A study of the native cell wall structures of the marine alga *Ventricaria ventricosa* (Siphonocladales, Chlorophyceae) using atomic force microscopy

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**Abstract**

A substantial proportion of the architecture of the plant cell wall remains unknown with a few cell wall models being proposed. Moreover, even less is known about the green algal cell wall. Techniques that allow direct visualization of the cell wall in as near to its native state are of importance in unravelling the spatial arrangement of cell wall structures and hence in the development of cell wall models. Atomic force microscopy (AFM) was used to image the native cell wall of living cells of *Ventricaria ventricosa* (*V. ventricosa*) at high resolution under physiological conditions. The cell wall polymers were identified mainly qualitatively via their structural appearance. The cellulose microfibrils (CMFs) were easily recognizable and the imaging results indicate that the *V. ventricosa* cell wall has a cross-fibrillar structure throughout. We found the native wall to be abundant in matrix polysaccharides existing in different curing states. The soft phase matrix polysaccharides susceptible by the AFM scanning tip existed as a glutinous fibrillar meshwork, possibly incorporating both the pectic- and hemicellulosic-type substances. The hard phase matrix producing clearer images, revealed coiled fibrillar structures associated with CMFs, sometimes being resolved as globular structures by the AFM tip. The coiling fibrillar structures were also seen in the images of isolated cell wall fragments. The mucilaginous component of the wall was discernible from the gelatinous cell wall matrix as it formed microstructural domains over the surface. AFM has been successful in imaging the native cell wall and revealing novel findings such as the ‘coiling fibrillar structures’ and cell wall components which have previously not been seen, that is, the gelatinous matrix phase.

**Key words:** atomic force microscopy, cell wall, cellulose microfibril, green algae, mucilage, sulphated polysaccharides, *Valonia, Ventricaria*
Introduction

The simplified concept of the complex dynamic plant cell wall (type I) is that it is a network of cellulose microfibrils (CMFs) cross-linked with hemicelluloses embedded in a pectin gel [1]. The mechanical and functional properties of the cell wall are defined by its detailed molecular architecture [2]. Even though the chemistry of the plant cell wall polymers is well known [3], there remains a lack of understanding regarding the arrangement of the polymers in the cell wall and of their functional role [4]. There are a few cell wall structural models that exist and these models are continually being refined [5,6].

Techniques that allow direct visualization of the cell wall structures and architecture are of enormous benefit as they have the potential to reveal how all wall polymers are related. The methods used in direct visualization studies have significant limitations in allowing the study of the cell wall in as near to its native state. Commonly, the cell wall is isolated from the plant cell and undergoes a variety of sample preparations necessary for the particular technique [7]. Unlike conventional electron microscopy techniques, the rapid freeze deep-etching electron microscopy technique [8] has been vital in the development of cell wall models, revealing the existence of the cross-linking nature of wall polymers such as the hemicellulosic polymer xyloglucan [9,10]. Histochemical and cytochemical labelling techniques have been useful in specific localization of some molecules in the wall but they are unable to provide information on the three-dimensional arrangement of the molecular network [8,11]. A relatively recent powerful technique which can be used in cell wall architectural and structural studies is atomic force microscopy (AFM).

AFM remains a relatively new form of microscopy in plant cell wall studies, even though it has been around for almost three decades [12] and has attractive advantages for its application in biological studies. A major advantage is that an AFM can be operated in liquid media and thereby allows the study of structures on living cells in their physiological environment [13,14]. Samples are imaged by a probing tip either in direct contact with the sample or in intermittent contact with the sample, allowing subnanometer resolution on proteins in reconstituted membranes and few tens of nanometers on live cells, due to their viscoelasticity [15,16]. Moreover, the AFM can also conduct nanomechanical measurements on cell surfaces and determine the relative elasticities of the sample [17]. For reviews on the application of AFM in cell biology studies see [18–20].

Commonly used models for plant cells are large unicellular green algae [21]. They are easy to work with and the area of disturbance by a probing device, e.g. an AFM tip, is insignificant in size compared with the cell [22–24]. Ventricaria ventricosa, formerly known as Valonia ventricosa [25] has had a long history in cell wall studies dating back to the early 1900s using spectroscopic and microscopic techniques [25–28]. The cell wall structures of this alga and its close relatives in the genus of Valonia are larger in size compared with related features in land plants; hence intricate polymer associations may be more easily discernible in such walls. The V. ventricosa cell wall is abundant in cellulose [29], which is in its highest form of crystallinity [25,30]. This has made it a popular sample used in the study of cellulose molecular structure [31–35]. The cellulose molecules are arranged in chains forming long ribbon-like structures, the CMFs [30]. The CMFs are arranged parallel to one another in each lamella of the cell wall and the overall cell wall of V. ventricosa displays a cross-fibrillar structure, having three main CMF directions with each lamella containing only one CMF direction [36]. There could be several layers of parallel CMFs in one lamella [37]. Unlike V. ventricosa the CMFs in the cell wall of land plants is usually in a helicoidal arrangement with a different arrangement in the cellulose synthesis enzyme complexes [38].

In this study, we aim to obtain a direct visualization of the intact native cell wall structures and architecture of a living cell utilizing the AFM. While there have been several AFM structural studies on isolated plant cell walls, either dry or partially hydrated [39–43], there has been only one structural study on a native plant cell wall [44]. In terms of the application of AFM in imaging algal cell walls, there has been no previous native cell wall study; however, isolated walls of Vaucheria terristris sensu Goetz have been imaged [45].

Materials and methods

Algal material

Large spherical cells of V. ventricosa (Siphonocladales, Chlorophyceae) [24] usually over 2 cm in diameter were collected from Heron Island on the Great Barrier Reef, Queensland, Australia. For experiments, sterile cultures were used where the aplanospores were propagated artificially and placed on sterile coral maintained in sterilized 1/2 medium with a pH of 8.0 [46]. Cultures were maintained at tropical temperatures (22–25°C) and grown in natural light cycles. The osmotic pressure of the medium was approximately 990 mOsmol kg\(^{-1}\). The osmotic pressure was measured with a cryoscopic Osmometer (Osmomat 030, Gallay Scientific, North Melbourne, Victoria, Australia).

Preparation for native cell wall imaging (live cells)

Cells with diameters ranging from 2 to 4 mm were selected for experiments. One week prior to the experiments, corals
containing cells were placed in 35 mm plastic Petri dishes for acclimatization to the experimental conditions in simplified artificial seawater (ASW), of 990 mOsmol kg⁻¹ and pH 8.0 [47].

Enzyme treatment on native cell wall

The surface cell wall layers including sulphated polysaccharide mucilage was removed by treating the cell surface with 1% Cellulysin (Tricoderma viridae) (Calbiochem-Novabiochem, Sydney, Australia) and 0.5% bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) in diluted 2-(N-Morpholino)ethanesulfonic acid (MES) (Sigma, St Louis, MO, USA) buffer solution containing 1:3 ASW/MES buffer (pH 5.8). The cells were incubated in the enzyme solution at 30°C for 10 min and then washed several times in ASW. As most commercially available wall-digesting enzymes are not highly purified, BSA was added to the enzyme digestion recipe to reduce the activity of proteases [48,49].

Preparation of cell wall fragment specimens

Cells were cut open and small fragments of cell wall were created and placed in a fixing solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer of pH 7.4) overnight at 4°C. The samples were washed three times with cacodylate buffer of pH 7.4 and the fixed cell walls were dehydrated with 70% ethanol.

Atomic force microscopy

The most commonly used imaging mode for live biological specimens is the contact mode (CMAFM) [50]. In contact mode imaging, the tip is in constant contact with the surface as it scans the sample. This mode usually produces the highest quality images if the applied force on the sample is able to be kept to a minimum to minimize sample damage, e.g. as in low force contact mode (LCMAFM) imaging [51–53]. We minimized imaging forces on the live cells by viewing the force curves in the AFM force mode prior to imaging and setting the imaging set point to correspond to minimal force. In terms of non-living biological specimens, tapping mode (TMAFM) [54–56] imaging is usually preferred. In tapping mode imaging, the cantilever is made to vibrate near its resonant frequency and then brought into contact with the surface which reduces shear forces as the tip is in intermittent contact with the surface.

In most AFM imaging studies, the amount of force applied to the sample is kept constant by a feedback loop system. Two types of images can be collected each time, one containing height information, called ‘height’ image, and the other called the ‘error signal’ image which is obtained by using the corrections of the AFM feedback loop system as it works to maintain the constant specified force set by the user [57]. The error signal image, referred to as ‘deflection image’ in CMAFM imaging and ‘amplitude image’ in TMAFM imaging, consists mainly of high-frequency components that are directly related to the fine topographic detail in the sample.

Native cell wall imaging in ASW

The cells were rigidly immobilized on the coral in ASW and were placed in the AFM isolation box overnight to acclimatize to the AFM room conditions. Live cell imaging was carried out using a Dimension 3100 AFM equipped with a Nanoscope IIIa controller (Veeco Instruments, Santa Barbara, CA, USA). The Petri dish containing the cells in ASW was mounted directly on the Dimension 3100 stage plate and held secure with double-sided tape. The fluid cell (DTFML, Veeco Instruments, Santa Barbara, CA, USA) was used for imaging in liquid. The Dimension 3100 AFM is equipped with a camera which facilitates positioning the tip onto the cell.

Cells were imaged in ASW, in contact mode (CMAFM) using a silicon nitride tip (DNP, Veeco Instruments, Santa Barbara, CA, USA) with a nominal spring constant of 0.32 N m⁻¹. An imaging scan rate of 0.3 Hz was used.

Cell wall fragments imaging in ambient conditions

Samples of the inner wall were prepared by gluing the wall fragment onto mica using araldite two-part glue. Excess ethanol was removed from the sample. Mica was stuck on an AFM metal sample puck. We used a Multimode™ AFM (Veeco Instruments™, Santa Barbara, USA) operating with a NanoScope IIIa™ controller and an E scanner. Tapping mode imaging (TMAFM) was applied on cell wall fragment specimens using silicon cantilevers, model tapping mode etched silicon probe (TESP, Veeco Instruments, Santa Barbara, CA, USA) operating at resonant frequencies of ~300 kHz. The drive frequency was set to ~8 KHz.

Surface measurements of structures in height images were made using the section analysis module of the AFM software (Veeco Instruments, Santa Barbara, CA, USA). Image flattening and filtering were also done using the AFM software (Veeco Instruments, Santa Barbara, CA, USA).

Results

Native cell wall

Most living cells contained patchy areas of elevated voluminous coating on their surface of thickness of hundreds of
nanometers and best viewed in large scan areas of 20 µm x 10 µm or more (Fig. 1). In areas of no coating, a flat and uniform wall surface was observed. Some cells did not contain voluminous coating but instead a thin layer of a substance with a microstructure of poorly defined domains (Fig. 2). Enzyme treatment was used to remove the thin coating substance and the underlying cell wall network was revealed (Fig. 3). Other cells revealed the cell wall network immediately, hence lacked a coating (Fig. 4). The height images were not clear in revealing the alignment of CMFs network; however, details of fibril alignments could be seen in the error signal images (Fig. 4). Moreover, image filtering emphasized the well-known cross-fibrillar cell wall structure of *V. ventricosa* (Fig. 5). Two main CMF directions were discernible and the images showed that the microfibrils were well aligned and straight.

Imaging at a higher resolution of cell wall surfaces showed ‘haziness’ in the images (Fig. 6). The haziness was created by a gelatinous phase substance. Also seen in the images is a glutinous phase fibrillar meshwork (Fig. 6 solid head arrows) overlying the CMF network. The meshwork was found only on areas of the wall with CMFs, voids in the CMF network corresponding to areas of no meshwork. Also revealed at this magnification were coiling fibrillar structures (Figs. 6 and 7 long open arrowhead). These coiling structures could possibly represent disorder in the fibril alignment, or most probably represent solidified fibril structures caused by features pointed by solid head arrows, which show glutinous phase meshwork fibrils draping over CMFs (Fig. 6 dotted solid head arrows). Some cell surfaces appeared solidified; the effect of the solidified phase matrix substances created a globular surface appearance (Fig. 8). Fibril outlines can be made out in the image. Higher magnification of the enzyme treated wall revealed fibril-like structures and cross-linking structures (Fig. 9).

![Fig. 1. Error signal image of large area of the native cell wall showing a voluminous non-transparent coating on its surface. Imaged in ASW using CMAFM. Contamination with plankton can be seen. Scale bar, 3.5 µm.](image1)

![Fig. 2. Error signal image of non-transparent coating appearing as a thin layer over the surface of the wall. Imaged in ASW using CMAFM. Scale bar, 0.8 µm.](image2)

![Fig. 3. Error signal image of native cell wall after enzyme treatment. Imaged in ASW using CMAFM. The CMF network can be seen. Scale bar, 0.5 µm.](image3)

![Fig. 4. Image of native cell wall without non-transparent coating imaged in ASW using CMAFM; height image (above), error signal image (below). The wall appears disordered in the height image. The cross-fibrillar wall network can be seen in the error signal image. Scale bar, 1.5 µm.](image4)
The measurements of the widths of the CMFs were within a broad range, from 80 to 250 nm. This suggests that the width of the CMFs may be affected by the amount of coiling fibrillar structures associated with them. The width of the thin coiling fibrillar structures ranged between 50 and 140 nm, with the more solidified surface having the smaller size polymer width.

Cell wall fragments

The inner surface of the cell wall imaged in TMAFM revealed the cross-fibrillar structure of the *V. ventricosa* wall (Fig. 10). The CMFs in the surface layers were sparse and this allowed imaging of the underlying layers of CMFs. In the deeper layers, the CMFs appeared to be more densely
packed. The cell wall lamellae were ~20 nm thick and the widths of the CMFs were in the range 40–60 nm. In addition to the CMFs, other cell wall components were visible. Higher-resolution images revealed that the CMFs had coiling thinner fibril-like structures along their lengths, comparable to the native cell wall images (Fig. 11, short arrows). The widths of these fibril structures were measured to be in the range 15–30 nm. Occasionally, we found areas of structureless material in the wall, located between adjacent wall lamellae and covering the underlying CMFs in that region (Fig. 11 long arrows). The location of the structureless material with reference to the surface layer could be seen much more clearly in the height images. Unusual swirl-like structures were found on the inner wall (Fig. 12) with diameters of ~700 nm. In larger area scans, multiple swirl-like structures were seen (Fig. 10 arrows).

**Discussion**

This study highlights the application of AFM in the study of native cell wall structures and architecture. Cell wall studies utilizing the AFM have been conducted on fragments of isolated wall, except for [44], using both contact mode and tapping mode imaging. The results from those studies reported mainly on the CMF part of the wall with little information on matrix cell wall components [58]. Studies of the native cell wall, abundant in matrix polysaccharides, are likely to provide more insights into cell wall mechanisms.

Native cell wall images revealed that the surfaces of some cells contained a coating of a substance which was either present in a voluminous amount or a thin layer, obstructing the visualization of the cell wall network, while other cells were coating-free. This coating was attributed to the sulphated polysaccharide extracellular mucilage that is commonly found on the surface of algal cells including *V. ventricosa* [59,60]. The existence of the mucilaginous substance over the surface of the cells, in smaller amounts, was identified by its microstructure. Similar sulphated polysaccharide mucilage microstructures have been observed on diatoms, also revealed by AFM [61]. Preliminary AFM physical measurements on the mucilaginous surface showed that it is an adhesive substance (data not shown). Enzymatic treatment of the wall surface removed this non-transparent coating and revealed the underlying cell wall network.

The cell wall network was directly observed on cells which lacked the coating. The height images appeared to show a complex CMF network, with no particular order, amongst amorphous soft phase matter. On the other hand, the error signal images, capable of revealing finer structural details, showed that the *V. ventricosa* outer wall is ordered with a cross-fibrillar structure. This finding concurs with previous studies on *V. ventricosa* wall [36,37,62–64].
Interestingly, our inner wall images of *V. ventricosa* also showed a cross-fibrillar ordering and, hence, as suggested by Preston [36], we conclude that the *V. ventricosa* wall is cross-fibrillar ordered throughout and the terms ‘primary wall’ and ‘secondary wall’ may not apply in this species. This is different from its close relative *Valonia-macrophysea* Kütz, which displayed a difference in the fibrillar phase in the primary and secondary walls [63]. This finding is of interest as it suggests more differences in their evolutionary lineages.

The complexity in the native wall network is augmented by the abundant presence of matrix polysaccharides. The matrix polysaccharides in the wall appeared in both an amorphous phase and a fibrillar phase with these two components appearing together, hence making it difficult to decipher whether this was one cell wall matrix component or two separate ones. The matrix polysaccharides in the wall appeared to have gelatinous characteristics appearing in varying ‘curing’ states, from glutinous fibrillar meshwork to solidified surface of ill-defined fibrils, having more of a globular feature. The matrix polysaccharides in its soft phase created hazy image artefacts due to its susceptibility to tip scanning. The soft phase matrix was present in regions that contained CMFs directly under it and not in areas that contained voids in the CMF network. These characteristics fit well with the pectin gel matrix which is known to exist in abundance in the *V. ventricosa* wall [28,65]. A similar finding of this behaviour for gelatinous pectin in the wall was found by Chanliaud and Gidley [66] in their study of the pectin/cellulose composite, which showed that pectin bonded with the CMF network, and voids in the pectin layer were seen to correspond with voids in the CMF network. Unlike the sulphated polysaccharide mucilaginous layer, we found this cell wall component not to have strong adhesive properties (data not shown). It appears that the gelatinous amorphous matrix component is susceptible to wall isolation and sample preparation techniques and, unlike the isolated or fixed cell wall, the native cell wall is abundant in glutinous matrix substances which provided optimal conditions for their observation.

The solidification of the matrix polysaccharides resulted in clearer images with no hazy artefacts. The glutinous fibrillar meshwork seen draping across CMFs solidified into coiling fibrillar structures. On some cells, these coiling structures are clearly seen and on others the coiling structures appear as globules as a result of possibly further solidification and tip broading artefact. It is known that cell wall pectin chemistry can undergo changes such as de-esterification [3], which would lead to wall hardening. There have been no previous reports of the coiling fibrillar structures in other high-resolution studies of the cell wall, utilizing AFM or electron microscopy, on *V. ventricosa* cell walls or its Valonia relatives. Interestingly, this fibril structure is also evident in our images of isolated cell wall. The visualization of these features in our imaging study of *V. ventricosa* wall, unlike previous reports, may be due to the larger cell wall structures of large algal cells compared with plant cells or the fact that these structures have not been seen on other Valonia species may suggest that it is native to *V. ventricosa*. Of interest, Baker et al. [33] found corrugated CMF surfaces in their AFM high-resolution study of purified CMFs from Valonia wall, which they attributed to sample preparation effects. The coiling fibrillar structure conformational association with CMFs suggests that they form a major load-bearing network in the wall, and hence may be a separate matrix polysaccharide from pectin, similar to the hemicellulosic polymer, xyloglucan, in the cell wall of land plants [67,68]. Xyloglucan is considered to be hydrogen bonded to cellulose. Morris et al. [69] have used AFM to study the desorption energies of xyloglucan from *V. ventricosa* cellulose which did not suggest a complex coiling interaction. However, studies in the conformation of xyloglucan have suggested that it can form into flexible to semi-flexible random coils [9,70,71]. There is no information on the hemicellulosic polysaccharides in the cell walls of *V. ventricosa*; however, possible candidates for the coiling fibrillar structures binding to CMFs in the cell wall of *V. ventricosa* are xyloglucan-like polysaccharides such as ulvan [72,73] or mixed linkage xyloglucan [74], which have been found in related algal species in the genus *Ulva*. While these polysaccharides are similar to xyloglucan, they differ in composition, structure and charge. Mannans also have the capability of bonding with CMFs [75]. Furthermore, a coiling fibril conformation associated with wall loosening may raise some questions in relation to the mechanism by which this change occurs (i.e. ease or difficulty of uncoiling of the polymer or does this coiling structure change into a different structure), issues which remain to be elucidated. Tepfer and Cleland [76] had previously demonstrated that the wall loosening response in *V. ventricosa* is less complex than in land plants, perhaps not involving enzymatic mechanisms but rather conformational changes in the structures in the cell wall.

Further evidence of the existence of the coiling structures is reflected in the measurements of the CMFs in our images. CMFs from green algae are larger in dimension than in land plants which are ~5–15 nm wide [3]. CMFs from *V. ventricosa* have been reported to be both rectangular and square in shape [27,62]. The majority of studies have found the width of the *V. ventricosa* CMFs to be ~20 nm using various techniques such as X-ray and electron diffraction and electron microscopy [27,62,77]. Similar findings have been reported using AFM [31,33], however, larger CMFs measurements have been reported (100 nm) and attributed...
to tip broadening effects [31–33]. In our case, in the native wall, even larger measurements of the CMFs were obtained. Although our measurements would have been susceptible to a certain degree of tip broadening, we suggest that our larger measurements for CMFs are a consequence of the association of the coiling fibrillar structures with them.

Peculiar swirl-like structures were observed on the inner wall. It is unclear as to what these were; we suggest they could be a site of protoplast (aplanospore) anchoring by cytoskeletal networks or an unknown cell substance. The study of Preston and Astbury [26] described modification of the cell wall into raised circular rims wherever holdfasts (rhizoids) or buds (aplanospores) had developed. Furthermore, V. ventricosa has a curious ability to eject zoospores, which form mitotically, directly through the cell wall [26, 78]. The cell wall is perforated enabling direct communication between the vacuole and the medium, and zoospores are ejected. However, Preston and Astbury [26] found no evidence in their polarizing light microscope study of V. ventricosa that the wall structure changes as a result of this process, the wall appearing to recover the original alignment of CMFs. The most likely explanation for the swirl structure is that it represents the region of rhizoid or aplanospore formation, as described by Preston and Astbury [26].

Identification of polymers is difficult using AFM alone and accompanying methods are necessary. While AFM is a powerful tool for unravelling structures in both the native state and fixed state, the identification of the polymers in a sample specimen is not possible. In cell wall imaging, the CMFs are obvious structures due to their abundance and appearance (long and thin) and well-known layout. The other structures seen in the AFM images of the cell wall have yet to be identified. AFM force measurements were conducted as an aid to obtain preliminary identification of the unknown cell wall components seen in our study. In particular, this method was used to aid in distinguishing between sulphated polysaccharide mucilage and pectin gel in the native wall (data not shown). Sequential extraction of polymers from the cell wall is often conducted to gain insight into the structures of cell wall components [40, 43, 45]. Measurements of CMF sizes before and after sequential extraction of certain targeted polymers would give an indication of the polymers’ association with the CMFs.

Conclusions

AFM has been a powerful tool in the study of the native cell wall allowing direct imaging of the wall’s structures and architecture. This has been of importance especially in revealing wall structures that are susceptible to wall isolation and fixation. Fine details of the wall structures were able to be seen in the error signal mode images. We found that cells contained different amounts of extracellular mucilage and that the presence of the extracellular mucilage could be identified by its distinctive microstructure and adhesion properties. We found the wall matrix to be associated with the CMF network and it existed in different curing states from a glutinous substance of amorphous matter and fibrillar matter to the solidified fibrillar phase of coiling structures. We surmise that the larger widths of CMFs found in the native cell wall is due to the amount of matrix coiling fibrillar structures associated with it. We have found that V. ventricosa has an ordered wall throughout, unlike its close relative Valonia macrophysa Kütz. Our images of the native V. ventricosa wall support the proposed cell wall models of land plants, in particular the tethered cell wall model [79] and multi-coat model [80]. The tethered model has cross-linking polymers to CMFs and the multi-coat model has matrix polysaccharides coating the CMFs. Further studies involving chemical analyses of the V. ventricosa wall would be of interest to reveal the identity of the thin fibril-like structures.

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