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High-throughput SEM preparation of proteinaceaous extracellular matrix

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Abstract Adhesives of aquatic organisms are of high scientific interest with a view to biomimicry. The visualization of their fine-structure, however, is difficult due to the fragility of the extracellular matrix (ECM). Care must be taken in its preparation for high-resolution SEM. Relating matrix structures to substrate properties demands a high throughput of samples for reliable comparisons. In order to acquaint ourselves with a suitable method we found an easy way to manage the critical steps of preparation for SEM. We show that thin layers of proteinaceous matrix can be satisfactorily prepared by combining freeze-drying and sputtering in a conventional sputter coater after one-step fixation (2.5% glutaraldehyde). The results were superior to CPD.

Introduction

Representatives from all animal phyla living in the sea attach permanently or temporarily to solid surfaces. The organisms secrete extracellular matrix (ECM) that acts as adhesive. Highly effective adhesives found in nature are being researched with the view to biomimicry (for reviews see [1–3]). In this context, the ultra and fine structures of various aquatic adhesives are currently being searched for (e.g. [4–6]). Barnacle adhesive (also called cement) can reach high adhesive and cohesive strength and is therefore a preferential study target. It is used in this study as the model material.

Bioadhesives may consist of a variety of substances, but proteins and carbohydrates feature prominently. The adhesive of barnacles is a proteinaceous material (>90% protein), while the remainder consists of carbohydrate, ash, and trace amounts of lipid [7].

It was previously shown [8] that the barnacle adhesive properties (including their water content) vary greatly with the physico-chemical properties of the substrate. Investigations of the ultra structure of barnacle cement revealed that the adhesive is composed of nanosized globular structures. These were suggested to be able to create specific superstructures in response to substratum characteristics. It was also reported in that study [8] that barnacles produce a highly hydrated adhesive on low energy surfaces. Similar observations have been made for other biomaterials (for algae [9]; for conditioning films [10]). The attempt to visualize these highly hydrated structures by SEM is hindered by the necessary preparatory steps, which may lead to artefacts of the very fragile matrix masking the original structure. For example, the fibrillar character of the *Enteromorpha* spore adhesive imaged by standard SEM was attributed as being dehydration artefacts after visualizing *Enteromorpha* spore adhesive by environmental SEM (ESEM) as a featureless, swollen gel-like adhesive pad [11]. In contrast to the traditional high-vacuum method the ESEM enables the imaging of samples in a partial pressure of gas [12]. It eliminates many of the sample preparation treatments discussed below. However, one of the main limitations cited by biological ESEM users is that effective resolution is reduced in ESEM compared to SEM [13, 14]. A very recent paper [15] is stressing the potential applications of ESEM in dynamic biological processes.

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Cryo-SEM is an alternative way to view biological specimens frozen-hydrated in the SEM (see the review [16]). Another paper compares cryo-SEM to ESEM and shows how the elimination of the need for coating can extend the potential applications of cryo-microscopy [17]. The authors concluded, however, that for high-magnification studies of static microstructure, traditional high-vacuum conditions and a coated specimen are generally preferable to either the low-voltage or low-vacuum imaging of uncoated specimens.

The method described below focuses on the traditional SEM, for which hydrated samples need to be dehydrated before viewing. The scanning electron microscope images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. Due to the way these images are created, SEM micrographs have a very large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample. It is the preferred technique in biology and material science for analyzing surface structures. Depending on the type of specimen and its preparation for SEM resolutions up to some nanometre can be obtained.

In order to compare bioadhesive structures formed on different substrates the scanning of numerous samples is necessary. In order to acquaint ourselves with a method allowing a high throughput of samples we found an easy way to manage the critical steps of preparation for SEM, which shall be presented here.

Method

Material collection

To avoid variations between samples, adhesives obtained from the same barnacle species released on the same substrate exposed in the sea at a specific location and time were used. Teflon panels were exposed in the sea at the island Norderney (North Sea) in early spring 2007. Individual barnacles were chosen for the experiment according to species (*Balanus crenatus* Bruguière, 1789) and size (base diameter 8–10 mm). Still being attached the barnacles and their substrates were cut out of the panel (pieces of approx. 1 cm × 1 cm). After cleaning, the barnacle was detached (barnacles stick to this material but can be released by bending the substrate) with the cohesive fracture occurring within the adhesive plaque. The thickness of the remaining adhesive on the substrate ranged between a few nanometres and 100 µm at maximum. The substrate pieces were rinsed and stored in Tris(hydroxymethyl)-aminomethane-buffer (Tris-buffer adjusted to pH 8.2—according to that of the seawater) in the cold (5 °C).

SEM sample preparation: in general

Sample preparation of biological specimens for SEM consists of a series of processes that aim to remove water but minimize changes in sample volume and morphology. Typically, the sample is first chemically fixed and then dehydrated. These steps are followed by sputter coating the sample with an electrically conductive material.

The chemical fixation preserves the structure of the sample by crosslinking the proteins within it and toughens the sample. Fixation is usually in glutaraldehyde. The disadvantage of the popular fixative is slow penetration of the specimen for which it is often used in combination with formaldehyde. Glutaraldehyde is more effective than formaldehyde regarding the fixation of free amino acids [18]. Lipids are indirectly stabilized by the fixation effects in proteins [19]. Due to the solubility of lipids after aldehyde fixation the samples may be post-fixed in osmium tetroxide and in uranyl acetate.

The sample must then be dehydrated. Air drying results in the sample collapsing, so alternative methods are employed. The most common technique is critical point drying (CPD). This process avoids artefact formation by never allowing liquid/gas interfaces to develop; in this way the material is not exposed to surface tension forces. The transition from liquid to gas at the critical point (determined by pressure and temperature) takes place without an interface because the densities of liquid and gas are equal at this point. In order to carry out this procedure at a convenient temperature and pressure, the water (which has a very high critical point) is replaced with another liquid (e.g. CO_2). Due to the limited miscibility of water and liquid CO_2 washing steps with an ascending series of alcohol and subsequent substitution with acetone are necessary to replace the water. CPD results in shrinkage of 10–15%, in some tissues even more, and the shrinkage may be spatially unequal [20].

An alternative technique is freeze-drying also known as lyophilization. This method has been well established for some time and the detailed procedures available are outside the range of this article. In any case they vary considerably in their application and users have a wide choice of what parameters to apply for their particular application. In its simplest terms it is the freezing of the wet specimen and the subsequent removal of water by the process of sublimation, from solid to vapour phase. The point at which the three phases (ice/ water/vapour) exist in equilibrium (called triple point in a phase diagram) has a unique value for pressure and temperature. For any temperature/pressure setting below the triple point, water can be changed from the solid to the vapour phase. When freeze-drying biological specimens for microscopic analysis, there are, however, limitations concerning the formation of ice

crystals (discovered by C. Birdseye), which damage the specimen structure (e.g. Geyer [19]). In order to prevent crystal formation freezing should be done quickly and at very low temperature; in addition, during sublimation the material should be kept below a critical temperature, above which amorphous ice may change into ice crystals. A temperature of −90 °C is reported suitable to minimize the risk for recrystallization of water [21–23]. Thus, the system pressure should be used much lower than the triple point pressure.

The non-conductive biological specimens require metal coating in order to avoid charging during the imaging process. This usually consists of sputter coating with gold though other materials can also be used to give a finer coating for high resolution work. Film thicknesses vary, but must be large enough to allow the coating to be conductive.

SEM sample preparation: experimental

According to the pros and cons discussed above a technique was searched for suiting the properties of the sample and enabling high throughput. Three methods were chosen to be compared (Table 1).

The 1st method

This is the conventional CPD method. The substrate pieces with their adhesive remains were fixed in 2.5% glutaraldehyde for 4 h. The glutaraldehyde was used in a solution of Tris-buffer. The fixation should be conducted at a stable pH in order to avoid acidification artefacts [24]. The pH of the buffer was set according to that of the seawater (pH 8.2). For the low thickness of the film (100 μm at maximum) the penetration by the glutaraldehyde fixative is fast; the addition of formaldehyde is not necessary. Postfixation may have been advantageous here; it was, however, dropped for the ambition of finding a satisfactory simple and quick way.

Dehydration followed in a series of ethanol in buffer of increasing strength (30, 50, 70, 80, 90, and absolute—10 min each) substituted with amylacetate (3:1, 1:1, 1:3, and absolute—15 min each) and subsequently dried with CO_2 in an Emitech K850 Critical Point Dryer. The specimens were mounted on stubs with a carbon-impregnated film and sputtered with a 15 nm layer of gold in a BAL-TEC SCD 500 Coating System (equipped with a quartz film thickness monitor QSG 100). The vacuum chamber was then vented with dry argon gas and specimens transferred into the SEM chamber or stored in an exsiccator.

The 2nd method

This replaces chemical fixation by immobilization through freezing in liquid N_2 (any excess liquid on the sample was removed before freezing). Cryo-protectants were not added. The sudden solidification of the liquid components during freezing immobilizes all free versatile components. Freeze-drying and sputter coating were conducted in combination with the above mentioned sputter coater, where specimens were coated without breaking the vacuum. It was important to transfer the samples quickly from the nitrogen into the vacuum chamber and to build up a vacuum of 10^{-4} mbar rapidly. (Recently, there are systems of controlled freeze-drying and subsequent coating commercially available making this step easier, e.g. BAL-TEC, Emitech.)

The 3rd method

The aquatic adhesive may contain high amounts of water resulting in the fragility of the sample. In order to stabilize the biomaterial the freeze-drying method was supplemented with prior fixation in glutaraldehyde (according to the procedure described in method 1).

Imaging conditions

SEM was carried out using a Philips XL30 CP operated in high vacuum mode. Imaging was performed at a range of accelerating voltages (for the best setting see "Results").

Results

With all methods a magnification of 30000 diameters yielded good insight into the fine structure of the samples suitable for comparison (Figs. 1, 2, 3).

Table 1 Essential steps of methods chosen for preparation of specimens for SEM analyses

Preparatory steps	1. Method	2. Method	3. Method
1. Immobilization			
Chemical fixation	Glutaraldehyde	–	Glutaraldehyde
Freezing	–	Liquid N_2	Liquid N_2
2. Dehydration	Chemical dehydration (ethanol, amylacetate) + CPD	Freeze-drying	
3. Coating	After transfer into vacuum chamber	In combination with dehydration without breaking vacuum	

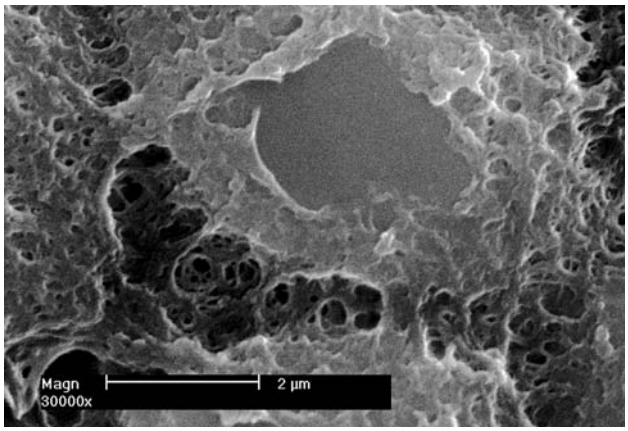

Fig. 1 SEM image of Barnacle adhesive (*B. crenatus*) released on Teflon substrate; sample preparation method 1 (CPD)

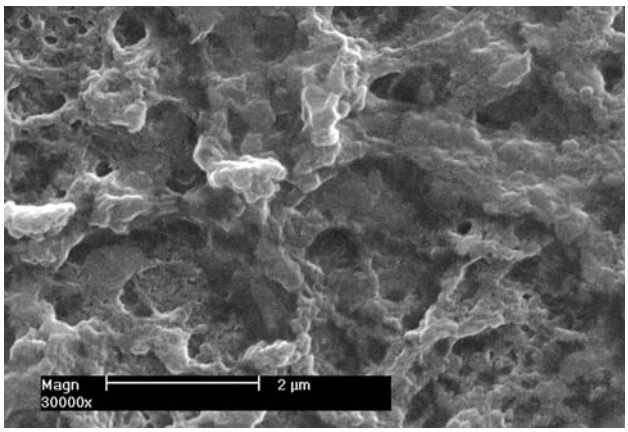

Fig. 2 SEM-image of Barnacle adhesive (*B. crenatus*) released on Teflon substrate; sample preparation method 2 (freeze-drying)

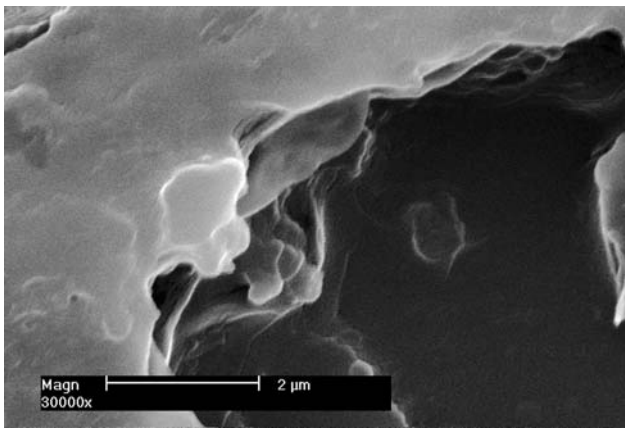

Fig. 3 SEM image of Barnacle adhesive (*B. crenatus*) released on Teflon substrate; sample preparation method 3 (freeze-drying + prefixation)

Samples treated according to the 1st method (CPD) exhibited a very rough surface and porous matrix (Fig. 1). In contrast, samples of the 3rd method (chemical fixation + freeze-drying) looked fairly homogeneous and had a smooth surface (Fig. 3). Due to the homogeneity an additional picture at lower magnification (5000×) is giving an overview (Fig. 4). Samples that were freeze-dried without fixation showed an intermediate situation—a slightly porous matrix and a rough, shrivelled surface (Fig. 2).

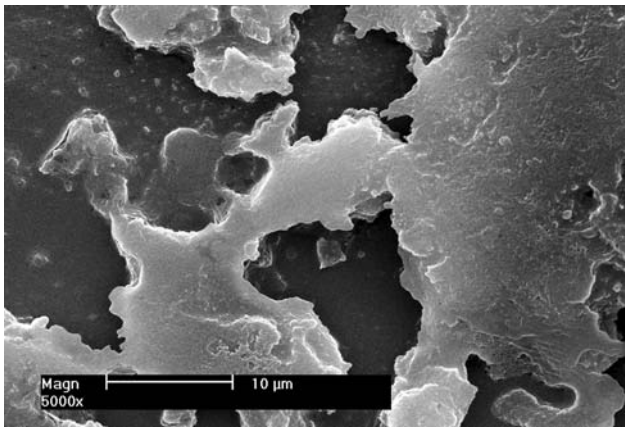

Fig. 4 Overview to preceding image (Fig. 3): SEM-image of Barnacle adhesive (*B. crenatus*) released on Teflon substrate; sample preparation method 3 (freeze-drying + prefixation)

A beam power of 10 kV was appropriate for all samples. However, when modifying method 2 by using adhesive with a higher content of water (e.g. adhesive secreted on PDMS-based coatings) and/or thinner metal coating it was only partially possible to visualize the samples. The material was too delicate to expose it to a beam power of more than 2 kV (and low magnification). Higher electron concentration resulted in the breaking of the sample surface.

Discussion

Fixation and dehydration may lead to artefacts masking the original structure. This is especially true for biological materials containing high percentages of water. In this study water containing barnacle cement was chosen as a model matrix. Cement secreted on Teflon can reach high cohesive strength and it was from previous experience expected to give a homogeneous appearance at the fine-structural level (see [8]). This expectation coincided with samples treated according to the 3rd method. Referring to samples of method 3 as being closest to the natural state leaves the samples of methods 1 and 2 to appear collapsed (Table 2).

The appearance of collapsed, porous structures are not to be confused with adhesive globules forming fibrils or threads, net- and foam-like structures as previously shown [8]. The differences are obvious in Fig. 5, which is printed again here for comparison.

Table 2 Comparison of appearance and artefacts of samples treated according to methods 1, 2, and 3

	1. Method	2. Method	3. Method
Appearance of matrix	Very rough surface/porous matrix	Rough surface/slightly porous matrix	Relatively smooth surface/homogeneous matrix
Interpretation	High degree of shrinkage/collapse of matrix	Shrinkage/collapse of matrix	Shrinkage extremely reduced—close to native state

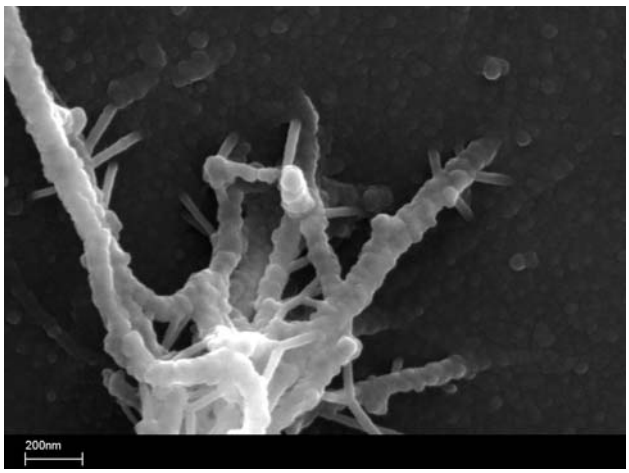

Fig. 5 Image from [8]: Barnacle adhesive (*B. crenatus*) composed of globular structures forming superstructures (compact matrix, threads, and loose networks)

The prominent artefacts occurring in the CPD method 1 are probably a result of chemical dehydration (ethanol/acetone series) while the artefacts through freeze-drying (method 2) were probably due to the instability of the biopolymer during the sublimation process. In contrast to the plain freeze-drying method the preceded chemical fixation (method 3) resulted in better conserved samples. The glutaraldehyde treatment had obviously toughened the proteins against the freeze-drying forces. It is agreed that careful prefixation can be used for the investigation of dimensional topography of macromolecules [25]. Glutaraldehyde has been shown to produce excellent preservation of cellular detail and is a primary fixative for electron microscopy [26]. Although it is a fast acting aldehyde, it has a slow rate of penetration and it is therefore suitable for small pieces of tissue.

The stabilizing effect of glutaraldehyde is not only advantageous for the freeze-drying process but also during imaging. A delicate specimen can be easily destroyed by charge building up on the sample surface. This may be avoided by using a lower primary beam voltage or by protecting the specimen with a thicker metal coating to eliminate charge [20]. As reported above, the very fragile samples with a high water content (obtained from a PDMS coating and freeze-dried without chemical fixation) were destroyed when the beam power was set to more than 2 kV. Glutaraldehyde fixation, however, showed a stabilizing effect against the exposition to the electron beam. Thus, the chemically fixed samples can be observed with a thinner metal coating revealing more details of the fine structure. However, preliminary trials are always necessary for finding the appropriate setting according to the fragility (water content) of the sample.

The drying process is the critical step in SEM preparation. Several studies have dealt with the advantages and disadvantages of critical-point drying versus freeze-drying. The results were contradictory (see e.g. [22, 27–29]) indicating that other circumstantial factors such as fixatives, additives, and settings of the drying process have a great impact. Nowadays, it is generally agreed that freeze-drying results in less shrinkage than CPD. The shrinkage of mouse liver tissue after freeze-drying (and prior glutaraldehyde fixation), for example, was estimated to be 7.5% [30].

In any case, freeze-dried samples are hygroscopic and pose the risk of rehydration when they are transferred to a coating machine. This step is critical and may limit high-resolution work. Some authors have addressed this problem by combining the steps of drying and coating using modified coating machines [23, 31]; such systems are now commercially available (see "Method"). The combination of freeze-drying and sputtering was possible for methods 2 and 3. After the transfer of the frozen specimens into the conventional sputter coater it was important that the vacuum of 10^{-4} mbar built up rapidly holding the low temperature. Therefore, any excess moisture had to be removed from the sample prior to freezing. After the initial sublimation of the outer layers, diffusion through the tissue is probably the more determining factor in the drying process. Therefore the low thickness of the sample was beneficial for a rapid dehydration process.

The importance of speed during freezing is an additional aspect favouring thin sections of specimens over bulk material to be used. The freezing in liquid N_2 (boiling point: −196 °C) seemed to be sufficient. There are, however, other methods available that allow even faster freezing (e.g. freezing in a slush of liquid and solid nitrogen to minimize the Leidenfrost-effect or "quick-freezing" against a block of ultrapure copper cooled to liquid helium temperature (−269 °C)).

Conclusion

This study demonstrates that quick and easy processing (one-step chemical fixation followed by combined freeze-drying and sputter coating) of ECM proteins for conventional SEM analysis is possible giving excellent results. This method is especially recommended for the fragile extracellular proteins with gel-like characteristic. Fixation with glutaraldehyde prior to freezing stabilized the protein matrix during sublimation and when exposed to the electron beam during SEM analysis.

There are several preparatory steps sensitive to the thickness of the sample. The slow penetration of glutaraldehyde, the necessities of fast freezing, and the diffusion process of liquid during sublimation favour thin specimens over bulk material to be used. Wettings of bioadhesives are perfect targets for the suggested technique.

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