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## Chalky versus foliated: a discriminant immunogold labelling of shell microstructures in the edible oyster *Crassostrea gigas*

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### ► To cite this version:

Vincent Mouchi, Franck Lartaud, Nathalie Guichard, Françoise Immel, Marc de Rafélis, et al.. Chalky versus foliated: a discriminant immunogold labelling of shell microstructures in the edible oyster *Crassostrea gigas*. *Marine Biology*, 2016, 163 (12), pp.256. 10.1007/s00227-016-3040-6 . hal-01400675

HAL Id: hal-01400675

<https://hal.sorbonne-universite.fr/hal-01400675>

Submitted on 22 Nov 2016

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1 **Chalky versus foliated: a discriminant immunogold labelling of shell**  
2 **microstructures in the edible oyster *Crassostrea gigas***

3

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26 **Key-words**

27 *Crassostrea gigas*; oyster shell; biomineralization; shell matrix extraction; polyclonal  
28 antibody; immunolocalization; in vitro crystallization; microstructures.

29

30 **Abstract**

31

32 Mollusc shells are organic-inorganic biocomposites, arranged in a limited number of  
33 superimposed calcified layers that generally exhibit very different organization of their  
34 crystallites. Because of their attractive mechanical and crystallographic properties, these shell  
35 layers have been the focus of several physical and biochemical characterizations. In  
36 particular, recent proteomic data obtained from individual layers suggest that their protein  
37 contents are different. However, the direct visual evidence that some macromolecular  
38 components are layer-specific is rather tenuous. This paper is based on a non-conventional  
39 immunogold labelling approach to localize proteins in the shell of the edible oyster  
40 *Crassostrea gigas*. The shell microstructure of this model organism is predominantly  
41 composed of foliated calcite, interspersed by discontinuous pockets of ‘chalky layers’, a  
42 porous microstructure typical of bivalves of the ostreid family. By developing a polyclonal  
43 antibody (in two rats) elicited against a proteinaceous shell fraction, we obtained differential  
44 staining of the two microstructures. We assert that our labelling is microstructure-  
45 discriminant. The difference in labelling of the two shell microstructures suggests either that  
46 they are formed by a variation of the secretory repertoire of the shell-forming cells of the  
47 calcifying mantle epithelium or that the chalky layer may be formed via a completely  
48 different mechanism. Our results allow a first glimpse on the subtle regulatory mechanisms  
49 that drive the process of chalky and foliated layers deposition.

50

## 51 **Introduction**

52

53 To protect their soft body, most molluscs secrete an external rigid exoskeleton, the  
54 shell. The shell is an inorganic-organic biocomposite, predominantly made of calcium  
55 carbonate, with a minor fraction of occluded organics, about 1% of the shell weight (Marin et  
56 al. 2012). This fraction, a mixture of proteins, glycoproteins and polysaccharides, collectively  
57 described as the shell matrix, is the main regulator of mineral deposition (Weiner and Traub  
58 1984; Lowenstam and Weiner 1989; Simkiss and Wilbur 1989). During calcification, the shell  
59 matrix is secreted by the calcifying mantle epithelium, together with inorganic precursor ions  
60 including calcium, bicarbonate and minor elements such as magnesium and strontium (Marin  
61 et al. 2012). All of these ingredients interact together at the interface between the mantle  
62 tissue and the growing shell and self-assemble to form crystalline architectures that are  
63 exquisitely crafted (Carter 1990).

64 Because of their mechanical properties, marine mollusc shells are often taken as a  
65 model for biomineralization studies (Addadi et al. 2006) and, more generally, as an  
66 inexhaustible source of inspiration for generating organic-inorganic composites with tailored  
67 mechanical properties and shapes (Cranford and Buehler 2010). In addition to these  
68 biotechnological applications, mollusc shells are studied for their capacity to record with high  
69 reliability the variations of physicochemical parameters of seawater (Rhoads and Lutz 1980).  
70 In recent years, this second aspect has become more pressing in the context of concern over  
71 global changes, in particular, of ocean acidification (Orr et al. 2005).

72 Oysters are excellent mollusc models for such environmental studies. Oysters are  
73 marine and brackish bivalves of the pteriomorphid subclass with a number of advantages;  
74 firstly, they are ubiquitous, occurring all over the world oceans and seas with the exception of  
75 polar regions, allowing comparisons at the global scale. Secondly, they withstand different

76 salinities (Bricteux-Grégoire et al. 1964) and different water depths (van Rooij et al. 2010),  
77 and can consequently be used as markers in different environments. Thirdly, their shell is  
78 entirely made of low magnesium calcite – with the exception the restricted myostracal layer  
79 that is aragonitic (Taylor et al. 1969) – and thus, resists diagenetic transformations better than  
80 most aragonite and high magnesium calcite shells (Ahr 2008). Oysters exhibit a large  
81 stratigraphical range that covers the last two hundred million years of the history of Earth  
82 (Marquez-Aliaga et al. 2005), allowing extensive palaeoenvironmental reconstruction studies  
83 at different geological periods. Finally, their shell is thick, *i.e.*, ideal from a practical  
84 viewpoint for measuring with high accuracy environmental proxies along transects, in both  
85 recent (Lartaud et al. 2010a; Mouchi et al. 2013) and fossil (Bougeois et al. 2014, 2016)  
86 specimens.

87         Our study focuses on one member of the ostreid family, *Crassostrea gigas*, the edible  
88 cupped oyster, also called the giant Pacific oyster, and a model of economic interest (Gosling  
89 2003). *Crassostrea gigas* can withstand huge environmental variations, including alternation  
90 of emersion/immersion, drastic change in salinity and rapid increase/decrease of temperature  
91 (Lartaud et al. 2010a). *Crassostrea gigas* shell allows a very precise temporal calibration,  
92 independently from the shell microstructures (Lartaud et al. 2010b). The shell of *C. gigas*  
93 exhibits two main textures: the foliated and chalky microstructures. Foliated calcite can be  
94 described as “*a laminar structure consisting of parallel calcitic laths arranged in sheet*  
95 *dipping at the same angle and in the same general direction over a large portion of the*  
96 *depositional surface*” (Carter and Clark II, 1985). The chalky microstructure is typical of  
97 Ostreidae and predominantly found in this bivalve family. It forms discontinuous, lenticular  
98 bodies that are intercalated between the folia. Numerous studies have reported different  
99 putative mechanisms for the formation of chalky layers (Orton and Amirthalingam 1927;  
100 Korringa 1951; Palmer and Carriker 1979; Vermeij 2014). So far, no consensus emerged.

101 In the present paper, we used an immunolabelling approach – complementary to that  
102 developed by one of us (F. L.) with manganese – to label the shell of *C. gigas*. To this end, we  
103 employed the following strategy: the shell matrix was extracted, characterized on  
104 monodimensional electrophoretic gels, and one fraction was further purified by preparative  
105 SDS-PAGE before being analyzed by proteomics, and tested for its ability to interact with the  
106 *in vitro* precipitation of calcium carbonate. This protein fraction was subsequently used to  
107 elicit polyclonal antibodies, which, after accurate testing, allowed immunolabelling of the  
108 shell. This labelling is microstructure-discriminant. The difference in labelling of the two  
109 shell microstructures suggests that their elaboration rests upon a variation of the secretory  
110 repertoire of the shell-forming cells of the calcifying mantle epithelium.

111

112

## 113 **Materials and methods**

114

### 115 Sample preparation

116 Fresh oysters were collected live in Dublin area, Ireland. For specimens used for matrix  
117 extraction, shells were emptied, the muscle scar scrupulously cleaned, and the outer surface of  
118 the shells mechanically abraded with a rotary tool (Dremel) to remove all epibionts that could  
119 act as a source of contaminant material. Whole shells were then chemically cleaned in dilute  
120 sodium hypochlorite (0.26 % active chlorine) for 48 h and rinsed thoroughly in deionized  
121 water several times. Shells were mechanically crushed in small fragments (4-5 mm) that were  
122 placed in sodium hypochlorite (0.26 % active chlorine) for 24 h. They were rinsed several  
123 times in Milli-Q water, dried at 37 °C and powdered using a mortar grinder (Pulverisette 2,  
124 Fritsch, Idar-Oberstein, Germany). The powder was sieved to select particles with a grain size  
125 below 200 µm.

126 For immunogold localization, additional shells were taken from the collection of the Institut  
127 des Sciences de la Terre de Paris (ISTeP, UPMC). These specimens had been previously bred  
128 for two years at Baie des Veys (Normandy, France) according to a published paper (Lartaud  
129 et al. 2010b). The shells were emptied and chemically cleaned with hydrogen peroxide (6 %)   
130 for 6 h followed by 0.15 N nitric acid for 20 min and washed in demineralized water. The  
131 hinge areas were cut from the shells, and hinge sections were glued with epoxy to glass plates  
132 and sawed to a thickness ranging from 500 µm to 1 mm, in order to visualize the chalky and  
133 foliated shell microstructures.

134

#### 135 Shell matrix extraction

136 The protocol used for matrix extraction was, with slight differences, used in previous papers  
137 (Osuna-Mascaró et al. 2014; Kanold et al. 2015): 25.08 g of shell powder was suspended in  
138 100 mL of Milli-Q water under constant stirring, at 4 °C. The powder was slowly decalcified  
139 overnight with cold dilute acetic acid (10 % vol.vol<sup>-1</sup>), with additions every 5 s (100 µl) using  
140 an electronic burette (Titronic Universal, Schott, Mainz, Germany). At the end of the  
141 decalcification, the resulting solution (>1 L) was centrifuged for 15 min at 3900 g. The  
142 supernatant containing the acid-soluble matrix (ASM) was separated from the pellet of the  
143 acid-insoluble matrix (AIM). The AIM was scrupulously rinsed by a series of resuspensions  
144 in milli-Q water/centrifugation (5 cycles). Each time, the resulting supernatants were added to  
145 the ASM. The AIM was finally lyophilized and weighed. The ASM was filtered on a Nalgene  
146 device with a 5 µm filter then its volume was reduced by ultrafiltration using a 400 mL  
147 Amicon cell with a 10 kDa cutoff membrane. At the end of the concentration process (final  
148 volume around 15 mL), the solution was dialyzed at 4 °C in a Spectra/Por tube (cutoff 1000  
149 Da) against milli-Q water, with 5 water changes in 4 days. The solution was lyophilized

150 overnight, and the resulting pellet, weighed. A second extraction was performed in similar  
151 conditions, with 30.03 g of shell powder.

152

### 153 Shell matrix analysis on 1D gel electrophoresis

154 ASM and AIM fractions were both analyzed by conventional mono-dimensional  
155 electrophoresis on 12 % polyacrylamide mini gels (Mini-Protean III, Bio-Rad, Hercules, CA,  
156 USA) following the manufacturer's instructions. Both matrices were resuspended in Laemmli  
157 sample buffer (LSB) containing  $\beta$ -mercaptoethanol, and denatured by heating at 100 °C for  
158 10 min. The solutions were cooled down on ice then centrifuged for 2 min. A fraction of the  
159 AIM was further solubilized by the Laemmli buffer and this soluble fraction was referred to  
160 as the LS-AIM (Laemmli-Soluble AIM). The proteins were fractionated on the gel for 15 min  
161 at 100 V, and then for about one hour at 150 V. The gel was stained with silver nitrate  
162 according to the protocol of Morrissey (1981), with the modification that the colour  
163 development was stopped with 3 M citric acid.

164

### 165 Protein extraction and fraction purification

166 Following the separation of the proteins in gel, one of the most abundant proteins was purified  
167 on a large scale, according to the procedure described in Marin et al. (2001) and Marin  
168 (2003): 2 mL of Milli-Q and 2 mL of LSB (2x) were admixed to 124 mg of extracted AIM  
169 and the preparation was denatured by heating at 100 °C for 10 min. Fractionation of the  
170 proteins of the LS-AIM was performed at 180-200 V for the stacking and 300 V for the  
171 running in a 12 % acrylamide preparative gel was cast in a Bio-Rad model 491 Prep Cell. The  
172 complete LS-AIM extract was eluted from the gel in about 13 hours and collected in 80 tubes  
173 (5 ml per tube, flow rate of elution: 0.5 ml/min). All 80 fractions were tested with dot-blot  
174 using a Bio-Rad Bio-Dot on a PVDF membrane. The membrane was subsequently treated



175 with polyclonal antibodies elicited against calprisin, a 37 kDa protein from the prismatic  
176 layer of the bivalve *Pinna nobilis* (Marin et al. 2005). This antibody presented a strong cross-  
177 reactivity with the shell matrix of *C. gigas* in preliminary tests and recognized epitopes of  
178 relatively abundant protein of the AIM in Western-Blot (results not shown here). Two  
179 consecutive fractions presenting such strong reaction were pooled, and the resulting solution  
180 dialyzed (Spectra/Por tube, 4 °C) and lyophilized. The purity of this extract, referred as F21-  
181 22, was tested on a 12 % polyacrylamide gel, similarly to what described above.

182

### 183 Proteomics on the purified fraction

184 The identification of the protein content of F21-22 was performed via a proteomic approach,  
185 according to an in-gel digestion, as previously described (Kanold et al. 2015). The fraction  
186 was denatured and run on a precast 12 % acrylamide mini-protean TGX gel (Bio-Rad). The  
187 gel was fixed overnight (colloidal Coomassie blue), then washed in Milli-Q water, and a band  
188 manually sliced near 30 kDa. The slice was cut into cubes, which were subsequently placed in  
189 an Eppendorf tube. Then, in-gel digestion was carried out with trypsin, according to a  
190 published procedure with minor adjustments (Shevchenko et al. 2001): the sample was  
191 destained twice with a mixture of 100 mM ammonium bicarbonate (ABC) and 50 % (vol.vol<sup>-1</sup>)  
192 acetonitrile (ACN) for 45 min at 22 °C and then dehydrated using 100 % ACN for 15 min,  
193 before being reduced with 25 mM ABC containing 10 mM DTT for 1 h at 60 °C and  
194 alkylated with 55 mM iodoacetamide in 25 mM ABC for 30 min in the dark at 22 °C. Gel  
195 pieces were washed twice with 25 mM ABC and dehydrated (twice, 15 min) and dried (10  
196 min) with 100 % ACN. Gel cubes were incubated with sequencing grade modified trypsin  
197 (Promega, USA; 12.5 ng.µl<sup>-1</sup> in 40 mM ABC with 10 % ACN, pH 8.0) overnight at 40 °C.  
198 After digestion, peptides were washed with 25 mM ABC, dehydrated with 100 % ACN and

199 extracted twice with a mixture of 50 % ACN–5 % formic acid (FA). Extracts were dried using  
200 a vacuum centrifuge Concentrator plus (Eppendorf).

201 For MS and MS/MS ORBITRAP, analyses were performed using an Ultimate 3000 Rapid  
202 Separation Liquid Chromatographic (RSLC) system (Thermo Fisher Scientific) online with a  
203 hybrid LTQ-Orbitrap-Velos mass spectrometer (Thermo Fisher Scientific). Briefly, peptides  
204 were dissolved in 4  $\mu$ L of 10 % ACN-0.1 % FA. Then peptides were loaded and washed on a  
205 C<sub>18</sub> reverse phase precolumn (3  $\mu$ m particle size, 100 Å pore size, 150  $\mu$ m i. d., 1 cm length).  
206 The loading buffer contained 98 % H<sub>2</sub>O, 2 % ACN and 0.1 % TFA. Peptides were then  
207 separated on a C<sub>18</sub> reverse phase resin (2  $\mu$ m particle size, 100 Å pore size, 75  $\mu$ m i. d., 15 cm  
208 length) with a 1 h gradient from 100 % A (0.1 % FA and 100 % H<sub>2</sub>O) to 50 % B (80 % ACN,  
209 0.085 % FA and 20 % H<sub>2</sub>O).

210 The Linear Trap Quadrupole Orbitrap mass spectrometer acquired data throughout the elution  
211 process and operated in a data dependent scheme with full MS scans acquired with the  
212 Orbitrap, followed by up to 20 LTQ MS/MS CID spectra on the most abundant ions detected  
213 in the MS scan. Mass spectrometer settings were: full MS (AGC:  $1 \times 10^6$ , resolution:  $6 \times 10^4$ ,  
214 m/z range 400–2000, maximum ion injection time: 500 ms) and MS/MS (AGC:  $5 \times 10^3$ ,  
215 maximum injection time: 20 ms, minimum signal threshold: 500, isolation width: 2 Da,  
216 dynamic exclusion time setting: 30 s). The fragmentation was permitted for precursors with a  
217 charge state of 2, 3, 4 and above. For the spectral processing, the software used to generate  
218 .mgf files was Proteome discoverer 1.3. The threshold of signal to noise for extraction values  
219 is 3. Database searches were carried out using Mascot version 2.4 (Matrix Science, London,  
220 UK) on “other metazoa” proteins (35,149,712 sequences) from the NCBI nr databank  
221 containing 12,374,887,350 residues (January 2014) ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) and an in-house  
222 shell protein databank (762 sequences containing 220,545 residues). The search parameters  
223 were as follows: carbamidomethylation as a variable modification for cysteins, and oxidation

224 as a variable modification for methionines. Up to 1 missed tryptic cleavage was tolerated, and  
225 mass accuracy tolerance levels of 10 ppm for precursors and 0.45 Da for fragments were used  
226 for all tryptic mass searches. Positive identification was based on a Mascot score above the  
227 significance level (i.e. 5 %). The reported proteins were always those with the highest  
228 number of peptide matches.

229

### 230 Antibodies production, ELISA testing and Western blots

231 The fraction was used to produce polyclonal antibodies (Eurogentec, Seraing, Belgium) in  
232 two rats, SER323 and SER324, following a standard immunization procedure: the rats were  
233 injected (60 µg of antigens per injection) at day 0, then at days 14, 28, 56 and 132, and their  
234 blood was collected at day 0 (pre-immune serum, PPI), 38 (small bleed, PP), 66 (large bleed,  
235 GP) and 142 days (final bleed, SAB). The titers of the different antibody solutions were  
236 checked by conventional ELISA (Clark and Adams 1977; Thresh et al. 1977): in brief, the  
237 antigens were incubated in a Nunc Maxisorp 96 well microplate (200 ng per well, 90 min, 37  
238 °C). After blocking step (0.5 % wt.vol<sup>-1</sup>gelatin in TBS), the microplate was incubated 90 min.  
239 with the antibody solutions (PPI, PP, GP, SAB, diluted 1/100 to 1/200000), then with the  
240 secondary antibody (goat anti-rat, Sigma A 8438, diluted 30000 times). The microplate was  
241 thoroughly rinsed with TBS/Tween 20 (using a manual Nunc Immuno Wash 12 microplate  
242 washer) between antigen incubation and blocking and after the 1<sup>st</sup> and 2<sup>nd</sup> antibodies  
243 incubations. The microplate was revealed with the substrate solution, consisting of p-  
244 nitrophenylphosphate (5 mg tablet in 10 mL) dissolved in a water:diethanolamine solution  
245 (10:1), pH 9.8. After short incubation at 37°C, it was read with a multichannel  
246 spectrophotometer at 405 nm. We checked that the two pre-immune sera gave no reactivities  
247 and consequently used the different sera for further characterization.

248 Western blots (Towbin et al. 1979) were used to test the specificity of the antibodies against  
249 the matrix of the shell of *C. gigas*. Both LS-AIM and ASM were tested on 12 %  
250 polyacrylamide mini-gels. After migration, the proteins from the gels were electro-transferred  
251 on a PVFD membrane (Immobilon, Millipore) for 90 min at 100 V in a Bio-Rad Mini Trans-  
252 Blot module. The membrane was then blocked in a TBS solution containing 1 % gelatin for  
253 30 minutes before placed in a TBS solution containing 1 % gelatin, 0.05 % Tween 20 and the  
254 antibodies diluted 1500 times. The membrane was incubated for 3 hours at 37 °C and then  
255 rinsed several times in a TBS/Tween 20 solution. It was subsequently incubated 90 minutes in  
256 a TBS/Tween 20/gelatin solution containing secondary antibodies (goat-anti-rat, Sigma, ref.  
257 A8438) coupled with alkaline phosphatase, diluted 30,000 times. Finally, the membrane was  
258 rinsed thoroughly (5 x 10 min) in TBS/Tween 20 and incubated for five minutes in the dark in  
259 CDP-Star (Sigma, ref. C0712) solution. The chemoluminescent signal was recorded by  
260 mounting the membrane between two write-on transparency sheets in a cassette and exposing  
261 it shortly to a X-OMAT Kodak film which was conventionally developed and fixed. In  
262 addition, the nitrocellulose membrane was stained with NBT/BCIP (Sigmafast tablets, Sigma,  
263 ref. B5655).

264

#### 265 Specificity of the antibody responses to microstructures

266 In order to check the extent to which the antibodies could differentiate the matrix of the  
267 individual shell microstructures of *C. gigas*, we performed specific ELISA with extracts from  
268 the chalky and foliated layers as previously described (Marin et al. 1999). In brief, left valves  
269 were cut in half to expose the hinge region showing the two microstructures. The chalky and  
270 foliated microstructures were collected separately using a dental drill. 40 mg of each powder  
271 were dissolved overnight in 4 mL EDTA solution (10 % wt.vol<sup>-1</sup>). After a short centrifugation  
272 (3900 g, 10 min), aliquots of the EDTA-extracts were directly incubated in 96-well

273 microplates (37°C, 90 min.). A conventional ELISA test, as described above, was performed  
274 with each of the sera (PP, GP, SAB) obtained from both rats. The microplate was read at 405  
275 nm. PPI (pre-immune serum) was used as a negative control.

276

#### 277 *In vitro* crystallization test

278 Both the ASM and the purified fraction of the AIM (F21-22) were tested for their capacity to  
279 influence the growth of calcium carbonate crystals, according to a procedure derived from  
280 that of Albeck et al. (1993). Briefly, calcite crystals were grown by the interaction between  
281 vapours of ammonium bicarbonate and CaCl<sub>2</sub> solution (10 mM) containing a small quantity  
282 of ASM or F21-22. The CaCl<sub>2</sub> solution (200 µL) with different quantities of matrix (from  
283 0.3125 to 20 µg.mL<sup>-1</sup>) was placed in the wells of a 16-well culture slide (Lab-Tek, Nunc). The  
284 cover of the slide was pierced to allow diffusion of ammonium bicarbonate vapours. The  
285 culture slide with its cover was sealed with Parafilm then placed at 4 °C in a 5 L closed  
286 desiccator containing crystals of ammonium bicarbonate for 72 h. Control scenarios of only  
287 CaCl<sub>2</sub> solution were tested in parallel. After incubation, the solution was carefully removed  
288 from each well using a blunt-ended needle connected to a vacuum system. The glassplate of  
289 the slide was dissociated from the well spare part and directly observed under a Hitachi  
290 TM1000 Tabletop microscope without carbon coating. This experiment was repeated four  
291 times to ensure homogeneity of the results.

292

#### 293 Protein localization by immunogold by SEM

294 Small freshly fractured (<5 mm) fragments were placed in sodium hypochlorite solution to  
295 remove superficial contaminants. They were rinsed (milli-Q water), dried then slightly etched  
296 in EDTA solution (1 % wt.vol<sup>-1</sup>) for 3 min to expose antigenic determinants. For the cleaning  
297 and etching steps, ultrasonic baths were not used, to avoid fragmentation of the shell pieces.

298 The samples were then washed for 1 min in TBS before being blocked in filtered TBS/gelatin  
299 (0.5 %) solution (pH adjusted to 7.5 with dilute NaOH solution) for 30 min. The shell samples  
300 were subsequently incubated 3 h in TBS/Triton/gelatin containing the antibodies (GP and  
301 SAB) diluted 500 times. They were rinsed 5 times in TBS/Triton for 10 min and incubated 3  
302 hours in TBS/Triton/gelatin (0.5 %) containing the secondary antibody (goat-anti-rat, 5 nm  
303 gold conjugate, BBI ref. EM.GTMA5, dilution 1/100). They were thoroughly washed in  
304 TBS/Triton then in water and slightly dried by capillarity. Fragments were finally silver-  
305 enhanced (BBI ref. SEKL.15) for 15 minutes before being rinsed in Milli-Q and dried at 37  
306 °C. Several negative controls were performed, by using PPI, or by replacing the antibodies  
307 (1<sup>st</sup> and/or 2<sup>nd</sup>) by TBS/Triton/gelatin. Observations were performed on the Hitachi TM1000  
308 Tabletop microscope without carbon coating. The test was repeated three times.

309

310

## 311 **Results**

312

### 313 Microstructures of the shell of *Crassostrea gigas*

314 As shown by Fig. 1, the shell of *C. gigas* is composed predominantly of two microstructures:  
315 the main one is foliated calcite, classically described by Taylor et al. (1969), Runnegar (1984)  
316 and Carter and Clark II (1985), and consisting of parallel calcitic laths arranged in sheets; the  
317 chalky layer, discontinuous, forming lenses in the hinge region (Fig. 1a) and intercalating thin  
318 layers in the other parts of the shell. From a microstructural viewpoint, chalky layers appear  
319 far more porous and made of a framework of blade-shaped (Margolis and Carver 1974)  
320 crystals that develop more or less perpendicularly to the mineral depositional plan. The blades  
321 are linked with each other by leaflets that branch at different angles (Fig. 1b), leaving a large  
322 amount of empty space. Between the framework, the space is filled by tangled crystals. In

323 addition to the foliated and chalky microstructures, a thin prismatic calcitic layer is observed,  
324 constituting the outermost part of the shell (not shown in Fig. 1).

325

#### 326 Shell matrix extraction and characterization on mini-gels

327 Similar amounts of ASM and AIM were obtained from the different fractions. From the first  
328 batch (25.08 g shell powder), we quantified 11.25 mg of ASM and 116.84 mg of AIM,  
329 representing 0.045 % and 0.465 % of dry weight of shell powder, respectively, representing  
330 together about half a percent of organics. The AIM/ASM ratio is about 10. When proteins  
331 were fractionated on a monodimensional gel and stained with silver, the profile (Fig. 2a)  
332 shows few proteins on each of the fractions distinct from some smearing material. ASM (Fig.  
333 2a lane 1) contains 3 main proteinaceous components at approximately 45, 27 kDa and 12  
334 kDa. The electrophoretic pattern of LS-AIM (Fig. 2a lane 2) exhibits similarities with that of  
335 ASM, since these 3 proteins are present, in addition to three other diffuse proteinaceous  
336 components at approximately 60, 34 and 22 kDa. In both extracts, the 45 kDa proteinaceous  
337 component is negatively stained. In the ASM, the upper part of the gel (above 130 kDa) and  
338 the zone between 17 and 30 kDa present also this particularity.

339

#### 340 Protein purification and testing of antibodies

341 The whole LS-AIM was fractionated on a preparative gel electrophoresis, and the fractions  
342 were dot-blotted. The fraction of interest was eluted in tubes 21-22, and further processed,  
343 including extensive dialysis and freeze-drying (Fig 2b). It was referred as F21-22 (Fig. 2c).  
344 After extensive dialysis and freeze-drying, 2.13 mg of purified protein was obtained from 30  
345 g of shell powder. The fraction, when tested on a mini-gel, is revealed as a thick  
346 proteinaceous component around 27 kDa (Fig. 2d). After production of polyclonal antibodies  
347 in two rats (SER323 and SER324), titers from the PPI, PP, GP and SAB bleeds were

348 determined in ELISA (Fig. 3). Pre-immune bleeds from both rats show no reaction to the  
349 targeted fraction. For rat SER323, the final titer (about 1:1500) is almost reached after the  
350 first injection (PP bleeding), and the differences of immunological reactivity between the  
351 successive bleedings are minimal. For rat SER324, we observe a progressive increase of the  
352 titer, in correlation with the successive immunizations. In this case, titers are 1:100, 1:500 and  
353 1:1000 for PP, GP and SAB, respectively.

354

355

#### 356 Western-blot of shell extracts with the anti-F21-22 antibody

357 The results of the Western blot of the shell extracts with the anti-F21-22 antibody from the  
358 rats SER323 and SER324 are shown on Fig. 4. For each of them, we present the data obtained  
359 with PPI (negative control, left), 2<sup>nd</sup> bleed (GP, center) and final bleed (SAB, right). For the  
360 clarity of the results, we only illustrate the Western blots obtained after the chemical staining  
361 of the membrane with NBT/BCIP. Entirely superimposable results, although more blurred,  
362 were obtained with the chemoluminescent CDP-Star. None of the two PPIs react with the  
363 shell extracts, ASM or LS-AIM. When tested on F21-22, the antibodies from the two rats  
364 successfully recognize this fraction, giving a high intensity signal. Although the antibodies  
365 were elicited against a discrete molecular weight fraction, their response against the whole  
366 ASM and LS-AIM encompasses a broad range of molecular weights, from above 170 kDa to  
367 about 10 kDa. For rat SER323 (Fig. 4a), we notice that the antibody allows visualizing  
368 proteins that cannot be discriminated on the silver-stained gel, in particular proteins of high  
369 molecular weights around 72, 130 and above 170 kDa. These proteinaceous components are  
370 observed both for ASM and LS-AIM from GP and SAB (Fig. 4a). Other proteins do cross-  
371 react around 50 and 40 kDa in the same lanes. For rat SER324, we obtain a different pattern,  
372 since the corresponding antibody stains preferentially the smear than the discrete proteins. In



373 the ASM (Fig. 4b, SAB), the F21-22 fraction is well marked. For both rats, the staining of LS-  
374 AIM is more pronounced than that of ASM. Signals given by GP bleeds are weaker than  
375 those of SAB bleeds, particularly for rat SER324. This finding is congruent with the ELISA  
376 results.

377

#### 378 Proteomics on the F21-22 fraction

379 The proteomic investigations, as summarized in Table 1, yielded a series of peptides that  
380 match with three proteins or protein families of *C. gigas* that are, respectively: Gigasin-6, and  
381 two of its isoforms Gigasin-6 X1 and Gigasin-6 X2; two nacrein-like proteins; a cell death  
382 abnormality protein 1-like. Gigasin and its two isoforms X1 and X2 were identified by an  
383 identical set of three different peptides. Each of the two nacrein-like proteins were also  
384 identified by three peptides, two of which being identical in the two proteins, while the third  
385 differed only by one amino acid residue (D or E). Two peptides – among which a 23 amino  
386 acid residues long hydrophobic peptide - could assign the cell death abnormality protein 1-  
387 like. The positions of these peptides along the different protein sequences are visualized in the  
388 supplementary Fig. 1. Additional *in silico* investigations in less stringent conditions (lower  
389 threshold, not shown in Table 1) generated six peptides – all located in the N-terminal region  
390 of the protein - that match with a transcription termination/antitermination protein NusA-like  
391 from the Mediterranean Fruit fly *Ceratitis capitata* (gi|498978467). The significance of these  
392 additional hits is not understood.

393

#### 394 In vitro crystallization in the presence of ASM and of F21-22

395 Results of the *in vitro* crystallization assay with fraction F21-22 and with ASM are shown on  
396 Fig. 5. The control scenario with no protein (Fig. 5a) produces single crystals that exhibit the  
397 typical rhombohedral morphologies of calcite. Effects are markedly different between the two

398 extracts. At low concentration ( $0.31 \mu\text{g}\cdot\text{mL}^{-1}$ ), ASM exhibits a pronounced effect on the  
399 crystal shape, with the formation of polycrystalline aggregates (Fig. 5e). At the same  
400 concentration (Fig. 5b), fraction F21-22 exerts almost no effect on the crystal morphologies.  
401 At  $5 \mu\text{g}/\text{mL}$ , the effect induced by ASM is strong (Fig. 5f) while it is limited with fraction  
402 F21-22 (Fig. 5c). At high concentration ( $20 \mu\text{g}\cdot\text{mL}^{-1}$ ), ASM induces only polycrystalline  
403 aggregates that are completely rounded: some of the crystals are overgrown on their edges  
404 (Fig. 5g); with F21-22, we notice the formation of polycrystalline aggregates, similar to that  
405 produced at low concentration of ASM. In summary, the effect of the ASM is more  
406 pronounced than that of the fraction F21-22, at equivalent concentrations.

407

#### 408 Response of the antibodies to microstructures

409 When tested by ELISA on extracts of the chalky and foliated microstructures, the antibodies  
410 of the two rats give different responses, illustrated by the histograms of Fig. 6: although the  
411 two series of antibodies cross-react with EDTA-extracts of both layers, SER323 antibodies  
412 recognize preferentially the one of the chalky layer (Fig. 6a), while SER324 antibodies give a  
413 stronger signal with the one of the foliated layer (Fig. 6b). For each of them, the most  
414 important differential response between the chalky and the foliated extracts is recorded with  
415 the GP bleeding, for which the reactivity ratio is about 2. The differences are attenuated with  
416 the final bleed (SAB).

417

#### 418 Immunogold staining of *C. gigas* shell microstructures

419 Results of the immunolocalization based on the antibody elicited against F21-22 fraction are  
420 indicated on Fig. 7. Sections (Fig. 7a, b) and fresh fracture surfaces (Fig. 7c-h) are illustrated  
421 and give congruent results. We observe a double phenomenon: firstly, a differential  
422 immunolocalization of the epitopes recognized by anti-F21-22 on the chalky and on the

423 foliated microstructures, respectively; secondly, different responses, due to the rats that  
424 generated the antibodies. While rat SER323 generated antibodies that recognize preferentially  
425 the chalky layer, rat SER324 produced antibodies that mainly target epitopes of the foliated  
426 layer and only few structures of the chalky layer.

427 The antibodies produced by SER323 and used on the fresh fracture surfaces (Fig 7c-d) mark  
428 the edges of the sheets composing the foliated structure. It is worth noticing that the surfaces  
429 of the sheets (parallel to the growth plane) are never stained. The SER323 antibodies stain the  
430 chalky layer more or less uniformly. SER324 antibodies produce intense staining of the  
431 foliated layer, together with a staining located specifically on a ‘chalky scaffold’  
432 perpendicular to the growth plane (Fig. 7e-h). SER324 antibodies stain none of the other  
433 structures present in the chalky layer.

434

435

## 436 **Discussion**

437

438 We have developed a non-conventional strategy for marking the shell of the edible  
439 cupped oyster *Crassostrea gigas*, one of the few bivalve models for which genomic data are  
440 available (Zhang et al. 2012). To this end, we have extracted the shell matrix, for  
441 characterization and selection of an immunogenic protein fraction, which is a potential  
442 appropriate marker of shell calcification. The resulting polyclonal antibody preparation has  
443 allowed us to perform differential immunogold staining of the shell microstructure.

444 The extracted matrix exhibits similar features to those extracted from other mollusc  
445 shells. Indeed, the proportions of ASM and AIM fractions to the dry weight of the shell enters  
446 the range observed for other bivalves (Marin et al. 2012). In particular, a 1/10 ratio between  
447 ASM and AIM quantified in this study is frequently found for the matrices of several macro-

448 prismatic bivalves (Marie et al. 2007). Our ASM:AIM values are also comparable to that of  
449 Marie et al. (2011) who extracted the equivalent of 0.4 % of AIM and 0.05 % of ASM from  
450 the same species. When fractionated using electrophoresis, the matrix – both ASM and LS-  
451 AIM – is constituted of a mixture of polydisperse ('smear') and few discrete macromolecules,  
452 *i.e.*, proteins, and the two fractions exhibit similar electrophoresis patterns. Although not  
453 tested in the present study, they may have overlapping protein compositions: our former  
454 proteomic investigations on different bivalve models demonstrated such similarities in protein  
455 compositions (Marie et al. 2009, 2010). This suggests that part of the AIM fraction may result  
456 from a polymerization/cross-linking of the ASM (Samata et al. 2008). Finally, we verified  
457 that the ASM interacted – in a dose-dependent manner – with the *in vitro* crystallization of  
458 calcium carbonate.

459           This overall characterization of the shell matrix served as a basis for further  
460 purification of an immunogenic protein fraction that was subsequently used for eliciting  
461 polyclonal antibodies. The purified fraction exerted an effect on the *in vitro* formation of  
462 calcium carbonate, but this effect was reduced in comparison to the one recorded with the  
463 whole ASM, suggesting synergistic effects of the different constituents of the ASM. When  
464 tested for proteomics, the fraction generated a short set of peptides that correspond to three  
465 proteins or protein families identified in the genome of *C. gigas*. The first protein family is  
466 that of gigasin-6 and its isoforms. Gigasin-6 is a 34 kDa (302 AA residue-long), leucine-rich  
467 protein with a basic *pI*, which exhibits a C beta-lactamase-like domain, a domain that  
468 catalyses the opening and hydrolysis of the beta-lactamine ring of this class of antibiotics,  
469 which include penicillins and cephalosporins. Interestingly, gigasin-6 was one of the eight  
470 proteins that were identified by us in a former study on the shell constituents of *C. gigas*  
471 (Marie et al. 2011). Its function in calcification remains unknown. The second family  
472 corresponds to nacrein-like proteins. Nacrein was initially identified and characterized in the

473 shell of the Japanese pearl oyster *Pinctada fucata* (Miyamoto et al. 1996). Then, several  
474 members, referred to as nacrein-like proteins, were identified in numerous molluscs and other  
475 metazoans. Nacreins and nacrein-like proteins exhibit similar primary structure: they possess  
476 a carbonic anhydrase (CA) domain, the function of which is to reversibly catalyse the  
477 conversion of carbon dioxide into bicarbonate (Le Roy et al. 2014). In addition, they exhibit a  
478 supernumerary domain, which is, in the present case, of the aspartic acid-rich type. Such a  
479 domain is likely to be involved in mineral interaction (Le Roy et al. 2014). Finally, our  
480 proteomic analysis identified a third member, a cell death abnormality protein 1-like  
481 belonging to a group of conserved proteins involved in cell apoptosis. This protein has a  
482 theoretical molecular weight of 28 kDa and is enriched in arginine and cysteine (about 12 %  
483 each) and in glycine and proline (about 9 %). The reason of the presence of such a protein in  
484 our shell fraction is obscure, and we cannot exclude the possibility that it was recruited for  
485 calcification to display a completely different function than that related to apoptosis. Note that  
486 this protein was not identified in our former proteomic study (Marie et al. 2011) on the whole  
487 matrix: we observed indeed that performing proteomics on electrophoresis fractions improve  
488 proteomics signals and allows the identification of rare proteins that are currently  
489 overshadowed by abundant ones in the mixture of the skeletal matrix macromolecules  
490 (Kanold et al. 2015). One intriguing aspect of our proteomic analysis is that the two nacrein-  
491 like proteins exhibit a molecular weight higher than that expected from the electrophoresis  
492 fraction (26-30 kDa), while that of the cell death abnormality protein 1-like and of gigasin-6  
493 fit approximately into this molecular weight range. This calls for two explanations that are not  
494 mutually exclusive: on one hand, we cannot rule out that nacrein-like proteins may have an  
495 anomalous migration due in particular to their acidic supernumerary domain and that they  
496 migrate 'faster' than expected; on the other hand, it is possible that these proteins may partly

497 degrade in the shell when occluded; consequently, what we detect by proteomics are simply  
498 degradation products that co-elute with gigasin-6/cell death abnormality protein 1-like.

499         The purified fraction was used to generate polyclonal antibodies in two rats.  
500 Interestingly, although the antibody batches (SER323 and SER324) cross-react with several  
501 discrete and non-discrete macromolecules of the ASM and LS-AIM fractions, as shown by  
502 Western blots, both gave different cross-reactivities, in term of specificity. Such a variation  
503 can be expected, as two animals immunized with the same antigens in identical conditions do  
504 not react similarly (Hanly et al. 1995). In addition, our antibody preparations cross-react with  
505 chalky and foliated extracts, suggesting partial overlaps in the protein compositions of these  
506 two microstructures. Interestingly, the two GP bleeding batches gave the highest difference  
507 between extracts of the chalky and of the foliated microstructures. We exploited these  
508 differential *in vitro* responses to perform *in situ* immuno-histological localization on *C. gigas*  
509 shell sections. The first antibody (SER323) marked predominantly, both on ELISA and on  
510 histological preparations, the chalky layer, while the second antibody (SER324), when tested  
511 with the similar techniques, marked the foliated layer, and some peculiar substructures of the  
512 chalky one. To our knowledge, this is the first time that this property can be subtly exploited  
513 for differential marking of shell microstructures. In former studies on nacro-prismatic  
514 bivalves (Marin et al., 2000; Marie et al., 2012), we identified protein markers that were  
515 present in one shell layer and absent in the adjacent layer.

516         How is the chalky layer synthesized and why is such a mechanically poor  
517 microstructure produced in the shell of *C. gigas*? As underlined in the introduction, the chalky  
518 layer typifies ostreid shell microstructures, although this peculiar microstructure is also  
519 present in other bivalve groups such as the spondylids (Vermeij 2014). Structurally speaking,  
520 the chalky layer consists of thin ‘bladelike structures oriented perpendicularly to the inner  
521 shell surface’ (Vermeij 2014). This structure is hollow, extremely light, and discontinuous,

522 *i.e.*, exhibits a lenticular shape. According to the extensive review of Korringa (1951) on  
523 chalky deposits in the shell of *Ostrea edulis*, ‘chalky layers are an economy building measure  
524 by the oyster (...) their function is to smooth out irregularities on the inside of the shell’.  
525 Further, Korringa defines chalky deposits as ‘cheap padding’. Interestingly, this author  
526 calculated that chalky deposits allow to fill the space with ‘one-fifth of the shell material that  
527 would be required if the folia layers were to be deposited’. Margolis and Carver (1974)  
528 consider that ‘deposition of calcite in the form of chalky deposits occurs as a specific  
529 physiological response to environmental stimuli, possibly during periods of maximum  
530 respiration’. It has been suggested that the chalky deposit is a rapid filling layer in periods of  
531 high growth rates (Palmer and Carriker 1979). Recently, Chinzei (2013) suggested that the  
532 function of chalky deposits is to lighten the shell as an adaptation to soft substrates.

533         From a physiological and cellular viewpoint, it is unclear by which mechanism chalky  
534 deposits are secreted: Orton and Amirthaligam (1927) assumed that they are formed in the  
535 places where the mantle loses contact with the shell. According to Palmer and Carriker  
536 (1979), all the mantle epithelial cells capable of depositing foliated layers have the ability to  
537 also deposit the chalky ones. In a very recent paper, Vermeij (2014) proposes a radically  
538 novel view for chalky layer (mocret) deposition: this process would occur remotely from the  
539 mantle tissues, which, in other words, strongly suggests that the deposition process is poorly  
540 controlled by the mantle epithelium and that this remote calcification is enhanced by  
541 carbonate-precipitating sulfate-reducing bacteria, which would ‘colonize and occupy spaces  
542 filled with a mixture of extrapallial fluid and seawater’, both, rich in sulphate. If so, this  
543 suggests that the deposition of chalky materials is mostly induced by an organic matrix of  
544 bacterial origin, which, in other words, means a completely different matrix as the one used  
545 for foliated shell deposition.

546           However, carbon isotope measurements from foliated and chalky deposits of oyster  
547 shells by Ullmann et al. (2010) tend to refute the influence of sulfate-reducing bacteria in the  
548 oyster shell mineralization. Indeed, isotopic signatures are identical in both microstructures (-  
549  $1.11 \pm 0.64$  ‰, n=83, and  $-1.02 \pm 0.32$  ‰, n=100, for foliated and chalky layers, respectively)  
550 while sulfate-reducing bacteria generally induce more negative values (around -10 ‰; Jia et  
551 al. 2015).

552           Our immunogold staining of the shell of *C. gigas* does not allow a firm and definitive  
553 conclusion on this matter. There is a clear differential immunogold marking of the two  
554 microstructures, as shown by the immunogold results obtained with antibodies from rat  
555 SER323. On the other hand, results obtained *in vitro* (ELISA) with chalky and foliated  
556 extracts and immunogold staining with antibodies from rat SER324 suggest that part of the  
557 epitopes of the chalky and of the foliated deposits are common to these two microstructures.  
558 In particular, the locally-restricted marking of the chalky layers by SER324 antibodies is  
559 limited to areas with a different aspect on those layers (Fig. 7e-h). These structures seem to  
560 expand vertically all the way through the chalky layer (Fig. 7e). They may represent some  
561 peculiar foliated structures that serve as “pillars” or “scaffolding” to help maintaining  
562 integrity of the porous chalky layer. It has also been observed (de Rafélis, unpublished data)  
563 that some micron-scale foliated layers were sometimes present in the chalky menisci of the  
564 hinge region of the shell.

565           One important point concerns the temporal and geometrical continuity between the  
566 foliated and chalky layers. Manganese labelling (Lartaud et al. 2010b) provided clear  
567 evidence that the thin (5 micrometres thick) manganese-rich layer that is marked in the  
568 foliated layer continues in the chalky one (see supplementary figure S2), although thickened  
569 and more diffuse (Langlet et al. 2006; Lartaud 2007). This provides evidence that the two  
570 microstructures are synthesized simultaneously, without temporal shift. We observed a similar



571 phenomenon, on a different mollusc model, the green ormer *Haliotis tuberculata* (Fleury et al.  
572 2008). In shell repair experiments, we observed the formation of very different  
573 microstructures in continuity to one another, suggesting, first, an extraordinary plasticity of  
574 the functioning of the calcifying epithelium, and secondly, the possibility that the secretion of  
575 similar (or partly overlapping) matrix repertoires can generate very different microstructures.  
576 For chalky *versus* foliated microstructures, the question remains open.

577

578

### 579 **Compliance with ethical standards**

580 We declare that raising polyclonal antibodies from rats was performed according to ethical  
581 standards. We declare no conflict of interest.

582

583

### 584 **Acknowledgments**

585

586 The work presented in this paper was made possible by the ENS PhD programme. The Earth  
587 and Natural Sciences Doctoral Studies Programme is funded under the Programme for  
588 Research in Third-Level Institutions Cycle-5 and co-funded under the European Regional  
589 Development Fund. The entire experimental work was performed in Dijon, via a financial  
590 support attributed to V. M. by the Irish Geological Association and the AllTech Innovation  
591 Competition. The work of F. M. was supported by INTERRVIE Program (INSU, CNRS) and  
592 OSU-Theta. The final publication is available at Springer via  
593 <http://link.springer.com/article/10.1007%2Fs00227-016-3040-6>.

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Table 1: summary of the LC-MS/MS analysis of the purified F21-22 fraction of the shell matrix of *C. gigas*. a: accession number of each protein hit according to NCBI database ; b: protein name according to NCBI database ; MW: theoretical molecular weight in Daltons, calculated from the identified protein (in parentheses, AA nb = number of amino acid residues); c: list of peptides identified by the analysis.

Accession number <sup>a</sup>	Protein identification <sup>b</sup>	Species	MW (AA nb)	Protein score	MS/MS peptides <sup>c</sup>	Peptide score
gi 317376184	Gigasin-6	<i>Crassostrea gigas</i>	34106 (302)	65	R.STIQEVYK.N K.NPGVIVSVVK.D K.NEIYTPLGMAK.S	35 8 21/(18)/(12)
gi 762132907	Gigasin-6 isoform X1	<i>Crassostrea gigas</i>	62490 (552)	65	Same peptides as for Gigasin-6	
gi 762132909	Gigasin-6 isoform X2	<i>Crassostrea gigas</i>	61546 (543)	65	Same peptides as for Gigasin-6	
gi 762104436	Nacrein-likeprotein	<i>Crassostrea gigas</i>	51244 (441)	63	K.TLSCLMEK.Y K.KPSDYFIK.N R.VEDTDNNPLK.E	12 9 42
gi 512134004	Nacrein-likeprotein	<i>Crassostrea gigas</i>	48258 (413)	58	K.TLSCLMEK.Y K.KPSDYFIK.E R.VEDTENNPLK.E	12 9 36/(18)/(9)
gi 762164175	Cell death abnormality protein 1-like	<i>Crassostrea gigas</i>	28195 (255)	56	R.SDFECPR.D R.AAGSISGGDPATGTEAADTGSGM.-	

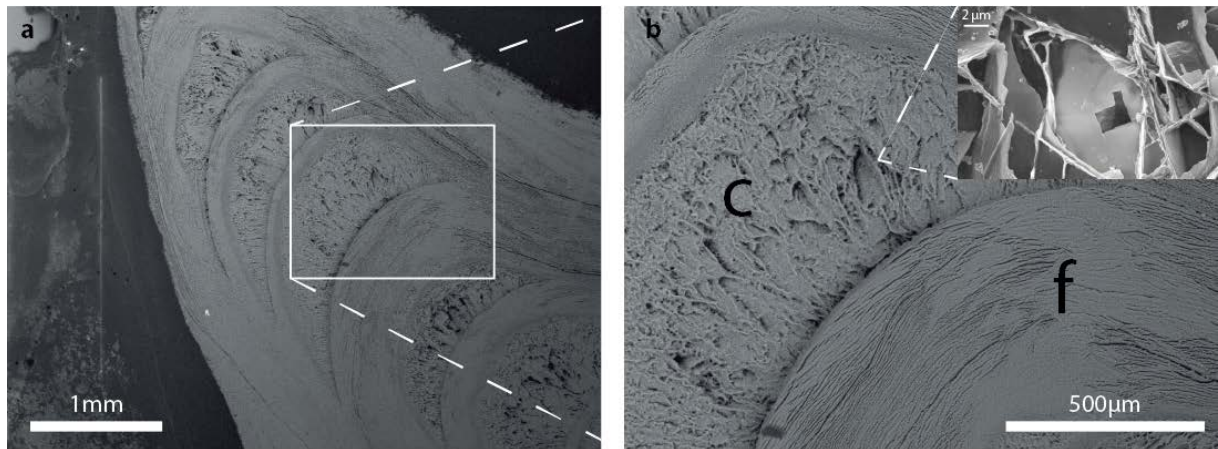


Figure 1: Microstructures in the umbo region of a *C. gigas* shell, longitudinal section, crossing the middle of the hinge region (perpendicular to the opening plan of the valves). **a**: Growth direction is from top left to bottom right. **b**: Detailed view (white square from a). “c” indicates the chalky structure while “f” corresponds to the foliated layers.

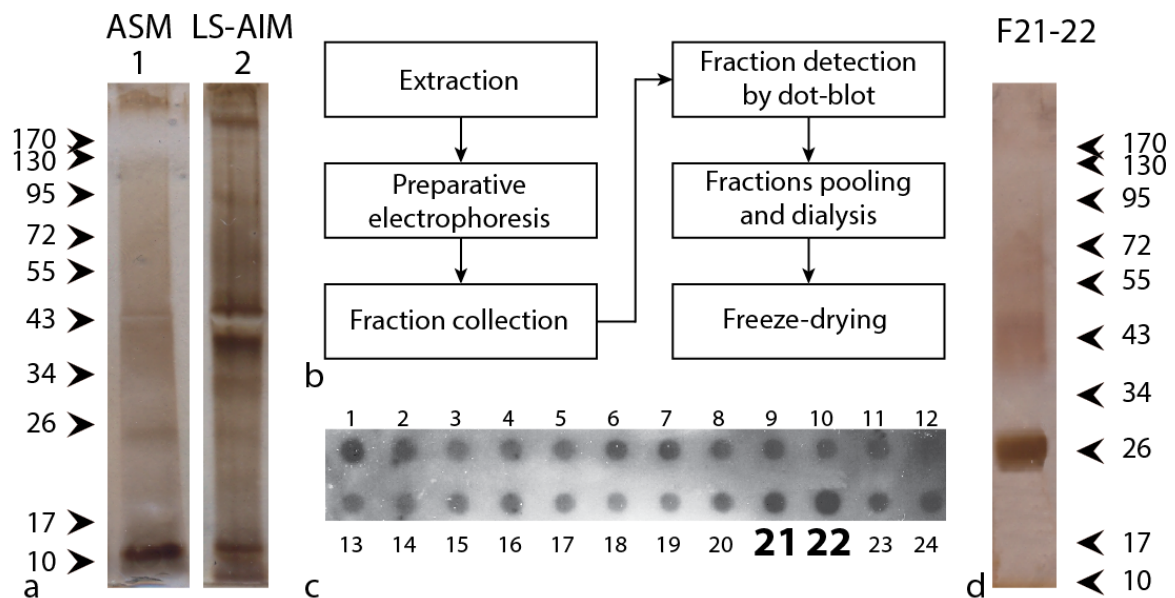


Figure 2: Characterization on monodimensional gels of the shell matrix of *C. gigas* and purification of one fraction. **a**: silver-stained gel electrophoresis of the acid-soluble (ASM, lane 1) and of the Laemmli-soluble acid-insoluble (LS-AIM, lane 2) matrices. Markers of different molecular weight (in kDa) are indicated on the left. **b**: Summary of the protocol used from the extraction to the freeze-drying of the purified fraction. **c**: dot-blot (performed after preparative electrophoresis) showing the cross-reactivities of the different fractions with anti-caspartin antibody (see text). Only the 24 first fractions are illustrated here. Note that the fractions of tubes 21 and 22 (F21-22) give the strongest signal. **d**: Gel electrophoresis of the F21-22 fraction, showing the apparent purity of this fraction, which was subsequently used for eliciting polyclonal antibodies. Markers of different molecular weight (in kDa) are indicated on the right.

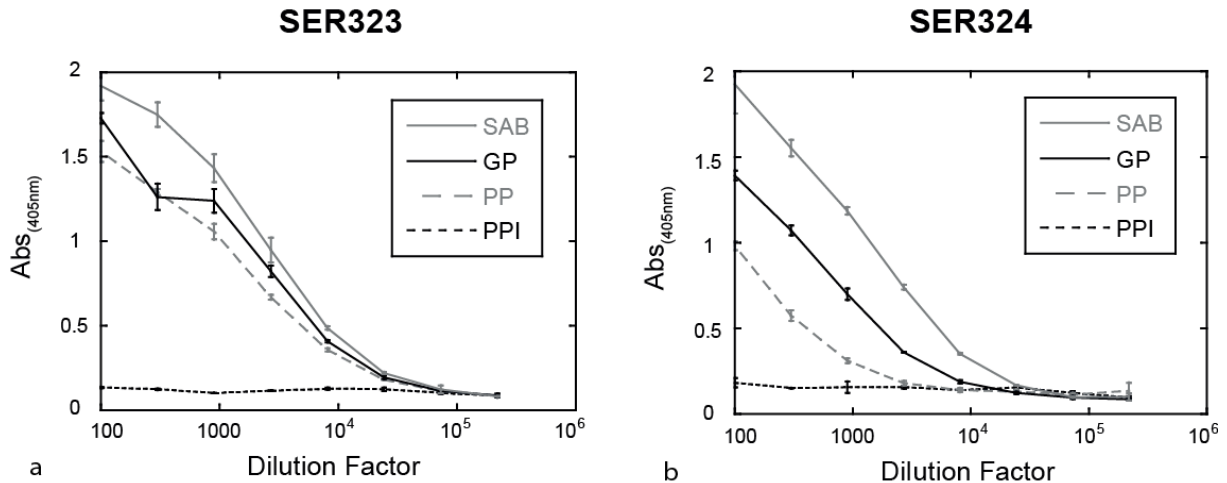


Figure 3: Titers of the ELISA tests performed on the antibodies produced by SER323 (a) and SER324 (b). PPI: pre-immune bleed. PP: small bleed. GP: large bleed. SAB: final bleed. Note that the two rats behave differently to the repetitive injections.

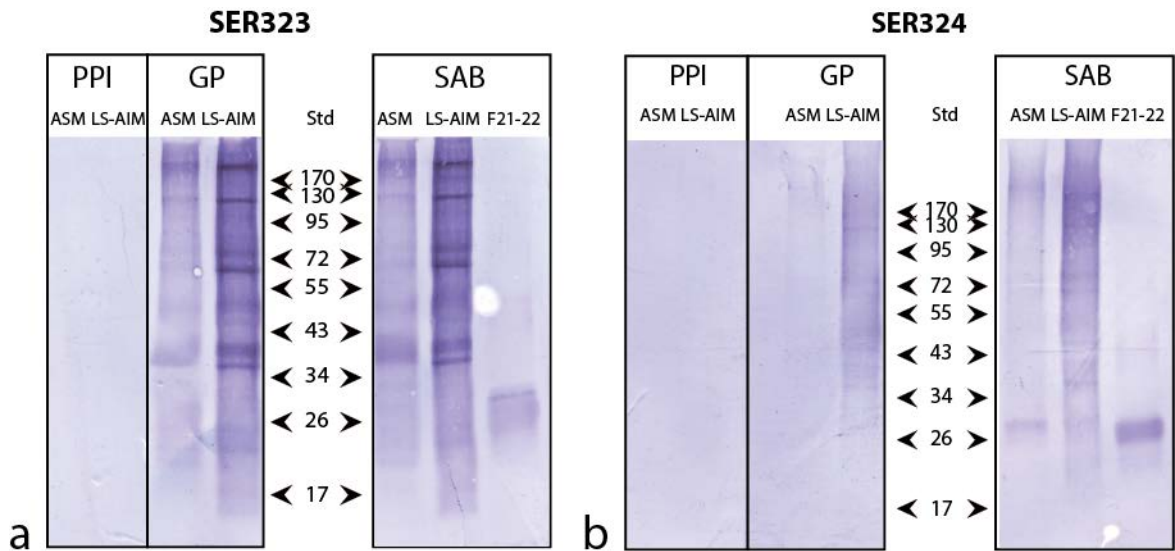


Figure 4: Western-blots of the ASM, LS-AIM and F21-22 fraction with polyclonal antibodies elicited against the F21-22 fraction. The PPI, GP and SAB antisera, produced in rat SER323 (a) and rat SER324 (b) were tested. Std: markers standard; the corresponding molecular weights (in kDa) are indicated on the right.

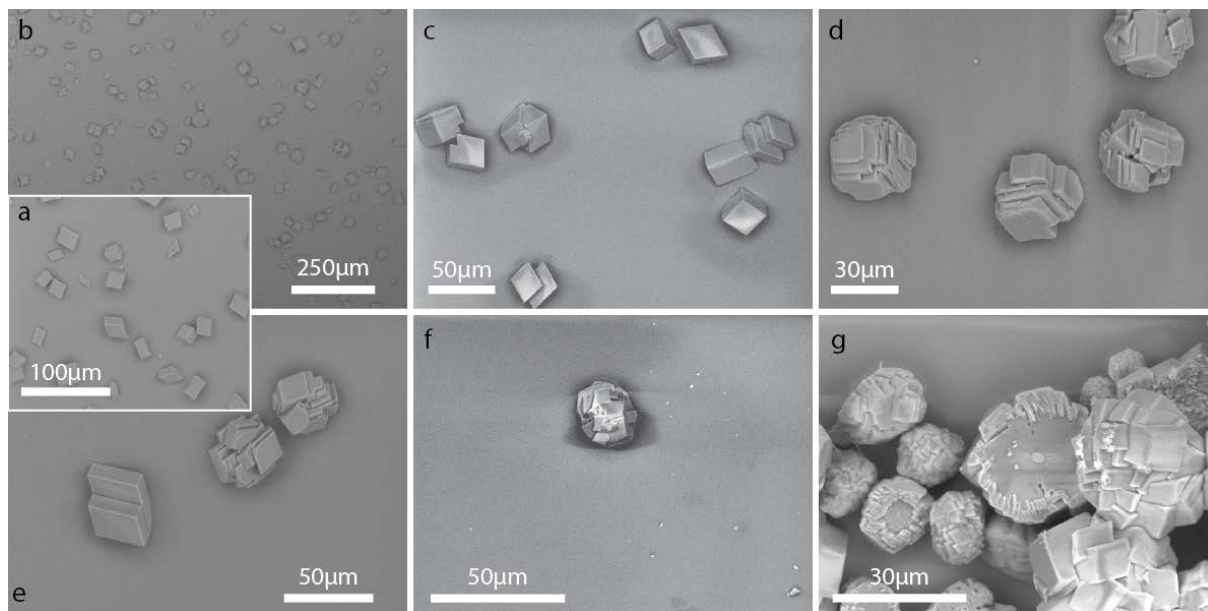


Figure 5: *In vitro* crystallization of calcium carbonate at different concentrations of F21-22 (**b-d**) and of ASM (**e-g**). **a**: 0 µg/mL. **b, e**: 0.3125 µg/mL. **c, f**: 5 µg/mL. **d, g**: 20 µg/mL.

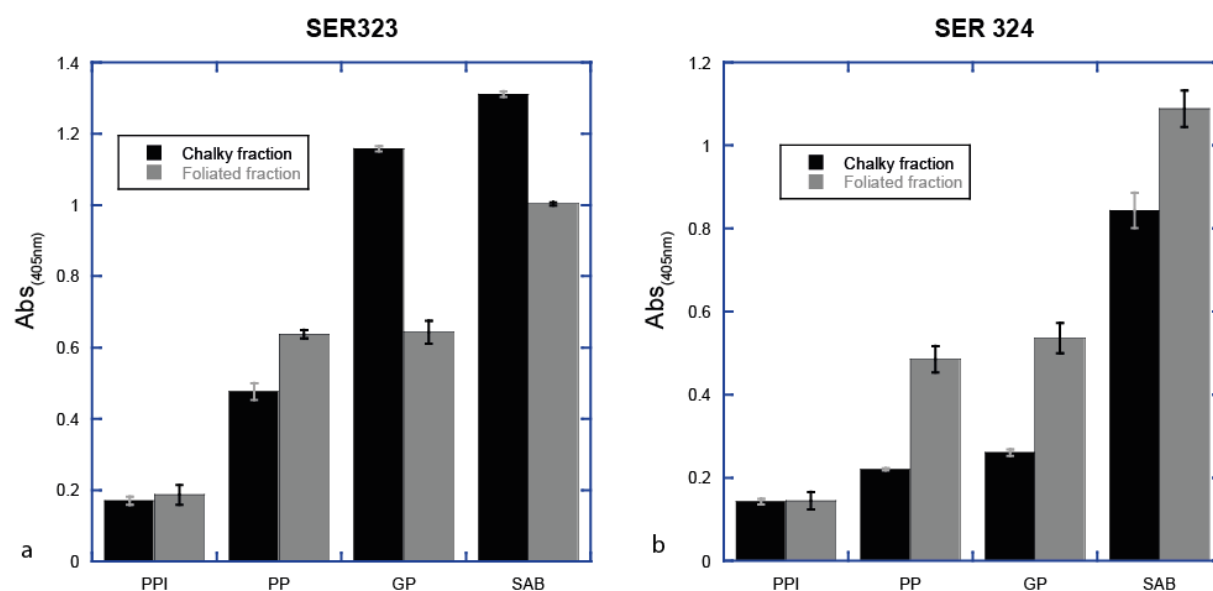


Figure 6: Differences in ELISA tests on chalky and foliated fractions with antibodies produced by SER323 (**a**) and SER324 (**b**) rats. Abs: absorbance, arbitrary units. PPI: pre-immune bleed. PP: small bleed. GP: large bleed. SAB: final bleed.



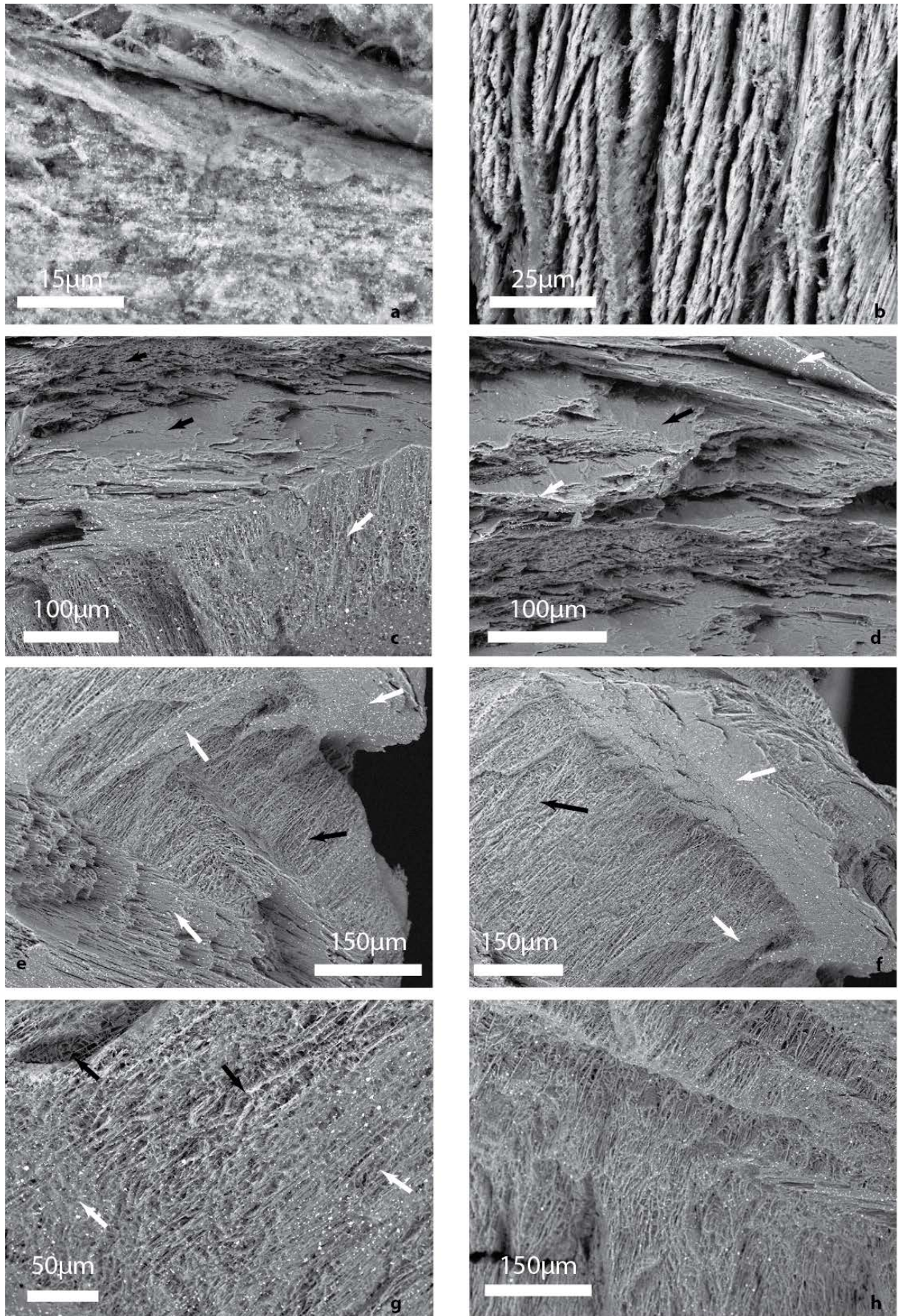


Figure 7: Immunogoldlocalization using the polyclonal antibodies raised against the F21-22 fraction on *C. gigas* shell. For evidencing the differential staining - according to the results of

Fig. 6 - we used GP antibodies produced by SER323 and SER324. **a-b**: Thin sections treated with SER323 antibodies. **c-d**: Fresh fractures treated with SER323 antibodies. **e-h**: Fresh fractures treated with SER324 antibodies. White arrows indicate positive immunolocalization while black arrows point to areas with no staining. For SER323 (**a-d**) only the chalky layers are stained. It is the opposite for SER324 (**e-h**) as the foliated areas are stained. Only specific areas of the chalky deposits, standing out from the rest of these structures, are stained by SER324.