UNIVERSITY
OF TRENTO - Italy
DEPARTMENT OF INDUSTRIAL ENGINEERING

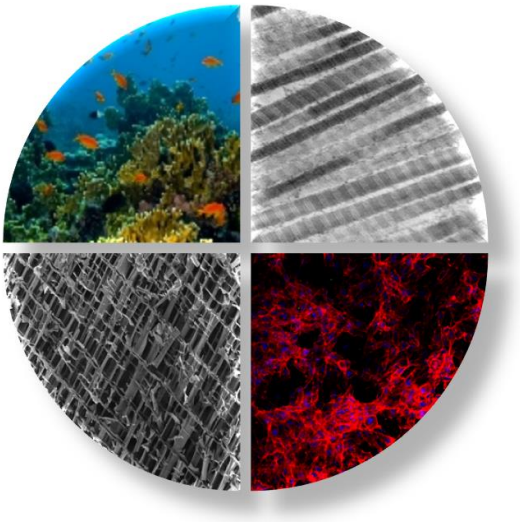
XXVIII cycle

Doctoral School in Materials Science and Engineering

Marine organisms as sources of materials for instructive scaffolds design

Natascia Cozza

Tutors:
Prof. Claudio Migliaresi
Prof. Antonella Motta



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Tutors:
Prof. Claudio Migliaresi
Prof. Antonella Motta

Ph.D. Commission:

Prof. Vincenzo Maria Sglavo,
Department of Industrial Engineering,
University of Trento, Italy.

Prof. Nuno M. Neves,
Department of Polymer Engineering 3B's Research Group,
University of Minho, Portugal.

Prof. Cekdar Vakifahmetoglu,
Department of Mechanical Engineering,
University of Istanbul Kemerburgaz, Turkey.

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Department of Materials Science and Engineering

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Chapter I

General introduction

1.1 Significance of the study

During the last years, there has been an even increasing interest for natural derived materials and for the study of the biophysical processes involved in their formation.
In fact, despite the possibility to fabricate home-made materials in reproducible way to meet specific performance demands, the complexity of biological systems suggested to consider nature as an inspiration for the design and synthesis of new types of materials.
In this context, marine biomaterials are a area of research with significant applications. In fact, the marine environment represents a unique resource of natural inorganic and organic materials with peculiar properties such as chemical and structural complexity, multifunctionality and miniaturization that are not possible to obtain in the laboratory.
Therefore, the isolation, characterization and processability of marine materials are crucial aspects for the development of the marine biotechnologies industry.
The aim of this study was to isolate and synthesize naturally-derived materials from marine organisms for biomedical use and namely for tissue engineering applications.
The study has been divided into two main parts.
The first part concerned the isolation and characterization of an important natural biopolymer: collagen. In particular, Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were isolated from *Loligo Vulgaris* squid mantle and comparatively characterized.
In the second part of the work a novel method to process cuttlefish bone powders for the production of highly bioactive ceramics formulations has been developed.

1.2 Objectives of the study

The first part of the present study aimed to:
1. Optimize the protocol for the extraction of collagen-derived materials from *Loligo Vulgaris* squid mantle;
2. Isolate and comparative characterize Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) fractions from *Loligo Vulgaris* squid mantle;

3. Provide a complete set of characterization techniques to evaluate the physico-chemical properties of the extracted collagens;
4. Investigate the dependence of ASC and PSC properties to the source and extraction method;
5. Describe a simple method for processing marine collagen fractions isolated from *Loligo Vulgaris* squid mantle, using genipin as natural crosslinking agent, to fabricate stable collagen matrices with no cytotoxic effects.

The main objectives of second part were:
1. Synthesize calcium phosphates-based materials starting from cuttlefish bone powders (*S. officinalis*);
2. Produce highly bioactive ceramic compositions through the co-sintering of naturally-derived hydroxyapatite (synthetized from cuttlefish bone powder) and Bioglass®-45S5;
3. Examine the structure, composition, mechanical properties and bioactivity (*in vitro*) of the new ceramic formulations in order to evaluate their potential use for bone tissue engineering applications.

1.3 Structure of the thesis

This thesis is organized in the following chapters:

Chapters II and **III** introduce some general concepts and provide the state of the art of naturally-derived biomaterials, drawing the attention on marine biopolymers and in particular on marine collagen.
Chapter IV describes the isolation and characterization of Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) fractions from *Loligo Vulgaris* squid mantle and provides a simple method for processing the isolated collagens using genipin as natural crosslinking agent.
Chapter V outlines the state of the art of bioceramics and underlines the importance of marine organisms as source and models for several bone tissue engineering applications.
Chapter VI describes a novel methods for fabricating highly bioactive ceramics compositions through the co-sinterization of naturally-derived hydroxyapatite (synthesized from cuttlefish bone powder) and Bioglass®-45S5; The characterization of the structure, composition and mechanical properties of the new ceramic formulations were provided and the *in vitro* bioactivity was also evaluated.
Finally, **chapter VII** presents the main conclusions of this thesis and discusses about further improvements and future perspectives of the research.

Chapter II

Naturally derived Biomaterial

2.1 Biomaterials and Biocompatibility

2.1.1 Biomaterials: history and definition

Several definitions have been developed for the term biomaterial. According to Williams [1] a biomaterial can be defined as *any substance (other than drug) or combination of substances synthetic or natural in origin, which can be used any time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body*. This definition includes a wide heterogeneous group of natural or synthetic compounds, placed in contact with the body with the aim of improving the function or replacing a diseased or injured tissue or organ.

In the last century, the evolution of biomaterials was very fast due to the knowledge gained from biology and materials science and, simultaneously, to the rapid advances in engineering (for example nanotechnology) that have allowed the production of materials with increasingly complex functions and sophisticated design [2]. In particular, biomaterials have moved from minimally interacting with the body to guiding the biological processes toward the goal of tissue regeneration [3].

At the beginning, biomedical materials were designed to perform largely mechanical functions: it was preferable being "inert" and not interacting with the host organism in order to be not harmful and prevent biological rejection.

The second generation of biomaterials begun around 1970 and overturned this concept: the materials started to assume a more “active” role, interacting with the biological environment and interfacing directly with cells and tissues through well-defined molecular pathways to direct biological responses [4]. The progress of technology have allowed to engineer biological activity into synthetic materials, improving the performance of biomaterials and increasing the number of their potential applications.

Later on, the efforts in the development of the latest generation of biomaterials were aimed to stimulate specific cellular responses at the molecular level, creating a strong interaction with the biological systems, through the design of bioactive/biomimetic materials [5]. These sophisticated materials are often designed to mimic the physicochemical properties of natural materials. In fact, materials used in living systems are multifunctional and dynamic. In addition, they are constructed by self-assembly, using 'bottom-up' fabrication methods, that allow the production of complex and information-rich structures, in reproducible way and with minimal

energy consumption [2]. For these reasons, both the materials and the processes involved in their formation, are a source of inspiration for the processing and design of new home-made biomaterials.

2.1.2 Basic principles of Tissue Engineering

Biomaterials represent a major component in various strategies of tissue engineering and regenerative medicine. Tissue engineering is an interdisciplinary field that combines the advantages of materials science and processing with the principles of life science, for the development of biological substitutes (scaffolds) that restore, maintain, or improve tissue or organ functions [6].
Biomaterials and scaffolds are fundamental parts of these strategy and they have been used for example as replacement of the extracellular matrix in a-cellular strategies, controlled delivery system for bioactive molecules, or as a guiding and supporting matrix for cell organization into a functional tissue [7].
The scaffolds act essentially as a template for tissue formation and are in general seeded with cells, and occasionally growth factors and/or other bioactive molecules, or can be subjected to biophysical stimuli in the form of a bioreactor. These cell-seeded scaffolds can be implanted directly into the injured site to induce the tissue regeneration (*in vivo*), or cultured *in vitro* in order to synthesize tissues which are subsequently implant in the damaged site [3].
The process of bioengineering 3D artificial tissues is very complex and differs based on the specific application. In particular, a crucial aspect is related to the choice of the biomaterial. In fact, the scaffolds have possibly to mimic the properties of the tissue to be substituted and, at the same time, provide structural support during the regeneration process. For these reasons, the design of the scaffold and the biomaterials synthesis and characterization are aspects that require a rigorous optimization.

2.1.3 Basic biomaterial design criteria

In general, the biomaterials for tissue engineering application should satisfy certain basic criteria. The first essential requirement is biocompatibility. Williams defined the biocompatibility as *the ability of a material to perform with an appropriate host response in a specific situation* [8].
In tissue engineering, this term may indicate the ability of the scaffold materials to support and guide the tissue regeneration process without negatively interfering with physiological processes or eliciting any undesirable local or systemic responses in the host [9].

However, in this broad definition no parameters are specified to test biocompatibility and the appropriate host response must be evaluated case-by-case because it is strongly related to the specific application and its context.
Ideally, the scaffolds should disappear from the host when the new healthy tissue has been formed and normal functions are restored [10]. For these purposes, the scaffolds should be biodegradable (for example through the degradation or dissolution of the matrix) and bioabsorbable. In addition, even the products of this process must be biocompatible and they have to be reabsorbed or discarded from the body by excretion [11].
Another important factor concerns the mechanical stiffness and strength of the material. In fact, scaffolds should have mechanical properties compatible with those of the host tissue to allow integration without interfering with its normal functionality [12]. In addition, they have to contain and support the seeded cells and, at the same time, preserve their structures under mechanical stress existing during cultivation or in the implantation site.
Finally, two further crucial aspects to consider in the scaffold design, regard the scaffold internal morphology and the fabrication process. In particular, when scaffolds are seeded with cells, the matrix should be porous (pores larger than 50 μm) and with interconnecting pore structure to enable cell-cell interactions and the vascularization of the construct [10]. At the same time, the scaffold should present cell recognition patterns to stimulate cell responses. As a consequence, the fabrication process should allow recreating the designed scaffold morphology without involving the use of toxic reagents or without adversely affecting the material properties (degradability, mechanical properties and cell recognition).

2.1.4 Naturally Derived and Synthetic Biomaterials

In recent years, biomaterials have been extensively investigated for several tissue engineering and regenerative medicine purposes and their market has expanded considerably together with the progress in medicine [7].
Based on their source origin, biomaterials are frequently classified in synthetic and naturally derived materials. Synthetic materials are synthesized artificially in the laboratory and, in general, they can be processed on a large scale in a reproducible way and with predictable and controllable properties in term of mechanical properties, microstructure and degradation rate [13]. However, in most cases, synthetic materials lack of the biological cues for promoting cell responses and their degradation by-products may be toxic for the cells. Therefore they can require to be functionalized or chemically modified to fulfil the desired properties in terms of biocompatibility [11].

Besides synthetic biomaterials, there are several materials derived from natural sources (animal or plant world) being considered for the use as biomaterials [14]. One of the advantages of using natural materials for biomedical applications is that they many of them are similar to materials familiar to the body and most of them, such as collagen and hyaluronan, present cell recognition patterns that stimulate cell response [15]. In fact, natural materials may carry specific protein binding sites and other biochemical signals that could induce the attachment and migration of cells from the surrounding biological environment, promoting the integration and tissue healing [16]. In addition, natural materials often do not present problems of toxicity, often found in synthetic materials [17].

Nowadays, biomaterials that are used in different medical applications are derived from multiple natural sources, including the extracellular matrix of mammalian tissue (e.g., porcine urinary bladder and small intestinal submucosa) as well as plants (e.g., cellulose), insects (e.g., silk), crustaceans (e.g., chitin) or other marine species (e.g., alginate) [18]. Due to the wide range of potential natural sources, their critical aspects are manly inherent to the purification processes, biological variability of the sources, pathogen transmission and immunogenicity. Another problem that could be associated with these materials, especially with natural polymers, is their tendency to denature or decompose at temperatures below their melting points [13].

However, despite the possibility to fabricate and engineering home-made materials in reproducible way to meet specific performance demands, the complexity of biological systems suggested to consider nature as an inspiration for the design and synthesis of new types of materials. Moreover, the materials used in biological systems are formed by a self-assembly processes that allow the construction of dynamic and multifunctional products. For these reasons, the field of biomimetics is growing and, during the last few years, there has been an even increasing interest for natural derived materials and for the study of the biophysical processes involved in their formation. The possibility to translate natural processes and products into technology represents one of the most promising ways to resolve and overcome different current technological and scientific limitations.

2.1.5 Bioinspired Synthesis of Composite Materials

During the evolutionary process, nature has developed a variety of highly sophisticated materials that generally show better physical properties than those of the equivalent synthetic materials, with analogous composition.

It is interesting to evidence that these natural materials are produced with relatively low energy consumption and at mild temperature and pressure conditions. In addition, despite their good mechanical performance, they are basically made of three basic 'building blocks': polysaccharides, proteins and minerals [19].

The final mechanical and functional performance of the natural composite materials are the result of their hierarchical structures and of the arrangement of their structural components [20]. In fact, biological composite materials are constituted by different layers, in which the components are organized in specific structures and controlled orientations, ranging from the molecular to the macroscopic level [21]. These layers are strongly interconnected to one another and are assembled through a 'bottom-up' approach mediated by cellular signals [22]. Inside this complex structural organization, the mineral component usually presents an anisotropic geometry and is often combined with a soft organic matrix [23]. For example, Figure 1 illustrates that bone and nacre have a similar nanostructure characterized by plate-like crystals in a soft organic matrix [24].

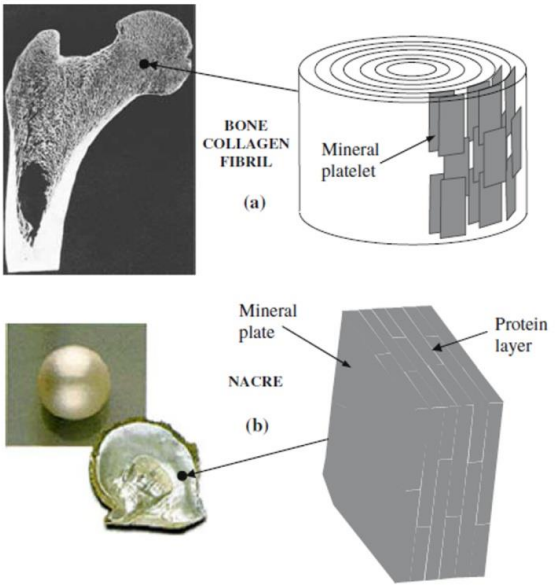

Figure II - 1. Nanostructures of nacre and bone. **(a)** The nanostructure of bone consists of plate-like mineral crystals 2–4 nm in thickness and up to 100 nm in length embedded in a collagen-rich protein matrix. **(b)** The elementary structure of nacre consists of plate-like mineral crystals 200–500 nm in thickness and a few micrometers in length with a very small amount of soft matrix in between. From [24].

As a consequence, the final behaviour and physical properties of the composite biomaterial are a result of the hierarchical organization as well as the surface-to-surface interactions between its components, including the interactions between the organic and inorganic components [25]. Therefore, in order to better understand the

structure of natural composite materials and the corresponding relationship with their peculiar properties and functions, the study of biomineralization is a crucial aspect. Two main process of mineralization have been described [26]. The first is an 'organic matrix-mediated' process. In this process the organism builds an organic template in which specific ions are introduced and then induced to the mineral phases formation. The second process is 'biologically induced' and is typical of some bacterial species and various green and brown algae. In this case the extracellular and/or intracellular mineral formation happens without prior formation of organic matrices. The mineral products in this case, are more similar to those obtained by precipitation from inorganic solutions.
The mechanisms that are used by organisms to produced minerals components could be used to develop novel processing strategies to produced high performance materials using mild conditions of temperature, pressure and solvent type. At the same time, the characteristics of some mineralized biological materials are very interesting for different biomedical applications, in particular for the regeneration of hard connective tissues [27].

2.2 Marine biomaterials

2.2.1 Introduction to Marine Biotechnology

Oceans covers more than 70% of the planet's surface and is a rich still unexploited source of chemical diversity. Because of the high variation of the physical and chemical conditions in terms of pressure, temperature and salinity of the marine environment, marine organisms are able to produce a variety of novel molecules with unique features and adapt their structures to survive in this complex living conditions [28]. As a consequence, in the oceans it is possible to find a structural complexity combined with biological potency and selectivity and a much higher chemical diversity that what can be obtained by synthesis or with the traditional technological approaches. Recent screening techniques have confirmed the immense biological diversity in the oceans and the presence of a large number of natural products with biological activities [29].
Many marine species are constituted by materials with a vast range of properties and are an economically important source for several industrial products, including food, medicine and various raw materials [30]. Among the different applications, marine biomaterials represent a significant fraction of the marine biotechnology industry and several marine species are proved to be good available sources of biomaterials for therapeutic and medical purposes.
In addition, the marine environment is rich in organisms and structures that present a functional design and can be consider as a source of inspiration for find efficient

strategies for solving technological and scientific problems. Figure 2 reports some examples of marine organisms characterized by interesting architectures that are often objects of biomimetic studies [31].

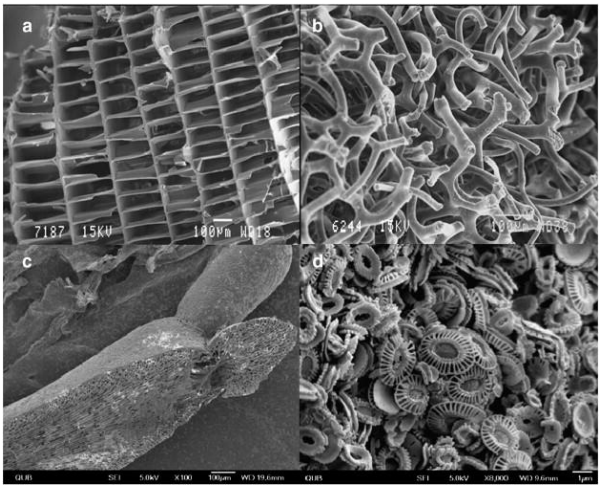

Figure II - 2. *Examples of marine organisms characterized by macro and microporous structures. Scanning electron micrographs of* ***(a)*** *cuttlefish (Sepia officinalis),* ***(b)*** *sponge (Spongia agaricina),* ***(c)*** *red algae (Corallina officinalis) and* ***(d)*** *coccolithophores (Emiliania huxleyi). From* [31].

In this regard, a sustainable exploitation of the marine resources can have several economic and environmental advantages, for example through the valorisation of the marine residues obtained after the food processing.

In recent years, the chemistry of marine derived compounds has become the object of a much greater research effort and an even increasing numbers of molecules and materials have been isolated from marine organisms and proposed as novel products for medical or health applications. However, marine biomaterials science is still a relatively new research area that offers an enormous potential for discovery, development, and marketing of novel marine by-products and for the development of new approaches finalized to the treatment of disease.

2.2.2 Marine Biopolymers

As major reserve of chemical diversity, the oceans are a rich source of polysaccharides, proteins and other polymeric compounds. In fact, due to the

variability of the living conditions in the ocean, marine organisms have developed polymers with a variety of properties to adapt at this complex environment. For example, polysaccharides have an important role as structural components in different species of algae or in crustaceans. Some proteins are involved in the mineralization processes for the formation of shells, other act as antifreeze agents allowing survival of some species at extremely cold temperature.

During the last few years, marine polymers have received a growing interest and have been increasingly used in industry and medicine [32]. Chitin and some algal polysaccharides such as agar, alginate and carrageenan are widely used for a variety of biomedical applications, with a market of several hundred million dollars annually [33].

Chitin (polymer of β(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose) derive principally from the organic matrix of exoskeletons of arthropods such as crustaceans and endoskeleton of mollusk. Chitin is the second most abundant natural polymer, after cellulose and is widely used in biomedical applications because of its biocompatibility, anti-bacterial and gel-forming properties [34]. Chitosan is the deacetylated form of chitin and is also widely employed in biomedicine.

The main steps that are involved in the production of chitin from raw material are reported in Figure 3 and include the consecutive removal of minerals (by acid treatment), proteins (by alkaline treatment) and pigments (using solvents or a mild oxidizing treatment) [35].

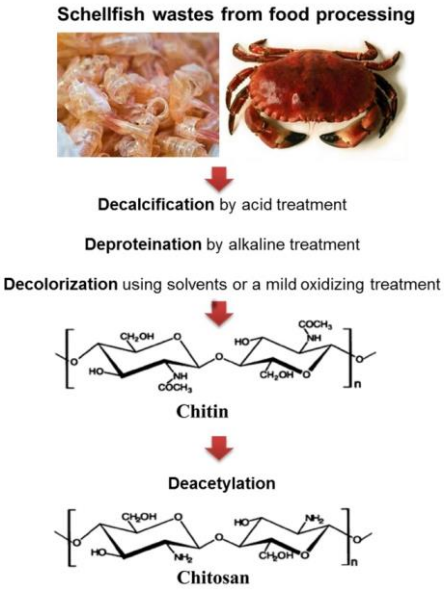

Figure II - 3. Steps for the industrial production of chitin from raw material.

Polysaccharides are a class of marine biopolymers constituted by carbohydrate monomers linked by glycosidic bonds. They have a wide range of applications and can be used directly as a biomaterial or can be subject to chemical modification, allowing a greater flexibility in the design of new co-polymers [30]. Red seaweed produce agars and carrageenans and brown algae produce alginates. All these natural sugars present excellent gelling and colloidal properties and are used in different tissue engineering applications.

In particular, agar is a complex polysaccharide present in the cellular walls of certain red algae and is used as a gel for cosmetics, medical ointments and inert drug carrier [36].

Alginate is a biopolymer extracted from algae using a treatment with aqueous alkali solutions, typically with NaOH, and a subsequent filtration process. At the end of the extraction, alginate can be obtained in the form of gelatinous alginic acid through the acidification of the alginate solution, or in the form of salt by precipitation with ethanol [37]. Alginate is composed of β-d-mannuronic acid (M) and α-l-guluronic acid (G) linked by 1-4 glycosidic bonds and its physicochemical properties are strongly influenced by the variability of the ratio and sequence of M and G units [38]. Due to its good gelling capacity, alginate is used as gel forming, stabilizing agent or in wound dressing applications [39].

Other marine-derived compounds, such as fucoidans, have been intensively studied for the formulation of new drug agents because of their varied biological activities, including anti-inflammatory, anticoagulant and antioxidant properties [40].
Marine organisms are also an important source of collagen. Between marine-derived proteins, collagen has been used in various kinds of drug delivery, cosmetics and tissue engineering purposes. In fact, collagen is the most abundant protein in mammals and due to its excellent biological characteristics, such as weak antigenecity, biodegradability and high cells affinity, is one of the most useful biomaterials [41]. Nowadays, the main industrial sources of collagen are bovine and porcine skins and bones. However, the high cost and complex purification procedures that are required to reduce the risk for transmitted diseases or structure alteration during the extraction process, have recently decreased mammalian collagen demands. In contrast, marine environment is rich of potential resources of collagen derived materials and marine collagen is safe and can be extracted following easy procedures [42]. In the last few years, several methods to obtain collagen based macromolecules from different marine species have been studied. However, the characterization and biomedical applications of marine collagen are still under development and efficient isolation techniques are yet to be discovered.

2.2.3 Marine Ceramics

Natural materials from marine origin such as sponges, nacres and corals provide also a rich source of inorganic materials with important applications for tissue replacement and regeneration [32].
Calcium carbonate (in aragonic or calcite forms) is quite abundant in the marine environment and can be found in different marine species such as corals, mollusk shells, sponges and fish bones. It is widely used in orthopaedics and dentistry, or as a bone filler. In addition, it can be used as the precursor material for obtain different calcium phosphates compounds, such as hydroxyapatite. Calcium phosphates are very interesting for the biomedical field because of their similarities with the mineral component of the bones and are currently used in drug delivery systems, as a components in bone graft substitute and tissue engineering scaffolding [43].
Another important class of ceramics are biosilica, which consist of glassy amorphous silica and are formed in many aquatic organisms, including sponges, diatoms and radiolarians [27]. Biosilica is being considered for bone replacement and regeneration strategies.
For example, an important source of biosilica are diatoms. Diatoms are unicellular organisms which have an exoskeleton made of silica nanoparticles assembled in a highly organized porous structure. Also some classes of sponges, like

demospongiae and hexactinellida, have a silica skeleton and in these organisms the process of biosilica formation is enzyme-mediated [42].

2.2.4 Applications of marine-derived biomaterials: Drug delivery and Tissue Engineering

Among the different biomedical applications of marine-derived biomaterials, drug delivery and tissue engineering have received a growing interest during the last decade [32]. A large number of drug delivery systems have been developed using marine biomaterials, in particular polysaccharides, and have been investigated for different therapeutic purposes. For example, polysaccharides-based nanoparticles, that act as injectable delivery systems for several kind of molecules or drugs, have been produced using various techniques such as covalent cross-linking or self-assembly processes [44]. Chitosan and alginate have been studied for the development of multi-layered nanostructured coatings and are probably the most used marine-derived polymers for the production of drug delivery particles [45] . The presence of acid or basic functional groups in several marine polysaccharides allows the design of pH-responsive hydrogels for the smart delivery of bioactive agents, which are able to swell or degrade as a consequence of changes in the pH of the physiological environment [46].

Different biodegradable marine-derived materials have been investigated even for the processing of scaffolds for tissue engineering applications. In this context natural materials, such as natural polymers, due to their similarity to the extracellular matrix and other biological macromolecules, are easily recognized by the biological environment and may avoid toxicity effects or immunological reactions [18]. However, in the processing of these kind of materials in general it is not possible to use the technologies that are commonly employed in the manufacture of synthetic materials. For example, severe conditions during the processing of the material, such as high temperature or organic solvents, may cause degradation or the loss of important properties of the material. For these reasons, mild treatments require to be developed in order to preserve the original characteristics of natural biomaterials.

Among the methods that are currently used, freeze-drying is a very used technique for processing different marine-derived materials, such as chitosan and collagen. This method allows the production of porous matrices through the freezing and subsequent lyophilisation of a solution containing the material. The structure and porosity of the obtained scaffold depends on some process parameters, including size and shape of the ice crystals formed during freezing. Microfibers from solution containing chitosan can be produced also by wet-spinning, where the polymer is precipitated into a coagulation bath [47]. Moreover, electrospinning have been used to fabricate non-woven matrices for tissues regeneration or delivery systems, made

of nanofibers of different marine biomaterials, like chitosan, collagen or alginate [48][49].

Also injectable systems, such as chitosan-based thermoreversible gels, carrageenan and alginate-based hydrogels for cartilage regeneration, are particularly interesting because they can be implanted in specific sites in the body using a minimally invasive way [50].

From these considerations, it is clear the great potential of marine-derived materials for tissue engineering applications. In addition, the potentiality in terms of chemical diversity and wide range of applications of these materials, can be further enhance by the development of strategies for improve their mechanical properties and stability or through specific physical or chemical modifications. Therefore, methods to obtain material from marine resources in a reproducible way must be develop and strong efforts are still needed to identified efficient systems for the processing and the evaluations of their physicochemical properties.

References

[1] D. F. Williams, "On the nature of biomaterials," *Biomaterials*, vol. 30, no. 30, pp. 5897–5909, 2009.

[2] N. Huebsch and D. J. Mooney, "Inspiration and application in the evolution of biomaterials," *Nature*, vol. 462, no. 7272, pp. 426–432, 2009.

[3] F. J. O' Brien, "Biomaterials & scaffolds for tissue engineering," *Mater. Today*, vol. 14, no. 3, pp. 88–95, 2011.

[4] B. K. D. Jandt and F. Jena, "Evolutions, Revolutions and Trends in Biomaterials Science – A Perspective," *Adv. Eng. Mater.*, vol. 9, no. 12, pp. 1035–1050, 2007.

[5] K. D. Park, X. Wang, J. Y. Lee, K. M. Park, S. Zhang, and I. Noh, "Research trends in biomimetic medical materials for tissue engineering: commentary," *Biomater. Res.*, vol. 20, no. 1, pp. 1, 2016.

[6] J. P. Vacanti and C. A. Vacanti, *The History and Scope of Tissue Engineering*, Fourth Edition. Elsevier, 2014.

[7] A. Atala, "Tissue Engineering and Regenerative Medicine: concepts for clinical application," *Rejuvenation Res.*, vol. 7, no. 1, pp. 15–31, 2004.

[8] D. F. Williams, *The Williams dictionary of biomaterials*. Liverpool University Press, 1999.

[9] Y. Luo, G. Engelmayr, D. T. Auguste, S. Ferreira, J. M. Karp, R. Saigal, and R. Langer, *3D Scaffolds*, Fourth Edition. Elsevier, 2014.

[10] P. X. Ma, *Biomaterials and Regenerative Medicine*. Cambridge University Press, 2014.

[11] L. G. Griffith, "Emerging Design Principles in Biomaterials and Scaffolds for

Tissue Engineering," *Ann. NY Acad. Sci.*, vol. 961, no. 1, pp. 83–95, 2002.

[12] B. P. Chan, and K. W. Leong, "Scaffolding in tissue engineering: general approaches and tissue-specific considerations," *Eur. Spine J.*, vol. 17, no. 4, pp. 467–479, 2008.

[13] R. Langer and D. A. Tirrell, "Designing materials for biology and medicine," *Nature*, vol. 428, no. 6982, pp. 487–492, 2004.

[14] T. Le, B. Ha, and T. M. Quan, "Naturally Derived Biomaterials: Preparation and Application," *Reg. Med.Tissue Eng.*, pp. 247–274, 2013.

[15] F. H. Silver and D. L. Christiansen, *Biomaterials science and biocompatibility*. Springer Science & Business Media, 2012.

[16] J. F. Mano, G. A. Silva, H. S. Azevedo, and P. B. Malafaya, "Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends," *J. Roy. Soc. Interf.*, vol. 4, no. 17, pp. 999–1030, 2007.

[17] J. Black and G. Hastings, *Handbook of biomaterial properties*. Springer Science & Business Media, 2003.

[18] O. O. Ige, L. E. Umoru, and S. Aribo, "Natural Products: A Minefield of Biomaterials," *ISRN Mater. Sci.*, 2012.

[19] G. M. Luz and J. F. Mano, "Mineralized structures in nature: Examples and inspirations for the design of new composite materials and biomaterials," *Compos. Sci. Technol.*, vol. 70, no. 13, pp. 1777–1788, 2010.

[20] P. Fratzl and R. Weinkamer, "Nature' s hierarchical materials," *Prog. Mater. Sci.*, vol. 52, no. 8, pp. 1263–1334, 2007.

[21] S. Weiner and L. Addadi, "Design strategies in mineralized biological materials," *J. Mater. Chem.*, vol. 7, no. 5, pp. 689–702, 1997.

[22] S. Zhang, "Fabrication of novel biomaterials through molecular self-assembly," *Nat. Biotechnol.*, vol. 21, no. 10, pp. 1171–1178, 2003.

[23] J. W. C. Dunlop and P. Fratzl, "Biological composites," *Annu. Rev. Mater. Res.*, vol. 40, pp. 1–24, 2010.

[24] H. Gao, "Application of fracture mechanics concepts to hierarchical biomechanics of bone and bone-like materials," *Int. J. Fracture*, vol. 138, no. 1-4, pp. 101–137, 2006.

[25] S. Weiner, "Organization of Organic Matrix Components in Mineralized Tissues," *Am. Zool.*, vol. 24, no. 4, pp. 945–951, 1984.

[26] H. A. Lowenstam, "Minerals Formed by Organisms," *Science*, vol. 211, no. 4487, pp. 1126–1131, 1981.

[27] A. J. Salgado, O. P. Coutinho, and R. L. Reis, "Bone tissue engineering: state of the art and future trends," *Macromol. Bioscience*, vol. 4, no. 8, pp. 743-765, 2004.

[28] D. W. Green, J. Lee, and H. Jung, "Marine Structural Biomaterials in

Medical Biomimicry," *Tissue Eng. Part B*, vol. 21, no. 5, pp. 438-450, 2015.
[29] B. Haefner, "Drugs from the deep: marine natural products as drug candidates," *Drug Discov. Today*, vol. 8, no. 12, pp. 536–544, 2003.
[30] *S. K. Kim, Marine Biomaterials: Characterization, isolation and applications.* CRC Press, 2013.
[31] S. A. Clarke, P. Walsh, C. A. Maggs, and F. Buchanan, "Designs from the deep: Marine organisms for bone tissue engineering," *Biotechnol. Adv.*, vol. 29, no. 6, pp. 610–617, 2011.
[32] T. H. Silva, A. Alves, B. M. Ferreira, J. M. Oliveira, L. L. Reys, R. J. F. Ferreira, R. A. Sousa, S. S. Silva, J. F. Mano, and R. L. Reis, "Materials of marine origin: a review on polymers and ceramics of biomedical interest," *Int. Mat. Rev.,* vol. 57, no. 5, pp. 276–306, 2012.
[33] J. B. Baker, *Marine Biomedicine: From Beach to Bedside.* CRC Press, 2015.
[34] M. Rinaudo, "Chitin and chitosan: Properties and applications," Prog. Polym. Sci., vol. 31, pp. 603–632, 2006.
[35] H. K. No, S. P. Meyers, and K. S. Lee, "Isolation and characterization of chitin from crawfish shell waste," *J. Agric. Food Chem.*, vol. 37, no. 3, pp. 575–579, May 1989.
[36] E. Percival, "The polysaccharides of green, red and brown seaweeds: Their basic structure, biosynthesis and function," *Brit. Phycol. J.*, vol. 1617, no. May, 2016.
[37] K. Yong and D. J. Mooney, "Progress in Polymer Science Alginate: Properties and biomedical applications," *Prog. Polym. Sci.*, vol. 37, no. 1, pp. 106–126, 2012.
[38] S. N. Pawar and K. J. Edgar, "Biomaterials Alginate derivatization: A review of chemistry, properties and applications," *Biomaterials*, vol. 33, no. 11, pp. 3279–3305, 2012.
[39] I. Donati and S. Paoletti, "Material properties of alginates," *in Alginates: biology and applications.* Springer Berlin Heidelberg, pp. 1–53, 2009.
[40] B. Li, F. Lu, X. Wei, and R. Zhao, "Fucoidan: Structure and Bioactivity," *Molecules*, vol. 13, no. 8, pp. 1671–1695, 2008.
[41] C. H. Lee, A. Singla, and Y. Lee, "Biomedical applications of collagen," *Int. J. Pharm.*, vol. 221, no. 1, pp. 1–22, 2001.
[42] S. K. Kim, *Marine proteins and peptides: biological activities and applications*. John Wiley & Sons, 2013.
[43] S. M. Best, A. E. Porter, E. S. Thian, and J. Huang, "Bioceramics: Past , present and for the future," *J. Eur. Ceram. Soc.*, vol. 28, no. 7, pp. 1319–1327, 2008.
[44] Z. Liu, Y. Jiao, Y. Wang, C. Zhou, and Z. Zhang, "Polysaccharides-based

nanoparticles as drug delivery systems," *Adv. Drug Deliv. Rev.*, vol. 60, no. 15, pp. 1650–1662, 2008.

[45] E. S. Miranda, T. H. Silva, R. L. Reis, and J. F. Mano, "Nanostructured Natural-Based Polyelectrolyte Multilayers to Agglomerate Chitosan Particles into Scaffolds for Tissue Engineering," *Tissue Eng. Part A*, vol. 17, no. 21–22, pp. 2663–2674, 2011.

[46] M. I. Giannotti, O. Esteban, M. Oliva, M. F. García-Parajo, and F. Sanz, "pH-Responsive Polysaccharide-Based Polyelectrolyte Complexes As Nanocarriers for Lysosomal Delivery of Therapeutic Proteins," *Biomacromolecules*, vol. 12, no. 7, pp. 2524–2533, 2011.

[47] G. C. East and Y. Qin, "Wet spinning of chitosan and the acetylation of chitosan fibers," *J. Appl. Polym. Sci.*, vol. 50, no. 10, pp. 1773–1779, 1993.

[48] Z. Chen, X. Mo, and F. Qing, "Electrospinning of collagen-chitosan complex," *Mater. Lett.*, vol. 61, no. 16, pp. 3490–3494, 2007.

[49] S. Van Vlierberghe, P. Dubruel, and E. Schacht, "Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review," *Biomacromolecules*, vol. 12, no. 5, pp. 1387–1408, 2011.

[50] N. Bhattarai, J. Gunn, and M. Zhang, "Chitosan-based hydrogels for controlled, localized drug delivery," *Adv. Drug Deliv. Rev.*, vol. 62, no. 1, pp. 83–99, 2010.

Chapter III

Literature Reviews: Collagen

3.1 The Collagen Molecule

3.1.1 Molecular structure

Collagen is the major structural material in vertebrates and the predominant mammalian protein, accounting about 20-30% of the total proteins in the body [1].
It provides several important functions. For example, collagen fibrils give structural and mechanical support to tissues such as tendon, skin and bone. In addition, they are involved in different biological functions, including the regulation of cellular activities and functionalities [2].
Collagen molecules structurally consist of three polypeptide chains intertwined to form a triple-helix structure. Each chain has an individual twist in the opposite directions and is characterized by the repetition of the proline-rich tripeptide Gly-X-Y (where X is mostly proline and Y hydroxyproline) [3]. In this conformation, only the N- and C- terminal regions of the chains, which consist of 15-26 amino acid residues and are called telopeptides, do not form triple helical structures. Figure 1 shows a graphical illustration of the molecular structure of the collagen triple helix.

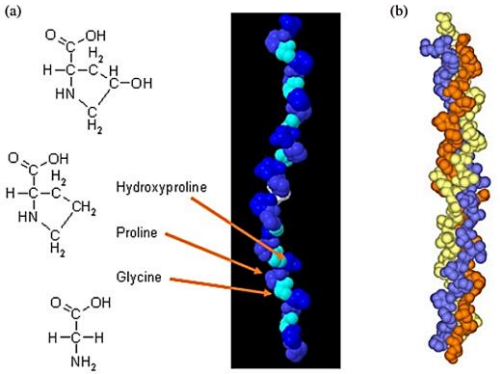

Figure III - 1. Illustration of the molecular structure of the collagen triple helix. **(a)** Model of part of a single collagen α-chain, with the typical amino acid residues Glycine, Proline and Hydroxyproline. **(b)** Model of the collagen triple helix. From [4]

The individual triple helices (tropocollagen molecules) inside the tissues are arranged to form fibrils, which can be further assembled and cross-linked (Figure 2). In particular the telopeptides have a very important role in the fibril formation. In fact, collagen intermolecular crosslinking generally involves a lysine aldehyde in the telopeptide of one chain and a hydroxylysine of the adjacent chain [4].

Additional crosslinks are formed between the telopeptide and adjacent helical domains of collagen molecules [5]. Therefore, through specific self-aggregation and crosslinking processes, collagen can form fibers.

This particular conformation confers the protein high tensile strength and flexibility and allows to support stress efficiently [6].

Generally, the degree of crosslinking inside the tissues increases with age and stress and as a consequence, it changes the properties of collagen material, making more difficult its solubilisation from the tissues under mild conditions.

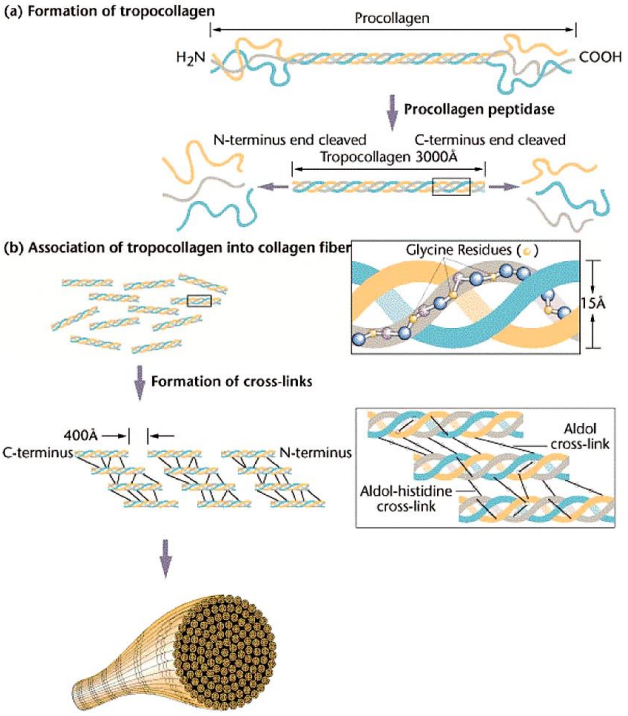

Figure III - 2. The individual triple helices or tropocollagen molecules, are arranged to form fibrils which are of high tensile strength and flexibility and can be further assembled and cross-linked. The figure has been reproduced from [7].

3.1.2 Amino acid composition

The basic collagen molecule contains three polypeptide chains intertwined to form a triple-helix structure, each consisting of more than 1000 amino acids [8].
The unique amino acid composition of collagen affects significantly the physico-chemical properties of the protein.
For example, the presence of glycine, which occurs at every third amino acid position in the sequence, is essential to stabilize the collagen triple helices through the formation of intra- and inter-chain hydrogen bonding [2].
About 35% of the non-glycine positions in the Gly-X-Y tripeptide are occupied by proline, predominantly in the X-position, and hydroxyproline in the Y-position [8].
Josse and Harrington proved that hydroxyproline and proline residues, which containing pyrrolidine ring, contribute to the structural stability of the protein. In particular, they reported that an high content of hydroxyproline is usually correlated to an high thermal stability [9].
Hydroxyproline derived from proline by post-translational hydroxylation mediated by prolyl hydroxylase during collagen synthesis [10]. It was demonstrated that collagen is the only protein rich in hydroxyproline (up then 10% in mammalian collagen) and for these reasons, its measurements offers a way to quantify collagen or its degradation products in the presence of other proteins .
Collagen also contains the unusual amino acid hydroxylysine that is formed from lysine in the endoplasmic reticulum via enzymatic hydroxylation by lysyl hydroxylase [8]. Both the imino acids, which represent approximately 23% of the residues, are involved in the stabilization of the triple helices. In fact, it was been proved that hydroxylysine, lysine and arginine residues and are involved to form additional hydrogen-bonds and are generally found at Y position of the tripeptide [3]. The X-position is instead usually preferred by leucine, glutamic acid and phenylalanine.
Another important characteristics in the amino acid composition of collagen, is the greater content of acidic and hydroxylated amino acid residues than lipophilic residues. As a consequence, collagen is a hydrophilic protein and swells in polar solvents [11].
Finally, it is interesting to note that, although the specific amino acid composition is strongly related to the type of collagen and its source, it is possible to make some general considerations. In fact, previous studies have reported that are no significant differences between the collagen from different vertebrate species [12]. Moreover, even fish collagens show a similar amino acid distribution to mammalian collagen but with decreased amounts of proline and hydroxyproline and increased serine, threonine and, in some cases, methionine and hydroxylysine residues. In particular, variations in the properties and composition in the case of marine collagens, seem to be strongly related to the temperature of the normal habitat, rather than to the zoological classification [13].

3.1.3 Biosynthesis

The majority of the collagen in connective tissues is produced by fibroblasts. Collagen pro-α chain is synthesized from a unique mRNA sequence and then transferred from the endoplasmic reticulum to the Golgi apparatus of the cell. During the transfer, some proline and lysines residues are hydroxylated and specific lysines are glycosylated. Then, the α chain, prior to be encapsulated in excretory vesicles, self-assemble to form protocollagen. Outside the cell the propeptides are cleaved to allow the auto-polymerisation. This process determines the start of tropocollagen self-assembly to form fibrils (10 to 300 nm sized) and the subsequent agglomeration of fibrils into 0.5 to 3 μm collagen fibers [14]. Fibril-forming collagens are the most commonly used as biomaterials.

3.1.4 Different kinds of collagen and their distributions

At present, 29 distinct collagen types have been identified and all display a typical triple helix structure. However, different kind of collagen are characterized by a considerable diversity in their structure, assembly and function [3].
Approximately there are twenty-five different α chain conformations (each produced by specific genes) and the combination of these chains, in sets of three, determines the formations of the different collagen types. The most common kinds of collagen are reported in Table 1.
In relation on their structure and supramolecular organization, collagens can be classified in fibril forming collagens, fibril-associated collagen (FACIT) and network-forming collagens. The fibril-forming collagens are the most abundant class of collagens (about 90% of the total collagens). This class include collagens types I and V that contribute to the structural backbone of the bone, and collagens types II and XI that are predominantly involved to form the matrix of articular cartilage.
Type III collagen is found in limited amount in tissues (mainly in scar tissue, arteries and intra-organ connections), usually in association with collagen type I. All these fibril-forming collagens contribute to give stability and integrity at the tissues due to their tensile strength and torsional stability. In addition, collagen types I, II, and III present large sections of homologous sequences independent of the species [15].
Fibril-associated collagens, like collagens IX and XII, have small chains which contain some non-helical domains and they associate as a single molecules with large collagen fibrils [16]. Types IV and VII are examples of network-forming collagens.

	Type	Molecular formula	Polymerized form	Cells of origin	Tissue distribution
Fibril forming (fibrillar)	I	$[\alpha1(I)]_2\ \alpha2(I)$	Fibril	Fibroblasts and reticular cells, smooth muscle cells, osteoblest, odontoblasts	Bone, skin, tendons, ligaments, cornea (represent 90% of total collagen of the human body)
	II	$[\alpha1(II)]_3$	Fibril	Chondrocytes, Retinal cells	Cartilage, invertebrate disc, notochord, vitreous humor in the eye
	III	$[\alpha1(III)]_3$	Fibril	Fibroblasts and reticular cells	Skin, blood vessels
	V	$[\alpha1(V)]_2\ \alpha2(V)$ and $\alpha1(V)\ \alpha2(V)\ \alpha3(V)$	Fibril (assemble with type I)	Fibroblasts, smooth muscle cells	As type I
	XI	$\alpha1(XI)\ \alpha2(XI)\ \alpha3(XI)$	Fibril (assemble with type II)		As type II
Fibril-associated	IX	$\alpha1(IX)\ \alpha2(IX)\ \alpha3(IX)$	Lateral association with type II fibril		Cartilage
	XII	$[\alpha1(XII)]_3$	Lateral association with type I fibril	Fibroblasts	Tendons, ligaments
Network-forming	IV	$[\alpha1(IV)]_2\ \alpha2(VI)$	Sheet-like network	Epithelial and endothelial cells	Basal lamina
	VII	$[\alpha1(VII)]_3$	Anchoring fibrils	Fibroblasts, keratinocytes	Beneath stratified squamous epithelia

Table III - 1. Collagen types, forms and distribution. Modified from [17].

3.1.5 Degradation and thermal denaturation

The various types of collagen show different resistance and mechanisms of degradation due to the specific conformation of the molecules and to varied thermal stability [16].

Probably due to its function as the primary structural protein in the body, collagen is particularly resistant to attacks by proteases and at neutral pH only specific collagenases are able to cleave the native structure of the protein [8]. In general, when the collagen molecules are aggregated to form fibrils, collagenases bind the native helix near the surface and the molecules are degraded starting from the exterior.

Another mode of protein degradation can occur through thermal denaturation. As previously described, the collagen triple helix is stabilized by the presence of

hydrogen bonds. Hydroxyproline is often involved in the formation of these bonds and contributes to the structural stability of collagen. In addition, the protein structure is further stabilized by intra- and intermolecular cross-links that allow the microfibril formation.

The process of thermal denaturation generally occurs under severe heating conditions and causes the rupture of the hydrogen bonds and the consequent rearrangement of the collagen triple helix into a random coil configuration [18]. It was proposed that the collagen denaturation process does not occur simultaneously along the entire length of the molecule but it starts in correspondence of a thermally labile region [19]. After that, the α-chains in this labile region are uncoupled, also all the rest of the structure starts to unfold. Hence, the thermal activation of the thermal denaturation process, is governed by the properties of the thermally labile region that usually is localized in correspondence of a hydroxyproline-deficient domain. Therefore, a high content of hydroxyproline is usually correlated to a high thermal stability of the protein.

From these considerations, it is clear the importance of hydroxyproline in stabilizing the triple helix and, in general, the important contribution of the collagen amino acids composition in determining the properties of the protein.

3.2 Properties of collagen

3.2.1 Functional properties

According to Gómez et al., the most important functional properties of collagen can be divided into two major groups: properties related to their surface behaviour and properties associated with its gelling behaviour [20]. The first group of properties include emulsion and foam formation and stabilization, adhesion and film-forming capacity. Charged groups in the protein side chains or the presence of sequences containing either hydrophobic and hydrophilic amino acids, are important factors that affect the surface properties of collagen. In addition these properties are strongly related to the characteristics of the row material. For example, it was found that acid-soluble collagen from Pacific whiting surimi refiner discharge, has higher emulsifying activity than both acid-soluble collagen from trout skin and the commercial emulsifier Tween-80 [21].

Soluble collagen has also suitable foaming properties because is able to reduce the surface tension at the liquid/air interface by increasing the viscosity of the aqueous medium [22].

On the other side, gelling and water binding properties are related mainly at the structure, molecular size and temperature of the system. In particular, changes in ionic strength, pH and temperature can induce the gelation process of collagen that

causes the microfibrillar aggregation of the molecules to form fibril, until equilibrium is reached. The gelation temperature of collagen is strongly related to the amino acid composition, physical properties and, consequently, to the extraction source. For example, the gelation process of collagen type I from vertebrate starts when temperature is increased from 20 to 28°C [23].
The swelling properties of collagen allow the production of hydrogels, which are characterized by their hydrophilicity and insolubility in water. As a consequence, collagen in solution can be covalently crosslinked to form matrices that are able to swell in presence of aqueous solutions, preserving their shape [24].

3.2.2 Bioactive properties

Besides its functional properties, collagen is also a promising source of bioactive peptides for various health related applications.
For example, antioxidant activity of several collagen-derived peptides has been observed. Various studies have demonstrated that they act as free radical scavengers, lipid peroxidation inhibitors and transition metal ion chelators [20].
The exact mechanisms of their action is still not completely clear, but it seems that antioxidant capacity could be mainly affected by some factors, including peptides conformation and molecular weight, abundance and position of specific amino acid in the peptide sequence and content of hydrophobic amino acid [25].
Gómez-Guillén *et al.* [26] have recently reported antimicrobial activity in peptides fractions obtained from tuna and squid gelatin. The hydrolysates were found to be highly active against different strains of Gram-positive and Gram-negative bacteria.
Antimicrobial peptides are able to kill target cells rapidly and play an important role in mediating innate immune responses to infection [27]. Usually they consist in short cationic peptides (less than 50 amino acids) and have positively charged lysine and/or arginine or histidine residues, and a large fraction of hydrophobic amino acids [28].
In addition, collagen has been shown to be a good source of antihypertensive peptides. These peptides are molecules which are able to lower blood pressure, through inhibition of vasoactive enzymes [29]. The antihypertensive activity of collagen hydrolysates may be related to the high concentration of hydrophobic amino acids, as well as to high proline levels [20]. Moreover, glyproline peptides, which are abundant in collagen, may also inhibit some factors involved in blood coagulation and platelet aggregation [30].
Collagen-derived peptides have been used even in the treatment of osteoarthritis and osteoporosis. In fact, some studies have proven the ability of collagen hydrolysates to stimulate bone collagen metabolism and improve bone mineral density [31].

Other important physiological activities include the enhancement of wound healing processes and a chemotactic activity to fibroblasts, peripheral blood neutrophils and monocytes [32][33].

3.3 Isolation and purification of collagen

Traditional sources of collagen and collagen-derived products, such as gelatin and collagen hydrolysates, are bovine skin and tendon and porcine dermis. [34] However, collagen is present not only in mammals, but throughout the entire animal kingdom including birds and fishes. For these reasons, recent studies have isolated and characterized varieties of collagen derived from numerous different sources, including human placenta and marine organisms [35][36][37].

Generally, tissues more rich in fibrous collagen are preferred as starting material for the extraction. However, collagen is not soluble in organic solvents and the main impediment to dissolution from native tissues is the presence of covalent crosslinks between molecules [38]. For these reasons, in the choice of the solvent for the extraction process, it is important to evaluate the natural crosslinks degree that is present in the specific tissue.

It was proved [39] that sources, extraction methods and pre-treatments affect the final characteristics of collagen, such as composition, rheological properties, solubility and thermal stability and consequently its biological activity.

Neutral salt-extraction and low concentration acid-extraction are the most commonly used methods to isolate collagen from natural tissues [40][41]. However, neutral solvents are able to solubilize only freshly synthesized and weakly crosslinked collagen molecules in the tissue. For these reasons, the yields of extraction using neutral salt solution are generally low and some tissues do not contain salt-extractable collagen.

Dilute acid solvents are more efficient than neutral salt solutions since they dissociate the intermolecular cross-links of the aldimine type causing the swelling of the fibrillar structures. [42] In fact, due to its amino acid composition and in particular to the higher content of acidic, basic and hydroxylated amino acid residues than lipophilic residues, collagen is a hydrophilic protein and swells in polar liquids [43]. As a consequence collagen can be directly extracted from tissues using organic or inorganic acids. Type and concentration of acid affect the yield and the final properties of the resulting protein. For example, the ionization constant (K_a) of the acid influences the swelling process and it was been proved that strong acids (high K_a) have effect in increasing collagen extraction rate [44]. On the other side, it was been demonstrated that extremely low pH could cause the denaturation and/or digestion of collagen during the extraction. Previous studies have reported that acetic acid, due to the small molecular size and low K_a, showed a good compromise

between extraction yields and properties of solubilized collagen that, after being reconstituted into fibrils, has similar properties to those of the native protein [45]. Consequently, solutions of acetic acid are in general preferred for the extraction process.
However, dilute acids solutions will not dissociate less labile cross-links such as keto-imine bonds, and solubilize approximately 2% of the tissue collagen.
Additional collagen can be solubilized using alkali solutions or enzyme treatment.
In particular, much higher yields can be achieved using proteases, i.e. pepsin that selectively cleaves peptides in the telopeptide region (Fig. 4), preserving the helix conformation [38]. In addition, it was been reported that the resulting material, named atelocollagen, benefits from the removal of the antigenic P-determinant located in the terminal non-helical regions of the protein and provokes milder immune response [46].

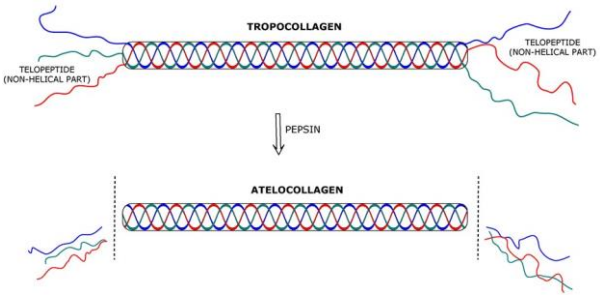

Figure III - 3. The cleavage of telopeptide region of collagen by pepsin. From [4].

Depending on the source and extraction process, the resulting collagen solutions are composed by varying proportions of monomer and high molecular weight aggregates. It was been reported that collagen isolated using proteases generally contains higher proportions of monomer than salt- or acid extracted material [47].
Finally, the remaining highly crosslinked insoluble collagen can be dispersed using mild denaturation agents and mechanical fragmentation, usually in acid conditions [48].

3.4 Collagen-Based Biomaterials

Collagen is the major component of the extracellular matrix and, as a consequence, is an ideal material to produce scaffolds or matrices for tissue engineering applications. In fact, collagen is an important structural component in various human tissues and organs, such as bone, skin, tendon, blood vessels, and cartilage. In addition, it is able to interact with the cells and plays an essential role for the

transmission of important signals that regulate cellular activities and functionality [49]. For these reasons, for decades, collagen-based materials has been widely used for different applications such as wound dressings, vascular graft coatings and injectable collagen for soft tissue augmentation [8].
Collagen based-biomaterials can be obtained through decellularization of a collagen matrix or by extraction, purification and processing of collagen to produce a functional scaffold [50].
The first method allows the preservation of the original extracellular matrix but requires the combination of physical, chemical and enzymatic treatments to achieve the complete decellularization of the tissue.
On the other side, different approaches were developed to extract collagen from biological tissues, mainly based on the solubilization of the protein using acid solutions, neutral salt solutions or proteolytic solutions [38].
However, both the approaches lead to obtain a product that in general requires to be stabilized or submitted to various cross-linking methods before use.

3.4.1 Collagen-based scaffolds

Decellularized scaffolds are obtained through the decellularization of tissues and organs, preserving in this way the original tissue shape and extracellular matrix structure. The obtained scaffold is formed by a complex mixture of structural and functional molecules, where collagen is present together with the other extracellular matrix components [51].
There are different techniques to remove cells from a tissue, but they can be basically grouped into three main decellularization methods: physical, chemical and enzymatic [52]. Physical methods include high pressure, snap freezing and agitation while chemical methods involve the use of reagents able to remove the cellular content of the extracellular matrix, such as ionic or non-ionic detergents, chelating agents, acid or alkaline solutions. Also the use of enzymes, that specifically remove DNA and RNA, are commonly employed. However, in order to completely remove the cells from a tissue, a combination of these techniques is often required. The origin of the tissue and the specific methods that are used for the decellularization process affect the efficiency of cell removal and determine the biochemical composition, ultrastructure and mechanical properties of the remaining extracellular matrix scaffold [51]. Extracellular matrices from various tissues, such as skin, ligaments, tendons, blood vessels, nerves and heart valves have been studied for regenerative medicine purposes [53][54].
Besides decellularization, another strategy to prepare collagen-based scaffolds for tissue engineering applications is to solubilize collagen from the tissue and then reconstitute and stabilize the isolated protein in the form and shape required for the specific application. In this way collagen can be prepared as porous scaffold, gel or as a hybrid scaffold in combination with other biomaterials.

For example, collagen sponges are usually fabricated by freeze-drying an aqueous solution of collagen or collagen gel. Using this technique, collagen porous structures can be create with a specific structural design that mimic the actual extracellular matrix of the specific tissue, through the control of the freezing process. In fact, the final morphology of the scaffold is affected by the process parameters, such as freezing rate, type of suspension medium and specific additives [55][56]. Due to their porous and tunable structures, collagen sponge scaffolds have been used for the replacements and regeneration of various tissues including articular cartilage, tooth and tracheal tissue [57].

Even collagen gels have showed interesting properties for tissue engineering applications such as the capability to retain cells and carry bioactive molecules [58].

Collagen gels can be prepared changing the collagen charge profile to a net positively charged or negatively charged protein, through a chemical modification, before hydrating the material to form a gel matrix. Another possibility is to shift the pH of dispersion away from its isoelectric point [59].

Collagen gels have been recently tested and showed promising results in different medical applications, including regeneration of cartilage or bony defects, skeletal healing and reconstruction, treatment of burn or chronic wounds, tissue engineering of heart valves and ligaments [60][61][62].

The drawback of collagen-based scaffolds and hybrid constructs where collagen is combined with other naturally derived polymers, is that they generally have poor mechanical properties. This makes difficult the medical manipulation of the constructs and the retention of the designed shape during the tissue regeneration. However, it was proved that these limitations can be overcome by chemical or photochemical cross linking of collagen, or by the combination with other mechanically strong materials into hybrid structures. For this purpose, biodegradable synthetic polymers like polyesters and polycaprolactones, or ceramics such as hydroxyapatite-based materials, are most commonly used for tissue engineering and for hybridization with collagen [63].

3.4.2 Crosslink methods

As previously described, a method to produce collagen based-biomaterials is the extraction, purification and processing of collagen from a natural tissue. However, reconstituted forms of collagen can present some limitations, such as poor tensile strength and low proteolytic resistance, due to the dissociation of the natural crosslinks during the extraction process [64].

In many cases, it is crucial to customize the rate of the scaffold biodegradation in relation to the specific application. For example, in tissue replacement, an implant has to maintain its properties while it is gradually replaced by the new-forming healthy tissue. As a consequence, the creation of exogenous crosslinking inside the

material is often required before use. Different chemical crosslinking agents have been proposed for this purpose.

For example, some trivalent metals or polyvalent cations, such as chromium and aluminum, have been used as crosslinking agents in absorbable medical products [65].

However, the most utilized reagents to introduce covalent crosslinks in collagen are aldehydes, in particular formaldehyde and glutaraldehyde. Formaldehyde reacts with the ε-amino groups of lysine and hydroxylysine forming an intermediate imine which is able to crosslink with tyrosine, glutamine or asparagine residues [38]. It was proved that after the formaldehyde treatment, collagen-based materials prolong their in vivo absorption. However, due to the potential toxicity of its crosslinked products, the use of formaldehyde for the production of medical devices is limited. Glutaraldehyde is a crosslinking agent commercially available, that can react with different groups of the collagen molecule (amino, carboxylic or amide groups) increasing its resistance to enzymatic degradation. Also in this case, concerns regard the cytotoxicity caused by the depolymerization of the glutaraldehyde polymer after the implantation [66].

Another strategy involves the use of hexamethylendiisocyanate (HDC) that forms crosslinks with two ε-amino groups of the protein. The treatment with HDC allows to obtain a strong and resistant collagen material, with lower cytotoxic effects in comparison with aldehydes [67]. Also epoxy compounds (ethylene glycol diglycidyl ether, glycerol polyglycidyl ether) are able to react with the amino groups on lysine and lead to the formation of more flexible collagen materials with acceptable cytotoxicity effects [68].

More recently, some studies have focused on the treatment with carbodiimides. These reagents promote the formation of amide bonds between carboxylic and amino groups on the collagen molecules without participate directly at the actual linkage [69].

Generally, the principal limitation in the use of chemical crosslinking agents is related to their potential cytotoxic effects and the possible release of toxic degradation products after implantation. For these reasons, naturally occurring crosslinking agents (such as genipin) are currently investigated and have been shown to be a valid alternative for crosslinking collagen-derived biomaterials [70].

As an alternative, even physical methods, including dehydrothermal treatment and exposure to ultraviolet light, could be used to improve the final characteristics of collagen-based materials [67].

3.4.3 Applications and recent advances

Most of the collagen-based biomaterials are produced using collagen type I and are used in fundamental studies in the laboratory or as scaffold materials in several medical applications.

For example, an important area of application regards bone and cartilage reconstruction. In fact, osteochondral defects frequently occur due to trauma or degenerative diseases, resulting in severe pain and disability. Most clinical approaches have been shown limited capacity to treat these lesions and tissue engineering has been proposed as an alternative strategy. Collagen-based scaffolds are generally implanted when autograft cannot be used for practical or pathological reasons or when the defects reach an important volume in order to promote the regeneration process. [71]. Especially in the production of scaffolds designed for the regeneration of hard connective tissues, in order to fulfil the mechanical requirements, collagen biomaterial can be hardened by crosslinking and/or combination with other materials (like calcium phosphates) [72]. The mechanical requirements are different in the treatment of cartilage injuries, where the scaffolds have to be more flexible, and are generally produced using collagen type II [73]. Future developments in bone tissue engineering applications, mainly aimed to optimize the pores size and distribution in the scaffolds and to obtain cells differentiation directly in the collagen based-biomaterials in order to permanently solve the disease, through promoting bone ingrowth in the material and eventually leading to a good incorporation of the material into host bone.. As alternative, also the decellularization of complex structures (such as meniscus) to produce replacements for specific osteochondral defects seems a promising strategy and is under investigation [74].

Collagen biomaterials are widely use even for wound dressing applications. Human skin is formed by two layers: dermal and epidermal. The epidermal layer is continually regenerated, while the dermal layer cannot completely regenerate after an injury. Collagen-based wound dressing have been extensively applied for the treatment of burn injuries and leg and foot ulcers [75]. Recently, innovative tissue-engineered skin substitutes have been proposed and commercialized. Most of them consist in monolayer of fibroblast cells embedded inside a matrix, generally composed of collagen or gelatin, that mimic the dermal layer of skin. Future strategies involve the combinations of collagenous materials and stem cells. This approach seems promising even for the treatment of corneal defects [76].

Other medical applications involve the use of collagen-based materials as dermal filler, delivery systems or to treat urogenital and cardiac diseases [50][77].

In addition, previous studies have been shown that different compositions of collagen-based biomaterials have positive effects in guiding the regeneration of peripheral nerve and promoting neuronal migration [50].

Therefore it is clear the great potential of collagen-based materials in the biomedical field and, in view of the large number of their potential applications, most of the current efforts of the research are aimed to optimize their properties, such as increase mechanical properties, biodegradability, and delivery characteristics.

3.5 Marine collagen

Nowadays, common sources of collagen and collagen-derived products, such as gelatin and collagen hydrolysates, are limited to bovine skin and tendons, and porcine dermis. [34]

However, in recent years, the risk of infection and transmission to humans of diseases (e.g., bovine sponge encephalopathy, transmissible spongiform encephalopathy, foot-and-mouth disease and avian influenza) have limited the use of collagen derived from land-based animals [78]. Therefore, many scientists have recently started to study alternative novel sources of collagen. Among the possible sources, marine organisms and marine by-products could be a valuable collagen resource being highly available, with no risks of disease transmission [79][80].

Therefore, extraction processes and functional characterizations have been reported for different marine species, including some under-utilized fish resources or residues of fish industry like fish scales or the solid waste from surimi processing [21].

For example, collagenous proteins have been isolated even from the musculature of crustaceans and mollusks and several jellyfish species (Aurelia aurita, Cotylorhiza tuberculata, Pelagia noctiluca, and Rhizostoma pulmo) have been studied as potential source of collagen [81][82]. In addition, also sponges contain fibrillar and nonfibrillar collagens, like vertebrates. However, collagen isolated from sponges is biochemically different from vertebrate collagen because it is highly glycosylated, insoluble and therefore more difficult to manipulate [83]. In fact, it was been proved that although even fish collagen can form the typical fibrillar structure, it differs from vertebrates collagen for the amino acid composition and sequence. In particular, the amounts of proline and hydroxyproline of marine collagen is generally lower than that of other species and this affects significantly the final properties of the material and its further applications. For example, the major observed difference between fish and mammal collagens includes compatibility to crosslinking as well as thermostability at high temperatures [84].

In this regard, different studies have been demonstrated that source, extraction methods and pre-treatments affect the final characteristics of collagen, such as composition, rheological properties, solubility and thermal stability [85] and, consequently, its biological activity.

As a consequence, considering the large number of new potential sources to be studied and the large number of parameters related at the extraction process, the chemical and biological characterization of isolated collagen is necessary and efficient systems of evaluation need to be developed [86].

References

[1] C. H. Lee, A. Singla, and Y. Lee, "Biomedical applications of collagen," *Int. J. Pharm.*, vol. 221, no. 1, pp. 1–22, 2001.

[2] K. Gelse, E. Pöschl, and T. Aigner, "Collagens - Structure, function, and biosynthesis," *Adv. Drug Deliv. Rev.*, vol. 55, no. 12, pp. 1531–1546, 2003.

[3] P. P. Fietzek, F. W. Rexrodt, P. Wendt, M. Stark, and K. Kühn, "The covalent structure of collagen," *Eur. J. Biochem.*, vol. 30, no. 1, pp. 163–168, 1972.

[4] D. J. S. Hulmes, "Collagen diversity, synthesis and assembly," in *Collagen*. Springer, pp. 15–47, 2008.

[5] L. E. Orgel, "Self-organizing biochemical cycles," *Proc. Natl. Acad. Sci.*, vol. 97, no. 23, pp. 12503–12507, 2000.

[6] U. G. K. Wegst and M. F. Ashby, "The mechanical efficiency of natural materials," *Phil. Mag.*, vol. 84, no. 21, pp. 2167–2186, 2004.

[7] W. S. Klug, M. R. Cummings, *Concepts of genetics*, no. Ed. 7. Pearson Education, Inc, 2003.

[8] W. Friess, "Collagen-biomaterial for drug delivery," *Eur. J. Pharm. Biopharm.*, vol. 45, no. 2, pp. 113–136, 1998.

[9] J. Josse and W. F. Harrington, "Role of pyrrolidine residues in the structure and stabilization of collagen," *J. Mol. Biol.*, vol. 9, no. 2, pp. 269–287, 1964.

[10] J. Weiss, "The collagens: biochemistry and pathophysiology." *Wiley Online Library*, 1993.

[11] A. M. Ferreira, P. Gentile, V. Chiono, and G. Ciardelli, "Collagen for bone tissue regeneration," *Acta Biomat.*, vol. 8, no. 9, pp. 3191–3200, 2012.

[12] R. Timpl, "Immunological studies on collagen," in *Biochemistry of collagen.* Springer, pp. 319–375, 1976.

[13] B. Y. J. E. Eastoe, "The Amino Acid Composition of Fish Collagen and Gelatin," *Biochem. J.*, vol. 65, no. 2, pp. 363–368, 1957.

[14] B. Nusgens, C. Merrill, C. Lapiere, and E. Bell, "Collagen biosynthesis by cells in a tissue equivalent matrix in vitro," *Collagen Rel. Res.*, vol. 4, no. 5, pp. 351–363, 1984.

[15] R. Timpl, "Structure and biological activity of basement membrane proteins," *Eur. J. Biochem.*, vol. 180, no. 3, pp. 487–502, 1989.

[16] R. Maynes, *Structure and function of collagen types*. Elsevier, 2012.

[17] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, "Cell junctions, cell adhesion, and the extracellular matrix," 2002.

[18] N. T. Wright and J. D. Humphrey, "Denaturation of collagen via heating: an irreversible rate process," *Annu. Rev. Biomed. Eng.*, vol. 4, no. 1, pp. 109–128, 2002.

[19] C. A. Miles and A. J. Bailey, "Thermal denaturation of collagen revisited,"

P. Indian As.-Chem. Sci., vol. 111, no. 1, pp. 71–80, 1999.

[20] M. C. Gómez-Guillén, B. Giménez, M. E. al López-Caballero, and M. P. Montero, "Functional and bioactive properties of collagen and gelatin from alternative sources: A review," *Food Hydrocoll.*, vol. 25, no. 8, pp. 1813–1827, 2011.

[21] J. S. Kim and J. W. Park, "Partially purified collagen from refiner discharge of Pacific whiting surimi processing," *J. Food Sci.*, vol. 70, no. 8, pp. c511–c516, 2005.

[22] R. Schrieber and H. Gareis, "From collagen to gelatine," *Gelatine Handb. Theory Ind. Pract.*, pp. 45–117, 2007.

[23] F. H. Silver and R. L. Trelstad, "Type I collagen in solution. Structure and properties of fibril fragments.," *J. Biol. Chem.*, vol. 255, no. 19, pp. 9427–9433, 1980.

[24] A. S. Hoffman, "Hydrogels for biomedical applications," *Adv. Drug Deliv. Rev.*, vol. 64, pp. 18–23, 2012.

[25] R. J. Elias, S. S. Kellerby, and E. A. Decker, "Antioxidant activity of proteins and peptides," *Crit. Rev. Food Sci. Nutr.*, vol. 48, no. 5, pp. 430–441, 2008.

[26] G. Guillén, M. E. López Caballero, A. Alemán, A. de Lacey, B. Giménez, P. Montero García, and others, "Antioxidant and antimicrobial peptide fractions from squid and tuna skin gelatin," 2010.

[27] P. Bulet, R. Stöcklin, and L. Menin, "Anti-microbial peptides: from invertebrates to vertebrates," *Immunol. Rev.*, vol. 198, no. 1, pp. 169–184, 2004.

[28] S. K. Kim and I. Wijesekara, "Development and biological activities of marine-derived bioactive peptides: A review," *J. Funct. Foods*, vol. 2, no. 1, pp. 1–9, 2010.

[29] K. Suetsuna and J.-R. Chen, "Identification of antihypertensive peptides from peptic digest of two microalgae, Chlorella vulgaris and Spirulina platensis," *Mar. Biotechnol.*, vol. 3, no. 4, pp. 305–309, 2001.

[30] G. Samonina, I. Ashmarin, and L. Lyapina, "Glyproline peptide family: review on bioactivity and possible origins," *Pathophysiology*, vol. 8, no. 4, pp. 229–234, 2002.

[31] R. W. Moskowitz, "Role of collagen hydrolysate in bone and joint disease," in *Seminars in arthritis and rheumatism*, 2000, vol. 30, no. 2, pp. 87–99.

[32] H. Hou, B. Li, X. Zhao, Y. Zhuang, G. Ren, M. Yan, Y. Cai, X. Zhang, and L. Chen, "The effect of pacific cod (*Gadus macrocephalus*) skin gelatin polypeptides on UV radiation-induced skin photoaging in ICR mice," *Food Chem.*, vol. 115, no. 3, pp. 945–950, 2009.

[33] A. E. Postlethwaite, J. M. Seyer, and A. H. Kang, "Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived

peptides," *Proc. Natl. Acad. Sci.*, vol. 75, no. 2, pp. 871–875, 1978.

[34] Y. Zhang, W. Liu, G. Li, B. Shi, Y. Miao, and X. Wu, "Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*)," *Food Chem.*, vol. 103, no. 3, pp. 906–912, 2007.

[35] T. F. Kresina and E. J. Miller, "Isolation and characterization of basement membrane collagen from human placental tissue. Evidence for the presence of two genetically distinct collagen chains," *Biochemistry*, vol. 18, no. 14, pp. 3089–3097, 1979.

[36] T. Nagai and N. Suzuki, "Isolation of collagen from fish waste material-skin, bone and fins," *Food Chem.*, vol. 68, no. 3, pp. 277–281, 2000.

[37] J. W. Woo, S. J. Yu, S. M. Cho, Y. B. Lee, and S. B. Kim, "Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin," *Food Hydrocoll.*, vol. 22, no. 5, pp. 879–887, 2008.

[38] W. Friess, "Collagen-biomaterial for drug delivery," *Eur. J. Pharm. Biopharm.*, vol. 45, no. 2, pp. 113–136, 1998.

[39] Z. Khiari, D. Rico, A. B. Martin-Diana, and C. Barry-Ryan, "Comparison between gelatines extracted from mackerel and blue whiting bones after different pre-treatments," *Food Chem.*, vol. 139, no. 1, pp. 347–354, 2013.

[40] A. Kessler, H. Rosen, and S. M. Levenson, "Chromatographic Fractionation of Acetic Acid-solubilized Rat Tail Tendon Collagen," *J. Biol. Chem.*, vol. 235, no. 4, 1960.

[41] D. Liu, G. Wei, T. Li, J. Hu, N. Lu, J. M. Regenstein, and P. Zhou, "Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin," *Food Chem.*, vol. 172, pp. 836–843, 2015.

[42] E. D. Hay, *Cell biology of extracellular matrix*. Springer Science & Business Media, 2013.

[43] I. V. Yannas, "Collagen and gelatin in the solid state," *J. Macromol. Sci. Macromol. Chem.*, vol. 7, no. 1, pp. 49–106, 1972.

[44] E. Skierka and M. Sadowska, "The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (*Gadus morhua*)," *Food Chem.*, vol. 105, no. 3, pp. 1302–1306, 2007.

[45] K. A. Piez, *Molecular and Aggregate Structures of the Coílagens I*. 1984.

[46] T. R. Knapp, E. Luck, and J. R. Daniels, "Behavior of solubilized collagen as a bioimplant," *J. Surg. Res.*, vol. 23, no. 2, pp. 96–105, 1977.

[47] H. F. Mark, *Encyclopedia of polymer science and technology, concise*. John Wiley & Sons, 2013.

[48] F. S. Steven and H. Thomas, "Preparation of insoluble collagen from human cartilage," *Biochem. J.*, vol. 135, no. 1, pp. 245–247, 1973.

[49] K. Gelse, E. Pöschl, and T. Aigner, "Collagens-structure, function, and

biosynthesis," *Adv. Drug Deliv. Rev.*, vol. 55, no. 12, pp. 1531–1546, 2003.

[50] R. Parenteau-Bareil, R. Gauvin, and F. Berthod, "Collagen-based biomaterials for tissue engineering applications," *Materials (Basel).*, vol. 3, no. 3, pp. 1863–1887, 2010.

[51] T. W. Gilbert, T. L. Sellaro, and S. F. Badylak, "Decellularization of tissues and organs," *Biomaterials*, vol. 27, no. 19, pp. 3675–3683, 2006.

[52] P. M. Crapo, T. W. Gilbert, and S. F. Badylak, "An overview of tissue and whole organ decellularization processes," *Biomaterials*, vol. 32, no. 12, pp. 3233–3243, 2011.

[53] S. L. M. Dahl, J. Koh, V. Prabhakar, and L. E. Niklason, "Decellularized native and engineered arterial scaffolds for transplantation," *Cell Transplant.*, vol. 12, no. 6, pp. 659–666, 2003.

[54] A. K. Gulati, "Evaluation of acellular and cellular nerve grafts in repair of rat peripheral nerve," *J. Neurosurg.*, vol. 68, no. 1, pp. 117–123, 1988.

[55] C. J. Doillon, C. F. Whyne, S. Brandwein, and F. H. Silver, "Collagen-based wound dressings: Control of the pore structure and morphology," *J. Biomed. Mater. Res.*, vol. 20, no. 8, pp. 1219–1228, 1986.

[56] I. V. Yannas, "Biologically active analogues of the extracellular matrix: artificial skin and nerves," *Angew. Chemie Int. Ed. English*, vol. 29, no. 1, pp. 20–35, 1990.

[57] J. F. Burke, I. V Yannas, W. C. Quinby Jr, C. C. Bondoc, and W. K. Jung, "Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury.," *Ann. Surg.*, vol. 194, no. 4, p. 413, 1981.

[58] N. Kasoju, S. S. Ali, V. K. Dubey, and U. Bora, "Exploiting the potential of Collagen as a Natural Biomaterial in Drug Delivery," *J. Proteins Proteomics*, vol. 1, no. 1, 2013.

[59] R. Jeyanthi and K. P. Rao, "In vivo biocompatibility of collagenpoly (hydroxyethyl methacrylate) hydrogels," *Biomaterials*, vol. 11, no. 4, pp. 238–243, 1990.

[60] F. Boccafoschi, J. Habermehl, S. Vesentini, and D. Mantovani, "Biological performances of collagen-based scaffolds for vascular tissue engineering," *Biomaterials*, vol. 26, no. 35, pp. 7410–7417, 2005.

[61] B. Chevallay and D. Herbage, "Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy," *Med. Biol. Eng. Comput.*, vol. 38, no. 2, pp. 211–218, 2000.

[62] L. Ma, C. Gao, Z. Mao, J. Zhou, J. Shen, X. Hu, and C. Han, "Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering," *Biomaterials*, vol. 24, no. 26, pp. 4833–4841, 2003.

[63] V. Guarino, M. G. Raucci, A. Ronca, and V. Cirillo, "Multifunctional scaffolds for bone regeneration," *Bone Substit. Biomater.*, p. 95, 2014.

[64] K. H. Stenzel, T. Miyata, and A. L. Rubin, “Collagen as a biomaterial,” *Annu. Rev. Biophys. Bioeng.*, vol. 3, no. 1, pp. 231–253, 1974.

[65] S. D. Gorham, “Collagen,” in *Biomaterials*, Springer, 1991, pp. 55–122.

[66] L. L. H. Huang-Lee, D. T. Cheung, and M. E. Nimni, “Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks,” *J. Biomed. Mater. Res.*, vol. 24, no. 9, pp. 1185–1201, 1990.

[67] E. Khor, “Methods for the treatment of collagenous tissues for bioprostheses,” *Biomaterials*, vol. 18, no. 2, pp. 95–105, 1997.

[68] H. W. Sung, J. S. Shih, and C. S. Hsu, “Crosslinking characteristics of porcine tendons: effects of fixation with glutaraldehyde or epoxy,” *J. Biomed. Mater. Res.*, vol. 30, no. 3, pp. 361–367, 1996.

[69] L. H. H. O. Damink, P. J. Dijkstra, M. J. A. Van Luyn, P. B. Van Wachem, P. Nieuwenhuis, and J. Feijen, “In vitro degradation of dermal sheep collagen cross-linked using a water-soluble carbodiimide,” *Biomaterials*, vol. 17, no. 7, pp. 679–684, 1996.

[70] A. Bigi, G. Cojazzi, S. Panzavolta, N. Roveri, and K. Rubini, “Stabilization of gelatin films by crosslinking with genipin,” vol. 23, no. 24, pp. 4827–4832, 2002.

[71] M. Geiger, R. H. Li, and W. Friess, “Collagen sponges for bone regeneration with rhBMP-2,” *Adv. Drug Deliv. Rev.*, vol. 55, no. 12, pp. 1613–1629, 2003.

[72] J. P. Gleeson, N. A. Plunkett, F. J. O’ Brien, and others, “Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration,” *Eur Cell Mater*, vol. 20, pp. 218–230, 2010.

[73] H. A. Breinan, S. D. Martin, H. P. Hsu, and M. Spector, “Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes,” *J. Orthop. Res.*, vol. 18, no. 5, pp. 781–789, 2000.

[74] T. W. Stapleton, J. Ingram, J. Katta, R. Knight, S. Korossis, J. Fisher, and E. Ingham, “Development and characterization of an acellular porcine medial meniscus for use in tissue engineering,” *Tissue Eng. Part A*, vol. 14, no. 4, pp. 505–518, 2008.

[75] D. Brett, “ A Review of Collagen and Collagen-based Wound Dressings,” *Wounds a Compend. Clin. Res. Pract.*, vol. 20, no. 12, pp. 347–356, 2008.

[76] P. Martin, “Wound healing-aiming for perfect skin regeneration,” *Science*, vol. 276, no. 5309, pp. 75–81, 1997.

[77] K. Panduranga Rao, “Recent developments of collagen-based materials for medical applications and drug delivery systems,” *J. Biomater. Sci. Polym. Ed.*, vol. 7, no. 7, pp. 623–645, 1996.

[78] E. Song, S. Y. Kim, T. Chun, H. J. Byun, and Y. M. Lee, “Collagen scaffolds derived from a marine source and their biocompatibility,” *Biomaterials*, vol. 27, no. 15, pp. 2951–2961, 2006.

[79] S. Xuan Ri, K. Hideyuki, and T. Koretaro, “Characterization of molecular species of collagen in scallop mantle,” *Food Chem.*, vol. 102, no. 4, pp. 1187–1191, 2007.

[80] S. Mizuta, R. Yoshinaka, M. Sato, and M. Sakaguchi, “Isolation and partial characterization of two distinct types of collagen in the squid *Todarodes pacificus*,” *Fish. Sci.*, vol. 60, no. 4, pp. 467–471, 1994.

[81] J. Zhang, R. Duan, L. Huang, Y. Song, and J. M. Regenstein, “Characterisation of acid-soluble and pepsin-solubilised collagen from jellyfish (*Cyanea nozakii Kishinouye*),” *Food Chem.*, vol. 150, pp. 22–26, 2014.

[82] P. Singh, S. Benjakul, S. Maqsood, and H. Kishimura, “Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*),” *Food Chem.*, vol. 124, no. 1, pp. 97–105, 2011.

[83] D. Swatschek, W. Schatton, J. Kellermann, W. E. G. Müller, and J. Kreuter, “Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum,” *Eur. J. Pharm. Biopharm.*, vol. 53, no. 1, pp. 107–113, 2002.

[84] S. Yunoki, N. Nagai, T. Suzuki, and M. Munekata, “Novel biomaterial from reinforced salmon collagen gel prepared by fibril formation and cross-linking,” *J. Biosci. Bioeng.*, vol. 98, no. 1, pp. 40–47, 2004.

[85] F. Pati, B. Adhikari, and S. Dhara, “Isolation and characterization of fish scale collagen of higher thermal stability,” *Bioresour. Technol.*, vol. 101, no. 10, pp. 3737–42, 2010.

[86] T. Silva, J. Moreira-Silva, A. Marques, A. Domingues, Y. Bayon, and R. Reis, “Marine Origin Collagens and Its Potential Applications,” *Mar. Drugs*, vol. 12, no. 12, pp. 5881–5901, 2014.

Chapter IV
Evaluation of alternative sources of collagen: Isolation and characterization of acid and pepsin-solubilized collagen from *Loligo vulgaris* squid mantle

Part of this chapter has been published in [1]***:***

Natascia Cozza[a], Walter Bonani[a,b], Antonella Motta[a,b], Claudio Migliaresi[a,b]

[a] Department of Industrial Engineering and BIOtech Research Center, University of Trento, Trento, Italy
[b] European Institute of Excellence on Tissue Engineering and Regenerative Medicine and INSTM Research Center, Trento Research Unit, Trento, Italy

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4.1 Introduction

Collagen material is a biopolymer used in a wide range of applications, in food, cosmetic and pharmaceutical industries [2]. It is frequently used as scaffold also in the field of tissue engineering due to its biocompatibility, biodegradability, low immunogenicity and cell-adhesive properties [3]. In particular, among the fibril-forming collagens, type I and V collagen fibrils have been extensively used as biomaterial for the development of tissue engineering constructs since they contribute to the structural backbone of bone [4].

Nowadays, the main sources of collagen and collagen-derived products, such as gelatin and collagen hydrolysates, are limited to bovine skin and tendon, and porcine dermis [5]. Collagen is however present not only in mammals, but throughout the entire animal kingdom including birds and fishes. Sources, extraction methods and pre-treatments affect the final characteristics of collagen, such as composition, rheological properties, solubility and thermal stability [6] and consequently its biological activity.

In the last years, concerns have been expressed about the use of collagen derived from land-based animals due to the risk of infection and diseases such bovine sponge encephalopathy, transmissible spongiform encephalopathy, foot-and-mouth disease and avian influenza [7][8] and increasing interest has been paid to alternative collagen sources. Marine organisms could be a valuable collagen source being highly available, with no risks of disease transmission [9][10].

Each member of the collagen family is characterized by the repetitions of the proline-rich tripeptide Gly-X-Y involved in the formation of trimeric collagen triple helices [4]. Fibrillar collagens are of two types, acid soluble or pepsin soluble. Neutral salt-extraction and low concentration acid-extraction are the most commonly used methods to isolate collagen from natural tissues [11][12]. Dilute acid solvents are more efficient than neutral salt solutions since they dissociate the intermolecular cross-links of the aldimine type causing the swelling of the fibrillar structures [13]. However, dilute acids will not disassociate less labile cross-links such as keto-imine bonds. Much higher yields can be achieved using proteases, i.e. pepsin that cleaves peptides in the telopeptide region [14][15]. Extraction and characterization of acid and pepsin-solubilized collagen have been reported for different fish species and fish collagen started to became a potential ingredient for cosmetic, food, pharmaceutical and biomedical applications [16][17][18]. The amino acid profiles of the two types of collagen may vary, depending on the source, as well as molecular weight and denaturation temperature. During the processing of the material, the presence of covalent cross-links between molecules represents the major impediment to dissolution of collagen from tissues. Therefore, native collagen must be pre-treated before it can be converted into a form suitable for extraction [19]. In addition, as a protein, collagen conformations are sensitive to different factors during the extraction process, which can induce the destruction of the native form and consequently cause the loss of specific functions. For example heat can induce the triple helix collapse and the thermal denaturation into gelatin [20].

In view of the large number of potential sources to be studied and the large number of parameters related at the extraction process, the chemical and biological characterization of isolated collagen is necessary and efficient systems of evaluation need to be developed [21].

Loligo vulgaris is a neritic, semipelagic species that occurs abundantly in coastal water from the North Sea to the west coast of Africa [22]. This species is commercially very important. In fact, it is extensively exploited by commercial fisheries during the whole year, with annual catches over 15,000 tons [23]. Due to the good availability, easy and fast storage conditions, there is the possibility to use this species for the extraction of collagen and as a model for the development of a simple and efficient system for study the quality of resulting protein.

Each anatomical part of a live organism has a specific function that can affect the amount of collagen and its degree of aggregation. Squid are fast-growing species and very active during their lifetimes. In particular, their mantle needs to be elastic to perform its propulsive function. Due to its peculiar musculature and the high degree of protein turnover [24], squid mantle presents a promising protein composition and collagen is present in a considerably large amounts inside the tissue (up to about 11% of total protein in the muscle of some squid species, like *Illex argentinus*) [25].

However, at present, there has been no investigation on the collagen extracted from *L. vulgaris*.

Therefore, in the present work, first acid-solubilized collagen (ASC) and then pepsin-solubilized collagen (PSC) from squid mantle (*L. Vulgaris*) were isolated and characterized in order to provide a parallel comparison of these collagens. A complete characterization of the physico-chemical properties of the resulting proteins was performed, exploiting different techniques. The extracted collagen was characterized using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Fourier Transform Infrared Spectroscopy (FTIR) and Gel Permeation Chromatography (GPC). Amino acid composition and solubility of collagen were also evaluated. Denaturation temperature was measured by viscosity change and confirmed with thermal analyses using Differential Scanning Calorimetry (DSC). No cytotoxic effect of isolated collagens was observed after the extraction process.

Finally, in order to test the processability collagen fractions isolated from *L. vulgaris* squid mantle, collagen-based films and sponges were produced using ASC and PSC. In fact, collagen-based films and sponges are frequently used as biomaterials [14]. However, for different technical applications, a cross-linking reagent is required to improve mechanical strength and degradation properties of collagenous matrices [26][27]. In this study, genipin was used as a naturally occurring crosslinking agent to fabricate the collagen matrices. The microstructures of the collagenous matrices produced using the marine collagen fractions cross-linked with genipin were investigated using scanning electron microscope (SEM) and the in vitro cytotoxicity of the constructs was also evaluated.

4.2 Materials

Acetic acid, Sodium hydroxide (NaOH), Sodium chloride (NaCl), Bovine collagen type I, Pepsin, Disodium hydrogen phosphate (Na_2HPO_4) and high molecular weight markers were purchased from Sigma-Aldrich (St. Louis, MO, USA).

All the chemicals and reagents were analytical grade and were used without further purifications.

4.3 Preliminary treatment and morphological characterization of L. vulgaris squid mantle

4.3.1 Pretreatment of the squid mantle e preparations of the samples

Squid *L. vulgaris* caught in the northern Adriatic Sea was purchased at Trento local market and kept in ice using a solid/ice ratio of 1:2 (w/w).
Samples for the morphological analysis and histological characterization were collected from the middle region of the mantle as shown in Figure1 (0.5 x 0.5 x 0.5 cm).

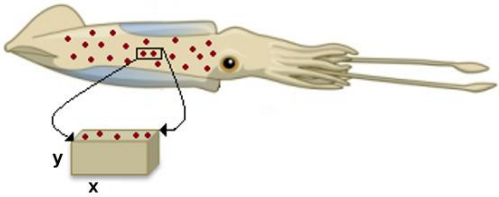


Figure IV - 2. Orientation relative to the surrounding tissue of the tissue blocks cut from the squid mantle for the morphological characterization and histological analysis. The longitudinal sections were cut parallel to the plane x-y.

Formaldehyde buffer (pH 7.0) was used for primary fixation for 24 h. The fixed samples were gradient dehydrated, dealcoholized, and embedded in paraffin. Longitudinal sections of the specimens were cut (10 μm thick) using a microtome. and mounted on glass slides for the morphological characterization of the tissue.
Figure 2 shows the main steps of the squid mantle preparation for the extraction process. After washing under iced tap water (0–2°C), skin and tentacles were discarded to isolated the mantle. Then, squid mantle was cut into small pieces (0.5 x 0.5 cm x 0.5 cm).

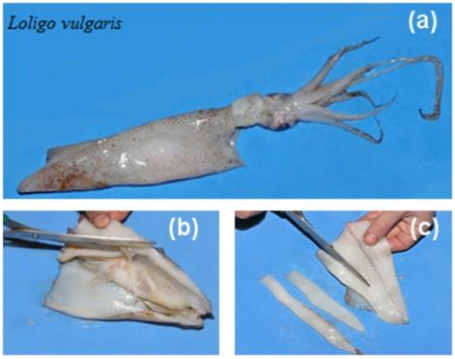


Figure IV - 2. Pretreatment of the squid mantle: **(a)** Squid *L. vulgaris* after washing with iced tap water (0–2°C); **(b)** Removing of skin and tentacles; **(c)** Cutting of the squid mantle into small pieces.

Squid mantle fragments were soaked in 0.1 M NaOH for 2 days to remove non-collagenous proteins, and then washed with DI water for one day. All the procedures were carried out at a temperature lower than 4°C.

4.3.2 SEM analysis of the squid mantle

Scanning electron microscopy (Supra 40, operating mode: high vacuum, secondary electron detector, Carl Zeiss, Europe) was used to observe the morphologies of longitudinal sections obtained from the squid mantle. Samples were previously sputter-coated with a thin gold layer under argon (SEM Coating Unit PS3, Assing S.p.A., Rome, Italy).

4.3.3 Histological characterization of the mantle

Longitudinal sections of the squid mantle (10 µm thick) were stained with 0.1 % solution of Sirius Red F3BA (Direct Red 80, CI 35780) (Sigma-Aldrich, St. Louis, MO, USA) in saturated aqueous solution of picric acid for 1 h, at room temperature. The sections were subsequently rinsed in acidified water for 1 min, twice, and in 70% ethanol for 45 sec. Then, the slices were dehydrated in ascending concentration of ethanol (1 x 80%, 1 x 90%, 2 x 100%, 5 sec each), cleared in xylene two times (5 sec each) and mounted in a glass slide with a drop of mounting medium. Finally, the ultrathin sections were examined and photographed by a light microscopy (DMIL microscope equipped with DFC420 camera Leica, Germany), using both bright field and polarized light.

4.4 Isolation of acid- and pepsin-solubilized collagen fractions

4.4.1 Optimization of the extraction protocol

Firstly, collagen fractions were extracted using three different extraction methods including acid [28], alkaline [29] and enzymatic methods [30]. The methods were compared on the basis of their effect on the extraction yield of collagen. After selecting the extraction method, the main parameters that influence the collagen extraction yield were identified and adjusted in order to obtain the best compromise between yield and final quality of the resulting protein.

4.4.2 Extraction of acid-solubilized collagen

Acid-solubilized collagen fraction (hereinafter ASC) was extracted according to [28] with some modifications. Briefly, pre-treated mantle fragments were finely minced and then treated with a 0.5 M acetic acid solution for three days under continuous stirring. Extraction was carried out at a temperature of 4°C. The mixture was later centrifuged at 80,000 *g* for 2 hours to pellet non-solubilized collagen fractions. Solid residues underwent a second extraction process under the same conditions. The filtrates obtained in the two processes were later mixed and collagenous molecules were precipitated by adding NaCl to a final concentration of 0.9 M. Precipitated proteins were recovered by centrifugation at 20,000 *g* for 30 min at 4°C and re-dissolved in a minimum volume of 0.5 M acetic acid. The solution was dialyzed against 0.1 M acetic acid for 2 days and then against DI water for 1 day in a Slide-A-Lyzer Cassette (MWCO 3500 Da from Pierce, Rockford, Illinois, USA). The resulting dialysate was freeze-dried to obtain the ASC fraction.

4.4.3 Extraction of pepsin-solubilized collagen

After ASC extraction, the remaining insoluble collagen was washed with DI water and treated with 0.5 M acetic acid with pepsin for three days at 4°C under continuous stirring. The mixture was centrifuged at 80,000 *g* for 2 hours to remove residues and dissolved collagen molecules in the supernatant were salted-out by addition of NaCl to a final concentration of 0.9 M. Precipitated proteins were then separated by centrifugation at 20,000 *g* for 30 min at 4°C, dissolved in 0.5 M acetic acid and dialyzed against 0.02 M Na_2HPO_4 solution for one day to inactivate pepsin and against 0.1 M acetic acid for two days. Finally, the solution was dialyzed against DI water for one day, and the resulting dialysates were freeze-dried to obtain the pepsin-solubilized collagen fraction (hereinafter PSC).

4.5 Characterization of extracted collagen fractions

4.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE kit and reagents were purchased from Invitrogen (Carlsbad, CA, USA). First, the lyophilized collagen powders were re-dissolved in NuPAGE® LDS Sample Buffer at a concentration of 0.5 mg/ml and incubated at 70°C for 10 minutes. Samples (15 µg protein) were analyzed by one-dimentional SDS-PAGE, with a XCell4 SureLockTM Midi-Cell (Carlsband, CA, USA), using NuPAGE® Tris-Acetate SDS Running Buffer, with a constant voltage of 150 V. Acrylamide SDS-PAGE NuPAGE® Novex Tris-Acetate Gels (3%-8% gradient) were used for electrophoresis. The acrylamide gels were stained using a Coomassie stain (Imperial Protein Stain). Gel separation patterns were digitalized by a GEL LOGIC 200 (Kodak Scientific Imaging Systems, Rochester, NY, USA) imaging system. SeeBlue® Plus2 Pre-Standard was used as Molecular Weight (MW) reference.

4.5.2 Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) analysis of the isolated collagens was conducted with a Shodex SB-805HQ column (Shodex OH pak®, Showa Denko, Munich, Germany). Freeze-dried ASC and PSC samples were re-dissolved in 0.5 M acetic acid to obtain a protein concentration of 0.5 mg/ml. The obtained collagen solutions were dialyzed against DI water, using a cellulose membrane (MWCO 3500 Da from Pierce, Rockford, Illinoys, USA). The chromatography system was operated with a flow rate of 1 ml/min and elution was detected with a Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 224 nm. Calibration curve was obtained with Low/High Molecular Weight Gel Filtration Calibration Kit (GE Healthcare Europe, Freiburg, Germany).

4.5.3 Amino acid analysis

The amino acid content of ASC and PSC lyophilized samples was determined with Waters AccQ-Fluor™ Reagent Kit using the AccQ-Tag™ amino acid analysis method (Waters Corp., Milford, MA, USA). Briefly, ASC and PSC samples were frozen in LN and freeze-dried using a Lio-5P lyophilizer (5Pascal, Milan, Italy). About 5 mg of lyophilized powders were hydrolyzed with 6 N HCl at 114 ± 2 °C in a silicone oil bath for 24 h. Air-dried hydrolysates were later reconstituted with 20 mM HCl to obtain a solution at a concentration in the range 4-200 pmol and then derivatized with Water AccQ Flour Reagent to obtain stable amino acids. The amino acid

content was determined by Reversed-phase high-performance liquid chromatography (RP-HPLC) using an AccQ-Tag™ column (Waters Corp.) with a gradient of Waters AccQ·TagTM Eluent A, Milli-Q water and Acetonitrile (HPLC grade) at a flow rate of 1 ml/min. The amino acids were detected with Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 254 nm. The chromatograms obtained were compared with Water amino acid hydrolysate standard to identify single amino acid residues.

Hydroxyproline content was determined with the Hydroxyproline Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). ASC and PSC lyophilized powders were first hydrolyzed with 6 N HCl at 120°C for 3 h. The hydroxyproline concentration was measured by the reaction of hydroxyproline with 4-(Dimethylamino)benzhaldehyde (DMAB, Sigma-Aldrich, St. Louis, MO, USA), which results in a colorimetric (560 nm) product proportional to the hydroxyproline present. All samples and standards were run in triplicate.

4.5.4 Fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectra of lyophilized collagen powders were collected using Spectrum One spectrometer with ATR correction (Perking Elmer, Waltham, MA, USA) with Zinc Selenide crystal. Sample spectra were averaged over 4 scans, ranging from 400 to 4,000 cm^{-1} at a resolution of 4 cm^{-1}.

4.5.5 Denaturation temperature

The denaturation temperature of ASC and PSC fractions was evaluated exploiting two different techniques: differential scanning calorimetry (DSC) and viscosity measurements. Differential scanning calorimetry (DSC) was conducted using a Mettler DSC 30 (Mettler-Toledo, OH, USA) to analyze the thermal stability of collagen. The ASC and PSC collagen powders were dissolved in 0.5 M acetic acid solution (sample/solution ratio of 1:40 w/v), and the mixtures were stored at 4°C for 2 day. For the DSC analysis the samples were accurately weighed into aluminum pans and sealed. The scan was performed over a range of 5-50°C, with a heating rate of 1°C/min. The denaturation temperature (T_d) was calculated from the endothermic peak of the DSC transition curve. The denaturation temperature was determined also using a Viscosimeter Physica MCR 301 (Anton Paar GmbH, Rivoli, Italy) according to the method of Zhang *et al.* (2007) with slight modifications. [31] A 2 mg/ml collagen solution in 0.5 M acetic acid was used to measure the viscosity. The thermal determination curve was obtained over a range of 16 to 30°C. The measurement was performed five times at each point. The fractional viscosity for each designated temperature was calculated with the following equation (Eq. 1):

$$\boldsymbol{v} = \frac{(\boldsymbol{v_{max}} - \boldsymbol{v(T)})}{(\boldsymbol{v_{max}} - \boldsymbol{v_{min}})} \qquad (\boldsymbol{Eq.1})$$

These values were plotted against the temperatures and the dinamic denaturation temperature (T_d) was evaluated as the temperature at which the fractional viscosity was 0.5.

4.5.6 Solubility of extracted collagen

The solubility of ASC and PSC extracted collagen in 0.5 M acetic acid was evaluated as a function of NaCl concentration and pH. Lyophilized collagen were re-dissolved in 0.5 M acetic acid at 4°C at a concentration of 3 mg/ml. The effect of NaCl on collagen solubility was evaluated by preparing various 0.5 M acetic acid solutions with different concentrations of NaCl (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M). The effect of pH on collagen solubility was evaluated by adjusting the pH of the 0.5 M acetic acid solution with 6 M HCl or 6 M NaOH to obtain a final pH values ranging from 1 to 10. The mixtures were kept at 4°C under stirring for 30 min and then centrifuged at 20,000 *g* at 4°C for 30 min to remove un-dissolved debris. Total proteins content in the supernatants was determined by means of Bradford Protein Assay (Sigma-Aldrich, St. Louis, MO, USA), using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) as standard. The relative solubility of the collagen samples was calculated as a percentage of the total collagen in acetic acid solution 0.5 M.

4.5.7 Cytotoxicity

Cytotoxicity of extracted collagens was evaluated following ISO 10993-5 (1999), by indirect cultivation of cells with collagen extracts. Embryonic mouse fibroblast NIH/3T3 cells (3T3) were expanded and cultured at 37ºC with 5% CO_2 in Dulbecco's Modified Eagle medium (DMEM) (Euroclone, Milan, Italy), supplemented with 10% fetal bovine serum (Gibco, NY, USA), 2mM L-glutamine, 1mM sodium pyruvate and 0.1% antibiotics (Gibco, Eggenstein, Germany). Culture medium was changed every 2 days until cells reached confluency, then cells were detached with 0.1% trypsin and re-suspended in culture medium. Later, 3T3 cells were seeded in polystyrene 24-well plate at a density of 1×10^4 cells per well and incubated under standard culture conditions. When cells reached about 70-80% of confluency, culture medium was removed and replaced with conditioned media containing collagen extracts. Conditioned media were prepared by soaking freeze-dried ASC and PSC samples in reduced medium at a concentration of 4 g of dry collagen per 20 ml of medium, as prescribed by the ISO standard (24 hours of soaking at 37°C with 5% CO_2). Reduced medium was prepared with Dulbecco's Modified Eagle medium without phenol red with 10% heat inactivated serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.1% antibiotics. Fibroblast 3T3 cells were cultured in medium conditioned with collagen samples in standard conditions for 72 hours. Five independent samples were tested for both ASC and PSC. Cells cultured in reduced medium were used as negative control. Lactate dehydrogenase assay (LDH) (TOX7, Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the cytotoxicity impact of collagen extracts on the cells. An aliquot of 100 µm of culture medium was then

collected from all wells and mixed with the LDH mixture, following manufacturers' instructions. Cells cultured in reduced medium and treated for 30 min with Triton x-100 were used as positive controls. Absorbance was measured using a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) at 490 nm, background absorbance was measured at 690 nm. Results were presented as mean ± standard deviation (n = 5).

4.6 Results and discussion

4.6.1 Morphological and histological characterization of *L. vulgaris* squid mantle

The SEM analysis of the longitudinal sections of *L. vulgaris* squid mantle (Figure 3) evidenced the presence of a fibrous connective tissue composed by a system of collagen fibers. As previously described, the collagen fibers run from the outer tunic to the inner tunic, with an angle of approximately 30° relative to the long axis of the animal [32].

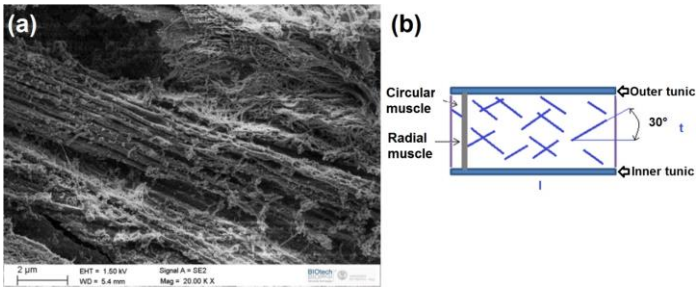


Figure IV - 3. (a) SEM analysis of the longitudinal sections of the squid mantle. **(b)** Organization of intramuscular fibers in the squid mantle: in longitudinal sections of the squid mantle, the fibers are seen to run from the outer tunic to the inner tunic at an angle of approximately 30 ° relative to the long axis [32].

Figure 4 reports the polarized light micrographs of the longitudinal sections of the squid mantle which evidenced the birefringence of the collagen fibers after picrosirius red staining of the tissue.

Bone et al. have reported that squid mantle is composed of a mass of circular muscle fibers, divided in a more or less regular way into rectangular blocks by thin partitions of radial muscle fibers [33]. The circular muscular fibers are characterized by a vascular bed and fibers with mitochondrial cores, while the radial muscle fibers

present similar characteristics of the circular fibers but are inserted on an inner and outer layer of robust collagen fibers called inner and outer tunics, respectively [34]. In addition, it has been proved that there is a second system of collagenous fibers, which runs through the thickness of the mantle with different orientations [35].

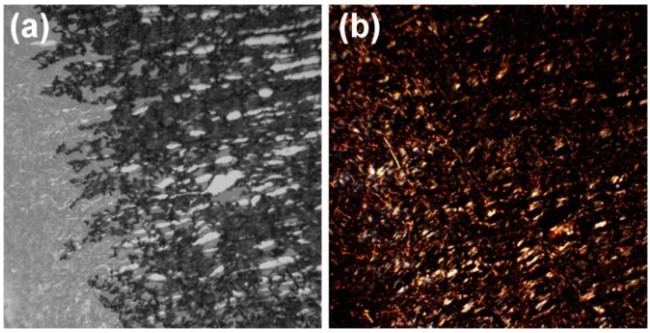


Figure IV - 4. (a) Standard light microscopy of the longitudinal sections of the L. vulgaris squid mantle; **(b)** Polarized light micrographs of the longitudinal sections of the squid mantle which evidenced the birefringence of the collagen fibers.

Our findings are in agreement with previous studies that have demonstrated that all the intramuscular fibers in the squid mantle are collagen fibers [32]. This complex system of collagen fibers has two important functions: structural support and storage of elastic potential energy during the motion of the animal. In addition, the shape and direction of these fibers seems to be a consequence of the different distribution of stress around the mantle [36].

4.6.2 Optimization of the extraction protocol

The aim of the first part of the study was to optimize the collagen extraction process from *L. vulgaris* squid mantle. In fact, the collagen extraction method have to be adjusted in relation to the starting raw material. Firstly, three different extraction methods (acid [28], alkaline [29] and enzymatic [30]) were compared and evaluated in term of the final yield of extracted collagen. Once selected the extraction method, the major parameters affecting the final yields of collagen extraction were investigated. Different process parameters were evaluated including the solid/solution ratio, concentration of enzyme and acid in the solution, extraction time and relative centrifugal force applied during the centrifugation for separate the solubilized collagen fractions from the solid residues. Single-factor test was employed to investigate the effects of different extraction methods and the influence

of the main parameters on the collagen extraction yield. Optimization of the process parameters was carried out by evaluating the best compromise between extraction yield and quality of the isolated collagen, in terms of solubility and denaturation of the resulting protein. The main steps of the method selected for the extraction process are reported in Figure 5.

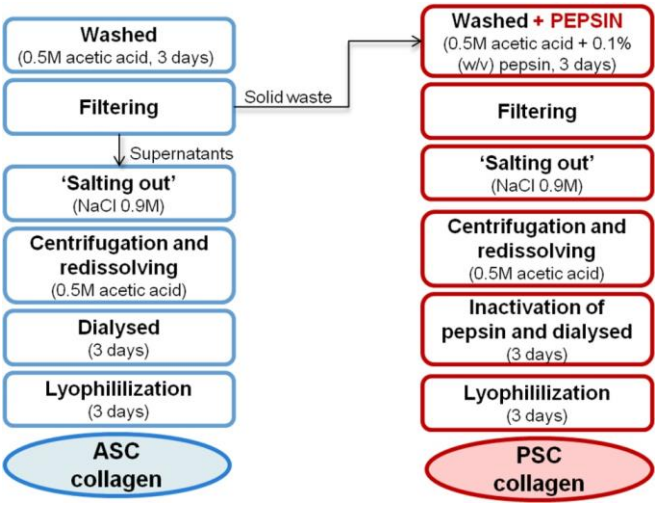


Figure IV - 5. Main steps of the methods used for the extraction of collagen fractions from *L.vulgaris* squid mantle.

Concentration of the enzyme and relative centrifugal force applied during the centrifugation to separate the solubilized collagen fractions from the solid residues were identified as the main factors that influence collagen yield. The optimal extraction conditions were obtained using a concentration of pepsin of 0.1% (w/v) and a centrifugal force of 80,000 *g*. In fact, higher pepsin concentration resulted in higher yield of extraction but caused the partial denaturation of the resulting protein. On the contrary, lower centrifugal force led to an increase of the extraction yield but also to a lower solubility of the isolated collagen, with a consequent minor reproducibility and more difficult subsequent processability of the material.

4.6.3 Collagen extraction yield

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were subsequently isolated from *L. vulgaris* squid mantle with yields of 5.1% and 24.2% (on dry weight basis), respectively. The low yield of ASC suggests that a large

amount of the collagen of the squid mantle was not solubilized by 0.5 M acetic acid solution. A higher yield was obtained with pepsin extraction. During the extraction process, the main impediment to collagen dissolution from tissues is the presence of covalent cross-links between molecules that decrease the solubility of collagen [37]. It was reported that it is possible to solubilize approximately 2% of the tissue collagen using dilute salt or acid solutions [14]. For example, dilute acetic acid solutions dissociate the intermolecular cross-links and induce swelling of fibrillar collagen structures. It is well known that the remaining insoluble collagen can be further extracted, without damaging the triple helix-structure, using strong alkali or enzymes [38]. According to Nalinanon *et al.* the enzymatic treatment can provide much higher yields compared with acid extraction but the characteristics of the extracted collagen, such as the size of the molecules and molecular weight distribution, strongly depend on the time of treatment and the enzyme concentration [39]. In particular, it was found that pepsin cleaved the cross-linked collagen molecules at the telepeptide region, thus allowing extraction with higher yield and without damaging the integrity of the triple helix [40]. Furthermore, the resulting material, so called atelocollagen, benefits from the removal of the antigenic P-determinant located on the non-helical protein sections and seems to provoke milder immune response [41][42].

In the present work we proved that pepsin can be used to increase the extraction yield of collagen from the *L. vulgaris* squid mantle.

The low yield of ASC suggests that the squid mantle has a high percentage of keto-imine bonds. In fact, dilute acids will not disassociate less labile crosslinks such as keto-imine bonds and consequently the amount of ASC inside the tissue is strongly related to the percentages of these bonds [43].

The total extractable yield (sum of yield of ASC and PSC) of squid mantle collagen, 29.3% (on dry weight basis), was higher than that of bigeye snapper skin (7.5%) and brownstripe red snapper (13.7%) [44][7].

4.6.4 Electrophoretic characterization and molecular weights determination

The electrophoretic profiles of ASC and PSC collagens are reported in Figure 6. The SDS-PAGE protein patterns of the isolated collagens were compared with the profile of commercial bovine type I collagen (Gibco Life Technologies Inc., Grand Island, NY, USA) which was used as a reference for the collagen type I protein pattern.

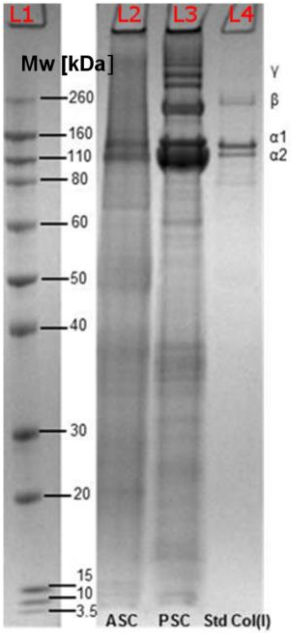
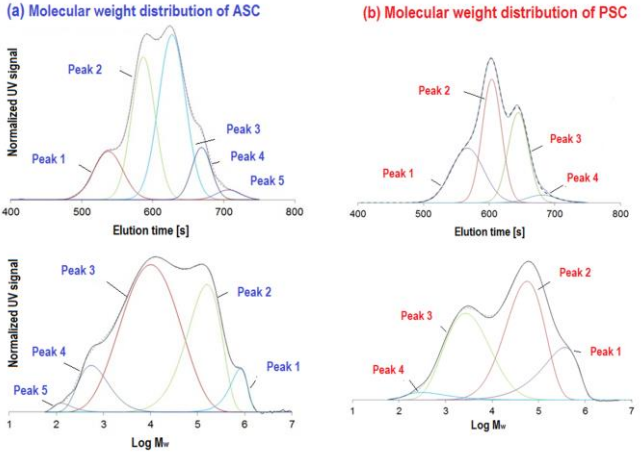


Figure IV - 6. SDS–PAGE pattern of collagens from the squid mantle (*L. vulgaris*). Lane 1(L1): high molecular weight marker; lane 2 (L2): ASC collagen from squid mantle; lane 3 (L3): PSC collagen from squid mantle; lane 4 (L4): commercial bovine collagen type I

The profiles of ASC and PSC showed the presence of two bands in correspondence of the α1 and α2 bands of collagen type I. These results suggested that both extracted collagens mainly consisted of two different α chains, α1 and α2. For both, ASC and PSC, the band associated with α2 chain was more intense than the α1 chain band. In addition, SDS-PAGE analysis of PSC revealed the presence of β- and γ-chain components, which are considered respectively a dimer and a trimer of the α-chains. [45] The protein profiles of ASC and PSC are typically associated with types I and V collagens that usually presented an heterotrimer structure in the forms $[\alpha1(I)]_2\alpha2(I)$, $[\alpha1(V)]_2\alpha2(V)$ or $\alpha1(V)\alpha2(V)\alpha3(V)$ [46] and were already detected in the mantle of other squid species [47][48][10].

Elutions profiles and molecular weight distributions of ASC and PSC measured by GPC are reported in Figure 7. The chromatograms showed that both the extracted collagens have a wide molecular weights distribution and ASC presented a broader and more complex elution profile than PSC. From Figure 7 it is possible to observe that severe peaks overlapping occurred and not all peaks are clearly distinguishable from the experimental data points. Therefore, the experimental curves were deconvoluted in order to analyse the molecular weight distributions of the different

collagen components. Deconvolution of the GPC profiles was performed by the fitting of the chromatograms into separate curves corresponding to the different molecular weights of the collagens fractions. The experimental data points were deconvoluted using the Multipeaks Fit deconvolution function of OriginPro software (OriginLab, OriginPro 8.5, USA). The peak fit analysis resulted in five peaks for ASC and four peaks for PSC, with a correlation > 0.997 between the experimental and the theoretical curves obtained by deconvolution. Peak I corresponds at the higher molecular weights peptides and it was found at Mn = 677 kDa (pdi = 5.51) for ASC and Mn = 154 kDa (pdi = 2.44) for PSC. In addition, ASC showed a second peak (Peak 2) at Mn = 95 kDa (pdi = 2.02) and three further peaks (Peak 3, Peak 4 and Peak 5) at Mn < 10 kDa. Peak 2 of PSC was found at Mn = 32 kDa (pdi = 3.21) and in this case, two peaks corresponding at the low molecular weights (Peak 3 and Peak 4, Mn < 10 kDa) were detected.



	Mn [kDa]	Mw [kDa]	pdi
Peak 1	677	823	5.51
Peak 2	95	191	2.02
Peak 3	4	23	1.21
Peak 4	<1	<1	1.26
Peak 5	<1	<1	1.76

	Mn [kDa]	Mw [kDa]	pdi
Peak 1	154	420	2.44
Peak 2	32	77	3.21
Peak 3	2	5	2.72
Peak 4	<1	<1	3.19

Figure IV - 7. Deconvoluted GPC curves of ASC **(a)** and PSC **(b)** from the squid mantle (Loligo vulgaris): elution profiles and differential molecular weight distributions. The curves represent the envelope smoothing function calculated from the experimental data; the picks are the Gaussian best-fit curves denoting the deconvolved populations. For each peak, the number-average molecular weight (Mn), weight-average molecular weight (Mw) and polydispersity index (pdi) are reported in tables.

These results suggested that both, ASC and PSC, are composed by peptides with different range of molecular weights. In particular, ASC seems to be composed of more complex components since it had a wider molecular weight distribution and comprised higher molecular weight aggregates than PSC. Is interesting to note that ASC and PSC collagens presented a peak, respectively Peak 2 at Mn = 95 kDa and Peak 1 at Mn = 154 kDa, that are probably related to the α chains. In fact, the presence of the α1 and α2 chains were clearly detected also in the SDS–PAGE pattern of the two collagens, which highlights two marked bands in correspondence with the same range of molecular weights. Furthermore, the electrophoretic profile of PSC showed some bands at low MW that are not present in the pattern of ASC and collagen type I. This result was confirmed by the presence of Peak 2 at Mn = 32 kDa in the GPC curve and could be due at the action of pepsin that might induce the partial hydrolysis of high MW cross-linked molecules in PSC collagen. The combination of gel electrophoresis and GPC can provide a deeper understanding of size, charge and polarity of the isolated proteins. Protein separations by SDS-PAGE are commonly used to determine the approximate molecular weights of a protein and the relative abundance of major proteins in a sample [49][50]. In fact, SDS-PAGE has a higher resolution in the bands separation and consequently allows the characterization of more narrowly distributed weights fractions [51]. However GPC enabled to investigate a wider range of molecular weights than SDS-PAGE and to gives some important information about the molar mass distribution and the construction of the molecules.

4.6.5 Amino acid composition of collagen from squid mantle

The amino acid compositions of ASC and PSC extracted from the squid mantle were determined by HPLC and are reported in Table 1. The content of single amino acids was expressed as number of residues per 1,000 amino acid residues. The amino acid composition found for both ASC and PSC were consistent with the amino acid composition of marine-derived collagens reported by other studies [7][52]. Glycine was the most abundant amino acid both for ASC and for PSC, representing 256 and 262 residues/1,000 residues, respectively. In addition, HPLC analysis found relatively high amounts of alanine, proline and glutamic acid. Both ASC and PSC presented a low content of methionine, cysteine, histidine; no tyrosine was detected. Also these results were in agreement with past analyses of collagens from different fish species [17][53]. The total amount of proline and hydroxyproline was higher for PSC than ASC (239 residues/1,000 residues and 266 residues/1,000 residues, respectively).

Amino acid residues	Samples	
	ASC	PSC
Hydrophobic amino acid		
Glycine (Gly)	256	262
Alanine (Ala)	106	98
Proline (Pro)	91	86
Valine (Val)	7	7
Isoleucine (Ile)	50	52
Leucine (Leu)	50	60
Phenylanine (Phe)	24	14
Methionine (Met)	7	6
Total content	**591**	**585**
Charged amino acids		
Aspartic Acid (Asp)	38	41
Glutamic Acid (Glu)	95	125
Arginine (Arg)	69	73
Lysine (Lys)	37	27
Total content	**239**	**266**
Polar amino acids		
Serine (Ser)	39	36
Histidine (His)	16	7
Threonine (Thr)	46	33
Cysteine (Cys)	8	0
Tyrosine (Tyr)	0	0
Total content	**109**	**76**
Hydroxyproline	**61**	**73**
Total residues	**1,000**	**1,000**

Table IV - 1. *Amino acid composition of ASC and PSC extracted from the mantle of squid L. vulgaris (number of residues per 1,000 residues).*

The amino acids were also functionally divided into three groups: (a) hydrophobic; (b) charged; and (c) polar amino acids. Both the collagens present a relatively high amount of hydrophobic amino acids, 591 residues/1,000 residues for PSC and 585 residues/1,000 residues for ASC. ASC presented a higher amount of polar amino acids than PSC (109 residues/1,000 residues and 61 residues/1,000 residues, respectively) and a lower amount of charged amino acid (239 residues/1,000 residues for ASC and 266 residues/1,000 residues for PSC). The high glycine content found in both the isolated collagens is due to the particular feature of collagen. In fact glycine regularly occurs in the helical part of the molecule in every third position, except for the first 14 amino acid residues from N-terminus and the first 10 residues from the C-terminus [54]. About 35% of the non-glycine positions in the repeating unit Gly-X-Y are occupied by proline or hydroxyproline [14]. Hydroxyproline is derived from proline by post-translational hydroxylation mediated by prolylhydroxylase [55]. The total amount of proline and hydroxyproline is an important factor because it is strongly related to thermal stability of the helix structure of collagen, and in particular, the higher the content, the more stable are the helices. [56] In fact the pyrrolidine rings of proline and hydroxyproline stabilize the secondary structure of the polypeptide chain, preventing possible changes in its conformation.

The structure is also maintained by the hydrogen bonds through the hydroxy groups of hydroxyproline. Consequently, the higher amount of proline and hydroxyproline in PSC suggested that PSC extracted from squid mantle presents a more stable conformation than ASC. However, in the ASC it was found a larger content of hydrophobic amino acids compare with PSC. This group includes amino acids with aliphatic side chains, such as alanine, isoleucine, leucine, proline and valine, that are non-reactive and rarely involved directly in protein function but play an important role in substrate recognition [57]. Therefore these largely non-reactive side chains can confer more stability at the collagen conformation and at the same time increase his capacity to bind/recognize specific ligands, such as lipids.

4.6.6 Fourier transform infrared (FTIR) spectra of collagen from squid mantle

FTIR spectra of collagen extracted from the squid mantle are presented in Figure 8. The major peaks in the spectra of ASC and PSC were similar to those of collagen isolated from others fish species. Similar FTIR spectra were observed between ASC and PSC. The amide I band was centered at 1643 for ASC and at 1648 cm^{-1} for PSC sample. As it is possible to see in Table 2, the amide I peak is assigned to C=O stretching vibration or hydrogen bond coupled with COO^-. The amide II band of PSC (1530 cm^{-1}) was found at lower wavenumbers than ASC band (1547 cm^{-1}) and represent N–H bending vibrations coupled with CN stretching vibration. The amide III band, assigned to C-H stretching, was found to be centered at 1236 cm^{-1} for the ASC and at 1240 cm^{-1} for PSC collagen. The band of amide A is typically assigned to N-H stretching mode and was detected at 3380 for ASC and 3378 cm^{-1} for PSC. Finally, the amide B band of ASC and PSC were found at 2960 cm^{-1} and is associated with the asymmetrical stretch of CH_2.

As previously reported by Payne *et al.* [58] collagen dry films show a characteristic FT-IR spectrum, with absorption bands of amide I at ~1650 cm^{-1}, amide II at ~1560 cm^{-1}, and a set of three weaker bands that represent amide III vibration modes centered at ~1245 cm^{-1}. Both ASC and PSC showed the characteristic peaks observed in collagen. In particular, the position of amide I band of ASC and PSC was similar to that reported for others marine-derived collagens, usually found in the range of 1600 - 1700 cm^{-1} [59] [60]. The amide I peak is associated to the stretching vibration of the carbonyl groups (C=O bond) along the polypeptide. When collagen is heated at higher temperature this peak undergoes a decrease in absorbance, becomes more broad and presents additional shoulders. Due to the similarity in the position, shape and amplitude, both collagens appeared not denatured after the extraction process. This was reconfirmed by the ratio between amide III and the peak observed at 1440 cm^{-1} for ASC and 1449 cm^{-1} for PSC. In fact, a ratio of approximately 1 between these two peaks can be considered as an index of the

triple-helical structure of collagen [61]. The position of amide II band for both the collagens was found at lower wave numbers if compare with the characteristic position of this peak in collagen [62]. This band represent N–H bending vibrations and a shift to lower wave numbers can be explained with a decrease in molecular order in the extracted collagens. Amide A band is typically assigned to N-H stretching mode and can prove that the NH groups in both extracted collagens were involved in hydrogen bonding, probably with a carbonyl group of the peptide chain [63]. In fact, N-H stretching vibration usually is observed in the range 3400–3440 cm^{-1}; however, amide A peak can shift to lower frequencies when the NH group of a peptide is involved in a hydrogen bond [60]. Finally, the amide B band of both ASC and PSC were found at 2960 cm^{-1} and is associated at the asymmetrical stretch of CH_2 [60].

The similarity between the IR-spectra of ASC and PSC suggested that pepsin hydrolysis had no apparent effects on the triple-helical structure of PSC. However, it is well known that pepsin extraction can cause some differences between ASC and PSC especially at the telopeptide region or in the secondary structural components (e.g., α-helix, β-sheet) of these collagens [64].

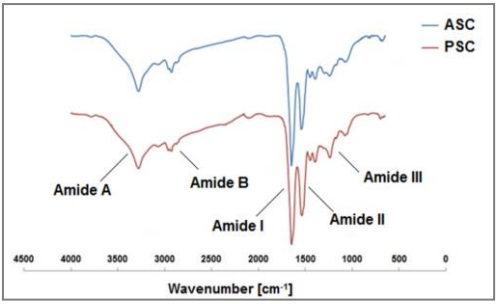


Figure IV - 8. *Fourier transforms infrared spectra of ASC and PSC from the squid mantle.*

Properties	Peak wavenumber (cm^{-1})		Assignment	Reference
	ASC	PSC		
Amide A	3380	3378	NH stretch and hydrogen bond	*Payne & Veis (1988)*
Amide B	2960	2960	CH_2 asymmetrical stretch	*Abe & Krimm (1972)*
Peak	2873	2929	CH_3 asymmetric strech mainly protein	*Abe & Krimm (1972)*
Amide I	1643	1648	C=O strech/hydrogen bond and COO^-	*Abe & Krimm (1972)*
Amide II	1547	1530	NH bend and CN stretch	*Payne & Veis (1988)*
Peak	1440	1449	CH_2 bending vibration	*Jackson et al. (1995)*
Peak	1408	1407	COO^- symmetrical stretch	*Jackson et al. (1995)*
Peak	1394	1394	CH_2 wagging of proline	*Jackson et al. (1995)*
Amide III	1236	1240	NH bend and CN stretch	*Payne & Veis (1988)*
Peak	1172	1080	COO-C asymmetric strech	*Jackson et al. (1995)*
Peak	1066	850	C-O stretch/C-O band	*Jackson et al. (1995)*
Peak	600	600	Skeletal stretch	*Abe et al. (1972)*

Table IV - 2. *Location of the FTIR characteristic peaks for ASC and PSC extracted from squid mantle and peak assignment.*

4.6.7 Thermal denaturation temperature of collagen from squid mantle

The thermal stability of extracted collagen samples was investigated with DSC and viscosity measurements. Dynamic viscosity measurement was performed using a cone/plate system (40 mm diameter). The temperature was controlled by a circulating water bath and the collagen samples were heated from 16 to 30°C. The measurements were performed over a shear rate range of 12.5 – 54.5 s^{-1}.
In Figure 9 we reported the change in viscosity of ASC and PSC solutions in 0.5 M acetic acid with temperature. During the helix-coil transition the intact trimers (γ) in the collagen solutions turn into individual chains (α) or dimers (β) causing the decrease of viscosity with the increase of temperature. The temperature at which the change in viscosity was half the maximum value was taken as the denaturation temperature of analyzed collagens solutions. In particular, the denaturation temperature (T_d) of ASC and PSC collagen from squid mantle was calculated to be about 22°C and 21°C, respectively.

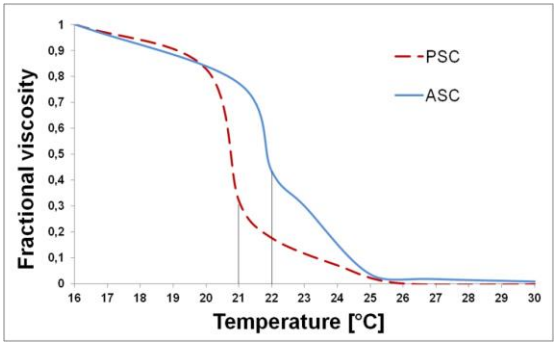


Figure IV - 9. *Thermal denaturation curves of ASC and PSC as shown by changes in fractional viscosity.*

The collagen denaturation process occurs with appreciable heat absorption. For this reason, differential scanning calorimetry (DSC) was also used to define collagen T_d, by measuring the endothermic heat flow. The thermograms of PSC and ASC are shown in Figure 10. The thermal denaturation process of extracted collagens was studied through the enthalpy change (ΔH) estimated from the denaturation endothermic peak of collagen and the minimum endothermic temperature (Td of ASC and PSC were 21°C and 24°C, respectively and ΔH about 0.9 J/g for the ASC and 0.8 J/g for PSC).

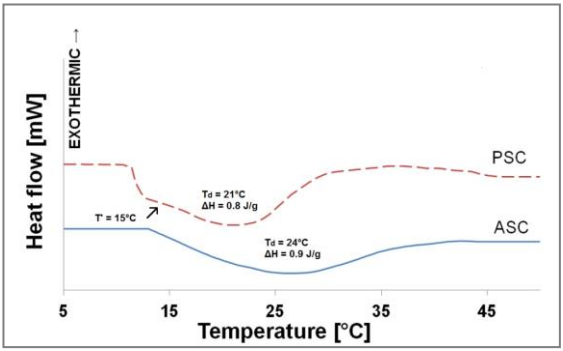


Figure IV - 10. *Thermal denaturation temperature of collagens from squid mantle: thermograms of PSC and ASC dispersed in 0.5 M acetic acid.*

Thermal denaturation temperature of extracted collagen in solution is an important parameter to understand the thermal stability of the material and to design the processing conditions. Indeed, thermal denaturation causes the irreversible transformation of the native triple helical structure into a more random (coiled) structure, thus changing several physical properties of the material, such as viscosity, elasticity, sedimentation, diffusion light scattering and optical activity [65]. Both the collagen extracted from squid mantle had a T_d value lower than mammalian-derived collagen; for example, collagen extracted from calf skin has a T_d of 37°C [66]. Denaturation temperature of collagen can be correlated with the different amino acids composition, but also at the environmental and body temperatures of fish species [67]. It was reported that the total amount of proline and hydroxyproline have a strong influence on thermal stability of collagen and in particular, a higher content of proline and hydroxyproline is associated at a higher thermal stability [68]. In addition, it was found that the difference content of proline and hydroxyproline amongst collagens isolated from different animals is correlated with the difference in the living environments of their sources, particularly habitat temperature [8]. For example, *L. vulgaris* typically lives in temperate seas and the T_d of collagen extracted from the squid mantle was lower than the T_d of tropical fish species, such as Brownstripe red snapper (30.5°C) [7] and bigeye Snapper (28.7°C) [69]. On the other side, the T_d of the squid collagens were higher than those of collagen extracted from cold-water fishes, such as Argentine hake (10°C), Baltic cod (15°C) [70] and Alaska pollack (17°C) [45]. These results confirm that some properties of isolated collagens, between others the T_d, are strongly related to the nature and living conditions of the organism used for the extraction and can be considered species-dependent. In addition, it is interesting to note that even if ASC and PSC collagens do not exist as separate forms inside the natural tissue, they presented different characteristics in relation to their specific amino acid

compositions and as a consequence of to the extraction method. For these reasons, a comparative characterization of ASC and PSC is necessary to evaluate how the different compositions can affect the physicochemical properties of the isolated proteins.

The heat transformation of collagen to gelatin is interpreted as a disintegration of the collagen triple helical structure into random coils. The overall helix to coil endothermic transition process of solubilized collagen has been monitored also by DSC. In the DSC thermogram of PSC a secondary endothermic peak was observed at T' = 15°C. A similar behavior is generally observed in the calorimetric curves of bovine hide collagen [71]. The minor peak was related to the breaking of hydrogen bonds among collagen molecules [31]. The presence of this minor peak can suggest that in the PSC the interactions between the collagen molecules are stronger than those of ASC. On the other side, the main endothermic peak in ASC curve was sharper compare with that of PSC, since ASC is composed of more complex components. The PSC collagen showed a denaturation temperature slightly lower than the ASC. Probably it is due to the action of pepsin, used during the extraction process. In fact, pepsin cleaves the cross-linked collagen molecules at the telepeptide region and this fact can facilitate the thermal denaturation process of PSC. However, DSC and viscosity measurements showed the helix-coil transition for both the extracted collagens, indicating that pepsin digestion did not affect the triple-helical structure of the protein. Previous studies have demonstrated that traces of acetic acid in solution have the effect to shift the denaturation temperature of collagen to a lower temperature [72]. These results suggested that when collagen is solubilized in a solution containing acetic acid, some of the intra-molecular hydrogen bonds that stabilize the triple helical structure can be disrupted for the repulsion between the collagen molecules dispersed in the acidic solution [16]. Therefore, a possible strategy to increase the thermal stability in order to extend the application area of collagen after the extraction process is to increase the degree of cross-linking inside the material [26][73]. Different ways to introduce exogenous cross-linking into the molecular structure of reconstituted collagen have been previously reported, most notably the use of aldehydes with formaldehyde and glutaraldehyde [74][75][76].

4.6.8 Thermal denaturation temperature of collagen from squid mantle

The solubility behaviour of ASC abd PSC with changes in pH and NaCl concentrations play a crucial role in their further processability and applications. Figure 11.(a) reports the solubility of ASC and PSC in a 0.5 M acetic acid solution with different NaCl concentrations. Both collagen samples showed similar solubility behavior. The solubility of ASC and PSC remained at a high level (more than 90%) in

the presence of NaCl at concentrations lower than 0.4 M, and decreased sharply when NaCl concentration was between 0.4 and 0.6 M NaCl. At higher NaCl concentration, for both collagen fractions, the solubility remained relatively stable but very low (around 10%). It is well known that the decrease of collagen solubility at high NaCl concentration is due to the so-called 'salting out' effect [77]. With the increase of NaCl concentration, the ionic strength of the solution increases. As a consequence, the solubility declined by enhancing hydrophobic–hydrophobic interactions between protein chains, leading to protein precipitation [2].

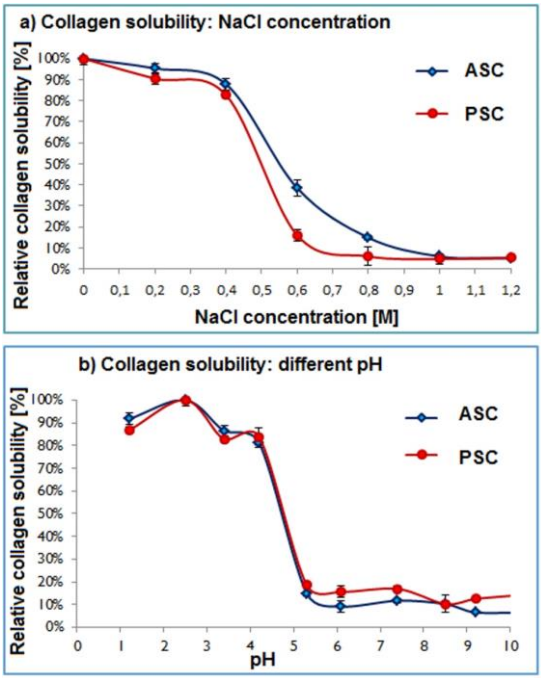


Figure IV - 11. (a) *Solubility of collagen solution from squid mantle in 0.5 M acetic acid with different NaCl concentrations;* **(b)** *Solubility of collagen solution from squid mantle in 0.5 M acetic acid at different pH values*

The effect of the pH of the solution on collagen solubility is shown in Figure 11.(b). Also in this case, both ASC and PSC were fully solubilized in 0.5 M acetic acid, and then the pH of the solution was adjusted to obtain a final pH ranging from 1 to 10. Both, the ASC and PSC had a high solubility in acid conditions and they reached the greatest solubility at pH between 2 and 3. A sharply decrease of the solubility was registered for both the collagens between pH 3 and pH 4 and between pH 4 and pH 5. At pH values higher than 5, the collagen solubility became low (under 20%) but relatively stable. ASC and PSC showed the lowest solubility at pH between 5 and 6. It is well known that a protein dissolved in buffer at its pI has no net charge, and thus, hydrophobic–hydrophobic interaction increases, and protein precipitation and aggregation are induced. Therefore, these results suggest that the extracted collagens had isoelectric points in the interval of pH between 5 to 6. This value was slightly lower than the values of pI previously reported for collagen, ranging from pH 6 to 9 [78]. The lower pI of ASC and PSC was probably related to a higher density of carboxyl groups inside the isolated collagens caused by the hydrolysis of some of the side amide groups during the extraction process.

Finally, we can observed that PSC showed a higher solubility than ASC for different NaCl concentrations and at pH higher than 5. This result confirmed that the content of the high MW cross-link of ASC was higher than in PSC and that ASC is composed of more complex components. The electrophoretic profiles showed that PSC presented a number of bands corresponding to the fractions with MW lower than those of the monomeric collagen chains. It could be due at the action of pepsin that might induce the partial hydrolysis of high MW cross-linked molecules, resulting in a greater solubility of PSC than ASC.

4.6.9 *In vitro* cytotoxicity evaluation of extracted collagens: LDH analysis

Cells were cultivated with culture medium conditioned with ASC and PSC powders. As shown in Figure 12 there was no significant difference in LDH release among the experimental groups at 72 hours. The results of LDH assay showed that the squid-derived collagens have no cytotoxic effect after the extraction process.

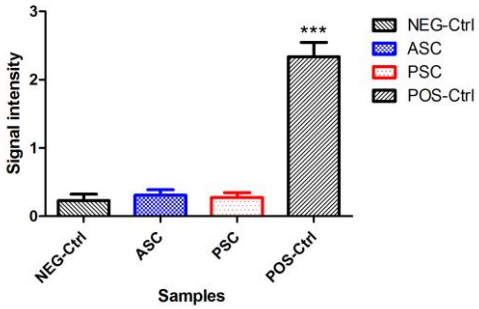


Figure IV - 12. *In vitro cytotoxicity evaluation of extracted collagens using the lactate dehydrogenase assay (LDH). NEG-Ctrl: standard culture medium; ASC: medium containing ASC collagen extracts; PSC: medium containing PSC collagen extracts; POS-Ctrl: medium containing Triton X-100.*

4.7 Production of marine collagenous fractions matrices cross-linked with genipin

4.7.1 Sample production

Collagen-based films and sponges are frequently used as biomaterials [14]. However, for different technical applications, a cross-linking reagent is required to improve mechanical strength and degradation properties of collagenous matrices [26] [27]. In this case, genipin was used as a naturally occurring crosslinking agent to fabricate collagen matrices.

Briefly, lyophilized ASC and PSC were redissolved in 0.5M acetic acid at 4°C to obtain a final concentration of 1% (wt/vol). ASC and PSC films were prepared by solvent casting on the bottom of a 6 cm petri dish starting from 10 ml of collagen solution. Collagen films were air-dried and then cross-linked with genipin by immersion in 0.1% (wt/vol) genipin solution in ethanol 70% at 4 °C for 48 h (pH adjusted to 7.4). Finally, cross-linked film were repeatedly washed with sterile PBS and air-dried at room temperature. In alternative, the collagen solutions were cast in 6 cm petri dishes, frozen at 80°C and freeze-dried for the preparation of the ASC and PSC sponges. The dry sponges were cross-linked in 0.1% (wt/vol) genipin solution in ethanol 70% at 4 °C for 48 h. The cross-linked sponges were then repeatedly washed with sterile PBS, frozen at -80°C and then freeze-dried for the subsequent characterization.

4.7.2 Microstructural analysis of the samples

Scanning electron microscopy (Supra 40, operating mode: high vacuum, secondary electron detector, Carl Zeiss, Europe) was used for the evaluation of collagen films and sponges morphologies. Samples were previously sputter-coated with a thin gold layer under argon (SEM Coating Unit PS3, Assing S.p.A., Rome, Italy). The micrographs obtained from scanning electron microscope (SEM) of the collagenous matrices were reported in Figure 13.

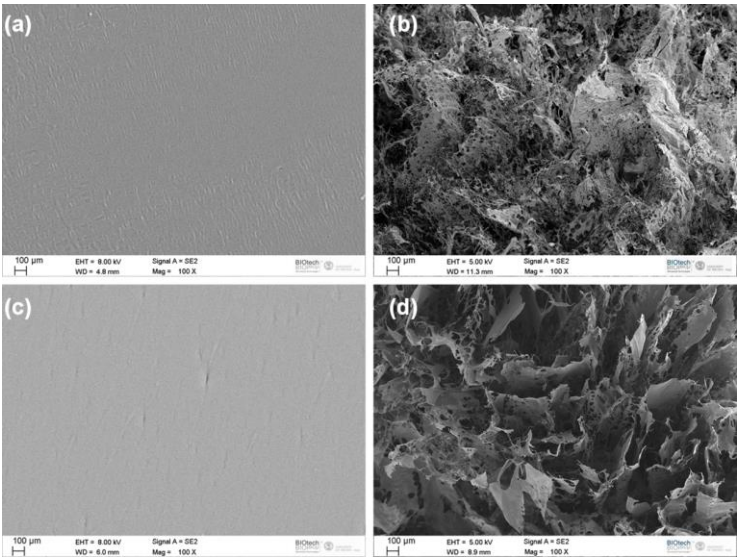


Figure IV - 13. *Genipin cross-linked collagen constructs:* ***(a)*** *ASC film;* ***(b)*** *ASC sponge;* ***(c)*** *PSC film;* ***(d)*** *PSC sponge.*

SEM observation evidenced that the surfaces of ASC and PSC films were smooth and uniform (Figure 13. (a) and (c)). The collagen sponges showed a three-dimensional porous structure, with irregular patterns and pores size (Figure 13. (b) and (d)). Both ASC and PSC sponges presented interconnected porosity, but with different pores size distributions. In particular, ASC sponges showed a pores size ranging between 10 and 150 μm, while PSC sponges had two types of porosity, one with a smaller pores size averaging 40 μm and the other with a larger pore size averaging 320 μm.

4.7.3 *In vitro* cytotoxicity evaluation of the samples

Cytotoxicity of the samples was evaluated following ISO standard 10993-5 (1999), by direct cultivation of cells with samples extracts. Embryonic mouse fibroblast NIH/3T3 cells (3T3) were expanded and cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle medium (DMEM) (Euroclone, Milan, Italy), supplemented with 10% fetal bovine serum (Gibco, NY, USA), 2mM L-glutamine, 1mM sodium pyruvate and 0.1% antibiotics (Gibco, Eggenstein, Germany). Culture medium was changed every 2 days. When cells reached confluence, they were detached with 0.1% trypsin and re-suspended in culture medium. Then, 3T3 cells were seeded in 24-well polystyrene plate at a density of 1×10^4 cells per well and incubated under standard culture conditions. At 70-80% of confluence, culture medium was removed and replaced with conditioned media containing samples extracts. Conditioned media were prepared by soaking the collagen constructs samples in reduced medium in a proportion of 4 g per 20 ml of medium, as prescribed by the ISO standard (24 hours of soaking at 37°C with 5% CO_2). Reduced medium was prepared with Dulbecco's Modified Eagle medium without phenol red with 10% heat inactivated serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.1% antibiotics. Fibroblast 3T3 cells were cultured in presence of conditioned media in standard conditions for additional 72 hours. Five independent measurements were acquired for each sample. Cells cultured in reduced medium were used as negative control. Lactate dehydrogenase assay (LDH) (TOX7, Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the cytotoxicity impact of samples extracts on the cells. An aliquot of 100 µm of culture medium was collected from all wells and mixed with the LDH mixture, following manufacturers' instructions. Cells cultured in reduced medium and treated for 30 min with Triton x-100 were used as positive controls. Absorbance was measured using a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) at 490 nm, background absorbance was measured at 690 nm. Results were presented as mean ± standard deviation (n = 5).

Figure 14 reports the in vitro cytotoxicity evaluation of the samples extracts. The cytotoxic effect was determined using the measurement of LDH release into the culture supernatant. The results show that there are no significant differences in LDH release among the experimental groups after 72 hours.

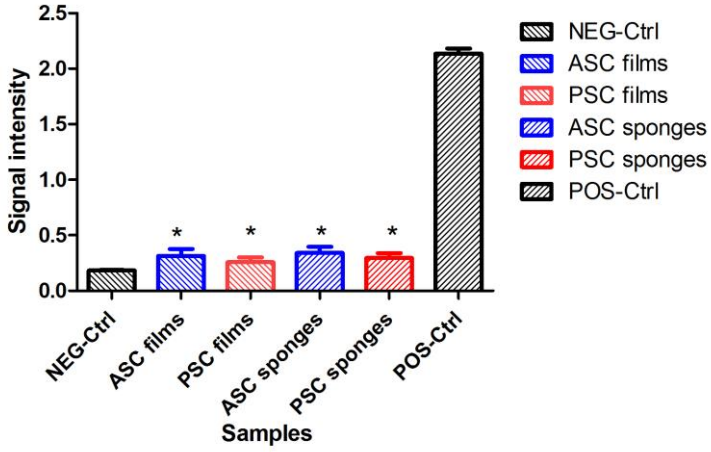


Figure IV - 14. *In vitro cytotoxicity evaluation of using the Lactate Dehydrogenase Assay (LDH). NEG-Ctrl: standard culture medium; ASC sponges: medium containing extracts of ASC sponges cross-linked with genipin; PSC sponges: medium containing extracts of PSC sponges cross-linked with genipin; ASC films: medium containing extracts of ASC films cross-linked with genipin; PSC films: medium containing extracts of PSC films cross-linked with genipin; POS-Ctrl: medium containing Triton X-100 (n=4, *p < 0.001, significantly* different from POS-Ctrl).

4.8 Conclusion

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) from squid mantle (*L. Vulgaris*) were successfully isolated and characterized. The results showed that the use of pepsin during the extraction process increase the yield of isolated collagen (by 4.7-fold) without damaging the integrity of the triple helix. DSC and viscosity measurements, showed the helix-coil transition for both the extracted collagens, indicating that pepsin digestion did not affect the conformation of the proteins. The SDS-PAGE patterns of ASC and PSC presented a protein profile characteristic of type I and V collagens, consisting of two α-chains (α1 and α2), β- and γ-components. GPC analysis showed that the extracted collagens are composed of different families of peptides which different molecular weight distributions. In particular ASC was composed by a higher content of high-molecular weight cross-links than PSC. These results were supported by the thermal analysis and the solubility behaviors that reported a lower solubility of ASC compare with PSC. Both the collagens had high solubility at acidic pH and lost solubility when the NaCl concentration increased above 0.4 M. The denaturation temperature of extracted collagens, in a 0.5 M acetic acid solution, was found to be lower than those of mammalian collagens since it is strongly influenced by the content of proline and hydroxyproline, environmental and body temperatures conditions. Moreover, the in

vitro cytotoxicity tests proved that after the extraction process the material did not release cytotoxic substances. Finally, stable cross-linked matrices (films and sponges) were produced starting from the marine-derived collagen fractions and using a natural crosslinking agent.

The results of this study demonstrated that squid mantle has a potential as an alternative source of collagen and could be considered for a further use in pharmaceutical or biomedical applications. In addition, we also demonstrated that the use of genipin as natural crosslinking agent could be a useful approach for create stable devices of marine collagen, without added cytotoxicity problems.

References

[1] N. Cozza, W. Bonani, A. Motta, and C. Migliaresi, "Evaluation of alternative sources of collagen fractions from *Loligo vulgaris* squid mantle," *Int. J. Biol. Macromol.*, vol. 87, pp. 504–513, 2016.

[2] A. Veeruraj, M. Arumugam, and T. Balasubramanian, "Isolation and characterization of thermostable collagen from the marine eel-fish (*Evenchelys macrura*)," *Process Biochem.*, vol. 48, no. 10, pp. 1592–1602, 2013.

[3] J. Glowacki and S. Mizuno, "Collagen scaffolds for tissue engineering," *Biopolymers*, vol. 89, no. 5, pp. 338–44, 2008.

[4] K. Gelse, E. Pöschl, and T. Aigner, "Collagens - Structure, function, and biosynthesis," *Adv. Drug Deliv. Rev.*, vol. 55, no. 12, pp. 1531–1546, 2003.

[5] Y. Zhang, W. Liu, G. Li, B. Shi, Y. Miao, and X. Wu, "Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*)," *Food Chem.*, vol. 103, no. 3, pp. 906–912, 2007.

[6] F. Pati, B. Adhikari, and S. Dhara, "Isolation and characterization of fish scale collagen of higher thermal stability," *Bioresour. Technol.*, vol. 101, no. 10, pp. 3737–42, 2010.

[7] A. Jongjareonrak, S. Benjakul, W. Visessanguan, T. Nagai, and M. Tanaka, "Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*)," *Food Chem.*, vol. 93, no. 3, pp. 475–484, 2005.

[8] A. A. Karim and R. Bhat, "Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins," *Food Hydrocoll.*, vol. 23, no. 3, pp. 563–576, 2009.

[9] S. Xuan Ri, K. Hideyuki, and T. Koretaro, "Characterization of molecular species of collagen in scallop mantle," *Food Chem.*, vol. 102, no. 4, pp. 1187–1191, 2007.

[10] S. Mizuta, R. Yoshinaka, M. Sato, and M. Sakaguchi, "Isolation and partial

characterization of two distinct types of collagen in the squid Todarodes pacificus," *Fish. Sci.*, vol. 60, no. 4, pp. 467–41, 1994.

[11] A. Kessler, H. Rosen, and S. M. Levenson, "Chromatographic fractionation of acetic acid-solubilized rat tail tendon collagen," *J. Biol. Chem.*, vol. 235, no. 4, pp. 989–94, 1960.

[12] D. Liu, G. Wei, T. Li, J. Hu, N. Lu, J. M. Regenstein, and P. Zhou, "Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin," *Food Chem.*, vol. 172, pp. 836–843, 2015.

[13] E. D. Hay, "Extracellular Matrix," *J. Cell Biol.*, vol. 91, no. 3, pp. 205s–223s, 1981.

[14] W. Friess, "Collagen-biomaterial for drug delivery," *Eur. J. Pharm. Biopharm.*, vol. 45, no. 2, pp. 113–136, 1998.

[15] F. Shahidi, *Maximising the value of marine by-products*. Woodhead Publishing, 2006.

[16] M. Ahmad and S. Benjakul, "Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket *(Aluterus monocerous*)," *Food Chem.*, vol. 120, no. 3, pp. 817–824, 2010.

[17] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, H. Kishimura, and F. Shahidi, "Isolation and Characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*)," *Food Chem.*, vol. 119, no. 4, pp. 1519–1526, 2010.

[18] S. Tamilmozhi, A. Veeruraj, and M. Arumugam, "Isolation and characterization of acid and pepsin-solubilized collagen from the skin of sailfish (*Istiophorus platypterus*)," *Food Res. Int.*, vol. 54, no. 2, pp. 1499–1505, 2013.

[19] M. C. Gomez-Guillen, B. Gimenez, M. E. Lopez-Caballero, and M. P. Montero, "Functional and bioactive properties of collagen and gelatin from alternative sources: A review," *Food Hydrocoll.*, vol. 25, no. 8, pp. 1813–1827, 2011.

[20] Z. Zhang, G. Li, and B. Shi, "Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes," *J. Soc. Leather Technol. Chem.*, vol. 90, pp. 23–28, 2006.

[21] T. Silva, J. Moreira-Silva, A. Marques, A. Domingues, Y. Bayon, and R. Reis, "Marine Origin Collagens and Its Potential Applications," *Mar. Drugs*, vol. 12, no. 12, pp. 5881–5901, 2014.

[22] S. K. Šifner and N. Vrgoč, "Population structure, maturation and reproduction of the European squid, *Loligo vulgaris*, in the Central Adriatic Sea," *Fish. Res.*, vol. 69, no. 2, pp. 239–249, 2004.

[23] C. F. E. Roper, M. J. Sweeney, and C. E. Nauen, "Cephalopods of the world: an annotated and illustrated catalogue of species of interest to

fisheries," *FAO Spec*, vol. 3, no. 125, 1984.

[24] Z. E. Sikorski and I. Kołodziejska, "The composition and properties of squid meat," *Food Chem.*, vol. 20, no. 3, pp. 213–224, 1986.

[25] I. Kołodziejska, Z. E. Sikorski, and C. Niecikowska, "Parameters affecting the isolation of collagen from squid (*Illex argentinus*) skins," *Food Chem.*, vol. 66, pp. 153–157, 1999.

[26] L. P. Yan, Y. J. Wang, L. Ren, G. Wu, S. G. Caridade, J. B. Fan, L. Y. Wang, P. H. Ji, J. M. Oliveira, J. T. Oliveira, J. F. Mano, and R. L. Reis, "Genipin-cross-linked collagen/chitosan biomimetic scaffolds for articular cartilage tissue engineering applications," *J. Biomed. Mater. Res. - Part A*, vol. 95 A, no. 2, pp. 465–475, 2010.

[27] I. Rault, V. Frei, D. Herbage, N. Abdul-Malak, and A. Huc, "Evaluation of different chemical methods for cros-linking collagen gel, films and sponges," *J. Mater. Sci. Mater. Med.*, vol. 7, no. 4, pp. 215–221, 1996.

[28] T. Nagai, "Isolation of collagen from fish waste material - skin, bone and fins," *Food Chem.*, vol. 68, no. 3, pp. 277–281, 2000.

[29] E. J. Miller, "Isolation and characterization of a chick cartilage collagen containing three identical chains," *Biochemistry*, vol. 10, no. 9, pp. 1652–1659, 1971.

[30] F. Guérard, F. Shahidi, and others, "Enzymatic methods for marine by-products recovery," *Maximising value Mar. by-products*, pp. 107–143, 2007.

[31] C. Mu, D. Li, W. Lin, Y. Ding, and G. Zhang, "Temperature induced denaturation of collagen in acidic solution," *Biopolymers*, vol. 86, no. 4, pp. 282–287, 2007.

[32] P. S. Macgillivray, E. J. Anderson, G. M. Wright, and M. E. Demont, "Structure and mechanics of the squid mantle," *J. Exp Biol.*, vol. 202, no. 6, pp. 683–695, 1999.

[33] Q. Bone A. Pulsford, and A. D. Chubb, "Squid mantle muscle," *J. Marine Biol. Ass. U. K.*, vol. 61, no. 02, pp. 327–342,1981.

[34] T. W. Moon and W. C. Hulbert, "The ultrastructure of the mantle musculature of the squid Symplectoteuthis oualaniensis," *Comp. Biochem. Phys. B,* vol. 52, no. 1, pp. 145–149, 1975.

[35] J. M. Gosline and R. E. Shadwick, "The role of elastic energy storage mechanisms in swimming: an analysis of mantle elasticity in escape jetting in the squid, *Loligo opalescens*," *Can. J. Zool.*, vol. 61, no. 6, pp. 1421–1431, 1983.

[36] M. Krieg and K. Mohseni, "New Perspectives on Collagen Fibers in the Squid Mantle," vol. 273, no. 6, pp. 586–595, 2012.

[37] E. A. Foegeding, T. C. Lanier, and H. O. Hultin, "Characteristics of edible muscle tissues," *Food Chem.*, vol. 3, no. 15, pp. 879–942, 1996.

[38] S. Nalinanon, S. Benjakul, W. Visessanguan, and H. Kishimura, “Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*),” *Food Chem.*, vol. 104, no. 2, pp. 593–601, 2007.

[39] S. Nalinanon, S. Benjakul, W. Visessanguan, and H. Kishimura, “Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor,” *Food Hydrocoll.*, vol. 22, no. 4, pp. 615–622, 2008.

[40] Y. U. Di, C. H. I. Chang-feng, W. Bin, D. Guo-fang, and L. I. Zhong-rui, “Characterization of acid- and pepsin-soluble collagens from spines and skulls of skipjack tuna (*Katsuwonus pelamis*),” vol. 12, no. 9, pp. 712–720, 2014.

[41] M. Chvapil, R. L. Kronenthal, and W. Van Winkle, “Medical and surgical applications of collagen,” *Int Rev Connect Tissue Res*, vol. 6, no. 1, 1973.

[42] T. R. Knapp, E. Luck, and J. R. Daniels, “Behavior of solubilized collagen as a bioimplant.,” *J. Surg. Res.*, vol. 23, no. 2, pp. 96–105, 1977.

[43] M. J. Beckman, K. J. Shields, and R. F. Diegelmann, “Collagen,” in *Encyclopedia of Biomaterials and Biomedical Engineering*. Taylor & Francis, pp. 324–334, 2013.

[44] A. Jongjareonrak, S. Benjakul, W. Visessanguan, and M. Tanaka, “Isolation and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin,” *J. Sci. Food Agric.*, vol. 85, no. 7, pp. 1203–1210, 2005.

[45] S. Kimura, Y. Ohno, Y. Miyauchi, and N. Uchida, “Fish skin type I collagen: wide distribution of an α3 subunit in teleosts,” *Comp. Biochem. Physiol. Part B Comp. Biochem.*, vol. 88, no. 1, pp. 27–34, 1987.

[46] L. Wang, Q. Liang, Z. Wang, J. Xu, Y. Liu, and H. Ma, “Preparation and characterisation of type I and V collagens from the skin of Amur sturgeon (*Acipenser schrenckii*),” *Food Chem.*, vol. 148, pp. 410–414, 2014.

[47] M. H. Uriarte-Montoya, J. L. Arias-Moscoso, M. Plascencia-Jatomea, H. Santacruz-Ortega, O. Rouzaud-Sández, J. L. Cardenas-Lopez, E. Marquez-Rios, and J. M. Ezquerra-Brauer, “Jumbo squid (*Dosidicus gigas*) mantle collagen: extraction, characterization, and potential application in the preparation of chitosan-collagen biofilms,” *Bioresour. Technol.*, vol. 101, no. 11, pp. 4212–9, 2010.

[48] J. Morales, P. Montero, and A. Moral, “Isolation and partial characterization of two types of muscle collagen in some cephalopods.,” *J. Agric. Food Chem.*, vol. 48, no. 6, pp. 2142–2148, 2000.

[49] A. L. Shapiro, E. Viñuela, and J. V Maizel, “Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels,” *Biochem. Biophys. Res. Commun.*, vol. 28, no. 5, pp. 815–820, 1967.

[50] K. Weber and M. Osborn, “The Reliability of Molecular Weight

Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis," *J. Biol. Chem.*, vol. 244, no. 16, pp. 4406–4412, 1969.

[51] S. R. Holding and E. Meehan, *Molecular weight characterisation of synthetic polymers*, vol. 83. iSmithers Rapra Publishing, 1995.

[52] P. Singh, S. Benjakul, S. Maqsood, and H. Kishimura, "Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*)," *Food Chem.*, vol. 124, no. 1, pp. 97–105, 2011.

[53] E. Jeevithan, Z. Jingyi, N. Wang, L. He, B. Bao, and W. Wu, "Physico-chemical, antioxidant and intestinal absorption properties of whale shark type-II collagen based on its solubility with acid and pepsin," *Process Biochem.*, vol. 50, no. 3, pp. 463–47, 2015.

[54] B. Brodsky and A. V. Persikov, "Molecular structure of the collagen triple helix," *Adv. Protein Chem.*, vol. 70, no. 04, pp. 301-339, 2005.

[55] E. J. Kucharz, *Biosynthesis of collagen*. Springer, 1992.

[56] T. Ikoma, H. Kobayashi, J. Tanaka, D. Walsh, and S. Mann, "Physical properties of type I collagen extracted from fish scales of Pagrus major and Oreochromis niloticas," *Int. J. Biol. Macromol.*, vol. 32, no. 3–5, pp. 199–204, 2003.

[57] M. A. Roseman, "Hydrophilicity of polar amino acid side-chains is markedly reduced by flanking peptide bonds.," *J. Mol. Biol.*, vol. 200, no. 3, pp. 513–522, 1988.

[58] K. J. Payne and A. Veis, "Fourier transform IR spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies," *Biopolymers*, vol. 27, no. 11, pp. 1749–1760, 1988.

[59] W. K. Surewicz and H. H. Mantsch, "New insight into protein secondary structure from resolution-enhanced infrared spectra," *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.*, vol. 952, pp. 115–130, 1988.

[60] Y. Abe, S. Krimm, and H. M. Randall, "Normal Vibrations of Crystalline Polyglycine I," vol. 11, no. 9, pp. 1817–1839, 1972.

[61] J. H. Muyonga, C. G. B. Cole, and K. G. Duodu, "Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*)," *Food Chem.*, vol. 86, no. 3, pp. 325–332, 2004.

[62] K. Belbachir, R. Noreen, G. Gouspillou, and C. Petibois, "Collagen types analysis and differentiation by FTIR spectroscopy," *Anal. Bioanal. Chem.*, vol. 395, no. 3, pp. 829–837, 2009.

[63] K. P. Sai and M. B. U, "Studies on Rana tigerina skin collagen," *Comp. Biochem. Phys. B.*, vol. 128, no. 1, pp. 81–90, 2001.

[64] T. Nagai, N. Suzuki, and T. Nagashima, "Collagen from common minke whale (*Balaenoptera acutorostrata*) unesu," *Food Chem.*, vol. 111, no. 2,

pp. 296–301, 2008.

[65] R. Usha and T. Ramasami, “The effects of urea and n-propanol on collagen denaturation: using DSC, circular dicroism and viscosity,” *Thermochim. Acta*, vol. 409, no. 2, pp. 201–206, 2004.

[66] M. Ogawa, M. W. Moody, R. J. Portier, J. Bell, M. A. Schexnayder, and J. N. Losso, “Biochemical Properties of Black Drum and Sheepshead Seabream Skin Collagen,” *J. Agric. Food Chem.*, vol. 51, no. 27, pp. 8088–8092, 2003.

[67] K. A. Piez and J. Gross, “The amino acid composition of some fish collagens: The relation between composition and structure,” *J. Biol. Chem.*, vol. 235, no. 4, pp. 995–998, 1960.

[68] K. H. Gustavson, “The Function of Hydroxyproline in Collagens,” *Nature*, vol. 175, no. 4445, pp. 70–74, 1955.

[69] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, T. Nagai, and M. Tanaka, “Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*),” *Food Chem.*, vol. 89, no. 3, pp. 363–372, 2005.

[70] B. J. Rigby, “Amino-acid Composition and Thermal Stability of the Skin Collagen of the Antarctic Ice-fish,” *Nature*, vol. 219, no. 5150, pp. 166–167, 1968.

[71] W. Liu and G. Li, “Non-isothermal kinetic analysis of the thermal denaturation of type I collagen in solution using isoconversional and multivariate non-linear regression methods,” *Polym. Degrad. Stab.*, vol. 95, no. 12, pp. 2233–2240, 2010.

[72] J. H. Muyonga, C. G. B. Cole, and K. G. Duodu, “Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*),” *Food Chem.*, vol. 85, pp. 81–89, 2004.

[73] S. Fernandes-Silva, J. Moreira-Silva, T. H. Silva, R. I. Perez-Martin, C. G. Sotelo, J. F. Mano, A. R. C. Duarte, and R. L. Reis, “Porous hydrogels from shark skin collagen crosslinked under dense carbon dioxide atmosphere,” *Macromol. Biosci.*, vol. 13, no. 11, pp. 1621–1631, 2013.

[74] A. Jayakrishnan and S. R. Jameela, “Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices,” *Biomaterials*, vol. 17, no. 5, pp. 471–484, 1996.

[75] K. S. Weadock, E. J. Miller, L. D. Bellincampi, J. P. Zawadsky, and M. G. Dunn, “Physical crosslinking of collagen fibers: Comparison of ultraviolet irradiation and dehydrothermal treatment,” *J. Biomed. Mater. Res.*, vol. 29, no. 11, pp. 1373–1379, 1995.

[76] E. D. Harris and M. E. Farrell, “Resistance to collagenase: a characteristic of collagen fibrils cross-linked by formaldehyde,” BBA-Protein Struct., vol. 278, no.1, pp. 133–141, 1972.

[77] S. Damodaran, *5 Amino Acids, Peptides, and Proteins.* Fennema's food chemistry, pp. 217, 1996.

[78] J. H. Highberger, “The Isoelectric Point of Collagen,” *J. Am. Chem. Soc.*, vol. 61, no. 9, pp. 2302–2303, 1939.

Chapter V

Literature Review: bioceramics for bone tissue engineering applications

5.1 Introduction to bone tissue engineering

5.1.1 Bone: structures and properties

Bone is an highly organised tissue that performs different mechanical, biological and chemical functions, including structural support, protection for the internal organs and mineral ions homeostasis [1].

At the macrostructure level, bone tissue is distinguished into trabecular, also called cancellous or spongy bone, and cortical or compact bone. Cortical bone has a compact structure with a high compressive and tensile strengths of about 180 and 100 MPa, respectively (compressive and tensile modulus around 17 and 13 GPa), and accounts approximately 80% of the total mass of bone [2]. Trabecular bone is arranged in a sponge-like form, with a higher porosity (between 50% and 90%) and a ultimate compressive strength around 20 times lower than that of cortical bone [3]. Bone mechanical properties and density however vary with the age, gender, body weight, specific bone location and more.

More in details, natural bone is a complex inorganic–organic nanocomposite material, in which a mineralized inorganic component (composed of hydroxyapatite nanocrystallites) and an organic component (mainly collagen type I) are organized in a hierarchical architecture overall several length scales (Figure 1). This complex structure confers to bone material heterogeneous and anisotropic properties, and appropriate compressive strength and high fracture toughness [2].

The mineral component of bone consists of a calcium-deficient apatite, with a Ca/P ratio < 1.67, which is the stoichiometric value of pure hydroxyapatite [4]. This non-stoichiometric apatite phase in natural bone is in the form of nanometer-sized needle-like crystals of approximately 5-20 nm width by 60 nm length, which presents a lower crystallinity in comparison with the synthetic hydroxyapatite. This is due to the small size of its crystallites and to the incorporation of ions into its lattice, such as CO_3^{2-}, Na^+, Mg^{2+}, SiO_4^{4-}, HPO_4^{2-} and other trace elements, which are naturally present in the physiological environment and extremely important in the biochemistry of bone formation and maintenance [5]. For example, it was been reported that Na^+ plays an essential part in bone metabolism and to prevent osteoporosis, Mg^{2+} is important in the calcification process and indirectly influences the mineral metabolism, while F^- contributes to the stability of the apatite phase.

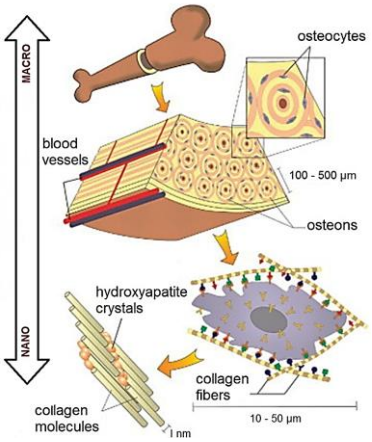


Figure V - 3. Hierarchical organization of bone over different length scales. (Modified from 2005 American Society for the Advancement of Science.)

In addition, the small size and non-stoichiometry of the bone crystals confer at the mineral phase of bone the solubility required for the resorption by osteoclasts, allowing the process of bone remodelling. In fact, as a dynamic tissue, bone continuously undergoes remodelling and repair throughout the lifetime of an individual. The elaboration, maintenance and resorption of this complex tissue are the result of the interaction of three cell types, with specific and essential roles: osteoblasts, osteocytes and osteoclasts [6]. The main bone cell types and their respective functions are summarized in Table 1.

Cell type	*Morphological characteristics*	*Function*
Osteoblasts	Cuboidal in shape, polarized and located, with their precursors, at the bone surface, where they form a tight layer of cells	Synthesis and regulation of bone ECM deposition and mineralization Respond to mechanical stimuli
Osteocytes	Stellate shaped	Calcification of the osteoid matrix Blood-calcium homeostasis
	Possess fewer organelles than the osteoblasts	Mechanosensor cells of the bone
Osteoclasts	Polarized cells Multinucleated cells	Bone resorption

Table V - 1. Bone cell types and respective functions. Compiled from [7].

5.1.2 Bone tissue engineering

Current therapies for bone defects or bone substitutes include mostly the use autologous bone grafts, autogenous bone grafts or as an alternative to these, metals and alloys [8]. However, the clinical use of the available grafts present some limitations, such as restrictions on the amount of available tissue in the case of autografts, or concerns about immunogenicity for allografts [9].

A promising strategy to overcome the limitations of the current graft and to improve bone regeneration, is to develop new kinds of bone substitutes through the combination of the principles of biomaterials and tissue engineering. In fact, the recent developments of tissue engineering allow to combine autologous cells and proteins that promote cell adhesion with osteoconductive materials to obtain osteoinductive products [10].

A key aspect in the tissue engineering approach concerns the design of the scaffold. In fact, there are many variables to be optimized that could affect the biologic response, including composition of the scaffold, architectural parameters (such as pore size and interconnectivity) and mechanical properties.

At this regard, a crucial aspect concerns the selection of the most appropriate scaffold material, since its properties will determine, to a great extent, the final properties of the scaffold. Theoretically, besides being biocompatible, an ideal scaffold for bone tissue engineering should be porous to allow cell penetration inside, osteoinductive to promote bone formation, and possess mechanical properties compatible with those of the native tissue [11]. In addition, the material has to induce cell attachment and promote the remodelling of the extracellular matrix to ensure the integration with the surrounding tissue. For example, it was reported that the interaction between osteoblast cells and the substrate can influence their ability to produce osteoid matrix around the scaffold and, consequently, determine the fate of the implant [12].

Scaffold for bone tissue engineering are currently made by polymers, ceramics, or their combination [13].

Between these two class of materials, polymers are generally used in the production of matrices to support cell growth and can be easily modified through the control of the polymerization process, or with the introduction of different functional groups [14]. In particular, several natural polymers such as polysaccharides (alginate, chitin/chitosan, starch) or proteins (collagen, silk, soy) possess highly organized structures, and are able to guide cells growth and show an innate affinity with the biological environment [7].

Ceramics and bioactive glasses, such as apatites and bioglass, have been increasingly used for bone tissue engineering applications due to their bioactive properties and high compressive strength [14]. It has been reported that after implantation, bioactive glasses and ceramics can modify their surfaces and develop

a biologically active hydroxy carbonate apatite layer which provides the bonding interface with tissues [8].
However, despite the analogy of certain synthetic bioceramic with bone mineral, it is important to remark that native bone presents a more complex composition. As previously described, besides calcium phosphate, it contains carbonate ions, hydrogen phosphate ions and other trace elements with important biological functions, such as magnesium, sodium and silicon [5]. For these reasons, in the search to improve the biological properties and mechanical strength of implant materials, attention has been directed towards the potential use of composites (such as polymer/ceramic or ceramic/ceramic materials) or certain ceramics derived from biological sources (e.g. coral-derived or seashell-derived apatites) which naturally show a chemical composition more similar to native bone.

5.2 Bioceramics in bone tissue engineering applications

Bioceramics is a class of advanced ceramic materials that are currently employed in a number of biomedical applications including dental restoration, filling and repair of bony defects, reconstruction of cranial bones [15]. They are produced by sintering or melting inorganic raw materials to obtain an amorphous or crystalline final product [16].
During the past 30–40 years, the demands of bioceramics have changed from performing basically biologically inert roles, such as provide parts for bone replacement, to providing a more integrated interaction with the host tissues [9]. According with their interaction with the host tissue and their bioactivity, they can be categorised as nonresorbable or relatively inert (such as alumina or zirconia), bioactive or surface active (such as hydroxyapatite or Bioglass®) and bioresorbable or noninert (such as tricalcium phosphates) [16]. In contrast to bioinert ceramics that cause a minimal level of response from the host tissue, bioactive and surface active ceramics are able to interact with the host tissue and promote bone growth on their surface. In addition, some porous bioactive ceramics are bioresorbable and can be replaced by the native tissue over time.
Representative bioactive ceramics are calcium phosphates, Bioglass® and glass-ceramics containing hydroxyapatite or its components, such as CaO and P_2O_5.

5.2.1 Calcium phosphates

Calcium phosphates materials have been utilized for treating bone defects as they are non-toxic to tissues, exhibit bioresorption and osteoconductive properties. In addition, it has been reported that calcium phosphates may bind directly to bone under certain conditions because of their chemical and crystal resemblance to bone

mineral [17]. In particular, after implantation, calcium phosphates are able to induce the formation at their surface of a bone like apatite layer from the ions present in the body fluids. It was been demonstrated that, when they are put in contact with the biological environment, several interactions occur. Firstly, the materials start to interact with collagen and the subsequent accumulation of proteins and cells at their surface leads to the gradual resorption of the material and then, to bone formation. During this process the composition of the crystals is an important factor that affects the solubility of the biomaterials and their resorption. For example, previous studies have reported that the non stoichiometric apatites containing both CO_3^{2-} and HPO_4^{2-} ions are generally highly resorbable [8].

However, in spite of their chemical and crystal similarity to bone mineral, one of the major limitations of calcium phosphate bioceramics is their poor mechanical strength under complex stress states. As in the case of other ceramic materials, the tensile and compressive strengths of calcium phosphates are mainly determined by the presence of pores or interstices, which are generated during the sintering, when densification occurs. Calcium phosphate based ceramic powders exhibit a poor sinterability probably because of their low surface area (typical 2–5 m^2/g) and therefore they are mechanically weak. As a consequence of the poor mechanical strength, their applications are often restricted to low-loaded implants and coatings, or as fillers for other composite biomaterials [18].

Among different forms of calcium phosphates (reported in Table 2), tricalcium phosphate ($Ca_3(PO_4)_2$, TCP) and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) have been intensively investigated for bone tissue engineering applications due to their excellent biological responses and affinity with the physiological environment [19].

Apatites	Chemical formula	Ca/P
Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2 \cdot H_2O$	0.5
Monocalcium phosphate anhydrous	$Ca(H_2PO_4)_2$	0.5
Dicalcium phosphate dihydrate (Brushite)	$CaHPO_4 \cdot 2H_2O$	1
Dicalcium phosphate anhydrous (Monetite)	$CaHPO_4$	1
Octacalcium phosphate	$Ca_8(H_2PO_4)_2(PO_4)_4 \cdot 5H_2O$	1.33
Tricalcium phosphate	$Ca_3(PO_4)_2$	1.5
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Tetracalcium phosphate monoxide (Hilgenstockite)	$Ca_2(PO_4)_2O$	2

Table V - 2. Main apatites used in biological applications. Modified from [19].

The recent efforts in bioceramic research aim to overcome the limitations of calcium phosphates, in particular hydroxyapatite based ceramics, and improve their biological properties.

5.2.1.1 Hydroxyapatite

Hydroxyapatite has been largely used as orthopaedic and dental material for its biocompatibility and chemical similarity with the inorganic component of natural bone. Hydroxyapatite actively takes part in bone bonding, forming strong chemical bonds with the surrounding tissue and it was found to be a more efficient osteoblast attractant than other calcium phosphates. In addition, it is the most stable phase of the calcium phosphates, being thermodynamically stable at physiological pH [15].

However, despite its good biological properties and its chemical similarity with natural bone, the bone mineral present a higher bioactivity compared to synthetic hydroxyapatite. Furthermore, the mechanical properties of synthetic hydroxyapatite are very poor compared to those of bone [20].

At this regard, many studies have reported that the mechanical strength and fracture toughness of hydroxyapatite can be improved by using different sintering strategies such as the use of nanoscale powders, the addition of sintering additives to improve densification through grain boundary strengthening or the addition of a low melting secondary phase to achieve liquid phase sintering for better densification [21] [22].

For example, it was demonstrated that there is a significant improvement of the mechanical properties of hydroxyapatite by adding glass or other kind of sintering additives containing trace amounts of calcium oxide (CaO). In particular, the sintering of appropriate hydroxyapatite/bioactive glass mixtures results in bioceramics with improved mechanical and biological properties [23].

Synthetic pure hydroxyapatite has a Ca/P ratio of 1.67 and presents a hexagonal structure. Wet chemical methods and solid-state reactions are the two main methods for the preparation of hydroxyapatite powders. These techniques involve the acid-base titration or co-precipitation from aqueous reaction between orthophosphoric acid solution and calcium hydroxide dispersed in water [24]. Other reagents can be added during the synthesis to introduce minor elements in the hydroxyapatite lattice.

For example, carbonate ions can be incorporated into hydroxyapatite structure, yielding carbonated apatites which are materials of great interest for biomedical applications due to their similarity with bone apatite.

Hydroxyapatite can also be obtained from biological sources. For instance, corals and seashells naturally contain calcium carbonate, which can be converted into hydroxyapatite with a reaction with an appropriate phosphorus source [25].

During the synthesis process, in order to obtained single phase hydroxyapatite, the Ca/P ratio has to be equal to the stoichiometric value of hydroxyapatite (1.67); on the other side, if during the synthesis process the ratio is equal to 1.5, single phase TCP is formed. Using Ca/P ratio between these two values, bi-phasic materials of hydroxyapatite-TCP can be produced [26].

β-tricalcium phosphate (β-TCP), chemical formula $Ca_3(PO_4)_2$, has a Ca/P ratio of 1.5 and presents a pure hexagonal crystal structure, while the related α-TCP is monoclinic; β-TCP turns in α-TCP around 1200°C.

According to previous studies, the dissolution rate of hydroxyapatite after the implantation is too low to allow bone bonding, while the dissolution of β-TCP ceramics is too fast. Therefore, a promising strategy to achieve the optimum dissolution rate is the use of biphasic calcium phosphate ceramics composed of both, hydroxyapatite and TCP. In fact, in the case of a biphasic composition the resorbability is mainly determined by the hydroxyapatite/TCP ratio [27].

5.2.2 Bioglass®

Bioactive glasses are a group of inorganic materials with interesting properties for bone tissue engineering applications [28]. Bioactive glasses are generally fabricated using traditional melting techniques or and sol-gel methods [29][30].
A common feature of all bioactive glasses is their bioactivity. In fact, after the implantation, they are able to react and create strong bonds with bone, through the formation of a bone-like hydroxyapatite layer. This bonding to living tissues occurs upon a sequence of reactions on the materials surface which leads to an effective biological interaction and fixation between the bone tissue and material surface [17].
Moreover, in the case of silicate bioactive glasses (*e.g.*, 45S5 Bioglass®), in parallel to the bone bonding, the materials release critical concentrations of soluble Si^{+}, Ca^{2+}, Na^{+} and P^{3-} ions. This process promotes favourable intracellular and extracellular responses and consequently a rapid bone formation [31]. In fact, recent studies have demonstrated that the dissolution products from bioactive glasses up-regulate the expression of genes that control osteogenesis, enhancing the bone regeneration process [17]. In particular, Bioglass® 45S5 (containing 45% silicon dioxide by weight and incorporating 24.5 wt % Na_2O, 24.5 wt % CaO and 6 wt % P_2O_5 as network modifiers) is one of the most bioactive and has been shown to stimulate osteogenesis in vitro by inducing the proliferation and osteogenic differentiation of human osteoblasts [32]. In addition, previous studies have shown that when Bioglass® 45S5 is used as sintering aid or as second phase in combination with hydroxyapatite, it enhances the bioactivity of the material, creating new ceramic phases which increase apatite deposition and osteoblast proliferation and differentiation in vitro compared to pure hydroxyapatite [33].

5.3 Fundamental aspects driving the regeneration of connective hard tissues

In recent years, new regenerative approaches to treat orthopaedic diseases based on the use of biomimetic and bioactive scaffolds have been increasing investigated.
However, the regeneration of native biological tissues often requires scaffolds with a complex structure and composition. In fact, in order to promote the regeneration of the tissues, scaffolds must be able to origin a correct sequence of biological events

and, at the same time, provide adequate chemical-physical, structural and morphological signals to guide the cells to express specific phenotypes.

Tissues are characterized by a hierarchical organization at different scale levels. Their highly organized and multifunctional structures allow them to function with a minimal energy consumption and a perfect optimization of the available resources [34]. Native tissues spontaneously assemble to form these complex organized structures following a 'bottom-up' scheme, up to the macroscopic scale, through the exchange of information at molecular level. This information regulates the formation of a mineral phase in contact with an organic matrix, that also contributes to regulate the nucleation process. In particular, in the generation of hard tissues, such as bone and osteochondral tissue, the heterogeneous nucleation of nanonuclei in the mineral phase occurs simultaneously with the development of a well-organized organic template. This mechanism results in the formation of three-dimensional organic-inorganic composites, characterized by superior mechanical and physicochemical properties, which are often difficult to reproduce using synthetic materials and the current manufacturing technologies [35].

As previously described, the mineral phase of bone consists in a nanoscrystalline calcium-deficient hydroxyapatite. It has been demonstrated that the incorporation of several foreign ions in the crystal sites of calcium (*e.g.*, Mg^{2+} , K^{+} , Na^{+} , Sr^{2+}, Si^{2+}) and phosphate (*e.g.,* CO_3^{2-}, SiO_4^{4-}) are extremely important for the biochemistry of bone formation and remodelling. For example, the substitution of carbonate ions for phosphate ions is the major source of structural disorder in bone mineral and causes the increase of its reactivity [5]. Among the substituting cations, magnesium is involved in the early stages of bone formation. In fact, the presence of magnesium enhances the kinetics of hydroxyapatite nucleation on collagen fibres and, at the same time, regulates its crystallization [36]. Also silicon is essential for healthy skeletal and cartilage growth and helps the stabilization of bone matrix molecules [37]. In addition, the calcium-deficiency and the presence of foreign ions, result in the formation of nano-sized crystals with a poorly crystalline phase in comparison with hydroxyapatite synthetized by conventional wet methods. The small size and non-stoichiometry of the crystals determine a high bioavailability and solubility at physiological pH, promoting the process of bone resorption [38].

Therefore, due to the complexity and large numbers of aspects that are involved in the regeneration of hard connective tissues, an effective regenerative process requires scaffolds exhibiting chemical and structural similarities with natural bone. In this respect, the development of biomimetic mineralization processes and the use of naturally-derived biomaterials may result in the generation of smart devices with designed, tailored functionalities.

5.4 Marine organisms: source and model for bone repair and regeneration

The limitations of most of the current synthetic bone-substitutes ceramic materials (such as their low bioresorbability and mechanical properties) together with the complexity of the chemical, biological and mechanical interactions involved in tissues regeneration, make difficult the design of a bone substitute material able to emulate bone functions. In fact, as previously described, human bone consists of a organic component (mainly collagen type I) within an inorganic matrix (calcium phosphate). These two components are intimately mixed or composed at all different hierarchical levels, starting at the nanometric scale, to form a complex inorganic–organic nanocomposite material with unique physico-chemical and mechanical properties [39]. However, this template is not unique in the animal kingdom. For example, in the marine environment, there are several marine organisms that use similar strategies to develop mineralized skeletons for support and function [40].

For these reasons, as a rich source of chemical diversity and organisms with unique bone-like structures, marine environment could be considered a viable resource for bone tissue engineering, particularly in the supply of biomimetic templates or materials that could be useful for bone repair.

In fact, even though some of the current synthetic bone substitutes (such as calcium phosphates) exhibit good bioactive properties and chemical similarity to the mineral phase of bone, these materials often present low mechanical strengths and are difficult to resorb for the body.

Recently, different marine derived and inspired bioceramics have been investigated showing good potential for bone tissue engineering applications, offering the desiderable physiochemical characteristics for bone repair [40].

There are different reasons why marine organisms could help to support the bone regeneration.

Firstly, marine organisms produce several kinds of minerals (Table 2), specially silica based and calcium carbonate (in the form of calcite or aragonite). For these reasons, they can be considered either as a precious resource of scaffolds materials or as a model for the study of the mineralization processes, in order to understand the mechanisms that allow mineral production at relative mild environmental conditions (at ambient temperatures and alkaline aqueous conditions) [40].

Phylum	Class	Common name	Skeletal mineral
Heterokontophyta	Bacillariophyta	Diatoms	Silica
Haptophyta	Prymnesiophyceae	Coccolithophorids	$CaCO_3$
Radiolaria	Polycystina	Radiolarians	silica
Foraminifera	Granuloreticulosa	Foraminiferans	$CaCO_3$
Rhodophyta	Florideophyceae	**Red algae**	$CaCO_3$/calcite
Ectoprocta	Stenolaemata	Bryozoans	$CaCO_3$
Porifera	Demospongia	**Sponges**	$CaCO_3$ and/or silica
Cnidarians	Anthozoa	**Corals**	$CaCO_3$/aragonite
Mollusca	Gastropoda	**Snails**, limpets	$CaCO_3$/aragonite
	Bivalvia	**Clams, mussels**	$CaCO_3$/aragonite
	Cephalopods	Squid, **cuttlefish**	$CaCO_3$/aragonite
Arthropoda	Crustacea	**Lobster, crab**, shrimp	$CaCO_3$/aragonite
Brachiopoda	e.g. Lingulata	Lampshells	$CaCO_3$ or CaPO4
Echinodermata	Asteroidea	**Starfish**	$CaCO_3$/calcite
	Echinoidea	**Sea urchins**	$CaCO_3$/calcite
	Ophiuroidea	Brittle stars	$CaCO_3$/calcite
	Holothuroidea	Sea Cucumbers	$CaCO_3$

Table V - 3. Examples of mineralizing marine organisms. Those that have already been investigated for bone tissue engineering are highlighted in bold. From [41].

Another reason why marine organisms could be useful for bone repair, is that many species represent a natural reserve of porous materials, with a wide pores size distribution [42]. It is well known that interconnectivity, pores size distribution and tortuosity that are naturally present in biological systems are difficult to replicate synthetically. For these reasons, marine organisms can be used as templates for materials design. For instance, one of the main limitations in the use of synthetic porous ceramic materials as bone substitutes is related to their low mechanical strength [43]. A possible strategy to overcome this limitation could be to mimic the complex natural structures of some marine organisms.

At this regard, nacre represents one of the naturally occurring biocomposites that has been largely investigated for the development of biomedical materials. Nacre is produced by molluscs and is characterized by a lamellar structure, consisting of alternating layers of aragonite and organic interlamellar membranes (made of chitin surrounded by acidic proteins) [44]. Nacre has found possible applications in the biomedical industry due to the superior biomechanical properties. In addition, its complex structure may offer the inspiration for novel design strategies for the development of advanced biomaterials [45].

As alternative, marine organisms can be used directly as bone substitute materials. For, example, the coralline porous structures produced by certain species of sea corals (such as Porite) are similar to human cancellous bone and have been already evaluated as bone graft substitutes. It was been demonstrated that the architectural properties of natural corals allow the bone regeneration process and that, after implantation, they are gradually reabsorbed and replaced by the newly formed tissue [46]. In addition, the exoskeleton of coral that is rich in calcium carbonate, has been shown to be biocompatible, bioactive, osteoconductive and biodegradable at variable rates depending on the species, porosity and site of implantation [47]. Another area

of great interest concerns the improvement of the calcium phosphate bone substitute materials. As previously discussed, the mineral phase of bone is composed by a non-stoichiometric hydroxyapatite that incorporates several minor ions (*e.g.*, CO_3^{2-}, SiO_4^{4-}, Mg^{2+}, K^+, Na^+, Sr^{2+}) which are of fundamental importance for the biochemistry of bone formation and remodelling. Therefore, ions substitution is considered as an additional value to the base ceramic scaffolds material and marine organisms are a rich natural source of these ions [40]. Hence, the direct use of the organisms structures as scaffolds can make these ions available in the form of their base mineral or, as alternative, these ions can be incorporated into a biomaterial after the conversion of the base mineral into calcium phosphates. Using both these approaches, it is possible to obtain biomaterials which naturally incorporate ion substitutions, using relatively simple manufacturing techniques compared to some manufacturing processes currently required to produce ion-substituted ceramics. For example, previous studies have investigated the hydrothermal conversion of the natural aragonite structure of cuttlefish bone into carbonated hydroxyapatite [48][49]. The obtained material has proven to be closer to the chemistry of natural bone than stoichiometrically pure hydroxyapatite and has been shown experimentally to have enhanced biological properties and bioactivity [42].

We have seen above that marine organisms could be useful for bone repair for several reasons, ranging from supplying bioactive mineral compounds or porous scaffolds materials, trough the study of the biomineralization process and the architectural design of some marine species, to the conversion of natural mineralized structures for the production of 'bone-like' ceramics. For these reasons, as a rich source of mineralising porous organisms, the marine environment could provide new directions for bone tissue engineering and provide several strategies for the development of advanced bioceramics materials.

References

[1] S. Weiner and H. D. Wagner, “The material bone: structure-mechanical function relations,” *Annu. Rev. Mater. Sci.*, vol. 28, no. 1, pp. 271–298, 1998.

[2] J.-Y. Rho, L. Kuhn-Spearing, and P. Zioupos, “Mechanical properties and the hierarchical structure of bone,” *Med. Eng. Phys.*, vol. 20, no. 2, pp. 92–102, 1998.

[3] K. Rezwan, Q. Z. Chen, J. J. Blaker, and A. Roberto, “Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering,” vol. 27, pp. 3413–3431, 2006.

[4] C. Rey, C. Combes, C. Drouet, and M. J. Glimcher, “Bone mineral: update on chemical composition and structure,” *Osteoporos. Int.*, vol. 20, no. 6, pp. 1013–1021, 2009.

[5] A. Tampieri, S. Sprio, M. Sandri, and F. Valentini, “Mimicking natural biomineralization process: A new tool for osteochondral scaffold development,” *Trends. Biotechnol.*, vol. 29, no. 10, pp. 526–535, 2011.

[6] S. C. Marks and S. N. Popoff, “Bone cell biology: the regulation of development, structure, and function in the skeleton,” *Am. J. Anat.*, vol. 183, no. 1, pp. 1–44, 1988.

[7] A. J. Salgado, O. P. Coutinho, and R. L. Reis, “Bone tissue engineering: state of the art and future trends,” *Macromol. Biosci.*, vol. 4, no. 8, pp. 743–765, 2004.

[8] T. Thamaraiselvi and S. Rajeswari, “Biological evaluation of bioceramic materials-a review,” *Carbon N. Y.*, vol. 24, no. 31, p. 172, 2004.

[9] S. M. Best, A. E. Porter, E. S. Thian, and J. Huang, “Bioceramics: past, present and for the future,” *J. Eur. Ceram. Soc.*, vol. 28, no. 7, pp. 1319–1327, 2008.

[10] A. Oryan, S. Alidadi, A. Moshiri, and N. Maffulli, “Bone regenerative medicine: classic options, novel strategies, and future directions,” *J. Orthop. Surg. Res.*, vol. 9, no. 1, p. 1, 2014.

[11] G. Khang, *Handbook of Intelligent Scaffold for Tissue Engineering and Regenerative Medicine*. CRC Press, 2012.

[12] B. D. Boyan, C. H. Lohmann, D. D. Dean, V. L. Sylvia, D. L. Cochran, and Z. Schwartz, “Mechanisms involved in osteoblast response to implant surface morphology,” *Annu. Rev. Mater. Res.*, vol. 31, no. 1, pp. 357–371, 2001.

[13] D. W. Hutmacher, J. T. Schantz, C. X. F. Lam, K. C. Tan, and T. C. Lim, “State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective,” *J. Tissue Eng. Regen. Med.*, vol. 1, no. 4, pp. 245–260, 2007.

[14] D. W. Hutmacher, “Scaffolds in tissue engineering bone and cartilage,” *Biomaterials*, vol. 21, no. 24, pp. 2529–2543, 2000.

[15] M. Vallet-Regí, “Revisiting ceramics for medical applications,” *Dalt. Trans.*, no. 44, pp. 5211–5220, 2006.

[16] L. L. Hench and J. Wilson, *An introduction to bioceramics*, vol. 1. World Scientific, 1993.

[17] L. L. Hench, “Bioceramics: from concept to clinic,” *J. Am. Ceram. Soc.*, vol. 74, no. 7, pp. 1487–1510, 1991.

[18] S. J. Kalita, A. Bhardwaj, and H. A. Bhatt, “Nanocrystalline calcium phosphate ceramics in biomedical engineering,” *Mater. Sci. Eng. C*, vol. 27, no. 3, pp. 441–449, 2007.

[19] K. K. Mallick and J. Winnett, “3D bioceramic foams for bone tissue engineering,” *Bone Substit. Biomater.*, p. 118, 2014.

[20] W. R. Moore, S. E. Graves, and G. I. Bain, “Synthetic bone graft

substitutes," *ANZ J. Surg.*, vol. 71, no. 6, pp. 354–361, 2001.

[21] G. Georgiou and J. C. Knowles, "Glass reinforced hydroxyapatite for hard tissue surgery-Part 1: mechanical properties," *Biomaterials*, vol. 22, no. 20, pp. 2811–2815, 2001.

[22] J. D. Santos, P. L. Silva, J. C. Knowles, S. Talal, and F. J. Monteiro, "Reinforcement of hydroxyapatite by adding P_2O_5-CaO glasses with Na_2O, K_2O and MgO," *J. Mater. Sci. Mater. Med.*, vol. 7, no. 3, pp. 187–189, 1996.

[23] F. Lin, C. Lint, H. Liut, Y. Huang, C. Wang, and C. Lu, "Sintered porous DP-bioactive glass and hydroxyapatite as bone substitute," *Biomaterials*, vol. 15, no. 13, pp. 187–189, 1994.

[24] N. Y. Mostafa, "Characterization, thermal stability and sintering of hydroxyapatite powders prepared by different routes," *Mater. Chem. Phys.*, vol. 94, no. 2, pp. 333–341, 2005.

[25] J. Hu, J. J. Russell, B. Ben-Nissan, and R. Vago, "Production and analysis of hydroxyapatite from Australian corals via hydrothermal process," *J. Mater. Sci. Lett.*, vol. 20, no. 1, pp. 85–87, 2001.

[26] S. Kannan, J. H. G. Rocha, J. M. G. Ventura, A. F. Lemos, and J. M. F. Ferreira, "Effect of Ca/P ratio of precursors on the formation of different calcium apatitic ceramics-an X-ray diffraction study," *Scr. Mater.*, vol. 53, no. 11, pp. 1259–1262, 2005.

[27] S. H. Kwon, Y. K. Jun, S. H. Hong, and H. E. Kim, "Synthesis and dissolution behavior of β-TCP and HA/β-TCP composite powders," *J. Eur. Ceram. Soc.*, vol. 23, no. 7, pp. 1039–1045, 2003.

[28] A. R. Boccaccini and P. X. Ma, *Tissue engineering using ceramics and polymers*. Elsevier, 2014.

[29] L. L. Hench and J. M. Polak, "Third-generation biomedical materials," *Science*, vol. 295, no. 5557, pp. 1014–1017, 2002.

[30] R. Gupta and A. Kumar, "Bioactive materials for biomedical applications using sol-gel technology," *Biomed. Mater.*, vol. 3, no. 3, p. 34005, 2008.

[31] L. Gerhardt and A. R. Boccaccini, "Bioactive Glass and Glass-Ceramic Scaffolds for Bone Tissue Engineering," *Materials*, vol. 3, no. 7, pp. 3867–3910, 2010.

[32] I. D. Xynos, A. J. Edgar, L. D. K. Buttery, L. L. Hench, and J. M. Polak, "Gene-expression profiling of human osteoblasts following treatment with the ionic products of Bioglass® 45S5 dissolution," *J. Biomed. Mater. Res.*, vol. 55, no. 2, pp. 151–157, 2001.

[33] H. Demirkiran, A. Mohandas, M. Dohi, A. Fuentes, K. Nguyen, and P. Aswath, "Bioactivity and mineralization of hydroxyapatite with bioglass as sintering aid and bioceramics with $Na_3Ca_6(PO_4)_5$ and $Ca_5(PO_4)_2SiO_4$ in a silicate matrix," *Mater. Sci. Eng. C*, vol. 30, no. 2, pp. 263–272, 2010.

[34] P. Fratzl and R. Weinkamer, “Nature’s hierarchical materials,” *Prog. Mater. Sci.*, vol. 52, no. 8, pp. 1263–1334, 2007.

[35] S. Weiner, “Organization of Organic Matrix Components in Mineralized Tissues,” *Am. Zool.,* vol. 24, no. 4, pp. 945–951, 1984.

[36] A. Bigi, E. Foresti, R. Gregorini, A. Ripamonti, N. Roveri, and J. S. Shah, “The role of magnesium on the structure of biological apatites,” *Calcif. Tissue Int.*, vol. 50, no. 5, pp. 439–444, 1992.

[37] S. R. Kim, J. H. Lee, Y. T. Kim, D. H. Riu, S. J. Jung, Y. J. Lee, S. C. Chung, and Y. H. Kim, “Synthesis of Si, Mg substituted hydroxyapatites and their sintering behaviors,” *Biomaterials*, vol. 24, no. 8, pp. 1389–1398, 2003.

[38] M. Vallet-Regi and J. M. González-Calbet, “Calcium phosphates as substitution of bone tissues,” *Prog. Solid State Chem.*, vol. 32, no. 1, pp. 1–31, 2004.

[39] S. Weiner and L. Addadi, “Design strategies in mineralized biological materials,” *J. Mater. Chem.*, vol. 7, no. 5, pp. 689–702, 1997.

[40] S. A. Clarke and P. Walsh, “Marine organisms for bone repair and regeneration,” *Bone Substit. Biomater.*, pp. 294–318, 2014.

[41] S. A. Clarke, P. Walsh, C. A. Maggs, and F. Buchanan, “Designs from the deep : Marine organisms for bone tissue engineering,” *Biotechnol. Adv.*, vol. 29, no. 6, pp. 610–617, 2011.

[42] H. Ivankovic, G. G. Ferrer, E. Tkalcec, S. Orlic, and M. Ivankovic, “Preparation of highly porous hydroxyapatite from cuttlefish bone,” *J. Mater. Sci. Mater. Med.*, vol. 20, no. 5, pp. 1039–1046, 2009.

[43] F. Zhang, J. Chang, J. Lu, K. Lin, and C. Ning, “Bioinspired structure of bioceramics for bone regeneration in load-bearing sites,” *Acta Biomater.*, vol. 3, no. 6, pp. 896–904, 2007.

[44] Y. Levi-Kalisman, G. Falini, L. Addadi, and S. Weiner, “Structure of the nacreous organic matrix of a bivalve mollusk shell examined in the hydrated state using cryo-TEM,” *J. Struct. Biol.*, vol. 135, no. 1, pp. 8–17, 2001.

[45] A. G. Checa, J. H. E. Cartwright, and M.-G. Willinger, “The key role of the surface membrane in why gastropod nacre grows in towers,” *Proc. Natl. Acad. Sci.*, vol. 106, no. 1, pp. 38–43, 2009.

[46] Y. Barbotteau, J. L. Irigaray, and J. F. Mathiot, “Modelling by percolation theory of the behaviour of natural coral used as bone substitute,” *Phys. Med. Biol.*, vol. 48, no. 21, p. 3611, 2003.

[47] G. Guillemin, J.-L. Patat, J. Fournie, and M. Chetail, “The use of coral as a bone graft substitute,” *J. Biomed. Mater. Res.*, vol. 21, no. 5, pp. 557–567, 1987.

[48] J. H. G. Rocha, A. F. Lemos, S. Kannan, S. Agathopoulos, and J. M. F.

Ferreira, “Hydroxyapatite scaffolds hydrothermally grown from aragonitic cuttlefish bones,” *J. Mater. Chem.*, vol. 15, no. 47, pp. 5007–5011, 2005.

[49] E. Battistella, S. Mele, I. Foltran, I. G. Lesci, N. Roveri, P. Sabatino, and L. Rimondini, “Cuttlefish bone scaffold for tissue engineering: a novel hydrothermal transformation, chemical-physical, and biological characterization,” *J Appl Biomater Funct Mater*, vol. 10, no. 2, pp. 99–106, 2012.

Chapter VI

Production and evaluation of naturally-derived hydroxyapatite/Bioglass® co-sintered bioceramics

6.1 Introduction

Several bioactive ceramics have been proven to be attractive candidates as scaffold materials for bone tissue engineering [1]. In particular, among largely used bioceramics, calcium phosphates based materials are preferred as bone graft materials due to their biocompatibility and chemical similarity to the inorganic matrix of natural bone [2]. Different phases and form of calcium phosphates (including HAP, β-TCP, α-TCP, biphasic calcium phosphate, monocalcium phosphate monohydrate and unsintered apatite) are currently used in the biomedical industry depending on the physical properties required for the specific application [3].

Hydroxyapatite (chemical formula $Ca_{10}(PO_4)_6(OH)_2$) is an ideal phase for applications in human body and has been widely studied for restoration of damaged hard tissue due to its osteoconductivity, bioactivity and chemical stability under physiological pH [4][5]. However, despite the chemical similarity with the mineralized bone of human tissue, the biological properties of synthetic hydroxyapatite significantly differ to those of natural bone. In fact, the mineral phase of bone has a complex composition and besides calcium phosphate phases, contains carbonate ions, magnesium, sodium, hydrogenophosphate ions and several other trace elements [6]. Therefore, the biocompatibility of apatites is strongly related to their composition and, in particular, the presence of various trace elements plays a crucial role in the overall physiological functioning and in the osseointegration process [7].

In recent years, various natural sources and bio-waste of calcium (including cuttlefish, corals and nacres) are receiving increasing interest because of the possibility to convert their calcium carbonate structures in hydroxyapatite via a hydrothermal reaction [8][9]. Among sea-derived fish skeletons, cuttlefish bone (CF_b), due to its structural resemblance to human bone, has been proposed as a suitable natural source of calcium phosphate. In fact, CF_b is a worldwide available and very low cost material of natural–biological origin that presents a mineral composition compatible with human bone tissue [10][6]. Previous studies have demonstrated that is possible to convert the calcium carbonate structure of CF_b into hydroxyapatite by using an hydrothermal transformation [11][9].

Hydroxyapatite obtained from natural sources is non-stoichiometric and can incorporate other ions, for example CO_3^{2-}, traces of Fe^{2+}, Na^+, Mg^{2+}, F^- and Cl^- [4]. For these reasons is more similar to the chemistry of the natural bone than the

stoichiometrically pure hydroxyapatite produced by synthetic methods [5] and has been proved to have enhance biocompatibility [6]. However, the major limitation of pure hydroxyapatite are the poor mechanical properties, that have restricted its applications to low-loaded porous implants and coatings, or as a filler for other composite biomaterials. In order to overcome these disadvantages hydroxyapatite has been combined with various ceramic, polymer or metallic reinforcements [12]. In addition, the conventional calcium phosphate based ceramic powders are difficult to sinter, probably in relation to their low surface area (typical 2–5 m^2/g) [13]. For these reasons, a strategy to improve the mechanical performance of synthetic hydroxyapatite is the addition of a secondary phase as sintering aid to increase the densification [14]. For example, it was demonstrated that there is a significant improvement of the mechanical properties of hydroxyapatite by adding glass or other kind of sintering additives containing trace amounts of calcium oxide (CaO). Previous studies have reported that the sintering of appropriate hydroxyapatite/bioactive glass mixtures results in bioceramics with improved mechanical and biological properties [15]. However, the reaction between hydroxyapatite and bioactive glasses depends on the time and temperature of the sintering process, but also on glass composition and each system requires in depth independent study.

Among bioactive glasses, Bioglass®-45S5 (consisting of 45 wt.% SiO_2, 24.5 wt.% Na_2O, 24.5 wt.% CaO, and 6 wt.% P_2O_5) is one of the most bioactive and is used in conjunction with hydroxyapatite either as a sintering aid or second phase [16]. Previous studies have shown that when Bioglass®-45S5 is combined with hydroxyapatite it enhances the bioactivity of the material, creating new ceramic phases, that increase apatite deposition and osteoblast proliferation and differentiation in vitro compared to pure hydroxyapatite [14].

In the present study, for the first time, calcium phosphate powders are synthesized starting from cuttlefish bone powder and at the same time co-sintered with Bioglass®-45S5 at 900°C, in order to develop highly bioactive ceramics compositions that present enhanced bioactivity, osteoconductivity and improved mechanical properties. Structure and composition of the new chemistry were examined using Fourier transform infrared spectroscopy, X-ray diffraction and scanning electron microscopy. Mechanical properties were evaluated using compression tests. In addition, human osteoblast-like cells (MG63) were cultured on these substrates and the cell activity and proliferation, after 1, 3 and 7 days cell culture, were investigated using PicoGreen® DNA quantification, Alamar Blue® and alkaline phosphate activity assays.

6.2 Materials

Synthetic hydroxyapatite powder (≥ 97% wt.%, particle size < 44 μm) with chemical composition $Ca_{10}(PO_4)_6OH_2$ was acquired from Sigma-Aldrich (St. Louis, MO, USA).

Bioglass®-45S5 powder, with particle size < 90 μm, consisting of 45 wt.% SiO_2, 6 wt.% P_2O_5, 14.5 wt.% Na_2O, and 24.5 wt.% CaO was purchased from US Biomaterials Corporation (Alachua, FL, USA).
All chemicals and reagents used were of analytical grade and were used without further modifications.

6.2.1 Cuttlefish powder preparation

Native cuttlefish bones of *S. officinalis* from the Adriatic Sea were cut in small pieces and treated at 300 °C for 3 h, to remove the organic component. For the thermal treatment only the internal lamellae part of the bone was used, since the aragonite in external shell during the pre-treatment at 300 °C could partially transform into calcite, which is more difficult to convert into hydroxyapatite [17]. After the thermal treatment bones were ground into a fine powder (particle size < 100 μm). In order to synthesize calcium phosphate powders, cuttlefish bone powders were ball-milled in 250 ml polyethylene bottles with zirconia (Y-TZP) ball media under an aqueous solution of $NH_4H_2PO_4$ (Ca/P ratio = 1.67) for 48 h, to break up agglomerates and achieve homogeneous mixing. After ball milling, the powder was treated in an air atmosphere at 900°C. In the preliminary stage, in order to optimize the process parameters, different durations of the thermal treatment were tested and the phase change after the treatment was examined by FT-IR analysis.

6.2.2 Production of the samples

Four different groups of samples were produced, using commercial hydroxyapatite powder (samples: HAP_st, HAP_30B) and cuttlefish bone powder (samples: CF_p, CF_30B). The formulations of the samples are summarized in Table 1.

Samples	Composition	Bioglass®45S5
HAP_st	commercial hydroxyapatite	/
HAP_30B	commercial hydroxyapatite	30 wt.%
CF_p	cuttlefish bone	/
CF_30B	cuttlefish bone	30 wt.%

Table VI - 4. Formulation of the samples.

Briefly, for the samples HAP_st and CF_p, hydroxyapatite and cuttlefish powders respectively, were ball milled with 0.6 M aqueous solution of $NH_4H_2PO_4$ (Ca/P = 1.67) in 250 ml polyethylene bottles, for 48h. After ball milling, the mixtures were pressed manually in a die with a diameter of 6.7 mm. For the formulation of samples HAP_30B and CF_30B, hydroxyapatite and cuttlefish powders were ball milled with

0.6 M aqueous solution of $NH_4H_2PO_4$ (Ca/P = 1.67). After 24 h a proper amount of Bioglass®45S5 powders (30 wt.%) was added and the mixtures were ball milled for other 48 h. All the samples were sintered at 900°C for 3 h with a heating rate of 10°C/min. The thermal treatment was followed by cooling at 10°C/min down to room temperature.

6.4 Materials characterization

6.4.1 Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) FT-IR spectra of sintered bioceramic mixtures were collected using Spectrum One spectrometer with ATR correction (Perking Elmer, Waltham, MA, USA) with Zinc Selenide crystal. Sample spectra were averaged over 4 scans, ranging from 600 to 3000 cm^{-1} at a resolution of 4 cm^{-1}.

6.4.2 X-ray diffraction (XRD) analyses

X-ray diffraction studies of sintered bioceramic mixtures were examined using a Bruker D8 Advance X-ray diffractometer using CuK_α radiation. The data were recorded over the 2θ range of 20-60° with a 0.04° step size and a dwell time of 1 s.

6.4.3 Scanning electron microscopy (SEM)

The microstructures in the bioceramic samples after sintering and subsequent cell culture studies were investigated using Hitachi S-300N VP-SEM and Supra 40 Zeiss scanning electron microscopes operated in high vacuum and secondary electron mode.

6.4.4 Compression test

The compression test were performed with a Bose AT 3300 servo electric material test machine. Four samples for each bioceramic composition were tested. The cylindrical samples were prepared according to ASTM C773-88 (2006) Standards with approximately 2 mean height/diameter ratio (height 12 mm and diameter 6 mm). The tests were run at a crosshead speed of 1 mm/min. Compressive strength of the samples was measured reporting load to failure divided by the cross-sectional area of the specimens.

6.5 Bioactivity (in vitro) characterization

6.5.1 Cell culture

MG63 cells (human osteosarcoma cells line) were used to evaluate the biocompatibility of the different bioceramics formulations. MG63 cells were expanded and cultured at 37ºC with 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) (Euroclone, Milan, Italy) containing 10% fetal bovine serum (Gibco, NY, USA), supplemented with 1mM sodium pyruvate, 2 mM L-glutamine, 0.1% antibiotics (Gibco, Eggenstein, Germany). Culture medium was changed every 2 days until cells reached confluency, then cells were detached with 0.1% trypsin and re-suspended in culture medium. Each samples for cell tests were sterilized in 70% ethanol for 3 hours and pre-conditioned with DMEM for 2 hours before seeding. Later, MG63 cells were seeded at a density of 7×10^3 cells per well on all the scaffolds, in individual wells of a polystyrene 96-well plate, and incubated under standard culture conditions.

6.5.2 Cell proliferation by DNA quantification

A PicoGreen® DNA quantification assay (Quant-iT PicoGreen® dsDNA Assay, InvitrogenTM, Carlsbad, USA) was used to quantify the amount of total DNA, that is correlated to the number of cells grown on the scaffolds. At predetermined time points (1, 3 and 7 days), culture medium was removed and the scaffolds were washed with PBS. The scaffolds were covered with 300 µL of 0.05% Triton-X in PBS, and the supernatants were collected and stored at -20°C until analysis. Later, samples were thawed at room temperature, transferred to a 1.5 mL tube and sonicated for 10 seconds with a Hielscher ultrasonic homogenizer (UP400S, 400 watt-24 kHz, amplitude 50%, from Hielscher Ultrasonics, Teltow, Germany). Extracts of 100 µL were subsequently placed in a black 96-weel plate, and mixed with 100 µL of PicoGreen® working solution, prepared following the manufacturer's instructions. Five independent samples were analysed for each experimental condition. Fluorescence intensity was measured with a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) using excitation wavelength 485 nm and emission wavelength 535 nm. Fluorescence measurements were taken in triplicate. A calibration curve was created using a double-stranded DNA standard provided by the kit and was used for the calculation of the DNA content. Finally, the approximate number of cells per sample was determined from DNA content by the conversion factor of 7.7 pg DNA per cell.

6.5.3 Cell morphological characterization by CLSM

After 1, 3 and 7 days of culture, the cells were fixed and permeabilized with 4% paraformaldehyde solution in PBS with 0.2% Triton X-100. Cells were then labelled with Rhodamine phalloidin (Molecular Probes®, Life Technologies, Monza, Italy) for the visualization of filamentous actin (F-actin) and 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI, Molecular Probes®) to stain the nuclei. The morphology of the cells adhered at the scaffold surfaces was observed by Nikon A1 confocal laser microscopy.

6.5.4 Cell metabolic activity (Alamar blue assay)

Cells viability after 1, 3 and 7 days of culture was determined using AlamarBlue® assay (InvitrogenTM, Carlsbad, USA), following the manufacturer's instructions. The AlamarBlue® dye is a low toxic redox indicator that causes a colorimetric change and a fluorescent signal in relation at the metabolic activity of the cells. In fact, when cells are alive they maintain a reducing environment in the surrounding culture medium while non-viable cells, that have an innate lower metabolic activity, generate a proportionally lower signal. AlamarBlue® reagent was added directly to each well as 10% of the sample volume. Then, the plates were incubated at 37°C in a humidified atmosphere at 5% CO_2 to allow cells to convert resazurin to resorufin. After 2 hours, 100 µL of solution for each samples were collected and the fluorescence signal was measured with a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland), using absorption at 560 nm and emission at 590 nm. Finally, results were analysed by plotting fluorescence intensity versus compound concentration.

6.5.5 Alkaline phosphatase activity

The functional activity of the proliferated cells was examined by measuring the alkaline phosphatase (ALP) activity expressed by the cells. At each culturing period (1, 3 and 7 days), the scaffolds were washed with PBS, followed by adding 300 µL of cell lysis buffer containing 0.2% Triton X-100 to the samples. Then, the supernatants were collected and frozen to - 20 °C until analysis. Later, the frozen samples were thawed at room temperature and the ALP activity was measured following the manufacturer's instructions (ALP fluorometric kit (ab83371), Abcam, Cambridge, UK). Fluorescence intensity was measured with a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) using excitation wavelength 360 and emission wavelength 440 nm. Five independent samples were analyzed for each experimental condition. The ALP activity was calculated from a standard curve which was generated using the reagents provided by the commercial kit.

6.5.6 Statistics

The *in vitro* MG63 cell proliferation and differentiation tests were performed on triplicate samples for each group of bioceramic compositions. All quantitative data were expressed as mean ± Standard Deviation (SD). Statistical analyses were performed using GraphPad Prism 5. A p value < 0.05 was considered statistically significant.

6.6 Results

6.6.1 Physical and chemical characterization

A scanning electron micrograph of the inner structure of the cuttlefish bone is reported in Fig. 1(b). Cuttlefish bone presented a unique microstructure characterized by layered regular sheets, with lamellar spacing ranging from 200 to 600 µm.

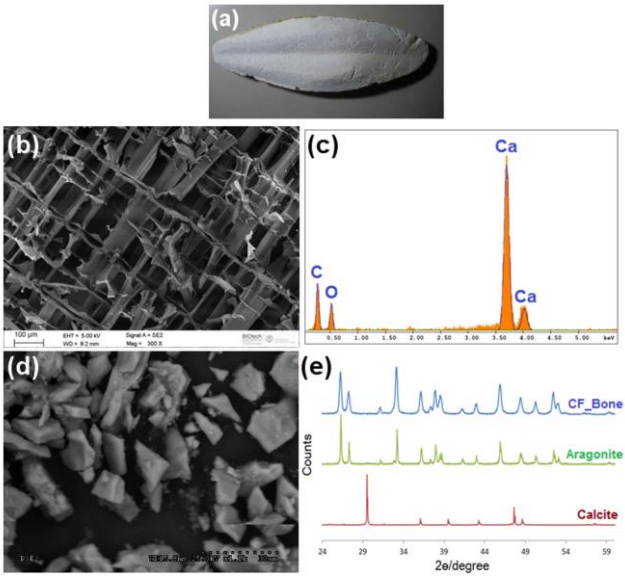


Figure VI - 5. ***(a)*** *Cuttlefish bone before processing;* ***(b)*** *SEM image of the lamellar porous structure of cuttlefish bone;* ***(d)*** *SEM image of the powder obtained after the grounding process of the cuttlefish bone;* ***(c)*** *EDS spectrum of cuttlefish bone powder;* ***(e)*** *XRD analysis of cuttlefish bone. The figure reports the spectrum of the cuttlefish bone powder in comparison with the spectra of pure aragonite and calcite.*

Pieces of about 1 x 1 x 2 cm^3 were cut from the internal bone matrix, treated at 300°C to remove the organic components, and mechanically fragmented. The powder obtained after the ball milling of the cuttlefish bone consisted of flake-like particles, with irregular shape and size ranging from 10 to 20 μm (Fig. 1(d)).

The EDS spectrum and XRD analysis of the cuttlefish bone powder (Fig. 1 (c) and (e)) confirmed the inorganic component of the starting material as crystalline $CaCO_3$ in the form of pure aragonite [10]. In particular, the XRD analysis clearly demonstrated that the relative intensity of the peaks of cuttlefish bone powder matches with the standard aragonite, while significantly differs from the standard calcite, plotted at the bottom side of the diagram. These results proved that the lamellae matrix of cuttlefish bone retained the aragonitic structure after the pre-treatment at 300°C.

The aragonitic structure of cuttlefish bone powder was converted in HAP after 90 minutes of thermal treatment at 900°C. The conversion of aragonite into hydroxyapatite powder was followed by FT-IR spectroscopy (Fig.2 (a)) and XRD analysis (Fig.2 (b)).

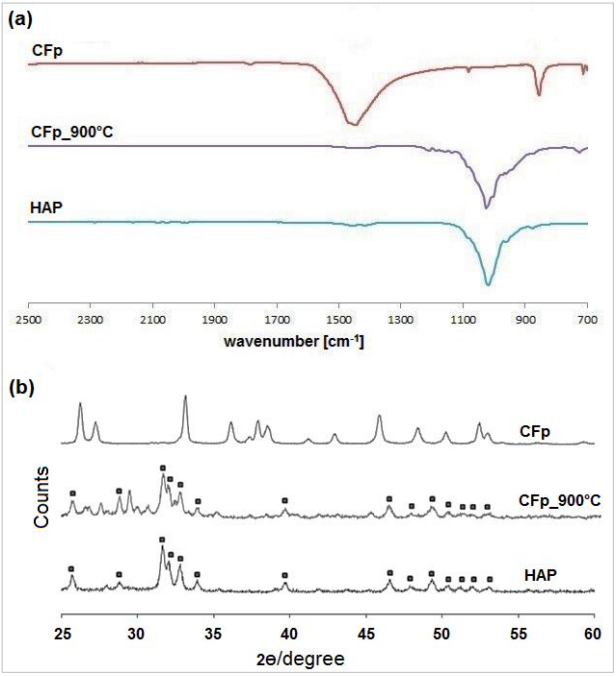


Figure VI - 2. *FT-IR (a) and XRD (b) spectra of cuttlefish bone powder before and after 90 minutes of thermal treatment at 900°C in comparison with the spectrum of synthetic hydroxyapatite powder (HAP).*

The results clearly showed that the characteristic bands of the functional groups of hydroxyapatite phase are absent in the FT-IR and XRD spectra of cuttlefish powder. After thermal treatment, the FT-IR of the powder presented the characteristic peak of hydroxyapatite at around 1021 cm-1, assigned to the vibrations of phosphate groups [18]. The characteristic peaks of hydroxyapatite can be detected also in the XRD spectra of the powder after the thermal treatment. In particular the three peaks of HAP at 2θ = 25.8° (0 0 2), 31.7° (2 1 1), and 32.9° (3 0 0) are present [19].

Fig. 3 shows the XRD patterns of the samples produced using commercial hydroxyapatite and cuttlefish bone powders, containing 30 wt.% Bioglass®45S5, after the thermal treatment at 900°C for a period of 3 hours.

When 30 wt.% Bioglass®45S5 is added to the cuttlefish powders (samples CF_30B), the phases detected after the thermal treatment at 900°C are hydroxyapatite, sodium calcium phosphate ($Na_3Ca_6(PO_4)_5$, JCPDS#40-0393) and β-TCP ($Ca_3(PO_4)$, JCPDS#09-0169). In this case there is no evidence of any crystalline silicate phases. However, when 30 wt.% Bioglass®45S5 is added to the commercial synthetic hydroxyapatite powders and treated at 900°C (samples HAP_30B) the main phases present in the product seem to be hydroxyapatite and $Na_2Ca_2Si_2O_7$ (JCPDS#10-0016).

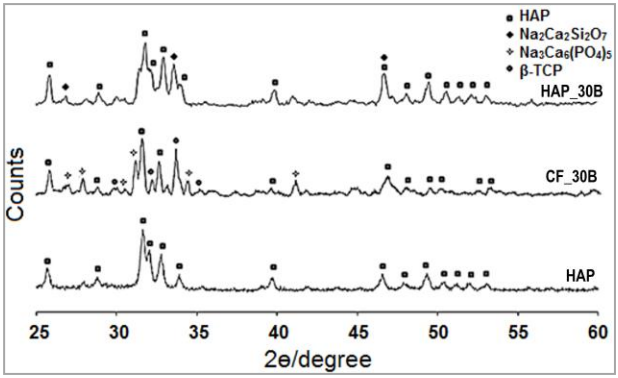


Figure VI - 3. XRD spectra of synthetic hydroxyapatite/Bioglass®45S5 (samples HAP_30B) and cuttlefish bone powder/Bioglass®45S5 (samples CF_30B) after 3 hours of thermal treatment at 900°C in comparison with the spectrum of synthetic hydroxyapatite powder (HAP).

The EDX element mapping analysis of the samples, after the thermal treatment at 900°C, are reported in Fig. 4. The Grey images in the figure are the base image of the local regions used for the EDX mapping of the samples, while the colored images show the corresponding O, Ca, P, Na and Si element mapping in the same regions. The images clearly show that, in both samples, O, Ca and P are evenly distributed throughout the entire region, while silicon is localized in specific areas of the surface. However, is interesting to note that, in the samples CF_30B, silicon is

localized in Ca deficient areas of the surface and it seems not combined with other elements to form new phases after the treatment at 900°C. However, the element mapping of the samples HAP_30B showed that, in this case, silicon is combined with Ca and Na, suggesting the presence of calcium silicate compounds in some areas of the surface.

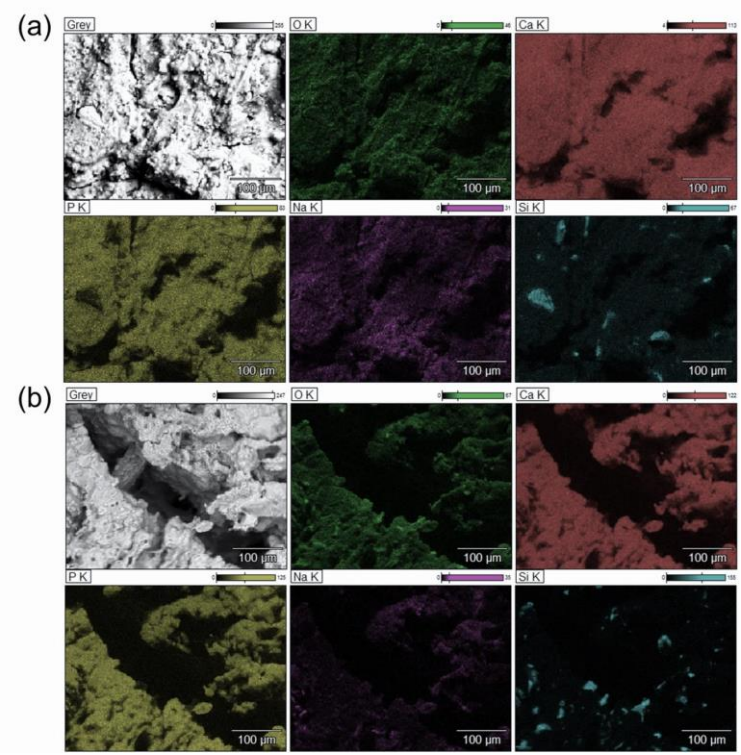


Figure VI - 4. *EDX element mapping of the specimens after the thermal treatment at 900°C:* ***(a)*** *samples HAP_30B;* ***(b)****samples CF_30B.*

6.6.2 Mechanical properties characterization

The compressive strength of the different samples sintered at 900°C for 3 hours is shown in Fig. 5. Compressive strength of cylindrical samples was evaluated measuring load to failure divided by cross-sectional area of the samples.

The samples CF_30B and HAP_30B presented the highest compressive strength values proving that the addition of Bioglass®45S5 significantly improved the average compressive strength of the sintered bioceramic samples.

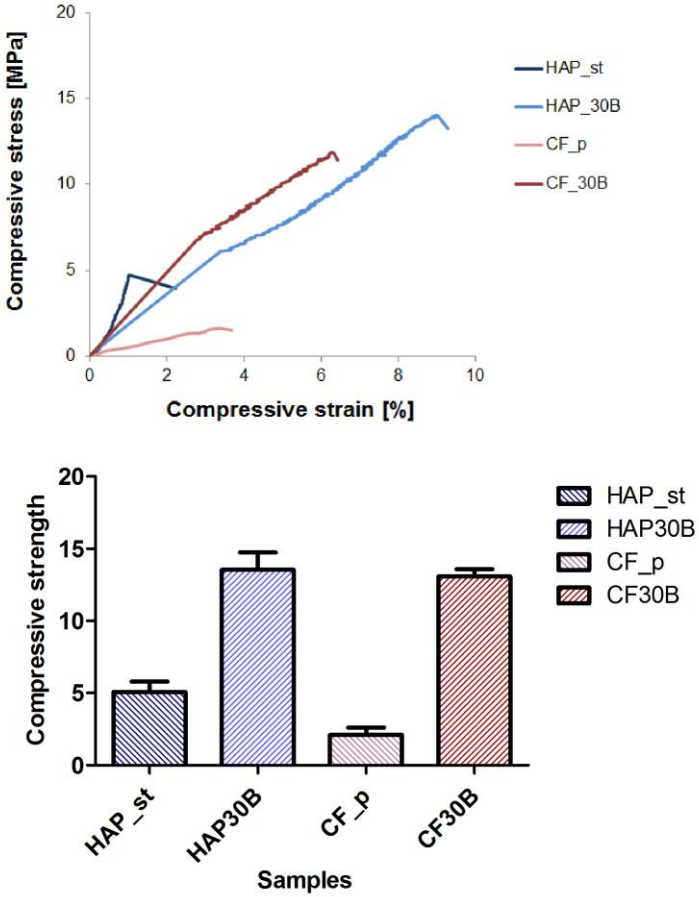


Figure VI - 5. Compressive strength of the different samples (HAP_st, HAP_30B, CF_p and CF_30B) sintered at 900°C for 3 hours; *p < 0.001, significant difference.

6.7 In vitro characterization

In order to study the ability to deposit apatite on the surfaces of the different bioceramics formulations, the samples were placed in media for 7 days without cells. Fig. 6 shows that, after 7 days of immersion in the culture medium, all the samples presented an apatite layer and in particular, the samples containing Bioglass® (HAP_30B and CF_30B), had the largest amount of apatite formation.

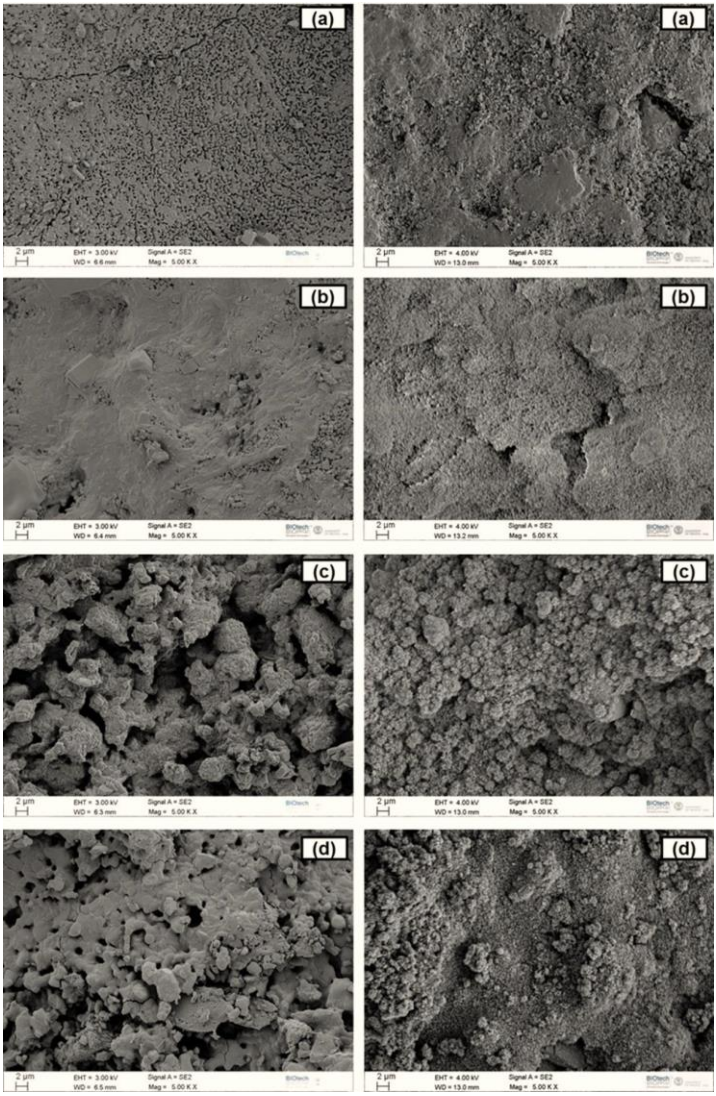


Figure VI - 6. Secondary electron scanning micrographs of the different bioceramic formulations: **(a)** samples HAP_st, **(b)** samples HAP_30B, **(c)** samples CF_p, **(d)** samples CF_30B. The SEM images of the samples incubated for 7 days in DMEM are reported in the left part of the figure.

6.7.1 Cell proliferation by DNA quantification

To investigate the ability of the different bioceramic formulations to promote cell proliferation and growth, MG63 cells were seeded on the scaffolds, and their behaviour was subsequently investigated. Cell proliferation studies were performed at different time points over a period of 7 days (Day 1, Day 3 and Day 7), using the PicoGreen® DNA quantification assay. According to the results (Fig.7(a)), the cell number increased gradually at each time point and reached its maximum at day 7, for all samples. At day 7, the samples produced using the cuttlefish bone powder as starting material (samples CF_p and CF_30B) presented a significantly higher cell number in comparison with the samples HAP_st and HAP_30B.

The actin microfilament cytoskeleton and nucleus of cells after 1, 3 and 7 days of cells culture were stained to visually explore the adhesion and spreading of MG63 cells on the different samples, results reported in Fig. 8. At day 1, Rhodamine-phalloidin and DAPI staining revealed a comparable number of cell attachment on all samples. Nucleus and cytoskeleton were visible. However, in the sample CF_p, cytoskeleton organization was not very prominent in comparison with the other samples formulations. After three days of cell culture, the cell started to growth and showed a stretched morphology of the cytoskeleton arrangement. At day 7, an appreciable cellular growth was evident in all the samples. In particular, samples CF_p and CF_30B presented a significant increase in cells number and cells covered the entire scaffold surface, forming a carpet of spread cells with well-developed actin filaments. These findings are in agreement with the PicoGreen® DNA quantification assay results, that revealed a larger cell growth for the scaffolds crated processing the cuttlefish bone powder than for those produced with the commercial stoichiometric HAP.

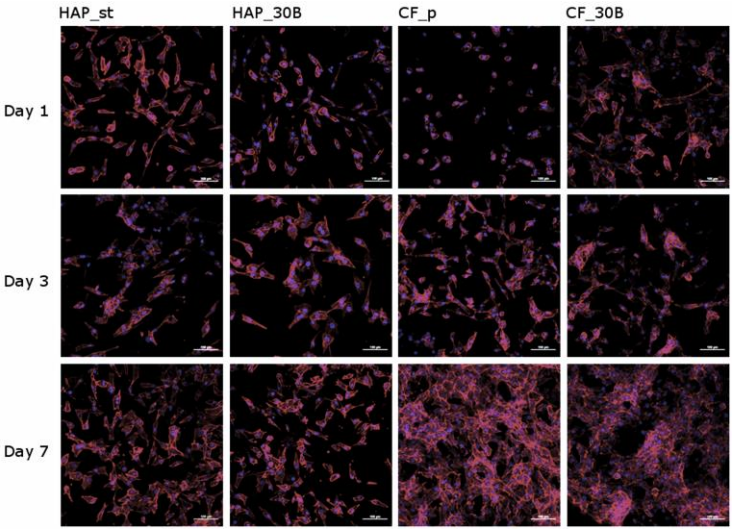


Figure VI - 7. *Rhodamine-labeled phalloidin (F-actin fiber in red) and DAPI (nuclei, blue) were used to visualize cell cytoskeleton organization and cell distribution respectively, in MG63 cultured on samples: HAP_st, HAP_30 B, CF_p and CF_30B.*

6.7.2 Cell metabolic activity by Alamar Blue® assay

Fig. 7(b) shows the metabolic activity, measured by using Alamar Blue® assay, of MG63 cells cultured for 1, 3 and 7 days on scaffolds produced with the different formulations. For all the scaffolds the cellular metabolic activity continued to increase after 7 days of cell culture. However, at day 7, cells grown on the scaffolds made with the commercial pure HAP (HAP_st) have a relatively lower metabolic activity compared to those of the other samples. In addition, it is interesting to note that the scaffolds containing Bioglass®-45S5 (HAP_30B and CF_30B) have a significantly higher metabolic activity with respect to scaffolds without bioglass.
These findings suggest that the formation of new chemistry compositions (sodium calcium silicate in the samples HAP_30B and sodium calcium phosphate with localized amorphous silica in the samples CF_30B) significantly influences the metabolic activity of MG63 cells.

6.7.3 Alkaline phosphatase activity

The activity of intracellular alkaline phosphatase (ALP) of MG63 cells line was monitored at 1, 3, 7 days as shown in Fig.7(c). ALP activity of the cells grown on all samples increased with increasing culture time, reaching a maximum at Day 7. It is

interesting to note that, at Day 7, the ALP activity for the samples produced using the cuttlefish bone powder as starting material (samples CF_p and CF_30B), resulted significantly higher than the ALP activity for the samples made with commercial HAP (HAP_st and HAP_30B). In particular cultures on CF_30B showed the highest ALP activity starting from the third day of cell culture.

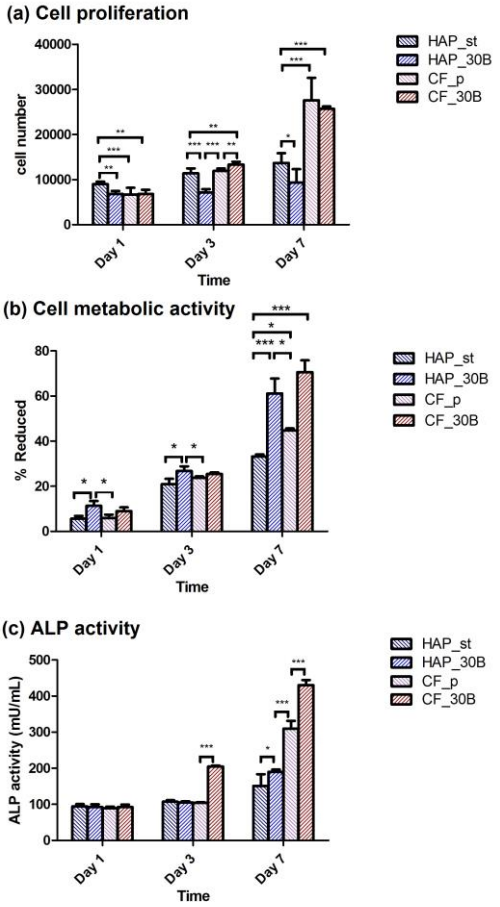


Figure VI - 8. ***(a)*** *PicoGreen® DNA quantification assay of MG63 cell cultured for 1, 3 and 7 days on the surface of the bioceramic samples (HAP_st, HAP_30B, CF_p and CF_30B);* ***(b)*** *Alamar Blue® assay of MG63 cell cultured for 1, 3 and 7 days on the surface of the samples: HAP_st, HAP_30B, CF_p and CF_30B;* ***(c)*** *ALP activity of the of MG63 cell cultured for 1, 3 and 7 days on the surface of the bioceramic samples. Results relative to the ALP activity were expressed in mU/mL. *p < 0.001, significant difference. All the data represents mean ±SD.*

6.8 Discussion

Among the requirements for scaffolds materials in bone tissue engineering applications, bioactivity and osteoconductivity are crucial aspects to achieve successful tissue regeneration. At the same time, the mechanical properties of the scaffold are critical in order to support the loads during the regeneration process.

Hydroxyapatite is the major mineral component of native bone and it has been widely used in several bone tissue engineering applications. However, it is well known that hydroxyapatite cannot be used alone as scaffold material because of its poor mechanical properties [20].

As previously suggested in literature, strong chemical bonds can be formed during the sinterization process of hydroxyapatite/bioactive glass formulations and the presence of a glassy phase can promote the decomposition of hydroxyapatite to β-TCP, thus accelerating atomic diffusivity and the kinetics of the sintering process [21]. As a consequence, hydroxyapatite-based constructs with enhanced mechanical properties can be obtained by sintering hydroxyapatite /bioactive glass powder formulations [22].

In addition, it has been demonstrated that the cosintering process can induce the formation of new ceramic phases. In particular, Demirkiran *et al.* have reported that the sintering process of hydroxyapatite with 10 wt.% Bioglass®45S5 resulted in the formation of calcium phosphate silicate ($Ca(PO_4)_2SiO_4$) and limited amount of β-TCP, while the composition with 25 wt.% Bioglass®45S5 originated sodium calcium phosphate ($Na_3Ca_6(PO_4)_5$) with no evidence of β-TCP [14].

Our study confirmed that the samples containing Bioglass®45S5 (HAP_30B and CF_30B) exhibited a significantly higher compressive strength when compared with those produced without Bioglass® (HAP_st and CF_p).

We also found that the addition of 30 wt.% Bioglass®45S5 to the samples HAP_30B triggered the decomposition of commercial stoichiometric hydroxyapatite during sinterization at 900°C into CaO and β-TCP, that further reacted with the Bioglass® with the formation of new phases. In particular, the XRD analysis of HAP_30B evidenced the presence of both hydroxyapatite and $Na_2Ca_2Si_2O_7$ (40.9 wt% SiO_2, 38.3 wt% CaO, 20.8% wt Na_2O) after sinterization. Furthermore, the element mapping of these samples showed that silicon was combined with Ca and Na and proved the presence of calcium silicate compounds in some areas of the surface.

On the other hand, the XRD analysis of sample CF_30B did not evidence any crystalline silicate phase. This is probably related to the fact that for this formulation hydroxyapatite was generated through the thermal conversion of aragonitic ($CaCO_3$) structure of CF powder, following the chemical reaction: $10CaCO_3 + 6(NH_4)_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6(NH_4)_2CO_3 + 4H_2CO_3$ [10]. At the same time, $CaCO_3$ was thermally decomposed to obtain CaO that reacted with P_2O_5 provided by Bioglass® to form β-TCP. The further incorporation of Na^+ cations into the structure

resulted in the formation of $Na_3Ca_6(PO_4)_5$. It is interesting to note that the elements mapping of CF_30B evidenced that silicon was localized in Ca-deficient areas of the surface, thus suggesting that silicon segregated as amorphous silica in localized regions of the samples surface during the thermal treatment.

In vitro biological evaluation was performed to investigate the effect of the new chemistry on the cellular response. MG63 cell line was used to evaluate the biocompatibility of the different bioceramics formulations. These cells are derived from a human osteosarcoma and are frequently used as an experimental model to study different osteoblast functions. In fact, these cells present a number of features typical of an undifferentiated osteoblast phenotype, including the synthesis of collagen type I and III, production of osteocalcin and the expression of alkaline phosphatase [23].

We showed that the scaffolds based on cuttlefish bone powder (samples CF_p and CF_30B) stimulated MG63 cells proliferation after 7 days of *in vitro* culture. In fact, analysis of DNA content and confocal images demonstrated a significant enhancement of the cell number, and a higher cellular proliferation for cuttlefish bone derived samples (CF_p and CF_30B) in comparison with samples produced using the commercial synthetic hydroxyapatite. We also noticed that the formation of new phases in the formulations with Bioglass® significantly influenced also the cell viability. This enhancement of the metabolic activity in the samples CF_30B and HAP_30B are consistent with previous works that studied the behaviour of bioceramic surfaces in presence of Bioglass®. In particular, Clark and Hench reported that a silica gel layer forms at the surface of bioceramics as a result of Bioglass® partial dissolution [24]. Such newly formed silica layer promotes cell proliferation and differentiation and at the same time improves the mineralization process through the formation of an apatite layer. Moreover, it has been shown that formulations containing bioactive glasses release silicon, calcium, sodium and phosphate ions during the degradation in the physiological conditions. Such ions are important because Ca, Si and P are the main components of biological apatite and play a crucial role in bone formation and resorption [25]. In particular, Ca is involved in the activation of the intracellular mechanisms during bone remodeling and Si is implicated in the metabolic processes of new bone matrix formation and calcification [26]. Therefore, the dissolution products of bioactive glasses can stimulate cells to produce new bone tissue and, at the same time, improve the mineralization process.

In this study, SEM analysis evidenced obvious calcium deposition on all the scaffolds after 7 days of immersion in DMEM and a highly developed apatite layer in particular for the samples containing Bioglass®. In the sample CF_30B, the dissolution of amorphous silica on the surface could also contribute to enhance the bioactivity thanks to the release of Si ions.

However, a suitable model for bone tissue engineering applications requires also that cells are able to differentiate into bone-forming cells. Alkaline phosphate (ALP)

is an enzyme that is associated with calcification and its activity increases when osteoblasts produce osteoid. In particular, ALP expression characteristically reaches a maximum level during the phase of matrix maturation, just before mineralization actually begins [27]. For this reason, ALP enzyme is considered an early marker of osteoblast differentiation. Furthermore, the ALP enzyme has the effect to increase local levels of inorganic phosphate, one of the components of apatite, revealing the activation of the early phases that will lead to the mineralization process [28]. The ALP measurements of the bioceramics formulations evidenced that the scaffolds CF_p and CF_30B were more effective to induce osteoblast ALP activity, than the scaffolds produced using commercial stoichiometric hydroxyapatite. These results suggested that the chemical composition of the non-stoichiometric hydroxyapatite synthetized from cuttlefish bone, could provide an adequate stimulatory effect on both cell proliferation and differentiation.

6.9 Conclusion

In this study, a method to produce highly bioactive hydroxyapatite-based ceramics compositions starting from cuttlefish bone powders, was studied. In particular, fragmented cuttlefish bone was co-sintered with Bioglass®-45S5, at 900 °C for 3 h, to obtain ceramics compositions with high bioactivity and enhanced mechanical properties. The composition and biological properties of the different bioceramics formulations were studied to evaluate the use of the material for bone tissue engineering applications. The X-ray diffraction analysis of the samples evidenced that the incorporation of the bioactive glass mixture promoted the sintering process and resulted in new phases formation. In particular, the addition of 30 % wt. Bioglass® to the cuttlefish bone powder resulted in the formation of sodium calcium phosphate ($Na_3Ca_6(PO_4)_5$), β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)$) and amorphous silica. The compressive tests of the samples proved that the combination with Bioglass®45S5 was very effective in increasing the mechanical properties of all the scaffolds. In addition, the biological tests showed that the addition of bioactive glass positively affected also the metabolic activity of MG63 cells, in vitro. Furthermore, the samples produced starting from cuttlefish bone powder showed the largest apatite deposition on the surface when immersed in DMEM and the highest proliferation of MG63 cells, after 7 days of cell culture. Finally, the measurements of ALP activity evidenced that naturally derived hydroxyapatite could be effective to promote both, cell proliferation and differentiation. These results suggested that cuttlefish bone provide a cost-effective and environmentally friendly source of hydroxyapatite and naturally derived hydroxyapatite/Bioglass® bioceramics are very promising materials for bone tissue engineering applications.

References

[1] M. M. Stevens, "Biomaterials for bone tissue engineering," *Mater. Today*, vol. 11, no. 5, pp. 18–25, 2008.

[2] W. Habraken, P. Habibovic, M. Epple, and M. Bohner, "Calcium phosphates in biomedical applications: materials for the future?," *Biochem. Pharmacol.*, vol. 19, no. 2, pp. 69–87, 2016.

[3] P. Wang, L. Zhao, J. Liu, M. D. Weir, X. Zhou, and H. H. K. Xu, "Bone tissue engineering via nanostructured calcium phosphate biomaterials and stem cells," *Bone Res.*, no. 2, pp. 14017, 2014.

[4] H. Yoshikawa, "Bone tissue engineering with porous hydroxyapatite ceramics," pp. 131–136, 2005.

[5] H. Zhou and J. Lee, "Nanoscale hydroxyapatite particles for bone tissue engineering," *Acta Biomater.*, vol. 7, no. 7, pp. 2769–2781, 2011.

[6] H. Ivankovic, S. Orlic, D. Kranzelic, and E. Tkalcec, "Highly Porous Hydroxyapatite Ceramics for Engineering Applicatios," *Adv. Sci. Technol.*, vol. 63, pp. 408–413, 2010.

[7] A. Oryan, S. Alidadi, A. Moshiri, and N. Maffulli, "Bone regenerative medicine: classic options, novel strategies, and future directions.," *J. Orthop. Surg. Res.*, vol. 9, no. 1, p. 18, 2014.

[8] K. S. Vecchio, X. Zhang, J. B. Massie, M. Wang, and C. W. Kim, "Conversion of bulk seashells to biocompatible hydroxyapatite for bone implants," *Acta Biomater.*, vol. 3, no. 6, pp. 910–918, 2007.

[9] E. Tkalčec, J. Popović, S. Orlić, S. Milardović, and H. Ivanković, "Hydrothermal synthesis and thermal evolution of carbonate-fluorhydroxyapatite scaffold from cuttlefish bones," *Mater. Sci. Eng. C. Mater. Biol. Appl.*, vol. 42, pp. 578–86, 2014.

[10] J. H. G. Rocha, A. F. Lemos, S. Agathopoulos, P. Valério, and S. Kannan, "Scaffolds for bone restoration from cuttlefish," vol. 37, no. 6, pp. 850–857, 2005.

[11] J. H. G. Rocha, A. F. Lemos, S. Kannan, S. Agathopoulos, and J. M. F. Ferreira, "Hydroxyapatite scaffolds hydrothermally grown from aragonitic cuttlefish bones," *J. Mater. Chem.*, vol. 15, no. 47, pp. 5007–5011, 2005.

[12] W. Suchanek and M. Yoshimura, "Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants," *J. Mater. Res.*, vol. 13, no. 01, pp. 94–117,1998.

[13] S. J. Kalita, S. Bose, H. L. Hosick, and A. Bandyopadhyay, "CaO – P_2O_5–Na_2O-based sintering additives for hydroxyapatite (HAp) ceramics," vol. 25, no. 12, pp. 2331–2339, 2004.

[14] H. Demirkiran, A. Mohandas, M. Dohi, A. Fuentes, K. Nguyen, and P. Aswath, "Bioactivity and mineralization of hydroxyapatite with bioglass as

sintering aid and bioceramics with $Na_3Ca_6(PO_4)_5$ and $Ca_5(PO_4)_2SiO_4$ in a silicate matrix," *Mater. Sci. Eng. C*, vol. 30, no. 2, pp. 263–272, 2010.

[15] F. Lin, C. Lint, H. Liut, Y. Huang, C. Wang, and C. Lu, "Sintered porous DP-bioactive glass and hydroxyapatite as bone substitute," vol. 15, no. 13, 1994.

[16] L. Gerhardt and A. R. Boccaccini, "Bioactive Glass and Glass-Ceramic Scaffolds for Bone Tissue Engineering," *Materials*, vol. 3, no. 7, pp. 3867–3910, 2010.

[17] H. Ivankovic, E. Tkalcec, S. Orlic, G. Gallego Ferrer, and Z. Schauperl, "Hydroxyapatite formation from cuttlefish bones: kinetics," *J. Mater. Sci. Mater. Med.*, vol. 21, no. 10, pp. 2711–2722, 2010.

[18] I. Rehman and W. Bonfield, "Characterization of hydroxyapatite and carbonated apatite by photo acoustic FTIR spectroscopy," *J. Mater. Sci. Mater. Med.*, vol. 8, no. 1, pp. 1–4, 1997.

[19] A. Ślósarczyk, Z. Paszkiewicz, and C. Paluszkiewicz, "FTIR and XRD evaluation of carbonated hydroxyapatite powders synthesized by wet methods," *J. Mol. Struct.*, vol. 744, pp. 657–661, 2005.

[20] M. Wang, "Developing bioactive composite materials for tissue replacement," *Biomaterials*, vol. 24, no. 13, pp. 2133–2151, 2003.

[21] X. Chatzistavrou, K. Chrissafis, E. Kontonasaki, T. Zorba, and P. Koidis, "Sintered Hydroxyapatite/Bioactive Glass Composites : Thermal Analysis and Bioactivity," In *Key Engineering Materials*. Trans Tech Publications, vol. 311, pp. 167–170, 2006.

[22] G. Goller, H. Demirkıran, F. N. Oktar, and E. Demirkesen, "Processing and characterization of bioglass reinforced hydroxyapatite composites," *Ceram. Int.*, vol. 29, no. 6, pp. 721–724, 2003.

[23] J. Clover and M. Gowen, "Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype?," *Bone*, vol. 15, no. 6, pp. 585–591, 1994.

[24] A. E. Clark, C. G. Pantano, and L. L. Hench, "Auger spectroscopic analysis of bioglass corrosion films," *J. Am. Cer. Soc.*, vol. 59, no. 1-2, pp. 37–39, 1976.

[25] P. Ducheyne and Q. Qiu, "Bioactive ceramics : the effect of surface reactivity on bone formation and bone cell function," *Biomaterials*, vol. 20, no. 23, pp. 2287–2303, 1999.

[26] A. Hoppe, N. S. Güldal, and A. R. Boccaccini, "A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics," *Biomaterials*, vol. 32, no. 11, pp. 2757–2774, 2011.

[27] B. D. Boyan, C. H. Lohmann, D. D. Dean, V. L. Sylvia, D. L. Cochran, and Z. Schwartz, "Mechanisms involved in osteoblast response to implant surface morphology," *Annual Rev. Mater. Sci.*, vol. 31, no. 1, pp. 357–371,

2011.
[28] P.D. Delmas, “Biochemical markers of bone turnover,” *J. Bone Miner. Res.*, vol. 8, no. S2, pp. S549–S555, 1993.

Chapter VII

Conclusions and future perspectives

Based on the enormous biodiversity of marine environment and the genetic uniqueness of marine organisms , many efforts are directed to obtain materials from marine resources in a reproducible way and to identify processing methods for suitable systems and applications.

The overall objective of this work was to isolate and synthesize naturally-derived materials from marine organisms and to characterize their properties looking at their potential use in biomedical applications.

The work was divided in two main parts.

In the first part of this study, acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) from *Loligo Vulgaris* squid mantle were successfully retrieved and characterized. The results showed that the use of pepsin during the extraction process increases the yield of retrieved collagen (by 4.7-fold) without damaging the integrity of the triple helix. At this regard, DSC and viscosity measurements showed the helix-coil transition for both the extracted collagens, indicating that pepsin digestion did not affect the conformation of the protein.

The SDS-PAGE patterns of ASC and PSC presented a protein profile characteristic of type I and V collagens, consisting of two α-chains (α1 and α2), β- and γ-components.

GPC analysis showed that the extracted collagens are composed of different families of peptides which different molecular weight distributions. In particular ASC contained a higher content of high-molecular weight cross-links than PSC. These results were supported by the thermal analysis and the solubility tests that indicated a lower solubility of ASC compared with PSC.

Both collagens were soluble at acidic pH water solutions and became fully insoluble when the NaCl concentration increased above 0.4 M.

Furthermore, in vitro cytotoxicity tests proved that after the extraction process the material did not release cytotoxic substances.

The denaturation temperature of extracted collagens, in a 0.5 M acetic acid solution, was found to be lower than that of mammalian collagen..

A possible strategy to increase the thermal stability in order to extend the application area of extracted collagen is to increase its degree of cross-linking. Genipin was used as natural crosslinking agent to introduce exogenous cross-linking into the molecular structure of reconstituted collagen.

The results demonstrated that squid mantle has a potential as an alternative source of collagen and could be considered for use in pharmaceutical or biomedical applications.

Nevertheless, it is interesting to underline that the relevance of this study was not only the identification of a new promising source of marine collagen but it has laid the foundation for more detailed investigation on this field. In fact, the thorough assessment of the main parameters affecting the collagen extraction process, in term of yields and quality of the isolated protein, has allowed the identification of simple procedures that could be easily adaptable also to other marine species.

On the other side, the detailed characterization of the isolated collagen fractions, obtained by comparison of different characterization techniques, could be used as a model for further investigations, providing fundamental tools for the evaluations of their physicochemical properties, future processability and potential applications.

In the second part of the research, a method for developing highly bioactive hydroxyapatite-based bioceramics starting from cuttlefish bone powders was investigated.

In particular, cuttlefish bone material was co-sintered with 30 wt% of Bioglass®-45S5 to synthesize hydroxyapatite-based powders with enhanced mechanical properties and bioactivity. Commercial synthetic hydroxyapatite was treated following the same procedure and used as a reference.

Structure and composition of the bioceramics formulations were characterized exploiting different techniques, including Fourier transform infrared spectroscopy, X-ray diffraction and scanning electron microscopy (SEM).

After the thermal treatment of the cuttlefish bone powder with Bioglass®, the chemical characterization of the samples revealed the presence of new phases with compositions of sodium calcium phosphate ($Na_3Ca_6(PO_4)_5$), β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)$) and amorphous silica.

In vitro cell culture studies were performed using human osteoblast-like cells (MG63) to evaluate the biological properties of the bioceramic formulations.

In particular, cell proliferation, metabolic activity and differentiation of MG63 cells were studied.

Our findings demonstrated that all the scaffolds produced using cuttlefish bone powder exhibited increased apatite deposition, ALP activity and cell proliferation compared to commercial synthetic hydroxyapatite. In addition, the new ceramic compositions obtained after the combination with Bioglass®, further enhanced the metabolic activity of MG63 cell and promoted the formation of a well-developed apatite layer after 7 days of incubation in DMEM.

However, further investigation is required, for example using the X-rays absorption near edge structure (XANES) spectroscopy to evaluate the crystalline as well as amorphous phases of the sintered bioceramic compositions.

In addition, a quantitative evaluation of the new phases formed during the treatment is necessary for the optimization of the process and to better understand the mechanisms that lead to the new phases formation.

To conclude, considering the hierarchical and complex structure of native bone, a promising approach to produce scaffolds for bone regeneration could be the combination of proteinic matrices (made for example of marine collagen) and naturally derived hydroxyapatite/Bioglass® bioceramics with the aim to design hybrid organic–inorganic composites with advanced functional and biological properties.

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