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# 3D Chitin Scaffolds of Marine Demosponge Origin for Biomimetic Mollusk Hemolymph-Associated Biomineralization *Ex-Vivo*

Marcin Wysokowski <sup>1,2,\*</sup>, Tomasz Machalowski <sup>1,2</sup>, Iaroslav Petrenko <sup>2</sup>, Christian Schimpf <sup>3</sup>, David Rafaja <sup>3</sup>, Roberta Galli <sup>4</sup>, Jerzy Ziętek <sup>5</sup>, Snežana Pantović <sup>6</sup>, Alona Voronkina <sup>7</sup>, Valentine Kovalchuk <sup>8</sup>, Viatcheslav N. Ivanenko <sup>9</sup>, Bert W. Hoeksema <sup>10,11</sup>, Cristina Diaz <sup>12</sup>, Yuliya Khrunyk <sup>13,14</sup>, Allison L. Stelling <sup>15</sup>, Marco Giovine <sup>16</sup>, Teofil Jesionowski <sup>1</sup> and Hermann Ehrlich <sup>2,17,\*</sup>

<sup>1</sup> Faculty of Chemical Technology, Institute of Chemical Technology and Engineering, Poznan University of Technology, Berdychowo 4, 60965 Poznan, Poland; tomasz.g.machalowski@doctorate.put.poznan.pl (T.M.); teofil.jesionowski@put.poznan.pl (T.J.)

<sup>2</sup> Institute of Electronics and Sensor Materials, TU Bergakademie Freiberg, Gustav-Zeuner str. 3, 09599 Freiberg, Germany; iaroslavpetrenko@gmail.com

<sup>3</sup> Institute of Materials Science, TU Bergakademie Freiberg, 09599 Freiberg, Germany; schimpf@iww.tu-freiberg.de (C.S.); rafaja@ww.tu-freiberg.de (D.R.)

<sup>4</sup> Clinical Sensing and Monitoring, Department of Anesthesiology and Intensive Care Medicine, Faculty of Medicine, TU Dresden, 01307 Dresden, Germany; roberta.galli@tu-dresden.de

<sup>5</sup> Faculty of Veterinary Medicine, Department of Epizootiology and Clinic of Infectious Diseases, University of Life Sciences, Głęboka 30, 20612 Lublin, Poland; achantina@op.pl

<sup>6</sup> Faculty of Medicine, University of Montenegro, Kruševac bb, 81000 Podgorica, Montenegro; snezap@ac.me

<sup>7</sup> Department of Pharmacy, National Pirogov Memorial Medical University, 21018 Vinnitsa, Ukraine; algol2808@gmail.com

<sup>8</sup> Department of Microbiology, National Pirogov Memorial Medical University, 21018 Vinnitsa, Ukraine; valentinkovalchuk2015@gmail.com

<sup>9</sup> Department of Invertebrate Zoology, Biological Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia; ivanenko.slava@gmail.com

<sup>10</sup> Taxonomy and Systematics Group, Naturalis Biodiversity Center, 2333CR Leiden, The Netherlands; bert.hoeksema@naturalis.nl

<sup>11</sup> Groningen Institute for Evolutionary Life Sciences, University of Groningen, 9747AG Groningen, The Netherlands

<sup>12</sup> Harbor Branch Oceanographic Institute, Florida Atlantic University, 5600 Old Dixie Hwy, Fort Pierce, FL 34946, USA; taxochica@gmail.com

<sup>13</sup> Department of Heat Treatment and Physics of Metal, Ural Federal University, Mira Str. 19, 620002 Ekaterinburg, Russia; juliakhrunyk@yahoo.co.uk

<sup>14</sup> The Institute of High Temperature Electrochemistry of the Ural Branch of the Russian Academy of Sciences, Akademicheskaya Str. 20, 620990 Ekaterinburg, Russia

<sup>15</sup> Department of Biochemistry, Duke University Medical School, Durham, NC 27708, USA; antistokes@gmail.com

<sup>16</sup> Department of Sciences of Earth, Environment and Life, University of Genoa, Corso Europa 26, 16132 Genova, Italy; mgiovine@unige.it

<sup>17</sup> Center for Advanced Technology, Adam Mickiewicz University, 61614 Poznan, Poland

\* Correspondence: hermann.ehrlich@esm.tu-freiberg.de (M.W.); marcin.wysokowski@put.poznan.pl (H.E.)

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**Abstract:** Structure-based tissue engineering requires large-scale 3D cell/tissue manufacture technologies, to produce biologically active scaffolds. Special attention is currently paid to naturally pre-designed scaffolds found in skeletons of marine sponges, which represent a renewable resource of biomaterials. Here, an innovative approach to the production of mineralized scaffolds of natural origin is proposed. For the first time, a method to obtain calcium carbonate deposition *ex vivo*,

using living mollusks hemolymph and a marine-sponge-derived template, is specifically described. For this purpose, the marine sponge *Aplysin aarcheri* and the terrestrial snail *Cornu aspersum* were selected as appropriate 3D chitinous scaffold and as hemolymph donor, respectively. The formation of calcium-based phase on the surface of chitinous matrix after its immersion into hemolymph was confirmed by Alizarin Red staining. A direct role of mollusks hemocytes is proposed in the creation of fine-tuned microenvironment necessary for calcification *ex vivo*. The X-ray diffraction pattern of the sample showed a high CaCO<sub>3</sub> amorphous content. Raman spectroscopy evidenced also a crystalline component, with spectra corresponding to biogenic calcite. This study resulted in the development of a new biomimetic product based on *ex vivo* synthesized ACC and calcite tightly bound to the surface of 3D sponge chitin structure.

**Keywords:** chitin; scaffold; sponges; hemocytes; hemolymph; biomineralization; calcite

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## 1. Introduction

Modern structure-based tissue engineering [1] urgently requires large-scale 3D cell/tissue-manufacture technologies, to create biologically active 3D scaffolds. Consequently, 3D scaffolds must possess biological, immunological, physicochemical, structural and mechanical cues at nanoscale, microscale and macroscale levels. Mostly artificially fabricated or prefabricated scaffolds must be bio- and eco-compatible [2] and should be suitable to support optimal cell growth, differentiation and proliferation [3], with the final goal of developing corresponding soft and hard tissues on request.

Recent focus in biomedicine has been concentrated on non-artificial, naturally prefabricated [4] skeletal structures of marine origin [5–11] that serve as examples of “marine biomimetics” [12]. This trend is well represented in the book by Choi and Ben-Nissan entitled *Marine-Derived Biomaterials for Tissue Engineering Applications* [13]. We completely agree with the statement of Professor Pierfrancesco Morganti that “the use of natural polymers in substitution to the petrol-derived ones seems the best way to produce skin-friendly healthy tissues and, slowing down the increased plastics production and waste, to try to save the Earth’s environment equilibrium and biodiversity” [2].

Special attention is currently paid to naturally pre-designed scaffolds found in skeletons of diverse marine sponges, which represent unique renewable resources for the sustainable development of novel 3D biomaterials [14–19]. The main players in this field are marine demosponges (phylum Porifera: class Demospongiae) of the orders Dictyoceratida (subclass Keratosa) and Verongiida (subclass Verongimorpha), which produce microporous proteinaceous (spongin)-based [16,20–23] and chitin-based [23–34] 3D skeletal constructs, respectively. Traditionally, it is mostly arthropods’ chitin that is recognized as the biological material suitable for application in biomedicine (for review, see [35–39]). Due to its biocompatibility, chitin is applied in dermocosmetics [2,40–43] and as wound-dressing biomaterial [44–46]. Therefore, reports concerning the successful clinical application of chitin in the form of dressings and membranes [47–51] are of crucial interest. Recently, Fang et al. [52] reported a method for producing 3D wound-dressing sponges made of quaternary chitin/partially deacetylated chitin nanofibers. Obtained material exhibited instant water/blood-triggered expansion and superabsorbent capacity. Moreover, authors proved such chitinous 3D sponges attract and stimulate blood cells/platelets, promoting blood coagulation and display superior hemostatic performance to traditional hemostatic materials [52].

The discovery of chitin in the skeleton of Cambrian demosponge *Vauxia gracilenta* (Walcott, 1920) [53], closely related to verongiids, confirmed a million-years-long success of sponges belonging to the order Verongiida, with respect to naturally optimized architecture of their chitinous, robust, fiber-based, microporous skeletons. The content of chitin in verongiids is species-dependent and ranges between 5% and 70% [4,24,27,29,30]. One of the most important advantages of sponge chitin, in contrast to traditionally used chitin sources, such as fungi [54], coralline algae [55], mollusks [56], corals [57],

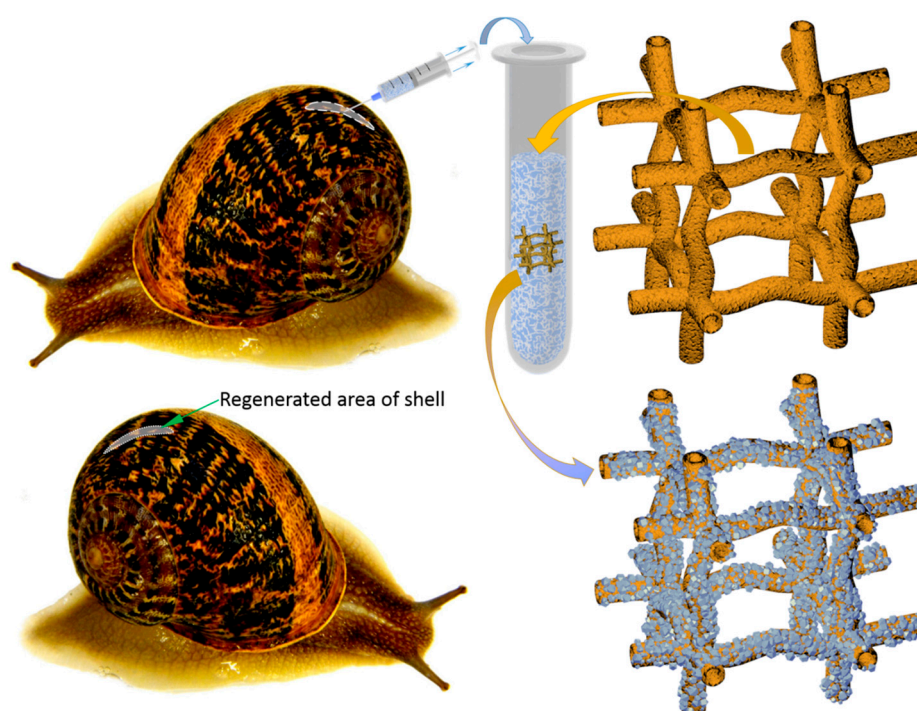
insects, spiders and crustaceans [58–61], is the size of sponges: they can reach up to 2 m in diameter (i.e., the elephant ear sponge *Ianthella basta* (Pallas, 1766)) [27,62], or up to 1.5 m length, as with the Caribbean stove-pipe sponge *Aplysina archeri* (Higgin, 1875) used in this study (Figure 1). Furthermore, verongiid sponges can be successfully cultivated under marine farming conditions [63–67] and possess the unique ability to regenerate their skeletal tissues up to 12 cm per year [62].



**Figure 1.** The giant Caribbean stove-pipe sponge *Aplysina archeri* (Demospongiae, Verongiida: Aplysinidae) produces up to 1.5-m-long skeletal tubes (of inner diameter  $\leq 10$  cm) made of mineralized chitin [4]. This image was made by V.N. Ivanenko, on June 12, 2017, in the coastal waters of Curaçao (Playa Marie Pampoen; 12°05′24″N, 68°54′19″W; depth 21 m).

Following the discovery of chitin content in sponges in 2007 [68,69], the first reports on the applications of 3D sponge-derived chitin scaffolds in tissue engineering appeared in 2010 [25,70]. Our scaffolding strategy [53,71] is based on the application of naturally prefabricated 3D chitinous scaffolds of poriferan origin as one of the forms of decellularized matrices [72]. Previously, we reported that the decellularized chitinous 3D matrices of the verongiid sponges *Aplysina aerophoba* [73–75] and *Ianthella basta* [76,77] can be applied in tissue engineering of selected human bone-marrow-derived mesenchymal stromal cells (hBMSCs) and human dermal cells MSCs (for overview, see [77]). Human chondrocytes [25,70], as well as cardiomyocytes [34], also showed good growth and proliferation features in experiments using 3D chitinous scaffolds isolated from the cultivated demospunge *A. aerophoba*.

It is well recognized that special multifunctional cells circulating in molluscan hemolymph, known as hemocytes, are crucial players in mollusk shell formation and repair processes [78–81]. These cells are responsible not only for calcium and bicarbonate ions transport but, due to a suitable microenvironment, also for biomineral crystal formation directly in the cell [82]. Consequently, such observations allowed us to create a methodological trend based on the development of calcium carbonate crystals deposition *ex vivo* by living mollusks hemolymph, using external templates [80,83–85]. For this purpose, both 3D chitinous scaffolds from *A. archeri* sponge and terrestrial snail *Cornu aspersum* (O.F. Müller, 1774) (previously known as *Helix aspersa*, (Mollusca: Gastropoda: Stylommatophora: Helicidae) (Figure 2) were used as an appropriate matrix and as the hemolymph donor, respectively. This mollusk is used as escargot meat and slime for cosmetic applications; thus, mollusks possess a high industrial potential as the most common breeding species on specialized farms—helicultures [86].



**Figure 2.** Principal schematic view of non-lethal manual extraction of *C. aspersum* hemolymph and its application for ex vivo biomineralization, using 3D chitinous scaffolds isolated from sponges with respect to obtain both amorphous and microcrystalline calcium carbonate phases (see also Figure 14).

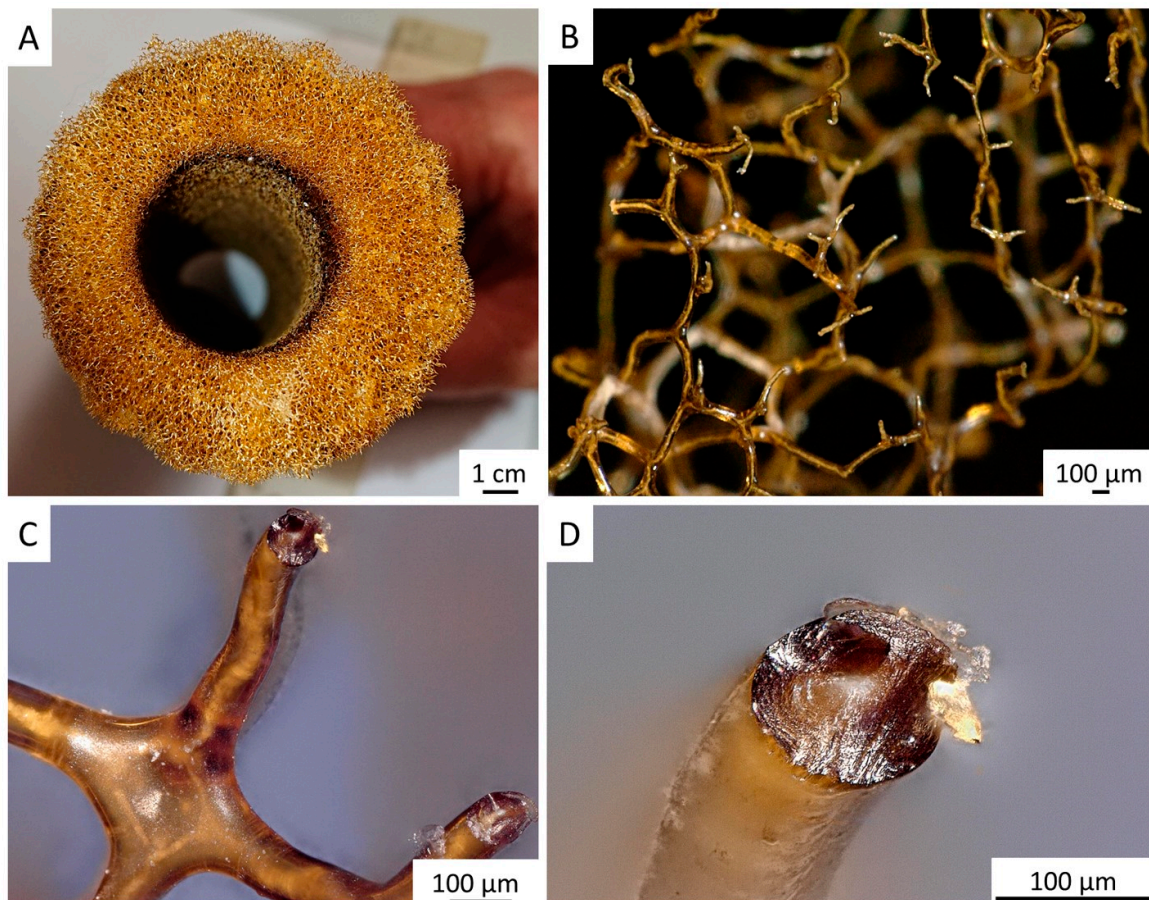
The aim of this paper is to use, for the first time, 3D chitin scaffolds isolated from the giant verongioid demosponge *A. archeri* (Figure 1) for molluscs hemolymph-associated biomineralization ex vivo. This experimental study was additionally motivated by the fact that both amorphous and crystalline phases (i.e., calcite) of calcium carbonates are known to be bio-compatible [87–89], biodegradable [90–92] and osteoinductive substrates for tissue engineering of bones and other hard tissues [93–105].

## 2. Results

The tube-like body (Figure 1) of the demosponge *A. archeri* represents a unique, naturally pre-designed, hierarchically structured fibrous construct (Figures 3 and 4). Such tubular constructs can be up to 150 cm long, with an inner diameter of up to 10 cm (for morphological details, see [106–109]). The color of the live sponge ranges from lavender through gray to brown; however, air-dried sponges turn reddish-brown (Figure 3) (see also [4]). Recently, we unambiguously showed the chitinous origin of *A. archeri* skeletal tubes [4]. The chitin content of *A. archeri* skeleton reaches ~5% by weight [4]. Consequently, now, we take the liberty of suggesting that such chitinous constructs represent the longest tubular chitin known. This unique source of pre-designed microporous chitin has a distinct advantage for practical applications: researchers, according to their aims, can use selected fragments (Figure 4) of specific size from the hard skeletal tube. For example, the long fragment of the tube can be fixed (Figure 3D) so that only the lower part is the subject for treatment (Figure 3A–C) with respect to the isolation of pure chitin scaffold (Figure 3F and Figure 6).



**Figure 3.** Step-by-step isolation of selected fragment of 3D tubular chitin scaffold from dried *A. archeri* segment. In step I, water-soluble salts were removed by pretreatment of the sponge skeleton with distilled water in the beaker placed on a magnetic stirrer (A) for 6 h. Afterward, in step II, the sponge was treated with 2.5 M of NaOH solution during 24 h, for deproteinization and depigmentation (B). In step III, the 3D scaffolds were treated with 20% acetic acid for 24 h, to remove residual calcium and magnesium carbonates, followed by washing with distilled water up to pH 6.8 (C). Steps II and III were repeated twice to obtain tubular scaffolds (D,E), which were cut off from the sponge and used for further studies (F).

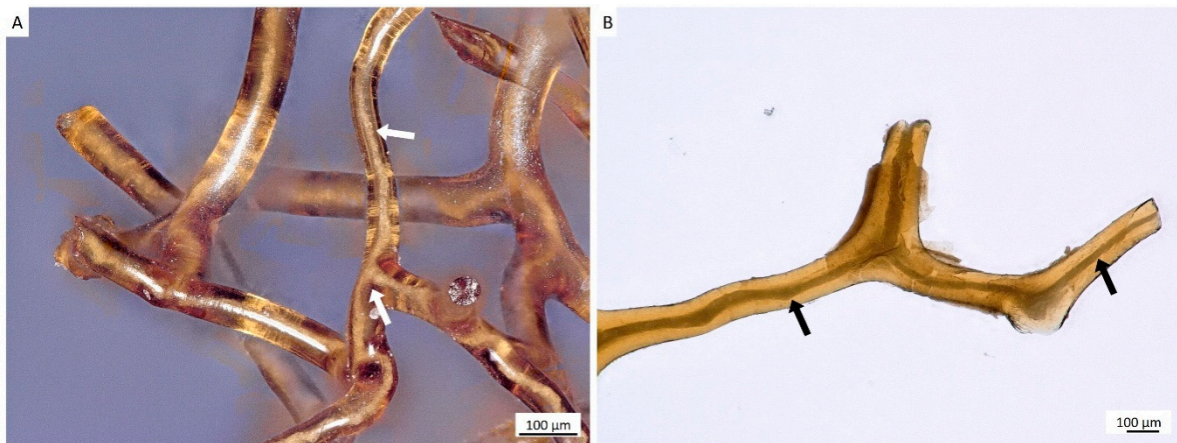


**Figure 4.** Decellularized chitinous skeleton of selected fragment of *A. archeri* (A) possesses anatomizing 3D microporous architecture (B) due to the presence of naturally mineralized, rigid fibers (C,D). The microstructure of skeletal fibers was characterized by us previously [4].

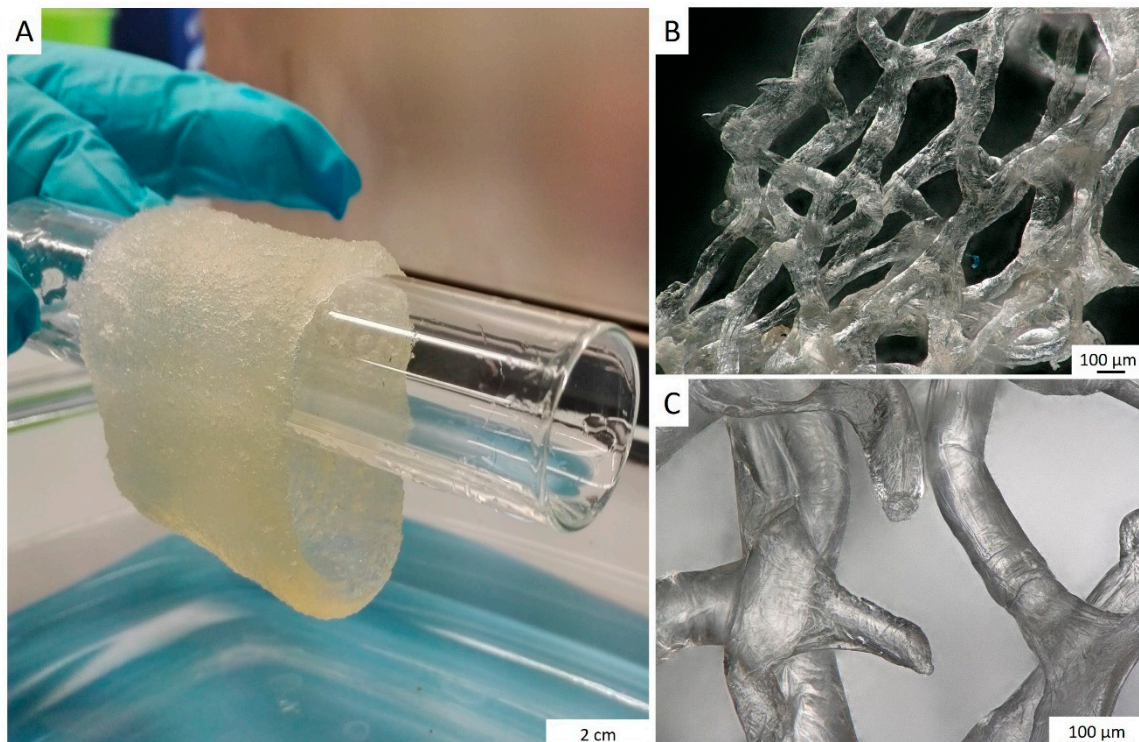
For the isolation of ready-to-use chitinous scaffold (Figure 6), we used the standard alternating alkaline-acid-based treatment [24], which took in this case up to four days at room temperature. During this procedure, the presence of interconnected inner channels located within skeletal microtubes could be well-visualized (Figure 5). Following this simple treatment, the tube-like skeleton of *A. archeri* was completely demineralized, cell- and pigment-free, mechanically flexible (Figure 6A) and transparent.

Recently, we reported [4] that 3D microtubular chitin of *A. archeri* sponge can function as an effective capillary system with respect to model liquids such as methylene blue dye, crude oil and pig blood. Consequently, there are all the necessary preconditions to suggest that 3D chitinous scaffold isolated in this study can also function as liquid delivery construct [28] for the cell-growth medium, which could be, for example, optimized for the cultivation of hemocytes of mollusks origin.

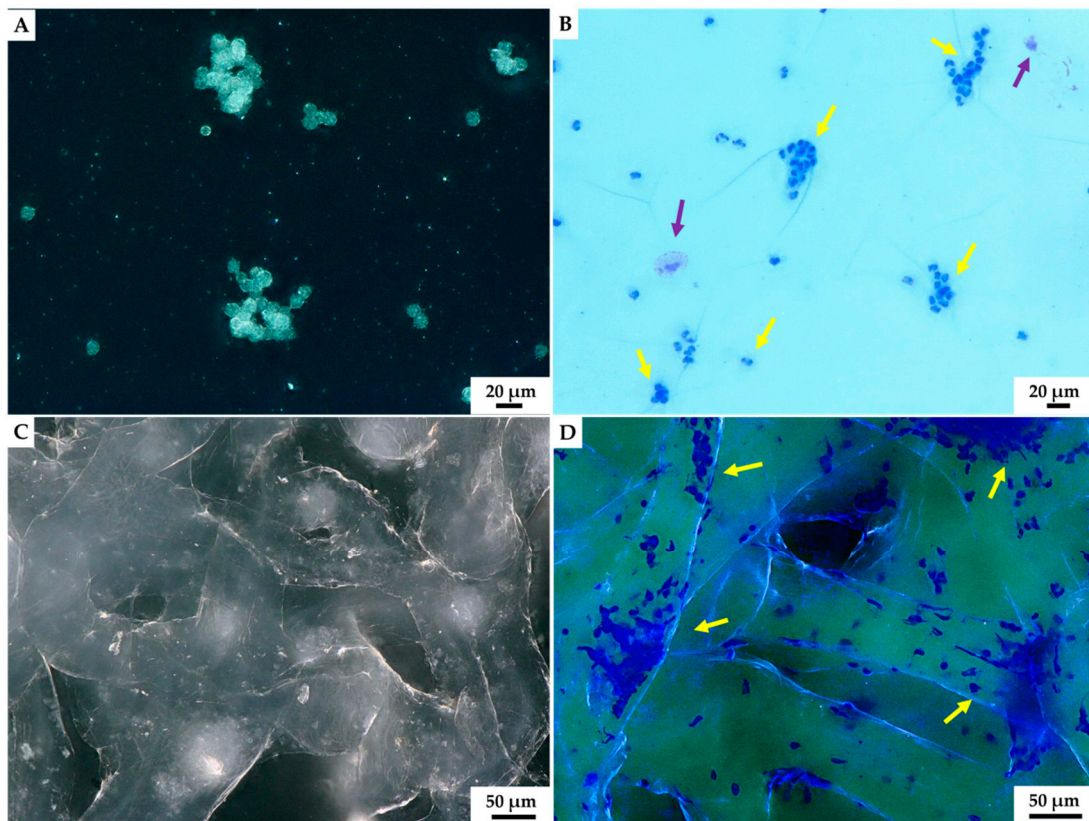
Herein, for the first time, we show how short-term cultivated *C. aspersum* hemocytes interact with the 3D chitinous scaffolds isolated from marine sponge *A. archeri* (Figure 7). According to Yoshino and co-authors [110], hemocytes cultured in vitro, without any additional culture medium, are able to survive for two to three days, and this was further confirmed by our results. The presence of hemocytes in the hemolymph of *C. aspersum* snail was shown by using eosin and methylene blue staining, according to the protocol of Grandiosa et al. [111]. At 24 h following the contact of hemolymph with sponge chitin, the formation of hemocytes clusters (see Figure 7C,D) at chitinous scaffolds became quite visible. Then, 48 h after the contact, hemocytes clusters enlarged in size (see Figure 8).



**Figure 5.** Alternating treatment of skeletal fibers of *A. archeri*, using alkali and acid solutions, leads to the visualization of inner channels (arrows) located within fibers (A,B). Such channels are responsible for capillary activity of these constructs with respect to water and other liquids. For details, see [4].



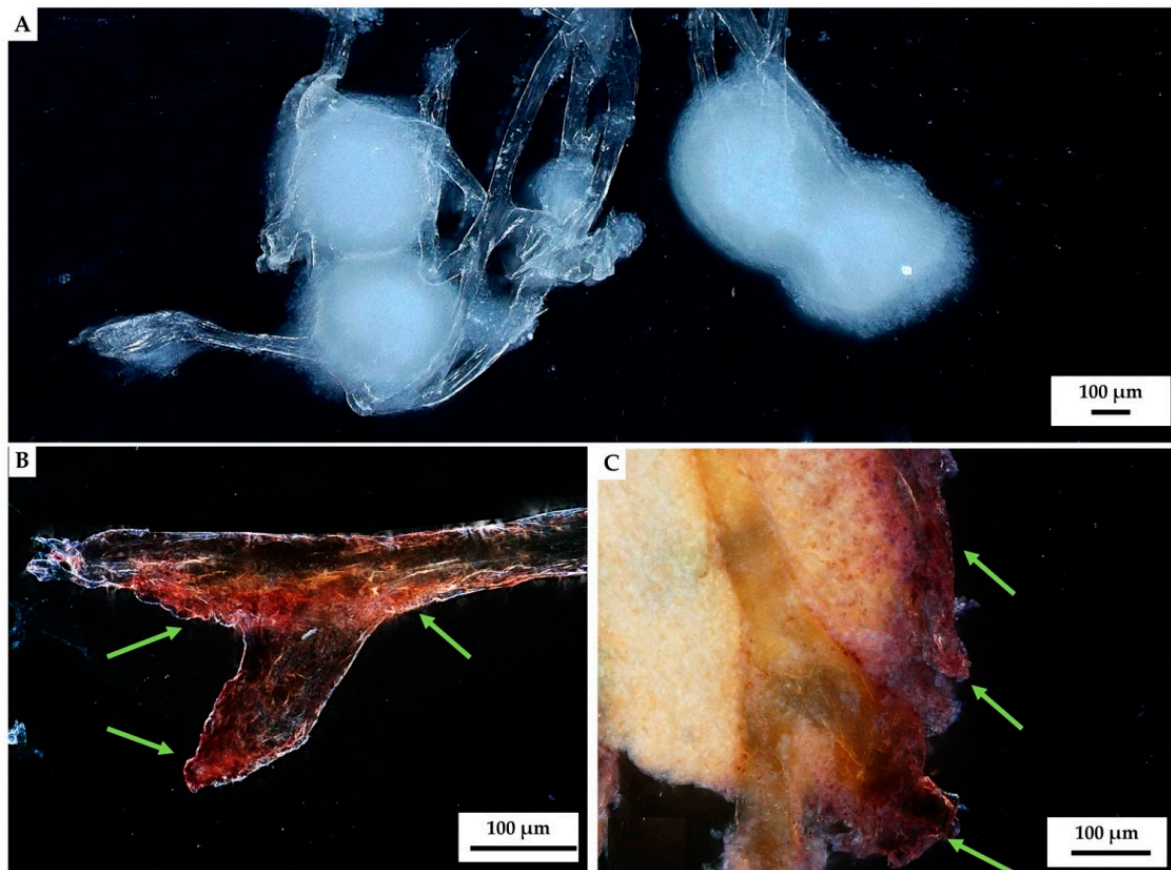
**Figure 6.** After purification, a translucent tube-like chitinous scaffold derived from *A. archeri* (see Figure 3) remains stable enough, but flexible after being saturated with water (A). It takes over the shape of respective hard surface, for example, this glass tube. The 3D architecture of the scaffold made of interconnected microtubes is very visible, using digital stereo microscopy (B,C). The porosity of such scaffold ranges between 300 and 800 µm.



**Figure 7.** Light microscopy imagery. Characteristic aggregates of hemocytes present in isolated hemolymph of *C. aspersum* snail was observed both without (A) and using eosin and methylene blue staining (B) on the glass slide. The formation of hemocytes-based clumps before (C) and after staining by eosin and methylene blue (D) became visible on the surface of *A. archeri* chitinous scaffold 24 h after the immersion in hemolymph. Two hemocytes types could be distinguished after staining: most dominant granulocytes (yellow arrow) and single hyalinocytes (violet arrow).

Our results indicated that hemocytes attached to the surface of external chitinous matrix. Most of the distinguished hemocytes were represented by granulocytes visible in light microscopy images (Figure 7B,D). Aggregation of hemocytes is natural and has been observed in 27 preparations of chitinous scaffolds immersed in hemolymph. Hemocytes are capable of ameboid movement, and they demonstrate the formation of nodules and positive chemotactic behavior in response to foreign substances. The appearance of long pseudopodia branching on the surface of tested chitinous material was visualized (Figure 7). Alizarin Red S staining confirmed the presence of calcium-rich deposits located on the chitin fibers (green arrow, Figure 8B,C), as well as on the cluster of disintegrated granulocytes, after 48 h (see Figure 8). It is possible that both the immune response of hemocytes and the disintegrations of their calcium-rich cytoplasm are crucial for biomineralization *ex vivo*.

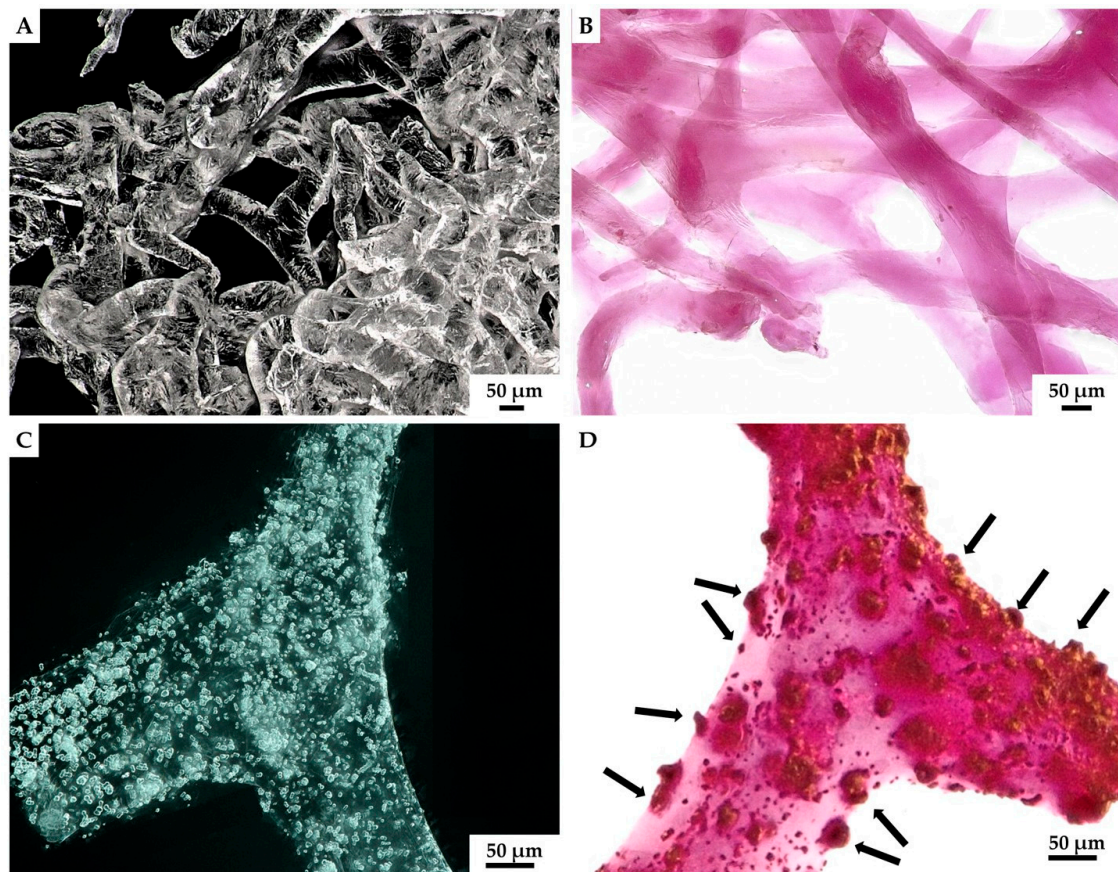




**Figure 8.** Stereo microscopy imagery of hemocyte clusters formed on the fibers of *A. archeri* chitinous scaffolds. Round white-colored clusters (A) of disintegrating hemocytes appeared 48 h after chitin immersion into the hemolymph isolated from *C. aspersum* snail (Figure 2 and Figure 14). Calcium-rich cytoplasmic dense microparticles (arrows) within the disintegrating granulocytes became visible after staining with Alizarin Red S on the chitinous fiber (B), as well as on the surface of the hemocytes cluster (C).

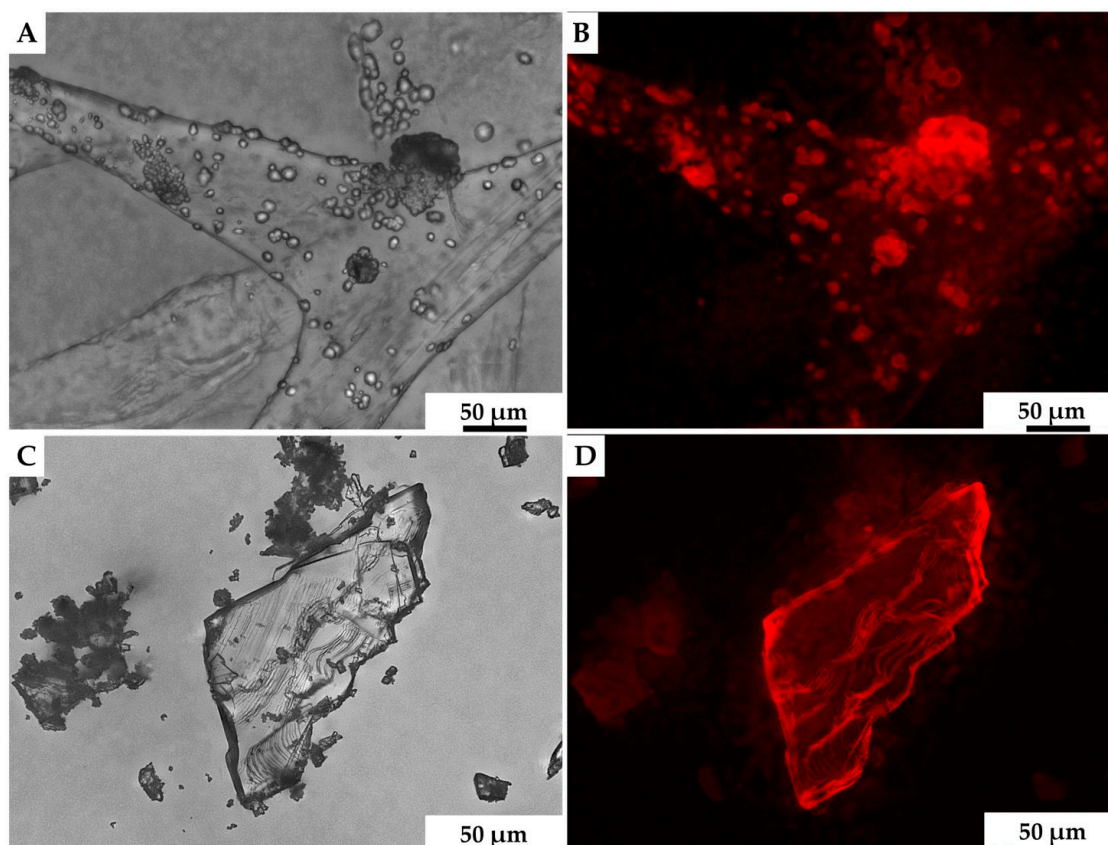
Thus, the formation of calcium-based phase on the surface of chitinous matrix after its immersion into hemolymph of *C. aspersum* snail can be easily confirmed by using Alizarin Red S staining (Figure 9). However, this raises an intriguing question regarding the nature of these calcium-containing granular deposits, which theoretically contain calcite, aragonite and vaterite, as well as amorphous calcium carbonate, according to previous reports [82,112,113].

We assumed that calcite was likely to be the biomineral formed ex vivo by hemocytes on the surface of 3D chitinous scaffolds (Figure 9C,D), and so we carried out the initial analysis of deposits, using fluorescence microscopy. The results obtained (Figure 10) clearly show a high similarity of red auto-fluorescence between calcite standard [114,115] and microgranular deposits formed after biomineralization ex vivo, using *C. aspersum* hemolymph. To analyze the obtained biomineral with respect to calcite composition, we used traditional analytical techniques, such as X-ray diffraction analysis (XRD) (Figure 11), FTIR (Figure 12) and Raman (Figure 13) spectroscopy.



**Figure 9.** Isolated 3D chitinous scaffolds from *A. archeri* prior to ex vivo biomineralization with *C. aspersum* hemolymph (A) became slightly violet after Alizarin Red S staining (B). However, the formation of granular calcium-based deposits on chitin surface (C) after biomineralization ex vivo is quite visible when using digital light microscopy due to the intensive red coloration (D) with the same stain.

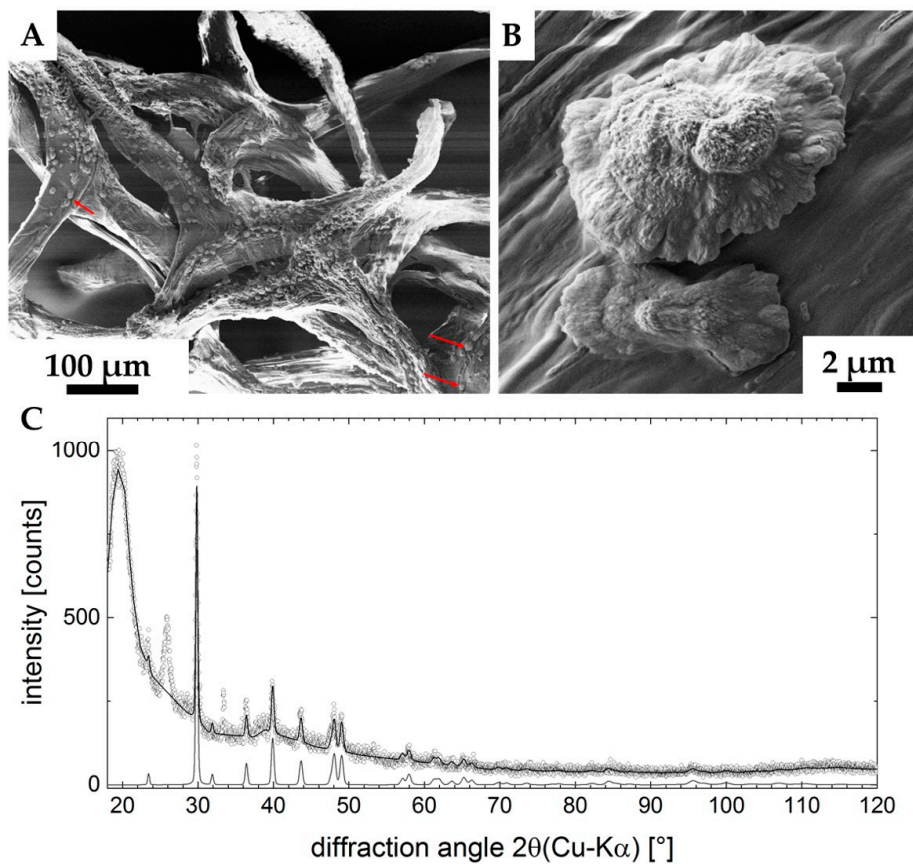
The X-ray diffraction pattern of the sample is represented in Figure 11 C. It shows the characteristics of a sample with a high amorphous content (big hump at  $2\theta \approx 20^\circ$ ). Superimposed, a set of peaks being characteristic for Calcite- $\text{CaCO}_3$  (ICDD #04-012-0489) were found. The pattern still shows two unidentified peaks ( $2\theta \approx 27^\circ$ ,  $2\theta \approx 33^\circ$ ), none of which provides evidence for the presence of a second  $\text{CaCO}_3$  modification (Aragonite, Vaterite or the structures containing crystal water). According to the database search, these two peaks may originate from a crystallized organic component in the system. XRD analysis (Rietveld refinement) pointed to the differences between the measured lattice parameters and the database entry. The refined lattice parameters are  $a = (4.973 \pm 0.001) \text{ \AA}$  (database:  $a = 4.987 \text{ \AA}$ ) and  $c = (16.987 \pm 0.001) \text{ \AA}$  (database:  $c = 17.058 \text{ \AA}$ ), corresponding to a shrunk lattice.



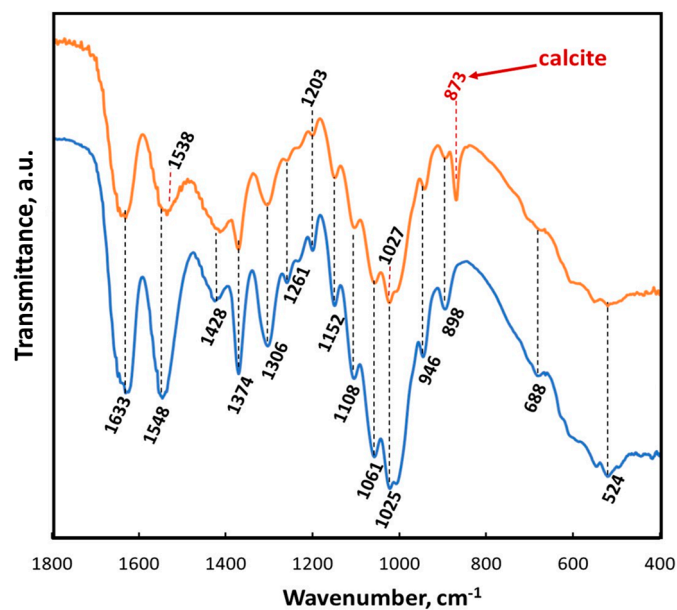
**Figure 10.** Both calcite mineral standard (A) and the mineral phase observed after ex vivo biomaterialization of 3D chitin scaffold, using hemolymph of *C. aspersum* snail (C), possess very similar features with respect to their auto-fluorescence [116] (B,D, respectively).

The results of ATR-FTIR analysis of the chitinous scaffold used in this study before and after ex vivo biomaterialization are represented in Figure 12. Both spectra show a characteristic split for  $\alpha$ -chitin, such as amide I at  $1633\text{ cm}^{-1}$  (see orange and blue lines). This band corresponds to the presence of stretching vibrations from intermolecular ( $\text{C}=\text{O}\cdots\text{HN}$ ) and intramolecular ( $\text{C}=\text{O}\cdots\text{HO}(\text{C}6)$ ;  $\text{C}=\text{O}\cdots\text{HN}$ ) hydrogen bonds [59,117]. The observation of such bands as amide II ( $\nu\text{N-H}$  and  $\nu\text{C-N}$ ) at  $1548\text{ cm}^{-1}$ , amide III ( $\nu\text{C-N}$  and  $\delta\text{N-H}$ ) at  $1306\text{ cm}^{-1}$  or characteristic intense band at  $898\text{ cm}^{-1}$  ( $\text{C-O-C}$  bridge as well as  $\beta$ -glycosidic linkage) additionally confirmed the presence of  $\alpha$ -chitin [24]. A sharp peak visible at  $873\text{ cm}^{-1}$  (red arrow) clearly indicated that calcium carbonate as calcite polymorph (monohydrocalcite) [117–120] appeared on the chitinous scaffold after ex vivo biomaterialization using hemolymph isolated from *C. aspersum* (see Figure 12).

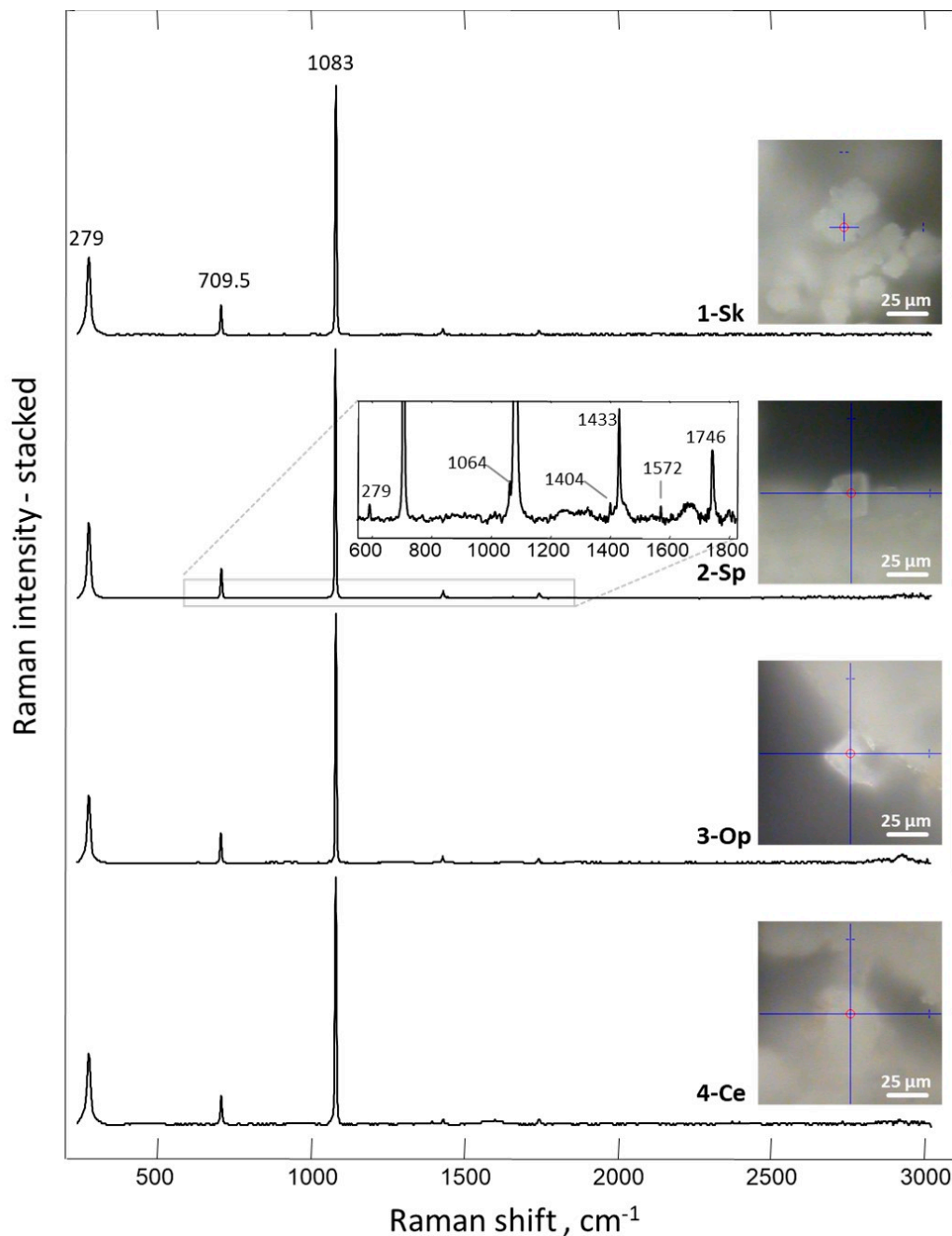
The results obtained by employing Raman spectroscopy clearly showed (Figure 13) that the spectrum recorded for crystals obtained after ex vivo biomaterialization corresponds to the spectra acquired for the biogenic calcite [121,122]. The most intensive band, with the maximum near  $1083\text{ cm}^{-1}$ , corresponds to  $\nu_1$  symmetric stretching vibrations  $\text{CO}_3^{2-}$  of calcite [121,123], where the peak detected at  $1433\text{ cm}^{-1}$  is related to  $\text{CO}_3^{2-}$   $\nu_2$  asymmetric stretching vibrations [124]. Additional evidence of calcite polymorph presence is provided by the bands at  $711$  and  $279\text{ cm}^{-1}$ . These two peaks are related to  $\nu_4$  in-plane bending vibrations of  $\text{CO}_3^{2-}$  and vibrational modes of calcite, respectively [124]. Moreover, all present bands are consistent with calcite, as reported for the natural mineral in the RRUFF database (<http://www.ruff.info/> (03.12.2019))



**Figure 11.** SEM images (A,B) of the ex vivo mineralized surface of 3D chitinous scaffold confirm the presence of microcrystallites (arrows). X-ray diffraction pattern of the sample is represented in the image C. Measured data are open dots; the refinement is highlighted by the solid line. The diffractogram of calcite- $\text{CaCO}_3$  is shown at the bottom, clearly verifying the presence of this phase in the sample. Two unassigned peaks remain in the data ( $2\theta \approx 27^\circ$ ,  $2\theta \approx 33^\circ$ ). The large feature at  $2\theta \approx 20^\circ$  indicates a high content of amorphous material in the sample (C).



**Figure 12.** ATR-FTIR spectra of *A. archeri* chitin scaffold before (blue line) and after ex vivo biomineralization (orange line) within the region of  $1800\text{--}400\text{ cm}^{-1}$ .



**Figure 13.** Raman spectrum of the mineral phase obtained on the surface of 3D chitinous scaffold after ex vivo biomineralization mediated by snail hemolymph.

### 3. Discussion

It is recognized that “the construction of novel polymer materials via physical approaches and green technology from raw chitin is a sustainable pathway and beneficial to the green development” [58]. However, this approach is connected with numerous preparative steps, including, for example, mechanochemical disassembly, particulate leaching, gas foaming, thermally induced phase separation, electrospinning, dissolution of chitin in special liquids [122,125,126], and its supercritical drying [127]. Alternatively, diverse fractions of flakes, powders and nano forms of chitin (i.e., nanowhiskers, nanofibrils and nanocrystals) [128–130] can be obtained from demineralized and deproteinated crustaceans’ chitin. Such scaffolding strategies [131], however, are connected with technological

difficulties and other disadvantages which can be a critical weakness in terms of cost and future clinical use [132].

As mentioned in the introduction, marine verongiid sponges produce practically ready-to-use 3D constructs made of microtubular, mechanically robust, yet flexible scaffolds, which are very well applicable for the cultivation of diverse human cells. However, would they be equally as good scaffolds for hemocytes of invertebrate origin?

The hemolymph of mollusks, including terrestrial snails, is mostly composed of water and contains hemocyanins, diverse inorganic ions, metabolites, lectins and enzymes, as well as the number of cells (i.e., blastocytes, hyalinocytes and granulocytes) [133–136] known as hemocytes. Granulocytes, making up over 95% of circulating hemocytes, are 9–50  $\mu\text{m}$  in size, have an irregular nucleus and cytoplasm containing numerous granules [137]. On a flat surface, they exhibit amoeboid motion and are able to form visible pseudopodia. These cells are capable of phagocytosis and its more complex forms, requiring collective participation of many granulocytes: encapsulation and nodulation [113]. Furthermore, granulocytes of the marine mollusk *Pinctada fucata* were reported to contain numerous calcium-rich vesicles and crystals serving as calcium pool [113].

Molluscan in vitro cell culture systems have a huge impact on our understanding of their complex physiological processes, functions and relation between tissue-specific cells [110]. Our study represents the first data concerning the ability of *C. aspersum* hemocytes to grow on chitinous matrices principally (Figure 7) and, furthermore, to build clusters (Figure 8). These phenomena may be explained as follows. Firstly, hemocytes are cells responsible for immune responses [138–141] recognizing sponge  $\alpha$ -chitin as a foreign body. Nodulation in terrestrial snails is a multi-stage process, requiring the presence of many granulocytes. This process is initiated by the contact of the granulocyte with a foreign substance. As a result of degranulation occurring in the granular cytoplasm, chemotaxis-related substances are released. They, in turn, attract other hemocytes to the site where the foreign matter is encapsulated by many layers of granulocytes (Figure 7). Moreover, this site becomes a condensation center of calcium ions present in hemolymph, as well as the ones released from cytoplasm of hemocytes.

A second explanation could be related to biomineralization. Previously, chitin within mollusk shells has been detected, next to proteins, being a part of organic membrane, which appears during shell repair or formation [81,142,143]. It is possible that hemocytes identify chitinous matrix as a substituent of this membrane. In the literature, we can find undeniable evidence concerning active participation of hemocytes in calcification in vivo [80–82,144,145]. For example, Kapur and Sen Gupta (1970) [146] described such a membrane as a specialized scaffold for the granulocytes, which lose their identity, leaving free nuclei and organic matter, and deposit their calcium-rich granules from cytoplasm. In this case, the organic membrane acts as a crystal's nucleation center. Similar explanations are to be found in numerous papers by Abolins-Krogis, who conducted studies on terrestrial snails from the *Helix* genus [79,144,147,148].

Biomimetic materials displaying the complexity of biominerals are artificially difficult to prepare [149], even though numerous in vitro attempts have been carried out previously [150–156]. Our results are in a good agreement with recently published data demonstrating that chitin– $\text{CaCO}_3$  and porous chitin– $\text{CaCO}_3$  scaffolds facilitated hMSCs attachment following cell osteogenic differentiation. However, such chitin scaffolds were prepared by dissolving crustaceans chitin flakes in LiCl/dimethylacetamide, following by gel formation and freeze-drying [157]. In contrast, the experimental results reported in this study unambiguously show a new biomimetic direction to apply already naturally prefabricated [18] 3D chitinous scaffolds of poriferan origin as templates for both the growth of mollusk and hemocytes and the functionalization of the surface of chitin fibers with respect to the formation of amorphous and crystalline (calcite) calcium carbonate-based layers. Our approach is based on ready-to-use 3D scaffolds of sponges' origin hence only the size of already naturally pre-designed pores could be a limiting factor. However, this can be neglected due to the ability of hemocytes not only to attach to the surface of individual chitin fibers, but also to build large-size clusters located between fibers that possess biomineralization activity (Figure 8).

Thus, our study resulted in the development of a new biomimetic product, utilizing mollusk hemocytes, which is based on ex vivo synthesized ACC and calcite tightly bound to the surface of sponge chitin used as 3D scaffold. The outlook of this biomimetic direction includes numerous open questions concerning the role of hemocytes in the creation of a fine-tuned microenvironment [158] which is necessary for calcification ex vivo. Without doubt, the mechanisms and kinetics of this kind of biomineralization should be investigated, in addition to carrying out further studies on mechanical properties of developed mineralized scaffolds aimed at practical application in biomedicine, in the near future.

## 4. Materials and Methods

### 4.1. Sample Location and Collection

#### 4.1.1. *Aplysina archeri* Sponges

The Caribbean sponges were collected and preserved (in 96% ethanol) as hosts of symbiotic invertebrates in the Bay of Pigs of Cuba (coordinates: 22°07'15.9"N, 81°07'07.2"W; depth: 21 m; date: 05 February 2019) and Playa Lagun of Curaçao (coordinates: 12°19'02"N, 69°09'09"W; depth: 20 m; date: 20 June 2017). Stove-pipe sponge *Aplysina archeri* (Higgin, 1875) (previously known as *Luffaria archeri* or *Verongia archeri* (see Van Soest et al., 2019) (phylum Porifera, class Demospongiae, order Verongiida, family Aplysinidae).

#### 4.1.2. *Cornu aspersum* Snails

One-year-old snails, *Cornu aspersum*, were obtained from a commercial heliculture farm from the central part of Poland (Snails Breeder—Hodowla Ślimaków, Małszyce, Poland). Snails were maintained in a glass aquarium, at room temperature. As food, lettuce, carrots and apples were used. Moreover, cuttlebone of *Sepia officinalis* cuttlefish was given to snails as calcium carbonate enrichment (CaCO<sub>3</sub>) in the diet. The snails were kept moist throughout the experimental period with wet humus. Animal rights statement is not required.

### 4.2. Isolation Procedure

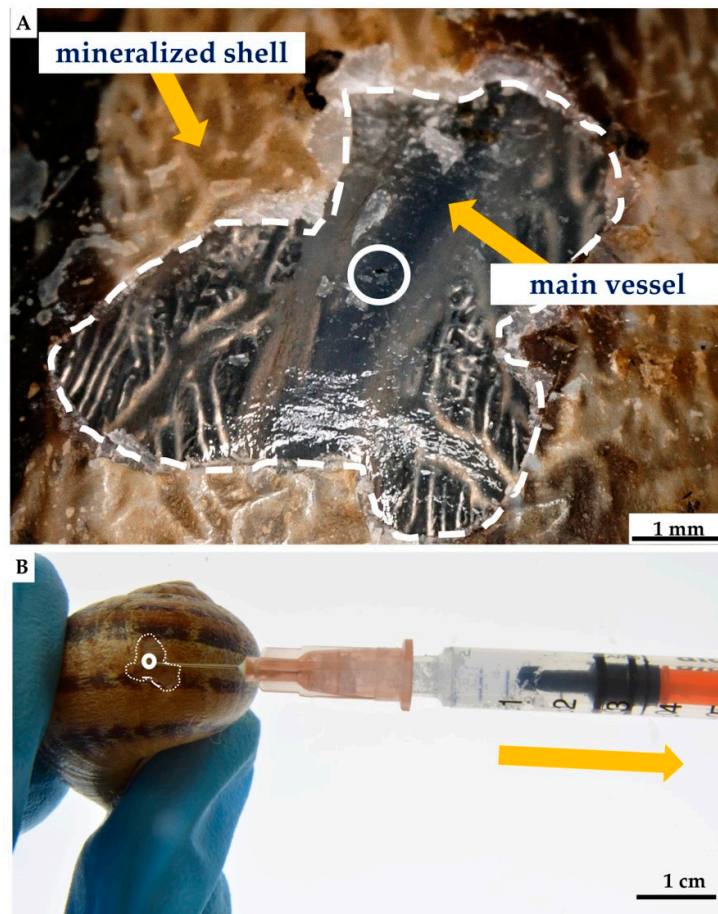
#### 4.2.1. Isolation of Chitinous Scaffolds from the *A. archeri* Demosponge Skeleton

The procedure for the isolation of chitin-based scaffolds from verongioid sponges, as reported by us [24], was followed here. In brief, it was performed accordingly to the following steps: (I) sponge skeleton was washed three times with distilled water in the vessel on a magnetic stirrer, to remove water-soluble compounds, for 6 h (Figure 3A). Afterward, the water was changed (step II) with 2.5 M of NaOH, at 37 °C, for 24 h, for deproteinization and depigmentation (Figure 3B). This step was then followed by (step III) treatment with 20% CH<sub>3</sub>COOH at 37 °C, for a period of 24 h, to remove residual calcium and magnesium carbonates and then washing in distilled water up to pH 6.8. Steps II and III were repeated two times, to obtain colorless tubular scaffolds (Figure 3D–F). The purity of isolated chitin scaffolds has been proved according to standard analytical procedures as described previously [4].

#### 4.2.2. Nonlethal Isolation of the Hemolymph from the *C. aspersum* Snail

For hemolymph collection, the modified nonlethal intravital method described in Polish patent P.410296 and article [159] was used. The shell surface of the selected *C. aspersum* snail was cleaned with 70% ethanol. After that, a piece of shell was removed, and 3 h later, about 1000 µL of hemolymph was isolated from an individual of *C. aspersum* by main-vessel puncture, using a sterile syringe and needle [159] (see Figure 14). Twelve hours post-isolation, an organic film was observed in the site of removed shell fragment, which was fully mineralized within the next few days [160]. During the next

three months after the procedure, the snails used in this study did not show any visible changes in their physiology or in behavior.



**Figure 14.** Nonlethal extraction of the hemolymph from cultivated *C. aspersum* snail, using syringe (A). Initially, the shell of the snail was disrupted mechanically by using a scalpel, with the aim of obtaining around 5 mm × 5 mm large shell-free surface. After this procedure, the location of the main “blood” vessel becomes visible (B) and accessible to use a needle for the hemolymph extraction (A).

#### 4.3. Ex Vivo Biomineralization of the *A. archeri* Chitinous Scaffolds

A square fragment (10 × 10 mm) of selected chitinous scaffold (Figure 10) was immersed in hemolymph of *C. aspersum* for one hour and then placed on a sterile slide, until completely dried, at room temperature. The procedure was replicated five times. The described technique aimed to simulate biomineralization of organic matrix by snail hemolymph under natural conditions via mimicking of physicochemical effects reported for shell regeneration of terrestrial snails previously [161,162]. Obtained in this way, mineral phases deposited on chitinous matrix were characterized, as represented below.

#### 4.4. Short-Term Cultivation of Hemocytes on Chitinous Matrix

For short-term cultivation of hemocytes, about 0.5 mL of the hemolymph was isolated, using the method described above, and placed in a 2 mL Eppendorf tube. Selected fragment of chitinous matrix was immersed in the hemolymph, at room temperature, for 24 and 48 h, respectively. To prevent possible contamination with bacteria streptomycin (100 µg/mL) and penicillin (60 µg/mL) have been used [163].



#### 4.5. Characterization of Obtained Materials

##### 4.5.1. Photography and Figures

Photographs and macroscopic images were performed by Nikon D-7100 camera with Nikon AF-S DX 18–105 mm f/3.5-5.6G and Nikon AF-S VR Micro-Nikkor 105 mm f/2.8G IF-ED objective lenses. Figures were prepared, using the GNU Image Manipulation Program GIMP and Microsoft Office tool PowerPoint 2016.

##### 4.5.2. Digital, Light and Fluorescence Microscopy

The obtained samples were observed, using an advanced imaging and measurement system consisting of Keyence VHX-6000 digital optical microscope and the swing-head zoom lenses VH-Z20R (magnification up to 200×) and VH-Z100UR (magnification up to 1000×). The light and fluorescence microscope modes were performed with a BZ-9000 (Keyence) microscope.

##### 4.5.3. Eosin and Methylene Blue Staining

We used Hemavet (Kolchem) as the combination of eosin and methyl blue dyes. Previously, this stain was successfully used for hemocytes detection and characterization [111,164,165]. Hemolymph cell monolayers (HCMs) were prepared by spreading the drop of hemolymph on sterile glass slide and drying it at ambient temperature. Staining was also used to detect hemocytes settled on chitinous scaffold (see Figure 7).

##### 4.5.4. Alizarin Red S Staining

Ex vivo mineralized chitinous scaffolds were stained with Alizarin Red S (Sigma-Aldrich) and compared with that of native *A. archeri* chitin as isolated. For the staining procedure, 40 mM of Alizarin Red S (pH 8.3) was used for staining of the samples, during 30 min, at room temperature (for details see [165]). Stained samples were washed with distilled water five times, to eliminate the unattached Alizarin Red S, as well as mineral particles which were not tightly attached to the surface of chitin fibers. Calcium deposits were detected as orange–red color particles, using digital microscopy.

##### 4.5.5. Fluorescent Microscopy Analysis

Materials in this study were observed with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan), in fluorescent and light microscopy mode. Calcite mineral standard was purchased from the International Institute of Biomineralogy (INTIB GmbH), Freiberg, Germany.

##### 4.5.6. ATR FT-IR and Raman Spectroscopy

Infrared spectroscopy techniques were used for the qualitative characterization of obtained mineralized scaffolds, as well as pure chitin isolated from *A. archeri*. The presence of expected functional group was confirmed by ATR FT-IR (attenuated total reflectance Fourier transform infrared spectroscopy) and verified, using Nicolet 210c spectrometer (Thermo Scientific). The investigation was performed over a wavenumber range of 1800–400  $\text{cm}^{-1}$  (resolution of 0.5  $\text{cm}^{-1}$ ). Raman spectra were recorded, using a Raman spectrometer (Raman Rxn1™, Kaiser Optical Systems Inc., Ann Arbor, USA) coupled to a light microscope (DM2500 P, Leica Microsystems GmbH, Wetzlar, Germany).

##### 4.5.7. XRD

The phases in the sample were identified through X-ray diffraction, using a SEIFERT-FPM URD6 diffractometer equipped with a sealed X-ray tube with Cu anode. The device operated in symmetrical Bragg–Brentano geometry with a secondary graphite monochromator in front of the proportional counter detector. For the measurement, the sample was fixed with Scotch Tape to a 'zero background' to a sample holder. Phase identification was performed with the ICDD PDF-4+ database linked to the

Panalytical HighScore+ software. Rietveld refinement for a more detailed analysis of the sample was conducted with the Maud software package.

#### 4.5.8. Scanning Electron Microscopy (SEM)

Scanning electron microscopy was performed using XL 30 ESEM Philips-Scanning Electron Microscope (FEI Company, Peabody, MA, USA). Samples were fixed on a sample holder with carbon patches and then covered with carbon or with a 5–10 nm gold layer using Edwards Sputter Coater S150B (BOC Edwards, Wilmington, MA, USA).

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