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Three-dimensional chitin-based scaffolds from Verongida sponges (Demospongiae: Porifera). Part II: Biomimetic potential and applications

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ABSTRACT

In order to evaluate the biomedical potential of three-dimensional chitinous scaffolds of poriferan origin, chondrocyte culturing experiments were performed. It was shown for the first time that freshly isolated chondrocytes attached well to the chitin scaffold and synthesized an extracellular matrix similar to that found in other cartilage tissue engineering constructs. Chitin scaffolds also supported deposition of a proteoglycan-rich extracellular matrix of chondrocytes seeded bioconstructs in an *in vivo* environment. We suggest that chitin sponge scaffolds, apart from the demonstrated biomedical applications, are highly optimized structures for use as filtering systems, templates for biomineralization as well as metallization in order to produce catalysts.

1. Introduction

This is the second paper on three-dimensional chitinous scaffolds of poriferan origin. The chitin isomorphs isolated so far from arthropods (crabs, lobsters, shrimps, crayfish, king crabs, and insects) as well as mollusks (e.g., squids) occur in the form of granules, sheets, or powders [1,2] and not as three-dimensional scaffolds. The unique chitin-based scaffolds [3–5] found in sponges may therefore find applications in biomedicine, bioengineering, and materials science. Natural or biomimetic materials are in general believed to be interesting alternatives for a number of synthetic materials in the field of biomedicine [6]. The skeletons of sponges (Porifera) appear to possess several unique and suitable properties: (i) The ability to become hydrated. This is favorable for cell

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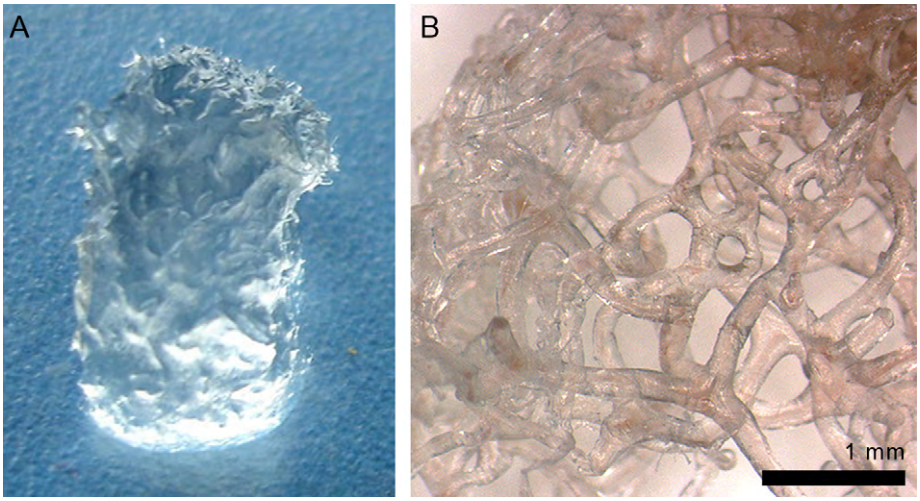

Fig. 1. Three-dimensional chitinous matrix isolated from *Aplysina cauliformis*. This matrix possesses a property to hold liquids including media for cell cultures (A). The practical value of this construct is due to it large internal surface area, which enables considerable liquid absorption to take place by capillary attraction (B).

adhesion. (ii) The presence of open interconnected channels created by the fiber network makes them an interesting host system, e.g., for cells. (iii) A tremendous diversity of skeletal architectures and fiber constructs within the phylum [7]. Collagenous marine sponges were already shown to be successful templates for the formation and support of musculoskeletal tissue *in vitro* and *in vivo* [7]. The skeleton of the commercial bath sponge, *Spongia* spp. (Class Demospongiae: Order Dictyoceratida: Family Spongiidae) is made of a regular network of spongin fibres with large surface areas and interconnected voids [8]. Apart from the aforementioned advantages of sponges, the presence of the biocompatible collagen (spongin) is another reason to make use of this material [7].

In addition to collagen, chitin has become a very attractive biomaterial for applications in tissue engineering and biomedicine during the last decade [1,2,9]. Chitin can be exploited as a biomaterial in two main ways: as bio-stable chitin or as a modified biodegradable material [1]. Because of the lack of "prefabricated" natural three-dimensional chitin scaffolds, several attempts have been made to process chitin into three-dimensional sponge-like materials with potential applications in tissue engineering. Abe et al. [10] produced a bioresorbable sponge-like β-chitin construct and used it as a scaffold for three-dimensional cultures of chondrocytes. The β-chitin was obtained from the pens of *Loligo* squid. Since this method results in pillar-shaped composites, it is possible to press-fit the material into articular cartilage defects without covering the periosteum or suturing the implant. The preparation of sponge-like materials from chitin has also recently been described by Suzuki et al. [11]. Several papers related to the application of chitinous scaffolds in tissue engineering have been recently published by the group of Professor Jayakumar [12–15]. Evaluation of the biomedical potential of the unique chitin-based scaffolds for tissue engineering purposes in regenerative medicine was the goal of the present paper.

2. Materials and methods

2.1. Sample preparation

Aplysina cavernicola was collected from the Mediterranean Sea by SCUBA diving from a population located between 14 and 16 m depth on a vertical, calcareous rocky wall substrate in Maire Island (Marseille, France). *Aplysina cauliformis*, *Aplysina fulva*, and *Aiolochroia crassa* were collected from the Caribbean Sea from a well illuminated coral reef located in the eastern part of Grand Bahama Island (Sweetings Cay, Bahamas) between at 4 and 8 m depth. *Aplysina aerophoba* was collected in the Adriatic Sea (Kotor Bay, Montenegro).

Sponge samples were put in ziplock bags underwater, brought back to the laboratory and frozen less than an hour after collection. The sponges were lyophilized prior to further treatment. The use of chemicals was avoided in the cleaning and preparing procedure of the skeletons.

Isolation of chitin-based scaffolds from Verongida sponges as well as identification of chitin is detailed described in the part I of this paper [16].

Isolation of chondrocytes, seeding of scaffolds, and cultivation of bioconstructs. The chitin-based scaffolds from *A. cauliformis* (Fig. 1) were used as a scaffold material for tissue engineering experiments. In all cell based experimental approaches, shape-stability and handling characteristics of the chitin scaffolds were excellent *in vitro* as well as *in vivo*.

Porcine articular cartilage was isolated from knees obtained from a local slaughterhouse. Chondrocytes were isolated from cartilage samples by collagenase B (1.5 mg/ml; Roche Diagnostics, Mannheim, Germany) and hyaluronidase (0.1 mg/ml; Serva, Heidelberg, Germany) digestion overnight at 37 °C. One million porcine chondrocytes were seeded into scaffolds immediately after isolation. In further experiments, porcine chondrocytes were expanded in low-glucose DMEM with 10% FCS and 100 units/ml penicillin–streptomycin and maintained in a humidified atmosphere of 6% CO_2 at 37 °C with 1.5×10^4 cells/cm^2 for two passages. Precultured chondrocytes were immersed in fibrinogen mixed with thrombin (Tissucol Duo S Immuno, Baxter, Germany) and immediately injected into chitin scaffolds. Fibrin gel was allowed to polymerize for 5 min. Cell-seeded chitin scaffolds were cultured in chondrogenic medium (DMEM supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid, 0.1 μM dexamethasone, 0.17 mM ascorbic acid-2 phosphate, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml BSA) to which 10 ng/ml TGFβ was added. After six weeks constructs were harvested. The cell distribution, cell vitality, and cartilage-like extracellular matrix synthesis were monitored.

2.2. Live/dead staining

To visualize cell vitality within the chitin–chondrocyte-constructs, living constructs were embedded into 2% low melting agarose in 1× PBS and cut into 50 μm sections using a vibratom (Leica VT 1000S, Germany). Slices were transferred to an object

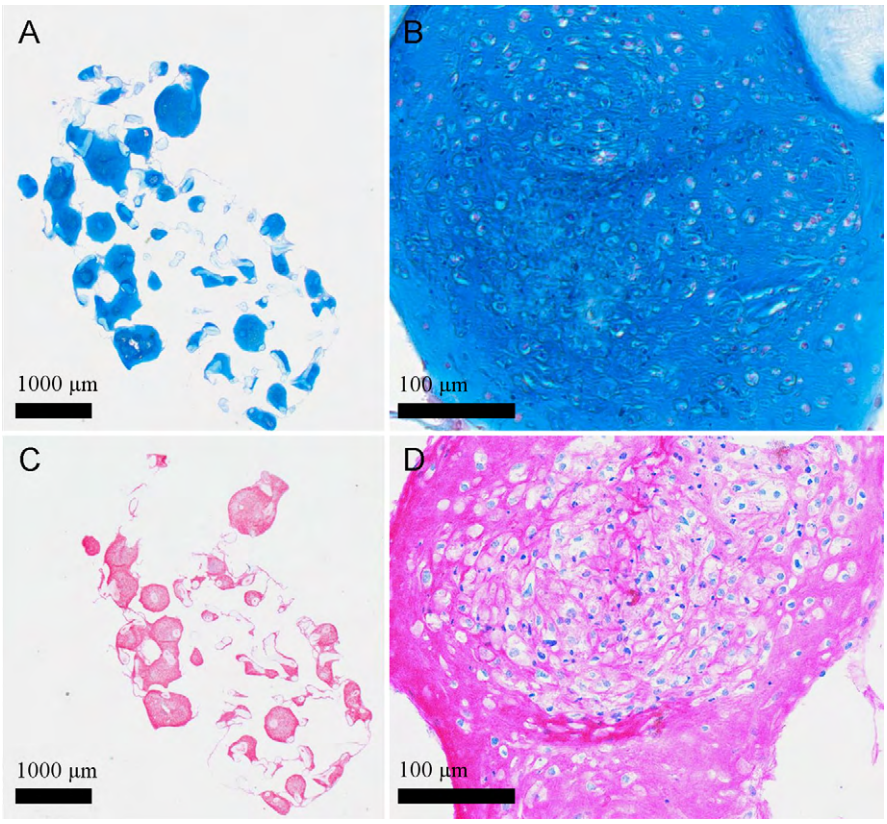

Fig. 2. Alcian blue and collagen type II staining of paraffin sections of primary porcine chondrocyte-seeded chitin scaffolds that were cultured under chondrogenic conditions for 6 weeks. (A) Positive alcian blue staining indicated a proteoglycan-rich matrix in all regions of the constructs. (C) Positive collagen type II immunohistochemistry (red) indicated production of a cartilage-like matrix. (B) and (D) are magnifications of (A) and (C), respectively. Scale bars represent 1000 μm in A, C and 100 μm in B, D. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

slide and stained with fluorescein diacetate (100 nM, FDA, Sigma) to visualize living cells in green and with propidium iodide (5 μg/ml, PI, Sigma) to stain dead cells in red. After incubation for 5 min in the dark at room temperature, slices were washed three times with PBS to remove excess dye, embedded in an aqueous mounting medium (Aquatex, Merck, Darmstadt), and analyzed immediately with a fluorescence microscope. FDA and PI were viewed at 380–490 or 465–550 nm excitation. Pictures were superimposed and aligned to reconstitute the whole specimen.

2.3. Histological and immunohistological evaluation of constructs

For histological evaluation, constructs were fixed in 4% formalin, dehydrated, and embedded in paraffin. Specimens were serially sectioned into slices of 5 μm. After deparaffinization, the sections were either stained with alcian blue or immunohistochemical staining was performed with an antibody recognizing collagen type II. Alcian blue (1%, Chroma, Köngen, Germany) staining was performed according to standard protocols, counterstained with nuclear fast red (Chroma, Germany), washed three times, dehydrated, washed with XEM and embedded in Eukitt. For collagen type II immunohistochemistry, sections were incubated with 2 mg/ml hyaluronidase (Merck, Germany, 700 WHO-U/mg) and 1 mg/ml pronase (Roche, Switzerland) in PBS at 37 °C for 15 and 30 min, respectively, followed by washing and blocking with 5% BSA (Sigma). Sections were then incubated with a primary mouse antitype II collagen monoclonal antibody (1:1000 in 1% BSA, ICN) overnight at 4 °C, washed, incubated with biotin–SP-conjugated goat antimouse IgG (1:500 in TBS, Dianova, Germany) and, finally, incubated with streptavidin–biotin complex/AP for 30 min at RT, washed and stained with fast red substrate (Roche). Nuclei were counterstained with hematoxylin and permanently mounted with Aquatex (Merck, Rahway, NJ).

2.4. Ectopic transplantation of bioconstructs

For ectopic transplants, human chondrocytes were isolated from articular cartilage that was obtained from knee samples derived from total endoprosthesis surgery. The study was approved by the local ethical committee and informed consent was obtained from all individuals included in the study. One million of freshly isolated human chondrocytes were immersed in fibrinogen, mixed with thrombin (Tissucol Duo S Immuno, Baxter, Germany), and immediately injected into chitin scaffolds. Fibrin gel was allowed to polymerize. After 30 min, bioconstructs were transplanted ectopically in subcutaneous pouches that were prepared on the backs of anesthesized male SCID mice (ages 8–10 weeks; Charles River, Sulzfeld, Germany). Mice were killed 4 weeks later and samples were harvested and evaluated by histology. The animal experiments were approved by the Local Animal Experimentation Committee Karlsruhe.

3. Results and discussion

3.1. Biomedical potential of three-dimensional sponge chitin

Chondrocyte culturing experiments (see Section 2) were performed on the described three-dimensional chitin-based Verongida sponge scaffolds in order to evaluate their biomedical potential. Deposition of a cartilage-like extracellular matrix was evident after six weeks of culturing primary porcine chondrocytes within a scaffold from *A. cauliformis* (Fig. 1A and B). Chondrocytes

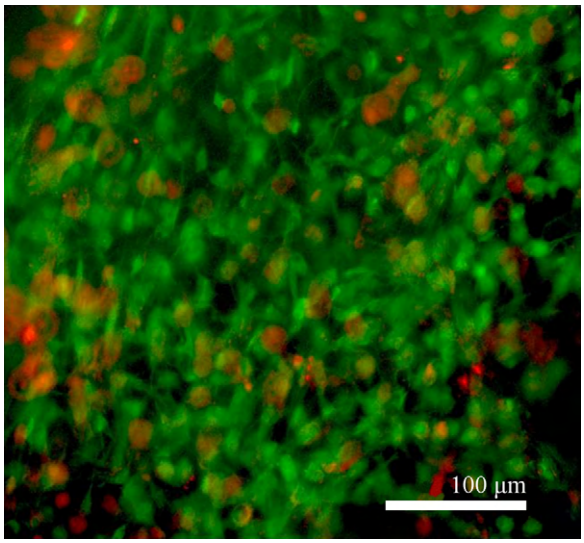

Fig. 3. Live/dead staining of chondrocyte-seeded chitin scaffolds after 6 weeks in vitro. A high number of living cells (FDA-staining, green) and a low number of dead cells (PI-staining, red) were present in the constructs. Scale bar represents 100 μm. A representative image of serial sections is shown. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

were present in all chitin-free regions. Positive alcian blue staining (Fig. 2A and B) indicated deposition of proteoglycan-rich extracellular matrix and the presence of cartilage-specific collagen type II was demonstrated by immunohistochemistry (Fig. 2C and D). Thus, freshly isolated chondrocytes attached well to the chitin scaffold and synthesized an extracellular matrix similar to that found in other cartilage tissue engineering constructs.

To evaluate cell vitality and matrix deposition for the case of precultured cells, chondrocytes were expanded for two passages, injected into chitin scaffolds, and constructs were cultured for 6 weeks. Cell vitality assessed by live/dead staining revealed a high number of viable green fluorescent cells within the scaffold and a few dead cells (red, Fig. 3).

Precultured chondrocytes also deposited proteoglycan-rich collagen type II positive extracellular matrix within the chitin scaffolds (Fig. 4).

3.2. Support of ectopic cartilage formation by chondrocyte-seeded chitin bioconstructs

In order to investigate, whether the chitin scaffolds may support ectopic cartilage formation, chitin sponges were seeded with

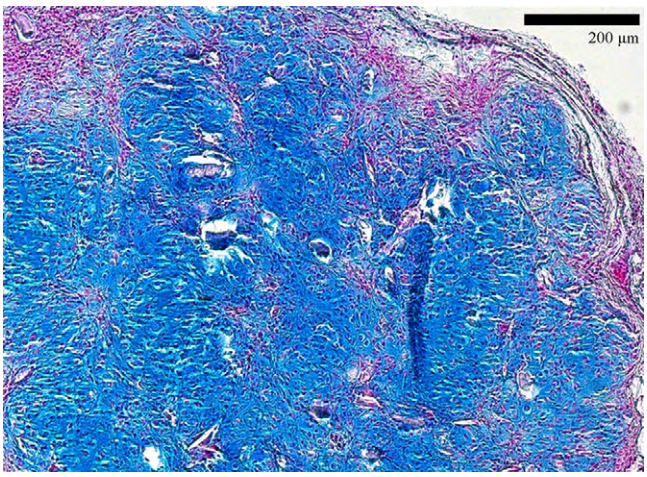

Fig. 5. Ectopic cartilage formation in chondrocyte-seeded chitin scaffolds. Alcian blue staining of paraffin sections of the constructs seeded with freshly isolated primary human chondrocytes after 4 weeks *in vivo*. The positive staining indicated proteoglycan-rich extracellular matrix deposition typical for cartilage in the explants. Scale bar: 100 μm.

primary human articular chondrocytes that were transplanted subcutaneously into SCID mice. Transplants were harvested 4 weeks later and histological evaluation revealed that in regions where human chondrocytes were present, an alcian blue positive cartilage-typical proteoglycan-rich matrix was deposited in the chitin scaffolds (Fig. 5). Thus, chitin scaffolds also supported deposition of a proteoglycan-rich extracellular matrix of chondrocytes seeded bioconstructs in an *in vivo* environment.

As demonstrated here, the unique scaffolds found in Verongida sponges may find interesting biomedical applications, e.g., in cartilage tissue engineering. One remarkable advantage of Verongida sponges is the possibility to culture them in primmorph-like cultures [17] as well as under marine ranching conditions. Thus, it was reported [18] that *A. aerophoba* is suitable for sponge-culture showing a survival rate of 80%. The peculiar growth of transported fragments suggests a potential role to improve sponge dispersal and recruitment for both conservation and biotechnological purposes. On the basis of the high biomimetic potential of the results obtained, we propose that biotechnological processes for the aquacultural cultivation of different Verongida sponges should be developed in the near future.

Apart from the demonstrated biomedical applications, the materials properties of chitin open the perspective to use the scaffolds as a support, e.g., for metals in order to produce catalysts.

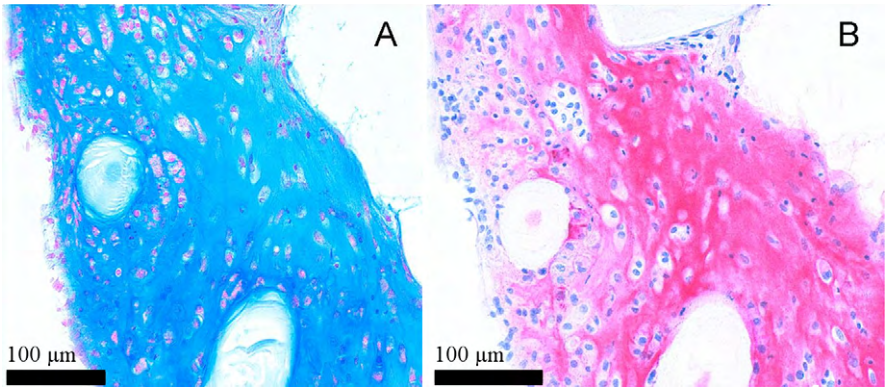

Fig. 4. Precultured porcine chondrocytes deposited an alcian blue positive proteoglycan-rich (A) and collagen type II positive (B) cartilage-like extracellular matrix in the chitin scaffolds. Cells were precultured for 2 passages and cell-seeded chitin constructs were cultured for 6 weeks in chondrogenic medium in the presence of 10 ng/ml TGF-β. Scale bars represent 100 μm.

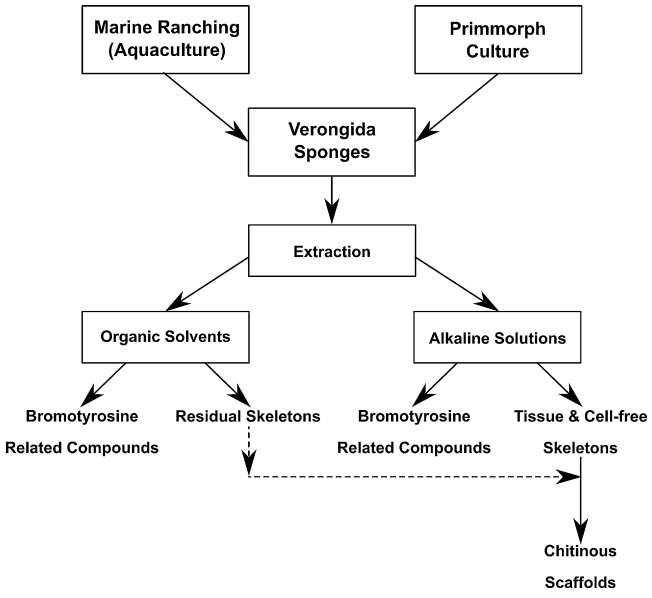

Fig. 6. Schematic view of the possible uses of Verongida sponges.

Thermogravimetric analysis of purified non-mineralized chitin revealed that this aminopolysaccharide may be stable up to 360 °C [19,20]. Surface metallization of chitin requires temperatures up to 300 °C and pH between 1 and 12 [21,22]. Therefore, chitin-based three-dimensional constructs with functionalized (e.g., metalized) surfaces seem to be feasible. In summary, it can be stated that Verongida sponges including their skeletons (Fig. 6) may be a natural resource for the production of diverse products in a biomimetic manner.

4. Conclusions

Increasing interest is currently devoted to the biotechnological potential of marine sponges: unique and innovative substances have been discovered in sponges which exhibit cytotoxic, antifouling, antitumoral, antibiotic, antiviral or cytoprotective, enzyme-inhibitory, anti-inflammatory and anti-Alzheimer activities [23]. Over the past 30 years, a huge number of biologically active secondary metabolites have been isolated from marine sponges, many of them from the order Verongida. Species of this order are biochemically characterised by the production of brominated tyrosine derivatives. However, three-dimensional chitin-based scaffolds isolated from sponges are promising candidates for practical applications in tissue engineering, especially for processes where chondrocytes are used.

It was shown for the first time in this work that freshly isolated chondrocytes attached well to the chitin scaffold and synthesized an extracellular matrix similar to that found in other cartilage tissue engineering constructs. Chitin scaffolds also supported deposition of a proteoglycan-rich extracellular matrix of chondrocytes seeded bioconstructs in an *in vivo* environment. Corresponding experiments with osteoblasts, osteoclasts, as well as fibroblasts are in progress.

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