

Chapter 5

The Mineralized Exoskeletons of Crustaceans

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Abstract The crustaceans constitute one of the oldest arthropod taxa, from which insects later evolved (Giribet et al., Nature 413:157–161, 2001; Regier et al., Nature 463:1079–U1098, 2010; Giribet and Edgecombe, Annu Rev Entomol 57:167–186, 2012). A typical feature that characterizes the Crustacea is their mineralized chitinous exoskeleton. The reinforcement of the chitinous exoskeleton with calcium salts and the formation of inorganic-organic composite materials by the crustaceans represent one of the oldest biomineralization mechanisms to have evolved in animals. The basic function of mineralization is to enhance the mechanical strength of the skeleton. When compared to other animals with mineralized skeletons, crustaceans face two distinct challenges inherent in the fact that their skeleton is external: first, the animal's locomotion abilities must not be compromised by its mineralized exoskeleton, and second, the growth mode by periodic molting requires intensive mobilization of minerals during the resorption of the old cuticle and the rapid recalcification of the new cuticle. These two demands are among the prime determinants that govern the various calcification patterns in Crustacea. This review focuses on the mineralogical aspects of the crustacean exoskeleton with emphasis on the controllable parameters of the mineral phase properties, namely, the degree of mineralization, the degree of crystallization, the phosphate/carbonate ratio, and the involvement of proteins. It also explores potential biomimetic applications inspired by the crustacean exoskeleton against the background of similarities between crustaceans and vertebrates, namely, both groups are the only groups in the animal kingdom that combine advanced locomotion with jointed mineralized skeletons. In addition, many crustaceans have the ability of calcium phosphate mineralization, like vertebrates. These similarities provide unique opportunities to compare different evolutionary solutions to similar functional challenges that, in turn, can inspire biomimetic approaches to the development of synthetic bio-composites for various skeleton-related medical applications.

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5.1 Introduction

The Crustacea and the Hexapoda are the two major groups of the Arthropoda. Recently, it has been shown that the Hexapoda are phylogenetically more associated with the Crustacea (and not with the Myriapoda as was previously assumed (Manton and Harding 1964; Wheeler et al. 2004)), and can actually be considered, phylogenetically, as “terrestrial crustaceans” (Regier et al. 2005). The two groups are thus now grouped into a clade known as the Pancrustacea (or the Tetraconata). The crustaceans, which inhabit marine, freshwater, and terrestrial habitats, are a highly heterogeneous group of animals in terms of morphology and living habits—from large lobsters and crabs that can exceed 1 m at maximum diameter (e.g., the Japanese spider crab) to microscopic Tantulocarida of less than 100 μm (e.g., *Stygotantulus stocki*), from agile shrimps to sessile barnacles, from benthic walkers to pelagic swimmers. A fundamental characteristic of crustaceans is their mineralized cuticle, and the heterogeneity of the crustaceans is reflected in their diverse cuticle properties, which differ not only between species but also between different body parts. The crustacean exoskeleton comprises four layers which are, from the outermost inward: epicuticle, exocuticle, endocuticle, and the membranous layer (Fig. 5.1). The outermost epicuticle is a thin layer, composed of proteins and lipids, that provides a mechanical barrier against pathogens and provides an impermeable layer (Mary and Krishnan 1974) that enable better ionic regulation. In terrestrial crustaceans (e.g. Isopoda) the epicuticle serves as a waterproofing layer (Hadley and Warburg 1986) probably with other functions (Vittori and Štrus 2014). The epicuticle is less calcified than the underlying exo/endocuticle (Dillaman et al. 2005) whereas the minerals are usually restricted to the pore canals (Hegdahl et al. 1978). The underlying exocuticle and the inner endocuticle represent the major part of the cuticle. These are the most mineralized layers and thus govern the mechanical properties of the crustacean exocuticle. On the proximal side, below the endocuticle, lies a non-mineralized “membranous layer” that overlies a single layer of epithelial cells which are primarily a secretory tissue, responsible for producing the overlying layers of the cuticle. The basic functions of the exoskeleton are to provide protection and to serve as an anchor for the muscles. In comparison with the mineralized endoskeleton of vertebrates, the exoskeleton of crustaceans provides better protection and possibly better leverage for the pull of muscles (Currey 1967; Taylor and Dirks 2012; Žnidaršič et al. 2012). Despite these advantages, the mineralized external skeleton presents two major challenges to the crustacean: First, the mineralized exoskeleton must not compromise the animal’s locomotion abilities (e.g., walking, swimming and tail-flipping), and second, the animal must facilitate the intensive mobilization of minerals associated with the growth process, which includes periodic shedding of the old cuticle through molting, followed by rapid recalcification of the new one. These two demands are among the prime determinants that govern the general calcification patterns in Crustacea.

The structural organization of the mineral – organic composites within the crustacean cuticle occurs over multiple length scales, from the nanometer to the

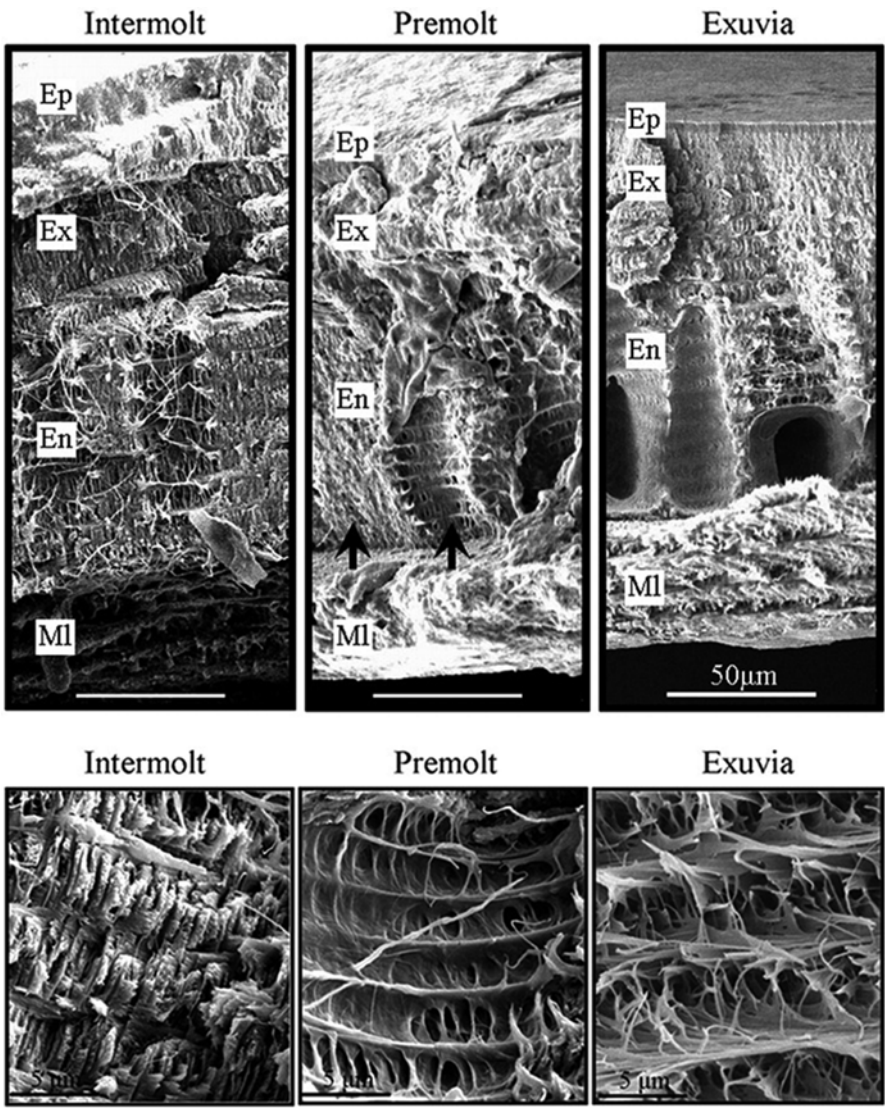


Fig. 5.1 Changes in mineralization patterns of intermolt and premolt cuticles, and of exuvia, in *Cherax quadricarinatus*. (a) Low-magnification SEM micrographs of cuticle cross sections. Ep, epicuticle; Ex, exocuticle; En, endocuticle; Ml, membranous layer. (b) High-magnification SEM micrographs of representative areas demonstrating matrix structure and mineral deposition in the cuticle during three stages of the molt cycle (Adapted from (Shechter et al. 2008a) with permission from the Marine Biological Laboratory)

millimeter scale. The building blocks of the cuticle are nanocomposite units of chitin–protein–mineral, which produce hierarchical structures with properties that are specifically tailored to carry out particular functions. Tight biological control over the properties of the mineral component of the composite is exerted via control of the degree of mineralization (from heavily mineralized to non-mineralized cuticle), the degree of crystallization (from amorphous to highly crystallized) and the nature of the mineral (ratio of calcium carbonate to calcium phosphate); this biological control over the mineral properties is probably mediated by various proteins, e.g. structural proteins, ions transporters and catalytic proteins. As discussed below, the fine biological control of the above parameters in different species and in different body parts produces various cuticular structures with different chemo-physical properties.

This review focuses particularly on the mineralogical aspects of the crustacean composite exoskeleton, with emphasis on the controllable parameters of the mineral phase. Thus, we refer the interested reader to previous review papers that have been written from a more physiological perspective (Luquet 2012) or from materials science perspective (Paris et al. 2013; Grunenfelder et al. 2014), respectively.

5.2 The Advantages of Mineralization

It is currently held that most lineages with exoskeletons originally had a tough non-mineralized exoskeleton, which became mineralized later in evolution: Many arthropods and arthropod-like animals (early Panarthropoda) that appeared during the Cambrian explosion had hard skeletons of purely organic cuticles with no incorporated biominerals (Bengtson 2004; Smith and Caron 2015). Thus, biomineralization in arthropods may be regarded an impregnation of an already developed cuticle, with minerals. We note that stiffness and hardness of organic skeletons can be attained without mineralization, for example, by protein tanning (as in insect cuticles) or by the incorporation of metals, but the strongest skeletons are those that incorporate biominerals (Bengtson 2004). Accordingly, in Crustacea, the mechanical properties of the exoskeleton are attributed mainly to the mineral phase (Vincent 2002). The incorporation of minerals into the cuticle makes it less prone to deformation and tearing and less susceptible to abrasion (Lichtenegger et al. 2002). While biominerals are typically stiff and hard, they are also brittle. This drawback is overcome by combining the minerals with structural organic matrices (proteins and polysaccharides) into composite materials. The inorganic–organic composite materials possess mechanical characteristics of stiffness, toughness, and strength that are superior to those of their constituent building blocks (Nikolov et al. 2010).

An additional advantage of mineralized exoskeletons over solely organic skeletons is that reinforcement by mineralization might be cheaper, in metabolic terms, than organic sclerotization. It is assumed that the main metabolic cost of shell construction lies in the creation of the proteins and polysaccharides required for the shell's composite structure and not in the precipitation of the mineral components

(Palmer 1992; Bengtson 1994, 2004; Cohen 2005). Thus, in order to stiffen the cuticle to a certain degree, it might be cheaper to use mineral reinforcement than enhancing sclerotization. The sources of the major salt used for reinforcement of the crustacean exoskeleton, CaCO_3 , are the ocean and freshwater bodies, in most of which the mineral is present in supersaturated concentrations. Therefore, in order to deposit CaCO_3 , the animal needs to kinetically enhance a process that is frequently favored thermodynamically. Various studies showed that ocean acidification, that decreases the saturation value of CaCO_3 , and consequently increases the metabolic cost of mineralization, may have an adverse effect on growth and survival of marine crustaceans (Cameron 1985; Kurihara et al. 2008; Arnold et al. 2009; Findlay et al. 2009; Whiteley 2011). It is assumed that although the crustaceans can compensate for the acidic condition by active ionic regulation, the metabolic cost of the compensatory responses that facilitate calcification have the potential to adversely affect growth, reproduction and survival. It is noteworthy that, since the chemistry of water bodies, is not stable (on the secular level), the changing metabolic price for different minerals and their various polymorphs, probably plays a major role in the mineralogy of crustaceans.

5.2.1 Mineralization and the Adaptations to Terrestrial Environment, Hexapods and Isopods

It is now widely accepted that the Hexapoda emerged from within the Crustacea, possibly from a malacostracan-like ancestor (Andrew 2011; Strausfeld and Andrew 2011; Ma et al. 2012). Thus, it is possible that hexapods as “terrestrial crustaceans”, that successfully invaded land as insects (Glenner et al. 2006; Mallatt and Giribet 2006), lost their mineralization capabilities in favor of a lighter cuticle, as part of their adaptation to terrestrial and aerial environments. Another group of crustaceans that successfully colonized land habitats is the terrestrial isopods (Oniscidea) that retained their mineralized exoskeleton. Comparison of the two groups shows different evolutionary pathways in the adaptation to terrestrial life. Two of the main physiological challenges in arthropods adaptation to terrestrial habitat are water retention and air breathing. The exoskeleton of aquatic crustaceans is relatively water-permeable so the animal is prone to dehydration at land. In addition, the typical external gills of crustaceans enhance water loss by transpiration, and without modification they would collapse in air under their own weight. Terrestrial arthropods were thus faced with two options: adapt or innovate (Dunlop et al. 2013). Hexapods innovated and developed an entirely new tracheal respiratory system as well as a non-mineralized highly impermeable waxed integument. On the other hand, terrestrial isopods still use their traditional gills for respiration (with special adaptations such as a complex water-conducting system and protected lung-like invaginations). They also retained the typical mineralized crustaceans-cuticle probably due to their passive defense strategy. There is also evidence that increased calcification of the

exoskeleton reduces permeability and water loss in crustacea (Edney 1960). Although the cuticle of Oniscidea have improved waterproofing (Bursell 1955) they lack the highly effective impermeable epicuticular wax layer (Barnes et al. 2009). Therefore, because of the water loss associated with respiration and integumental transpiration, terrestrial isopods are usually nocturnal and are mostly restricted to cryptozoic moist niches (Edney 1960; Little 1990).

5.3 Degree of Mineralization

A major tunable parameter that is highly variable in crustaceans, both in different body parts (Fig. 5.2) and between species, is the degree of mineralization. The change in mineral density is used to control hardness vs flexibility, to form graded structures, and to control the general density of the animal as an adaptation to different environments, e.g., pelagic vs benthic habitats.

5.3.1 Degree of Mineralization in Different Body Parts

Mineral content is a dominant factor in cuticle stiffness. As mentioned above, mineralization is not uniform in the different body parts, the basic trade-off being flexibility vs hardness. The most mobile parts, such as joints, legs and arthrodial membranes, are lightly calcified if at all (Cribb et al. 2009), whereas the carapace and claws are more heavily mineralized, and the mandibles are usually the most heavily mineralized cuticular element. A comparison of mineral contents in the carapace, claw, and finger of the American lobster *Homarus americanus* and the edible crab *Cancer pagurus* revealed a positive correlation between the degree of mineralization and hardness and a negative correlation between the degree of mineralization and elasticity (Boßelmann et al. 2007). In both species, the mineral content increases from the carapace through the claw to the finger, as is to be expected from the different requirements for hardness of these body parts: The movable finger, being an incising puncturing device, is highly mineralized and very hard. In contrast, the fixed claw, which serves as the cutting base, is less mineralized and hence softer and more elastic. Such an arrangement can be viewed as a hard sharp knife working on a stationary softer cutting board.

5.3.2 Degree of Mineralization and Graded Structures

Control over the degree of mineralization is also used to produce mechanically graded structures. As discussed above, a basic problem in mineral reinforcement is the brittleness of biominerals, which may be exploited by predators. When a

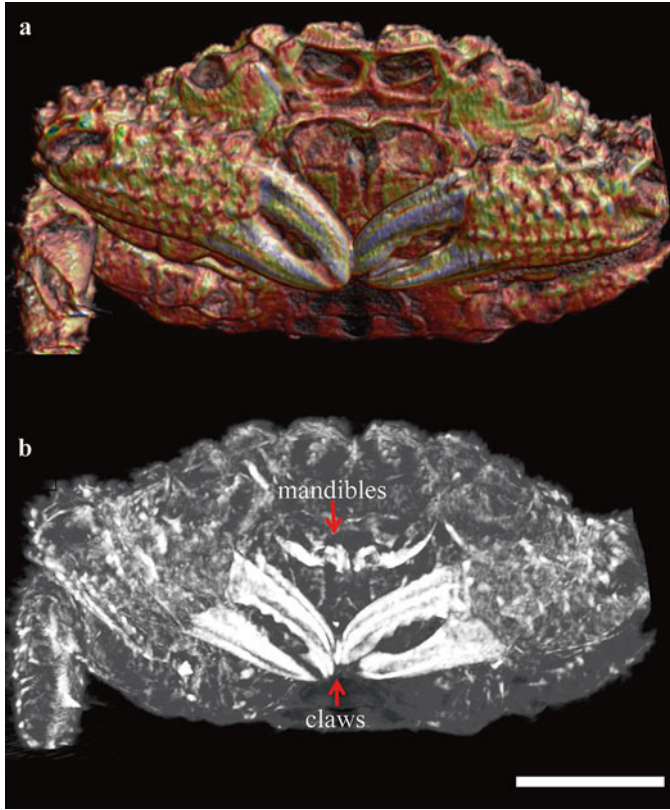


Fig. 5.2 Volume rendered micro-CT scan reconstruction of the crab *Trapezia* spp., showing high variability in the degree of mineralization. (a) 3D model surface rendering showing densitometry information for the mineral phase. The image was pseudo-colored according to an RGB palette, with low mineral density in red and high density in blue. In this setting the organic matter is transparent. (b) Maximum intensity projection (MIP) image (voxels with the highest attenuation value) show that the claws and the mandibles are the most highly mineralized parts of the exoskeleton. Scanning and volume rendering reconstructions were performed using a Skyscan1 1172 micro-CT machine and CTVox software (Skyscan). scale bar = 3 mm

predator exerts a compression force on the outer layer of the cuticle, a rigid layer would be likely to crack under the stress, exposing the underlying soft tissues. One of the solutions to this problem is the formation of a graded structure, with a hard surface layer being mounted over a more compliant layer, an arrangement that can dissipate deformation energy without the propagation of cracks (Nedin 1999). In Crustacea, the degree of mineralization plays a major role in the formation of such structures. Generally, as the degree of mineralization is increased, greater stress is required to harmfully deform the cuticle, and conversely the less mineralized the cuticle, the greater the deformation required to damage it irreversibly (Nedin 1999). Accordingly, the exocuticle layer is more mineralized and hence harder and stiffer than the endocuticle (Sachs et al. 2006). The less mineralized endocuticle is

correspondingly softer but tougher: it resists tensile stresses but is liable to failure under compression (Raabe et al. 2005; Chen et al. 2008). It is noteworthy that in many decapods, in addition to the higher mineral content, the exocuticle shows a higher stacking density of the chitin–protein layers (Raabe et al. 2005) and enhanced sclerotization when compared to the endocuticle (Neville 1975; Paris et al. 2013).

5.3.3 Mineralization and Flexibility

The degree of mineralization is usually positively correlated to hardness and elastic modulus, and negatively correlated to flexibility. Thus, more flexible crustaceans tend to have a less mineralized exoskeleton than rigid species: crabs (*Brachyura*), for example, have developed a heavily mineralized exoskeleton that provides good protection but decreased flexibility. The tradeoff between mineralization and flexibility is demonstrated in the tail flip escape mode prevalent in decapods. This mechanism allows crustaceans to escape predators through rapid abdominal flexions that produce powerful swimming strokes, thereby pushing the animal backwards and away from potential dangers. This escape behavior requires some flexibility that enables bending of the carapace. The carapace, which covers the main body, must therefore have a certain degree of elasticity to allow the movement and some bending of the animal. A study that provides a good demonstration of the tradeoff between mineralization and flexibility is the above-mentioned comparison of the mineral contents in the carapace of *H. americanus* and *C. pagurus* (Boßelmann et al. 2007). In particular, the different degrees of mineralization of the carapace of these two species are reflected in their escape behavior: the increased mineralization of the crab carapace prevents the flexural bending that is required for the tail flip, and therefore upon attack the crab clings to the ground, utilizing its highly mineralized armored exoskeleton to protect itself. In contrast, the lobster with the more elastic cuticle escapes rapidly and hides between rocks.

5.3.4 Degree of Mineralization and Habitat Zone Within the Water Column

More mineralized crustaceans are heavier. Consequently, there is a clear correlation between the degree of mineralization, on the one hand, and the habitat zone and the locomotion style, on the other hand (pelagic vs benthic and swimmers vs walkers). Benthic species tend to have a more mineralized exocuticle than pelagic species (Pütz and Buchholz 1991), a characteristic that correlates with the traditional division of the decapods into Natantia (comprising the families that move predominantly by swimming, such as the shrimps) and the Reptantia (e.g., crabs and lobsters that move chiefly by walking on the bottom of the water body).

It is noteworthy that the degree of mineralization is also associated with the general breeding and molting strategy of the different Crustacea. In highly calcified species, intermolt periods are longer than those in poorly calcified species (Aiken and Waddy 1980). A short life span and a high growth rate mean that biomineralization is relatively more costly in metabolic energy terms and therefore light calcification is advantageous. Accordingly, calcified species are associated with K-strategies (i.e., extended life span, slow maturation, brooding of young, relatively small numbers of offspring), while weakly calcified species are associated with r-strategies (short life span, rapid maturation, broadcast spawning, greater mobility, and higher population density) (Sastry 1983; Sardà et al. 1989).

5.4 Reinforcement with Stable Amorphous Phases

The Crustacea is the only major taxon in the animal kingdom that widely uses amorphous phases – amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP) – for the structural purpose of cuticle stiffening (Addadi et al. 2003). Other species use more stable crystalline minerals, namely, calcite and aragonite in invertebrates and apatite in vertebrates. Amorphous phases are less stable than their crystalline counterparts and are usually present as a transient phase. When required permanently, such phases must therefore be stabilized by additives that inhibit crystallization. Compared to calcite, ACC has a lower hardness and a lower modulus of elasticity, but ACC has a number of potential biological advantages: (1) an isotropic structure with no preferred growth direction, which enables it to be molded into any desired shape; (2) a lack of cleavage planes, which makes it less brittle than its counterpart crystalline materials (Aizenberg et al. 2002); (3) a high solubility combined with a high surface area, which provides improved resorption potential; and (4) the ability to incorporate much higher levels of trace elements and impurities than crystalline phases (Bentov and Erez 2006).

5.4.1 Resorbability

In order to grow, crustaceans periodically shed their exoskeleton and build a new larger one. During the premolt stage, massive resorption of the ‘old’ skeleton takes place (Fig. 5.1), and ions (Ca^{2+} , CO_3^{2-} , and PO_4^{3-}) are stored in various ways, e.g., as gastroliths, hepatopancreas granules and sternal deposits (Luquet and Marin 2004); these ions are later used for calcification of the new cuticle. It is thus entirely plausible that a soluble polymorph, which enables better and faster resorption, is advantageous. It is noteworthy that in addition to thermodynamic solubility, another advantage of ACC lies in the size of the mineral spheres, usually 50–100 nm, implying a very high surface area that would further enhance efficient resorption.

Measurements of resorption yield show that up to 75 % of the cuticle can be resorbed (Skinner 1962). The resorption process is executed by the innermost epidermal layer and thus the endocuticle is frequently more resorbed, than the exocuticle (Travis 1955; Roer and Dillaman 1984). Both exocuticle and endocuticle show enhanced resorption at the “ecdysial suture” which is a region that splits to allow the animal to emerge during ecdysis (Priester et al. 2005).

5.4.2 *Mechanical Advantages*

The typically small size of the ACC spheres might also confer a mechanical advantage, as the nanometer size of the mineral particles contributes to optimum strength and maximum robustness (Gao et al. 2003). In addition, the small size enables nanomechanical heterogeneity, which is typical of the chitin-protein-mineral nanocomposite. Such nanoscale structural heterogeneity might also enhance ductility and energy dissipation (Tai et al. 2007). Finally, as mentioned above, the lack of cleavage planes in ACC, due to its isotropic properties, probably makes it less brittle than calcite (Aizenberg et al. 2002).

5.4.3 *Phosphate Incorporation*

A factor that possibly contributes to the wide use of stable ACC for exoskeleton reinforcement is the ability of the amorphous phase to incorporate higher amounts of trace elements and impurities than crystalline phases (Bentov and Erez 2006). In Crustacea, the major co-precipitate with the bulk calcium carbonate in the exoskeleton is calcium phosphate (the possible advantages of calcium phosphate biomineralization are discussed below). Since calcite crystal lattices barely accommodate phosphate ions, this co-precipitation is restricted to amorphous phases where ACC-ACP (designated ACCP) behaves as a solid solution having various PO_4/CO_3 ratios. It is believed that the phosphate in the ACCP solid solution is used, together with other additives, for ACC stabilization (Weiner et al. 2003; Bentov et al. 2010). In addition, as discussed below, phosphate incorporation also plays a mechanical role in ACCP: It has been shown that an increased PO_4/CO_3 ratio in ACCP is associated with enhanced mechanical properties, such as improved hardness and modulus of elasticity (Figs. 5.3 and 5.4) (Currey et al. 1982; Bentov et al. 2012) and reduced solubility (Bentov et al. 2016). Importantly, as shown in Fig. 5.3, ACCP has mechanical properties (hardness and Young's modulus) that are comparable to those of vertebrate bone. Thus, crustaceans seem to have overcome some of the limitations of ACC (e.g., solubility and low hardness) via the incorporation of phosphate.

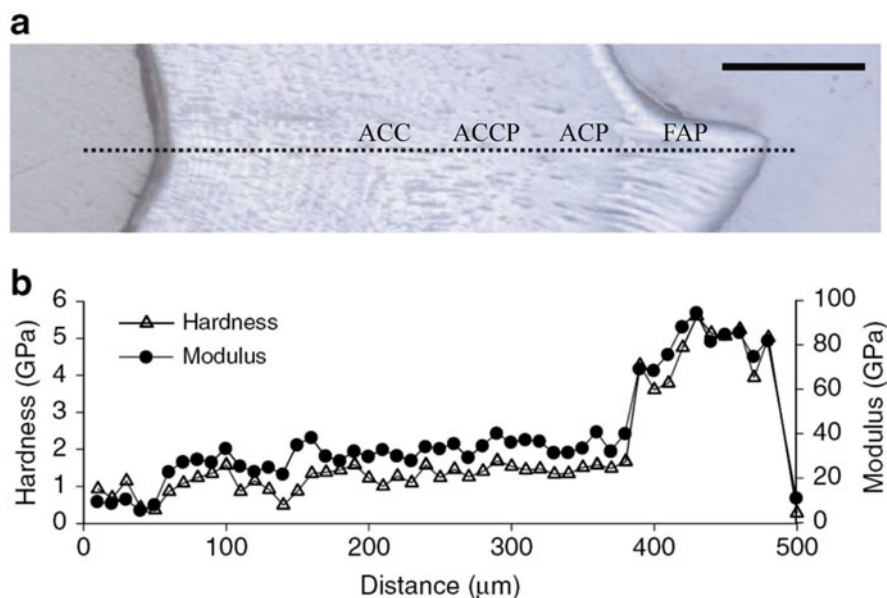


Fig. 5.3 Nanoindentation testing of the anterior crayfish molar. (a) Light micrograph of the measured region in cross-section. bar, 100 μm. (b) Hardness (*open triangles*) and reduced Young's modulus (*filled circles*) for the dotted line in a. Both properties show an increase towards the surface (ACC < ACCP < ACP) and much larger values within the fluorapatite (FAP) distal layer (Adapted from (Bentov et al. 2012) with permission from the Nature Publishing Group)

5.5 The Combination of Calcium Carbonate and Calcium Phosphate

An intriguing question in the evolution of biomineralization is why and how specific minerals were adopted by the different taxa. Mineralization with specific minerals has proved to be a conserved trait. Once a specific mineral is selected, taxa rarely switched mineralogies (Porter 2007). From the beginning of biomineralization in the major animal phyla, during the Cambrian explosion approximately 525 MYA ago, the minerals used by most animals for skeletal reinforcement were calcium salts, either calcium phosphate or calcium carbonate. Consequently, these two minerals remained the most important bioinorganic materials throughout the Phanerozoic eon (until present times), in terms of both phylogenetic distribution and biomineral quantities (Lowenstam and Weiner 1989).

According to the general scheme of biomineral distribution in animals, vertebrates adopted calcium phosphate, while invertebrates adopted calcium carbonate (Knoll 2003). However, in crustaceans the picture seems to be more complex, with various pieces of evidence suggesting that in many crustaceans controlled biomineralization of calcium phosphate takes place in addition to the formation of the bulk

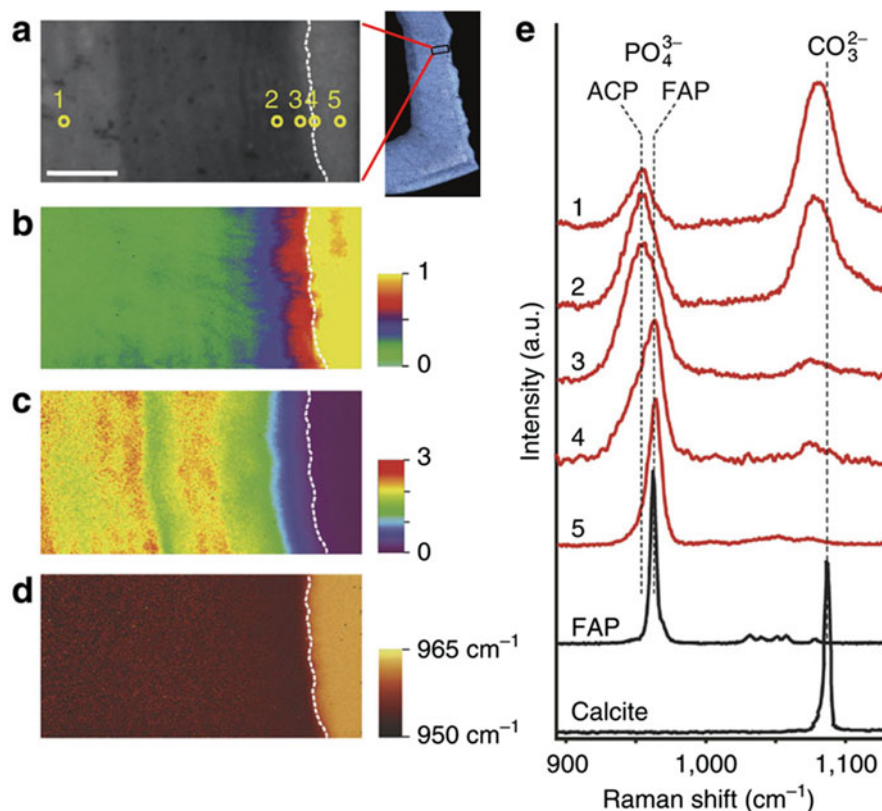


Fig. 5.4 Raman spectroscopic imaging of the *Cherax quadricarinatus* molar. (a) Light micrograph of the analyzed area. Scale bar, 20 μm . The measured area on a molar cross-section covers the transition zone between the apatite and the amorphous mineral (see inset). (b) Raman spectroscopic image of the ν_1 phosphate peak. The normalized integrated intensity ($IPO_4/IPO_{4\max}$) shows that phosphate is found in the entire mapped region. (c) The intensity ratio (ICO_3^-/IPO_4) of the carbonate (ν_1) to the phosphate peak shows a pronounced gradient with superimposed fluctuations. (d) The position of the ν_1 phosphate peak shows a sharp transition from the crystalline fluorapatite (FAP) zone (higher values) to the amorphous regions (lower values). The boundary between the apatite layer and the amorphous phases is indicated by the white dashed lines in b–d. (e) Normalized single Raman spectra at positions 1, 2, 3, 4 and 5 as indicated in a, compared with synthetic reference materials, FAP and calcite. The changes in the peak intensities show the gradual decrease/increase in carbonate/phosphate contents. The broadening of the phosphate peak as well as the shift to lower wavenumbers, when passing from position 5 to position 3 across the sample, represents the transition from FAP to ACP (Adapted from (Bentov et al. 2012) with permission from Nature Publishing Group)

calcium carbonate. Unlike other invertebrates (e.g., Mollusca and Echinodermata), many crustaceans contain different amounts of phosphorous within their carbonate skeletons (Huxley 1884; Clarke and Wheeler 1922; Prenant 1927; Vinogradov 1953; Lowenstam and Weiner 1989; Bentov et al. 2010; Kunkel and Jercinovic 2013; Vatcher et al. 2015). This phenomenon was observed as long ago as the 1850s

by Darwin, who noted that crustacean calcareous shells show high variability of phosphate content, whereas molluscan shells have virtually no phosphate (Darwin 1851). As mentioned above, the presence of phosphate in crustacean skeletons has been attributed mainly to co-precipitation with ACC (Raz et al. 2002; Becker et al. 2005; Hild et al. 2009). The presence of apatite crystals in the skeletal tissues of crustaceans has long been considered to be unusual and was thought to be restricted to the barnacle group of Iblidae that contain small crystals of carbonated apatite (Lowenstam et al. 1992). However, it has recently been demonstrated that calcium phosphate mineralization is widely prevalent in crustacean mandibles and that calcium phosphate teeth are frequently formed at specified locations in the mandible in the major crustacean class, Malacostraca (which includes lobsters, crayfish, prawns and shrimps) (Bentov et al. 2016). In these structures, calcium phosphate is not merely co-precipitated with the bulk calcium carbonate but rather creates specialized structures in which a layer of calcium phosphate, frequently in the form of crystalline fluorapatite, is mounted over a calcareous “jaw.” A study of the mandible of the crayfish *Cherax quadricarinatus* showed that the molar teeth are reinforced with large oriented crystals of fluorapatite. This phosphatic cover is mounted over a carbonate basis, forming a graded structure of multi-phase composite material that produces superior mechanical properties, comparable to those of vertebrate enamel (Fig. 5.3) (Bentov et al. 2012). Another example of a particular structure that is mineralized with crystalline apatite is the raptorial appendage of the mantis shrimp (Weaver et al. 2012; Amini et al. 2014). The ability to produce sophisticated structures with superior mechanical properties suggests that many crustaceans possess two biomineralization machineries, one for calcium carbonate and the other for calcium phosphate (Bentov et al. 2016). The wide occurrence of this phenomenon further suggests that the phosphate mineralization mechanism did not evolve independently in the different groups but rather that it has older evolutionary roots.

The phosphatic “inclination” of crustaceans seems to go back to the very beginning of crustacean biomineralization. According to fossil records, calcium phosphate skeletons were more predominant than calcium carbonate skeletons during the appearance of early biomineralizing animals (Lowenstam 1972). The first arthropods frequently had a phosphatic skeleton (e.g., Bradoriida, Aglaspidida and phosphatocopines). In fact, at least until the Ordovician, arthropods exhibited the most advanced calcium phosphate skeletons, which preceded the specialization of vertebrates (Chordata) in terms of calcium phosphate mineralization (Lowenstam 1972). During the Cambrian, the relative abundance of these groups of minerals was reversed, and by the mid-Cambrian, carbonate minerals had become the most common bioinorganic constituents of skeletal elements and remain so to date (Lowenstam and Margulis 1980; Lowenstam and Weiner 1989). The reason for this shift is unclear, although it might have been associated with high phosphate concentrations in the early Cambrian oceans, as a result of the extensive phosphogenic events that took place across the Precambrian-Cambrian boundary and ended by the close of the Cambrian (Rhodes and Bloxam 1971; Cook and Shergold 1984; Cook 1992). This shift is best demonstrated within arthropods fossils, which are among the most dominant Cambrian fossils (e.g., at Burgess Shale in the Canadian Rockies).

The phylogenetic nature of this Cambrian shift from calcium phosphate to calcium carbonate is subject to dispute. According to Lowenstam (1972), the shift from phosphate to carbonate calcification represents an evolutionary adaptive change within the arthropods, namely, a change that reflects the rising metabolic cost of phosphate usage. Other authors argue that a mineralogical shift within a clade is not likely and that the “shift” merely reflects the evolutionary success of various early lineages with different mineralogies. It has further been argued that it is more likely that the apparent replacement of phosphatic minerals with carbonate was the result of secondary diagenetic (post-mortem) phosphatization of carbonate shells (Bengtson and Runnegar 1992). However, support for the option of early phosphatic arthropods may be drawn from several studies showing that fossils of Cambrian arthropods that had previously been considered to be secondarily phosphatized actually had a primary (original) phosphatic cuticle (Bachra et al. 1965; Muller 1979; Briggs and Fortey 1982; Lin et al. 2010). In addition, the above-mentioned frequent finding of dual mineralization systems in recent crustaceans demonstrates the feasibility (in evolutionary terms) of an intra-lineage mineralogical shift within the crustaceans.

The possession of dual mineralization “toolkits” for calcium carbonate and calcium phosphate has provided the crustaceans with (additional) evolutionary plasticity in designing exoskeletal properties. During evolution, the balance between the mechanical requirements of cuticular elements (e.g., the carapace and feeding tools) and the metabolic cost of phosphate and carbonate use (as derived from the animal’s environment) probably dictated the extent of phosphate distribution in the largely calcareous cuticle.

Comparing the biomineralization of calcium carbonate and calcium phosphate from a physiological perspective shows that, theoretically, these biomineralization mechanisms can share some basic features. The concentrations of calcium, carbonate, and phosphate are under tight cellular control. Calcium supply to the calcification site can be similar in both systems. It is likely that carbonate and phosphate have different transport systems, but both serve as important cellular buffers, and their global concentration is under the same cellular regulation system of pH homeostasis. In addition, in both systems, the relative concentration of the required deprotonated species, CO_3^{2-} and PO_4^{3-} , is enhanced by alkalization. The two mineralization systems also share similar crystallization inhibitors (e.g., phosphoproteins, ATP and magnesium) (Blumenthal et al. 1977; Raz et al. 2000; Bentov et al. 2010; Qi et al. 2014).

The major conflict between the two systems is their mutual inhibitory effect on the crystallization process: phosphate ions interfere with the crystallization of calcium carbonate and (to a lesser extent) carbonate ions with that of apatite. This may be one of the reasons that crustaceans frequently use fortification with non-crystallized ACC and ACP, thereby bypassing the structural lattice constraints that are not inherent in the amorphous phases. Thus, an animal can maintain these two mineralization systems utilizing, partially, the same physiological mechanisms (i.e., calcium supply, pH elevation and crystallization inhibitors), with relatively simple adaptations, such as using different matrix proteins (Tynyakov et al. 2015b).

The high variability in the phosphate/carbonate ratio suggests that many arthropods do indeed maintain two biomineralization “toolkits,” one for calcium carbonate and the other for calcium phosphate. This dual system provides an adaptable and versatile biomineralization mechanism that is able to produce separately or simultaneously all the minerals along the calcium carbonate–calcium phosphate spectrum, namely, calcite, ACC, ACP, and apatite, as demonstrated in the mandible of *C. quadricarinatus*. Calcium phosphate mineralization can be beneficial in both the amorphous and crystalline phases. In its crystallized form, apatite offers the hardest mineral option from the range of calcium carbonate/phosphate salts [on the Mohs hardness scale, apatite is 5 while calcite is 2.5–3 (Broz et al. 2006)]. Likewise, in the amorphous phase, calcium phosphate probably plays a major role in enhancing the mechanical properties and chemical resistance of the exoskeleton, in addition to its role in ACC stabilization. Mechanical analysis of the crayfish mandible (Bentov et al. 2012) and the mantis shrimp dactyl limb (Currey et al. 1982) showed that there is a positive correlation between the ACP/ACC ratio, on the one hand, and hardness and the modulus of elasticity, on the other hand (Figs. 5.3 and 5.4). In addition, ACP probably contributes to the chemical resistance of the skeletal element due to its lower solubility than ACC and calcite (Dorozhkin 2010). As mentioned above, one of the natural solutions for reinforcement of the skeletal structure with the aim of withstanding massive impact, is the formation of a mechanically graded structure, in which a hard material is mounted over a softer base that absorbs any impact and deflects potentially dangerous cracks (Currey 2005). It seems that the dual mineral system provides the animal with an efficient method of forming mechanically graded structures through a gradual change in the phosphate/carbonate ratio, as demonstrated in the mandibular structure of crustaceans (Bentov et al. 2012).

In summary, the advantage of calcium phosphate mineralization lies in improved mechanical qualities (such as hardness, stiffness, and wear resistance) and enhanced stability and acid resistance. These advantages are off-set by the higher metabolic cost of calcium phosphate (phosphorus typically present in oceans and freshwater bodies in much lower concentrations than carbonate (Smith 1984; Mackenzie et al. 1993; Urabe et al. 1997; Wu et al. 2000)), which is amplified by less efficient resorption (due to lower solubility) during the premolt stage. Therefore, at the evolutionary level, there seems to be a species-specific tradeoff between the improved mechanical and chemical resistance properties of calcium phosphate and its higher metabolic price. In many species, the balanced solution seems to be the deployment of calcium phosphate only at the wear-prone sites of the skeleton.

5.6 Involvement of Proteins and Genes

Since crustaceans are unique in their ability to rapidly mineralize and demineralize their exoskeletal matrices, they have been designated the “champions of mineral mobilization and deposition in the animal kingdom” (Lowenstam and Weiner 1989) and extensive studies have been performed on this subject (Travis and Friberg 1963;

Ueno 1980; Ueno and Mizuhira 1984; Lowenstam and Weiner 1989; Luquet and Marin 2004; Shechter et al. 2008b; Nagasawa 2012). Key players in the formation of the mineralized exoskeleton are proteins, which are involved in almost every aspect of this process, namely: chitin assembly and metabolism (Buchholz 1989; Abehsera et al. 2015); control of mineralization (Inoue et al. 2008; Bentov et al. 2010); determination of elastic and structural properties (Tynyakov et al. 2015a); and sclerotization (Kuballa and Elizur 2008). It has also been suggested that proteins are involved in mediating the interactions between the chitinous and the mineral phases and the assembly of nanocomposites during skeletogenesis (Glazer et al. 2015).

Studies on the role of proteins in cuticle formation in arthropods were originally performed on insects (Chihara et al. 1982; Snyder et al. 1982), which share cuticle similarity with crustaceans, although their cuticles are non-mineralized. Today, the field of interest has shifted to cover the entire pancrustacean clade, including decapods, as discussed in a recent review article (Roer et al. 2015b). The latter review emphasizes the high similarity across the clade in cuticular structures and content, especially in the proteins involved in cuticle formation. In the different arthropod species, the proteins related to cuticle formation have common features. An important example is the abundant chitin-binding domain found in many cuticular proteins (Ardell and Andersen 2001; Inoue et al. 2001). One such domain is the cuticle-specific chitin-binding domain known as the Rebers-Riddiford (RR) consensus sequence (Rebers and Riddiford 1988; Rebers and Willis 2001). Another chitin-binding domain found in many phyla, including the arthropods, is the type 2 cysteine-rich chitin-binding domain (cys-CBD2), which is present, for example, in the cuticular obstructor protein family (Behr and Hoch 2005). Although many proteins bind to the chitinous scaffold directly, several proteins that lack a chitin-binding domain, such as the important knickkopf protein (Chaudhari et al. 2011), bind to the scaffold through a mediator protein, such as a member of the chitin-binding Obstructor family (Chaudhari et al. 2011; Pesch et al. 2015). An example of this type of indirect scaffold binding is the key role played by protein complexes based on a single core chitin-binding protein in the formation of the gastroliths of the freshwater crayfish *C. quadricarinatus* (Glazer et al. 2015). In addition, proteins lacking a chitin-binding domain can be involved in the formation of non-chitinous cuticular matrices, such as the epicuticle (Roer and Dillaman 1984). Another important process that involves protein-protein interactions is sclerotization, the cross-linking of proteins with phenolic materials (Andersen 2010). Finally, the presence of a signal peptide is probably obligatory in most cuticle-forming proteins, due to the fact that cuticular matrices are extracellular and do not contain any living cells (Roer and Dillaman 1984). Indeed, most of the known cuticular matrix proteins do contain a signal peptide (Ardell and Andersen 2001; Takeda et al. 2001).

Up to this point we have discussed proteins that are involved in the formation of the crustacean cuticle but are not associated directly with the biomineralization process. In comparison to the extensive body of research on the role/s of proteins in biomineralization in vertebrates or echinoderms, knowledge of the protein basis of biomineralization in crustaceans is limited. There are only a few known crustacean proteins that have been shown to be involved in biomineralization, and for most of

these the mechanism of action has not been elucidated. Such proteins exhibit characteristics that are common to many biomineralization-related proteins, e.g., they are proteins having regions that are predicted to be intrinsically disordered or acidic in nature (Addadi and Weiner 1985; Evans 2012; Wojtas et al. 2012). A handful of studies have been conducted on proteins that take part in biomineralization in crustaceans. In the crayfish *Procambarus clarkii*, for example, an acidic protein was found to have an inhibitory effect on calcium carbonate precipitation (Inoue et al. 2008). Also in *P. clarkii*, the acidic gastrolith matrix protein (GAMP), which is predicted to have intrinsically disordered regions consisting of many repeats, is believed to play a key role in mineral precipitation (Ishii et al. 1996; Tsutsui et al. 1999). Similarly, several acidic proteins have been found in the gastrolith disc of the crayfish *C. quadricarinatus* and are known or suggested to be involved in the calcification of the gastroliths (Glazer and Sagi 2012). The molar tooth of *C. quadricarinatus* (see Sect. 5.5) provides a unique opportunity to study the protein basis of calcium phosphate biomineralization in crustaceans (Bentov et al. 2012). In this process, a putative chitin-binding acidic protein that was predicted to be disordered was shown to induce precipitation and mineralization of calcium phosphate *in vitro* (Tynyakov et al. 2015b).

From a genomic point of view the regulatory machinery and set of promoters during molting is poorly known in crustaceans. Since ecdysone plays a key role in regulating molting of pancrustaceans (Qu et al. 2015) the ecdysone pathways is of interest with this respect. Recently it was found in the decapod shrimp *Neocaridina denticulate* that the ecdysteroid pathways known from insects are conserved and suggested to be present in the pancrustacean common ancestor (Sin et al. 2015). Temporal expression during molting provides a powerful tool for the study and mining of molt-related genes. For example genes involved in chitin metabolism and extracellular matrices formation were found to have a molt-related pattern of expression in several studies done on crustaceans (Kuballa and Elizur 2008; Seear et al. 2010; Yudkovski et al. 2010; Rocha et al. 2012; Tynyakov et al. 2015a). Next Generation Sequencing (NGS) provides the ability to perform multigene studies based on molt-related transcriptomic libraries. Several such studies performed recently gave unique insights into the molecular mechanism of molt in crustaceans (Abehsera et al. 2015; Gao et al. 2015; Das et al. 2016). From a future perspective since designating functions in NGS studies is based on annotations, that originates mainly from insect cuticular proteins (Roer et al. 2015a), the temporal expression approach might enable mining of genes unique to crustaceans.

5.7 Potential Biomimetic Applications Inspired by the Crustacean Exoskeleton

Biomineralization in crustaceans is particularly efficient, with the few examples covered above suggesting the utility of studying the proteinaceous machinery in crustaceans. Therefore, understanding the uniqueness that makes crustaceans the

“champions of mineral mobilization and deposition” could be of great importance in biotechnological areas such as biomimetics; such an understanding could provide clues to potential bio-medical applications related to the field of regenerative bone and teeth applications.

The field of biomaterials is in the midst of a transformation in which the life sciences are being combined with materials science and engineering for the formation of bio-inspired materials (Huebsch and Mooney 2009). As part of this process, there has been renewed interest in arthropods as a guide to new biomimetic materials (Paris et al. 2013).

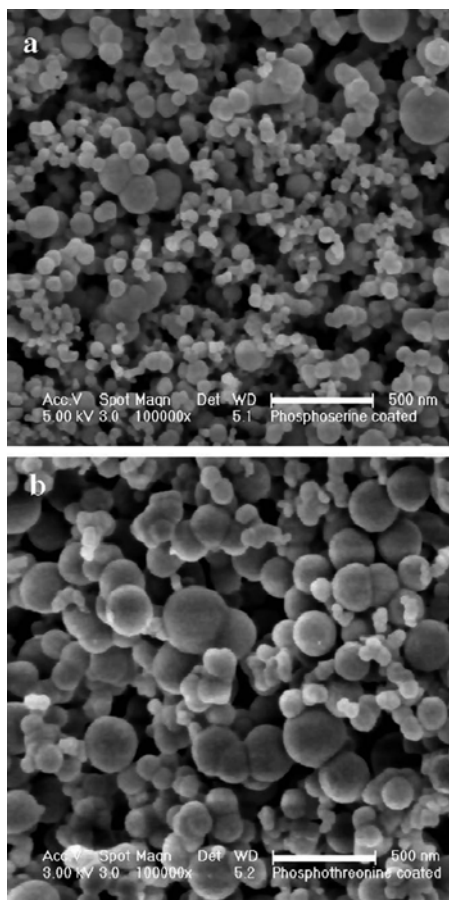
Inspiration for the development of synthetic bio-composites for various skeleton-related medical applications may be drawn by examining the differences and similarities between the crustaceans and the vertebrates, the two major groups in the animal kingdom that combine articulated mineralized skeletons with advanced locomotion. The recent findings that many crustaceans can form elaborate calcium phosphate structures with properties that are comparable to those of vertebrate bones and teeth (Bentov et al. 2016) further suggests a convergent evolution that reflects similar solutions to similar mechanical challenges. One major difference between vertebrate and crustacean skeletons is the organic scaffold that serves as a template for biomineralization: The crustacean scaffold is composed mainly of the polysaccharide α -chitin, while the vertebrate scaffold is made of collagen proteins. However, chitin and collagen are considered as universal templates in biomineralization (Ehrlich 2010). In nature, chitin was also found to serve as a template for calcium phosphate precipitation, as was shown in the chitinophosphatic skeletons of brachiopods (Williams et al. 1994). In pre-clinical and clinical studies, chitin and its deacetylated counterpart chitosan were found to be suitable biomaterials in the field of human calcified tissue engineering, showing good properties of biocompatibility, biodegradability, nontoxicity, nonimmunogenicity, osseointegration and adequate porosity (Khor and Lim 2003; Kim et al. 2008; Costa-Pinto et al. 2011).

There are several potential applications for amorphous mineral phases – stabilized ACC, stabilized ACP, and the combination of calcium carbonate and calcium phosphate mineralization as discussed below.

5.7.1 *Stabilized ACC*

The highly soluble ACC has a potential use as an available calcium supplement for bone health. However, ACC is a metastable phase that readily transforms to the more stable crystalline calcium carbonate, and therefore, understanding stabilization mechanisms is a precondition for any biotechnological application of this mineral. A study of the stabilization mechanism of ACC in the gastrolith (a calcium storage receptacle of premolt crayfish) showed that phosphorylated amino acid moieties probably play a major role as kinetic inhibitors of crystallization (Bentov et al. 2010). The elucidation of a “simplified” biogenic mechanism of ACC stabilization by phosphoamino acids (Fig. 5.5) inspired the development of synthetic stable

Fig. 5.5 Synthetic ACC produced by a biomimetic approach. **(a)** SEM micrographs of ACC comprising 20–200-nm nanospheres produced in the presence of phosphoserine (bar = 500 nm). **(b)** ACC, composed of 50–300-nm nanospheres, induced by the presence of phosphothreonine (1 mM) (bar = 500 nm) (Adapted from (Bentov et al. 2010) with permission from Elsevier)



ACC, which has been tested as a dietary supplement of available calcium (Bentov et al. 2014). Clinical trials showed that the stabilized ACC does indeed have improved absorption properties in humans (Meiron et al. 2011; Vaisman et al. 2014).

5.7.2 *Stabilized ACP*

Due to significant chemical and structural similarities with calcified mammalian tissues, as well as excellent biocompatibility and bioresorbability, ACP is a promising candidate for implementation in artificial bone grafts, bone cement, and bone fillers (Combes and Rey 2010; Dorozhkin 2010). A major challenge in bone regeneration is to adjust the degradation kinetics of the calcium phosphate bone filler with the rate of bone tissue formation. In this context, the insolubility of hydroxyapatite might be problematic, and a possible solution is to use ACP (Barrère et al. 2006).

ACP is a thermodynamically unstable compound that tends to transform spontaneously to crystalline calcium phosphates, mainly to apatite. Thus, the expertise of crustaceans in ACP formation and stabilization (Bentov et al. 2016) could be mimicked for the above applications.

5.7.3 *Combination of Calcium Phosphate with Calcium Carbonate*

As mentioned above, a basic toughening feature observed in the crustacean cuticle is the formation of different layers with different mechanical properties, e.g., the hard exocuticle that covers a more compliant endocuticle. This arrangement probably evolved to withstand compression loading and to restrict crack propagation. In addition to the macro-layering, there is a gradation of heterogeneities on the nanoscale, e.g., the gradual decrease in the PO_4/CO_3 ratio from the distal hard surface to the proximal more compliant region that is found in the crayfish mandible (Fig. 5.4) (Bentov et al. 2012) and the smashing limb of the Stomatopoda (Currey et al. 1982; Weaver et al. 2012). This gradation in mechanical properties may be attributed to the lack of long-range atomic order of the amorphous phases, which allows the formation of ACCP solid solutions with various PO_4/CO_3 ratios. A biomimetic approach could therefore be adopted for the synthesis of mechanically graded biomaterials comprising ACC and ACP, which are both biocompatible with human tissues (Tolba et al. 2016).

5.7.4 *Concluding Remarks*

Finally, an as yet unexploited biomimetic direction lies in the exoskeleton's basic building block comprising a chitin–protein–mineral nanocomposite. The intimate association of these three types of materials within the nanocomposite plays a key role in the properties of the skeleton. It is generally believed that proteins are involved in the fundamental functions of chitin binding, calcium binding, formation of nucleation sites, elastic properties, and inhibition or enhancement of crystallization. It seems therefore that elucidation of the role of proteins in the formation and functioning of the nanocomposite represents a major challenge and, once successfully addressed, could promote the development of future generations of biomaterials inspired by the crustacean exoskeleton.

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