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Characterization and *in vivo* biological performance of collagen-like from marine sponges: A review

Caracterização e desempenho biológico *in vivo* do colágeno de esponjas marinhas: Uma revisão

Caracterización y desempeño biológico *in vivo* del colágeno de esponjas marinas: Una revisión

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Abstract

The use of collagen-based biomaterials in tissue engineering has surged in recent decades due to collagen's advantages, including biocompatibility, controlled biodegradation, and support for cell adhesion and differentiation. Traditional collagen sources, often from bovine or porcine origins, pose challenges such as zoonotic risks, immunogenic reactions, and ethical concerns. To address these limitations, researchers are exploring innovative collagen sources, such as marine sponges. Collagen from marine sponges, known as spongin (SPG) or spongin-like collagen (SC), exhibits biocompatibility and is considered a natural component for tissue regeneration, serving as a cell-matrix adhesion framework. Our group has conducted experiments over several years to extract SPG from sponges, assess its biocompatibility and cytotoxicity, as well as its *in vitro* and *in vivo* biological effects. This research aims to review the data obtained from our research on characterization and *in vivo* biological performance of collagen-like from marine sponges. Furthermore, combining SPG with ceramics like hydroxyapatite and bioactive glasses has demonstrated beneficial biological properties. Despite ethical and regulatory challenges, marine sponge collagen shows promise as a natural biomaterial that could improve patients' quality of life, particularly in bone injury treatments. This review highlights the innovative use of marine sponges and their collagen-based components in tissue

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engineering, emphasizing their potential as a promising alternative for bone injury treatment. Additionally, it underscores the need for further research to fully harness this natural biotechnological resource.
Keywords: Bioactive Compounds; Collagen; Marine Sponges; Spongin.

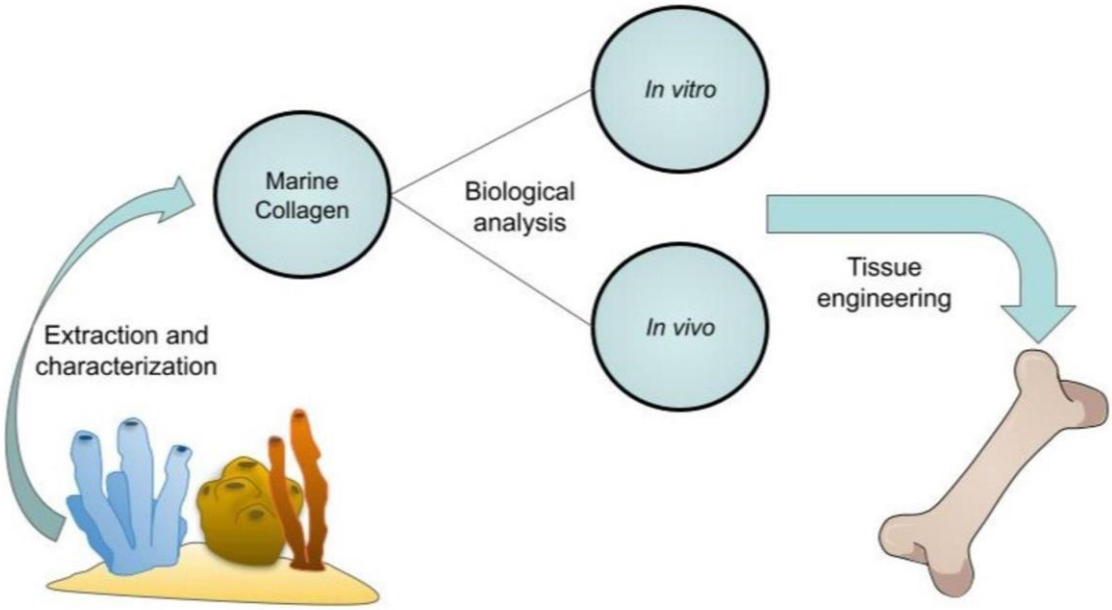
Resumo

O uso de biomateriais à base de colágeno na engenharia de tecidos tem aumentado nas últimas décadas devido às vantagens do colágeno, incluindo biocompatibilidade, biodegradação controlada e suporte à adesão e diferenciação celular. As fontes tradicionais de colágeno, frequentemente de origem bovina ou suína, apresentam desafios como riscos zoonóticos, reações imunogênicas e questões éticas. Para superar essas limitações, pesquisadores estão explorando fontes inovadoras de colágeno, como as esponjas marinhas. O colágeno das esponjas marinhas, conhecido como espongina (SPG) ou colágeno tipo espongina (SC), apresenta biocompatibilidade e é considerado um componente natural para a regeneração tecidual, atuando como uma matriz de adesão celular. Nosso grupo conduziu experimentos ao longo de vários anos para extrair SPG de esponjas, avaliar sua biocompatibilidade e citotoxicidade, bem como seus efeitos biológicos *in vitro* e *in vivo*. Esta pesquisa tem como objetivo revisar os dados obtidos em nossa pesquisa sobre a caracterização e o desempenho biológico *in vivo* de colágeno de esponjas marinhas. Estudos *in vitro* e *in vivo* sugerem que a SPG promove o crescimento celular e a regeneração tecidual, particularmente em fibroblastos e osteoblastos, facilitando a integração dos tecidos. Além disso, a combinação de SPG com cerâmicas como a hidroxiapatita e vidros bioativos demonstrou propriedades biológicas benéficas. Apesar dos desafios éticos e regulatórios, o colágeno de esponjas marinhas se mostra promissor como um biomaterial natural que pode melhorar a qualidade de vida dos pacientes, especialmente no tratamento de lesões ósseas. Esta revisão destaca o uso inovador de esponjas marinhas e seus componentes à base de colágeno na engenharia de tecidos, enfatizando seu potencial como uma alternativa promissora para o tratamento de lesões ósseas. Além disso, ressalta a necessidade de mais pesquisas para explorar plenamente esse recurso biotecnológico natural.
Palavras-chave: Compostos Bioativos; Colágeno; Esponjas Marinhas; Espongina.

Resumen

El uso de biomateriales a base de colágeno en la ingeniería de tejidos ha aumentado en las últimas décadas debido a las ventajas del colágeno, incluyendo su biocompatibilidad, biodegradación controlada y soporte para la adhesión y diferenciación celular. Las fuentes tradicionales de colágeno, a menudo de origen bovino o porcino, presentan desafíos como riesgos zoonóticos, reacciones inmunogénicas y preocupaciones éticas. Para superar estas limitaciones, los investigadores están explorando fuentes innovadoras de colágeno, como las esponjas marinas. El colágeno de las esponjas marinas, conocido como esponjina (SPG) o colágeno tipo esponjina (SC), exhibe biocompatibilidad y se considera un componente natural para la regeneración tisular, actuando como una matriz de adhesión celular. Nuestro grupo ha llevado a cabo experimentos durante varios años para extraer SPG de esponjas, evaluar su biocompatibilidad y citotoxicidad, así como sus efectos biológicos *in vitro* e *in vivo*. Esta investigación tiene como objetivo revisar los datos obtenidos en nuestra investigación sobre la caracterización y el desempeño biológico *in vivo* del colágeno-like de esponjas marinas. Estudios *in vitro* e *in vivo* sugieren que la SPG promueve el crecimiento celular y la regeneración tisular, particularmente en fibroblastos y osteoblastos, facilitando la integración del tejido. Además, la combinación de SPG con cerámicas como la hidroxiapatita y los vidrios bioactivos ha demostrado propiedades biológicas beneficiosas. A pesar de los desafíos éticos y regulatorios, el colágeno de esponjas marinas muestra un gran potencial como biomaterial natural que podría mejorar la calidad de vida de los pacientes, especialmente en el tratamiento de lesiones óseas. Esta revisión resalta el uso innovador de las esponjas marinas y sus componentes a base de colágeno en la ingeniería de tejidos, enfatizando su potencial como una alternativa prometedora para el tratamiento de lesiones óseas. Además, subraya la necesidad de realizar más investigaciones para aprovechar completamente este recurso biotecnológico natural.
Palabras clave: Compuestos Bioactivos; Colágeno; Esponjas Marinas; Esponjina.

Graphic abstract - A review of the extraction, characterization and biocompatibility analyses of marine collagen extracted from sea sponges for bone tissue engineering.



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1. Introduction

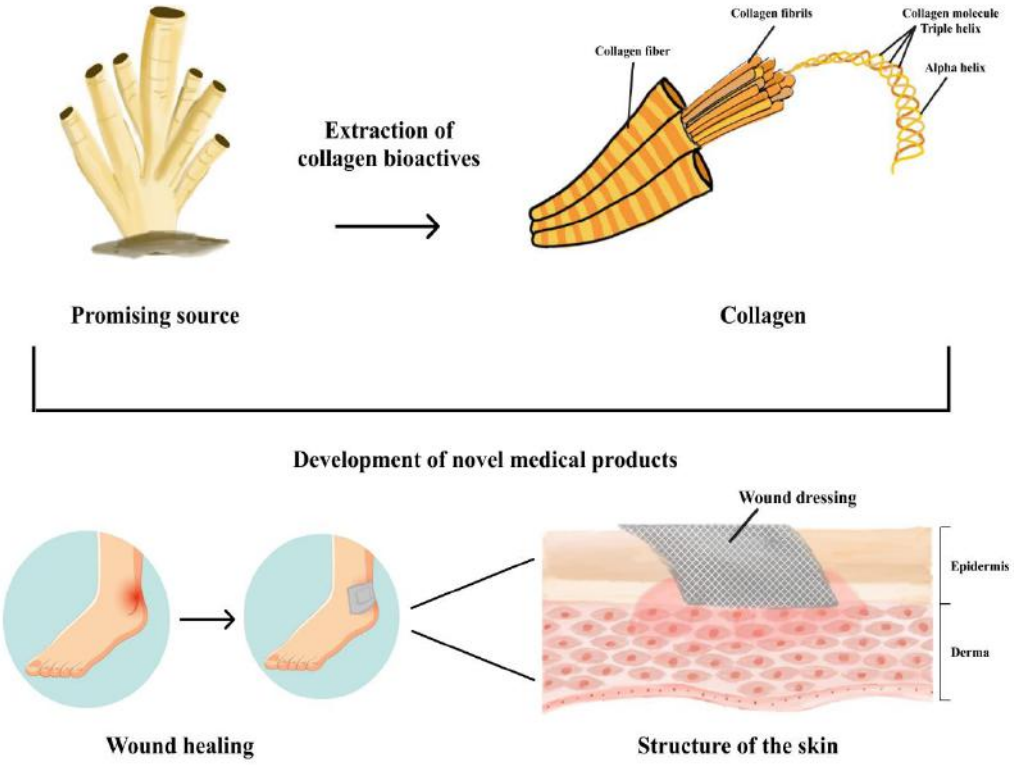
During the last decades, the use of collagen-based biomaterials has been significantly growing for tissue engineering proposals. It has been used for producing bone defect grafts, treatment of burns and wounds, antithrombogenic surfaces and therapeutic enzymes immobilization (Zheng et al., 2023). As is widely known, collagen is the major component of skin and bone tissues (Li et al., 2021). Moreover, until now, 29 different kinds of collagens were characterized and all of them are comprised of three α chains, each chain being composed of more than a thousand amino acids (Silvipriya et al., 2015). In addition, collagen is known for its biocompatibility, high affinity to water, appropriate biodegradation, hemostatic properties, lack of inflammatory host response and ability of attracting and supporting the proliferation of many cell types (Patel et al., 2020).

Also, it is worthwhile pointing out that collagen can be originated from many different sources mainly porcine and bovine skin and bones (Chilakamarthi et al., 2014; G. S. Silva et al., 2014; T. Silva et al., 2014). However, the use of these collagens may involve some issues such the extremely high costs of extraction, the risk of transmission of diseases and some religious concerns (Salvatore et al., 2021). As a way of overcoming these problems, the use of collagen from alternative sources has been investigated (Rodríguez et al., 2017). In this context, marine biodiversity has been showing a promising source of natural bioactive compounds with efficient biological interaction with tissues promoting their growth and repair (Araujo et al., 2021; C. Parisi et al., 2020; Rezvani Ghomi et al., 2021). Among them, the marine sponges (phylum Porifera) are one of the most promising sources of biocompounds and molecules, with a wide potential for different applications including antitumor, antiviral and anti-inflammatory effects (Tassara et al., 2023).

Marine sponges present a unique structure and composition, being composed by many different bioactive components, including spongin (SPG) or spongin-like collagen (SC), which represent a structure with an analogous composition of the vertebral collagen (Liu et al., 2022). Different studies have demonstrated that SPG has biocompatibility and can be used as a cell–matrix adhesion framework, working for tissue bioregeneration (Ali & Dubey, 2010). In addition, SPG appears to be more thermally resistant than standard fish collagen, has molecular similarities to calf skin type-I collagen

(Panagiotis Berillis, 2015) and it can be the base material to produce bioactive peptides, collagen-based hydrogels and collagen-based cosmetic products (Araujo et al., 2021; Pozzolini et al., 2018) (Figure 1).

Figure 1 - Skin wound dressings using collagen bioactives from marine sponges.



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For example, Pozzolini et al. (2018) extracted SPG from *Chondrosia reniformis* and used it as a raw material to manufacture skin dressings. Through *in vitro* studies, they demonstrated its ability to support fibroblast and keratinocyte proliferation when cultured in the presence of these dressings. Recently, Araújo et al. (2021) compared different protocols of SPG extraction in 2 different marine sponges and found a fibrillar structure for the extracts of *Chondrilla caribensis*, with the presence of GAGs, a marked antioxidant activity and biocompatibility in fibroblast cells, demonstrating the positive biological effect of the extracted SPG.

Additionally, the exploitation of SPG for developing medical products for tissue engineering proposals will undoubtedly culminate in more efficient and innovative therapeutical interventions, involving a reduced cost of production, which can be regarded as breakthrough in increasing competitiveness of the industry related to the technological area (Davison-Kotler et al., 2019). Moreover, many advantages for the population can be associated with these new approaches such as the increase of patient recovery rate, reduction in the duration of treatments and improvement of quality of life of the population. Thus, the bioactive compounds from marine sponges are a 'gold mine' regarding the diversity of their secondary metabolites, which can culminate in new products for society, improving the quality of life of people (Hernández-Rangel & Martin-Martinez, 2021).

In this context, our group has been developing for many years now, different experiments to develop protocols for extracting SPG from marine sponges and investigating the biocompatibility, cytotoxicity and its *in vitro* and *in vivo* biological effects. Thus, this research aims to review the data obtained from our studies. Both *in vitro* and *in vivo* studies suggest that

SPG promotes cell growth and tissue regeneration, particularly in fibroblasts and osteoblasts, facilitating tissue ingrowth.

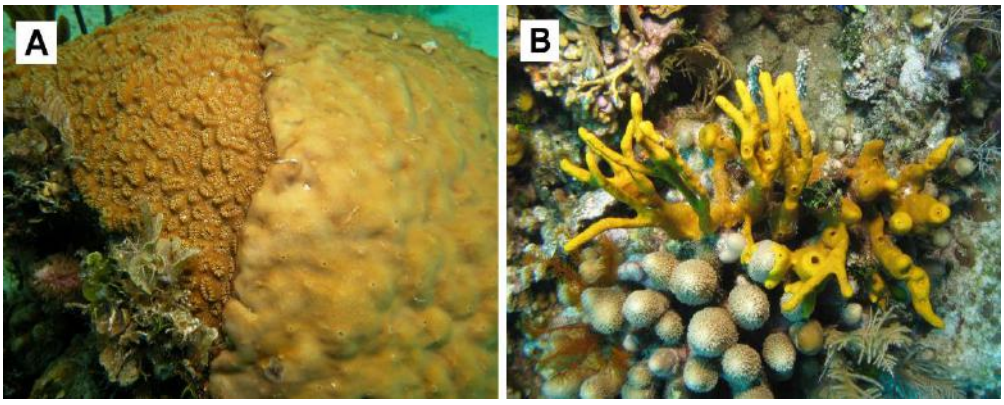
2. Methodology

A descriptive, qualitative, and quantitative experience report was conducted (Pereira et al., 2018; Gil, 2017), aiming to present a synthesis of the investigation carried out by our research group. Over several years, the group conducted experiments to extract SPG from sponges, evaluate their biocompatibility and cytotoxicity, and assess their biological effects *in vitro* and *in vivo*.

3. Marine sponges

Different species of marine sponges have been investigated along the years by our group (Araújo et al., 2021; Cruz et al., 2020; Fernandes et al., 2021; C. Parisi et al., 2020; J. R. Parisi et al., 2019; Santana et al., 2021). Mainly, the specimens of marine sponges *C. caribensis* (Figure 2A) are well known for having a predominant inorganic part, allowing the extraction of SPG like compounds. Similarly, the *Aplysina fulva* marine sponge (Figure 2B) also have a structure constituted by an inorganic part. After the registration in the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), species were collected along the coast of Praia Grande (23°49'23.76"S, 45°25'01.79"W, São Sebastião, Brazil). Samples were placed in containers with seawater and transported to the laboratory for processing. The marine sponges were washed 3 times with distilled water to remove cell debris and were stored at -20 °C.

Figure 2 - 2A corresponds to the marine sponge *C. caribensis* and 2B to the marine sponge A. fulva.



Source: The Sponge Guide.

4. Protocols of extraction of SPG

The first protocol of SPG extraction used by our group was based on the methodology of Swatschek et al. (2002). This protocol was based mainly on placing the sponge samples in Tris-HCl buffer (100 mM, pH 9.5, 10 mM EDTA, 8 M urea, 100 mM 2-mercaptoethanol) and adding NaOH for adjusting the pH (up to 9). Afterwards, the solution was centrifuged (5000 g; 5 minutes and 2 oC) and the pH was adjusted again to 4 using acetic acid solution, the precipitated re-suspended in Milli-Q water and centrifuged and lyophilized for storing (Araújo et al., 2021; Fernandes et al., 2021; J. R. Parisi et al., 2019).

However, the efficiency of this protocol to extract SPG from the sponges, there is a continuous need for optimizing protocols of SPG extraction, focusing on obtaining a faster, inexpensive and more efficient process (Araújo et al., 2021; Pozzolini et al., 2018). In this context, one work of our group was developed focusing in comparing 4 different protocols for SPG extraction from 2 marine sponge species (Chondrilla caribensis and *Aplysina fulva*) (Araújo et al., 2021). Briefly, one of the protocols

(P1) was based on a standard protocol described in the literature (Swatschek et al., 2002). For more details, please check Araújo et al. (2021). For the other protocols (P2, P3, and P4), the samples were cryogenically milled, and extraction was performed using different solvents: 0.1% trypsin/100 mM ammonium bicarbonate (pH 8.5) for P2, 0.1 M Tris-HCl buffer (pH 7.5) for P3, and deionized water (pH 6.8) for P4 (Table 1).

Table 1 - Protocols of extraction and description of the reagents.

Reference	Species	Protocol	Extraction reagents	Methodology summary
Swatschek et al., 2002	*Chondrosia reniformis*	P1	Tris-HCl buffer; EDTA; Ureia; 2-Mercaptoethanol; NaOH; Acetic Acid	Agitation at room temperature with distilled water pH adjustment (NaOH) Centrifugation steps Suspension in distilled water Lyophilization of the material.
Araújo et al., 2021b	*Chondrilla caribensis* *Aplysina fulva*	P2	0.1%trypsin/100 mM ammonium bicarbonate (pH 8.5)	Cryogenic milling Solubilization with a vortex-mixer at room temperature Centrifugation Suspension of distilled water Dialysis against deionized water (P2 and P3) Lyophilization of the material
		P3	0.1M Tris-HCl buffer (pH 7.5)	
		P4	Deionized water (pH 6.8)	

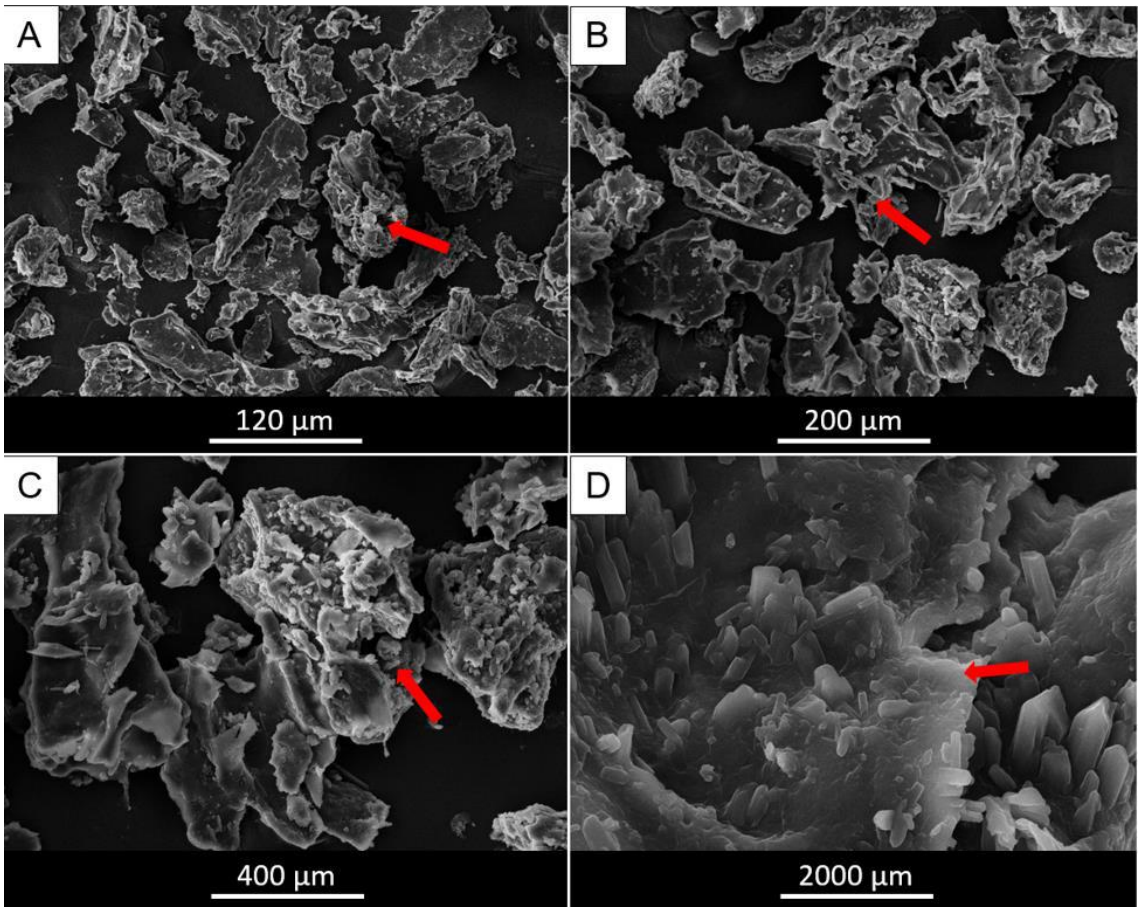
Source: Created by the Authors.

Many different analyses were used for evaluating the efficiency of the protocols. A higher extraction yield when using P2, P3 and P4 for both species compared to P1. The quantification of SPG was another analysis used for evaluating the efficiency of the different protocols. In this analysis, a higher amount of SPG was obtained using the modifieds protocols of extraction compared to the standard P1 for *C. caribensis*. The same behavior was found for the GAG evaluation and antioxidant assay. For the A. fulva, no statistically significant difference was observed among the experimental groups. All these analyses demonstrated that the change in the reagents performed in the protocols was efficient to optimize the SPG extraction from the marine species being more effective for the *C. caribensis*. Also, it was demonstrated that fibroblast cell proliferation also increased in *C. caribensis* extracts. In summary, the proposed protocols P2 and P4 have optimized the extraction of nearly 50% of SC, and the amount of SPG, protein, GAGs and an antioxidant activity. The results suggest that protocols that are trypsin and water-based were more efficient than others tested in this work for extracting SC from small particles of *C. caribensis.*

5. Characterization of SPG

To progress the investigation of the characteristics and properties of the SPG, one of the first studies of our group investigated the physicochemical and morphological properties of the material by using Energy dispersive X-ray spectroscopy (EDS), FTIR, scanning electron microscopy (SEM), mass loss and pH. Disks manufactured with SPG-like extracted from the *Aplysina fulva* marine sponge were used to perform the characterization. SEM analysis demonstrated that SPG-like presents a granular aspect and an irregular structure (Figures 3A and 3B). Moreover, in a higher magnitude, it is possible to observe the smooth surface of the particles, with a heterogenous morphology (Figures 3C and 3D).

Figure 3 - Scanning Electron Microscopy (SEM) image of SPG. Arrows highlight the SPG fibrils.



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Also, degradation tests were performed. For this purpose, samples were weighed and placed in 3 ml of phosphate buffered saline (PBS, 10 mM, pH 7.4) in a Falcon tube for 3, 7 and 14 days. Mass loss and pH were evaluated in these periods. The data from this work demonstrated that pH measurements, after 14 days of immersion in PBS, an increase in the values was seen along the experimental periods, reaching a value of 8.34. Mass loss measurements for SPG have not demonstrated a significant decrease along the experimental period of 14 days after immersion.

In a separate study conducted by Fernandes et al. (2021) FTIR was employed to identify five distinct peaks associated with SPG-like structures derived from marine sponges. The first peak corresponds to O-H stretching at 3522 cm-1, the second relates to amide A at 2900 cm-1, while the third, fourth, and fifth peaks are associated with amides I, II, and III at 1644 cm-1, 1510 cm-1, and 1258 cm-1, respectively (Figure 4).

Figure 4 - FTIR spectra of SPG. Dashed lines show peaks associated with SPG (OH stretching, NH axial deformation, C=O stretching, NH bending and CN stretching).

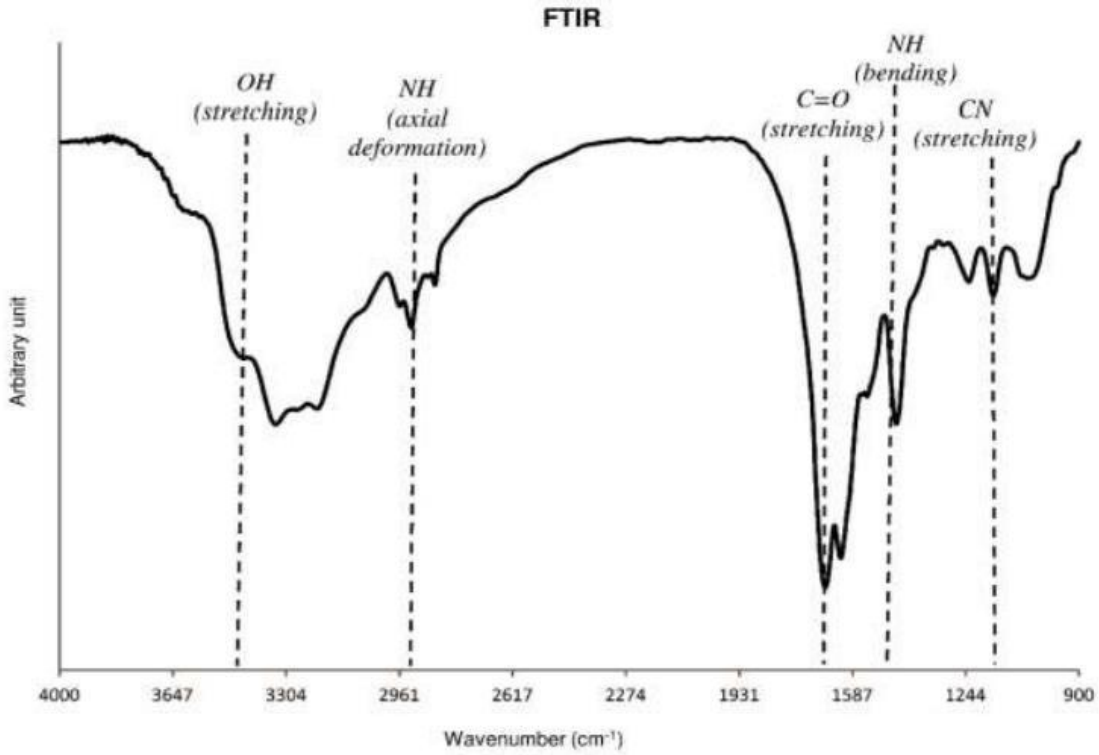

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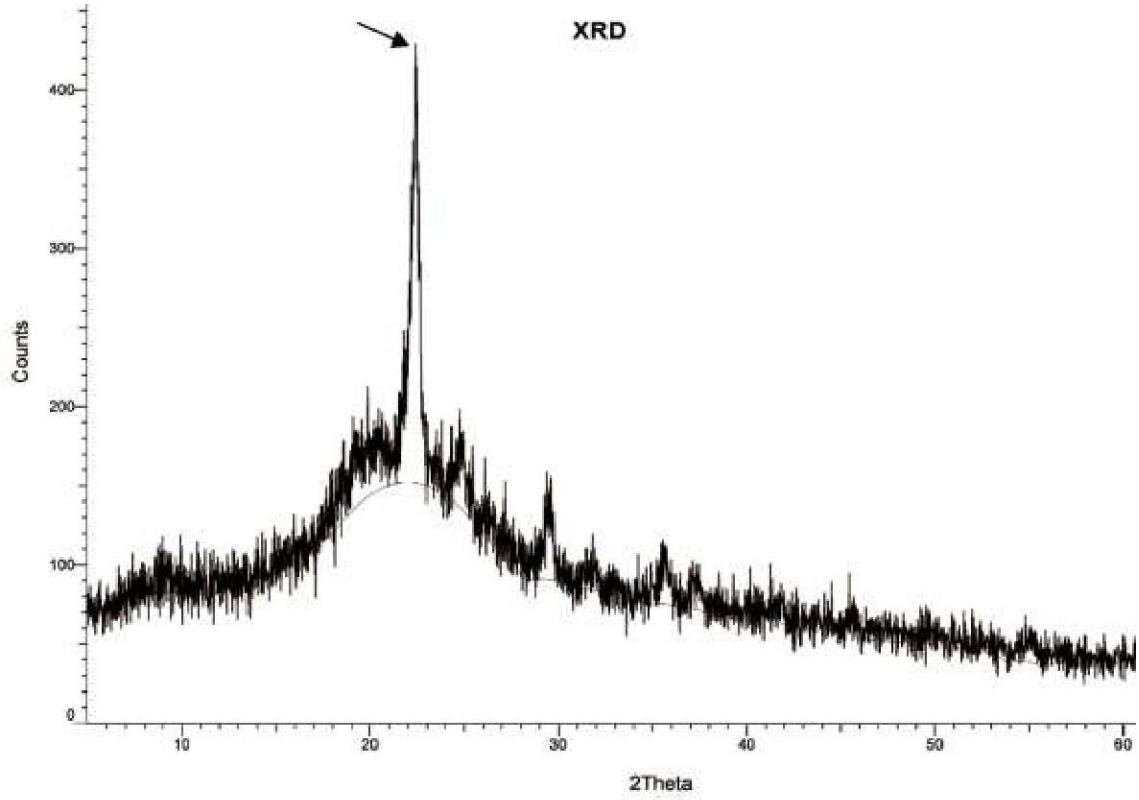
Table 2 - Characteristic FTIR absorption peaks of SPG.

Peak	Wavenumber (cm^{-1})	Description
1	3522	O-H stretching
2	2900	Amide A (N-H)
3	1644	Amide I (C=O)
4	1510	Amide II (N-H)
5	1258	Amide III (C-N)

Source: Created by the Authors.

Also, the X-ray diffraction (XRD) confirmed the predominantly amorphous profile of SPG (with a crystalline at 2-Theta Degrees of 22.40). This crystallinity can also be observed in the SEM analysis of the same sponge extracted using the same protocol as previously demonstrated. These data demonstrate the semi-crystalline character of this SPG-like material (Figure 5).

Figure 5 - XRD diffraction pattern of SPG. A reduced crystalline peak was found at 2-Theta Degrees of 22.40, as indicated by the arrow.



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6. Biocompatibility of SPG

To continue the investigations about the biocompatibility and cytotoxicity of SPG from marine sponges, our group performed a study with the aim of investigating the cytotoxicity and genotoxicity of SPG, through *in vitro* (cell viability and comet assay) and *in vivo* studies using a model of bone defect in rats (Santana et al., 2021). For cell viability, fibroblasts (L929) and osteoblasts (MC3T3) cells were used and SPG, in different percentages were used (SPG25%, SPG50% and SPG100%) and compared to a control group (CG). For fibroblast cells, after 3 days of culture, CG and SPG 25 groups showed significantly higher values compared to SPG 50 and SPG 100. Interestingly, on day 7, CG and SPG 50 showed higher value compared to SPG 25 and SPG 100. On the last period analyzed, cell viability of L929 for CG presented significantly higher values compared to the other groups. Also, SPG25 demonstrated a statistically significant difference compared to SPG 50 and SPG 100. For osteoblast cells, CG demonstrated statistically higher values compared to all SPG groups (at all concentrations) on days 3 and 7. At the same experimental periods, cell viability for SPG 100 was statistically lower compared to SPG at 25 and 50%. A significant difference was observed between SPG 25 and 50. On day 14, osteoblast cell viability of CG demonstrated higher values compared to SPG 50 and 25. Significantly difference was demonstrated compared to SPG 50 and SPG 25. The genotoxicity analysis (comet assay) demonstrated statistically lower values were observed for CG compared SPG 25 at all set points, demonstrating the lack of genotoxic effects of this biocompound (Santana et al., 2021). Also, Fernandes et al (Fernandes et al., 2021) performed *in vitro* studies evaluating the biocompatibility of SPG using fibroblast and osteoblast cells. For fibroblasts, cell viability in the groups cultured in the presence of SPG was higher on day 7 compared to day 1. For

osteoblast viability assay, all SPG groups demonstrated a higher cell viability at day 7 in comparison to day 1. These results demonstrate that SPG from sponges was able of supporting cell growth *in vitro*, showing its biocompatibility.

The *in vivo* biocompatibility of SPG was also investigated using an experimental model of tibial bone defect in rats. Santana et Santana et al. (2021) analyzed the bone response 7 days post SPG implantation. For CG, bone defects were filled mainly by granulation tissue, with some areas of newly formed bone, especially at the periphery. For SPG treated animals, biomaterial particles and granulation tissue could be seen at the region of the defect. Areas of newly formed bone were observed for some animals. There was no inflammatory process or capsule formation between the implant and the edges of defect. Also, for CG, Runx2 immunostaining was observed in the granulation tissue all around the bone defect. For SPG, Runx 2 immunostaining was predominantly detected in the granulation tissue at the center of the bone defect and around the particles of the material, 7 days post – surgery. Fernandes et al. (2021) performed an experimental model of noncritical tibial bone defect treated with SPG like and evaluated the effect of the grafts for 15- and 45-days post-surgery. The histological results demonstrated that SPG treated animals presented material degradation, presence of granulation tissue ingrowth and areas of newly formed bone tissue after 15 days. After 45 days of implantation, it was observed that bone defects were filled mostly with newly formed bone, without signs of the presence of material particles. The authors state that SPG from the sponges can be considered as an alternative source of collagen for tissue engineering proposals.

7. Composite biomaterials with SPG for bone tissue engineering

To continue the investigation of the effects of SPG for bone tissue engineering proposals, our group initiated the development of studies investigating the effects of SPG mixture with ceramic materials such as hydroxyapatite (HA). The aim of these studies was to develop an innovative bone graft with similar composition of bone tissue (an organic and inorganic part) trough *in vitro* studies to evaluate the effects of composites in cell proliferation and cytotoxicity. Briefly, HA is a crystalline phase of calcium phosphate, with osteoconductive and angiogenic properties (Pang et al., 2015; Parizi et al., 2013; Wang et al., 2007). Following this line, Parisi et al. evaluated the *in vitro* response of the association of HA and SPG (J. R. Parisi et al., 2019). It was demonstrated that a higher fibroblast cell proliferation was found for HA/SPG 70/30 compared to HA and HA/SPG 90/10 on days 3 and 7 days. Furthermore, HA/SPG 70/30 presented lower MC3T3 cell viability compared to HA. on the experimental period of 3 days. The results of the present study indicate that the composites HA/SPG demonstrated improved biological properties, especially the one mimicking the composition of bone (with 70% of HA and 30% of SPG), highlighting that the introduction of SPG into HA has improved the performance of the graft for bone regeneration applications.

Moreover, another experiment was performed investigating the orthotopic *in vivo* response into HA/SPG samples. For this purpose, composites were implanted into tibial bone defects of rats and bone tissue response was evaluated (2 and 6 weeks after implantation). Two weeks post-surgery, for HA treated animals, some particles of the material still could be observed, surrounded by granulation tissue and with some areas of newly formed bone. For HA/SPG, it was possible to observe particles of the material residual and some areas of granulation tissue but with an intense presence of newly formed bone in the defect. Six weeks post-surgery, for HA, it was possible to observe a complete degradation of the material and formation of mature bone at the edges of the lesion. For HA/SPG, a complete material degradation was observed, with some areas of granulation tissue but with the bone defect filled with newly formed bone. The histomorphometric analysis demonstrated that a higher value of % BV/TV was found for HA/SPG compared to HA, in both experimental periods. For Ob.S/BS, a significantly higher value was found for HA/SPG compared to HA, after 2 weeks. After 6 weeks, it was possible to verify a higher value of Ob.S/BS for HA/SPG compared to CG (J. R. Parisi et al., 2019).

In the same line, another ceramic material (Biosilicate) was added to SPG to manufacture a composite. Bioactive glasses and glass-ceramics (including Biosilicate®) are one of the most bioactive materials to be used as a treatment for stimulating fracture healing (Granito et al., 2009). They are a class of synthetic silica-based bioactive materials able of bonding to bone tissue and forming an active apatite layer on their surface, which acts as a template for newly bone formation (Renno et al., 2013). For example, Biosilicate® (BS) has shown very positive effects on bone metabolism and on the acceleration of fracture healing. In this context, Fernandes et al raised the hypothesis that the association of BS and SPG would constitute a bone graft with optimized osteogenic properties for accelerating cell proliferation (Fernandes et al., 2019). The *in vitro* studies demonstrated that BS/SPG groups higher fibroblast proliferation after 3 and 7 days of culture. Based on the present *in vitro* results, it was suggested that the incorporation of SPG into BS produced an improvement in the physical-chemical characteristics and in the biological performance of the graft.

In another study, Parisi et al. (2020) hypothesized that the addition of SPG might improve the biological performance of BS *in vivo* and had the aim of evaluating the orthotopic *in vivo* response to BS/SPG composites in the process of bone healing in rats (Parisi et al., 2020). For this purpose, scaffolds in different formulations were implanted in cranial bone defects in rats and their effect was measured after 15- and 45-days post-surgery. Two weeks post-surgery, inflammatory process was not detected for any group. For BS treated animals the defect was filled with granulation tissue and newly formed bone at the edges. Degradation process started in the implanted biomaterial. For BS/SPG, granulation tissue was seen in most of the defect area, with some osteoids and newly formed bone and particles of the biomaterial. At the second experimental period (6 weeks) for BS, degradation of the material was observed in the defect with granulation tissue and some newly formed bone. For BS/SPG, an intense degradation of the material was seen, as well as portions of osteoids and newly formed bone. The immunohistochemistry analysis demonstrated that for BS and BS/SPG, VEGF immunostaining was observed after two and six weeks. The results corroborate the hypothesis that BS and BS/SPG scaffolds could support bone formation in a critical bone defect in rats.

Another study associated the scaffolds of SPG with photobiomodulation (PBM), highlighting the hypothesis that the treatment using both therapies would be able of accelerating tissue metabolism, stimulating bone healing. PBM is known for being able of modulating inflammatory processes after an injury, stimulating neoangiogenesis and accelerating tissue healing after an injury (Lima et al., 2010; Hamblin, 2017; Ruh et al., 2018). Its action can be explained mainly by the interaction of light and tissues, culminating in a series of modifications in the metabolism of cells, stimulating mitochondrial respiration and increasing in the synthesis of ATP (Fernandes et al., 2016; Khadra et al., 2005). All the modifications increase expression of genes related to protein synthesis, cell migration and proliferation, anti-inflammatory signaling and antioxidant enzymes (Freitas & Hamblin, 2016; Khadra et al., 2005). In this context, Cruz et al investigated the biological effects of scaffolds of SPG associated with PBM on newly formed bone using a calvarial bone defect model (Cruz et al., 2020). As a result, for SPG, at 15 days post-surgery, bone defect was filled mainly by scaffolds, but some connective tissue and inflammatory infiltrate could be seen with some areas of newly formed bone. In the second experimental period, degradation of the material was observed as well as neoformed bone tissue, with some scarce areas of connective tissue and material particles. For SPG/PBM, at 15 days post-surgery, bone defect was filled with some connective tissue around the material, material particles, some inflammatory infiltrate areas and newly formed tissue areas. In the second experimental period, a few material particles and areas of newly formed bone were seen in the defect. For the histomorphometry analysis, higher values were found for N.ob/T. Ar (mm2) for SPG/PBM 15 days post-surgery in comparison to SPG, 45 days post-surgery. For TGF-β immunostaining, a similar pattern of TGF-β immunoexpression could be observed for the groups. Additionally, 45 days post-surgery, it is possible to observe that TGF-β immunolabeling continues in the granulation tissue, mainly at the center of the defect and around the

particles of the materials. In conclusion, authors state that the association of SPG and PBM can be a promising treatment for stimulating bone growth and fracture healing.

8. Future trends for the use of marine spongin in tissue engineering

As detailed in this review article, SPG, the collagenous biomaterial, has been successfully extracted from marine sponges, characterized for its physicochemical properties, evaluated for its biocompatibility, and employed in various composites developed for use in tissue engineering. Established as one of the main pillars in this field, biomaterials serve as structural support for cell adhesion, proliferation, differentiation, and anabolic activity (Wubneh et al., 2018).

Cells play a central role in constructing a new tissue by actively synthesizing the proteins that will later constitute it (Ho-Shui-Ling et al., 2018). Therefore, future research involving SPG should, from this point forward, focus on its influence on cellular metabolism, in a more detailed manner. Organic materials like SPG offer the advantage of providing a biointeractive surface conducive to cell adhesion and, consequently, their synthetic activity (Lin et al., 2011). In this sense, new studies should also involve the development of constructs that include multiple cell types mimicking the complex physiological microenvironment in which these cells function, communicate, interact, promoting tissue growth, integrity, and functionality over time. Thus, the use of SPG in constructs involving multiple cell types constitutes the next crucial step in creating artificial tissues that resemble natural tissues in terms of complexity, functionality, and responsiveness to the environment. This expands therapeutic applications and enhances the effectiveness of tissue engineering-based therapies.

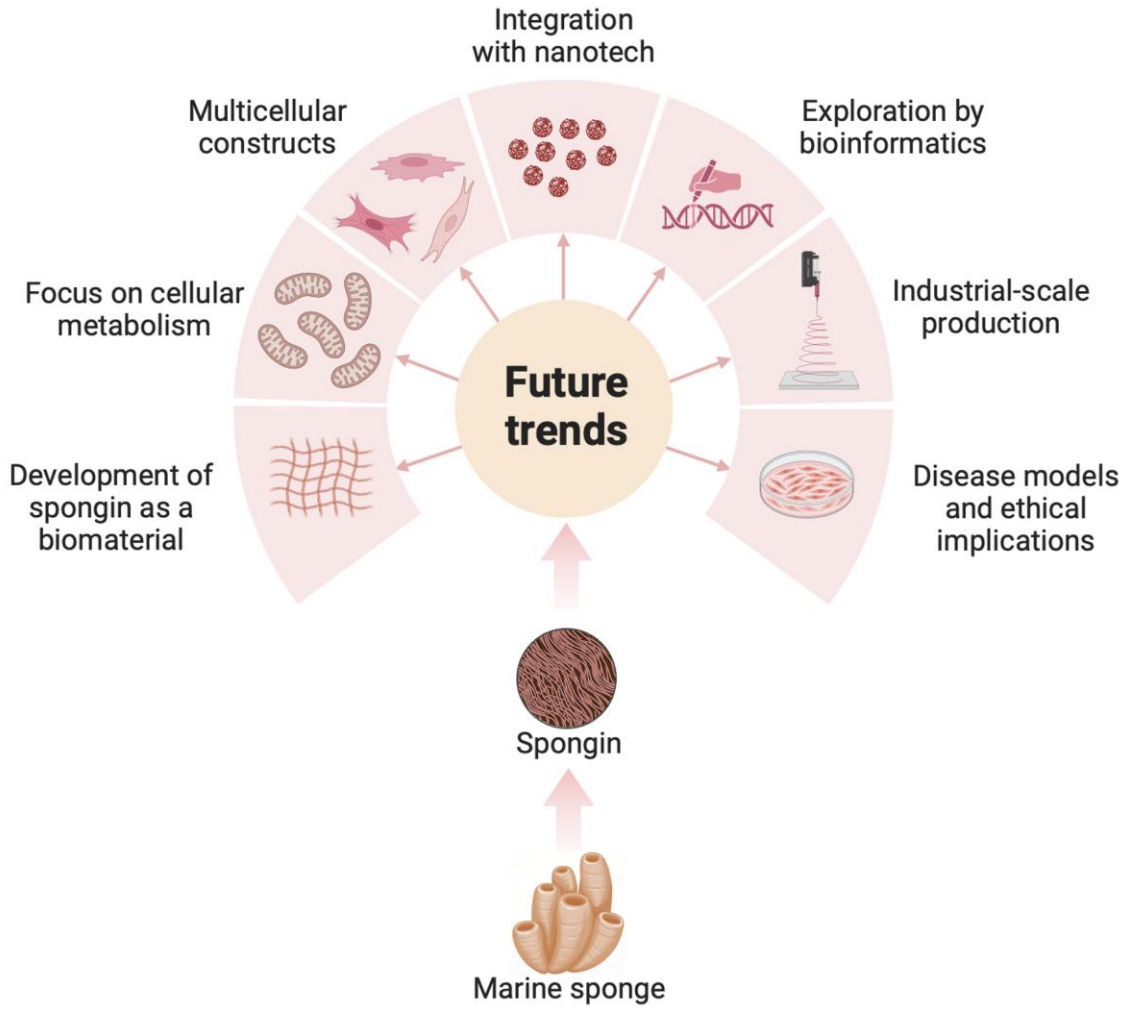
In addition to the combined use of cells and biomaterials, the third pillar of tissue engineering covers the control of the tissue microenvironment (Berthiaume et al., 2011). In this regard, natural biomaterials like SPG can be designed to act as carriers for the direct transport of growth factors to the repair site, facilitating regenerative therapy. Furthermore, more precise control of the microenvironment in which cells grow, including factors such as nutrients, oxygen, and mechanical tension, can be achieved to optimize cell growth and differentiation.

Moreover, SPG can be structurally modified and adapted for various applications. It can be employed in different forms, such as gels, membranes, sponges, or fibers, and its structure can be adjusted to meet the specific needs of an application. Personalized medicine is certainly one of the main advancements that has been achieved in recent years but must be expanded to reach healthcare systems globally.

The industrial-scale production of tissues and organs for transplantation is also a goal to be accomplished and involves the development of large-scale production methods, such as cell culture in bioreactors and 3D bioprinting. The latter interestingly allows the precise creation of tissue structures (Matai et al., 2020). In the future, improvements in precision, speed, and the variety/combination of biomaterials and cells used are expected, to enable the creation of more complex and functionally diversified tissues. Furthermore, beyond the creation of individual tissues, the engineering of complete organs is an ambitious objective for the field in the coming years.

Finally, it is possible to SPG for the development of *in vitro* disease models, which would enable the study of various diseases, drug screening, a better understanding of the underlying mechanisms of these diseases, as well as the feasibility of genome editing techniques to genetically correct tissues before implantation. This may be accompanied by work involving ethical and regulatory issues, which will become increasingly important, including topics related to safety, intellectual property, and equity in access to medical advancements (Figure 6).

Figure 6 - Future trends for the use of marine spongin in tissue engineering.



Source: Created by the Authors.

These are just some of the future directions in the field of tissue engineering that could involve the application of natural organic biomaterials like SPG. It is an ever-evolving field, and innovations in this area have the potential to revolutionize regenerative medicine and significantly improve the quality of life for patients worldwide.

9. Conclusion

Taking all the results together, the good osteogenic performance reached by marine sponges and/or their extracted components encourage the development of new studies that could lead to the development of SPG graft materials, which could constitute a promising alternative for the treatment of injuries. The aim of this review was to present the innovative use of marine sponges in the tissue engineering field, mainly due to their appropriate structure and composition. In the studies presented herein, the authors demonstrated that a series of different marine sponges seems to have appropriate porosity, surface chemistry, *in vitro* stability and no cytotoxicity, being also able to induce cell growth. Although more studies are warranted to investigate the safety and the biological performance of sponges, the development of natural biotechnological products for bones is a promising strategy that deserves further attention.

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