ISOLATION OF CHITIN FROM *APLYSINA AEROPHOBA* USING A MICROWAVE APPROACH

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Abstract

Chitin of poriferan origin represents a unique renewable source of three-dimensional (3D) microtubular centimetre-sized scaffolds, which have recently been recognized as having applications in biomedicine, tissue engineering, and extreme biomimetics. The standard method of chitin isolation from sponges requires concentrated solutions of acids and bases and remains a time-consuming process (lasting up to seven days). Here, for the first time, we propose a new microwave-based express method for the isolation of chitinous scaffolds from the marine demosponge Aplysina aerophoba cultivated under marine farming conditions. Our method requires only 41% of the time of the classical process and does not lead to the deacetylation of chitin to chitosan. Alterations in microstructure and chemical composition due to the microwave treatment were investigated using various analytical approaches, including Calcofluor White staining, chitinase digestion, scattering electron microscopy, and Raman and ATR-FTIR spectroscopy. It was demonstrated that microwave irradiation has no impact on the chemical composition of the isolated chitin.

Keywords: *chitin isolation, marine sponge, microwave treatment, Aplysina aerophoba*

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1. Introduction

It is well known that chitin—an aminopolysaccharide consisting of β(1,4)-linked N-acetyl-glucosamine units—is one of the most widespread biopolymers on the Earth. In nature, chitin can be found in three different allomorphic forms, named α, β, and γ [1], which differ in crystalline structure. Although the most abundant form of chitin is the α-polymorph, β- and γ-polymorphs have already been described in flexible structures, such as cocoon threads, periotrophic membranes of locust, and cockroach or squid pens [2]. However, only the α-polymorph has practical importance, which is isolated from fungi or exoskeletons of selected crustaceans—mostly shrimps and crabs—to obtain the final product in the form of flakes, powders, or whiskers, which are then used for various applications in material science and engineering [3]. Chitin in this form can be easily transformed into chitosan and used for cosmetic purposes. For biomedicine applications, crustacean and fungal chitin has to be manufactured to obtain a material with the strictly desired three-dimensional architecture [3].

Sponges are currently among the most investigated marine invertebrates due to the possibility of their cultivation under marine ranching conditions, as well as their remarkable content of unique secondary metabolites and biological materials, such as collagen and chitin, which have great relevance for technological, biomedical, and cosmetic applications [3–15]. These organisms have even been described as a gold-mine for marine pharmacology and bioinspired materials science [16]. Among marine demosponges, only representatives of the order Verongiida are known to synthesize both biologically active substances (bromotyrosines and quinones) as well as skeletons made of chitin. Since 2007, when chitin in the form of three-dimensional (3D) scaffolds was isolated for the first time from the marine demosponge *Verongula gigantea* [17], its presence has been verified in 19 marine species, mostly from the order Verongiida (for overview, see Ehrlich et al., [18] and Żółtowska-Aksamitowska et al., [19]), and in two freshwater species [20,21]. The chitinous scaffolds of keratose sponges have been investigated by various bioanalytical and physicochemical techniques to determine their morphology, composition, and structure [6,22]. Chitin-based sponge scaffolds are extremely interesting in materials science since the processing of chitin into sponge-like materials or foams is technologically difficult and expensive. These naturally prefabricated constructs can be isolated from the sponge skeletons using a stepwise extraction procedure mainly based on the use of 2.5 M NaOH. This procedure was recently patented [23]. It has also been shown that the unique 3D architecture of poriferan chitinous skeletons opens the door to their application as adsorbents (of uranium) [24], as scaffolds for tissue engineering [25–27], and in extreme biomimetics [28–35].

The marine demosponge *Aplysina aerophoba* (Verongiida, Aplysinidae) was proposed as a potential source of both chitin and bioactive bromotyrosines in 2010 [22]. Nowadays, this sponge is cultivated on a marine farming facility that is 100 m^2 in area in Kotor Bay, Montenegro [36], providing an adequate supply of both promising components. The anti-tumorigenic and anti-metastatic potential of bromotyrosines, such as Aeroplysinin-1 (AP-1) and Isofistularin-3, isolated from this demosponge on pheochromocytoma cells was recently evaluated for the first time [37]. However, the long time required for the isolation of both substrates is a major drawback, excluding the possible industrial use of chitin scaffolds and bromotyrosine-based compounds. Therefore, the development of new express methods for simultaneous isolation of chitin and bromotyrosines from cultivated verongiid demosponges is currently an important trend in research.

The successful isolation of chitin from the Caribbean demosponge *Aplysina archeri* [38] inspired us to apply a combined method of microwave irradiation with alkali-based pre-treatment to isolate chitinous scaffolds together with bromotyrosines from *A. aerophoba*. Our next goal was to evaluate the time of chitin extraction using this method

and to investigate the microstructure and chemical nature of the obtained chitinous scaffolds.

2. Materials and Methods

2.1. Samples

Dried samples of *Aplysina aerophoba* (Schmidt, 1862), originating from Kotor Bay (Montenegro), were supplied by BromMarin GmbH, Germany. We used a chitin standard from the snow crab *Chionoecetes opilio* (INTIB GmbH, Germany, catalog number 8845.2) and a chitosan standard from Sigma-Aldrich (Germany, catalogue number 417963).

2.2. Microwave Approach for Chitin Isolation

Selected fragments of *A. aerophoba* were cut into pieces that were 2 × 2 cm in size (**Fig. 1**). Pre-treatment with 2.5 M NaOH for 24 hours at 37°C led to a deproteinized and partially depigmented skeleton. The obtained scaffolds were rinsed with distilled water to reach a pH of 6.5. The fragments were then treated with 2.5 M NaOH under microwave irradiation for 1.5 minutes. After rinsing with distilled water to pH 6.5, the fragments were placed in 20% acetic acid and again treated under microwave irradiation for 1.5 minutes. These two steps were carried out alternately until the fragments were completely colourless and demineralized. The state of demineralization was monitored using SEM/EDX, as described previously [38].

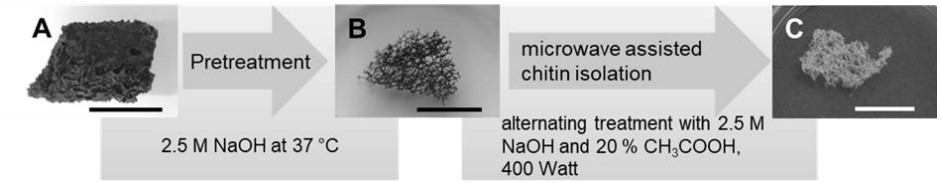

Figure 1. Fragment of *Aplysina aerophoba* (A). Fragment of the sponge skeleton pre-treated with 2.5 M NaOH for 24 hours under ambient conditions to remove pigments and proteins (pre-treatment time of 24 h) (B). Triple alternating treatment with 2.5 M NaOH and 20% CH_3COOH at 400 W resulted in a completely demineralized, soft, and colourless chitin matrix (C) within 37.5 min. Scale bar is 1 cm.

2.3. Light and Fluorescent Microscopy Analysis

For observation of the isolated chitin fibres, a BZ-9000 microscope (Keyence) was used. This may be operated in fluorescent and light modes.

2.3.1. Calcofluor White Staining

The fluorescence of polysaccharides is enhanced when Calcofluor White (CFW) (Fluorescent Brightener M2R, Sigma-Aldrich, Taufkirchen, Germany) is bound to them [39,40]. In view of the polysaccharide nature of chitin, the demineralized scaffold was stained with Calcofluor White. For this purpose, 20 µL of a solution composed of 10 g NaOH and 10 g glycerine in 90 mL water was applied. After 15 seconds, a 0.1% CFW solution was added. After 30 minutes in darkness, the fibres were washed with distilled water to remove unattached CFW and then dried at room temperature. The isolated fibres were then analysed using the fluorescent microscope before and after CFW staining to investigate the increase in fluorescence.

2.3.2. Chitinase Digestion

The chitinase digestion test is a well-established method for chitin identification [6,17,18,39-42]. To observe the chitin digestion process, a single fibre was rinsed with 50 mL phosphate buffer (pH 6.0). When the phosphate buffer was removed, chitinase from *Streptomyces griseus* (3.2.1.14, No. C-6137, Sigma-Aldrich) was added and the first microscopy images were taken. The progress of chitin digestion was investigated by observation of the sample every 30 minutes over 10 hours with fluorescent and light microscopy (Keyence BZ-9000).

2.4. ATR-FTIR

Qualitative analysis of the isolated fibres was performed using infrared spectroscopy. Spectra were recorded using a Nicolet 380 Fourier transform infrared spectrometer (Thermo Scientific) and analysed with appropriate software (OMNIC Lite Software). By way of comparison, the spectra of a chitin standard and a chitosan standard were additionally recorded. Further post-processing of the spectra was performed with OriginLab 2015.

2.5. Raman Spectroscopy

A Raman spectrometer (Raman Rxn1™, Kaiser Optical Systems Inc., Ann Arbor, USA) was used to record the Raman spectra of the isolated fibres, chitin standard, and chitosan standard. The applied laser beam had a power of 110 mW at the sample, and the analysis was performed between 200 and 3250 cm^{-1} with a resolution of 4 cm^{-1}. The signal-to-noise ratio was improved by setting the integration time to 1 s, and 50 spectra were summarized. Baseline implementation was performed with MatLab (MathWorks Inc., Natick, USA), and post-processing was carried out with OriginPro 2015.

2.6. ESI-MS

For ESI-MS analysis, samples were prepared as follows. The isolated chitin matrix was hydrolysed in 6 M HCl for 24 hours at 90°C. The resulting solution was filtered with a 0.4-micron filter, and the filtrate was freeze-dried to remove excess HCl. The solid residue was dissolved in water for the ESI-MS analysis. As a control, a D-glucosamine standard was used (Sigma-Aldrich, Taufkirchen, Germany). All ESI-MS measurements were performed using an Agilent Technologies 6230 TOF LC/MS spectrometer (Applied Biosystems, Foster City, CA, USA). Nitrogen was used as a nebulizing and desolvation gas.

2.7. Scattering Electron Microscopy

For scattering electron microscopy, an S4700 instrument (Hitachi) was applied. The fibres were fixed on a sample holder with carbon patches and covered with a 5–10 μm platinic layer using the Edward Sputter Coater S150B.

3. Results and Discussion

In 2007, a method of chitin isolation from verongiid sponges was reported for the first time [17]. Recently, this standard method has been improved by using microwave irradiation to isolate chitin from the verongiid sponge *Aplysina archeri*, collected in the Caribbean [38]. This new approach enables the isolation of the chitinous scaffolds in less than 1% of the treatment time in comparison with the standard method. The method did not result in any chemical or microstructural changes. In the present work, the impact of this new express method on the chemical and morphological properties was tested using the example of the marine sponge *A. aerophoba* from the Adriatic. Chitin from this sponge

species has previously been isolated by the standard procedure [6]. In that case, demineralization and deproteinization of the sponge skeleton under ambient conditions were carried out for 66 hours. To preserve the bromotyrosines, which are promising agents in experimental tumour research, the organics were extracted using 2.5 M NaOH at 37°C for 24 hours (Fig. 1) [37]. However, the isolated skeleton was still stiff and pigmented (Fig. 1A, Fig. 2A). During alternating treatment with 2.5 M NaOH and 20% acetic acid under microwave irradiation (2450 MHz, 400 W, 1.5 minutes per treatment), the scaffold lost its stiffness and colour (Fig. 2A–D).

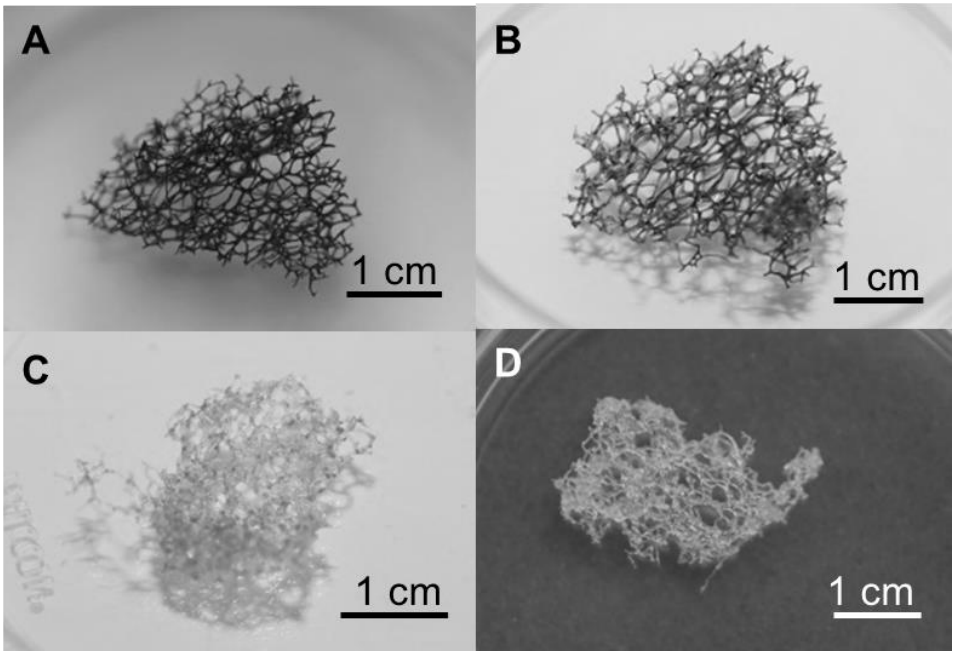

Figure 2. The pigmentation of the mineral skeleton (A), isolated with 2.5 M NaOH under 24 h of pre-treatment, visibly decreases after only 1.5 minutes of alkaline and 1.5 minutes of acidic treatment under microwave irradiation (B). However, the greater part of the scaffold is still mineralized. After additional treatment for 1.5 minutes with 2.5 M NaOH and for 1.5 minutes with 20% acetic acid under microwave irradiation, the scaffold shows only weak pigmentation and is now flexible (C). A further 1.5 minutes of alkaline treatment under microwave irradiation yields a completely demineralized and flexible scaffold (D).

In total, the process of isolation using microwave irradiation required 7.5 minutes to produce a soft and colourless three-dimensional scaffold (Fig. 2D). Taking into account the time required for bromotyrosine extraction and for rinsing between alkaline and acidic treatment (30 minutes), this method still saves about 59% of the treatment time compared with the standard method.

With regard to the harsh treatment conditions and reports concerning the deacetylation of chitin under microwave irradiation [43–45], the identification of chitin was a main objective of this research. Calcofluor White staining was used as a first step towards identifying the chitinous nature of the isolated fibres. Fluorescent microscopy imaging showed strong fluorescence under an exposure time as short as 1/3700 s (Fig. 3) [17,46].

Application of chitinase enzyme leads to the degradation of chitin to low-molecular-weight oligomers such as N-acetyl-D-glucosamine. The results of such treatment can be displayed using light microscopy. Chitinase treatment has previously been applied to isolated scaffolds of diverse sponges [4,17,19,47]. Fig. 4 shows the digestion of chitin by means of endo-hydrolysis of N-acetyl-β-D-glucosamine-β-(1→4) linkages over 17 hours.

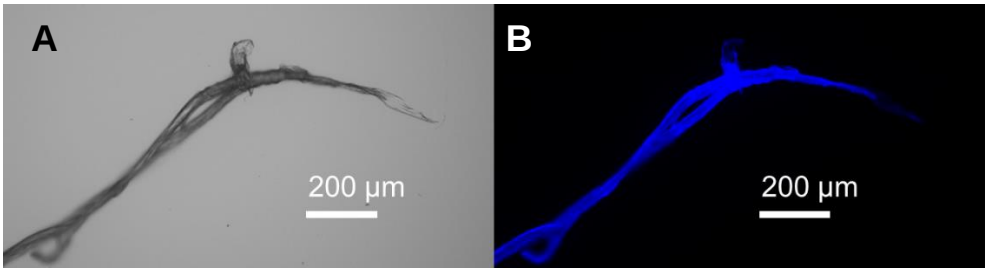

Figure 3. Images obtained by light microscopy (A) and fluorescence microscopy (B) showing fibres after CFW staining. The blue fluorescence is visible even at an exposure time of 1/3700 seconds.

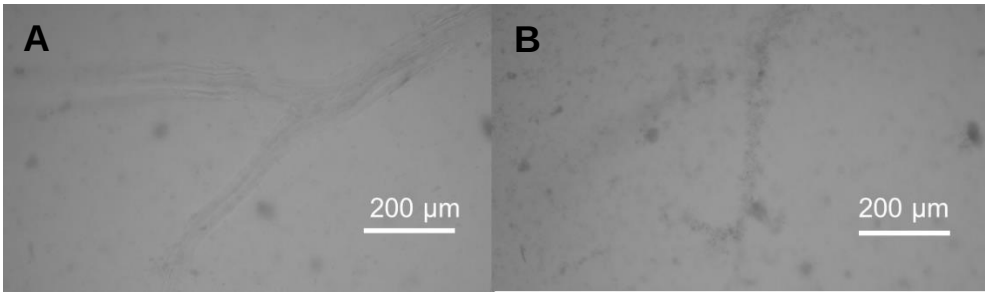

Figure 4. Chitinase digestion of an isolated fibre of *Aplysina aerophoba* by microwave irradiation at the start of treatment (A) and after 17 hours of treatment (B).

Interestingly, the microwave approach does not have an influence on the content of N-acetyl-d-glucosamine (NAG) released during chitinase treatment. The measured content of NAG was equal to 560 µg/1 mg of the dry skeleton, which is in accordance with already published results [22].

In addition to these methods, ATR-FTIR, Raman spectroscopy, and ESI-MS were applied to further analyse the isolated scaffolds. In a previous paper on the microwave-assisted isolation of chitin from *A. archeri* [38], it was shown that these analytical methods are suitable for the determination of chitin and for excluding the formation of chitosan during the treatment. The ATR-FTIR spectrum (Fig. 5) and Raman spectrum (Fig. 6) of the isolated scaffold are compared with the spectra of α-chitin and chitosan standards.

The ATR-FTIR spectrum of the purified chitinous matrix of *A. aerophoba* was compared with the spectra of both α-chitin and chitosan standards. The spectra of chitin of *A. aerophoba* origin and of the α-chitin standard display the typical split amide-I band, which is a consequence of stretching vibrations of C=O bonds (1620 cm^{-1} and 1651 cm^{-1}), while the amide-II band at 1556 cm^{-1}, related to N–H bending vibrations, and the amide-III bands between 1308 cm^{-1} and 1201 cm^{-1} are also visible.

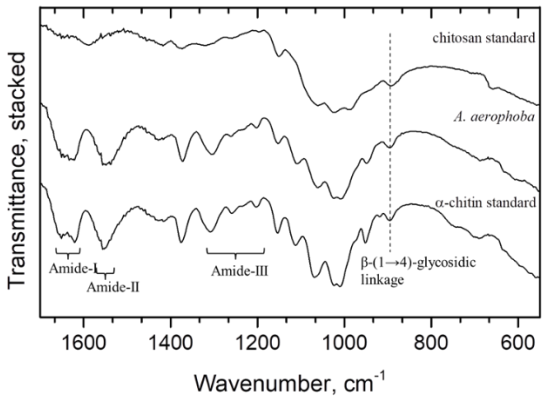

Figure 5. ATR-FTIR spectrum of the isolated scaffold from *Aplysina aerophoba* compared with the spectra of the α-chitin and chitosan standards.

By contrast, the chitosan standard spectrum displays the characteristic disappearance of the band at 1556 cm^{-1} (amide-II) and decreased intensity of the bands at 1621 cm^{-1} (amide-I), 1376 cm^{-1} [δ (CH3)], and 1308 cm^{-1} (amide-III). These changes arise due to deacetylation, which changes the amide group to an amine group. All spectra show the typical band at 895 cm^{-1} originating from the (1→4)-β-glycosidic linkages. Additionally, the spectrum of the matrix isolated from *A. aerophoba* by the microwave approach shows good agreement with the spectrum of the matrix isolated from the same sponge using the standard method. **Fig. 6** shows the Raman spectrum of chitin isolated from *A. aerophoba* using the microwave approach compared with those of the α-chitin and chitosan standards.

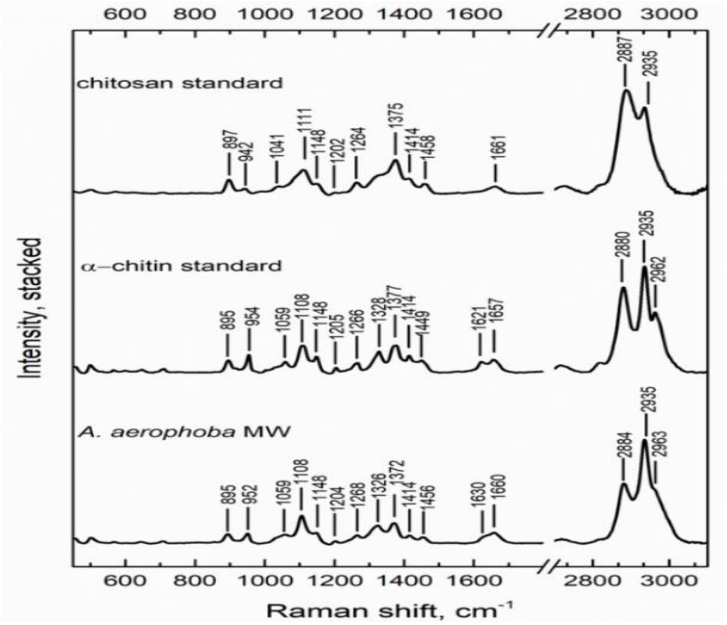

Figure 6. Raman spectrum of chitin isolated from *Aplysina aerophoba* using the microwave approach compared with the spectra of the α-chitin and chitosan standards.

The Raman spectrum of the scaffold isolated from *A. aerophoba* using the microwave approach displays good agreement with the spectrum of α-chitin. Given that chitosan is the deacetylated form of chitin, its spectrum displays the chemical changes as alterations of the intensity of the relevant peaks. Significant alterations are visible for the bands at 2280 cm^{-1} and 2962 cm^{-1}. The asymmetric vibrations of CH_3 at 2962 cm^{-1} disappear and the intensity of the peak at 2935 cm^{-1} decreases. Additionally, the amide peaks are less intense in the chitosan spectrum. For example, the peak at 1661 cm^{-1}, which in the spectra of both α-chitin and *A. aerophoba* chitin, is split into two different peaks (1657 cm^{-1} and 1621 cm^{-1}). The spectrum of the matrix isolated from *A. aerophoba* by the microwave approach does not display these changes in intensity, which indicates that no conversion from chitin to chitosan took place. Based on the high similarity of this spectrum to the spectrum of α-chitin, the material can be identified as α-chitin.

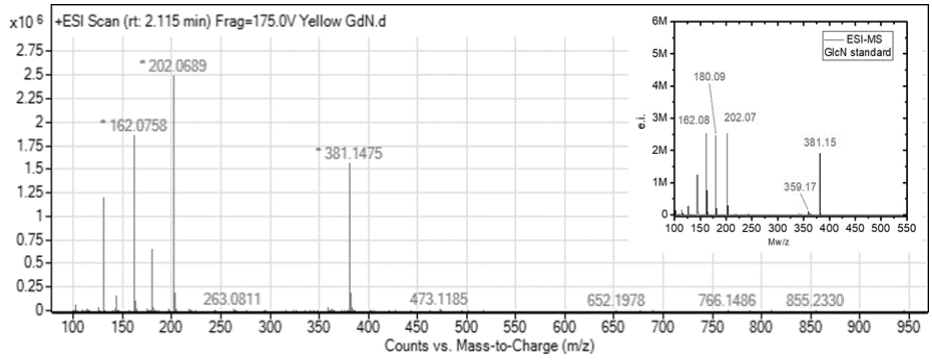

Figure 7. ESI-MS spectrum of chitin isolated from *Aplysina aerophoba* skeleton using the microwave approach. ESI-MS spectrum of the D-glucosamine standard (inner plot).

Electrospray-ionization mass spectroscopy (ESI-MS) is a standard method commonly used for the identification of D-glucosamine (dGlcN), which is a product of acidic hydrolysis of chitin [41]. The spectrum obtained after acid hydrolysis of the *A. aerophoba* chitinous matrix is in good accordance with the spectrum of the D-glucosamine standard and contains three main signals with $M_{w/z}$=162.08, 202.07, and 381.15 (Fig. 7). The signal at $M_{w/z}$=162.08 corresponds to a [M + H+] species with a molecular weight of 160.85, namely a dGlcN ion [$M-H_2O + H^+$] without H_2O, which is very common for molecules of this type [41,42]. The signals at $M_{w/z}$=202.07 and 381.15 correspond to [$M + K^+$] and [$2M + K^+$] species, namely potassium-bound dGlcN monomer and noncovalent dimer. A smaller, unlabelled signal appearing at $M_{w/z}\approx$180 corresponds to the form [$M + H^+$] of the dGlcN molecule.

Although it has been demonstrated that microwave irradiation has no impact on the chemical composition of the chitin isolated from *A. aerophoba*, SEM reveals significant microstructural changes. The isolated fibres show the typical wrinkled surface that occurs after the use of alkaline solution [48]. The harsh microwave treatment leads to the breakup of the multiple concentric layers that form the fibre, as has already been shown for *A. aerophoba* (Fig. 8).

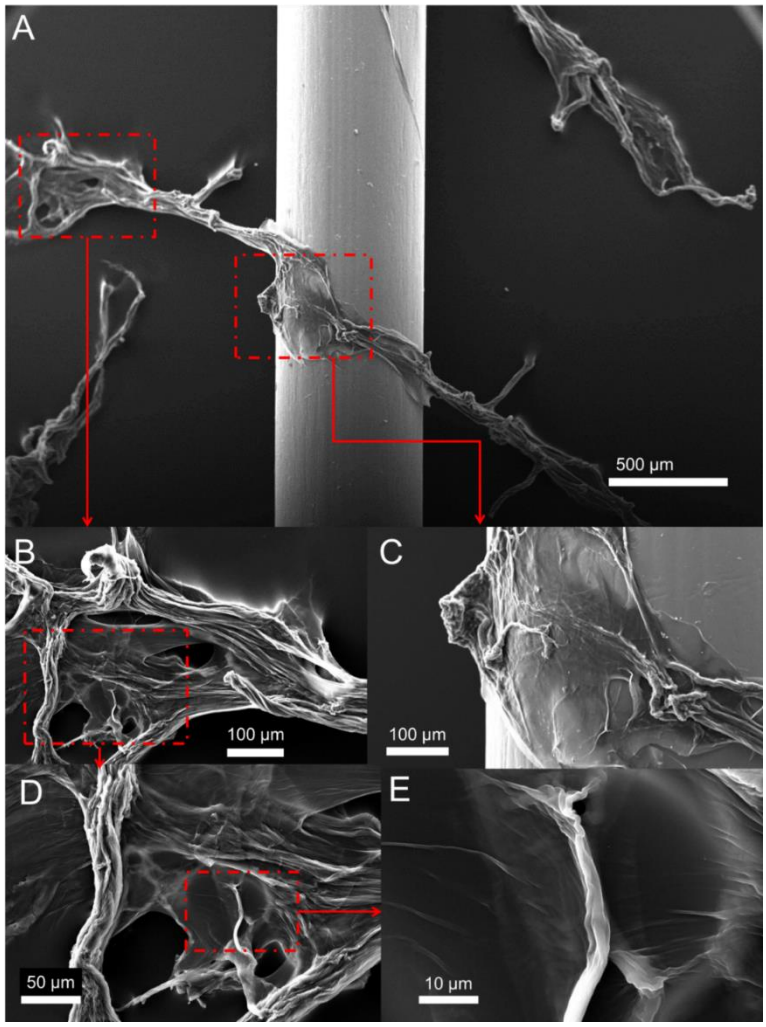

Figure 8. SEM microphotographs of the chitinous scaffold isolated using a microwave approach from *Aplysina aerophoba* display the breakup of the typical tubular microstructure. As a consequence, there exist both tubular parts (A) and parts that are fanned out (B–E).

Microwave irradiation has recently been proven to be a suitable tool for various processes in green chemistry. It is an advantageous technique for the extraction and modification of polysaccharides [49]. In that case, the benefits of microwave-assisted extraction are the reduction of extraction time and solvent consumption due to improved energy transfer, improved solvation reaction, and disruption of hydrogen bonds [50]. In the majority of cases, microwave irradiation is used for the deacetylation of chitin of various origin. A patent from 1979 describes the activation of chitin [51]. In this study, we have successfully isolated chitin from *A. aerophoba* by microwave-assisted extraction, preserving its chemical properties but altering its microstructure. A study from 2010 on the microstructure of *A. aerophoba* describes a multi-layered tubular structure that supports the absorption of liquids. Consequently, it has been applied as a liquid delivery

construct after the swelling of zirconyl salt solution [31]. Furthermore, it has been proven that the chitinous scaffold of *A. aerophoba* possesses cytocompatibility, which facilitates the attachment, growth, and proliferation of human stromal cells (hMSCs) [26,27]. The unique microstructural alteration of the isolated chitin scaffold by the microwave approach will affect such properties. As a result of the breakup precipitated by microwave irradiation, the usually concentric layers are fanned out. This can lead to better accessibility for cells or microorganisms. Therefore, further studies should concentrate on possible applications of these rapidly isolated renewable 3D chitinous scaffolds in biomedicine and tissue engineering, as well as in water and wastewater treatment—for example, as an adsorbent of pharmaceutically active compounds. Another important task relates to the detailed characterization of bromotyrosines isolated with this method. In the next stage, the mechanical properties of the chitinous matrix isolated using this approach should also be evaluated in detail.

4. Conclusion

In this study, it has been shown that it is possible to isolate chitin from the skeleton of the marine demosponge *A. aerophoba* using microwave irradiation. The entire isolation process required less than 41% of the treatment time of the standard method. It has been shown that no chemical changes occur as a result of the microwave irradiation. However, in regards to the microstructure of the three-dimensional scaffold, the harsh conditions lead to the breakup of the multiple concentric layers that form a fibre. Besides the microstructural alteration, the swelling capability of the isolated scaffold is preserved. Future investigations will focus on the impact of the morphological alterations on properties of the substance, supporting new fields of application.

5. Acknowledgements

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