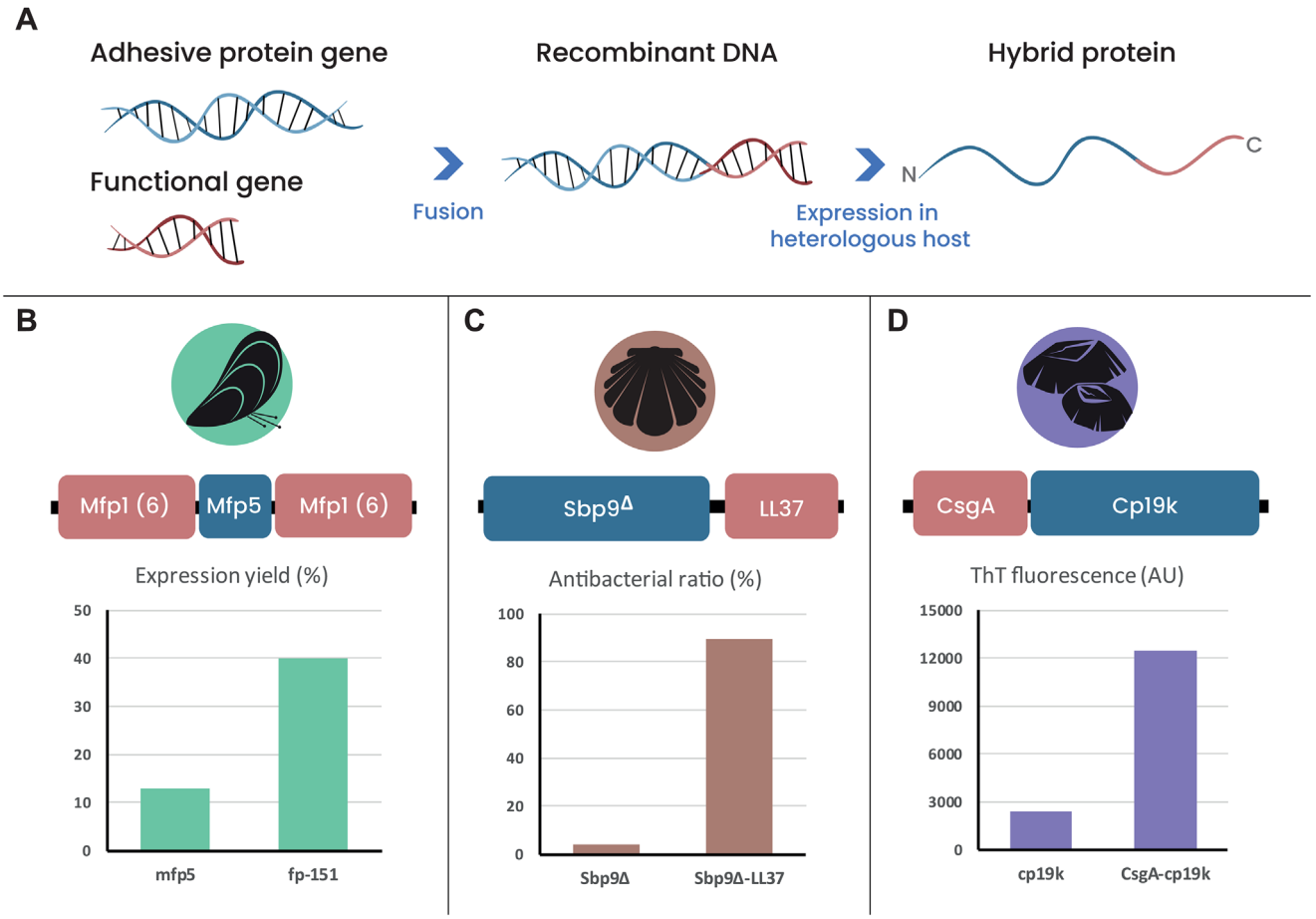

Figure 10. Hybrid protein design and their improved performances. (A) The DNA sequence coding for an adhesive protein can be cloned into an expression vector in frame with a sequence coding for another protein. After production, the resulting hybrid protein usually displays new functionalities. (B) The hybrid protein fp-151, obtained by the fusion of decapeptides from the mussel cuticle protein mfp1 at each terminus of the interfacial adhesive protein mfp5, increased expression yield three-fold compared to recombinant mfp5 alone (data from[221]). (C) Coatings made up of the hybrid protein Sbp9$^{\Delta}$-LL37, obtained by the fusion of the partial scallop adhesive protein Sbp9$^{\Delta}$ with antimicrobial peptide LL37, displayed antimicrobial activity whereas coatings consisting of Sbp9$^{\Delta}$ alone did not (data from[222]). (D) The hybrid protein CsgA-cp19 k, obtained by the fusion of bacterial curli protein CsgA with barnacle cement protein cp1 9k, demonstrated improved self-assembly into amyloid fibrils compared to recombinant cp19k alone as evidenced by Thioflavin T fluorescence after a 16 h incubation in artificial seawater (data from[223]).

In marine adhesion, the first hybrid proteins were designed to overcome the low production yield, low purification yield, and high levels of post-purification insolubility of recombinant mussel adhesive proteins. The most investigated mussel hybrid protein is fp-151. Recombinant fp-151 consists of the fusion of the interfacial adhesive protein mfp5 flanked at each terminus by decapeptides of the cuticle protein mfp1 (Figure 10B).[221] Its expression yield accounted for ≈40% of *E. coli* total protein, corresponding to an extraction yield of about 100 mg/l of batch-type flask culture, compared to ≈13% (about 3 mg/l) for mfp5 (Figure 10B; Table S1, Supporting Information).[221] The yield was further increased by co-expression of fp-151 with *Vitreoscilla* hemoglobin (VHb) which facilitates oxygen utilization by bacterial cells.[254] For another hybrid protein, fp-353, which results from the fusion of the interfacial adhesive protein mfp3 to each terminus of mfp5, the production yield was not higher than mfp3A alone, a previously studied recombinant made by the same research group.[237] Nevertheless, fp-353 was more soluble than mfp3 or mfp5 alone, permitting the preparation of a concentrated protein solution and the formation of viscous glue for large-scale adhesion strength measurements.[237] All the above hybrid proteins were still produced in inclusion bodies, however. Using a system in which hybrid proteins were co-expressed with the molecular chaperones SUMO and Trx, Wang et al. (2023) were able to increase soluble expression of fp-31, fp-33, and fp-35 (i.e. mfp3 with mfp1, -3, or -5), avoiding the formation of inclusion bodies.[238] Recently, yet another hybrid mussel recombinant protein, HRfp-1, was produced in *E. coli*. This hybrid recombinant protein consists of 12 mfp1 decapeptide repeats flanked at the N- and C-termini by the histidine-rich (HR) domain of mfp4, reporting higher adhesive strength than mfp1 alone.[243]

To address the post-translational modification issue, fp-151 was produced in Sf9 insect cells using a baculovirus-based expression system allowing the in vivo modification of tyrosine residues to DOPA, along with the modification of serine and proline residues to phosphoserines and hydroxyprolines,

Table 2. Hybrid recombinant proteins constructed by fusing marine adhesive proteins with other proteins or peptides, with details about their production and use.

Name of hybrid protein	Recombinant construct	MW (kDa)	Host for expression	Use of hybrid protein	References
Mussels					
Fp-151	Fusion of 6 mfp1 decapeptide repeats at the N- and C-termini of full-length mfp5	≈ [24-30	B	Fabrication of bioadhesives, antibacterial coatings, and hemostatic patches	[101,221,224–229]
		23.6	IC	Surface coating	[182]
Fp-151-RGD	Fusion of fp-151 with Arg-Gly-Asp peptide	≈ 28	B	Fabrication of a cell adhesion material	[230,231]
r-fp-151-VT	Fusion of fp-151 and vitronectin	≈ 27	B	Development of a therapeutic agent to treat skin inflammation	[232]
MAP-SP	Fusion of fp-151 and substance P peptide	24.3	B	Fabrication of hydrogel for nerve repair	[233]
MAP-FPs	Fusion of fp-151 and various antimicrobial peptides	≈ 26	B	Antibacterial compounds	[234]
RAP	Fusion of fp-151 and various antimicrobial peptides	≈ 26	B	Fabrication of RAP-coated skin patches	[235]
MAP-VEGF, MAP-QK, MAP-FGF2, and MAP-RGD	Fusion of 12 decapeptide repeats of mfp1 with different biofunctional peptides	≈ 14-16	B	Fabrication of an adhesive microneedle bandage	[236]
Mfp-353	Fusion of full-length mfp3A at the N- and C-termini of full-length mfp5	≈ 22	B	Bioadhesive fabrication	[237]
Fp-31	Fusion of full-length Mgfp3B with a partial 6-decapeptide mfp1	≈ 27	B	Sustainable supply of protein adhesive material	[238]
Fp-33	Fusion of two full-length mfp3	≈ 26	B	Sustainable supply of protein adhesive material	[238]
Fp-35	Fusion of full-length Mgfp-3B with full-length Mgfp-5	≈ 26	B	Sustainable supply of protein adhesive material	[238]
CsgA-mfp3	Fusion of the bacterial curli protein CsgA and full-length mfp3	≈ 25–28.5	B	Amyloid fibrils formation	[45,239]
CBD-CsgA-mfp3	Fusion of the chitin-binding domains from *Bacillus circulans* chitinase, the bacterial curli protein CsgA and full-length mfp3	≈ 32	B	Amyloid fibrils formation	[240]
Mfp3-GvpA	Fusion of full length mfp3 and cyanobacterial gas vesicle protein GvpA	≈ 31	Y	Fabrication of high performance bio-inspired biomaterial	[241]
A-S-Mefp3-P	Fusion of full-length mfp3 and a gel-forming protein comprising a leucine-zipper, an unstructured polyelectrolyte and a helical coiled-coil domain	≈ 30	B	Fabrication of hydrogels for use as a cardiac patch	[242]
HRfp-1	Fusion of the partial, histidine-rich domain of mfp4 at the N- and C-termini of 12 mfp-1 decapeptide repeats	≈ 16.2	B	Fabrication of hydrogels	[243]
$Mfp5^{(2)}$	Fusion of two full-length mfp5	≈ 20	B	Fabrication of graphene oxide-mfp composites	[244,245]
BC-MAP	Fusion of domains B and C of protein A with full-length mfp5	≈ 23.5	B	Fabrication of ELISA platforms	[246,247]
Mfp5-CsgA	Fusion of full-length mfp5 and the bacterial curli protein CsgA	≈ 28.5	B	Amyloid fibrils formation	[45]
Mfp5-CsgA-CBD	Fusion of full-length mfp5, the bacterial curli protein CsgA and the chitin-binding domains from *Bacillus circulans* chitinase	≈ 37	B	Amyloid fibrils formation	[240]
VE-mfp5	Fusion of full-length mfp5 and the C-terminus of VE-cadherin extracellular domain EC1-2 protein	56	B	Fabrication of coatings for vascular stents	[248,249]
TLC-M	Fusion of the LC domain of the transactive response (TAR) DNA binding protein of 43 kDa (TDP43 LC) and full-length mfp5	nr	B	Liquid-liquid phase separation and amyloid fibril formation	[250]
8xKLV-mfp-5	Fusion of the zipper-forming domain of an amyloid protein, flexible spider silk sequences and full-length mfp5	≈ 35.9	B	Fabrication of an underwater adhesive protein hydrogel	[251]

(*Continued*)

Table 2. (Continued)

Name of hybrid protein	Recombinant construct	MW (kDa)	Host for expression	Use of hybrid protein	References
Mfp5-GvpA	Fusion of full-length mfp5 and cyanobacterial gas vesicle protein GvpA	≈ 32	Y	Fabrication of high performance bio-inspired biomaterial	[241]
A-S-Mefp5-P	Fusion of full-length mfp5 and a gel-forming protein comprising a leucine-zipper, an unstructured polyelectrolyte and a helical coiled-coil domain	≈ 32	B	Fabrication of hydrogels for use as a cardiac patch	[242]
^{N}M-16xFGA-^{C}M	Fusion of Mfp5 fragments to the termini of a 16-repeat of artificially-designed amyloid-silk protein 16xFGA	≈ 60	B	Fabrication of fibres using spinning protocol	[252]
Scallops					
Sbp9$^{\Delta}$	Fusion of 4 Epidermal Growth Factor-like repeats of Sbp9 and antimicrobial peptide LL37	nr	B	Fabrication of coatings for wound-healing	[222]
Barnacles					
CsgA-cp19k	Fusion of the bacterial curli protein csgA with full-length cp19k	≈ 30	B	Amyloid fibrils formation	[223]
Cp19k-MaSp1	Fusion of cp19k with *Nephila clavata* dragline silk protein	≈ 37	Y	Fabrication of protein fibre scaffolds	[253]

Abbreviations: B, bacterium (*E. coli*); IC, insect cells (*Sf9*); Y, yeast (*P. pastoris*)

respectively.[182] Compared to the fp-151 produced in *E. coli*, the transgenic insect cell-produced fp-151 exhibited a ≈2-fold higher coating ability. In another approach a co-expression system was used in bacteria, with the production of fp-151 and mushroom tyrosinase with a dual vector system. The in vivo modification efficiency was higher than that in vitro, leading to an increased adhesive strength.[101]

Adhesives can also be fused with other types of proteins or peptides to bring new functionalities. For example, fp-151 was produced fused to an Arg-Gly-Asp (RGD) peptide at its C-terminus. RGD is a cell adhesion recognition motif and the hybrid fp-151-RGD protein presented superior cell spreading and cell-adhesion abilities compared to commercial cell culture coatings.[230] The RGD peptide has also been fused with mfp1 and the resulting hybrid protein was used to fabricate a cardiac patch that allows rapid and efficient anchoring of viable cardiomyocytes as well as recruitment and migration of endothelial cells into cardiac infarcted areas (Section 5).[236] Similarly, fp-151 has also been fused with the neurotransmitter peptide substance P, creating a bioadhesive hydrogel for sutureless nerve repair[233] while mfp1 has been fused with several biofunctional peptides (VEGF, QK, FGF2, see also Section 5) as a therapeutic in cardiac pathology.[236] Mfp5 has been fused with two domains (B and C) of protein A (antibody-binding protein) for efficient antibody immobilization on diverse surfaces.[246] Additionally, fp-151 and sbp9 have been fused with various antimicrobial peptides and used for skin treatment (Figure 10C).[222,234,235]

Mussel adhesive proteins have also been fused with the bacterial curli protein CsgA or gas vesicle protein A (GvpA). Mfp5-CsgA and mfp3-GvpA have been produced in the yeast *P. pastoris*[241] while CsgA-mfp3 and mfp5-CsgA have been produced in the bacterium *E. coli*.[45,239] The latter have also been produced in fusion with a chitin-binding module.[240] In these studies, the bacterial protein partners, both known to form amyloid-like fibrils, were added to potentially improve self-assembly of the hybrid proteins. This effect was demonstrated in the study of Li et al. (2022) in which hybrid proteins made up by the fusion of CsgA and the barnacle cement protein cp19k can rapidly self-assemble into amyloid fibrils in artificial seawater (Figure 10D).[223] Amyloid fibrils can additionally provide interesting material properties such as resistance to degradation and mechanical strength. In the same vein, Kim et al. (2021) designed a complex hybrid protein comprising a zipper-forming sequence from Aβ amyloid protein, a flexible sequence from the Nephila clavipes dragline spider silk protein MaSp1, and mfp5.[251] The amyloid and spider silk sequences were repeated 8 times to obtain a sufficient chain-length for self-assembly into stable β-crystals under aqueous conditions. The hybrid protein formed a semi-crystalline hydrogel that exhibited high strength and toughness, as well as strong underwater adhesion to a variety of surfaces.[251] Other hydrogels were created by fusing full-length mfp3 or mfp5 with a gel-forming protein comprising a leucine-zipper domain and a coiled-coil domain.[242]

5. Recombinant Protein Complexes for Biomaterial Design

Biomaterials, by definition, are biological and/or synthetic substances introduced into living systems to solve a medical issue. Native adhesive proteins display intrinsic properties that have been exploited to produce increasingly complex biomaterials with added functionalities, such as wearable electronics or drug delivery systems (**Figure 11**). Many of these materials utilize recombinant forms, usually from mfps, combined with metal coordination or bio-mimicked fabrication techniques. Ma-

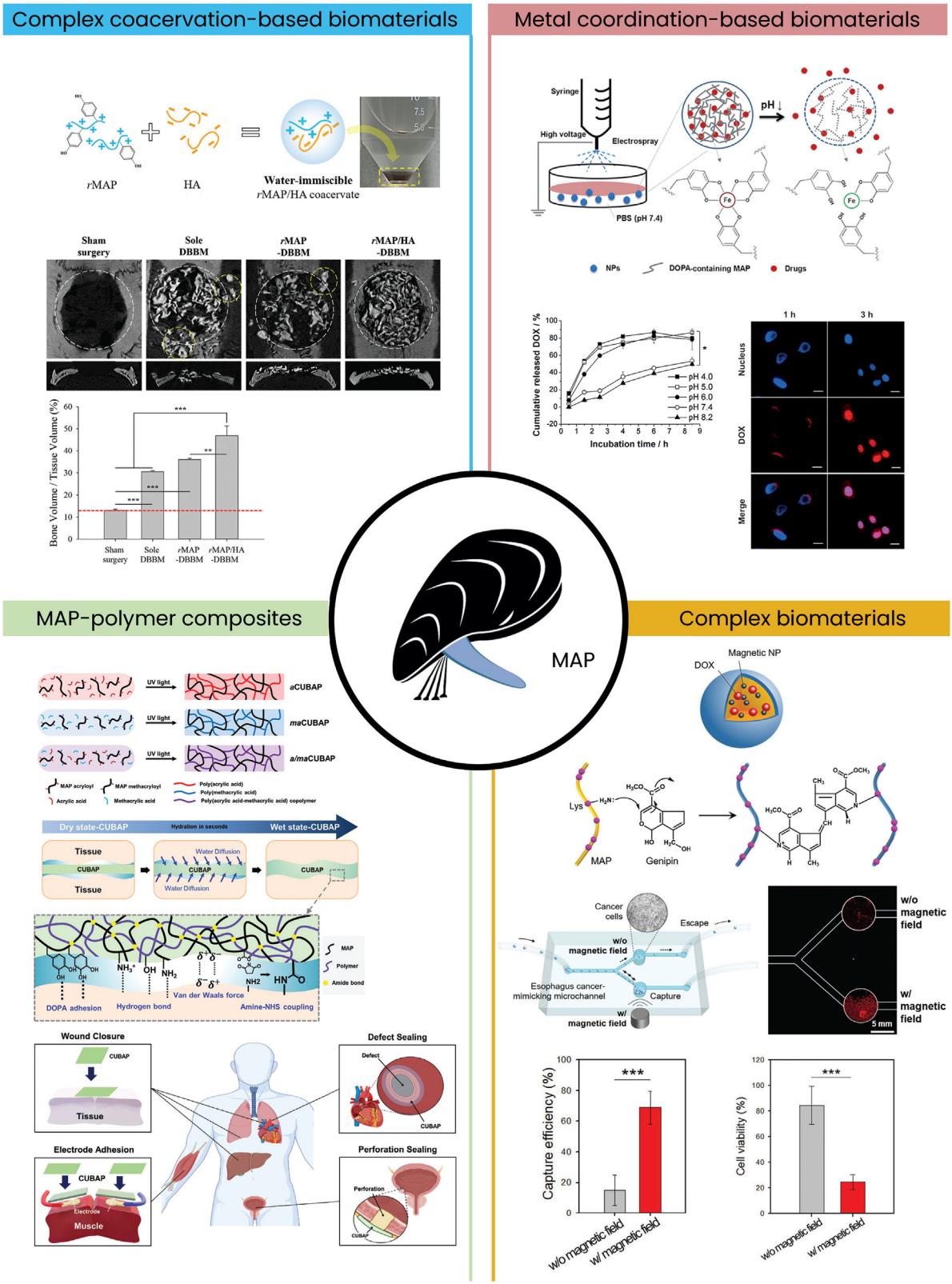

Figure 11. Examples of biomaterials fabricated through the association of mussel adhesive proteins (MAPs) with other components such as polysaccharides, ions, or synthetic materials. The hydrogels, fibers, or films assembled in this way were further processed or combined to fabricate complex biomaterials for various medical applications. (A) A bone graft binder has been obtained through the complex coacervation of negatively charged hyaluronic acid (HA) with a positively charged recombinant MAP. The *r*MAP/HA coacervate stabilized the agglomerated deproteinized bovine bone

jor themes stemming from observations of natural systems are self-assembly and complex coacervation.

Directly inspired by marine adhesive systems, complex coacervation lends itself to the production of materials incorporating recombinant adhesive proteins.[259] Complex coacervation is the liquid–liquid phase separation (LLPS) of macromolecules in solution.[31,260] This process is often driven by electrostatic interactions but may involve other types of interactions. In marine adhesives, it was first described in the tubeworm *P. californica* in which the association of oppositely charged adhesive proteins results in their condensation within the cement glands.[259] LLPS can also occur in a solution containing only one type of macromolecule, and some native mussel proteins (e.g., mfp1, -3 and -5) have also been reported to form such coacervates in vitro.[85,105,261] Coacervates are useful in many applications because their fluid-like properties enable surface wetting through low interfacial tension.[260] Complex coacervates have often been formed by associating the positively charged recombinant protein mfp-151 with negatively charged hyaluronic acid, a chief component of the extracellular matrix (Figure 11A).[225,231] These coacervates have then been applied as drug-carrier microcapsules,[262] as a bioadhesive sealant for urinary fistula repair,[263] as a bone graft binder[255] (Figure 11A), as an injectable material with encapsulated mesenchymal stem cells for cardiac regeneration,[264] or as a drug-loaded bioadhesive for skin or cardiac regeneration.[227,265]

The intrinsic ability of some native adhesive proteins to self-aggregate in the presence of metal ions is another way to form bio-inspired materials. For example, protein-metal coordination bonds allowed the formation of extensible fibers when recombinant scallop protein sbp5-2 was mixed with Ca^{2+}. The introduction of conductive graphene during the fiber drawing process allowed the fabrication of wearable or implantable electronic motion sensors.[142] Similarly, coatings for skin wound healing were self-assembled by mixing $sbp9^{\Delta}$ with Ca^{2+}.[108] In another example, the formation of DOPA-metal complexes between the recombinant mussel protein mfp1 and Fe^{3+} ions can lead to the formation of self-healing hydrogels.[266] By using coaxial electrospraying, this same material can be turned into uniform-size drug-loaded nanoparticles which can then be used for drug delivery by exploiting the pH-dependent changes in the structure of DOPA-Fe^{3+} complexes (Figure 11B).[256] In a similar way, a fp-151 – Fe^{3+} hydrogel loaded with the antibiotic gentamicin was used as an antibacterial coating for titanium bone implants.[228] Implants were coated through a simple dip-coating process before being placed surgically in mice. The gentamicin-loaded coating exhibited complete inhibition of bacterial growth in vivo against *Staphylococcus aureus* by enabling bacterial concentration-dependent antibiotic delivery in response to infection-induced acidification.[228] However, as DOPA residues cannot be involved in both hydrogen bonding with the surface and metal coordination in the bulk material, surface adhesion is reduced when cohesion is enhanced. To circumvent this problem, Maeng and others (2024) designed the hybrid mussel protein HRfp-1 (Section 4) in which the histidine-rich domain of mfp4 allows hydrogel formation owing to reversible metal coordination bonds with Zn^{2+} ions, while the DOPA moieties of the mfp1 repeats are preserved for surface coupling.[243] The developed self-healing hydrogel thus exhibits both high adhesion and cohesion in underwater environments.

Recombinant adhesive proteins have been combined with synthetic polymers to produce materials with improved properties. Recombinant fp-151, recombinant mfp1 – Fe^{3+} complexes, and the hybrid protein cp19k-MaSp1 have been electrospun with polycaprolactone or poly L-lactide-co-caprolactone to produce stiff nanofibers that can be used to engineer tissue scaffolds.[253,256,267] Recombinant mfp5 was combined with graphene oxide to make thin high-strength and -toughness films, while recombinant fp-151 was conjugated to poly(meth)acrylic acid to fabricate adhesive patches promoting effective wound healing in diverse internal organs (Figure 11C).[257,268] Other types of bi-layer patches were produced using the hybrid leucine-zipper mfp3/5 protein hydrogels or combining mfp-151 with silkworm silk fibroin, and were tested on heart or liver tissues, respectively.[229,242]

Other novel ways to incorporate marine adhesives in medicine and surgery include the use of bioengineered mussel adhesion proteins (MAPs) to effectively deliver treatments to difficult-to-target tissues, such as the esophagus and heart.[236,258] In 2021, Choi and others developed a method of embedding iron oxide nanoparticles within bioengineered genipin-mfp1 microparticles for localized anticancer drug delivery (Figure 11D). Genipin is a natural cross-linking agent derived from gardenia fruit.[269] Here it was used to cross-link the lysine residues of mfp1 to create a cuticle around the iron-oxide nanoparticles while keeping the DOPA residues free for adhesion with the target tissue. Therefore, the microparticles can be magnetically guided and kept in place until adhesion has occurred.[258] In a different 2021 study, Lim and others designed and constructed microneedle patches to deliver growth factor-fused biofunctional mfp1 locally to myocardial tissue for repair post infarction. The patch consists of a thin regenerated silk fibroin-based coating deposited on a mfp1/hyaluronic acid coacervate-based hydrogel, the two layers being intra- and inter-crosslinked via dityrosine crosslinks. Once placed on the surface of the heart, the microneedles swell within

minerals (DBBM)and promoted in vivo bone regeneration. Reproduced with permission.[255] 2016, Wiley. (B) Nanoparticles (NP) based on Fe^{3+}–DOPA complexation with recombinant DOPA-containing MAP were synthesized using an electrospraying process and loaded with doxorubicin (DOX). In vitro, the DOX-loaded NPs released the associated drug by changing the pH. Fluorescence microscopy images of HeLa cells incubated with DOX-loaded NPs for one and three hours showed the cellular uptake behavior of DOX. Reproduced with permission.[256] 2015, Wiley. (**C**) Customized underwater bioadhesive patches (CUBAPs) have been fabricated by ultraviolet (UV) crosslinking of MAP-(meth)acryloyl with (meth)acrylic acid to form poly((meta)acrylic acid). CUBAPs display switchable underwater adhesiveness: initially dry and nonadhesive, they absorb moisture when applied to living tissues and develop strong surface adhesion based on different molecular interactions. CUBAPs have been evaluated ex vivo and in vivo for healing wounds in diverse internal organs and implanted electronic devices. Reproduced with permission.[257] 2024, Wiley. (D) Drug-loaded magnetic microparticles have been prepared by incorporating iron oxide (IO) magnetic nanoparticles and DOX in a MAP matrix (MAP@IO MPs). The bioengineered MAPs were crosslinked through their lysine residues by using genipin. Esophageal cancer-mimicking microchannels were used to evaluate the magnetic capture of the MAP@IO (red fluorescence). The high capture efficiency of this material led to decreased cancer cell viability. Reproduced with permission.[258] 2021, Wiley.

tissue due to the water-absorbing ability of the coacervate hydrogel, which contributes to effective tissue adhesion. Growth factors, such as vascular endothelial growth factor (VGEF), promote vascularization but currently have limits in their clinical application due to their short half-life and rapid clearance.[236] The mfp1-VGEF and hyaluronic acid coacervate might enhance retention of the therapeutic factor at the infarcted zone, partly due to the inherent properties offered by MAPs. By delivering factors directly to the tissue, and keeping them there, therapeutic action can be prolonged at the site and high systemic dosing avoided.

6. Perspectives and Prospective Targets

As stated in Section 2, the usual goal of recombinant adhesive protein production is to synthesize sufficient quantities of relatively pure protein for the employment of downstream characterization methods. In the case of invertebrate marine adhesion, characterization has usually been restricted to experiments to determine coating ability, adhesion, or other mechanical aspects. This downstream testing, however, requires obtaining recombinant proteins that are structurally and conformationally as close as possible to their native counterparts. A look at the literature on recombinant adhesive proteins from marine invertebrates (Table S1, Supporting Information) indicates that, in the vast majority of the studies, protein production has been done in *E. coli*. For many years, this bacterium has been considered as one of the best recombinant protein expression systems as it is easy to genetically alter.[270] Protein production in *E. coli* can be relatively simple, fast, inexpensive, robust, and scalable. However, it can result in the formation of inclusion bodies, which have both advantages (i.e., ease of isolation of the recombinant proteins) and disadvantages. A disadvantage is that unfolded/misfolded insoluble proteins can require extensive denaturation/renaturation processes for refolding and subsequent recovery of activity. This misfolding can be due to the rapidity and scale of protein production in bacteria which, together with the highly reductive *E. coli* cytosol, prevents correct disulfide bond formation. In some studies, this problem has been addressed by fusing adhesive proteins with a Trx tag (e.g., mfp3 in mussels or cp19k in barnacles).[155,238] Other systems have been developed, such as the CyDisCo (cytoplasmic disulfide bond formation in *E. coli*) system, that have been used successfully to produce large extracellular matrix proteins but have rarely been tried with adhesive proteins, except for sea star sfp1.[217,271] This method may be useful for the large multimodular proteins found in some marine adhesives (e.g., scallop sbp5-2 or sbp9, sea anemone TSRL).

A well-recognized limitation of recombinant production of eukaryotic proteins in prokaryotes is the inability to perform PTMs.[272] In mussels, many protocols have been designed to produce recombinant mfps incorporating DOPA but the most widely used solution is still the in vitro modification of the protein after production. However, because there is no such method for all PTMs, a switch to other expression systems might aid PTMs such as hydroxylation, phosphorylation, and glycosylation. Large marine adhesive proteins have been produced in eukaryotic host cells (Table 1 and Table S1, Supporting Information). For example, mussel mfp1, -3, preCol-D and barnacle cp19k, as well as the hybrid proteins mfp3/5-GvpA and cp19k-MaSp1 have been produced in yeast;[78,98,111,241,253] the mussel mfp-151, barnacle SIPC and tunicate ASP1 have been produced in insect cells;[124,181,182] and the tunicate APAP-1 and -2 have been expressed in mammalian cells.[193] Thus far, alternative heterologous hosts have been largely under-utilized in marine adhesion research.

Another mainstay justification of research in this area is the oft-cited future potential of bio-inspired, adhesives for biomedical applications (surgery being a typical example). Section 5 presents a selection of the innovative biomaterials incorporating recombinant marine adhesive proteins that have been designed for cutting-edge applications. Biomaterials are required to undergo extensive biocompatibility studies. For example, cytotoxicity and biocompatibility results have been reported by Choi et al. (2014) in their study of the hybrid mussel protein mfp-151;[273] by Jiang et al. (2022) in their work on a bilayer hydrogel cardiac patch;[242] and by Lee et al. (2024) in their work on a mussel/silk fibroin adhesive complex.[229] As biomaterials based on recombinant proteins advance, in-depth biocompatibility, immunogenicity, and biodegradability trials will surely be more forthcoming.

Beyond bio-inspired applied research, there are still many fundamental lessons to be learned from natural systems. As we decode legacy data from the rise of omics in recent decades, it becomes more apparent that while the code may be almost broken, the message must also be deciphered, and the message is the protein interactome. It is important to note that the recombinant proteins presented herein are being studied in isolation from the secreted milieu that forms the adhesive in totality. It is known these adhesives are protein complexes, therefore, purifying a single protein to study in the absence of the sum is akin to studying a single cog to determine the function of a machine. Except for mussel byssus,[274] very few studies on marine invertebrate adhesives have undertaken experiments to fully decrypt interactions, this despite the fact that these secreted proteins provide an opportunity to decipher protein-protein interactions within a model likely evolved from the extracellular matrix.[25] By use of, for example, the yeast two hybrid system or affinity mass spectrometry, researchers could take advantage of the finite complexity of these systems to comprehensively map interactions.[44,275] Moreover, the recent development of AI prediction tools could also help decipher the protein interaction networks underlying the organization of marine adhesives. Recently, these tools have been leveraged to propose a mechanistic model for the reversible formation of fibers in the adhesive projectile slime of velvet worms.[276] In this terrestrial invertebrate, structural predictions suggested a receptor-like binding of Pro-rich high-molecular weight proteins by leucine-rich repeat proteins, providing potential avenues for fabricating bio-inspired materials. These methods could be applied, for example, to the catalogue of proteins from sea star adhesive, which provides a potential interactome of 16 proteins with 21 different protein domains implicated in various putative functions across biological literature.[162] Yet, in this organism, only the functional domain interaction between two subunits within the protein sfp1 has been reported so far.[219] As many protein domains are shared across phyla, the data gleaned may translate to functional interactions of domains in other systems, including humans.

The bulk of the research thus far has focused on mytilid mussels and while their strategy for adhesion is impressive, their adhesive components (i.e., short, disordered proteins without functional domains) are often markedly different from one mussel

genus to another. Moreover, mfps also differ from adhesive components (long multimodular proteins) in other phyla and even other bivalves. By comparing and contrasting the adhesive strategies of the various marine invertebrates, we can begin to focus our attention on the similarities that emerge from the data, for example, proteins sharing similar domains (EGFL, TSP1, etc), cysteine-rich proteins, or proteins that are also involved in biomineralization. This literature review begins this work by cataloguing recombinant adhesive proteins from marine invertebrates to date in order to clarify some of the best targets for future biomimetic research, while also paying just homage to the master strategists of underwater adhesion: marine invertebrates. As once eloquently stated by Oscar Wilde, "Imitation is the sincerest form of flattery that mediocrity can pay to greatness".

All species names are correct at the time of publication and checked against the online database at World Register of Marine Species (WoRMS, 2024).[277] Several of the species' names presented herein differ from associated cited works. Where possible, care has been taken to present both current species and common names to avoid confusion.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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