Biologically-Inspired Systems

Hermann Ehrlich

Marine Biological Materials of Invertebrate Origin

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Biologically-Inspired Systems

Volume 13

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Stanislav N. Gorb, Department of Functional Morphology and Biomechanics,
Zoological Institute, Kiel University, Kiel, Germany

Motto: Structure and function of biological systems as inspiration for technical developments

Throughout evolution, nature has constantly been called upon to act as an engineer in solving technical problems. Organisms have evolved an immense variety of shapes and structures from macro down to the nanoscale. Zoologists and botanists have collected a huge amount of information about the structure and functions of biological materials and systems. This information can be also utilized to mimic biological solutions in further technical developments. The most important feature of the evolution of biological systems is multiple origins of similar solutions in different lineages of living organisms. These examples should be the best candidates for biomimetics. This book series will deal with topics related to structure and function in biological systems and show how knowledge from biology can be used for technical developments in engineering and materials science. It is intended to accelerate interdisciplinary research on biological functional systems and to promote technical developments. Documenting of the advances in the field will be important for fellow scientists, students, public officials, and for the public in general. Each of the books in this series is expected to provide a comprehensive, authoritative synthesis of the topic.

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Hermann Ehrlich
Institute of Electronic and Sensor Materials
TU Bergakademie Freiberg
Freiberg, Sachsen, Germany

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Preface

Biological materials – hard, soft, and hybrids of both phases – first appeared in marine environments in ancient oceans. Some of them can be found only in the forms of fossils, especially in cases where they are exceptionally preserved over millions of years. Others were so successful in their survival strategies that we now dwell in their amazing abundance which include unicellular (protists, diatoms, radiolarians) and multicellular (sponges, corals, molluscs, worms, echinoderms, arthropods) invertebrate organisms.

The origin of biological materials is closely connected with the formation of the first biominerals which occurred in the first ancestral unicellular organisms that evolved under the harsh environmental conditions of ancient aquatic niches. Both biologically induced and controlled mineralization promoted the development of protective biocomposites to shield cells from thermal, chemical, and ultraviolet stresses. Such conditions also allowed adaptation of unique extremophilic and polyextremophilic biomineralizers, which are still found today (i.e., hydrothermal vents). In most cases, the mechanisms of these specialized biomineralogical phenomena remain unknown. Traditionally, most attention has been paid to biomineralization – in the last 50 years, the following topics have been studied: diverse aspects of biomineralization strategies, processes and mechanisms, biomineralizing phyla, evolutionary history and the Cambrian explosion, problems of the vital effect, and principles of control and design, including shaping of crystals with biomolecules and inspiration for biomaterials science. Especially, extreme biomineralization provides crucial information for progression in such modern scientific direction as extreme biomimetics. This exciting area of modern research holds promise for being the next step in creating the next generation of composites using organic-templating approaches under biologically extreme laboratory conditions.

Today, the principal strategy for research which deals with biological materials of marine invertebrates' origin is based on the following steps:

- Collection of specimens (Fig. 1)
- Isolation of the corresponding material
- Identification of the material

- Determining the mechanisms of its formation
- Using this knowledge for creation of novel material(s)

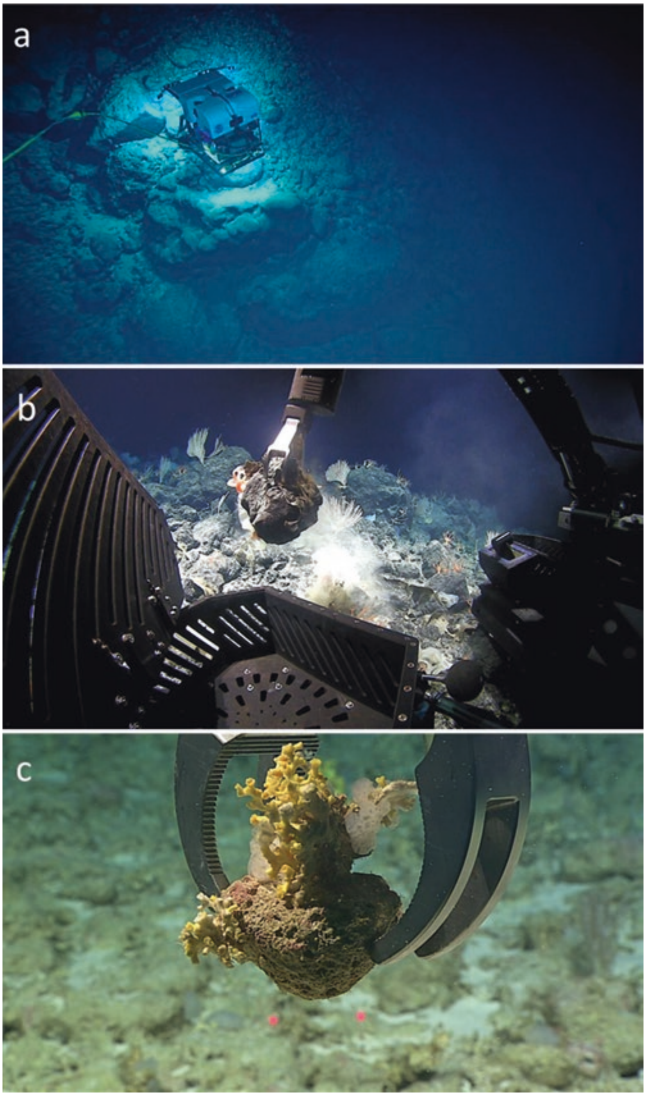

Fig. 1 The sea bottom remains to be the fascinating source of diverse invertebrates which are carefully collected using modern underwater devices (**a**) and robotic systems (**b** and **c**) for scientific aims only

Challenges, solutions, and future directions of modern biological materials science as an interdisciplinary research field are represented in 24 chapters of this monograph. For brevity, some aspects will only be briefly discussed, but interested readers are referred to pertinent references which include time stamps from the nineteenth century till May 2019. Principally, I took the liberty to discuss the current state of the work related to biomimetics, taking inspiration from biomineralization and biopolymers-related science and their niches in modern biomimetic-oriented materials science. It is to be hoped that interested readers will be motivated enough to visit corresponding websites of my colleagues listed below to be familiar with the state of the art in marine biomaterials today):

Group leader	Topics	Links
Andrew Smith	Adhesives	https://faculty.ithaca.edu/asmith/
Ali Miserez	Bioelastomers, biomechanics	http://www.ntu.edu.sg/home/ali.miserez/
Azizur Rachman	Chitin	http://utoronto.academia.edu/AzizurRahman
Christine Ortiz	Marine biomaterials	https://dmse.mit.edu/people/christine-ortiz
David Kaplan	Collagen, silk, chitin	
David Kisailus	Biomaterials, crustaceans	https://intra.engr.ucr.edu/~david/kisailus.php
David William Green	Spongin, biomaterials	https://www.researchgate.net/profile/David_Green55
Haneesh Kesari	Sponge biomechanics	https://vivo.brown.edu/display/hkesari
Heinz Schröder	Biosilica, sponges	https://www.unimedizin-mainz.de/physiolchemie/forschung/univ-prof-dr-dr-h-c-w-e-g-mueller/mitarbeiter.html
Henrik Birkedal	Byssus	https://pure.au.dk/portal/en/persons/henrik-birkedal(e5418e07-b317-4ecc-a9df-8bdedaa32f5b).html
Herbert Waite	Bioelastomers, biopolymers	https://www.mcdb.ucsb.edu/people/faculty/waite
Hermann Ehrlich	Extreme biomimetics, chitin, spongin	https://tu-freiberg.de/esm/institut/institutsleitung/biomineralogie-und-extreme-biomimetik
James Weaver	Biosilica, sponges	https://mediatedmattergroup.com/james-weaver
Jan Michels	Biomaterials microscopy	https://www.sgorb.zoologie.uni-kiel.de/?page_id=15
Joanna Aizenberg	Biosilica, sponges	https://aizenberglab.seas.harvard.edu/
Marcin Wysokowski	Extreme biomimetics	https://tu-freiberg.de/esm/mitarbeiter
Marco Giovine	Sponge collagen	http://www.distav.unige.it/drupalint/user/52
Marina Pozzolini	Sponge collagen	http://www.distav.unige.it/drupalint/user/163
Markus Buehler	Collagen	https://cee.mit.edu/people_individual/markus-j-buehler/
Matthew J Harrington	Byssus	https://www.mpg.de/11091566/mussels-byssus-threads

(continued)

Group leader	Topics	Links
Michael A Monn	Sponge biomechanics	https://vivo.brown.edu/display/hkesari
Patrick Flammang	Adhesives	http://staff.umons.ac.be/patrick.flammang/pubsfr.html
Peter Fratzl	Biomechanics	http://www.mpikg.mpg.de/biomaterials/director/peter-fratzl
Riu R Reis	Sponge collagen, gelatin	https://www.3bs.uminho.pt/
Sheila Patek	Biomechanics	https://biology.duke.edu/people/sheila-n-patek
Stas N Gorb	Adhesives, resilin, biomechanics	https://www.sgorb.zoologie.uni-kiel.de/?page_id=15
Teofil Jesionowski	Spongin, chitin	http://www.fct.put.poznan.pl/kadra/73
Thomas Scheibel	Byssus, silk	https://www.epo.org/learning-events/european-inventor/finalists/2018/scheibel.html
Wang Xiaohong	Biosilica, sponges	https://www.unimedizin-mainz.de/physiolchemie/forschung/univ-prof-dr-dr-h-c-w-e-g-mueller/mitarbeiter.html
Werner Müller	Biosilica, sponges	https://www.unimedizin-mainz.de/physiolchemie/forschung/univ-prof-dr-dr-h-c-w-e-g-mueller/mitarbeiter.html
Yael Politi	chitin	https://www.mpikg.mpg.de/staff-members/22386

I strongly believe that numerous open questions raised in this book will inspire a younger generation of biologists, chemists, engineers, physicists, and materials scientists to research marine biological materials. I hope that in the future they will shine a light on the current gaps in the knowledge related to this fascinating but still poorly investigated materials world.

Finally, I am thankful to Mehdi Rahimi-Nasrabadi, Marcin Wysokowski, Iaroslav Petrenko, Alona Voronkina, and Yevgen Karpov for taking excellent care of the manuscripts and proofs. I would like to thank numerous colleagues who have supported me during the preparation of this work including Prof. Markus Buehler, Prof. Catherine Skinner, Prof. David Kisaulis, Prof. Andy Knoll, Prof. Edmund Bäeuerlein, Prof. Victor Smetacek, Prof. Dan Morse, Prof. George Mayer, Prof. Hartmut Worch, Prof. David Rafaja, Prof. Teofil Jesionowski, Prof. Alex Ereskovsky, Prof. Konstantin Tabachnik, and Prof. Yvonne Joseph for their permanent interest in my research. I am also grateful to Dr. Allison Stelling for editing the manuscripts. I am under deep obligation for the patience and strong support during hard times to my parents, my wife, and my children.

Freiberg, Sachsen, Germany Hermann Ehrlich
May 2019

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Part I
Biomaterials

Chapter 1
Biomaterials and Biological Materials

> *The union of biology with materials science and engineering represents one of the most exciting scientific prospects of our time. As currently few biologists know much about engineering and even fewer engineers know much about biology, the expectations of future advances seem unbounded.*
>
> Robert O. Ritchie, 2008

Abstract Broad diversity of biological systems including cells, tissues and organisms are able to produce individual organic, inorganic as well as composite-based materials known as biomaterials. Structure and chemistry of corresponding phases within biocomposites have been recognized on a microscopic or macroscopic size scale using modern analytical instruments. Nowadays, not only bio-inert materials, but mostly biocompatible and biodegradable materials attract attention of experts in regenerative medicine, wound healing, surface coatings and tissue engineering. This short chapter deals with classification and history of biomaterials.

Scientific discipline known as Materials Science and Engineering was born in1950s, and showed progress mostly in such directions as ceramics, polymers and metals. Only in 1990s "*biological materials*" are being added to this area (Doherty et al. 1992; Williams 1995) and promises intriguing future development (Williams 2008;Meyers et al. 2008; Wnek and Bowlin 2008; Beldjilali-Labro et al. 2018; Peeters et al. 2018; Brovold et al. 2018; Hou et al. 2019; Zhang 2019; Zhang et al. 2019). Nowadays, as bioengineering has become in engineering (Ritchie 2008), biomaterialogy has become one of the fastest growing directions in materials science. Interdisciplinary oriented biomaterials research is strongly dependent on corresponding progress in highly sophisticated bioanalytical and structural methods as well as on better understanding of the biological and chemical mechanisms which are responsible for biosynthesis and gene-based regulation of diverse naturally occurring individual and composite materials. The requirements of biomaterials have been grouped into following categories: (1) Biocompatibility; (2) Sterilizability;

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(3) Functionability and (4) Manufacturability (Teoh 2004). Recent ecological problems with non-degradability of artificial materials (i.e. microplastics) suggest further progress in biomaterials related science, especially due to their formation (Jones et al. 2019), biodegradability (Sheikh et al. 2015; Prakasam et al. 2017) and bioresorbtion (Perale and Hilborn 2016) and regulated application of corresponding renewable sources.

1.1 Definitions and History: Biomaterial and Biological Material

There are few terms concerning biomaterials, biological materials and biomaterials science (Park and Lakes 2007). For example, according to Williams (1999), "***biomaterials science*** *is the study of the structure and properties of biomaterials, the mechanisms by which they interact with biological systems and their performance in clinical use."*

Hench and Ethridge defined **biomaterial** as *„any material used to make devices to replace a part or a function of the body in a safe, reliable, economic and physiologically acceptable manner*".

Alternatively, a **biomaterial** must be "*a systemically and pharmacologically inert substance designed for implantation within or incorporation with living systems*" (cited by Park and Lakes 2007).

Tranquilli Leali P and Merolli A (2009) used the definition for "*biomaterial*" proposed by the European Society for Biomaterials Consensus Conference II quotes: "*A biomaterial is a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body*".

According to MORDOR Intelligence website from 2019 https://www.mordorintelligence.com/industry-reports/global-biomaterials-market: "*Biomaterials are substances that has been engineered to interact with biological systems for a medical purpose, either a therapeutic (treat, augment, repair, or replace a tissue function of the body) or a diagnostic one*".

However, a ***biological material*** is a material produced only by a biological system (collagen, spongin, silk, keratin, conchiolin, chitin, cellulose (tunicin), dentin, enamel, bone etc.). Consequently, *viability* remains to be the main difference between biomaterials (i.e. implants, or metal biomaterials (Niinomi 2008)) and biological materials (Fig. 1.1).

If it is possible to detect distinct phases within one material on a scale larger than the atomic, and in which mechanical features are significantly altered in comparison with that of a monolithic, homogeneous material, we have to do with a ***Composite materials*** (Fig. 1.2) Readers will find in this book interesting examples of composite materials of biological origin within section regarding so called "*multiphase biomineralization*". Also metal-organic composites with self-healing features remain to be in focus of researchers (Andersen et al. 2019; Speck and Speck 2019; Zechel et al. 2019; Qin et al. 2019a, b).

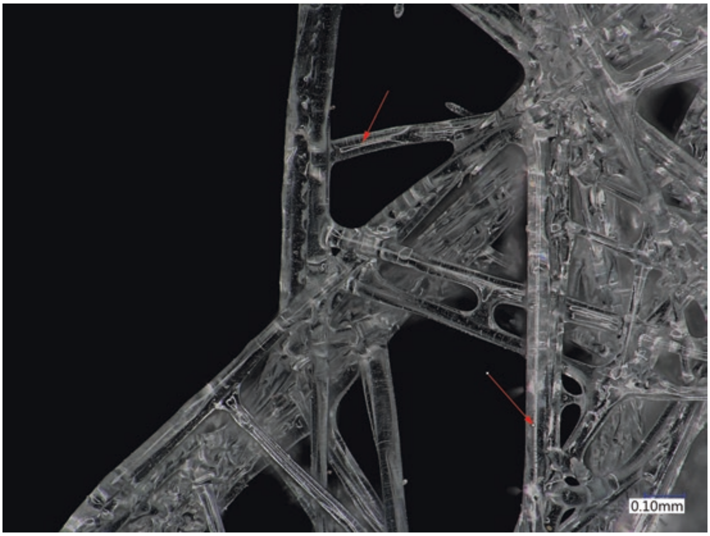
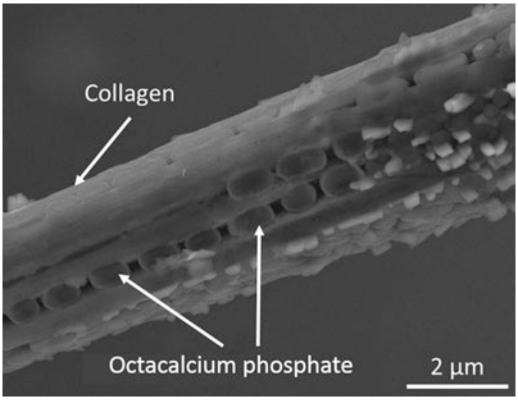


Fig. 1.1 Highly complex skeleton of the glass sponge *Euplectella* sp. is made of tube-like glassy spicules which are interconnected through axial channels (arrows). This is typical example of biosilica- based construct as biological material which has been manufactured by the sponge at temperatures between 0 and 4 °C

Fig. 1.2 SEM image: Collagen fibre mineralized with octacalcium phosphate phase in vitro represents an example of composite material



I suggest that biomaterials together with bioenergy are principally well integrated today into global bioeconomy (see for review Aguilar et al. 2019). Consequently, it is not surprising that such novel generation of biomaterials termed as "*bioinks*" which are necessary for 3D printing technologies for biomedical aims including manufacturing of cell therapies (Abdeen and Saha 2017), tissues (Lindsay et al. 2019) and functional organs (Pastino et al. 2018) is in trend now. Such topics as biodegradable metals (see for overview Qin et al. 2019a, b) for biomedical application and biomaterials for drug delivery (Fenton et al. 2018) will not lost their priority in the near future.

Analysing the historical background of biomaterials as well as biological materials, I can only agree with the statement by Dawid Green made in 2016 that biomimicry remains to be the main driving force in biomaterials design and regenerative medicine (Green et al. 2016). Since ancient times human mimics diverse mechanisms from the "*living organisms as the ultimate survivalists*" (Green et al. 2019) also with respect to biomaterials. The historical overview concerning biomaterials science can to be found in works by Albee and Morrison (1920), Lambotte (1932), Popp (1939), Weinberger (1948), Harkins and Koepp Baker (1948), Ray et al. (1952), Baden (1955), Sivakumar (1999), Kühn (2000), Ratner and Bryant (2004), Teoh (2004), Staiger et al. (2006), Park and Lakes (2007), Orive et al. (2009), and Suzuki (2019). Special section about bioglasses in the history of biomaterials is to be thanked to Larry Hench (Hench et al. 1971; Hench and Paschall 1973; Hench and Andersson 1993; Hench and West 1996; Hench and Polak 2002) and his followers (Thomas et al. 2005; Ashby and Greer 2006; Abou Neel et al. 2009; Schroers et al. 2009; Ma et al. 2014; Kaur et al. 2016; Montazerian and Zanotto 2017; Alhalawani and Towler 2017; Mancuso et al. 2017;Tripathi et al. 2019; Farano et al. 2019).

Corresponding information with historical background about biomaterials in prosthesis (Sevy and Arriaga 2018), in joint replacement (De Meurechy et al. 2018), in tendon engineering (Beldjilali-Labro et al. 2018), in eye treatment (Tran et al. 2018), in neurodegenerative diseases (Silva Adaya et al. 2017) is to be found now. Huge literature concerning history and applications of calcium phosphate bioceramics has been reviewed in several excellent works (see Weiss 2003; Dorozhkin 2009, 2010, 2011, 2012, 2013a, b, 2015a, b, 2017; Eliaz and Metoki 2017; Pujari-Palmer et al. 2018; Degli Esposti et al. 2018; Khan and Syed 2019). Recent review by Heinrich and co-workers covers the history of bioprinting (Heinrich et al. 2019).

1.2 Classification of Biomaterials

Diverse proposals have been made with respect to classification of biomaterials (Table 1.1). Corresponding information including explanations, terms and discussions can be extracted from the numerous books represented in the Table 1.2. Some

Table 1.1 Classification of biomaterials

Classification of biomaterials			
Natural biomaterials	Metallic biomaterials	Ceramic biomaterials	Polymeric biomaterials
	Metals and alloys	Biostable ceramics	Composites
		Bioactive ceramics	Elastomers
		Bioresorbable ceramics	Cellular materials
		Ceramic bone	
		Cements	

Table 1.2 Books related to biomaterials

Year	Title	Author(s)	Publisher
1948	**An introduction to the history of dentistry with medical and dental chronology and bibliographic data**	Weinberger BW	St. Louis, The C.V. Mosby Company. D.D.S., New York
1967	**Cell wall mechanics of wood tracheids**	Mark RE	Yale University Press, New Haven
1968	**On growth and form. 2nd ed.**	Thompson DW	Cambridge University Press, Cambridge
1970	**Strength of biological materials**	Yamada H (Edited by Evans FG)	Williamd and Wilkins (Company, Baltimore)
1970	**Physical properties of plant and animal materials**	Mohsenin NN	Gordon and Breach scientific Publishers
1971	**Organic chemistry of biological compounds**	Barker R	Englewood Cliffs, NJ, Prentice-Hall
1971	**Biophysical properties of the skin**	Elden HR	Wiley, New York
1972	**Biomechanics: its foundation and objectives**	Fung YC, Perrone N, Anliker M	Englewood Cliffs, NJ, Prentice-Hall
1972	**Keratins: their composition, structure, and biosynthesis**	Fraser RDB, MacRae TP, Rogers GE	Springfield, Thomas
1974	**The structure and function of skin. 3rd ed.**	Montagna W, Parakkal PF	New York: Academic press
1974	**The physical biology of plant cell walls**	Preston RD	London: Chapman and Hall
1975	**Structural materials in animals**	Brown CH	London: Pitman
1975	**Biology of the arthropod cuticle**	Neville AC	New York: Springer-Verlag
1976	**Mechanical design in organism**	Wainwright SA, Biggs WD, Currey JD, Gosline JW	Princeton: Princeton University Press
1976	**Wood structure in biological and technological research**	Jeronimidis G. In: Baas P, Bolton AJ, Catling DM	Leiden: The University Press
1977	**Chitin**	Muzzarelli RAA	Pergamon Press, UK, Oxford
1980	**Guidelines for physicochemical characterization of biomaterials. Devices and technology branch national heart, lung and blood institute**	Baier RE	NIH Publication No. 80-2186
1980	**Mechanical properties of biological materials**	Vincent JFV, Currey JD	Cambridge: Cambridge University Press
1980	**Introduction to Composite Materials**	Tsai SW, Hahn HT	Technomic Pub. Co.: Westport, CT
1981	**Mechanical properties of bone**	Cowin SC	New York: American Society of Mechanical Engineers

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
1983	**Biomaterials in reconstructive surgery**	Rubin LR	The C.V. Mosby Company: St. Louis, MO
1984	**Mechanical adaptations of bone**	Currey JD	Princeton: Princeton UP
1984	**The mechanical adaptations of bones**	Currey JD	Princeton: Princeton University Press
1985	**Cellulose chemistry and its applications**	Nevell TP, Zeronian SH	New York: Wiley
1985	**Cellulose chemistry and its applications**	Nevell TP, Zeronian SH	New York: John Wiley and Sons
1986	**Cellulose: structure, modification, and hydrolysis**	Young RA, Rowell RM	New York: John Wiley and Sons
1990	**Biomechanics: motion, flow, stress, and growth**	Fung YC	New York: Springer-Verlag
1990	**Handbook of bioactive ceramics, volume II - calcium phosphate and hydroxylapatite ceramics**	Yamamuro T, Hench L, Wilson J	CRC Press: Boca Raton
1991	**Biomaterials: novel materials from biological sources**	Byrom D	Macmillan
1991	**Structural biomaterials**	Vincent JFV	New Jersey: Princeton University Press
1992	**Materials selection in mechanical design**	Ashby MF	Oxford: Butherworth-Heinemann
1992	**Allografts in orthopaedic practice**	A. Czitrom and Gross A	Williams & Wilkins, Baltimore
1992	**Biological performance of materials: fundamentals of biocompatibility**	Black J	New York: Marcel Dekker
1992	**Biomaterials - an introduction, 2nd Edition**	Park JB and Lakes RS	Plenum Press, New York
1992	**Biological performance of materials, 2nd Edition**	Black J	Marcel & Dekker, New York
1993	**Biomechanics: mechanical properties of living tissues. 2nd ed.**	Fung YC	New York: Springer-Verlag
1993	**Composite materials for implant applications in the human body**	Jamison RD and Gilbertson LN	American Society of Testing and Materials, Philadelphia, USA
1994	**Composite materials: engineering and science**	Matthews FL and Rawlings RD	Chapman & Hall
1994	**Applied dental materials**	McCabe JF	Blackwell Science Publications, Oxford

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
1994	**Implantation biology: the host response and biomedical devices**	Greco RS	CRC Press, London
1994	**Hierartchical structures in biology as a guide for New materials technology**	National Materials Advisory Board, Comission on engineering and Technical systems, National research Council, NMAB- 464	National Academy Press, Washington DC
1994	**Hierarchical structures in biology as a guide for new materials technology**	Tirrell DA	Washington, DC: National Academy Press
1995	**Proteins at interfaces II. fundamentals and applications**	Horbett TA, Brash JL	American Chemical Society: Washington,DC
1995	**Self-reinforced bioabsorbable polymeric composites in surgery**	Rokkamen P, Törmälaö P	Tampereen, Pikakapio: Tampere, Finland
1995	**Biomedical applications of synthetic biodegradable polymers**	Hollinger JO	CRC Press
1996	**Biomaterials science: an introduction to materials in medicine**	Ratner BD, Hoffman AS, Schoen FJ, and Lemons JE	Elsevier Sci., New York
1996	**An Introduction to composite materials**	Hull D and Clyne TW	Cambridge University Press, Cambridge, UK
1997	**Biomechanics: circulation. 2nd ed.**	Fung YC	New York: Springer-Verlag
1997	**Protein-based materials**	McGrath KP, Kaplan DL	Boston: Birkhäuser
1998	**The chemistry, biology, and medical applications of hyaluronan and its derivatives**	Laurent TC	London: Portland Press
1998	**Biomaterials in surgery**	Walenkamp GHIM, Bakker FC	Stuttgart: New York
1998	**Design engineering of biomaterials for medical devices**	Hill D	John Wiley & Sons, New York
1999	**Basic transport phenomena in biomedical engineering**	Fournier RL	Taylor & Francis, PA, Philadelphia
1999	**A primer on biomechanics**	Lucas GL, Cooke FW, Friis EA	New York: Springer
2000	**The history of metallic biomaterials, metallic biomaterials, fundamentals and applications**	Sumita M, Ikada Y, and Tateishi T	ICP, Tokyo
2000	**Bone cements**	Kühn K-D	Springer, Berlin
2001	**Structural biological materials**	Elices M	Pergamon
2001	**Bone biomechanics, 3rd**	Cowin SC (ed)	CRC Press: Boca Raton, FL

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
2001	**Chitin: fulfilling a biomaterials promise**	Khor E	Oxford: Elsevier
2002	**Heterogeneous materials: microstructure and macroscopic properties**	Torquato S	Springer: New York
2002	**Integrated biomaterials science**	Barbucci R	New York: Kluwer Academic/Plenum
2002	**Biomaterials**	Bhat SV	Narosa Publishing House, New Dehli, India
2002	**An introduction to tissue-biomaterial interactions**	Dec KC, Puleo DA, and Bigirs R	John Wiley & Sons, NY
2002	**Bones: structure and mechanics**	Currey JD	New Jersey: Princeton University Press
2003	**Calcium phosphate bone cements: a comprehensive review**	Weiss DD	Journal of Long-Term Effects of Medical Implants, 13(1)41-47
2003	**Failure in biomaterials, in comprehensive structural integrity series, Vol. 9**	Teoh SH	Elsevier, London, UK
2004	**Engineering materials for biomedical applications**	Teoh SH	World Scientific Publishing Co. Pte. Ltd.
2005	**Biomaterials science: an introduction to materials in medicine**	Ratner BD, Hoffman AS, Schoen FJ, Lemons JE	New York: Academic Press
2005	**Surfaces and interfaces for biomaterials**	Vadgama P	Woodhead Publishing Ltd
2005	**Medical textiles and biomaterials for healthcare**	Anand SC, Miraftab M, Rajendran S, Kennedy JF	Woodhead Publishing Ltd
2006	**An introduction to biomaterials**	Guelcher SA, Hollinger JO	CRC Taylor & Francis
2006	**Mechanics of biological tissue**	Holzapfel GA, Ogden RW	New York: Springer
2007	**Cellular transplants: from lab to clinic**	Halberstadt C and Emerich DF	Academic Press
2007	**The gecko's foot**	Forbes P	London: Fourth Estate
2007	**Biomedical polymers**	Jenkins M	Woodhead Publishing Ltd
2008	**Cellular response to biomaterials**	Di Silvio L	Woodhead Publishing Ltd
2008	**Shape memory alloys for biomedical applications**	Yoneyama T, and Miyazaki S	Woodhead Publishing Ltd
2008	**Orthopaedic bone cements**	Deb S	Woodhead Publishing Ltd

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
2008	**Natural-based polymers for biomedical applications**	Reis RL, Neves NM, Mano JF, Gomez ME, Marques AP, Azevedo HS	Woodhead Publishing Ltd
2008	**Bioceramics and their clinical applications**	Kokubo T	Woodhead Publishing Ltd
2008	**Dental biomaterials: imaging, testing and modelling**	Curtis RV and Watson TF	Woodhead Publishing Ltd
2009	**Orthodontic biomaterials**	Matasa CG and Chirita M	Technica-Info Kishinev
2009	**Bulk metallic glasses for biomedical applications**	Schroers J, Kumar G, Hodges TM, Chan S and Kyriakides TM	JOM, 61, 21-29
2009	**Mechanical behaviour of materials**	Meyers M, Chawla C	Cambridge University Press
2009	**Biomaterials and regenerative medicine in ophtalmology**	Chirila TV	Woodhead Publishing Ltd.
2009	**Bone repair biomaterials**	Planell JA, Best SM, Lacroix D, Meroli A	Woodhead Publishing Ltd
2009	**Biomaterials and tissue engineering in urology**	Denstedt J and Atala A	Woodhead Publishing Ltd
2009	**Biomaterials for treating skin loss**	Orgill DP, Blanco C	Woodhead Publishing Ltd
2009	**Materials science for dentistry (Ninth edition)**	Darvell BV	Woodhead Publishing Ltd
2009	**Biomedical composites**	Ambrosio L	Woodhead Publishing Ltd
2010	**Injectable biomaterials: science and applications**	Vernon B	Woodhead Publishing Ltd.
2010	**Biomaterials for artificial organs**	Lysaght M	Woodhead Publishing Ltd.
2010	**Bioactive materials in medicine: design and applications**	Zhao X, Courtney JM and Qian H	Woodhead Publishing Ltd.
2010	**Surface modification of biomaterials: methods, analysis and applications**	Williams R	Woodhead Publishing Ltd.
2010	**Biotextiles as medical implants**	King MV and Gupta BS	Woodhead Publishing Ltd.
2010	**Novel biomedical hydrogels: biochemistry, manufacture and medical implant applications**	Rimmer S	Woodhead Publishing Ltd.
2010	**Regenerative medicine and biomaterials for the repair of connective tissues**	Archer C and Ralphs J	Woodhead Publishing Ltd.

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
2012	**Biomimetic, bioresponsive, and bioactive materials: an introduction to integrating Materials with tissues**	Santin M and Phillips G.	John Wiley & Sons, Inc.
2012	**Biomaterials: an introduction**	Park J.B.	Plenum Press
2012	**Biomimetic approaches for biomaterials development**	Mano, J.F.	Wiley-VCH
2012	**Biomaterials and stem cells in regenerative medicine**	Ramalingam M., Ramakrishna S., Best S.	CRC Press, Taylor & Francis Group
2012	**Optical biomimetics: materials and applications**	Large M.	Woodhead Publishing/Elsevier
2013	**Biological and biomimetic adhesives challenges and opportunities**	Santos R, Aldred N., Gorb S., Flamming P.	RSC Publishing
2013	**3rd edition biomaterials science – an introduction to materials in medicine**	Ratner B.D., Hoffman A.S., Schoen F.J., Lemons J.E.	Academic Press/ Elsevier
2013	**Marine biomaterials: characterization, isolation and applications**	Kim S.-K	CRC Press, Taylor and Francis Group
2013	**Biomaterials associated infection. immunological aspects and antimicrobial strategies**	Fintan Moriarty T., Zaat S.A.J., Busscher H.J.	Springer
2013	**Biomimetic biomaterials. Structure and applications**	Ruys A.	Woodhead Publishing/Elsevier
2013	**Bio-inspired asymmetric design and building of biomimetic smart single nanochannels**	Hou X.	Springer
2013	**Biomimetic biomaterials: structure and application**	Ruys A.J.	Woodhead Publishing
2014	**Biomineralization sourcebook: characterization of biominerals and biomimetic materials**	DiMassi E. and Gower L.B.	CRC Press, Taylor&Francis Group
2014	**Biomaterials innovation. Building technologies and life**	Styhre A.	Edward Elgar Publishing Limited
2014	**Biomaterials for bone regeneration. Novel techniques and applications**	Dubruel P., Van Vlierberghe S.	Woodhead Publishing/Elsevier
2014	**Essential biomaterials science**	Williams D.	Cambridge University Press
2014	**Biomaterials**	Migonney V.	John Wiley & Sons, Inc.
2015	**Biomimetic and biohybrid systems**	Wilson S., Verschure P., Mura A., Prescott T.	Springer

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
2015	**Biomaterials and their applications. SpringerBriefs in materials**	Rezaie H.R., Bakhtiari L., Öchsner A.	Springer
2015	**Biological materials of marine origin. Vertebrates**	Ehrlich H.	Springer
2015	**Biotechnologies and biomimetics for civil engineering**	Torgal F.P., Labrincha J.A., Diamanti M.V., Yu C.-P., Lee H.K.	Springer
2015	**Evolution of lightweight structures. Analyses and technical applications**	Hamm C.	Springer
2016	**Biomechanics and biomaterials in orthopedics- second edition**	Poitout D.G.	Springer
2016	**Biomimetic principles and design of advanced engineering materials**	Xia Z.	John Wiley & Sons, Inc.
2016	**Biomaterials from nature for advanced devices and therapies**	Neves N.M. and Reis R.L.	John Wiley & Sons, Inc.
2016	**Biomimetic design method for innovation and sustainability**	Cohen Y.H. and Reich Y.	Springer
2016	**Handbook of biomaterial properties**	Murphy W., Black J. and Hastings G.	Springer
2016	**Marine sponges: chemicobiological and biomedical applications**	Pallela R, Ehrlich H	Springer
2016	**Biological adhesives**	Smith A.M.	Springer
2017	**Monitoring and evaluation of bimaterials and their performance in vivo**	Narayan R.J	Woodhead Publishing/Elsevier
2017	**Comprehensive biomaterials II**	Ducheyne P., Healy K., Grainger D.W., Kirkpatrick C.J.	Elsevier
2017	**Biomaterials science and tissue engineering**	Basu, B.	Cambridge University Press
2017	**Advances in biomaterials for biomedical applications**	Tripathi A. and Melo J.S.	Springer
2017	**Biomaterials: the intersection of biology and materials science**	Temenoff, J.S.	Content Technologies Inc.
2017	**Engineering of biomaterials**	Dos Santos V., Brandalise R.N. and Savaris M.	Springer
2017	**Extreme biomimetics**	Ehrlich H	Springer
2018	**Fundamentals of biomaterials**	Hasirci V	Springer
		Hasirci N	
2018	**Functional hydrogels as biomaterials**	Li J	Springer
		Osada Y	

(continued)

Table 1.2 (continued)

Year	Title	Author(s)	Publisher
2018	**Liquid metal biomaterials**	Liu J	Springer
	Principles and applications	Yi L	
2019	**Biomaterials science and technology: fundamentals and developments**	Dahman Y	CRC Press

of proposals are oriented to application fields (i.e. Orthopedic, Cardiovascular, Dental, Wound Healing Plastic Surgery, Tissue Engineering, Neurology, Ophthalmology etc.). I personally accept the version that is based on the nature of biomaterials (see Table 1.1.) where "biological materials" definitively belong to the "natural biomaterials" (Fig. 1.2).

1.3 Conclusions

Biomaterials research is one of the fastest developing fields. This is to be proved by literature analyses (Table 1.2) including patents overviews, review papers (Elisseeff 2019) and, especially diverse biomaterials market reports where the need in biomaterials is easy to see. The progress of the global biomaterial industry depends on situation with cardiovascular diseases, the increasing demand on hip and knee replacements and, principally, on growing geriatric population. Also modern technological advancements will influence this process.

For the better understanding, I take the liberty to represent some remarks from selected citations to be found in corresponding recent market reports as follow:

> The biomaterials market is projected to reach USD 149.17 Billion by 2021 from USD 70.90 Billion in 2016, at a CAGR of 16.0%. (Source:https://www.marketsandmarkets.com/Market-Reports/biomaterials393.html?gclid=EAIaIQobChMIyq68sMS84gIVh8x3Ch2gOQMwEAAYAiAAEgJFKfD_BwE)

> The global biomaterial market size is expected to reach USD 250.4 billion by 2025, according to a new report by Grand View Research, Inc., exhibiting a CAGR of 14.7% during the forecast period. (Source: https://www.grandviewresearch.com/press-release/global-biomaterials-market)

> The global biomaterial market size is projected to cross $175.9 billion by 2022, growing at a CAGR of 16.1% during the forecast period. (Source: https://www.psmarketresearch.com/market-analysis/biomaterials-market)

> Global Biomaterials Market was valued US$ 70.98 Bn in 2017 and is expected to reach US$ 251.28 Bn by 2026, at a CAGR of 17.12% during a forecast period. (Source: https://www.maximizemarketresearch.com/market-report/global-biomaterials-market/20349/)

> Global biomaterials market was valued at USD 82.6 billion in 2017, and is likely to expand to USD 215.3 billion by 2024, witnessing a CAGR of 14.4% over the forecast period. (Source:https://www.marketwatch.com/press-release/global-biomaterials-market-to-witness-a-cagr-of-144-during-2018-2024-2019-02-01)

Thus, the tendency is clear. Two biological materials of marine origin are also constantly under observations. I mean marine collagens and chitin/chitosan. For detailed information see: https://marketresearch.biz/report/chitin-market/request-sample/

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Part II
Biomineralization and Biominerals

Chapter 2
Biominerals

Abstract Biominerals can be found usually in the neighbouring environment of the organism or within its body. The natural selection is responsible for the shaping of the skeleton's functions and forms, as well as for the physiological manners, by which minerals precipitation is possible. The expressions of these skeleton formations and metabolic pathways were not changed during evolution. Majority of marine invertebrate's biomineralized tissues can be assumed as composites, comprising an organic matrix of biological origin and micro- or nanosized crystalline or amorphous minerals. In present part, the broad variety of biominerals formed by marine invertebrate's origin is presented and discussed. Redear can find a comprehensive description of marine biominerals composed of such elements as: Ca, Mg, Ba, Fe, V, Sr, B, Ti, Cu, Zn, Mn, Ge as well as Si.

Nowadays, one of the most fascinating and challenging scientific directions that is becoming more and more popular is the formation of biominerals using the organic templates. However, these biominerals do not differ from classical minerals and meet the typical "mineral" definition, although they could have different morphologies and possess special inputs to common life forms (Skinner 2005; Skinner and Ehrlich 2013).

Until 1900s, only ten minerals have been found in the living creatures, but after paper published by Heinz A. Lowenstam (1962a) where the existence of magnetite in chitons was evaluate, the situation has been changed. Previously chemists thought that due to the relatively high hardness of magnetite, it can be only formed at very high temperatures and pressures. In this study, Lowenstam proved that the magnetite plays crucial functional role in chiton radula. This discovery has resulted in finding many new biominerals, with various chemical composition, that can be found in the book by Lowenstam and Weiner (1989).

In this book, 38 "typical" biominerals of metazoan origin are described. During formation of biominerals the Ca, Zn, Ba, K, Cu, Mn, Mg, Ni, Na, Fe, Sr, Si, and Pb cations, have been determined. These cations usually exist as sulphides hydroxides, oxides, or as carbonates, phosphates and sulphates. Interestingly, the same classifi-

H. Ehrlich, *Marine Biological Materials of Invertebrate Origin*,
Biologically-Inspired Systems 13, https://doi.org/10.1007/978-3-319-92483-0_2

cation for biominerals as in the case of minerals has been proposed (Skinner 2005). In metazoans, representatives of various minerals classified in the 78 different classes based on the anionic constituents, can be found (Gaines et al. 1997).

Depending on the metabolism of living creature biominerals can be accumulated within the organism or within its immediate environment, or both (Skinner 2000). The natural selection is responsible for the shaping of the skeleton's functions and forms as well as for the physiological manners, by which minerals precipitation is possible. The expressions of these skeleton formations and metabolic pathways were not changed during evolution (Knoll 2003). The broad diversity of biomineralizers, as reviewed by Ehrlich et al. (2008), is related to the fact that 128,000 molluscs species, around 700 calcareous red, green and brown algae species, as well as over 300 deep-sea benthic foraminifera species, approximately 800 corals species, more than 5000 sponges species (including 525 glass sponges species), as well as 200,000 species of diatom have been reported (Mann and Droop 1996).

2.1 Biominerals of Marine Invertebrates Origin

2.1.1 Calcium-Based Biominerals

Due to millions years of evolution, calcium-based marine invertebrate's skeletons have been optimized to physically support and physiologically maintain various types of tissues encompassing a diversity of functions (Green et al. 2002) (Figs. 2.1

Fig. 2.1 Crustaceans antennule represent typical example of calcium carbonate-based structure

Fig. 2.2 Representatives of Calcarea sponges contain skeletons made of calcium carbonates

and 2.2). Due to the plenty of brilliant experimental papers, as well as books and reviews published during last 120 years (Herdman 1884; Dreschel 1896; Leitmeir 1910; Vinogradov 1933; Chave 1954; Lippmann 1973; Milliman 1974) dedicated to the calcium carbonate (Dhami et al. 2013; Flores and Livingston (2017) and calcium phosphate biominerals of marine invertebrate origin (see e.g. publications by listed in my previously published reviews (Ehrlich et al. 2008, 2009), here I intend to focus reader attention on the very rare biominerals, which have received less attention of the scientific community, but play important role as the basic materials for skeletal constructs of various marine invertebrates.

2.1.1.1 Calcium Oxalate (Weddellite)

In his pioneer work, Cramer (1891) proved chemically that the weddellite ($CaC_2O_4{\cdot}2H_2O$) mineral occurred as constituent of *Bornetella sphaerica* marine algae skeletons. This mineral was showed using scanning electron microscope for the first time by Flajs (1977a, b) in the in *B. nitida* (Dasycladales), he also demonstrated that it is not a contaminant, but a part of the algae skeleton. Twenty years later it has been proven by Berger et al. (1997) that the skeletal structures of above mentioned specimen also contain $CaCO_3$ in form of aragonite; Mg- and Sr-based carbonates have been also detected.

In the gizzard plates of the deep water gastropod *Scaphander cylindrellus,* weddellite has been recognized (Lowenstam 1968), what for the first time proved no pathological nature of the deposition of this mineral within the body of this animal species. Lowenstam discovered weddellite in the Weddell's Sea sediments, supporting the earlier assumption that, the weddellite is an authentic component of the sediments located in Antarctica. Additionally, both the anhydrous calcium carbonate phase and weddellite were recognized in mollusca and as constituent in the ascidian tunicate *Molgula manhattensis* rental sac (Saffo and Lowenstam 1978). The existence of weddellite together with urate in the concretions implies a similarity to human kidney stones, however, the *Molgula* concretions do not appear to be pathologic precipitates. Recent overview concerning Ca oxalates has been presented by Ruiz-Agudo et al. (2017).

2.1.1.2 Fluorite (CaF_2)

Fluoride (Fig. 2.3) is usually deposited in the skeletons of both invertebrates as well as vertebrates. However, no or slight accretion in edible, tender tissues, aside from fish skin (Wright and Davison 1975).

The presence of fluorine in invertebrate skeletons as well as in calcareous marine sediments has been reported (Monniot et al. 1995). The existence of fluorine in all shells of molluscs has been described by Vinogradov (1953) then he discovered that, the concentration of this element in freshwater species is lower than in marine ones. This element was also found in various calcium- based formations of marine invertebrates: mussels and crustaceans (Wright and Davison 1975), holothurians (Lowenstam and Rossman 1975); polychetes (Vovelle et al. 1989); ascidian spicules (Monniot et al. 1995).

Based on the results obtained from XRD, it was proved that, in some tectibranch gastropods, fluorite can be found in the gizzard plates, additionally this element also build statoliths obtained from the marine mysid crustaceans (Lowenstam 1968) The

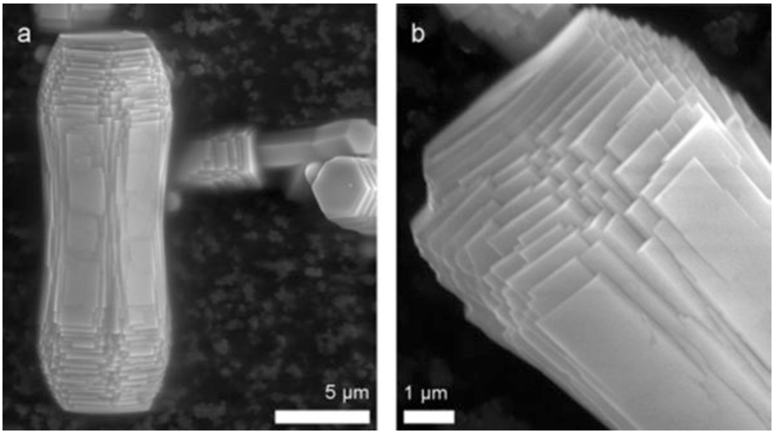


Fig. 2.3 Fluorapatite crystals (**a**) possess typical features on the crystal surface (**b**). (SEM images courtesy Paul Simon)

deposition of fluoride within the marine invertebrates' hard tissues differs in relation to its location in the oceans (lower in the bathyal zones and widespread in shelf waters). Interestingly, shrimp *S. spiritus* from the genus Schistronysis is able to precipitate 3.60 µg fluoride and 3.79 µg calcium. This is equalled 2788 mg ambient water or 9.3 mg CaF_2, respectively. With reference to the statocyst, the concentrations factors in relation to the mass of water are 183.000 or 610 respectively. Therefore, the total content of fluoride per weight of dry body is so high (4500 ppm) (Wittmann and Ariani 2008).

2.1.1.3 Calcium Sulphate-Based Biominerals

The identification on calcium sulphate dihydrate as biomineral forming statoliths in scyphozoan medusa (*Aurelia* sp.) was reported for the first time in 1968 by Spangenberg and Beck. In 1985 Chapman (1985) proved that statoliths from cubomedusan are comprised of gypsum, he also showed the lamellated nature of statolith; although it was assumed as the gypsum cleavage. Interestingly, the unique biomineral – bassanite (calcium sulphate hemihydrate, $CaSO_4 \cdot \times 1/2\ H_2O$) has been found in the statoliths obtained from various scyphozoan medusa (*Cyanea capillata*, *C. lamarckii Aurelia aurita*,– Semaeostomeae; *Rhizostoma octopus* – Rhizostomeae; *Periphylla periphylla* – coronata;) (Tiemann et al. 2002; Becker et al. 2005).

2.1.2 Magnesium-Based Biominerals

High amount of magnesium carbonate can be found in animal organisms (Chave 1954) as well as calcite skeletons of marine algae (Böhm 1973). The magnesium content is related to the temperature of water and this percentage differs distinctly in carious taxonomic entities (Milliman et al. 1971). The Corallinaceae is the best example, they possess almost linear correlation of magnesium content with temperature and the highest concentration of magnesium (up to 30% $MgCO_3$). It is possible that, the changes in the rate of algae growth can result in changes in the concentration of magnesium in the calcite by affecting the formation of a template or organic matrix diffusion and a rate of magnesium diffusion through the cell wall (Kolesar 1978).

Interestingly, magnesium is a key element in geotaxis of some ctenophores or comb jellies (Aronova 1987). In most animals, the main chemical element of the otolith apparatus is known to be calcium that occurs in form of carbonates, sulphates, phosphates and fluorites. Additionally, cations of following elements: Mg, Na, K, Fe, Sr, P, Be, etc. (Vinnikov et al. 1981) are found as well. Based on the X-ray structural analysis reported by (Aronova 2009) the highest percent of electrolyte composition of the comb jelly *Beroe cucumis* otolith apparatus, is in magnesium and calcium carbonates, where the highest percentage belongs to Mg—44.8%.

For the corals, Mg is also essential constituent of their skeletal constructs (Dodd 1967). Therefore, the ion beam microprobe imaging proved that in the reef-building coral *Pavona clavus* aragonite skeleton, the magnesium distribution is related to layered structure of the aragonite fibers, which can have even ten times higher concentration of magnesium and is associated with the fine-scale skeleton arrangement (Meibom et al. 2004). This is an evidence that the formation of magnesium in all structural components is over biological control. These authors proposed that coral can use magnesium to regulation of the formation of the various crystal components of the skeleton.

Brucite (magnesium hydroxide) is the next form of magnesium-based biominerals, which has been found within marine organisms. The brucite can be form in nature only in environment with low concentration of CO_2 and high pH (Noreen and Holmes 2006; Nothdurft et al. 2005). However, the brucite biomineralization is poorly studied, in the literature examples of brucite formation in red algae (Schmalz 1965) and in nudibranch spicule (Cattaneo-Vietti et al. 1995) can be found.

Identification of brucite within the skeletons of various coral's genera ensures data about processes occurring within their carbonate skeletons, under the coral's living surface. It was reported that, brucite is located within green bands that can be found in the skeletons of some *Montastraea faveolata* corals (Nothdurft et al. 2005). Moreover, it has been reported (Tesson et al. 2008) that some of the marine diatoms developed mechanism for the formation of brucite – as a defending mechanism against alkalization of the living environment. The alkalinization of the growth medium of the diatom *Phaeodactylum tricornutum* results in formation of microbialite. The existence of brucite crystals around the cells was observed after 4 days of the diatoms growth, due to an alkalization of medium by photosynthetic activity of the microalgae. The authors proposed that the sorption of magnesium onto cell walls of diatoms is related with the existence of carboxylated and sulphated polysaccharides and has biologically-mediated nature. It was proposed that carboxyl groups can act as active site of Mg sorption.

2.1.3 Barite-Based Biominerals

Barite is formed when zooplankton consumed the surface plankton, and in consequence constitutes microenvironments where the precipitation of barite is caused by it supersaturation. The surface plankton is able to concentrate barium in their organic matter and shells, therefore their decomposition in the presence of sulphate is responsible for the formation of supersaturated conditions essential for the precipitation of barite (Puchelt 1972; Hubert et al. 1975). Important role in precipitation of barite have surface protist Acantharia, that form their shells from celestite (strontium sulphate) (see below). Xenophyophorea build exoskeletons from barite and they are one more available barite source, which may be agglutinated from falling barite crystals in the ocean biomineralizes (Schulze and Thierfelder 1905; Hopwood et al. 1995).

Based on the electron diffraction, X-ray diffraction as well as electron microscopy investigations of the crystals of barium sulphate found in three species of xenophyophore (*Reticulammina labyrinthica, Aschemonella ramuliformis, Galatheammina lamina*) located at abyssal and bathyal depths in the north-eastern Atlantic (Gooday and Nott 1982) two different crystalline forms have been reported. The first formation of crystals have small particles (<0.5 μm) with poorly developed crystalline structure, the second were tablets-shaped, bigger in length (~2 μm) and hexagonal or rhombic in outline.

Interestingly, it was found that near the warm sulphur spring system in Northwestern Utah (USA) the diatoms participate in precipitation of barite in the calcified microbial mats (Bonny and Jones 2007). Diatoms are able to create microbial mats together with sulphate reducing bacteria and cyanobacteria. Usually they reproduce in the saline, warm (~48 °C), sulphur-rich and bicarbonate spring water. Microcrystalline barite is usually related to the frustules of diatom, because barite can replace silica in diatoms, and commonly precipitates in haloes surrounding the diatom bundles and then fulfils the diatom frustules. Barium is bioaccumulated in diatom cells, and extracellular polysaccharides with barium can adsorb onto diatoms structures, and in consequence is immediately reliable for the primary barite precipitation at the Stinking Hot Springs as well as intermediate in the saturation of barite within lithifying microbial mats (Bonny and Jones 2007). This phenomenon suggests the marine diatoms living in barium rich environmental niches might be able to produce barium containing frustules.

2.1.4 Fe-Based Biominerals

Numerous invertebrate representatives use crystallline metal-based biominerals to harden their cutting as well as grinding gear. The teeth of marine molluscs, are characterized by presence of various Fe-based biominerals (Webb et al. 1990). The chitons (Polyplacophora) and limpets (Gastropoda: Acmaeidae, Patellidae) are the most popular representatives in these groups, having mineralized teeth. The second lateral tooth in chitons is mineralized mostly by magnetite (Fe_3O_4) (Lowenstam 1962a). Although, lepidocrocite (γ-FeOOH), ferrihydrite ($5\ Fe_2O_3 \times 9\ H_2O$), goethite (α-FeOOH), apatite (crystalline calcium phosphate) as well as maghemite (γ-Fe_2O_3) also occur (Lowenstam 1967; Towe and Lowenstam 1967; Kirschvink and Lowenstam 1979; Mizota and Maeda 1986;Kim et al. 1989; Webb et al. 1989; St. Pierre et al. 1992; Shaw et al. 2008). Biomagnetite in marine vertebrates has been recently reviewed by Ehrlich (2015).

Only opal ($SiO_2 \times nH_2O$) and goethite impregnated the radular teeth In limpets (Lowenstam 1962b; Mann et al. 1986; Sone et al. 2005). In *Falcidens* sp. (Caudofoveata) the existence of hydroxyapatite and amorphous iron oxide within the highly modified radular apparatus has been described (Cruz et al. 1998). Recently, results of pulsed-laser atom-probe tomography show 3D chemical maps of nano-crystalline magnetite (Fe_3O_4) mineral which covers organic fibres with a

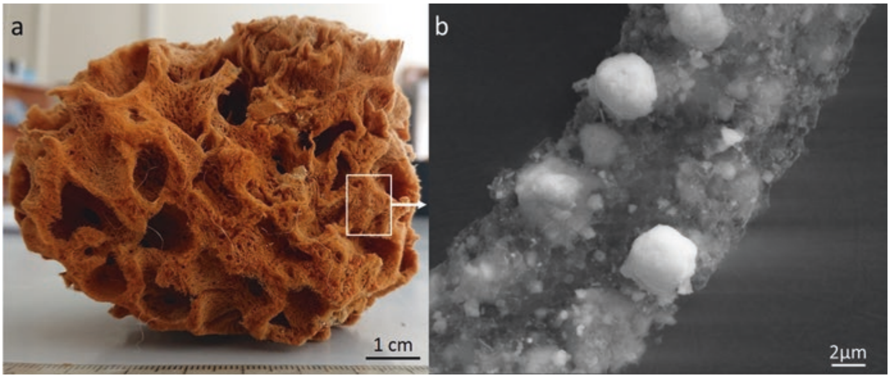


Fig. 2.4 Rusted keratosan demosponge *Hippospongia communis* (**a**) is habituating in Middeteranian Sea. SEM imagery of he surface of individual spongin fibres shows that they are tightly covered with Fe-based biomineral nanoparticles (**b**)

diameter of 5–10 nm in the tooth of the *Chaetopleura apiculata* marine mollusk (Gordon and Joester 2011). Nowadays, the iron-related biomineralogy of radula in diverse molluscs is well represented (for review see Sounders et al. 2009; Weaver et al. 2010; Nemoto et al. 2012; Wang et al. 2013; Emmanuel et al. 2014; Grunenfelder et al. 2014; Ukmar-Godec et al. 2017).

Marine sponges (Porifera) are recognized as organisms building their fascinating skeletal structures using calcium carbonate or silica. However, within the phylum Porifera the first determination of the formation of hard tissue as well as the first appearance of crystalline iron mineralization and among the Keratosa demosponges have been reported (Towe and Rützler 1968). Thus, reddish-brown microparticles integrated into the spongin fibers of some keratosa sponges are composed of lepidocrocite (Fig. 2.4). It was proven that these small iron granules are fixed with fiber skeleton of sponge and are not of foreign origin (Towe and Rützler 1968). In 1988, Vacelet and co-workers detected calcite, lepidocrocite and goethite (α-FeOOH) within spongin fibers of *Spongia officinalis* bath sponge (Fig. 2.5). However, until these days the biological importance of this type of biomineralization remains unknown.

2.1.5 Vanadium (Biomineral?)

We still do not understand the physiological role of vanadium, but in the nature we can found many examples of vanadium biomineralization. For example several ascidians are able to accumulate vanadium at concentrations ten million times higher than those in seawater (Ciereszko et al. 1963; Brand et al. 1989; Michibata et al. 2003). The following hypothesis were recently discussed (Ciancio et al. 2004; Odate and Pawlik 2007): (i) vanadocytes may produce the tunic cellulose; (ii) in

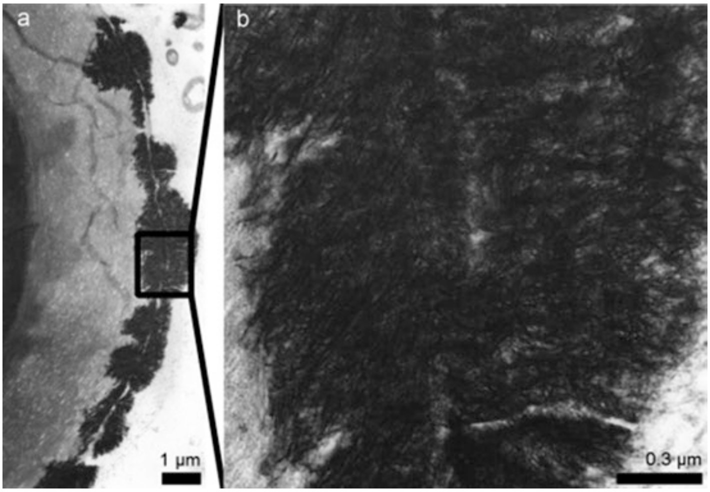


Fig. 2.5 SEM images: collagen fibers mineralized with iron. (Adapted from Vacelet et al. 1988)

ascidians vanadium can be an antimicrobial agent or may be responsible for their protection against fouling, (iii) in environment with low oxygen tension, vanadocytes containing vanadium may act as a trap for oxygen. Although, majority of the proposed explanations do not appear to be appropriately evidenced. Consequently, still, we cannot find any explanation to evaluate the role of vanadium in the ascidians physiological processes. The solution of this clue can provide more information about this unique deposition of vanadium in only one class of marine organisms. Vanadium related biomineralization inspired researchers to design corresponding nanoparticles for practical application. For example, it was reported that vanadium pentoxide nanowires function like vanadium haloperoxidases found in nature, and can be used to prevent marine biofouling (Natalio et al., 2012).

2.1.6 *Strontium-Based Biominerals*

Radiolarians and molluscs are recognized as major invertebrates involved in strontium cycle in the world ocean (Odum 1949, 1951a, b).

Acantharians secrete cystic and skeletal structures (Bütschli 1906) comprised of celestite ($SrSO_4$), therefore, from the chemical point of view, they are exceptional group of organisms (Odum 1951a; Veizer 1978; Bernstein et al. 1987, 1992). Bernstein et al. (1987) proved that acantharians are important for the flow of the strontium in the oceans. Taking into account that previous investigations shown Ba as a major component in acantharian celestite (Rieder et al. 1982; Bernstein et al. 1992) it has been hypothesised that acantharians are the key players in the biogeochemical cycles of both Ba and Sr (Bernstein et al. 1998).

However, strontium together with a traces of barium were found in the *Remanella* species, too (Rieder et al. 1982). Recently, the report on Sr-rich biominerals within coccolithophores (*Scyphosphaera* and *Pontosphaera*) excreting very large scale coccoliths have been published (Hermoso et al. 2017). The occurrence of Sr-rich coccolith species in the geological record should be important for palaeoceanographic reconstruction.

2.1.7 Boron

Boron is recognized as an abundant and an essential trace element in the ocean (Harriss 1969). Mostly procaryotes possess ability to insert boron in their metabolism, however it also plays a significant role as carbohydrate cross-linker in stabilizing algal (i.e. diatoms) (Lewin 1965, 1966a) cell walls (Black and Mitchell 1952). The biological activity of boron has been recently reviewed (Pizzorno 2015).

The role of boron within molluscs shells remains unknown. Interestingly, there is a close relation between salinity of marine water and boron concentration. Molluscan shells grow by incremental accretion and preserve within them geochemical records of their environments. This suggests that boron can be used as a measure of salinity. By analysis of the B content is shells of selected molluscs it is possible to reconstruct the environment salinity (Roopnarine et al. 1998).

2.1.8 Titanium-Based Biominerals

Titanium is an element that can be bioaccumulated by diatoms (Riley and Roth 1971), which are able to incorporate traces of Ti (1254 ppm) of titanium into siliceous frustules (Martin and Knauer 1973). Jeffryes et al. (2008) successfully incorporated metabolically titanium to frustules of *Pinnularia* sp. by controlled feeding with soluble titanium and silicon species. Interestingly, formed frustule preserved the highly organized structure. Titanium was favourably deposited as a nanophase coating the base of each frustule pore. To achieve the maximum level of titanium incorporation, the co-uptake of titanium and silicon was required. This is an example of forced biomineralization.

Accordingly, to my best knowledge, only one titanium-based skeletal structure was found in natural environment. This structure was reported in one species of foraminifera, *Bathysiphon argenteus*, which employs a titanium mineral in its test (Cole and Valentine 2006).

2.1.9 Copper-Based Biominerals

Formation of copper-based biominerals by marine invertebrates is unfortunately driven by the contamination of the environment wih heavy metals (Bryan 1976). The regulation of the copper biosorption is a complex phenomenon and is closely related with water temperature and salinity. It has been shown (Ozoh 1994) that accumulation of Cu ions by *Hediste diversicolor* at low temperatures (12 °C) increase with low salinity. In contrast, at temperatures 17–22 °C bioavailability of copper to the worms increased irrespective of salinity gradient and can reach even 784 µg/g dry weight.

Copper-based biminerals have been identified in the form small mineralized fibers made of atacamite ($Cu_2Cl(OH_3)$) in the *Glycera dibranchiata* jaws (Gibbs and Bryan 1980; Gibbs et al. 1981, Lichtenegger et al. 2002). However, the jaws system is more complex and contains of ~40 wt % protein (glycine and histidine rich), 40 wt % melanin, and <10 wt % metals including: Zn, Fe, and unmineralized Cu compounds as well (Lichtenegger et al. 2005). The unmineralized metals are concentrated on the tip of the jaw.

Overall, as reported recently (Moses et al. 2008), *Glycera* jaws are composed metals and minerals, the latter mostly in the form of unmineralized copper (Cu) with small amounts of mineralized atacamite fibers. The Cu in all forms is concentrated at the tip of the jaw, typically within ~30 µm of the surface.

2.1.10 Zinc-Based Biominerals

The biosynthesis of Zn-containing biominerals has been suggested as pathway for the Zn detoxification, especially in plants where Zn-substituted calcite, Zn-containing silica and phosphate have been occurred (Sarret et al. 2006). The biomineralization of hydrozincite and Zn-silicate in microalgae and bacteria have been reported in long-term adapted biocenose of Naracauli stream (Sardinia). The mechanism governing the formation of these Zn-based biominerals at the molecular level remains unclear. It is suggested that hydrozincite biomineralization is an example of epicellular biomineralization where electronegatively charged extracellular polysaccharides show templating activity (for details see Podda et al. 2014; Wanty et al., 2013; De Giudici et al. 2014).

Similarly to copper, zinc is a main inorganic constituent in jaws of the Nereid (Bryan and Gibbs 1979). For example, the content of Zn in the jaws of *Nereis versicolor* ranged between 0.5% and 2.4% of the dry weight (Howard and Brown 1983). It has been proven that mechanical properties of jaws (hardness and stiffness) strongly depend on the local concentration of zinc, and this confirms that zinc plays structural role (Lichtenegger et al. 2003; Birkedal et al. 2005; Khan et al. 2006). The presence of zinc anions is always accompanied with presence of chlorine. Results made with use of the X-ray absorption spectroscopy show that zinc and chlorine ions are coordinated by histidine (Broomell et al. 2006). Broomell et al. (2006) proved by using nanoidentation that Zn plays a crucial role in the mechanical properties of histidine-rich *Nereis* jaws. Removal of Zn by chelating agents from jaws resulted in 65% decrease of the hardness.

Zinc silicate, is another zinc-based biomineral investigated in marine invertebrates, especially in the diatoms. For details, see studies by Ellwood and Hunter (2000) and Jaccard et al. (2009).

2.1.11 Manganese Oxides

Global biogeochemical cycle of manganese in aquatic environments is driven generally by microorganisms (Keim et al. 2015). Mn (II)-including oxidizing bacteria and fungi are the key players in ancient and modern biogeochemical environments. Mn (II)-oxidizing microorganisms have the unique ability to catalyse the oxidation of soluble Mn (II) to insoluble manganese oxides of the general formula MnO_x (where $x = 1, 2$). Therefore, marine organisms contribute to formation of minerals in polymetallic nodules or hydrothermal vents (Wang and Müller 2009a, b), where free-living and biofilm-forming bacteria provide the organic template for Mn-based mineral deposition (Glasby 2006). It was also reported that some bivalve molluscs can accommodate manganese in its aragonitic shell (Soldati et al. 2016).

2.1.12 Germanium-Based Biominerals

Germanium is a trace element in the Earth's crust (about 1.6 ppm Ge crustal average). Interestingly, plants are able to replace B with Ge, when boron is missing, but some signs of deficit will be visible with a lag period of approximately 1–3 weeks (Rosenberg 2008). Germanium practically does not participate in biogeochemical reactions, and in addition in nature Ge is usually found in the form of hydroxyl acids and germanium tetravalent complexes. Naturally, germanium exists as an organic compound in form of mono- and dimethylgermanium. It was assumed that these compounds are synthesised during microbes activity in places rich in germanium minerals, and then are excreting to the rivers and finally to the seas (Rosenberg 2008).

Siliceous organisms treat germanium ions as if they were a very heavy silicon isotopes (Lewin 1966b). Performed studies showed that Ge radioisotopes were mostly incorporated into the diatom cell with low Ge/Si ratios (0.01) (Azam et al. 1973).

At low concentrations, the metabolic pathway of Ge simulates the silicon pathway, and influences the uptake and transport of silicon because, at high concentrations it is working as a typical competitive inhibitor. This kind of behaviour has been reported in all organisms that undergo silification and which need silicon as a fundamental element. For instance, the influence of the presence of germanium on the siliceous spicules secretion in *Spongilla lacustris* the freshwater sponge was studied by exposing of hatching gemmules to different germanium concentrations in the presence of silicon (Simpson 1979). Then, it was proved that germanium acid at concentration levels of 1–25 mg l^{-1} is able to destroy the formation of siliceous spicule in freshwater sponges (Simpson et al. 1983).

The results of qualitative and quantitative analysis show that a molar ratio of [Ge]/[Si] equalled 1.0 totally inhibits the deposition of silicon. Middle ratios (0.5, 0.1, 0.01) lead to obtain shorter, thinner and fewer spicules, with relatively less microscleres in a short bulbous megascleres. The length of bulbous megascleres decreases with increasing of [Ge]/[Si] ratio as well as increase of [Ge]/[Si] ratio lead to increase size of the bulb. This germanium effect may be defined by its capability to detach the growth of the surrounding silicalemma from the growth in length of the axial filament. The excess of silicalemma is formed in these conditions because the silica is able to deposit in short spicules in the form of bulbs. Also, Ge exposure terminates lorica formation and coastal strip synthesis in choanoflagellates, resulting in disruption to cytokinesis and fatal build-up of biosilica (Marron et al. 2016).

Results described above are encouraging to use sponges for the biotechnological development of Ge-based biominerals.

2.1.13 Silica-Based Biominerals

In nature, the bio-constructs based on silica are widely distributed (Figs. 2.6 and 2.7).

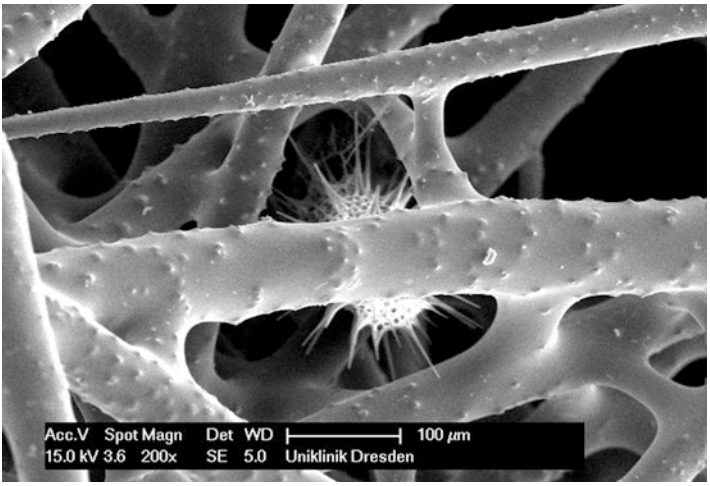


Fig. 2.6 SEM image: siliceous radiolarian within siliceous skeletal framework of glass sponge

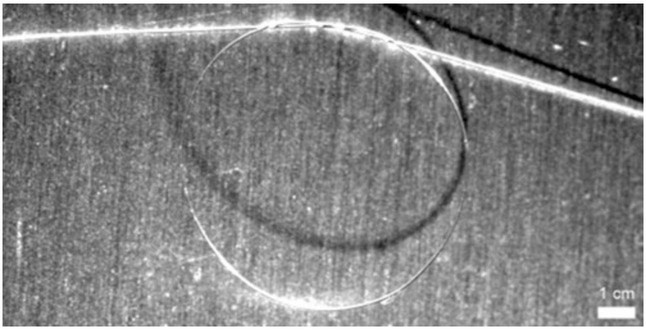


Fig. 2.7 Highly flexible anchoring spicule of glass sponge *Hyalonema sieboldin*

Thanks to the Si supersaturation in the geothermal springs, we know that microorganism's cell membranes can act as seed crystals for Si precipitation (Sommer et al. 2006). In the lumens, cell walls and intercellular voids of plants, silicic acid which is actively or passively taken up from soil solution usually is precipitated as amorphous silica (Ma et al. 2006). On the surface of hulls, leaves, and stem, the double layers of silica-cuticle and silicacellulose were observed (Yoshida 1965). Silicon can also be found in cystoliths of some plant which are covered in a sheath of siliceous material (Watt et al. 1987). Silica in plants possess variety of functions including lending the plant structural rigidity by supporting the shoot, giving lodging (falling over) resistance giving mechanical strength and rigidity to leaves, for review see Neethirajan and co-workers (2009).

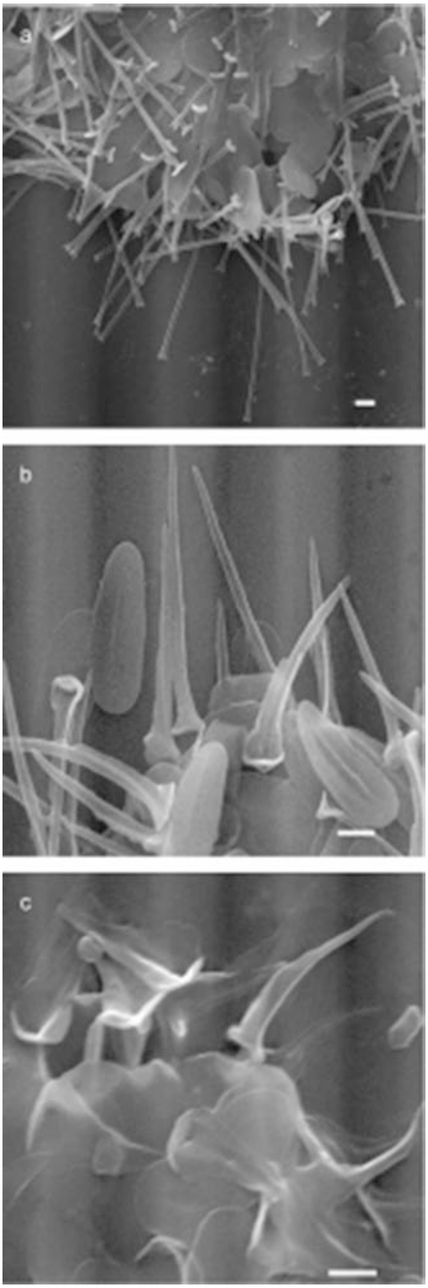
Mechanisms of plant biosilicification are excellently reviewed by Kumar et al. (2017). Thus, for details reader is referred to these articles.

In soils, skeletons of diatoms as well as sponge's spicules, the major component of biogenic silica is the phytoliths (phytogenic silica) (Clarke 2003).

Siliceous spicules and skeletons can be found in different Protozoa (Fig. 2.8), e.g. silicoflagellates (Ehrenberg 1830), sarcodines (Dujardin 1841) and radiolarians (Hertwig 1879). However, in other animals the existence of silica-based skeletons is not only restricted to the demosponges spicules and glass sponges (Uriz 2006); they can be found in the boreal copepod's opalized mandibular blades (Sullivan et al. 1975) as well as within the epidermis of some brachiopod larvae where silica form the micron-scale tablets (Williams et al. 2001). Siliceous microparticulated structures with size 50–100 μm were also identified within the Henze precipitate of ascidians (Monniot et al. 1995). Both, the occurrence of Si and its role in ascidian blood is still unclear.

The IR spectroscopy performed by Lowenstam (1971) shown the presence of silica in form of opal located in radula teeth of the Patallecea (Mollusca, Gastropoda). The opalized plate-like structures were found only in the families Acmaeidae and Lepetidae. Additionally, silica-based penial structures with various morphologies and specular formations within cuticle were also discovered within several representatives of mollusca (e.g. *Onchidiella celtica*) (Labbé 1933a, b, 1934a, b).

Fig. 2.8 SEM imagery: The heliozoan *Placocystis* sp. possess highly special scales and spines (bar scales – 250 nm (**a**), 300 nm (**b**, **c**). (Images courtesy Ludmila Gaponova)



The coccolithophores (Calcihaptophycidae) produce an extracellular covering of ornate calcium carbonate plates (coccoliths) and are major contributors to biogenic calcification in the ocean. Surprisingly, Durak and co-workers (Durak et al. 2016) discovered that an expanded family of diatom-like silicon transporters (SITs) are present in *Prymnesium neolepis* coccolithophores.

Biosiliceous constructs with sophisticated architecture formed by marine glass sponges (Figs. 2.3 and 2.9.) are described and discussed in Chap. 8).

2.2 Conclusion

To sum up, most of the biomineralized tissues are complex biocomposites combining organic biomacromolecules and amorphous or crystalline inorganic phases (mostly SiO_2 or $CaCO_3$) on various hierarchical levels, from nano- to macroscale (Fig. 2.10). During the biomineralization, the organic matter behaves differently as cooperative modifier, nucleator, or as the mineral ions template. The properties of pure minerals are completely different than the properties of obtained tissue. The organic matrix precludes the cracking of the mineral and the rigid mineral averts the organic matrix (lipids, peptides, proteins, polysaccharides) from yielding (Treccani et al. 2003). However, we still do not know why several marine organisms use as a structural material germanium or silica instead of goethite or calcium carbonate. Perhaps, it is related with external environmental factors, physico-chemical peculiarities of biomacromolecules produced during different stages of ontogenesis?

Unfortunately, chemical contamination of the oceans can leads to formation of new, unknown and “bizarre” forms of biominerals, in response to the environmental stress. Example of such phenomenon, is presented on Fig. 2.5. SEM image presents the formation of crystalline iron-based mineral on collagenous nanofibers of marine sponge living in environment with high concentration of Fe ions.

I support the proposal of Livingston et al. (2006), which showed that the developmental and genetic mechanisms of biomineralization can be repeatedly employed even if the biomineralized structures per se occurred independently in various lineages. This phenomena is likely one of the most popular theme in modern materials science biomedicine, biomimetics and geochemistry (Estroff 2008). The following chapters summarize the fundamental knowledge available for the general and alternate organic templates with reference to both silicification as well as calcification in vitro and in vivo, and allow to put attention of the field of biological materials of marine origin.

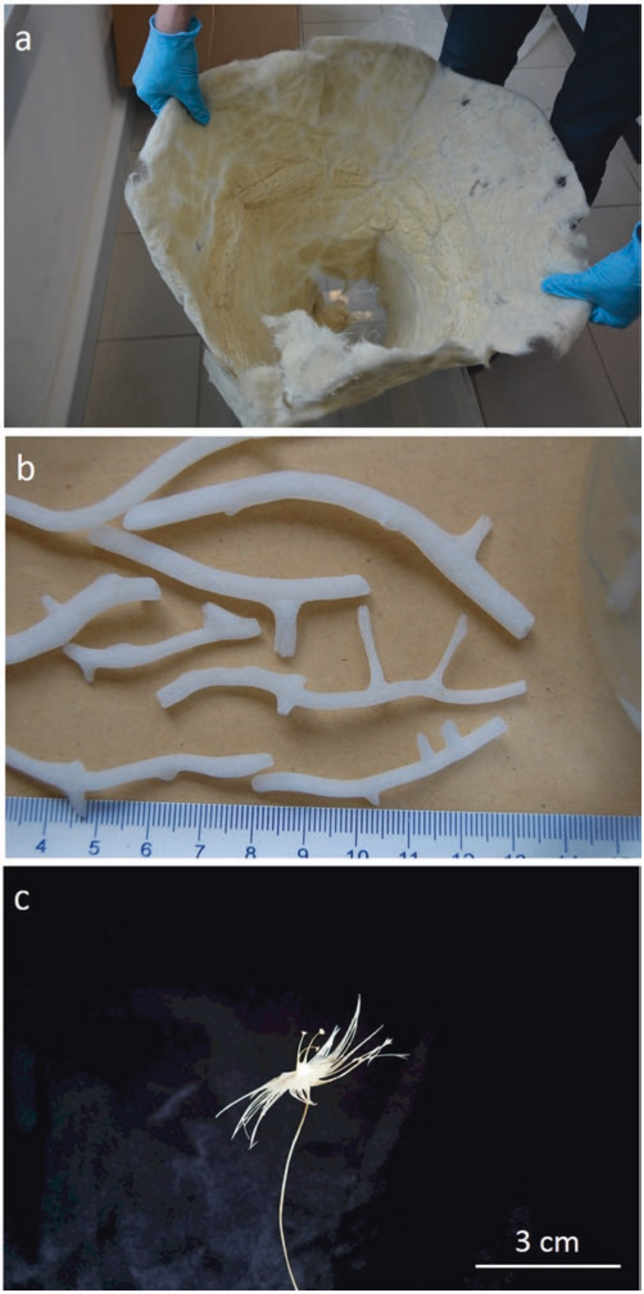


Fig. 2.9 Glass sponges (**a**, **b**) and demosponges (**c**) possess broad variety of silica-based hierarchical skeletal constructs

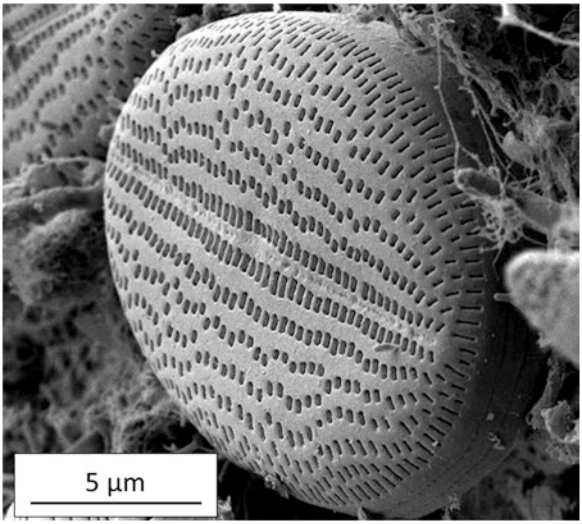


Fig. 2.10 Microarchitecture of siliceous marine diatom is well visible on this SEM image

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Chapter 3
Biomineralization

Abstract Usually during biomineralization, organisms gather the precursors (such as ions of metals) essential to formation of biominerals directly in their natural environments. Marine invertebrates both fossil and recent represent amazing sources of biominerals which mostly arose at the early stages of evolution. Consequently broad diversity of hypothesis and speculations concerning the mechanisms of biomineralization of amorphous and crystalline phases within biominerals has been established during last 50 years of intensive research. The organic matrix has a fundamental role in biomineralization, and is inspiration for biomimetics, bioengineering and for the oncoming nanotechnologies. This chapter includes the list of most recent references related to the modern trends in biomineralization.

Biomineralogy is an interdisciplinary research field dealing with phenomena of biomineralization, demineralization, and remineralization, which have been naturally occurring since life began. The best way to understand the basic principles of biomineralization on both molecular and nano level lies through multidisciplinary experience, knowledge and sophisticated technologies by a coherent synergetic collaboration of biologists, chemists, physicians, and materials scientists.

Usually during biomineralization, organisms gather the precursors (such as ions of metals) (Currie et al. 2006) essential to form the biominerals directly in their natural environments (water, soil, food). After their transfer into organisms (or cells), precursors are stored (mostly within special organelles, or vesicles) and, finally, turned into biominerals.

In accordance with classical nucleation theory (Volmer 1939; Nancollas 1982), the minerals crystallization begins from the precursor's ions, which form small clusters of crystals during dynamic process of growth and disintegration, adequately to their ionic complementarity. After reaching the critical size, abovementioned clusters evolve into stable forms. Although in opposite to classic theory of nucleation, it was proved that formation of the calcium carbonate crystals begin from a transient amorphous phase of the precursor, in biomimetic as well as biological systems (see for review Nudelman and Sommerdijk 2012; De Yoreo et al. 2015). Hence, the discovery of formation of the calcitic spicules by sea urchin from amorphous calcium carbonate phase was described in 1997 (Weiner et al. 2009). Since this year it was

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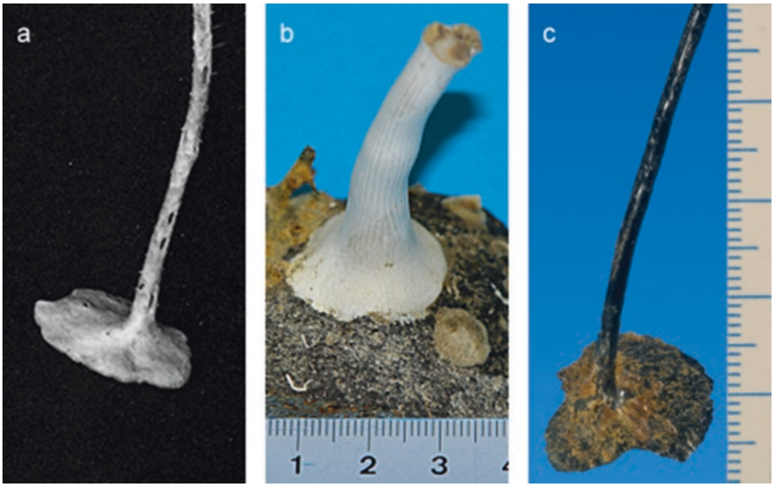

Fig. 3.1 The foot-like structures of Glass sponge *Walteria leucarti* (**a**) octocoral of *Isidella* sp. (**b**), and the black coral (*Antipatharia*) show similar behaviour with respect to their attachment to hard substrata, however differ in mechanisms of this phenomenon

reported that other animals are able to use this strategy for both calcite as well as aragonite. Nowadays, it was shown that the bone mineral matrix of vertebrate origin can be composed from amorphous calcium carbonate as a precursor phase (Weiner 2006). Papers by Politi et al. 2004, 2008; Beniash et al. 2006 confirmed this strategy. Currently, the major challenge is related to understanding the detailed manner of the formation of the primary unstable phases, as well as the mechanism of their temporary stabilization, followed by destabilization and the final formation of the crystalline products (Weiner et al. 2009). So called nanoscopic prenucleation clusters may play a crucial role being in conflict to the rules of classical nucleation theory for calcium carbonates (Pouget et al. 2009; Walker et al. 2017). Interaction between intracrystalline biomolecules leads to anisotropic distortion of crystal lattices in biological aragonite and calcite (Pokroy et al. 2004, 2007). Diverse biomineralizing systems contain diverse set of pathways (Lakes 1993; Mann 1993; Mann 1995; Aizenberg et al. 2005; Meyers et al. 2006; Fratzl 2007; Pouget et al. 2007; Dey et al. 2010; De Yoreo et al. 2013; Boskey and Villarreal-Ramirez 2016) which are dependent from both the reaction dynamics as well as complexity of the free-energy landscapes (Cartwright et al. 2012; De Yoreo et al. 2015; Jiang et al. 2018).

Taking into consideration the biological environments, the process of biomineralization is divided into an intercellular, extracellular or intracellular process (Subburaman et al. 2006). The knowledge about the mechanisms of selection, localization, and concentration of these elements by organism is achieved by evaluation of the biologically controlled biomineralization. It was accepted as a fact that the organic matter has a significant role in the formation and growing of crystals, as well as is important to the formation of the mineralized tissue biomechanical features (Weiner 1984). Corresponding organic matter in several mineralized tissues, is able to create two- or three-dimensional templates on which (or into) the crystalline phase is formed. Therefore, the oriented adhesion - one of the most important biomineralization mechanisms - is responsible for the structure formation and development of the characteristic physicochemical features of biological matrixes (Li et al. 2012; (see also Fig. 3.1). From this view especially those biomineralizing organisms (biomineralizers) which are able to produce structures where the organic component of the biomineral possesses properties of crystals and the inorganic part remains amorphous, are of crucial scientific interest. Typical examples of such biomineralizers include broad variety of unicellular and multicellular organisms which produce amorphous biosilica (see for review Wysokowski et al. 2018).

It is well recognized that, especially in calcified tissues, the crystal growth and orientation are influenced by a distinctive assemblage of special glycoproteins and acidic proteins, or both (see for overview Veis and Dorvee 2013; Chen et al. 2014; Branson et al. 2016; Polowczyk et al. 2016; Deymier et al. 2017; Kim et al. 2018; Jiang et al. 2017; Xu et al. 2018).

One of the fundamental questions formulated by Stephen Mann is "*why some organisms utilize, for example, silica rather than calcium carbonate as a structural material is unknown*" (Mann 1995).

The reason for corresponding selection may be based on the atomic and molecular features of the inorganic phases. Predestination to insert Ca and not Ba into biominerals producing protozoans as *Acantharia*, *Remanella* and *Loxodes* (Karyorelictida) has been studied previously (Rieder et al. 1982; Fenchel and Finlay 1984, 1986). This phenomenon is based on ability of these protists possess a specialized system that distinguish the light elements from the IIa group of the periodic system. For organisms, the incorporation of Ba from fresh water is easier than from seawater, due to a fact that the ratio of Sr to Ba is 5:1, and 400:l in seawater (Rieder et al. 1982).

In this group of elements there is a great difference between the ionic radius. The radius increases proportionally with mass of the ion. Therefore, it is assumed that there is one general mechanism of the recognition of ions, which has not been changed during evolution significantly. This manner may apply the various ions radius. Thus, some related organisms could not differentiate between the elements such as *Loxodes* or *Remanella.* Other specimens from the Karyorelictida order, like *Trachelocerca* or *Geleia,* modify the naturally occurring ratio of Ca:Sr:Ba to a large extent, although they could not discern as well against the lighter elements.

Although, good discrimination of elements from the IIa group is reported in all protozoa tested except of one *Trachelocerca* species which is able to embed Mn.

Why are Ba and Sr embedded towards above-mentioned unicellular creatures? Why do they not embed calcium (exclude *Prorodon),* that they can achieve without difficulty? The authors suggested that *Remanella* as well as *Loxodes* embedded Sr or Ba due to their mass (higher than Ca,) and thus these minerals are more beneficial for a mechanoreceptor. However, other question has to be clarified, why is Sr embedded in acantharia, and while Ba, Sr or Ca are incorporated in *Trachelocerca and Geleia?*

Any explanation was found for the *Trachelocerca* species derived from island Sylt in Germany that embeds Ba, Sr, Ca and probably small amount of Mn, while other specimens found in northern Italy absorbs Sr, Mn and Ca. It is still not specified which manganese compounds are incorporated into the animal. Probably, in this case different incorporation mechanism occurs, another than this one that incorporate Sr, Ca, and/or Ba (Rieder et al. 1982).

The question about the ability of organisms to select cations is also related to phenomenon known as "*multiphase biomineralization*" proposed by us for the first time in 2010 (Ehrlich et al. 2010). We found that organic matrices (i.e. chitin) template the formation of both amorphous (silica) and crystalline (aragonite) phases in skeletal fibres of selected demosponges of the Verongiida order (Ehrlich et al. 2010). Multiphase biomineralization occurs in skeletal formations of diverse marine invertebrates including radula in molluscs (Rinkevich 1993; Brooker et al. 2003), shells in brachiopods (Schmahl et al. 2004a, b, 2008; Merkel et al. 2007; Goetz et al. 2009), as well as teeth of copepods (Miller et al. 1990; Becker et al. 2005; Michels et al. 2012, 2015; Michels and Gorb 2015).

3.1 Conclusion

The literature on biomineralization is amazing. A general principles of biomineralization that addresses diverse levels of this phenomenon has been proposed by Nancollas (1982), Lowenstam and Weiner (1989), Mann (1995, 2001), Simkiss and Wilbur (1989), Addadi and Weiner (1992), Addadi et al. (2002), Cölfen and Mann (2003) and the excellent overall sources of information on this and related topics have been provided by Bäuerlein (2007), Meyers et al. (2006), Weiner (2006), Estroff (2008), Cölfen (2010), De Yoreo et al. (2015), Niu et al. (2013), Smeets et al. (2017), and Wood et al. (2017). Nowadays attention is paid on biomineralization of viruses (Wang et al. 2018), in foraminiferans (Tyszka et al. 2019), in sponges (Voigt et al. 2017), in corals (Mass et al. 2017), in plants (He et al. 2014), fungi (Li and Gadd 2017) as well as to biophysical (Rao and Cölfen 2016), medical (Kim et al. 2018; Götz et al. 2019) and diverse practical (Ramesh et al. 2017; Evans 2019) aspects of this phenomenon.

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Chapter 4
The Circle: Biomineralization-Demineralization-Remineralization in Nature

Abstract The mineral-biomacromolecule cycle can be divided into three fundamental stages namely: (i) biomineralization; (ii) demineralization and (iii) remineralization occurring in various organisms and environments. It is obvious that equilibrium between demineralization and biomineralization is required. Therefore, in the biomineralogy these two processes should be considered as two sides of the same coin. Interestingly, the reagents of both natural and artificial origins, mechanisms and principles of chemical dissolution that have been reported to exist in natural environments discussed in this chapter comparatively.

In biological systems which are involved in biomineralization, the alternative process known as demineralization also occurs. Demineralization remains to be one of the fundamental processes in nature (Ehrlich et al. 2008, 2009, 2010). It takes place both in organisms as well as in the surrounding environment (Liu and Lim 2003).

Demineralization is based on removing mineral ions from hard tissues of organisms (physiological and pathological demineralization) and from the hard substrates (rocks) in the case of bioerosion. Artificial demineralization deals with the dissolution of mineral phases under laboratory conditions with the aim to isolate corresponding organic matrix using chemical reagents. Physiological demineralization has been well studied in animal and human organisms, including marine invertebrates. Traditionally, significant attention has been paid tooth development, resorption of bone tissue and fracture healing (Ehrlich et al. 2008, 2009). For example, tooth caries and bone remodeling in vivo share the same initial step – “dissolution of the inorganic phase by the generation of low pH solutions” (Collins et al. 2002), nevertheless the origin of demineralization processes in both hard tissues are diverse.

It is well recognized that bioerosion in marine environment, which is based on mechanical abrasion, biologically mediated demineralization and physicochemical dissolution, has history as long as that for biomineralization (Wisshak et al. 2005; Davidson et al. 2018). Consequently, both ancient and modern bioerosion of limestones, shells and corals (including coral reefs) remains to be in trend.

H. Ehrlich, *Marine Biological Materials of Invertebrate Origin*,
Biologically-Inspired Systems 13, https://doi.org/10.1007/978-3-319-92483-0_4

Three routes of demineralization in the natural environment with respect to biomineralized structures have been recognized as follows:

- *Chemical deterioration of the organic phase;*
- *Chemical deterioration of the mineral phase*
- *Biological (microorganisms, enzymes) attack of the composite*. (Collins et al. 2002)

The first of these three pathways occurs in environments that are geochemically stable for the inorganic component due it is extremely difficult to dissolve or alter the organic phase without first or simultaneously effecting the closely associated mineral phase. Although this kind of biocomposite destruction may yield useful biomolecular information because rates of biomolecular deterioration in the burial environment are rather slow. In the majority of environments, biocomposites undergo total chemical deterioration what is related with the lack of thermodynamic equilibrium with the soil solution. The dissolution of mineral "shield" reveals the biomacromolecules to deterioration by microbes (biodeterioration), thus in most cases after the dissolution of the initial phase microbial attachment occurs (the third pathway).

The phenomenon of dark decalcification is another example of naturally occurring demineralization (Tentori and Allemand 2006). Kawaguti and Sakumoto (1948) observed "output of Ca^{2+}" in all scleractinian corals exposed to dark and in "intake of Ca^{2+}" all corals exposed to light. Authors claimed that the formation of skeleton occurs preferentially at alkaline pH (8.84–9.15). The decrease of pH to 8.00–7.80 under exclusion of light resulted in "*resolution of the* skeleton [sic]" (Kawaguti and Sakumoto 1948). Phenomenon of dark decalcification, which has been observed in coralline algae located at various depths Chisholm (2000). It was suggested that this is the result of acidification caused by cell respiration or the previous light exposure. It is also possible that the decalcification was due to tissue injury as well as tissue recovery verified visually underwater was overestimated. It was proved that such soft corals species as *Sinularia* sp. and *Sarcophyton* sp.) possess dark decalcification. According to Tentori and Allemand (2006), "diurnal calcification–decalcification cycles may be responsible for controlling coral sclerite shape and size in both species".

However, principles, mechanisms as well as the agents of chemical dissolution that take place or are found in natural environments described above appear parallel to clarification of demineralization which takes place in vitro. According to Ehrlich and co-workers, understanding of the mechanisms underlying of natural demineralization will give us possibility to develop subtle demineralization techniques for identification of biomacromolecules involved in biomineralization and understand the mechanism of this fascinating phenomenon (Ehrlich et al. 2008).

Diverse biological systems "*seem to create specialized environments in together with the biomineralized tissues formations and probably have been doing so since life first appeared*" (Skinner 2005). It is to note that both biomineralization and demineralization are rather two components of the mineral–biomacromolecule cycle which has been founded in these specialized local environments in nature (Fig. 4.1). Consequently, remineralization remains to be the third component of this biochemical cycle.

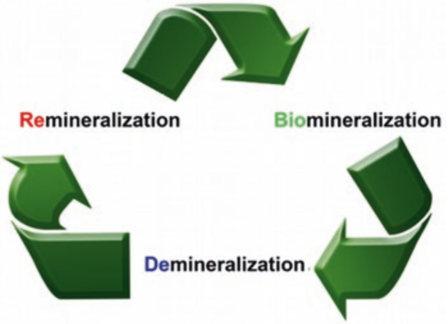

Fig. 4.1 Schematic representation of "biomineralization–demineralization–remineralization" circle which occurs in natural environment

According to our definition, "*remineralization is the process of rebuilding the solid minerals – throughout the transfer of cations and anions– to sites of nucleation where the lattices leading to mineral structures are generated. Remineralization usually follows demineralization and can be observed both in vivo in a host of natural environments*" (Ehrlich et al. 2009).

Interesting example of remineralization of foraminiferan tests in natural environments has been described by Le Cadre et al. (2003). If a specimen of *Ammonia beccarii* with a partially demineralized test was put to a solution at normal pH, organism was capable to regenerate this structure. Remineralization was accompanied, in most cases, by morphological abnormalities, which was the main difference for original calcification (e.g. different chamber sizes, wall with concave structure, abnormal expansions). Such structural abnormalities can be also caused by ocean acidification including anthropogenic impacts. Recalcification phenomenon has been also described for the spicules of gorgonian coral *Leptogorgia virgulata* (Watabe et al. 1986). It was reported that under selected conditions, exposed organic matrices from demineralized spicules, may induce the remineralization with respect to ($CaCO_3$) and impact on the mineral form (Watabe et al. 1986).

Also the formation of crystalline hydroxylapatite structures in the remineralized human enamel has been observed (Tohda et al. 1990). Recently, Neel et al. (2016) made a review on dynamics of demineralization-remineralization in bone and teeth.

The understanding of the driving forces and mechanisms of biomineralization–demineralization–remineralization related processes at the molecular level will open the way to evaluate more advanced methods for the developing of new medical tools and approaches.

4.1 Principles of Demineralization: Isolation of Organic Matter

Traditionally, scientists who have the task to isolate some organic matrix from biomineral-based structure (i.e. bone, teeth, shells, spicules, etc.) take the decision with respect to some "quick and deep" demineralization technique. However such kind of demineralization is usually based on aggressive chemicals (i.e. HF) which led to partial, or complete destruction of both mineral as well as organic phases and to obtaining of artifacts (Ehrlich et al. 2010). For instance according to remark made by Croce and co-workers, using of hydrofluoric acid for dissolution of biosilica might have strong impact on the molecular structure of spicular proteins from Hexactinellida (Croce et al. 2004). Consequently, in 2008 we have proposed the following strategy: "*the isolation of an organic component from any natural biomineralized material whether mineralized with silica- or calcium-containing compounds is crucial, however in our minds the most effective and efficient method should be based on a slow, biomimetically inspired process that could spare the organic component in the biomineral-based naturally occurring composites and not result in artefacts, instead of fast dissolution of the inorganic component*"(Ehrlich et al. 2008, 2009, 2010).

Interestingly that Nature uses similar, long-term strategy of gentle biological demineralization. For example, diverse unicellular (Garcia-Pichel 2006) and metazoan organisms show so called *calcibiocavicole* activity (Carriker and Smith 1969) with respect to calcium carbonate-based substrates to get access for the organic nutrients hidden under the mineral cover (Carriker and Smith 1969). Diverse aerobic heterotrophic bacteria as well as nitrifying and sulphide-oxidizing strains can synthesize carbonic, organic, nitric, and sulphuric acids, respectively, and in this way may dissolve acid-labile minerals (Ehrlich 1996). Diverse organic acids are secondary metabolites produced by fungi and lichens in soils could change pH in weathering solutions and in result inhibit or promote etching (Kalinowski et al. 2000).

Organic compounds may act as chelators for metal cations in solution, lowering the saturation index in solution and inhibit precipitation or enhance dissolution. Diverse organic ligands can also adsorb onto the surface of corresponding minerals (rock). So called ligand-promoted dissolution has been reported for oxides (Pokrovsky et al. 2005), carbonates (Pokrovsky and Schott 2001; Jordan et al. 2007) and silicates (Golubev et al. 2006; Golubev and Pokrovsky 2006).

Boring marine foraminiferans are reported for bioeroding capability to infest various organic and inorganic substrates (Venec-Peyre 1996; Venec-Peyre 1987; Wisshak and Rüggeberg 2006).

Also some species of sponges (Porifera) are related to bioeroding organisms. According to Zundelevich and co-workers the biological reason of bioeroding activity of poriferans is based on "*the strategy to carve into the carbonate subgrade that is unreachable for majority of predators, with the subsequent advantage of using a space out of reach to their competitors*" (Zundelevich et al. 2007). Especially representatives of the Clionidae family, which bore in the calcareous rocks, valves of living and dead molluscs, as well as corals, remain to be typical bioeroders (Cobb 1969; Risk et al. 1995; Calcinai et al. 2000; Schönberg 2002a, b, 2006). As reported by Cobb special type of amebocytes possess that are able for etching of calcareous materials forming cavities and tunnels (Cobb 1969). It has been suggested (Pomponi 1980) that in bioeroding sponges such enzymes as carbonic anhydrases and phosphatases are involved in this process. It cannot be excluded that also symbiotic zooxanthellae (Vacelet 1981; Schönberg 2006), however Zundelevich and co-authors suggested that these unicellular organisms have no impact on the boring rate of sponges (i.e. *Pione cf. vastifica* (Zundelevich et al. 2007).

Annelids have been also reported as bioeroders (Lunz 1940; Haigler 1969; Blake and Evans 1973; Liu and Hsieh 2000). For example, a small polychaete *Polydora websteri* known also as „*a pest of bivalves"* (Lunz 1940) lives in the shells of oysters and is able to penetrate "*all layers of the oyster shell*"(Haigler 1969). Blake and Evans proposed following mechanisms for *Polydora* caused bioeroding: (i) chemical – involving specific glands able for secreting of acidic solutions and in consequence dissolving the substrates; and (ii) mechanical, where the modified setae on the 5th setiger scrape the substrate. Both chemical and mechanical mechanisms can occur synergistically (Blake and Evans 1973).

Another polychaete, *Polydora villosa* has been reported habituating only in living corals colonies (Liu and Hsieh 2000). This species are able to produce so-called the U-sharped passages (Williams and Margolis 1974).

Some gastropod species are known as bioeroders of the bivalves (Carriker (1961); Carriker and Williams (1978); Carriker et al. (1967, 1974). According to Carriker and his group, the bioeroding mechanism includes two alternating phases:

(i) *chemical, in which an accessory boring organ (ABO) or demineralization gland, secretes an uncharacterized substance that etches and weakens the shell at the site of penetration;*
(ii) *mechanical, during which the radula rasps off and swallows some of the weakened shell as minute flakes*. (Ehrlich et al. 2009)

Troschel (1854) described, for the first time the ABO in *Dolium galea* (Naticidae). Schiemenz (1891) first proposed that this ABO excretes an acid. Ankel (1937), hypothesized the presence of a *calcase*, special enzyme with location within ABO. According to Carriker and Williams "*a combination of various chelating agents, enzymes and HCl in a hypertonic mucoid secretion discharged by the ABO dissolve shell during boring of hole*" (Carriker and Williams 1978).

In these experiments, the fine structure of shell etched by the excretion was compared with shell artificially solubilized and normal shell. As a standard for ultrastructural interpretations of the dissolution pattern, a synoptic series of scanning electron photographs of model regions of the normal shell of *Mytilus edulis* were prepared (Carriker 1978). It was proposed that, ABO excretion, which leads to favored dissolution of the shell matrix is functionally beneficial to boring gastropods what is related to increasing of the surface area of mineral phase exposed to the etching and enables discharge of shell units from surface of the borehole by the radula.

Scleractinians mostly located in shallow-water subtropical to tropical reefs. Majority of them calcify promptly and are present on reefs as a consequence of symbiosis with zooxanthellae. They are one-celled algal symbionts, which exist in the endodermal tissues of their coral host. Their existence is responsible for stimulating promptly calcification (Stanley 2003). Lately, approach of coral dissolution and physiological response for increased atmospheric CO_2 was presented by Fine and Tchernov (2007). Thirty coral fragments of the scleractinian Mediterranean species from the five coral colonies, were cultivated in the seawater (pH equaled 7.3–7.6 or 8.0–8.3 (ambient)) for 12 months, at the temperature of seawater, in the flow-through indoor system with photoperiod. After 30 days in acidic environment, the changes in morphology of corals were well visible, especially the elongation of the polyp, as well as the colony dissociation followed by total dissolution of the skeleton. Interestingly, after 1 year, and moving back to the natural conditions, the investigated polyps were able to calcify and rebuild the colonies.

Furthermore, Johnston (1980) notified that a contradistinction could be done between "skeletal organic material" as well as "skeletal organic matrix", and implied that the first is related to the skeletal organic matrix and all other contaminating components like trapped tissues and endoliths. Therefore, to prevent this kind of contamination, the corals chemical treatment was rigorously carried out using powders of skeletons (usually prepared with bleach and/or sodium hydroxide) in attempt to obtain only the skeletal organic matrix. Cuif et al. (1999) described the common procedure. In this study they used 24 species of corals from different environments, including deep and/or cold seas and tropical lagoons of Polynesian atolls. Coral samples were exactly washed using sodium hypochlorite, cleaned with water, and dried in oven (40 °C) overnight. Then, samples were powdered. The obtained powder was thoroughly calibrated in order to adjust the decalcification, which was carried out in standardized conditions. To conduct the characterizations of full sequence of mineralizing matrix, powder (3 g) was suspended in ultrapure water (25 ml) under constant magnetic stirring, and in next step ultra-pure acetic acid under permanent pH control was added to begin decalcification. After the complete dissolution of inorganics, a low-speed centrifugation was applied to separate soluble and insoluble matrices. The desalination of soluble components was carried out using low-pressure standard system on Sephadex G25 gel-based, which allows removing the low molecular organic compounds. These two succeeding preparation steps have an important role in obtaining reliable biochemical information, because this method leads to remove majority of the insoluble external contaminants as well as low-weight soluble compounds (Cuif et al. 1999).

In comparative study on *Stylophora pistillata* leafy complex coral, widely known as *Pavona cactus*, branched robust coral, or hood coral, the same decalcification method was applied (Puverel et al. 2005). It was proved that soluble organic matrix of both coral species consist of high amounts of glycine and other acidic amino acids. Although, the results of the proteins SDS–PAGE analysis obtained from soluble organic matter and proportions of glycosaminoglycans differed from each other. For each species, sequences of internal peptide of two matrix proteins were obtained. However, *S. pistillata* sequence is extraordinary because it is consisted of a long poly-aspartate domain, as noticed for proteins from molluscan species and proteins belonging to the calsequestrin family.

Nowadays, it is recognized that such enzymes as carbonic anhydrase (Chave 1984), phosphoprotein phosphatase (Kreitzman et al. 1969; Kreitzman and Fritz 1970), vacuolar-type H+-ATPase (Ziegler et al. 2004) as well as alkaline phosphatase, play crucial roles in biomineralization/demineralization/remineralization phenomena. Alkaline phosphatase detaches phosphorous from organophosphorous compounds, while carbonic anhydrase increases the rate in the CO_2–H_2O reactions system. Both of them can be commonly found in phosphate and carbonate sites of mineralization, as well as in the majority of noncalcifying sites and organisms.

Recently, it has been discovered that *Didymosphenia geminata* nuisance diatoms are able to biosynthesize a network of calcite nanofibers within their adhesive stalks (Ehrlich et al. 2016). The nanofibrous framework in the mineralized polysaccharide matrix is responsible for the mechanical support to the stalks. Biomineralization is controlled by periplasmic carbonic-anhydrases (CAs). CAs in microalgae are mainly responsible for the regulation of carbon concentrating mechanisms and respiratory functions in response to environmental CO_2 changes, but in multicellular organisms their additional functions of decalcification were described (for review see Ehrlich et al. 2009; Meyran et al. 1987; Tresgurres et al. 2013). The activity of external carbonic-anhydrase was identified to be more than 50% of the total CAs activity; this highlights its role in anchoring this bioeroding diatom on hard surfaces and controls availability of calcium ions. Therefore, it has been suggested that the formation of $CaCO_3$ (calcite) within the adhesive stalks of *D. geminata* is driven by the activity of external CAs (Ehrlich et al. 2016).

Enzymatic demineralization is not only limited to calcium carbonates and phosphates, surprisingly enzymatic desilicification is also occurring in natural environments. Indeed, demineralization of biosilica on the surface of the spicules of *Suberites domuncula* marine demosponge can be observed using SEM (Fig. 4.2.). It has been suggested that special enzyme termed as silicase remains to be able to depolymerise amorphous SiO_2 (Schröder et al. 2003).

Based on its intriguing ability to dissolve or to etch siliceous substrates, the silicase is of interest for biotechnology. Isolation, purification as well as cloning of the gene encoding silicase are patented (Müller et al. 2007).

Thus, the natural mechanisms of demineralization are discussed above. Below I will describe briefly the present state of the art with the man-made approach.

Application of aqueous solutions of HF for chemical wet etching of silicate glasses is an important issue which has been evaluated for many years. The first report derives from the HF discovery by Scheele in 1771 (Scheele 1771). The presence, in solution of HF, the fluorine-containing species such as HF_2, HF, and F^-, are responsible for attack and etching the glass surface. The mechanism of dissolution, and the role of the various fluorine-containing species are excellently described and discussed by Spierings (1991, 1993).

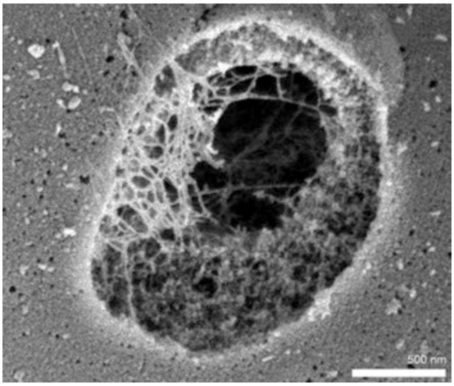

Fig. 4.2 Naturally occurring desilicification of the surface of *Suberites domuncula* demosponge spicule. (SEM image courtesy Carsten Eckert)

HF has been used in dissolution of biosilica since nineteenth century. As pointed out early by Bütschli (1901), this express method may produce artifacts (Kono et al. 1992; Croce et al. 2004; Schröder et al. 2006). However, the structural organic matrix was described for *Euplectella* sp. spicules that had been treated using HF gas (Travis et al. 1967). Also, silicateins were isolated from siliceous spicules of *Tethya aurantia* by treatment with the HF/NH_4F (Cha et al. 1999). Despite the reported formation of artefacts, the HF-based deminieralization protocols are still in use (Weaver and Morse 2003).

The use of HF to dissolve biosilica of the diatoms (Brunner et al. 2009) (Fig. 4.3) and sponges origin is represented in details in few reviews (Ehrlich et al. 2010; Ehrlich 2011; Wysokowski et al. 2018). Remarks on corresponding "collateral damages" due to the use of HF for isolation of organic matrices from skeletal structures of diverse biological objects are also to be found in these works.

In order to overcome diverse possible obstacles which could be related to HF-based dissolution of biosilica, alternative, slow-etching approach with the use of alkaline (mostly 2.5 M NaOH) solutions has been developed in 2006 (Ehrlich et al. 2006).

The following mechanism can be put forth on the basis of the reaction of alkaline desilicification of organic-silicon materials (Fig. 4.4.). Hydroxyl ions primarily attack and subsequently break the stronger siloxane bonds (Si–O–Si) located on the surface of the siliceous component.

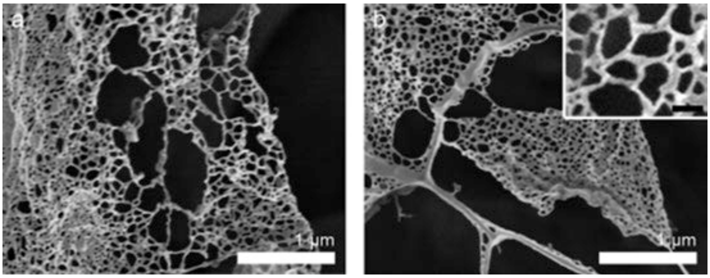

Fig. 4.3 SEM imagery: NH_4F-based demineralization in vitro of the *T. pseudonana* diatom frustule leads to isolation of filigree network (**a**, **b**) made of chitin nanofibers

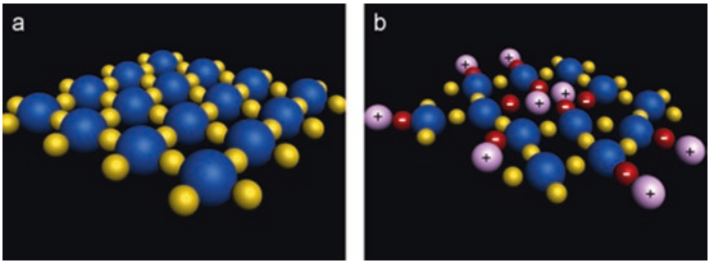

Fig. 4.4 Schematic view of the alkali-silica-reaction (left – crystalline, right – amorphous silica)

The positively charged alkali cations are able to balance the negative charge which is a consequence of bond breakage:

$$\mathrm{Si-O-Si+2NaOH \rightarrow 2Si-O-Na+H_2O}$$

The mechanism of alkaline desilicification reaction is related to the penetration of SiO_2 particles by hydroxide ions, which lead to lessening of the latter structure. This kind of the lattice disintegration using alkaline solution is practically impossible for the highly crystallized structures such as quartz; but, the amorphous silicates (likeopal A) can be easily digest because of the presence of particles with irregularly open structure and increased surface area (Ferraris 1999).

4.2 Conclusion

The process of demineralization is related to removal of inorganic part of the biominerals in organisms, and takes place in nature via chemical, physiological or pathological pathways. Naturally occurring demineralization known as bioerosion provides chemical deterioration of the mineral and organic phase using organic and inorganic acids, chelators as well as diverse specialized enzymes. Bioeroders are represented by endolithic unicellular (bacteria, cyanobacteria, fungi) and multicellular (sponges, annelids, mollusks, echinoids and fish. Artificial demineralization (in vitro) is aimed to obtain understanding of location, structure, nature and possible role of the organic matrix within skeletal structures as biocomposites (Fig. 4.5). Today, development of especially effective but gentle methods of demineralization remains to be still in trend.

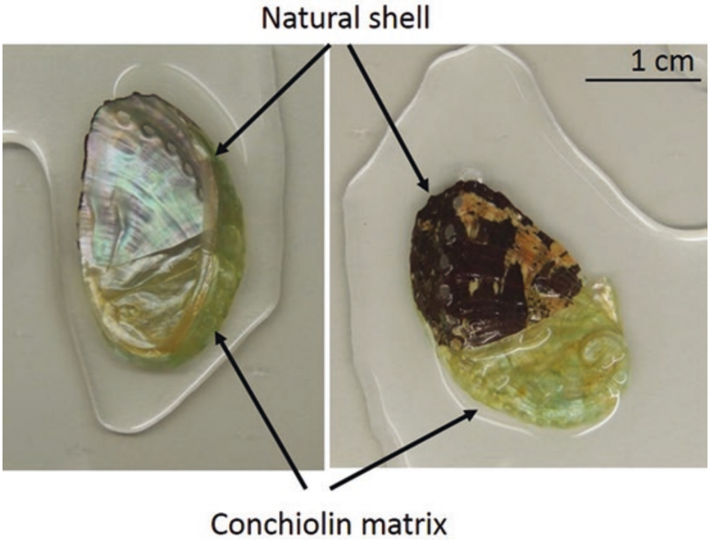

Fig. 4.5 Partial demineralization of the abalone shell using EDTA-solution at room temperature leads to visualization of the mineral-free organic matrix

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Part III
Biocomposites and Biomineralized Structures

Chapter 5
Hierarchical Biological Materials

> *One of the main driving forces in studying biological materials from the viewpoint of Materials Science is to use the discovered natural structures and processes as inspiration for developing new materials.*
> Peter Fratzl

Abstract Today bioinspiration stimulates the development of new generation of advanced functional materials and constructs with sophisticated architecture and exceptional properties. Due to large diversity, marine invertebrates (i.e. radiolarians; diatoms; molluscs; corals and sponges) are inexhaustible source of inspiration for development of different types of rigid; and flexible materials. Their biomineralized cellular tissues with anastomosing hierarchical complex microstructure combine high strength and stiffness with low weight. Cellular materials can be assumed as multiphase composites that comprise of the fluid and solid phases, while this fluid has gaseous nature. From the morphological point of view, these cellular composites can be divided into 2-D solids, like honeycomb structures comprising hexagonal cells, as well as 3-D foams, like sponges.

In natural environment, biological materials are developing through growing or self-assembling controlled naturally, applying the commonly existing materials and adapting the environmental conditions. These materials are formed by applying the prescriptions involved in the genetic code as well as under pressure of environmental conditions (Grunenfelder et al. 2018). Consequently, at all levels formation of the tissues and biological materials by hierarchical structuring is possible. Thus, to adjust structure and form to the desired function, there must be self-healing as well as the ability of adapting to variable conditions and environmental stress (Fratzl and Weinkamer 2007; Nosonovsky and Bhushan 2008).

Obviously, nature is able to use hierarchical materials, containing the nanostructures in different situations, to obtain the appropriate effectiveness (Nosonovsky and Bhushan 2008; Zhang et al. 2011). Development of flexible and low cost meth-

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ods of production of hierarchical structure and comprehension its role could simplify their biomimetical applications (Christian 2009; Grunenfelder et al. 2014; Dunlop and Fratzl 2015; Yaraghi and Kisailus 2018).

Biomimetics is related to the resembling of nature or biology. Biomimetics (in Greek "biomimesis"), as a word was developed in 1957 by Otto Schmitt, who based on the observation of the electrical nerve action evolved a device which was able to imitate this action. The biomimetics can be interchangeably used with biomimicry; bionics or biognosis (Bhushan 2009). Due to complexed mechanisms of formation of natural biomaterials the field of biomimetics/bio-inspired materials science needs to be highly multi- and interdisciplinary field of science (Hench and Thompson 2010; Bigi and Boanini 2017; Poppinga et al. 2017; Ding et al. 2017; Wood 2019).

It allows understanding the biological principles, actions, as well as structures of diverse matter discovered in nature by various scientists; and the evaluation and production of various materials and components by engineers and designers. Nevertheless, I agree with George Mayer view (Mayer 2005): "*Generally, what has been copied from nature for building synthetic structural composites has been the architectal configurations and the material characteristics rather than the specific natural materials that were originally found. This approach has limitations. A complicating and difficult issue has been the enormous potential problem of copying architectural features that are found in nature, at the micro and nano scales, into real, macroscale structural materials at reasonable cost*".

Complex combination of historical coincidences, developmental limitations and environmental stresses were crucial in the evolution of biological constructions. The variety of architectures observed in nature may initiate new clues and hypothesis (Poladian et al. 2009; Przekora 2019). Nowadays bioinspiration stimulates the development of new generation of advanced functional materials and constructs with sophisticated architecture (Wei and McDonald 2016; Schaffner et al. 2018) and exceptional properties (Aizenberg and Fratzl 2013; Gu et al. 2016; Frank et al. 2016; Gorb and Speck 2017; Ling et al. 2017; Zhou et al. 2017; Gagliardi 2017; Yang et al. 2018; Berglund and Burgert 2018; Bowles and Setton 2018; Khan and Tanaka 2018; Cremaldi and Bhushan 2018; Si et al. 2018; Yang et al. 2019; Speck and Speck 2019; Wat et al. 2019; Peeters et al. 2019; Perera and Coppens 2019; Huss et al. 2019).

5.1 Cellular Structures

Living systems including that of biomineralizing organisms have developed diverse keyways of succeeding in extraordinary structural efficiency from a somewhat limited set of building constituents/precursors. Hierarchical cellular sandwich structures are examples of nature's mostly efficient constructs and cellular designs (for review see Burgueño et al. 2005; Dunlop and Fratzl 2013; Bhate 2019).

The cellular materials differs from conventional composites not only on various levels of structural organization, morphology and their mechanical performance is rather unusual. These features additionally include surface properties like porosity and surface free energy. Some topographies of natural cellular materials are highly optimized as channels for efficient transport of nutrients and removal of waste products and to provide proper mechanical properties that often decide about the survival of the organisms, particularly in marine environments. Consequently, it is not surprising that diverse marine invertebrates are example of various cellular materials with outstanding mechanical performance. Their low weight, unique strength and toughness (Mayer and Sarikaya 2002), make cellular scaffolds attractive objects for mimicking and design of artificial structures. Cellular materials can be assumed as multiphase composites that comprise of the fluid and solid phases, while this fluid has gaseous nature. From the morphological point of view, these cellular composites can be divided into 2-D solids, like honeycomb structures comprising hexagonal cells, as well as 3-D foams, like sponges (Huang et al. 2002). Theory and practice of hierrachically sructured biocomposites have been recently reviewed in the group of Prof. Peter Fratzl (Guiducci et al. 2014; Fratzl et al. 2016; Zlotnikov et al. 2017).

Owing to the impressive strength of honeycomb structures, they are commonly applied in various structural functions. The increasing demand for the lighter and stronger materials, necessary for the aerospace and astronautic applications made these industries the main users of these honeycomb structures. A present state-of-the-art of the multidisciplinary efforts to better understand the design principles for structures with honeycomb architecture, including their properties, optimization of design principles and methods for their fabrication has been recently published (Zhang et al. 2015). According to the modern view, diverse bioinspired honeycomb structures based on repeating unit cells must possess high elastic stiffness and low density (Berger et al. 2017). Such constructs represent some of the strongest, stiffest, but lightest materials available nowadays. Additionally, improvement of the manufacturing of these composites lead to make them more viable and affordable for various industries including modern 3D printing (for review see Jammalamadaka and Tappa 2018).

5.2 Honeycomb Matrices

The variety of honeycomb architectures in nature is astonishing. For example, on the basis of observations made with light microscopy radiolarians have been excellently presented in the essential drawing by Haeckel (Fig. 5.1). Comprehensive study of such kind of biological structures has been made by Sir D'Arcy Thompson using mathematical knowledge from 1917. He drawled information from the fundamental works from Aristhoteles, Plateau, Euler or Kepler (D'Arcy Thompson 1942).

The honeycomb-like cellular matrixes are broadly employing in a various industries and purposes such as lightweight structures able for high energy absorption during last century. Recent progress of honeycomb structure fabrication materials is well represented in the literature (Heng et al. 2013; Guiducci et al. 2014, 2016; Huang et al. 2017).

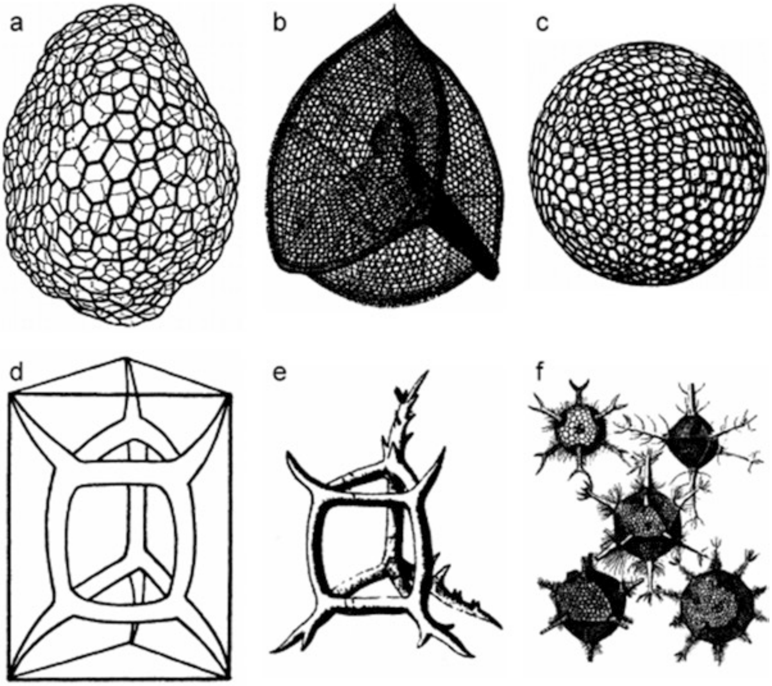


Fig. 5.1 The microarchitecture of radiolarians in glance: (**a**) *Reticulum plasmatique*. After Carnoy.; (**b**) A Nassellarian skeleton, *Callimitra agnesae* (0.15 mm diameter); (**c**) *Aulonia hexagona*; (**d**, **e**). Schematic representation and skeleton of *Prismatium tripodium*, respectively; (**f**) Drawings of radiolarians skeletons, after Haeckel. (Adapted from Thompson 1942)

The rules of geometry are common for natural as well artificial materials (Ashby et al. 1995) independently of their dimension (Du and Hao 2018) (Fig. 5.2). However, when we speak about naturally honeycomb materials, we should keep in mind that such “primitive” organisms are able to create such sophisticated, well-defined architectures without of access to mathematical modelling, tools and computers used by humans. Nevertheless, progress in engineering arising from the need to use honeycomb architecture for various practical applications, become a driving force in the analytical studies on these natural constructs. In fact, establishment of analytical methods for evaluating the elastic properties of various honeycomb materials is of huge importance for various scientific communities (Fortes and Ashby 1999; Masters and Evans 1996; Warren and Kraynik 1987; Pan et al. 2017).

Gibson and Ashby (1997) proposed the cell-edge bending model, which can be used to evaluation of the mechanical features of the cellular materials. These features are related to the material properties, geometry of the cell and relative density of the solid from which they are build. In this model, they proposed a solution for one hexagonal cell, which was considered as repeated unit (Gibson and Ashby (1988) (see Figs. 5.3 and 5.4).

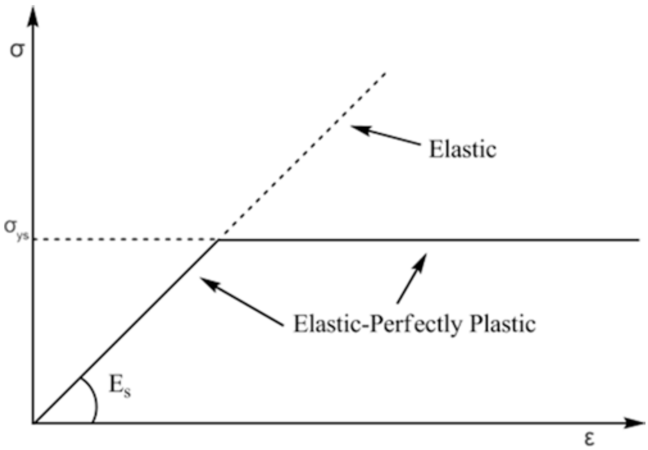
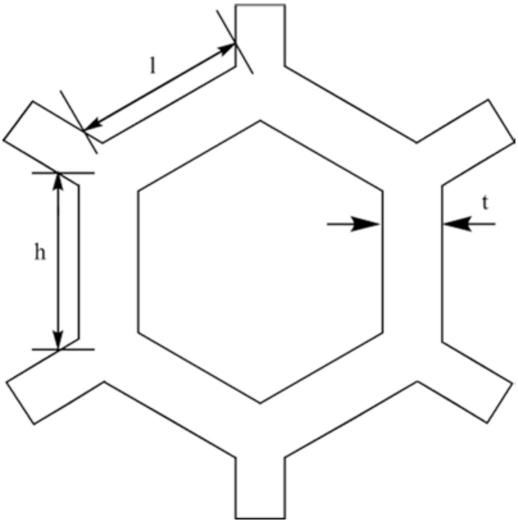


Fig. 5.2 The stress-strain curve of the solid cell wall material in the honeycombs

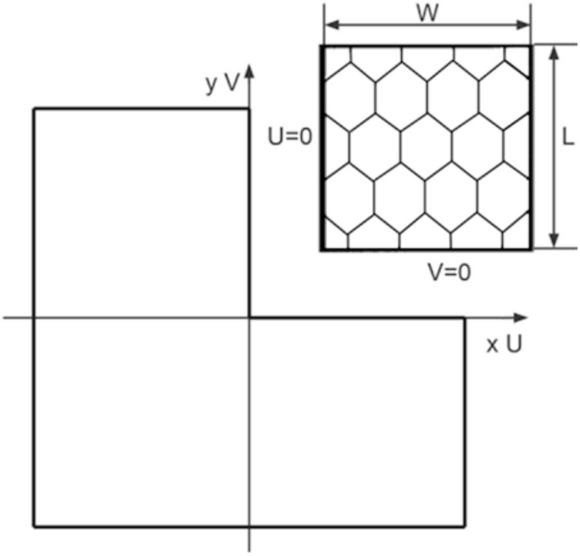
Fig. 5.3 Classical geometry of honeycomb



Taking into consideration the definition of the stiffness, the load-deformation relations, as well as the equilibrium conditions, Gibson and Ashby (1988) proposed following equations:

$$\frac{E_x}{E_s} = \frac{E_y}{E_s} = 2.3\left(\frac{t}{l}\right)^3$$

Fig. 5.4 Finite element model



$$\frac{E_x}{E_s} = \left(\frac{t}{l}\right)^3 \frac{\cos(\theta)}{\left(\frac{h}{l} + \sin(\theta)\right)\sin^2(\theta)}$$

$$\frac{E_y}{E_s} = \left(\frac{t}{l}\right)^3 \frac{\left(\frac{h}{l} + \sin(\theta)\right)}{\cos^3(\theta)}$$

$$\mu_{xy} = \frac{\cos^2(\theta)}{\left(\frac{h}{l} + \sin(\theta)\right)\sin(\theta)}$$

$$\mu_{yx} = \frac{\left(\frac{h}{l} + \sin(\theta)\right)\sin(\theta)}{\cos^2(\theta)}$$

$$\frac{G_{xy}}{E_s} = \left(\frac{t}{l}\right)^3 \frac{\left(\frac{h}{l} + \sin(\theta)\right)}{\left(\frac{h}{l}\right)^2\left(1 + \frac{2h}{l}\right)\cos^3(\theta)}$$

where *l* is the length of inclined strut, *h* is the height of vertical strut *t* is the strut depth, E_s is the Young's modulus for the strut material, E_x is the Young's modulus for the foam material in the X direction, q is the strut inclination with the horizontal, μ_{yx} is the Poisson's ratio for loading in the Y-dir, G_{xy} is the shear modulus for the foam material in the X -Y plane, μ_{xy} is the Poisson's ratio for loading in the X-direction and E_y is the Young's modulus for the foam material in the Y-direction.

Mostly biological architectures based on biosilica might be considered as the brittle honeycombs. Indeed, different models for mathematical analysis of materials properties of honeycomb cellular materials are of huge significance and commonly can be used, but we need to keep in mind that those of marine origin are made of biocomposites and in fact their mechanical resistance is not only associated with the specific honeycomb structure but also with unique hierarchical organization on the nano-; micro- and macroscale. Similar to the natural cellular structures, several microstructural defects (like non-uniform cross-section of the cell edge, curved cell edges and non-periodic microstructures), that occurs owing to the formation process, exist also in biological materials. For review on biomechanic of diverse cellular solids see Gibson (2005) and Yang et al. (2008).

Unfortunately, we do not have any information regarding the mechanisms which are responsible for the remodelling; remineralization and reinforcement processes performed by marine invertebrates when the honeycomb construction of their skeletons was damaged.

Because diatoms are recently one of the mostly studied objects in biometics (Gordon et al. 2009), I made an attempt to perform a brief analytical essay on their structures accordingly to observations made by different independent research groups.

5.3 Siliceous Honeycombs in Diatoms

During last 200 million years of diatoms evolution (Benoiston et al. 2017), nature developed and optimized hierarchical strategies to produce unique cellular structures on the nano- and microscale. Diatoms possess almost unique structural organization of their cell walls (Terracciano et al. 2018) and, consequently, are recognized as examples where harmonization and unification between structure and function (Heng et al. 2013) seems to be perfect. For instance, *Triceratum favum* (Fig. 5.5) possesses nano- and microscaled hierarchical designs characteristic for the cellular constructs. The complex design of such microsized constructions is done by distribution of biomaterial especially in the areas where it is mostly needed, for example in that of which are exposed for high stress. Structural hierarchy can maximize the efficiency of load-bearing component and of the resulting material (Burgueño et al. 2005; Sen and Buehler 2011).

I believe that unique structural features in diatoms are advantageous in terms of sieving and selection of nanoparticles due to their unique photonic properties. They are also crucial for photosynthesis.

In diatoms, the honeycomb-like motive allows for a more economic use of biosilica, especially in environments where the access to dissolved silica in form of

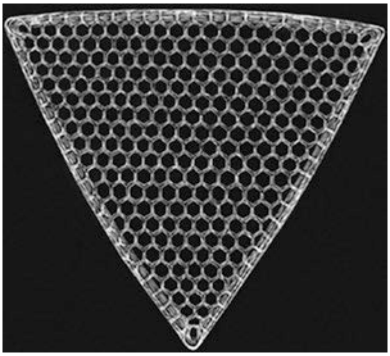


Fig. 5.5 Nanoorganized honecomb cellular structures in the shape of *Triceratium favum* with triangular motif. (Image courtesy Christina Brodie)

silicic acid is limited. It has also been demonstrated that there is a significant difference in the SiO_2 content in marine and freshwater diatom species (Vrieling et al. 2007). In both *T. punctigera* and *T. weissflogii*, the siliceous frustules possess higher density at the lower salinity (Vrieling et al. 2007).

The content of silica in seawater is insufficient, but diatoms preclude their silica dissolution through an outermost organic layer in the cell wall. Therefore, it is well visible that the relationship between organic layer that is covering this construct and siliceous honeycomb structure possess crucial importance for surviving of these microalgae. We suppose that, the structural integrity, in particular the silica-based honeycomb architectures, determines the diatom survival in natural environment. Specific frustule perforations provide the considerable compressive strength of diatom (Hamm et al. 2003), which clarify the ability of frustules to remain being undamaged under diverse mechanical challenges, such as solid sediments and abrasive particles, or changes in osmolality, which may results in bursting or swelling of non-protected cells (Losic et al. 2009).

The silica-based valves of diatoms represent examples of lightweight structures (Ferrara et al. 2014) which expected to offer excellent strength (Sterrenburg et al. 2005).

According to Yamanaka and co-workers, "*the diatoms use both blue and red wavelength regions for photosynthesis. An excess supply of blue light, however, gives rise to active oxygen, which is harmful to organisms. Thus, one of the roles of the nanostructures inside the frustules may be the reduction of such light. Furthermore, the blue light may be scattered in the frustules to uniformly irradiate chlorophylls inside diatoms for the purpose of an efficient enhancement of photosynthesis*" (Yamanaka et al. 2008).

The theoretical hypotheses and analyses regarding diatoms optical properties are confirmed experimentally and show a strong interaction between blue light and inner biosilica (Yamanaka et al. 2008).

The unique mechanical performance of the honeycomb materials are resulting in their broad application in industry (Nguyen et al. 2018). For example, honeycombs structures made of ceramic are applied as the diesel particles filters and catalytic converters for automotive industry, in the filters for continuous casting plants, in medical prosthetic implants, and in the plates for gas burners. In the Hubble telescope, as space mirrors lightweight supports glass honeycombs have been used. In majority of cases, these ceramic frameworks (Quintana Alonso and Fleck 2009) are disposed in a configuration of sandwich plates with strong and stiff face sheets.

5.4 Conclusion

Such scientific disciplines as biomimetics, bioinspired materials science and bioinspired materials chemistry have been developed due to significant scientific interest to understand the principles, mechanisms and driving forces of naturally designed hierarchically structured composites. Complex biological structures, which have risen from hundreds of million years of bioorganic evolution, have been on the agenda of materials science and engineering during last decades. The advantage of modern biomimetics that it is possible to moderate complexity and diversity of biomaterials to a selected number of basic principles of general character. The best way to understand such basic principles on a molecular level is by a coherent synergistic collaboration using explicit reasoning and well-tested explanatory principles of multidisciplinary experience, knowledge and sophisticated technologies.

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Chapter 6
Paleodyction- Enigmatic Honeycomb Structure

Abstract For the first time the *Paleodictyon* trace fossil comprises of hexagonal meshes was described in 1850. According to paleontologists, *Paleodictyon* is recognized to be a part of the trace fossils group called graphoglyptids, those fossils casts are extremally organized and usually discovered in the lower surface of distal turbidites. It is theorised that *Paleodictyon* might be of the glass sponge origin. Images made in my group strongly confirms that skeletons of some glass sponge are nothing else as silica-based tubular structures with honeycomb structure and visible axial channels. It is hypothesized that mechanism of formation of such sophisticated cellular skeletons in Hexactinellida is genetically controlled, and that these features were preserved during about last 700 Myr.

Large diversity of nano- and microsized honeycomb structures is reported for unicellular organisms. Also some honeycomb patterns have been found in macroscale including the *Paleodyction*-related "creatures", and also some specimens of the glass sponges (Hexactinellida). In contrast to the currently well-known glass sponges, Paleodyctions are very mysterious. Until recently, these deep-sea animals have not been capture. Only one characteristic is visible – the hexagonal pattern of small holes. However, until the scientist will find the real animal, this intense discussion will be continued. Although, the function of these six-sided patterns is still unknown, they can be parts of body, animal remains, burrows or vacant residue.

Menghini (1850) first described the *Paleodictyon* trace fossil and showed that, they comprise of a hexagonal meshes. Consistent with palaeontologists opinion, *Paleodictyon* is recognized to be a part of the trace fossils group called graphoglyptids (Fuchs 1895), those fossils casts are extremally organized and usually discovered the lower surface of distal turbidites (Fig. 6.1a). Usually, *Paleodictyon* is preserved in pelagic and turbidite rock layers (Garlick and Miller 1993).

Paleodictyon-like structures have been described not only in samples which range the Early Cambrian through the Miocene, but also in the recent time in the depths of Atlantic (Ekdale 1980; Rona et al. 2003; Rona 2004).

The detailed data on Paleodictyon's morphology including corresponding ichnospecies have been described by Ksiazkiewicz (1970) Seilacher (1977), and Uchman

H. Ehrlich, *Marine Biological Materials of Invertebrate Origin*,
Biologically-Inspired Systems 13, https://doi.org/10.1007/978-3-319-92483-0_6

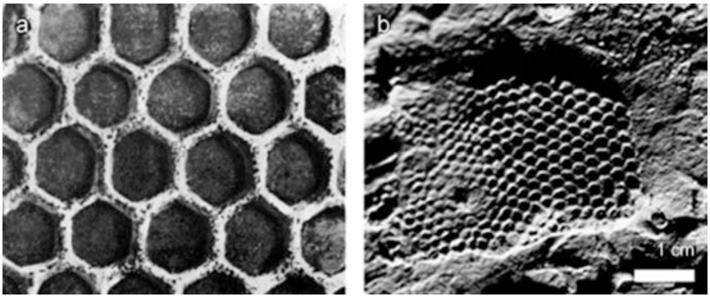

Fig. 6.1 Imagery of *Paleodictyon* (*Glenodictyon*) *hexagonum*. (Adapted from Mark 1873) (**a**). Recent image: Paleodictyon (**b**) observed in the deep-sea. (Image from the IMAX film "Volcanoes of the Deep Sea", courtesy Rutgers University and The Stephen Law Company)

(2003). According to Ksiazkiewicz, "individual mesh elements (Fig. 6.1) vary in linear dimensions from millimeters to centimeters, whereas entire mesh patterns can cover areas up to a square meter. The edges or threads that make up the mesh are usually cylindrical or ellipsoid in cross-section" (Ksiazkiewicz 1970). Diverse hypothesis concerning the function and origin of Paleodyction as some kind of trap for suspended food, or a foraging path (Seilacher,1977); structure which may be associated with xenophyophoran protists (Swinbanks 1982), or traces of a very special growth of an unknown organism (Plotnick 2003; Honeycutt and Plotnick 2005) exist up today.

It seems, however, that Paleodyction is even today surviving. Thus, in 1976 a field of strikingly symmetric, small (diameter 2.4–7.5 cm) hexagonal patterns was depicted using camera on the lower east wall of the Mid-Atlantic Ridge (water depth 3415–3585 m) in order to study the hydrothermal activity of the axial valley (Rona and Merrill 1978). In the sediment, the visualised motif consists of the three sets of the parallel black dots rows, at that time considered as a holes, that cut across at angles of 120°. Due to a fact that, these row's sets are collateral to the two sides of the bounding equilateral triangle, in general they create hexagonal pattern comprising of hexagonal cells network. In an area of 3 km at water depths of 3200–3600 m thousands of those patterns, typically several per m^2, were imaged. However, this pattern refused comprehensible ranking as some benthic organism or its product. Additionally, the pattern sizes as well as symmetry were recognized to be very similar to a compacted glass sponge adjusted to a loose substrate (Rona and Merrill 1978). Consequently, the pattern was recognized as the fossil *Paleodictyon nodosum* inferred surface expression detected in Eocene age flysch sediments near Vienna, Austria, and other areas. Explanation of the fossil body form, as well as a hexactinellid sponge, create the large foraminiferan test, either an oraxenophyophore, astrorhiziid, or part of the protistan super group Rhizaria as recently reviewed in Rona et al. (2009). The classification of the *P. nodosum* as a hexactinellid sponge of agglutinated sediment is supported (Reiswig and Mackie 1983) by:

1. form similar to disk (2.4–7.5 cm diameter) outstanding above the surface of the sediment (0.5 cm);
2. a hexagonal pattern of the narrow tubes which penetrated an outer wall (1 mm diameter; pores); tubes connection by the vertical, short (1–2 mm long) pits to a flattened body cave comprising of a hexagonal tubes which formed constant horizontal network;
3. the passive ventilation of the sieve of the body burrows increased by the marginal ridges, at margins flow (Reiswig and Mackie 1983).

Recently, finding of *P. nodosum* has been reported from abyssal equatorial Pacific area with polymetallic nodule fields (Durden et al. 2017). The regular pattern of small circular openings on the seafloor with a mean size of about 45 mm (n = 841) were partially interrupted by nodules.

Luckily, in May 2009, my research group analysed the structural features *Aphrocallistes beatrix* – representative of glass sponges. After reading Peter Rona's paper (Rona et al. 2009) I decided to inform him Hexactinellids possessing scaffolds with honeycomb structure indeed exist. So, I sent him images of *A. beatrix* obtained by our lab using stereo and scanning electron microscopy (Fig. 6.2.). Images made in my group strongly confirm that skeletons of these sponge are nothing else as silica-based tubular structures with honeycomb structure and visible axial channels. It is hypothesized that mechanism of formation of such sophisticated cellular skeletons in Hexactinellida is genetically controlled, and that these features were preserved during about last 700 Myr.

6.1 Conclusion

In fact, it can be hypothesized that similar genomes might be present also in *Paleodyction* species. Various exemplars of fossilized *Paleodyctions* and recent glass sponges possessing specific honeycomb cellular skeletal structures strongly suggests that these unique skeletal form play a vital role during evolution of biogenic cellular structures.

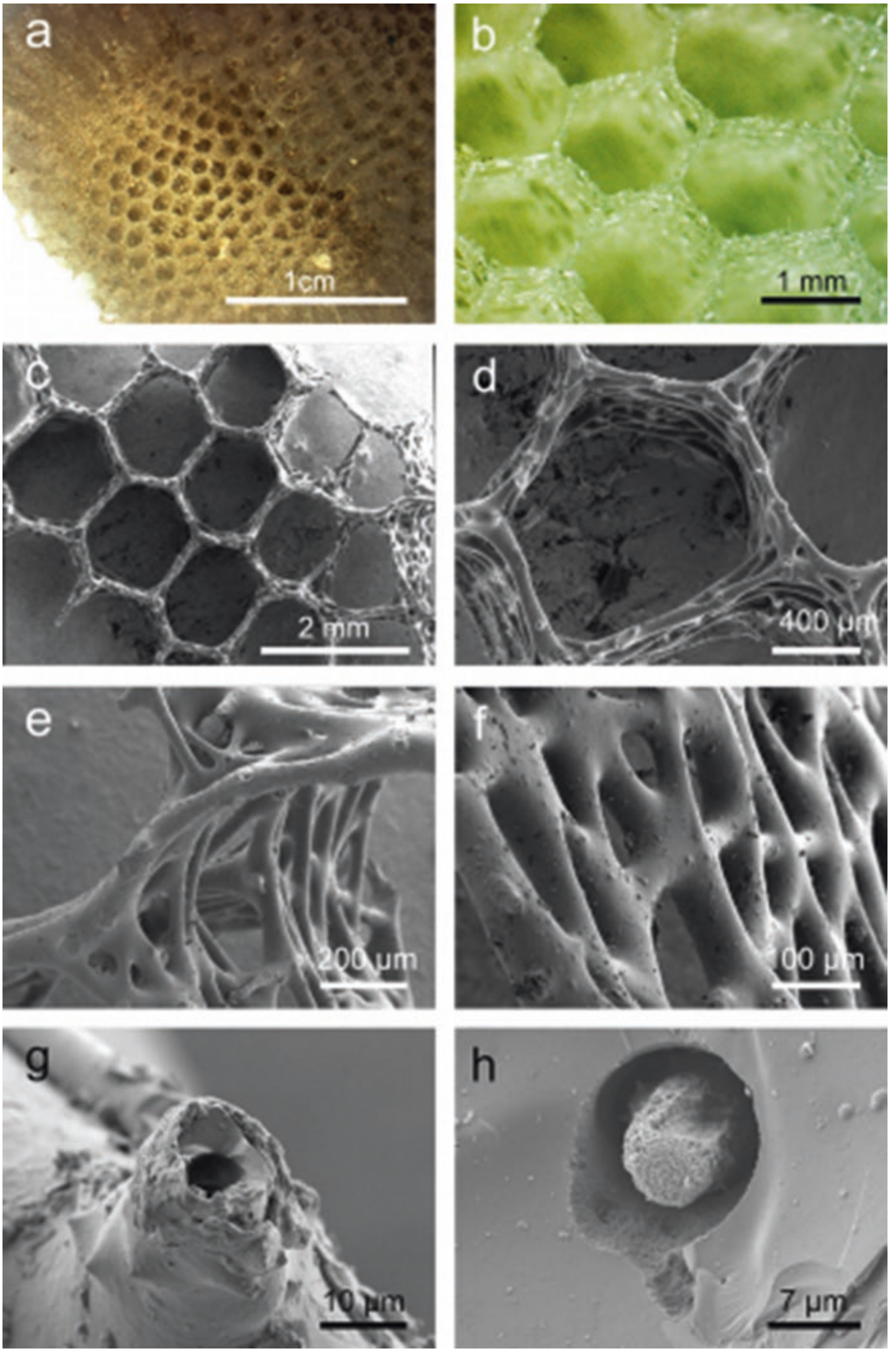

Fig. 6.2 Hexactinellid sponge *Aphrocallistes beatrix* possess unique 3D honeycomb architecture that is easy visible using stereo (**a**, **b**) as well as scanning electron microscopy (**c**–**f**). Both, axial channel (**g**) and axial filaments (**h**) are well visible, too

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Chapter 7
Sponge Biosilica- Perfectionism in Glass

Abstract Skeletons of only marine hexactinellids (glass sponges) display an amazing amount of sizes, complexity and diversity due to their ability to produce silica-based spicules of triaxonic (cubic) and mostly hexactinic symmetry. These structural repetitive motifs are to be found in up to 2 m large and highly hierarchical structured skeletons of selected hexactinellid species. Recent data confirmed, however, the presence of crystalline phases of calcium carbonates origin within glassy spicules of some hexactinellids. Hexactinnelds are still in trend as objects of investigations which are carried out by experts in materials science, architecture, photonics and biomimetics oriented scientific directions.

Poriferans, or sponges, are the most simple and ancient multicellular animals on earth and live attached to the seabed or another substratum. Sponges diverged from other animals earlier in evolutionary history than any other known animal group, extant or extinct, with the first sponge-related record in earth history found in 1.8 billion year old sediments (Nichols and Wörheide 2005). The amazing diversity with respect to their natural habitat is probably the reason for the estimated number of approximately 15,000 different sponge species (Hooper et al. 2002). According to the data represented in Simpson (1984) some species are able to pump through their bodies huge amounts of seawater (170–72,000 × their own body volume per day) filtering it to capture food particles, such as bacteria, micro algae, other unicellular organisms or dead organic particles. The whole sponge body is structurally and functionally predestined for efficient filtration of the surrounding seawater.

The phylum Porifera is divided into four classes (see for review van Soest et al. 2012; Morrow and Cardenas 2015). Hexactinellida, Demospongiae and Homoscleromorha are comprised of a silica-based skeleton, and the Calcarea possess only calcareous skeletal network (Bergquist 1978). Unlike Silicispongea, calcareans lack morphologically distinct microscleres, and their calcitic spicules are secreted intercellularly within an organic sheath (vs. formation of silica spicules onto an intracellularly secreted axial organic filament in Silicispongea (Botting and Butterfield 2005). The presence of the true tissues in poriferans is still questionable (Cavalier-Smith 2017).Sponges are characterized only by a presence of a few different cell types. For example, the sclerocytes are responsible for formation of the

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often needle-shaped structures known as spicules which mostly assemble a distinct skeleton or appear as loosely dispersed structures within the sponge body. Systematics of sponges is still based on description of their spicules with respect to size, type, shape, combination of spicules, within corresponding skeletal arrangements (Erpenbeck et al. 2006; Boury-Esnault and Rützler 1997).

7.1 Glass Sponges (Hexactinellida)

Representatives of exceptionally marine sponge class Hexactinellida usually form silica-containing spicules of hexactinic symmetry including triaxonic motifs (Fig. 7.1) within syncytial tissues. Hexactinellids are viviparous and, from detailed study of a single species, produce distinctive trichimella larvae. It is possible to distinguish two subclasses which can be recognizing by distine microsclere forms – amphidiscs and hexasters. Hexactinellids include about 700 described species, 7% of all sponges, distributed in five orders, approximately 18 families and approximately 119 genera (Schulze 1886, 1904; Sollas 1888; Okada 1928; Reiswig 1971, 2002a,b; Lévi et al. 1989; Mehl 1992; Leys 2003).

In comparison to demosponges, most hexactinellids spicules are more luxuriously architecture and larger (see Figs. 7.2 and 7.3) (Tabachnick 2002).

Principally, hexactinellids are belonging to psychrophilic organisms with unique ability to synthetize giant biosilica-based skeletons at under temperatures level between minus 2 and 4 °C (Tabachnick et al. 2017). Up to 2017, some one of the

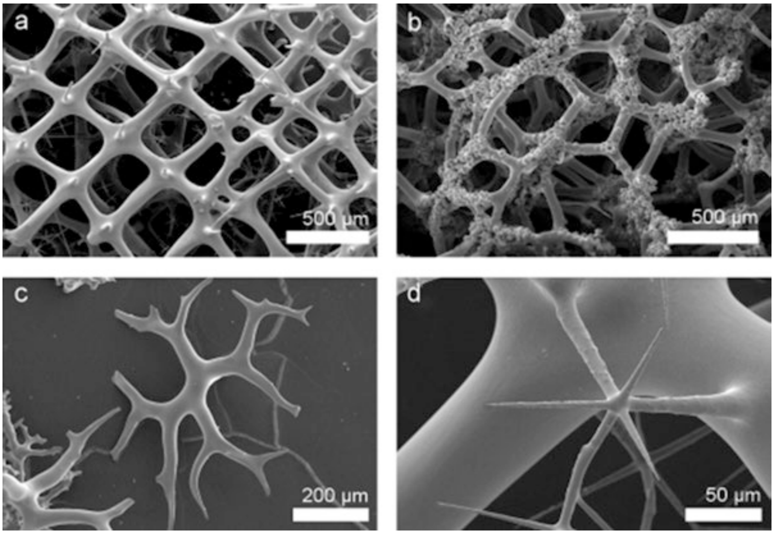

Fig. 7.1 SEM imagery: fascinating and complex skeletal networks can be visualized in glass sponges like *Farrea occa* (**a**, **d**) as well as in lithistid sponges like *Neopelta* sp. (**b**, **c**)

Fig. 7.2 Up to 1 m-long bundles of anchoring spicules of *Hyalonema (Corynonema) populiferum* Schultze 1899 collected by Dr. Kamikawa in the coastal waers of Oregon, USA. (Samples courtesy Dan Kamikawa)

largest dimensions reported for any poriferans were those of a colony of glass sponge *Aphrocallistes vastus* found in cold coastal waters in western Canada. The dimensions of such colonies were, as example, 3.4 m in length, 0.5 m in width and 1.1 m in height (Austin and Conway 2007).

In 2017, one hexactinellid species has been discovered and recognized as the largest with the size 2.0 m in height, over 3.5 m in length, and 1.5 m in width (Wagner and Kelley 2017). This species is belonging to the subfamily Lanuginellinae within family Rossellidae.

It is suggested that glass sponges are extremely slow-growing biosilica-producing invertebrates (Dayton et al. 1974), with a lifespan, about 1500 years (Dayton 1979; Gatti 2002). After the death of the glass sponges, spicules mats which range between 50 cm thick and 2 m in thickness have been reported (Koltun 1968; Dayton et al. 1974). Sponges appear to be significant gamers in various geobiological processes (Reitner 2004). For instance, in prehistoric times reefs of siliceous sponges were widely distributed and at one time formed the largest reef system known on our planet. According to Krautter et al. (2001), during the Upper Jurassic there existed

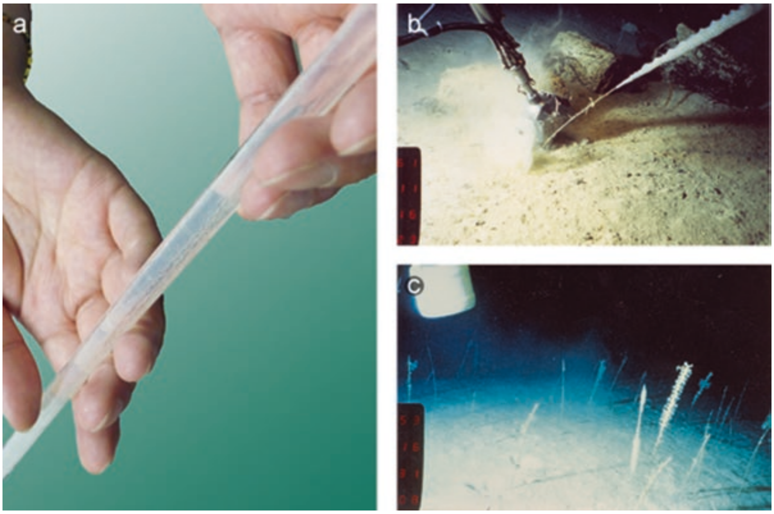

Fig. 7.3 (**a**) Giant glass sponge *Monorhaphis chuni* possesses the longest siliceous spicule. (**a**) (Image courtesy Werner E.G. Müller). (**b**, **c**) Precisious feeling is needed to sample individual stalks from the sea bottom. (Images courtesy Konstantin Tabachnick)

a deep-water reef belt on the northern Thetis shelf that was 7000 km long. Nowadays representatives of the Hexactinosida order have formed reefs at several localities off the coast of British Columbia, Canada (see for overview Conway et al. 2004, 2005). These reefs contain such hexactinosidan species as *Aphrocallistes vastus*, *Farrea occa* and *Heterochone calyx* and represent stable communities that have been developing for up to 9000 years (Conway et al. 2004). These unique biosilica constructed reefs can cover areas of more than 300 km^2 and occur in 90–240 m depth range (Conway et al. 2001; Conway et al. 2005; Lehnert et al. 2005). It is not surprising that since 2017 Canada's tentative list for World Heritage Sites includes the British Columbia's Hecate Strait and Queen Charlotte Sound glass sponge reefs.

7.2 Demosponges (Demospongiae)

The most diverse and largest group of sponges is represented by the class Demospongiae (Sollas 1885), with 95% of all existing porifer-ans. With around 500 valid genera, 88 families, as well as15 orders, (Hooper and Van Soest 2002; van Soest et al. 2012) these sponges habituating today both marine (mostly) and freshwater areas. One of the characteristic taxonomical features of demosponges is based on their monaxonic or tetraxonic (never triaxonic) organization of the siliceous spicules which are joined together with structural protein termed spongin. Major taxonomic signs which are different in each species are based on microscopic analysis of the spicules morphology. There is still lack of information (Uriz et al. 2003;

Uriz 2006) concerning the molecular mechanism(s) that define the arrangement and position of the spicules within the skeleton of demosponge.

7.3 Lithistid Sponges

Lithistid sponges possess siliceous skeletons comprising spicules similar to glass sponges. These spicules known as desmas (Kelly 2000, 2003; Pisera 2003, 2006) interlock, rendering the skeleton rigid and often stony. Unfortunately, till now there is no detailed information concerning mechanisms of silica deposition in this taxonomic order (Schuster 2017).

Lithistids are commonly found on tropical and temperate seamounts and continental margins down to depths of about 1000 m (Kelly 2000). Several southwest Pacific species form large cups, bowls, and plates that extend linearly from the sponge margin. Close inspection of the lamellae show irregular or concentric ridges, indicating that silica deposition is not continuous but rather variable in nature. Ellwood et al. (2007) presented detailed records of trace metals and carbon isotopes to understand siliceous spicule formation in the deep-sea lithistid sponge *Corallistes undulatus* (Demospongiae: Corallistidae). The overall age established using ^{32}Si analysis indicated that the sponge was between 135 and 160 years old.

7.4 Cellular Structures in Glass Sponges

Hexactinellids represent huge diversity in form, shape and size with respect to their skeletal constructs. One of the largest glass sponge species, the *Aspidoscopulia* sp. (Fig. 7.4) contains up to 2 m high and of about 75 cm width skeleton with a quadrangular meshed biosilica-based structural motif (Tabachnik et al. 2011). With regards to *Aspidoscopulia*, it must be stated that “*structure in Nature is a strategy for design*” (Pearce 1978). Especially in such case as highly hierarchical 3D skeletons of *Aspidoscopulia* and related speceis, “*stealing Nature’s best ideas*” for our inspiration is a good strategy.

It is well recognized that, for the morphology of all biological crea-tures, the hexagonal array seems to be the most typical cellular monodispersions pattern (Ozin 1997). While the equivalent tensions appear in all contact surfaces, the cellular pattern can balance to this where all shared surfaces run across at angles of 120° and all vertices are joined likewise at angles of 109°28′. Thompson, in the work about the source of radiolaria and diatom hexagonal patterns, sug-gested that, a layer of the cells with similar size (i.e., a protoplasmic froth) formed into hexagons meshwork by their reciprocal tensions (Thompson 1917). This can act as a form for the silica deposition within this intercellular positions.

Sir D’Arcy Thompson described this geometrical motif in his classic work from 1917 entitled On Growth and Form. This hypothesis about physicogeometrical cau-

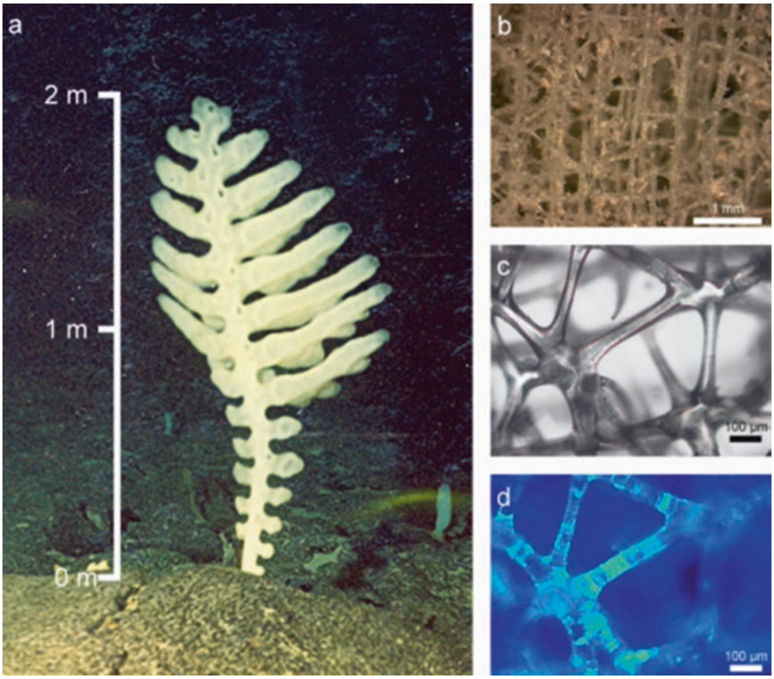

Fig. 7.4 Complex hierarchical 3D glassy skeleton is characteristic for the huge hexactinellid sponge *Aspidoscopulia* sp. (**a**). (Image courtesy Konstantin Tabachnick). The microarchitecture can be observed using stereo (**b**), light (**c**) as well as fluorescence (**d**) microscopy

sation was proposed to clarify the concept of the forming of various marvelous protozoan agglutinated structures. In this work, the common laws of the cellular surface energy minimization and close-packing surrendered to inter- and intramolecular force fields were invoked, to evaluate the existence of a various one-cell microskeletons. The physico-mathematical claims as well laws which control the arrays and forces of surface tension in the cells groups seem to be regulated by the processes of adsorption and deposition of inorganics to form complex arrangements which resulted in formation of the various microskeletal structures in nature. D'Arcy Thompson reported that the protoplasmic organisms are able to use the minimization of the surface energy to find and excrete the solid particles in the form of agglutinated shell (Thomp-son 1917; 1992).

Another example are the cells in the pith of Juncus which are stel-late in a radiating pattern, where the cross-section of the tissue is similar to a six-rayed stars network (Fig. 7.5), joined together by the rays tips, and divided by symmetric, filled with air intercellular spaces, which result in the snow-like white colour of the pith. Interestingly, the 12-rayed, solid dodecahedra-star can be observed using the binocular micro-scope with big clearness in thick sections. According to Thompson (1917), understanding of these arrangements is not difficult. Visualise, a system of similar, joined together spheres that each of these is in contact with other 12 nearby residents, and six of them is touching the equatorial.

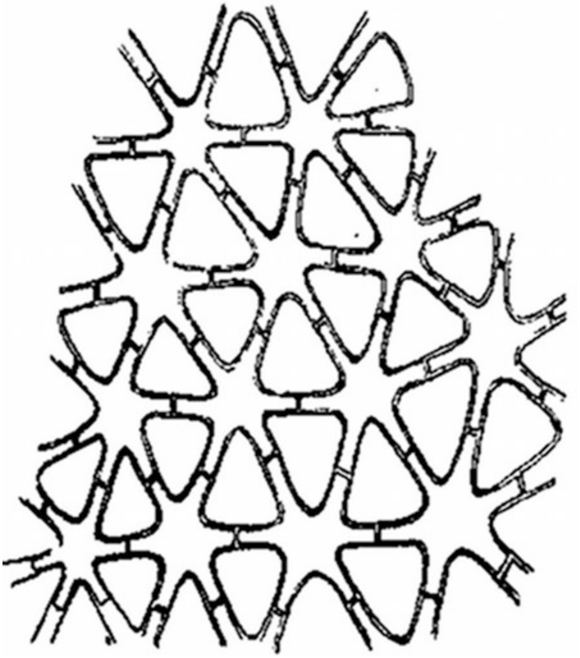

Fig. 7.5 Stellate cells in pith of *Juncus*. (Adapted from Thompson 1942)

What really takes place promptly is alike, however not completely identical. The boundary wall which surrounds pith-cells, instead of a few pith-cells that are able to shrink accordingly to a boundary with permanent size, is able to expand continuously even after the inhibi-tion of the pith-cells growth or multiplication. The attachment points on the surface of one another pith-cell are pushed back, although the cell filling does not evenly enhance; and the residual surface sections shrivels inwards. This fancy symmetrical figure was named by Kepler as a stardodecahedron, which under the new and altered conditions is still a surface of minimum area (Matzke 1935).

Stellate cells- based construct are characteristic to the plant world. However, very similar structural motifs are to be found in such hexactinellids as *Sarostegia* (Fig. 7.6) that belongs to the Farreidae family. Also such formations as quadrangular meshed dictyonalia wchich remain to be fused (Fig. 7.7) are typical for farreids.

Psychrophilic glass sponge *Farrea occa* is a typical deep sea dweller with a depth range from 80 to more than 5000 m (Krautter et al. 2006). As reported by Prof. Manfred Krautter "*this organism is able to form tremendously large deepwater reefs larger than 30 m in diameter. Its body shape is a dichotomously branching and anastomosing system of subcircular to ellipsoidal and terminal open thin walled (no thicker than 2 mm) tubes, which are up to 10–15 cm long* (Fig. 7.8). *The siliceous skeleton of this species contains maximally two layers of fused hexactin spicules*" (Krautter et al. 2006).

Bioanalytical studies with regard to identification of organic matrix which may be responsible for biosilicification in farreids shown with strong evidence the pres-

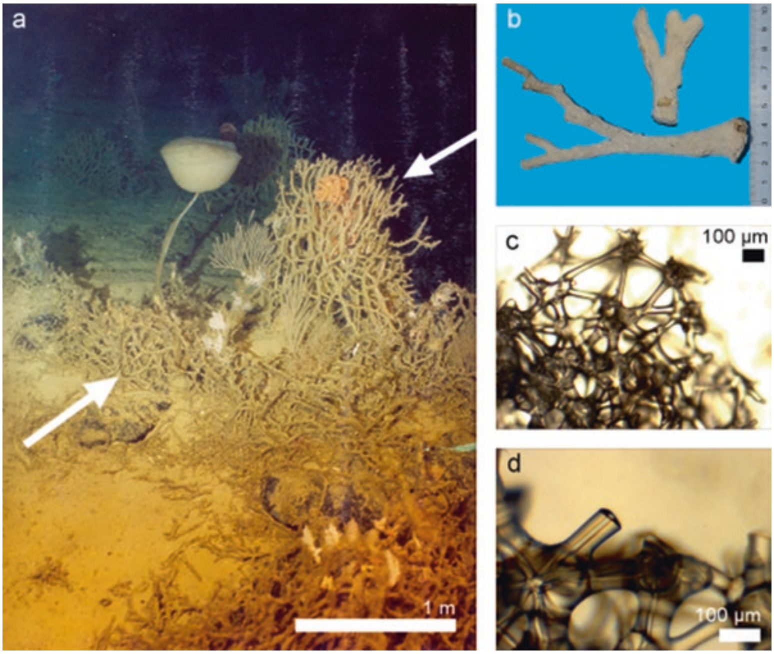

Fig. 7.6 (**a**) Very rigid, glassy 3D skeletons are characteristic for the (arrows) is probably a unique reef-building sponge *Sarostegia oculata.* (Image courtesy Konstantin Tabachnick). (**b**). Even light microscopy observations (**c**, **d**) seems to be enough to confirm hierarchical structure of its skeleton

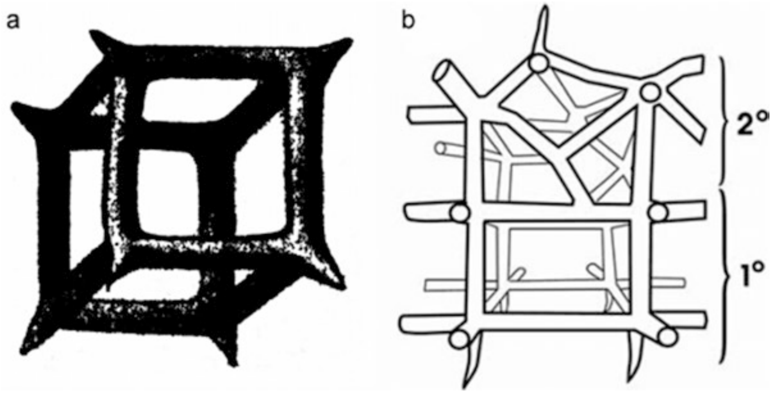

Fig. 7.7 Structural units in biosilica: from radiolarians. (**a**) (Adapted from Thompson 1942) to farreid glass sponge dictyonal frame with two regular, quadrangular, primary layers (1°) and irregular additional secondary layers (2°). (**b**) (Adapted from Reiswig 2002a)

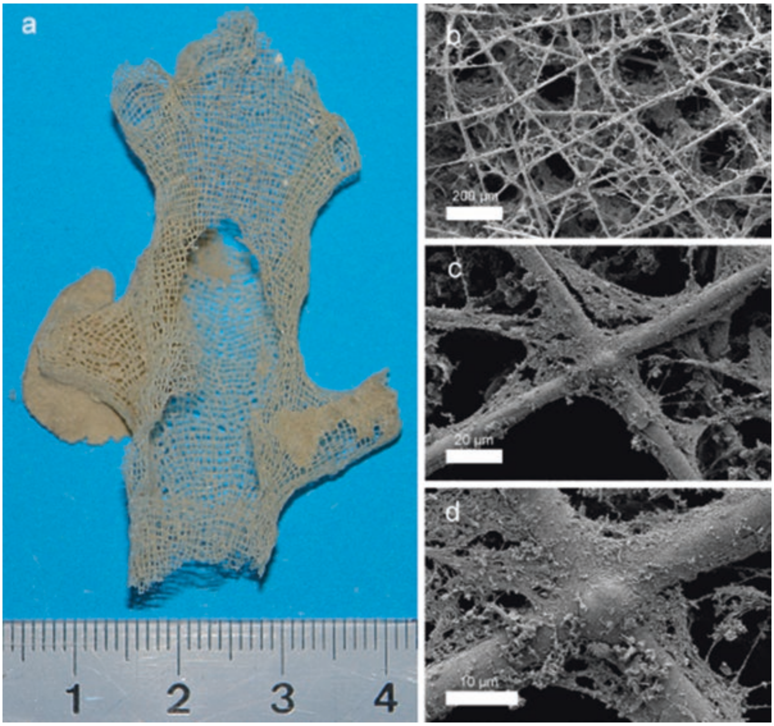

Fig. 7.8 Diverse kinds of geometrical proportions can be observed within skeleton of the reef-building hexactinellid *Farrea sp*. (**a**) using SEM (**b**–**d**)

ence of chitin (Ehrlich et al. 2007). It was shown that chitin in mostly to be detected using fluorescence microscopy method (Fig. 7.9) in the locations where spicular structures join each other. Chitin has been also detected within skeletons of such glass sponges as *Euplectella aspergillum* (Ehrlich and Worch 2007) as well as in psychrophilic hexactinellid *Rossella fibulata* (Ehrlich et al. 2008a).

According to the hypothesis (Ogasawara et al. 2000; Ehrlich et al. 2008a, 2016), silicate ions and silica oligomers preferentially interact with glycopyranose rings exposed at the chitin surface, mostly by polar and H-bonding interactions. According to Schwartz, "*silicon was found associated with glycosaminoglycans bound as an ether or ester-like silicate with C-O-Si or C-O-Si-O-Si-O-C bonds, in amounts of one Si atom/130-280 repeating units of the organic*" (Schwartz 1973).

In some cases hexactinellids skeletal frameworks possess very complex architecture (similar to that of Eiffel tower; Fig. 7.10) and seems to be constructed through thousands of glued microscleres (Fig. 7.11). Often seen are spiny surfaces of the hexactinellids skeletons. Recently, we have reported that such features can be based on the existence of club-like structures located within spicular joints (Ehrlich et al. 2011). Such structures play the crucial role in surviving of the whole organism due to ensuring of skeletons integrity. Intriguingly, the main mineral phase within this

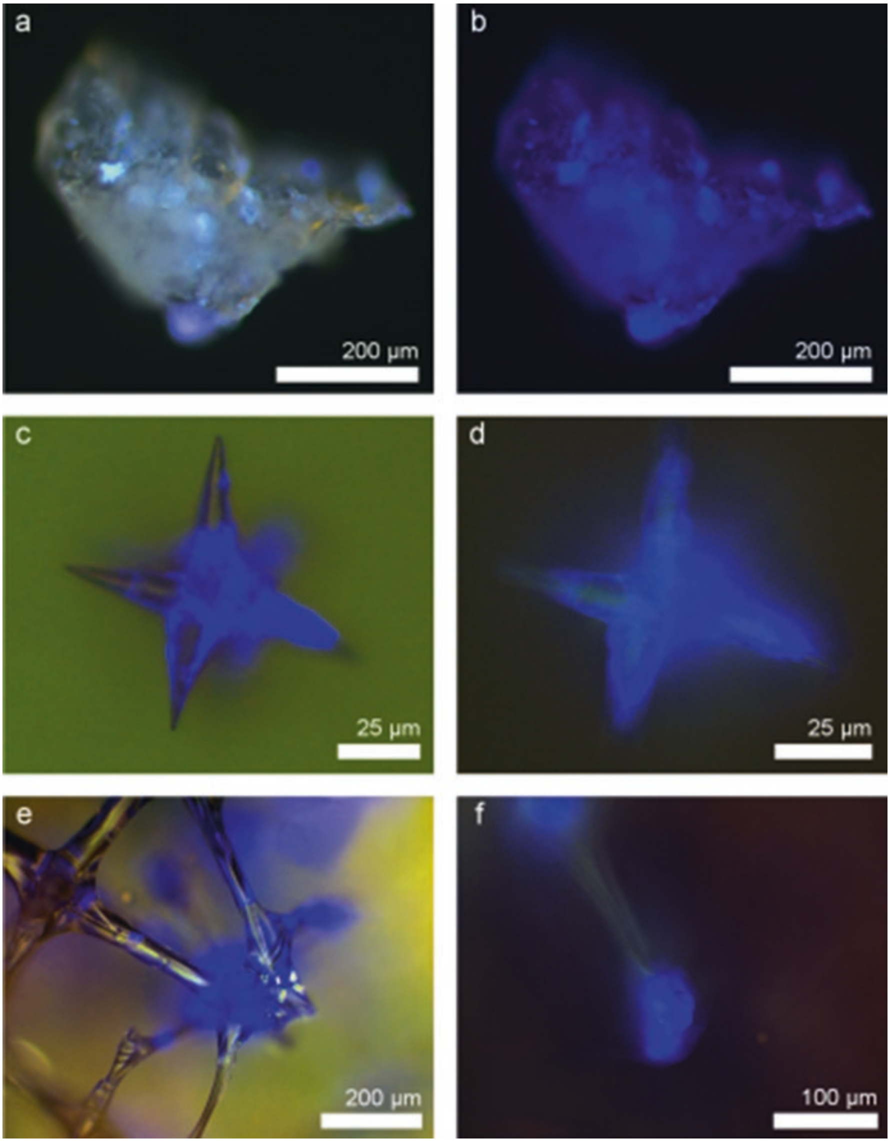

Fig. 7.9 Identification of chitin microparticles within silicate-based composites become observable using fluorescence modus both prior (**a**) and after staining with special Calcofluor White stain (**b**). The method has been successfully used to localize the chitinous residues on the surface of *F. occa* skeletal fragments (**c**, **d**, stained). Even after 6 months of insertion in alkali residual chitin, which prevents silica against dissolution, is still well visible (**e**, **f**, both stained)

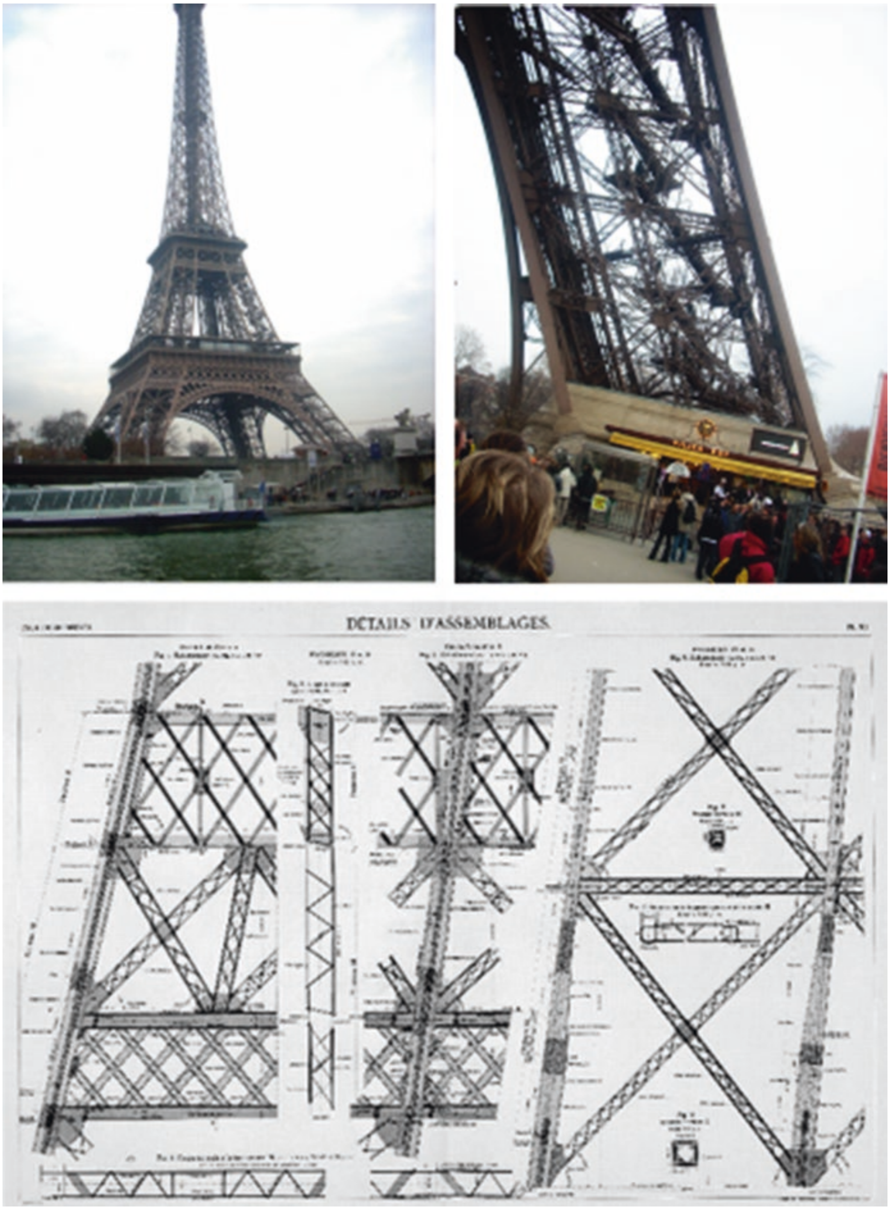

Fig. 7.10 An example of man-made hierarchical construct- the Eiffel tower. (Photos courtesy Vasily V. Bazhenov)

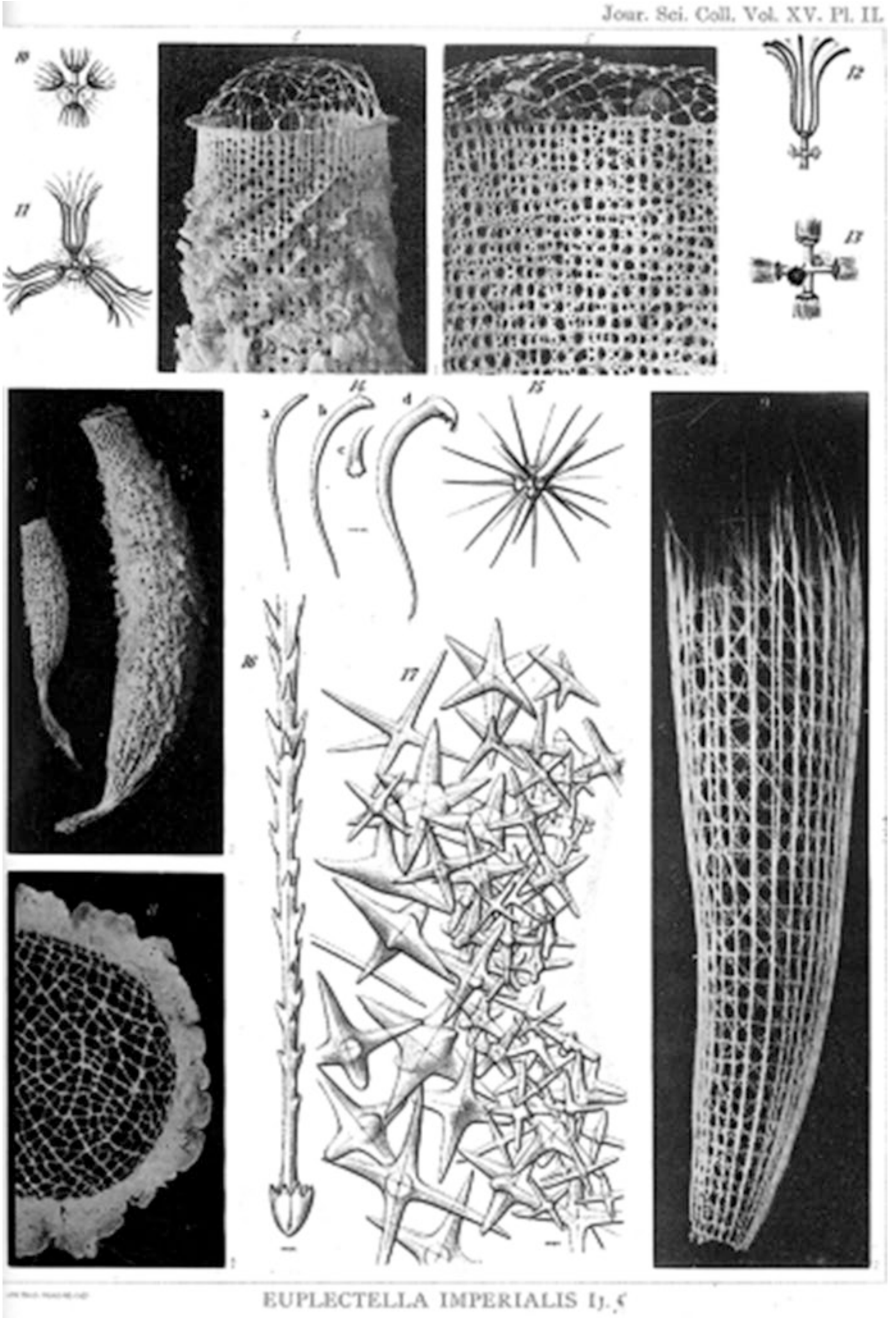

Fig. 7.11 Also in the drawings of *Euplectella* glass sponge made by Iijima in 1901 the Eiffel tower motif is easily recognizable

Fig. 7.12 The *Caulophacus sp.* glass sponge possess mushroom-like morphology. (Image courtesy NOAA)

biosilica has been represented by crystalline calcite (see for overview Figs. 7.12, 7.13, 7.14, 7.15, 7.16, 7.17, and 7.18). In spite that *Farrea* sponges and *Euplectella* belonging to different families, our investigations on "glass tower" of *Euplectella* sp. confirmed (Fig. 7.19) very similar structural features also within this sponge.

7.5 Spiculogenesis

Diverse and complex skeletal frameworks, desmas and, especially, spicules, are the main forms of the biosilica deposition in poriferans. The history of discoveries connected to spicules of sponge is well represented by (Vosmaer and Wijsman 1905). As was stated by these researchers, in 1819 Schweigger proved that in some sponges species spicules are not comprised of calcium carbonate, in 1826 Grant proved that

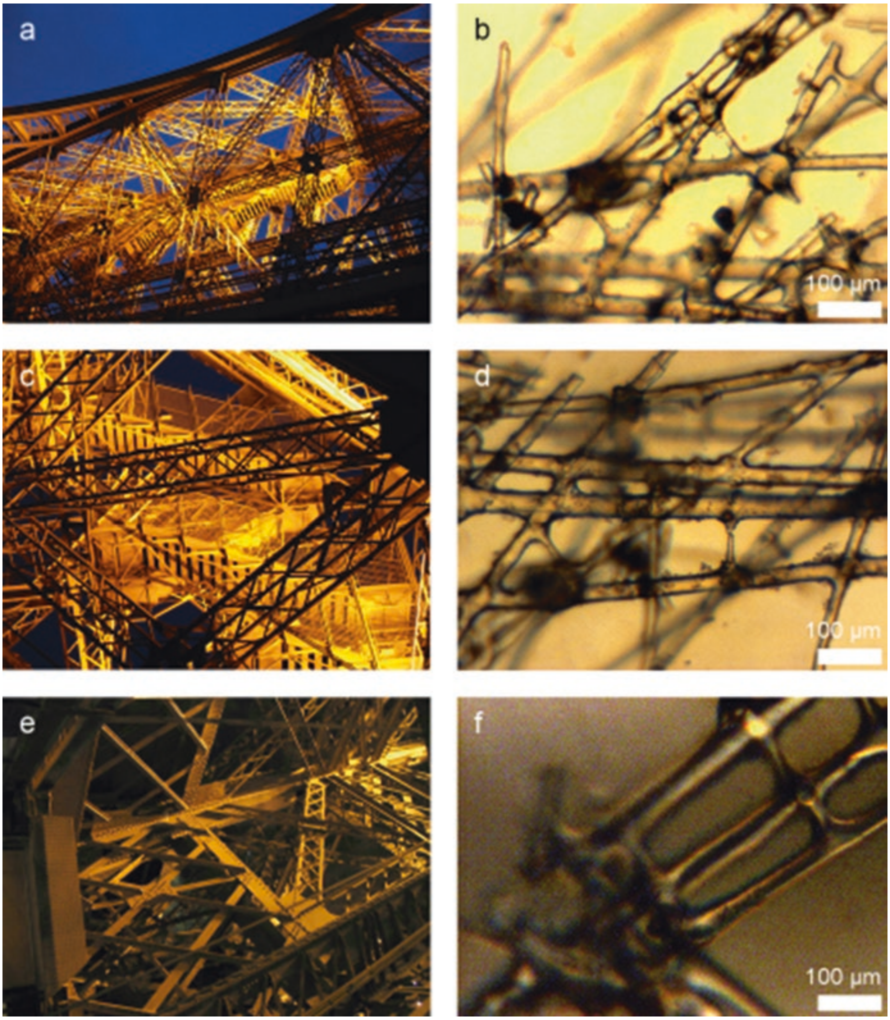

Fig. 7.13 Characteristic similarities between structural fragments in Eiffel tower (**a**, **c**, **e**) and within siliceous framework of *Caulophacus* sp. stalk (**b**, **d**, **f**) are visible under light microscopy modus

they contain silica, then in 1841 Bowerbank demonstrated that, except to silica, organic material also exists. Furthermore in 1885 Sollas discovered that the silica from sponge is similar to opal. He reported that the refractive index of biosilica from sponge is closer related not to quartz but to colloidal silica or to opal. Corresponding term " *spicopal* " has been proposed by Vosmayer and Wijsman, although F.E. Schulze named the spicule organic matter "*spiculine*". In the case of structure of spicules, in 1835 Gray stated that in Hyalonema hexactinellid sponges spicules comprised of layers which came pronounced during heating. In 1864 Kölliker

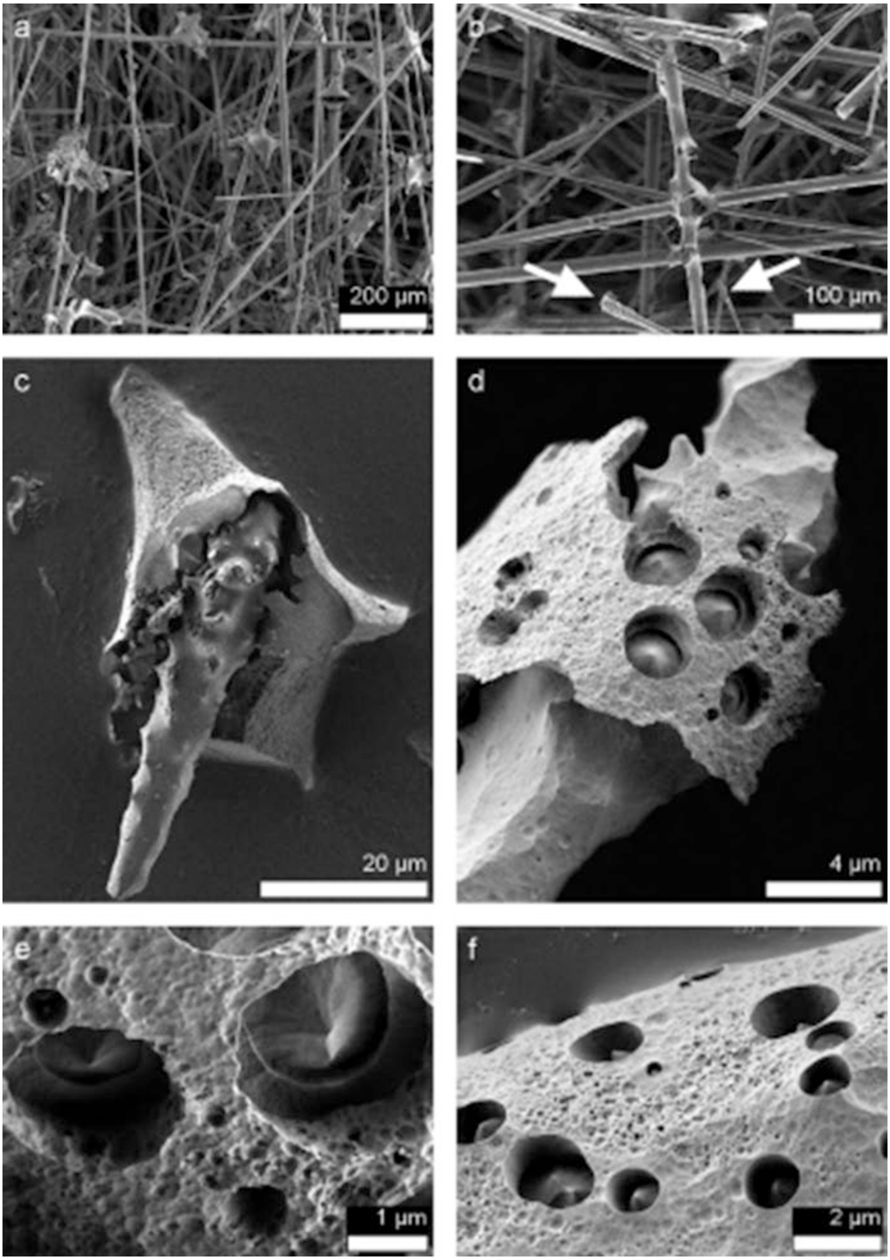

Fig. 7.14 SEM imagery: interconnected spicular network within stalks of *Caulophacus* sp. sponge (**a**). It was possible to isolate individual club-like spicules (**b**, arrow) using pincers. After 14 days of insertion in the 2.5 M NaOH, the club-like constructions could be visualized as were they walled within the articulations (**c**, **d**). The corrosion on the surface of siliceous layer that covers the club-like structure is well visible (**e**, **f**). The spines remain to be resistant to NaOH

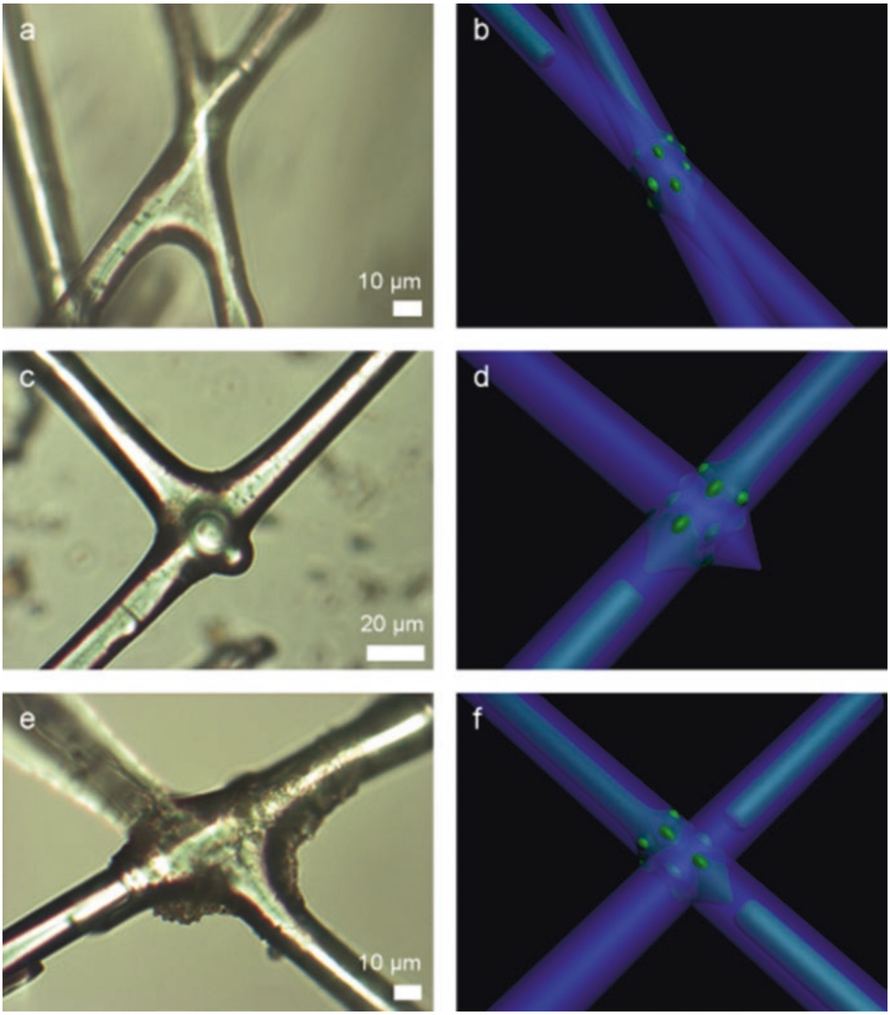

Fig. 7.15 Light microscopy helps to visualize the diversity in articulations between spicules of *Caulophacus* sp. (**a**, **c**, **e**). Corresponding computer simulations of club-like formations are represented in (**b**, **d**, **f**)

showed that, these layers surround concentrically a "*central canal*", which is filled out by the axial rod in the form of organic mass. The next term known as the "*axial cylinder*" was proposed by Claus in 1868. It represents homogenous silica which surrounds directly this axial rod. Thus, the spicule of sponge comprises of a central organic axis, encircled by concentric opal layers, which the outermost is involved in a spicule sheath of organic matrix (Vosmaer and Wijsman 1905) (see Fig. 7.20), or rather, of organic matrix in close connection with biosilica.

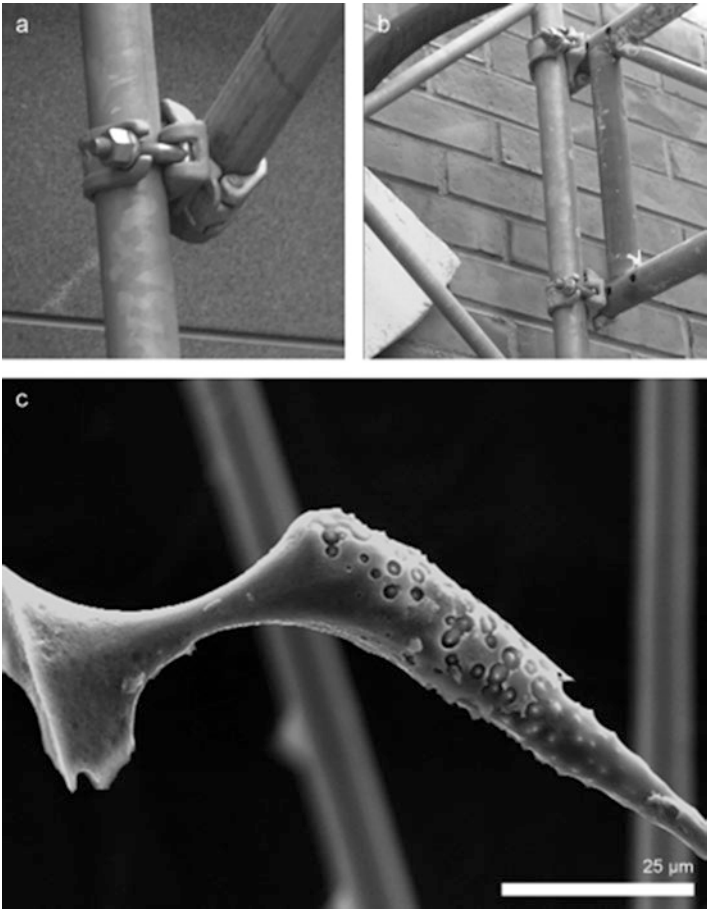

Fig. 7.16 Strategies against the slippage: (**a**, **c**) man-made, (**c**) – made by *Caulophacus* sp. glass sponge

Freshwater sponge *Ephydatia fluviatilis* has been used in studies by Weissenfels and Landschoff (1977) and Weissenfels (1989) to show that the spicules formation begins in sclerocytes inside a specific vesicle. Silicon is deposited around an axial organic filament, after its production, and the complete spicule forming process (6–8 μm in diameter and 190 μm in length) is finished after 40 h, at 21 °C.

Traditionally, poriferan spicules have been divided into two catego-ries defined, according to their size, microscleres and megascleres (e.g., Lévi 1973). In sponges, spicules are highly diversified and the selection pressures are tough to imagine. Thus, 12 basic types of megasclere as well as 25 types of microsclere are reported in Demospongiae, in Hexactinellida 20 basic types of megasclere, as well as 24 types of microsclere can be distinguished (for morphological details see Lévi 1973, 1993; Garrone et al. 1981; Simpson 1984, 1990; De Vos et al. 1991; Bavestrello et al. 1993; Boury-Esnault and Rützler 1997; Tabachnick and Reiswig 2002).

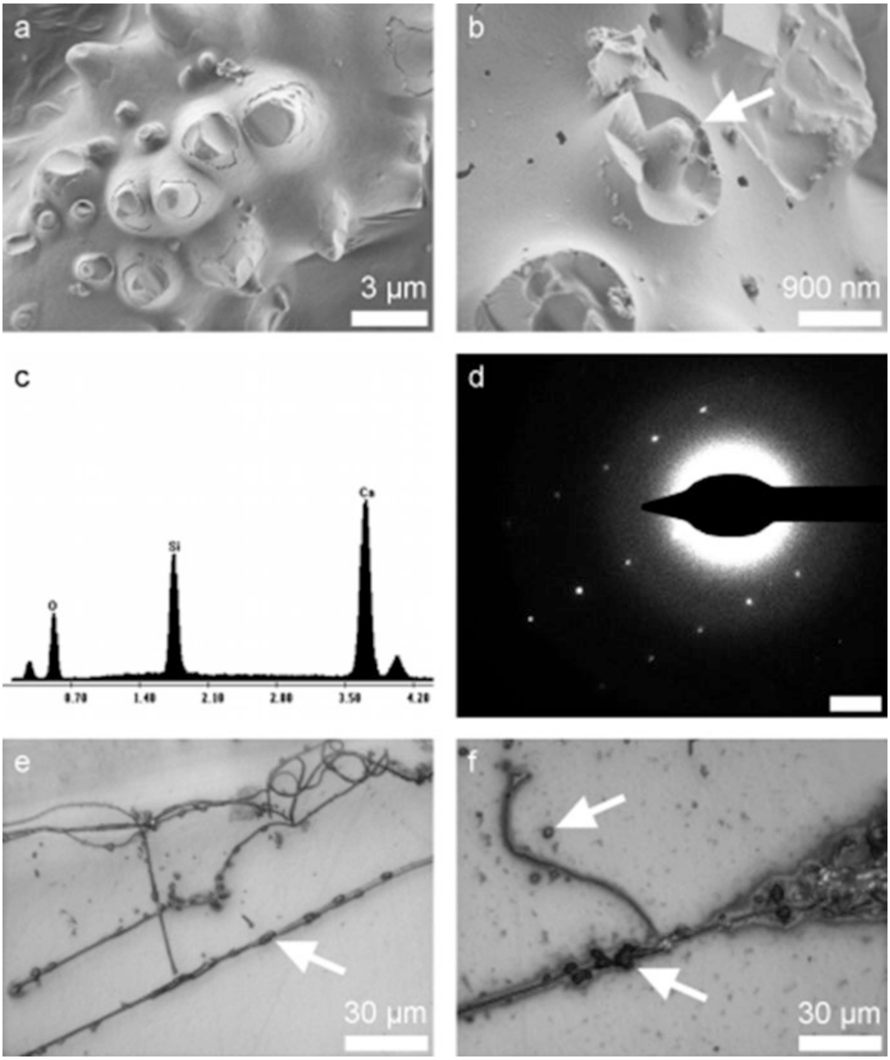

Fig. 7.17 SEM imagery: seed-like structures, covered with a layer of silica are visible within the broken spine of *Caulophacus* sp. (**a**, **b**). Energy-dispersive X-ray spectroscopies (**c**) as well as electron diffraction (**d**) show with strong evidence that crystalline calcium and not silicon is the seed-like structure. After demineralization in alkali, the nanocrystals of calcite can be visualized as attached formations on the surface of axial filaments (**e**, **f**)

In contrast to characteristic spicules of demosponge desmas always demonstrate irregularity and frequently complex sculpture as well as morphology, and secondly they are connected by articulation. Possibly, the most essential differences is the fact that the axial or crepis filament is mostly very short (or sometimes invisible) and stretches only for a short distance from the centre of spicule. Increase desists

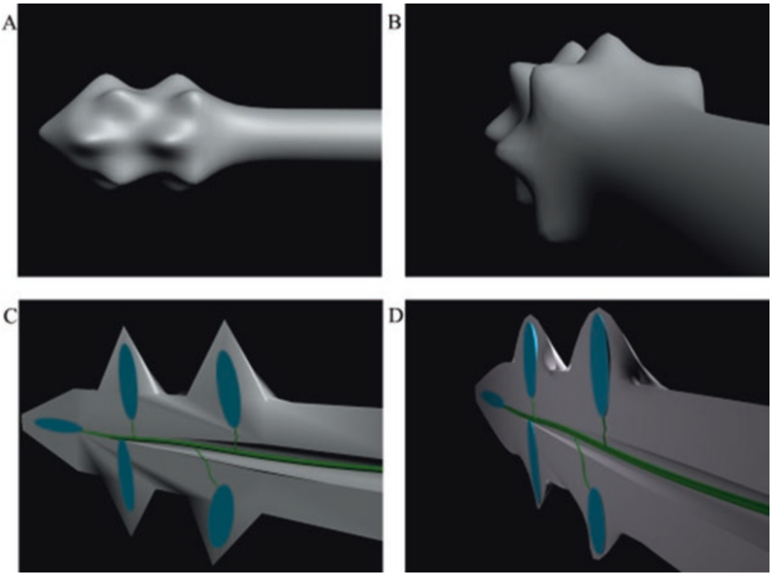

Fig. 7.18 Computer simulations of the club-like constructs within spicules of *Caulophacus* sp. (**a**, **b**) represent the cross-sectional view (**c**, **d**) of this formation. (Images courtesy Denis Kurek)

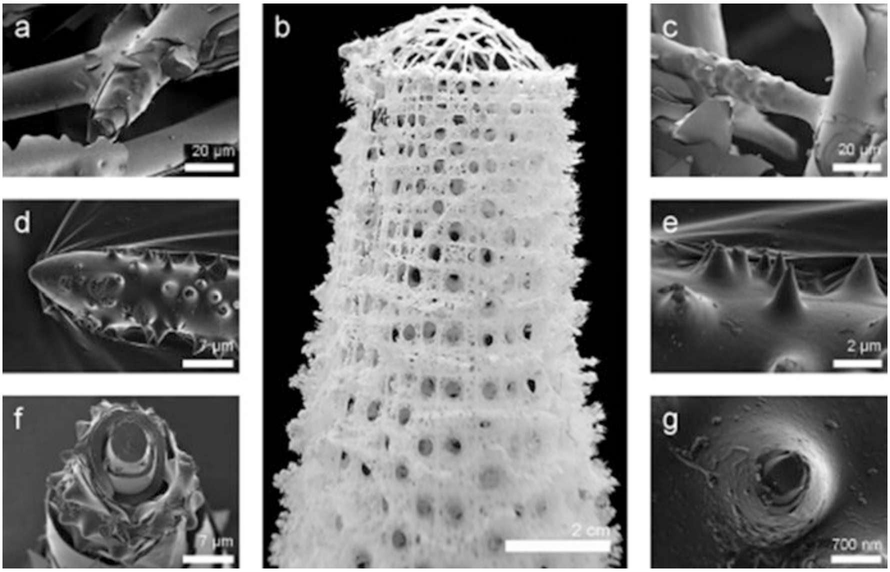

Fig. 7.19 The *Euplectella* sp. glass sponge (**b**) also possess club-like structures within its spicules (SEM images: **a**, **c**, **d**, **e**, **f**, **g**), which should be needed for the skeletal integrity

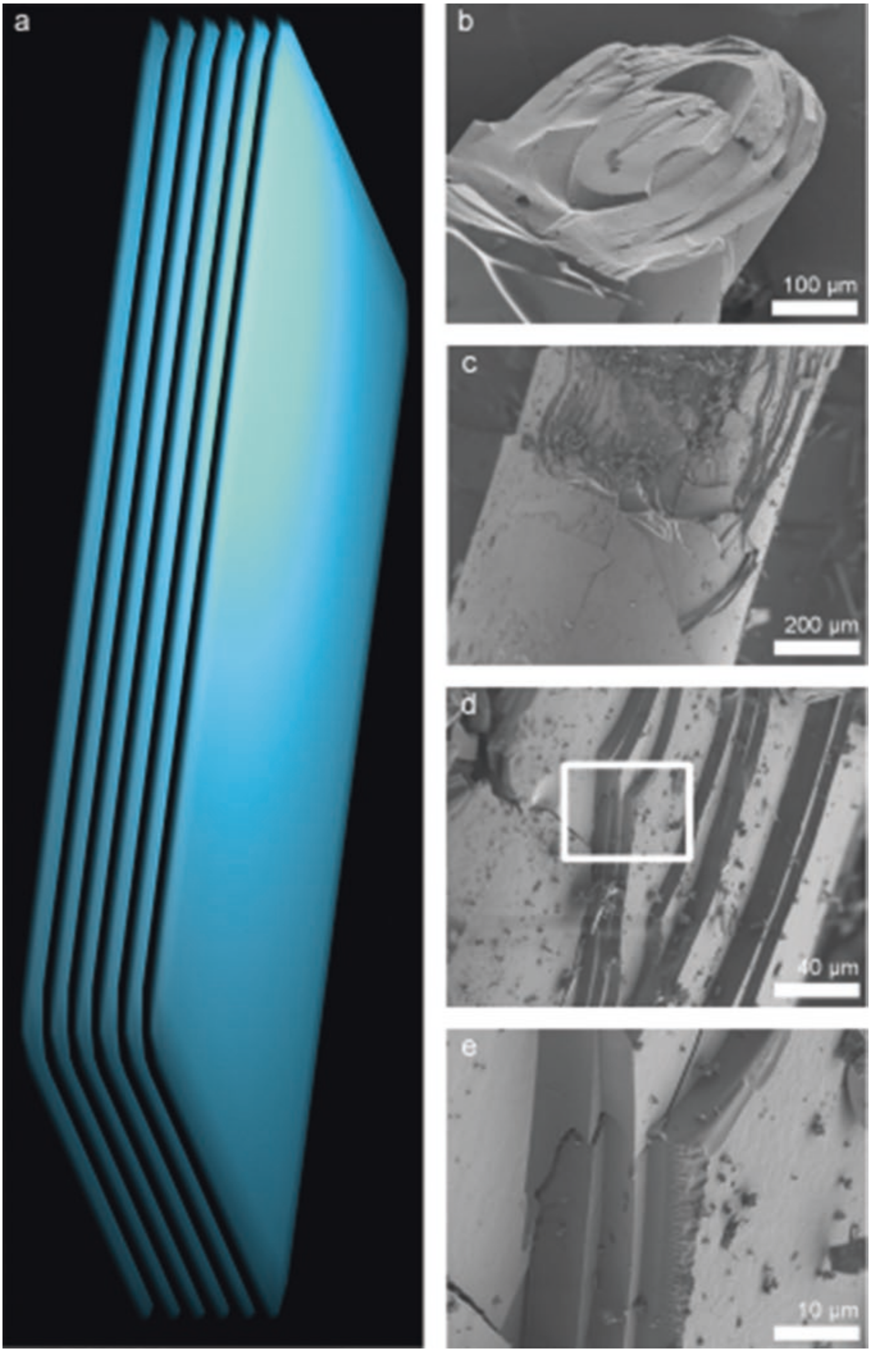

Fig. 7.20 Computer simulation of the multilayered structure typical for the hexactinellid spicule (**a**) is based on the observation which has been carried out using SEM (**b**, **c**, **d**, **e**). However, no organic layers are visible

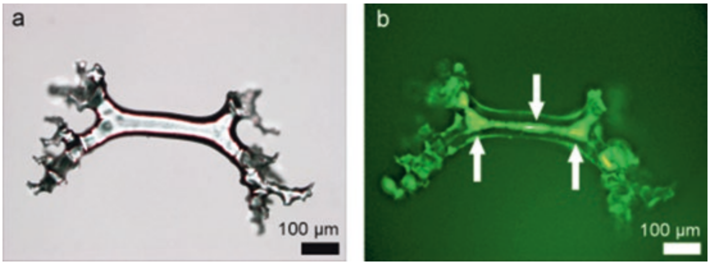

Fig. 7.21 The desmas of fossilized lithistid *Neopelta* sp. are easily observable using light microscopy (**a**). Intriguingly, fluorescence microscopy image (**b**) of the same desma confirms the presence of residual organic material within this glassy structure (arrows)

very fast, and therefore an essential desma part, together with elements of sculptures as well as secondary branches, does not depend on the existence of axial filament (Sollas 1888; Lévi 1991). Our joint observations together with Andrzej Pisera on unknown organic matrix within selected skeletal fragments of the 37 Myr old fossil litistid sponge using fluorescence microscopy confirmed occurrence of some organic matter with very strong autofluorescence within desmas of this sponge (Fig. 7.21). Unfortunately, till now it was not possible to isolate this organic matter and to carry out corresponding identification.

I take the liberty to recommend motivated materials scientist to learn more about diversity in form, size and shape of sponge spicules with axial, reticulate, hymedesmoid, radiate, and plumose miocroarchitecture from the works by Schulze and von Lendenfeld (1889), Uriz et al. (2000) and Boury-Esnault and Rützler (1997).

Scientific interest on mechanisms of spicules formation in diverse poriferan classes arose very early (see in Bütschli (1901) and Minchin (1909). However, a breakthrough was achieved in 1998 with finding of silicateins (Shimizu et al. 1998; Cha et al. 1999; Weaver and Morse 2003) as the main player in biosilicification in sponges. Till now, the silicatein theory is widely represented in the literature (Weaver and Morse 2003; Schröder et al. 2004; Müller et al. 2006, 2007; Fairhead et al. 2008; Riesgo et al. 2015; Wang et al. 2009,2018; Shimizu and Morse 2018; Aguilar-Camacho and McCormack GP 2019). In contrast to this well recognized theory, I still believe that silicateins are nothing else as one group of cathepsins which have been decorated with this intriguing term. My scepticism has been recently confirmed in the work by Povarova et al. (2018). Also, 10 years ago silicatein genes have been detected within spicule-free marine demosponges (Kozhemyako et al. 2009).

Back to the silicatein way for spiculogenesis in sponges proposed by Werner Müller as follows:

1. **Intracellular phase** (initiatory rise): It was proved that cells (sclerocytes) can actively absorb silicic acid using the Na^+/HCO_3^- $[Si(OH)_4]$ cotransporter (Schröder et al. 2004). At the same time, ripe silicatein is formed/created and then accumulated with silicic acid within the silicasomes – the special organelles of sclerocytes. The formation of axial filaments around the silica occurs inside the silicasomes, and then silica is enzymatically deposited. After first layer for-

mation (or several layers), young spicules are excreted to the extracellular space, and there, they rise in diameter and length via deposition of silica lamellae layer by layer (Müller et al. 2005). Within the extracellular space, spicules get their final shape through the terminal globular swelling.

2. **Extracellular phase**: in the extracellular space, silicatein exist in an enzymatically active form (Müller et al. 2005). Then, using immunogold electron microscopic analysis, it was proved that molecules of silicatein formed larger units. Silicatein particles are localized lengthwise to the filaments located parallel to the surface of the spicule (Schröder et al. 2006). In addition, they contain galectin – protein which is able to oligomerize when Ca^{2+} cations are present. Inside the organic cylinder which surrounds the forming spicule, the silica structure rises step by step, by the parallel lamellae layering. Interestingly, the extraspicular silicatein is able to drive both axial growth ("elongation") as well as thickening (centrifugal growth) of spicules. Similar situation is observed inside the extracellular space, also radial and axial spicules growth is powered by silicatein particles that enclose the already formed silica lamellae surface. It was found that, even 1000 of the layered silica lamellae, can be located in the hexactinellids (Wang et al. 2009).
3. **Extracellular phase** (final morphogenesis): To these days, al-ready described processes do not clarify the species- specific spicules formation. This last spiculogenesis stage (called morpho-genesis) is still enigmatic.

Consequently, when the demosponges (Eckert et al. 2006) as well as and hexactinellids (Müller et al. 2008) spicules are enclosed or even inserted within a fibrous structure of collagen or another proteins, thus we can propose that the molecules which are excreted by the functional sponge cells serve as a scaffold which can be used for organization of the galectin strings. Results imply that galectin strings formed net of collagen fibers (Schröder et al. 2006). The collencytes – specialized cells that released these fibers, are responsible for the organization of special platform for the spicules morphogenesis. The oblong spicules growth might be clarified by the fact that during the formation and elongation of the axial canal the silicatein/galectin complexes are embedded into biosilica deposited at the spicules tips.

Interestingly, the intra- versus extracellular secretion of spicules in Demosponges has been extensively discussed over the last century and is still a matter of speculation (Simpson 1984). Microscleres could be secreted both intra- and extracellularly (Simpson 1968) and the most recent studies seem to support the intracellular secretion of megascleres (Garrone 1969; Garrone et al. 1981; Simpson and Vaccaro 1974).

Thus, recent knowledge about the spiculogenesis in glass sponges is related to the microscleres secretion as well as small megascleres in a few species. It appears to be physically unreasonable for one cell of sponge which has 20 μm in diameter, to contain a big megasclere such as in the case of Astrophorid, Axinellid, Spirophorid, or some Hadromerid sponges (Uriz et al. 2000). But in this unusual case, the implication of some sclerocytes in excreting spicule appears to be essential, therefore, extracellular excretion could not be excluded.

For instance, the extracellular excretion of megasclere occurs in Crambe crambe sponge (Uriz et al. 2000). It was proved that the process of silicification is completed successfully in an extracellular space limited by the sclerocyte pseudopodia, when the axial filament is discarded to the mesohyl. Interestingly, Simpson (1984) suggested that the silicalemma seems to be nothing more than the plasmalem-ma. Maldonado and Riesgo (2007) in his investigation of *Corticium candela-brum* homosclerophorid of Demospongiae class proved that the sclerocytes as well as two types of epithelia (pinacocytes) comprise intracellular spicules. The ultrastructure of the layers of silica as well the size of the intracellular spicules, clearly show that the spicules silicification is incomplete, and promote the concept that the epithe-lial produce them "in situ", instead of their incorporation from the intercellular mesohyl. The descent of the choanocytes cytoplasm as well as the small spicules which rarely exist inside the sclerocytes nucleus is more indefinite. In this sponge, the spicules structure and location is untypical. The spicule cross-section indicates a subcircular axial filament surrounded externally by a siliceous layer, coated with two concentric extra-axial organic layers, which each of it is envel-oped by siliceous ring. This structural motif is interpreted by Maldonado (2007) as a consequence of a distinctive three-step pro-cess, comprising of a preliminary (axial) silicification process around the axial filament, followed by two (extra-axial) silicification processes. Obtained results show that the cellular pathways of spic-ule formations differ between the poriferan species.

7.5.1 Chitin- and Collagen-Based Silicification Versus Silicatein-Based Way

Structural aminopolysaccharide chitin has a nanofibrous structure and the chain of pyranose ring arranges almost parallel to the (100) plane and extends along the fiber axis (Iijima and Moriwaki 1990). The chitin molecule has C=O, O-H, and N-H groups and oxygen atoms. These have affinity for the calcium, phosphate, carbonate, and hydroxyl ions of the corresponding calcium phases as well as for silicate ions. Consequently the existence of naturally occurring silica-chitin composites in biomimeralizers was hypothesized by us in 2007 (Ehrlich et al. 2007) and confirmed later including both sponges (Ehrlich and Worch 2007; Ehrlich et al. 2008b, 2016) and diatoms (Brunner et al. 2009).

First report on collagen-like fibrillary matter observable during desilicification og the spiocules of glass sponge *Monorhaphis chuni* (Fig. 7.22) was published by Ehrlich et al. in 2008. Discovery of previously unknown hydroxylated collagen (Ehrlich et al. 2010) from psychrophilic, deep-sea hexactinellid *Hyalonema sieboldii* that possess repeating [Gly-3Hyp-4Hyp] motif introduce the new player into biosilicification "big game" and make trouble for adepts and follower of the silicatein theory.

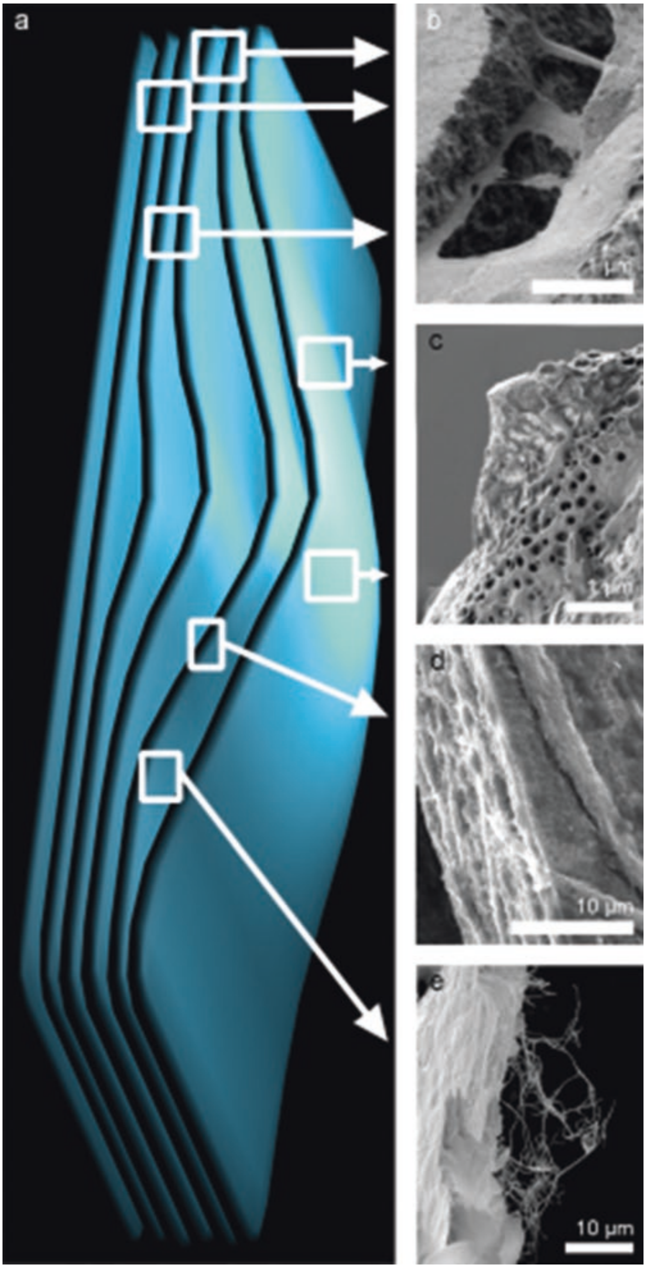

Fig. 7.22 Computer simulation of the multilayered structure of the *Monorhaphis* spicule after partial demineralization in 2.5 M NaOH (**a**) is based on the SEM observations (**b**, **c**, **d**, **e**). In contrast to intact spicule represented in the Fig. 7.20 the fibrous organic matter is well visible

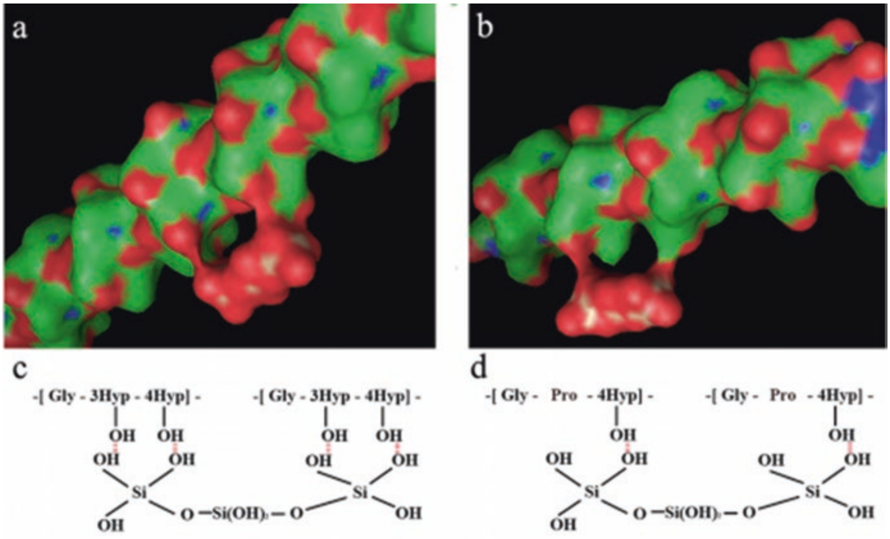

Fig. 7.23 Simulated model of interaction between Gly-3Hyp-4-Hyp polypeptide and polysilicic acid (**a**) and/or with Gly-Pro-4-Hyp polypeptide (**b**) typical for classical collagen triple helix. (Green- carbon atoms; red-oxygen, blue-nitrogen, white – silica white). Mechanisms for these interactions are shown schematically in (**c**) and (**d**). (Image courtesy Denis Kurek)

The collagen pattern found in *Hyalonema* is similar to the Schumacher et al. (2006) model which depicts the 3(S)-hydroxyproline residues in the Xaa position. Thus, the formation of an interaction between Gly-3Hyp-4-Hyp polypeptides and polysilicic acid isolated from glass sponge collagen (Fig. 7.23) is possible. Additionally, it is reported that the interaction between hydroxyl groups and orthosilicic acid can be assumed as hydrogen bond (Tilburey et al. 2007). This assumption indicates that the formation of stable complex between polysilicic acid hydroxyl groups and surface's hydroxyls groups of 3-Hyp and 4Hyp is possible due to existence of hydrogen bonds. In this model the role of 3-Hyp in the silification of collagen is also proposed. Collagen, which is characterized by the presence of the surface hydroxyl groups, may react with silicic acid molecules (by means of condensation reactions) which result in loss of water. Therefore, analogical to the Hecky et al. (1973) model, the primary condensed silicic acid layer will be stable joined to the hydroxylated collagen template, in such geometric array that will allow further silicic acid polymerization.

7.6 Conclusion

The fact is that during million years of evolution nature produced fascinating silica-based 3D skeletons with sophisticated joint systems (Fig. 7.24) to hold them as integrative and mechanically robust constructs (Schulze 1886,1904; Iijima 1901;

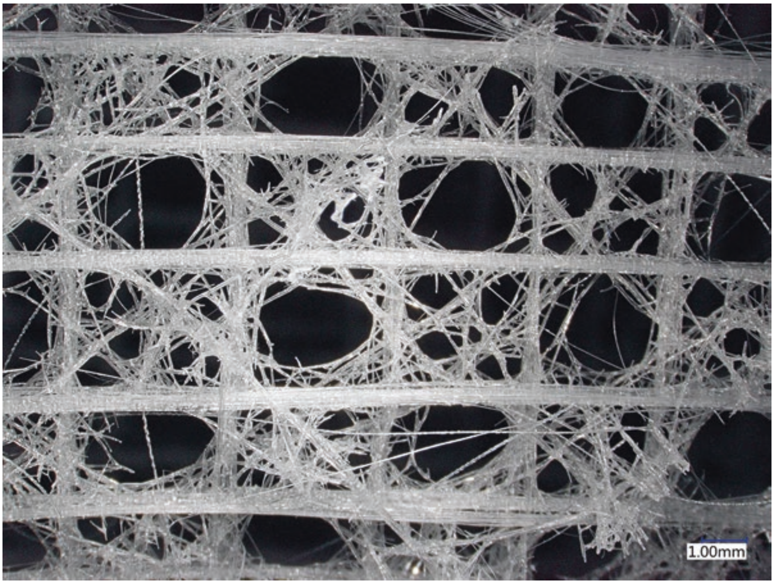

Fig. 7.24 Light microscopy image of the skeletal fragment of *Euplectella* sp. represent fascinating 90° angle-geometry made through biosilicification. Regulations mechanisms of naturally occurring manufacturing of such highly sophisticated structures are still unknown

Levi et al. 1989; Janussen et al. 2004; Aizenberg et al. 2005; Weaver et al. 2007; Tabachnik et al. 2011, 2017) under diverse conditions within marine environments (i.e. deep-sea underwater current flows, temperatures around 0 °C, etc.) (Heezen et al. 1966; Bavestrello et al. 1993). Unfortunately, the fact is also the dissatisfied situation with our many years of trying to obtain true knowledge concerning the mechanisms of biosilicification in sponges. Thus, except silicatein theory, the recent discovery of a histidine-rich protein named "*glassin*" (Shimizu et al. 2015) and cathepsins (Povarova et al. 2018) confirmed the existence of alternative players in this kind of poriferan biomineralization. Furthermore, according to recent opinion by Ozaki et al. (2018): "*The advances in peptide-driven mineralization suggest that it will soon be possible to completely control the silica mineralization process using peptides*". It is still hard to believe that giant skeletons of some hexactinellids (i.e. *Aspidoscopulia* (Fig. 7.4.), or 3 m long *Monorhaphis*) (Wang et al. 2009), have been manufactured in vivo using only low molecular weight peptides, or proteins. In this case the role of such biopolymers as chitin and collagen in biosilicification of these large-sized sponges remain enigmatic.

Moreover, the detailed analysis of all images of so called axial filament commonly located within siliceous spicules (for details see recent review by Wysokowski et al. 2018) leads to understanding that this structure is not completely "arrested within silica", but is free from the mineral phase. Therefore, I take the liberty to suggest that some other that collagen, or silicatein-like fibrillar protein represents axial filament in all classes of sponges. This protein is not involved in biosilicifica-

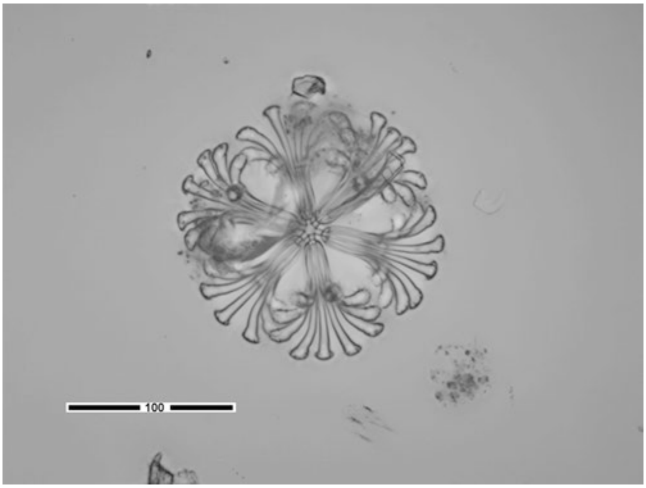

Fig. 7.25 Light microscopy image represent the highly symmetric multirayed microsclere of some sponge. Challenging task remains to understand the cellular mechanism of formation of such siliceous microscleres

tion, however it may play the crucial role in oriented growth of the spicules including their dichotomic as well as unique ray-like morphology (Fig. 7.25).

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Chapter 8
Interspace Mineralization Within Bilayered Organic Matrix of Deep-Sea Bamboo Coral (Anthozoa: Gorgonacea: Isididae)

Abstract Bamboo corals are the representatives of the Gorgonacea order within Isidids octocorals. Opposed to corals from the Scleractinia order, in which the biomineralization process has been well evaluated, the mineralization in the Isididae corals is still not fully understood. The interspace mineralization process in the deep-sea bamboo corals bilayered organic matrix is described and analyzed.

It is well known that, the mineralization phenomena in corals is a process where the organism is able to create an organic structure that provokes the nucleation and development of crystals, together with the framework microarchitecture and appropriate crystallographic orientation (Goldberg 2001). Opposed to corals from the Scleractinia order, in which the biomineralization process has been well evaluated (Cuif and Sorauf 2001; DeCarlo 2018; Tambutte et al. 2011), the mineralization in the Isididae corals is still not fully understood. These are known as long-lived psychrophilic gorgonin octocorals (Frenkel et al. 2017).

Bamboo corals are the representatives of the Gorgonacea order within Isidids octocorals (for overview see Etnoyer 2008; Watling and France 2011; Watling et al. 2011; Alderslade and McFadden 2012; Dueñas et al. 2014; Frenkel et al. 2017). They are the deepest occurring habitat-forming deep-sea coral family, with a maximum depth of over 3800 m. Over 50% of Northeast Pacific coral organisms are usually located at a depth higher than 1000 m (Etnoyer and Morgan 2003). In the Isididae family, 138 species within 38 genera have been described by S. Cairns and F. Bayer from the Smithsonian Institute in Washington D.C. (Bayer 1990). The unbranched as well as abundantly branched taxa can be easily found. In some species, branching might appear at the internodes, as well as at the nodes in another species (Fig. 8.1a). The skeleton is coated by the coenenchyme which is the colonial living tissue comprised of calcareous sclerites and epidermal layers surrounding mesoglea, similar to polyps. Sanchez (2002) characterized Isidids as classic representatives of Calcaxonia suborder with branching tree-like networks, calcareous joints and continuous multilayered axis (Fig. 8.1c, d). In 2006 we conducted research to understand the nanotopography of gorgonin intersurfaces and node surfaces, of the coral axial internode together with the morphology of the nanoscale mineralized as well as observed patterns. We evaluated the function of the gorgonin

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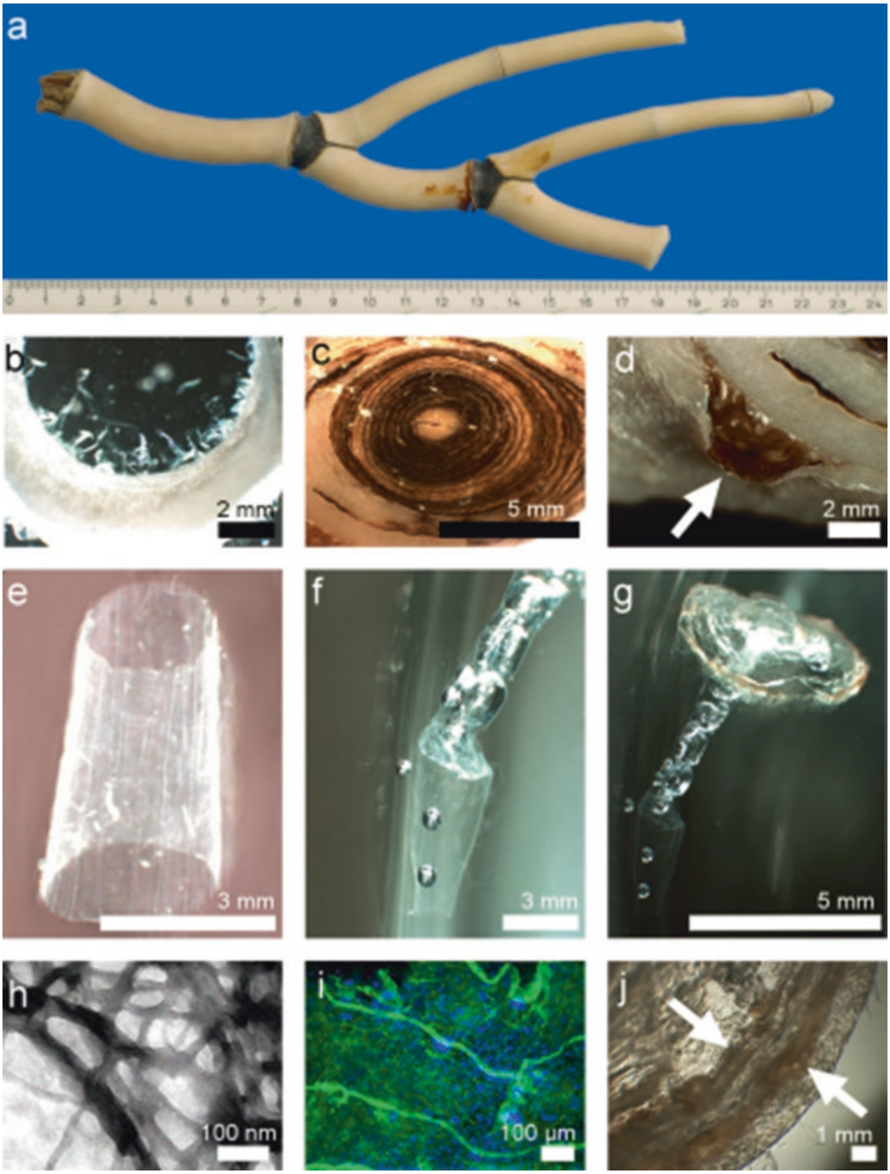

Fig. 8.1 Finger-like architecture is typical for the bamboo corals of Isididae family (**a**). The cutted surface of the skeletal nodes helps to visualize the continous multilayered axis (**c**, **d**). Osteosoft treatment leads to the isolation of the organic matrix (**b**, **e**, **f**, **g**). It possesses noncollagenous, but nanofibrillar structure (**h**). Layers made of gorgonin are usually visible in fluorescence (**i**) as well as light (**j**) microscopy

mineralization, as well as the biomimetic capability of the bamboo corals for the possible progress in obtaining novel biological materials for the biomedical purposes (Ehrlich et al. 2006).

In order to understand the nanostructure as well as nature of the axial organic matrix obtained from corals, the process of decalcification with Osteosoft™ was applied (Fig. 8.1b). After 7 days of decalcification, the total lysis of the calcite axis internode, and the presence of a transparent gelatinous pellicle were observed (Fig. 8.1e–g). The fibrillar protein behaviour of the organic matrix was confirmed using SEM, TEM (Fig. 8.1h) and AFM, respectively. The amino acid analysis shows that the proline and glutamine (24.0, 28.9% and respectively) are the main building blocks of this matrix (Ehrlich et al. 2006). The low glycine content (2.5%) excludes the eventuality that the fibrillar structure has collagen origin. This is contrary to the collagen detection within the organic matrix obtained from *Corallium rubrum* (Allemand et al. 1994). Thus, we found that the Isididae's axial internode organic matrix can be assumed as an acidic fibrillar protein. These findings are in the line of the popular Lowenstam and Weiner theory, which stated that these proteins are essential during the biomineralization (Lowenstam and Weiner 1989). It is worth to notice that, in a distal gorgonin-containing node, any disruption effects were observed, even after 3 months of the analogous decalcification procedure with Osteosoft™. Interestingly, after 72 h of alkali treatment the fragmentary disruption and dissolution this sample was achieved.

The light microscopy analysis of the dark brown Isidid node from the distal part of sample (Fig. 8.1i, j) indicates the existence of yearly alternating rings of mineral and horny (gorgonin) origin.

Data of the chemical characterization of sample, performed with use of EDX/ESEM, demonstrate the existence of C-57.73, N-20.91, O-10.94, Br-2.54, S-1.96, Na-1.86, P-1.05, Cl-1.65, Mg-0.87, Ca-0.21, Fe-0.20 Zn-0.08 (At/%). Recently, gorgonin (known also as chitinous- iodine- bromine-, mucopolysaccharide-containing protein or tanned-collagen (Ehrlich et al. 2003)) had no determined chemical composition. In 1855 Valenciennes first noticed that the Mediterranean Gorgonia internal skeleton consisted of a horn-like protein, therefore it receives the name gorgonin (Block and Bolling 1939). In 1939, Block and Bolling demonstrated the abnormally large amounts of phenylalanine (6.5%), cystine (9%) and tyrosine (13%) in gorgonin obtained from *Gorgonia flabellum*.

The coating of the organic matrix middle channel from the gorgonin distal node observed using SEM (Fig. 8.2a, b) is brownish under light microscopy. Differences among these two structures emerge from the unique organization of the nanostructure (Figs. 8.3a, b and 8.4a, b). In the bilayered epithelium of the gorgonin-comprising middle channel node, the mineralized fibrous structures could be observed on both interfaces (thickness of the layer about 150 nm). The spherical formations of calcium carbonate, about 70 nm large, are closely spread on microfibrils to form the intersurface of gorgonin (Fig. 8.5a, b). Within the microfibrils-free surface of the gorgonin layers, no formation of the mineral phase was observed. Therefore, gorgonin layers themselves can act as patterns for the formation of proteinaceous microfibrils, which can be used as a template for the biomineralization. These results

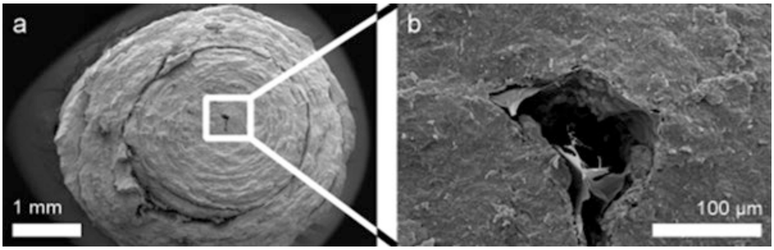

Fig. 8.2 SEM image: transverse view of the distal gorgonin-containing node

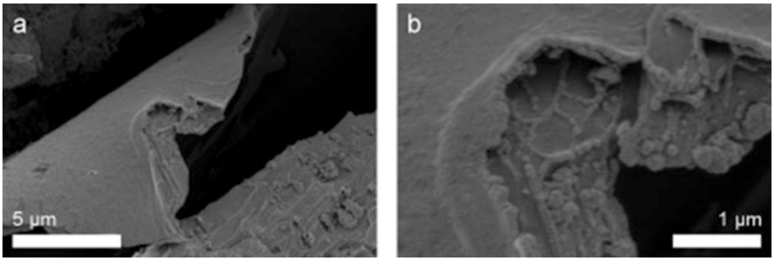

Fig. 8.3 SEM images of the bi-layered gorgonin-containing epithelium covering the middle channel of distal node

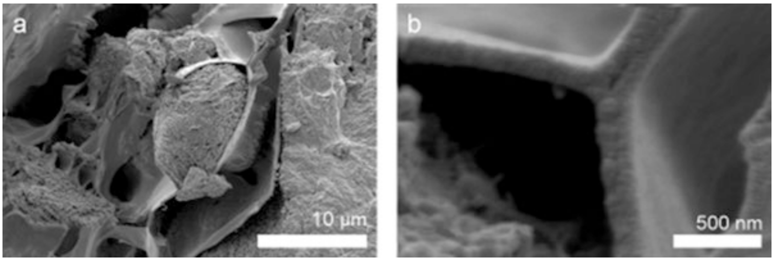

Fig. 8.4 SEM images of the bi-layered gorgonin-containing epithelium covering the middle channel of distal node

corresponds with those obtained by Bayer and Macintyre (2001). They described the submicron spheres of carbonate-comprising hydroxylapatite found in some gorgonian species within their chambered axial core. We supposed that on the nanolevel the interspace biomineralization process can be typical for octocorals.

The pigment recognition amenable for brownish gorgonin-comprising internodes was conducted using FTIR and UV/Vis- spectroscopy. FTIR spectra (Ehrlich et al. 2006) accurately correlate with the IR-spectra obtained for the quinone-tanned Mefp-1 ft protein received from the *Mytilus edulis* sea mussel by Suci and Geesey (2001). Results of the UV/Vis analysis of the yellow, sticky supernatant (Fig. 8.6, right below) from the alkali-treated gorgonin node (data not shown) received after

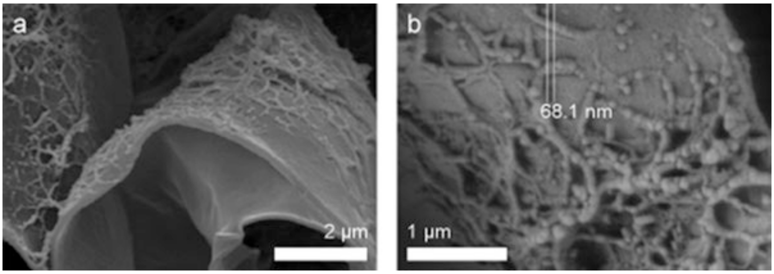

Fig. 8.5 (**a**) SEM Image of gorgonin-containing epithelium that function as a template for microfibrils on which mineralization occur (**b**)

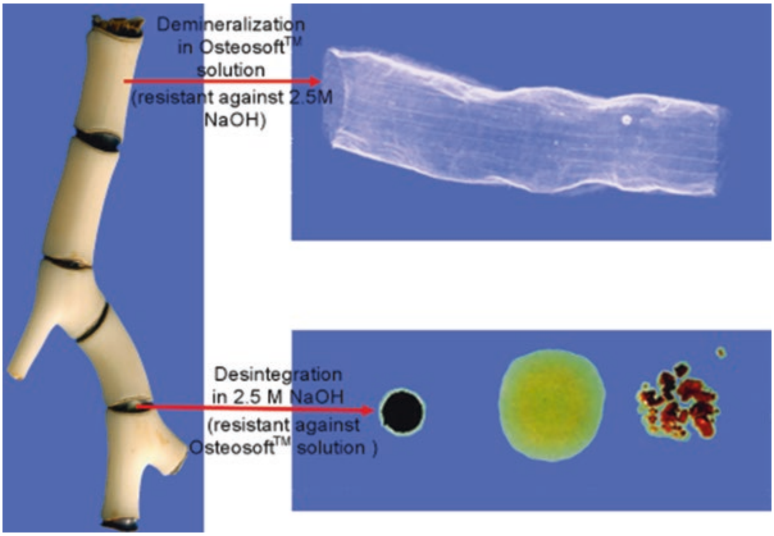

Fig. 8.6 Both fibrillar organic matrix (right above) and dark colored gorgonin-based extracts (right below) can be isolated after selective chemical treatment of Isididae sp. coral fragment

centrifugation (15,000 g, 30 min) were alike to the spectra of the quinone compounds isolated from the dark pigment of the capsules skate egg, what was reported by Koob and Cox (1990).

A gorgonin tanning potential mechanism can be determined as follows: a stable cross-linked protein is formed by the reaction of the quinones with sulphydryl and terminal amino groups. The polymerisation of quinones during the synthesis of the melanin-like biopolymers is responsible for the brow colour of the tanned Isidid coral nodes. A graphical representation (Fig. 8.7) of the likely polyphenolic compounds of the gorgonin, elucidate the unavailability of these non-reactive oxygen residues for any interaction with gorgonin's Ca-ions. The detailed comparison of the other references similar to various quino-proteins of marine origin like antipathin (Goldberg et al. 1994) or byssus (Coyene and Waite 2000; Zhao and Wait 2005)

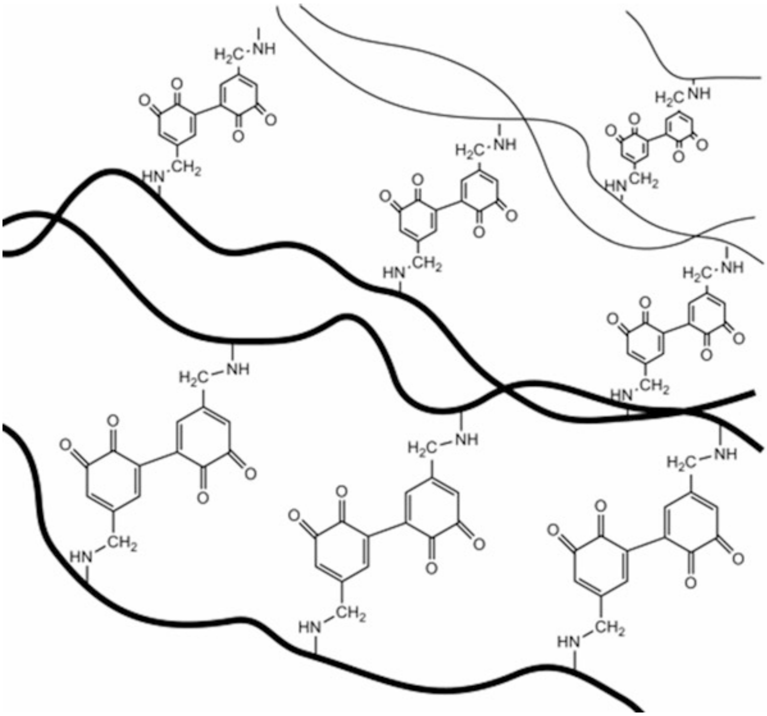

Fig. 8.7 Schematic view of the possible gorgonin-based cross-links in Isididae corals (image courtesy Denis Kurek)

validates our hypothesis that majority of the proteins comprising polyphenol are basic and hydrophobic, thus the mineralization under natural conditions is impossible. Therefore, in Isididae corals, the presence of gorgonin nodes is related to their flexibility, not to hardness, as opposed to calcite-containing axial internodes.

8.1 Conclusion

Thus, for the first time, we showed that within the Gorgonacean family: Isididae, the quinones-containing compounds play important role in the gorgonin nodes. A versatile knowledge about gorgonin-containing nodes and their structure, composition and mineralization can be used to create new pathway of biomimetic formation of hybrid materials with characteristic bioelastomeric features essential for their application in biomedicine. The biopolymers based on polyphenolic compounds can be applied in the formation of novel blood vessel implants and calcification resistant biomaterials.

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Chapter 9
Living Bone Implants of Bamboo Corals Origin

Abstract The structure of Porites, widely applied coral, can be compared to cancellous bone, and their mechanical features also resemble the bone properties. Porites exoskeletons which can be assumed as scaffolds with high amount of calcium carbonate are osteoconductive, biocompatible and biodegradable what is related to the porosity of exoskeleton, species and the inoculation site. Due to their microarchitecture and joint-like anatomy octocorals belonging to the Isididae family remain to be perspective model organisms for biomedicine. Thus, the development of biotechnological cultivation processes of Isididae corals according to the aquacultural conditions to obtain "living bone implants" is the next challenging task.

Already in the 1970s scientists tried to use corals as possible bone graft implants in mammals, and then, in 1979 in humans (see for review Demers et al. 2002). The reason is based on the structure of Porites corals, which morphology can be compared to that of cancellous bone, and their mechanical features also resemble the bone properties. Porites exoskeletons which can be assumed as scaffolds with high amount of calcium carbonate are osteoconductive, biocompatible and biodegradable what is related to the porosity of exoskeleton, species and the inoculation site. It has to be noticed that, corals do not have osteogenic or osteoinductive properties, but their grafts serve as an active growth factors carrier and let attachment, growth, spreading and differentiation of the cells. After appropriate selection of coral implant and application method to obtain similar resorption rate to the rate of bone formation on the implantation site, it was proved that exoskeletons of the natural coral are effective bone graft implants.

Hydrothermal conversion of the calcium carbonate skeletons of selected corals into hydroxyapatite (HAP) has been used for manufacturing of so called coralline hydroxyapatite (CHA) (Damien and Revell 2004). Corresponding overview concerning the preparation steps of coral for bone grafting and CHA for bone replacement for clinical applications is represented in Fig. 9.1. Unfortunately, the biomedical applications of CHA can be limited owing to its reduced biodegradation as well as inherent mechanical weakness. On the other side, such properties of CHA as its biocompatibility, safety, and osteoconductivity (Coughlin et al. 2006) confirmed possibility of its applications as a substitution biomaterial for bone.

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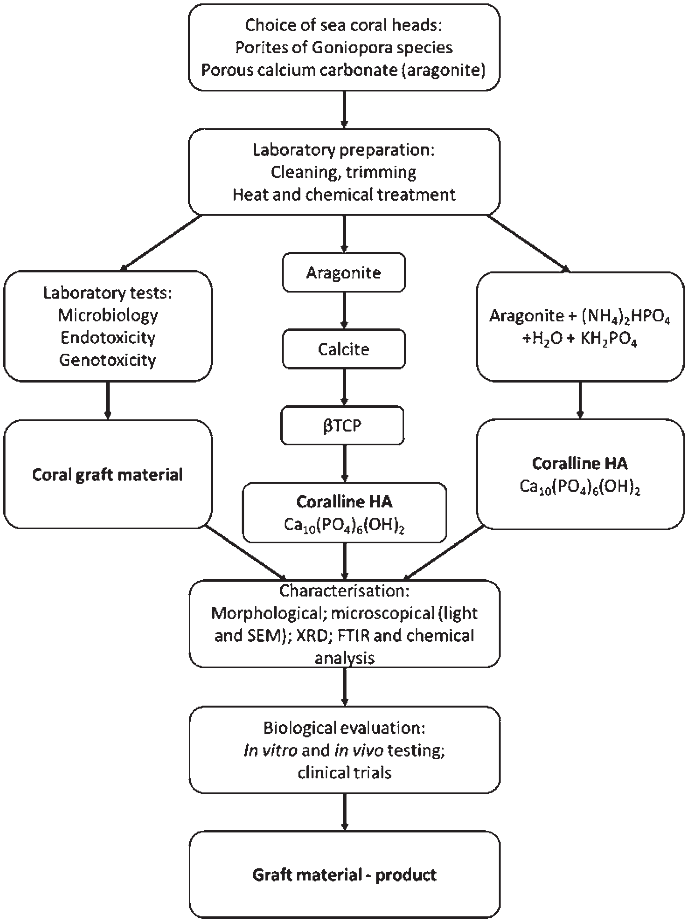

Fig. 9.1 Overview for the development of bone grafting using sea coral. (Adapted from Damien and Revell 2004)

HAP of coralline origin has been used also for designing of the so called "bio-eye" (Jordan et al. 1998). In opposite to the majority of usually applied implants, after porous hydroxyapatite implantation into the eye socket, organism do not treat it as a foreign substance, but allow integration with tissue and blood vessels (Dutton 1991; Jordan et al. 2004). Consequently, this ingrowth into the orbital tissues results in the resistance of implant to the migration and rejection.

Selected dogs bones have been used as model systems for implantation of coral fragments of different sizes (Guillemine et al. 1987). For example, a cortical defect (width 5 mm, length 8 mm; see Fig. 9.2.) was made in both ulnae (30 defects) in 15 dogs, and repaired with highly porous fragments isolated from Porites coral.

Thus, coralline-derived bone grafts substitutes of the stony corals origin have a long track record as biocompatible and osteoconductive biomaterials (see for

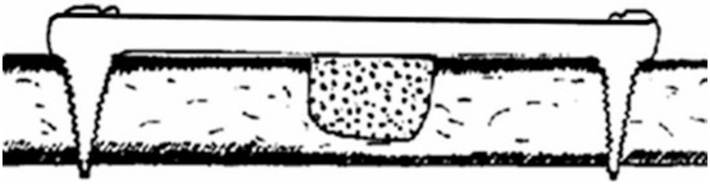

Fig. 9.2 Schematic representation of a cortical defect (length 188 mm, width 5 mm) grafted with a coral fragment and stabilized with a bone plate. (Adapted from Guillemine et al. 1987)

overview Gao et al. 2009; Wu et al. 2009; Zhang et al. 2011; Tran et al. 2011; Geng et al. 2013; Puvaneswary et al. 2013; Pountos and Giannoudis 2016).

What's about octocorals? In order to evaluate the possibility of applying the *Isididae* octocorals (Dueñas et al. 2014; Watling 2015; Moore et al. 2016; Cairns 2018) calcite-containing internodes, we put into the tibia of the dog a corresponding fragment (Ehrlich et al. 2006). Corresponding oval aperture within the animal's tibia has been made under intravenous anaesthesia conditions using a milling cutter on low circulations. The specimen was put into the hole and was quite observable in the X-ray image after 24 h, which is a consequence of higher optical density in comparison with the bone cortical layer. After 10 months, the biotransformation of the implant into the dog's bone tissue as well as the complete resorption was observed, because the locality where the coral specimen has been implanted was no more visible. It has been proposed that, in resorption process of the coral fragment used the main factor is an enzyme, carbonic anhydrase which is abundant in osteoclasts (Guillemine et al. 1987; Ehrlich et al. 2009). The enzyme dissolves the bone matrix consisted of calcium carbonate and reduces the pH at the interface between osteoclast and implant. In the case of other fragments of natural coral, the resorption proceeds centripetally and was most active in the locations of the tight bone-matrix contact.

After intriguing results obtained in experiments with dogs, one Russian team from Samara State Technical University, on February, 22, 2006, inserted a centimeter large bamboo coral fragment into the dental plate of the 17 year old male patient as represented in the Fig. 9.3 with success.

According to the personal communication from Russian colleagues, any rejection of coral implant as well as infection was observed in this case. Interestingly, the resorption of the calcite-containing implant of the coral origin was faster compared to that of bioceramics .

9.1 Conclusion

Based on the impressive biomedical potential for the results reported above, we suggest that biotechnological aquaculture-based cultivation of the *Isididae* corals to obtain "living bone implants" (Ehrlich et al. 2006) should be proposed and

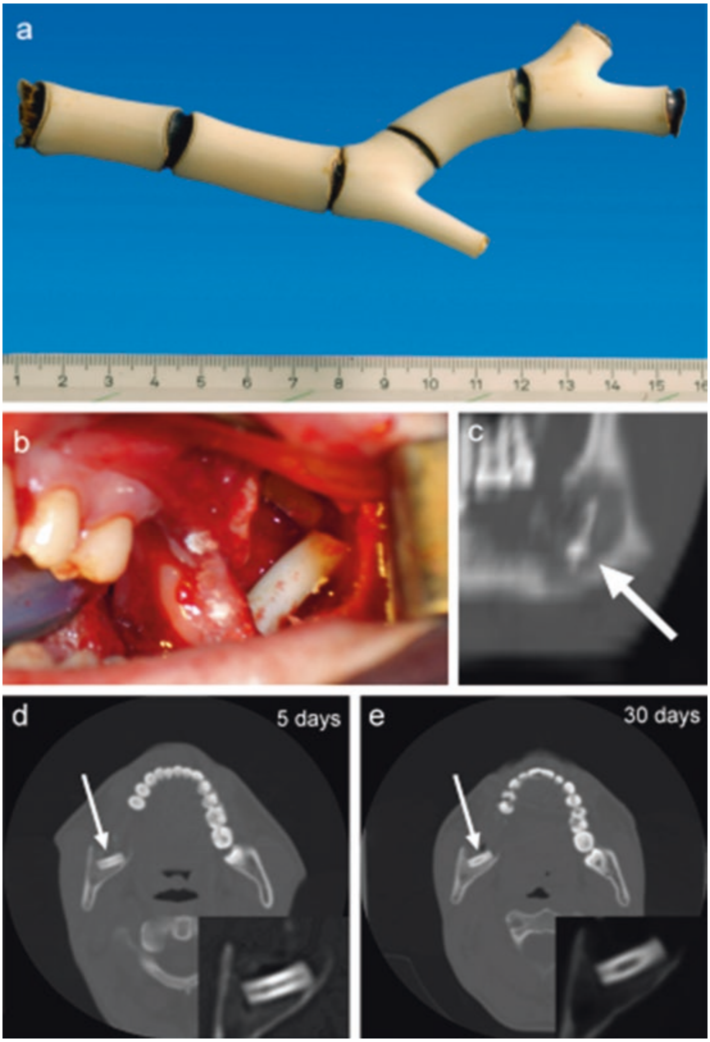

Fig. 9.3 Selected fragment of the branched section of *Isidella tentaculus* bamboo coral (**a**) has been successfully implanted with the goal to repair the mandible defect of a patient (**b**). More detailed view is observable on X-ray images (**c**–**e**)

established in the near future. According to Ehrlich and co-workers "*Isididae growth under natural conditions suggest that the growth rate of these corals was estimated to be between 19 and 120 cm per year*" (Ehrlich et al. 2006). The determination of aquaculture-based farming for the cultivation of these unique octocorals should open a new keyway for the designing of bone implants with natural origin.

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Chapter 10
Spicular Structures in Molluscs

Abstract Biosynthesis, structural and functional diversity of spicules and spicule-like constructs in marine molluscs remain to be hot topic for biomaterials community. Especially scare information has been obtained with regards to the nature of organic phases within both calcareous and siliceous spicules. This chapter includes discussions concerning unique spicular formations in selected species of Aplacophora, Nudibranchia, Polyplacophora as well as Onchidiella molluscs. Special attention has been paid to onchidiid sea slugs where possible existence of siliceous spicules in genital organs is debated since the first report in 1934.

Calcium carbonate- based spicules are well distributed structures in marine invertebrates including sponges (class Calcarea) (Woodland 1905; Jones 1970, 1978; Ledger and Jones 1977), corals, plat worms, molluscs, echinoderms and ascidians (Loewig and Kölliker 1846; Giard 1872; Woodland 1907; Lambert 1979; Kniprath and Lafargue 1980). In the overview made by Kingsley (1984) we can find as follows: "*Mature spicules appear to be extracellular structures. Sponge spicules initiate intercellularly then become extracellular. Alcyonarian, turbellarian, echinoid and ascidian spicule deposition begins intracellularly and then becomes extracellular. The continuation of growth in the extracellular environment has not been documented except for the echinoids. Placophoran spicules initiate and remain as extracellular structures. Early spicule growth seems to occur from or within a single cell. However, cell aggregation and/or neighboring cells appear to be important to the process of spicule formation. The spicule forming cells, in general, are found in a collagenous medium which may be associated with spicule growth.*" (Kingsley 1984).

There are 7 Molluscs taxons, 6 of which (the Cephalopoda, Gastropoda (except order Nudibranchia), Bivalvia, Monoplacophora, Scaphophoda, and Polyplacophora) are shell-bearing (Hyman 1967; Treves et al. 2003). The Aplacophora usually possesses spicules deposited within mantle tissue, instead of shell, which can be assumed as composite material build of organic framework covered by mineral phase.

Calcium carbonate-based spicules have been detected in such molluscs as Solenogasters (Aplacophora) which belonging to primitive organisms (Kingsley and Marks 2008) with location at the basis of the phylogenetic tree. Interestingly,

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these tiny molluscs are characterized by the presence of discontinuous cluster of calcium-containing spicules inserted in, or outstood upper their cuticle, instead of continuous shell.

10.1 Spicules of Nudibranchia

Representatives of Nudibranchia also called “sea slugs” (Fig. 10.1) with more than 3000 species worldwide) attracted attention of numerous scientific groups (Alder and Hancock 1845–1855; Garstang 1890; Todd 1981; Behrens 1991, 2004; Gosliner 1994). Common caracterization has been given by Wägele and Willan (2000) as follow: “*nudibranchs are characterized by the lack of the operculum and shell, existence of papillae on the notum, head distinct from foot, obligate cross-fertilization involving semelparity, copulation as well as 1 3 haploid chromosomes, contempo-*

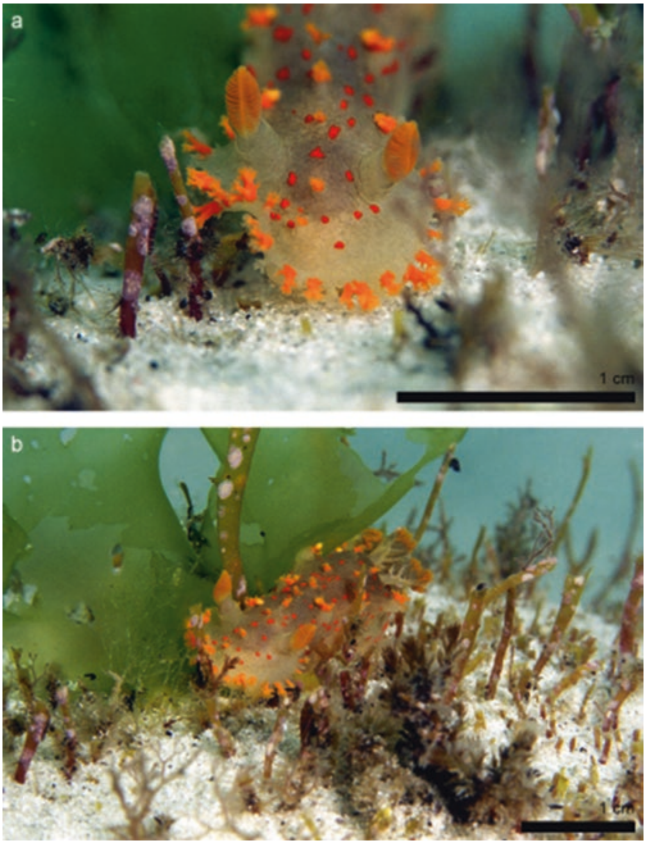

Fig. 10.1 See bottom is the usual habitat of diverse Nudibranch species (**a**, **b**). (Images courtesy A.V. Ratnikov)

rary development of the notum over the dorsal body surface during metamorphosis, multiple bilaterally symmetrical g d s, extensive detorsion which causes almost total (external) bilateral symmetry, hermaphroditic reproductive system with contemporary gametes maturation, paired gustatory oral tentacledveil, paired dorsal chemosensory tentacles (rhinophores)" (Wägele and Willan 2000). There are several defence mechanisms of the soft body such as chemical (*in situ* formation of synthesis repugnant fluids as toxins and utilization of toxins excreted by prey), behavioural (crypsis, autotomy) as well as morphological (spicules and kleptoplasty of cnidarian nematocysts). These marine animals (only *Anylodoh baicalensis* is freshwater organism) are free living, exisitng in various ecological niches.

These organisms have a varied diet comprising of different animal groups (Cnidaria, Porifera, Crustacea, Bryozoa, Ascidiacea Mollusca), but single species can have great specificity.

They have different size, from small *Amiwa cinnabareu* (adult form has 4 mm in length) to *Hexabrunchus sanguineus,* which adult organism has 600 mm in length (Wägele and Willan 2000), but majority of species have no more than 30 mm.

In his study on *Proneomenia aglaopheniee* Woodland (1907) described formation of calcareous spicules from granular, spherical concretion (Fig. 10.2) which becomes a rod. According to Woodland (1907), "*the median portion of the adult spicule is formed first (evident when this is thickened), and the tapering extremities growing out from this. The scleroblast, i. e. single nucleus, never divides, so that the spicules are purely unicellular products. In most cases the body of the scleroblast (the small mass of protoplasm immediately surrounding the nucleus) is constantly situated midway in the length of the spicule, i. e. in the vicinity of where the spicules will be the thickest.*

Chemistry and structural features of diverse nudibranch spicules which biological reason is to defense against predators (Penney 2006) are described in details by Prenant (1924), Schmidt (1924, 1944), Foale and Willan (1987), and Cattaneo-Vietti et al. (1995). Morphologically calcareous spicules are slightly curved, slender, and sometimes spiny (Fig. 10.3). Mechanically tested they are brittle, being

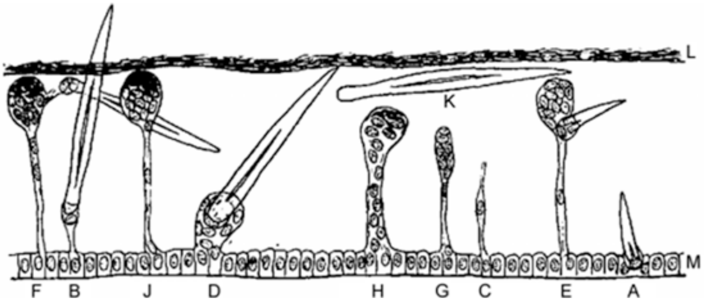

Fig. 10.2 The spiculiferous cells and spicules by the hypodermal papillae of *Hiehypodermis* and cuticle of *Proneomenia aglaopheniee* are represented on this semi-diagrammatic drawing. The figure (X 400 diam.) is composed of drawings of the actual structures, brought together into one field. For detailed description see Woodland (1907)

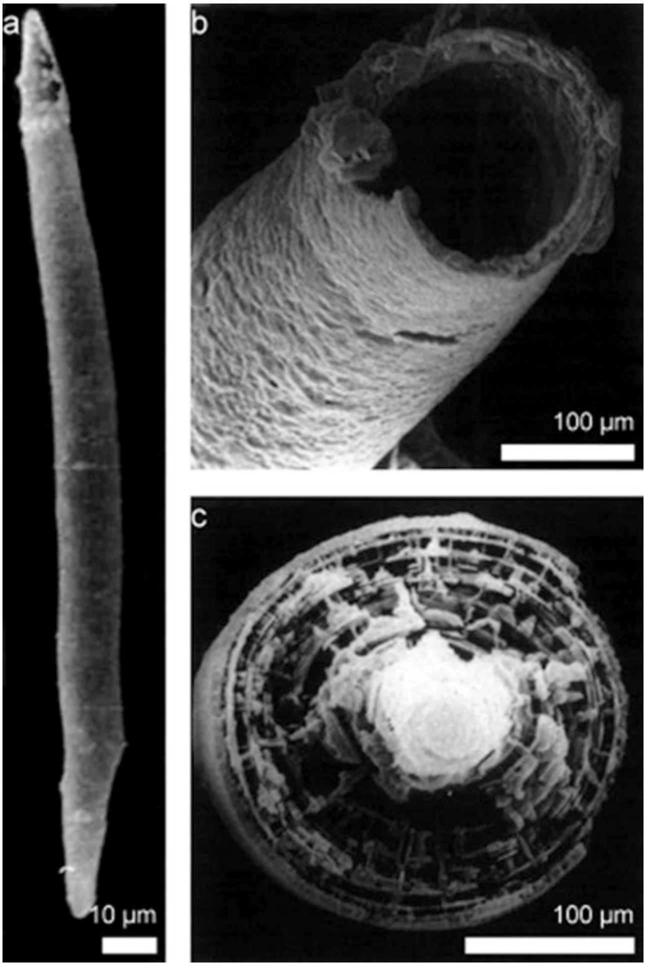

Fig. 10.3 (**a**) Empty fusiform spicule of the *Discodoris atromaculata*,; (**b**) the internal structure of a fusiform spicule of *Austrodoris kerguelenensis* shows concentric layers and radial pillars; (**c**), internal structure of a fusiform spicule of *Anisodoris tessellate.* (Adapted from Catteneo-Vietti et al. 2004)

hollow in the centre (Fig. 10.3a). However, some of them content quite complex structure which is based from concentric layers (Fig. 10.3b).

It is suggested that structural features of spicules can be used for a phylogenetic analysis (Wägele and Willan 2000).

Jorunna sp. and *Rostanga arbutus* dorid nudibranchs possess huge amount of caryophyllidia (even several thousand) evenly located on the whole upper surface. Caryophyllidia are small (diameter of 40–50 µm) with upright nodules internally supported by several (four to seven) perpendicular, calcium-based spicules that appear as a circumfluent crown surrounding on an apical knob (Fig. 10.4). These

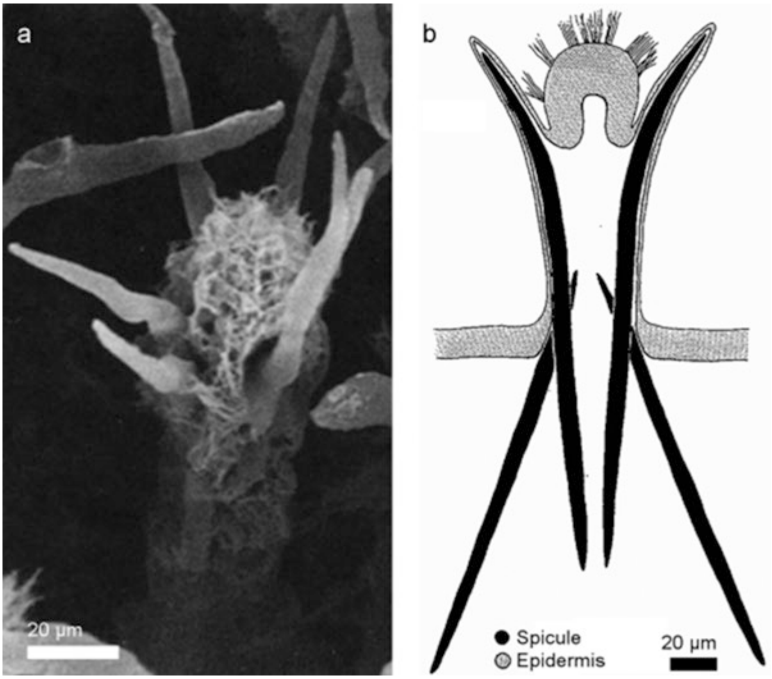

Fig. 10.4 SEM image of the spicular formation of *Rostanga arbutus*. (**a**) Epidermal cap from broken tip of emergent spicule. (**b**) The longitudinal section of a caryophyllidium represents spicule organization including the epidermis. (Adapted from Foale and Willan 1987)

arrangements are characterized by a complex muscle and advanced organized spicular systems. Epidermis of the capping sub-epithelial ganglion forms the apical knob. The caryophyllidium structure shows its significance as a new sign in the phylogeny and taxonomy of dorid (Foale and Willan 1987).

It seems that, the caryophyllidium is created by the epidermis which is stretched on the skeletal structures of spicules. Thus, the caryophyllidium interior part is detached from the epidermis by the epidermal basement membrane as well as continuously covered the mantle connective tissue (Fig. 10.5). Thus it can be assumed that, spicules cannot infiltrate this membrane. Despite their existence at the apex, spicules are still enclosed in an epidermis.

Surprisingly, some molluscs possess ability to synthesise calcium carbonate-containing spicules (Fig. 10.5) as well as simultaneously to secret acid (Fig. 10.6a, b).

The protective secretion of acid by various prosobranch and opisthobranch molluscs was described for the first time by Garstang (1890) in the opisthobranch *Pleurobranchus membranaceus*. Later, Thompson and Slinn (1959) reported that this fluid is released by mantle cells as well as specific gland and has pH 1. A substance with pH 1 or 2 was also found to be excreted by the skin of the various molluscs such as *Stylostomum ellipse* (Dalyell), the ophiuroid *Ophiocomina nigra* Abildgaard (Fontaine 1964) and polyclad turbellarians *Cycloporus papillosus* Lang

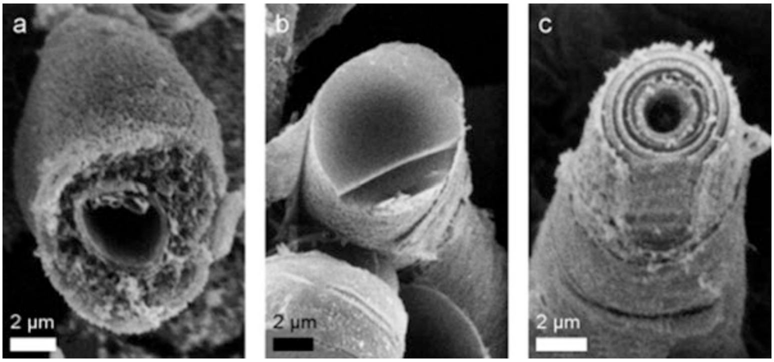

Fig. 10.5 SEM imagery of nudibranchs spicules. (**a**) Epidermal cap from broken tip of emergent *Rostanga arbutus* spicule. (**b**, **c**) crystalline forms of these structures. (Adapted from Foale and Willan 1987)

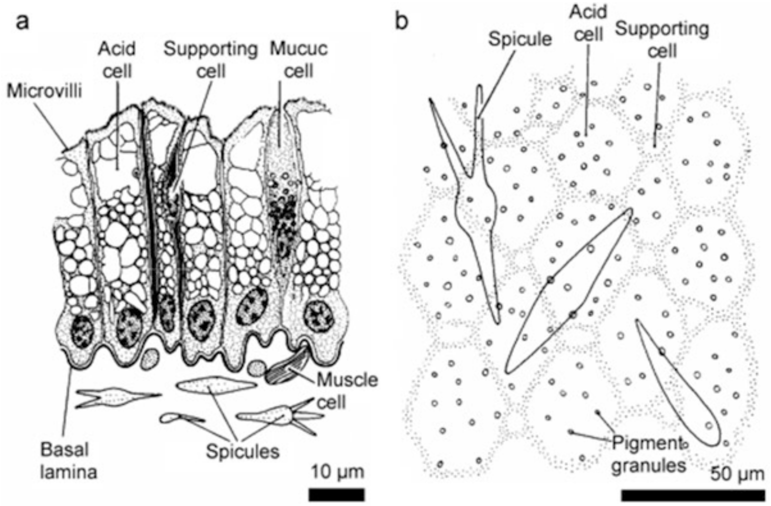

Fig. 10.6 Special acid glands are located within epithelia of gastropod molluscs. (**a**) Cut through the epidermis of *Pleurohrunchu s.* (**b**) Calcareous spicules within the living acid epidermis of *Pluurohranclzus.* (Adapted from Thompson 1983)

and (Thompson 1965). Fluid secreted by the *O. nigra,* is a strongly sulphated mucopolysaccharide, thus may be simply stained using appropriate histochemical and histological reagents. Thompson showed that, in the glands of polyclads and molluscs, which could be responsible for acid excretion of acid seem to be clear in histological sections. Several evidences was found (Thompson and Slinn 1959; Thompson 1961) that, the fluid obtained from *Lamellaria, Trivia* and *Pleurobranchus,* comprises sulphuric acid. Probably, spicules are covered with ultrathin organic layer (Schmidt 1944) that protect them against acids.

10.2 Spicules in Aplacophora

Spicule-covered molluscs belonging to Aplacophora are most numerous (Vinther et al. 2011) and possess the greatest species diversity at depths greater than 200 m in the sea (Kingsley et al. 2013) including hydrothermal vents. Numerous works on their morphology, biology, ecology and evolution have been published during last 150 years (Koren and Danielsen 2009; Hubrecht 1881; Kowalewsky and Mahion 1887; Hansen 1889; Wiken 1892; Thiele 1897; Nierstrasz 1902; Thiele 1913; Heath 1911, 1918; Baba 1938, 1940, 1951, 1999; Salvini-Plawen 1968, 1972, 1978; Scheltema et al. 2012; Sutton et al. 2012; Telford 2013). They are characterized by simple anatomy in comparison to other mollusc with the vermiform shape (Scheltema 1993, 1996), which can be result of progenesis. Within Aplacophora two taxa the Chaetodermomorpha (Caudofoveata) and the Neomeniomorpha (Solenogastres) can be distinguished (Scheltema et al. 1994).

Majority of hermaphroditic neomenioids crawl use a narrow foot or octocorals and hydroids which they can also use to feed. Chaetoderms are dioecious, and eating foraminifera or organic detritus. Records on aplacophoran anatomy are to be found in papers by Salvini-Plawen (1985) and Scheltema et al. (1994).

Aragonite-based spicules are characteristic for Aplacophorans. It was observed that in *Epimenia babai* (Neomeniomorpha), aragonitic spicules, pedal pit and a ciliated foot evolve from the final ectoderm of the embryo (Okusu 2002). At the beginning, spicule forms a solid tip, following by creating a spicule with open-ended hollow and finally closes its hollow. See diversity of forms and sizes on SEM images represented in Fig. 10.7.

Diversity in spicules forms has been reported by Scheltema and Ivanov (2002) a tiny neomenioid postlarva (Fig. 10.8). There is still lack of knowledge about biochemistry and ultrastructure of these postlarval spicules.

Classification of epidermal spicules can be found in Scheltema and Schander 2000) as follow:

1. *skeletal* (5tangential)—the spicules are located inside the cuticle, in one or more layers, at right angles to each other, spiraling from ventroposterior to dorsoanterior as well as from dorsoposterior to ventroanterior;
2. *upright* (5radial)—formed only one layer, almost upright, with their distal ends commonly extending above the epidermis; and
3. *adpressed,* with overlapping spicules, which form a flat, single layer lying on the body wall epidermis (e.g., *Tegulaherpia;* see Scheltema 1999).

Different arrangement, such as single array or a combination of (1) and (2) or (2) and (3) (e.g., *Acanthomenia)* (Scheltema 1999) can be found in various species. Several types of spicules occur within one species, which can be rounded with empty space inside, solid, rounded or flat, with different sizes and shapes. Near the pedal groove, the spicules are rather arched, or curved on one side, and on the each side they form a single, oblong row; they usually have a “handle,” or *root.*

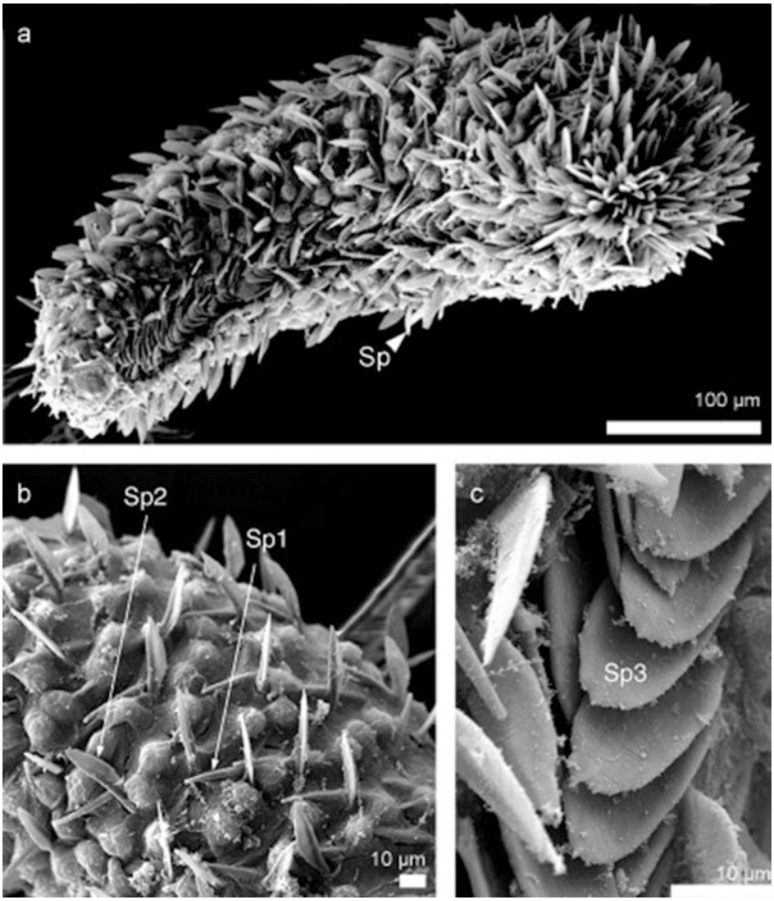

Fig. 10.7 SEM imagery: (**a**) Structural diversity of larval spicules (Sp) of postmetamorphic juvenile of *Epimenia babai*. (**b**) The hollow upright spicules (Sp1) lie against the body, while bladelike solid spicules (Sp2) project outwards from the body. (**c**) Another larval spicule (Sp3) is solid and broad. (Adapted from Okusu 2002)

Aplacophora possess aragonitic spicules which are are ornamented to varying degrees (Scheltema 1976). Such kind of species specific ornamentation is represented in the (Fig. 10.9). Different kinds of spicules within one species (Fig. 10.10) are typical for Solenogaster molluscs (known as *Crystallophricon)* (Schwabl (1961a, b).

Solenogaster species and related molluscs (Fig. 10.11) remain to be interesting objects to study calcification.

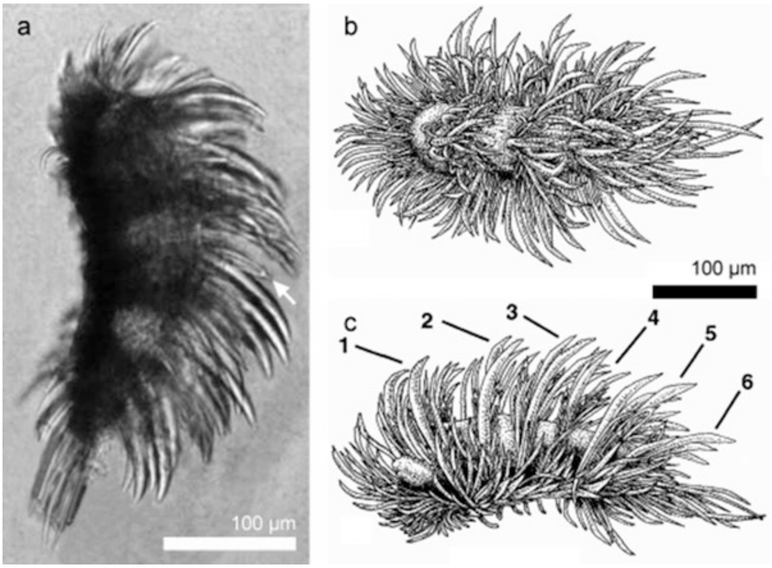

Fig. 10.8 Transverse groups of spicules (anterior to the left) are visible on postlarva (**a**) of a neomenioid aplacophoran from Fieberling Guyot. The barbed spicule is marked with arrow. Drawings of the same postlarva are represented in (**b**, **c**). (Adapted from Scheltema and Ivanov 2002)

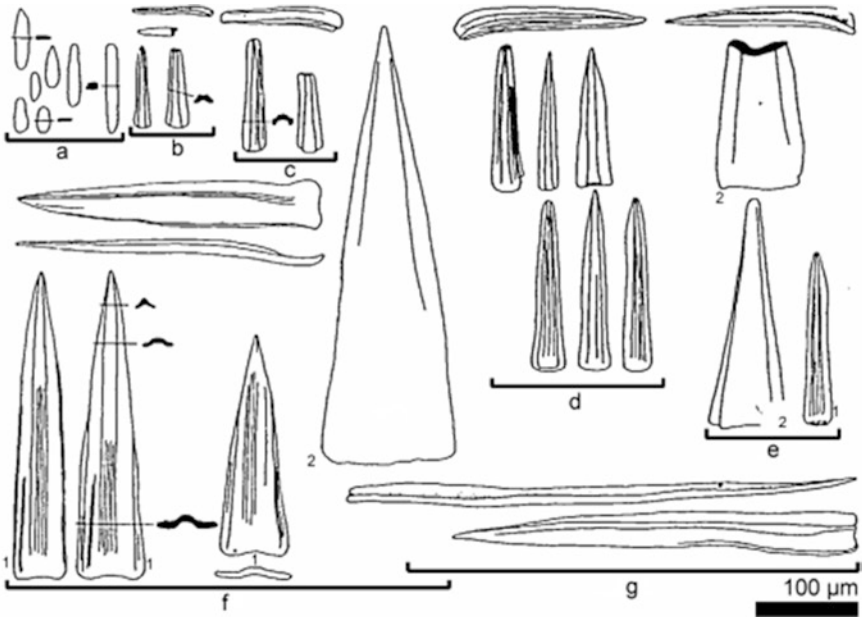

Fig. 10.9 Overview of the spicules isolated from *Chaetoderma abidjanense n. sp.* (Adapted from Sheltema 1976)

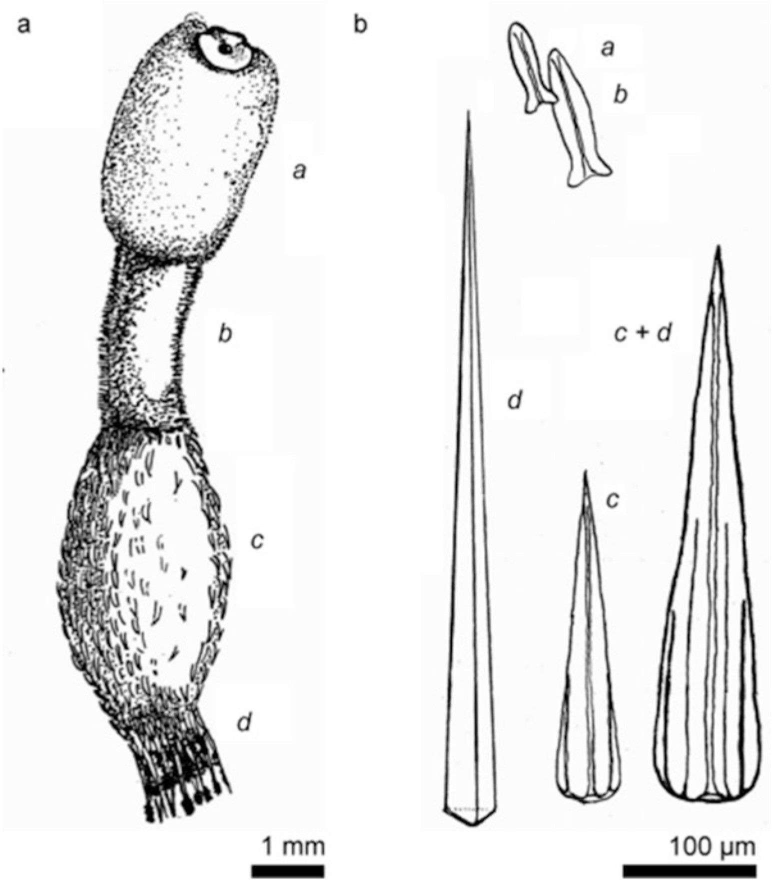

Fig. 10.10 (**a**) The entire *Crystallopbrisson recisum* is shown in side view, showing the body regions (*a, b, c, d),* from which the spines represented in (**b**) have been taken. (Adapted from Schwabl 1961)

Spicules which are needed in copulation are known for Neomenioids. For example, sometimes more of calcitic *copulatory spicules* are presented in various neomenioids species (Figs. 10.12), which are excreted in deep, pocked of epidermal mantle cavity. However, their function is still unknown. Interestingly, they are ephemeral or some species could resorb them, but they can also provide 1/3 of the total length of the organism.

Fig. 10.11 Spicule-forming molluscs. (Images courtesy Dmitry Ivanov)

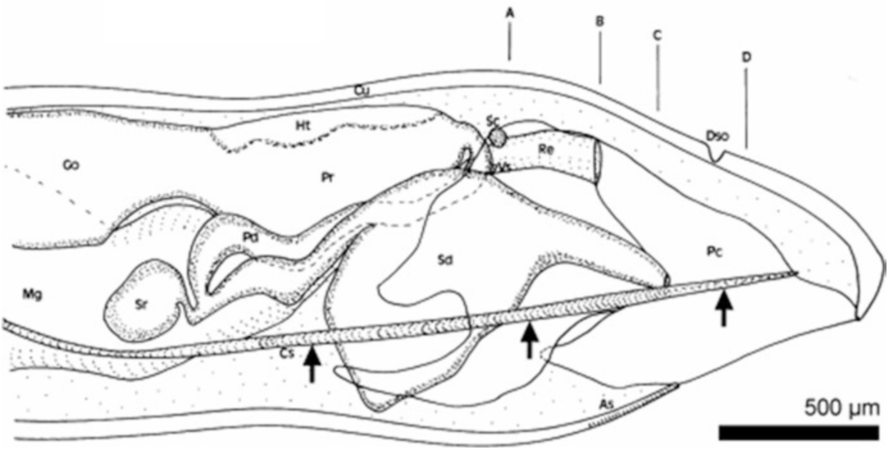

Fig. 10.12 Overview of the posterior body of *Dorymenia troncosoi* (*As* abdominal spicule, *Cs* copulatory spicule, *Cu* cuticle, *Dso* dorsoterminal sense organ, *Go* gonad, *Ht* hearth, *Mg* midgut, *Pc* pallial cavity, *Pd* periocardioduct, *Pr* pericardium, *Re* rectum, *Sc* suprarectal commissure, *Sd* spawning duct, *Sr* seminal receptacle, *Vs* seminal vesicle

10.3 Spicules in Polyplacophora (Chitons)

Shells of the chitons (Polyplacophora) are comprising of eight aragonitic plates (Fig. 10.13), which explains the name polyplacophora as holder of many plates (Checa et al. 2017). Only some part of body is covered by shell, the rest called girdle, can be protected by scales spicules or might be nonmineralized (Pilsbry 1892–1894; Thiele 1909a, b; Van Belle 1999). Mostly, spicules are embedded in the cuticle of this molluscs (Rieger and Sterrer 1975) and, probably, are secreted by corresponding single epidermal cells.

More detailed information concerning the development of the spicular structures during metamorphosis was described in detail in *Mopalia muscosa* by Leise (1984) as follows:

> *After five days the trochophore has around 350 μm length and barrel-shaped with simple columnar epithelium, without spicules. The apical fringe with uniform microvilli is presented in all epidermal cells, even in the ciliated ones, and acts as a brush border, which is maintained till metamorphosis. At this stage no cuticle is observed, but epidermis contained goblet cells with mucus.*
>
> *A six-day-old larvae can have cells with 6 μm in length, supranuclear and conical calcareous primary spicules at the edge of the mantle, this feature is typical for mantle field. A one spicule is formed by a spiniferous cell. The mantle field is surrounded by the five or six rows of spiniferous cells. Until settlement of larvae (day nine or ten) these spicules are retained inside the cell and grow up to 3.0 μm in width and 9.0 μm in length. After six days the shape of gridle cells is still columnar, but they shorten (20 μm) until differentiation of papillae occurs.*
>
> *A nine- or ten-days old, 400 μm in length and dorsoventrally flattened trochophore is able to settle and metamorphose. After first day of settlement the larva becomes shorter (up to 310 μm) and changes into a juvenile and secretes a hairs, shell plates and cuticle, loses*

Fig. 10.13 Sea bottom is also the habitat of well armored with dorsal plates and microspicules chiton. (Image courtesy A.V. Ratnikov)

> *the apical tuft and prototroch as well as discards the primordial spicules. The scalloped cuticle is excretes above the shell, which is located in the middle of the mantle field. The gridle spicule tips are protruded into the shell, and lie within the cuticle. Two days after settlement, they are discarded and covered by the cuticle, which is ventrally thin and dorsally thick. Primordial spicules do not have central pigmented granules (typical for adult spicules), shaft as well as the dense chitinous cup but possess thin cups.* (Leise and Cloney 1982)

The study about the formation of aragonite in adult chitons girdle was carried out in the group of Lia Addadi (Treves et al. 2003). Four chitons genera were investigated and collated: *Acanthopleura uaillantii*, *Nuttalina fluxa*, *Ischnochitonina* sp. and *A. spinigera*. The first three exanimated species have girdles decorated by spicules with a typical sturdy elongated shape. Dark and light bands of spicules, which differ in colour were typical for *A. uaillantii*.

Based on IR spectroscopy and X-ray diffraction, it was proved that aragonite is the only mineral component in the plates and spicules. Under polarized light as well as X-ray diffraction, individual spicules or plates act as polycrystalline materials. Surely, examination of broken spicules using the SEM microscope clearly shows that, they possess a specific, polycrystalline aragonite morphology. The single crystallites look like an individual, prolonged prisms and are of sub-μm thickness and few μm in length and packed in clusters. The lengthened prisms forming a compact material because they have aligned long axes within a bundle.

Interestingly, in the case of all samples (Treves et al. 2003), the insoluble spicule contents seem to be consisted of heavily glycosylated proteins. These form a reticulum of fibers (Fig. 10.14) which differ from the other in the investigated samples. Especially, white spicules of *A. uaillantii* and scale matrices of *Ischnochitonina* sp. appear as lamellae, without a favoured spacious orientation. Interestingly, the *A. spinigera* fibrous matrix (Fig. 10.14a) and black spicules of *A. uaillantii* (Fig. 10.14b) form a pattern with a specified favoured orientation.

Partial dissolution of spicules were carried out to get more clear diffraction pattern of organic material, which indicate the components from the fibers of organic

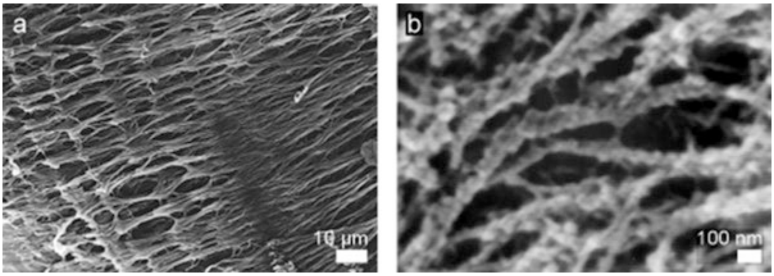

Fig. 10.14 SEM imagery: demineralised girdle hard parts of chitons. (**a**) *A. spinigera* spicule matrix. (**b**) *A.ualantii* black spicule nanofibrillar matrix. (Adapted from Treves et al. 2003)

matrix and lead to obtain reflections in form of dots and arcs. The dots are in accordance with the (111) and the (021) reflections of aragonite, the arcs are compatible to the (002) reflection of the aragonite crystals. Thus, these authors showed a well-defined spatial connection between mineral and matrix. Unexpectedly, these results are not in accordance with chitin, or other identifiable fiber structure visibly corelated with each other (Treves et al. 2003).

Unlike the most mollusc's mineralized tissues, the content of organic matrix is noticeably higher (2 ± 3% in comparison to approximately 1% by weight of dry mineral (Treves et al. 2003). But, the protein content in scales and spicules is much lower and varies between 0.07% and 0.23%, suggesting the polysaccharide nature of the organic matrix. This material also builds specific fibrous or lamellae-like meshwork, not similar to those found in mollusk shells. Based on abovementioned results, it can be assumed that the mechanisms of the chiton girdle mineralization are different than those known for other part of the same organism and other molluscs as well.

10.4 Onchidiella Spicules

> *"Onchidium is a small, naked, pulmonate mollusk which is remarkable because of several conspicuous peculiarities. The habitat of Onchidium is littoral-marine and the animal divides its existence between life in the water and in the air."*
> Leslie B. Arey

Gastropods which can produce siliceous spicules are known as Onchidiidae (Pulmonata: Gastropoda) (Baker 1938). After first report on *Onchidium* by Buchannan in 1800, Onchidiidae, remain to be poorly-known taxon including its systematics (Dayrat 2009; Dayrat et al. 2016) however few papers on anatomy and morphology of this group have been published in XIX century (Plate 1893, 1894: von Wissel (1898), however most reports appeared in XX century (Stantschinsky

1908; Awati and Karandikar 1948; Marcus 2011, 1979; Tillier 1983; Britton 1984; Weiss and Wägele 1998). One of "*conspicuous peculiarities*" of these molluscs has been reported in several works by French scientist Alphonse Labbé (1933, 1934a, b, c, 1935) who suggested existence of siliceous spicules in these organisms.

Thus, Buchanan in 1800 described the *Onchidium typhae* as the first onchidiacean species. Four years later a second species of the pulmonates group was added by Cuvier. But in 1818 Blainville stated that, both are opisthobranchs.

Also some unusual anatomical records have been observed such as inverted type retinulae in the mantle of several bears eyes species (Semper 1877; Stantschinsky 1908; Hirasaka 1912). Interestingly, for the long time, the existence of lungs (omitting the analogues formed form kidney) was contradicted, but now this mistake is revised.

The lung in *Onchidium* was firstly described by Cuvier (1804) but then this finding was changed. But Milne-Edwards (1857) noted that pouch hitherto which was previously described as lung is kidney, as in the case of the rest gastropods. It is known that in some marine forms, the *Onchidium* lungs are closely related to cloacal part of the broadened kidney. He supposed that, this organ have changed the function from a secreting a respiratory sac.

Due to their habitat on the interface between water and air, respiratory behaviour of oncidiids is more specific in comparison to that ofterrestrial species. Such species as *Onchidium floridanum* possess numerous short papillae in its mantle which enable the mollusc to respire cutaneously (Arey and Crozier 1918).

Unfortunately, there is still a lack of experts that can identify onchidiids species in details, thus in museums majority of them are labelled as "Onchidiidae"(Dayrat 2009). Several monographs regarding European collections: Berlin, Frankfurt and London (Plate 1893); Stockholm as well as Copenhagen (Hoffmann 1928a, b); Paris (Labbé 1934a) can be found.

Onchidiids do not have the internal shell, thus they can be assumed as the true snails. Majority of them are living in marine waters in the upper intertidal zone, in muddy sandy or even rocky habitats, including mangroves. Although, two specimens: *Onchidium typhae* Buchannan, 1800, and *Labella ajuthiae,* was found to exist in brackish habitats and they also can be found in fresh water environment (Labbé 1935). Interestingly in the mountain rain forests, three terrestrial specimens have been found: *Platevindex ponsonbyi* (Collinge 1901); from Borneo; *Platevindex apoikistes* (Tillier 1983), from Mindoro, Philippines as well as *Semperella montana* (Plate 1893), from Sibugan Island, Philippines. In the literature the highest found specimen of the onchidiid is the *Platevindex ponsonbyi* (850–1060 m), but the author has studied also specimens found up to 1850 m from Panay as well as Mindoro Islands (Phillipines). These animals are seasonal – the highest number of them is recorded in the summer (Dayrat 2009). Onchidiids can be found worldwide, they do not occur only in the Arctic and Antarctic (Hoffmann 1928a, b, 1929; Pinchuck and Hodgson 2010).

Fig. 10.15 Celtic sea sluges (**a**, **b**, **c**, **d**) habituating within water/air interface of the rocky substrata. (Images courtesy Peter Barfield)

In 1817 for the first time, Cuvier (see Goulding et al. 2018) described the *Oncidium celticum* snail, found in the Brittany coast, and nowadays this specimen is knowns as *Onchidella celtica* – the celtic sea-slug (Fig. 10.15). Peter Barfield in his communication supposed that the name derives from latin adjective, which describes the western Europe inhabitants – today's France. Although, 'Celt', can also denote 'a warrior', thus the *O. celtica* appearance (with armor) could be taken into consideration as a warrior, but this celt-slug eats only small and microscopic algae and prefer a rock crevices habitats, to be protected from sunlight and wave action. These gatropods during high-tide are hiding in large amounts within so called "nests", naturally occurring cavities in the rock (Arey and Crozier 1918; Russell 1925; Tween 1987). Usually when their stomachs are empty, they leave the nests and catch the diatoms and small algae from the tide. Also these animals can be located on mussel's beds or barnacles encrustations. When the wave covered them, they will digest the stomach content, but due to a lack of cellulase the algal filaments will not be

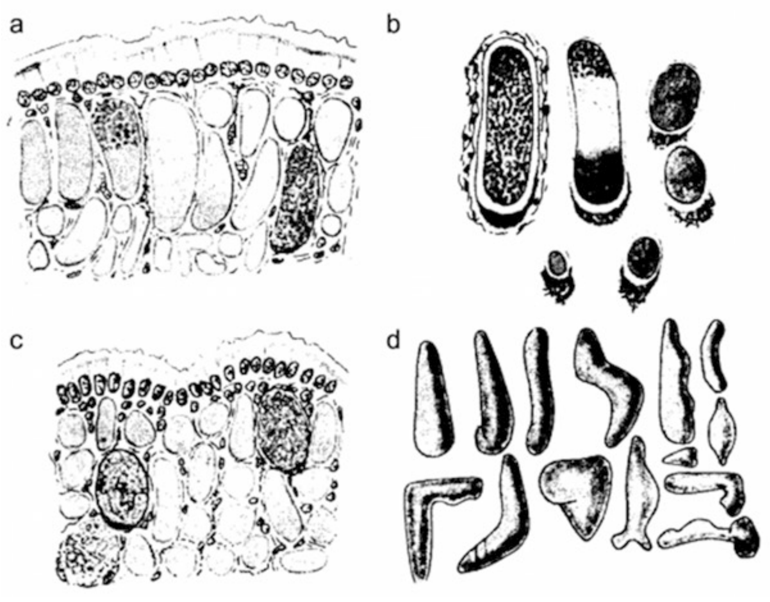

Fig. 10.16 Spicules of *Oncidium leopoldi*. (**a**) Section of integuments of *O. leopoldi* showing the arrangement of the spicules. (**b**) Spicules of *O. maculate*. (c) Silica gland. (**d**) Diverse morphologies of spicules observed in *O. leopoldi.* (Adapted from Labbe 1934)

largely affected. The diatoms are the most important part of their diet and are mostly digested. Some sponges and foraminifera parts and well as detritus and sand is taken up. The sand has special role of milling the food, thus it can be assumed as a physical aid to digestion. Of course, the intriguing question about the origin of silica (or biosilica) in *O. celtica* attracted attention since the first paper by Labbé in 1934.

This author wrote as follow:

> *The silica is here mostly in the form of spicules: subspherical, elongated, cylindrical, even or dented sticks with an average length of 40-60 μm for a width of 8-12 μm. Some are smaller and don't exceed 10-12 μm, but they are less common. Each spicule is wrapped by a thin conjunctive capsule; packed parallel to each other, these spicules form a kind of a palisade tissue with two or more layers. These spicules were resistant and not deformable by concentrated nitric acid and strong acids. It is therefore, indisputably, silica.* (Labbé 1934)

Related images are represented in the Figs. 10.16, 10.17, 10.18, and 10.19.

All Onchidiidae species are hermaphrodite, and their genital pores are well separated. Close to the right cephalic tentacle the male pore can be found, and the female pore is located at the body end, sometimes markedly offset the right of the mid-line. Several specimens do not have spines of the inner edge of the curve as well as the penis gland, and the penis has spines in separate distal or proximal groups (Britton 1984).

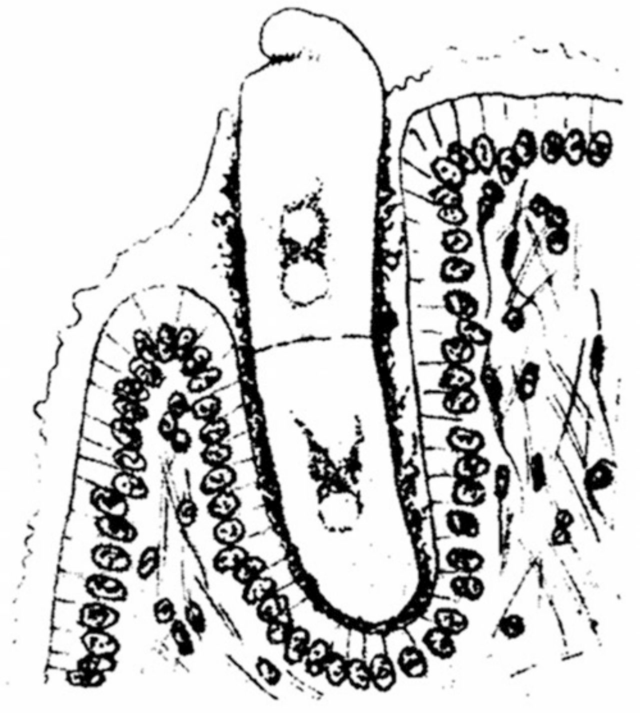

Fig. 10.17 External extra-epidermal spicules of *Oncidium peronii* surrounded by pigment-containing cells. (Adapted from Labbe 1934)

There are no doubts concerning the unique spinose shape of the penial structures in Onchidiidae (Fig. 10.20), however the real inorganic nature of such sharp, hook-like structures remains to be unknown.

It seems to Labbé that siliceous structures in Onchidiidae are to be found everywhere including dorsal eyes (Hirasaka 1912). He reported as follows:

> *Indeed I believe that considering their resistance to the strong acids the lens cells of the dorsal eyes are also made of silica glands. Semper had already insisted on the similar nature of the cells in the lens and the skin glands, to which he gave a role in the development of the eye. Although it is the opinion of Hirasaka, I don't think that these are optoblasts that transformed in lens cells; these cells have a great similarity with the siliceous skin glands that are probably also ectodermic. It would anyway be noteworthy if the crystalline lens was formed by a substance close to glass. What seems certain is that there's a connection between the siliceous spicules and the dorsal eyes: in O. leopoldi that has a dorsal shell of spicules and eyes, there are no spicules in the small areas where the eyes are.*

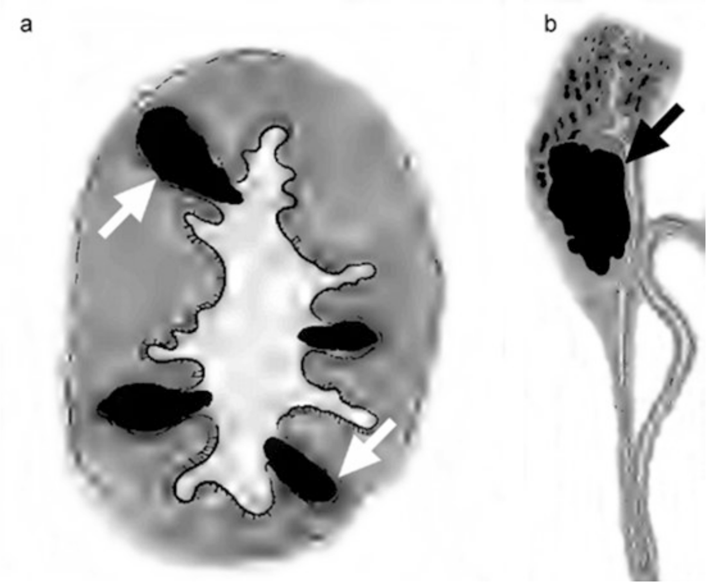

Fig. 10.18 Dark colored mineral formations (arrows) within and on the surface of mollusks "chondroid"(cartilage-like) penises under light microscope as described by Plate in 1893. (Adapted from Plate 1893)

Labbé also calculated that 0.044 g of silica can be isolated from a 1 cm long *O. celtica* weighting 0.37 g (Labbé 1934c).

In spite of recent discoveries and descriptions of novel onchidiid slugs species (Sun et al. 2014; Dayrat et al. 2017; Goulding et al. 2018) the truth about existence of silica-based spicular structures located within these mollusks is still not cleared up.

10.5 Conclusion

Thus, chemical composition including organic phases in spicules and spicule-like formations in molluscs remain to be non-investigated in details which are comparative with that of sponges (both classes as Calcarea and Demospongiae). Also the selection mechanisms for biosynthesis of calcareous or siliceous structures are not clear. It could be hypothesized that the large diversity of spicules suggests different mechanisms of their formation within cuticular layers, or other tissues. Additional

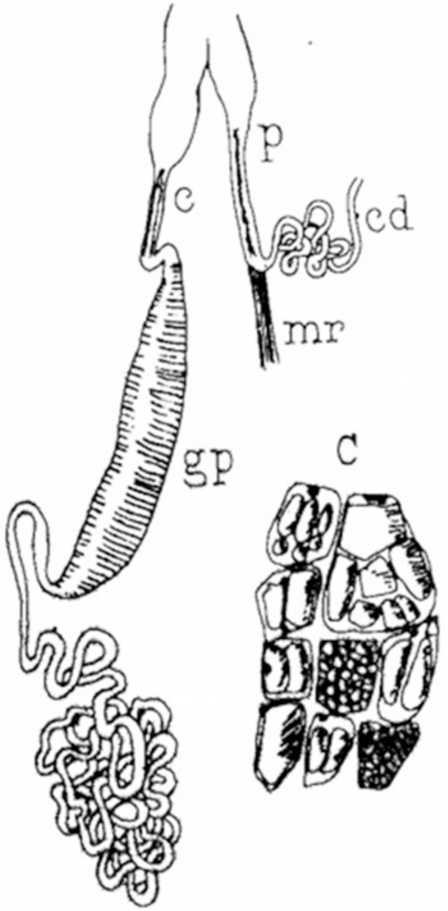

Fig. 10.19 Genital system of *Oncidium straelenii*. (*P* penis, *gp* penial gland), *C* siliceous microplates, which cover the penis. (Adapted from Labbe 1934)

limiting factor to obtain more detailed information is based on the scantiness to obtain corresponding biomaterial from museum's collections for destructive analysis. This is especially true for representatives of onchidiid slugs.

However, I still trust that experts in biomineralization as well in bioinspired materials chemistry will pay more attention in the future to resolve numerous open questions reported above.

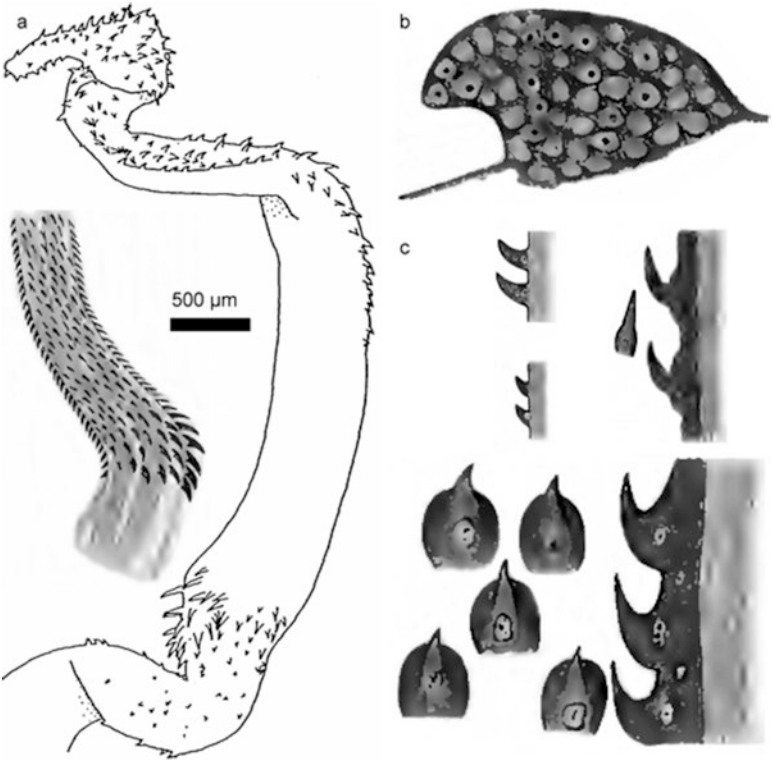

Fig. 10.20 The penis of oncidiides bears spines in distinct proximal and distal groups, sometimes almost merging on the outer surface of the curve, while the inner edge of the curve is always devoid of spines (**a**). (Images adapted from Britton 1984); (**b** and **c**). (Images adapted from Plate 1893)

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Part IV
Non-mineralized Structures

Chapter 11
Enigmatic Structural Protein Spongin

Sponges appeared to me only as skeletons
Peyssonel (1758)

Abstract In the orders Dendroceratida, Verongida and Dictyoceratida, the characteristic for most Demospongiae siliceous skeleton is replaced by proteinaceous, fibrous like spongin skeleton. These spongin fibers can be anastomosed in order to create a network which provides support for skeleton of the sponge's cell tissues. This network represents sets of diverse unconnected, mostly dendritic three dimensional structures. From chemical point of view, spongin remains to be an enigmatic proteinaceous biomaterial than contains halogenated residues and cannot be sequences till now. Consequently it was defined previously as pseudoceratin, euceratin, horny or sclerotized protein, iodospongin, silk-, or gelatin-like protein, etc. State-of the art concerning diversity, biological functions, and material features of spongin are described and discussed in this chapter.

Bath sponges (Fig. 11.1) also known as commercial sponges (Jesionowski et al. 2018) have been used since ancient times (Schulze 1879; de Laubenfels and Storr 1958) for "bathing, painting, cleaning, medical uses, padding for battle armor, and as a vessel for drinking water" (Cresswell 1922). Chemical, structural and material features of micro- and macro-porous fiber-based exoskeletons of these sponges are responsible for broad variety of their practical applications till now.

In the orders Dendroceratida, Verongiida and Dictyoceratida, the characteristic for most Demospongiae siliceous skeleton is replaced by fibrous like spongin skeleton. These skeletal fibers have been anastomosed in order to create a network which provides support for skeleton of the sponge's cell tissue. This network can be organized as sets of diversely branched, mostly dentritic and unconnected up to 100 µm-thick spicule-free fibers. Dendritic skeletons occur in representatives of both Dendroceratida and Verongiida orders, whereas 3D skeletons with recticulate morphology has been observed in all three orders (Bergquist 1980; Maldonado 2009). Due to recent discovery of chitin within skeletal structures of Verongiida demosponges (Ehrlich et al. 2007, 2010; Brunner et al. 2009; Wysokowski et al. 2013; Żółtowska-Aksamitowska et al. 2018a, b; Shaala et al. 2019; Klinger et al.

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Fig. 11.1 Commercial sponge as source of spongin

2019) it is necessary to carry out corresponding revision with respect to identification of chitin and spongin in diverse orders of Demospongiae now. For example, the finding of chitin in the skeleton of dendroceratid sponge originally defined as *Dendrilla lacunosa* leads to renaming of this sponge as a novel species *Ernstilla lacunosa* in the new family Ernstillidae (Vacelet et al. 2019; Fromont et al. 2019).

11.1 Spongin as a Halogenated Scleroprotein

Probably, the first report on structural similarity between bath sponges fibres and silk has been published by Geoffroy (1707). He made this suggestion after chemical experiments which have been carried out at that time. However, after this report, the interest on chemistry of bath sponge skeletons was not significant because special attention has been paid to use them as source for pharmacological applications. Marine sponge *Spongia usta*, commonly called "Coventry Remedy", which has been used already by the ancient Chinese, it a typical example in this case. Professor Andrew Fyfe from Aberdeen was the first who reported large quantities of iodine in this sponge (Fyfe 1819). Intriguingly, bath sponges have been intensively studied as source of iodine till 1914!

The name "keratose sponges" for bath sponges was proposed in 1841 because of presence of keratose fibrous matter in their skeletons (Bowerbank 1841). However, in 1843, Croockewit suggested, as before, that keratose fibres of sponges are chemically similar to silk due to close analogy to the *sericin* and *fibroin* – two main structural components of silk (Croockewit 1843). That was not to be overlooked that horny, keratose fibres contain iodine, sulphur, and phosphorus. Consequently, Croockewit proposed the chemical formula of this proteinaceous material as: $20(C_{39}H_{62}N_{12}O_{17}) + J_2S_3P_{10}$.

Later, the possible silk origin of horny skeletons has been dethroned in experimental works by Schlossberger (1859), as well as by Städeler (1859) who introduce the name *spongin* for this biological material.

The first histological studies on sponges were carried out. For the first time, the structural features of spongin fibres from the histological view have been studied by von Kölliker (1864).

The experiments on iodine-containing spongin flare up again in the laboratories of Hundeshagen (1895) and Harnack (1898) who introduced the name "jodospongin" and postulated that in such protein iodine must exist in combination with organic components. In some cases it was possible to obtain "jodospongin", which contained over 9.4% nitrogen and 8.5% iodine (Harnack 1898).

In 1900 Minchin suggested the existence of two different forms of spongin, which, however, were nearly identical in their chemical composition (Minchin 1900). One has been located within the internal, fibrous skeletons and another one was synthesized intracellularly and secreted. Only in 1926, corresponding report on spongin identification has been published by Clancey. He suggested that the composition of spongin is related to its origin, and that the compounds which build the main framework of the Euceratosa and enclose the Pseudoceratosa spicules are not the same, as many physiologists reported already (Clancey 1926). Many reports regarding spongin composition have been published especially for spongin of *Hippospongia equina* origin, or the "Turkey cup sponge" *Euspongia officinalis*. Obtained results indicated significant differences related to the faulty analytical methods as well as the commercial sponges used as a source of spongin have been bleached and macerated in different manner (Strauss 1904; Abderhalden and Strauss 1906).

Previously obtained results are not consistent due to the existence of the complex of iodine. This complex was obtained by Wheeler and Mendel (1909) after a hydrolysis with barium hydroxide and they indicated so called iodogorgonic acid isolated by Drechsel from *Gorgonia cavolinii* (1896). They showed also that this compound has structure of the 3,5-diiodotyrosine.

It was also proved that 3,5-diiodotyrosine does not give all characteristic reaction for tyrosine: the presence of iodine gives negative result of Milton reaction, but the xanthoproteic reaction, characteristic for benzene ring, occurs.

To carry out the productive hydrolysis and obtain relative content of the amino-acids succeeding methodology was applied.

Three grams of the spongin, was boiled (under a reflux) with 20% hydrochloric acid for 30 h to obtain any increase of amino-acids in the resulted solution.

Acid hydrolysis of spongin leads to obtain iodogorgonic acid together with other amino-acids. The amount of iodogorgoic acid found in the protein was between 4% and 7% (Clancey 1926). Clancey (1926) has reported also the lack of hydroxyproline in the *Hippospongia aquina* skeleton purified with water followed by acid and alkali treatment. He described huge content (18.4%) of glutamic acid as well as 5.7% proline, 14% glycine, 11% tryptophane or histidine, 2.8% tyrosine and a small content of cystine. Prior to 1956, when Gross et al., published results about two different spongins it was assumed that mostly spongin is a halogenated scleroprotein (Ackermann and Burchard 1941; Ackermann and Müller 1941; Low 1951; Roche 1952). It cannot be excluded that the very high resistance of this structural protein to enzymatic treatment (including proteases, trypsin, pronase collagenase, amylase or lysosymes) (Junqua et al. 1974) is based on the presence of both iodine and bromine which have never been identified in natural keratins or collagens. However, in natural environment, spongin can be enzymatically destroyed by various microorganisms (Gaino and Pronzato 1989). Therefore, isolation, purification and identification of these specific enzymes is a challenging task which will be helpful in detailed proteomic analysis and sequencing of probably last enigmatic proteinaceous biopolymer – spongin (Jesionowski et al. 2018)

11.2 Spongin as a Collagenous Protein

Recently, Ehrlich et al. (2018) published comprehensive review where the chemistry, biochemistry as well as structural diversity of collagens in sponges have been analysed and discussed.

Now it is recognozed that so called spongioblasts – the cells which form spongin, are derived from the epithelium. It was found that (Minchin 1900) the cuticular fibrils of spongin are intracellularly excreted, but the skeletal fibers are formed extracellularly.

Gross et al. (1956) proposed two forms of spongin fibers which differ morphologically and named them as "A" and spongin "B", and classify them to the class of collagen. The proposed finding was confirmed by analysis of the general amino acid pattern especially the glycine and hydroxyproline content and as well as by the structural analysis using electron microscopy and x-ray diffraction. For spongin "A" the glycine to hydroxyproline content was equalled 1.6 and for spongin "B" 1.8. Corresponding unbranched fibril of 20 nm width, with an axial period of 625 Å are typical for spongin "A". Spongin "B" a largely branched fiber has between 10 and 50 μm in width, and is comprising mainly of fibrillary bundles which contain 10 nm-wide unbranched filaments. Fragments of "B" fiber in the collagen range can sometimes exhibited an axial period, but a low-angle pattern was not observed.

For both tested fibers as well as for amorphous matter, the content of hexosamine, pentose, uronic acid as well as hexose, was evaluated. For amorphous matter and spongin "A", a glucosamine, glucose, galactosamine, uronic acid, mannose, fucose galactose and arabinose were chromatographically determined. In spongin "B", a low content of aminated sugar together with galactose and glucose were shown.

Interestingly, spongin "A" and "B", in opposite to collagen of mammal origin, cannot be dissolved in the collagenase *(Clostridium hystolyticum)* or pepsin as well as by significant excess of alkali or dilute acid solutions (Gross et al. 1956)

Diversity of spongins has been well represented by Exposito et al. (2002). Thus, taking into consideration the resemblance between collagen from nematode cuticular, spongin, and type IV collagens of basement membrane as well as the lack of sponge's basement membranes, it can be suggested that "two evolutions line are observed within the spongin family. First may represent exocollagens (like spongins) which binding sponges to their substrata (e.g. mussel byssus threads, worm cuticles, the Selacians egg capsule). The second can be related to the collagens internalization, which results in the differentiation of the basement membrane of collagens. Finally, nematode cuticular collagens seem to be evolutionarily related to vertebrate FACIT and FACIT-related collagens. All possess various short collagen domains, with similar C-terminal noncollagenous (NC1) domains as well as preserved residues of cysteine at the COL1–NC1 junctions" (Exposito et al. 2002).

Therefore, fibrils of vertebrates origin are engaged in the dermis protection in body, providing the physical properties of cornea and ensuring protection against mechanical strength in tendons. Next attractive feature is the occurrence of the collagenous fibrils within the vertebrates skeletons. Additionally, spongins can be accepted as exocollagens that ensure the attachment of the sponge body to corresponding substratum, this appear to be essential, because of the loosely arranged pinacoderm cells. During evolution, these had a defensive function, as in the annelids and nematodes cuticle. This protective effect may be associated with its resilience to a various proteases. Additionally, the trimeric structure of the collagen molecules is a consequence of their engagement in the polymers formation (with or without various compound of collagen origin. From the evolutionarily point of view, spongins possess some similarities with the collagens basement membrane, additionally in sponges the existence of collagens type IV was reported, which role is related to the formation of the basement membranes scaffolds. Collagen type IV is also involved in the attachment of endothelial and epithelial layers (Exposito et al. 2002).

Classification of spongins proposed by R. Garrone in his book published in 1978, is represented here in the form of a scheme (Fig. 11.2).

Firstly, there exists spongin of the *spiculated fibres*, which is mostly related to inorganic, but endogenous skeleton of the sponge. Such spongin can be found only at the nodes of a delicate spiculous network, or forms wide fibres with a very thin mineral element in the core, in the group of *Reniera* sponges. Also this spongin is resistant to alkaline hydrolysis as well as to action of mild acids, pepsin and most bacterial collagenases. However, at room temperature, a solution of cuprammonium hydroxide appears able to destroy spongin.

Secondly, the *spongin fibres which constitute the skeleton of the horny sponges*: the compactness and abundance of the spongin, and practically complete disappear-

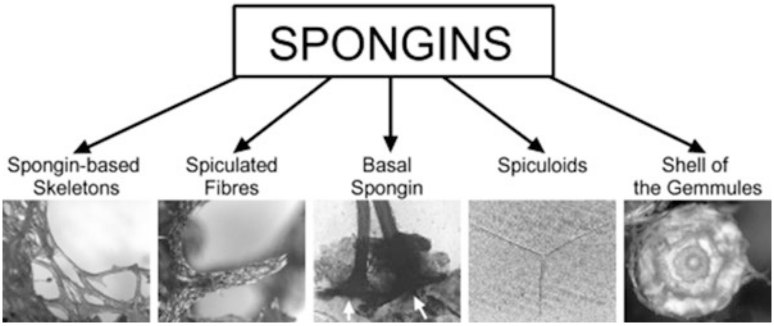

Fig. 11.2 Schematic drawing: diversity of spongins

ance of its own biominerals, which are replaced to foreign micro-particles and specular remnants, testify to the originality of this spongin.

11.2.1 The Basal Spongin

Due to mostly sessile behavior of sponges, the functional importance of the basal spongin is crucial for survival of these invertebrates. In sponges which do not possess organized inner skeleton, the external spongin excreted by basopinacocytes, form more or less continuous layer and is responsible for animal attachment to substratum.

However, sponges which are characterized by the existence of an organized skeleton, build from spongin or speculated fibres, the basal spongin, together with inner spongin, form a continuous layer. In *Chondrosia reniformis*, a sponge which does not have internal spongin as well as spicules, the basal spongin fixes the organism to the hard substrate so strongly that it cannot be removed entirely. However, the basal spongin in erect sponges, remains to be discontinuous, thus in these point the organized internal skeleton can be formed.

The spiculoids represent organic elements whose shape is structurally similar to that of mineral-based spicules. Such structures are encountered in certain Dendroceratida belonging mostly to the genera *Darwinella* and *Igernella* and they can be partly joined to skeleton fibers or free. The elasticity and flexibility of spiculoids is well known phenomenon (see for overview Dandy 1916).

For example, here the original description of spiculoids from *Darwinella* sponges made by Dandy: "*The spicules consist of three or four slender, tapering rays diverging from a common centre, and they are quite detached from the ordinary spongin skeleton, which coexists with them. No axial thread or canal has been observed, though I have examined them carefully, by different staining methods, from this point of view. Nevertheless it seems almost certain that the spongin must be deposited around some axis, and the fact that this has not yot been shown to exist may perhaps be explained by its close agreement in chemical and physical properties with the enveloping spongin. The spongin appears to be secreted by a, surrounding sheath of spongoblasts as in the case of the ordinary fibres of the horny skeleton. There is no evidence at all of origin within, or envelopment by a single "mother cell*" (Dandy 1926).

The detailed description of spiculoids from the skeleton of *Igernella* species ranging from 7 to 67 μm in diameter with rays up to1000 μm in length is to be found in the work by Maldonado and Uriz (1996).

Finally, there is an example of some kind of protective organic *shell on the surface of the gemmules*. These biological constructs are asexual reproductive bodies, located inside the tissues of some marine as well as majority of the freshwater sponges. They are spherular, consisted of a dense mass of indistinguishable cells, with diameter between a few tenths to more than 1 mm in, enclosed by an organic coat called the shell, which in some cases is covered with very sharp spicules. The shell of gemmules is comprised of collagenous protein (which may be referred to spongin) and chitin (Ehrlich et al. 2013).

11.3 Role of Spongins in Natural Environments

Additionally, majority of demosponges possess a fibrous internal matrix formed by spongin (Bergquist 1978). Sometimes, sandy particles and external spicules can be integrated within the fibers of spongin (Bergquist 1978; Wainwright et al. 1982). The orientation and morphology of spicules (Koehl 1982; Wainwright et al. 1982) together with the fibers thickness and density influence the toughness and rigidity of the tissue of sponge (Storr 1964). It was proved that spongin may be a major part of the sponge biomass; for instance, in the *Mycale* sp. Reiswig (1973) showed that 30% of the dry mass is composed from spongin. It is also assumed that spongin is hardly digestible (Chanas and Pawlik 1996). Thus, the merger of indigestible silica with hardly digestible spongin can lead to form the tissue that not be chosen as a source of food (Chanas and Pawlik 1995).

In spite of previously reported resistance of spongin to diverse proteases studied in the Lab's (Junqua et al. 1974; Junqua 1978) it can be destroyed very well in the natural habitats of sponges by some bacteria and fungi. For example, the identification of pathogen responsible for marine sponges disease was described by Webster et al. (2002). The digestion of the fibers of spongin from the Great Barrier Reef sponge *Rhopaloiedes odorabile* by action of the bacterial pathogen was reported. As a result of infection the covering of the sponge surface with epiphytic algae as well as increasing of softness and fragileness of sponges is observed. These symptoms were the same as those observed in the Mediterranean commercial sponges, where the bacteria were inside the fibers of spongin. This similarity together with microscopic finding clearly showed that bacterial disease in sponge can be a worldwide problem. Interestingly, the analogy in to the disintegration and softening of the *Xestospongia muta* skeleton (in the last stages it can be kept together only by a huge spicules amount), the algal epiphytes on *Geodia* sp and weakening of *Verongula gigantea* implies that the bacteria can be embedded into fibrous skeleton.

11.4 Mechanical Properties of Spongin-Based Skeletons

Traditionally the quantitative quality testing of commercial sponges has been based on their structure, porosity and mechanical properties (stiffness, elasticity, firmness, resilience, compressive strength, etc.) (Cresswell 1922; Louden et al. (2007). The mechanical properties of spongin are excellently summarized in review by Jesionowski et al. (2018) where authors showed that large spongin-based scaffold is resistance to compression due to its unique elasticity and flexibility. Thus, the scaffold was able to return to its original volume and form in 60 s even after being compressed under a weight of around 50 kg. Taking into account that spongin scaffolds become interesting for various technological applications, i.e. tissue engineering, the various mechanical parameters, like tensile and compressive strength, compression modulus, elastic strain, elastic limit, modulus of resilience as well as modulus of elasticity, have to be carefully evaluated and analyzed (Jesionowski et al. 2018).

11.5 Spongin as a Three Dimensional Scaffold for Tissue Engineering

Functional tissues are organized and supported by highly specified natural skeletons, which ensure essential information for the design and formation of the novel, effective tissue-engineering scaffolds (Green et al. 2002). Consequently, the evaluation of inexpensive, simply fabricated scaffolds for transplantable nonimmunogenic tissues and large cell carriers for kidney, liver, bladder, bone, muscle esophagus as well as connective tissue is still an important clinical requirement (Langer and Vacanti 1993).

Nowadays, the main aim in the designing and synthesis of a scaffold with sponge-like structure and ability to easily biodegradation in order to obtain three-dimensional material with interconnected macroporosity, with strictly designed pore sizes and interconnections is a main goal of tissue-engineering. The application of the scaffolds which ensure the appropriate support for tissue is main clinical problem, but the tissue-engineering and biomedicine can find appropriate solution (see Green et al. 2002).

The spongin-based skeletons of commercial sponges seem to have a various of needed features, such as (i) the high degree hydration capability, which improves the adhesion of the cells, (ii) the existence of interconnected, open channels formed by the fibrous network – providing the proper nutrients diffusion to the cells, as well as (iii) the tremendous variety in the fiber construction and skeleton architecture in this phylum (Green 2008). Spongin-based scaffold possess internal surface area which ranges between 25 and 34 m^2 for a construct of 3–4 g dry weight. Due to corresponding capillary attraction this enables considerable liquid absorption of the scaffold (Garrone 1978).

Most successes with respect to tissue engineering was accomplished with collagenous templates obtained from collagen-based marine sponge skeletons which were used in a formation and support of musculoskeletal tissue (mainly bone tissue) in vivo and in vitro (Green 2008). *Spongia* (Class Demospongiae: Order Dictyoceratida: Family Spongiidae) representative of collagen marine sponges creates the fibrous structures with big surface area and interconnected canals (Green et al. 2003). A presence of the various collagen forms leads to obtain scaffold which is highly consistent with growing human cells and favors attachment and proliferation, as well as ensures the maintenance of cell phenotype and enhances the lineage differentiation.

Three-dimensional scaffolds of marine sponge origin have favored position in comparison to already described synthetic materials like fibrous fleece of polyglycolic acid (PGA) applied for the reparation of cartilage (Freed et al. 1993). Application of collagen-based marine sponge skeletons for bone tissue engineering showed that human cells are able to stick to the fibres of sponge and adjust onward the *c*-axis. This, hierarchical, network structure of sponge skeletons ensures appropriate conditions for proliferation, thus in consequence the growth inside the interfibre spaces, followed by differentiation into initially calcified human osteoid tissue occurs. Similar results can be achieved using cells of human kidney, liver, fat, cartilage, foetal cell cocultures and mesenchymal stem cells (Green et al. 2003).

The success of the skeletons of marine sponges in tissue engineering applications can be explained in two ways, firstly being the fibrous and micro-and meso-porous structure (Fig. 11.2), and due to the well-known biocompartibility of collagen (spongin) (Green 2008).

Interestingly, it have been reported that osteoblast-like MG-63 cells differentiating on spongin obtained from *Hymeniacidon sinapium* (Kim et al. 2009), and with primary osteoblasts of mouse origin on spongin isolated from marine demospongesof Callyspongiidae family (Lin et al. 2011). Moreover, recent studies by Nandi et al. (2015) prove that spongin isolated from *Biemna* sp. marine demosponge, can be proposed as a potential biological material for bone augmentation and bone repair.

11.6 Conclusion

It can be summarized that spongin is, probably, not a pure structural protein like collagen, silk, or keratin, with well-known sequences but some kind of collagen-based, halogenated fibrillary composite material resulting from a super-compaction of nano- fibrils and unique filaments. The relationship between bromine, iodine, sulfur, chlorine and collagenous part within spongin, remains unclear. I take the liberty to suggest that the ability of selected marine microorganisms to damage the structural integrity of spongin in keratosan demosponges is based initially on the activity of their dehalogenases and not proteases. It seems that the way to isolate pure collagen from spongin using modern tools in vitro must be started with application of such dehalogenases on the base of corresponding biomimetic strategy.

Definitively, investigations of material properties, biochemistry as well as chemistry of the sponginoius skeletons in sponges, are becoming more and more popular nowadays; which is a consequence of the lack of knowledge regarding sponge diseases and various applications possibilities of spongin skeletons in modern technologies (Norman et al. 2018; Szatkowski et al. 2018) including extreme biomimetics (described in the last chapter of this book).

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Chapter 12
Gorgonin

Abstract Gorgonians can be found in all type of ecosystems between the poles and the equator as well as in shallow or deep waters. Skeleton of the gorgonian coral, in opposite to the Scleractinia corals, comprised of the gorgonin – protein-based and polyphenol-containing organic material. The role of gorgonin is related to provide the flexibility, thus this organism can exist even in environments with strong current. Similar to spongin, gorgonin has been described during his history by the different researchers as jodotyrosin-based protein, sclerotin, fibrillar scleroprotein, tanned collagen, pseudokeratin as well as eukeratin, etc. Unfortunately, there is still the big lack of knowledge concerning the chemistry, biochemistry and genomics of gorgonin. Here, the role of gorgonin in paleooceanographic dynamics as well as selected materials properties of this biological composite material are discussed.

According to Goldberg (1976), antipatharian and gorgonian coral skeletons are among the most chemically resistant and resilient tanned proteinaceous materials reported.

Gorgonians can be found in all type of ecosystems between the poles and the equator as well as in shallow or deep waters. Skeleton of the gorgonian coral, in opposite to the Scleractinia corals, comprised of the gorgonin – protein-based organic material. However, in Gorgonians calcite is also present, but as mineralized locular forms (Lewis et al. 1992). In contrast to crystalline calcite which provides stability of gorgonian skeletons, biopolymer gorgonin delivers elasticity, enabling these corals to survive even in special, current-affected environments. In order to get enough food, these animals filtrate a huge amount of water through their body, thus usually they are located in places where strong currents occur. The skeletons of the gorgonian are able to ensure sufficient amount of food and resist strong water current. However, the currents velocity as well as their pattern are major elements influencing the gorgonians shape and prosperity (Wainwright and Dillon 1969; Grigg 1972; Genin et al. 1986; Weinbauer and Velimirov 1995). Understanding of the orientation and shape of the gorgonian colonies can supply important knowledge about the current patterns, as well as lead to evaluate the relationship between hydrodynamic conditions and colony structure especially when the currents patterns are established (Mortensen and Buhl-Mortensen 2005).

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It was observed that, the calcitic increases surround the gorgonian skeleton when it achieves the critical size. These accretions built from carbonate crusts, improve the axis stability by enclosing the nodes (Grasshoff and Zibrowius 1983).

Thus, calcium carbonates remain to be the main biomineral phases within skeletal structures of gorgonians. What about gorgonin? In contrast to the chemistry of the gorgonian's secondary metabolites which is well studied (Lei 2016), the origin, biosynthesis including genomics, chemistry and content of gorgonin as a skeletal substance remain to be still under study over the last 150 years.

12.1 Introduction into the History and Chemistry of Gorgonin

For the first time, gorgonian corals on example of *Gorgonia viminalis* have been described by Pallas in 1766 (Pallas 1766). However, studies on chemistry of gorgonin as a substance started by Balard in 1825 when he reported for the first time the occurrence of so called "iodogorgic acid" (Balard 1825). The term "gorgonin" has been introduced by Valenciennes (1855) in corresponding monograph on gorgonians. In 1896 Drechsel obtained from *Gorgonia cavolinii* an iodinated amino acid which has been named "*Jodgorgonsäure*", or iodoaminobutyric acid (Henze 1907). Following studies have confirmed the crucial role of iodine in metabolism of gorgonians especially in the building up of the framework of their axial skeleton (Mendel 1900; Cook 1904; Mörner 1907, 1908; Oswald (1908); Sugimoto 1928). Wheeler and Jamieson (1905), established the fact that iodogorgoic acid is identical with the 3,5-diiodotyrosine that had been prepared by them under laboratory conditions.

The input in chemistry of gorgonin as a halogenated biopolymer was given in 1931 when Block and Vickery investigated the *Plexaurella dichotomu* and *Gorgonia flabellum* undigested skeletons, and reported the existence of arginine, histidine and lysine within *Gorgonia flabellum* skeleton with molecular ratios of 12:1:6 but the molecular ratios of these amino acids in *Plexaurella dichotoma* were equalled 12:1:8. Due to clear similarity to the content of these amino acids in other eukeratins (12:1:4), at that time authors proposed that gorgonin is a eukeratin. More detailed studies with better purified gorgonin samples showed higher content of histidine and lysine, but lower of arginine. Based on obtained results the molar rations of histidine, lysine, and arginine were not 1:4:12 but were equalled 1:4:6.

Despite the fact that the amount of isolated from gorgonin arginine was lower than for typical eukeratin as well as the lysine content was higher, but taking into consideration the ratio of arginine to histidine of 1:12, in 1931 this protein was called eukeratin. Later other keratin-like proteins (so called pseudokeratins) have been studied. They had similar ratio of lysine to arginine 4:6 (Block and Boiling 1939).

After that, 10 years later, Marks et al. (1949) studied the X-ray diffraction properties of gorgonin and suggested that it is nothing else as a fibrillar collagen-type protein. It was also hypothesised that due to a very specific cross-linkage determined by iodine this collagen-like protein cannot be digested by pancreatic nor gastric juice. Such aminoacids as monoiodotyrosine, diiodotyrosine, as well as traces of thyroxine (see also Table 12.1) have been identified in extracts from the axial skeletons of diverse gorgonians too (Roche and Eysseric-Lafon 1951; Roche 1952; Roche et al. 1959, 1963). The iodine content differed with species and was related with the amount of tyrosine in the axial skeleton (Roche and Eysseric-Lafon 1951). Roche (1952) also showed that in the older parts of the skeleton the amount of iodine was less. Perhaps, the iodinated tyrosine residues react with phenolic molecules or other iodinated tyrosine, leading to a loss of iodine.

Then, Roche et al. (1959) using radiochromatography tried to prove that, in the axial epithelium of these corals, the iodination of the tyrosine residues took place prior to the assembling of amino acids into proteins. In accordance to Roche et al. (1963) in gorgonians the iodination occurs within the living tissue, and is able to concentrate iodide to a high degree.

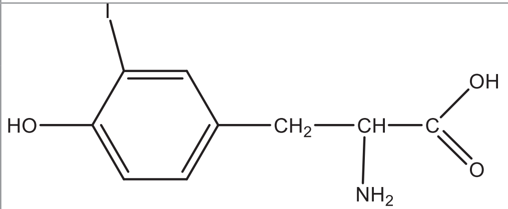
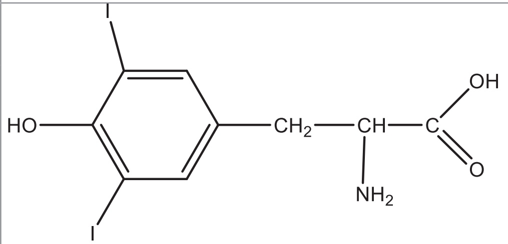
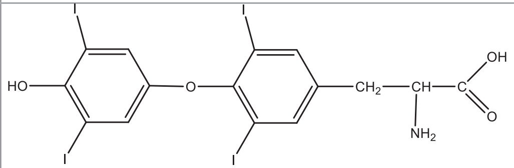
The biological function of iodine which occurs in scleroproteins of cuticles (annelinds, arthropods), perisarcs of hydroid polyps, poriferan spongin and mollusk periostracum (Gorbman et al. 1954), is still unknown. Probably, iodine is in some way involved in promoting of inter-molecular bonds between that protein chains which are involved in such biological processes as hardening (sclerotization) and tanning similar to function of quinones as well as polyphenols as the cross-linking agents in arthropods (Brunet 1967).

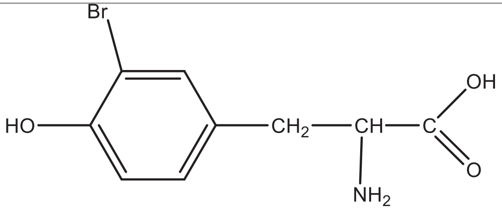
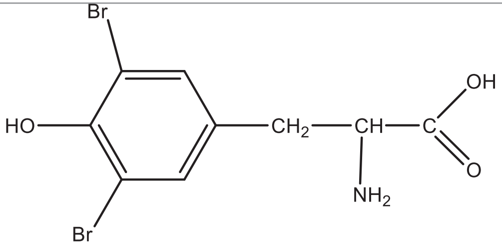
With exception of the paper by Leversee's (1969) in which he reported that the gorgonin is major constituent of the axial skeleton, which is consisted mostly of proteinaceous matrix with collagen fibers, the chemical composition of the gorgonian axial skeletons has been insufficiently studied. According to Szmant (1970), the skeletal material of *Muricea californica* (Gorgonacea) is "inert and persists long after the death of the animal". Glucose and galactose, at approximately 1% by weight, have been also identified within axial skeleton of this species (Szmant 1970). This skeleton was resistant to treatment with anhydrous formic acid, 0.1 M mercaptoethanol, 8 M urea and 2 M hydroxylamine.

Gorgonin became more recognized as a collagenous matrix and as a sclerotized collagen with a favoured axial orientation after studies made by Goldberg (1973, 1974, 1976, 1978).

Both a cytochemical and ultrastructural analysis of the cellular basis for tyrosine derived collagen crosslinks have been reported by Tidball (1982) on example of gorgonin isolated from *Leptogorgia virgulata.*

Table 12.1 Possible modifications of tyrosine in antipathines, gorgonines and spongines

Name	Chemical structure	Proteins
Monoiodotyrosine		Antipathines Gorgonines Spongines
Diiodotyrosine		Antipathines Gorgonines Spongines
Thyroxine		Gorgonines

Monobromotyrosine		Gorgonines
Dibromotyrosine		Gorgonines Spongines

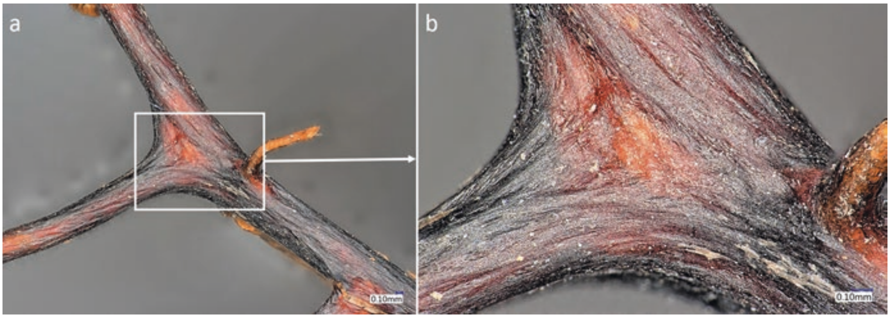


Fig. 12.1 Dark colored gorgonin (**a**, **b**) is typical for gorgonian corals

Up today, gorgonin is known as polyphenol-containing fibrillar protein (Ehrlich et al. 2006), or fibrillar scleroprotein (Noé et al. 2008) (Fig. 12.1).

Thus, during his history "Gorgonin" was defined by the different scientists listed above as an jodotyrosin-based protein, pseudokeratin, or eukeratin, sclerotin or tanned collagen. However, some features can be defined as common for this unique biopolymer:

- the presence of iodine;
- the presence of basic amino acids;
- hydrophobic properties;
- fibrillar nature;
- high resistance to enzymatic and chemical treatment;
- it has never been reported to induce calcification.

In contrast to the lack of data on biosynthesis and chemistry of gorgonin, its function with respect to selected mechanical features of gorgonian axial skeletons has been well studied.

12.2 Mechanical Properties of Gorgonin-Based Skeletons

According to Szmant-Froelich (1974), "the axial skeleton of gorgonian *Muricea* can be described as a tubular structure with two parts: an outer solid cortex, and an inner hollow core which is divided into chambers by convex cross-walls. The axial epithelium is presumed to synthesize the axial skeleton. The outer cortex is made up of many thin laminae of dark colored gorgonin. Each lamina extends only partially around the circumference of the axial skeleton; it overlaps and is overlapped by adjacent laminae. The density of these laminae varies: they are more numerous in the lighter colored layers of the axial skeleton, but are less numerous and more closely packed in the darker layers" Szmant-Froelich (1974).

The similarities between structure of collagen and gorgonin led to propose biomineralization model of the isidid (bamboo corals) gorgonian endoskeleton: endodermal epithelium cells produced gorgonin, which during the process of mineralization is an insoluble structural template, and the viscous slime enclosing the skeleton serves as soluble polypeptide in form of ß-sheet. Here, I take the liberty to stress that gorgonin functions a scaffold, rather than a template with respect to calcification.

Unfortunately, there is no compressive study regarding the skeletogenic cells which are responsible for secreting of calcium carbonate in gorgonian axes as well as controlling of the crystal deposition. In the study about *Leptogorgia virgulata* Kingsley and Watabe's (1984) indicated that transport of calcium ions occurs from the external environment to the axis, and then using Ca-ATPase as a mediator from the axis to the axial epithelium. Then, these authors successfully obtained carbonic anhydrase activity from the adjoining structures of calcifying axis, these results showed that the fluid supersaturation exists in gorgonians and the formation of mineral crystals possibly occurs on an external structures of polysaccharides and proteins.

Goldberg (1976) found within the heavily mineralized gorgonian axial skeletons, huge content of glycosaminoglycans (GAGS), which can cover the single fibers of collagen (gorgonin), therefore begin the granular crystals nucleation.

In different species of octocorals, corresponding diversity with respect to the calcification step has been reported and discussed (Sherwood and Risk 2007). For example, the Red tree corals (family Primnoidae) depose a two-part "horny axis" builds from calcite-gorgonin, towards the inner segment of the axial skeleton, and later on, a solid calcite matrix. In representatives of the Bamboo corals (family Isididae), gorgonin nodes are localized between internodes made of massive calcite. Such structures look like the joints of a finger. Thus, in the material and mechanical properties, skeletons of various gorgonian species vary drastically from each other.

Flexibility may plainly be modulated or controlled by the collagen sclerotization within the axial skeleton. As described by Jeyasuria and Lewis (1987), in gorgonians, a commonly applied manner of hardening axes is carbonates extracellular deposition within the interstitial spaces of collagen.

Surprisingly, the data to be found in the paper by Jeyasuria and Lewis (1987) show a wide spectrum of mechanical properties for the axial skeletons of gorgonian. Meaningful interspecific differences are apparent from the figures of Torsion as well as Young's modulus tests, which examine the resistance to shear and tensile strain, respectively. It was reported that the Young's modulus is above that for tendon and lies between 2×10^8 and 9×10^9 N/m^2, which suggests that collagen within axial skeleton is a stiffer form of this structural protein (Jeyasuria and Lewis 1987). Such difference has functional significance, because octocorals axial skeletons function in contrast to ligaments or tendons of vertebrates, as the primary support formations that retain the separation as well as elevation of the branches of the colony. Biological material with the stiffness similar to ordinary collagen could be too flexible to maintain proper separation of the branches and hold the colony of branches off an abrasive substratum, even with the support provided by H_2O.

Torsion modulus is in the range from 6.7×10^7 to 8.55×10^8 N/m^2 (Jeyasuria and Lewis 1987). It must be noted that the resistance to shear varied quite drastically and is not completely correlate interspecifically with Young's modulus. To put it differently, a high Torsion modulus for a species can not mean a high Young's modulus for the same species. This can be connected with the feeding technique applied by individual species and the location of corresponding polyps on the skeletal branches.

These authors found that the tensile stiffness of the axial skeleton along axis is directly proportional to the content of carbonates. The evaluated Torsion modulus for the axial skeleton correlated with calcareous content. However, this is a more complex polynomial function, not a simple relationship.

Although, it appears to be eminently reasonable when the relationship between greater stiffness and rapid change in water movement becomes evident. The Young's modulus data of the axial skeletons of 13 selected holaxonian octocorals species, from 12 various genera, have been reported by Jeyasuria and Lewis (1987). These authors determined that they vary from 0.2 to 90 Gdynes/cm^2. Additionally, the axial stiffness is well corelated with zone-related water movement. Within the axial skeletons, relative quantities of calcareous material were highly correlated with Young's modulus, implying a significant role of this material in adjusting the axial skeleton mechanical features. Therefore, using calcification to adjustment the axial stiffness can be consider as an efficient method for managing the various hydrodynamic forces hit on different depths.

12.3 Gorgonin-Based Skeletons and Paleooceanographic Dynamics

Both bamboo corals as well as primnoid gorgonians were recently studied as possible model organisms in studies on paleoaceanographic dynamics, age estimation including growth rates (Andrews et al. 2002; Sherwood et al. 2005a, b; Thresher et al. 2004; Noé et al. 2007, 2008). Recently, it was recognized that the gorgonin-based skeletons of deep-sea octocorals may be crucial sources of high temporal-resolution, geochemical-based climate reconstructions of both intermediate/deep-water and surface water processes (Heikoop et al. 2002; Thresher et al. 2004; Sherwood et al. 2005a, b). For example, it was proved that the rate of growth of the skeletons of bamboo coral can fluctuate from 0.05–0.1 mm year^{-1} (Roark et al. 2005) to 1.9–4.4 mm year^{-1} (Andrews et al. 2005). These results provide evidence that the rate of growing can be different between individual species, and may be controlled by variations in oceanographic conditions of the coral surrounding environment.

The changes in the gorgonin:calcite ratio is responsible for synthesis of the rings located in the octocorals horny regions as well as it can be also related to the alternations in the tanning protein content (Szmant-Froelich 1974). The rings of calcite are produced as repeated crystal growth and nucleation events (Risk et al. 2002; Marschal et al. 2004; Sherwood 2002). The periodicity of the annual ring in the *Primnoa resedaeformis* red tree coral has been studied using bomb-^{14}C (Sherwood et al. 2005c) and ^{210}Pb-dating (Andrews et al. 2002). Within this species, lunar-originated, bigger growth rings also have been reported (Risk et al. 2002; Sherwood 2002). Also diffused banding patterns have been found in other publications (Druffel et al. 1990; Roark et al. 2005; Andrews et al. 2005). It is supposed that in scleractinians, the timing and look of the rings is related to the properties of the environment, such as the organic matter flux. Octocorals can live hundreds of years (Druffel et al. 1990; Risk et al. 2002; Andrews et al. 2002; Thresher et al. 2004; Roark et al. 2005), one of the oldest specimen of *P. resedaeformis* was a 700 years old (Sherwood et al. 2006).

The appearance of the colonies of bamboo coral is distinctive. Their skeletons are characterized by existence of proteinaceous nodes and calcitic internodes which build the articulated axis. They are ensured by non-retractile polyps equipped with large needles and rod-shaped sclerites. Keratoisidinae species are able to exist at abyssal depths; however they were not found in the Arctic Ocean (Kükenthal 1924; Bayer 1956). The Keratoisidinae subfamily comprises of four genera (Isidella, Acanella, Keratoisis and Lepidisis) and two genera (Australisis as well as Tenuisis) that could be submitted also to Isididae (Bayer 1990). According to Noé and Dullo (2006), skeletons of isidids can be used also as high-resolution archive of paleoceanographic dynamics in deeper water masses.

In the dendrochronology of the isidids nodes is also useful to evaluate the age of the *Isidella tentaculum* colonies, thus the stable Pb and U/Th isotope from the basal nodes growth rings were applied (Etnoyer 2008), based of obtained results it was assumed that they are 75–100 years old (Roark et al. 2005; Andrews et al. 2005). Interestingly investigation of these rings shows that they grow in lunar cycles, as well as some oceanic events like nuclear bombs testing are clearly visible (Roark et al. 2005). Keratoisidinae axes are distinctive and durable (Di Geronimo et al. 2005), and they possess a fossil history.

12.4 Conclusion

Gorgonian octocorals are characterized by a divided endoskeleton, formed from enclosing coenenchyme. They have a compositional heterogeneity: a gorgonin-based dark organic nodes together with a vertical alternation of heavy mineralized internodes, that are usually found in these marine animals. Gorgonin, as biopolymer with clear chemical definition remains to be enigmatic. State of the art knowledge is based on it acceptance as fibrous, collagen-like, halogenated cross-linked biopolymer, which is not involved in the calcification. However, its rigidity and flexibility enable gorgonian corals to survive in high-energy and current-affected aquatic niches. Gorgonin-based biomaterials are exceptional examples for experts in extreme biomimetics because most deep-sea gorgonians are distributed within the psychrophilic coral ecosystems with temperatures around 4 °C. Better understanding of biomaterials formation under such conditions is still in trend.

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Chapter 13
Antipathin

Abstract Antipatharians are characterized by an erect, rigid chitinous skeleton that create long unbranched whip-like coil or tree-like, unbranched colony. The skeleton of black corals represents a structure typical for a laminated composite. However, the detailed inorganic-organic composition can differ from one species to another. Different elements that can be found in the skeleton are carbohydrates, lipids, sterols and phenols. Bromine as well as iodine seem to be the main single elements. In the antipatharian, during the skeletal formations the dominant structural components are represented by chitin and an antipathin, some kind of halogenated scleroprotein. Antipathin appears to be related with specific material properties of skeletons of black coral. The antipatharians skeletons are less rigid and more elastic in comparison to another biomaterials used as structural components in the nature such as bone, wood, insect cuticle and mollusc shell. Simultaneously, the density of antipathin is lower than bone or shell and higher than wood, but almost similar to cuticle of arthropods.

13.1 Brief Introduction in to Antipatharia

The clonial cnidarians black corals (Antipatharia) are marine organisms existing world-wide. Within the Antipatharia order (Milne-Edwards and Haime (1857) (Cnidaria, Anthozoa) there are 235 species (Schultze 1896; Totton 1923; Brugler and France 2007; Wagner et al. 2012) belonging to 39 genera and 6 families (Opresko 1974). In the 1700s, the systematic studies regarding black corals have been performed, then at the beginning of 1900s a few monographs regarding taxonomy of black corals were published such as a ground-breaking works prepared by Brook (1889), Schultze (1896), and van Pesch (1914), most of them were based on diverse oceanographic expeditions (see for overview Delage and Hérouard 1901; Cooper 1909; Kinoshita 1910; Pasternak 1977; Warner 1981; Grigg and Opresko 1977; Echeverria 2002; Loiola 2007). Both the ecology and biology of black corals have been recently reviewed by Bo (2008), Prouty et al. (2011), Wagner et al. (2012), Brugler et al. (2013), Carreiro-Silva et al. (2013), Cardona et al. (2016), and Álvarez-Valero et al. (2017).

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All species are characterized by slow growth, low natural mortality in adulthood and, consequently, by a long life. They achieve reproductive maturity rather after 10 years, which is much later in comparison with other similar marine organisms. The output of planula (lasrvae) as well as the recovery of organism is slow. Polyps possess dimensions in the range of 0.5–5 mm (Nowak et al. 2009). The age of such deep-sea black coral as *Leiopathe*s sp. has been estimated using radiocarbon analysis of around thousands years old. The oldest known organism *Leiopathes* sp. was 4265 years old (Roark et al. 2009).

Antipatharians possess a straight, stiff, chitinous skeleton in the form of a branched colony or an extended lash-like reel (similar to wire) (Fig. 13.1). The skeleton structure of antipatharians can be consider as a laminated composite, comprised of the fibrils of chitin together with the non-fibrillar protein, but the detailed chemical composition can be different for different species. The carbohydrates, lipids sterols and phenols can also exist within the skeleton, but the bromine and iodine are the major single component (Juárez de la Rosa et al. 2007).

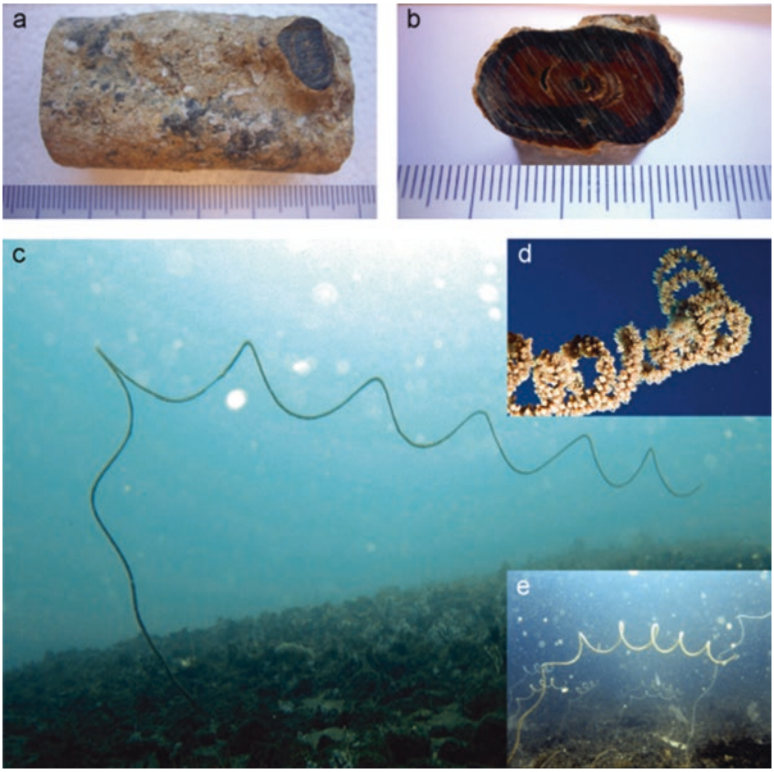

Fig. 13.1 Black corals contain gorgonin- and chitin- based very rigid skeletons (**a**, **b**) that forms a tree-like and branched colony or a huge meters-long whip-like coil that remains to be unbranched (**c**–**e**). (Images courtesy Marzia Bo, Giorgio Bavestrello and Mark Spencer)

Now, it is well recognized that the age of antipatharian corals can be estimated using the characteristic ring structures well visible on the cuts of the skeletons (Goldberg 1978). Similar to selected species of red corals, special information (e.g., on the salinity and water temperature in chronological order,) which can be potentially stored in the rings could be useful for corresponding scientific aims.

The importance of black corals for commerce and the special field of jewellery is well known since ancient times (Hickson 1924). In spite of still existing consumption of black corals in the jewellery trade, the tools of the black coral origin are listed in the Convention for the International Trade in Endangered Species (CITES) (Wood and Wells 1988).

13.2 Chemistry of Black Corals

The major structural constituents of the antipatharian skeletal formations are chitin and a halogen-containing scleroprotein known as antipathin. For instance, based on the NMR results the content of organic matter within the load bearing skeletal plate of *Antipathes fiordensis* (the New Zealand black coral) was established to be 70% protein, 15% diphenol, 10% chitin, and 5% lipid by weight (Holl et al. 1992). The *A. fiordensis* tips or younger pinnules possess not more than 3% diphenol by weight.

Hydrochloric acid treatment led to extract 3,4-dihydroxybenzaldehyde (DOBAL) and 3-(3,4-dihydroxyphenyl)-IX-alanine (DOPA) from the coral skeleton. The base of *A. fiordensis* has more DOPA the tips, additionally DOPA acts as a peptidyl component in the skeletal protein. The DOBAL and DOPA oxidation lead to obtain quinones which can be responsible for the structural proteins cross-linking to chitin or other proteins which provides mechanical stabilization of the coral skeleton (Holl et al. 1992; Kim et al. 1992).

The “antipathin” is commonly used definition to discern the “gorgonins” from these proteins. This name has been proposed for this phenol-based biological material prior to the discovery of corresponding chitin fraction (Roche and Eysseric-Lafon 1951). The highest histidine content of any known protein has been reported for antipathin (Roche and Eysseric-Lafon 1951). Additionally, iodohistidine was also detected in the skeletons of black corals (Goldberg 1976).

Antipathin, also possesses all the characteristic properties for sclerotized protein, as in the case of gorgonin (Brown 1950): proteolytic agents does not affect it, high in tyrosine, mostly dark in color, and dissoluble only in chlorine bleach. The autoclaving do not make antipathin soluble. Collagenous origin of this biomaterial has not be confirmed (Goldberg 1976). The extraordinary stability of antipathin can be explained by the existence of crosslinking between iodinate histidine and tyrosine, but the role of huge amount of histidine is still unknown. The typical stain for connective tissues such as the Mallory’s, Gomori’s or van Gieson’s gave negative results. The only histochemically detectable polysaccharide was a sialic acid, the function of which remains to be known. Thus, the material composition, reactivity together with structure implies that it is not composed of keratin or collagen

(Goldberg 1976). Also, there is lack of any kind of scientific information in the available literature concerning the hypothetical role of antipathin in both biosilicification, or calcification.

Similar to diverse demosponges, iodine is to be found in a number of antipatharians (Roche et al. 1963; Goldberg 1976). Probably, due to high amounts of iodine in *A. salix* some kind of iodine dependent cross-linking mechanisms exists within skeletal structures of this species. It has been reported that also bromotyrosine as well as bromohistidine may also be present as in the skeletons of gorgonian corals (Roche et al. 1963). However, these compounds have yet to be isolated and identified in antipatharians.

The skeletons of all species of black corals possess significant chitin amount, which varied between 6% and 18% of the total mass of the skeleton (Nowak et al. 2009). In *Antipathia salix* the amount of chitin is equalled 14.5% of the skeletal mass (Goldberg 1976). Although, significant differences in the amount of both chitin and antipathin have been shown for diverse species of black corals. For instance, the amount of chitin in skeleton of *A. salix* estimated by NMR was two times higher that of *Antipathia fiordensis* (Holl et al. 1992). Determination of chitin content by the measuring of glucosamine content was applied by Kim et al. (1992). Authors showed that tips of *A. salix* contained about 29% more chitin than *A. fiordensis.*

Recently, the presence of morphologically distinctive multilayer hybrid structures in black corals from three different locations was reported by Nowak et al. (2009). The matrix consists of α−chitin, which content is equalled approximately 15% of its weight, supplemented by a various of carbohydrates and proteins. The chitin crystallites locations varied in different zones; the fibrils of chitin have 4 μm in width and they are located along the cells long axes in the interior zone. However, in the skeletal hydrolysates of antipathins studied previously, tyrosine is not prominent (Goldberg 1976). However, a relatively large amount of tyrosine is tightly bound with the chitin of the skeleton origin, implying an important role of covalent bonding between protein and chitin.

It was proved by morphological observation that, the axial structure of antipatharians is composed of a core encased in a number of special cells (for details see Nowak et al. 2009) related to intermediate gluing layers known also as "*layers of organic cement*" (Holl et al. 1992). Corresponding clusters of periodic opaque cement can be responsible for the growth-ring pattern and lines between skeletal layers (Goldberg 1991). The variations in chemical composition of the black coral and periodicity of its structure can be related to fluctuations in environment and thus could act as a record of local environmental conditions (Holl et al. 1992; Goldberg 1978).

Guerriero et al. (1988) discovered so called leiopathic acid (hydroxydocosapenthaenoic acid) in the black coral *Leiopathes* sp. Unfortunately, there is no suggestion concerning the possible role of this fatty acid with respect to formation of some skeletal structures of antipatharians.

13.3 Material Properties of Antipathin-Based Skeletons

Skeletons of black corals can be assumed as laminated composites formed from antipathin and chitin fibrils (Bo et al. 2012). However, the layers in antipatharian skeleton remain not to be simple laminated constructs. It seems that the helically wound skeleton is fixed at multiple points due to a fact that, the spines are inserted and cemented layer upon layer (Goldberg and Taylor 1989a, b). Kim and co-workers hypothesised that "the occurrence of spines increases the surface area for cementing one skeletal layer to the other. Moreover, they could have an important role as continuous rivets, resisting delamination of shear forces produced by skeletal bending and torsion. If this statement is true, the occurrence of spines might eliminate or reduce the demand of small fibril biases between helically wound layers" (Kim et al. 1992).

The antipatharians skeletons are less rigid and more elastic than other biomaterials chosen and used in the nature as structural components such as bone, mollusc shell, wood as well as the insect cuticle. Simultaneously, the density of antipathin is lower than bone or shell higher than wood, but almost the same as cuticle of arthropods (Wainwright et al. 1976).

The specific modulus is recognized as the ratio of Young's modulus to density (E/p), and is used for evaluation of the stiffness per unit weight of corresponding materials. Low values of specific stiffness are usually considered to be inferior to high values because, these high values enable construction of lighter but stiffer structures. Although, in the case of antipatharians (Goldberg 1976; Kim et al. 1992), higher flexibility per unit of density seems to be more significant than stiffness. Consequently, black corals possess a lower specific modulus in comparison to values characteristic for insect cuticle presented by Wainwright et al. (1976).

There is an increasing amount of evidence describing a relationship between ecological function and the biomechanics of skeleton (Kim et al. 1992). Orientation to flow can reduce drag forces and increase suspension feeding efficiency in organisms with flexible skeletons (reviewed by Wainwright et al. 1976). In some gorgonian corals, acclimatization to flow can be observed in the skeleton of fan-like species as a variation in preferred orientation (Wainwright and Dillon 1969; Grigg 1972). For example, deposition of calcium carbonates within the skeleton of some branched gorgonians can lead to perpendicular reinforcement to the flow direction (Wainwright et al. 1976; Wainwright and Koehl 1976).

There are no doubts that the mechanical and structural features of the skeleton of black coral appear to be overdesigned for the hydrodynamically more docile, deeper zones where antipatharians are usually occur.

However, fitting the skeletal design to ecological function is unclear, the contradistinction between these two studied specimens has indicated that *A. salix* is harder, more hydrophobic, darker, denser, and stiffer than *A. fiordensis* (Kim et al. 1992; Holl et al. 1992). The differences in material properties of this species seem to reflect higher commercial request of *A. salix* as the subject of jewellery.

High stiffness which enables the construction of light and strong skeletons is definitively a benefit. The hardness of the skeleton of both antipatharians is hard enough to scratch calcite and is equalled 3 on the Mohs scale (Nowak et al. 2009). It seems to be bizarre that invertebrates which live in conditions which do not allow to get a lot of energy have decided to build such an energy-consuming and refined skeleton construct made of chitin instead pure mineral-based skeleton. It can be suggested that the elastic organic skeleton can obey the natural wave movements of the water currents. Additionally, the organism saves the energy, after the initial expending of material and energy on formation of the skeleton, with a "large total surplus over the scale of a life time (~40 years for colonies reaching a maximum of 1.8 m in height)" (Nowak et al. 2009).

13.4 Conclusion

Mechanical and physical properties of the skeletons of black corals are likely to imitate the nature, architecture and relative abundance of corresponding biopolymers, which stabilize their inner structure. Comprehension this high degree of variety with reference to the chemical and structural properties of antipatharians is a challenging point. The commercial harvest for jewellery purposes together with deep-fishing is a big risk for the conservation of deep-sea coral colonies including black corals. I take the liberty to agree with Brendan Roark, who recently noted that "*in light of the unusual longevity of black corals, a better understanding of deep-sea coral ecology and their interrelationships with associated benthic communities is needed to inform coherent international conservation strategies for these important deep-sea habitat-forming species*" (Roark et al. 2009).

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Chapter 14
Rubber-Like Bioelastomers of Marine Origin

Abstract Opposite shells of bivalve molluscs articulate due to existence of the hinge ligament. This by the mantle isthmus secreted structure is of proteinaceous nature. The hinge ligament possesses two parts. The outer flexible part, which serves as a hinge, grips the two valves tightly along the hinge line. The elastic material in the form of block acts as the inner part, which role is making a gape to relax the adductor muscle. Hereby, I take in account the description of the chemistry and structural properties of molluscs hinge ligaments as bioelastomers of marine invertebrates origin.

Usually, the flexible proteins possess extraordinary and optional properties, for example: ligaments which are made of abductin in clams (bivalve molluscs) are responsible for shell opening after the relaxing the muscles thus generating an action of swimming. However, byssus threads bind mussels to substratum and have flexible as well as stiff spheres to withstand the wave current.

The recent progress in better understanding of protein elasticity is related to our capability to evaluate knowledge from molecular biology(i.e. the molecular sequences) and the structural biology of protein's three dimensional constructs and to correlate them with the mechanical features of macromolecular arrays and single molecules, the latter using novel methods, like an atomic force microscopy and laser tweezers, which drive to fast advances, but have inevitably also raised new and challenging questions. Corresponding data about diverse bioelastomers are to be found in the book edited by Peter R. Shewry, Arthur S. Tatham and Allen J. Bailey and entitled "*Elastomeric Proteins: Structures, Biomechanical Properties, and Biological Roles*" (Cambridge University Press, 2001). Consequently, following chapters are dedicated only to bioelastomers of marine invertebrate origin like hinge ligaments of molluscs, the unusual whelk egg capsules biopolymer as well as resilin identified in crustaceans.

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14.1 Hinge Ligament

Molluscan bivalved shells, serve as a models to study biomineralization, biomimetics, as well as bionic (David 1998). The complex structure of the shell and existence of several macromolecules such as silk, acidic proteins and chitin are responsible for the extraordinary mechanical properties of the shell (tensile strength, stiffness, fracture toughness) in comparison to other composite materials. Nowadays, it is possible to find several reports regarding the complex mineral phases arrangement as well as their crystallographic orientation within corresponding layered structures (see for review Marin et al. 2008; de Paula and Silveira 2009).

The well-known proteinaceous semi-rigid structure of bivalve molluscs termed as hinge ligament can be found in the region where the two opposite shells articulate (Fig. 14.1). The anatomical records together with and materials features of hinge ligament in diverse mollusc species are represented in details in papers by Trueman (1949, 1950a, b, 1951, 1953, 1969), Owen et al. (1953), and Alexander (1966, 1968, 2002).

Thus, within the hinge ligament two parts can be distinguished (Trueman 1953). The outer flexible part, which serves as a hinge and grips to the two valves. The elastic material in form of block acts as the inner part, which role is making a gape to relax the adductor muscle. For the inner ligament Dall (1889) suggested the name "resilum" in order to convey its compressional function. It has the calcified lateral – in this place where attachment to the valve occurs, however mostly it is consisted of noncalcified protein. It does not show any section's birefringence (in *Pecten*

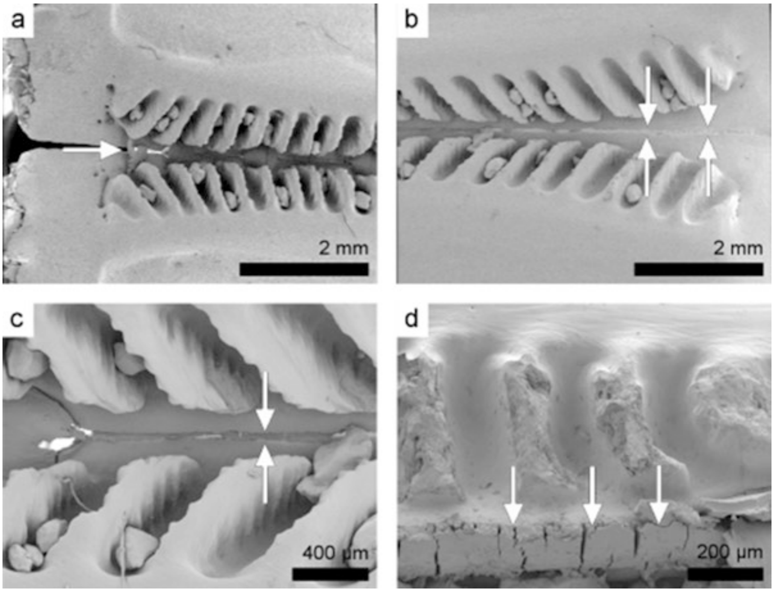

Fig. 14.1 SEM imagery of the hinge ligament (**a**–**d**) (arrows) of *Solen* sp. collected in Tunisia. (Image courtesy Lothar Brehmer)

Pectinidae (Lamellibrancbiata)) as well as breaks down with a conchoidal rupture. Thus, unstrained it can be assumed as amorphous, while the cut out ligament is able to rebound similarly to the dropped on a hard surface rubber.

In some marine molluscs, hinge ligament is also crucial for their swimming behaviour. Typical examples are Scallops (*Pecten*, etc.) with exceptional ability among bivalves to swim including very cold Antarctic waters. They can ejecting jets of water on either side of the hinge due to rapidly and repeatedly opening and closing their shells. Special muscle is responsible to open the shell, however there is no muscle to open it. This is the function of an elastic hinge ligament just inside the hinge (Fig. 14.2). This system is based on the rubber-like protein abductin. This term has been proposed by Kelly and Rice in 1967 for the protein of the internal ligament of *P. magellanicus* and *A. irradians* (Kelly and Rice, 1967). The abductin is compressed when the shell closes, but elastically recoils to open it. According to Alexander: "*The resonant frequency of the animal, due to the interaction of the compliance of the hinge ligament with the masses of the valves of the shell and the added masses of water that move with them, matches the frequency of the swimming movements*" (Alexander 2002).

It is to note, that, in contrast to non-swimming bivalves, the hinge ligament of scallops has low hysteresis, suiting its role in swimming (Trueman 1953, 1969).

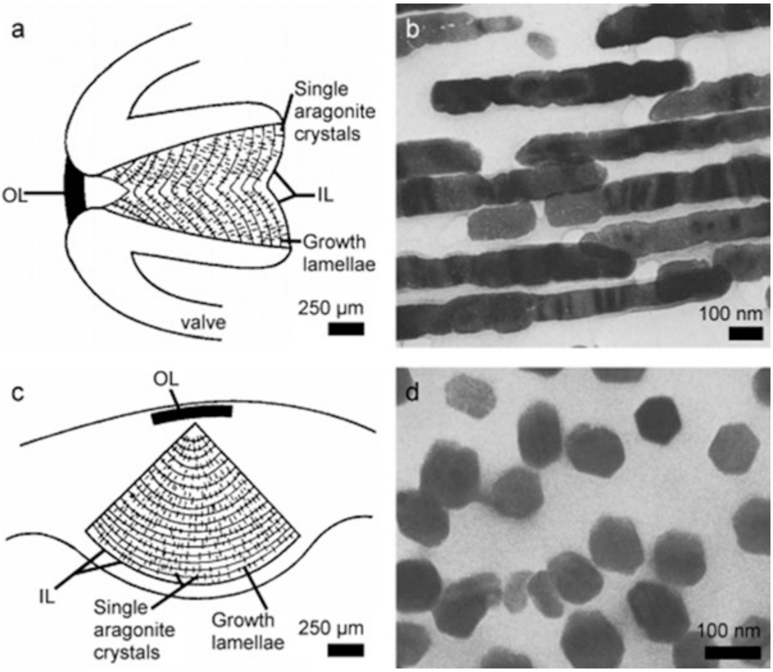

Fig. 14.2 The inner structure of the hinge ligament of *Spisula solidissima*: (**a**) transverse and (**c**) sagittal views. TEM images (**b**, **d**) shows orientation of aragonite nanocrystals within organic matter. (Adapted from Kahler et al. 1976)

14.2 Chemistry of the Hinge Ligament

Scientific information about chemistry of hinge ligaments in molluscs is broad, but sometimes curious (Suzuki et al. 2015; Kubota et al. 2017; Poitevin et al. 2018).

Therefore, birefringence in selected fragments of the ligaments of *Tellina tenuis* (Trueman 1949) and *Mytilus edulis* (Trueman 1950b) was described by Trueman. Author proposed that some compound of lipoid nature which is localized within the ligament is responsible for the birefringence of the *Tellina* ligament. Overall, the ligament, likewise the shell, is consisted of some combination of calcium carbonate and protein, but in some species (i.e. *Aequipecten*) the latter phase cannot exist. The organic phase of *Anodonta* hinge ligament was suggested by Trueman (1950a) as a quinone-tanned protein.

Unprecedented high content of glycine has been noticed in the ligaments of *Mytilus californianus, Placopecten magellanicus,* and *Aequipecten irradians* (Hare 1963; Kelly and Rice 1967). The composition of the inner hinge ligament of *Spisula solidissima* (Bivalvia) was investigated by Kahler et al. (1976a, b). Authors showed that in a hydrated protein matrix, calcium carbonate is embedded. Results of the amino acid composition of the matrix proved that glycine represents more than 62% of corresponding residues.

There is possibility that shell soluble proteins are associated with hinge ligament proteins.

It have been reported (Kahler et al. 1976a) that the amino acid composition of the *Mercenaria* ligament crystalline sheaths is more closely associated to the soluble protein of the *Mercenaria* shell, than to the bulk ligament protein in which these sheaths are located. According to Kahler and co-workers, “the ligament crystal sheaths consists of 20.0% aspartic and 16% glycine compared to 11% aspartic and 29% glycine in the bulk ligament protein and 29.7% aspartic acid and 15.6% glycine in shell protein. Such resemblances demonstrate that the bulk protein elastomer, which contains the ligament, is not involved in the process of protein mineralization and the protein from both, ligament crystal sheaths and shell are aligned in similar phases of mineralization which mechanism can proceed in a similar manner” (Kahler et al. 1976a).

However, the crystal sheaths of ligament comprise only 17% of the amide residues in amide form. These data collates well with 16% acid residues in the ligament protein, in contrast to the shell protein of *Mercenaria* (Kahler et al. 1976a), which is known as completely amidated (Crenshaw 1972).

Corresponding amides constitute 50% of the acid residues in the crystal sheaths of *Spisula*. The subtracting of the amides leads to obtain the sheaths of *Spisula* with only 5.5% acidic residues.

Suzuki and co-workers isolated from the ligament of *Pinctada fucata*, small acidic peptide, termed LICP (ligament intra-crystalline peptide which consists of ten amino acid residues with N-terminal pyroglutamic acid (Suzuki et al. 2015). It was hypothesized that LICP has a regulative role in the formation of the crystals identified as aragonite in the ligament due to inhibition of the growth of such crystals in the c-axis direction. Another ligament-specific protein has been isolated from the organic matrix of fibrous nature located in the ligament of the same species between aragonite crystals. This tissue inhibitor of metalloproteinase (PfTIMP), has a molecular weight of 13,580.4 and consists of 143 amino acid residues (Kubota et al. 2017). It was suggested that PfTIMP can regulate the formation of organic, fiber-based ligament structure.

The presence of diverse kinds of cross-links within molluscan hinge ligaments cannot be excluded. For example, Andersen (1967) isolated 3.3′-methylenebistyrosine from of *M. edulis* by demineralization of the ligaments in 1 M CH_3COOH, followed by hydrolysis in 6 M HCl. He suggested that in this case phenolic compounds resemble dityrosine. In corresponding *Nature* paper, Anderson claimed the existence of some kind of 3D network where dityrosine-based compounds are able to link the protein chains in the ligament together. However, the re-examination of the data reported in Anderson's work by Kikuchi and co-workers (1982, 1987, 1988) leads to the knowledge that isolated compound was an artefact. These authors investigated the hinge ligament cross-linking constituents of a Sakhalin surf clam *Pseudocardiurn suchalinensis* (family Mactridae). They proved that the resilium possesses around 65% aragonite and 35% protein crystals calculated for dry mass. Within the 100 mol of the resilium total amino acids content, approximately 50 mol glycine and 20 mol methionine (mostly in the form of methionine S-oxide) can be found. This is characteristic for the representatives of the Mactridae family.

The results of hydrazinolysis indicated that the protein is composed of almost 1800 amino acid remains with glycine at its carboxyl terminal. The existence of desmosin together with isodesmosin as crosslinking components in the resilium protein also have been proved. After HCl hydrolysis of the protein the 3,3′-methylenebistyrosine was extracted. Although, based on obtained results it was assumed that during the hydrolysis the derivative of the tyrosine can be synthesised from formaldehyde and tyrosine as an artefact (Kikuchi et al. 1987, 1988).

Interestingly, hinge ligament of molluscan origin is heavily resistant to enzymatic as well as chemical treatment. It is to note that 0.5 N alkali or acid at room temperature has no effect on *Spisula* inner ligament protein as well as no visible changes are observed when the ligament protein is inserted into boiling water for 5–6 h, in contrast to collagen (Kahler et al. 1976). However, mechanisms of digestion of hinge ligaments were developed by some species of marine bacteria. The resilium, one of the two structural elements forming the oyster hinge ligament, is indictable for the valves opening as well as is also the most frequent site of erosive lesions. Therefore, the numerically dominant or codominant cultures of cytophaga-like gliding bacteria (CLB) have been obtained as components of bacterial populations related to the *Crassostrea gigas* juvenile Pacific oysters proteinaceous hinge ligaments (Dungan 1987). From the morphological point of view, isolated microorganisms were similar to elastic and long, bacilli existing within degenerative lesions in oyster hinge ligaments. From all isolated strains only CLB were able to growth and used the matrix of the hinge ligament as a nitrogen and organic carbon source. The proliferation and penetration of CLB bacteria into ligament matrices were achieved after in vitro incubation of the ligament resilium with ligament

CLB. The bacterial proliferation resulted in total destruction of resilium mechanical and structural integrity together with total liquefaction, at temperatures in the range of 10–20 °C (Dungan and Elston 1988; Dungan et al. 1989).

14.3 Structural Features of Hinge Ligaments

The morphology and structural features of bivalve ligaments have been down in the early investigations by Trueman (1949, 1950a, b, 1951, 1964). A lamellar continuity seems to be between the adjacent ligament and shell (Owen et al. 1953). Originally, the lamellae of the ligament have been recognized as special modification of the adequate shell lamellae (Trueman 1964). The electron micrographs of inner ligament of *Crassostrea virginica* were published by Galtsoff (1964). Author stated that" *images from sections tangent to the growth lamellae exposed 500 Å holes in a hexagonal array. Further image from sections, exhibited fibrils varying in diameter from 370 to 500 Å, at right angles to these holes*" (Galtsoff 1964).

The ligament organic phase is most frequently related with an inorganic phase, $CaCO_3$. The identification of crystalline aragonite in the *C. virginica* ligament was described by Stenzel (1962) and in the *Mytilus californianus* ligament by Hare (1963). The transmission electron microscopy images of this aragonite located in the ligament of *M. edulis* have been published by Bevelander and Nakahara (1969). However, in the ligament, calcite has never been detected, including in species with a calcite-based shell (Kähler et al. 1976b).

Microscopy (polarized light), X-ray diffraction and electron microscopic data reported by Marsh et al. (1976) and Kähler et al. (1976b) exhibit the formation of individual aragonite crystals of several thousand angstroms in length and with diameter about 1000 Å. The crystallographic orientation of the crystals with respect to c-axis remains to be normal to the observable growth lamellae, however the a- and b-axes are oriented randomly (Fig. 14.2).

The detailed analysis of the light reflectivity and ultrastructure of a dry as well as wet *Pinctada maxima* ligament was performed by Zhang (2007), and he proposed a lamellate structure. The lamellate has thickness around 35 μm where fibers of aragonite (ca. 78 nm) are evenly fixed in the protein matrix every 127 nm, in a similar manner as a photonic crystal. The described ligament (6.5 cm long, 2 cm wide and 0.2 cm thick) contains remaining proteins and about about 70 wt% of fibrous aragonite. It is coated with black horny layer. As represented in Fig. 14.3a, b the ligament is formed by of about 35 μm thick lamellae in which the fibers are highly aligned.

Observations of hinge ligaments rigidification properties were also previously described. The *S. solidissima* hydrated inner hinge ligament powder achieved constant weight after drying over $CaC1_2$ in vacuo for 24 h at 20 °C with a weight loss equalled 11.9 + 0.44% (Kähler et al. 1976a). Upon drying at room temperature, whole ligament gets brittle and quite hard. Thereby, rehydration brings back the typical resilience of the hinge protein. However, after several days of exposure to glycerol the *S. solidissima* ligament loses its resilience and shrinks markedly (Kahler et al. 1976b).

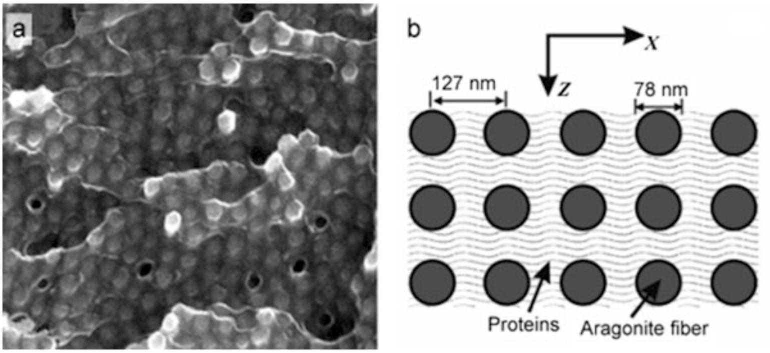

Fig. 14.3 SEM imagery of cross section of the ligament fibers (**a**) and schematic view (**b**) representing the 2D lattice model for theoretical simulation where Z indicates the propagation direction of light. (Adapted from Zhang 2007)

In a seminal work published by Alexander (1966), comparison of material features as well as thermodynamics of resilience and elasticity between bioelastomers like elastin and resilin with that of molluscan filament has been excellently represented.

14.4 Conclusion

Progress in marine bioelastomers including molluscan hinge ligaments is based on their biological and biomedical significance, particularly because of selected human diseases. Also design and development of novel artificial hybrid composites are dependent on the knowledge of the unusual properties of these biological materials. Both, chemistry and biomechanics play here very important role. To understand the principles of the bivalve hinge ligament biomineralization and the relationship between organic and mineral phases, there is a definite need for additional information which could be obtained due to genomics and molecular biology approaches.

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Chapter 15
Capsular Bioelastomers of Whelks

> *The eggs of the whelks are laid enclosed in protective capsules which, after the hatching of the eggs, are frequently found cast up by the sea along the shoreline. These capsules have a horny sclerotized appearance and are semitransparent and brownish in colour.*
> S. Hunt, Nature, 1966

Abstract Diverse whelks which represent a group of marine caenogastropod snails localize their embryos in unique biocomposite-based egg capsules. These multilaminate and capsular proteins-containing constructs are highly resilient. Capsular protein possesses comprehensive flexibility with ability to fast recovery which is proved by the decrease in the magnitude of elastic modulus (seeming damage) which starts at 3–5% strain. Take into consideration of the mechanical reaction to strain, this material is dissimilar to typical elastomeric proteins such as elastin or collagen. It is suggested that capsular elastomers possess high biomimetic potential for design and development of novel artificial hybrid materials.

In majority of marine invertebrates, the eggs, which are developed in the benthos, are deposited with characteristic surrounding structure such as multilaminated capsule or gelatin-based belts and masses (Fretter and Graham 1994). The formulation of these encapsulating structures, which is typical for the gastropod molluscs and polychaetes, allows developing of their embryos in benthos (Pechenick 1979).

Elastic capsules function as protectors of embryos against predation, microorganisms, desiccation, osmotic stress as well as UV irradiation (Ojeda and Chaparro 2004). In some species they can be very large in size. Thus, the eggs of *Busycon* are yielded in the form of a 1 m in length, helical strand usually called the mermaid necklaces, which can have almost 160 capsules (Fig. 15.1). They are subjected to the breakers with velocities reached even 10 ms^{-1} along the banks of the North America east coast (Miserez et al. 2009). Eggs capsules are acellular, with the structure and morphology closely connected with their functional roles. To carry out appropriate interpretation of the evolutionary aspect, function, origin of the structural patterns of the capsule as well as for material science and biomimetics

H. Ehrlich, *Marine Biological Materials of Invertebrate Origin*,
Biologically-Inspired Systems 13, https://doi.org/10.1007/978-3-319-92483-0_15

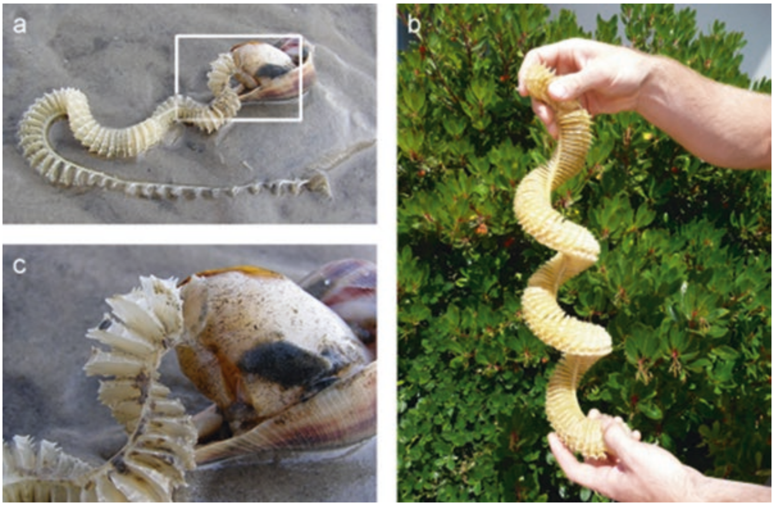

Fig. 15.1 Whelks are able to synthesise unique egg capsules (**a**, **c**) with interesting mechanical features (**b**). (Image courtesy Ali Miserez)

applications, the knowledge regarding the capsule wall's chemical structure is essential.

As assumed in the literature (Gathercole 1969; Rapoport 2003), the formation of the egg capsules can be described as below. A pliable soft sack (precapsule) is full of embryos and made of proteinaceous material. This sack is formed before leaving the genital tract by the nidamental (capsule) gland mesodermal secretory cells. It is supposed that, the precapsule in the pedal pore underwent physical shaping processes and enzymatically catalysed post-translational modifications that conceive the final chemical and morphological properties of the rigid rubbery capsule that can be observed *in situ*. According to Rappoport and Shadwick, "*gradations in snail size within a species produce egg capsules that are scaled versions of similar morphology, while differently shaped pedal glands finally form unique egg capsule morphologies*" (Rapoport and Shadwick 2007).

Thus, it was suggested (Tamarin and Carriker 1967) that the formation of these capsules is an example of the exocrine secretory activity, and lead to obtain stable structure strictly related to the species – that is unusual in comparison to typical amorphic secretion. In the snail *Urosalpinx cinerea,* the vase-shaped egg capsule comprise of four laminas, which three are anisotropic and have various transversal arrangements in comparison to the longitudinal plain, and one of them is isotropic. From the structural point of view, the birefringent matter clearly indicates a fine periodicity of variable dark (240 Å) and light (290 Å) regions, in each five subzone striae (Fig. 15.2a). The isotropic lamella together with the basal core comprises of distracted, 50 Å in width fibers, that can merge to form periodic fibrils (Tamarin and Carriker 1967). Goldsmith and co-workers in their investigation named the striated filaments as "pre-capsulin" and "capsulin" what was a consequence of the different

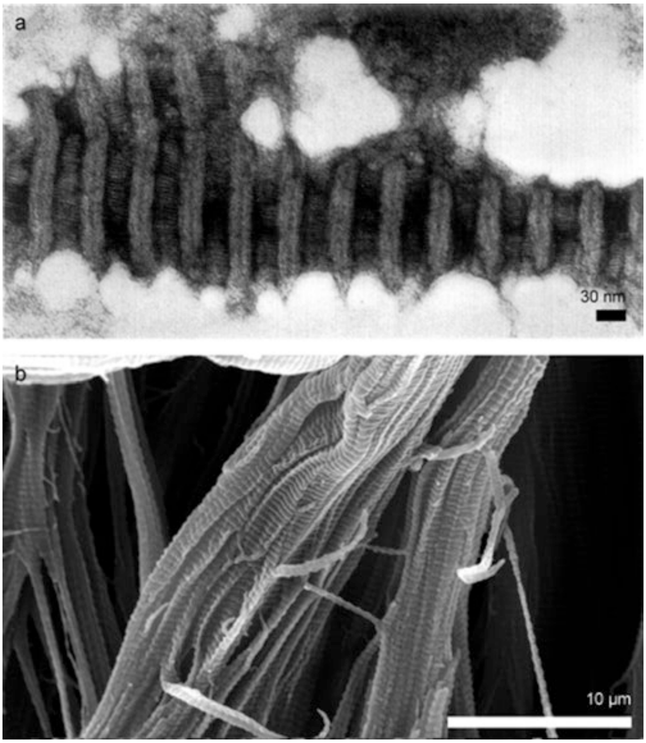

Fig. 15.2 Ribbon-like fibres of the whelks. (**a**) TEM image of stripped *Urosalpinx cinerea* ribbons stained with uranyl acetate, x 158,000. (Adapted from Flower et al. 1969). (**b**) Fibres within the egg capsule of *Busycon canaliculum* visualized using SEM. (Image courtesy Ali Miserez)

solubility of the individual pretranslationally modified matrix and the final product which is sclerotized (Goldsmith et al. 1978). It was hypothesized that both density as well as the cross-linking mode of the egg capsule biomaterial can influence its mechanical properties (Rapoport 2003). Firm fibre-like protein from the egg capsules of the *Buccinum undatum* has been characterized as resistant to both enzymatic (bactertial proteinases, chymotripsin, trypsin, pepsin) as well as to chemical (4 N NaOH, 0.5 M NaCl, 45%, 4% trichlor acetic acid at 60 °C, phenol at 90 °C,) treatment (Hunt 1966).

The alpha configuration of this protein has been proved (Rudall 1968; Flower et al. 1969) as well as in some features is similar to the proteins of the connective tissue origin of other invertebrates and vertebrates (Price and Hunt 1973). Studied on the chemistry of selected egg capsules showed that predominantly protein with minor amounts of lipid and carbohydrate is the main structural component (e.g., Hunt 1966). Amino acids as glutamic acid, aspartic acid, leucine, and lysine represent the dominant phase of the protein (Rapoport 2003).

Like elastin and some other structural proteins, the *B. undatum* capsule protein possesses yellow colour and under UV light displays a strong blue-white fluorescence in native state and after solubilization. Price and Hunt (1974) evaluated the possible role of the fluorophore-chromophore during the capsule protein cross-linking and that possesses aldehyde functional groups. It seems that di- or trityrosines are not to be present in the egg capsules of the whelks.

Comparative data on basic mechanical and biochemical egg capsules features obtained from different whelk snails species, such as the Kellet's whelk snail, *Kelletia kelletii*, characteristic to the West Coast of USA the *Busycon canaliculatum* channelled whelk snail usually found in the East Coast, are to found in corresponding papers (Rapoport and Shadwick 2000, 2001, 2002, 2007).

Research evaluated the qualified response of the material to stretching. The most interesting was the measuring of the energy diffused during the returning of material to its previous shape. Shadwick and Rappoport, stated that in all examined snails species, the material of capsule was technically identical, which proves that, during evolution this material has not changed and therefore, enhances survivorship of the snail.

Based on Rapoport and Shadwick (2002, 2007), capsular protein possesses comprehensive flexibility with an ability to fast recovery which is proved by the decrease in the magnitude of elastic modulus (seeming damage) which starts at 3–5% strain. Take into consideration the mechanical reaction to strain, this material is dissimilar to typical proteins such as elastin or collagen. However, the qualitative affinities of keratin, another common structural protein, in strain/stress behaviour are more than accidental in relation to structure and mechanical quantification. This suggests that the matrix organization and alpha-helical structure may resemble these two proteins. In fact, the protein in egg capsule can be tightly connected to keratins of vertebrate origin like intermediate filaments. Using the SEM microscopy, Rapoport and Shadwick (2007) confirm the fibrous hierarchical arrangement of *K. kelletii* and *B. canaliculatum* capsules. It was implied that the primary step in formation of such arrangement is responsible for binding of the fibrous components together. It is based on some still undetermined mechanism of sclerotization occurring in the ventral pedal gland. These researchers proposed a model (Fig. 15.3) for the mechanical and structural properties of whelk egg capsule biopolymer based on decomposing the mechanical behaviour of this biomaterial (WECB) under diverse chemical and physical treatments.

Recently, it was proposed (Fischer et al. 2013) that hysteresis during cyclic loading within this pseudoelastic biomaterial is based on a mismatch between the rate at which the phase transition occurs and the speed of the corresponding mechanical driving force. Some precursor proteins that make up this material have been reported too (Wasko et al. 2014). Thus, the behaviour of welk capsular protein is extraordinary in the recovery speed as well as the value of the dissipated energy, and is similar to the crystallization induced by strain in some polymeric fibres and deformations of superelastic character related to the transitions of the diffusionless phase known for shape-memory alloys (Corbett 2006; Harrington et al. 2012). Consequently, few attempts have been undertaken to develop constitutive models for this class of biomaterials (Kazakevičiutė-Makovska and Steeb 2011).

Diffusion of oxygen within egg capsules seems to be crucial from biological view. Thus, the egg capsules which mainly should save the embryos from predators,

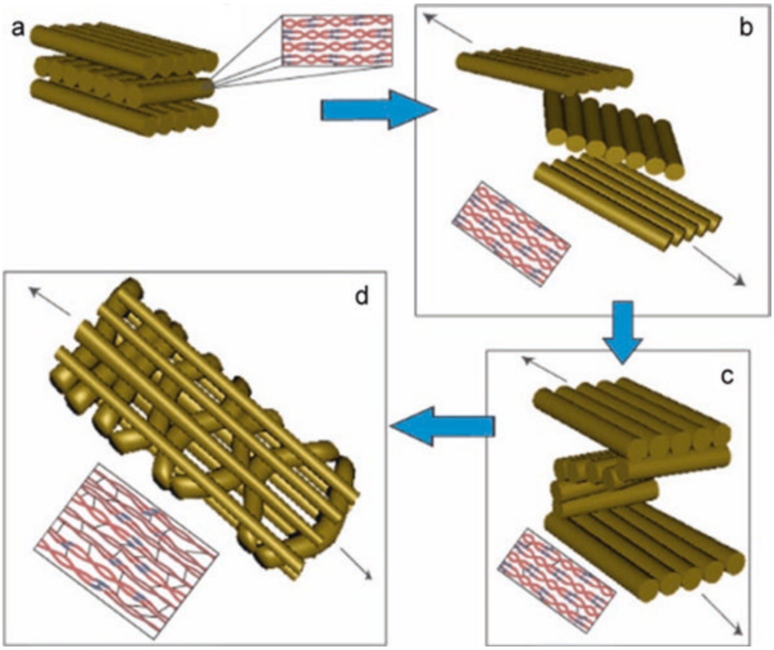

Fig. 15.3 Model of WECB mechanics through maturation: (**a**) generalized structure; (**b**) pre-ventral pedal gland (**c**) ventral pedal gland, and (**d**) post-ventral pedal gland. See detailed information in the text. (Adapted from Rapoport and Shadwick 2007)

also may pose physiological issues because these capsules provide a barrier to the gases diffusion (Gutowska and Melzner 2009). It was showed that the coefficients of oxygen diffusion in the egg capsules of marine animal are usually only 10–20% that of pure H_2O (Brante 2006). During molluscs development, the rate of the oxygen consumption increases greatly (Cronin and Seymour 2000; Brante 2006). Therefore, to enhance the oxygen fluxes, in many molluscan the eggs bulge during the development increases the surface areas, and reduces the thicknesses of their walls (Cronin and Seymour 2000), and thus enhances the oxygen conductance. Additionally, inside the capsules fulfilled with fluid embryos are able to produce some currents to preclude the development of pO_2 gradients (Cronin and Seymour 2000). Molluscs species which do not have active brooding behaviour (Gutowska and Melzner 2009), their eggs or capsules can be located in such niches where exchange of water and oxygen is high.

Additionally, the construction of the capsule has to provide the possibility to survive, grow and escape of the encapsulated embryos. To get the detailed information about fulfilments of these different requirements may be achieved the evaluation of composition and physicochemical features both of capsules and gas exchange during onthogenesis.

15.1 Conclusion

It was showed that the capsules of the molluscs egg (especially *Busycon* species) may be used as a source of novel bioelastomeric material with greater extensibility and fast recovery than it was supposed. Better understanding of naturally occurring impact-absorbing properties and reversible extensibility (>100%) for the insulation of molluscan damage-prone tissues remains to be a crucial point. The understanding of corresponding mechanisms can be applied to formation of new encapsulants for tissue implants, pharmaceuticals, the cells transplantation or other tissues in various medical researches. Interestingly, these naturally existing encapsulation strategies are not a point of interest of engineers, however they are well organized.

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Chapter 16
Byssus: From Inspiration to Development of Novel Composites

Abstract Fibrous shock-absorbing biopolymer byssus is responsible for attachment of diverse marine molluscs. This structure is recognized as an extra-corporeal thread made of tiny tendons which functions as organic anchor to a foreign surface. This fibrous, acellular construct is located outside of the living mollusc's tissue. Human exploitation of byssus is known since ancient cultures where corresponding threads isolated from this biomaterial were woven into textile. Nowadays, this biopolymer remains to be an object for both bioinspired materials chemistry and biomaterials science especially in the field of adhesives that function effectively underwater. This chapter is dedicated to better understanding of the chemistry and selected materials features of byssus.

Both, cementation and adhesion using byssal threads remain to be two different attachment strategies which have been developed during the evolution of molluscs. The adhesion is recognized as typical for marine mussels, which successfully habituate in wind- and wave-swept rocky shores worldwide. The survival of these invertebrates in such special niches is based on their ability to attach strongly to diverse hard (rocky) substrates using byssus, the biopolymer with shock-absorbing structure. The extra corporeal origin of byssusthreads which are responsible for anchoring of the animal to diverse (mostly hard) su is well known (Fig. 16.1). Byssus location is outside the boundaries of living tissue and does not possess any cells for repair or maintenance. The role of the biomaterial lies in supplying secure attachment to rocks and pilings for mussels. It participates in contact between a very soft living tissue and very a hard inert material such as ship hull or rock. Each individual thread of byssus mussel provides a unit of attachment, with the proximal end embedded into tissue of living mussel as well as the distal end joined to rock. Depending on the species, each thread measures 50 μm in diameter and between 2 and 6 cm in length (Fig. 16.2). The investigation of byssus as a biopolymer is an interdisciplinary endeavour encompassing diverse scientific disciplines (biochemistry, materials science, biomedicine, biomimetics). Since the early fiftieth of twentieth century and up today the byssus oriented studies have been mostly dedicated to:

H. Ehrlich, *Marine Biological Materials of Invertebrate Origin*,
Biologically-Inspired Systems 13, https://doi.org/10.1007/978-3-319-92483-0_16

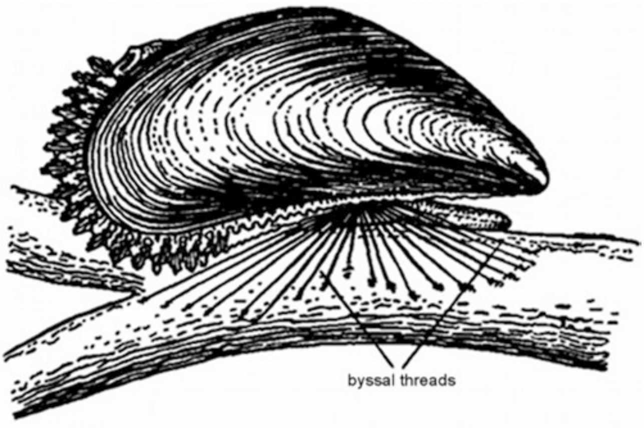

Fig. 16.1 Drawing of the Bivalvia mollusc that is attached to the surface of dead wood through byssus fibres

Fig. 16.2 Overview of dried byssal threads isolated from deep-sea molluscs. (Samples courtesy Anatoly Drosdov)

- material features;
- byssus precursor-proteins, their molecular biology, genomics and biochemistry;
- mechanism of cross-linking reactions and adhesion;
- chemical properties of mussel adhesives with respect to diversity of surfaces;
- development of antifouling strategies;
- biomedical applications as tissue adhesives.

Because of amazing volume of publications including books and patents worldwide, I take the liberty to recommend readers to visit web sites of scientific groups of S. Haemers, P. B. Messersmith, J. H. Waite, P. Fratzl, H. Birkedal for obtaining state-of-the art information on this intriguing topic.

16.1 Byssus: An Ancient Marine Biological Material

Since ancient times the attachment of the mussels to the substratum was reported as one of the earliest bioadhesion example. The holdfast in the fan mussel (*Pinna*) was described by Aristotle (transl. 1910) as a strong filaments bundle with adhesive tips. He used well accepted name byssus (Greek "bysso" means flax linen) for this holdfast (van der Feen 1949). It is to note that, this name was known prior to Aristoteles. For more detailed information about the history of byssus, I take the liberty to recommend papers by F. Maeder which are listed in the references.

The Greek name "*bussos*" for the modern term byssus originated from "*bus*" in the Aramaic language. In Latin language "byssus" means the fine sea silk ("sericum"). Also Egyptians know byssus as sea silk and manufactured clothes using byssal fibers isolated from the *Pinna nobilis* or pen shell (Fig. 16.3.). The Greek historian Herodotus visited Egypt in 500 b.C. personally and described the tunic that was found in a sarcophagus at Thebes as follow:" *it is made of a loose fabric of exceedingly fine thread, as thin as that used in the manufacture of lace. It is finer than a hair, twisted and made of two strands, implying either an unheard of skill in hand-spinning, or else machinery of great perfection.*"

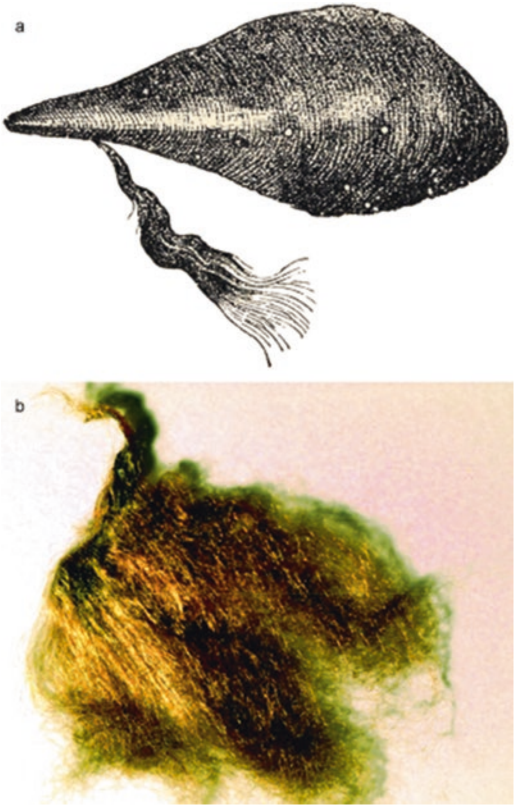

Fig. 16.3 Mollusc *Pinna nobilis* (**a**) – a recognized producer of byssus as sea silk since ancient times (**b**)

It's suggested that the "*golden fleece*", sought by the Greek legendary hero Jason, was woven from the pen shell's threads too. Tertullian (200 a.C.) has written about the byssus: *"Nor was it enough to plant and sow your tunic, unless it had likewise fallen to your lot to fish for raiment. For the sea withal yields fleeces, inasmuch as the more brilliant shells of a mossy wooliness furnish a hairy stuff."*

Later, strong but supple fabric called "cloth of gold" has been manufactured until the Middle Ages. Some locations in Turkey (Smyrna/Today Izmir), Greece, France (Corsica), and Italy (Sardinia, Golfodi Taranto/Calabria, Sicily), have been involved in manufacturing of byssus-based textiles as stockings and gloves. This biological silk-like material is not only transparent, but very light and fine. For example, a pair of gloves made from byssus could be multiple times folded and inserted into a walnut shell.

In the eighteenth century some regions in the South Italy in the south of France have experienced a rebirth of byssus manufacture.

In his famous novel "20,000 leagues under the sea", published in 1870, Jules Verne wrote *"…I felt so great a heat that I was obliged to take off my coat of byssus!"*

Today, there are only about 30 exemplars of the byssus cloth in European museums. Interestingly, one pen shell can synthesize about 1–2 g of byssus threads as raw material. To obtain amounts between 200 and 300 g of fine byssus silk about 1.000 mussels must have been collected and processed! Unfortunately, nowadays not only the volume of living colonies of *P. nobilis* have been dramatically decreased, but also the art of making byssus cloth has been partially lost.

Several images on byssus manufacturing which can be still observed nowadays in Sardinia are represented in the Fig. 16.4.

16.2 Why Molluscs Produce Different Kinds of Byssus

Ontogenesis, ecology, evolution, and biomechanics of byssus producing mussels from Mytilidae family remain to be in the focus of international scientific community. For example, Pearce and La Barbera (2009a) critically analysed these topics from a biomechanical point of view.

Histochemical and immunohistochemical techniques as well ultrastructural analyses strongly confirm that diversity in forms of byssal fibres (Diana et al. 2017), their nature as well in their quantities are dependent on specificity of corresponding environments. It has been observed that semi-infaunal mytilids species (Meadows and Shand 1989) form a plenty of thin threads, an interestingly epifaunal specimens form not so many thicker threads. Additionally, mytilid threads have collagenous nature (Golser and Scheibel 2017), but the anomiids, dreissenids and pinnids threads do not have it. In fact the remarkable chemical and structural differences between the byssus of various bivalve groups origin (Jackson et al. 1953; Pujol 1967; Pujol et al. 1970; Ohkawa et al. 2004) translate into differences in mechanical properties. Therefore, a comprehensive and rigorous biomechanical analysis and comparison

Fig. 16.4 Manufacturing of byssus nowadays. (**a**–**c**) the raw byssus threads have been washed, dried and combed; (**d**) byssus microfilaments must be twisted together to form a single thread; (**e**–**g**) – a pindle – a tapered stick of wood was the earliest spinning tool; (**h**) treatment with the lemon juce gives the byssus its typical gold-brown shining color; (**i**) raw byssus threads and byssus filaments; (**j**) – sea byssus spindles; (**k**) detail of byssus tie. (Adapted from www.designboom.com)

of the semi-infaunal and epifaunal bivalves threads from a diverse orders will enable to find the link between life habits and the biomechanical features of byssal fibres.

Pearce and La Barbera (2009b) performed comprehensive analysis of the mechanical properties of the byssal threads of *Atrina rigida* and *Ctenoides mitis*, two representatives outside the Mytilidae. Interestingly, their byssal threads show significant differences in mechanical properties in comparison to mytilids, but in the case of both molluscs threads were significantly weaker than in the species of the Mytilidae family. The threads of *C. mitis* shown the highest malleability of the distal region than other tested byssal threads. The material properties similarities have been found in various taxonomic groups. For instance, the *Modiolus modiolus* and *A. rigida* threads showed a low extensibility, high stiffness and remarkable double-yield behavior. It seems that the material features of the semi-infaunal thread in some specimens probably evolved at the same time. Environmental impact on the properties of byssus is excellently reviewed by Dinessen and Morton (2014). It is worth to highlight that ocean acidification as a significant impact on mussel byssuss attachement. O'Donnell et al. (2013) reported that mytilid byssal fibre exhibited 40% reduced mechanical performance when secreted under high pCO_2 (>1200 atm) conditions.

In contrast to non-mineralized byssus known from representatives of Mytilidae, Henrik Birkedal and co-workers has founded a non-typical,mineral-containing byssus. Thus, *Anomia* sp., is the only representative of the Anomiidae family in which byssus mineralized by aragonite and calcite (>90%) has been discovered (Eltzholtz and Birkedal 2009; Birkedal et al. 2013). The attachment behaviour of this species has been described as follow: "The animal attaches to small stones or shells from other mollusks and lies on the side so that the right shell is turned towards the substrate, while the left shell is presented to the environment" (Eltzholtz et al. 2009).

16.3 Chemistry of Byssus and Related Proteins

Italian Professor Lavini in 1835 published, probably, the very first report on chemistry of byssus (Lavini 1835). After alkali-based hydrolysis of byssus isolated from *P. nobilis),* he identified J, Br, Na, Mg as well as residual amounts of Fe and Si. In early works on biochemistry of byssus, it has been classicified to something between proteins and polysaccharides (chitin) (Müller 1837; Tallberg 1877; Carriere 1879). Byssus as a silk-like protein has been proposed by Aderhalden (1903).Diverse structural proteins of Mylitidae have been investigated in details only by Brown (1952).Today, it is recognized that byssal collagen (PreCol D) as well as primarily six proteins (known as ***mefp*** 1–5) are the main components within byssal fibers of *M. edulus*. It was proposed to divide them corresponding to special locations:

1. " **the root** that is embedded in the base of the muscular foot;
2. **the threads** (proximal and distal regions) that are produced along a ventral groove that runs the length of the foot; and
3. **the attachment disc**, or plaque, which mediates adhesion to the substratum." (Waite et al. 1998, 2005; Harrington and Waite 2007).

Thus, *mefp-1*, a thin, securing protein coating (*Mytilus edulis foot protein* -1), preserves the byssus from microbial demage and abrasion due to sandy microparticles. The *mefp*-1 to *mefp*-5 proteins are also located distally on each byssus fibre, within an adhesive plaque, which permit to anchor to such solid surfaces which remain to be wet (Suci and Geesey 2001). It was suggested (Rudall 1955) that the distal parts of byssus thread contain about 50% unidentified ß-pleated sheets and 50% triple helices of collagen. Later this biomaterial was named (pre)Col-D) and has been partially sequenced (Qin and Waite 1995). A block copolymer, which is called PreCol, comprises a central collagen domain that covers approximately ½ of the precool (Coyne 1997).

The characterization of diverse *Mefp* proteins is to be found in the review prepared by Deshmukh (2005).

Mytilus edulis adhesive protein-1 (or “mussel adhesive protein” as well as “polyphenolic protein”) has been characterized on the basis of gel chromatography and mass spectrometry. It has molecular weight equal 130 kDa and 115 kDa, respectively. *Mefp*-1 protein is mostly composed of two building blocks 12 hexa-peptides containing Ala^{1}-Lys^{2}-Pro^{3}-Thr^{4}-Tyr^{5}-Lys^{6} and 71 deca-peptides (Waite et al. 1985) containing the residues Ala^{1}-Lys^{2}-Pro^{3}-Ser^{4}- Tyr^{5}-Pro^{6}-Pro^{7}-Thr^{8}-Tyr^{9}-Lys^{10}.

In the deca-pepetide part, the transformation of Pro to dihydroxyproline, as well as convertion of Pro into a hydroxyproline has been reported by Taylor and coworkers (Taylor et al. 1994). However, crucial importance possess the modification which is related to transformation of Tyr to DOPA (3,4-dihydroxyphenyl-L-alanine) (Waite and Tanzer 1981). In the hexa-peptide, Tyr at the fifth position is also transformed to DOPA. A clearly high percentage of *mefp*-1 protein constitutes these hydroxylated amino acids. As in the case of the hexa- and deca-peptide, the non-repetitive, N-terminus domain is rich in Lys residues and is hydrophobic. The composition of the decapeptide seems to remain approximately unhanged in different mussel’s populations. In this sequence, the location and presence of the Lys, Tyr and/or DOPA is absolutely unchangeable.

However, hydroxyproline is incorrectly considered to be characteristic of collagen-like proteins, majority or all of the hydroxyproline remains related with the Gly-deficient collagenase-resistant fragment, in the polyphenolic protein (Waite 1983).

In the byssus *Mefp-1* protein, which is applied as a lacquer-like coating; the addition of Fe- iron can lead to obtain “ironclad” finish and is partially responsible for the corresponding intractability of the bio material (Taylor et al. 1996) (see

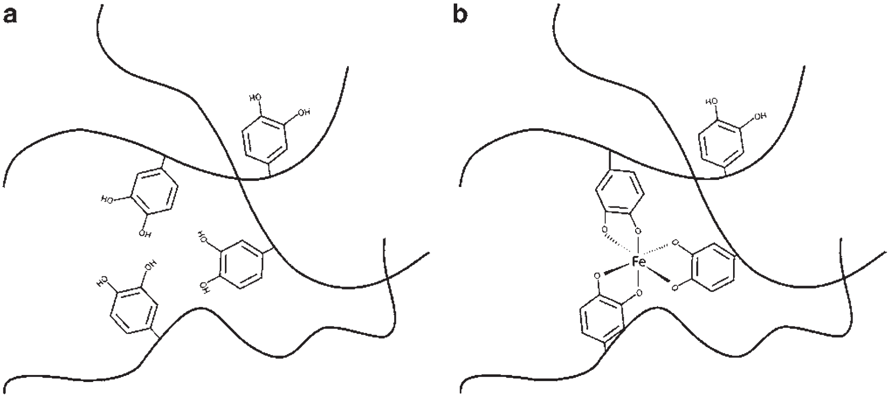

Fig. 16.5 Schematic drawing: *mefp-1* protein (**a**) forms in the presence of ferric iron an extremely complicated macromolecular coordination of ferric ion (**b**)

Fig. 16.5a, b). When ferric iron exists in the environment, *mefp*-1 creates an highly complicated macromolecular complex of ferric ion. Regardless of previous tests, *mefp*-1 acts likewise as a low molecular weight catecholates such as the siderophores. This protein is able to solubilize ferric iron even in the presence of polynuclear hydrolytic species unreachable to simple complex agents.

16.3.1 (mefp-2) Mytilus Edulis *Adhesive Protein*-2

This adhesive substance is a next main blue mussel DOPA protein and it seems to be only the plaque structural component (Rzepecki et al. 1992), and it is a part of up to 25% of plaque protein. It is a multi-domain, tandemly repetitive 45 kD protein, rich in Cys and possesses acidic, short, DOPA containing C- and N-terminal locations as well as a large central domain limited by quasi-periodic internal –S-S-bridges to consistent conformation which is responsible for stability of the protein to proteolysis. The *mefp*-2 peptide motifs are quite irregular than those known for other structures. The *mefp*-2 composition, including DOPA and Cys, is responsible for certain role comprising the plaque matrix stabilization by quinine derived and covalent disulphide cross-links. *Mefp*-2 is able to create oligomeric aggregates with inter-molecular cross-links that can be stabilized by –S-S- bridges. The residues of DOPA can be used to cross-link the obtained homopolymer of disulphide-linked *mefp*-2 to different proteinaceous domains in the plaque.

Taking into account the amino acids composition of *mefp*-1 and 2 as well as the terminal adhesive plaques of byssal fibres we can imply that *mefp-1* and *mefp*-2 content in plaque protein represent about 5% and 25%, respectively.

16.3.2 (mefp-3) Mytilus edulis *Adhesive Protein*-3

This compound represents a non-repetitive, small (6 kDa) protein. In DOPA content and basicity, *Mefp-3* resembles other proteins which have been characterized as precursors. Different forms of the *mefp*-3 family can be preferentially spread onto plastics, polyethylene, artificial glass, as well as stainless steel. It was suggested that due to high amount of arginine and its hydroxylated derivatives (Papov et al. 1995) the *mefp*-3 is the only evident family protein close to the plaque-substrate interface with action similar to that of DOPA (Vreeland et al. 1998).

16.3.3 (mefp-4) Mytilus Edulis *Adhesive Protein-4*

The *mefp*-4 protein has a mass of 70–80 kDa and belongs to this proteins family. All contain detectable levels of H, G and R as well as have common N terminus. Within the plague, this protein is located in the adhesive pulp. The DOPA content of in this protein is equalled 5 mole% (Vreeland et al. 1998).

16.3.4 (mefp-5) Mytilus edulis *Adhesive Protein-5*

This is another example of adhesive protein to be found in the foot of the *M. edulis*. The protein is inserted into the attachment pads of byssal thread (Waite and Qin 2001). The peptide mapping and *c*DNA sequencing was applied to purification and determination of primary structure of *mefp*-5. The protein has a mass of about 9500 Da and is 74 residues long. The *Mefp*-5 composition indicates a significant amount of non-aromatic amino acids, Gly and Lys constitute 65 mol% of the composition. The biochemical production of *mefp*-1 to 5proteins is complex, as well as the extraction from the mussels) is difficult, due to poor yields. Therefore, the in vitro experiments (Taylor and Weir 2000) remain to be unique and requiring large amounts of these bioadhesives.

PreCol-D, is known as the sixth mussel adhesive protein (Waite (2002). Its collagen domain contains175 Gly-XY repeats. Corresponding flanking silk-like domains of this protein include six polyalanine clusters (Qin et al. 1997).

Byssus is an example of biocomposite-based material where not only proteins, but also fatty acids (up to 8% w/w in *M. edulis* (Cook 1970) and *M. galloprovincialis* (Holten-Andersen and Waite 2008)) have been reported. Additionally, ions of calcium, silica, aluminium as well as iron are known constituents. Especially the possible functional role of iron binding is still under investigations. Diverse byssal threads of mussels isolated from the environment are devoid of Fe, but seem to be mechanically strong enough in tension.

As biological material which is resistant to enzyme- and chemicals-based treatments (see similar properties of spongin, gorgonin and antipathin) byssus remains to be of interest for experts in materials science and biomedicine. However, there are reports on microbial destruction of this polyphenol-containing biomaterial. Byssus threads have been attacked mostly by marine fungi (Kohlmeyer 1972; Vitellaro-Zuecarello 1973; Franchini et al. 2005) as well as some bacteria as *Pseudoalteromonas peptidolytica* sp. *nov* (Venkateswaran and Dohmoto 2000) which must possess some kind of very special proteases.

The modern view on chemistry standing beyond the mussel robust adhesion is comprehensively reviewed in article published by J. Herbert Waite (2017), therefore readers are referred to this excellent piece of work.

16.4 Biomechanics and Materials Properties of Byssus

Molluscs produce byssus threads to keep their body firmly joined to the surface in order to avoid the action of waves and attack of predators. Thus, these treads should be strong, and be able to take up the energy of waves and strong flows.

According to Smeathers and Vincent (1979), single byssal thread can defy a tensile force of around 0.25N, but if is dry this magnitude is doubled, therefore, when organism use many thread to defend itself will form a strong attachment. Based on the conservative estimation, the mussel's strength of anchorage using 50 threads (with a radial threads arrangement), that have contact angle of 20° with the substratum an ultimate strain of 0.4 is calculated to be 9–10 N and in the case of parallel location to the substratum around 4N. Then the estimations of the drag forces for a shell (60 mm long) show that in order to get a 4N drag force the water flow have to be higher than 5.7 m s^{-1}. Such velocities can exist only during winter storms, normally over the mussel beds current velocity is lower than 3–4 ms^{-1} thus the flow over the shells has to be lower because of the velocity gradient.

The destroyed byssal threads can be easily substitute by formation of the new material at the stem base. However, byssus is able to be an active absorber of energy, due to its ability to overcome from the effects of the softening stress of previous strains when it can relax over a period of time as is possible in the intertidal zones (Smeathers and Vincent 1979).

In his study on byssus composition-structure-function relationship Sun (2002) proposed to accept this biomaterial as an extrinsic connective tissue with interesting mechanical features that can be evaluate to study the mechanism of gradients formation. Thus, the protective outer cuticle of byssal threads possesses *mfp*-1with DOPA content at 10–15 mol %. As reported by Sun and Waite in 2005: "amino acid analysis of mfp-1 extracted from successive foot sections of *M. galloprovincialis* reveals a post-translationally mediated gradient with highest DOPA levels present in mfp-1 from the accessory gland near the tip of the foot, which decrease gradually toward the base. The DOPA content of successive segments of byssal threads decreases from the distal to the proximal end and thus reflects the trend of mfp-1 in the foot" (Sun and Waite 2005). Based on the results of the inductively coupled plasma analysis it is assumed that metal ions, such as iron, are deposited in similar manner as DOPA within the byssus. Results of XRD analysis clearly indicated that iron was deposited in the threads cuticle or rarely in the core, however the gradient of deposition was not correlated with those of DOPA. The treatment of mussels with byssus threads with seawater with addition of Fe^{3+} indicated that the threads cannot use the iron ions from seawater. Thus, mussels absorb soluble iron ions and particles during feeding and then, iron is deposited within byssus during their formation. In consequence, the mussels are able to use the interactions between metals and DOPA to adjust the mechanical properties of various threads parts (Harrington and Waite 2007).

Data concerning the mussel tenacity as well as byssal adhesive and tensile strength are well represented in the literature. I recommend to read about in the papers by Smeathers and Vincent (1979), Price (1981), Qin and Waite (1995), Waite et al. (1998), Vaccaro and Waite (2001), Gosline et al. (2002), Aldred et al. (2007).

In 2008 Holten-Andersen and Waite reported interesting results on mechanical properties of byssal cuticles. SEM imagery of the surface of, for example, cuticle on *M. galloprovincialis* showed densely packed mottled microgranules. This feature suggests such cuticles as naturally occurring structures which resemble sandpaper. About 50% of the byssal cuticle volume is fulfilled with nano-domains (20–40 nm)-based and up to 800 nm-large granules. The origin and biosynthesis of such structures remain unknown. The selected mechanical properties of byssal cuticle from *M. galloprovincialis* have been analysed using nano-indentation techniques (Holten-Andersen et al. 2005, 2007). Thus, the granular coating is able to absorb damage induced by strain (Holten-Andersen et al. 2007) due to it redistribution to high volume (even up to 70% before rupture). It is assumed that, the micro-tears are convertible, because threads are able to extend without visible changes in their structure.

The importance of the above-mentioned structure was confirmed in two studies. According to Holten-Andersen et al. (2007),the *P. canaliculus* possesses homogeneous cuticle, and similar values of H and Ei to the cuticle of *M. galloprovincialis,* but it broke at strains of 30% and even lower . Additionally, the *M. californianus* cuticle which has granule with diameters equalled 200 nm (1/4 of the *M. galloprovin/cialis* granule), demonstrates disastrous breaking at strains bigger than 120%.

16.5 Conclusion

Many organisms are living in such turbulent environments like wind and wave rocky shores usually forming huge colonies as in the case of mussels. The mixing of nutrient, removal of waste products and gasses diffusion are important advantages of those environments, but as a risk the abrasion, anoxia, desiccation and high drag can be assumed. Both mineral-containing byssus and pure organic byssus threads are the main players in corresponding effective strategies responsible for success of marine mussels during long time of their evolution. Adhesive related biomimetics is mostly based on discoveries of DOPA related compounds and specific *mef*-proteins. Hereby, I prefer the terminology proposed by Herbert Waite in this special case, who spoke of "*biomimetic attempts*". Therefore such biomimetic analogues are recently under continuous studies – especially the *mefp*- 1 and the decapeptides which are tandemly repeated. (see Swerdloff et al. 1989; Olivieri et al. 1989; Taylor and Weir 2000). Interestingly, Yamamoto (1987) achieved significant improvement in this field by applying the strategy of the condensation of short fragments to obtain a structure with a decapeptide repeats.

It can be agreed that:

1. "*mfp*-1 adsorbs rapidly and strongly to a variety of surfaces;
2. the strength of adsorption is closely linked to the presence of DOPA;
3. the oxidation of DOPA before adsorption leads to weaker adsorption to mineral surfaces, but to stronger interactions with other organic macromolecules; and

4. the oxidation of DOPA after adsorption provides an effective platform for adlayer formation" (Holten-Andersen and Waite 2008).

Such features of mussel byssus as self-healing, biorenewability and stiffness in marine environments including cold Arctic and Antarctic waters have attracted the interest of ecologists, zoologists, chemists and material scientists (Montroni et al. 2017). Byssus from industryal mussels remain to be renewable waste material with intriguing potential as a raw starting material useful for developing diverse functional materials with perspectives to be applicable in technologies including biomedical engineering (Liu and Xu 2014, Krogsgaard et al. 2016). For example, the biotechnological production of byssus-derived collagen – like materials is also reported (Golser and Scheibel 2017).

The tendency to design novel byssus-like compounds and structures is based on the necessity to use them in pH-triggered cross-linking, abrasion-resistant coatings, giant self-assembling mesogens, and self-healing polymers (Hassenkam et al. 2004; Harrington and Waite 2007; Holten-Andersen et al. 2007).

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Chapter 17
Abductin

> *Another protein rubber is abductin found in the shell-opening ligaments of bivalve mollusks. One or two adductor muscles hold the two half shells or valves of a bivalve closed (the edible part of a scallop is one of these muscles). Closing compresses the ligament, so its elastic resiliency can reopen the shell if the muscles relax. Interestingly, scallops, which swim by repeatedly clapping their valves together, recover a greater fraction of the work done on their abductin than do clams and other more sedentary forms.*
> Steven Vogel 2003

Abstract Abductin remains to be the only naturally occurring elastomer that shows compressible elasticity. The analyses of amino acid composition of abductin obtained from *Placopecten magellanicus* – the swimming scallop showed the occurrence of three major amino acids: phenylalanine, methionine and glycine. Additionally, it was proved that, in the primary sequence of abductin from *Argopecten* the repeating pentapeptide with sequence FGGMG, was observed. The main similarity between abductin and elastin is based on the presence of many repeating sequences with glycyl residues. The biomimetic potential of abductin is mostly related to design and development of abductin-like biomaterials including recombinant forms with their future applications as vascular prostheses.

Abductin is an example of naturally occurring elastomer, which is the main building element of the abductor ligament in Bivalvia. The abductor ligament remains to be hydrated and it is characterized by rubber-like texture (Kelly and Rice 1967), dark brown tinge and triangular shape (Thornhill 1971). The ligament can be found in the hinge area of the bivalve and works alike to a coil spring, because by relaxation of the adductor muscle, the ligament opens the shell which is tightly closed. Additionally, it has important role in the swimming of scallops. The energetic and mechanics of scallop adductor muscle during swimming has been investigated in details (Vogel 1997; Morton 1980; Rall 1981; Marsh et al. 1992; Marsh and Olson 1994; Denny and Miller 2006). Due to the presence of the abductor ligament, the shell can repeatedly open at a frequency of 4 Hz, after relaxation of the adductor muscle, what is related to discharge of water dorsally. This feature allows the scal-

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lop to swim several meters at once to avoid the predators which move slowly (i.e. starfish).

In the file shells and scallops, swimming skill depends on the ability to rapid release of fluid (in other words, the more often the animal producing thrust) and subsequently to reinflate, this is typical for all jet-propelled animals. The more often the animal is able to carry out this cycle, the more thrust is performed at once. Thus, animal can produce more power to move the body. Theoretically, the increasing of the power of thrust has valuable effects: the greater the power, the bigger the mass that may be raised against gravity, thus the animal moves faster. Therefore, the thrust power accessible to a jet-propelled bivalve enhances, if the stiffness of the springs is increased or the damping coefficient or the mass is decreased.

The scallop adductor muscle (which is responsible for closing the valves together) has striped fibers which shrink rapidly in comparison to the muscles found in other bivalves and represents an exceptionally large part of the overall mass of this animal (usually ~25%) (Rall 1981; Marsh et al. 1992; Marsh and Olson 1994). In the swimming bivalves, the mass of the shell is relatively reduced in comparison to the species which are attached to the substrates. This modification both reduces the need for thrust and increases simultaneously the power of thrust, because the mass that must be lifted against gravity decreases (Gould 1971). Lastly, an elastic and stiff abductin forms the resilium, which is responsible for rapidly opening the shell after it was closed (Gould 1971; Cheng and DeMont 1996). The reduction of the damping of the system is related to the mechanical resilience of abducin (ability to store the potential deformation energy with little loss to viscous processes).

What about the sequences of abductin? The conceptual sequence of amino acid, determined for *Argopecten irradians,* is to be found in the paper by Cao and co-workers (Cao et al. 1997). The analyses of amino acid composition of abductin obtained from *Placopecten magellanicus*, the swimming scallop, showed the occurrence of the three major amino acids: phenylalanine, methionine and glycine (Cao et al. 1997). The existence of FGGMG repeating pentapeptide primary sequence in *Argopecten* abductin, was confirmed too.

Tripeptides like FGG and tetrapeptides such as GGMG and MGGG are also replicated but at a lower frequency, however the nonapeptide GGFGGMGGG and the decapeptide FGGMGGGNAG appear to be the main repeating sequences. Generally, the major feature of abductin sequence is the occurrence of majority of repeating sequences, which all containing glycyl residues (Bochiccio et al. 2005), analogically to elastin. It is to note here that the gene encoding abductin is not the same of the resilin gene. The GGFGGMGGGX repeating sequence remains to be characteristic for abductin, which does not contains tyrosine that is necessary for the formation of dityrosine links which are typical for resilin (see Chap. 20 in this book). The knowledge of such special repeating sequences id crucial for development of abductin-like synthetic biopolymers (see, for example the United States Patent entitled, "*Molluscan ligament polypeptides and genes encoding them*").

Adamussium colbecki the Antarctic scallop can evaluate the ability to swim. This animal lives in frosty environment (water temperature –1.8 °C) where water is about 43% more viscous in comparison to the temperature of typical habitat of the most scallops (10 °C). However, low temperature could decrease the adductor muscle power output. Simultaneously, higher viscosity enhances the required power to push the body through the water column and to form a propulsive jet. Additionally, at low temperatures the resilience of rubber-like polymers (like abductin) usually decreases. For instance, one of the reasons of the space shuttle *Challenger* crash was related to the decrease in resilience of a synthetic rubber ring (Denny and Miller 2006) during the launching at temperatures near freezing. A decrease in the resilience of the abductin pad in the Antarctic scallops, can potentially reduce the power accessible for thrust.

Also elastin is recognized as bioelastomer (Gosline and French 1979) that is able to maintain high resilience at low temperature. However, unlike elastin, abductin from *A. colbecki* shows behaviour when its volume decreases at low temperatures.

17.1 Conclusion

Understanding of the genetic mechanisms which are responsible for synthesis of unique highly repetitive domains in elastomers like abductin represents a big challenge. Mostly, because this knowledge can opens the way for corresponding biomimetic strategies. It was suggested that abductin-like elastomers and composite materials, including recombinant forms (Su et al. 2013), could be used, for example, in development of diverse matrices useful for human tissues, as well as vascular and organ prostheses (Shewry et al. 2003).

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Chapter 18
Resilin

Abstract Proteins similar to resilin comprise repetitive distinct domains containing tyrosine residues. Corresponding cross-linking between residual tyrosines is responsible for formation of di- and trityrosines, which are molecular markers for resilin identification. Traditionally, resilin related studies have been focused on it identification, function and properties in diverse terrestrial arthropods. Investigations concerning resilin in marine crustaceans are still in progress now. Especially, better understanding of the mechanical features and function of resilin in marine environment, including high salinity, pH around 8.8 and psychrophilic conditions, remain to be crucial. One of the modern trends in resilin research is dedicated to engineering of corresponding recombinant resilin-like proteins for further technological and biomedical applications.

Bioelastomer named resilin, has been isolated and identified in many locations in insects (Andersen (2010), Bayley et al. (2012), Burrows (2016), Burrows and Sutton (2012), Burrows and Dorosenko (2014), Burrows et al. (2008), Michels and Gorb (2012), Schmitt et al. (2017), in some crustaceans ((Burrows et al. 2008) and worms (Wong et al. 2013) where full and rapid recoil is needed, or corresponding energy must be stored. Data concerning the history of resilin discovery and research are to be found in the paper by Bennet-Clark (2007). He pointed out the first observation made by Weis-Fogh (1960) concerning the perfect elasticity of this bioelastomer in the resilin-based tendon of a dragon fly. In 1964, Sven Olav Andersen and others, reported about possibility to visialize resilin due to existence of dityrosine and trityrosine, the aminoacids which show characteristic autofluorescence (Andersen 1964). According to Andersen and Weis- Fogh 1964 and Elvin et al. 2005, for example, dityrosine possess UV light autofluorescence after light excitation at 315 nm as well as maximally radiating at 430 nm. The method provides a simple way of non-destructive identification of this rubber-like biopolymer (see Fig. 18.1).

The trend to produce resilin and resilin-like proteins using gene engineering started after success of experiments by Chris Elvin and co-workers (Elvin et al. 2005). Corresponding pro-resilin genes have been inserted into *Escherichia coli*. After that developed gene product has been cross-linkinked and transformed into quite large constructs (Bennet-Clark 2007) with remarkably high resilience.

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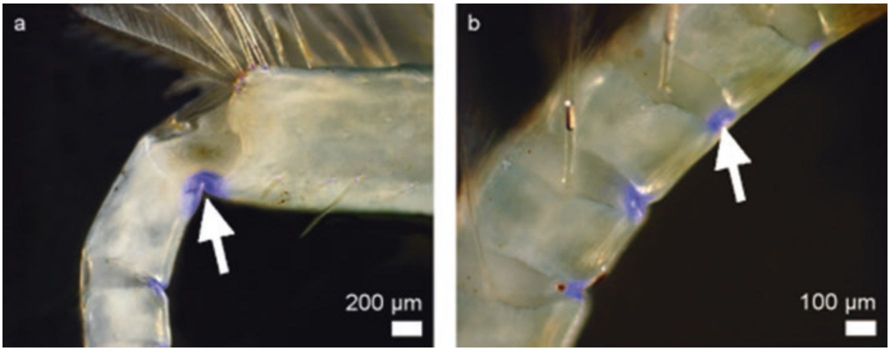

Fig. 18.1 UV light helps to visualize the location of resilin in crayfish (**a** and **b**). (Images courtesy Malcolm Burrows)

Nowadays, numerous studies on development of recombinant resilin and its properties have been carried out (for details see Qin et al. 2009; Balu et al. 2015; Girotti et al. 2015; Li et al. 2016; Weitzhandler et al. 2017; Sutherland et al. 2018).

Elvin and co-workers used a recursive stepwise approach (Lyons et al. 2009), to obtain oligomeric expression structures encoding periodic peptides using the *Drosophila melanogaster* resilin gene. Similarly to recombinant Rec1-resilin, created 16-repeat polypeptides were stable during heating as well as hydrophobic.

In contrast to the fact that most resilin related studies have been dedicated to terrestrial organisms (Bennet-Clark and Lucey 1967; Haas et al. 2000), several investigations on this bioelastomer with respect to marine invertebrates exists too (Tynyakov et al. 2015). I take the liberty to recommend readers diverse publications as well as videos made by Malcolm Burrows (University of Cambridge, UK) about properties and function of resilin in walking legs of crabs and crayfish (Burrows et al. 2008; Burrows 2009). Probably, the next step in such investigations must be to carry out comparative study on structure and functional behavior of resilin in crustaceans habituating in fresh water and marine environments (Burrows and Dorosenko 2014). Development of resilin-like bioelastomers which could be useful in sea water at low temperatures and differences in pH (Fang et al. 2017) and salinity remains to be of high challenge.

Biomedicine is that scientific direction where resilin-like biopolymers with high fatigue lifetime and high resilience as well as resilin-based hydrogels are still needed (Li and Kiick 2013; McGann et al. 2013, 2016; Sanami et al. 2015; Kim et al. 2016; Li et al. 2018). Without doubts, resilin remains to be a source for bioinspired materials science (Nairn et al. 2008; Qin et al. 2012; Li and Kiick 2014; Su et al. 2014; Whittaker et al. 2015; Rajabi et al. 2016; Aghaei-Ghareh-Bolagh et al. 2016; Khandaker et al. 2016; McGann et al. 2016; Wang et al. 2016; Fang et al. 2018) and biomimetics (Lapidot et al. 2012; Balu et al. 2014; Beckmann et al. 2015; Fang et al. 2017; Heepe et al. 2018; Schmitt et al. 2018; Kovalev et al. 2018; Su et al. 2019).

18.1 Conclusion

The remarkable resilience and extensibility of resilin as rubber-like biomaterial are that features which attract attention of international scientific community, mostly due to the trend in designing of resilin-like composites, flexible joints for micro-robotics, multifunctional and hybrid hydrogels and wearable sensors (e.g. Patent WO/2008/055931). Due to these favorable attributes, also studies on recombinant and genetically engineered resilin-like polypeptides will be still in trend. One of the challenging tasks is to find the key way for replacement of a soft resilin-dominated cores between artificial, or biopolymer -based (silk, keratin, collagen, chitin, chitosan) materials to manufacture functionalized devices including that for underwater applications. Additional attention will be focus on the understanding of specific resistance of resilin against chemical and enzymatic treatment.

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Chapter 19
Adhesion Systems in Echinodermata

Abstract Several biological adhesion systems (e.g. permanent, transitory, temporary and instantaneous) have been developed during evolution of diverse marine invertebrates including representatives of Echinodermata. Both, protein-based solid or highly viscous adhesive secretions are responsible for attachment of these marine organisms to substrata with diverse surface architecture, stiffness and naturally occurring changes of air to water phases. Here, broad variety of adhesive systems in echinoderms has been discussed. Bioadhesives of echinoderms origin still possess intriguing biomimetic potential especially for applications in underwater construction in marine environment as well in dentistry, surgery, tissue repair and other fields of the modern biomedicine.

Environmentally durable and strong adhesion remains to be the prerequisite for survival of broad diversity of marine invertebrates habituating in areas with rocky intertidal shores where water velocities are often in excess of 10 m·s^{-1}. If such organisms want to withstand the hydrodynamic forces, they must have adhesive mechanisms (Nachtigall 1974). Marine sessile invertebrates must developed very special survival strategies being adhered underwater and attached to the hard surfaces with diverse surface chemistry and topology (Waite 1987). Shielding from microbial degradation and aqueous erosion, ability for self-organization, adsorption to various materials including plastics, metals and wood, are only few functions which have been observed in aquatic organisms (Kamino 2003).

According to Flammang (2006) diverse adhesion systems can be classified as follow:

Permanent adhesion, which is based on the ability of some adult organisms (e.g. barnacles) to secrete a cement-like material. Usually such species remain in the same place of their habitat.

Transitory adhesion, which allows simultaneous locomotion and adhesion due to synthesis of a viscous film they lay down to the substratum. For example, some turbellarians creep on this organic film which they leave behind as they move.

Temporary adhesion which has been observed on adhesive behaviour of echinoderm podia. It allows invertebrates to attach firmly but momentarily to the surface of diverse substrata.

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Instantaneous adhesion. This type of adhesion comprises such adhesive systems which do not fit into the types described above.

In contrast to recently well described mechanisms of temporary adhesion of marine flatworms (Wunderer et al. 2019), similar aspects on molecular level in echinoderms remain to be under study. There are examples like gastropod molluscs which can use suction together with either transitory adhesion when they are moving (Smith et al. 1999) or temporary adhesion when stationary for a longer period of time. In Echinoderms different adhesive systems may be employed in their onthogenesis and metamorphosis from larvae to adult stages (for details see Flammang 2006). Especially, interest on sea urchins is based on their ability to attach to substratum very strongly but in a reversible manner.

Diverse examples of echinoderm adhesives are represented below.

19.1 Sea Urchins

These echinoderms are able to stick to the surface using many autonomous adhesive organs such as the adoral tube feet. These structures are perfectly formed for interim adhesion (Lebesgue et al. 2016a, b). Sea urchins have one characteristic feature, the flattened and enlarged apical disc that generates an adhesive as well as a de-adhesive secretion which is used, respectively, to attach the animal to the surface or to voluntary detach of sea urchin. Additionally, they possess an extensible stem (Flammang and Jangoux 1993; Flammang 1996) which is connected to this disc, and endures the tensions causes by hydrodynamic forces.

This adhesive excretion is transferred to the distal surface of the tube foot by the disc cuticle, and thus attached it to the substratum by forming a thin film (Flammang et al. 2005). Within the cuticle, the deadhesive excretions are discharged, and it is suggested that, they remove the outermost layer of cuticle, named fuzzy coat, what is related to their enzymatic activity. Therefore, after detachment, the occurrence of footprint is observed. The adhesive biomaterial that is strongly attached to the substratum is to be found within the footprint (Flammang and Jangoux 1993; Flammang 1996; Flammang et al. 1998).

There are several reason for that, the features of the temporary adhesive of sea urchin are extraordinary. Tube feet (1) are efficiently attached to the surfaces of diverse chemical contents and roughness (Santos et al. 2005; Santos and Flammang 2006); (2) possess highly specific epidermal adhesive regions consisted of diverse secretory cells that are able to discharge separately the deadhesive and adhesive excretions, thus allowing for the occurrence of successive attachment–detachment events (Santos and Flammang 2006); (3) have a high adhesive strength ranging between 0.09 and 0.54 MPa (Santos et al. 2005; Santos and Flammang 2006, 2008), in comparison to data estimated in other marine organisms (0.5–1 and 0.1–0.5 MPa for permanent and nonpermament adhesives, respectively (Smith 2006) thus fulfil well the technological conditions for artificial underwater adhesives (0.2–0.7 MPa; Waite 2002).

Up to 2016, based only on the histochemical studies, the information about the composition and nature of the sea urchin adhesive were obtained. These studies proved that, the echinoid footprints (circular-shaped residues of secreted adhesive remaining on the substratum after detachment) can be specifically stained not for proteins, however for acid mucopolysaccharides (Flammang and Jangoux 1993). These footprints are consisted of proteins (6.4%), lipids (2.5%), neutral carbohydrates (1.2%), and mostly inorganic residues (45.5%).

In the sea star species *Asterias rubens* (Flammang et al. 1998) the footprints are consisted of much higher amount of proteins (20.6%) but similar values of inorganic residues (40%), lipids (5.6%) and neutral sugars (3%). In *A. rubens* footprints, another carbohydrates like uronic acids (3.5%) and amino sugars (1.5%) were also detected. Interestingly, lack of DOPA in the footprints show that in contrast to the cements of polychaetes and mussels, in the sea urchin, cross-links made of DOPA do not appear (Santos et al. 2009).

Modern proteomics report on sea urchin tube feet differential proteome and the secreted adhesive proteome has been published in 2016 (Lebesgue et al. 2016a, b). It was suggested that approximately 70% of the adhesive proteome is related with microbial protection and exocytosis. 163 highly over-expressed disc proteins have been reported, too. However, the main player is a sea urchin tube foot adhesive protein is represented by Nectin (Lebesgue et al. 2016a, b). Corresponding Nectin genes has been recently characterized by Toubarro and co-workers (Toubarro et al. 2016).

19.2 Sea Cucumbers

The Cuvierian tubules represent a peculiar and specialized defense system in sea cucumbers (class Holothuroidea) (Flammang et al. 2002). The function of the system becomes visible after mechanical stimulation of the organism, resulting in the discharge of a few white colored tubules (Fig. 19.1). These structures become stickly in seawater upon contact with any substratum during less than 10s (for details see Flammang 2006).

In such species as *Holothuria forskali*, the secreted adhesive was composed of 40% neutral carbohydrate and 60% residual protein (De Moor et al. 2003). The biological material is sensitive to proteolytic enzymes which can reduce the adhesive strength of Cuvierian tubules in this sea cucumber (Zahn et al. 1973). Amino acid glycine, and uncharged and polar amino acids have been found in the proteinaceous component of adhesives from diverse holothurians. The soluble proteinaceos fraction consists of about ten different proteins with molecular masses ranging from 10 to 220 kDa (De Moor et al. 2003).

Demeuldre et al. (2014) used various microscopic techniques for studying of the mesothelium (epithelium with adhesive properties)—as well as the sticky biomaterial isolated from *H. forskali* again. Performed microscopic analysis demonstrated the adhesive layer is composed of very adhesive nanoglobules (diameter ~70 nm) in contrast the collagen fibers which have been also detected. The adhesive studied presenting a high affinity for glass surface, mica or Teflon®.

Fig. 19.1 The sea cucumber (*Bohadschia argus*) successfully uses its *Cuvierian tubules* for attachment to diverse surfaces. (Image courtesy Patrick Flammang)

19.3 Sea Stars

However, in the sea stars (Fig. 19.2) only temporary adhesion is observed. The sea star probably detaches on its own at an early stage, if the substratum does not let to form a strong holdfast (similar to Teflon) (Hennebert et al. 2008), without leaving a significant parts of the bioadhesive on the substratum.

Previous studies have clearly showed that, in the sea stars, two cell types responsible for secretion of adhesive, which are located in the disc epidermis of the tube feet, release the adhesive material (Flammang et al. 1994, 1998). Although the formation mechanism of the complex micro-structure of the footprint during the secretion is unknown. The morphology of cells, with an apical process packed with intact granules, is different that of adhesive cells type 1. By the way, these conspicuous cells can be easily divided into corresponding sections. Notwithstanding, adhesive cells type of 2 are not very conspicuous in comparison to tube feet which remains to be unattached. Their apical processes containing fewer granules and they are always smaller. This implies that, this type of cells excrete their contents as first, thus they are responsible for the development of the homogeneous film that covers the substratum. In fact, the appearance of the released material and the material which forms this film is the same. As was mentioned previously, this priming film is able to bind strongly to the surface and displace water. At the time, type 1 cells begin to excrete a heterogeneous electron-dense material. In this stage, the two components excreted by their respective cells, alternate in the secretion of adhesive.

It is well visible using TEM and SEM analyses that, the material from type 1 adhesive cells, in some cases, can be excreted in the form of spheroidal structures such as granules clusters released together. This feature can be explained by the fact that, this material appears to evolve progressively after its release or inside the cells. Certainly, the excreted material extends and fussed with this secreted from other cells which leads to the formation of a meshwork in selected regions on the surface of the detached tube feet. Therefore, cells type of 1 could be treated as a source of the meshwork pattern, and the system of their secretory pores on the surface of the

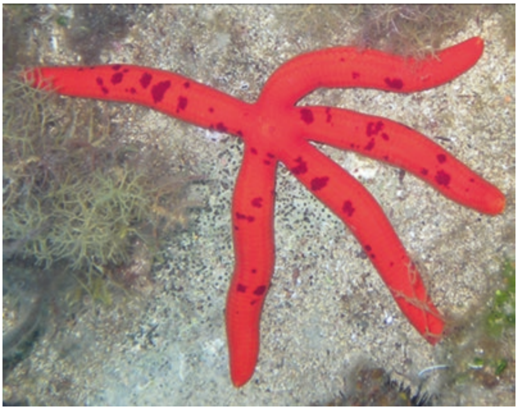

Fig. 19.2 The adhesion of sea stars in the sea remains to be temporary. (Image courtesy Zoran Kljajic)

disc can be considered as the responsible for the formation of the template of this pattern. The third component of the adhesive coating traditionally termed as "*the loose electron-luscent material*" is localized between the different materials excreted by the two types of adhesive cells and originated, probably, from the cuticle outer layer that covering the disc epidermis. It was suggested that this "fuzzy coat" layer can be removed from the surface of disc and embedded into the footprints by the discharge of the de-adhesive excretion (Flammang et al. 1998).

The SEM, AFM, LM and TEM methods were applied to observe the ultrastructure of the adhesive derived from the sea star *A. rubens* and deposited on different substrata (Hennebert et al. 2008). Regardless of the methods used, this structure always resembles a sponge-like network located on a homogeneous thin film.

It was proved that the adhesive structure is not modified by the separation fluctuations like cavitation or fingering instabilities as well as by discharge of the de-adhesive excretion which appears after corresponding detachment of the tube foot. The appearance of the adhesive material does not depend on the attaching of the footprints to the substrata as well as hydration or dryness of these footprints (Flammang et al. 1998, 2005).

The substratum on which footprint is deposited affects its thickness. Certainly, albeit footprints left on mica, Teflon and glass are characterized by the same size and shape, the thickness of the layer of the material deposited by the tube feet differs depending on the substratum. Tube feet deposits thinner layer of the adhesive material on Teflon (a low-energy surface) than on mica or glass (two high-energy surfaces). Both, the material and the homogeneous film form the meshwork which is consisted of a consecutive globular nanostructures, at the nanometre scale. For the dry footprints, the diameter of these structures estimated using AFM or SEM (Hennebert et al. 2008) is practically the same (50–90 and 50–100 nm, respectively). Interestingly, AFM studies on incompletely hydrated footprints indicate that a diameter of globule can be rich 80, or even 200 nm. Probably, this small difference is based on the shrinking of adhesive biomaterial during drying.

19.4 Conclusion

The present state of art regarding adhesion systems in echinoderms is reviewed by Flammang et al. (2016). There is, definitively, fundamental interest on evolution of diverse adhesive systems in Echinodermata as well as the biomimetic aspect with the aim to find possible technological and biomedical applications. Trend in studies on practical application is mostly oriented to development of new antifouling strategies and development of water-resistant adhesives including that for sea water conditions. In this context, echinoderm adhesives may serve as the starting point for the design of artificial reversible adhesion systems for industrial and medicinal application.

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Chapter 20

Adhesive Gels of Marine Gastropods (Mollusca) Origin

Abstract Synthetic glues differ from analogous biopolymer-containing adhesive gels of gastropod origin in their chemistry and properties. Artificial glues contain diverse synthetic polymers or cross-linked materials and can be obtained in semi-solid and solid states. The consistence of highly deformable adhesive gels is determined by more than 95% water and, consequently, by diluted polymer networks. Diverse biochemical changes are crucial for detachment mechanisms of marine gastropods. The interplay between special proteins and mucous polysaccharides remain to be principal for substrate dependent attachment-detachment phenomena which are based on adhesive gels.

Gastropods (slugs, snails, limpets and whelks) represent the largest and most distributed (with freshwater, marine, and terrestrial species) class of molluscs. As reviewed in Béguinot (2017), this class includes herbivores, carnivores and parasitic species.

With respect to adhesion in the sea water, attention has been paid especially to limpets which have been successfully used as model organisms (Grenon and Walker 1981) (Fig. 20.1). Mostly the wave-swept rocky shores are that areas where limpets are commonly found although these molluscs can be subjected to up to 20 ms^{-1} high water velocities (Denny 2000). The biological reason of adhesion as fast -curing nature phenomenon is to protect limpets from dislodgement by predators such as shorebirds due to appearance of adhesive strength. The interest on adhesive gels of marine limpets is, partially based on the ability of these organisms to possess attachment forces which are strong enough that organism can be really difficult to detach from the selected surfaces by hand. Limpets excrete their gel-like glue under the foot sole. These gels containing up to 97% water, are used to produce tenacities (attachment force per unit area) from 100 and up to 500 kPa (see for details Smith 1992; Smith and Morin 2002; Smith 2002, 2006). These properties are similar to the adhesive strength parameters (500–1000 kPa) of the barnacles and bivalves solid cements (Waite 1983; Yule and Walker 1987). Smith (1991, 1992, 2002) and Smith et al. (1999, 2009) carried out detailed characterization of the physiology of gel production in various gastropods such as limpets in accordance to the attachment-detachment, suction-adhesion, and variety in chemistry and functionality of corre-

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Fig. 20.1 Limpets possess excellent “techniques” to be strongly attached to diverse surfaces

sponding mucus-like substances. The role of mucus in marine gastropods has been recently described by Hannides and Aller (2016).

Within different gastropod species Smith and co-workers (Smith et al. 1999, 2009; Smith and Callow 2006; Smith 2010), observed structural similarities in the glue proteins. In these proteins an acidic isoelectric points and a large proportion of polar and charged amino acids are similar. For instance, in proteins derived from the sea snail *Littoraria irrorata*, the two glue-related proteins possess an isoelectric point of 4.75 (Smith and Morin 2002), while the *L. limatula* proteins exhibited isoelectric points between 4.7 and 5.3 (Smith et al. 1999). Although, one relatively uncommon protein (53 kD), possesses a basic isoelectric point at 8.6. The different sizes of the glue proteins also have been observed. Their masses decreased from 118 kD for the primary limpet glue protein to 14 kD for the primary glue protein isolated from slug (Smith et al. 1999; Pawlicki et al. 2004).

In opposite to the majority of glue proteins, a 68-kD protein is involving in detachment of the limpet *L. limatula* (Smith et al. 1999). Another one protein with molecular weight of 140 kDa is glycosylated (Smith et al. 1999). Detailed investigations of the eventual function of these special proteins during adhesive gastropods gels cross-linking has been also carried out. Hence, highly stable 40 kDa protein of *Arion subfuscus* origin remained bind to the large polysaccharides-protein complexes even after addition of 8 M urea with non-ionic detergent (Smith et al. 2009). It is believed that metals might be mainly in charge for the stiffness and strong cross-links (Braun et al. 2013; Smith 2013) in various bioglues (Werneke et al. 2007; Filippidi et al. 2017). Additionally, even the removal of metal from the mature glue derived from *A. subfuscus* has no influence on the capability of the 40 kDa protein to crosslink into large complexes containing polymer.

Because the topic of adhesive gels with high bonding strength is excellently represented in the recently published book by Andrew M. Smith entitled "*Biological Adhesives*"(2016), I strongly recommend readers to acquaint with the state of the art in this intriguing field of research by their self.

20.1 Conclusion

The studies on gastropods adhesive gels have clear implications with respect to both biological reason and practical applications. The ability of aquatic species to attach themselves using biopolymer-containing glues to substrates of diverse surface chemistry and microstructure seems to be crucial for surviving of these animals under diverse environmental conditions (wind, waves, currents, gravity), and to overcome to be "collected" by predators. The biochemical mechanisms of such phenomena occurring in wet, irregular environments are still under investigations (Waite 2002; Smith 2016; DeMartini et al. 2017). The modern trend in the adhesive biomimetic engineering is dependent on development of gels with unusual properties (Clancy et al. 2016; Guo et al. 2017; Kord Forooshani and Lee 2017; Lu et al. 2017) and is still inspired by diverse adhesive gels of marine gastropod origin.

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Chapter 21
Biocements

Abstract Marine crustaceans with actively swimming larvae and sessile adult forms are represented by barnacles (Cirripedia). These organisms developed three types of efficient biological adhesive systems which enable them effectively use special proteinaceous cements for strong attachment to diverse hard substrata including that of natural (rocks, wood) and artificial (human made underwater devises and constructions) origin. This chapter includes brief overview on chemistry, structure and function of adhesives containing repetitive sequence blocks which have been studied up today. Better understanding of the mechanism of adhesive systems in barnacles could help in solving both ecological (marine biofouling) and biomedical (glues for bone and tissue repair) problems.

According to Kamino (2008), marine underwater bioadhesives represents the vital link between biology and bioinspired materials science. Barnacles(Cirripedia: Maxillopoda: Crustacea: Arthropoda) with more than 1280 species (Pitombo 2004) represents the next group of marine invertebrates which have developed specific biopolymer-based strategies uniquely to "glue" themselves to diverse substrates (for review see Kamino 2008; von Byern and Grunwald 2010). Adhesive biomaterials are able to displace water (Waite 1987), or form and spread adhesive bonds with the corresponding substratum (Li et al. 2016). The life span of barnacles is between 1 and 10 years (Ruppert and Barnes 1994). The scientific field that deals with barnacles is recognized as cirripedology.

21.1 Barnacles: Crustaceans That Mimic Molluscs

Calcareous plates of volcano-shaped shells remain to be well known areas where barnacles (Fig. 21.1) are usually occurring.

Until 1830 barnacles were thought to be molluscs (Ruppert and Barnes 1994) because of similarities of their body plan to that from molluscs. However, they produce free-living larvae, the phenomenon that defined them as crustaceans.

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Fig. 21.1 Barnacle as adult organism. (Image courtesy Gary H. Dickinson)

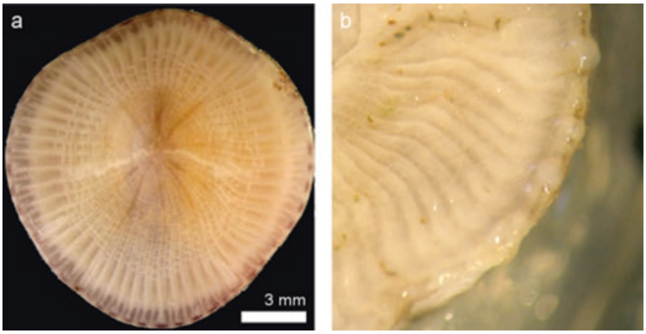

Fig. 21.2 Representation of the barnacle base. (Images courtesy Gary H. Dickinson)

The stalkless and stalked barnacles represent two main groups. One of typical examples of the stalked barnacles is goose barnacle, or gooseneck (order Pedunculata), which can grow up to 75 cm in length. This animal attaches themselves to substrata with the help of the cement glands which are located within flexible, muscular stalk-like peduncle.

In contrast to stalked barnacles, so called acorn barnacles (order Sessilia) are permanently attached to diverse substrates including the skin of whales or carapaces of sea turtles. Some species can rich up to 23 cm in height and 8 cm in diameter. A vertical wall of chitin-based calcified plates completely covers the soft body of the strong attached animal (Fig. 21.2). Special feathery appendages are responsible to filter feed.

Because of their behaviour to attach any kind of hard surfaces including man made constructions (pier pilings, buoys, and ship bottoms) it is not surprising that barnacles can create many problems related to marine fouling (see for details Larsson et al. 2010; Demirel et al. 2017; Song et al. 2019).

21.2 “First-Kiss” Adhesion Behaviour in Barnacles

It is well recognized that also for barnacles a planktonic larvae stage remains to be a most critical moment in their survival. Prior to metamorphosis to juveniles, larvae must find some kind of “excellent” place for their attachment on the hard substrate (Walker et al. 1987; Satuito et al. 1996; Maruzzo et al. 2012). According to Morse (1990) and Pawlik (1992) specific chemical cues are thought to be most crucial for the larval settlement. Some species (e.g. *Balanus improvises*) possess seven planktonic larval stages prior to metamorphoses into a sessile animal (Ödling et al. 2006).

There is broad diversity of both natural and artificial factors which can affect larval settlement and the following metamorphosis. It is to find in the literature that bacterial films, footprints by cyprid antennular secretion, synthetic peptide-based analogs of barnacle attachment pheromones (Yamamoto et al. 1999; Dreanno et al. 2006), water movement (Rittschof et al. 1984), or even surface colour (Yule and Walker 1984) can play significant role for both phenomena. Additionally, such endogenous factors as dopamine and serotonin can influence the searching behaviour of the *Balanus amphitrite* larvae (Yamamoto et al. (1996, 1999).

It is known that cyprids explore a substrate by “walking” using pair of antennules, which are nothing else as attachment organs (Walker and Yule 1984; Yule and Walker 1985). The pair of antennulules contains unicellular secretory glands which produce an adhesive (Okano et al. 1996). The main players here are the adhesive-secreting cells which are located in the muscular cement sacs (Walker 1971).These sacs are connected to the four segments-based antenna using cement ducts. The attachment of the cyprid larvae by itself to the selected surface area only after the secretion of the adhesive biopolymer is started (Harrison and Sandeman 1999).

Ödling et al. (2006) reported that during exocytotic secretion the cement granules of cyprid larvae of *B. improvisus* in vivo undergo a dramatic swelling.

The cyprids, in the exploratory phase, must be able to detach leaving temporary adhesive “footprints” in a form of blobs. This temporary adhesive is resistant to bacterial damage, acts as a signalling molecule to provoke the additional cyprids settlement as well as it does not diffuse in water (see also Fears et al. 2018). Cyprids exhibited a pronounced attachment response induced by pheromone, in the presence of polymerizing cement (Dickinson 2008). Therefore, it was proposed that cement polymerization that is essential for the growth of barnacle, leads to release the peptide pheromones which are able to attract predators and provoke the settlement of the cyprid. It is impossible to exclude that the formation of intertidal communities is related to the growth of barnacle and consequently the associated release of cement (Dickinson et al. 2009).

Thus, after selection of an appropriate surface on which to settle (Aldred et al. 2018), the organism stands on its head and secretes protein-based cement onto the paired antennules. The cyprid cement flows permanently during 3 h after that some kind of a discrete matrix is formed. Corresponding metamorphoses into the adult barnacle with mineralized walls can be happened only after that (Callow and Callow 2002).

21.3 Barnacles Cements

The major limiting factor in biochemical evaluations of the nature of the barnacle cement is related to its insolubility. It is worth to note (Kamino 2006) that, polymerized biocement of barnacles origin still has not been completely dissolved under any conditions. Recently, Dickinson et al. (2009) reported that the barnacle cement comprises of up to 90% protein (see also Naldrett 1993; Kamino et al. 2000) as well as lipid (1%), inorganic ash (4%; where 30% of the inorganic ash is calcium) and carbohydrate (1%) (Walker 1972). Barnacle cement is considered as an conglomerate of at least ten main proteins (for reviews see Kamino 2006, 2008). Recently, two (Bamcp20k-1 and Bamcp20k-2) homologous 20 k-cement proteins have been identified within secretory cells in the cyprid cement gland of *Amphibalanus amphitrite* (He et al. 2013). Abundant charged amino acids, such as histidine as well as conservative, repeated Cys domains have been confirmed in both proteins. It was demonstrated, however, that these proteins have distinct functions in barnacle cementing. The presence of phosphoproteins in barnacles adhesives has been recently confirmed (Dickinson et al. 2016). Also the isolation of one 100 kDa protein has been reported (He et al. 2018). Intriguingly, the recently reported sequence basis of barnacle cement nanostructures can be defined by proteins with silk homology (So et al. 2016). The attempts to understand the biochemical mechanisms of cement polymerization in barnacles are still in progress (Chen et al. 2011; Burden et al. 2012; Kamino et al. 2012; Aldred et al. 2013; Nakano and Kamino 2015; Jonker et al. 2015; Golden et al. 2016; Senkbeil et al. 2016; Liu et al. 2017; Raman et al. 2017; Yap et al. 2017; So et al. 2017). Interestingly, proteins (Petrone et al. 2015) and lipids can play synergistic roles in the permanent bioadhesive of barnacle larvae (Gohad et al. 2014).

It is not excluded (Dickinson et al. 2009; Barlow et al. 2009) that in the barnacle *A. amphitrite* (Pitombo 2004) the polymerization mechanism of it adhesive cement can be biochemically similar to clotting of blood and could be accepted as some kind of wound healing. This hypothesis is confirmed due to the presence of proteins and hemocytes characteristic for hemolymph (Dreanno et al. 2006) which might work as a cement (Dickinson 2008).

Thus, it is proposed that covalent cross-linking, induced by transglutaminase a hemocyte-released enzyme, is capable to reinforce the cement (Fig. 21.3). Lastly, activated structural proteins covalent cross-linking with their subsequent transformations led to obtain an insoluble network of interwoven fibrous proteins (Fig. 21.4).

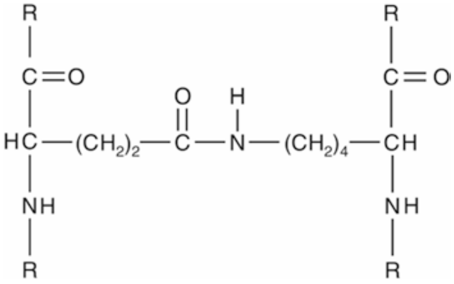

Fig. 21.3 ε-(γ-Glutamyl) lysine cross-link is to be found in barnacle cement. (Adapted from Dickinson et al. 2009)

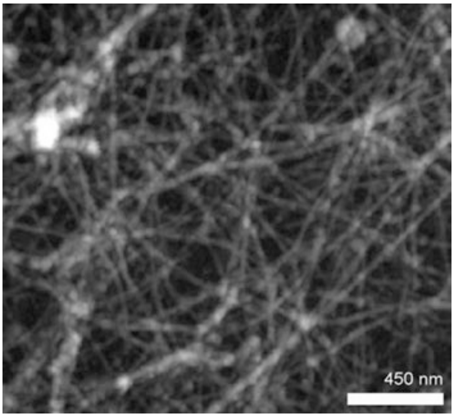

Fig. 21.4 Nanostructural organization of the unpolimerized cement of *Amphibalanus amphitrite* becomes visible using AFM. (Adapted from Dickinson et al. 2009)

21.4 Conclusion

Remarkable strength and durability of the adhesives of barnacles origin represent stimulating factors to study them in details. There are no doubts that production of bioadhesives in the form of cement is crucial for their survival in diverse marine environments, however it represents also serious problem with respect to global biofouling. Corresponding control and prevention of marine biofouling required more than US$ 5.7 billion annually (Callow and Callow 2002; Townsin 2003; Sur 2008; Demirel et al. 2017). Without understanding of the biosynthetic mechanisms related to the cements it would be impossible to control the settling of barnacle's larvae on, or removing of the adult colonies from artificial marine underwater constructions. Another direction of barnacle's related research includes the biomimetical aspect and biomaterials inspired chemistry (see for details Sullan et al. 2009; Sangeetha et al. 2010; Yang et al. 2013; Zheden et al. 2014a, b; So et al. 2016) as well as genetics investigations (Jonsson et al. 2018), especially that on gene expression of diverse cement proteins (Lin et al. 2014; Perina et al. 2014; Wang et al. 2015).

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Chapter 22

Halogenated Biocomposites

Abstract Parallel to calcified hard tissues, halogen- and metal–halogen-based biological materials are to be found in diverse marine invertebrates. There is still lack on information about the diversity in form and structure, biosynthesis, genetics their role in the evolution, ecology and behaviour, of invertebrates habituating in marine environments. This chapter is dedicated to few selected examples (jaws of polychaete and halogenated cuticles of crustaceans) of halogen-based biological materials of marine origin.

The amount of organic and mineral phases in the biological creations is an evolutionarily adaptable continuum. There is no doubt that, nature has the cases for each step of this continuum, between all-organic and all-mineral. For example, the structural, mechanical and biochemical features of three impact structures: the beak of the squid (*Dosidicus gigas*) as well as the jaws from the marine polychaetes (*Nereis* species and *Glycera dibranchiata*) have been described in the work by Broomell et al. (2007). From above-mentioned, merely jaws from the Glycera possess some amount of mineral. In the *Nereis* jaws instead of mineral phase, only Zn ions can be found, while in the *Dosidicus* beak no inorganic content can be detected (Broomell et al. 2007).

Teeth and other heavy mineralized (more than 50%) formations have been extensively investigated in morphological, chemical, mechanical, and clinical details. However, we still do not have many information about the more varied, slightly or non-mineralized structures. Surely, non-mineralized tissues commonly have been discounted – at least as the bio-inspired template for designing rigid and abrasion resistant composites, due to the popular opinion that the mechanical stiffness is conveyed by the occurrence of crystalline minerals.

There are lot of examples in nature, when mechanical features of diverse biological composite materials have been determined by incorporation of metals into the structures of their organic (mostly proteinaceous) parts (Lee et al. 2009). Thus, halogens (Br, Cl)- and heavy metals (e.g., Cu, Zn, Mn) have been detected in large amounts within the tarsal claws, stings, jaws, and other skeletal constructs of arthropods. In contrast to Ca within mineralized cuticles, the content of corresponding metal and halogen is usually lower (Schofield 2005; Schofield et al. 2009). Existence

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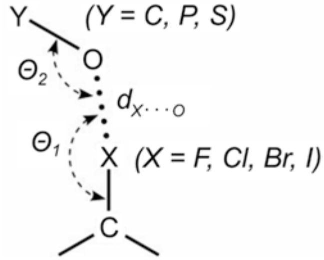

Fig. 22.1 Drawing of the short halogen (*X*) interactions with various oxygen-containing functional groups (where O—*Y* can be a carbonyl, hydroxyl, or carboxylate when *Y* is a carbon; a phosphate when *Y* is a phosphorus; or a sulfate when *Y* is a sulfur). The geometry of the interaction is defined by the normalized $R_{X\ldots O}$ distance [$R_{X\ldots O} = d_{X\ldots O}/R_{vdW(X\ldots O)}$], the Θ_1 angle of the oxygen relative to the C—*X* bond, and the Θ_2 angle of the halogen relative to the O—*Y* bond. (Adapted from Auffinger et al. 2004)

of metal-halogen-based biocomposites (MHBs) seems to be an ancient phenomenon. Cyanobacteria were the first living cells to produce oxygen, as well to accumulate iodides as antioxidants more than 3 billion years ago (Venturi et al. 2000). MHBs represent the naturally occurring alternative to biomineral-based structures (e.g. calcium-containing biominerals). In contrast to biominerals, studies on genetic, biosynthesis, diversity of forms and functions of MHBs are mostly in embryonic stage with exception of few examples represented below.

Iodine-containing biocomposites have been reported in diverse marine invertebrates including sponges (Ueberlein et al. 2017), worms, molluscs (Gorbman et al. 1954). Corresponding “success” of halogen bonds in biomacromolecules (chitin, spongin, gorgonin, etc) during evolution could be based on hypothesis proposed by.

Auffinger et al. (2004):“*thyroid hormones represent a class of naturally iodinated molecules for which halogen bonds appear to play a role in their recognition, as evident by the short I-O contacts between tetraiodothyroxine and its transport protein transthyretin. These short contacts, originally called charge-transfer bonds, were attributed to the transfer of negative charge from an oxygen, nitrogen, or sulphur (a Lewis base) to a polarizable halogen (a Lewis acid). They are now referred to as halogen bonds* (Fig. 22.1)”. These bonds can play crucial role in stabilization of inter- and intramolecular interactions in folding of proteins and nucleic acids (see olso Scholfield et al. 2013, 2015; Cavallo et al. 2016; Danelius et al. 2017). Corresponding to Auffinger et al. (2004), “*a halogen bond within biomacromolecules can be accepted as a short C-X---O-Y interaction (C-X is a carbon-bonded iodine, bromine, or, chlorine, and O-Y is a hydroxyl, carbonyl, charged carboxylate, or phosphate group), where the X---O distance is equal to the sums of the respective van der Waals radii (3.37 Å for Br---O, 3.27 Å for Cl---O, and 3.50 Å for I---O) and can conform to the geometry seen in such molecules where the C-X---O angle is approximatelly 165° and the X---O-Y angle around120°*”.

Consequently, the diversity of halogen-based biocomposites in marine invertebrates can be based on the diversity of the interacting partners of halogen bonds and their specific geometry. Especially corresponding posttranslational modification of amino acids (Birkedal et al. 2006), including that which can be multiply halogenated by bromine, or iodine, and chlorine is responsible for occurrence of non-mineralized but mechanically hard biomaterials. Examples of these modified amino acids, namely bromoiodotyrosine, chloroiodotyrosine, chlorodityrosine, dibromohistidine, bromoiodohistidine, and other related molecules are to be found in papers by Herbert Waite and Henrik Birkedal (see references below). Diverse MHBs have been studied in details also by other researches (Lichtenegger et al. 2002, 2003; Broomell et al. 2006, 2007; Khan et al. 2006). For example, Robert Schofield and co-workers even introduce the new term "*heavy-element biomaterials*" describing MHBs isolated from marine crustaceans (see for details Schofield (1990, 2001, 2005), Schofield et al. 2002, 2003)

22.1 Polychaetes Jaws

Worms known as Polychaetes (Annelida) are the most abundant multicellular invertebrates habituating in marine environments today. They can be found in diverse waters (bathyal, sublittoral and abyssal) as sessile, parasitic as well as pelagic forms (Eriksson and Elfman 2000). Some of species are really large, for example the North American Pacific bloodworm, *Glycera dibranchiata* Ehlers, whose length reaches up to 37 cm (Klawe and Dickie 1957). Such phenomena as digging and feeding in representatives of the orders Eunicida and Phyllodocida can function due to the presence of powerful, composite-based jaws. Due to their chemistry numerous fossilized and well preserved jaws have been found in fossilized matter in Palaeozoic rocks as well as in the early Cambrian (ca. 540 Ma) remnants (Bergmann 1979). Scolecodonts contain Si, S, Cl, Na, Ca, Fe, Cu, Ti, Ni, Cr, and sometimes P, Br, I and Mn (Eriksson and Elfman 2000). The chemistry of the recent specimens differs from that of the fossil specimens (Fig. 22.2). The morphological and structural features of selected polychaete jaws have been described by Michel et al. (1973) and Elfman et al. (1999).

The complexity of the jaws of *Nereis virens* is based on their structure, microarchitecture and chemical composition (Broomell et al. 2008; Cribb et al. 2008). For example, the structure is similar to fibre-reinforced materials with bunches of fibres collaterally distributed according to the direction of the long axis of the jaw. Results of the SAXS analysis clearly show that these fibres build constructs of tightly packed 50–100 nm fibrils in diameter (Lichtenegger et al. 2003). The jaw is closed within an amorphous coating (3–10 mm thick) of unknown composition. Metal ions, protein, and the halogens such as Cl, Br, and I; are heterogeneously spread within the structure of *Nereis* jaws (see for details Lichtenegger et al. 2003; Khan et al. 2006; Birkedal et al. 2006). The arrangements of Cl, I and Br, were examined close to the jaw tip by SIMS method from the surface to about 10 mm of depth. Obtained results indicated the presence of shortages in the first 1–2 mm. Protein remains to be the most widespread constituent with up to 90% of the total mass. The increase of histidine concentrations towards toothed-edge and the tip of the jaw

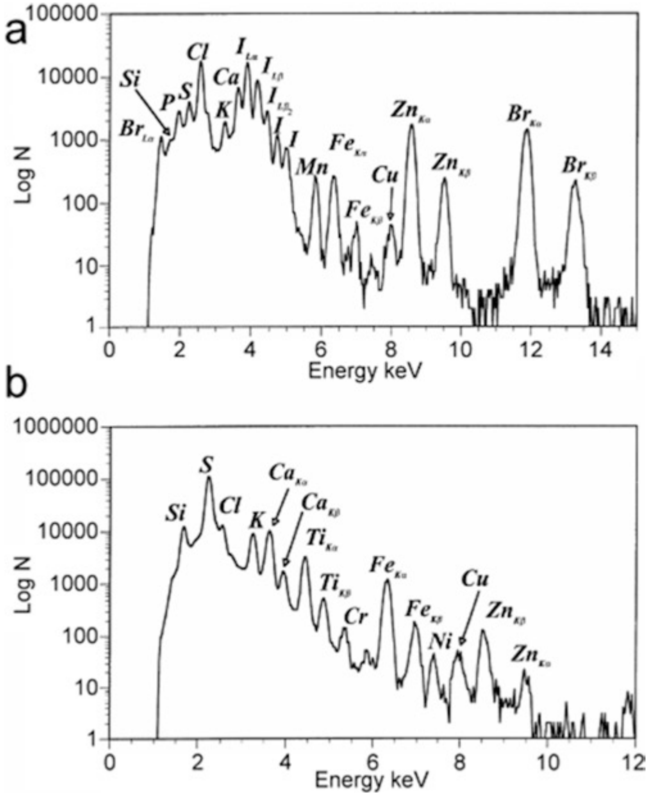

Fig. 22.2 Chemistry of polychaete jaws: the difference with respect to the elemental content in the jaws of the recent (**a**) and fossilized (**b**) organisms is detectable. (Adapted from Eriksson and Elfman 2000)

have been reported by Birkedal et al. (2006). The contrary tendency is observed for alanine; towards the jaw base the alanine levels increase. The content of another dominant amino acids (aspartate and tyrosine) does not differ within the jaw. Obtained results indicate that the jaw is consisted of two, but possibly more, proteins with individual distribution profiles and compositions. Only 0.1% of the total mass of the jaw represents the extractable protein. The primary extracted protein has a mass of about 35 kDa (Broomell et al. 2007). Concentration of both glycine and histidine in it approximates 30 mol%. This protein probably dominates in the tip.

Recent result implies that an appreciable post-translational modification of proteins derived from nereid jaw usually occurs. The halogenation of tyrosine and histidine is widespread, however products of aryl coupling such as di- and trityrosines as well as DOPA (Birkedal et al. 2006) are also noticeable.

However, the biochemical processes which are in charge of these modifications in the jaws still have not been established. It has been histochemically detected *in situ*, that peroxidase mediated in the cross-linking as well as in the halogenation of tyrosine residues (Broomell et al. 2007). But, the modification can be a byproduct of the other (i.e. inadvertent halogenation results in intentional cross-linking or *vice versa*). Due to the pronounced distribution of each halogen it might be suggested that modification of the jaw protein requires majority of specific processes.

Due to the fact that the first studies concerning the composition of *Nereis* jaws were carried out on species obtained from polluted estuaries in the UK (Bryan and Gibbs 1979), it was suggested that the jaws should act as a metal sink, separating the Zn absorbed onto the sediment from the living tissue. Subsequent studies clearly indicated that content of Zn in jaws were high, independently of environmental conditions, thus it can be hypothesised that metals can improve their mechanical properties. Many evidences are in agreement with this hypothesis. The content of Zn enhances (up to 10%) towards the toothed-edges and tip of the jaw – these regions have to be hard *in vivo*. The chemical composition of the halogens in the jaws of Nereis was described by Khan et al. (2006). They showed that Cl exists in two modes Cl–C and Cl–Zn with the first comprising 40% of the Cl content, in opposite to single chemical environment of I and Br. This clearly indicates that, in the absence of a metal, at least one halogen is able to form a significant structural component.

Both modulus and hardness enhance with Zn amount in the jaws of this worm species (Lichtenegger et al. 2003). In addition, elimination of Zn by chelation using EDTA leads to a reduction of about 80% in both E (Young modulus) and H (hardness) (Broomell et al. 2006). These two features can be almost entirely restored after reintroduction of Zn into the organic scaffold of the jaw. Therefore, these results confirm the hypothesis that Zn is an important constituent for providing the mechanical robustness of the *Nereis* jaw.

It has been assumed that in *Nereis*, a main matrix protein which contains significant content of histidine (30 mol%) seems to be a Zn^{2+} polydentate ligand (Broomell et al. 2007). The stiffness and hardness are remarkably reduced by the elimination of Zn^{2+} and restored after reintroduced of Zn^{2+}. Zn^{2+} can serve as a reversible cross-linker, in opposite to the dityrosines also detected in the structure.

Glycera jaws differ from that of *Nereis* as a composite of tanned (with melanin) protein, metal ions (Cu) and mineral phase -[$Cu_2Cl(OH)_3$]- known as atacamite (Gibbs and Bryan 1979; Lichtenegger et al. 2002, 2005; Moses et al. 2006). The content of melanin is about 40% of the jaws after drying. The amount of atacamite mineral in the form of nanofibres is less than 10% of the jaws by dry weight (Lichtenegger et al. 2002). Despite of the atacamite, Cu ions also seem to occur within the near-surface layers in the jaw tips (Lichtenegger et al. 2005). It was suggested (Moses et al. 2006) that the Cu ions together with the atacamite fibres serve an important structural components in the jaw tip.

Materials features of jaws from both worms species were compatible with that from mineral-based biomaterials. Thus, that the resistance to abrasion of the *Glycera* jaws was equivalent to that of the tooth enamel, however with only a tenth as much mineral (Lichtenegger et al. 2002). In another study (Lichtenegger et al. 2003), it was proved that the stiffness and hardness of the totally non-mineralized *Nereis* jaw approximate those of tooth dentin. Both above-mentioned examples validate the suggestion (Broomell et al. 2007, 2008) that non-mineral factors are able to provide biological materials with the abrasion resistance, hardness, and stiffness.

22.2 Crustaceans Alternative Cuticles

It is well known that grasping crabs including species as *Metopograpsus frontalis* are feeding on algae which they can scrape from the hard, rocky surfaces using the tips of their chelipeds (Shaw and Tibbetts 2004). These structures are tipped with a light brown but translucent biological material where no minerals have been detected. Such "*alternative cuticles*" (Cribb et al. 2009) with complex morphological layering have attracted scientific attention very well (Schofield et al. 2003).

Possible correlation between origin and form of corresponding halogens and such material features as elastic modulus (E) and hardness (H) in the exoskeleton of *M. frontalis* has been also studied. No correlation has been found between mechanical properties of cuticle and metal-free halogens occurring there. In the case of the jaws of *Nereis* halogens seems to be not a contributors to features of hardness and wear (Khan et al. 2006). However, Br-rich tips of calcium carbonates-based claws of the crab *Pachygrapsus crassipes* are less hard but more resistant to fracture (Schofield et al. 2009). The energy of fracture of Br-rich cuticle is greater than for calcified cuticle of about an order of magnitude. Bromine has been detected also in tarsal claws of isopods, some representatives of land-based crustaceans (Schofield 2001). Intriguingly, a comparison with other natural and man-made materials indicated that the brominated cuticle of the studied crustaceans remains to be stiffer and harder than the hardest tested acrylic plastics (Schoefild et al. 2009).

According to Robert Schofield, "*fracture resistance is of greater importance in smaller organisms, and we speculate that one function of heavy elements in mechanical biomaterials is to reduce molecular resonant frequencies and thereby increase absorption of energy from impacts*" (Schoefild et al. 2009).

22.3 Conclusion

Halogens do play significant roles in marine biological systems. Number of enzymes (peroxidases, dehalogenases) is involved in halogenation of nucleic acids and proteins in marine invertebrates from sponges to crustaceans. Halogen-based "*heavy-element biomaterials*" (Schoefild et al. 2009) possess materials features similar to calcified structures, and, consequently, represents the key alternative to biomineralization. Such biomaterials have been established during the evolution of biocomposites in skeletons of marine organisms parallel do biomineralized hard tissues. Crustaceans represent the group of biomineralizers where "*metal – protein – halogen*", "*metal-protein*" and "*protein-halogen*" systems exist together with calcified matrices within the same organism. Diverse halogen-based biocomposites briefly discussed above possess high biomimetic potential to be studied in the nearest future using more sophisticated bioanalytical approaches.

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Chapter 23
Chitin-Protein-Based Composites

Abstract Fur-like biological material made of densely distributed setae with unknown function has been reported here for some representatives of decapod crustaceans, both marine and fresh water habituating species. Such crabs as *Kiwa hirsute*, *Shinkaia crosnieri* as well as *Eriocheir sinensis* and *E. japonica* are described in the chapter as organisms producing setaous structures, which are made of chitin-protein-based composites. The presence of silk-like fibrillar protein within the inner pars of the setae has been discussed in comparison with marine silk of the amphipod's origin.

Sclerotized chitinous cuticle which represents the outermost layer of the skeletons in insects and Crustacea functions as a protector. This cuticle can possess broad diversity of hair-like structures known as setae, cuticular mechanoreceptors or mechano-sensory hairs responsible for sensory relationships with the environment (Howse 1968; Young 1970; Kouyama et al. 1981; Kouyama and Shimozawa 1982). The involvement of these structures as sensors which are connected to the nervous system of crustaceans and can influence their behaviour has been intensively debated since the middle of the XIX century (see for overview Leydig 1851; Claus 1891; Rath 1891, 1892; Weatherbya and Lenz 2000).

In few species of crustaceans the density of setae is so high that researchers introduced the term "*fur*". This structure has been observed on the external surfaces of the decapods *Kiwa hirsuta*, *K. puravida, K. tyleri* and *Shinkaia crosnieri* (all habituating near hydrothermal vents) (see for overview Roterman et al. 2013; Thatje et al. 2015; Watsuji et al. 2015; Shen et al. 2016) as well as on claws of mitten crabs *Eriocheir sinensis*) (Tian and Jiao 2019; Li et al. 2019) and *E. japonica*) (Zhang et al. 2018). The possible function of this setae-based fur as specialized sensilla-like structures or mechanoreceptors is still unknown.

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23.1 The Highly Flexible Setae of Hairy Lobster *Kiwa hirsuta*

Within about 70 species of decapods which habituate around the hydrothermal vents (Wolff 2005), only 15 species are belonging to four families of Anomura. One of these species, known as "Yeti" crab, or *Kiwa hirsuta* (Fig. 23.1a) is omnivorous and possess well developed setae- base fur on its walking legs and the chelipeds (Fig. 23.1b) (Macpherson et al. 2005; Baba et al. 2008) that is used as special 3D constructs by corresponding bacteria (see for review Goffredi et al. 2008). This white and up to 15 cm-large "hairy" crab belongs to the superfamily Galatheoidea. The setae-associated microorganisms are strongly attached to the chitinous surface of the setae and belong to e-*Proteobacteria, g-Proteobacteria* and *Bacteroidetes* (~56%, ~25% and ~10% of the recovered ribotypes, respectively (Goffredi et al. 2008).

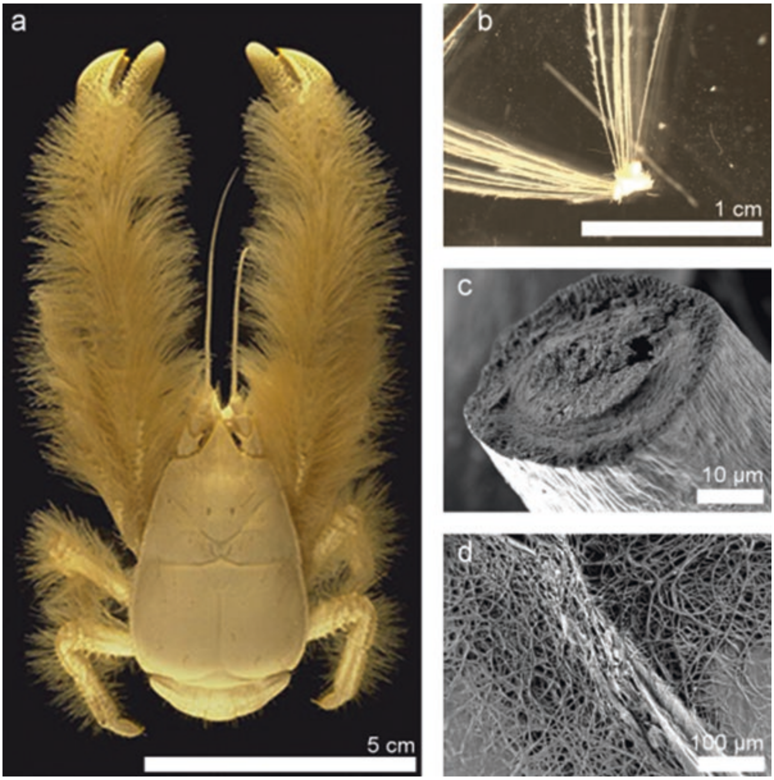

Fig. 23.1 "Yeti" lobster (*Kiwa hirsuta*) covers it chelipeds and walking legs with extraordinarily setose fur (**a**). Chitin and fibrillar protein are the main structural components of these setae (**b**). Chitinase treatment leads to digestion of the chitinous outermost layer (**c**) and uncovers proteinaceous microfibrils (SEM image (**d**)). (Samples courtesy Michel Segonzac)

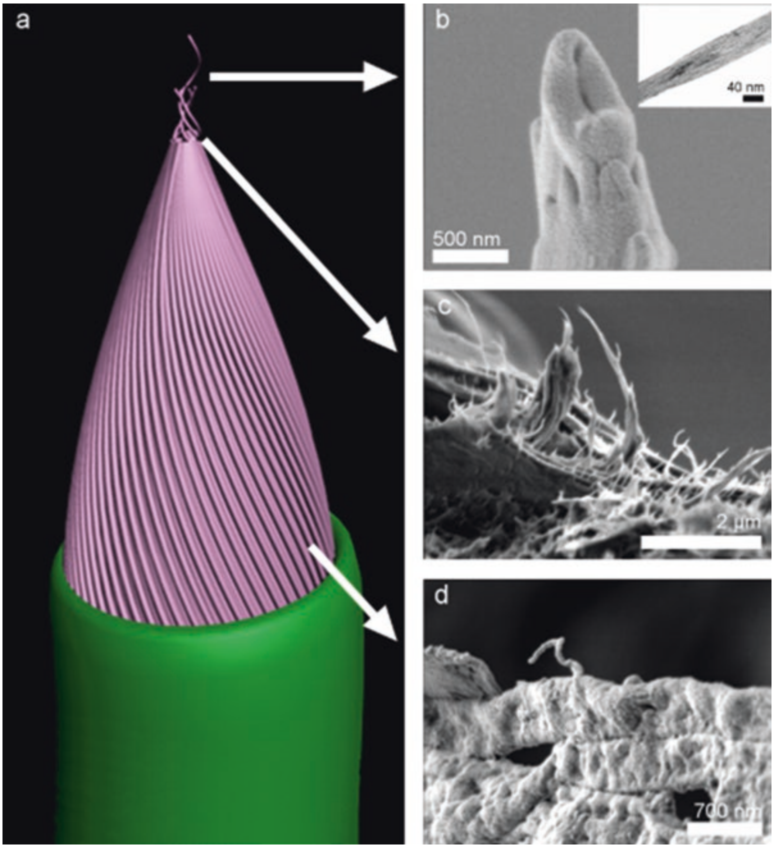

Fig. 23.2 The structure of *K. hirsuta* setae (**a**) as proposed by Denis Kurek. The nanostructural organization (**b**–**d**) of the fibrillar core protein is clear

Additionally to chitin, these approximately 10–20 mm in length fibres contain protein (Fig. 23.1c). Chitinous layer can be easily removed from the surface of setae using the enzymatic digestion with chitinase (Fig. 23.1d). While the inner, proteinaceos part can be dissolved using alkaline treatment. The fibrillary nature of the protein becomes visible under electron microscope observations (Fig. 23.2). Preliminary investigations using Raman, FTIR, and X-ray diffraction analysis confirmed corresponding similarity of this protein to silk. This event is interesting because decapods are evolutionary older than silk-producing arachnids. It seems that these chitin-protein biocomposites in the form of setae possess a very specific function. Due to the absence of any kind of detectable microtubuli within *K. hirsuta* setae, which are characteristic for mechanoreceptors of insect's origin (Kouyama and Shimozawa 1982) possible sensoric function of these structures could be excluded.

The presence of silk within inner parts of setae should be confirmed using proteomics and genomics approaches as soon as possible.

23.2 Shinkaia Crosnieri

Another hairy crab *Shinkaia crosnieri* (Decapoda: Anomura: Galatheidae) (Fig. 23.3a–c) has been discovered at the Bismarck Archipelago and in the Okinawa and identified by Baba and Williams (1998). Later this species has been observed in hydrothermal vents area near Taiwan (Chan et al. 2000). Probably, similar species habituated oceans in Eocene (Schweizer and Feldmann 2008). It was suggested (Ohta and Kim 2001) that similar to *K. hirsuta*, this crab is also able to "culture" filamentous bacteria on the exoskeletal surfaces. These heterogeneous filamentous bacterial communities are represented by the *Epsilonproteobacteria* (belonging to the Sulfurovum group) and the metanotrophic and thioautotrophic *Gammaproteobacteria.*

S. crosnieri collected from corresponding habitats survived in aquarium under artificially designed sulfidic and aerobic environment for more than 5 months.

Unfortunately, because of the absence of representative material of setae and spines from *S. crosnieri* in my Lab, we took the decision to study similar structures of the Mitten Chinese Crab *Eriocheir sinensis* (Fig. 23.4), which was easy to collect due to abundance in Germany.

23.3 Structural Features of *Eriocheir sinensis Setae*

Originally Mitten crabs have to be discovered in Asia as crustaceans habituating mostly in limnic and intertidal areas. As reviewed by Tang et al. (2003), the genus *Eriocheir* (Varunidae: Grapsoidea) (Hoestlandt 1948; DeLeersnyder 1967) consist of five taxa, viz. *Eriocheir sinensis* H. Milne Edwards, 1853, *Eriocheir japonica* de Haan, 1835, *Eriocheir leptognatha* Rathbun, 1913, *Eriocheir hepuensis* Dai, 1991, and *Eriocheir formosa* Chan, 1995. Mitten crab *E. sinensis*, is known as invasive species in Europe and North America (Ingle and Andrews 1976; Cohen and Carlton 1997; Dittel and Epifanio 2009).

The patches of brown setae on the inner and outer surface of white-tipped chelae of *E. sinensis* (Fig. 23.4) are typical also for other representatives of the genus *Eriocheir* (Veldhuizen 2001).

The size of the claws is roughly equal. The carapace that is wider than longer, possesses four spines on its former lateral edge. Usually, the width of the carapace of the adult mitten crab is approximately 80 mm, but sometimes larger animals can be found (Siegfried 1999). The pigmentation of the crab's carapace differs from a greenish-brown – observed in the newly molted and the adult crabs to brownish-orange, found in the juvenile crabs (Zhao 1999). The width of the carapace is usually twice as short as the length of mitten crab's legs and, among adult crabs and

Fig. 23.3 Small spines (arrows) on the lateral margins of the rostrum (**a** and **b**) remains to be characteristic for *Shinkaia crosnieri*, which can form large colonies (**c**) in deep-sea hydrothermal vent areas

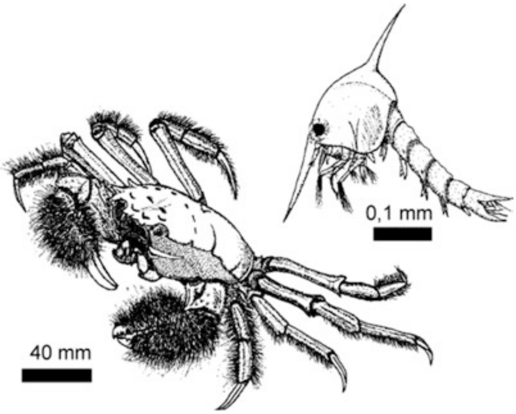

Fig. 23.4 Chinese Mitten Crab (*Erioheir sinensis*)

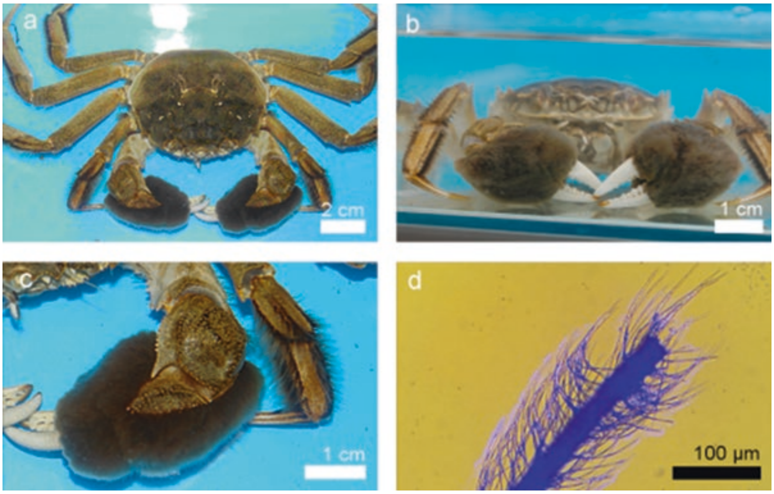

Fig. 23.5 Typical feature of *E. sinensis* claws is the presence of fur-like patches of brownish colored setae (**a**–**c**). The presense of chitin within setae has been confirmed using Calcofluor White staining after observation in fluorescence microscopy modus (**d**). (Image courtesy Thomas Hanke and Sascha Heinemann)

older juveniles, on the distal segments of the legs the existence of hairs along the lateral edges was determined (Fig. 23.5). The female and male crabs can be diversify based on the abdomen shape, only after reaching a carapace size equalled 10 mm. The male carapace has a narrower abdomen in the shape of an inverted funnel while, the female's carapace is rounded and takes up the majority of the thorax area.

China mitten crabs are able to reproduce only once (Normant et al. 2002); majority of them reproduce during the autumn, although some of them can wait until the spring. Females usually bear from 250,000 to 1,000,000 eggs (Hymanson et al. 1999) fastened to the abdominal appendages known as pleopods, which are located on the bottom of their abdomen. After reproduction, the mitten crabs back to the coastal banks; the females die after the hatching of eggs and males die after copulation (Zhao 1999).

The life of mitten crabs begins in marine pelagic zoeae. The crabs are subjected to 1 megalopal and 5 zoeal stages, and they need approximately 15 days to gain the megalopal stage, under the laboratory conditions (Kim and Hwang 1995). In this stadium, the crabs start migrating towards mainland to find fresh water niches.

The megalopal crabs are fast swimmers, looking for waters with lower salinity to gain the salty water (Zhao 1999). After reaching the megalopal stage, these animals embed on the substrate and then proceed their wandering as juveniles. Juvenile mitten crabs take on tremendous excursions during their migration (Dan et al. 1984), and have been detected 1250 km away from the coast in freshwater streams. Prior to start their wandering downstream as adults, the Juvenile crabs stay from 1 to 5 years growing and feeding in the China's freshwater niches, although most of them maturing within 1–2 years (Hymanson 1999; Zhao 1999).

The European investigations concerning this species (Anger 1991) show that, the adult animals start migration downstream in winter or late fall, the males descending to salty waters, a month before females.

As juveniles, *E. sinensis* migrate from the saline environment (Krogh 1938) upstream to fresh water. As adults, they live in burrows in the banks of rivers (Shaw 1961; Olsowski et al. 1995; Onken 1999; Silvestre et al. 2004, 2005a, b). Information concerning historical migration of Mitten crabs from Asia to Europe is to be found in numerous papers (e.g. Marquard 1926; Peters and Panning 1933; Panning 1939; Carlton and Geller 1993; Dittel and Epifanio 2009). In the first half of 1935, Panning (1938) noticed occurrence of over 12,000 kg (3,444,680 mitten crabs individuals) at a dam on the Weser River in Germany. At this moment, the plague was so intensive that the crabs cropped out of the rivers and were walking down city streets and going into houses! First reports on distribution of Chinese Mitten Crab in North America have been published in 1973 (Nepszy and Leach 1973).

Our preliminary experiments confirmed structural and chemical similarities between setae of *E. chinensis* to that from *K. hirsuta*. Mitten crab "hairs" contain chitin and nanofibrillar protein (Fig. 23.6) which became visible after digestion with chitinase (Fig. 23.6c, d). Also the presence of unidentified bacteria attached to the surface of setae is visible (Fig. 23.7a). The *E. chinensis* setae can be dissolved using 35% H_2O_2 under intensive lighting at room temperature (Fig. 23.7c).

Our suggestion concerning silk-based fibres in decapods is not without reason. Silk is to be recognized as biological materials also in representatives of the crustaceans order Amphipoda. So called "amphipod silk" which is produced in corre-

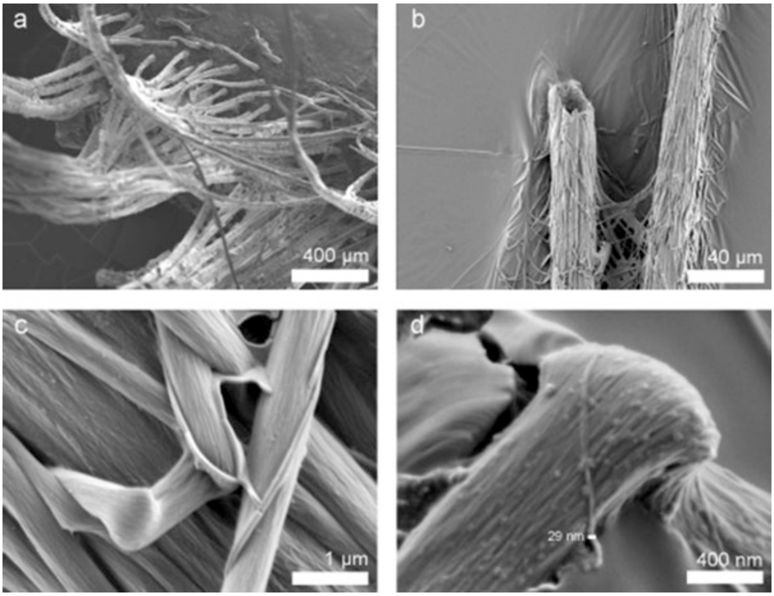

Fig. 23.6 Nanostructural organisation of both chitin and fibrillary protein becomes well visible using SEM investigations of the *E.sinensis* setae (**a** and **b**). Corresponding chitinase treatment leads to better visualization of the proteinaceous nanofibrils (**c** and **d**)

sponding silk glands is known since nineteenth century (Nebeski 1880). Amphipods, including diverse marine species, are able to produce and construct their differently architecture nests, whips or masts (for review see Holmes 1901; Sattler 1958; Cerda et al. 2010; Kakui and Hiruta 2014; Neretin 2016; Neretin et al. 2017) made of this self-secreted, adhesive biopolymer. Especially tube-building by amphipod species within the Corophiidea, Ampeliscoidea and Aetiopedidea is based on silk biosynthesis (Kronenberer et al. 2012a, b). Such silk threads-constructed tubes and nests can be produced in short time, often in less than a half hour (Holmes 1901). It was observed that this fibrous material hardens soon in water after its emergence. The chemical structure of amphipod silk has been studied on example of *Crassicorophium bonellii* Milne Edwards 1830 (Kronenberger et al. 2012a).

It was reported about the ability of this crustacean to spin underwater gossamer threads that are sticky and salt-water resistant. These properties are, probably, based on the structural peculiarities of amphipod silk that remain to be a mixture of protein and mucopolysaccharides (Kronenberger et al. 2012b). It was suggested that processing system of silk nests building amphipods seems to combine the internal extrusion spinning duct characteristic for arachnids with the barnacle's adhesive glue-cement exudate production glands (Kronenberger et al. 2012a). Recent research on amphipod silk is mostly focused on structural investigations (Kaji et al. 2016; Neretin et al. 2017) as well on estimation of evolutionary significance and possible biomimetic potential (Kamalathevan et al. 2018) of this biological material.

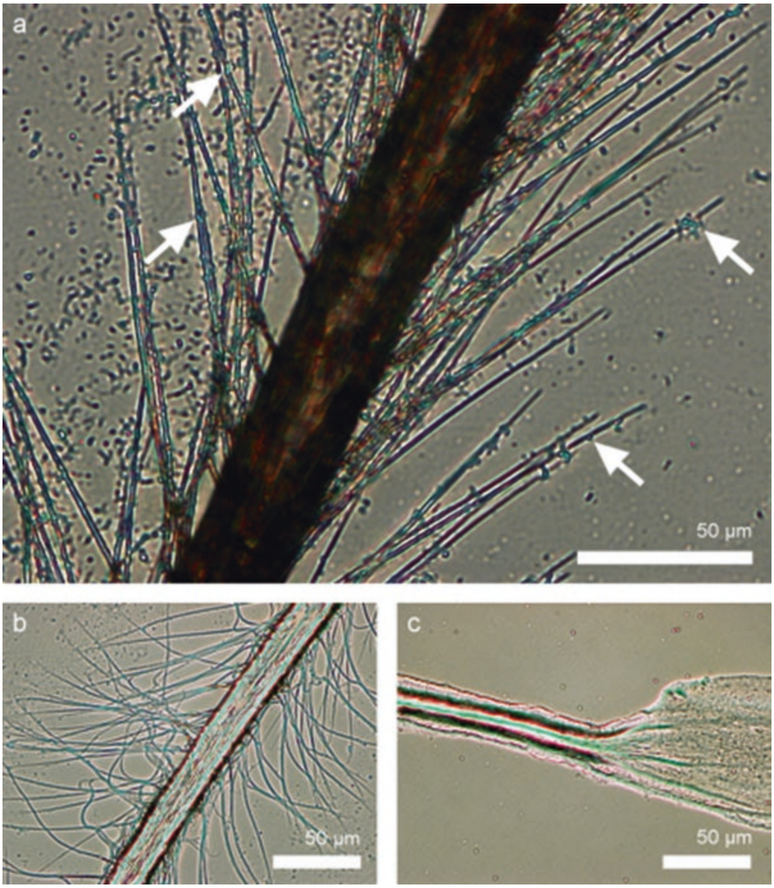

Fig. 23.7 Similar to the case of *K. hirsuta* (Goffredi et al. 2008), bacteria (**a**) (arrows) colonized the surface of *E. sinensis* setae. Chitinous components can be depigmented (**b**) and depolymerised (**c**) after treatment with 35% hydrogen peroxide

23.4 Conclusion

Crustaceans belonging to the decapods are interesting organisms not only because of their ecology (hydrothermal vents) and symbiosis with diverse marine bacteria, but also due to the nature and origin of their setae as examples on nanostructured chitin-protein-based biocomposites. The chemistry and structural features of setae from marine and fresh water species should be studied in details especially with respect to identification of silk or silk-like domains. The true function of decapod's setae reported above remains to be unknown. However, some spider crabs (Fig. 23.8) are able to use the setose surface of their carapaces to attach diverse micro stones with respect to mimic the surrounding sandy sediment.

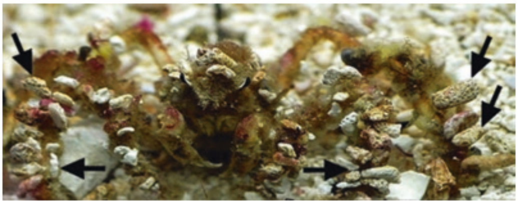

Fig. 23.8 Setose surface of the spider crab is used by the animal to attach microstones (arrows)

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Part V
Macromolecular Biopolymers

Chapter 24
Chitin

Abstract Chitin of marine invertebrate's origin (cephalopod molluscs, crustaceans) has been traditionally well studied as the main source for manufacturing of its derivate chitosan. Nowadays, the trend is directed to naturally prefabricated three dimensional (3D) chitinous scaffolds which can be simply isolated from diverse marine demosponges. Such ready to use constructs have been reported as applicable as adsorbents, scaffolds for development of composite materials and biocompatible matrices for tissue engineering of diverse human mesenchymal stromal cells. This chapter includes huge list of references on chitin properties and applications including recently published books and reviews.

24.1 Chitinous Scaffolds of Marine Sponges Origin

Sponges (Porifera) are still recognized as the first metazoans as well as the earliest branching animals (Reitner and Wörheide 2002; Philippe et al. 2009; Love et al. 2009).Consequently, the presence of structural aminopolysaccharide chitin in their skeletal structures confirmed also its ancient origin. In spite of chitin discovery in 1823 by Antoine Odier (Odier 1823), chitin in marine sponges has been detected only in 2007 in my Lab (Ehrlich et al. 2007a, b; Ehrlich and Worch 2007). Later, in 2013 we succeed in confirmation of it presence also in fossilized skeleton of 505 MYR old demosponge *Vauxia gracilents* (Ehrlich et al. 2013b). Also in 2013, we discovered that skeletal structures of fresh water sponges such as *Spongilla lacustris* (Ehrlich et al. 2013a) and *Lubomirskia baicalensis* (Ehrlich et al. 2013c) contain α-chitin. It was hypothesized that chitin identified within the holdfast of *L. baicalensis* is responsible mainly for strong attachment of the sponge body to the hard substrata (Ehrlich et al. 2013c).

Thus, since 2007 a systematic monitoring on the presence of chitin among the diverse sponge species is still in progress. This polysaccharide has been found in 19 species of sponges (for overview see Brunner et al. 2009; Cruz-Barraza et al. 2012; Ehrlich et al. 2010b, 2013a, b, 2017, 2018; Ehrlich 2013, 2018; Fromont et al. 2019; Klinger et al. 2019; Shaala et al. 2019; Wysokowski et al. 2013a; Żółtowska-Aksamitowska et al. 2018a, b; Vacelet et al. 2019). One of the common features of

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Fig. 24.1 Typical chitinous skeleton of marine demosponge from the Verongiida order. Skeletal fibres contein chitin, mineral phases and pigments, mostly bromotyrosines and uranidine

poriferan chitin is that it resembles the size, shape, porosity and 3D architecture of originally occurring sponges. We can speak about existence of naturally prefabricated, tubular 3D chitinous scaffolds, most of which are to be found in representatives of marine demosponges belonging to the Verongiida order (Fig. 24.1).

Discovery of chitin in various representatives of sponges prove that this biomacromolecule is a key structural biopolymer in both calcification and silicification processes (Ehrlich 2010; Wysokowski et al. 2018) but also provide useful information for revolutionary classification sponge species.

It is worth to note that demosponges, including well known bath, or commercial keratosan sponges are organisms capable of regenerating damaged tissues. This ability has been observed also in chitin-producing verongiid demosponges. For example, Rohde and Schupp found that *Ianthella basta* (Fig. 24.2) is able to regenerate 20 cm of damaged tissue per year. This naturally occurring phenomenon open the key way for recultivation of *I. basta* under marine farming conditions and for their establishment as renewable sources of specially prefabricated chitin.

Isolation of chitin from demosponges is a very simple procedure. Due to resistance of chitin (in contrast to proteins and pigments) to alkali, it has been isolated after treatment with 10% NaOH at 37 °C during 7–14 days (Ehrlich et al. 2007a, b, 2010a, b). The results of such kind of treatment on example of unique, flat 3D skeleton of *I. basta* are represented in the Figs. 24.3 and 24.4a. It should be noted that to avoid deacetylation and possible base-induced transformation of β-chitin in α-chitin the NaOH concentration during deproteinization should be around 10%

Fig. 24.2 Giant verongiid demosponges *Ianthella basta* also possess skeleton masde of chitin fibres. (Underwater image courtesy Mark Spencer)

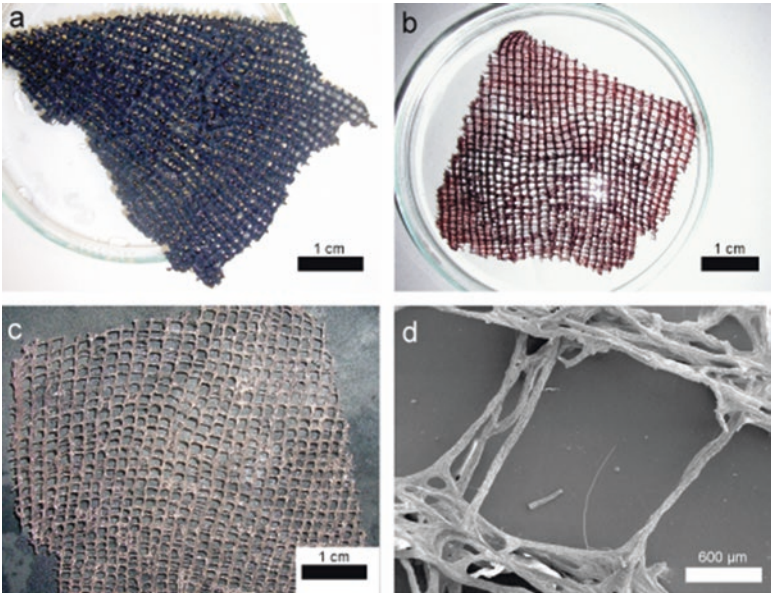

Fig. 24.3 Flat 3D skeleton of of *I. basta* (**a**) is the source of chitinous matrix that can be easily obtained after 2.5 M NaOH- based treatment (**b**, **c**). The micro- and nano-organization of such chitinous material is well detectable using SEM (**d**). (Samples courtesy Peter Schupp)

(Noishiki et al. 2003). Thanks to that, both the integrity as well as the crystalline structure of the sponge chitin is not influenced by alkaline treatment.

Alkali-based treatment leads to extraction of bromotyrosines (Fig. 24.4a) as well to visualization of unique spherulocites, cells which are responsible to synthesize these biologically active substances with antibiotic, antiviral and antitumor effect.

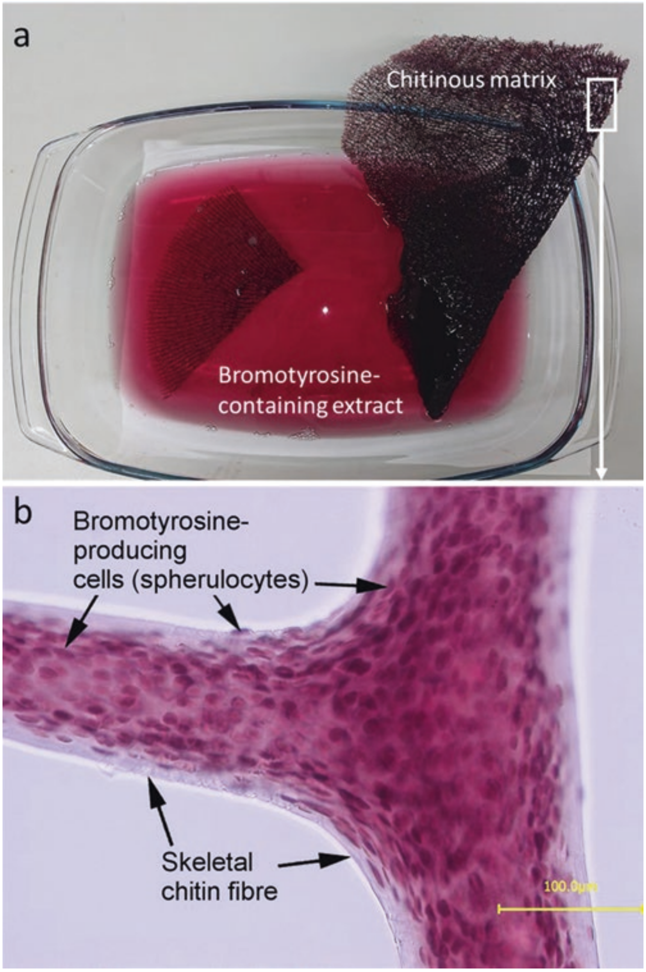

Fig. 24.4 Alkali-based treatment of skeletons of verongiid demosponges (i.e. *I. basta*) leads to depigmentation of chitin (**a**) and visualization of the bromotyrosines secreting cells located within chitin fibres (**b**). Bromotyrosines possess inhibitor activity against chitinolytic microorganisms

Consequently, chitin scaffolds in the form of 3D constructs can be isolated also from finger-like marine demosponge *A. aerophoba*. Nanostructural organization of such chitinous scaffolds becomes well visible on SEM microphotographs (Fig. 24.5). Chitin nanofibrils of similar diameter and distribution within tube-like fibres have been observed in all tested verongiids (Ehrlich et al. 2007b; Brunner et al. 2009; Wysokowski et al. 2013a; Żółtowska-Aksamitowska et al. 2018b).

One of the common morphological features of verongiid's chitin is their hollow, pipe-like (Fig. 24.6) translucent architecture with species specific interconnected macroporosity. These properties remain to be unchanged also after ultrasound, or microwave irradiation of sponges bodies selected for chitin isolation (Klinger et al. 2019).

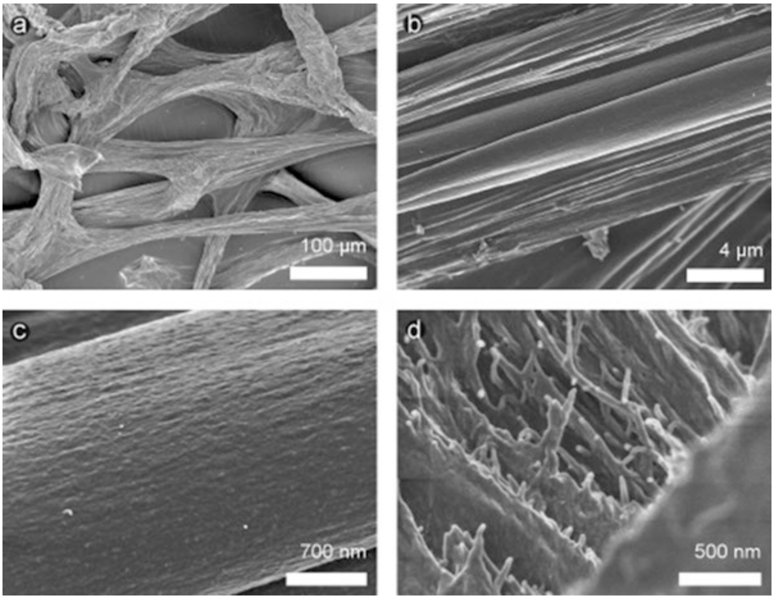

Fig. 24.5 SEM imagery: nanostructural organization of chitin isolated from the *A. aerophoba* marine demosponge

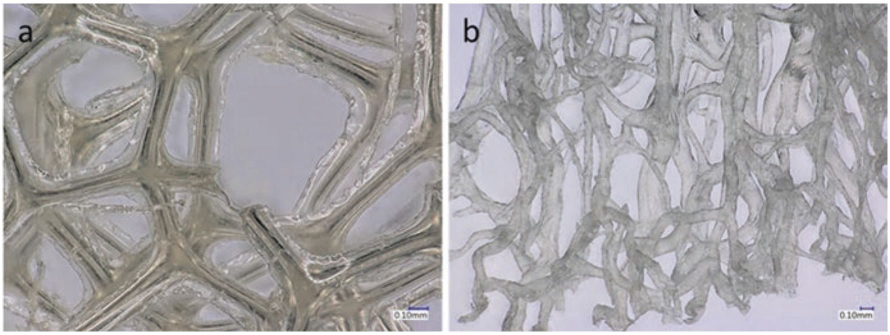

Fig. 24.6 In contrast to monolithic artificial sponges (**a**), chitinous scaffolds (**b**) are tube-like interconnected structures with excellent capillary effects. (For details see Klinger et al. 2019)

Furthermore, such kind of chitin-based scaffolds possess high biomimetic potential as alternative solution for development of artificial 3D constructs for tissue engineering (Green et al. 2002; Green 2008). Some strategies concerning the practical applications of chitin from representatives of the Verongiida order are represented in Fig. 24.7.

Due to its biodegradability (Khor 2001), chitin is well recognized as a very attractive and promising biological material (Yeul and Rayalu 2013) in modern tissue engineering (see reviews by Jayakumar et al. 2010; Yang 2011; Anitha et al. 2014). Previously, diverse attempts to develop chitin-based 3D scaffolds from pow-

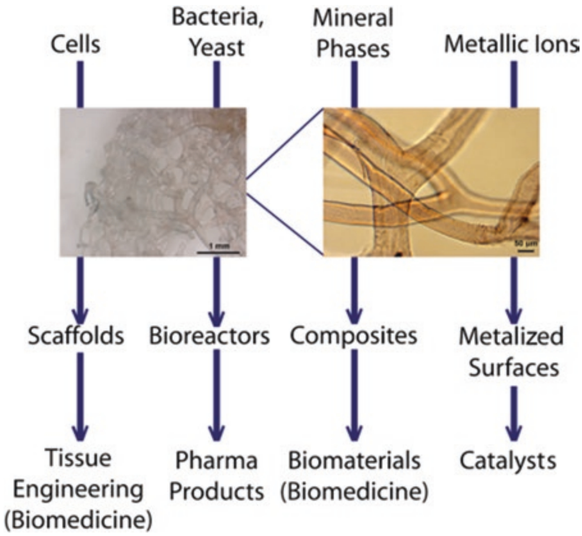

Fig. 24.7 Perspectives of practical application of 3D chitinous scaffolds isolated from verongiid sponges

dered chitin through it transformation into sponge-like forms have been carried out (Abe et al. 2004). However, in contrast to synthetic biomaterials (Mir et al. 2018) isolation of "ready to use" chitinous scaffolds from Verongiid demosponges seems to be the cheapest way. Except applications in tissue engineering, they can be used for designing of filtering systems and composite materials with mineralized as well as metalized surfaces applicable as catalysts (Ehrlich and Worch 2007).

Exceptional thermostability of chitin up to 400 °C (Köll et al. 1991; Stawski et al. 2008, 2017) opens the key way to use it in development of novel composite materials according to the rules of Extreme Biomimetics (Wysokowski et al. 2015; Petrenko et al. 2017).

One of the advantages to isolate chitin from Verongiida demosponges is based on their ability to synthetize broad variety (more than 60) different bromotyrosines as secondary metabolites with well recognized antimicrobial, antiviral, antiparasitic and antitumor activities (see for review Bechmann et al. 2018). Thus, it is time to develop and to establish some kind of a non-waste technology (Fig. 24.8) for the renewable use of verongiids as sources of substances and biomaterials, which could find practical applications in technology, pharmacology and medicine.

24.2 Biological Features of Chitin

Chitin in the bio-stable form (Khor 2001) is commonly used for preparing coatings onto blood contact tubing as well as wound dressings principally. The implant category includes tissue engineering applications and bone substitutes. These applications need the biodegradable chitin, to prevent a second operation of the restoring the implant.

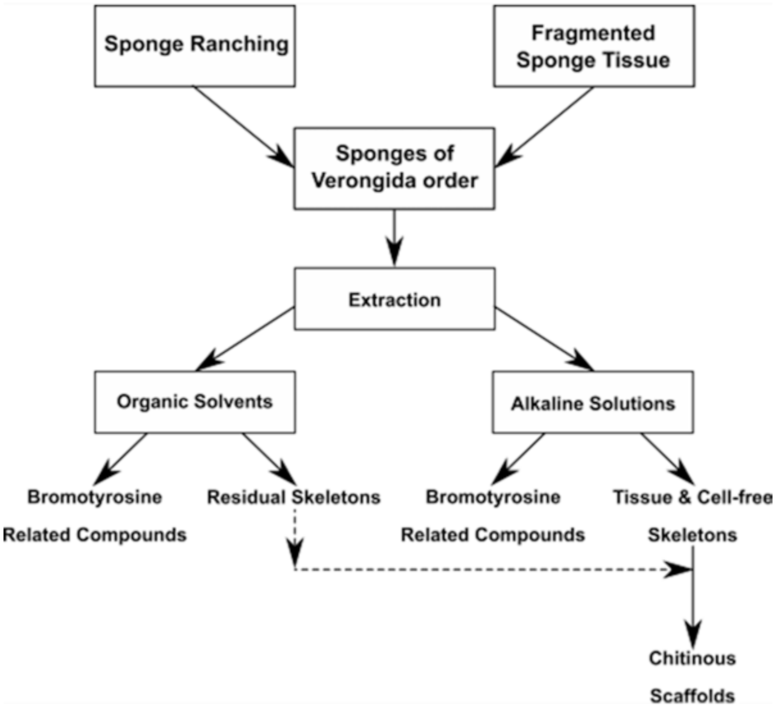

Fig. 24.8 Utilization of Verongiida demosponges can be proposed as non-waste technology

The chitinous wound dressing is produced as an non-biodegradable external device (Khor 2002). However, it is hypothesised that the lysozyme, which abundantly occurs in wound beds, should be able to degrade the chitinous wound dressings, but the biodegradation could appear only at the interface, leaving the dressing mainly unharmed. In the case of using chitin as a coating for blood-contact tubing, the chitin should be embedded on the internal surface of the tube and provide proper anchoring of the biomaterial into the surface of the tube. Chitin in corresponding wound dressings (e.g. flexible films) (Mushi et al. 2014; Zhang et al. 2015; Kadokawa 2016) did not incite the abnormally high inflammatory response typical for some biodegradable materials (Muzzarelli 2010; Elieh et al. 2018) and possess, consequently, infection-free healing rate (Jayakumar et al. 2011; Muzzarelli et al. 2014; Younes and Rinaudo 2015; Azuma et al. 2015). Also a low toxicity of chitin has been reported (Knorr 1984; Okamoto et al. 1993; Tanaka et al. 1997; Kwak et al. 2005; Aranaz et al. 2009; Badwan et al. 2015). However, there are different opinions concerning digestion (Tabata et al. 2018) and immune response to chitin in the form of microparticles (Ozdemir et al. 2006; Da Silva et al. 2010; Kogiso et al. 2011; Alvarez 2014) as well as chitin-producing invertebrates and microorganisms (Elias et al. 2005; Reese et al. 2007; Da Silva et al. 2009; Lee 2009; Koller et al. 2011; Roy et al. 2012; Bueter et al. 2013; Li et al. 2013; Van Dyken et al. 2014;

Wiesner et al. 2015; Koch et al. 2015; Shen et al. 2015; Khosravi and Erle 2016; Becker et al. 2016; Elieh et al. 2018; Arae et al. 2018; Dworkin 2018; Nakashima et al. 2018).

Diversity in interpretations regarding to the chitin-based immune response had motivated me to ask such renowned expert on this topic as Prof. Jack Elias (Yale University School of Medicine, USA), directly. Here, I take the liberty to represent his answer:

"*I wish I could give you a simple answer. There is no question that chitin has been used in implants and has been reported to be inert in that setting. On the other hand, our studies and the work of others suggest that chitin can drive inflammation. Our best guess is that, once we know enough, we will have defined the criterion that determines these different results. One appears to be the size of the chitin fragment with very large polymers tending to be inert and smaller fragments inducing cytokine production and inflammation. We also have evidence that chitin can act as an adjuvant and an antigen.*"

Thus, I can only postulate that the exact immune regulatory effects of chitin observed in vivo remain controversial. True mechanisms of corresponding immune responses described in the papers listed above are still poorly defined.

What about the biodegradability of chitin?

In human body, few enzyme systems, which are able to break down chitin can be found. Firstly, macrophages as a part of their phagocytic arsenal possess lysozyme and chitinases (Boot et al. 1995). Additionally, the non-reducing terminal N-acetylglucosamine chitin residue can be hydrolysed by N-acetyl-ß-D-glucosaminidase. The chitinase-like enzymatic activity in human leukocytes and serum has been reported by Escott and Adams (1995). It was demonstrated that the chitinolytic activity remains be distinct from that of lysozyme. *In situ* digestion of chitin during 14 days that was subcutaneously implanted in dogs by lysozyme has been reported by Saimoto et al. (1997). Similar effects have been described in experiments with a rat (Tomihata and Ikada 1997).

Onishi and Mashida (1999) informed that after intraperitoneal injection of chitin into mice, the 50% of the deacetylated chitin was passed with the urine in a form of small molecular weight materials. These results undoubtedly show that chitin is in vivo degraded and is not accumulated in the body tissues. One of the patents entitled as "*A human chitinase, its recombinant production, its use for decomposing chitin, its use in therapy of prophylaxis against infection diseases*"is dedicated to this intriguing topic (for details see Aerts 1996).

What about the biocompatibility of chitin? The capability of a biomaterial to act with a proper host response in a specific application is defined as biocompatibility. Biocompatibility is related to the interactions between host tissue and biomaterial (Khor 2001). In order to induce the biomaterial interactions and toxicological aspects of biomaterials, the cell culture methods have been designed. Investigations concerning the macrophage activity assay as well as the effect of chitin on fibroblast cells proliferation were conducted by Schmidt et al. (1993). Obtained results clearly indicated that, the presence of chitin did not support cell proliferation because of low cell efficiency in comparison to control at day 3, with further decrease at day 6.

Chitin was non cytotoxic because the cells during exposition remained to be still viable. In addition, it was found that chitin is not an inhibitor of the macrophage activity.

Kojima et al. (1998) evaluated the application of a feline in vivo model in wound healing to study the formation of the granulation tissue on the wounds after implantation of chitosan and chitin containing implants in a 14-day period. The granulation tissue, cut out from place where chitin containing implant was applied, was thin and exhibited insignificant foreign body reaction. However, the formation of the excessive granulation tissue, which extends into the surrounding tissues, was observed in the wound exposed to chitosan. These results indicate that chitosan induced a continuous and greater inflammatory response adverse for appropriate wound healing while chitin provided better wound stimulation due to increase of preliminary collagen synthesis.

Very speculative photographs (Fig. 24.9) concerning application of nanofibrillar chitin in wound healing have been represented by Morganti and Morganti (2008). These authors suggested that especially nanofibrils of chitin possess an enormous surface and give corresponding space for interaction with diverse cell compounds including platelets and enzymes. Such form of chitin stimulating type IV collagen synthesis, the regular deposition of collagen fibers and angiogenesis, increasing cytokine production and lead to successful repair of dermo-epidermal lesions and healing (Fig. 24.9). Nanostructured chitin remains to be still in trend in skin remodeling and cosmetic dermatology (Morganti 2010; Morganti et al. 2011; Aranaz et al. 2018) both in pure form as well as important component of corresponding composite materials (Morganti et al. 2012; Coltelli et al. 2019).

Biocompatibility of chitin (Roberts 1992) stimulated the progress in development of wound dressing (Huang et al. 2014; Kojima et al. 2004; Okamoto 1995;

Fig. 24.9 Chitin scaffold show excellent repairing activity in wound healing. (Adapted from Morganti and Morganti 2008)

Singh et al. 2008). In the amazing paper published by Prudden et al. (1970) the history of the beginnings of the chitin application as the wound dressings can be found. Their study clearly showed that NAG was the active component in the application of cartilage in wound healing. In order to check their hypothesis, powdered chitin derived from shrimps and fungi were used directly on wounds. Finally, obtained results validated the chitin's ability to accelerating the wound healing. It has been suggested that the chitin powders were able to release NAG during the digestion of chitin using lysozyme, plentifully occurred in the fresh and healing wounds.

Ohshima and co-workers (1987) successfully studied chitin as a non-woven fabric-type dressing. In order to produce the non-woven dressing, several steps were applied: firstly the preparation of chitin fiber followed by cutting these fibers to appropriate length, then the fibers were dispersed in water contained binders to obtain non-woven sheets which were finally cut to dimensions adequate for a dressing. Such dressing was evaluated to be efficient in treating skin ulcers and burns. Moreover, the dressing has been well adhered to the wound and maintained wound to be dry. Also the treatment of deep ulcers using a chitinous-sponge-like material was possible for longer period of time. The dressing in the form of the sponge-sheet reinforced with the gauze shows good ability to absorb exudates (Ohshima et al. 1991).

Chitin-based therapy has been described by Dr. Collini as follow:

> While at the Mayo Clinic in 1988, I had the opportunity to use the original form of chitin on the skin graft donor sites of 4 patients. My experience was favourable. Each patient had adequate pain relief, the wounds showed suitable drying and complete healing took place in 5-7 days. I found that if topical thrombin was first applied to the donor site, adherence of chitin was almost immediate and no further dressing was necessary. As re-epithelization took place, the chitin painlessly separated from the wound. No side effects were observed and patient satisfaction was dramatic. (Collini 1991)

To improve the wound healing properties, chitin is now combined with various antibacterial compounds including zinc oxide (Sudheesh Kumar et al. 2013) or silver (Madhumathi et al. 2010; Singh and Singh 2014) nanoparticles. For example, Wysokowski et al. (2013b) developed an extreme biomimetic approach as anew powerful tool for the development of β-chitin-based hybrid inorganic–organic nanostructured (chitin/ZnO) dressings in the form of films with special antimicrobial properties against Gram positive bacteria. Consequently, Sudheesh Kumar et al. (2013) developed β-chitin hydrogel/nano ZnO composite bandage using freeze-drying method. In results they prepared bandage with the interconnected micro-porous structure that possesses enhanced the swelling ratio and is beneficial in absorbing exudates from the wound interface.

Very recently, a chitin film containing bFGF (so called basic fibroblast growth factor) with a chitin-binding domain (ChtBD) as wound dressings has been developed by Wang et al. (2017). This construct showed exquisite ability to promote both cellularization and vascularization.

24.3 Chitin Scaffolds for Application in Tissue Engineering

In the orthopaedic applications, chitin has been used in native form as well as in combination with mineral (mostly Ca-based) phases. Maeda et al. (1984) applied chitin in the form of powders, rods, and braided filaments. They found that these forms of chitin were potentially appropriate for temporary synthetic ligaments and sutures for the knee joint.

The method of the calcium deposition onto the porous chitin scaffold by precipitation of the calcium phosphate directly on the chitin scaffold was reported by Wan et al. (1998). Around 55% by mass of Ca was adsorbed on the chitinous scaffold. May be this method might be a useful approach for the production of materials comprising calcium and chitin for tissue engineering purposes.

Mutsenko et al. (2017a, b) reported novel approach to produce "ready-to-use" tissue-engineered products based on chitin isolated from *I. basta* and *A. aerophoba* demosponges. Such 3D scaffolds (Fig. 24.10) showed exceptional biocompatibility and were cytocompatible with human mesenchymal stromal cells (hMSCs) in vitro. Cells cultured onto chitin scaffolds were able to differentiate into the chondrogenic, osteogenic and even adipogenic lineages, respectively.

Chitin based materials have been also proposed as neural scaffolds for effective treatment of peripheral nerve injuries (Freier et al. 2005; Wu et al. 2017). In their experiments with bone marrow mesenchymal stem cell (BMSC) combined with modified biodegradable chitin conduits, Xue et al. (2015) examined they restorative effect after right spinal cord hemisection injury. The conduits provide a microenvironment conducive to BMSC proliferation, migration and differentiation in the spinal cord, protect neurons and promote axonal growth, effectively preventing the invasion of scar tissue. The transplanted BMSCs survive and differentiate into Schwann-like cells, thus promoting nerve regeneration. The results of their study proved that after repair of spinal cord hemisection injury with a biological chitin conduit combined with BMSCs, the spinal cord retained its tubiform shape until 14 weeks. The majority of sleeve conduits degraded by this time indicating the excellent biocompatibility of conduit in the spinal cord. Very recently, Wu and co-workers revealed the chitin-carbon nanotubes hydrogels have good biocompatibility without cytotoxicity and neurotoxicity and accelerate proliferation of neuronal cells and Schwann cells in vitro (Wu et al. 2017).

24.4 Conclusion

Nowadays, the main scientific direction and the key factor driving the rising demand for marine chitin with respect to its application as medically relevant, sustainable biomaterial is the biomedicine including tissue engineering (see for overview Khor and Lim 2003; Kurita 2006; Struszczyk 2006; Kim 2010; Suginta et al. 2013; Alvarez 2014; Badwan et al. 2015; Koch et al. 2015).

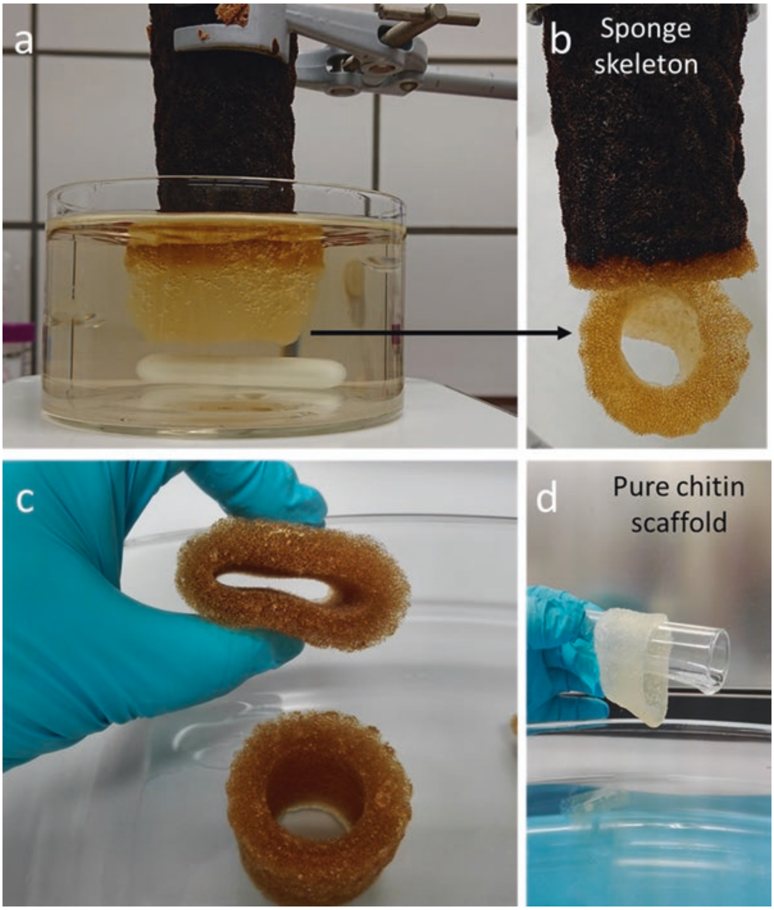

Fig. 24.10 Brief overview of step by step (**a**–**c**) isolation of ready to use 3D chitinous scaffold (**d**) from *Aplysina archeri* marine demosponge

According to the report published on http://www.reportlinker.com/p04806925/Chitin-and-Chitosan-Derivatives-Technologies-Applications-and-Global-Markets.html

> The global chitin and chitosan market should reach $4.2 billion by 2021 from $2.0 billion in 2016 at a compound annual growth rate (CAGR) of 15.4%, from 2016 to 2021.

Monitoring of novel, renewable sources of chitin is still in trend. In contrast to traditionally used powdered chitin of fungi (Wu et al. 2004) and arthropods origin poriferan chitin possess unique advantages to be originally prefabricated in the form of interconnected, anastomosing tubular 3D scaffolds (Fig. 24.11). Due to ability of selected marine demosponges to regenerate their chitin, to grow under marine ranching conditions and to produce bromotyrosine-related secondary metabolites in large amounts the biotechnological, biomimetic and pharmacological potential of these invertebrates remains to be very high.

Fig. 24.11 Ready to use chitinous scaffolds of poriferan origin seems to be intriguing biological materials for plastic surgery and wound healing of skin

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Chapter 25
Collagens from Marine Invertebrates

Abstract Collagens represent the suprafamily of more than 28 main types which have been isolated and described in diverse marine organisms including invertebrates. Especially collagens from glass sponges (Hexactinellida) and horny sponges (Demospongiae) attract attention because of their very ancient origin and unique structural features. Domains typical for collagen have been detected as main structural segments in other structural marine proteins including spongin, gorgonin, byssus, conchiolin. Collagen is to be found as crucial player in diverse mineral-based biocomposites as well as within non-mineralized, cartilage-like tissues of invertebrates. Due to their biocompatibility, marine collagens remain to be biological materials in trend for applications in biomedicine, regenerative medicine, wound healing, cartilage and hard tissue engineering. Soft corals collagens have been recently recognized as a new potential group pf marine collagens for biomedicine.

25.1 Poriferan Collagens

Nowadays, it is recognized that collagens represent the suprafamily of structural proteins (Sorushanova et al. 2018). The presence of collagens in sponges (Porifera), which are recognized as the first metazoans on our planet, confirms their fundamental significance in evolution of all biological systems. It has been suggested (Exposito et al. 2008) that the gene of ancestral fibril-containing collagen arose before the divergence of sponge and eumetazoan lineages. Thus, this event happened at the dawn of the Metazoa when the duplication events leading to the formation of the three fibrillar collagen clades known as A, B, and C occurred. By the way, only the B clade of fibrillar collagens exceptionally preserved their typical structural features from poriferans to human (Exposito et al. 2008). These structural, fibrillar proteins with unique triple-helical segment are most abundant extracellular matrix proteins and, consequently, have been well studied with respect to better understanding of their amino acid composition, structure-function relationship being

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Biologically-Inspired Systems 13, https://doi.org/10.1007/978-3-319-92483-0_25

involved into naturally occurring development of biomineral-containing as well as mineral-free biocomposites. I am not surprised about existence of numerous reviews (e.g. Gross et al. 1956; Bailey 1968; Adams 1978; Tanzer 1978; Garrone 1999; Engel 1997; Exposito et al. 2002; Ehrlich et al. 2018; Jesionowski et al. 2018), books and book chapters (e.g. Garrone 1978; Bairati and Garrone 1985; Ehrlich and Worch 2007; Pallela and Ehrlich 2016; Ehrlich et al. 2018) which have been dedicated especially to collagens of marine sponges origin.

Because of the two recently published reviews on poriferan collagens (Ehrlich et al. 2018; Jesionowski et al. 2018), I see no necessity to discuss additionally the state of the art concerning these collagens here. However, I take the liberty to suggest that the future research in this direction will be focused on diversity in molecular and supramolecular organization, as well as on the better understanding of the origin, genetics, biosynthesis and functions of highly specialized collagens such as:

- Hydroxylated collagen in Hexactinellida as template for biosilica formation (Ehrlich et al. 2008, 2010);
- Glycosylated collagens (Hennet 2019) in demosponges;
- Type IV collagens (Wu and Ge 2019) in demosponges;
- *Chondrosia reniformis* collagen (Fig. 25.1) especially with respect to its practical applications (see for overview Garrone et al. 1975; Heinemann et al. 2006, 2007a, b, c; Fassini et al. 2017; Pozzolini et al. 2015, 2018; Gökalp et al. 2019)
- Collagens within spongin (Ehrlich et al. 2018; Jesionowski et al. 2018),

Also, such perspective scientific and future-oriented technologies as processing (Meyer 2019) and spinnability (Bazrafshan and Stylios 2019) of sponges collagens will be in trend. However, the large scale manufacturing of collagen from spongin (e.g. from commercial sponges) is still limited due to strong insolubility of this biopolymer.

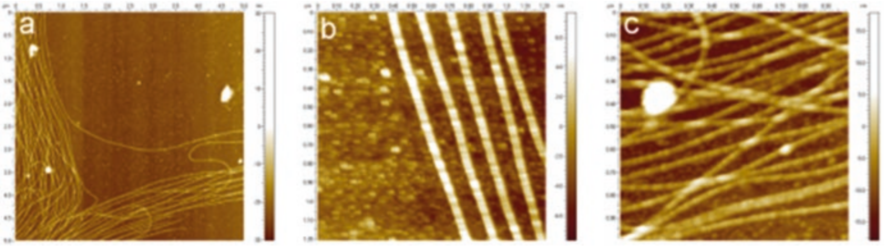

Fig. 25.1 Structural features of the collagen fibers isolated from marine demosponge *Chondrosia reniformis* are well depicted in atomic force microscopy images (**a**–**c**). (Images courtesy Sascha Heinemann)

25.2 Coelenterates Collagens

Collagen polymorphism has been reported in diverse representatives of Cnidaria (corals, sea anemones, sea pens, jellyfish) (Shpirer et al. 2014; D'Ambra and Malej 2015; Mandelberg et al. 2016) and mostly haloplanktonic Ctenophora (comb jellies known as Sea Walnut, Sea gooseberry, Venus' Girdle) (Coelenterata) (Richardson et al. 2009). Some of cnidarians are only a few mm in diameters (i.e. hydromedusae. However, there are species as scyphozoan jellyfish with up to 2 m in diameter (Purcell et al. 2007; Pauly et al. 2009).

Detailed information about diversity of Coelenterate collagens is to found in the work of Franc (1985). In sea anemones, collagen is localized within water rich and thick extracellular layer termed as the mesoglea (Piez and Gross 1959). Corresponding differences in content of basic amino-acids and of hydroxylated proline and lysine have been reported for the collagens of anthozoans origin (Young 1971), the siphonophore *Physalia* (Piez and Gross 1959) as well as on the float of and of the hydroid *Corynactis* (Chapman 1973).

Collagen plays important role in calcification of axial rod of some species pennatulid species (Marks et al. 1949) including *Veretillum cynomorium* (Franc et al. 1974) where the main mineral phase is represented by calcite (Ledger and Jones 1977; Wilbur 1976; Ledger and Franc 1978).

Some kind of flexible collagen fibers, embedded in a proteinaceous, pliant matrix, have been described in octocorals (Kingsley and Watabe 1984) including gorgonians (Coelencerata: Octocorallia) (Leversee 1969; Goldberg 1974; Kingsley et al. 1990).

Recently, it has been proved that some representatives of octocorals (for example *Sarcophyton ehrenbergi*) possess unique, long collagen fibres (unprecedented length of tens of centimeters) (Benayahu et al. 2013, 2018; Sharabi et al. 2014; Orgel et al. 2017). These fibres with concern to their mechanical properties (rigidity and highly reversible extensibility) remain to be very similar to mammalian collagen fibres.

It is worth to highlight that collagen fibers of *S. ehrenbergi* provided structural support in the mesoglea and featured an extraordinary stretching ability, to a high strain (19.4 ± 4.27%), without failing or undergoing irreversible damage. Orgel et al. (2017) pointed that this collagen contains structural domains of both types I and II.

Mandelberg et al. (2016) confirmed the presence of collagen fibres also in the polyps of the reef-dwelling octocoral *S. auritum* using specific histological staining (Masson Trichrome), which were also homologous with that of mammalian collagen.

Sharabi et al. (2014) described the designing of a novel composite material that includes unique long collagen fibers extracted from a soft coral and alginate. The developed bio-composite is characterized by hyper elastic behaviour.

Isolation as well preparations of biocompoistes based on collagen isolated from octocorals are topics for patents i.e. by Benayahu et al. (2013). Taking into account that corals are protected, I have serious doubts regarding legality of utilization of soft corals as a source of collagen for biomaterials science. Definitively soft coral farming technologies should be developed to support drug discovery (Leal et al. 2013) however coral farming could as well be an alternative option to fabricate these unique collagens for biomedical uses (Benayahu et al. 2018). Although captive breeding and propagation of corals is a well-known activity among aquarium hobbyists, the connection between coral science and aquaculture is still poorly developed (Leal et al. 2014).

Collagen of edible for human quality has been reported in diverse species of jellyfisch (e.g. *Stomolophus meleagris*, *Lobonema smithi*, *L. gracilis*, *Nemopilema nomurai*, *Rhopilema esculentum* (Nishimoto et al. 2008), where it content can rich up to 40% of the dry weight (Miura and Kimura 1985; Nagai et al. 1999). As reported by Richardson et al. (2009): "*Jellyfishes have been eaten by humans since 300 AD in China, and about 425,000 tonnes y*$^{-1}$ *are harvested globally (1996–2005) for human consumption in Southeast Asia. Jellyfish fisheries exist in 15 countries, including China, India, Indonesia, Japan, Malaysia and the Philippines, with export industries in Australia and the USA*". According to Khong et al. (2016), "*total collagen content of edible jellyfish varied from 122.64 to 693.92 mg/g D.W., accounting for approximately half its total protein content*".

One of the largest jellyfish with species a bell diameter of up to 1.5 m is represented by *Nemopilema nomurai* (Scyphozoa) (Fig. 25.2). These up to 200 kg by the weight species are able to build dense aggregations which swim near coastal waters of Japan and represent potential source of collagen as well (Iizumi 2004; Honda et al. 2005, 2009; Honda and Watanabe 2007).

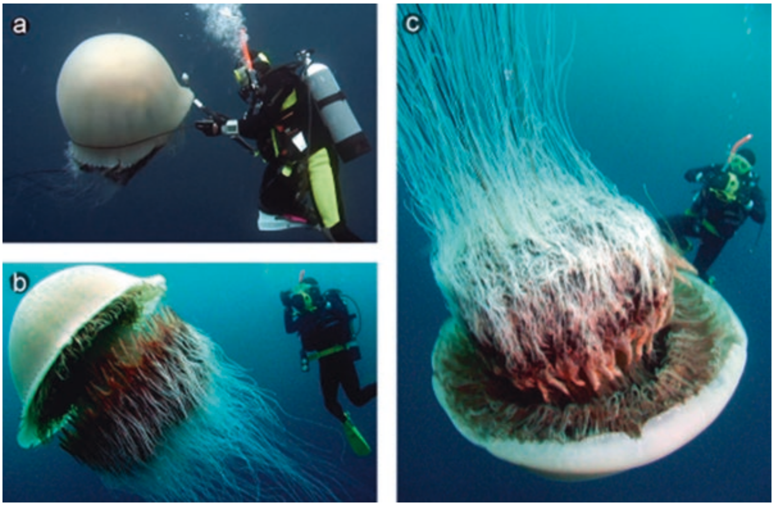

Fig. 25.2 Giant jellyfish *Nemopilema nomurai* (**a** and **b**) rich up to 1.5 m in diameter (**c**) and possess high potential as source of special collagen. (Images courtesy Naoto Honda)

Hsieh et al. (2001) reported the jellyfish processing as follows:

> *Jellyfish are cleaned with sea water, scraped to remove mucus membranes and gonadal material. Traditional methods of processing involve a step-wise reduction of the water content using salt (NaCl) and alum ($AlK[SO_4]_2$ x 12 H_2O). A salt mix containing about 10% alum is used for initial salting of jellyfish, using about 1 kg salt-alum mix for 8–10 kg of jellyfish. Salted jellyfish are then left in the brine for 3–4 days, followed by several transfers to another container salted with a fresh mix containing a smaller amount of alum. The salted jellyfish can then be heaped and left to dry on a draining rack at room temperature for 2 days, and the heap is turned upside down several times during that period to allow excess water to drain out through compression from its own weight. The entire process requires 20–40 days to produce a salted final product with 60–70% moisture and 16–25% salt. The processed jellyfish has a yield of about 7–10% of the raw weight depending on the species and processing formula.*

Diverse modern isolation and purification approaches for jellyfish collagens including their characterisation have also been recently reported (Jin 2008; Caleo et al. 2009; Shen et al. 2009; Addad et al. 2011; Zhang et al. 2014; Miki et al. 2015; Cheng et al. 2017; Khong et al. 2018).

Additionally to their use as food, jelly fish collagens recently attracted attention due to their potential for diverse applications in biomedicine (Song et al. 2006; Kämmerer et al. 2017; Felician et al. 2018, 2019; Widdowson et al. 2018), tissue engineering (Hoyer et al. 2014; Sewing et al. 2017), and protein delivery (Calejo et al. 2012).

Also jelly fish collagen-based hybrid and biphasic composites are still in trend (see for details Jeong et al. 2007; Pustlauk et al. 2016, 2017; Bernhardt et al. 2018).

It is well recognized that there is no risk of infection with bovine spongiform encephalopathy (BSE)) or foot and mouth disease (FMD) in case of the use of jellyfish collagens. However, there are certain worries due to both possible toxicity and allergenic activity of jelly fish collagens (Hsieh et al. 2001; Nishimoto et al. 2008; Putra et al. 2014; Cañas et al. 2018).

25.3 Molluscs Collagens

Similarity in structure and chemistry of connective tissues between the molluscs and vertebrates (Bairati 1985) suspects corresponding similarities for their collagens (Tanzer 1978). For example, the heterotrimeric collagen isolated from the cranial cartilage of the cuttlefish, (*Sepia officinalis*), remains to be similar to minor collagens of vertebrates (Sivakumar and Chandrakasan 1998). This phenomenon has been also describes for two collagens isolated from the mantle muscle and skin of the common squid *Todarodes pacificus* (Mizuta et al. 2009). Both possess heterotrimeric chain compositions termed as $[\alpha1(SQI)]_2\alpha2(SQ\text{-}I)$ and $[\alpha1(SQ\text{-}II)]_2\alpha2(SQ\text{-}II)$, respectively.

The collagens of diverse types have been were isolated and described mostly from representatives of Bivalvia (Mizuta et al. 2004, 2005; Tabakaeva et al. 2018) as well as from Cephalopoda (Shadwick 1985; Bairati et al. 1989, 1999; Mizuta et al. 1994a, b; Rigo and Bairati 1998, 2002; Sivakumar and Chandrakasan 1998; Nagai et al. 2001; Sivakumar et al. 2003; Mizuta et al. 2009).

Recently, the amount of collagen of two marine species of edible bivalve molluscs has been measured in the range between 30.5 and 39 mg/g wet weight, accounting for about the half of their total protein content (Tabakaeva et al. 2018). However, the most industrially oriented sources of molluscan collagens are cephalopods with their well-developed connective tissues (Sivakumar et al. 2003) including mantle (Krieg and Mohseni 2012; Cozza et al. 2016).

Highly resistant to hot water extraction collagen-containing crude fractions have been obtained from the mantle muscles of such cephalopods as *Sepia esculenta*, *S. longipes*, *Photololigo edulis*, *Todarodes pacificus*, and *Sepioteuthis lessoniana*, using extraction with 0.1 M NaOH (Mizuta et al. 2009). Descriptions concerning diverse extraction procedures of squids collagens are well represented in the recent literature (see for details Uriarte-Montoya et al. 2010; Ramasamy et al. 2014; Nakchum and Kim 2016; Coelho et al. 2017). Also biomimetic (Torres et al. 2014) and biomedical potential (Dai et al. 2018) of squids collagens, especially in wound healing (Jridi et al. 2015; Veeruraj et al. 2019) and tissue engineering (Coelho et al. 2017) was opportunely detected.

25.4 Echinoderm Collagens

The reason why collagens of echinoderms origin attracted significant scientific attention since first study by Jickeli (1884) is based on two events. First of all, diverse collagen-rich representatives of this phylum are edible (for overviews see Matsumura 1974; Bailey et al. 1982; Saito et al. 2002; Cui et al. 2007). Secondly, their collagen fibrils remain to be a good model in comparative studies with vertebrate's collagens (Trotter et al. 1994; Thurmond and Trotter 1996; Rainey et al. 2002; Heim et al. 2006; Eppell et al. 2006) and, consequently, are studied as source for biomedical applications (Ferrario et al. 2017). Here, I recommend enjoying the review prepared by Szulgit (2007) with intriguing title "*The echinoderm collagen fibril: a hero in the connective tissue research of the 1990s*".

Collagens have been isolated, purified and characterised in such echinoderm as sea urchins (Nagai and Suzuki 2000; Benedetto et al. 2014; Barbaglio et al. 2015), sea stars (Kimura et al. 1993; Schwertmann et al. 2019), and mostly in sea cucumbers (Holothuria) (Butler et al. 1987; Trotter et al. 1994; Liu et al. 2010, 2018; Qin et al. 2016) including their integument (Abedin et al. 2013), skin (Adibzadeh et al. 2014; Liu et al. 2017) and body wall (Zhong et al. 2015; Dong et al. 2019).

There are reports on chemically modified naturally occurring collagen fibres in echinoderms (i.e. with proteoglycans) (Graham et al. 2004). Recently, collagen modified with fucosylated chondroitin sulfate (fCS) has been detected in sea cucumber *Apostichopus japonicas* (Wang et al. 2018). This polysaccharide was covalently attached to collagen fibrils via O-glycosidic linkages and located within their gap regions in the form of ellipsoidal or globular dots well visible on TEM images. The presence of fCS is responsible for the negative charge of the in this way decorated collagen fibrils.

One of the well-studied directions with respect to echinoderm's collagen is related to the unique connective tissues (Mutable Collagenous Tissues, MCTs) (Jickeli 1884,) which are responsible for rapid reversible changes in the tissue mechanical properties (Ovaska et al. 2017). MCT is able to change its mechanical features in a time scale between less than 1 s to a few minutes (Wilkie 1984). However, the experimental results show that the mechanical flexibility of MCT is a consequence of to the shaping of the interactions between the components of the extracellular matrix. The occurrence of the muscle within some mutable collagenous structures implies the suggestion that contractile cells can have an essential role in the process of the variable tensility, thus a re-evaluation of the MCT concept is essential (Wilkie 2002; Wilkie et al. 2004; Wilkie 2005). It was determined that, the majority of the mutable collagenous structures demonstrate reversible stiffening and de-stiffening (Ribeiro et al. 2011; Mo et al. 2016), and do not have a muscle cells. According to the modern view, the sliding action of collagen fibrils in the MCT is regulated by the nerve-control system (Goh and Holmes 2017).

Nowadays, echinoderms have been proposed also as an innovative source of collagen for designing of collagen barrier-membranes for so called Guided Tissue Regeneration (GTR) (Ferrario et al. 2017).

25.5 Conclusion

Principally, the global collagens research now is concentrated on such modern biomedical directions (Gu et al. 2019; Liu et al. 2019; as 3D printing using collagen-based materials and dressings for wound healing (Mandla et al. 2018; Pallaske et al. 2018), cartilage- (Irawan et al. 2018; Freedman and Mooney 2019) and hard tissue engineering (Marques et al. 2019), nerve conduits (Rbia et al. 2019) and dermatology (Choi et al. 2019).

I have no doubts that there is a necessity to prepare the separate book on diversity, genetics, structural biology, biochemistry as well as materials science of collagens as fundamental biological materials from marine invertebrates in the future. Consequently, the goal of this chapter was to represent and to discuss only some selected examples which seem to be interesting for technology and biomedicine. Collagens of marine vertebrates origin have been described by me earlier (Ehrlich 2015).

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Part VI
From Extreme Biomineralization to Extreme Biomimetics

Chapter 26
Extreme Biomimetics

Abstract The origins of extreme biomineralization are found in the first ancestral unicellular organisms that evolved under the harsh environmental conditions of ancient oceans. Such conditions also allowed adaptation of unique extremophilic and polyextremophilic biomineralizers, which are still found today Psychrophilic, thermophilic, anaerobic, alkaliphilic, acidophilic, and halophilic conditions, as well as forced biomineralization arising in environments with very high or toxic metal ion concentrations are considered. In most cases the mechanisms of these special biomineralogical phenomena remain unknown. Nevertheless, extreme biomineralization provides crucial information for progression in extreme biomimetics. This exciting area of modern research could be the next step in creating the next generation of composites using organic-templating materials of marine invertebrates origin under biologically extreme laboratory conditions.

After pioneering works by Heinz A. Lowenstam on biomineralization as global natural phenomenon, experts have reviewed current knowledge in numerous books and papers. However, the Achilles' heel of this field is that most biomineralization investigations are carried out under ambient conditions, and biologically extreme conditions have been overlooked.

Under which extreme conditions do biomineralizers occur today? Extreme environments by definition are characterized by "conditions that are far outside the boundaries in which most organisms live comfortably. Conditions include: pH, air pressure, temperature, salinity, radiation, dryness (desiccation), and oxygen levels" (Amils et al. 2007). Underwater hydrothermal vents, geothermal waters and terrestrial hot springs all fall under this definition due to acidic or alkaline pH, high hydrostatic pressures and temperatures, the presence of toxic compounds, a reducing atmosphere, and the absence of light. Despite these harsh conditions, it has been reported that a number of various thermophilic and hyperthermophilic organisms (Taylor and Roterman 2017), including biomineralizers (mollusks, crustaceans, worms), are widely distributed at these specific environmental locations (Juniper et al. 1992; Cary et al. 1998; Luther et al. 2001; Warén et al. 2003; Zbinden et al. 2003; Suzuki et al. 2006; Le Bris and Gaill 2007; Watanabe et al. 2018; Lee et al. 2019).

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Special kind of biomineralization, so called forced biomineralization could be proposed to exist in such environments. Forced biomineralization is the case of induced biomineralization when toxic concentrations of metal ions forced the development of biomineral-based structures (shells, layers etc.) that contribute to the survival of extremophiles. Biominerals formed due to forced biomineralization may serve to protect cells, for example, from metal toxicity.

Psychrophilic biomineralization is intertwined with cryobiology, as well as exobiology and includes chemical, biogeochemical and biochemical processes at very low temperatures. Diverse psychrophilic biomineralizers are well distributed on Earth and represent a community of active players in biocalcification, biosilicification, and iron related biomineralization (see for details Ehrlich 2017). For example, broad species diversity of biominerals producing corals, molluscs, echinoderms, crustaceans and sponges is known for Antarctic seas (Arndt and Swadling 2006; Clark et al. 2007; Chu and Leys 2010; Flores et al. 2012; Assmy et al. 2013; Sleight et al. 2015).

Today the adaptation of principles derived from biomineralization research to the synthesis of inorganic materials with controlled properties, is one of the most extensively investigated topics in modern materials science in relation to biomimetics. Biomimetic mineralization deals with utilization and selection of biological or synthetically created macromolecules with templating activity, which predestinate nucleation, thermodynamic and kinetic of the inorganic crystal growth (Xu et al. 2007).

The “traditional biomimetics”means reactions at near-neutral pH and in the range of temperatures between 20 °C and 40 °C .It is wrongly assumed that, for example, proteins will be not stable enough to be applicable as templates at temperatures higher than 40 °C. However, above-mentioned examples of polyextremophilic biomineralizers shine new light on the main concepts of biomimetics and encourage us to rethink the role of specific organic templates in biomineralization and bioinspired processes under special conditions.

Extreme biomimetics fits perfectly into the basic assumptions of “*soft solution processing*” (Yoshimura and Suchanek 1997), which is a very general concept and covers wide range of synthetic procedures at temperatures below 200 °C.

26.1 Templates for Extreme Biomimetics

It is well recognized that polysaccharides play a crucial role as templates in biomineralization to be found in skeletal structures of broad diversity of marine invertebrates (from unicellular protists to giant crustaceans). The aminopolysaccharide, which is most commonly found in the biomineralized tissues of such marine organisms as diatoms, corals, mollusk, coralline alga, crustaceans, polychaetes, freshwater, and marine sponges is chitin (see corresponding chapter above). This biopolymer is proposed as unified template for biomineralization in nature (Ehrlich 2010). However, the mechanisms of the templating activity of chitin in biosilicification in psychrophilic glass sponges and diatoms as well as in thermotolerant diatoms are still unknown. Open questions with regard to the role of chitin also in multiphase biomineralization still exist.

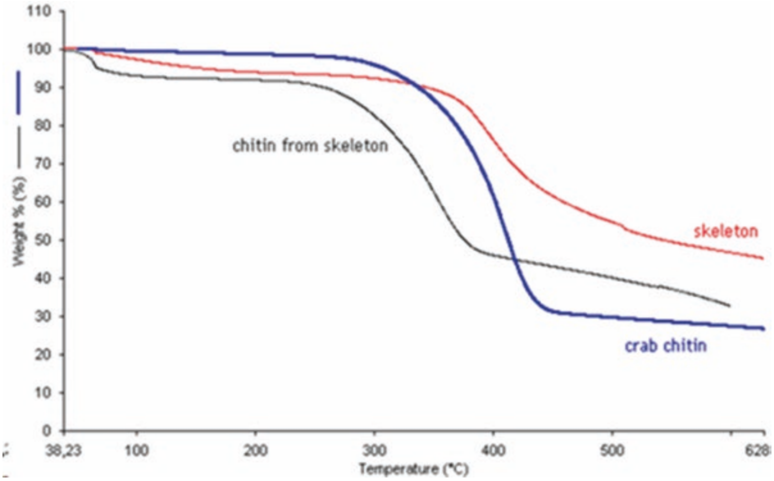

Fig. 26.1 Thermogravimetric features of the crab chitin, Verongiida sponge chitin and Verongiida sponge skeleton. (Courtesy Dawid Stawski)

The crucial property of chitin with respect to extreme biomimetics is its thermal stability. The first studies related to chitin thermal stability were performed by Köll and Metzger (1979) and Köll et al. (1991). Authors proved by thermogravimetry, that the two-step degradation process of chitin with an acetylation degree is 62% when started at 200 °C. Stawski et al.(2008) confirmed that the thermal stability of chitin is associated with crystallinity and that some species related chitins can be thermally stable even up to 400 °C (Moussout et al. 2016) (Fig. 26.1).

The above mentioned unique thermal and hydrothermal stability of chitin predestinates its potential as organic template in wide range of synthesis reactions occurring under hydrothermal conditions in vitro. Such functional groups as (–OH, C=O, and –NH) in its molecule can interact with a corresponding precursor solution by forming covalent bonds. Alternatively, chelation reactions under hydrothermal conditions of metal ions lead to formation of hybrid organic-inorganic materials. Diverse examples of how to use chitin in hydrothermal synthesis of novel composites which contain silica (Wysokowski et al. 2013a, b; zirconium oxide (Ehrlich et al. 2013; Wysokowski et al. 2013c), zinc oxide (Wysokowski et al. 2013d), and iron oxide (Wysokowski et al. 2014, 2015a), have been recently reported. It was possible to develop chitin-based composite materials in vitro which even never have been reported before. Thus, the thermal stability of nanofibrillar sponge chitin recently allowed us to obtain from the precursor germanium ethoxide a crystalline phase of hexagonal GeO_2 at 185 °C. This 3D construct resamples the shape, size and form of the chitinous centimeter-large sponge skeleton (Wysokowski et al. 2015b). Such solid GeO_2-chitin-based composite possess functionalized surfaces with intriguing photoluminescence and fluorescence features.

Life under extreme conditions (i.e. hydrothermal vents) suggests existence of corresponding thermostable proteins. The understanding the mechanisms contributing to their exceptional stability has been a long-standing topic for investigation (Kumar et al. 2000). Such understanding, of the proteins of the thermophilic organism's origin, is not only essential for a theoretical description of the physicochemical principles that underlie their stability at the molecular scale, but is also crucial to get insight into their role in biomineralization. Finally, it also opens new possibilities in designing efficient enzymes that can work on service of extreme biomimetics and modern materials science. The key to protein function, in extreme temperature environments, is the maintenance of an appropriate balance between molecular stability on the one hand and structural flexibility on the other.

Advances in knowledge regarding thermophilic proteins allows for Kobatake and co-authors (2000) to publish a milestone paper reporting gene engineering synthesis and creation of an extremely thermostable protein with biological activity (retained over 90%) after autoclaving at 120 °C. Nowadays, thermostable proteins are used in broadly defined biotechnologies (including the food industry, biocatalysis etc.). Latza et al.(2015), by using thermostable "*suckerins*" – the proteins isolated from sucker ring teeth of some cephalopod molluscs, opened the way for the molecular creation of peptide-based thermoplastic biopolymers applicable, for example, in the modern 3D printing of composite materials. Suckerins isolated from sucker ring teeth from cephalopods and are first known reported exception of thermoplastic biopolymers (see for review Andreeßen and Steinbüchel 2019).

Structural proteins with high levels of thermostability (up to 300–350 °C) as silk (Ishikawa et al. 1972) and spongin seems to be extraordinarly interesting for extreme biomimetics. Unfortunately, there are no reports on practical application of marine silk for the aims of extreme biomimetics. However, spongin of the poriferan origin seems to be excellent candidate.

Spongin definitively can be ordered to the "collagen suprafamily" which representatives are still in the focus of modern science due to their unique nanofibrillar organization, biomechanical behavior and high potential for materials engineering (Jesionowski et al. 2018; Ehrlich et al. 2018).

The structure of collagen-like spongin has multiple levels comprising single fibers up to 100 μm thick, composed of nanofibers, which are combined into complex hierarchical 3D networks of high macroporosity (Fig. 26.2) exhibiting specific structural and mechanical properties (for review see Jesionowski et al. 2018). In view of spongin's thermostability up to 360 °C and its resistance to acids, spongin-based scaffolds have only recently been used in hydrothermal synthesis reactions aimed at developing novel Fe_2O_3- and TiO_2-based composites for electrochemical and catalytic purposes (Szatkowski et al. 2017, 2018). Preliminary experiments on the carbonization of spongin scaffolds at 650 °C have demonstrated their mechanical stability and excellent chemical functionalization, allowing their use in the design of an effective centimeter-scale MnO_2-based supercapacitor (Szatkowski et al. 2017) (Fig. 26.3).

These unique properties of biological proteinaceous material motivated us to develop new 3D carbonized spongin scaffolds capable of withstanding temperatures as high as 1200 °C, without loss of their hierarchical architecture. We hypothesized

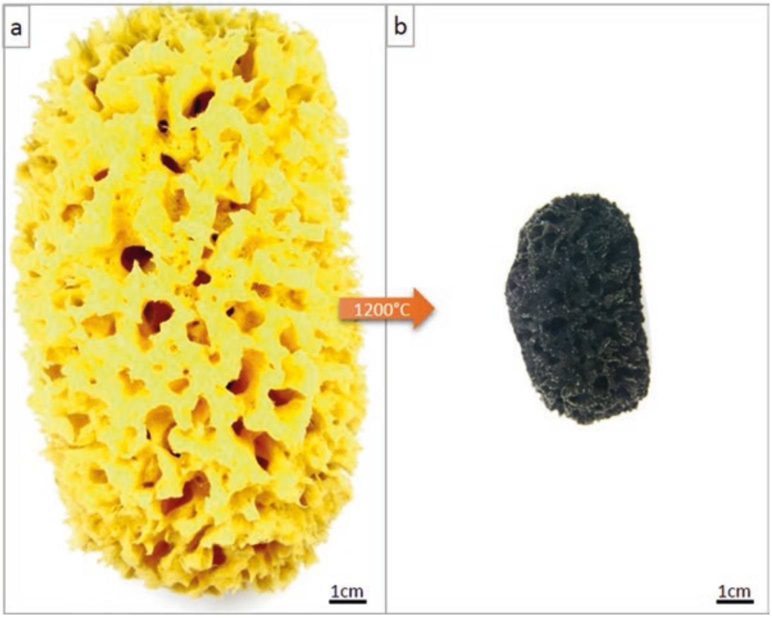

Fig. 26.2 Macroporous spongin-based scaffold (**a**) remains to resemble it inner structure even after heating up to 1200 °C in inert atmosphere, however lost up to 70% of its volume (**b**)

that spongin would resist such high temperatures without loss of its form and structural integrity, and that its specific surface area would probably increase due to the appearance of nanopores, favoring its further functionalization as a catalyst.

26.2 Conclusion

Extreme biomimetics represents a scientific niche, fostering the study of natural and artificial phenomena that occur below the human comfort zone. This scientific direction born at the cross-roads of biology, biochemistry, chemistry, physics and materials science in 2010 in Germany (Ehrlich 2017) (Fig. 26.4). Work in these research fields requires a radically new approach, to create inorganic-organic hybrid composites under harsh chemical and thermal microenvironments, using as templates thermostable and chemically resistant biopolymers with naturally prefabricated three-dimensional architecture. The field has gained big attention, especially in the development of hierarchically structured 3D composites using renewable, non-toxic and biodegradable organic scaffolds isolated from marine sponges.

The sources for inspiration in Extreme Biomimetics are represented by extremophiles including: anaerobes, thermophiles, acidophiles, alkaliphiles, halophiles and polyextremophiles. Their survival is linked to the production of biominerals under

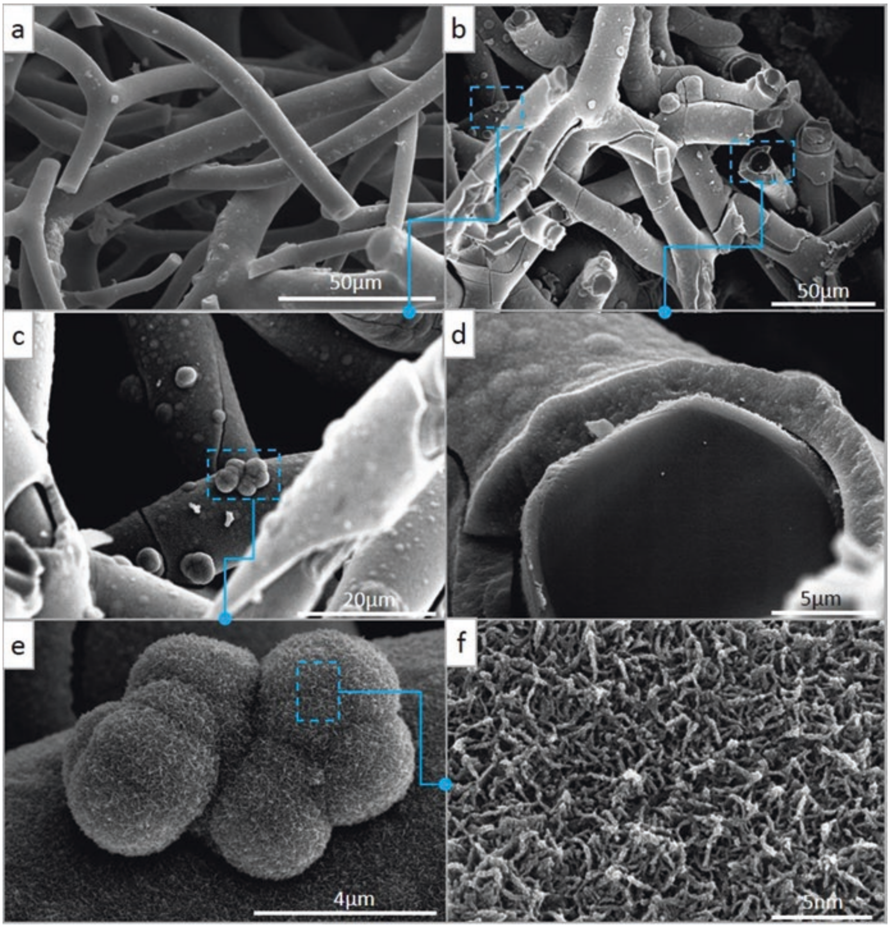

Fig. 26.3 Therrmal stability od spongin open the unique way to develop carbonized sponge-like structures (**a**) and cover them with Mn-oxide nanocrystals (**b**–**f**) using hydrothermal synthesis reactions. Finally, the designed construct showed excellent properties as catalyst. (For details see Szatkowski et al. 2018)

toxic concentrations of metal ions, as they live under complex environmental extremes such as anaerobic-acidic, thermal-alkaline, etc. conditions. Consequently, their skeletal structures are examples of exceptional biocomposites where biopolymers with high resistance to harsh chemical and thermal extreme conditions are the specialized templates. Rethinking of the unique chemical and biochemical pathways of biomineralization processes performed by organisms living in extreme environmental niches will lead to better understanding of their survival strategies. In parallel, it stimulates the creation of innovative tools for the synthesis of new-generation of advanced materials with entirely novel physicochemical properties.

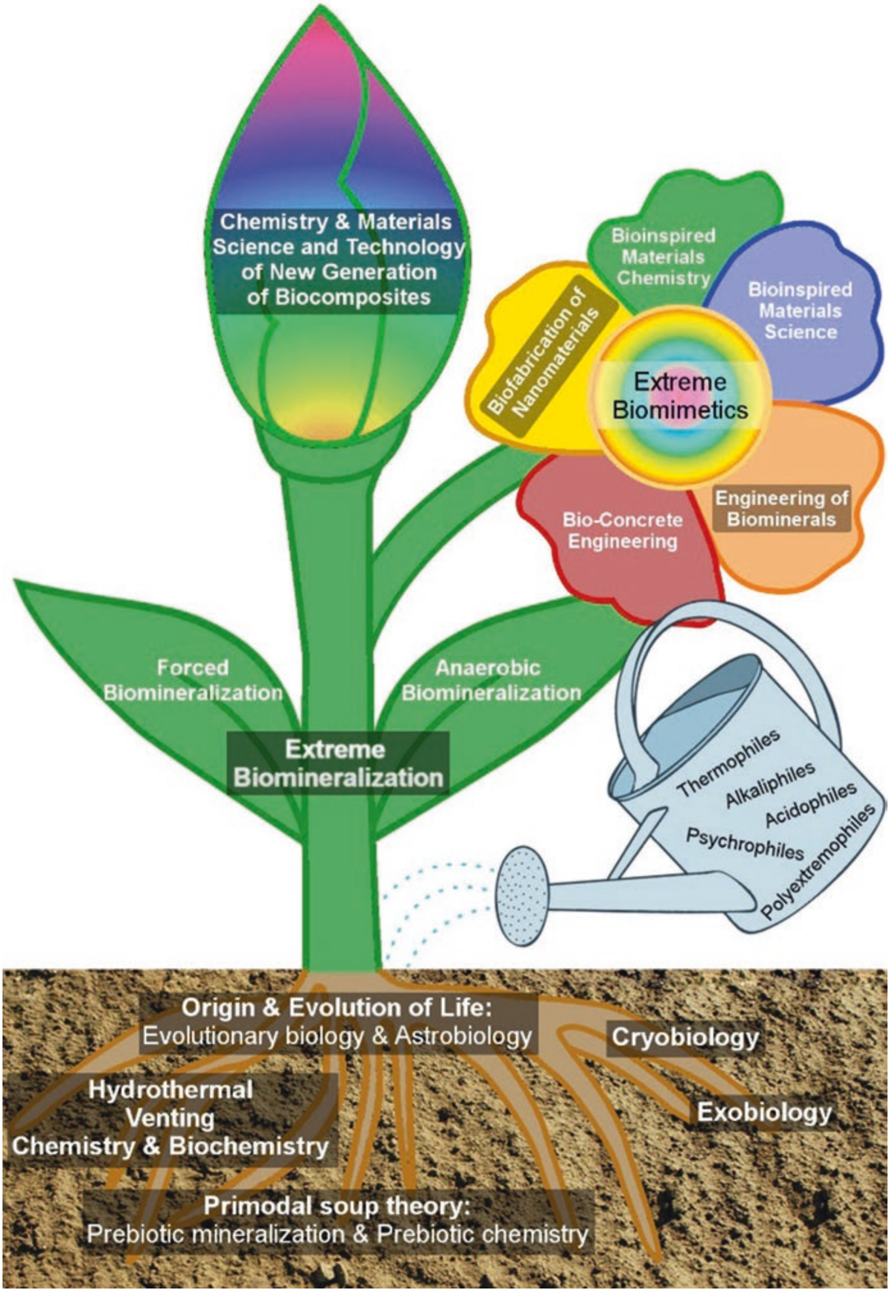

Fig. 26.4 Extreme biomimetics has been created as interdisciplinary scientific direction. (For details see Ehrlich 2017)

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Chapter 27
Epiloque

Biomaterialogy is an interdisciplinary research field dealing with the phenomena of biomineralization and mineral-free sclerotization (rigidification) of soft tissues into diverse skeletal structures which have been naturally occurring since life began (Mann 2001). Today, biomineralization is defined as an ancient (Conway-Morris 2003; Wähtershäuser 2007), fundamental and highly dynamic biological process by which living organisms produce minerals with multifunctional properties. These include hardening existing subcellular organic templates, scaffolds and tissues, producing protective armours and shells against external environmental damages (predators, UV irradiation, toxic metals, etc.), as well as to help organisms with magnetic navigation (Monteil et al. 2019).

The mechanisms of biomineralization in diverse uni- and multicellular marine invertebrates remain hotly debated, especially because the knowledge that can be extracted from biomineralization is the driving force for recent progress in biomimetics, bioengineering and biomedicine. One of the challenging tasks here is how to translate biological, structural, and mechanical features of marine biocomposites into an engineered material. For example, how to mimic the simultaneously hard, hyper-extensible and durable cuticle of mussel that shields byssal threads from environmental exposure (Monnier et al. 2018) in an artificial composite material which should be designed for similar functioning but *in vitro*?

Hereby, I take the liberty to propose several future directions that highlight the strong potential for applications for biological materials of marine origin.

27.1 Biomedicine and Bioengineering

A novel trend for biomedical and bioengineering applications is to discover so called naturally prefabricated, ready-to-use (Klinger et al. 2019) skeletal constructs in the form of porous 3D structured scaffolds (Neto and Ferreira 2018) with anastomosing, hierarchical microarchitecture and large special surface areas within broad

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diversity of marine invertebrates. Definitively, only those organisms which are renewable (i.e. sponges) and cultivable under marine ranching conditions (i.e. molluscs shells), and which do not have negative impacts on existing environmental niches, are of potential interest.

The need for 3D scaffolds of marine origin for tissue engineering, remodelling, repair and regeneration of bone tissue is still high (Diogo et al. 2018). Novel data have been reported recently in the review by Macha and Ben-Nissan (2018). Some researchers still trying to use naturally occurring biominerals (i.e. biosilica from sponges) (Gabbai-Armelin et al. 2019) as a supporting biomaterial. Others succeeds in incorporation of the biopolymer spongin into hydroxyapatite (Parisi et al. 2019). Even "*organo-biomaterials*" which are based on water soluble extracts from nacre have been recently developed (Zielak et al. 2018). There are no doubts that we could speak about success in practical application of such biocomposites only after long-term studies.

Studies on biomimetic adhesives inspired by the marine glues will also be actively continued. Recently, glues fabricated by the marine sandcastle worm have been used as a model system for development of a biocompatible sealant for soft tissue repair (Costa et al. 2019). Also efforts in functionalization of biomimetically developed hydrogels (i.e. that from proteinaceous jaw of *Nereis virens* marine worm) have been based on understanding of the metal-ions dependent sclerotization mechanisms which occur *in vivo*. It was shown that, especially divalent Zn cations are responsible for reversible sclerotizing of the hydrogels due to their ability to form coordinated cross-links (Gupta et al. 2018).

An absolutely novel trend for the application of marine biomaterials has been appeared recently. I refer to their application in 3D bioprinting for manufacturing of structural bioscaffold. For example, alginate-based hydrogel has been used as a 3D bioprinting system with high cell viability (Zhang et al. 2018). Another marine biopolymer, aneroin, the silk-like protein originating from the sea anemone has been used as a biocompatible and mechanically integrative bioprinting ink with good printability for designing of the vascular graft for a simulated human ear (Park et al. 2019).

27.2 Marine Biomaterials and Microplastic

Nowadays, our seas and oceans represent the giant aquatic space where man made materials in all possible dimensions, sizes and origins (Jabeen et al. 2017; Kroon et al. 2018) meet biological materials represented in this book. Unfortunately, this "meeting" leads to dramatic ecological events such as contamination with marine debris (Fig. 27.1) (Moore et al. 2001, 2002) where microplastics play the crucial role due to the presence of diverse toxic molecules such as polystyrene-based (PS) oligomers, phenanthrene, polychlorinated biphenyls, styrene mono-, di- and trimers, etc. Information about the "progress" in this kind of contamination can be obtained through the comparison of data from such documents as *Science* paper by Carpenter and Smith from 1972 entitled "*Plastics on Sargasso Sea surface*" and recent work by Jambeck et al. (2015) entitled "*Plastic waste inputs from land into*

Fig. 27.1 Marine biological materials meet man-made materials. Plastic and microplastic pollution in the ocean (**a**) is a source of anxiety for Captain Charles Moore from Algalita Marine Research Foundation (**c**). Nature struggles to survive and to adapt to the changes forced on it by these synthetic materials (**b**), but is this truly the future of our oceans?

the ocean", or "*Plastic Debris in the World's Oceans*" published by Greenpeace on the November, 02, 2006 and a corresponding report from the United Nations website from 2016: https://www.un.org/Depts/los/global_reporting/WOA_RPROC/Chapter_25.pdf

Numerous modern reviews concerning distribution and influence of micro-, meso- and nanoplastics in diverse marine localities have been already published (Andrady 2011; Browne et al. 2011; Cole et al. 2014; Eriksen et al. 2014; Thompson 2015; Galloway and Lewis 2016; Lönnstedt and Eklöv 2016; Taylor et al. 2016; Auta et al. 2017; Cózar et al. 2017; Boucher and Friot 2017; Salvador Cesa et al. 2017; Jamieson et al. 2019).

Calculations of perspectives with respect to increase of microplastics-based pollution in marine environments have been recently made by Isobe et al. (2019) in their *Nature Communications* paper entitled "*Abundance of non-conservative microplastics in the upper ocean from 1957 to 2066*"(!!!).

The dimensions of selected microplatics, for example, that with diameter of 12–16 μm (De Falco et al. 2019), suggest that they could pass through wastewater filtering systems and pose a threat for sea habitants. Examples of marine invertebrates ingesting microplastics are well known (Goldstein and Goodwin 2013; Jamieson et al. 2019). Some crustaceans are known for "*turning microplastics into nanoplastics through digestive fragmentation*" (Dawson et al. 2018). Consequently, it is not surprising that in the nearest future we will isolate these from marine invertebrates as well, and study novel and unique hybrid materials which originate from both biological materials and nanoplastics as products of metabolismus. For example, exopolxsaccharide (EPS)- microplastic- based hetero-aggregates have been recently reported for two marine microalgae (*Tetraselmis* sp. and *Gloeocapsa* sp.) (Cunha et al. 2019). The *Gloeocapsa* sp. was able not only to adhere onto the surface of microparticles, but also incorporated them into the EPS. This leads to appearance of novel composite material with unknown properties.

To take an optimistic view, I hope that these novel composites will not open the door for more dangerous scenarios.

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