Is the snail shell repair process really influenced by eggshell membrane as a template of foreign scaffold?

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ABSTRACT

Biominerals are inorganic-organic hybrid composites formed via self-assembled bottom up processes under mild conditions. Biominerals show interesting physical properties, controlled hierarchical structures and robust remodeling or repair mechanisms. Biological processes associated with biominerals remain to be developed into practical engineering processes. Therefore, the formation of biominerals is inspiring for the design of materials, especially those fabricated at ambient temperatures. The study described herein involves the influence of chicken outer eggshell membrane on the type of calcium carbonate ($CaCO_3$) polymorph deposited on the shell of the land snail *Helix aspersa* during the repair process after an injury. A piece of snail shell was removed by perforating a hole from the largest body whorl. The operated area was left either uncovered or covered with either a thermoplastic flexible polyolefin-based film Parafilm® or a piece of chicken eggshell membrane.

The repaired shells of control and experimental animals were analyzed using SEM, EDS, Raman and FTIR spectroscopies.

We found that in the presence of eggshell membrane, the polymorph deposited on the substratum during the first hours resembles calcite, the polymorph present in eggshell normal formation, but at 24 and 48 h, when snail mantle cells produced their normal organic matrix (mainly β-chitin plus proteins and proteoglycans), the polymorph deposited is aragonite, the characteristic polymorph of *Helix* shell.

Therefore, the eggshell membrane influences the type of polymorph, but only in the initial stages of biomineral deposition, before an organic matrix layer is deposited by the snail.

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1. Introduction

Biominerals are deposited in elaborate forms and hierarchical structures for functional use. They are formed by an intriguing interaction at the organic-inorganic interface, where cells (bioreactors) regulate the rate of crystal formation by controlling the micro-environment in which such mineralization events take place, thus regulating where, when, to what extent, and in what form mineralization occurs. Biominerals are inorganic-organic hybrid composites which are formed by self-assembled bottom up processes under mild conditions, and which possesses interesting properties, controlled hierarchical structures and mechanisms for remodeling or repair biominerals still remain to be developed into practical engineering processes (Heuer et al., 1992). Therefore, biominerals provide a unique and innovative guide for the design

of materials, especially those that must be fabricated at ambient temperatures. In biominerals, the small amount of organic component not only reinforces the mechanical properties of the resulting composite, but exerts crucial control on the mineralization process contributing to the determination of the size, crystal morphology, specific crystallographic orientation, polymorph or amorphous phase stabilization, and superb properties of the particles formed (Arias and Fernandez, 2003). Therefore, biological routes of structuring biominerals are becoming valuable approaches not only for understanding such fundamental process but also for synthesizing novel materials and for designing mechanisms to avoid undesired biomineralization processes such as pathological mineralization or incrustations of pipes by invertebrates.

In spite of the great variety of proteins and polysaccharides that have been shown to be involved in controlling biomineralization, the precise role of specific chemical groups and their interaction with other active groups in a spatio-temporal ordered cycle of events is far for being fully understood (Aizenberg et al., 1999; Arias and Fernandez, 2008; Falini and Fermani, 2013). In fact, a great

Fig. 1. Photographs illustrating the procedure for perforating a hole in the snail shell.

variety of pure chemicals have been tested *in vitro* on their ability to control crystal formation (Beato et al., 2015; Gebauer et al., 2009; Rao et al., 2015). However, in nature some of the different chemical groups do not act separately but more frequently they work together in the form of a solid substrate in constrained spaces on which mineralization occurs (Arias and Fernandez, 2003).

Therefore, if the specific types and distribution of functional chemical groups in a polymeric matrix and their confined spatio-temporal deposition influence *de novo* nucleation, growth and morphology of a biomineralized crystalline phase, then the study of the comparative effects induced on a specific experimental biomineralization process by different organic matrices, in which different chemical groups act together, represents an appropriate integrative approach for understanding the topology of biomineralization in constrained spaces. In this context three main approaches have been followed to understand processes of biomineralization: (i) characterization of biomineralized structures, (ii) characterization of the assembly process during biological development of such structures, and (iii) characterization of the assembly process occurring during wound healing and repair of biomineralized systems (Arias and Fernandez, 2008).

Since the beginning of the 20th century, and especially during the development and utilization of electron microscopic techniques, the process of shell repair in molluscs, mainly land pulmonate snails, has been extensively studied, emphasizing the cells involved in CaCO$_3$ transfer (Abolinš-Krogis, 1958, 1961, 1963a,b, 1968, 1972, 1973, 1976; Andrews, 1934; Beedham, 1965; Bevelander and Nakahara, 1969; Durning, 1957; Istin, 1970; Istin and Masoni, 1973; Kapur and Gupta, 1970; Manigault, 1939; Saleuddin and Chan, 1969; Saleuddin, 1970, 1971; Sioli, 1935; Timmermans, 1969; Travis et al., 1967; Wada, 1961; Watabe, 1965; Wagge, 1951, 1952; Wagge and Mittler, 1953; Wilbur, 1964, 1972). These studies have shown that histochemically differentiated regions of snail outer mantle epithelium and underlying calcium gland cells secrete the several layers comprising the gastropod shell, formed basically of β-chitin, proteins and proteoglycans, formerly called acid mucopolysaccharides (Pavat et al., 2012). This suggests that the nature of the organic substrate determines the polymorph of CaCO$_3$ formed. In fact, after short-term experimental induction of shell repair in the land snail *Otala lactea* employing several substrata, including the periostraca of four species of molluscs and the outer membrane of the eggshell of the hen, Meenakshi et al. (1974) showed that the pattern of mineral deposited by *O.lactea* on the eggshell membrane corresponded closely to that of the inner surface of the hen eggshell.

The chicken eggshell is a bioceramic fabricated by combining specific extracellular matrix molecules with crystalline calcite (Arias et al., 1993). Ultrastructurally, it is composed of shell membranes, mammillae, palisade, and cuticle. The shell membrane fibers, that do not mineralize, are composed of a core, containing type X collagen (Arias et al., 1991a,b), and are surrounded by a
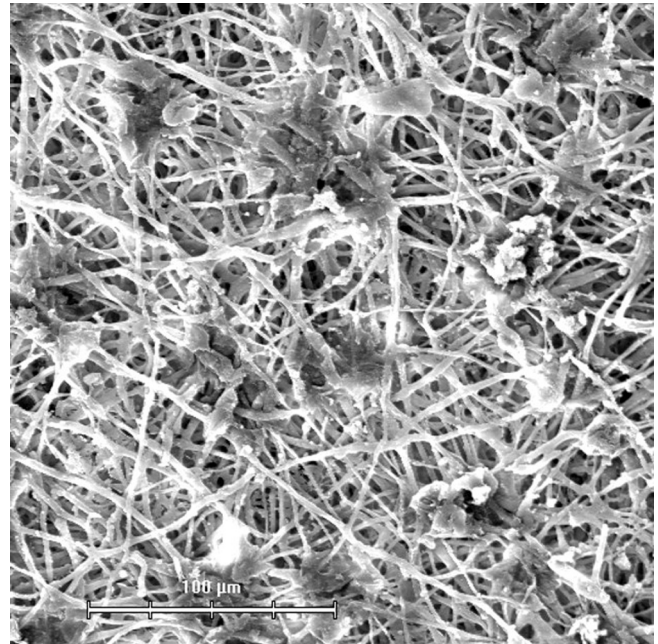

Fig. 2. Chicken fibrillar eggshell membranes showing the outer side, which was used as substrate for snail shell repair.

fuzzy material referred to as a mantle (Simons, 1971). In addition to the collagenous composition of eggshell membranes, genomic and proteomic approaches have demonstrated the concomitant content of, among others, a cysteine-rich ESM protein (CREMP), EDIL3 homologous protein, clusterin, ovocalyxin 32 and 36 and fibrillin (Kodali et al., 2011; Kaweewong et al., 2013; Du et al., 2015; Marie et al., 2015; Cordeiro and Hincke, 2016). Sparsely deposited on the outer side of the shell membranes are the mammillae (mammillary layer), consisting of mammillan, a keratan sulfate-rich proteoglycan, which are the sites for calcite nucleation (Arias et al., 1993).

In order to separate the effect of a foreign substrate from that of the inherent snail mechanisms of normal repair, we studied the shell repair process of *Helix aspersa*, using eggshell membrane, at several times during regeneration.

2. Materials and methods

A total of 54 specimens of *H. aspersa* were obtained from a local farm and were maintained in a black painted glass aquarium at room temperature. The snails were fed with lettuce, carrots and ground eggshell as a source of calcium and were kept moist throughout the experimental periods with wet humus. Cylinders
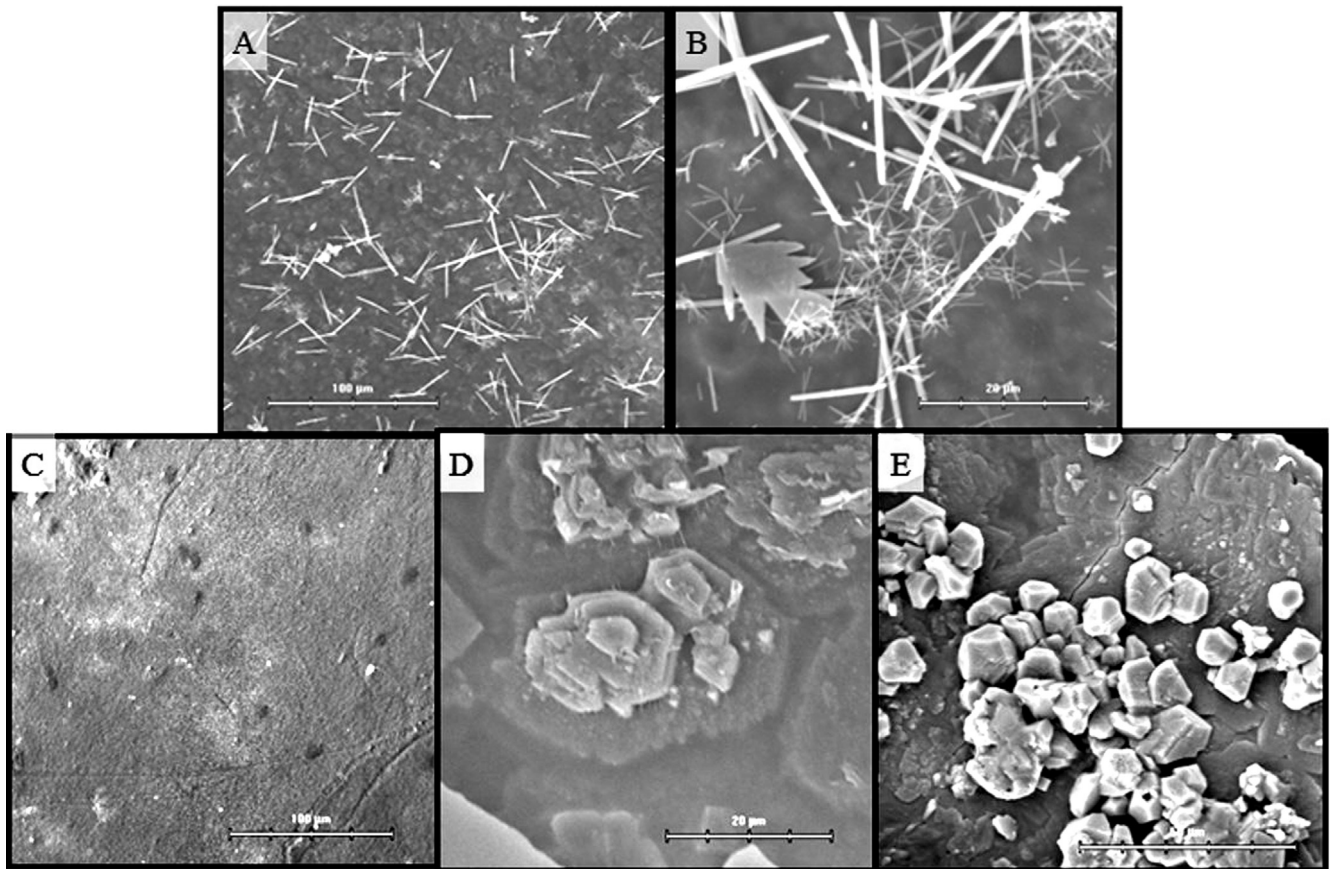
Fig. 3. Normal snail shell repair of an uncovered injury. (A) and (B): 24 h of repair. Aragonite spicules. (C) to (E): 48 h of repair; (C), chitin sheet; (D), piles of crystalline tablets; (E), rhombohedrical crystals near the border of the injury.

of polyvinyl chloride (PVC) pipes 10 cm in length and 10 cm in diameter were put in the aquarium as shelters for the snails.

Regeneration was initiated by removing a piece of shell by perforating a hole approximately 5 mm in diameter from the largest body whorl using a dental diamond bur (Fig. 1). Lesion was done faraway from de shell border where mantle cells produce periostracum to avoid repair contamination of periostracum material. Care was taken to avoid injury to the mantle which lies next to the shell. After removal of the piece of shell, the mantle always remained beneath the hole. The operated area was left uncovered (18 snails) or covered with either a thermoplastic flexible polyolefin-based film Parafilm®, (Bemis, Neenah, WI) (18 snails), or avian eggshell membrane (18 snails) as experimental materials which served as substrata for the crystals deposited during repair. Eggshell membranes of White Leghorn hen eggs were obtained as described elsewhere (Arias et al., 2008). Briefly, the eggshell was opened at the end of the shell opposite the air chamber, and the albumen and yolk were poured out. The emptied eggs were thoroughly washed with distilled water several times and then were filled with 1% acetic acid and allowed to stand for 10 min. The membranes were manually extracted, rinsed with distilled water, dried at 37 °C for 24 h and cut into sheets of 1 cm^2 (Fig. 2). The outer side of the shell membranes, that is, the side facing the crystal columns of the eggshell, was laid on the snail shell hole facing the mantle. The snails were identified with a number written on the shell with a permanent marker.

After 24 or 48 h of repair, snails were euthanized by isoflurane inhalation and the repaired area was harvested by cutting the shell with scissors around the repaired area. Samples of repaired shell

were mounted on aluminum stubs with Scotch double-sided tape and coated with gold. Crystals were observed in a scanning electron microscope TESLA BS 343A at 15 kV. SEM-EDS micrographs of crystals deposited during shell repair were captured by using a Hitachi TM3000 tabletop microscope (Hitachi High Tech) coupled with an energy-dispersive X-ray microanalysis (EDS) spectrometer Quantax 70 (Bruker, Germany) and equipped with a high-sensitivity semiconductor BSE detector. The morphologies and EDS studies of the crystals were carried out by using "analysis" as observation condition and the "charge-up reduction mode" as observation mode.

A DeltaNu Advantage Systems Raman spectrometer was used to obtain the Raman spectra of parallel shell repair samples. Analysis of the Raman spectra was done to determine the polymorphism of the crystals that formed compared to standard spectra published elsewhere (Gopi et al., 2013; Wehrmeister et al., 2010).

Fourier transform infrared spectroscopy (FTIR) analysis was performed on dried shell repair samples. FTIR spectra were obtained with an FTIR benchtop spectrometer Interspec 200-X (Estonia). FTIR of $CaCO_3$ from experimental samples were compared with standards reported by Vanegas et al. (2003).

3. Results

Samples of normal snail shell repair were obtained from the uncovered hole where the exposed mantle formed new shell material. After 24 h, a large amount of spicule-like crystalline material, 20 μm long and lying on a sheet of chitin, was observed and confirmed by FTIR. The shape of the crystals resemble aragonite
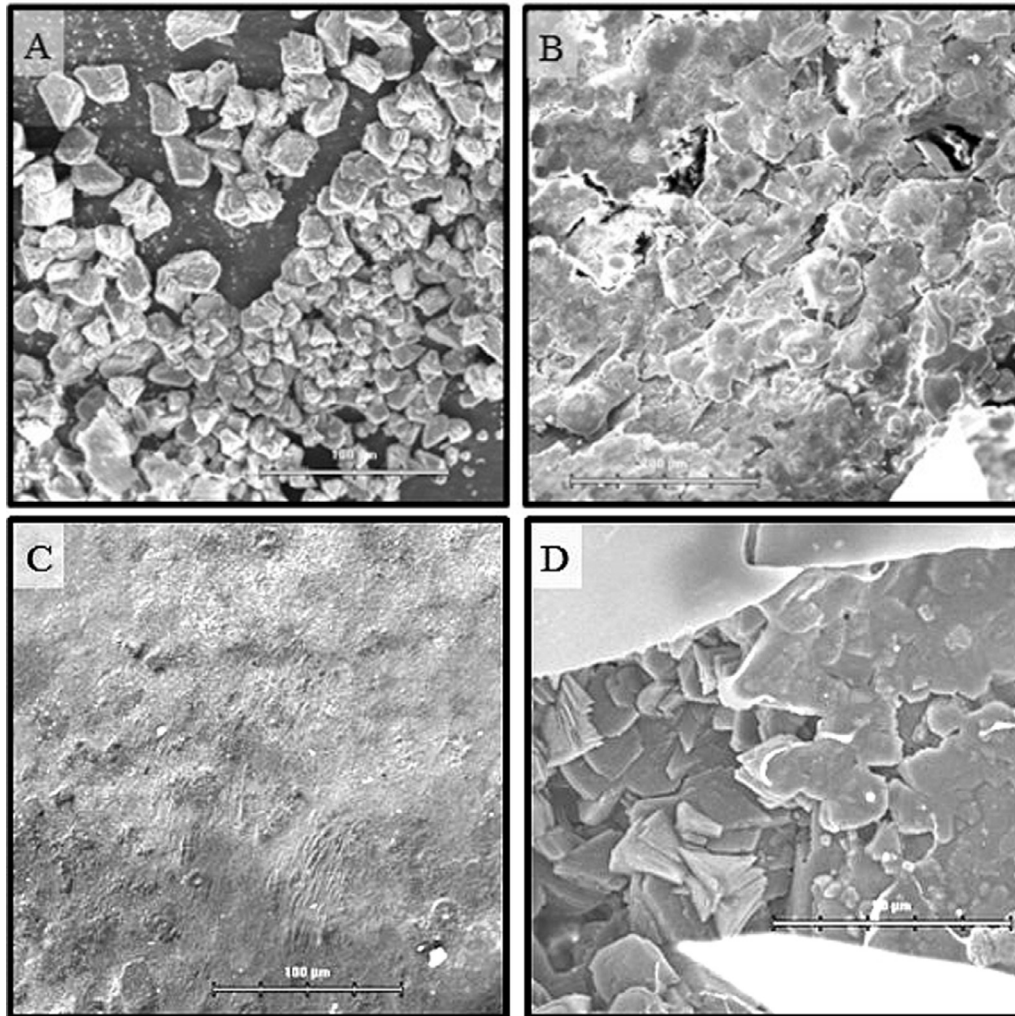
Fig. 4. Snail shell repair on Parafilm® substrate. (A-B) 24 h of repair. (A) Clusters of rombohedral crystals deposited on the Parafilm® layer. (B) In the center of the repair, a chitin sheet covering the clusters of rhombohedral crystals. (C-D) 48 h of repair. (C) β-chitin layer covering all the previous crystalline clusters. (D) Piles of crystalline aragonite tablets growing between the chitin sheets.

(Fig. 3A, B). At 48 h some sectors showed only chitinous layers (Fig. 3C), while nearby, piles of crystalline tablets, showing increasing diameter from top to bottom (Fig. 3D) were observed and were sometimes surrounded by rombohedral crystals toward the borders of the injury (Fig. 3E).

At 24 h after the start of shell repair, clusters of rombohedral crystals deposited on the Parafilm® layer can be observed (Fig. 4A). In the vicinity of these clusters, close to the center of the repair, a thin chitin sheet was observed covering the clusters of rombohedral crystals (Fig. 4B). At 48 h after injury, a thicker chitin sheet covered the crystalline layer (Fig. 4C), and piles of crystalline aragonite tables were observed growing between the chitin sheets (Fig. 4D).

Before 24 h of snail shell repair on eggshell membrane substrate, spherulitic crystal growth was observed (Fig. 5). It consists of a collection of crystal individuals extending radially in all direction from mammillary centers of the eggshell membranes. At 24 h after the start of shell repair on the eggshell membrane substrate, different results were observed depending on the site of the observations. However 24 h is to late for observing crystal deposits on eggshell membranes because they are covered by snail-produced chitinous layers. In some sites, microscopic examination showed a thin chitin layer deposited on columns of crystals (Fig. 6A). In other places, the same chitin layer was the substrate for deposition
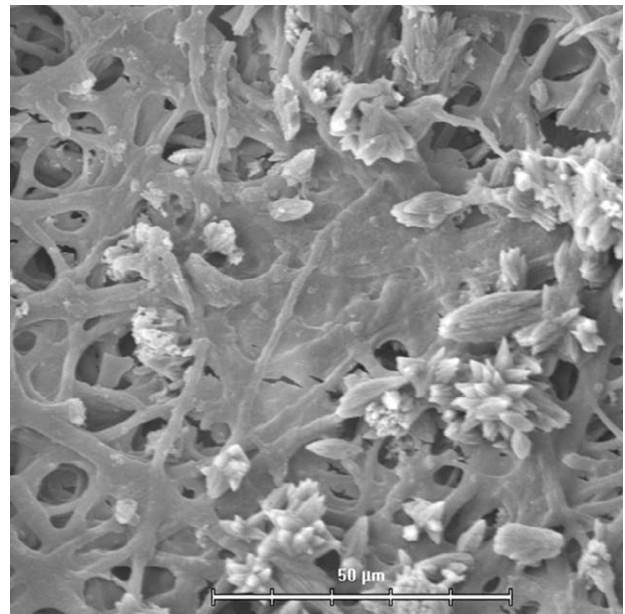

Fig. 5. SEM image of spherulitic crystal growth on mamillary knobs of eggshell membrane substrate before 24 h of snail shell repair.
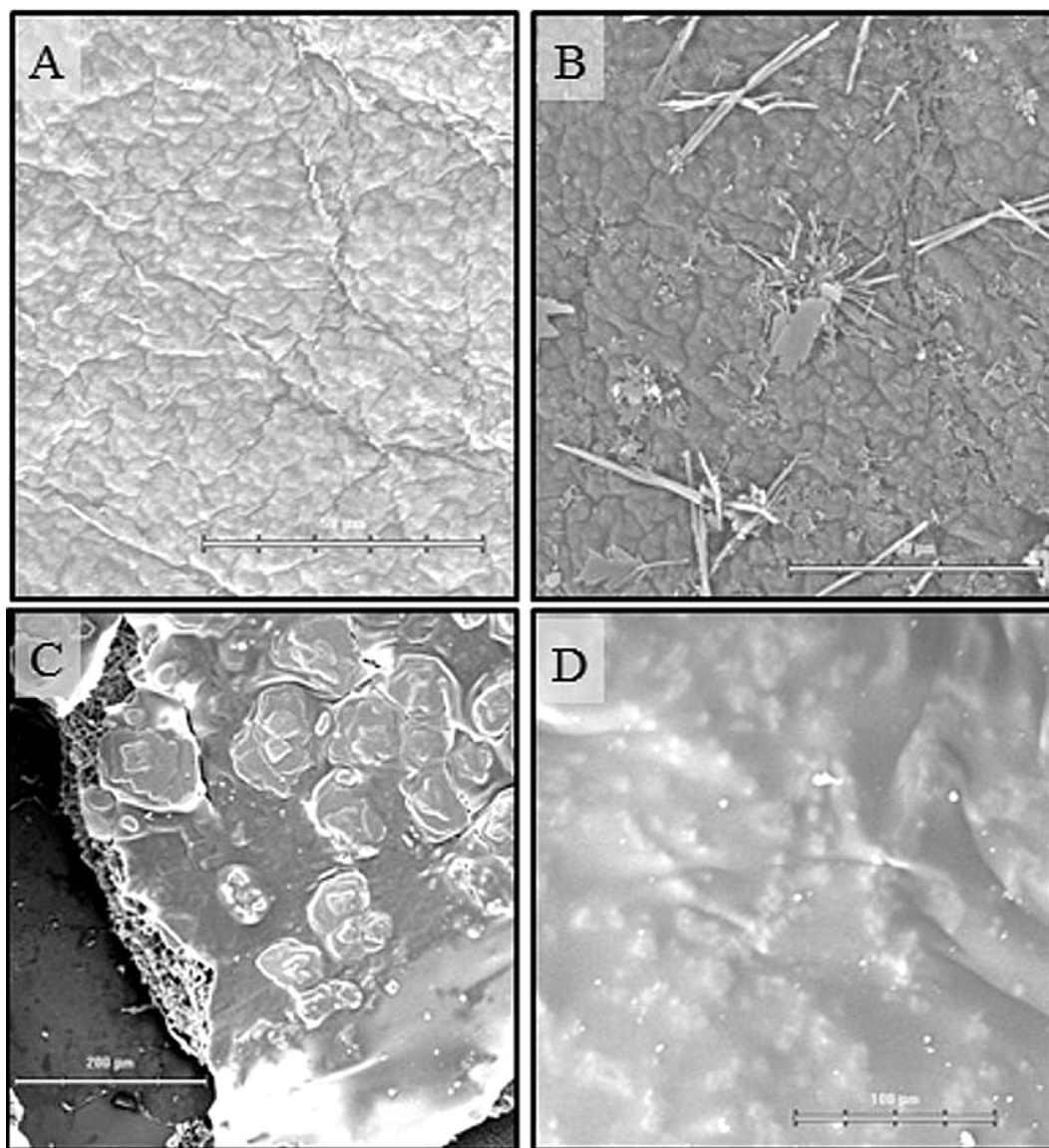
Fig. 6. Snail shell repair on eggshell membrane substrate. (A–C) 24 h of repair. (A) Thin chitin layer deposited on columns of crystals (B) Spicule-like crystals deposited on the chitin sheet. (C) Piles of crystalline aragonite tablets growing on the chitin sheet. (D) 48 h of repair. Chitin layer covering the piles of crystalline aragonite tablets.

of aragonite spicule crystals (Fig. 6B) or crystalline tablet piles (Fig. 6C). After 48 h of repair, a thick sheet of chitin covering the whole crystalline layer was observed in all the samples (Fig. 6D).

Samples at 48 h of snail repair on eggshell membranes, showing regions where an ordered delamination occurred were observed and analyzed (Fig. 7). The analysis of these regions showed an ordered stratification of the materials deposited as a function of time. First, it was possible to observe the outer side of the eggshell membranes that covered the snail shell injury (Fig. 7A). On a subsequent stratum, small column-shaped crystals were deposited perpendicularly on and between the eggshell membrane fibrils (Fig. 7B). In the next stratum, these columns increase in number and size, reaching approximately 10 μm in height. On the top of this crystalline stratum, a sheet of chitin is deposited (Fig. 7C), and on the surface of this sheet, piles of crystalline tablets grow (Fig. 7D). Then a new sheet of chitin is secreted on the top of the growing crystalline tablets (Fig. 7E).

Electron dispersive spectrometry of scanning electron microscope (SEM-EDS) samples corresponding to uncovered or covered snail shell repair showed typical elemental composition of $CaCO_3$ (Fig. 8).

Raman and FTIR spectroscopic analysis identified selective polymorphism of aragonite during snail shell repair at 24 and 48 h (Figs. 9–11). Raman spectra showed stretching (v_1) bands at 1085 cm^{-1} corresponding to crystalline polymorphs of $CaCO_3$ (Fig. 9). When both Parafilm and eggshell membrane were used for covering the shell injury, characteristic Raman (v_4) bands (in-plane bending) at 703 cm^{-1} corresponding to aragonite was observed. However, in the uncovered injury condition, the Raman (v_4) band displayed a shift from 703 to 684 and 697 cm^{-1} at 24 and 48 h respectively. Nevertheless, FTIR of the same uncovered shell injury showed absorption bands at 712 and 700 1085 cm^{-1} corresponding to aragonite crystals. In a complementary way, FTIR of both Parafilm® and eggshell membrane covering experimental specimens (Fig. 10), showed spectra combining absorption bands of the substrates used together by bands corresponding to chitin as compared with control samples (Fig. 11).
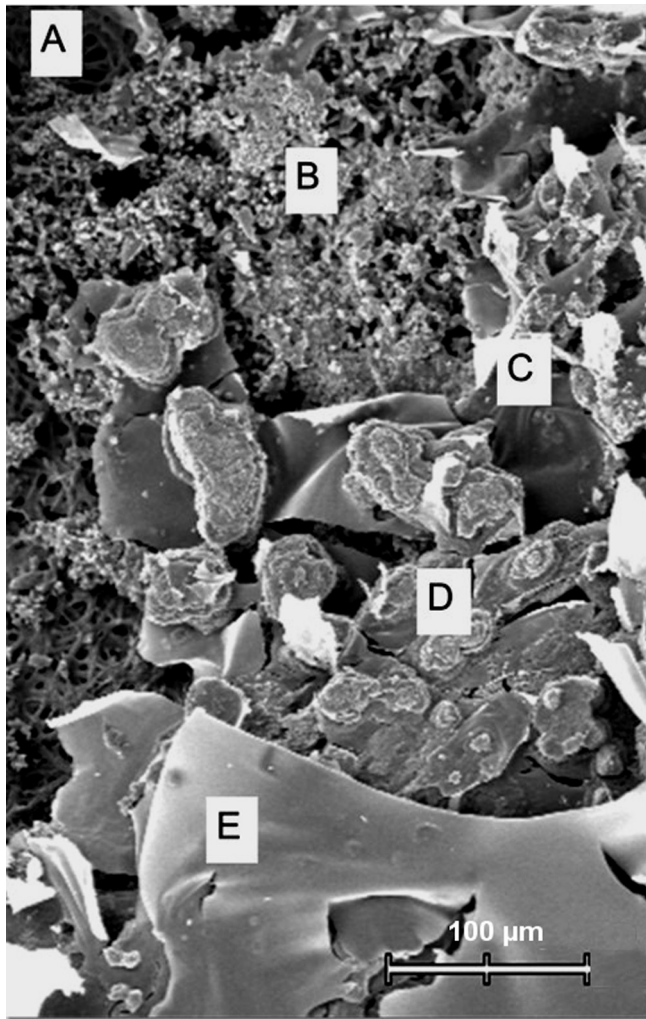
Fig. 7. Stratification of the different layers formed by the snail shell repair process using eggshell membranes as a foreign substrate. (A) Outer side of the eggshell membranes. (B) Small column-shaped crystals deposited perpendicularly on and between the eggshell membrane fibrils. (C) A sheet of chitin deposited on top of the crystalline stratum. (D) piles of crystalline tablets grow on the surface of this sheet. (E) A new sheet of chitin is secreted on top of the growing crystalline tablets.

4. Discussion

Meenakshi et al. (1974) have previously found that if an experimental substratum is inserted in the area of snail shell repair, the crystalline material deposited will be different from normal regeneration and will conform closely to the microtopography of the surface of the substratum inserted in the repair area, given the idea that this happened during the complete process of snail shell repair. These authors also showed that normal repair of a hole cut in the shell of the land snail *O. lactea*, that is without the addition of any foreign substratum, is initiated by deposition of a sheet of organic matrix secreted by the mantle on which small aragonite crystals were deposited after two to three hours, and the area of repair was completely covered by an aragonitic layer within 24 h or less. Similar features have being described for *Helix* sp shell regeneration and the chitin and acid mucopolysaccharide nature of the organic sheets has being established (Saleuddin, 1970, 1971; Saleuddin and Chan, 1969). Our results on the normal repair process after making a circular injury keeping the resulting hole uncovered, agree with

the observations of previous authors (Meenakshi et al., 1974; Saleuddin, 1970, 1971; Saleuddin and Chan, 1969). Complementary to Meenakshi et al. (1974) results, here we show that if the hole is covered with a substratum, i.e. Parafilm® or eggshell membranes, the crystalline material deposited in the beginning is morphologically different to the normal snail shell repair. These experiments demonstrate that if the substratum is crystallographically inert, as Parafilm® is, the very early secretion of calcium and carbonate ions crystallizes to the *most stable* polymorph, that is calcite. The same happens when eggshell membranes are used as substrate, but in this case the crystals formed resemble the same calcite crystalline polymorph and spherulitic growth that normally occurs during chicken eggshell formation (Simkiss and Wilbur, 1989; Simons, 1971; Arias et al., 1993; Nys et al., 1999), which is highly influenced by the proteoglycans associated with the eggshell (Rao et al., 2015). However, contrarily to Meenakshi et al. (1974) conclusions, this is true only in the first hours of repair, because once the first layer of organic matrix is deposited by the snail mantle, the mechanism of repair closely resembles normal gastropod shell growth, as has been described by Nakahara (1991). That is, the aragonitic crystal tablets grow within multilayered compartments and are arranged as a pyramid-shaped stack. These pyramid stacks are separated by thicker chitin sheets forming a columnar-type nacre. As expected, when normal snail shell macromolecules are subsequently secreted, the resulting microenvironment is able to stabilize the natural gastropod aragonitic structure.

The ordered stratification observed in samples at 48 h of repair and covered with eggshell membranes is consistent with a mechanism that is well regulated spatio-temporally and is further evidence that the process of biomineralization occurs in precise consecutive steps (Arias and Fernandez, 2003).

Infrared (FTIR) and Raman spectroscopy have proven to be valuable methods for distinguishing polymorphs of $CaCO_3$ (Andersen and Brecevic, 1991; Raz et al., 2000; Urmos et al., 1991; Wehrmeister et al., 2010). However, when $CaCO_3$ crystals are intermixed with organic materials, the vibration bands of the mineral are masked by the organic material, as has been shown by Hasse et al. (2000) when they studied mineralized structures of a freshwater snail. However, that masking effect does necessarily affect FTIR or Raman vibrational bands simultaneously or to the same extent. Therefore the complementary use of both spectroscopies is very valuable when there is a mixture of organic and inorganic materials.

The characterization of the assembly process occurring during wound healing and repair of biomineralized systems should be a promising approach to understand how mineralization is controlled in a micro- or nanosized constrained space or how it is possible to regulate the spatiotemporal deposition of specific solid interfaces or macromolecules in solution to produce self-assembled mineralized structures.

5. Conclusions

It is reasonable to conclude that on the deepest layer of eggshell membrane, the first crystals deposited were calcite crystals with a columnar shape, while piles of aragonite crystalline tablets only appeared after secretion of sheets of chitin (and other associated macromolecules) that originated from the snail mantle. Therefore, the influence of eggshell membranes as a template of foreign scaffold for mineralization only appears at the very beginning of the repair process, and then this process proceeds independently of such influence once macromolecular sheets from the snail mantle start to be secreted.
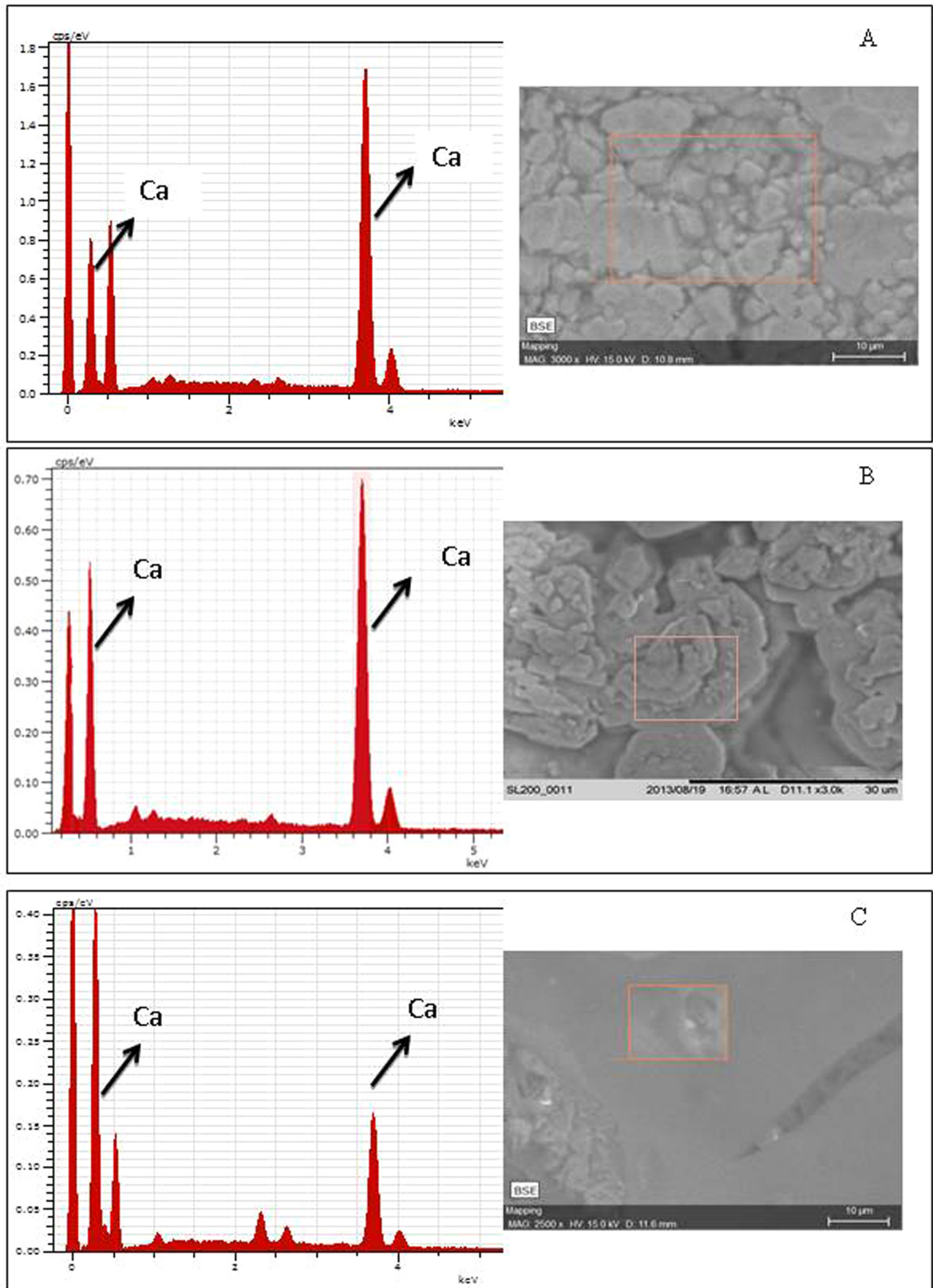
Fig. 8. SEM-EDS of uncovered shell injury (A), Parafilm® covered injury (B) and eggshell membranes covered injury (C) showing elemental composition of CaCO₃.
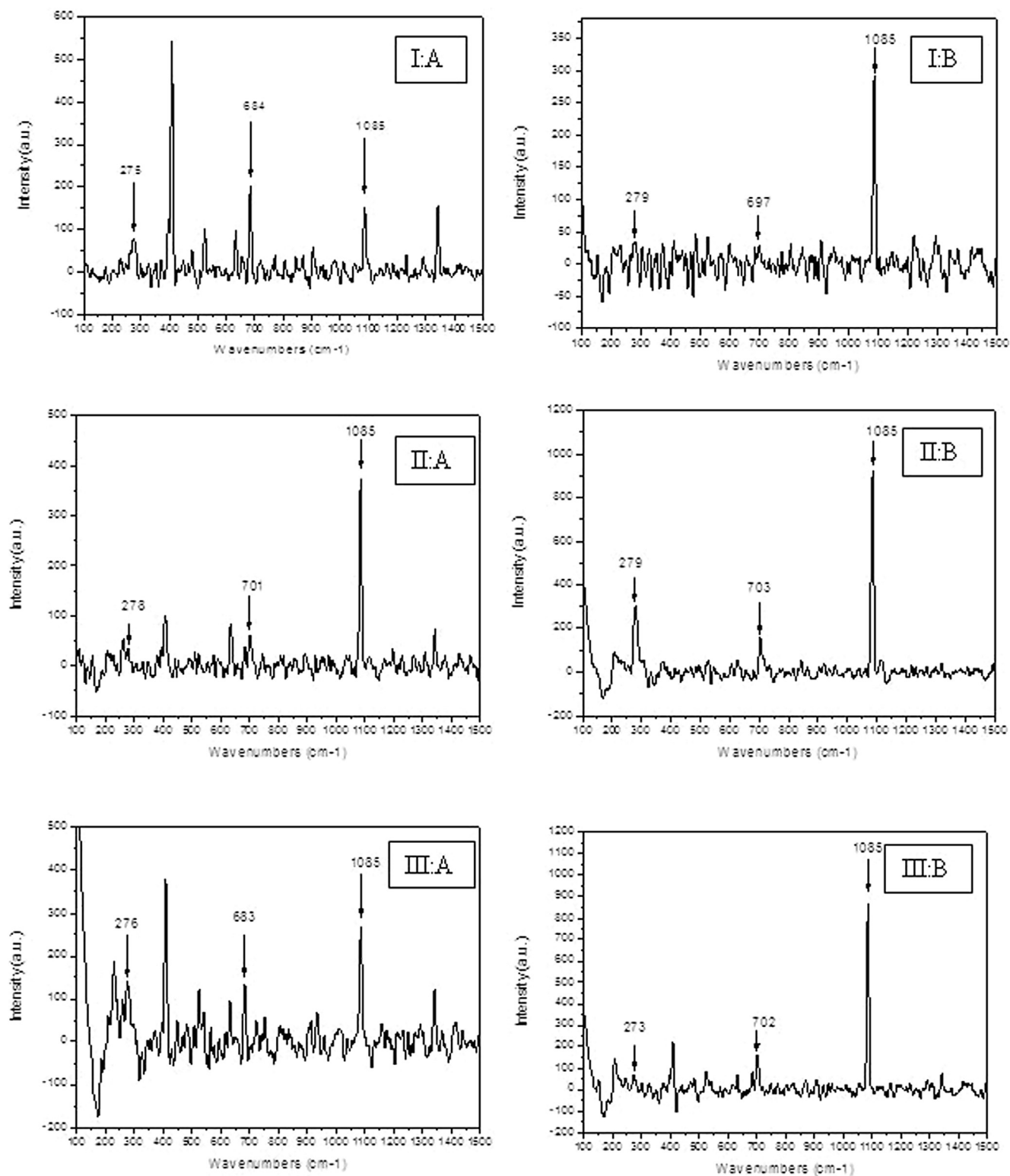
Fig. 9. Raman spectra of uncovered or Parafilm® or eggshell covered shell injury at 24 or 48 h of repair showing stretching bands at 1085 cm^{-1} corresponding to crystalline polymorphs of CaCO$_3$ and in-plane bending bands at 703 cm^{-1} corresponding to aragonite.
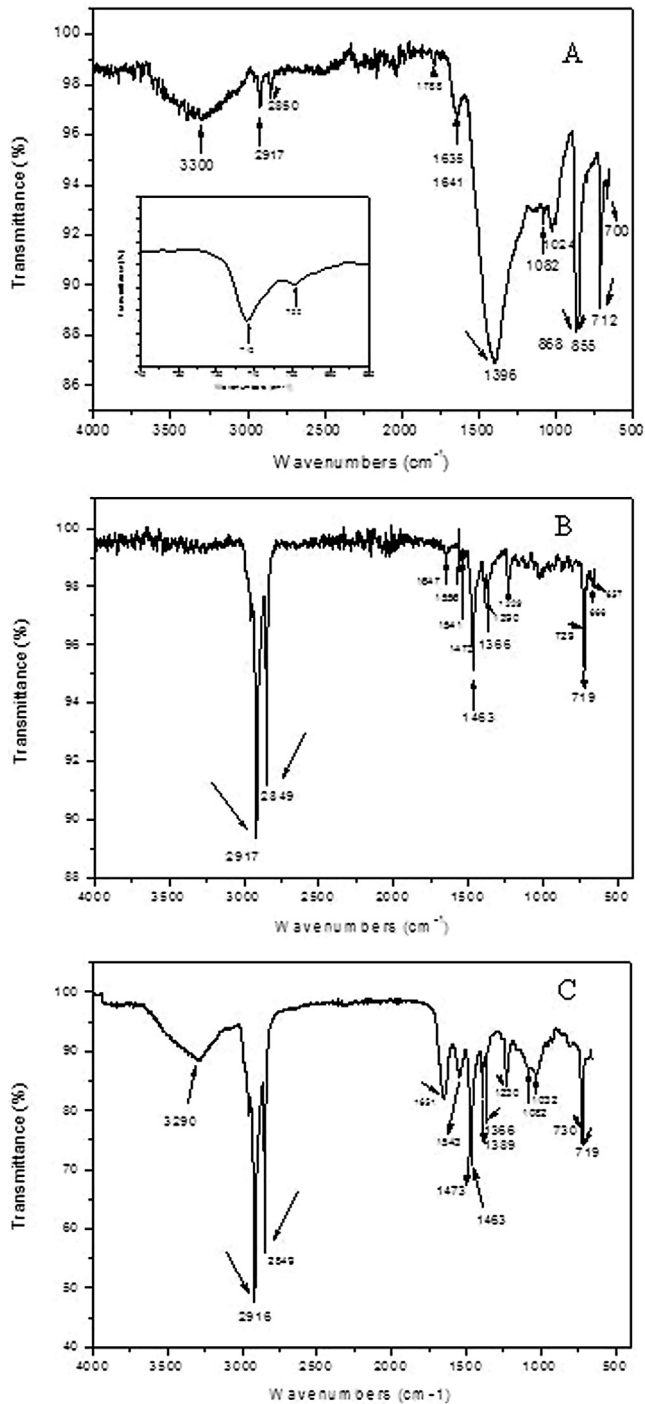
Fig. 10. FTIR spectra of snail shell repair at 24 h of uncovered shell injury showing chitin bands together with aragonite absorption bands at 712 and 700 cm^{-1}.

Fig. 11. FTIR spectra of control samples of Parafilm® and eggshell membranes substrate(A) and commercial chitin standard (B).

Acknowledgment

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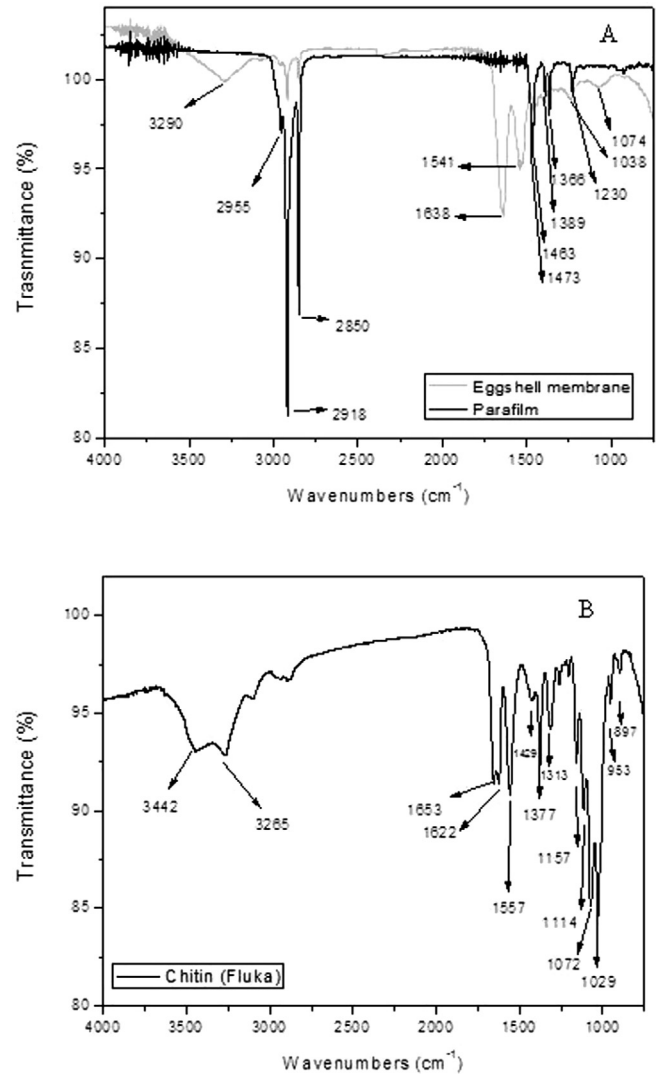
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