Testing the best method to prepare recent and fossil brachiopod shells for SEM analysis

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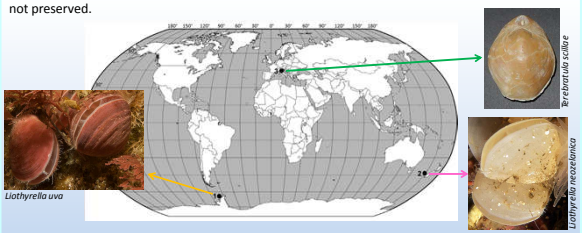
Introduction

The analysis of shell and skeleton microstructures by Scanning Electron Microscope (SEM) is a fundamental step in the study of the mineralised parts of marine and terrestrial organisms and it provides invaluable information in different fields of palaeontology, from the comprehension of evolutionary taxonomy and of biomineralisation processes to the detection of shell diagenetic alteration.

In precipitating their low-magnesium calcite shells in isotopic equilibrium with ambient seawater, brachiopods are excellent archives of past seawater temperature and ocean chemistry. However, diagenetic processes may alter the original microstructure (in the form of recrystallisation, amalgamation and/or dissolution of the fabric) and geochemical composition; the SEM analysis of the microstructure represents one of the most common method used to test fossil shell preservation and eventually exclude diagenetic alteration. Notwithstanding the importance of this analysis, only few, scattered data have been published about the preparation and cleaning of brachiopod shells for SEM analyses. Here, we aim to identify a general protocol for the preparation of recent and fossil brachiopod shells for the study at the SEM, besides checking the response of the shell mineral fabric to: a) the resin used to embed the valves before cutting and b) different times of exposure to hydrochloric acid (HCl), hydrogen peroxide (H_2O_2) and bleach (Crippa et al., 2016).

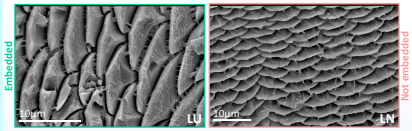
Material

Recent taxa analysed include *Liothyrella uva* (Broderip, 1833) (LU) and *Liothyrella neozelanica* (Thomson, 1918) (LN), respectively collected from Antarctica and New Zealand; fossil shells belong to *Terebratula scillae* (Seguenza, 1871) coming from the lower Pleistocene Stirone River sedimentary succession in Northern Italy. Terebratulid brachiopods have usually a two- or three layered mineralised shell (primary, secondary and tertiary layers). The secondary layer has a higher organic content compared to the primary and tertiary ones. The removal of the organic matrix (OM) is essential to obtain clear and distinct images of the mineralised shell fabric of recent brachiopods at the SEM. The problem does not arise in the case of fossil shells, as OM is generally not preserved.

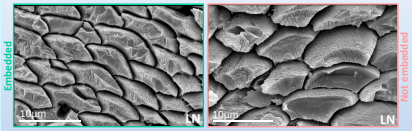


Resin: embedding vs not embedding

In valve sections **not treated** with H_2O_2 or bleach, the OM is abundant and forms coverages and/or filaments which do not allow to clearly distinguish the fabric. These coverages/filaments occur both in the specimens embedded in araldite and in the ones without the resin.

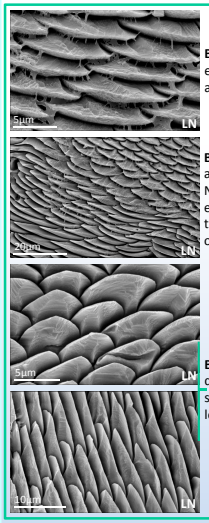


Shells **treated** with bleach or H_2O_2 appear cleaner due to the effect of the chemical solution which dissolve the OM. No difference is observed between specimens embedded or not embedded in the resin and experimenting the same procedure.



Organic matrix removal

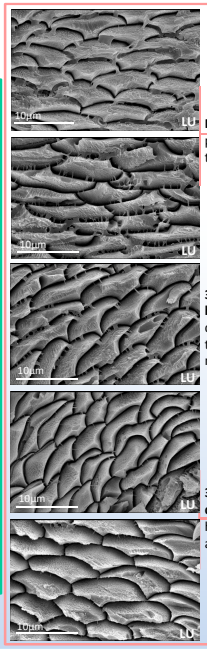
In order to remove OM two procedures have been used: 1) immersion in diluted commercial bleach (5% v/v) for two hours and one day; 2) immersion in hydrogen peroxide (H_2O_2) with different concentrations for different time intervals: a) 36 volume (11%) H_2O_2 for two hours and for one day; b) 12 volume (3.6%) H_2O_2 for one day and for three days. After the treatment sections were rinsed with distilled water.



Bleach for **2 hours**; the shells exhibit filaments of OM around the fibers

Bleach for **2 hours**; the shells are not clean from the OM. Note that the OM is more evident around fibers in transversal section than in oblique/parallel ones

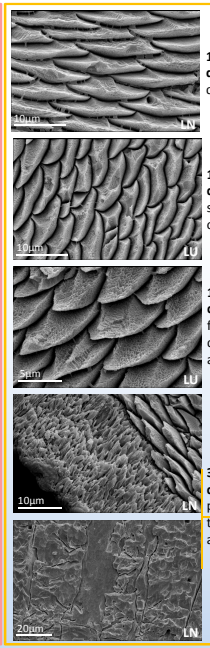
Bleach for **1 day**; OM seems dissolved. The surface of the secondary fibers showed a low grade of dissolution



No H_2O_2 treatment; OM is present around the fibers of the secondary layer

36 volume (11%) **H_2O_2** for **2 hours**; the content in OM decreases due to the H_2O_2 treatment, but the shell is not clean

36 volume (11%) **H_2O_2** for **1 day**; OM is dissolved; however, a slight dissolution appears on the fiber surface



12 volume (3.6%) **H_2O_2** for **1 day**; the shell is not completely free of OM

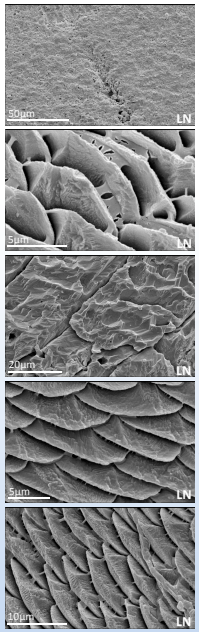
12 volume (3.6%) **H_2O_2** for **3 days**; OM is dissolved, but a slight dissolution is present on the fiber surface

12 volume (3.6%) **H_2O_2** for **3 days**; the surface of each fiber is dissolved in correspondence of the attachment sites of the OM

36 volume (11%) **H_2O_2** for **1 day**; the crystallites and prisms of the primary and tertiary layers are not affected by H_2O_2 dissolution

Hydrochloric acid effect

To better understand the effect of HCl on brachiopod shells, valve sections are immersed in the acid for different times (0, 3, 15 and 30 seconds).



No HCl treatment; Silicon Carbide (SiC) residues remain on the valve surface masking the fabric

HCl for **3 seconds**; the surface is clean from SiC residues and the fabric is distinct; the OM is clearly visible around the fibers of the secondary layer

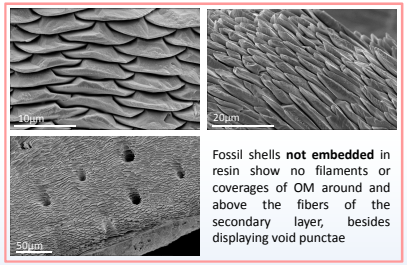
HCl for **3 seconds**; in the tertiary layer residues of OM have not been observed

HCl for **15 seconds**, corrosion appears on the surface of the fibers

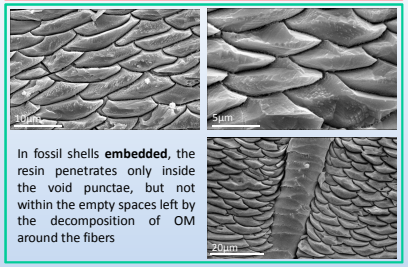
HCl for **30 seconds**, the fibers are corroded; note the sheaths of OM around the fibers

Fossil shells

Fossil shell sections are not treated with diluted bleach or H_2O_2 as their shell usually do not contain OM. We therefore check the degree of penetration of the araldite resin into the shell substance from the section. The scheduled time of shell etching (15 seconds) does not cause damage or corrosion of the fabric.



Fossil shells **not embedded** in resin show no filaments or coverages of OM around and above the fibers of the secondary layer, besides displaying void punctae



In fossil shells **embedded**, the resin penetrates only inside the void punctae, but not within the empty spaces left by the decomposition of OM around the fibers

Which is the best method to reveal the details of the shell fabric?

1 Resin embedding and cutting
Resin: araldite DFB + hardener HY956 (10:1 or 8:2). Give strength to valves to avoid shell breakage during cutting with a low speed saw with a thin diamond blade

2 OM removal
Only for recent specimens. Best treatment: bleach for 1 day, 36 volume H_2O_2 for 1 day or 12 volume H_2O_2 for 3 days. Although this causes a slight dissolution of the fiber surfaces, this does not compromise the morphology of the fabric and the analysis at the SEM. Rinse with distilled water

3 Smoothing
This step has to be done employing Silicon Carbide (SiC) with two different granulometries: first, the coarser one (400) to remove the scratches left on the shell surface by the blade during the cutting, then the finer one (1000) to complete the smoothing. Rinse with distilled water to remove SiC residues

4 5% HCl etching
Essential step to remove the mechanically disturbed surface layer - due to SiC residues - but also to highlight the details of the fabric. Time of etching: 3 seconds for recent shells, 15 seconds for fossil ones. After etching, immediately rinse the shells with abundant water to stop the effect of the acid

Some final considerations...

Coverages and filaments of OM are more evident in the fibrous secondary layer, particularly when fibers appear in cross sections; in the primary and tertiary layers, OM was not detected at this scale of analysis.

Chemicals cause a slight dissolution of the surface of the secondary fibers; in contrast, the crystallites and prisms of the primary and tertiary layers do not exhibit dissolution.

In testing the different procedures used to remove OM, shell sections of *L. uva* seem more difficult to clean than the ones belonging to *L. neozelanica*.

These observations can be explained with the different content in OM of the fabric of the three shell layers. The secondary layer has a high OM content, both intercrystalline and intracrystalline (e.g., Gaspard, 2007; Pérez-Huerta et al., 2009). The primary and tertiary layers have, instead, a lower OM content; in fact, they do not exude organic sheets between primary crystallites or tertiary prisms (Williams et al., 1997; Schmahl et al., 2012). In having a higher organic content, coverages and filaments of OM are more developed in the secondary layer. If not fixed with organic compound, as glutaraldehyde (Gaspard et al., 2007; Immel et al., 2015; Casella et al., 2017), or dissolved with appropriate chemical solutions, OM represents an obstacle to examine shell microstructures at the SEM.

Fibers cut in transverse section show a preferential orientation which allow to better expose the organic membranes which surround each fibers; this does not occur in fibers with parallel or oblique orientation.

Chemicals, in dissolving intercrystalline OM, leave a depression in correspondence of the attachment sites of the organic membranes on the surface of the fibers, producing dissolution.

L. uva has only a primary and a secondary layer, whereas *L. neozelanica* has a primary, a secondary and also a tertiary layer (Peck et al., 1997). The higher OM content of the shell of *L. uva* (Watson et al., 2012) results in a greater difficulty to clean it.

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Acknowledgements

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