

Chapter 13

Chitinous Scaffolds from Marine Sponges for Tissue Engineering



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Abstract Chitin as a biological material which has been identified in skeletal structures of a broad variety of unicellular (yeast, protists, diatoms) and multicellular (sponges, corals, worms, molluscs, arthropods) organisms is recognized as natural template with good perspectives in modern biomedicine. This chapter provides first insights into prospective applications of naturally prefabricated three-dimensional chitinous scaffolds from selected marine sponges in tissue engineering. This became possible only owing to the recent discovery of poriferan chitin which provoked

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renewed multidisciplinary interest driven by growing demand in novel biomimetic materials. Here, we focused on both demosponges of Verongiida order as a renewable source of chitin scaffolds with jewelry designs, and human mesenchymal stromal cells having high therapeutic potential. The chapter covers approaches for isolation of scaffolds from the chitin-bearing marine sponges, nuances of their interaction with human cells and cryopreservation potential.

Keywords Marine sponges · Chitin · Chitinous scaffolds · Tissue engineering · Stem cells · Cytocompatibility · Biomimetic approaches · Cryopreservation

13.1 Introduction

Tissue engineering is a multidisciplinary research field based on principles of engineering and biology with the final goal to design functional tissue bioequivalents for subsequent grafting and regeneration of damaged tissue [1]. Recent trends in this highly promising discipline are keeping pace with the growing clinical demand of new scaffolding materials worldwide (reviewed in [2]). Both synthetic and natural biomaterials offer a broad complementary spectrum of characteristics one may choose to design scaffolds with target application requirements (for review see [3]). However, to pre-shape the biomaterials into bulk three-dimensional (3D) structures and confer their appropriate structural and functional advantages, technically complex fabrication processes are necessary. In this regard, marine sponges represent a rich source of evolutionary refined 3D scaffolds with unique properties for biomedical application [4, 5] which makes them attractive candidates in particular for bone tissue engineering applications [6]. However, their overall tissue engineering potential is still largely underexplored.

Chronologically, first reports addressing cytocompatibility of skeletons isolated from marine sponges with mammalian cells were described for so-called commercial sponges, also known as bath sponges. According to Laubenfels and Storr, “the commercial sponge is the macerated and dried skeleton of one of the sponge animals that has no proper spicules. It must be from a species whose skeleton consists of spongin fibers, and furthermore, these fibers must continue to be elastic or ‘spongy’ even after having been dried” [7]. Intriguing historical information has been recently reported in the review by Jesionowski and co-workers: “successful attempts to use spongin in the form of commercial sponges as biomedical implants have been reported since the 18th century. Fragments of the bath sponge skeleton were used as small prostheses in early “plastic surgery”. Revolutionary results can be found in the paper published by Hamilton in 1881 under the title “*On sponge-grafting*”. In the reported case, a woman underwent surgery for the removal of a mammary tumor, during which a large area of skin was removed. This skin was replaced with a thin slice of aseptic sponge skeleton, which, ten days after the surgery, was observed to be vascular, and three months later, was covered with epithelial tissue” [8].

Table 13.1 Examples of cytocompatibility of collagenous marine sponges with mammalian cells

Sponge species	Type of mammalian cells	Reference
Species of <i>Spongia</i> (later classified as <i>Spongia officinalis</i>)	Human osteoprogenitor cells	[9]
<i>Chondrosia reniformis</i>	Mouse osteoblasts (immortalized cell line 7F2)	[12]
Unidentified sponge species of the genus <i>Hippospongia</i> , the genus <i>Callyspongia</i> , and the family <i>Chalinidae</i>	Mouse calvarial-derived primary osteoblasts	[13]
<i>Spongia officinalis</i>	Human bone marrow stromal cell; human fetal tissue-derived mesenchymal stem cells	[14]
Family <i>Callyspongiidae</i>	Mouse primary osteoblasts	[15]
<i>Spongia agaricina</i>	Primary human endothelial cells derived from umbilical vein, human dermal microvascular endothelial cells, osteoblast-like MG63 cell line	[16]
<i>Biemna fortis</i>	MC3T3 (osteoblast precursor cell line derived from mouse calvaria)	[17]
<i>Spongia agaricina</i>	Human foetal osteoblasts	[18]
<i>Petrosia ficiformis</i> , <i>Agelas oroides</i> and <i>Chondrosia reniformis</i>	Immortalized mouse lung fibroblast cell line (L929)	[19]
<i>Chondrosia reniformis</i>	L929 mouse fibroblast cell line, human keratinocyte cell line	[11]

In the recent literature, spongin-based sponges have been called “collagenous sponges” by David Green [9]. The direction to use such sponges for the aims of tissue engineering and biomedicine is well in trend up today (Table 13.1). In contrast to spongin-based sponges, such species as *Chondrosia reniformis* possess ability to synthesize collagenous networks (see for review [10]) with a good chance to be applicable in tissue engineering [11].

The later discovery of chitin in marine [20, 21] and freshwater [22] sponges opened not only a novel source of this attractive biomaterial but also a renewable source of unique 3D scaffolds [23, 24] due to the possibility to cultivate chitinous demosponges using aquaculture technologies. Richness and diversity of scaffold designs uncovered in chitin-producing sponges created numerous opportunities to advance their application in biomedical field. Analogous to silica-collagen biocomposites of poriferan origin [25, 26], their chitin counterparts [27] hold great promise due to increased mechanical stability and great variety of scaffold templates that could be easily adapted to tissue engineering needs using biomimetic approaches.

Demonstrated possibility to functionalize poriferan chitinous scaffolds with desired compound of biomedical interest as in the case with SiO₂-chitin [28, 29], ZrO₂-chitin [30, 31] and Fe₂O₃-chitin composites [32, 33] considerably increases

their tissue engineering value. The further development and standardization of purification protocols based on alkali-acid treatment led to discovery of chitin scaffolds in new sponge species, e.g., *Aplysina fistularis* [34]. Collectively, all this paved the way to first in vitro and in vivo cytocompatibility studies involving chitinous sponges and various cell types. For instance, it was shown that the scaffolds derived from *A. cauliformis* supported long-term growth and functionality of primary porcine and human chondrocytes. They were capable of depositing proteoglycan-rich collagen type II positive extracellular matrix in vitro as well as forming ectopic cartilage after subcutaneous transplantation into mice with severe combined immune deficiency [24]. This stimulated further investigations which demonstrated the cytocompatibility of Verongiida sponges *Aplysina fulva*, *Aplysina aerophoba* and *Ianthella basta* with human adipose tissue-derived MSCs [35]. The most recent identification of chitin in new Verongiida species such as *Pseudoceratina purpurea*, in non-Verongiid marine demosponges *Mycale euplectellioides* [36, 37] as well as in Red Sea demosponges sponges *Acarnus wolffgangi* and *Echinoclathria Gibbosa* [38] which can be cultivated at large scales using marine aquaculture stimulates future evaluation of their tissue-engineering capabilities.

13.1.1 Chitinous Scaffolds from Marine Sponge Ianthella Basta: Prospects for Tissue Engineering

Decellularization of three-dimensional organ matrix and its repopulation using stem cells is a fundamental concept of tissue engineering [39]. Acellular matrix can act as a natural platform providing mechanical support and microenvironment required for cell infiltration, proliferation as well as differentiation into target tissues. From a number of marine sponges in which chitin has been discovered, one species of so called “elephant ear sponge” *Ianthella basta* deserves particular attention for its chitinous matrix structurally defined in a unique manner [40]. This matrix can be easily isolated and characterized in a controllable manner using standardized processing workflow as proposed by Ehrlich et al. [20]. This method ensures complete removal of sponge cellular debris and inorganic components while maintaining structural integrity and biomechanical properties of scaffold framework. One commercial prerequisite for *Ianthella basta* application in tissue engineering is its big size and relatively high growth rates. *Ianthella basta* colonies reaches sizes up to 35,000 cm² in its natural environment in the Indo-Pacific region [41].

Figure 13.1 shows representative pictures of original *Ianthella basta* sample (a) and purified chitinous framework made up of numerous repeating units of square-shape macropores with the diameter up to 2 mm (b). Such gauze bandage-like scaffold geometry and a broad use of chitin in production of wound healing coatings [42] make these scaffolds attractive especially for wound management. Excellent hemo- and bacteriostatic activity of chitin successfully demonstrated in numerous animal and

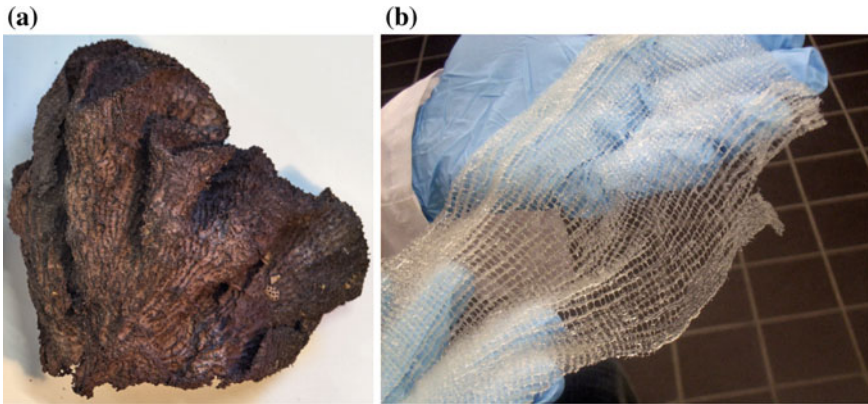


Fig. 13.1 Photograph of 15 cm-large fragment of freeze-dried *Ianthella basta* demersponge as collected (a) and bulk chitinous 3D scaffold obtained after step-wise purification procedure (b) which resembles the original shape of the sponge skeleton. The building block of the mesh is represented by square chambers firmly linked to each other via tubular, intercalated chitin microfibers (for details see Brunner et al. [40] and Ehrlich [4, 5])

clinical studies on wound healing (review in [43]), led to commercializing of chitin dressings such as Chitopack S[®], Chitopack P[®], Chitopack C[®] and Bechitin[®].

Figure 13.2 is an illustration of a single square shaped cell having complex capillary network formed by translucent interconnected chitin fibers (a) with open axial channels inside (b). Such a fiber composition and overall large internal surface area of sponge's skeletons enables considerable liquid absorption and transport of gases and nutrients throughout network by capillary forces (for details see [30]). This provides favorable microenvironment necessary for cell attachment and expansion as well as dynamic cell-cell and cell-matrix interactions within 3D chitinous matrix. Capillary-like structure of *Ianthella basta* fibrous scaffolds and overall beneficial effects of chitin on angiogenesis may trigger proper oxygenation and nutrient supply favorable for neovascularization which plays a crucial role in tissue engineering (see for overview [44]) and is very challenging [45]. We could further assume that remarkable fluid-absorbing properties shown for marine sponges biopolymers-based skeletons may be most likely applicable to wound healing field due to effective absorption and retention of wound exudates.

Sterilization is an essential step in manufacturing of tissue-engineered scaffolds to provide contamination-free *in vitro* cultivation in cell culture plates or bioreactors and engineered products of high quality [46], ideally, not compromising scaffold composition and mechanical properties. Chitin from marine organisms is a highly thermostable material having the range of the thermal degradation of 300–460 °C [47] and, because of that, it is extensively used as a versatile template for extreme biomimetics approaches (for review see [48]). Furthermore, according to the literature chitin has high glass transition temperature around 335 °C [49] and is insoluble in most regular organic solvents. In our disinfection studies, we used autoclaving

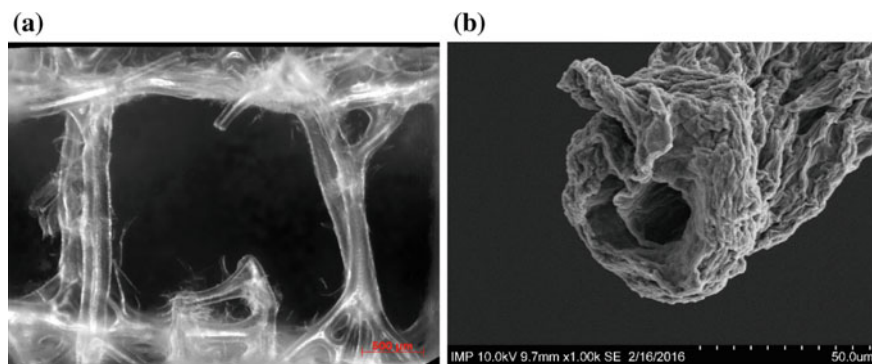


Fig. 13.2 Microimagery of *Ianthella basta* chitinous scaffold. Stereomicroscope image of a typical scaffold cell composed of translucent capillary-mimicking chitin skeletal fibers (a) and SEM image of a single chitin fiber with a well discernable internal axial channel. Such composition of poriferan chitin structural elements may favor nutrient supply for cell ingrowth in 3D environment

[50], 70% ethanol and supercritical carbon dioxide treatments [51]. Neither damage to chitinous scaffolds derived from *Ianthella basta* nor change of their cytocompatible properties was observed after sterilization. Moreover, they were stable in long-term culture.

As the next step, such sterilized scaffolds were seeded with human mesenchymal stromal cells (hMSCs) and their viability was determined using live-dead staining. As can be seen from Fig. 13.3, chitinous scaffolds from *Ianthella basta* are non-toxic to cells on day one (a) as well as day 14 (b) after seeding. An increase in the number of marked cell colonization on the scaffolds as time progresses suggests there are biological cross talks, which supports high cell viability and promoting cell proliferation. Such 3D platforms constructed from chitinous scaffolds and selected human cells may serve as an *ex vivo* engineered skin model for drug screening and cosmetic testing that would better mimic physiological conditions compared to cell expansion in 2D monolayer culture [52]. To support this hypothesis, recent publication revealed that alpha-chitin nanofibers and nanocrystals prepared of crab shell have protective effect on epithelial cells and reduce proinflammatory cytokine content as demonstrated in 3D human tissue-engineered skin model [53]. Later, the same research group reported that chitin nanofibers have not only anti-inflammatory and antioxidant properties but also are protective against ultraviolet radiation [54]. The similar future research on anti-inflammatory effects of poriferan chitin is of high fundamental and practical importance.

Composition, stiffness, nanotopography and specific biomechanical signals provided by certain 3D scaffolds types govern alignment, proliferation, migration and, importantly, differentiation of stem cells to certain lineages (for review see [55]). All these characteristics are important to consider in order to design scaffolds with suitable microenvironment in which cells can reside and acquire corresponding phenotypic patterns. In our experiments with chitinous scaffolds from *Ianthella basta*,

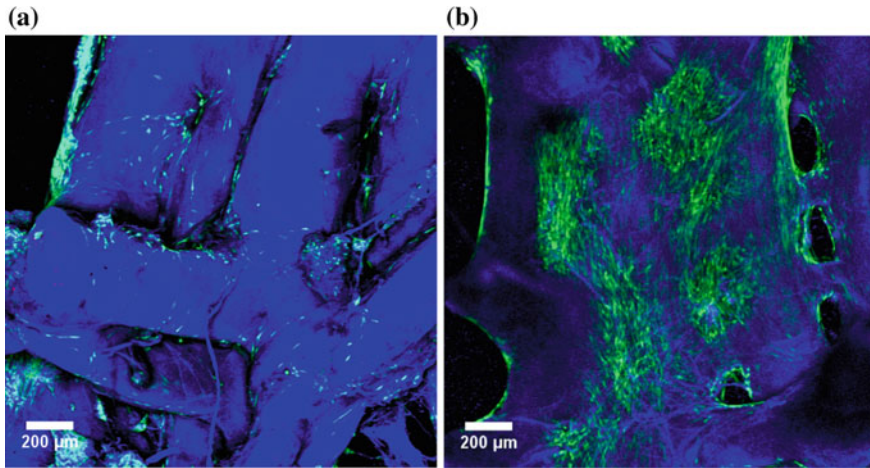


Fig. 13.3 Representative fluorescent images of chitinous scaffolds derived from *Ianthella basta* seeded with hMSCs on day one (a) and day 14 (b) in culture. Chitin is stained with specific dye Calcofluor white and exhibits intensive blue fluorescence even after very short light exposure time (lower than 1/500 s). Live cells are stained with fluorescein diacetate and exhibit green fluorescence. Dead cells are stained with ethidium bromide and have red fluorescence. Only few dead cells, spread green cells and visible increase in cell number with time indicates excellent scaffold cytocompatibility with hMSCs

two differentiation lineages adipogenic and osteogenic, were successfully generated. Figure 13.4a demonstrates sheets of adipogenic-stimulated hMSCs in 3D chitinous scaffolds with clearly discernable lipid droplets characteristic for successfully differentiated adipocytes. Similarly, Fig. 13.4b displays sheets of osteogenic-differentiated cells expressing alkaline phosphatase differentiation marker. Such formation of tissue-like structures with differentiated cells could be used for studying differentiation in 3D culture systems compared to cell monolayer cultures.

New advances in transplantation of stem cells, tissues and organs require development of efficient methods for their long-term storage [56]. Cryopreservation process usually includes incubation of a sample with cryoprotective solution, cooling to sub-zero temperatures, storage for a certain period of time, thawing, and removal of the cryoprotectants and recovery of cells under physiological conditions.

During cooling, crystallization event occurs which in turn comprises of ice nucleation and crystal growth. In this context, we were the first who reported on cryopreservation of human cells within scaffolds derived from marine sponges [57] with a moderate cell recovery following cryopreservation (higher than 50%). These results were significantly improved in follow-up studies using sucrose as extracellular cryoprotectant in combination with dimethyl sulfoxide (DMSO) as an intracellular one (unpublished data). Similar results were reported on cryopreservation efficiency of engineered epithelial sheets based on keratinocytes seeded and frozen on chitosan-gelatin membranes [58] where DMSO-based cryopreservation was con-

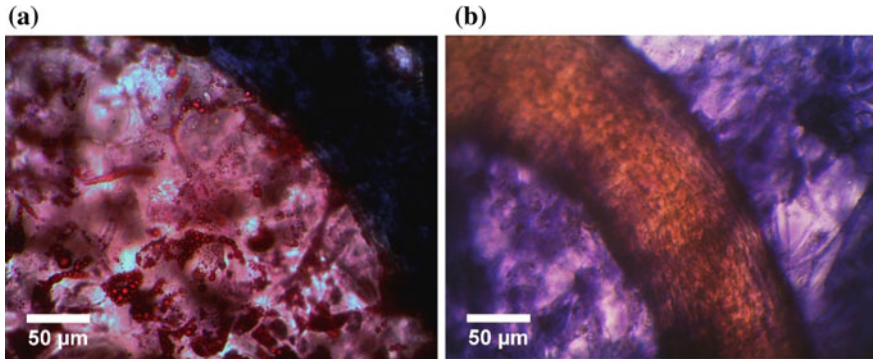


Fig. 13.4 Light microscopy pictures of hMSCs, those differentiation towards adipogenic and osteogenic lineages was induced. Accumulation of lipid droplets is visualized by bright red color after classical Oil Red O staining (a) and alkaline phosphatase gives intense blue color after Fast blue staining (b) indicating early osteogenesis, respectively. This finding shows that easy-to-isolate chitinous scaffolds from *Ianthella basta* support assembly of hMSCs into cell sheets in long-term culture and promote their multilineage differentiation in this unique naturally prefabricated 3D microenvironment

siderably improved through the introduction of another disaccharide trehalose. In our cryomicroscopy studies, we did not reveal any dramatic changes in scaffold structure after cryopreservation utilizing 10% DMSO (Fig. 13.5).

The main rationale behind cryopreservation studies in tissue engineering implies the ability to prepare functional tissue substitutes in advance and develop effective storage strategies for their retrieval upon demand in addition to enhancing the logistics by implementing continuous ‘in-line process’. Interestingly, it has recently been shown that inherent properties of a material, more specifically its glass transition temperature and alignment of scaffold fibers as well as viscoelastic properties, drastically influence the fate of cryopreserved adherent cells [59]. The authors showed the interesting observation that increase in scaffold elasticity mitigates differential thermal contraction as compared to more stiff scaffolds of the same material and thereby enhances post-thaw cell recovery. Consequently, to prevent excessive shrinkage during freeze-thawing, the elasticity of poriferan scaffolds could be adjusted by modifying purification parameters and it is a point worth investigating. Other promising cryopreservation tools such as vitrification and directional freezing can be examined on *Ianthella basta* scaffolds for the preservation of stem cells in 3D environment [60].

Thus, we showed that demosponge *Ianthella basta* due to its ability to produce unique, flat 3D scaffolds (Fig. 13.1b) is an appealing objective for tissue engineering applications. The feasibility of culturing these sponges in sea-ranching conditions and their high growth rates may provide a sustainable source of chitinous porous scaffolds. These scaffolds could be purified and produced with different size and shape in a controlled manner, sterilized and seeded with corresponding stem cells. The derived chitinous scaffolds support attachment, proliferation and differentiation

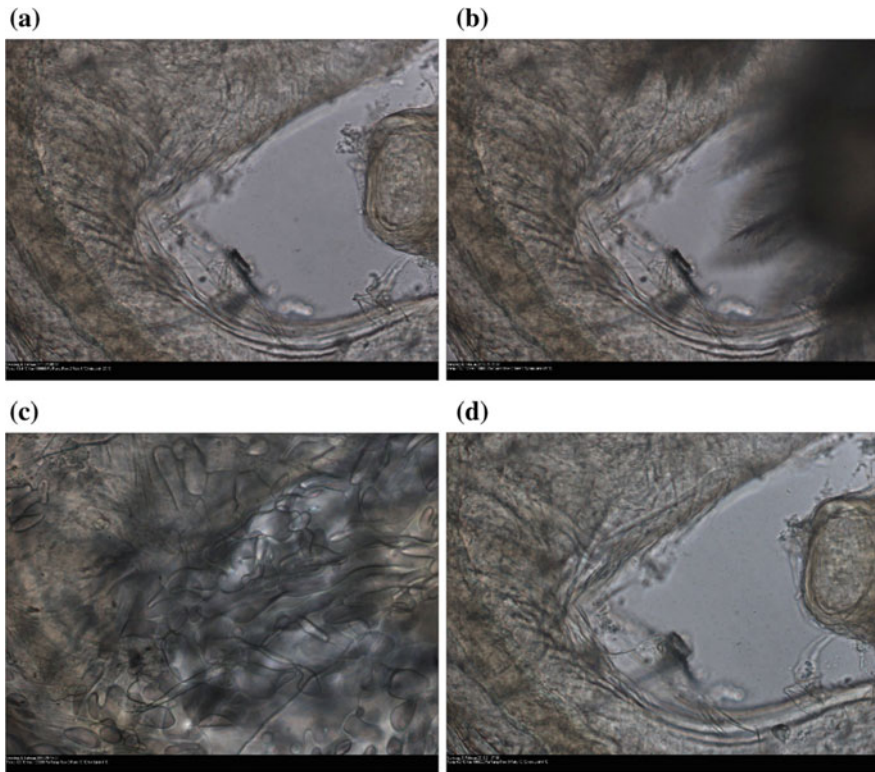


Fig. 13.5 Cryomicroscopy images of the cell-free chitinous scaffolds derived from *Ianthella basta*: **a** before the ice nucleation; **b** nucleation event at $-13\text{ }^{\circ}\text{C}$ with typical dendritic ice crystals; **c** ice melting at $-3\text{ }^{\circ}\text{C}$; and **d** after thawing back to $+4\text{ }^{\circ}\text{C}$. No apparent alterations in scaffold shape and structure after thawing have been observed

of hMSCs and should be attractive as wound dressings, 3D drug screening platforms as well as cryobiological models.

13.1.2 3D Chitinous Scaffolds from Marine Sponges Aplysina Aerophoba and Aplysina Fulva: Interspecies Biocompatibility with Human Cells

Another area of interest to many multidisciplinary research teams is motivated by the fascinating microarchitecture and structural properties of other demosponge *Aplysina aerophoba* belonging to *Aplysinidae* family (order Verongiida). These sponges represent a renewable source of scaffolds due to their abundance and standardized cultivation in laboratory and mariculture, for instance, in the Adriatic Sea [61]. In particular, in Boka Kotorska Bay of the Adriatic Sea, significant amounts

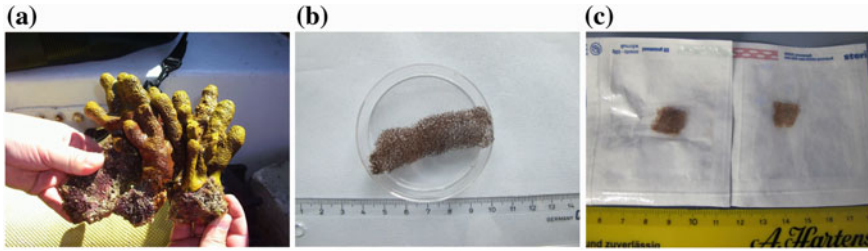


Fig. 13.6 Technological route for derivation of chitinous scaffolds from *Aplysina aerophoba* and their preparation for subsequent in vitro culture. **a** *Aplysina aerophoba* fragment from the marine farming facility after collection in the Adriatic Sea (Kotor Bay, Montenegro); **b** Purified 3D skeleton of *Aplysina aerophoba* with preserved original shape; **c** Derived chitinous scaffolds in vacuum sealed packages before (left) and after (right) sterilization using supercritical carbon dioxide treatment

of this sponge could be obtained in a sustainable way. Furthermore, ready-to-use chitinous scaffolds from this sponge have recently become commercially available from BromMarin GmbH in Germany.

In a natural habitat, this sponge possesses finger-like cylindric body (Fig. 13.6a). After lyophilization of sponge skeleton, additional processing steps include step-wise removal of water-soluble salts and impurities, proteins and residual pigments, calcium and magnesium carbonates in respective treatment solutions [23]. Such isolation results in obtaining bulk volumetric mesh (Fig. 13.6b) which could be subsequently sterilized, for example, by subjecting to supercritical carbon dioxide treatment (Fig. 13.6c). This technique is highly effective for sterilization of decellularized biomaterials and does not exert any damaging or cross-linking impact on them [62].

To better comprehend the structural features of the scaffolds obtained from *Aplysina aerophoba*, a more detailed characterization of fiber morphology is provided hereinafter. SEM analysis revealed that the resulting macroporous finger-like latticework of *Aplysina aerophoba* has a very complex naturally branched spatial design (Fig. 13.7a) and is totally different from a more planar *Ianthella basta* scaffolds. Morphologically, the derived scaffolds resemble human cancellous bone. The stratified composition of chitinous fiber clearly visible on Fig. 13.7b gives reasons to assume that its external layers might provide substrate for cell anchoring and distribution while the inner layers endows fibers with increased mechanical strength. The biomimetic strategies aiming at producing multi-layer scaffolds are increasingly being used in tissue engineering, for example, for musculoskeletal regeneration or small intestine [63] to mimic native layered tissue [64]. The presence of microchannels within the fibers of *Aplysina aerophoba* chitin (Fig. 13.7c) and apparent fiber interconnectivity may ensure effective circulation of nutrients over the chitinous ducts sufficiently deep into central parts of the scaffolds to facilitate corresponding cell ingrowth. The complete diversity of intriguing structural peculiarities of scaffolds isolated from *Aplysina aerophoba* demosponge convinced us to evaluate their behavior in vitro.

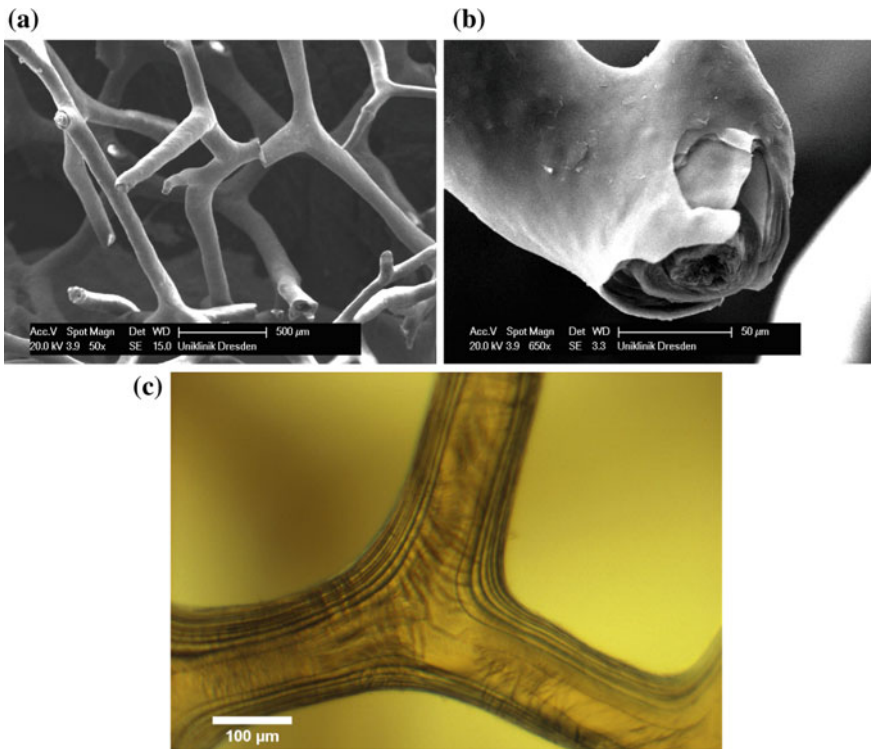


Fig. 13.7 Detailed representation of *Aplysina aerophoba* chitin scaffold microstructure. **a** SEM image of spatial arrangements of interconnected chitinous fibers as the basic structural matrix of the 3D scaffold; **b** SEM image of an individual chitinous fiber with typical radially oriented multilayers. Bright field image (**c**) shows the internal structure of selected chitinous fiber with tubular inner part

Cell adhesion is a fundamental feature which serves for cell communication with a substrate and other surrounding cells and is a pivotal requirement to be met when designing 3D scaffolds (for review see [65]). Moreover, exploring how cells adhere, flatten and spread in a 3D environment are more appropriate compared to 2D culture in an attempt to understand cell behavior in physiological conditions. It is known that in a standard monolayer culture, cells possess a lower vertical height whereas in a 3D scaffold cells are able to spread in all three dimensions (for review see [66]). The overall affinity of a cell for a scaffold determines its future fate achieved through cell migration, proliferation and differentiation. As synthetic materials may lack sites for cellular adhesion, they are often functionalized using specific attachment-enhancing molecules, treated by a variety of chemicals to modify their surface or to increase scaffold hydrophilicity [67]. In the context of decellularization, an adequate control over the treatment type and time is critically important in order to preserve cell adhesion sites for target cell populations, their differentiation capability and recapitulate a functional target organ. Common methods for enhancement of cell

attachment to decellularized tissues are conjugation of bioactive molecules such as antibodies and RGD peptides [68]. Irrespective of considerable structural difference between *Ianthella basta* and *Aplysina aerophoba*, similar positive tendency in cell-scaffold interactions was detected from the first in vitro studies. This eliminates any need in cost-consuming modifications of the scaffold surface to increase efficiency of attachment at least for hMSCs. Specifically, confocal microscopy shows high cellular viability in the core and peripheral regions of chitinous scaffolds on day 7, which is a clear evidence of the scaffold non-toxicity (Fig. 13.8a). In addition, Fig. 13.8b presents intact cellular cytoskeleton and numerous cell nuclei reflecting proliferation and propagation of hMSCs throughout intricate labyrinth of naturally prefabricated chitinous fibers. Moreover, as shown on Fig. 13.8c cells were able to attach, spread and migrate when using fibrous network as a solid support for further colonization of this scaffold. It is relevant to note that in these experiments hMSCs have been seeded using perfusion method to provide better cell saturation of the decellularized chitinous scaffolds.

Surprisingly, the quality and rate of hMSCs growth over the decellularized chitinous skeletons of *Aplysina aerophoba* was totally different from that of *Ianthella basta*. As depicted in Fig. 13.9, at around day 14 typical cell assemblies stretching between fibers appears (a) and by day 21–28 (depending on the initial seeding density), they almost completely refill the available voids between the chitinous fibers (b). We strongly believe that when using dynamic culture in perfusion bioreactor systems, 3D expansion of such pre-seeded constructs would even more accelerate the cell growth efficiency analogous to what was shown for synthetic scaffolds [69].

This assumption is expected because marine sponges live under constant water flow and their exoskeletons, in terms of tensile strength, are adapted to withstand strong waves and currents as well as any resultant shear stresses present. Cell bridging phenomenon might be related to variable 3D organization and biomechanics which distinguish these Verongiid demosponges morphologically from one another. This type of cell connectivity and migration is not fully understood and definitely merits in further investigation. In case of synthetic scaffolds, they would require laborious modifications to achieve such cellular communication and formation of cell-scaffold channels [70]. Thus, chitinous scaffolds from *Aplysina aerophoba* raise many fundamental biological questions and could find application as alternative 3D tissue-engineered model for instance to reduce animal usage in research and drug testing (for review see [71]).

Previously, we have revealed the multilineage differentiation capacity of hMSCs sheets grown within chitinous scaffolds derived from *Aplysina aerophoba* cultivated via marine ranching [72]. Such cells were able to differentiate into three canonical lineages, i.e. adipogenic, osteogenic and chondrogenic. Representative pictures of hMSCs with adipogenic phenotype characterized by lipid droplet accumulation in cell cytoplasm (Fig. 13.10a) and osteogenic phenotype (Fig. 13.10b) characterized by calcium deposition are shown below.

Maintenance of the differentiation potential of hMSCs seems to be a distinctive property not only for *Ianthella basta* and *Aplysina aerophoba* but also for many other Verongiida sponges. Here, for comparison purposes, we would like to high-

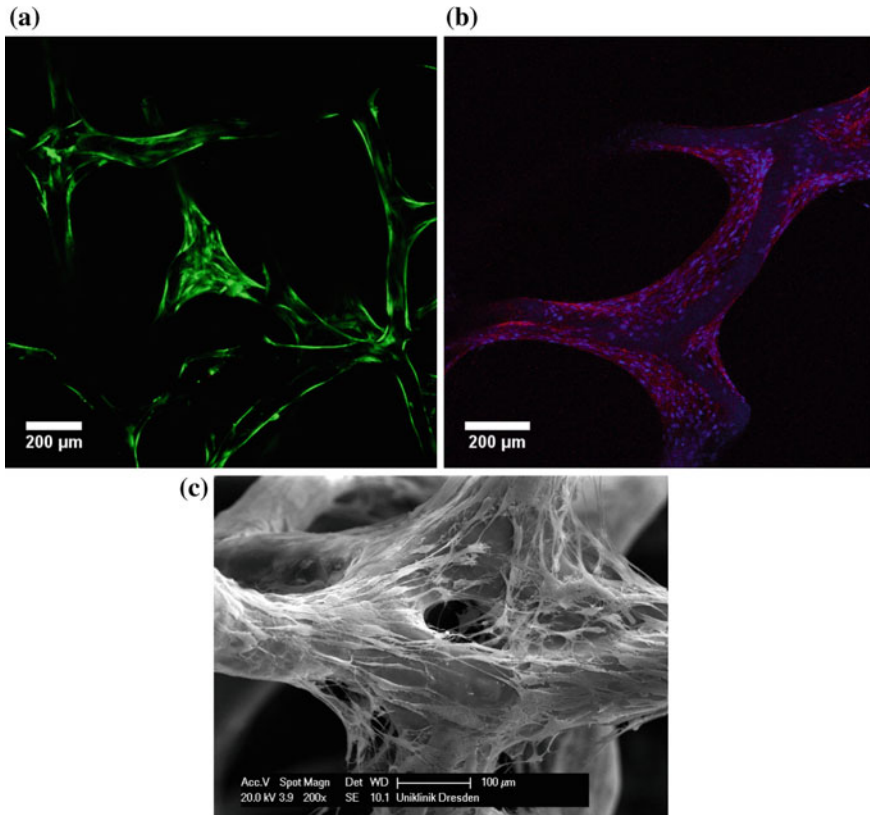


Fig. 13.8 Representative confocal fluorescence microscopy images (a, b) and SEM image (c) of the cell-seeded *Aplysina aerophoba* chitin scaffolds on day 7 in culture as a proof of interspecies compatibility of poriferan chitin with selected human cells. **a** Live-dead cytotoxicity assay shows high survival rate of hMSCs when grown on the chitinous scaffolds. Viable and dead cells are depicted in green and red, respectively; **b** DAPI/Phalloidin staining of cell nuclei and cytoskeleton. F-actin is shown in red and cell nuclei are counterstained in blue; **c** SEM analysis of cell attachment and spreading suggests that neither purification procedure nor poriferan chitin per se elicit any negative impact on human cells used in the study

light the exciting structure [23], phylogeny [73], and excellent biocompatibility of another demosponge *Aplysina fulva* (Verongiida: Demospongiae). The reticulate structure of this sponge mimics trabecular bone with a naturally diversified porosity (Fig. 13.11a, b). Furthermore, hMSCs were able to attach (Fig. 13.11c) and proliferate filling interfibrillar gaps between chitinous fibers from peripheral to central scaffold compartments. They were homogeneously distributed and characterized by enhanced alkaline phosphatase activity upon osteogenic stimulation (Fig. 13.11d).

From design and biocompatibility point of views, chitin scaffolds from both *Aplysina aerophoba* and *Aplysina fulva* demosponges are potential candidates for bone tissue engineering applications. In this regard, the organic part of their skele-

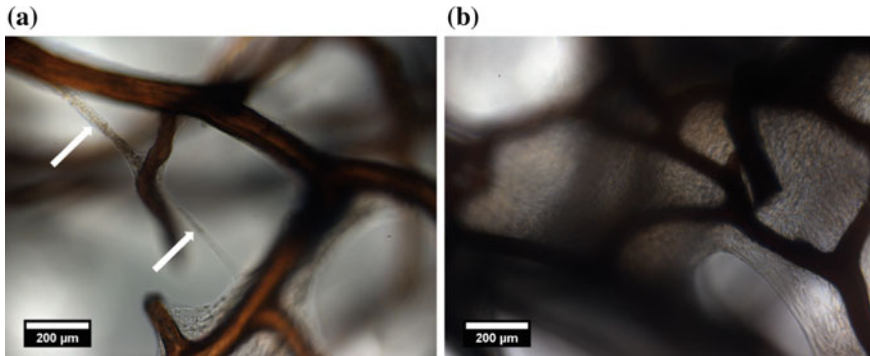


Fig. 13.9 Light microscopy images showing population of scaffolds derived from the skeletons of *Aplysina aerophoba* with selected hMSCs. White arrows indicate formation of bridging cell sheets between chitinous struts (a) which further develop into continuous membranous sheets wrapping the scaffold framework (b)

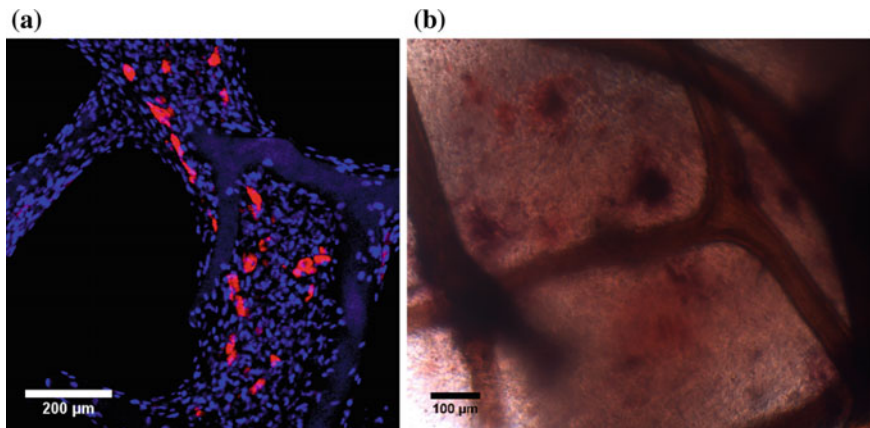


Fig. 13.10 Confocal fluorescence microscopy image (a) of adipogenic-induced hMSCs accumulating lipid droplets stained with Nile Red (red fluorescence); cell nuclei are counterstained with Hoechst (blue fluorescence). Bright-field microscopy image (b) of osteogenic-induced hMSCs sheets stained with Alizarin Red S contrasting calcium deposits by deep red color. Successful adipogenic and osteogenic differentiation further confirms the biocompatibility of this chitin scaffolds

tons can be functionalized with osteogenically active components of their inorganic part such as biosilica. For example, biogenic polymers polyphosphate and biosilica of poriferan origin were shown to be beneficial for application as biomimetic bone substitution materials (for review see [74]). These molecules exert osteoinductive and morphogenetic effects on cells revealed by significant increase in the expression of alkaline phosphatase and bone morphogenetic protein 2 [75]. Apart from that, it was demonstrated that polyphosphate induces accelerated tube formation of HUVEC (human umbilical vein endothelial cells) and, therefore, could be used in preparation

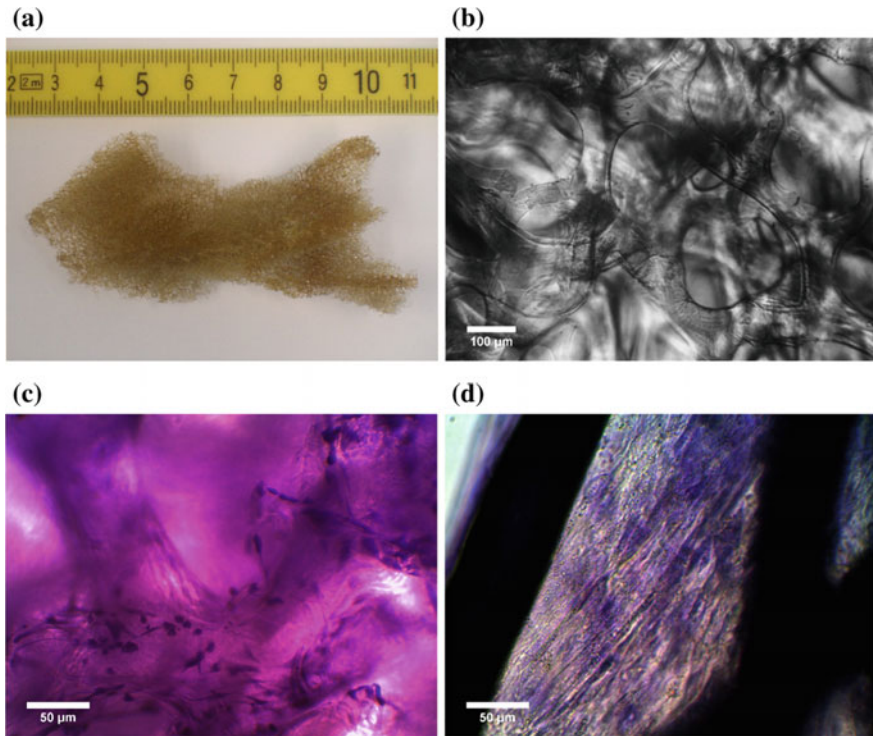


Fig. 13.11 General appearance of a cell-free skeleton from *Aplysina fulva* which was harvested in the Caribbean Sea and decellularized using alkali treatment (a); light microscopy image of uniform chitinous construct without cells (b), with hMSCs stained with Azur-Eosin (c) and for alkaline phosphatase on day 14 of in vitro culture (d). Chitinous scaffolds from *Aplysina fulva* roughly resemble trabeculae of spongy bone and support osteogenic capability of hMSCs

of bone tissue substitutes with intrinsic vascularization potential [76]. Gaharwar et al. [77] as well as Yang et al. [78] reported the positive effect of silica nanoparticles on induction of osteogenic differentiation of hMSCs. In these publications, the activity of alkaline phosphatase was significantly enhanced due to cellular uptake of silica nanoparticles as revealed in 2D cell culture studies. Likewise, similar results were observed in relations to the stimulatory effect of silica-based nanoparticles on murine osteoblasts in vitro and enhancement of bone mineral density in mice in vivo [79]. Since controlled generation of silica-chitin composites has already been successfully established for numerous chitinous sponges such as glass sponge *Farrea occa* [21] or Verongiida sponges *Aplysina cauliformis* [29], *Ianthella basta* [28] and *Aplysina aerophoba* (data not published), further evaluation of their osteoconductivity is of great interest. Other important point is that chitin scaffolds with incorporated silica nanoparticles may hypothetically have better cryopreservation properties providing multiple nucleation sites and improved thermal conductivity. Both topics are envisaged in future multidisciplinary research projects.

Other interesting aspect associated with the use of biomimetic approaches in tissue engineering is the fabrication of scaffolds for 3D direct or indirect co-culture systems that reflects more realistically the *in vivo* situation it is trying to address (for review see [80]). In practice, chitinous scaffolds derived from *Aplysina aerophoba* and *Aplysina fulva* could be used as a support for generation of 3D co-culture of osteogenically pre-stimulated hMSCs with endothelial cells to study neovascularization process in natural scaffolds and develop cryopreservation protocols that, to our best knowledge, have not yet been reported for mammalian co-culture systems.

Yet another prospective application where chitinous scaffolds from *Aplysina aerophoba* and *Aplysina fulva* can be useful is anti-tumor drug testing (for review see [81]). Such systems could integrate 3D scaffolds and anti-tumor compounds derived from the same marine sponges, for instance, Aeropylsinin-1 from *Aplysina aerophoba* (for review see [82]). Interestingly, the results of a recent publication revealed pronounced effect when they examined the anti-tumorigenic and anti-metastatic activities of aeropylsinin-1 and isofistularin-3 derived from *Aplysina aerophoba* against pheochromocytoma, with aeropylsinin-1 [83].

This subsection show that chitinous scaffolds derived from demosponges *Aplysina aerophoba* and *Aplysina fulva* possess a number of exclusive properties making them promising for use in tissue engineering. Among them, scaffold macro- and microdesigns, cytocompatibility with human mesenchymal stromal cells and support of their multilineage capacity. Using biomimetic approaches, these properties could be further enhanced according to biomedical needs.

13.2 Conclusion

The macro- and micro-designs of poriferan chitin scaffolds, their cytocompatibility with human mesenchymal stromal cells and support of their multilineage capacity make sponge chitin to be the intriguing alternative to scaffolds made of the structural proteins (collagen, keratin, and spongin) as well as artificial (polymers) materials. Fundamental questions about the pathways of demosponges chitin biosynthesis and its relationship with key biochemical reactions involved in the biosynthesis of diverse secondary metabolites (i.e. bromotyrosines) in the same species are still unanswered. There is a lack of knowledge concerning the mechanisms of chitin halogenization and biomineralization under natural conditions. What are the functional roles of halogens and mineral phases for rigidification of chitin-based skeletons of verongiid demosponges? Also, the challenging task of isolating and application of such mineral-containing and mechanically stable 3D chitinous scaffolds from selected sponges remains to be addressed. The key ways for oxygen-free methods for carbonization of 3D chitin constructs isolated from diverse demosponges ought to be investigated.

There is also a lack in comparative study on pores diversity within diverse species of Verongiida sponges with respect to their potential as highly appropriative chitinous scaffolds. Consequently, the ultimate goal of our research is to answer questions

about the existence of the relationship between specificity of pore size, stiffness and fiber density of poriferan chitin scaffolds with respect to the aims of modern tissue engineering.

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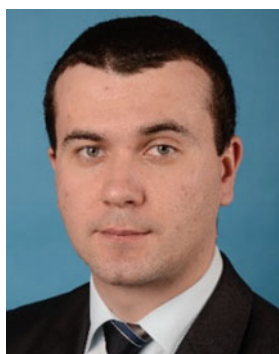
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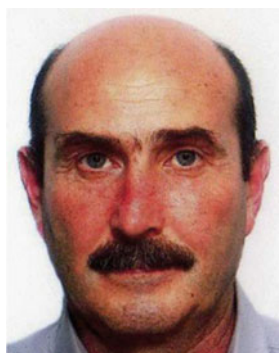
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