





# The Physiological Inorganic Polymers Biosilica and Polyphosphate as Key Drivers for Biomedical Materials in Regenerative Nanomedicine

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**Abstract:** There is a need for novel nanomaterials with properties not yet exploited in regenerative nanomedicine. Based on lessons learned from the oldest metazoan phylum, sponges, it has been recognized that two previously ignored or insufficiently recognized principles play an essential role in tissue regeneration, including biomineral formation/repair and wound healing. Firstly, the dependence on enzymes as a driving force and secondly, the availability of metabolic energy. The discovery of enzymatic synthesis and regenerative activity of amorphous biosilica that builds the mineral skeleton of siliceous sponges formed the basis for the development of successful strategies for the treatment of osteochondral impairments in humans. In addition, the elucidation of the functional significance of a second regeneratively active inorganic material, namely inorganic polyphosphate (polyP) and its amorphous nanoparticles, present from sponges to humans, has pushed forward the development of innovative materials for both soft (skin, cartilage) and hard tissue (bone) repair. This energy-rich molecule exhibits a property not shown by any other biopolymer: the delivery of metabolic energy, even extracellularly, necessary for the ATP-dependent tissue regeneration. This review summarizes the latest developments in nanobiomaterials based on these two evolutionarily old, regeneratively active materials, amorphous silica and amorphous polyP, highlighting their specific, partly unique properties and mode of action, and discussing their possible applications in human therapy. The results of initial proof-of-concept studies on patients demonstrating complete healing of chronic wounds are outlined.

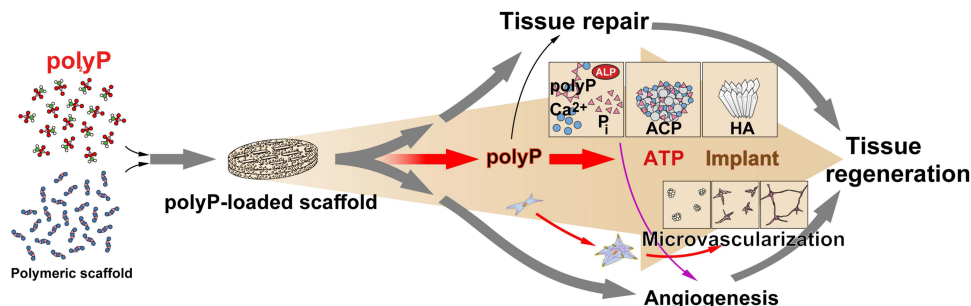
**Keywords:** biomaterials, nanoparticles, biosilica, polyphosphate, silicatein, regenerative medicine

## Introduction

Over the course of its life, the integrity of any metazoan, from the simplest organisms to humans, is compromised to varying degrees by injury or chronic degenerative changes, leading to dysfunction. In humans, when the physiological repair capacity is insufficient, medical intervention is required to prevent a collapse of the fragile cellular homeostasis and the tuned organization of cells and tissues. There is a need for new concepts in regenerative therapy. Animals that have physiologically extensive regenerative abilities, such as sponges, are possible models for copying these concepts from nature. During the evolution of the metazoans from their basal phylum, the sponges (Porifera), with their ~40,000 predicted protein-coding loci,<sup>1</sup> to the “crown taxa”, humans, with 20,000 to 25,000 genes,<sup>2</sup> a gradual decrease in the genetic complexity is observed. This fact supports the view that the basal animals like sponges have a higher redundancy potential, combined with a variety of alternative metabolic circuits that provide them with a higher evolutionary stability. In turn, the regeneration capability of basal animals is more extensive compared to the “more advanced” taxa, including humans.

In animals, particularly in humans, some defects are not adequately repaired especially when physiological functional restoration is not supported by the inherent regeneration potential. If tissue/cell homeostasis is not maintained, the repair processes are insufficient with the result that the affected, damaged tissue is only replaced by tissue-like entities that lack physiological and

## Graphical Abstract



biochemical functions. Or, as in present-day solutions, the defective parts are replaced or augmented by largely inert or only partially biological substituting materials. For example, the deficiencies of the bone-supporting implants can be overcome with tissue regenerating materials that restore not only the mechanical but also the biological and functional properties of the damaged tissue in the defect region. It is important to emphasize that any regeneration process requires an optimal physiological functioning of the cells, which depends on molecular and mechanical signals from the environment.<sup>3</sup> Both the cells in the tissue surrounding the defect, and particularly the cells within the areas to be repaired, must be supplied with growth factors and nutrients that support the anabolic repair pathways and, importantly, with metabolic energy (ATP or another energy-carrying molecule).<sup>4</sup> Not only intracellularly but also extracellularly, a sufficient supply of biochemically useful energy is important for the regeneration of the extracellular matrix (ECM). The number of cells in these regions is often low, for example in cartilage, which contains only ~10% chondrocytes.<sup>5</sup> During regeneration, biochemical reactions in the ECM are driven by the cell-based synthesis of fibrous mats (collagen) or hydrogels (hydrogel-forming polypeptides). These reactions are anabolic reactions that require the conversion of chemical fuels like glucose into ATP (energy). In addition, enzymes are needed that break down larger precursor molecules in order to drive anabolic ones in bone, cartilage, and muscle regeneration.<sup>6</sup> Accordingly, the two cornerstones, enzymes and energy, contribute significantly to the regeneration pathways. The initial basis for this gain came from studies from the basal animals, the sponges.

This review has a special focus on the regenerative abilities of two inorganic biomaterials in the formation and repair of both soft and hard tissues, amorphous silica (“biosilica”) and amorphous polyphosphate (polyP). Both biomaterials or biopolymers are regeneratively active only in their amorphous form and, in accordance with their physiological function, exhibit properties that the conventional organic biopolymers used in regenerative medicine do not have, such as in the case of polyP, the delivery of metabolic energy required for energy-dependent repair processes, as discussed in this review. In particular, the role that sponges, as the most basal phylum, have played in understanding the critical function of enzymes in the energy-dependent tissue regeneration/repair processes, including the formation of biomineralized structures, is discussed, while previous reviews on these biomaterials primarily focus on either only one of these materials<sup>7,8</sup> or on their physicochemical properties or more mechanistic aspects.<sup>9</sup>

## Regeneration Capacity Across Animal Taxa

The basic principles of regeneration processes have been enigmatic for a long time. In 1606, it was stressed that bone formation and the regeneration of this organ depend on spiritual forces, *virtus naturalis*, based and localized in the lower body area, which is characterized by a “warm and humid” local environment.<sup>10</sup> Later, especially by Trembley, first evidence-based experiments were performed, which revealed remarkable regenerative abilities especially in the more basal Metazoa such as the phylum Cnidaria.<sup>11</sup> The sponges (Porifera), the basal phylum of Metazoa, traditionally served as a model system for regeneration.<sup>12</sup> Animal taxa that evolved later from sponges have a reduced regeneration capacity mainly due to the presence of lower amounts of stem cells.<sup>13</sup> Thomas Hunt Morgan introduced the perspective of functional genomics by studying the regeneration of segments in earthworms as a model.<sup>14</sup>

In Table 1, a summary of the regeneration capacity across various animal taxa is shown. The ability to regenerate damaged or lost tissue greatly varies among different species and can include the whole body, the primary body axis, or only certain structures or tissues.<sup>15</sup> Whole-body regeneration is particularly found in the basal metazoans such as sponges, cnidarians and ctenophores.<sup>16,17</sup> The regeneration of the sponge body or sponge tissue after an injury can either start from small body fragments or occur via aggregation of dissociated sponge cells.<sup>16</sup> Sponges as sessile marine organisms are particularly prone to wounding, eg, by grazers or mechanical injury, which may lead to the occurrence of chronic wounds if repair fails and homeostasis is not retained. Investigations of the response of the sponge *Aplysina aerophoba* to damage caused mechanically or by a spongivorous opisthobranch using differential gene expression analysis based on RNA-sequence data revealed a repair mechanism involving enzymes/proteins such as metalloproteases, transglutaminases, and integrins, and signaling pathways (Wnt and mitogen-activated protein kinase – MAPK), similar to that involved in wound healing in higher animals, including humans.<sup>18</sup>

## Taking Concepts from Nature as a Blueprint for Regeneration

Intense efforts over the past 40 years have uncovered, in a scientific, causal-analytical manner, the strategies of how living systems function with the aim of exploiting them to meet the urgent needs in engineering tissue replacement

**Table 1** Regeneration Capacity Across Various Animal Taxa

		Taxon	Whole Body Regeneration	Primary Body Axis Regeneration	Structure/Tissue Regeneration	
		Porifera (Sponges)	X			
		Cnidaria (Cnidarians)	X	X	X	
		Ctenophora (Ctenophores)	X	X	X	
Bilateria	Protostomia	Acoela (Acoels)	X	X	X	
		Mollusca (Molluscs)	–	X	X	
		Annelida (Annelids)	X	X	X	
		Platyhelminthes (Platyhelminthes)	X	X	X	
		Nematoda (Nematodes)	–	–	–	
			Arthropoda (Arthropods)	–	–	X
	Deuterostomia		Echinodermata (Echinoderms)	X	X	X
			Hemichordata (Hemichordates)	?	X	X
			Cephalochordata (Cephalochordates)	–	X	X
			Urochordata (Urochordates)	X	X	X
			Amphibia (Amphibians)	–	X	X
			Reptilia (Reptiles)	–	X	X
			Aves (Birds)	–	–	–
			Mammalia (Mammals)	–	–	X

**Notes:** Data from these studies.<sup>15,19</sup> X, Regeneration; –, no regeneration; ?, unknown.

biomaterials. Biomimetic and bioinspired approaches based on these achievements have contributed considerably to a paradigm change in the field of tissue engineering.<sup>20–22</sup> Studies on sponges, the evolutionarily oldest metazoans, showed that these animals produce a range of organic biomolecules that they effectively defend against bacteria and viruses.<sup>23–25</sup> Then, the application of molecular biology techniques made it possible to show – what was not expected before – that these basal animals have in their protein toolkit, eg, building blocks even for basic immune defense systems that are successfully used by humans against foreign invaders. The existence of the Rhesus factor<sup>26</sup> or the polymorphic immunoglobulin molecules,<sup>27</sup> members of the acquired immune system, are mentioned here as examples. Even more, analyses of the formation of the skeletal system of the siliceous sponges, made of amorphous silica (“biosilica”), enabled the discovery of principles underlying tissue regeneration including biomineral formation, which were later found also in higher vertebrates including humans. In particular, it was discovered that biomineral formation is driven by enzymes, just like the biological formation of organic biomolecules and the functioning of metabolic pathways.<sup>28,29</sup> Thus, sponges served as a model to progress advances in osteochondral regeneration.<sup>30</sup> In addition, due to their excellent mechanical but also light transmitting properties,<sup>31</sup> which are particularly evident in the siliceous sponge spicules, sponges can be considered and used as blueprints for the design of functional composite materials not only for nanobiomedical but also for technological applications.

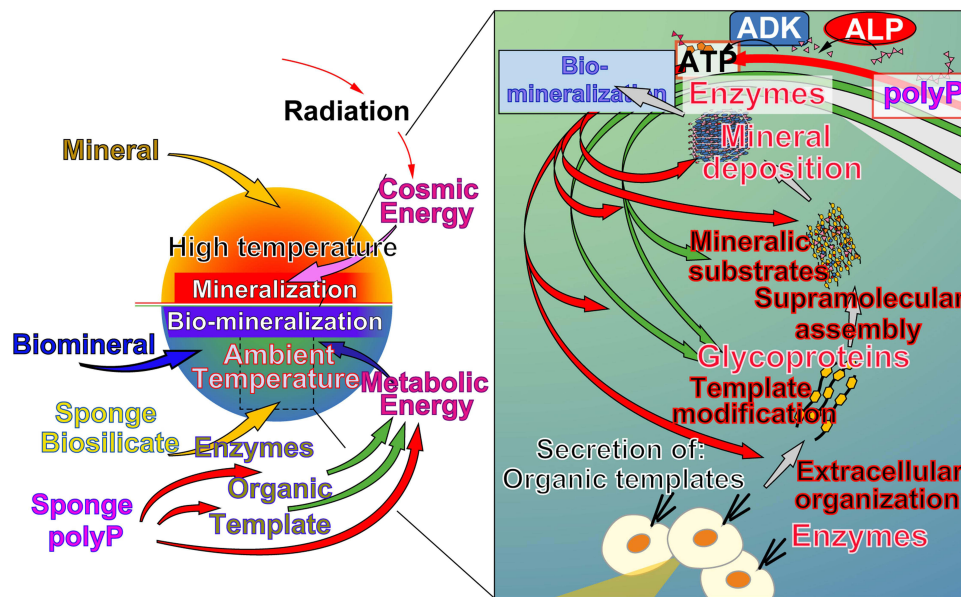
In general, scaffolds formed from natural biopolymers provide an excellent matrix and niche for cell attachment, growth, migration, and differentiation, mimicking the ECM of native tissues (for a recent review, see Ref.<sup>32</sup>). In these properties, natural biomaterials differ from bioinert synthetic materials used in tissue engineering and regenerative medicine that do not show bioactivity. Only by modifying them according to the design of natural polymers can synthetic bioinspired materials with biological activities be obtained.<sup>33</sup> Natural biomimetic biomaterials also have the advantage that they are non-toxic and do not exhibit genotoxic or teratogenic effects. In contrast to the natural biomaterials currently used, which are organic biopolymers such as polysaccharides (eg, hyaluronic acid, alginate, chitosan, and cellulose), polyesters (eg, polylactic acid and polyhydroxyalkanoates), and polypeptides/proteins (eg, collagen, gelatin, and fibroin),<sup>32</sup> the two inorganic materials described here, biosilica and polyP, although occurring physiologically, are also readily available by chemical methods, thereby overcoming the problem of batch variability shown by many natural polymers.

## Sponges (Porifera): Their Seminal Contributions Towards Understanding Biomineralization

Based on the lessons learned from sponges, the two inorganic polymeric materials amorphous biogenic biosilica (“biosilica”) and inorganic polyphosphate (polyP) have attracted increasing interest in regenerative medicine due to their unique properties. While biosilica forms a sponge skeleton, polyP is a ubiquitous polymer, found from bacteria to men, in sponges particularly in the environment of biosilica. Findings in sponges have been of crucial importance for an understanding of the biological mineralization processes taking place in organisms, as compared to abiotic mineralization.<sup>34,35</sup> While abiotic mineralization solely relies on chemical processes, biomineralization is based on chemical reactions that take advantage of genuine biochemical conversion processes. Biochemical reactions are principally catalyzed by enzymes, following the rules of thermodynamics. They often link individual processes together, where endergonic, non-spontaneous processes can be driven by exergonic, energy-releasing reactions.

More specifically, two principles have been revealed in sponges as are outlined in this review: First, that the vast majority of biochemical reactions, including biomineralization reactions, are enzyme driven and dependent. From sponges, the first enzyme that mainly contributes to inorganic mineral formation was discovered: silicatein.<sup>28,36,37</sup> While chemical reactions only strive to reach an equilibrium, biological processes proceed in open systems and are attuned to non-equilibria allowing both continuous and discontinuous flux of matter and energy.<sup>38</sup>

Biosilica, enzymatically synthesized *in vivo* in sponges (Figure 1), has become a paragon for a mineralic biomaterial. Later, it became increasingly aware that enzymes are also involved in the synthesis and degradation of the inorganic scaffold of other skeletal systems such as bone.<sup>39</sup> Of note here is the alkaline phosphatase (ALP), a ubiquitous, membrane-bound tetrameric enzyme that is attached via glycosyl-phosphatidylinositol moieties to the outer cell surface and involved in osteoid formation and mineralization.<sup>40</sup> The tartrate-resistant acid phosphatase and cathepsin K, an



**Figure 1** Sponges, the earliest animals on Earth, have proven to be a valuable model system for understanding the basic principles of biomineralization. In these organisms, in siliceous sponges, the first enzyme involved in mineral formation (formation of the sponge skeletal elements, the spicules) was discovered: silicatein. Centers for ATP consumption were found adjacent to the spicules. Subsequently, the polymer inorganic polyphosphate (polyP) was identified as an extracellular storage for metabolic energy, which serves to generate ATP by successive enzymatic degradation via alkaline phosphatase (ALP) and adenylate kinase (ADK). In contrast, mineralization processes require higher temperatures for the chemical reactions instead of enzymes and ATP. The process of biomineralization is facilitated by organic templates/sheets, often collagen, secreted from the cells and undergoing modifications (often glycosylation), allowing the formation of supramolecular assemblies, and finally biomineral deposition.

osteoclast-specific enzyme, are involved in bone resorption.<sup>41</sup> Furthermore, it has been suggested that bone formation also involves a carbonic anhydrase that synthesizes amorphous Ca-carbonate bioseeds during the course of Ca-phosphate bone mineral deposition.<sup>42</sup>

In contrast to enzymatic, biotic biomineralization, abiotic mineralization, eg, geothermal mineralization, is driven by heat derived from, eg, cosmic rays/ionizing radiation (Figure 1). In addition, it was shown that, in addition to enzymes, bone biomineralization as well as the organization of the bone architecture also require energy in the form of ATP,<sup>43,44</sup> without specifying the extent.<sup>45,46</sup> In fact, the energy issue was largely ignored until approaches to regenerate or repair tissue through biochemically based substitution therapy were addressed. Both osteoblasts and osteoclasts release ATP into the extracellular space in amounts that depend on the proliferation and differentiation state of the cells.<sup>43</sup> Despite a variety of ATP export channels into the extracellular space, the ATP concentration there is very low at ~10 nM, in contrast to the large intracellular pool at ~100 μM (see Ref.<sup>5</sup>). Since the level of ATP in human blood is also low at ~100 nM, an additional energy source for ATP generation had to be postulated.

Based on the therapeutic success with polyP, after application of this polymer for the repair in different organs, bones, and chronic wounds,<sup>30,47–49</sup> it was proposed and then proved that polyP with its energy-rich acid anhydride linkages could serve as a source for metabolically useful energy in the form of ATP and/or ADP,<sup>50,51</sup> reviewed in Ref.<sup>5</sup> As reported later, the energy stored in polyP can be converted to ADP/ATP in a stepwise enzymatic reaction chain.

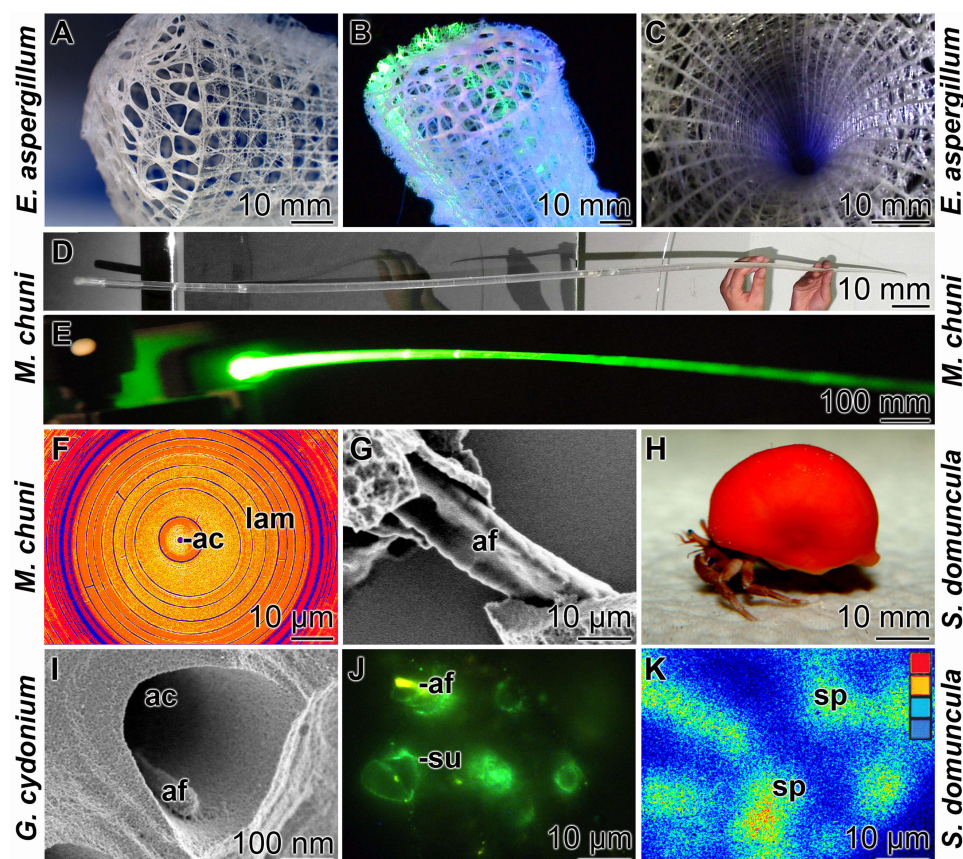
In addition to the two corner stones, (i) enzymes and (ii) metabolic energy, organic templates are usually required for the deposition of minerals during biomineralization. In bone, there are the collagen matrices built from collagen bundles that act as a platform for the apposition of mineral aggregates. Their post-translationally modified side chains, processed by hydroxylation and glycosylation, provide suitable deposition sites.<sup>52</sup>

## Sponge Biosilica

The sponges (Porifera) share a common body plan with the other, more evolved metazoans.<sup>53</sup> This fundamental finding, based on extensive studies of the (expressed) sponge genome, together with the discovery that these animals synthesize their mineral skeletons with the help of enzymes, as mentioned above,<sup>28,36,37</sup> formed the reason why these animals have

become metazoic model taxa for the development of biomimetic materials and processes applicable for both nanobio-medical and engineering purposes for the benefit of humans. Equally important was the discovery that biomineral formation is an energy-consuming process that requires an energy (ATP) source.

Based on their skeletons, the Porifera are subdivided into the classes of the siliceous Demospongiae and Hexactinellida and the calcareous sponges, the class Calcarea.<sup>54</sup> The siliceous spicules of most Demospongiae remain individualized and the secreted cellular tissue units are formed around them. In these sponges, the spicules are linked together with organic molecules (collagen-related spongin and lectin) that form a bulky extracellular matrix.<sup>55</sup> It is the siliceous scaffold that directs the particular, species-characteristic form of the sponge tissue. The siliceous skeleton of the hexactinellids is composed of discrete spicules, which often fuse to a basket-like scaffold as in the hexactinellid *Euplectella aspergillum* (Figure 2A).<sup>56</sup> In particular, the siliceous skeleton of this class of sponges transmits light along the outer surface of the cage (Figure 2B), but also within the inner fan pocket (Figure 2C). Particularly impressive are the central giant spicules of the deep-sea sponge *Monorhaphis chuni* with a length of up to 3 m (diameter 1.7 cm), which represent the largest biosilica structures on Earth (Figure 2D).<sup>57</sup> Light can pass efficiently along these giant spicules (Figure 2E). These spicules are composed of up to 800 lamellae, each 5–10 µm thick, which are arranged concentrically around the axial canal (Figure 2F). In the center, the axial canal harbors a 10 µm thick proteinaceous filament, the axial filament (Figure 2F).



**Figure 2** The siliceous sponges and their silica skeletons. (A–C) The hexactinellid sponge *E. aspergillum*; in (B), the silica cage is illuminated with a laser beam. (D and E) The hexactinellid *Monorhaphis chuni* with its up to 3 m large giant basal spicule; in (E), the silica rod is illuminated with green laser light. (F) Cross break through a giant basal spicule showing the central axial canal (ac) and the surrounding lamellae (lam). (G and H) A tylostyle spicule harboring (G) the central axial filament (af) of (H) the demosponge *S. domuncula*. (I) A broken aster spicule from the demosponge *G. cydonium* exposing the axial filament (af) in the axial canal (ac). (J) Cross break of a tissue unit from *S. domuncula* showing the immunostained silica-forming enzyme, silicatein, in the axial filament (af) and also on the surface (su) of the tylostyle. (K) Cryosection through a *S. domuncula* tissue unit; the transition in color from blue to orange reflects the increase in ATP level around the spicules (sp).

## Enzymes Involved in Sponge Biomineralization

It is a distinctive feature of both classes of siliceous sponges, the demosponges and the hexactinellids, that their skeletal elements, composed of amorphous biosilica, are synthesized by an enzyme, silicatein. With silicatein, the first enzyme was discovered that catalyzes the formation of an inorganic “polymeric” material, here biosilica, from an inorganic precursor, here monomeric silicic acid.<sup>37,58–60</sup>

Silicatein is an enzyme protein found exclusively in Porifera. Three isoforms of silicatein are found in the axial filaments.<sup>28,37</sup> Molecular sequence analyses revealed that the silicatein family of proteins originates from the cathepsin family of proteases, more precisely from cathepsin L.<sup>61</sup> Like the silicateins, the cathepsins are hydrolytic enzymes.<sup>62</sup> Since sponges are suspension/filter feeders, our group had proposed and then identified this major catabolic enzyme, cathepsin L.

Among the siliceous sponges, the demosponge *Suberites domuncula* (Figure 2H) has been used in most cell biological and molecular studies, because this sponge species can be readily kept in aquaria and allowed the establishment of a cell culture, the primmorphs.<sup>63</sup> In the demosponges, the axial filament is voluminous (Figure 2G and I). The silicatein filaments determine the morphology of the spicules, either rod-shaped (as in *S. domuncula*) or star-shaped (as in *Geodia cydonium*).

Cross fractures through the spicules revealed that not only the central silicatein rod but also the surface of the spicules reacts with antibodies against silicatein (Figure 2J). Interestingly, when cryosections through *S. domuncula* are studied, a regional distribution of ATP is measured (Figure 2K). The highest ATP levels are found in the vicinity of the spicules. The latter finding reflects the crucial role of ATP in the biomineralization processes and the dependence of the formation of skeletal elements on metabolic activity.<sup>64</sup> Biomineralization requires not only an enzyme that lowers the activation energy of this process but also metabolic energy in the form of ATP.

The other class of sponges, the calcareous sponges, Calcarea, are stabilized by calcareous spicules that are formed of calcite.<sup>65</sup> Following the general rule that any physiological crystalline structure is built from amorphous precursors, calcite formation starts with amorphous Ca-carbonate (ACC).<sup>66</sup> Our group found that the Ca-carbonate-based spicules are synthesized enzymatically by a carbonic anhydrase,<sup>29</sup> a finding that has been suggested earlier.<sup>67</sup> Also of interest is the finding that the ACC precursor is stabilized by polyP.<sup>68</sup> Consequently, incubation of ACC with the polyP-hydrolyzing enzyme ALP causes the transformation of the ACC phase into calcite.<sup>69</sup>

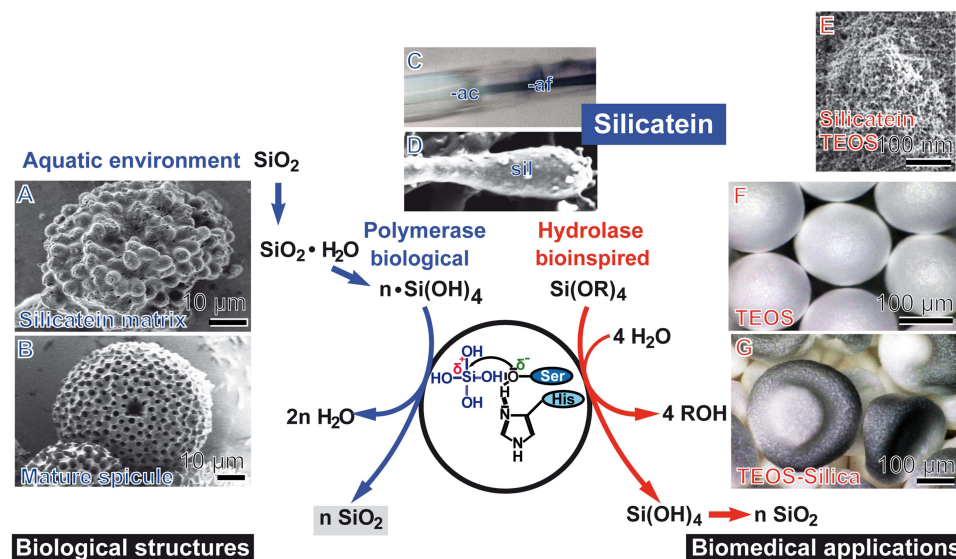
## The Substrate and Mechanism of Silicatein Reaction

Sponges take up silicon from seawater after conversion of  $\text{SiO}_2$  to soluble, biological biosilica ( $\text{SiO}_2 \cdot n\text{H}_2\text{O}$ ) and from there to readily soluble  $\text{H}_4\text{SiO}_4$  (aq).<sup>70</sup> Silicic acid is transported via a Si transporter into the sponge cells,<sup>71</sup> where it serves as a substrate for silicatein for the formation of amorphous biosilica. The synthetic silicon compound tetraethyl orthosilicate (TEOS), as a silicic acid precursor, was used for the functional analyses.<sup>28,58,72</sup>

Silicatein catalyzes the (poly)condensation/polymerization of its physiological substrate, ortho-silicic acid, at low concentrations (<1 mM).<sup>59,73</sup> Higher concentrations are required for chemical, non-enzymatic condensation reactions (>1 mM).<sup>73–75</sup> In bioinspired fabrication processes, silicatein acts as a hydrolase (silicic acid esterase) that facilitates the hydrolysis of alkoxysilane compounds (cleavage of Si–O–C “ester” bonds), eg, of TEOS.<sup>28,37,76</sup>

Among demosponges, the first deduced amino acid sequences of silicateins were published for *S. domuncula*<sup>28,77</sup> and *T. aurantium*.<sup>37</sup> The catalytic triad (catalytic center) of silicatein consists of the amino acids Ser, His, and Asn. The cathepsins have a Cys residue instead of Ser. Another characteristic sequence of silicatein is a Ser-rich cluster.

The mechanism of biosilica formation with silicatein has been outlined.<sup>78</sup> In the marine environment, the concentration of silicon is around  $\sim 10 \text{ mg mL}^{-1}$ . Silicic acid is taken up by cells via a specific transporter where it acts as a substrate for silicatein (Figure 3).<sup>71</sup> The reaction starts with nucleophilic attack of the catalytic triad Ser–OH group at a silicic acid substrate molecule, supported by hydrogen bridge formation to the His imidazole group in the catalytic center (Figure 3). The third amino acid of the catalytic triad of silicatein, the Asn residue (not shown), binds to the OH leaving group of the substrate, which is released as a water molecule. This step enables the formed Ser-bound silicic acid, again facilitated by H-bridge formation to His, to undergo two condensation reactions, resulting in the formation of an enzyme-bound disilicic acid and then a trisilicic acid species, which is then released from the enzyme after cyclization as cyclotrisilicic acid (cyclotrisiloxane). Next, a purely chemical condensation process takes place, initiated by preferential



**Figure 3** Physiological silicic acid polymerase reaction and bioinspired hydrolase reaction catalyzed by the biosilica-forming enzyme silicatein. Silicatein mediates the condensation of monomeric ortho-silicic acid  $[\text{Si}(\text{OH})_4]$  to polymeric amorphous silica ( $n \cdot \text{SiO}_2$ ; formation of Si–O–Si bonds). In a second further reaction, silicatein acts as a hydrolase, which catalyzes the hydrolytic cleavage of the alkoxy silane compound TEOS  $[\text{Si}(\text{OR})_4$ ; R = ethyl] (cleavage of Si–O–C bonds) allowing a bioinspired synthesis of artificial amorphous silica-based (nano)materials for biomedical applications. Shown in the center (enclosed in a ring) are the two amino acids of the catalytic triad, Ser and His, which interact with silicic acid and mediate the condensation. (A) Silicatein core protein of an aster from *G. cydonium*. (B) Complete sponge aster after incubation of silicatein with TEOS. (C) A spicule from *S. domuncula* with the axial canal (ac) centered by the silicatein-containing axial filament (af). (D) An axial filament with thorny silica (sil) protrusions. (E) Silicatein deposits layered onto thorny template, after reaction with TEOS. (F) Microspheres lacking silicatein after incubation with TEOS. (G) Silicatein-containing microspheres after TEOS incubation.

addition of further silicic acid species to this cyclic silicon compound. The subsequent steps of silica formation basically follow the Stöber synthesis, allowing a controlled growth of spherical silica particles to condensed silica deposits by hydrolysis of alkyl silicates with subsequent polycondensation of the produced silicic acid units.<sup>79</sup>

The biosilica synthesized by silicatein is deposited onto a structured organic template, the enzyme (Figure 3A and B) which also forms a mature siliceous spicule. Consequently, silicatein existing in the axial canal (the axial filament) predetermines the morphology of the spicules (Figure 3C and D). If the spicules are thorned, the surface of the silicatein axial filament shows thorny protrusions (Figure 3D). Using TEOS as a synthetic substrate, silicatein has been used to coat various surfaces/templates (Figure 3E), such as microspheres (Figure 3F and G).

## Biosilica: Bioinspired Application as a Morphogenetic Polymer

The field of application of (bio)silica and the bioinspired/biomimetic materials and biomineral structures based on it is wide, ranging from the use in regenerative medicine, improved bacterial fermentation to optical fibers and even as a construction model for architectural buildings.

Biosilica has been considered as a paradigm for biological mineral morphogenesis and evolution not only in sponges<sup>80</sup> but also in diatoms.<sup>81</sup> In both systems, the morphology of the structures formed is driven by genetically controlled mineralization. The morphogenetic biosilica effect is attributed to its gene-inducing effect.<sup>82</sup> In a series of applied experiments, it could be demonstrated that biosilica is a potent material for osteogenic stimulation and differentiation.

## Biosilica: A Generic Template for Bone and Tooth Repair

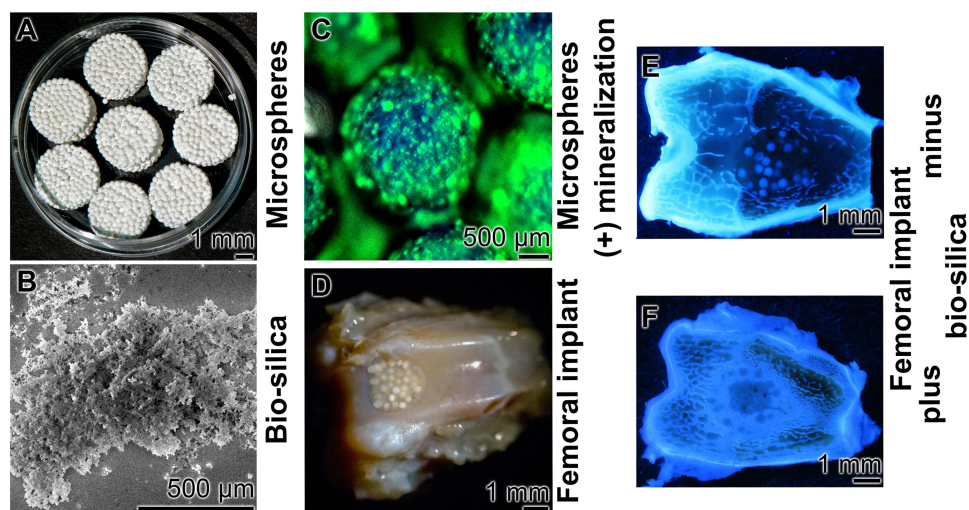
The property of biosilica to be morphogenetically active and supports and likewise accelerates regeneration/repair processes in humans was proven in vitro and in animals (rabbits).<sup>80,83</sup> In a first bioinspired approach, the sponge biosilica was applied in in vitro cell systems to evaluate and then to document an increased mineralization potential, first by measuring the expression of respective genes, like the structural proteins within the tooth matrix proteins, amelogenin, ameloblastin, and enamelin, which were strongly upregulated.<sup>80</sup> The rationale for this line of study came from Carlisle in her report in 1972.<sup>84</sup> Carlisle showed that chicks fed a silicon-rich diet grew and developed substantially faster than



control animals. These data were confirmed later<sup>85</sup> by demonstrating that the bone strength of broilers is strongly enhanced by dietary supplementation with bioavailable silicon. In continuation, in a series of in vitro studies, it was measured that both biosilica and silicatein induce the growth of mineralizing cells on silicatein/biosilica-coated matrices and cause under these conditions a strong increase in hydroxyapatite (HA) mineral formation;<sup>86</sup> in the absence of biosilica, no HA mineral nodules were detected.

Consequently, animal studies were performed using New Zealand White rabbits by inserting 600- $\mu\text{m}$ -large microspheres pressed into pellets (Figure 4A) and implanted into the anterior patella (between the medial and lateral femoral condyles). As an active ingredient, either silicatein ( $0.8 \mu\text{g g}^{-1}$ ) or biosilica ( $100 \mu\text{g g}^{-1}$ ) was added (Figure 4B). The biosilica pellets, added into PLGA (poly(D,L-lactide-co-glycolide))-based microspheres, were implanted.<sup>83</sup> After termination of the study, the implants reacted strongly with OsteoImage (Figure 4C), reflecting the increased regeneration/mineralization activity caused by biosilica.<sup>87</sup> The PLGA-based microspheres, supplemented with biosilica, were placed into the drilled space of the patellar grooves of the rabbits (Figure 4D).<sup>83</sup> After a total healing period of 100 days, the progression of bone regeneration was inspected by in vivo staining the samples with oxytetracycline dihydrate. The intensity of the blue fluorescence along a bone slide reflects the degree of regeneration.<sup>88</sup> The images showed that in the control samples with microspheres, supplemented with  $\beta$ -TCP ( $\beta$ -tricalcium phosphate), only a homogenous tissue is visible around the microspheres that did not brightly stain with oxytetracycline dihydrate and was not flashing under ultraviolet light (Figure 4E). In contrast, in the sections with the implanted biosilica-supplemented spheres, the microspheres are surrounded by bone tissue and this area lights brightly up almost homogeneously in blue (Figure 4F).

In general, crystalline biominerals such as bone HA are formed from the amorphous precursors.<sup>66</sup> The mechanism underlying the morphogenetic activity of biosilica is not fully understood. The release of orthosilicic acid from amorphous biominerals could contribute to the stimulating effect on the mineralization of bone-forming cells as in bioglass. The mechanism proposed for bioglass involves an exchange of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  with  $\text{H}^+$  ions, leading to the formation of silanol groups and an increase in the surrounding concentration of  $\text{OH}^-$  ions.<sup>89</sup> The cleavage of Si–O–Si bonds by  $\text{OH}^-$  then results in the release of orthosilicic acid and the formation/re-condensation of further silanol groups, which – similar to biosilica – leads to a hydrated, silica-rich layer on the glass surface, which is a suitable matrix for mixed carbonated HA deposition by invading  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$ ,  $\text{OH}^-$  and  $\text{CO}_3^{2-}$  ions. It is known that low silicate concentrations (0.05–0.5 mM) promote HA nucleation.<sup>90</sup> Silica has also been shown to stabilize and prevent the crystallization of ACC, the precursor of amorphous calcium phosphate (ACP), and of crystalline HA.<sup>91</sup> In addition,



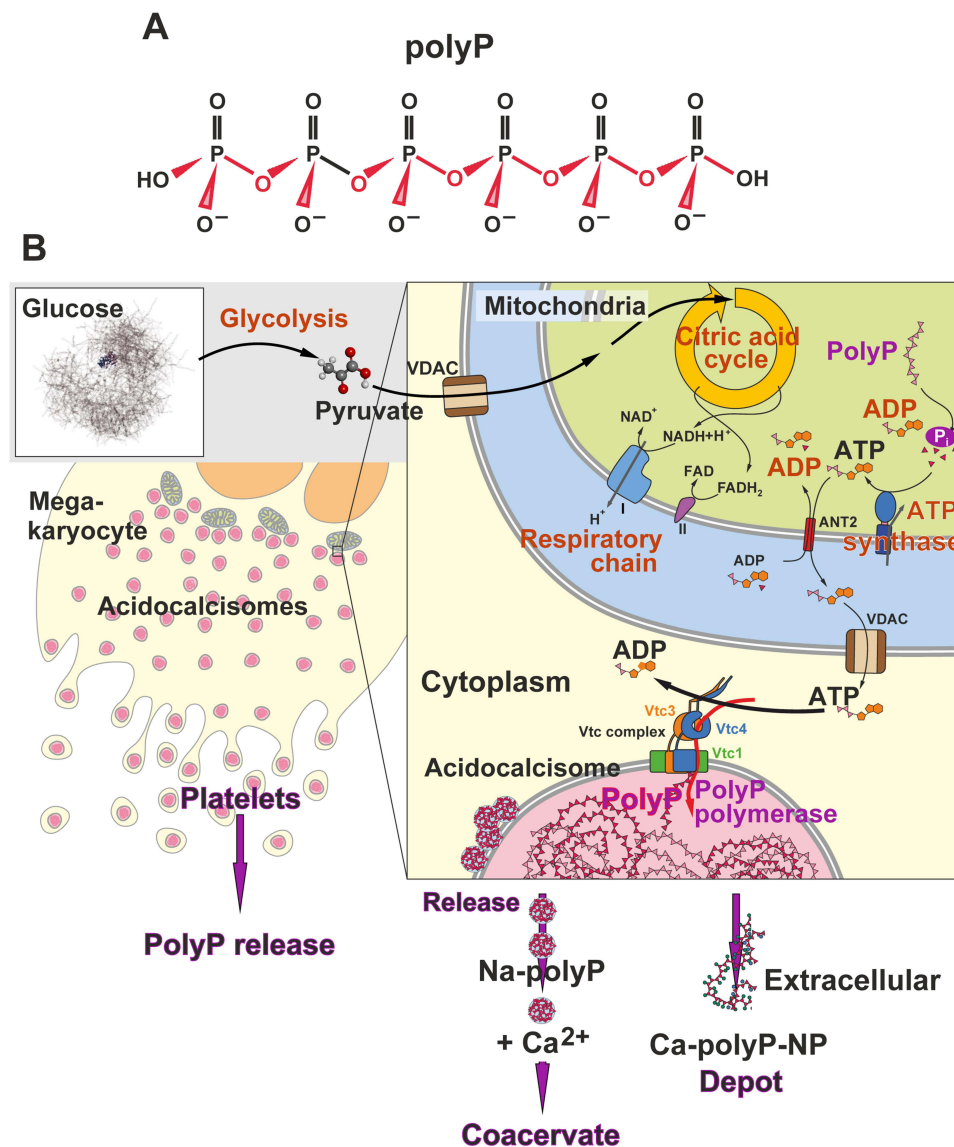
**Figure 4** Morphogenetic activity of biosilica in animal experiments (rabbits) - regeneration of bone in holes drilled into patellar grooves. (A) Microspheres embedded in pellets and (B) biosilica prepared by silicatein for the implant experiments. (C) The microspheres were fabricated together with biosilica. Staining of the mineral nodules formed in rabbits after insertion for 5 days. Staining with OsteoImage (fluorescence flashing). In the (D) femoral implant experiments, new bone formation was detected after in vivo staining with (E and F) oxytetracycline dihydrate under UV light. A striking difference was found between  $\beta$ -TCP controls (E) and biosilica-supplemented microspheres (F). Adapted from *Bone*, volume 67, Wang SF, Wang XH, Draenert FG, et al. Bioactive and biodegradable silica biomaterial for bone regeneration. 292–304. © 2014, with permissions from Elsevier Inc.<sup>83</sup>

modeling studies showed that the silanol groups of cyclic trisilicic acid motifs on the silica surface stereochemically mimic the  $\text{Ca}^{2+}$ -binding HA nucleation site on bone sialoprotein (BSP).<sup>92–94</sup>

These data underscore that biosilica shows morphogenetic activity on bone-forming cells not only in vitro but also in vivo, in animal experiments.<sup>95</sup>

## Inorganic Polyphosphate

The second physiological inorganic polymer, identified in sponges,<sup>96</sup> that has attracted increasing attention, is polyphosphate (polyP). Originally, polyP was identified in bacteria and yeast (for a review, see Ref.<sup>97</sup>), and later in animals, like in sponges<sup>96</sup> and higher vertebrates/humans.<sup>98</sup> This apparently ubiquitous polymer<sup>99,100</sup> exhibits a unique property that no other inorganic material useful for human therapy possesses - the delivery of metabolic energy. PolyP (Figure 5A) has



**Figure 5** (A) Chemical structure of polyphosphate (polyP). (B) Intracellular synthesis of polyP. Glucose is the body's main source of energy. After cellular uptake, glucose is metabolized to pyruvate during glycolysis, which is then channeled via the voltage-dependent anion channel (VDAC) to the mitochondrial intermembrane space and then to the matrix, where it undergoes oxidative metabolism. The generated reduced coenzymes (NADH and FADH<sub>2</sub>) drive redox reactions and the electron transport chain builds an electrochemical transmembrane proton gradient whose energy is converted to ATP. ATP is released into the cytoplasm via the adenine nucleotide translocator-2 (ANT2) and VDAC. Subsequently, ATP is channeled through the vacuolar transporter-chaperone complex (VTC) in the acidocalcisomal membrane, which functions in yeast as a polyP polymerase. From the acidocalcisomes in the megakaryocyte-platelets, polyP is released to the extracellular space either as soluble Na-polyP or as membrane-associated Ca-polyP nanoparticles (Ca-polyP-NP). Adapted from Müller WEG, Schröder HC, Wang XH. Inorganic polyphosphates as storage for and generator of metabolic energy in the extracellular matrix. *Chem Rev.* 2019;119:12337–12374. © 2019 American Chemical Society. Creative Commons.<sup>5</sup>

emerged as a prime example of a physiological polymer that not only fulfills the structural but also the energy-supplying requirements for a successful biomedical regeneration process.

Physiologically, polyP is stored intracellularly in organelles, now termed acidocalcisomes, which have been intensively studied in trypanosomatids, protozoan parasites.<sup>101,102</sup> Earlier they have been identified as metachromatic granules<sup>103</sup> or volutin granules.<sup>104</sup> In the blood platelets, polyP is accumulated in the dense granules, as identified by Ruiz et al.<sup>105</sup> The discovery that ATP and polyP harbor metabolic energy came from Meyerhof and Lohmann (cited in Ref.<sup>106</sup>). The unequivocal identification of the chemical structure was described by Lohmann and Langen.<sup>107,108</sup>

Recently, as described later, the proof-of-concept of the therapeutic benefits in the clinic for polyP could even be successfully provided. As a hydrogel, the natural polymer not only provides a platform for cell proliferation, cell differentiation, and cell migration but also provides the metabolic energy required for maintaining the molecular and supramolecular organization of the extracellular matrix and cell function.<sup>48,49</sup>

## Polyphosphate: Cell-Based Synthesis

The reaction chain by which polyP is synthesized in bacterial cells is fairly well understood (reviewed in Ref.<sup>109</sup>). Less is known about polyP synthesis in higher vertebrate cells.<sup>98,110</sup> For the synthesis of polyP in mammals, ATP is required, which is generated intracellularly in the mitochondria (Figure 5B).<sup>111–113</sup> There, in the respiratory chain, ATP formation is linked with complex V, the F<sub>1</sub>F<sub>0</sub>-ATPase. For the chemical synthesis of the phosphoanhydride bonds in ATP, heating to >100°C for ~10 h is required.<sup>114</sup> Accordingly, for an intracellular, physiological synthesis of ATP at 37°C, with its high-energy anhydride bonds, an activation energy of 110 kJ mol<sup>-1</sup> has to be expended, as determined using Arrhenius plot.<sup>115</sup> Therefore, the reactions in the mitochondria must be mediated by enzymes. It is the ATP synthase, localized in the inner mitochondrial membrane, which catalyzes the synthesis of ATP from ADP. In turn, the biosynthetic pathway for the formation of polyP with its >30 high-energy anhydride bonds must involve enzymes as well. In mammalian systems, the genuine polyP-synthesizing enzyme has not yet been discovered. Experimental evidence in yeast suggests that polyP is formed enzymatically from ATP during import into the acidocalcisomes (Figure 5B).<sup>116,117</sup> Indicative is the fact that, in the dense granules of the platelets that correspond to the yeast acidocalcisomes, the concentrations of ADP (600 mM), ATP (400 mM), and pyrophosphate (300 mM) are exceedingly high,<sup>118</sup> while the polyP content is comparably low (130 mM; based on P<sub>i</sub>).<sup>119</sup> Therefore, it might be assumed that phosphatases or phosphotransferases present in platelets<sup>120,121</sup> could be involved in polyP synthesis through backward reactions from ATP, driven by these enzymes. From the platelets, polyP is released in two forms, either in a soluble form, as a chain with Na<sup>+</sup> as counterion, or in an “insoluble” form, as a NP with Ca<sup>2+</sup> as counterion (Figure 5B).<sup>118,122</sup> Since polyP compartmentalized into the acidic dense granules (pH 5.4) is released in a controlled manner,<sup>123,124</sup> it is likely that the Ca<sup>2+</sup> gradient (Ca<sup>2+</sup> concentration in the dense granules is 2.2 M)<sup>119</sup> determines the formation of the two forms of polyP as proposed.<sup>118</sup>

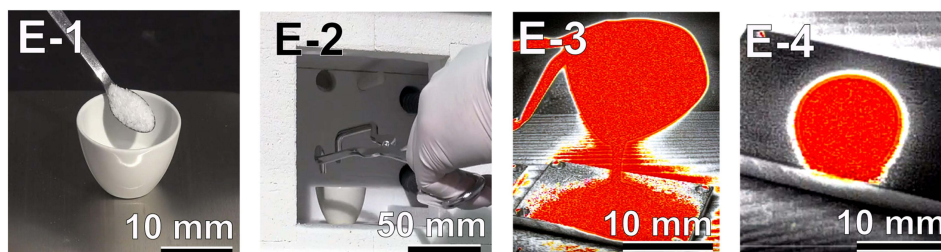
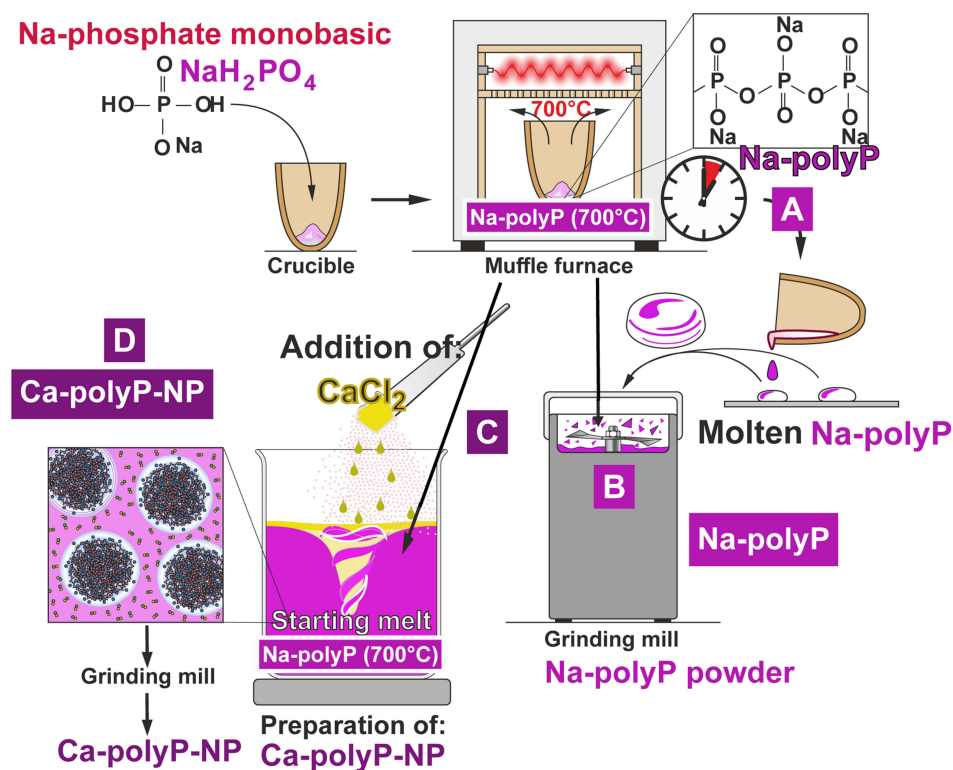
After full platelet activation, the concentration of polyP in the blood is relatively high with 0.5 to 3 μg mL<sup>-1</sup>.<sup>118,125</sup> There, the polymer has a physiological chain length of ~50 phosphate (P<sub>i</sub>) units. In the particulate Ca<sup>2+</sup> form, the polyP chains are longer with ~250 P<sub>i</sub> units. However, this value is very variable since there are high levels of ALP in the blood, which hydrolyzes the polymer from the chain termini as an exopolyphosphatase.<sup>126</sup>

A biocompatible polymer can be particularly beneficial to patients when transported within the body to the target site for tissue repair. Here, too, polyP follows the route of a biomimetic medical compound. In vivo, under physiological conditions, polyP is efficiently distributed to the injured sites in the body with the blood circulation. There, the polymer is delivered by the blood platelets to the damaged tissue regions, where the polymer initiates the regeneration process.<sup>127</sup> In fact, platelets with the stored polyP are hallmarks of regeneration.<sup>128</sup> It was Julius Bizzozero who discovered platelets as small medicinal pellets of a size between 1 and 2 μm.<sup>129</sup> He described them as the initiators of blood clotting. PolyP is a major constituent of platelets.<sup>105</sup> Using these vehicles, polyP is distributed throughout the body in order to onset regeneration/repair. The distribution of polyP is flanked by macrophages, which bind or internalize polyP, independently of their signaling roles, via their P2Y<sub>1</sub> and RAGE receptors.<sup>130</sup> Besides acting on the clotting cascade and enhancing hemostasis, platelets are activated and release polyP into the extracellular space when certain growth factors, such as epidermal growth factor or platelet-derived growth factor, are released.<sup>122</sup> In this environment, platelets bind directly to exposed collagen fibers, as well as to von Willebrand factor, fibronectin, and other adhesive proteins.<sup>131</sup> This efficient

distribution mechanism of polyP in the body contributes to the prominent position that polyP has achieved in regenerative medicine, as described later.

## Polyphosphate: Chemical Synthesis

Chemically, polyP can be prepared in sufficiently large quantities.<sup>132</sup> Na-polyP with its energy-rich phosphoanhydride bonds (Figure 6A) is obtained by melting of  $\text{NaH}_2\text{PO}_4$  at temperatures up to  $700^\circ\text{C}$  (Figure 6B). For the fabrication of Ca-polyP, both an enzymatic/wet chemical approach<sup>133</sup> and a calcination process have been introduced.<sup>134</sup> By shortening the high-temperature processing protocol, it is possible to prepare both amorphous Na-polyP and Ca-polyP-NP almost in parallel (Figure 6B and C). The process runs at  $700^\circ\text{C}$ . At the end of the polycondensation reaction, polyP, with a chain length of  $\sim 50\text{--}100$   $\text{P}_i$  units, is supplemented with  $\text{CaCl}_2$  and heated for an additional period of time to obtain the amorphous Ca-polyP-NP with a diameter of  $\sim 100$  nm (Figure 6D). When Na-polyP is synthesized, the material is ground to  $50$   $\mu\text{m}$  powder (Figure 6B). Different steps of the procedure, starting from  $\text{NaH}_2\text{PO}_4$ , are summarized in Figure 6E-1-E-4.



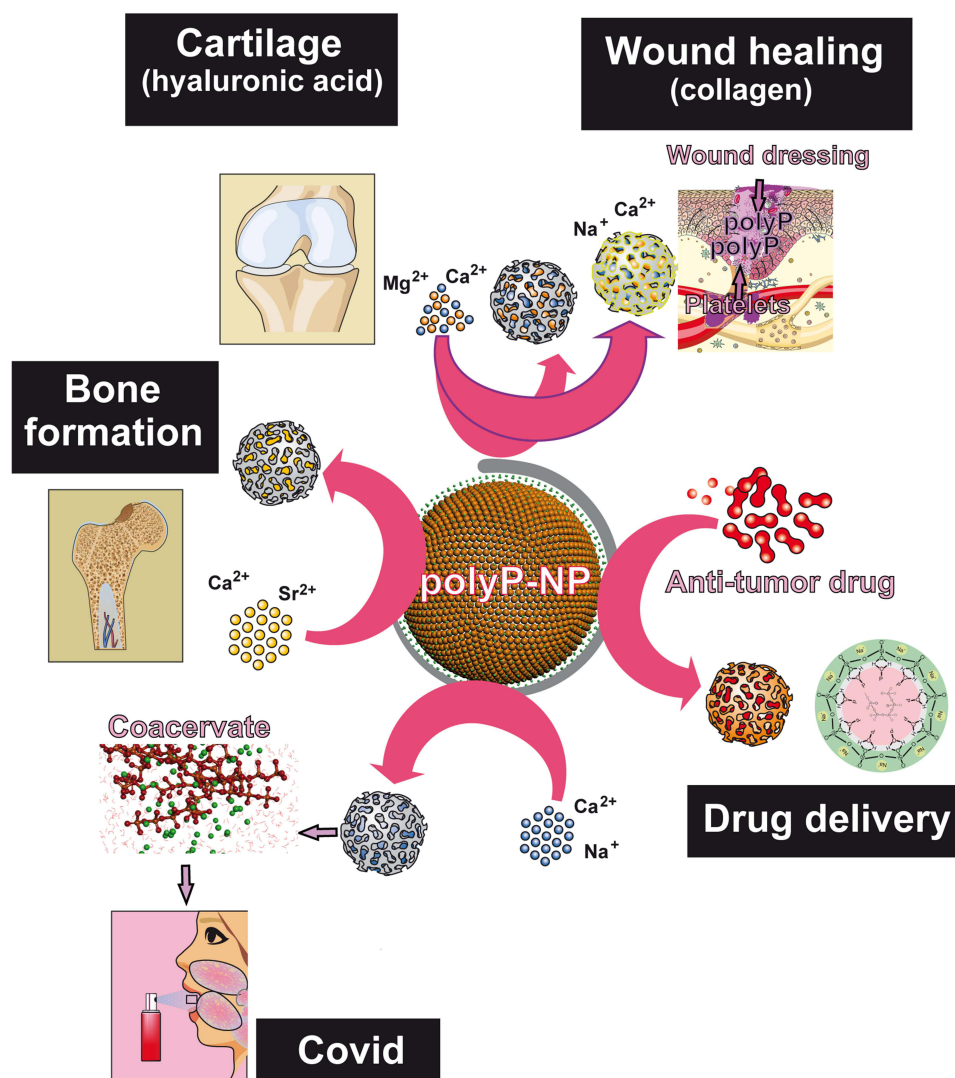
**Figure 6** Chemical preparation of polyP. PolyP is prepared by heating the starting material  $\text{NaH}_2\text{PO}_4$  to  $700^\circ\text{C}$ . Both amorphous (A and B) Na-polyP, and (C and D) Ca-polyP-NP can be prepared in parallel. (A) Na-polyP is formed during heating to  $700^\circ\text{C}$ . The glass-like melt is (B) ground to Na-polyP powder. Ca-polyP-NP is prepared from Na-polyP by addition of  $\text{CaCl}_2$  (C and D). By this, polyP of a physiological chain length of  $\sim 50\text{--}100$   $\text{P}_i$  units ( $\text{Na-polyP}_{50\text{--}100}$ ) is obtained. (E-1–E-4) Preparation process of Na-polyP and Ca-polyP-NP. (E-1) Start of preparation; (E-2) sliding in the furnace; (E-3) The molten glass poured from the crucible onto (E-4) a steel plate (images with infrared camera).

## Polyphosphate: A Biomimetic Molecule for Human Therapy

PolyP as a polyanion can be present in tissues as salt with various cations as counterions. The biological regeneration function of polyP differs depending on the counterion chosen. In addition, the functional activity of polyP depends on the form of the polyP salts, which can exist both in a soluble form, such as the sodium salt (Na-polyP), and in an insoluble nanoparticulate form that can be used as a storage (depot) form, such as salts with various divalent cations, eg,  $\text{Ca}^{2+}$  ions. The latter salts can also form a gel-like coacervate phase, a physiologically active form, as outlined later.

### Cation-Specificity of Polyphosphate: A Smart Nano/Micro Biomaterial

Besides the thermal method described above, polyP can be fabricated in a biomimetic way as nanoparticles (NP) as in vivo in human cells using a wet chemical procedure.<sup>118,135</sup> The biological activity of the polyP nanoparticles is determined, at least in part, by the counter-cations used for a particular scaffold (Figure 7). For cartilage repair,  $\text{Ca}^{2+}$ - and also  $\text{Mg}^{2+}$ -polyP complexes are more suitable,<sup>136</sup> while for bone regeneration, the Ca-polyP forms might be favored due to their higher stability. In addition, the  $\text{Sr}^{2+}$ -polyP complex shows strong regenerative activity both in vitro and in animal experiments.<sup>137</sup> Drugs that are effective, eg, against bone tumors/metastases, such as the bisphosphonate zoledronic acid, can be conveniently integrated into the Ca-polyP-NP and used as implant particles.<sup>138</sup> For another



**Figure 7** PolyP is a genuine, smart nano/micro biomaterial whose properties and applications depend on the selected counterion. PolyP-NP, polyphosphate nanoparticle. Reproduced with permission from Wang XH, Schröder HC, Müller WEG. Amorphous polyphosphate, a smart bioinspired nano-/bio-material for bone and cartilage regeneration: Towards a new paradigm in tissue engineering. *J Mat Chem B*. 2018;6:2385–2412. © 2018 The Royal Society of Chemistry. Creative Commons.<sup>142</sup>

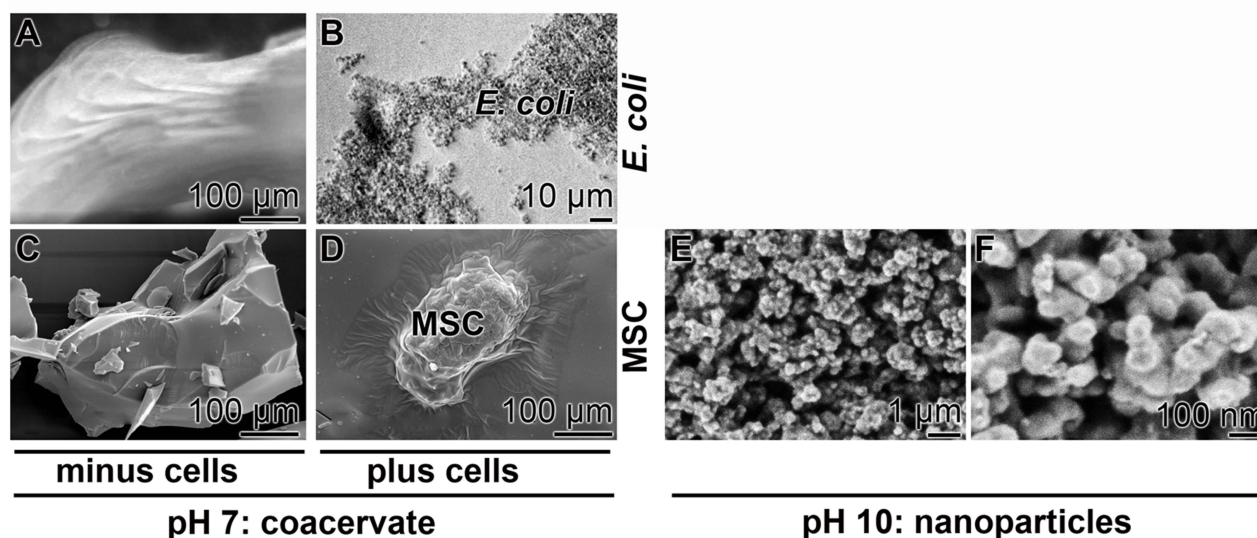
application target, chronic wound healing,<sup>48,49</sup> a combination of Na-polyP and Ca-polyP-NP acts most efficiently on the regeneration process. In addition, polyP particles, when applied in an aerosol form, have a protective effect on the respiratory epithelium, after conversion into the coacervate form upon contact with mucin, the protein of the airway mucus.<sup>139</sup> As a result, viral particles such as SARS-CoV-2 are entrapped and inactivated, in addition to a direct inhibitory effect on this virus.<sup>140,141</sup>

### Polyphosphate Coacervate Formation

It is an exceptional feature of polyP (as Na-polyP) to undergo coacervation in the presence of  $\text{Ca}^{2+}$  or other divalent cations, at physiological pH. In principle, polyP nanoparticles, including non-processed Ca-polyP-NP, are biologically inert. They have to be converted into a biologized form, a polyP-coacervate. During this phase transition, polyP provides its physiological functions. At pH 7,  $\text{Ca}^{2+}$  together with polyP forms a viscous aqueous phase, the coacervate (Figure 8A and C). These aggregates are formed through liquid–liquid phase separation, resulting in a denser phase and a dilute phase that are in thermodynamic equilibrium.<sup>143</sup> In the coacervate phase, polyP shows morphogenetic activity as well as its function to generate ATP.<sup>144</sup> In our approach, the two phases and their interconversion were studied using in silico simulation studies, as well as (physico)chemical analyses. The data showed that Ca-polyP coacervate formation occurs at pH 7 and is slower, compared to Ca-polyP-NP formation at pH 10 (Figure 8E and F). Interestingly, if  $\text{CaCl}_2$  is dropped into a Na-polyP solution at pH 7, the Ca-polyP coacervate initially forms and becomes then converted to NP at pH 10. Conversely, when Na-polyP is added to a  $\text{CaCl}_2$  solution, only the coacervate phase is obtained at both pH values.<sup>144</sup>

The coacervate is biocompatible and allows the infiltration of mesenchymal stem cells (MSC) into the gel matrix, where polyP promotes cell proliferation and differentiation,<sup>143</sup> the MSC become completely embedded in the matrix (Figure 8D). When the coacervate ( $\text{Ca}^{2+}$ -polyP) forms during the liquid–liquid phase separation, different local densities arise causing turbulences during which bacteria, such as *E. coli*, are wrapped (Figure 8B) and then killed.<sup>49</sup>

PolyP particles are taken up by endocytosis, as determined by inhibition studies with trifluoperazine dihydrochloride.<sup>51</sup> Intracellularly, the particles begin to transform into a coacervate. The free polyanionic polyP (not in a salt form) is not able to traverse cell membranes. Only after caging the polymer, eg, into a guanidinium/oligocarbonate vehicle, polyP can be channeled into the cells.<sup>145</sup>



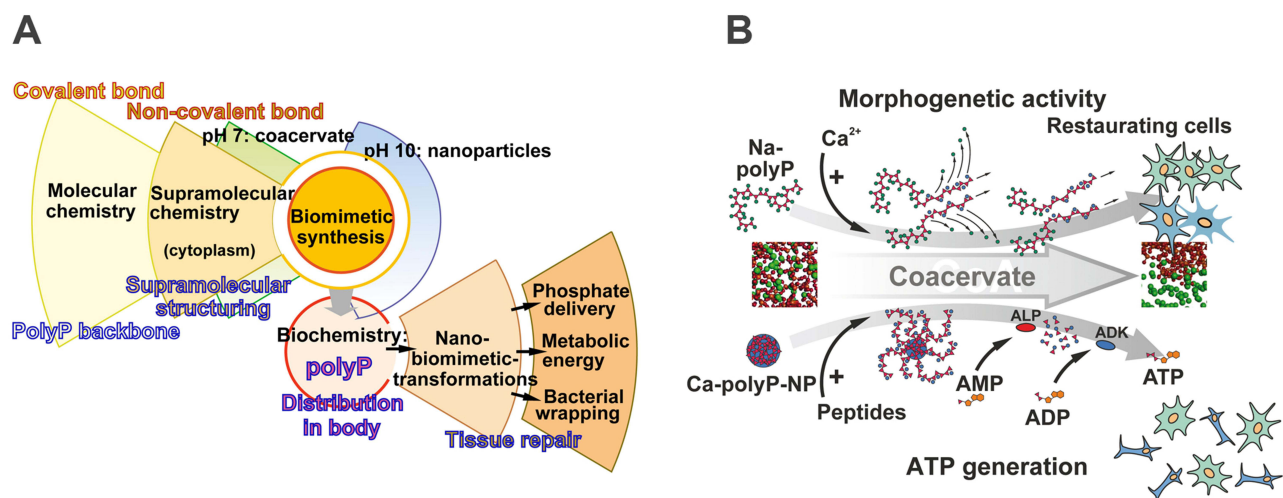
**Figure 8** PolyP / Ca-polyP phases. (A–D) At pH 7, the polyP coacervate is formed from  $\text{Ca}^{2+}$  and Na-polyP. During this process, the aqueous liquid-liquid gelatinous phase envelopes bacteria such as (B) *E. coli* and/or (D) attracts mesenchymal stem cells (MSC) and allows them to nest and differentiate there. (E and F) NP formed from  $\text{Ca}^{2+}$  and Na-polyP at pH 10.

## Supramolecular Extracellular Matrix Organization: Importance of Polyphosphate Coacervation and Energy Requirement

Biomimetic materials used for soft or hard tissue regeneration can be classified according to the four tissue categories: covering tissue, connective/and supporting tissue, muscle tissue, and nervous tissue.<sup>146</sup> The ECM of the different organs has a different percentage of cells, such as the liver with a high (>90%) and cartilage with a low cell density (<10%). A common feature of their ECMs is that the cells are embedded into a fibrillar, often collagenous, hydrogel, which allows architectural stability and simultaneous diffusion of nutrients as well as cell movement. To ensure biocompatibility, a physiological or close-to-physiological hydrogel is preferably used in regenerative medicine. Surely, such hydrogels can elicit signals stimulating tissue regeneration. Normally, hydrogels lack the ability to directly supply the cells with metabolic energy, preferably in the form of ATP.<sup>5</sup>

Supramolecular hydrogels have been introduced into regenerative medicine because of their unique dynamic properties for self-healing and injectability based on the non-covalent crosslinking organization of their macromolecules. Hydrogels based on this fabrication technology are held together by forces arising from non-covalent bonds such as electrostatic interactions, hydrogen bonds, metal coordination, aromatic stacking, and hydrophobic and van der Waals forces (Figure 9A).<sup>147,148</sup> The individual molecules are synthesized by covalent bond formation (“Molecular chemistry”) and become subsequently organized into complex supramolecular chemistry-based systems. There (usually) more than one chemical unit interacts via non-covalent bonds, as in the case of receptors or enzymes. Consequently, ligands or substrates in the next higher hierarchy level typically associate with receptors in turn via noncovalent bonds and form an active state of the system. In this complex arrangement, signal transduction pathways (with the respective ligands) are initiated or enzymatic reactions follow, through which new covalent bond(s) are introduced into the reaction chain.<sup>148</sup>

Due to its ability to form a coacervate promoting immigration and embedding of cells in the presence of  $\text{Ca}^{2+}$  or other divalent cations, as well as its ability to induce stem cell growth and differentiation, polyP is an excellent material for mimicking the conditions in the ECM of human tissues. In particular, polyP has the unique property of not only exerting a morphogenic effect but also providing the metabolic energy needed for tissue regeneration (Figure 9B). Organic polymers used with polyP included negatively charged polysaccharides such as *N,O*-carboxymethyl chitosan (*N,O*-CMC), alginate,<sup>149</sup> chondroitin sulfate,<sup>150</sup> hyaluronic acid,<sup>136</sup> and karaya gum,<sup>151</sup> as well as collagen<sup>150</sup> and poly(vinyl alcohol) (PVA).<sup>151</sup> These in vitro results were confirmed by animal experiments (rat muscle) showing



**Figure 9** Formation of a physiological hydrogel in mammalian cells. **(A)** The different steps of physiological hydrogel formation through the steps of molecular chemistry (formation of covalent bonds) and supramolecular chemistry (non-covalent bonds) to the biomimetic synthesis of a coacervate (pH 7) or NP (pH 10). During these reactions and pathways, polyP is distributed to the target tissues where it serves as a source for  $\text{P}_i$  delivery, metabolic energy supply, and bacterial wrapping. **(B)** The bidirectional functional activity of polyP. Na-polyP forms at pH 7 a coacervate by compartmentation of polyP molecules after  $\text{Na}^+/\text{Ca}^{2+}$  exchange. The transformation of Ca-polyP-NP, the storage (depot) form of the polymer, is facilitated in the presence of peptides. The coacervate is the functional form of polyP, which exhibits morphogenetic activity. During the progression of Ca-polyP-NP to the coacervate, the polymer is (partially) hydrolyzed by ALP under formation of ADP, which in turn is up-phosphorylated to ATP to bio-fuel the cells.

a replacement of the implants by granulation tissue already after an implantation period of 2 to 4 weeks.<sup>151</sup> The most advanced polyP-based bio-ink, which provides metabolic energy in addition to morphogenetic activity, was developed using an *N,O*-CMC/alginate/gelatin hydrogel enriched with soluble Na-polyP- and Ca-polyP-NP (see below). This bio-ink enabled the successful 3D bioprinting of MSCs that remained functionally active and able to migrate, grow, and differentiate to mineralizing osteoblasts.<sup>152</sup>

In tissues, the organization of both the intra- and extracellular supramolecular structures requires an energy input.<sup>153,154</sup> Self-assembly reactions are usually considered to be independent of energy supply; however, the subsequent downstream processes certainly require ATP, such as the organization of collagen fibers into bundles.<sup>155</sup> Until recently, the source of metabolic energy in the ECM was uncertain. It should be mentioned that the concentration of ATP in the extracellular space is only ~10 nM, while intracellularly the ATP level is high, varying between 0.5 and 5 mM depending on the cell type.<sup>156,157</sup> ATP is required for many functions in the ECM. There, ATP is involved in the cell-cell communication, particularly in the nervous system, but also for the activation of T cell receptor (TCR) signaling.<sup>158</sup> ATP is also required, as a chemotactic agent, for endothelial cells to direct them to micro-vessels along an ATP gradient.<sup>51</sup> In addition, ATP-consuming kinase reactions also run extracellularly and chaperones are also present (reviewed in Ref.<sup>5</sup>).

## Physiological Polyphosphate: A Key in Regenerative Medicine

Autologous platelet concentrate, platelet-rich plasma, is rich in polyP and is used for the prevention and treatment of bone and cartilage defects and for wound closure, especially in small ulcers.<sup>159</sup> Interestingly, fibrin (which contains platelets with their polyP) shows the same accelerating effect on regeneration processes.<sup>160</sup>

So far, the main focus of the polyP-correlated biomimetic application for human therapy is in the field of repair of osteochondral (bone and cartilage) and skin and soft tissue (chronic wounds). The common denominator for these targets are the MSC which promote both bone/cartilage<sup>161,162</sup> and wound repair.<sup>163</sup> Furthermore, in vitro studies have shown that polyP is a potent inducer of cell proliferation and differentiation<sup>164–166</sup> as well as mineralization.<sup>167</sup> There are two features that qualify the metabolizable polyP as a distinguished polymer in regenerative medicine; first, it is based on phosphate, an osteoconductive biomaterial required for bone cell growth,<sup>168</sup> and second, the inherent function of polyP to dissipate metabolic activity.<sup>5,99</sup>

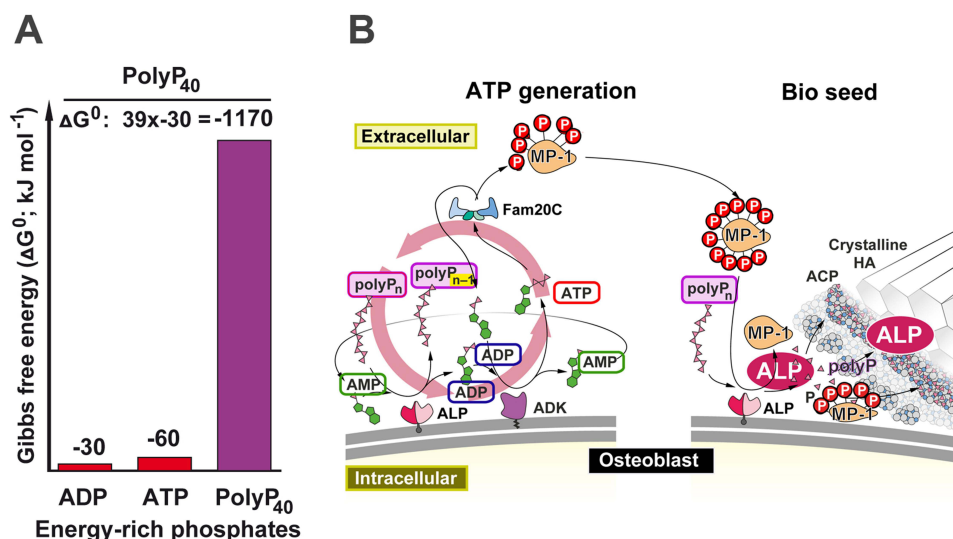
## Principal Distinctive Feature of Polyphosphate: Generation of Metabolic Energy

The distinguished feature of polyP is that its orthophosphate units are condensed via energy-rich phosphoanhydride bonds, forming a linear polymer with a physiological chain length of ~50–100 P<sub>i</sub> units.<sup>169</sup> The energy (Gibbs free energy;  $\Delta G^0$ ) stored in each bond and released during hydrolysis from the ends of the polymer is about  $-30 \text{ kJ mol}^{-1}$ .<sup>170</sup> The law of energy-constancy in a closed system implies that energy is converted into different forms, while the sum of energy remains constant. The energy simply converts from one form to another. In a biochemical reaction, a part of the energy is dissipated as heat and the rest is used as metabolic energy that drives biochemical reactions.<sup>171</sup> In turn,  $\Delta G^0$  for ADP hydrolysis is about  $-30 \text{ kJ mol}^{-1}$  and for ATP (both energy-rich bonds), it is about  $-60 \text{ kJ mol}^{-1}$  (Figure 10A). In contrast, polyP with a chain length of, eg, 40 P<sub>i</sub> units (39 energy-rich bonds) comprises a  $\Delta G^0$  of approximately  $-1170 \text{ kJ mol}^{-1}$ . Experimental evidence from SaOS-2 cells, after incubation with polyP, showed that an increased amount of energy-rich nucleotides (ADP and ATP) accumulates extracellularly.<sup>50</sup> During the incubation of the cells with polyP, vesicles with both ALP and adenylate kinase (ADK) activities are released. These two enzymes are plasma membrane associated.<sup>172,173</sup> Inhibition studies indicate that initially ADP is formed (during degradation of polyP with ALP), which is further up-phosphorylated to ATP by ADK.<sup>51</sup> So far, a quantitative evaluation of the yield of ATP production from polyP is missing.

## Kinases: An Essential Transfer Element

The outlined generation of ATP from polyP would cease when an equilibrium defined by the equilibrium constants between polyP, AMP, ADP, and ATP is reached. Consequently, metabolic energy must flow out of this equilibrium to keep the system running (Figure 10B). At this point, kinases achieve a crucial function. Extracellularly, a number of proteins involved in mineralization are phosphorylated, in particular osteopontin, dentin matrix protein-1, and dentin sialophosphoprotein.<sup>174,175</sup> As the only (major) enzyme that catalyzes the phosphorylation of these proteins, the Golgi-





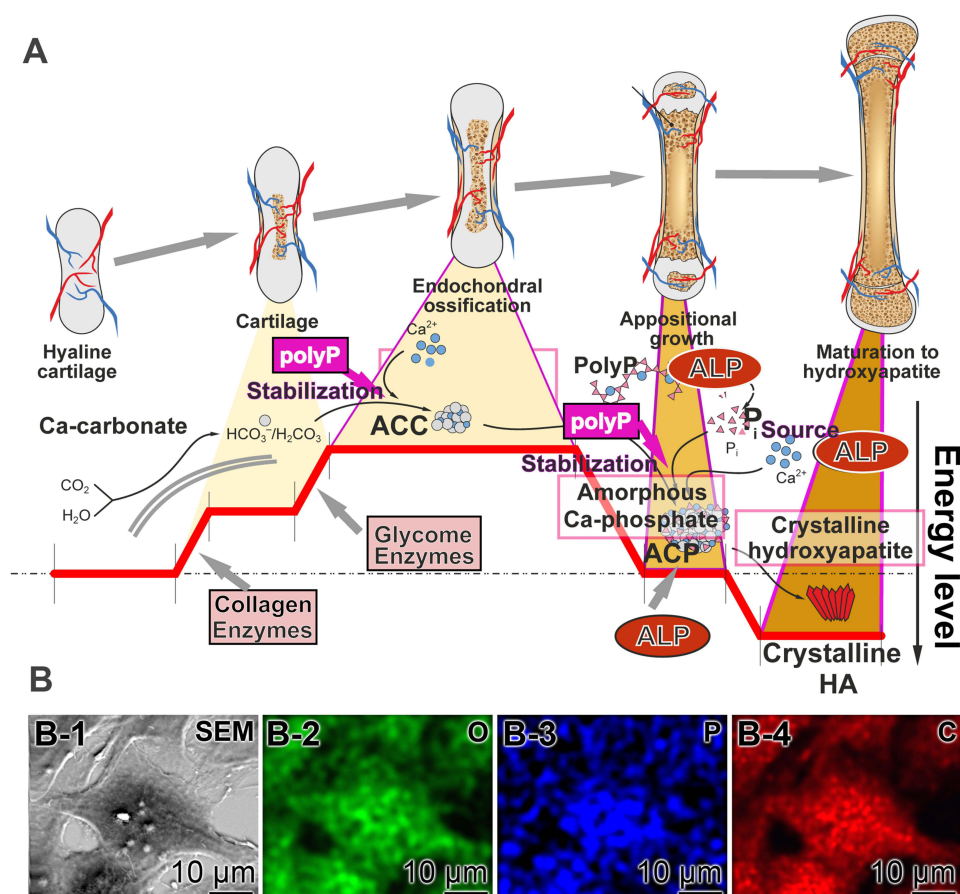
**Figure 10** Role of ATP during bone mineralization. **(A)** PolyP acts as an extracellular generator for ATP (for polyP<sub>40</sub>:  $\Delta G^0$ :  $-1170 \text{ kJ mol}^{-1}$ ). A polyP molecule can generate a multifold of the energy present in ADP ( $-30 \text{ kJ mol}^{-1}$ ) or ATP ( $-60 \text{ kJ mol}^{-1}$ ). **(B)** Bone mineralization is an energy-dependent process and proceeds extracellularly (Scheme). This process can be divided (left panel) into an ATP generation section and a bioseed formation for initiating of bone/HA synthesis (right panel). On the surface of osteoblasts, polyP is enzymatically hydrolyzed by ALP to form ADP from AMP. During cleavage of the high-energy acid anhydride bonds in polyP<sub>n</sub>, Gibbs free energy is released; in each step, the polyP chain is shortened by one P<sub>i</sub> unit (polyP<sub>n-1</sub>). The enzyme ADK forms one mole of ATP from two moles of ADP. This process is coupled to the Fam20C kinase, which phosphorylates the dentin matrix protein I (MP-I), among other proteins. The phosphorylated MP-I is an essential key in bone mineralization. The molecule acts as seed for the deposition of ACP (amorphous Ca-phosphate). Subsequently, after hydrolysis of polyP via ALP, ACP transforms into crystalline hydroxyapatite (HA).

localized protein kinase Fam20C, a serine kinase, has been identified.<sup>176</sup> Failure of the function of Fam20C often leads to lethal osteosclerotic bone dysplasia, known as the Raine syndrome.<sup>177</sup>

### Repair of Osseous Hard Tissues by Polyphosphate

Already Lowenstam suggested that the calcareous invertebrate skeletons from the Early Cambrian  $\approx 560$  mya are made of ACC, a view that has been later corroborated by dissolution experiments.<sup>178,179</sup> In sponges, the class of calcareous sponges (Calcarea) forms their hard skeletons of Ca-carbonate, which is individually composed of calcite formed enzymatically with the sponge-specific carbonic anhydrase.<sup>29</sup> The inorganic biomaterial of the otoliths in the human inner ear also completely consists of Ca-carbonate, which is present in the form of calcite.<sup>180</sup> It is not yet clear which enzymatic processes are involved in Ca-carbonate biosynthesis in human systems. Experiments with human osteogenic SaOS-2 cells suggested that in this system the carbonic anhydrase CA-IX, a cytosolic enzyme localized on cell membranes, is upregulated after exposure of SaOS-2 cells to bicarbonate.<sup>142</sup> HA/bone formation proceeds through the phases of hyaline cartilage (hyaline, translucent cartilage), “smooth” cartilage (smooth type of connective tissue), endochondral ossification (embryonic bone tissue formation), appositional growth (increase in diameter), and finally, maturation of the mineral deposits to crystalline HA (Figure 11A).<sup>181</sup> Element mapping using SaOS-2 cells by scanning electron microscopy of the surfaces of mineral deposits showed that the Ca minerals show signals for phosphorus, as expected, but initially also for the element carbon (Figure 11B-1–B-4). These data corroborate the assumption that mineralization in human bone also passes through the Ca-carbonate stage before the conversion to Ca-phosphate can take place.<sup>42</sup> Therefore, it was proposed that the initial step in bone formation starts with the deposition of Ca-carbonate, perhaps catalyzed by the carbonic anhydrase. The matrix onto which bone mineral deposition takes place is a collagen meshwork. Surely, the first mineral deposits are amorphous.<sup>66</sup> The transformation of the enzymatically formed (carbonic anhydrase-mediated) amorphous Ca-carbonate (ACC) towards amorphous Ca-phosphate (ACP) proceeds as an exergonic reaction and is not enzyme-driven.<sup>182,183</sup> After that, ACP transforms into crystalline hydroxyapatite (Figure 11A).

A huge amount of phosphate is required for bone formation. Based on in vitro data, it had been suggested that it is the organic phosphate,  $\beta$ -glycerophosphate, which, after hydrolysis by the ALP, provides the inorganic phosphate for bone formation.<sup>184</sup> Later, when the crucial role of the platelets in bone synthesis and bone remodeling was revealed,<sup>185</sup> it



**Figure 11** Ca-phosphate/bone formation. **(A)** Steps during bone formation: amorphous Ca-carbonate (ACC), amorphous Ca-phosphate (ACP) and finally crystalline Ca-phosphate/hydroxyapatite (HA). The different phases of bone initiation, growth and maturation are outlined in the upper trace. At least the final step, the transition from ACC to ACP, is driven enzymatically by ALP. There, the enzyme ALP hydrolyzes polyP and, by this, eliminates the stabilizing polymer polyP from the amorphous ACC and ACP phases. This process leads to the maturation of ACP to crystalline HA. During this process, the initial endergonic reactions are pulled by the subsequent exergonic processes. **(B)** Element mapping of the surfaces of Ca mineral deposits formed on human osteoblast-like SaOS-2 cells (**B-1**; SEM) for the elements oxygen (**B-2**), phosphorus (**B-3**) and carbon (**B-4**). Adapted with permission from Wang XH, Schröder HC, Müller WEG. Amorphous polyphosphate, a smart bioinspired nano-/bio-material for bone and cartilage regeneration: Towards a new paradigm in tissue engineering. *J Mat Chem B*. 2018;6:2385–2412. © 2018 The Royal Society of Chemistry. Creative Commons.<sup>142</sup>

became evident that polyP is the main source of  $P_i$ .<sup>186</sup> In this study, it was also reported that the gene expression of ALP,<sup>187</sup> a marker enzyme for bone formation, is upregulated after polyP exposure.<sup>186</sup> The enzyme-driven mineralization chain then proceeds to the ACP step and allows an appositional growth of the bone.<sup>188</sup> In ACP, polyP acts as a stabilizing polymer of the amorphous phase during its transition to crystalline HA.<sup>167</sup> After hydrolysis of polyP by the ALP, maturation from amorphous to crystalline Ca-phosphate can take place.<sup>69</sup>

Following these new scientific insights, polyP has been tested for its regenerative activity in vivo. Using the rat calvarial defect system as a model of bone repair, the polymer was integrated into PLGA microspheres. After eight weeks, the material was fully resorbed and the bone defect was completely restored, while  $\beta$ -TCP caused only limited repair. A comparative gene expression study revealed that polyP also significantly upregulates the expression of the collagen gene, in contrast to the  $\beta$ -TCP material.<sup>189</sup>

As mentioned above, crystalline biomaterials have only limited morphogenetic, regenerative activity, while amorphous materials exhibit regenerative potential.<sup>66</sup> This differential effect can be demonstrated with the polyP-stabilized amorphous phase of Ca-phosphate, ACP. In the presence of small amounts of polyP (5% by weight), ACP still proceeds to the crystalline Ca-phosphate state (CCP). However, after increasing the polyP level to 15%, the ACP mineral remains frozen in the amorphous state.<sup>167</sup> Therefore, a comparative animal study was performed with crystalline Ca-phosphate (containing 5% polyP), cCa(5)polyP, versus amorphous ACP/15% polyP particles (aCa(15)polyP).<sup>30</sup> The Ca-phosphate formulations were integrated into PLGA microspheres. The starting material cCa(5)polyP shows a hexagonal columnar

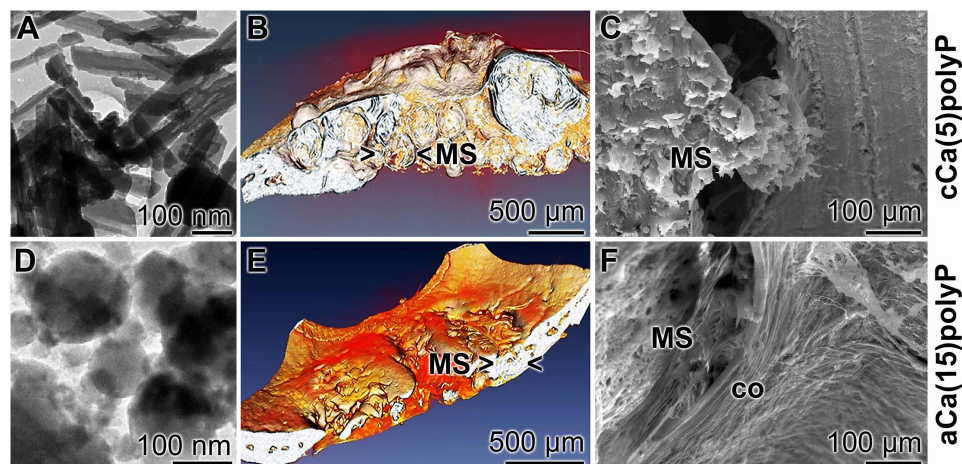
crystal morphology<sup>190</sup> (Figure 12A). In the in vivo model (rabbit calvarial bone defect model), cCa(5)polyP only marginally repaired the bone defect after 6 weeks (Figure 12B). Interesting was the fact that the association (acceptance) of the microspheres to the tissue environment with cCa(5)polyP was very poor or even absent (Figure 12C).<sup>30</sup> In comparison to crystalline ACP, the amorphous ACP (aCa(15)polyP) deposits appear as globular spheres (Figure 12D). Those ACP deposits accelerate the processes of migration, microvascularization, and mineralization in SaOS-2 cells. The repair process was dramatic; the newly formed tissue covered the regenerating zone and showed only remnants of the microspheres (Figure 12E). Even more, the microspheres in the regenerating area of the aCa(15)polyP-treated animal group readily attracted and interacted with the newly formed collagen bundles (Figure 12F). These data show that aCa(15)polyP combines osteoinductive activity with the morphogenetic potential to build the HA calvaria.

### Repair of Soft Tissue: Chronic Wounds

It is established that the supply of blood, and hence platelets, is crucial for bone tissue development and repair, and for healing of osteoporotic fractures.<sup>191–193</sup> During these processes, platelets surround the affected site and become activated, and release growth factors and certainly also polyP. Even more obvious is the role of platelets in wound healing. It has been reported that platelet deficiency plays an adverse, critical role in skin healing and that its failure prevents healing of chronic wounds.<sup>194</sup> In all phases of wound repair, hemostasis, followed by inflammatory reactions, up to the proliferative and remodeling phase, platelets decisively promote the regeneration process.<sup>195</sup>

Wound healing is energy-intensive. Most of the metabolic energy is consumed for positioning of new cells in the wound area.<sup>196</sup> The epithelial cell motility, which is controlled by different members of the Rho family of GTPases, which act as molecular switches, consumes energy-rich nucleotides.<sup>197</sup> In addition, wound healing is decisively dependent on new vessel formation, beginning with a polyP-induced microvascular network,<sup>198</sup> which is required for optimal tissue perfusion and oxygenation. Finally, ATP acts as a stimulus for the endothelial cells to follow an ATP gradient and guide the cells via an autocrine sensing mechanism during the initial chemotactic formation of 100  $\mu\text{m}$  circular endothelial cell rings.<sup>51,199,200</sup> This ATP originates from glycolysis and not from the respiratory chain.<sup>201</sup>

Chronic wounds are becoming an increasingly common clinical problem, especially in patients with diabetes mellitus.<sup>202</sup> In these patients, severe hyperglycemia due to an impaired insulin supply leads to reduced gene expression of enzymes involved in mitochondrial oxidative phosphorylation.<sup>203,204</sup> In addition, an imbalance between angiogenic factors, angio-inhibitory factors, and abnormal apoptotic potential is observed, which reduces the proliferation of cells



**Figure 12** Bone healing with crystalline Ca-phosphate (cCa(5)polyP; containing 5% polyP). (A) Crystalline Ca-phosphate formed during aging in the presence of only 5% polyP. (B and C) Healing studies of encapsulated Ca-phosphate microspheres (MS) in vivo using the rabbit calvarial bone defect model. (B) After 6 weeks of insertion of MS filled with cCa(5)polyP, only a slightly repaired bone defect was seen in animals; (C) the interaction between the microspheres (crystalline phosphate implant) and the adjacent bone is poor. In contrast to the crystalline Ca-phosphate, containing only 5% polyP, (D) addition of 15% polyP to the starting substrates for the preparation of Ca-phosphate,  $[(\text{NH}_4)_2 \text{HPO}_4 \text{ and CaCl}_2]$ , freezes the product Ca-phosphate at the amorphous phase. (E) Those MS elicit a strong healing process, the MS fused with adjacent regenerating bone and (F) formed a tight collagen (co) around the MS. Adapted with permission from *Acta Biomater.* Volume 18, Müller WEG, Ackermann M, Al-Nawas B, et al. Amplified morphogenetic and bone forming activity of amorphous versus crystalline calcium phosphate/polyphosphate. 233–247, copyright 2020, with permission from Elsevier.<sup>30</sup>

and their remodeling capacity. In diabetic wounds, the cells remain in a chronic inflammatory state and do not switch to the proliferation and remodeling phase. In turn, the use of drugs that generate metabolic energy in the wound is indicated. Besides ATP<sup>205</sup> and a hydrogel supplemented with 0.02% adenine,<sup>206</sup> it turned out that the physiological polymer polyP accelerates the healing rate of both experimental wounds in diabetic animals<sup>207</sup> and chronic wounds in patients.<sup>48,49</sup>

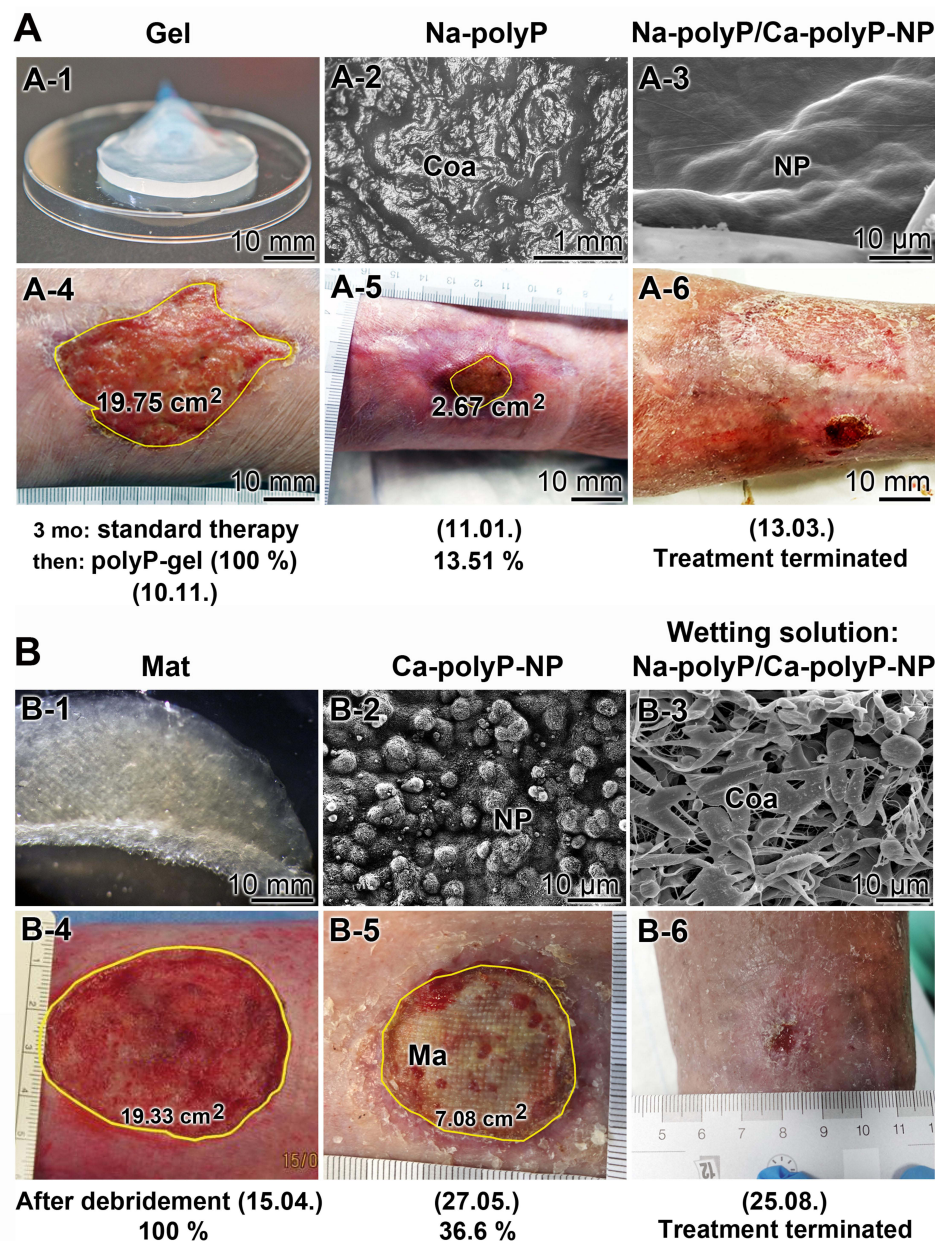
In animal studies, polyP was applied into 8-mm wounds of diabetic mice and normal non-diabetic animals punched in the interscapular region down to the underlying fascia muscularis.<sup>207</sup> In both animal groups, the polymer was applied once. While in normal mice (untreated), the physiological regeneration proceeded by 31% re-epithelialization during a 7-day healing period, the polyP-treated animals showed a strong acceleration of the regeneration potential to 72% during 7 days. In diabetic controls, re-epithelialization was even less, only about 20% by day 7, while with polyP-NP, healing was greatly accelerated and was complete after 14 days.

These very promising results prompted our group to conduct off-label studies in patients. Depending on the depth of the wound, either more superficial or deeper to the hypodermis along the muscle or down even to the bone, the active substance has to be integrated into a hydrogel or alternatively into a collagen-based mat.<sup>208</sup> In the first human trial with chronic wounds, a hydrogel (with hydroxyethyl cellulose) containing  $600 \mu\text{g mL}^{-1}$  Na-polyP (to elicit immediate regenerative activity) together with  $60 \mu\text{g mL}^{-1}$  Ca-polyP-NP (acting as a depot) was prepared (Figure 13A).<sup>49</sup> The Na-polyP in the gel formulation undergoes direct coacervation after application onto the wound, taking up the  $\text{Ca}^{2+}$  from the wound bed (Figure 13A-1). The Ca-polyP-NP ingredient (depot form) must form a coacervate before becoming active (Figure 13A-2 and A-3). A clinical example is illustrated in Figure 13A-4–A-6. The 55-year-old female patient was suffering from a ventral ulcer on the tibia for 3 months. This patient had been treated with cortisone over a long period due to a number of pre-existing illnesses. Treatment of the wound with the hydrogel started on 10.11. (Figure 13A-4). After 8 weeks of treatment, the wound area was reduced by 87% (Figure 13A-5). The patient was released from the clinics 10 weeks later (Figure 13A-6).

Deep wounds can extend through the epidermis, dermis, hypodermis, and even to the cutaneous trunci muscle covering the skeletal muscle. These wounds require a wound dressing that elicits low antigenicity, suitable biocompatibility, and hemostatic properties to allow for cell proliferation and adhesion. One choice is collagen mats, which imitate the ECM, since collagen stabilizing the skin is present in the mats with an abundance of 60% to 70%. The collagen-based mats used here for application to deep wounds of patients<sup>48</sup> were modified by compressing the collagen fibers in order to transfer the unstable collagen sheets to a strong wound-covering layer. For this purpose, bovine collagen samples were processed through a controlled pH shift series. The mats were then supplemented with 8 mg of Ca-polyP-NP per 1 mL collagen solution ( $10 \text{ mg mL}^{-1}$ ). The thickness of the mats was 1 to 1.3 mm (Figure 13B-1). The NP within the mats were oriented along the orientation of the collagen bundles (Figure 13B-2). Every second day, after application onto the wounds, the mats were moisturized with 1 mL of wetting solution composed of  $300 \mu\text{g g}^{-1}$  Na-polyP and  $30 \mu\text{g g}^{-1}$  (w/w final) Ca-polyP-NP to improve the healing process.<sup>48</sup> After moisturizing the mats, a coacervate developed from the Na-polyP component (Figure 13B-3). As with the hydrogel treatment, the results of the off-label studies with the wound mats have been very encouraging. An example is shown in Figure 13B-4–B-6. The 79-year-old male patient suffered from a chronic wound resulting from surgical resection of a squamous cell carcinoma. The treatment of the wound, with a dimension of  $\sim 20 \text{ cm}^2$ , started in April 2021 (Figure 13B-4). After debridement by surgery and ultrasound, the wound was covered with the polyP-collagen mat. After 6 weeks of treatment, the wound diameter had shrunk to 37% (Figure 13B-5), and after a further 12 weeks, the patient could be released home (Figure 13B-6).

### Repair of Soft Tissue: Respiratory Epithelium

The epithelium of the respiratory tract (from the trachea to the bronchi) has an important barrier function against inhaled pathogens. The mucus gel layer produced by the goblet cells of the respiratory epithelium plays a crucial role in this defense mechanism by the entrapment of respiratory bacteria and viruses such as the coronavirus SARS-CoV-2. This epithelium is prone to damage not only by these pathogenic agents but also by environmental factors such as pollution by particulate matter. PolyP was found to induce the expression of mucins, the main protein component of the mucus.<sup>139</sup> In addition, it was found that polyP exhibits a strong antiviral activity against SARS-CoV-2.<sup>140,141</sup> The finding that polyP elicits regenerative activity in the epithelial respiratory tract was not unexpected. The way it inhibits SARS-CoV-2 was surprising. Based on modeling studies, it has been demonstrated experimentally by *in vitro* studies and also supported by *in vivo* studies that polyP covers the tips of the coronavirus SARS-CoV-2 viral envelope spike proteins by binding to an



**Figure 13** Application of polyP for the treatment of chronic wounds. **(A)** Application of the polymer in **(A-1)** a hydrogel supplemented with Na-polyP and Ca-polyP-NP to cure more superficial wound. **(A-2)** Formation of a coacervate after contact with  $\text{Ca}^{2+}$  in the wound bed. **(A-3)** The presence of Ca-polyP-NP served as a depot for retarded polyP release. The chronic wound was treated with **(A-4)** the hydrogel. **(A-5)** After a 8 weeks of treatment, the wound dimension shrunk by 87%. **(A-6)** After a total of 18 weeks, the patient could go back home **(B)** For deeper wounds, **(B-1)** collagen-based mats were fabricated after compressing the collagen fibers. **(B-2)** Orientation of Ca-polyP-NP on the collagen bundles. **(B-3)** Every second day, a wetting solution composed of Na-polyP and Ca-polyP-NP was used and dropped onto the wound to keep it moist. There the Na-polyP underwent coacervation. Treatment success with the polyP-containing mats. **(B-4)** Initial state after debridement; **(B-5)** a strong reduction was observed after 6 weeks of treatment; **(B-6)** final state prior to patient discharge. **(A)** Adapted with permission from *J Mat Sci Technol*. Volume 135, Müller WEG, Schepler H, Neufurth M, et al. The physiological polyphosphate as a healing biomaterial for chronic wounds: Crucial roles of its antibacterial and unique metabolic energy supplying properties. 170–185. Copyright 2023. Creative Commons.<sup>49</sup> **(B)** Adapted with permission from Schepler H, Neufurth M, Wang SF, et al. Acceleration of chronic wound healing by bio-inorganic polyphosphate: In vitro studies and first clinical applications. *Theranostics*. 2022;12:18–34. © 2022 Ivyspring. Creative Commons.<sup>48</sup>

unusually conserved cationic groove composed of basic amino acids (Arg, Lys, and His).<sup>139,140</sup> Interestingly, the spacing of the basic amino acids on the spike protein perfectly matches the distances of 2.0 to 2.5 Å between the  $\text{P}_i$  units within the polyP molecule. In subsequent studies, a complete prevention of viral infection by polyP was experimentally proven. Due to this property, polyP can be classified as a component of the innate immune system and as a “smart” biomaterial (see Figure 7).<sup>209</sup>

## Application of Biosilica and Polyphosphate in Emerging Tissue Engineering Techniques

The two biomaterials, silica and polyP, can also contribute significantly to the field of tissue regeneration through the application of new tissue manufacturing techniques such as 3D printing technology. Among the existing 3D fabrication techniques such as selective laser sintering, stereolithography, fusion deposition modeling, digital light processing, and 3D bioprinting (for a review, see Ref.<sup>210,211</sup>), the latter in particular is one of the most advanced techniques. The development of 3D bioprinting is undergoing rapid development. This extrusion-based additive manufacturing technique is not only suitable for the creation of customized bone implants but also the fabrication of even large-sized tissue constructs and complex organs.

Nevertheless, this technique still has a number of limitations that await novel solutions. These include, for example, the fabrication of hollow structures such as blood vessels that would collapse as a result of gravitational forces during the layer-by-layer printing process. This makes, among others, the production of vascularized tissues difficult. One way to circumvent this problem is to use embedded bioprinting,<sup>212</sup> a gel-in-gel manufacturing technique in which the bio-ink is printed into a microgel or granular support bath. In the case of cortical bone implants, the printed material should have the required porosity and mechanical properties. Recapitulating the different mechanical properties and biological functions of the different parts of bone is a challenging task. Another requirement specifically for 3D cell printing (3D bioprinting) of tissues is the availability of a suitable bio-ink. This bio-ink, which usually consists of a hydrogel, certain other factors such as growth factors and the cells, must be able to protect the cells suspended in the hydrogel not only during the printing process but also afterwards, after curing (usually achieved by printing in a hardening solution containing  $\text{Ca}^{2+}$  ions), to maintain essential cell functions such as vitality, proliferation, migration, and differentiation abilities. Furthermore, the cells must be able to be printed in a density that corresponds to the high cell densities of native tissues and organs.

The introduction of the two biomaterials, silica and polyP, brought decisive progress, particularly with regard to the latter properties. Firstly, both amorphous biomaterials are regeneratively active themselves, they support cell vitality and promote cell proliferation/differentiation and do not require the addition of growth factors. The activity of such biodegradable growth factors/proteins is often difficult to control. Secondly, polyP in particular is able to provide the metabolic energy required for cell function, supporting cell migration and the synthesis, organization, and maintenance of the ECM structure. Cells can be printed at sufficiently high concentrations required for tissue regeneration/repair and formation of functional tissue constructs.

Taking advantage of these unique properties of silica and polyP/polyP-NP, the first bioprintable biosilica and polyP-containing hydrogels were developed. Embedding SaOS-2 cells in an alginate hydrogel resulted in a 3D printable material that showed increased HA formation and expression of bone morphogenetic protein-2 (BMP-2) and osteoprotegerin (OPG) genes.<sup>213</sup>

The first cell-free, polyP-enriched bio-ink suitable for 3D printing consisted of Ca-polyP-NP embedded in a poly( $\epsilon$ -caprolactone) (PCL) matrix. The scaffolds printed with this bio-ink supported the ingrowth of SaOS-2 cells, accompanied by increased expression of the cell-attracting chemokine stromal cell-derived factor-1 (SDF-1).<sup>214</sup> The first cell-containing polyP-bio-ink consisted of Na-polyP, gelatin, and alginate complexed by  $\text{Ca}^{2+}$  ions. The cells (SaOS-2) embedded in this ink remain alive and grow efficiently in the 3D printed construct.<sup>215</sup> The best results were achieved – as described above – with a bio-ink based on *N,O*-CMC/alginate/gelatin containing both soluble polyP (Na-polyP) and Ca-polyP-NP. MSC contained in this matrix remained functionally active and showed increased cell migration and propensity to grow and differentiate into mineralizing osteoblasts.<sup>152</sup>

As a further method, the electrospinning method has been used to fabricate advanced biosilica- or polyP-containing scaffold materials. Electrospun nanofiber mats prepared from poly(D,L-lactide) (PLA) and modified by enzymatically (silicatein)-formed biosilica were found to promote the growth and mineralization of SaOS-2 cells.<sup>216</sup> In addition, the synergistic effect of amorphous Ca-polyP-NP and retinol on cell growth has been exploited by manufacturing electrospun PLA fiber mats containing both components in view of their beneficial effects as antimicrobial and regeneratively active wound dressing materials.<sup>217</sup>

## Preferred Fields of Application

Both biomaterials, biosilica and polyP, have a wide range of potential applications. The most important ones identified are summarized in Table 2. Although there are some potential applications in the technical field, the preferred application

of both materials is in tissue engineering and regenerative medicine. For the latter applications, the amorphous nature of the material is necessary. As shown in animal studies (rabbits; see Figure 4), silica encapsulated in PLGA microspheres alone or together with silicatein strongly stimulates bone regeneration after implantation in the femur compared to  $\beta$ -TCP controls.<sup>83</sup> As stated, a similar mineralization-promoting effect was also found with polyP, both with soluble polyP<sup>189</sup> and with polyP-NP, but also with polyP-stabilized ACC<sup>68,218</sup> and polyP-stabilized ACP.<sup>30,69,167</sup> The effect of the polyP-NP is significantly influenced by the counteraction of the polyanionic polyP. While Ca, Sr, and Mg are the preferred counterions in inducing bone regeneration,<sup>135,137,142,189</sup> the Mg- and Ca-salts of the polymer are more suitable for cartilage regeneration.<sup>136</sup> Using the calvarial bone defect model, polyP-stabilized ACC<sup>218</sup> and polyP-stabilized ACP<sup>30</sup> encapsulated in PLGA and embedded in cranial defects of rats or rabbits significantly increased the formation of new bone mineral.  $\beta$ -TCP and crystalline Ca-phosphate, used as a controls, were much less active.

Instead of encapsulation in microspheres, silica and polyP can also be administered after integration into a hydrogel-forming organic polymer matrix that can be hardened in the presence of divalent cations. In this way, biohybrid materials are created that combine the regenerative properties of the inorganic biomaterials/biopolymers with the advantageous mechanical and water-binding properties of the organic polymers. SaOS-2 cells embedded in an alginate-based hydrogel matrix containing biosilica formed by silicatein<sup>213</sup> or grown on 3D printed biosilica-alginate scaffolds<sup>219</sup> showed increased growth, mineralization, and gene expression of BMP-2, collagen type 1, and OPG, but not of RANKL (receptor activator of NF- $\kappa$ B ligand). Increased osteogenic activity was also found with enzymatically formed biosilica on a chitosan-graft-polycaprolactone matrix.<sup>234</sup> Organic polymers used together with polyP mainly included negatively charged polysaccharides, as well as collagen and PVA. In the presence of divalent metal cations such as Ca<sup>2+</sup> (used for bone implants) or Mg<sup>2+</sup> ions (for cartilage), these materials can be cured by forming metal bridges to produce materials with suitable hardness and viscoelastic properties for potential use in bone and cartilage regeneration/repair. PVA is hardened by intermolecular cross-linking by freeze-thawing.<sup>151</sup> The addition of Ca<sup>2+</sup> to the polyP-containing matrices can also lead to the in situ formation of Ca-polyP-NP,<sup>150,151</sup> which upon contact with the body fluid are converted into a biologically active coacervate, as confirmed in animal studies.<sup>151</sup> Furthermore, due to their regenerative activity, biosilica and polyP can also be used in dentistry for tooth restoration/repair,<sup>226</sup> but also as dental sealants.<sup>80,227</sup> Other identified potential applications of both polymeric biomaterials include their use in drug delivery, biologization of inert materials and protection against biofouling, including caries prevention (Table 2).

The most advanced application of polyP and polyP-NP in regenerative medicine is in the field of wound healing, specifically for the treatment of chronic wounds, where this polymer has already been successfully used in initial human studies, as described here. The material can be in the form of a wound gel<sup>49</sup> or incorporated into a collagen-based wound

**Table 2** Preferred Applications of Amorphous Biosilica and Amorphous polyP

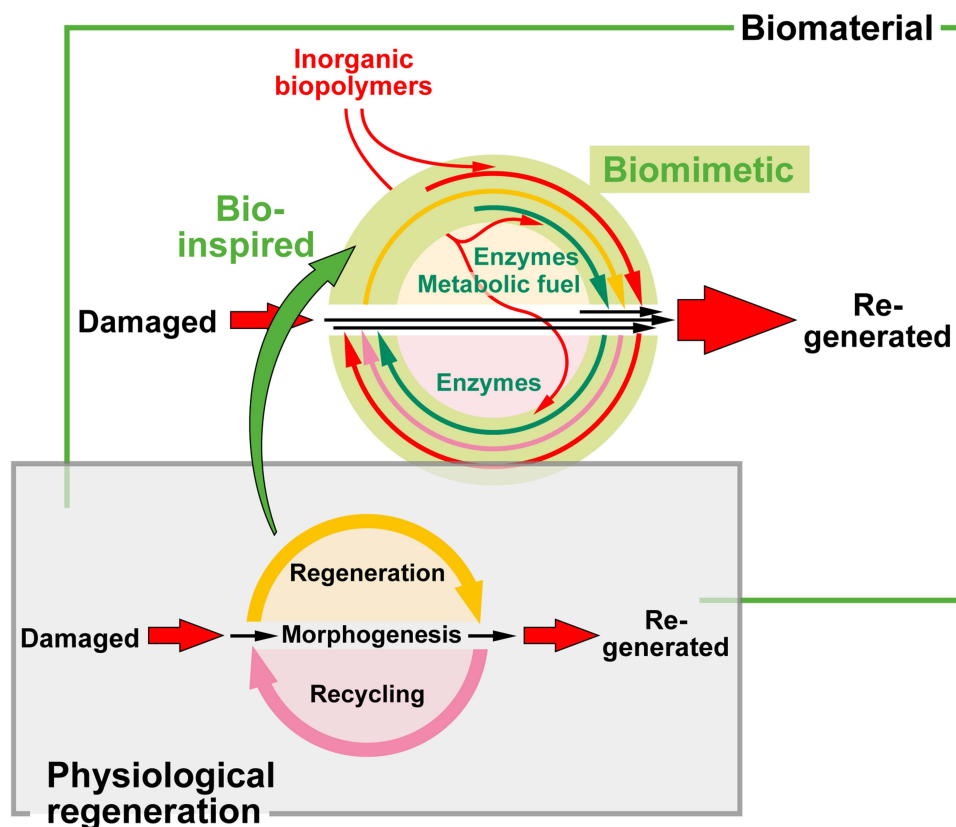
Bio-Material	Application Field	Specific Application/Formulation	References
Biosilica	Bone regeneration/repair	Silica microspheres	[7,83]
	Dentistry	Dental sealant	[80]
	3D printing	Silica/alginate	[219]
	Electrospinning	Silicatein/silica/polycaprolactone	[216]
	Encapsulation	Enzymes	[220]
		Bacteria	[221,222]
	Biofouling protection	Antibacterial activity	[223]
	Others	Light transmission	[31,57]
Polysiloxane synthesis by silicatein		[62,76]	

(Continued)

Table 2 (Continued).

Bio-Material	Application Field	Specific Application/Formulation	References
PolyP	Bone regeneration/repair	PolyP-NP (Ca, Sr, Mg)	[135,137,142]
		Ca-polyP/N,O-CMC	[224]
		Ca-polyP/collagen/chondroitin sulfate	[150]
		Ca-polyP/poly(vinyl alcohol)/karaya gum	[151]
		PolyP-stabilized ACC	[68,218]
		PolyP-stabilized ACP	[30,69,167]
	Cartilage regeneration/repair	Mg-polyP/hyaluronic acid	[136]
		Ca-polyP/alginate/N,O-CMC	[149]
		Ca-polyP/poly(vinyl alcohol)/karaya gum	[151]
	Wound healing	Wound gel	[49]
		Wound mat	[48,217]
		PolyP-stabilized ACC	[225]
		Ca-polyP-NP	[207]
		Ca-polyP-NP/retinol	[217]
	Dentistry	Tooth restoration/repair	[226]
		Tooth sealant	[227]
	Drug delivery	Bisphosphonate/Ca-polyP-NP (bone tumors/metastases)	[138]
		Core-shell particles	[228]
	3D printing	3D-printing	[214]
		3D-bioprinting (cells)	[152,215]
	Artificial vascular grafts	Ca-alginate/N,O-CMC/polyP/silica (extrusion method)	[229]
	Biologization	Allogeneic bone grafts	[47]
		Hernia nets	[230]
Entrapment	Mesenchymal stem cells encapsulation	[143]	
Microvascularization	Stimulation of microvascularization	[51]	
Blood coagulation	Procoagulant	[169,231,232]	
Antimicrobial activity	Antiviral	[125,139–141]	
	Antibacterial	[49,233]	





**Figure 14** A suitable biomaterial is formed enzymatically and drives the expression of genes encoding for enzymes in the surrounding tissue. During regeneration, the structural proteins and the newly developed cells require metabolic energy for functional arrangement and positioning.

mat.<sup>48,217</sup> In addition, the use of the remarkable antiviral activity of polyP,<sup>125</sup> especially against SARS-CoV-2,<sup>139,140</sup> also proven in vivo,<sup>141</sup> in the form of a nasal spray is promising.

## Conclusion and Outlook

At first glance, both the soft tissues, such as cartilage or fibrous connective tissue such as the dermal layer of the skin, and the bones or teeth, the hard tissue, are largely composed of non-cellular ECM structures that regulate almost all cellular functions. Especially during regeneration, these tissues are subject to continuous remodeling mediated by enzymes such as the catabolic-acting matrix-degrading enzymes<sup>235</sup> or the anabolic-acting ALP.<sup>236</sup> The dysregulation of the energy metabolism in cells of the ECM, such as fibroblasts (dermis layer of the skin), chondrocytes (cartilage), or bone cells, such as osteoblasts, osteocytes, and osteoclasts, disrupts the balance between various growth factors, proteoglycans, mineralizing cells, and enzymes.<sup>237</sup> Focusing on cells of the mineralized tissue, the central role of enzymes was first discovered in sponges, as summarized in this review. Furthermore, the connection of the metabolism of the ECM and the skeletal elements with bioenergetics was first disclosed in these evolutionary oldest animals. The mineralized elements in sponges are formed in an ATP-rich environment. A suitable biomaterial, in turn, must provide these two landmarks, enzymes and metabolic energy, to enable regeneration, morphogenesis, and repair of damaged tissue (Figure 14). Here again, the sponges offered a solution, directing the bioinspired/biomimetic studies towards an inorganic biopolymer, polyP, which from then on became a distinguished biomaterial for engineering scaffolds and supplements for successful repair of damaged tissue with graded structural, mechanical, and compositional properties. The anionic polyP, in combination with cationic ions, acts as a structural and reinforcing biomaterial that tends to transform into a hydrogel-like coacervate in the presence of peptides.<sup>143</sup> In this water-rich phase, polyP undergoes enzymatic degradation and processing into ADP and ATP.<sup>5</sup>

Also, wound healing is an energy-intensive process. Sponges are already provided with basal mechanisms involved in wound healing, including enzymes and signaling pathways that are found in higher animals and humans.<sup>18</sup> Sponges as sessile animals are sensitive to injury and tend to develop chronic wounds when repair is compromised. However, they can survive even in adverse conditions with low oxygen levels, possibly by using polyP accumulated in these animals.<sup>238</sup>

Although biomineral formation in sponges and human bone shares some similarities in terms of enzyme involvement and energy requirements, it should be noted that both the chemical nature and the steps in which enzymes are involved are different. The sponge biosilica consists of covalently linked  $\text{SiO}_4$  tetrahedra that share an edge with each other (some edges of the tetrahedra remain unlinked as free silanol Si-OH groups) and is an amorphous material, while HA is a crystalline material composed of  $\text{PO}_4^{3-}$ ,  $\text{OH}^-$ ,  $\text{CO}_3^{2-}$ , and  $\text{Ca}^{2+}$  units linked together by ionic forces. In nature, in living organisms, both are composites, with silica as silica spicules consisting of multiple silica lamellae surrounding a central axial filament of silicatein and separated from each other by thin layers made of silicatein and some other proteins.<sup>239</sup> Bone, on the other hand, consists mainly of HA and collagen, which forms the matrix for the deposition of the HA crystals, but also of some other proteins involved in the regulation of the biomineralization process. The steps in which enzymes are involved in mineral deposition and the nature of the protein framework for biomineral deposition are also different. In the sponge spicules, the protein scaffold around which silica is deposited, itself carries the enzymatic, silica-forming activity, while in bone, the proteinaceous collagen scaffold is enzymatically inactive and enzymes (ALP) are only involved in providing the required energy and phosphate (released from polyP or some other sources, eg, ATP), as well as in the formation of the ACC precursor of ACP and crystalline HA (carbonic anhydrase). As a regeneratively active biomineral for human tissue regeneration/repair, silica consists only of the inorganic amorphous material.

Biosilica derived from sponges has a wide range of bioinspired applications, ranging from orthopedic implants, as summarized in this review, to dental sealants<sup>80</sup> and light transmission systems.<sup>31</sup> The exploitation in human therapy is remarkable and based on the morphogenetic and regenerative activity of the material. Closely related to biosilica are bioactive glasses, also an amorphous material that was introduced in 1971.<sup>240,241</sup> However, the chemical composition of biosilica is different from the CaO-SiO<sub>2</sub>-P<sub>2</sub>O<sub>5</sub> composition of bioactive glasses prepared by sol-gel methods. Biosilica formed enzymatically by silicatein consists largely of a (water-rich) network of covalently linked SiO<sub>2</sub> units, while 45S5 bioglass, for example, consists of 24.5 wt% CaO, 45.0 wt% SiO<sub>2</sub>, 6.0 wt% P<sub>2</sub>O<sub>5</sub>, and 24.5 wt% Na<sub>2</sub>O.<sup>242,243</sup> As shown here, the morphogenetic activity of biosilica can be exploited in implants as demonstrated by the differential expression of various genes required for cell growth and differentiation in response to the biomineral.<sup>80</sup> In addition, from the natural biosilica, the blueprint, the fabrication of distinctive hierarchical porous structures could be learned, which even enabled the production of fibers with specific optical properties.<sup>244</sup>

PolyP has a unique property to act both as a structural and degradable biomaterial for bone regeneration in orthopedics and simultaneously as a generator for extracellular metabolic energy. The application of polyP is even more advanced in the area of wound healing, especially the healing of chronic wounds. Healing of chronic wounds caused by metabolic diseases (diabetic foot ulcers), inadequate blood flow (venous leg ulcers), sustained pressure (bedsores), infections or cancer is still an unsolved medical challenge and a major cost driver in medical care. Initial applications in human patients with chronic wounds led to promising results, as described here. PolyP can be administered both in soluble form (as Na-polyP, immediately active) and as Ca-polyP-NP (depot form, prolonged effect), either as a wound creme or integrated into a collagen mat. Since the proof of concept for this biopolymer could be elaborated in human studies, its central position for physiological regeneration has been established.

Future tasks will also include using the two biomaterials silica and polyP to develop stimuli-responsive materials that are sensitive to specific signals from the tissue environment (pH, temperature, presence of certain biomolecules, etc.). Such materials may be endowed with the ability to decompose, swell or shrink, undergo sol-gel transition, or release certain drugs, growth factors, or other biomolecules in response to pH or other changes in the surrounding tissue.<sup>245</sup> In particular, pH changes (acidosis) that occur, for example, in the course of wound healing,<sup>246</sup> at tumor sites<sup>247</sup> or during ischemia<sup>248</sup> can be used to trigger the release of these molecules from the implanted or administered material. In recent years, a number of pH-dependent delivery systems have been developed, based, for example, on *N,O*-CMC,<sup>249</sup> alginate-dialdehyde-gentamicin/chitosan,<sup>250</sup> or poly(*N*-isopropylacrylamide-*co*-acrylic acid) hydrogels.<sup>251</sup> PolyP, as described in this review, is particularly suitable for the development of such delivery systems because it can transform from the

nanoparticulate state (polyP-NP) to the coacervate state in a pH-dependent manner. Using bisphosphonate zoledronic acid, Ca-polyP-NP carrying this drug for potential treatment of tumors/metastases has already been synthesized.<sup>138</sup> A challenging task will be the development of silica- or polyP-based materials that carry multiple bioactive molecules or drugs, released at different times or on demand, and controlled by specific biochemical signals during multi-step repair processes such as the phases of wound healing (hemostasis, inflammation, proliferation, and maturation/remodeling) or the stages of bone regeneration (hematoma formation, inflammation, soft callus formation, hard callus formation, and remodeling). This could be realized by incorporating these biomolecules into various carrier systems such as core-shell particles, and nanoparticles (polyP-NP) with different counterions or nano/microspheres. Recently, a core-shell drug delivery system has been developed consisting of a polyP-NP core and a polyP-coacervate shell loaded with two different bioactive molecules (dexamethasone and ascorbic acid) that are released with different kinetics.<sup>228</sup> Such systems would also be of interest for the long-term treatment of osteoporosis caused by a reduced ratio of expression of the two cytokines OPG and RANKL, leading to increased osteoclastogenesis/bone resorption, or rheumatoid arthritis, which is associated with overexpression of inflammatory cytokines such as tumor necrosis factor  $\alpha$  or interleukins 1, 6, or 7 causing bone destruction. Both polyP and biosilica have been shown to increase OPG expression,<sup>9,213</sup> and polyP to decrease proinflammatory cytokine levels.<sup>141</sup> Finally, the beneficial effects of the regenerative activity of silica and polyP in other pathological conditions such as neurodegeneration need to be elucidated.

As summarized in this review, bioinspired/biomimetic thinking has the potential to reconcile the present-day demands of our comparatively very young species with its deficits. The human species (*Homo sapiens*) with an age of ~0.3 million years can benefit by learning from other metazoan taxa such as sponges (age 800 million years),<sup>252</sup> and also from the world of microorganisms, which can be traced back to over 3465 million years of the Earth's history.<sup>253</sup> Keeping in mind that every organism on earth has a long, rich history driven by adaptation and survival of the fittest.<sup>254</sup> The biosilica and polyP materials discussed here have the groundbreaking potential for further scientific, biomedical, and technical innovations. Surely, these two materials will push ahead sustainable progress of our society in science, medicine, and technology.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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