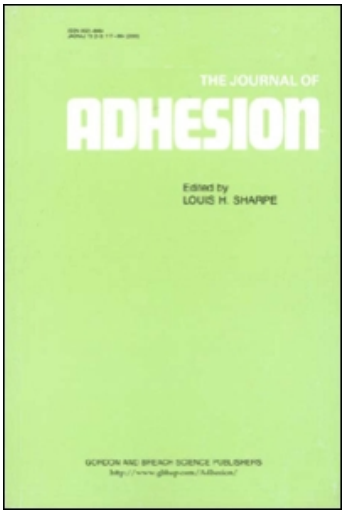

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Rapid Biopolymerisation During Wound Plug Formation in Green Algae

Matthew Welling[a]; Georg Pohnert[a]; Frithjof C. Küpper[b]; Cliff Ross[c]
[a] Institut für Anorganische und Analytische Chemie, Friedrich-Schiller-Universität, Jena, Germany [b] Scottish Association for Marine Science, Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland, UK [c] Department of Biology, University of North Florida, Jacksonville, Florida, USA

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Matthew Welling[1], Georg Pohnert[1], Frithjof C. Küpper[2], and Cliff Ross[3]
[1]Institut für Anorganische und Analytische Chemie, Friedrich-Schiller-Universität, Jena, Germany
[2]Scottish Association for Marine Science, Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland, UK
[3]Department of Biology, University of North Florida, Jacksonville, Florida, USA

Organisms living in the marine environment contain a number of primary and secondary metabolites which are involved in bioadhesive processes. Much progress has been made regarding the characterization of underwater adhesive structures utilized by sessile invertebrates such as barnacles, mussels, and tubeworms. The structural components and biochemical mechanisms involved in the wound-plug forming process in marine siphonous green algae have received far less attention. This review focuses on the lectin-carbohydrate and protein cross-linking strategies that serve as the basis for wound plug formation in siphonous green algae. Based on structural considerations it should be noted that cross-linking mechanisms are ubiquitous features of a variety of marine taxa that have been previously overlooked.

Keywords: *Bryopsis plumosa*; *Caulerpa taxifolia*; Cellular signaling; Cross linking; *Dasycladus vermicularis*; Natural product chemistry; Wound response

I. INTRODUCTION

Natural products that specifically evolved to perform adhesive functions represent attractive and well-publicized targets for bioengineering. The marine environment is a vast resource for such primary

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Address correspondence to Cliff Ross, Department of Biology, University of North Florida, 1 UNF Drive, Jacksonville, FL 32224, USA. E-mail: cliff.ross@unf.edu

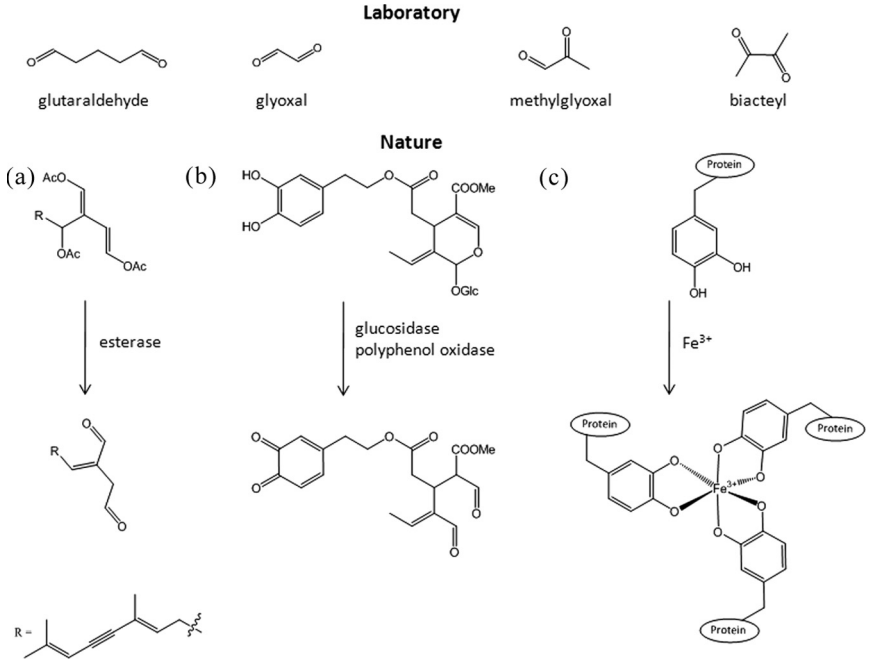

FIGURE 1 Laboratory: Selected cross-linker molecules. Nature—Controlled release of natural products capable of cross-linking: (a) Esterase-mediated activation of caulerpenyne to oxytoxin 2 in the green alga, *Caulerpa taxifolia*; (b) Enzymatic activation of oleuropein to yield reactive catechol and aldehyde functionalities in the privet tree *Ligustrum obtusifolium*; and (c) Fe^{3+} chelated protein-bound-dopamine secreted to form adhesive basal threads in the mussel, *Mytilus edulis*.

and secondary metabolites which may have or mediate bioadhesive capabilities. Frequently, adhesive properties are based on polymerization reactions that occur in seawater. Rapid biopolymerization under water is, however, not only restricted to bioadhesive processes but also found in the polymer plugs that are produced by siphonous algae after wounding. This review focuses on the recent advancements, in particular the structural components and mechanisms that drive the responses to wounding and especially the plug formation in marine siphonous green algae, with emphasis on the role of specialized secondary metabolites.

Understanding principles of biopolymer formation can directly contribute to novel materials that may advance biomimetic (nano) technology [1,2] and also aid in the development of ecologically

compatible materials which are of an ever pressing need in a world of massive population growth with a substantial ecological footprint [3]. Much progress has been made regarding the characterization of the underwater adhesive mechanisms employed by sessile marine organisms such as barnacles, mussels, and tubeworms [4–7]. A common theme for the adhesive process in these examples is that of metal-centered coordination systems that mediate the adhesion and polymerization of biomacromolecules, such as proteins. This mechanism is found in the dopamine-rich protein secretion from the common blue mussel, *Mytilus edulis*, and aids its affixation to surfaces [8–10] (Fig. 1). A second common theme involves the enzymatic oxidative transformation of catechols to reactive quinones and their covalent reaction with proteins [11–15]. The tanning mechanism is based upon the reactivity of quinones which are capable of Schiff base reactions and of forming Michael addition products with the nucleophilic groups of biomacromolecules. These processes are well conserved across phyla and even geography as terrestrial forms of sclerotization in arthropod silks, exoskeletons, and eggcases have been reported to rely on similar mechanisms [12,16,17]. Interestingly, the principle of a rapid recruitment of proteins into a polymer material is also found in the wound plug formation of siphonous green algae, where secondary metabolites are transformed enzymatically to generate potent protein cross-linkers. In addition, the wound plug material is supported by sugar binding lectins.

II. SIPHONOUS GREEN ALGAE

Algae in a variety of forms are present as primary producers at the base of the marine food web and, therefore, are crucial to the existence of higher trophic levels within an ecosystem. Of particular interest are the characteristics displayed by some of the members contained in the division Chlorophyta. Siphonous green macroalgae have an extraordinary structure: they are comprised of a giant single cell without interrupting cross walls, allowing cellular contents to move about freely. Multiple identical nuclei are often present in one cell that can reach several meters in length. However, some species of the genus *Acetabularia* (Dasycladales) remain uninucleate throughout most of their life cycle. According to the fossil records, giant-celled architecture dates back well into the Silurian era (*ca*. 440 million years ago) [18–20] and, in some remarkable cases, cell structures have remained essentially unchanged morphologically since the Cretaceous era (*ca*. 140 million years ago) [21]. Yet, many algal lineages originate from the Cambrian (500 millions years ago) [22] or the crown diversification

of eukaryotes and predate their predators [23,24]. Thus, it can be postulated that repair mechanisms are evolutionarily old.

Considering the character of their native environment, siphonous green algae face deleterious pressures from an array of events in an unrelenting struggle for existence. Cellular disruption or wounding may occur through herbivorous grazing, fragmentation during storms, parasites, epiphytes, and/or sand abrasion [25,26]. Upon injury, these unicellular organisms are presented with two alternatives: limit the damage as quickly as possible or perish. Unlike in multicellular organisms, a hypersensitive response or apoptosis is not an option and, therefore, a multitude of protective mechanisms have evolved. For example, the cytoplasmic retraction away from the wound site concurrent with turgor pressure loss enables the sealing off of the wound-healing vesicle and allows the deposition of a new cell wall [27]. Some systems have coupled this with the formation of a wound plug.

III. GENERAL PRINCIPLES OF WOUND PLUG FORMATION

Plug material in a wounded siphonous alga was first reported at the end of the 19th century by Noll [28] in a paper entitled "Die geformten Proteine im Zellsaft von *Derbesia*" ("The shaped proteins in the cellular liquid of *Derbesia*"). For over a century now, scientists have documented the unique ability of some siphonous green algae to rapidly form gel-like wound plugs that might harden over time when inflicted with physical damage [26]. Upon injury, a biochemical cascade of events is initiated that triggers the immediate agglutination of intracellular contents into the wounded region (Fig. 2). This immediate wound response can efficiently prevent subsequent hemorrhaging of the cell that would clearly be lethal, conferring a major selective advantage in the natural environment where these organisms would experience considerable damage [29]. Under the protection of this temporary gelatinous plug, the intricate regeneration of a new cell wall may commence.

Throughout the 1970s and 1980s, pioneering microscopic and cytochemical investigations were carried out chronicling the events during plug formation. This work was chiefly based upon the microscopic and the (bio)chemical evaluation of wound plug material from selected siphonous algae [27,30–34]. The most elaborate advancements by the scientific community truly recognizing the adhesive nature of wound plugs were reviewed by Menzel [26]. Subsequently, research on this area came to a halt for nearly a decade. The specific biochemical moieties involved in algal wound plug formation and the precise mechanisms as to how an underwater biopolymer forms were not addressed

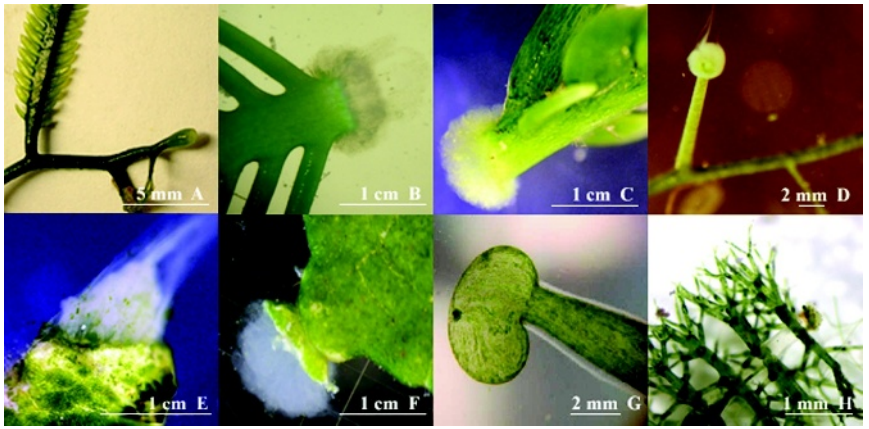

FIGURE 2 Wound plugs from marine chlorophytes: (A) *Caulerpa taxifolia*; (B) *Caulerpa sertularioides*; (C) *Caulerpa mexicana*; (D) *Caulerpa verticillata*; (E) *Halimeda tuna*; (F) *Halimeda incrassata*; and (G) and (H) *Dasycladus vermicularis*.

in these early studies. Only within the past several years, with the increased use of specific fluorescent probes and other high powered analytical tools such as liquid chromatography/mass spectrometry, significant advancements have been made that have offered novel insight into the biochemical mechanisms that drive plug formation. The general picture that arises during wound plug assembly in green algae follows two major mechanisms: lectin-carbohydrate interactions and protein cross-linking.

IV. LECTIN-CARBOHYDRATE INTERACTIONS

Lectins represent a broad category of proteins that have the ability to bind to selected carbohydrates in a specific yet reversible manner. Lectin-carbohydrate complimentary systems serve as the basis of numerous cellular adhesion-based processes encountered across Kingdoms [35–38]. With respect to algal wound plugs, Dreher *et al*. already observed that the polymer material is rich in saccharides [32]. Ross *et al*. [39] investigated the early steps involved in plug formation in *Dasycladus vermicularis* using adhesive fluorescent microspheres and biotinylated lectins with anti-biotin fluorescent isothiocyanate (FITC) conjugates. Rapid underwater gelling was shown to require a complimentary lectin-carbohydrate ligand system where the addition of free D (+) glucose and D (+) galactose competitively

inhibited the formation of nascent gel plugs. Plugs that were permitted to form for 10–30 minutes (post-injury) could not be dissociated with the sole addition of exogenous sugars as it would be expected for a reversible lectin/carbohydrate interaction. Only with the simultaneous addition of metal chelators, chaotropic agents, and non-ionic detergents could plugs be dissolved. This suggests that, besides lectin binding, an increasing series of biochemical interactions were occurring as a function of plug age.

Kim *et al*. [40] demonstrated that a similar lectin-carbohydrate mediated process was occurring in the protoplast formation of *Microdictyon umbilicatum* (Cladophorales). D-galactosamine, D-glucosamine, and α-D-mannose were all capable of inhibiting protoplast regeneration. Furthermore, FITC-labelled lectins such as *Ricinus communis* agglutinin and concanavalin A were capable of labelling protoplasmic particles. Complementary studies have suggested that a similar process is occurring in the genera *Chaetomorpha* [41] and *Codium* [42].

Perhaps the most well characterized lectin-based wound response involves the case study of the green alga *Bryopsis plumosa*. Work by Kim *et al*. [43] has demonstrated that when specimens of *B. plumosa* were injured, the cellular contents exuded into the surrounding medium and rapidly agglutinated to form protoplasts. Within 15 minutes a gelatinous envelope forms around sub-protoplasts under which a lipid membrane is slowly assembled. N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were both capable of inhibiting protoplasmic aggregations [43]. Upon further examination a novel lectin (Bryohealin) was purified and identified as the major component of the lectin-carbohydrate complementary system [44]. Subsequent work has demonstrated that the C-terminal domain of this lectin contains antibiotic activity, suggesting that not only does this protein mediate wound plug formation but it can also serve a protective role from bacterial contamination for successful protoplast regeneration [45].

V. CHEMICAL CROSSLINKERS

Nature provides a wealth of examples of proteins that can be amassed with either advantageous or deleterious consequences. Examples include the hardening of insect cuticles [46,47] and the formation of brainstem Lewy bodies which are related to Parkinson's disease [48]. Most types of protein cross-linking are mediated *via* covalent bonds to cross-linker molecules [49,50]. Inter and intra protein disulfide bonds and metal-centered protein coordination present some of the exceptions to this model. However, although cross-linking can vary in nature, the overall concept holds true, that is, proteins are secured

by the outstretched "arms" of cross-linkers, thereby holding them together. This process is repeated many times giving rise to a large complex matrix of cross-links and proteins: a biopolymer (Fig. 3). Protein cross-linking by the help of small molecules has been routinely employed in research labs for decades to fix cells and tissues in order to prolong their storage, to observe *via* microscopy, or to gain an insight into a particular metabolic state [51,52]. For the purpose of this review, a protein cross-linking system mediated by a cross-linker molecule shall be focused on in detail as this lies at the core of the concept of wound plug formation.

Among the widest utilized and best investigated reactions is that of protein cross-linking by means of glutaraldehyde. The addition of glutaraldehyde to cells facilitates covalent reactions of this bi-functional compound with free amino acid functionalities of proteins [53,54]. This can have a wide range of effects, from denaturation and suppression of activity to improved mechanical properties of the resulting polymer [55]. The mechanism by which this proceeds takes place in two steps. Firstly, as an electrophile, a carbonyl group of glutaraldehyde is readily attacked by any nucleophile bearing side chain present on the protein and undergoes a nucleophilic addition elimination type mechanism. Groups such as primary amines ($R\text{-}NH_2$) are ideal candidates and as such, lysine, which is usually abundant in proteins, is commonly found at the site of attachment. The result of this first reaction is a protein to which an aldehyde is covalently linked. The second step proceeds much like the first, with nucleophilic attack of another amino acid nucleophile at the second carbonyl group, resulting in the formation of either an intra- or inter-protein linkage. With more complex cross-linkers containing additional functional groups, thiols, namely cysteine, can undergo Michael addition reactions thereby furthering the cross-linked matrix (Fig. 4). These features are even

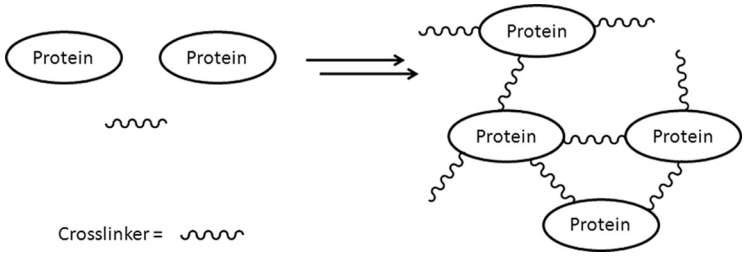

FIGURE 3 With relatively few components a biopolymer can be readily assembled consisting of multiple proteins linked *via* cross-linker molecules.

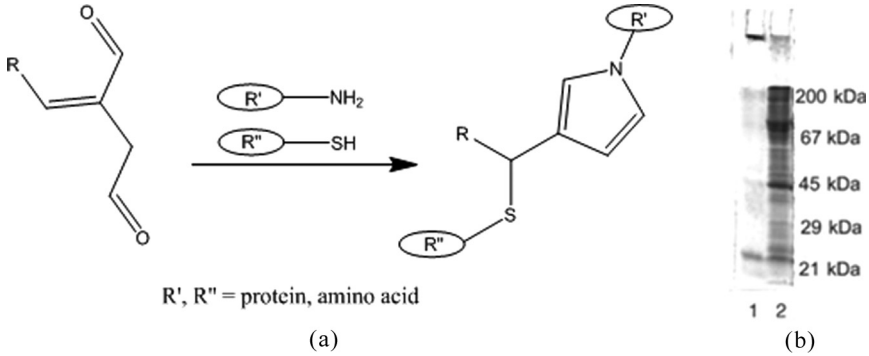

FIGURE 4 a) Glutaraldehyde-like cross-linkers can form covalent bonds with nucleophiles; b) SDS-PAGE separation of cellular proteins from *C. taxifolia* cross-linked (lane 1) and in their native form (lane 2) (modified from [68]. Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission).

more salient when viewed in terms of a protein's tertiary structure containing hydrophilic amino acid residues at the surface that are poised to interact with the surrounding environment. Other metabolites with dicarbonyl functionalities have also been shown to react with free amino acid groups from proteins and peptides (Fig. 1) [56,57]. This can be further generalized to suggest that a candidate cross-linker molecule has to be capable of binding at least two functional groups of proteins (Fig. 4) [51,52,58].

Many secondary metabolites bear functionalities that fulfill this criterion. These include quinones and dialdehydes that can react directly with proteins (Fig. 1). What all these protein cross-linkers have in common is that they are very reactive and, as a consequence, they have to be carefully controlled in the living cells. In nature, enzymatic activation of less reactive storage forms has been proven a successful concept. For example, enzymatic transformation of oleuropein, a secoiridoid glycoside from the leaves of the privet tree *Ligustrum obtusifolium*, results in the release of an extremely potent protein cross-linker containing both quinone and aldehyde functionalities (Fig. 1) [59].

Some siphonous algae deploy the same principle of polymerization by the cross-linking of proteins to rapidly form a wound plug upon mechanical damage to their single cell. The first detailed investigations were reported for the invasive tropical green alga *Caulerpa taxifolia*, which has spread throughout the Mediterrarean, the coast of Australia, and the North American Pacific [60–62]. The *C. taxifolia* plug-forming process is not only a means of protection, but also the

basis of an efficient proliferation strategy (Fig. 2). For example, primary cells may form fragments *via* mechanical disruption. These fragments may immediately seal and are subsequently capable of growing independently. Since the cells have multiple nuclei bearing all the same genetic information, the generated fragments represent clones of the parent cells. One key player involved in the plug formation is the acetylenic sesquiterpene caulerpenyne. It is the major secondary metabolite present in *C. taxifolia* representing up to 1.3% of the algal fresh weight [63,64] (see Fig. 1 for structure). Caulerpenyne has been shown to inhibit both basal and sodium-induced activity of the Na^+/K^+-ATPase in leech touch neurons [65], interfere with the main ionic signals involved in the cell dynamics during sea urchin egg cleavage [66], and to deter feeding from the sea urchin *Echinometra lucunter* [67]. This multifunctional secondary metabolite also has another, more striking role. By acting as a cross-linker, the wound-activated form is able to covalently bind to proteins causing them to aggregate and form an insoluble biopolymer [68]. This initially sticky and, following a couple of hours, hardened plug is a vital contingency plan for such coenocytic algae after cell disruption. For this extremely rapid and effective mechanism to take place, caulerpenyne is enzymaticaly transformed to oxytoxin 2, a more reactive di-aldehyde upon wounding (Fig. 1). The nearly quantitative transformation occurs *via* cleavage of acetyl groups by an esterase within a few minutes after wounding [69]. The final product, oxytoxin 2, is reactive containing a labile 1,4-dialdehdye moiety with a conjugated double bond. It has, thus, a striking resemblance to technically used cross-linkers and, as with these reagents, the aldehyde functionalities are readily attacked by nucleophiles. This can be demonstrated by incubating caulerpenyne and an esterase in the presence of cysteine [68]. The structure of the first cross-linking product, which was confirmed by 2D NMR spectroscopy, confirms that amino acid-derived nucleophile groups react with oxytoxin 2 according to a Michael Addition of a S nucleophile to the conjugated double bond and condensation of a N nucleophile with the 1,4-dialdehyde moiety (Fig. 4a). Most likely, according to the same mechanism by which cellular proteins are involved in cross-linking after wounding. The size-distribution of proteins contained in *C. taxifolia* changes dramatically upon wounding and plug generation, resulting in the formation of a protein-based polymer material (Fig. 4b). In *in vitro* experiments, this transformation can be disrupted by wounding the cell in the presence of an exogenous lysine-rich solution that competes with the naturally occurring nucleophilic amino acids for oxytoxin 2. As a result, the wound plug is not readily formed and the cell contents are rapidly leaked out into the

surrounding medium indicating that a recruitment of proteins is essential for the sealing of the cells. The resulting biopolymer is highly insoluble, even after excessive boiling in sodium dodecyl sulfate and dithiothreitol [68]. A potential application for the caulerpenyne/esterase system in the targeted initiation of protein cross-linking reactions was suggested.

This enzymatic transformation has recently been shown to play a second role in the wound response of *C. taxifolia*; by diminishing its food quality and, thus, reducing herbivory [70]. Active reduction in herbivory is brought about by the depletion of available proteins or essential amino acids due to the cross-linking reaction. This conversion, from a palatable to an unpalatable food source, rendering the alga undesirable to herbivores, might be part of a large cascade of signals within a cell that initiates an intricate defense mechanism enabling these unicellular sessile marine organisms to defend themselves against an array of herbivorous predators.

VI. SIGNALING THAT DRIVE UNDERWATER POLYMERIZATION PROCESSES

Ross *et al*. [39] described the chronological events in the rapid wound plug formation exhibited by the siphonous green alga, *D. vermicularis*, and suggested a two-part generalization on the sequential steps involved in previously characterized plug formation [26]. Within an hour post injury, significant browning and increased hardening was observed in the plug material. Ross *et al.* [71] reported an oxidative burst and a release of nitric oxide species in relation to injury. The chemical signals triggering the signal transduction chain in *Dasycladus* and *Acetabularia* have been identified most recently: Torres *et al*. [72] found that extracellular nucleotides trigger all downstream signalling and wound-healing steps. During injury, cytosolic ATP is released into the extracellular space, where it likely binds to purinoceptors, activating a chain of signal transduction events leading to the induction of an oxidative burst of reactive oxygen species and an NO burst. The oxidative burst has been found to play a key role in the secondary stage of *Dasycladus* wound plug formation [73], while NO has a coordinating role of the oxidative burst and upregulation of peroxidase activity [71].

VII. OVERLOOKED PROTEIN CROSS-LINKER POTENTIAL?

An assortment of metabolites that contain functionalities, which, upon activation, could contribute to the biochemical aspects driving wound

plug formation, have been reported from other siphonous green algae. Inhabitants of tropical reef ecosystems suffer intense grazing from herbivorous fish and, thus, are commonly chemically and physically defended [74]. Algae of the genus *Halimeda* are no exception and they contain elaborate secondary metabolites [75]. The enzymatic activation of halimedatetraacetate yields halimedatrial which has previously been shown to deter grazing [76]. The unique trialdehyde functionally (of which one is conjugated to a double bond) would suggest that, at least chemically, it is capable of cross-linking reactions. The plug-forming green alga, *Chlorodesmis fastigiata* [77], inhabits the Great Barrier Reef and has been shown to produce Chlorodesmin, a diterpenoid tetraacetate, and other related metabolites [78]. Diterpenoid diacetates (Udoteal [79] and Udoteal B [80]) are also common in *Udotea*, another wound plug-forming alga [34]. All these masked di-aldehydes can potentially be viewed as an evolutionarily conserved class of compounds among siphonous green algae that have the potential to undergo enzymatic transformations and enzymatic cross-linking.

In a first survey of other *Caulerpa spp.* it was shown that the principle of wound-activated caulerpenyne transformation is apparently more widely distributed, since *Caulerpa prolifera* and the also invasive *Caulerpa racemosa* exhibit comparable transformations of caulerpenyne [81]. Caulerpenyne is, in fact, one of a series of similar secondary metabolites produced by *Caulerpa* species [75,82]. For example, the sesquiterpenoid flexilin and the diterpenoid trifarin were isolated from the southern Australian *C. flexilis* and *C. trifaria* species, respectively [83]. Both metabolites are related to caulerpenyne structurally and contain bis-enoylacetate moieties. Although, from a chemical perspective, they would be predicted to be less potent protein cross-linkers due to the lack of a conjugated aldehyde functionally in the resulting transformation product. Highly sulfated polysaccharides, rich in glucose, isolated from wound plugs produced by *Caulerpa simpliciuscula* suggest a participating role in the alga's wound response [32] and possibly, therefore, a different or additional plug assembly mechanism.

In the green alga, *Bryopsis hypnoides*, wound plug composition has been shown to be largely a proteinaceous biopolymer [84]; however, as of yet, no protein cross-linking system has been reported. This in contrast to the wound response displayed in *B. plumosa* in which a lectin-carbohydrate system dominates [44].

The major secondary metabolite, 3,6,7-trihydroxycoumarin (THC), found in *D. vermicularis* and *Cymopolia barbata* [85] has been proposed as an ideal candidate for protein cross-linking. The oxidation

of THC, presumably from the rapid onset of an oxidative burst [73], would produce reactive quinone intermediates capable of undergoing nucleophilic attack. *D. vermicularis* could, therefore, adopt both a lectin-carbohydrate and a protein cross-linking mechanism to seal its wounds, providing a useful model for more sophisticated underwater adhesive systems.

VIII. CONCLUSIONS

This review focused on lectin-carbohydrate and protein cross-linking *via* secondary metabolites that serve as the basis for wound plug formation in green algae. The marine environment still contains a wealth of wound-healing systems found in many lineages of algae that have evolved to counteract the events associated with fatal cellular damage. These processes have yet to be examined in greater detail. Based on structural considerations, we anticipate that related mechanisms are wide-spread in nature. Plug formation itself, once initiated, usually proceeds in an undirected fashion. By understanding how these processes are initiated and coordinated, a variety of biotechnological advancements can ensue.

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